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Aerobic granulation and resource production under continuous and intermittent saline stress

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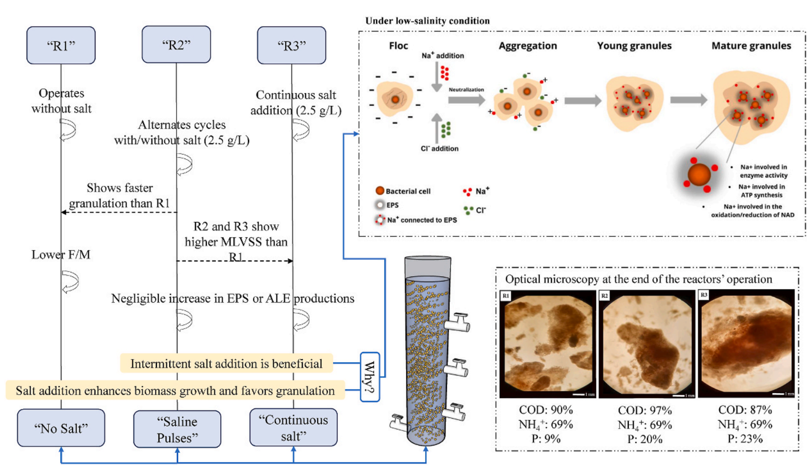
HIGHLIGHTS

- Aerobic granulation and resource production were investigated in AGS systems.
- Continuous and intermittent salt addition was evaluated.
- Intermittent osmotic pressure favors the granulation process and granule stability.
- *Thauera* was the most abundant genus found in the saline reactors.
- *Blastopirrellula, Phaeodactylibacter,* and *Rodhopirellula* could also be found in the saline reactors.

GRAPHICAL ABSTRACT



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ABSTRACT

Three sequential batch reactors (SBR) were operated to evaluate salt addition's impact on granulation, performance, and biopolymer production in aerobic granular sludge (AGS) systems. System R1 was fed without adding salt (control); system R2 operated with saline pulses, i.e., one cycle with salt (2.5 g NaCl/L) addition followed by another without salt; and R3 received continuous supplementation of 2.5 g NaCl/L. The results indicated that the reactors supplemented with salt presented higher concentrations of mixed liquor volatile suspended solids (MLVSS) and better settleability than R1, showing that osmotic pressure contributed to biomass growth, accelerated granulation, and improved physical characteristics. The faster granulation occurred in R2, thus proving the beneficial effects of intermittent salt addition through alternating pulses. Salt addition did not impair the simultaneous removal of carbon, nitrogen, and phosphorus. In fact, R2 showed better carbon removals. In conclusion, continuous or intermittent (pulsed) supplementation of 2.5 g NaCl/L did not lead to increased production of extracellular polymeric substances (EPS) and alginate-like exopolymers (ALE). This outcome could be attributed to the low saline concentration employed, a higher food-to-microorganism (F/M) ratio observed in

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R1, and possibly greater endogenous consumption of biopolymers in the famine period in R2 and R3 due to the greater solids retention time (SRT). Therefore, this study brings important results that contribute to a better understanding of the effect of salt in continuous dosing or in pulses as a selection pressure strategy to accelerate granulation, as well as the behavior of the AGS systems for saline effluents.

1. Introduction

The excess sludge produced in Sewage Treatment Plants (STPs) presents a major problem, considering the environmental impacts and economic aspects of treatment and final disposal. In this context, the reuse and recovery of resources related to sewage treatment have stood out in the technical and scientific fields in recent years (Semaha et al., 2023; Hamza et al., 2022; Ferreira et al., 2021). Given this new perception, STPs are no longer seen as a waste stream and are considered potential resource recovery factories that fuel the circular economy (Kehrein et al., 2020).

Numerous resources can be recovered from wastewater and transformed into value-added products, such as phosphorus, nitrogen, biopolymers, methane, and hydrogen (Batstone et al., 2015). Among the emerging sewage treatment technologies that allow contaminant removal and resource recovery, aerobic granular biomass can be considered an effective and promising environmental biotechnology (Nancharaiah and Sarvajith, 2019). Aerobic granular sludge (AGS) systems demonstrate advantages over conventional activated sludge, presenting a higher concentration and better biomass sedimentation capacity, reduced reactor size, energy consumption, and environmental impact (Bou-Sarkis et al., 2022).

In the AGS system, different redox conditions (anaerobic, anoxic, and aerobic) occur within the layered granule structure, favoring the processes of simultaneous nitrification, denitrification, and phosphorus removal (He et al., 2020). Granule formation involves the microbial production of extracellular polymeric substances (EPS), which have a complex structure and are mainly formed by proteins (PN), polysaccharides (PS), nucleic acids, and humic acids (Felz et al., 2019). Research has shown that a large fraction of AGS EPS consists of alginate-like exopolymers (ALE), a biomaterial with several industrial applications (Ferreira et al., 2021; Rollemberg et al., 2020).

ALE can be a raw material in the paper and construction industries, with potential use in agriculture, horticulture, and medicine (Van Leeuwen et al., 2018). Furthermore, it can be used in the textile sector as a sustainable biosorbent for removing dyes from aqueous solutions (Ladnorg et al., 2019). In a study by Sarvajith and Nancharaiah (2023), ALE was the dominant biopolymer in the AGS EPS matrix, accounting for 44% on a weight basis. Therefore, the recovery of ALE as a biomaterial with high added value as a renewable resource in the AGS process presents itself as a promising trend, with possibilities for environmental and economic benefits.

EPS is considered the most important aspect of AGS's granulation, stability, and performance (Ou et al., 2018). Some of the main factors that help in the granulation are the hydrodynamic shear force and the regimes of substrate abundance (feast) and scarcity (famine) in the sequential batch reactors (SBR), in addition to the short sedimentation time (Nancharaiah and Reddy, 2018). However, the long granulation time and low granular stability are the main obstacles to AGS technology (Hamza et al., 2022). In general, the formation period of aerobic granules is longer than 30 days (Zhang et al., 2019a,b). Given this, the AGS literature has presented several strategies to accelerate granulation and improve granule stability by adding chemical compounds or establishing operational conditions that facilitate cell aggregation and impose a selection pressure that stimulates EPS production (Han et al., 2022).

Campo et al. (2018) demonstrated that salinity can positively affect aerobic granulation, allowing bacteria to cluster and form stable granules with gradually increasing salinity (0.30–38 g NaCl/L). Therefore, research has also evaluated the effect of AGS cultivation with salt as a selection pressure strategy since Na^{+} is involved in several transport processes and can promote enzymatic activity if maintained at appropriate concentrations (Cui et al., 2015). Frutuoso et al. (2023a) evaluated the effects of adding salt to maximize resource recovery. They reported improved ALE production after the granulation phase of up to 7.5 g NaCl/L. However, a reduction was found with 10 g NaCl/L, being related to granule fragmentation and collapse.

In this context, a possible strategy to accelerate the granulation process and stimulate the production of resources without causing the breakdown of aerobic granules and consequent instability of the system is the addition of salt in alternating pulses since the dispersed addition of NaCl is capable of causing diffuse stress throughout the operation, also configuring itself as a selection pressure. Moreover, this operational strategy is little reported in the scientific literature for AGS. Given the abovementioned issues, more research is needed to evaluate the effects of adding NaCl concentrations in conventional systems and pulses to convert aerobic flocs into granules and observe the effects on system performance and resource production.

Therefore, the present work aimed to investigate the influence of adding 2.5 g NaCl/L continuously and in saline pulses on granulation, performance, biopolymer production, and microbiology in AGS systems. Therefore, this study brings important results that contribute to a better understanding of the effect of low salt concentration, either in continuous dosing or in pulses, as a selection pressure strategy to accelerate granulation and the behavior of the AGS system for saline effluents.

2. Material and methods

2.1. Experimental setup of the system, inoculum sludge, and synthetic effluent

The experiment consisted of operating three reactors in sequential batches for 57 days of operation, using propionate as a carbon source [1000 mg chemical oxygen demand (COD)/L] to be considered as the substrate that provides greater stability in granulation and maturation, in addition to being more efficient in the production of resources (dos Santos et al., 2022). The basal medium was prepared using drinking water, macro and micronutrient solution, and sodium bicarbonate as a buffer, maintaining a C:N ratio of 20 (dos Santos et al., 2022).

The reactors differed in terms of salt concentration in the feed; system R1 was fed without adding salt (control); system R2 operated with saline pulses, i.e., one cycle with salt (2.5 g NaCl/L) addition followed by another cycle without salt; and R3 received continuous supplementation of 2.5 g NaCl/L.

The three research reactors were made of acrylic, with a useful volume of 7.8 L, a height of 1 m, and a diameter of 100 mm. They were inoculated with aerobic sludge from a carousel-type activated sludge system from a domestic sewage treatment plant located in Fortaleza, Ceará, Brazil. This sludge had an initial concentration of mixed liquor volatile suspended solids (MLVSS) of 5 g/L and a sludge volumetric index in 30 min (SVI_{30}) of 152 mL/g.

The operating cycle lasted a total of 360 min, distributed over anaerobic feeding (20 min), anaerobic period (100 min), aerobic period (215–225 min), anoxic period (10 min), and sedimentation (15-5 min). To stimulate granulation, the settling time was reduced from 15 to 10 min (in 7 days of operation), and to 5 min (in 15 days of operation), with the subtracted time added to the aerobic period. During periods of aeration, oxygenation was supplied through an air compressor, with an aeration rate of 10.0 L/min and an ascending superficial gas velocity of

2.1 cm/s. The adopted volumetric exchange ratio was 50%, which resulted in a hydraulic retention time (HRT) of 12 h. The applied volumetric organic load was 2.0 gCOD/L/day. In this study, sludge retention time (SRT) was not controlled with intentional sludge discharge. However, the average SRT was calculated based on the biomass present in the SBR and the one lost with the final effluent (Frutuoso et al., 2023b).

2.2. Analytical methods

The reactors' performance in removing organic matter and nutrients was evaluated twice a week through analyses of influent and effluent samples, COD, ammonia (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), and phosphate (PO_4^{3-}-P), all following the recommendations of the Standard Methods (APHA, 2012). Dissolved oxygen and pH were monitored using a probe (YSI 5000, YSI Inc., USA).

The physicochemical characterization of biomass was carried out weekly through analyses of MLVSS and sludge volumetric index in 30 min (SVI_{30}) and 5 min (SVI_5), according to APHA (2012). The formation and development of granules were evaluated weekly by granulometry and optical microscopy. In analyzing the particle size, three sieves with an opening of 0.2 mm were used; 0.6 mm and 1 mm, according to dos Santos et al. (2022). The granules were visualized using optical microscope images (Opton).

The EPS extraction procedure combines sodium hydroxide and heating (Hong et al., 2017). The PS and PN fractions in the EPS were analyzed using the phenol-sulfuric (DuBois et al., 1956) and folin-phenol (Lowry, 1951) methods, respectively. The total EPS was considered as the sum of PN and PS, with the results 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 by dos Santos et al. (2022).

ALE was determined through alkaline extraction with sodium carbonate in its acidic form at high temperatures (Lin et al., 2010; Felz et al., 2016). The mass value was expressed in mg ALE/g VSS, following the recommendations of Felz et al. (2016).

2.3. Microbiological analyzes

Molecular biology analyses to identify microorganisms present in the sludge were segmented into three stages: DNA extraction, 16S rRNA gene sequencing, and data processing. Using the PowerSoil® DNA isolation kit (MoBioLaboratories Inc., USA), following the manufacturer's instructions, DNA extraction was performed from mixed liquor samples from the reactors, collected during aeration at the end of the operation, with a wet weight of 0.25 g. Amplicon sequencing of the V4 region of the 16S rRNA gene was prepared using region-specific primers (515F/806R), according to Ilumina (2013). Sequencing and data processing were carried out as described by dos Santos et al. (2022).

2.4. Statistical analyzes

In order to compare the performance of the reactors, the Kruskal-Wallis non-parametric test was applied, assuming a confidence level of 95%. Thus, for $p \leq 0.05$, the data groups were considered statistically different.

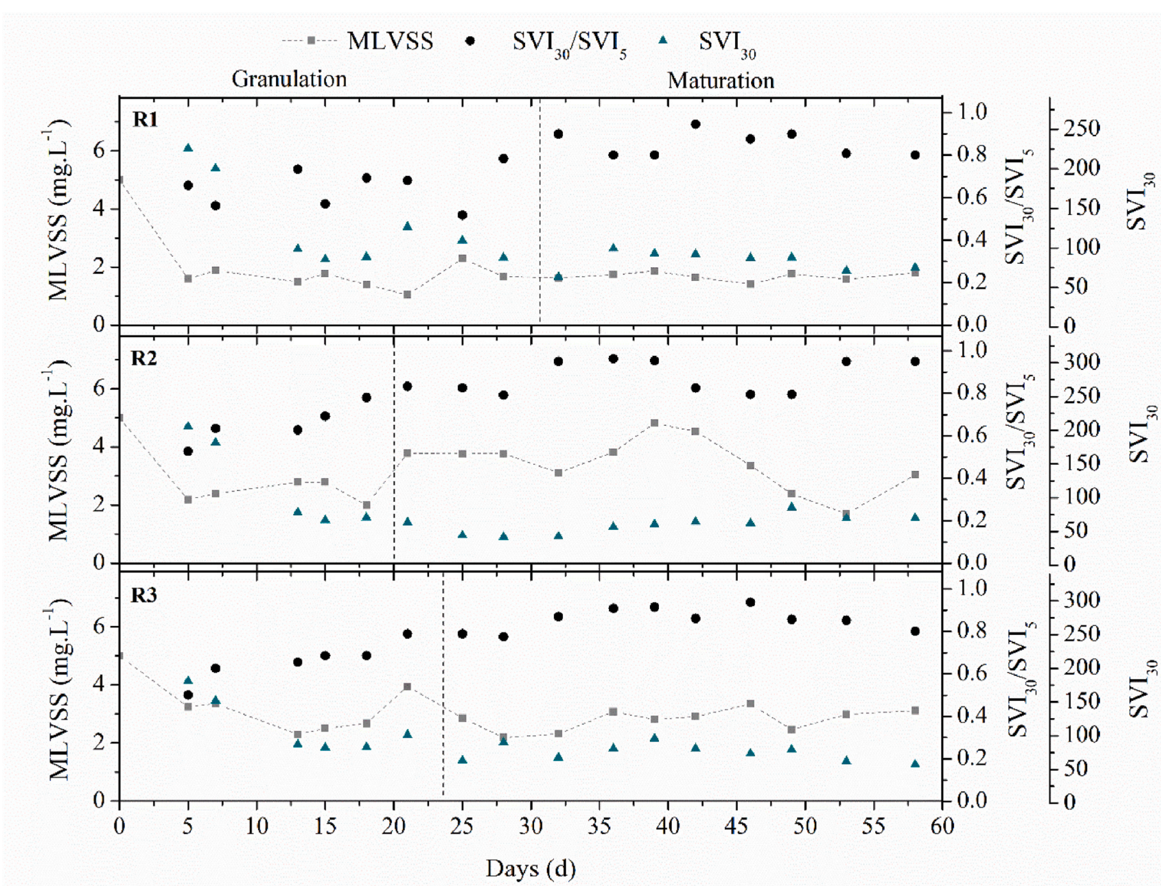

Fig. 1. MLVSS, SVI_{30}, and SVI_{30}/SVI_5 ratio in reactor R1 (control), R2 (addition in alternating pulses of 2.5 g NaCl/L), and R3 (continuous addition of 2.5 g NaCl/L) throughout the operation.

3. Results and discussion

3.1. Granulation process and physical properties of aerobic granules

MLVSS, SVI_{30}, and SVI_{30}/SVI_5 data over the days of reactor operation are presented in Fig. 1. It is possible to observe a reduction in the initial concentration of MLVSS in all reactors after inoculation due to filamentous biomass washout. Other studies have made similar observations (Frutuoso et al., 2023a,b; dos Santos et al., 2022; da Silva et al., 2021; Rollemberg et al., 2020).

In R1, the control reactor operated without salt addition did not display significant growth in MLVSS concentrations, stabilizing at approximately 1.7 g/L by the end of the operation. Throughout system maturation, the SVI_{30}/SVI_5 ratios and SVI_{30} values remained around 85% (±6) and 84 mL/g (±12), respectively. Conversely, salt-supplemented reactors exhibited higher MLVSS concentrations, reaching 3.5 g/L (±0.9) in R2 and 2.9 g/L (±0.5) in R3. Additionally, they demonstrated improved sedimentation performance, reaching SVI_{30} values of about 60 mL/g (±13) in R2 and 74 mL/g (±14) in R3. Although the SVI_{30}/SVI_5 ratio was similar to R1 at approximately 85% during granule maturation, this stage was reached earlier in R2, followed by R3. This behavior indicates that osmotic pressure contributed to biomass growth and enhanced sedimentation characteristics, with intermittent osmotic pressure showing better effectiveness.

A progressive reduction in MLVSS can be noted in R2 between days 42 and 53. Also, there was a drop in the SVI_{30}/SVI_5 ratio between days 42 and 49 due to operational problems caused by disturbances in aeration, which resulted in biomass loss and, consequently, affected system settleability. However, after this period, an increase in MLVSS in the reactor was noted, and the SVI_{30}/SVI_5 ratio reached a value close to 1.0, indicating the system's recovery capacity and resilience of AGS technology, which adapts to changes and different environmental conditions due to their complex microbial communities (Nancharaiah and Reddy, 2018).

It is worth highlighting, therefore, that biomass showed an improvement in sedimentation capacity in the presence of NaCl, a result that was also obtained in the study carried out by Campo et al. (2018) at different salinity concentrations (0.30–38 g NaCl/L). Other studies also corroborate the positive influence of NaCl on sludge settleability. For instance, by comparing three SBR reactors with salinities of 0 g NaCl/L, 10 g NaCl/L, and 30 g NaCl/L, Meng et al. (2019) concluded that a better sedimentation performance was achieved with increasing salinity. Finally, Frutuoso et al. (2023a) obtained stable aerobic granules in settleability with up to 7.5 g of NaCl/L.

The morphology of the mature granules can be visualized through images obtained by optical microscopy at the end of the reactors' operation, as illustrated in Fig. 2. The three reactors presented granules with an irregular shape and a rough surface. In R2 and R3, the biomass showed a larger granule size than in R1. In research conducted by Campo et al. (2018), who studied the effects of the gradual increase in salinity on granule formation in an AGS system, found granules with an irregular shape and an average diameter of approximately 1.1–1.2 mm when a concentration close to 2.5 g NaCl/L was tested.

Regarding the SRT, R1 consistently maintained low values, around 6 (±2) days throughout the operational period. Conversely, R2 exhibited an increase, reaching 15 (±3) days during the maturation phase, while R3 achieved 11 (±2) days.

Complete granulation in the AGS system is characterized by more than 80% of the biomass forming aerobic granules with a diameter greater than 0.2 mm, resulting in a ratio close to one between SVI_{30} and SVI_5, as described by Adav et al. (2008), De Kreuk et al. (2005), and Schwarzenbeck et al. (2004). In the present study, this complete granulation was observed between days 32 and 36 in R1, between 21 and 25 in R2, and between 25 and 28 in R3.

The main factors influencing granule formation include EPS synthesis, quorum sensing, bacterial surface hydrophobicity, and ionic bridging (Sarma et al., 2017; Liu et al., 2003). Salt facilitates this process by compressing the electrical double layer and promoting proton translocation, which reduces surface electronegativity and enhances aggregation (Frutuoso et al., 2023b; Lu et al., 2023; Sarma et al., 2017; Li et al., 2017). Additionally, Na^+ ions are essential for enzymatic activities and redox reactions; however, high concentrations can disintegrate granules by substituting divalent cation bonds and weakly bound EPS with Na^+ ions (Cui et al., 2015). Therefore, it is believed that supplementation of 2.5 g/L of NaCl in R2 and R3 contributed to particle aggregation, reducing the time required for complete granulation.

In the salt-supplemented reactors, R2 achieved complete granulation more quickly by applying salt in alternating pulses. This method introduced diffuse stress throughout the operation, establishing a dynamic selection pressure that varied the environmental conditions. These salt stress cycles fostered microbial adaptation and enhanced system stability during non-salt periods, thereby improving the formation and maturation of aerobic granules. Similarly, studies by Chen et al. (2015) and Niu et al. (2017) showed improved granulation in upflow anaerobic sludge blanket (UASB) systems with variable stimuli. Chen et al. (2015) employed intermittent Mg^{2+} supplementation, whereas Niu et al. (2017) explored the effects of changing C/N ratios under semi-starvation to optimize granule development.

3.2. Performance of the reactors

During the operation period, the pH of all reactors remained close to 7, and the dissolved oxygen (DO) at the beginning of the aerobic period presented an average of 2.8 mg/L in R1 and an average of 3.2 mg/L in R2

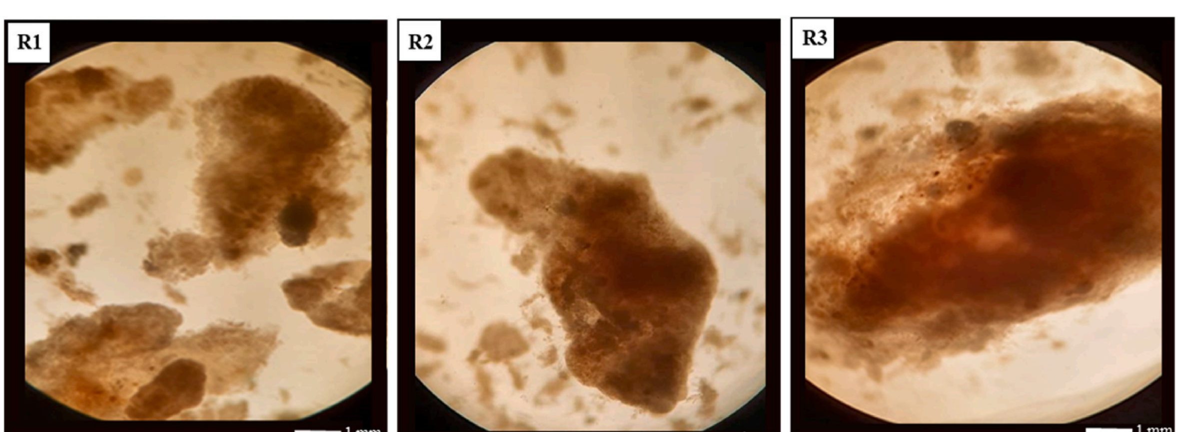

Fig. 2. Optical microscopy photograph of reactors R1 (control), R2 (addition in alternating pulses of 2.5 g NaCl/L), and R3 (continuous addition of 2.5 g NaCl/L).

and R3. All systems achieved excellent COD removals (Table 1). These results align with those reported in the literature, in which satisfactory COD removals were observed in low salt concentrations (Frutuoso et al., 2023a; Meng et al., 2019). Despite satisfactory performance across all systems, R2 demonstrated significantly better efficiency ($p < 0.05$), likely due to the rapid granule development facilitated by pulsed osmotic pressure, which enhanced organic matter removal.

Large, structurally stable aerobic granules during the maturation period favored the removal of nutrients, in which the average total nitrogen (TN) removal was above 60% (Table 1). No statistically significant difference was observed between the reactors ($p = 0.98$), indicating that intermittent or continuous saline stress did not affect nitrogen removal, which is good. In all systems, a small improvement in TN removal efficiency was observed between the granulation and granule maturation phases, achieving an average TN removal of 69% with the granule maturation. As a result of granule development, an environment with aerobic and anoxic/anaerobic zones is formed within the granule itself, which allows the coexistence of nitrifying and denitrifying bacteria (He et al., 2020).

Higher TN removal was limited by nitrogen fraction accumulation, with concentrations around 7 mg NO_2^--N/L (±0.9) and 9 mg NO_3^--N/L (±1.6) across all systems. According to Da Silva et al. (2021), incomplete denitrification may result from rapid depletion of the carbon source, insufficient biodegradable organic matter, and inadequate accumulation of polyhydroxyalkanoates (PHA). Additional factors include excessive aeration oxygen or unsuitable granule sizes preventing the formation of internal anaerobic and anoxic zones (Layer et al., 2020; Peng et al., 2018). However, granule size was likely adequate in this study as over 50% of the biomass during the maturation period had diameters greater than 1 mm. Dissolved oxygen levels were 2.8 mg/L in R1 and 3.2 mg/L in R2 and R3, supporting simultaneous nitrification and denitrification mechanisms (Peng et al., 2018).

Granulation significantly enhanced nitrification efficiency, with ammonia removal rates exceeding 90% across all reactors and no significant differences among them ($p > 0.05$). During the granule maturation phase, effluent ammonia concentrations were 1.3 mg-N/L in R1, 2.4 mg-N/L in R2, and 1.0 mg-N/L in R3, demonstrating nitrification rates above 97%. This performance aligns with the findings by Quartaroli et al. (2019), which reported consistently high ammonia removal across a range of salinities (5–40 g NaCl/L). These results, particularly during granule maturation, indicate robust simultaneous nitrification and denitrification (SND) despite the presence of NOx.

Phosphorus removal efficiencies in the maturation phase were approximately 9% for R1, 20% for R2, and 23% for R3, with R2 and R3 showing improvement throughout the operation, whereas R1 experienced a slight decline. The overall phosphorus removal was low, particularly in systems with an anaerobic phase, likely due to the predominance of glycogen-accumulating organisms (GAOs) over phosphorus-accumulating organisms (PAOs). GAOs compete with PAOs for organic matter but do not aid in phosphorus removal, thereby hindering this biological process (Lopez-Vazquez et al., 2009). Factors such as DO concentration, carbon sources, temperature, and pH significantly influence the competition between PAOs and GAOs (Carvalheira et al., 2014; Lopez-Vazquez et al., 2009). Molecular biology analysis confirmed GAOs' dominance over PAOs in all reactors.

3.3. EPS and ALE productions and characterization

EPS matrix was analyzed in the form of total PN and total PS (Fig. 3). An increase in PN and PS contents was observed in all systems shortly after start-up in comparison to the activated sludge flocs used as inoculum, indicating that favorable hydrophobic conditions were created for microbial aggregation and stability of the granule structure (Nancharaiah and Reddy, 2018). PN contents are the predominant component in the EPS matrix in relation to the PS contents. According to Campo et al. (2018), proteins are considered the main polymeric substances responsible for maintaining granule structure.

The PN/PS ratio is an important parameter for analyzing granulation, as it expresses the combined effect of PN and PS on forming aerobic granules. During the operation of the three reactors, the PN/PS ratio varied greatly, presenting values of 1.8–11.6 in R1, 0.9–7.4 in R2, and 2–8.2 in R3. It is worth noting that the highest PN/PS ratio (11.6) was observed in reactor R1 on the 21st day of operation. During this period, there is also a peak of greater total EPS production, probably due to the instability of the granular structure, considering that microorganisms usually secrete more EPS to maintain their shape in adverse conditions (Nancharaiah and Reddy, 2018).

During start-up, after increasing the EPS concentration in comparison with the inoculum sludge, a decrease was noted in the three reactors, similar to other AGS research, in which EPS production peaks are commonly observed in the initial period of granulation, with subsequent drops (dos Santos et al., 2022; Frutuoso et al., 2023a). Excessive EPS production can compromise granule structure through core mineralization or pore blockage (Lemaire et al., 2008). While low EPS levels can impede granulation, high levels may hinder long-term granule stability (Corsino et al., 2016).

During maturation, a reduction in EPS production is also observed in all reactors, similar to findings by dos Santos et al. (2022), who linked this reduction to several possibilities, including increased endogenous consumption due to higher SRT (Rollemberg et al., 2020) and reduced EPS production due to increased granule diameter caused by oxygen limitation and carbon diffusivity (Verawaty et al., 2013). Notably, in this study, approximately 50% of the biomass had diameters exceeding 1 mm, considered relatively large. Moreover, average EPS productions

Table 1
Operational performance of reactors R1 (control), R2 (addition in alternating pulses of 2.5 g NaCl/L), and R3 (continuous addition of 2.5 g NaCl/L) over the 53 days of operation.

Reactor		R1		R2		R3	
Stage		Granulation	Maturation	Granulation	Maturation	Granulation	Maturation
COD	Influent (mg/L)	1129.3 (215.7)	1122.6 (145.7)	1019.5 (154.2)	1059 (149.3)	1101 (163.3)	1047.5 (139.4)
	Effluent (mg/L)	43.2 (43.3)	117.6 (64.5)	21.6 (28.4)	33 (29.3)	105.9 (83.3)	155.8 (88.3)
	Efficiency (%)	96 (4)	90 (6)	98 (4)	97 (3)	91 (6)	87 (6)
Nitrogen fractions	NH_4^+ influent (mg-N/L)	59.1 (8.3)	56.4 (0.94)	58.1 (7.9)	59.6 (4.8)	58.1 (2.6)	55.1 (3.48)
	NH_4^+ effluent (mg-N/L)	5.8 (2.1)	1.3 (0.5)	3.9 (1.9)	2.4 (0.7)	4.5 (3.7)	1.0 (0.7)
	NO_2^- effluent (mg-N/L)	8.3 (1.3)	7.1 (0.9)	7.5 (1.4)	7.3 (0.9)	7 (1.2)	7 (0.6)
	NO_3^- effluent (mg-N/L)	7.8 (0.6)	9.1 (1.55)	9.5 (1.6)	8 (0.4)	9.7 (2)	9.2 (1.4)
	Nitrification efficiency (%)	92 (4)	98 (1)	95 (3)	97 (2)	92 (1)	98 (1)
	Nitrogen removal (%)	64 (6)	69 (4)	65 (2)	69 (4)	63 (10)	69 (5)
Phosphorous	Influent (mg PO_4^{3-}-P/L)	13.5 (1.2)	14.3 (0.4)	13.9 (1.4)	15 (1)	14.3 (1.3)	16.7 (2.8)
	Effluent (mg PO_4^{3-}-P/L)	12 (1)	13.1 (0.7)	12.7 (1.2)	12.1 (1.2)	12.2 (1.7)	12.9 (0.9)
	Efficiency (%)	11 (3)	9 (5)	8 (5)	20 (5)	13 (6)	23 (12)

Note: Standard deviation is shown in parentheses.

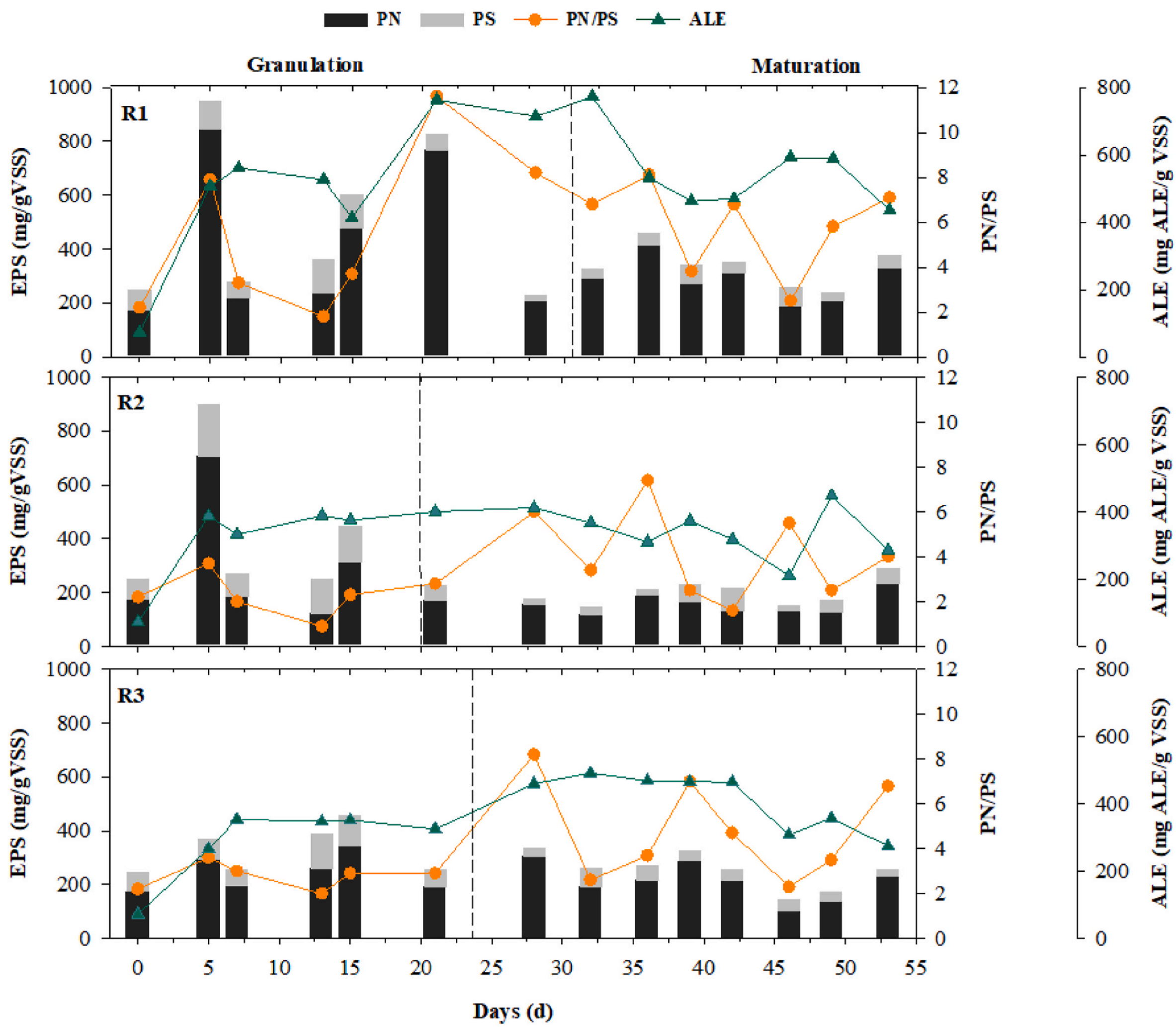

Fig. 3. Production of EPS and ALE from reactors R1 (control), R2 (addition in alternating pulses of 2.5 g NaCl/L), and R3 (continuous addition of 2.5 g NaCl/L) throughout the operation.

were 286 (±84), 172 (±60), and 209 (±68) mgEPS/gVSS, respectively, in R1, R2, and R3, values close to those observed in other studies (Campo et al., 2018, 2022; Wang et al., 2021).

Statistical analysis revealed significant differences in total EPS production among the reactors ($p = 0.024$), with R1 producing more EPS than the others. Adding salt at 2.5 g NaCl/L, either continuously or in pulses, did not enhance EPS production, underscoring that EPS levels are affected by variables such as SRT, food-to-microorganism ratio (F/M), inoculum type, and extraction protocol (Lu et al., 2023; Rollemberg et al., 2020). Wang et al. (2023) demonstrated that higher F/M ratios boost EPS production, as shown in R1, which had a significantly higher F/M ratio of 1.3 (±0.3) compared to R2 (0.7 ± 0.2) and R3 (0.8 ± 0.1), indicating that under lower saline conditions (≤2.5 g/L), the F/M ratio may be more impactful. Furthermore, prolonged SRT typically encourages endogenous biopolymer consumption, particularly when exceeding 20 days. Although this study's duration fell short of that threshold, R1 remained notably lower than R2 and R3 (Frutuoso et al., 2023b).

When comparing the production of EPS between R2 and R3, slightly higher production was found in R3, possibly due to larger salinity stress, as the saline concentration in R2 was diluted by intermittent operation. This observation is noteworthy, considering that the operational conditions between these reactors were similar (including F/M ratio, STR, inoculum type, and extraction protocol).

Meng et al. (2019) found an increase in total EPS production in the continuous presence of 10 and 30 g NaCl/L. Ou et al. (2018) also verified that increased salinity stimulated bacteria to secrete EPS, with its highest production being 725.5 mg/gVSS with 50 g NaCl/L. In the study carried out by Frutuoso et al. (2023a), the phase with the continuous addition of 2.5 g NaCl/L showed a lower average production of EPS compared to the other phases that used higher salt concentrations (5, 7.5, and 10 g/L). Therefore, based on the findings, very low saline stress, as observed in this study, positively influences granulation due to charge neutralization. However, this stress may not have a direct impact on EPS production.

Regarding ALE production, a high ALE concentration is observed immediately after activated sludge inoculation, similar to the EPS behavior (Fig. 3), corroborating the verification that EPS contain large proportions of ALE. The three reactors showed significant ALE productions, in agreement with values reported in the AGS literature (Sarvajith and Nancharaiah, 2023; Zahra et al., 2022; Rollemberg et al., 2020).

The results also noted a downward trend in ALE production in all systems, approximately from the 28th day of operation. This differs from a study by Schambeck et al. (2020), who obtained an increase in ALE concentration with granule maturation, with an enrichment of around 29% of ALE. However, our results align with data found in other studies, which also showed a drop in ALE production upon granule maturation (Frutuoso et al., 2023a; dos Santos et al., 2022). Because ALE is the main EPS component, the EPS content also showed the same declining trend in the maturation period.

Generally, ALE is a fraction of EPS characterized by its ability to form hydrogels (Schambeck et al., 2020). However, on some days of operation, the ALE fraction was observed to be greater than the EPS production. One explanation for this fact is that the quantification of ALE is equivalent to structural EPS, and its composition is composed not only of polysaccharides and proteins but also of humic acids and lipids (Lin et al., 2010).

The statistical analysis found a significant difference ($p < 0.05$) between ALE production in the reactors. In general, the results show that ALE production was higher in reactor R1. Therefore, it is inferred that adding 2.5 g NaCl/L of salt did not result in greater ALE production. Similar to the observed EPS trend, the higher F/M ratio and lower SRT in R1 may have favored the greatest ALE production. This result contrasts with the research conducted by Frutuoso et al. (2023a), in which they observed that ALE production was favored by adding up to 7.5 g NaCl/L after the granulation step. In another study, Meng et al. (2019) found that ALE was highly enriched at moderate and continuous salinity (10 g NaCl/L).

Some components in the EPS matrix are aromatic and have distinct fluorescence properties. 3D-EEM fluorescence spectra were used to analyze the EPS composition of each reactor at the end of the operation, at neutral pH and basic pH, as shown in Fig. 4. A total of eight fluorescence peaks were identified in the different samples, as described by Wang and Zhang (2010), in which the EEM spectrum can be divided into regions, and each EEM peak can distinguish the chemical composition of EPS samples. Regions I and II were attributed to substances similar to the amino acids tyrosine (I) and tryptophan (II), and it was found that the peaks in region I were found only in neutral pH samples.

Tyrosine- and tryptophan-containing proteins were identified in regions III and IV, respectively. Notably, the peaks in region III are more intense in neutralized samples, as high pH can favor the protein denaturation process. Polysaccharides (region V) were well identified in the basic EPS extract. Therefore, sample neutralization can change the chemical properties of the polysaccharides' functional groups. Regions VI, VII, and VIII were identified as containing substances resembling fulvic acid and humic acid, found in greater abundance in the neutral EPS extract. Therefore, in general, the results indicate that the presence of salinity did not modify the chemical structure of EPS.

3.4. Microbiological characterization

Different compositions and distribution of bacterial communities were observed through microbiological characterization in inoculum sludge (I) and biomass samples from reactors R1, R2, and R3 at the end of the operation (Fig. 5). In the activated sludge of the inoculum, 43 bacterial phyla were classified, being *Planctomycetota* (24.6%), *Chloroflexi* (15.6%), *Actinobacteriota* (13.1%) and *Proteobacteria* (9.2%) the most abundant. With the granulation process, a large reduction in the phyla *Planctomycetota*, *Chloroflexi*, and *Actinobacteriota* was observed in all reactors since they comprise some groups of filamentous microorganisms that are disadvantaged during this process.

On the other hand, there is an enrichment of the phylum *Proteobacteria*, which has become the most dominant phylum, followed by *Bacteroidota*. *Proteobacteria* are recognized for their ability to secrete EPS, one of the main components in granule formation (Meng et al., 2019; Hay et al., 2014). Likely, this phylum played a key role in the granulation process. This result aligns with the literature, which indicates that the *Proteobacteria* phylum is one of the most common in AGS systems (Ely et al., 2022; Quartaroli et al., 2019). *Proteobacteria* presence was higher in reactors supplemented with salt, with abundances of 73.3% and 70.7%, in R2 and R3, respectively. Thus, it is evident that this phylum has developed a good adaptation to the saline environment.

The phyla *Myxococcota* and *Verrucomicrobiota* showed higher abundance in R1 compared to the inoculum and reactors supplemented with salt, indicating that granulation provided suitable conditions for their development and salinity inhibited their growth. Thus, salt's presence negatively affected these microbial groups' growth and metabolic activity. Studies by Frutuoso et al. (2023a) and Quartaroli et al. (2019)

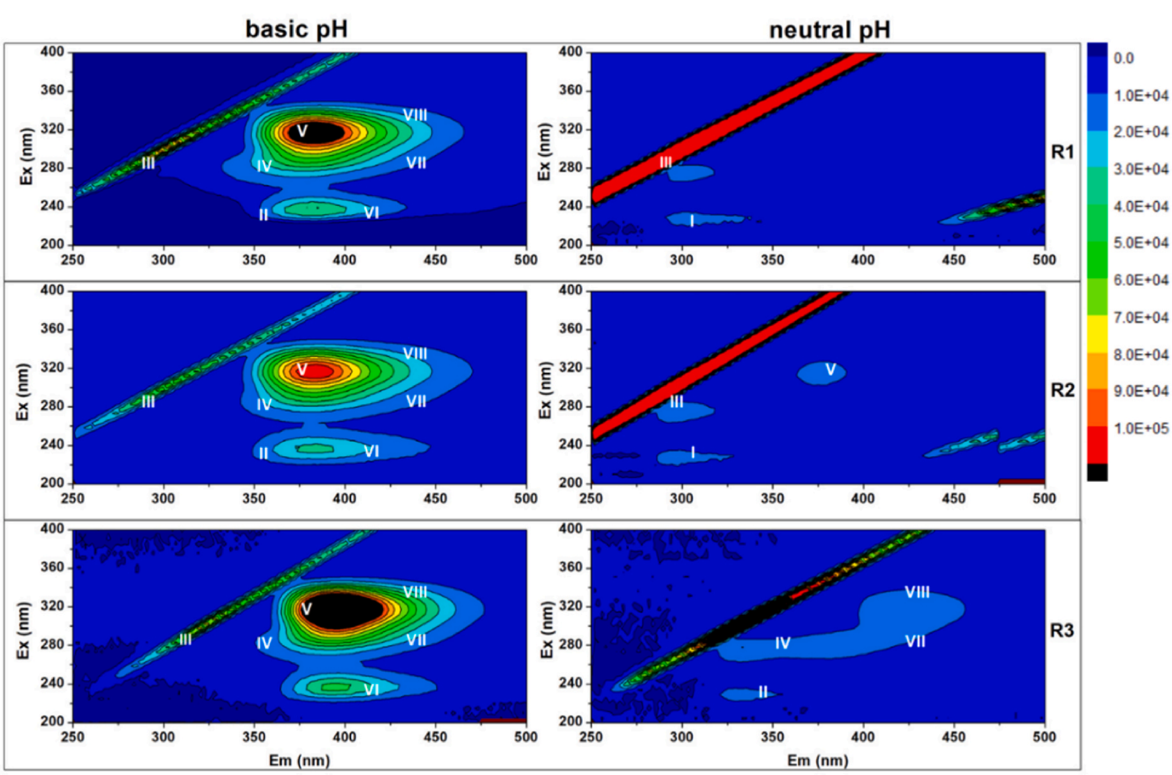

Fig. 4. Fluorescence spectra of EPS emission and excitation matrix extracted from mature aerobic granules at basic pH and neutral pH. The X and Y axes represent the emission (Em) and excitation (Ex) spectra, respectively. Contour lines represent fluorescence intensity.

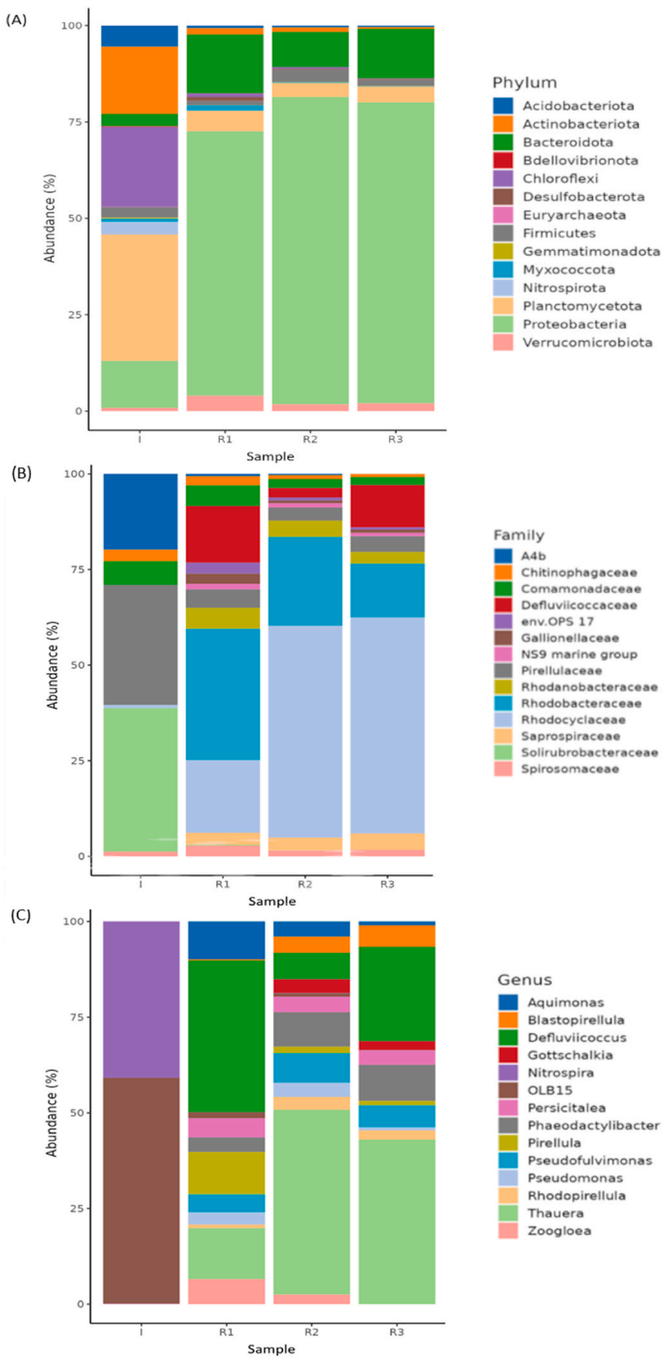

Fig. 5. Relative distribution of microbial diversity of inoculum sludge (I) and AGS cultivated in R1 (control), R2 (addition in alternating pulses of 2.5 g NaCl/L), and R3 (continuous addition of 2.5 g NaCl/L), distributed at the level of phylum (A), family (B) and genus (C), with relative abundance ≥1.

also reported a reduction in these phyla with increasing salinity due to their vulnerability to osmotic pressure. According to Xu et al. (2023), bacteria belonging to the phylum *Myxococcota* are responsible for significantly increasing EPS production.

The *Rhodobacteraceae* and *Rhodocyclaceae* families were predominant in the AGS samples and are related to EPS production and granule development, possibly stimulating the production of signaling molecules such as cyclic di-guanosine monophosphate (c-di-GMP) (Han et al., 2022). In reactors with the presence of salinity, the *Rhodocyclaceae* family presented a higher proportion, representing 36.4% (R2) and 39.4% (R3), in contrast to 11.7% in R1. The *Rhodobacteraceae* family showed a reduction in its abundance in reactors with salinity and was more abundant in reactor R1 (21.1%). Such observations indicate a differentiated adaptation of bacterial families to saline conditions, suggesting that *Rhodocyclaceae* were favored by salinity while *Rhodobacteraceae* were negatively affected.

Defluviicoccus was identified in abundance at the genus level in R1, while in the R2 and R3 reactors, *Thauera* was the predominant genus. The study by Meng et al. (2019) reported that the presence of *Thauera* increased as salinity concentration increased. Reports indicate that these microorganisms can produce and secrete EPS (Yu et al., 2020; Pronk et al., 2017) and improve denitrification efficiency (Liu et al., 2013). It is worth mentioning that, in addition to the genus *Thauera*, saline environments in reactors R2 and R3 stimulated the growth of *Blastopirellula*, *Phaeodactylibacter*, and *Rodhopirellula*, which belong to genera of marine bacteria.

According to Hay et al. (2014), the genera *Pseudomonas* and *Azotobacter vinelandii* of the phylum *Proteobacteria* are recognized for producing ALE. However, in this study, only *Pseudomonas* was detected, suggesting that other bacteria also produce ALE. Frutuoso et al. (2023a) indicated that genera such as *Pirellula*, *Thauera*, *Pseudofulvimonas*, and *Rodhopirellula* possibly contributed to greater ALE production in the AGS systems studied. Therefore, the presence of these genera in the R1, R2, and R3 samples suggests a probable relationship with ALE production.

Fig. 6 presents the functional groups involved in removing carbon and nutrients at the family level, separated into ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), denitrifying bacteria (DNB), glycogen-accumulating organisms (GAOs), and phosphorus-accumulating organisms (PAOs). An abundance of AOB, NOB, DNB, GAOs, and PAOs is generally observed after the granulation process, which suggests that granulation creates favorable conditions for them to grow.

Concerning nitrifying and denitrifying bacteria, a greater diversity of NOB and DNB was identified in reactor R1, suggesting that the growth of some microorganisms was inhibited by salinity.

A greater abundance of AOB, NOB, and DNB was also observed in reactors supplemented with salt, although nitrifying bacteria are more sensitive to the environment. A similar result was found by Frutuoso et al. (2023b) while studying the AGS performance, stability, resource production, and microbiological dynamics during the continuous addition of salt, in which an increase in the abundance of AOB and NOB was observed between concentrations of 2.5 and 10 g/L of NaCl. Despite these differences between microbial populations, the efficiency in nitrogen removal was similar among the systems studied (63–69%). Therefore, it is likely that some bacteria are inactive, not contributing to the removal processes.

Some studies have established a positive link between ALE production and PAOs (Schambeck et al., 2020; Meng et al., 2019); however, more in-depth investigations are required to evaluate this relationship. The abundance of PAOs was similar in the three reactors; however, a predominance of GAOs over PAOs is noted. Competition for substrate between these two groups disfavors phosphorus removal (Barros et al., 2020). Therefore, the analysis of bacterial communities reinforces that the low efficiency in removing phosphorus resulted from the competitive advantage of GAOs to PAOs in obtaining substrate.

Among the families of bacteria that play essential roles in the removal of carbon and nutrients, the *Rhodocyclaceae* and *Rhodobacteraceae* families of proteobacteria stand out, which were detected in greater abundance in the three reactors, corroborating observations from previous studies (Frutuoso et al., 2023a; dos Santos et al., 2022). The *Rhodobacteraceae* and *Rhodocyclaceae* families play an important role in the degradation of organic matter, with *Rhodobacteraceae* being the dominant microorganisms in the one-step anaerobic process, with the ability to accumulate phosphorus during denitrification (Zheng et al., 2016).

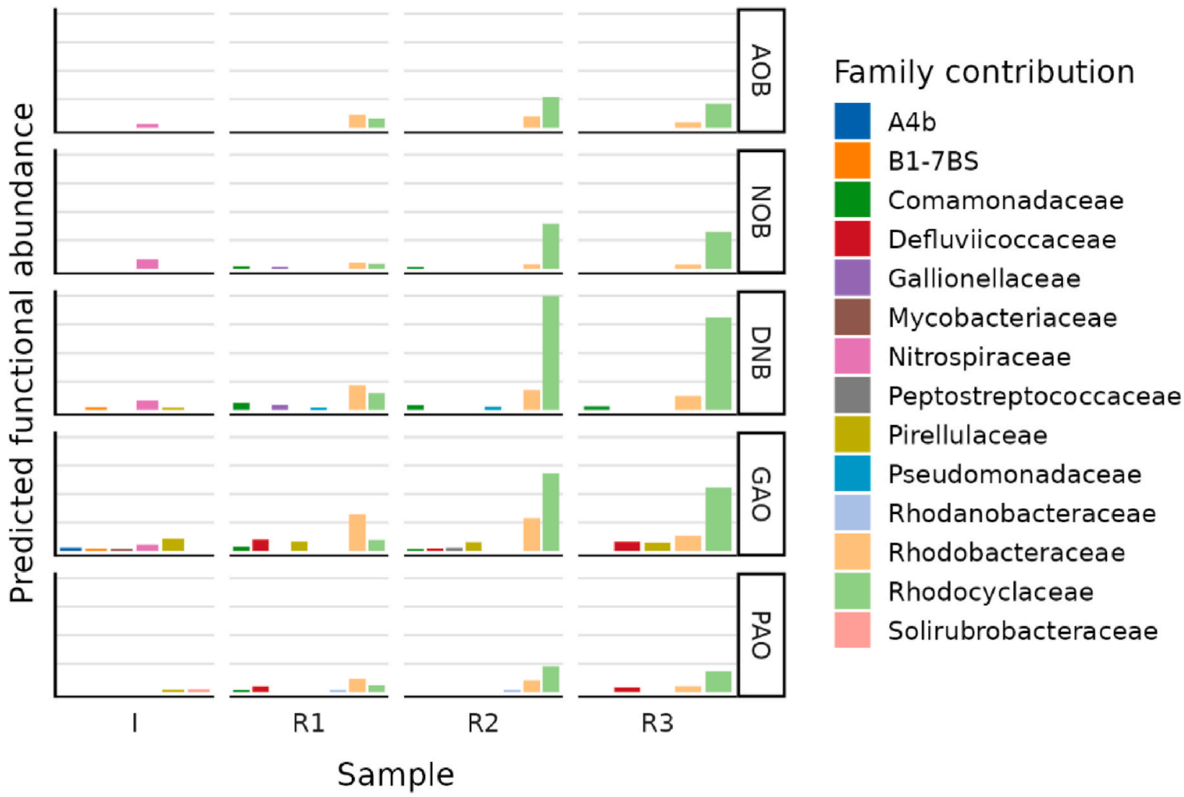

Fig. 6. Functional groups identified at the family level in the inoculum and experimental systems, with absolute abundance of functions ≥10.

4. Conclusions

The reactors adding 2.5 g NaCl/L in alternating pulses (R2) or continuous saline dosage (R3) showed higher concentrations of mixed liquor volatile suspended solids and better granule settleability. These reactors also had a faster granulation, especially R2, thus confirming the positive effect of adding salt through alternating pulses.

Salt addition did not favor greater EPS and ALE productions, possibly due to the low concentration of salt used. Endogenous EPS and ALE consumption due to the lower F/M ratio and SRT in R2 and R3 also contributed to this result.

Finally, 2.5 g NaCl/L did not interfere with the reactors' performance in terms of simultaneous C, N and P removals in AGS systems operated as SBR. In fact, the reactor with intermittent osmotic pressure (R2) showed better carbon removals.

Therefore, this study brings important results that contribute to a better understanding of the effect of low salt concentrations, either with continuous dosing or in pulses, as a selection pressure strategy to accelerate granulation, as well as the behavior of the AGS system for saline effluents.

CRediT authorship contribution statement

Francisca Denise Pereira Almeida: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Clara Bandeira de Carvalho:** Writing – review & editing, Writing – original draft, Investigation. **Antonio Ricardo Mendes Barros:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Francisca Kamila Amancio Frutuoso:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **André Bezerra dos Santos:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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.

Data availability

No data was used for the research described in the article.

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