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Aerobic granulation and bioresource production under intermittent saline stress

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ABSTRACT

Aerobic Granular Sludge (AGS) systems have gained prominence for liquid waste treatment and bioresource recovery. In this regard, various strategies are employed to accelerate granulation processes in sequencing batch reactors (SBRs) and maximize stability and bioresource production. Intermittent stress supplementation can facilitate granule development, promoting slow-growth microorganisms. At the same time, osmotic stress may enhance bioresource production. In this study, an AGS reactor control with no saline stress (R1) was compared to two AGS systems operated under constant (R2) and intermittent (R3) saline stress in terms of efficiency, stability and bioresource productions (alginate-like exopolysaccharides-ALE and tryptophan). Granulation occurred around day 59 and 21 in R2 and R3, respectively, while complete granulation was not achieved in R1 within the 80-day operational period. However, SBRs performed well after 45 days, with 70% of the biomass being granular. The production of the biopolymer ALE remained more stable in reactors with osmotic stress compared to R1. The microbiological analysis revealed specific responses and adaptations to environmental factors, with genera such as *Thauera* and *Pseudofulvimonas* showing higher abundance in reactors with complete granulation, indicating a positive correlation with saline conditions (5 g/L). These insights are crucial for optimizing the performance of AGS systems in future applications, emphasizing the benefits of intermittent saline stress in promoting rapid granulation, stability in bioresource production, contaminant removal, and robust granule formation.

1. Introduction

In recent years, wastewater treatment plants have been considered potential resource factories [34,2]. Not only is water a valuable resource due to water scarcity, but there is also the possibility of recovering nutrients (nitrogen and phosphorus), biosolids, energy, and other bioresources with high added value, such as alginate-like exopolymers (ALE), tryptophan (TRY), bioplastics, among others [64].

Biopolymers recovery from extracellular polymeric substances (EPS) has gained potential with the development of Aerobic Granular Sludge (AGS) systems. Compared to traditional activated sludge processes, this technology has shown considerable benefits such as increased capability to eliminate carbon, nitrogen, and phosphorus concurrently, and higher biopolymer production [49]; [55]; [58]; [59]. Furthermore, a reduction in ecological footprint and energy costs has been reported. Bio-ALE recovery at Nereda® plants in the Netherlands demonstrated gains associated with operating costs (OPEX), with a 20–30% reduction in biomass produced, lower energy consumption, and lower CO_2 emissions [33].

Biopolymer production is closely linked to the system's stability, successful granulation, and controlled sources of stress. The EPS matrix, crucial for the immobilization and aggregation of cells that form granular biomass, is a complex material consisting mainly of polysaccharides, lipids, nucleic acids, glycoproteins, and humic substances, essential for cellular metabolic processes [12,34,2].

A stress source is very important to form aerobic granules [22]. However, the stress must be controlled not to compromise system stability, which has been reported as a challenge in AGS systems [39]; [49]; [55]. For this reason, various strategies have already been adopted to improve AGS system stability, accelerate the granulation process, and enhance bioresource production, such as: (1) addition of divalent or trivalent cations; (2) supplementation with powdered dry sludge; (3)

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step-feeding; and (4) cultivation of AGS in saline medium [16,36,14,25,26].

In general, the presence of salinity in biological wastewater treatment systems can be unfavorable to the process, as it can lead to high osmotic pressure, resulting in cellular dehydration, plasmolysis, and release of intracellular constituents [27,63]. However, one of the advantages of AGS technology is the production of more resistant granules, in which microbial strains use adversities as a selection and survival mechanism, tolerating extreme environmental conditions (including NaCl). Wang et al. [67] pointed out that salinity creates greater resistance to precipitation due to higher buoyancy forces, contributing to the selection of microorganisms in the inoculum sludge with better sedimentation capacity.

Thus, salt stress does not appear to negatively affect the physical characteristics of aerobic granules. However, AGS system efficiency relies on the microbial composition, and the literature does not demonstrate a clear picture. For instance, Pronk et al. [54] found that ammonia oxidation was not affected at a salinity level of 20 g/L. On the other hand, He et al. [29] found that the phosphorus removal process collapsed at the same salinity level. Finally, Tang et al. [62] showed that AGS could be successfully cultivated in a hypersaline environment under different organic loading rates (OLRs) of 2.4, 3.6, 4.8, and 7.2 kg COD/m^3·d.

Simultaneously with these processes, when aerobic granular sludge is exposed to high osmotic pressure, the granules can resist salt stress by secreting relatively larger amounts of EPS [10]. The change in salinity prolongs the microbial endogenous respiratory period, subsequently leading to intracellular material expulsion and secretion of a large amount of EPS to relieve the osmotic pressure in cells [45,10].

Tang et al. [63] and Niu et al. [51] also point out that the highest EPS content corresponds to proteins (PN), which act as a buffer layer to strengthen granule stability by reducing the negative charge on the cell surface and decreasing the electrostatic repulsion between cells. Thus, bioresource recovery becomes promising. Sui et al. [61] obtained stable ALE content, with an average value of 50.8 ± 1.7 mg/gVSS and a positive correlation with the sedimentability and diameter of aerobic granules. Chen et al. [10] also reported that EPS accumulation in saline conditions favored tryptophan synthesis and coincided with improved pollutant removal efficiency.

However, most relevant studies found in the literature have only discussed the impact of saline conditions on mature aerobic granules without investigating the advantages that saline stress can bring to the technology, especially during the granulation process and bioresource production. For example, considering the long-term salinity assimilation strategy, it would be more advantageous to cultivate the aerobic granules directly with saline wastewater. This intermittent supplementation would create conditions for the gradual adaptation of microorganisms to the osmotic pressure caused by salinity and would weaken the adverse effects of salinity on the microbial composition. Furthermore, another strategy of adding salt in alternate cycles could favor bioresource recovery by creating a high-stress condition that would stimulate the production of EPS while making the granules more resistant and tolerant to salt shocks.

Therefore, saline conditions can easily impact the granulation process, treated effluent quality, and bioresources production by altering EPS secretion, functional enzymes, and other relevant substances. To understand the effect of salt in these scenarios, this work sought to evaluate the engineering and microbiological aspects of AGS systems subjected to intermittent and constant saline stress compared to a conventional control reactor.

2. Materials and methods

2.1. Experimental system configuration

Three sequential batch reactors (SBRs), measuring 1 m in height and 100 mm in diameter with a useful volume of 7.6 liters and constructed from acrylic, were utilized to study the effects of saline stress. The operational setups were as follows: R1, the control system, operated without any saline addition; R2, subjected to continuous saline stress with the feed solution consistently prepared at a concentration of 5 g/L; and R3, which experienced intermittent saline stress. In R3, the feeding alternated between the control solution and a solution containing 5 g/L of salt. Specifically, within a single day, four feedings were conducted - two without salt and two with salt, in an alternating pattern. This pattern of intermittent feeding was maintained throughout the operational period.

The operational cycle comprised sequential phases: initially, a 120-minute anaerobic period, followed by an aerobic phase lasting between 220 and 225 minutes. This was succeeded by a non-aeration period of 0–10 minutes, aimed to induce an anoxic period, akin to the approach of Rollemberg et al. [58], who found that such a period - constituting approximately 2.5% of the total cycle - was conducive to enhanced production of ALE and TRY. Furthermore, according to Silva et al. (2023a), a short anoxic phase at the end of the cycle favors denitrification, in which intracellular polyhydroxyalkanoate (PHA) can be used as an electron donor to support denitrification in this phase. The settling time was adjusted from 20 minutes down to 5 minutes during the initial three weeks of operation, with the reduced time being reallocated to extend the anoxic period (non-aeration) initially and, subsequently, the aerobic phase. This is concluded with a discharge in less than 1 minute, which cumulatively results in a 6-hour cycle.

Mixing of the sludge during the anaerobic period was achieved through 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, resulting in a gas superficial velocity of 2.1 cm/s.

The volumetric exchange adopted was 50%, the organic loading rate (OLR) applied was 2.0 g/L/day, and the reactors were operated at local ambient temperature (28±2ºC). The solids retention time (SRT) was determined based on Frutuoso et al. [26]. However, it is important to note that intentional sludge discharges were not employed to control the SRT.

2.2. Inoculum sludge and synthetic influent

The inoculum sludge was collected from a domestic wastewater treatment plant of an aerated biofilter system located in Fortaleza, Ceará, Brazil. Around 3.8 liters of sludge with a concentration of approximately 5.0 g/L and a sludge volume index at 30 minutes (SVI_{30}) of 368 mL/g were used in each reactor during start-up.

The systems were fed with propionate as a carbon source (1000 mgCOD/L), which was considered favorable for granule stability and bioresource production [22]. The feeding solution included a basal medium with macro and micronutrients, buffered according to dos Santos et al. [22]. Moreover, a C:N ratio of 20 was maintained, known to enhance ALE production, as identified by Rollemberg et al. [58]. All reagents in this study were used in their original state without further purification.

In the study of saline systems, a salt concentration of 5 g/L NaCl was strategically chosen for the feed solution to optimize granule stability and bioresource production efficiency, following the findings of Frutuoso et al. [26]. The R3 system was subjected to intermittent osmotic stress by alternating its feed between the control system (R1) and the continuously stressed system (R2), effectively interspersing cycles of osmotic stress with stress-free periods. This research aligns with broader findings, such as those reported by Panagopoulos [52], noting that saline concentrations in wastewater can range widely from 0.5 to 150 g/L. A concentration of around 5 g/L is particularly common in co-treatment systems or mixed effluents. For instance, cities like Hong Kong and Qingdao in China utilize seawater for flushing toilets, thereby elevating the salinity levels of municipal waste [37].

2.3. Physicochemical analysis

The reactor's operational efficiency was evaluated by analyzing the removal of organic matter and nutrients in terms of Chemical Oxygen Demand (COD), ammoniacal nitrogen (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), and phosphate (PO_4^{3-}-P). In addition, dissolved oxygen (DO) levels (maintained between 4 and 6 mg/L) and pH in the mixed liquor were monitored using a YSI Pro1020 probe (YSI Pro1020, YSI Inc., USA). All analyses were performed three times per week, according to the Standard Methods for the Examination of Water and Wastewater [1].

The biomass assessment covered a range of parameters, including mixed liquor volatile suspended solids (MLVSS), mixed liquor total suspended solids (MLTSS), sludge volume index in 5 min (SVI_5), and SVI in 30 min (SVI_{30}) [1]. Granule size distribution analysis utilized a granulometry method with four sieves of different openings (0.2 mm, 0.6 mm, 1.0 mm, and 2.0 mm), as described by Bin et al. [5]. EPS, ALE, and TRY were also evaluated.

The structure of mature granules was analyzed by scanning electron microscopy (SEM) combined with energy-dispersive X-ray spectroscopy (Inspect S50, FEI Company, USA). The pre-treatment involved fixation, washing, and lyophilization, following the methodology described by Motteran et al. [48].

The EPS was fractionated into loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS). The extraction of both fractions was carried out following the method by Hong et al. [32]. Protein content (PN) was determined using the modified Lowry method [42], while the polysaccharide content (PS) was assessed using the sulfuric acid-phenol method [23]. Furthermore, the analysis of EPS composition included an assessment of the EPS extract's fluorescent properties, as per Liu & Cinquepalmi [40], with adaptations outlined in dos Santos et al. [22].

Previously documented methods for extracting and quantifying ALE were adopted from prior studies [38,24] and adapted according to dos Santos et al. [22]. The extraction of TRY was the same as that conducted for EPS, considering the total TRY content encompassed both LB-EPS and TB-EPS fractions, and the quantification process followed the instructions previously described by dos Santos et al. [22].

2.4. Microbiological analyses

Molecular biology analyses were performed to characterize the microorganisms present in the inoculum sludge and mixed liquor from SBRs (R1, R2, and R3). This involved DNA extraction, sequencing of the 16 S rRNA gene, and subsequent data processing. The collection and DNA extraction were conducted in the same manner as described by dos Santos et al. [22]. Sequencing and data processing were conducted as described by da Silva et al. [17]. After sequencing, raw sequence data were archived in the National Center for Biotechnology Information (NCBI) BioProject database, ID PRJNA836880.

2.5. Statistical analysis

The Kruskal-Wallis and Mann-Whitney tests were employed to compare the reactors' performance with the assessment based on the resultant p-values. A p-value of ≤ 0.05 indicated the rejection of the null hypothesis and statistical disparity between the data groups. Additionally, the Pearson test was used to evaluate the interrelationships among bioresource production parameters. In other words, the relationships between ALE and EPS, TRY and EPS, and ALE and SVI_{30} were assessed. These correlations were classified as weak ($0.3 < r < 0.5$), moderate ($0.5 < r < 0.7$), and strong ($r > 0.7$), following the standard classification found in the literature [19].

3. Results and discussion

3.1. Formation and stability of aerobic granules

The SBRs were inoculated with biomass removed from a secondary settling tank of a submerged aerated biofilter system, which had an SVI_{30} of 368 mL/g. In each reactor, 50% of the useful volume of MLVSS biomass was inoculated at a concentration of 5.0 g/L. In all systems, most of this biomass was washed out shortly after the start due to poor biomass settleability, as shown in Fig. 1. Similar observations have been made in other studies [14,18,22,25,58]. Chen et al. [10] also found that NaCl addition acted as a selective pressure, eliminating poorer quality sludge and resulting in an initial reduction in MLSS followed by a biomass increase.

The criteria used for successful granulation are when over 80% of the biomass is larger than 0.2 mm, the SVI_{30}/SVI_5 ratio is greater than 80%, and SVI_{30} is less than 80 mL/g, values well-documented in the literature [16,25,12]. In this regard, in R1, a system operated without salt addition, complete granulation was not observed within the 80-day operation. However, the stability was achieved after 45 days, resulting in MLVSS of 2.6 g/L (±0.13), MLTSS of 2.9 g/L (±0.14), and a decreasing trend in SVI_{30}, reaching approximate values of 70 mL/g (±9). However, only 70% of the granules measured more than 0.2 mm, and the SVI_{30}/SVI_5 ratio was also around 70%, although the SVI_{30}/SVI_{10} ratio showed values higher than 80%.

The delayed granulation in R1 is likely a consequence of the inoculum used. The literature reports that the granulation time in AGS systems will depend on the inoculum quality and the selection pressures imposed in the new environment [53]. Additionally, other similar operations achieved complete granulation around 30 days using activated sludge biomass [22,25,26], commonly used in aerobic granulation due to its ease of obtainment, high density, and microbiological diversity [53].

On the other hand, in R2, the system with constant saline addition, complete granulation was achieved around day 59, with 83% of granules larger than 0.2 mm, SVI_{30} of 55 mL/g (±13), and an SVI_{30}/SVI_5 ratio close to 100%. Although providing salt in the environment can help microbial consortia adapt to saline conditions, a higher start-up period may be required to achieve complete granulation. For instance, when analyzing the SVI_{30}/SVI_5 ratio in the work of Carrera et al. [6] on a pilot scale under saline conditions, granulation was only achieved from day 80 onwards.

The concentration of solids reached values around 5.3 g/L of MLVSS and 6.7 g/L of MLTSS at the end of the operation. The rapid increase in biomass, especially in inorganic materials, is possibly due to the saline addition, similar to what was observed by Meng et al. [47] and Frutuoso et al. [26]. In contrast to R1, observations in R2 suggest osmotic pressure contributed to granulation, as the system achieved complete granulation, albeit delayed (59 days). This is consistent with the findings of [25,26], which indicated that low saline concentrations, up to 7.5 g/L, can help reduce electronegativity present on the cell surface, promoting granulation and system stability.

Also similar to that indicated by the anaerobic granulation literature, the following mechanism has been proposed [43]: at low levels of salinity, the negatively charged sludge granules show electrostatic repulsion, making it difficult to aggregate, and in this phase, the granule formation primarily relies on EPS acting as a bridge between granules. However, with increasing salinity, the biggest concentration of Na^+ ions compress the colloid electric bilayer, neutralizing the electrical charge of the granular sludge. When electrostatic repulsion becomes weaker than the van der Waals attraction, the repulsion barrier disappears, allowing aggregation. The process heavily relies on electro-neutralization and EPS bonding, playing key roles in facilitating granulation.

The best results in terms of granulation were observed in R3, i.e., the system operated under alternating saline stress, reaching the stage of

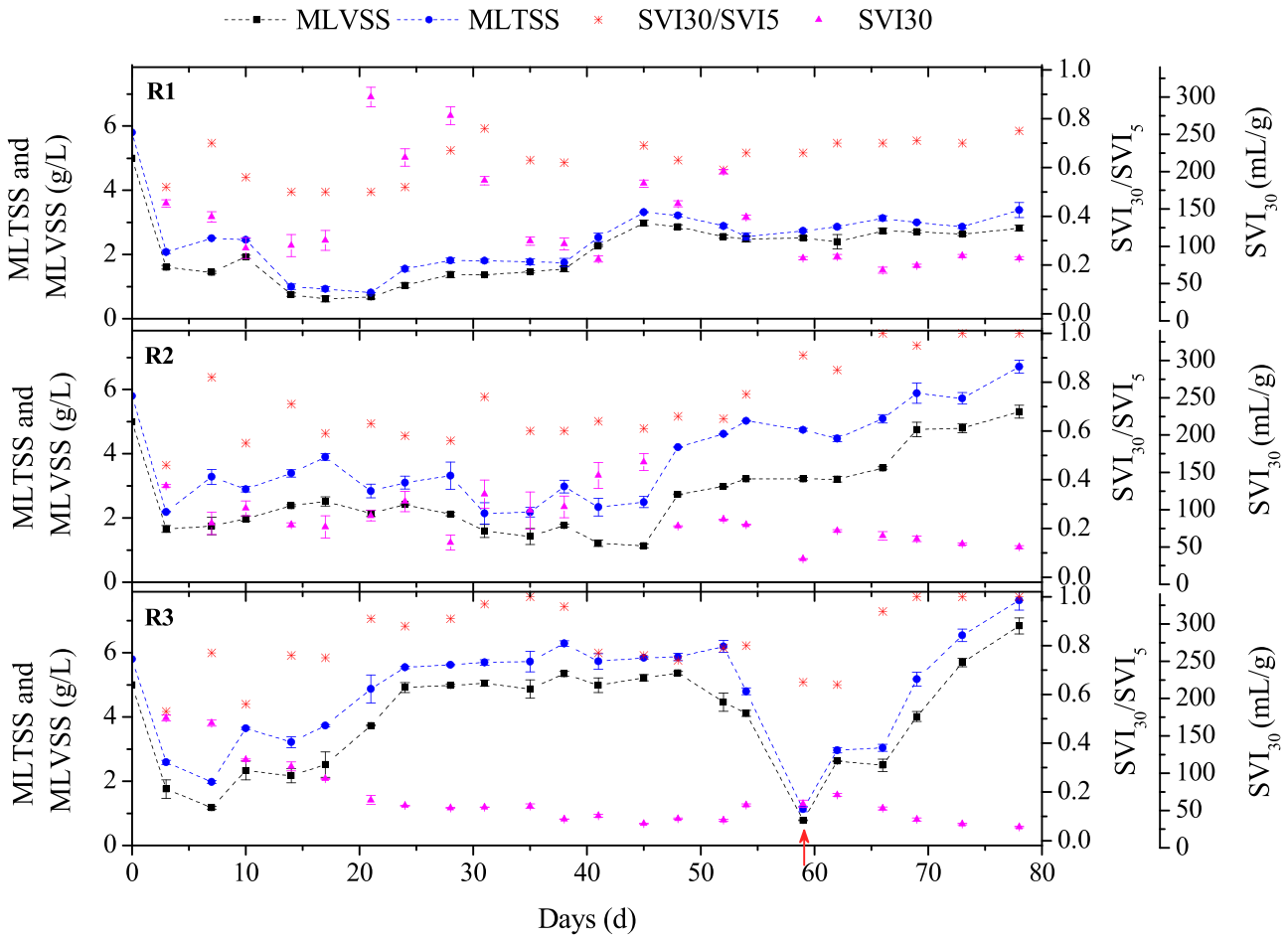

Fig. 1. Mixed liquor volatile suspended solids (MLVSS) and total suspended solids (MLTSS), SVI_{30}/SVI_{5} ratio, and SVI_{30} in the three reactors over 80 days of operation.

complete granulation around day 21. At this point, the system already had 91% of granules larger than 0.2 mm, an SVI_{30}/SVI_5 ratio of 91%, and an SVI_{30} of 58 mL/g (±6), reaching even lower SVI_{30} values (< 40 mL/g). Solids accumulation remained stable between days 25 and 55, with 5.0 (±0.4) g/L of MLVSS and 5.8 (±0.5) g/L of MLTSS. Around day 55, operational problems due to aeration disturbances caused severe biomass washout, resulting in granule breakage and compromising the system's good settleability, as can be observed and indicated by a red arrow in Fig. 1. However, shortly after, the system experienced a rapid recovery.

In general, in both R2 and R3, salt addition favored the granulation process. However, the greater stress caused in R3 by salt addition in alternate cycles caused a microbial selection and played a crucial role in ensuring that complete granulation was achieved in shorter periods. Under saline stress, the microbiota produces adaptation mechanisms, such as increased EPS production. In the subsequent cycle without saline stress, granule stability may be favored.

Moreover, alternating conditions allow for dilution of the saline load, as evidenced by the lower accumulation of inorganic materials in R3. Fig. 1 shows a noticeable difference between MLTSS and MLVSS in R2, followed by R3 and R1. Similarly, Chen et al. [8] and Niu et al. [50] observed improved performance in UASB system granules when subjected to alternating stimuli. The former authors worked on granule cultivation with intermittent Mg^{2+} supplementation, and the latter studied semi-starvation fluctuating C/N ratios.

It is interesting to note that in all three reactors, a strong positive Pearson correlation was observed between SVI_{30} data and the food to microorganisms (F/M) ratio in R2 ($r = 0.81$), and a positive and moderate correlation in R1 ($r = 0.69$) and R3 ($r = 0.56$), indicating that F/M is an important parameter for granulation control and maintaining good stability of AGS systems. Wang et al. [68] demonstrated good treatment efficiency and good sedimentation when the AGS systems operated under F/M conditions of < 1.38 kgCOD/(kgMLVSS·d). In this study, R1 exhibited values higher than this range, around 35–40 initial days, due to the high biomass loss.

Regarding the SRT, R1 maintained low values throughout the operational period, around 9 (±2) days. In contrast, R2 reached 18 (±2) days during the maturation phase, and R3 reached 19 (±5) days after 30-day operation period, although with a decline after day 55 (4 ±2 days).

Granule morphology showed that around 47-day operation period, only R3 presented well-formed granules with well-defined spherical structures. R2 exhibited transparent spheres resembling a gelatinous material, while R1 displayed biomass with irregular morphology (Fig. 2). At the end of the operation (day 73), in R1, well-formed granules were visible, albeit still with filamentous biomass. In R2, a more brownish biomass was formed, with large and dense granules, although still irregular in shape. On the other hand, R3 still showed many well-rounded granules, but larger ones and some irregularly shaped granules were also observed. Importantly, despite their irregular shape at the end of the experiment, the granules in reactors R2 and R3 exhibited excellent settleability.

3.2. *Reactors' performance*

In Fig. 3, the performance of the systems over the 80-day operation period can be observed in detail. Regarding COD removal, the first 30

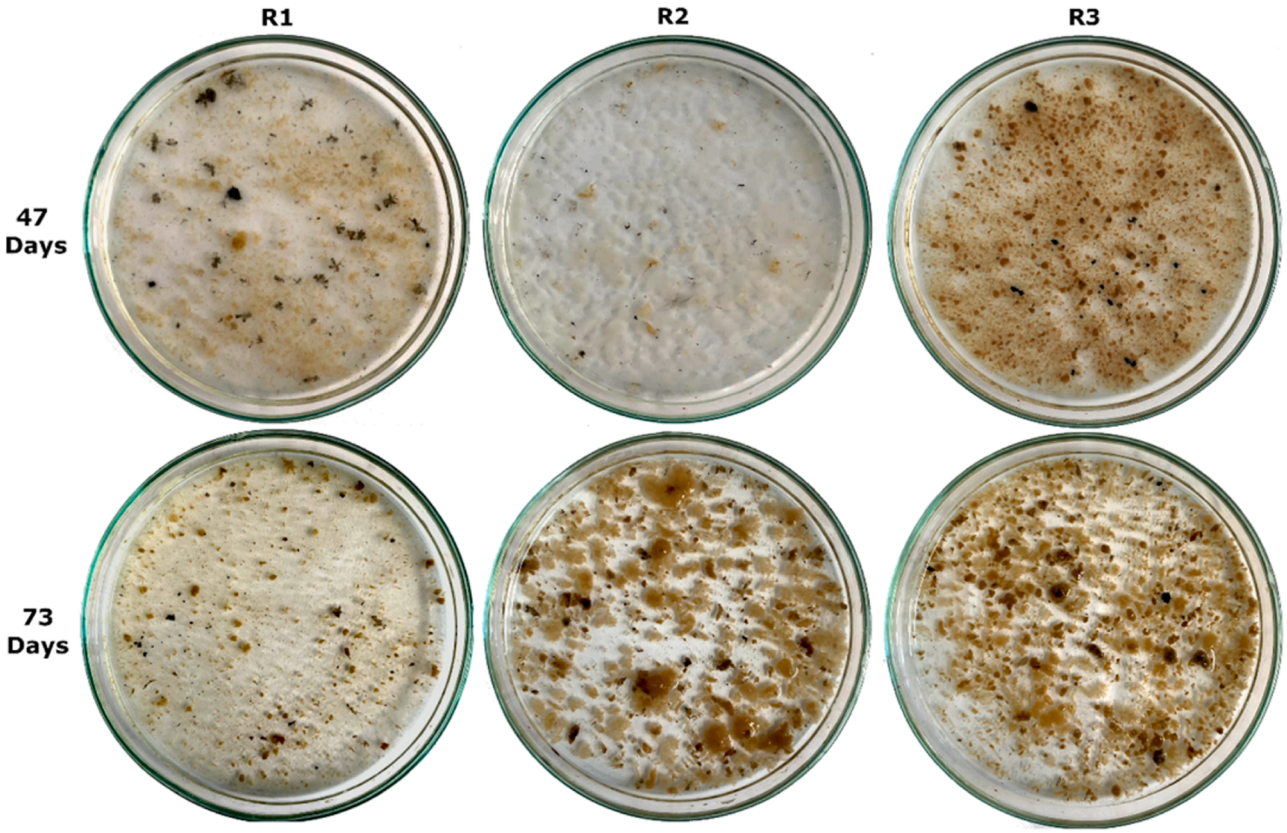

Fig. 2. Granule morphology in the middle and end of the 80-day operation.

days revealed no statistically significant differences in removal rates across the systems, with R1, R2, and R3 recording average efficiencies of approximately 89% (±5%), 91% (±7%), and 92% (±7%), respectively. This period exhibited considerable performance variability, likely due to the biomass adaptation phase. Stability in COD removal was observed in R1 from day 45, with an efficiency of around 96% (±3). Although R2's performance was generally similar to R1's, showing no significant difference ($p > 0.05$) across all data, a noticeable decline to 87% (±5%) was recorded upon granule maturation. Conversely, R3 achieved early stabilization and improved performance after 20 days, sustaining an efficiency rate of 96% (±3). These observations are in agreement with the findings from Santos et al. [22,25,26], Wang et al. [69], and Han et al. [28].

Nitrification efficiency is improved with granulation, stabilizing values after 45 days of operation in R1 and with maturation in R2 (59 days) and R3 (21 days). R3 showed a significantly higher average removal (93 ± 10%) compared to R1 ($p = 0.007$) (83 ± 16%) and R2 ($p = 0.0002$) (78 ± 17%). This likely occurred because the system exhibited better granulation and faster development, allowing it to maintain good efficiency from day 20 of operation, supporting the hypothesis that intermittent stimuli favor better granule development [8,50]. However, considering only the values after stabilization, no significant differences between the reactors were found, with values around 97% (±3), 95% (±3), and 99% (±1) in R1, R2, and R3, respectively.

Denitrification, on the other hand, failed to demonstrate a significant difference ($p > 0.05$) among the systems, with average removals of 67% (±8), 67% (±8), and 62% (±6) for R1, R2, and R3, respectively. Higher removals were not achieved due to the accumulation of nitrogen fractions (NOx). Shi et al. [60] also reported nitrite accumulations on the order of 20 mg/L when creating sodium-based saline conditions. At 3% salinity, from day 75 onwards, Meng et al. [46] also observed accumulations of nitrogenous fractions. Additionally, nitrite and nitrate accumulation (Fig. 3) is linked to the high rate of carbon source consumption, especially in R1, as evidenced by the cycle analysis (Fig. 4), where, after one hour of aeration, 95%, 77%, and 87% of COD had been consumed in R1, R2, and R3, respectively. In line with Silva et al. (2021), these findings suggest that accumulating polyhydroxyalkanoates (PHA) during a short COD depletion period in the aerobic phase may be insufficient for subsequent denitrification. Furthermore, the absence of anoxic conditions in all three systems could have compromised the Simultaneous Nitrification and Denitrification (SND) process. Literature suggests that anoxic conditions are typically induced in mixtures, keeping low levels of dissolved oxygen (DO), around 2–4 mg/L [21,35], a condition that was maintained only during the first hour of aeration, as shown in the cycle analysis.

This study is consistent with the findings of Frutuoso et al. [25] and Wang et al. [69], which demonstrated that the progressive adaptation of AGS to saline conditions or low saline concentrations allowed for stable and complete removal of carbon and nitrogen. Although the present study did not achieve complete nitrogen removal, the deficiency was not caused by osmotic pressure. In addition, studies have demonstrated that salinity higher than 1% could cause a severe negative effect on the activity of NOB, compromising full nitrification [73].

Interestingly, in R1, despite incomplete granulation, COD and nitrogen removal stability are evident after day 45, coinciding with stability in the system's solids concentration. This suggests that even without achieving complete granulation, 70% of the granules present in the system were sufficient to maintain good performance. In R3, a drop in COD, nitrification, and denitrification performance can be observed around day 59, which can be attributed to a biomass reduction during this period. However, a swift re-adaptation followed.

The phosphate removal throughout the experimental period was relatively low in all systems, with statistically equal efficiency ($p > 0.05$). Removal rates of 28% (±6), 25% (±4), and 25% (±6) were achieved in R1, R2, and R3, respectively. The limited phosphate removal

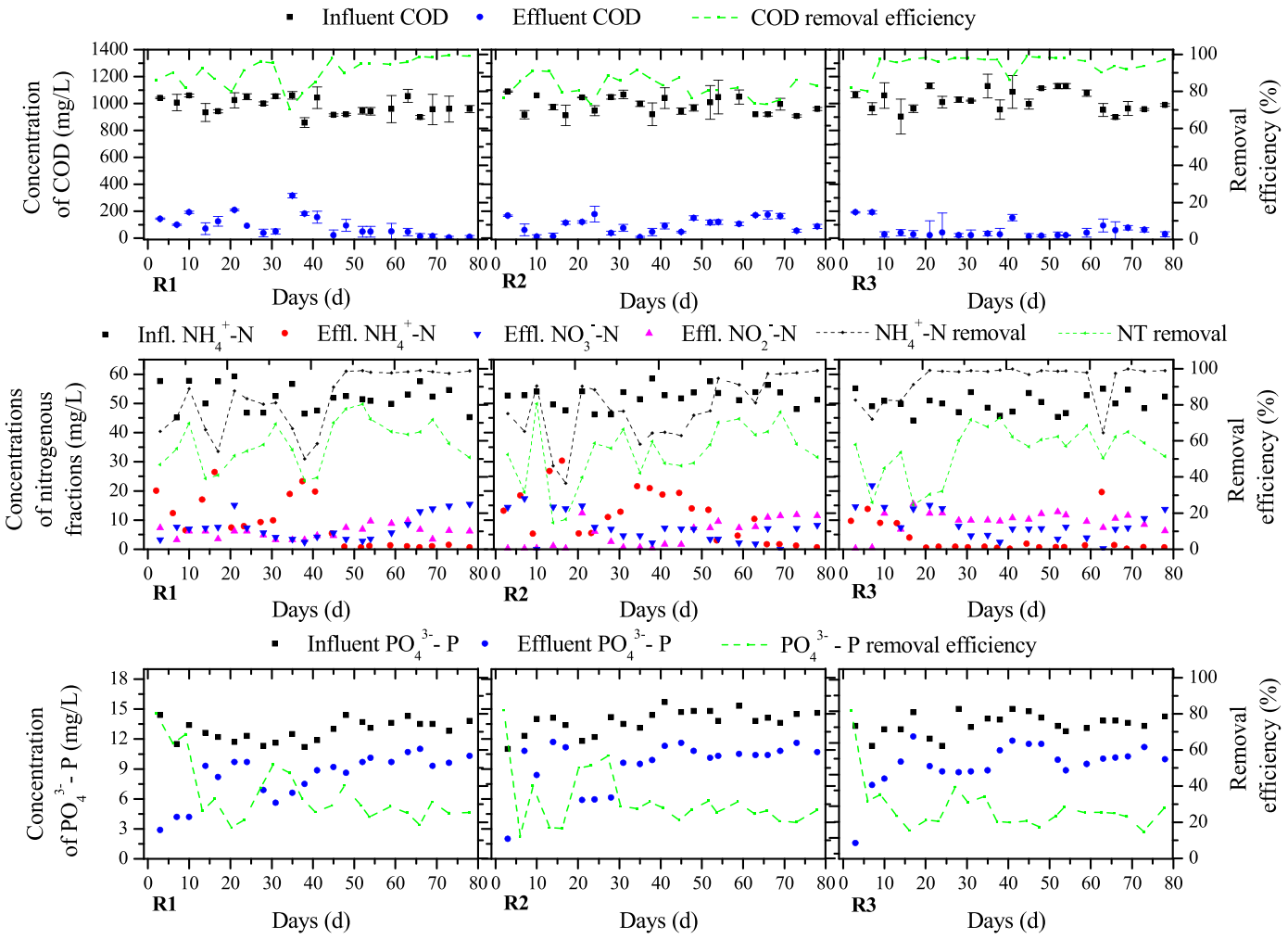

Fig. 3. Performance of the reactors over the 80 days of operation.

efficiency observed in the study can be attributed primarily to two mechanisms, which are consistent with the findings of Carvalheira et al. [7], Rollemberg et al. [57], Bassin et al. [4], and Liu et al. [41]. Firstly, nitrite and nitrate accumulation may have disrupted the phosphate release mechanism during the anaerobic phase by interfering with polyphosphate-accumulating organisms (PAOs) activity. Denitrifying bacteria, which can use nitrite and nitrate as final electron acceptors for denitrification, appear to outcompete PAOs, inhibiting their usual PHA accumulation and phosphate release processes. Secondly, the dominance of glycogen-accumulating organisms (GAOs), as demonstrated through molecular biology analyses, further exacerbates this issue by competitively disadvantaging PAOs in substrate utilization, leading to less efficient phosphate removal.

3.3. Cycle analysis

The consumption profile of organic matter and nutrients throughout an operational cycle has been examined and is depicted in Fig. 4. DO analysis during the cycle revealed that DO remained between 3.0 and 4.5 mg/L during the first 30 minutes of aeration in R1, while in R2, this value persisted for approximately two hours, and in R3, for about one hour. The higher DO demand in R2 and R3 could be associated with two possible reasons: (1) a higher amount of biomass in these systems, leading to higher endogenous activity and consequently a greater DO demand; (2) the presence of salt influenced the microbiota's metabolism, indicating that the degradation of organic matter was slower, thus maintaining the DO demand for degradation over a more extended period.

After this period, DO levels were maintained above 5 mg/L in all reactors, reaching values close to 7 mg/L. This suggests that ceasing aeration towards the cycle's conclusion was insufficient for diminishing DO levels to achieve a fully anoxic environment. These elevated DO levels occurred during the famine period - characterized by the absence of soluble COD, as previously reported [26]. It is during this phase that microorganisms enter an endogenous metabolic state, thus requiring lower DO concentrations for their metabolic activities.

A slower decline in COD was observed in R2 during the anaerobic period compared to other reactors, with a total removal at the end of this period of approximately 60%, while in R1 and R3, this removal was around 80%. After one hour of aeration, 95% of COD had already been removed in R1; however, only 77% in R2 and 87% in R3. These data agree with the oxygen demand in each system and indicate that the addition of salt possibly influenced the degradation rate of heterotrophic microorganisms; however, there was no detrimental effect on the overall efficiency of the systems.

Regarding ammonia removal, after 2 hours of the aerobic period, all ammonia had already been consumed in all systems. A similar behavior is also achieved regarding nitrite and nitrate accumulation. It is noted that reactors R1 and R3, at the end of the cycle, reached an accumulation of NO_3^- and NO_2^- of 15 (±2) mg-N/L and 6 (±1) mg-N/L, respectively. On the other hand, R2 presented a slightly higher accumulation of NO_2^- (14 ±1 mg-N/L) than NO_3^- (11 ±1 mg-N/L). This same pattern is observed in the systems' performance data in the last weeks of operation, with nitrite and nitrate accumulation. Despite the high COD/TN ratio, there was

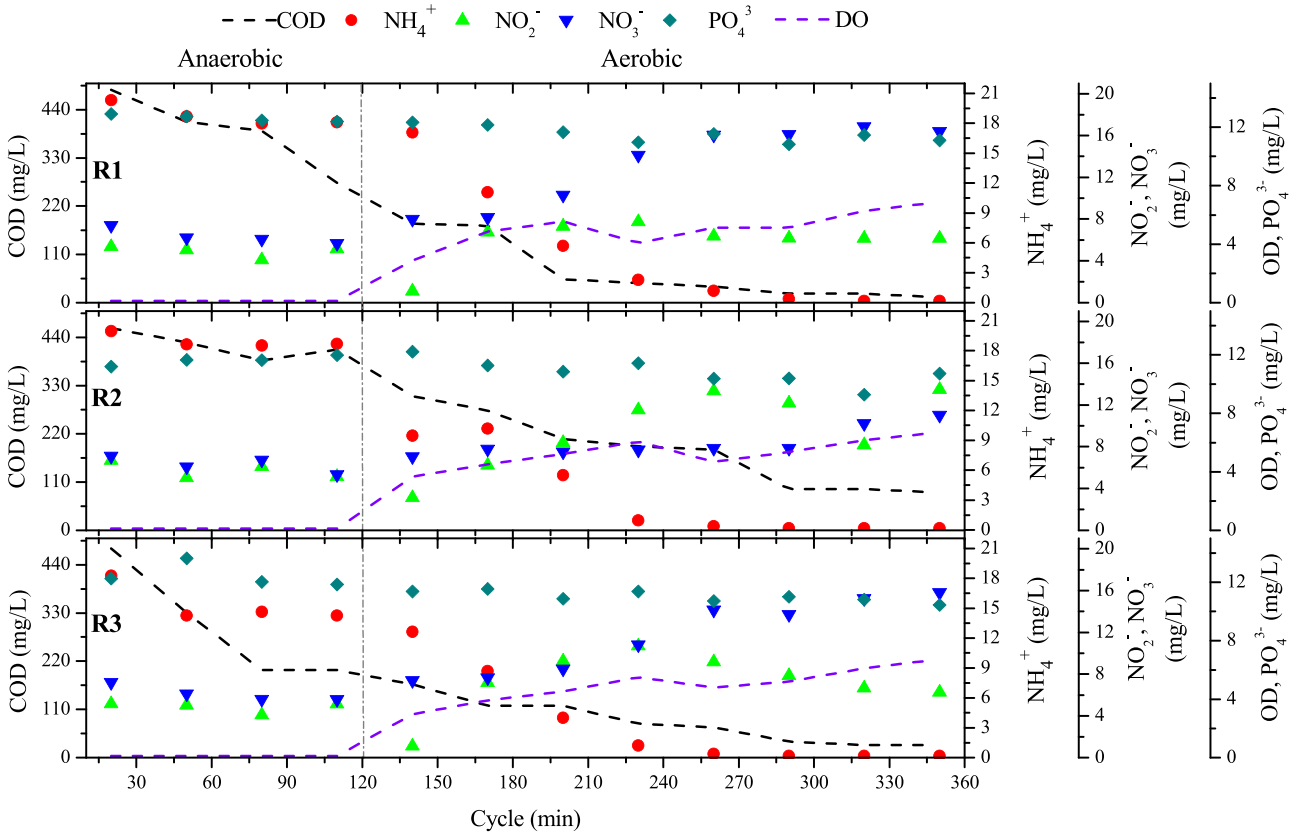

Fig. 4. Cycle analysis of the reactors R1 (no saline stress), R2 (constant stress), and R3 (intermittent saline stress) at the end of the operational period.

carbon consumption during the anaerobic phase, probably to reduce the accumulation of NOx from the previous cycle since the concentrations of these nitrogenous fractions were inconsistent in this phase. Thus, the remaining carbon was quickly consumed at the beginning of the aerobic period, becoming unavailable for complete denitrification to occur in the remainder of the cycle. During this short period of COD depletion in the aerobic phase, PHA accumulation is not sufficient to sustain the denitrification process (the phosphorus data allows us to infer this). Additionally, the available carbon is used for microbial growth and denitrification, resulting in partial and incomplete nitrogen removal.

No significant phosphorus removal was achieved in either system. This is probably due to two reasons: (1) During the anaerobic phase, the presence of NOx impaired the metabolism of PAOs since denitrifying bacteria outcompete PAOs. This fact is clearly visible in R2, where the presence of DNB is greater than that of PAOs. In the other reactors, despite PAOs being more dominant than DNBs, the results converge to believe that the DPAOs route was the preferred one; (2) According to microbiological analysis (see Section 3.5), the GAOs group was predominant to the PAOs group in all reactors, i.e., the major carbon flow was being directly to GAOs.

Cheng et al. [11] observed that influent concentrations exceeding 60 mg/L led to NOx accumulation, inhibiting PAOs' activity, in their investigation of various influent ammonia loads in an AGS system. This led to a reduction in the phosphorus removal rate to approximately 49.7%. Barros et al. [3] reported a similar behavior, relating the kinetic advantage of heterotrophic denitrifying microorganisms compared to PAOs. Da Silva et al. [16] also observed a comparable trend, although they did not explicitly discuss this behavior. Notably, as noted in their work, the systems with higher NOx accumulation did not exhibit a significant phosphate buildup.

Furthermore, the literature [57,4] has suggested that controlled sludge removal (bed or bottom) is crucial for removing PAO-saturated granules and improving phosphorus removal. However, controlled and selective biomass removal was not adopted for this study.

3.4. EPS and resources productions

The bioresources production (EPS, ALE, and TRY) over the 80 days of operation is shown in Fig. 5. Analysis through a Pearson correlation indicated that EPS and ALE share a moderate correlation, with coefficients of 0.4, 0.4, and 0.6 in reactors R1, R2, and R3, respectively, highlighting ALE as a major component of EPS, as reported by Carvalho et al. [20], Frutuoso et al. [26], and Bahgat et al. [2]. Research identifies ALE's unique property to form hydrogels in the presence of divalent cations like Ca^{2+} and Mg^{2+}, representing a key structural element of EPS ([24]; Zahra et al., 2022; [2]).

When considering the initial granulation days (~45 days), a significantly lower PS production ($p = 0.019$) was observed in R1. Literature has related [43] that certain bacteria tend to elevate their cellular and extracellular polysaccharide content in anaerobic granules under salt shock. This phenomenon is believed to be a defense mechanism, protecting bacteria against phagocytosis, predation, and stress. As expected, a significantly higher production ($p < 0.05$) of TB fractions compared to LB was noted, similar to Liu & Cinquepalmi [40]. Interestingly, the LB fraction in R2 was higher than in R3 (Table 1), possibly due to higher osmotic pressure. The literature [43] has reported that the LB-EPS fraction tends to increase with increasing salinity while the TB-EPS fraction decreases.

Although higher PN-TB production was observed in R3, followed by R2 and R1 (Table 1), the differences among the samples did not reach statistical significance. Similarly, no significant disparity was observed in overall EPS production among the reactors ($p = 0.43$). In general, fluctuations in osmotic pressure induced by elevated salinity levels may stimulate microbial cells to release EPS, enabling them to counteract these alterations [15,13,47,43]. However, several factors can influence its production, such as SRT, F/M ratio, inoculum sludge, and substrate

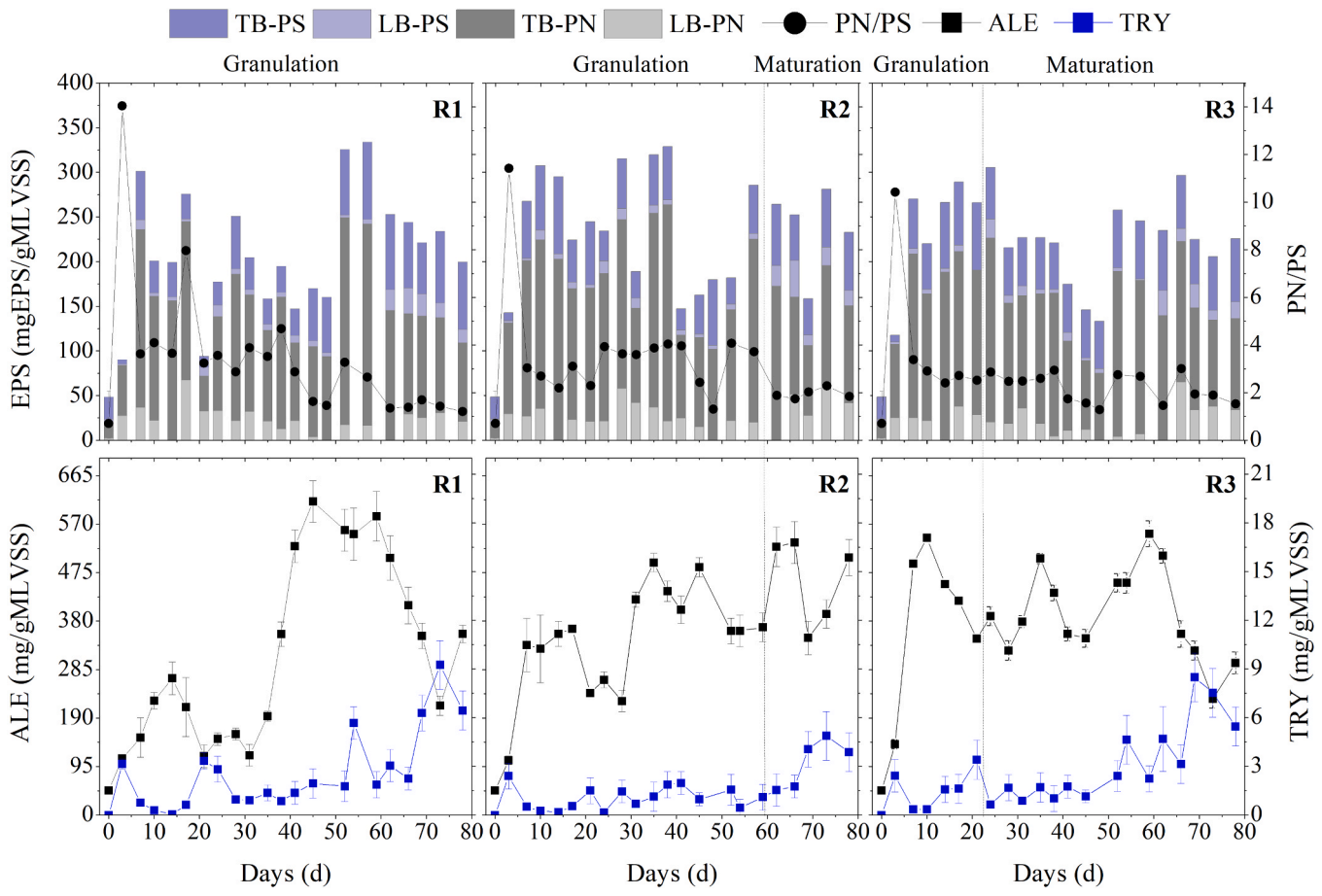

Fig. 5. EPS, ALE, and TRY production over the 80-day operational period.

Table 1
Average EPS production in the inoculum, R1, R2, and R3 during the granulation and maturation phases.

		PN (mg/gMLVSS)		PS (mg/gMLVSS)		PN/PS
		LB	TB	LB	TB	
Inoculum		2 (1)	18 (6)	3 (1)	25 (7)	0.7 (0.2)
Granulation	R1	28 (13)	129 (47)	6 (3)	34 (16)	4 (3)
	R2	24 (11)	157 (49)	6 (4)	55 (21)	3 (2)
	R3	25 (4)	168 (39)	5 (2)	63 (26)	3 (3)
Maturation	R1					
	R2	46 (14)	120 (35)	23 (11)	58 (12)	2 (1)
	R3	24 (7)	133 (36)	11 (9)	58 (7)	2 (1)

used [58,26,43]. According to the literature [58,26], higher SRT stimulates endogenous consumption of biopolymers, especially at SRTs exceeding 20 days. Although this study did not reach SRT values exceeding 20 days, SRT in R1 was significantly lower than in R2 and R3. Additionally, Wang et al. [68] demonstrated that EPS production is directly related to the F/M ratio. It was evident that in R1 (1.2 ± 0.7) and R2 (0.9 ± 0.4), the F/M ratio was significantly ($p < 0.05$) higher than in R3 (0.7 ± 0.5). Therefore, it is inferred that EPS production in R2 and R3 is lower due to the lower F/M ratio and higher SRT; however, osmotic pressure likely favored EPS production, compensating for endogenous consumption.

Fig. 6 shows the three-dimensional fluorescence spectrum of the emission-excitation matrix (3D-EEM) for samples from each reactor at different operational times. The literature [40,22,25] has described each spectrum region as a specific substance commonly found in the EPS matrix (Table 2).

Observations around day 7 of operation indicate that all three reactors exhibited similar characteristics with the production of tyrosine amino acid (A), tryptophan amino acid (B), tyrosine-containing proteins (C), tryptophan-containing proteins (D), polysaccharides (E) and fulvic acid-like substances (F). Predominantly, during this initial phase, the selective stressors were the major contributors, leading to an increase in these key substances crucial for granulation. Similar results were found by dos Santos et al. [22] and Frutuoso et al. [25]. However, effective and rapid granulation was only achieved in R3. As seen around day 35 of operation, it was the only system to maintain an intense production of all these substances.

At the end of the operation, around the 78th day, there is a recovery of diversity and intensity of substances in R1 and R2. In R3, an increase in intensity is observed, showing a particularly higher production of tyrosine and tryptophan proteins in R2 and R3. This aligns with the findings of Cui et al. [15] and [25,26], highlighting that systems under osmotic stress positively influence EPS production, particularly proteins.

Concerning ALE production (Fig. 5), a significantly lower yield is observed in R1 ($p = 0.013$) when considering the initial granulation days, similar to what was found in the EPS data. Furthermore, R2 and R3 showed greater stability over time compared to R1, suggesting that the imposed osmotic pressure possibly induced this behavior, similar to the findings of Frutuoso et al. [25]. ALE production was also stable in the experiments by Sui et al. [61] with salt stress, averaging 50.8 ± 1.7 mg/gVSS. These values were much lower than those found in this research under similar salinity conditions, possibly due to the quantification methodology that was different from the present study.

In R1, a higher production of ALE was found, which was proportional to the improvement in biomass quality and settleability characteristics. Statistically, a moderate Pearson correlation is observed ($r = -0.47$)

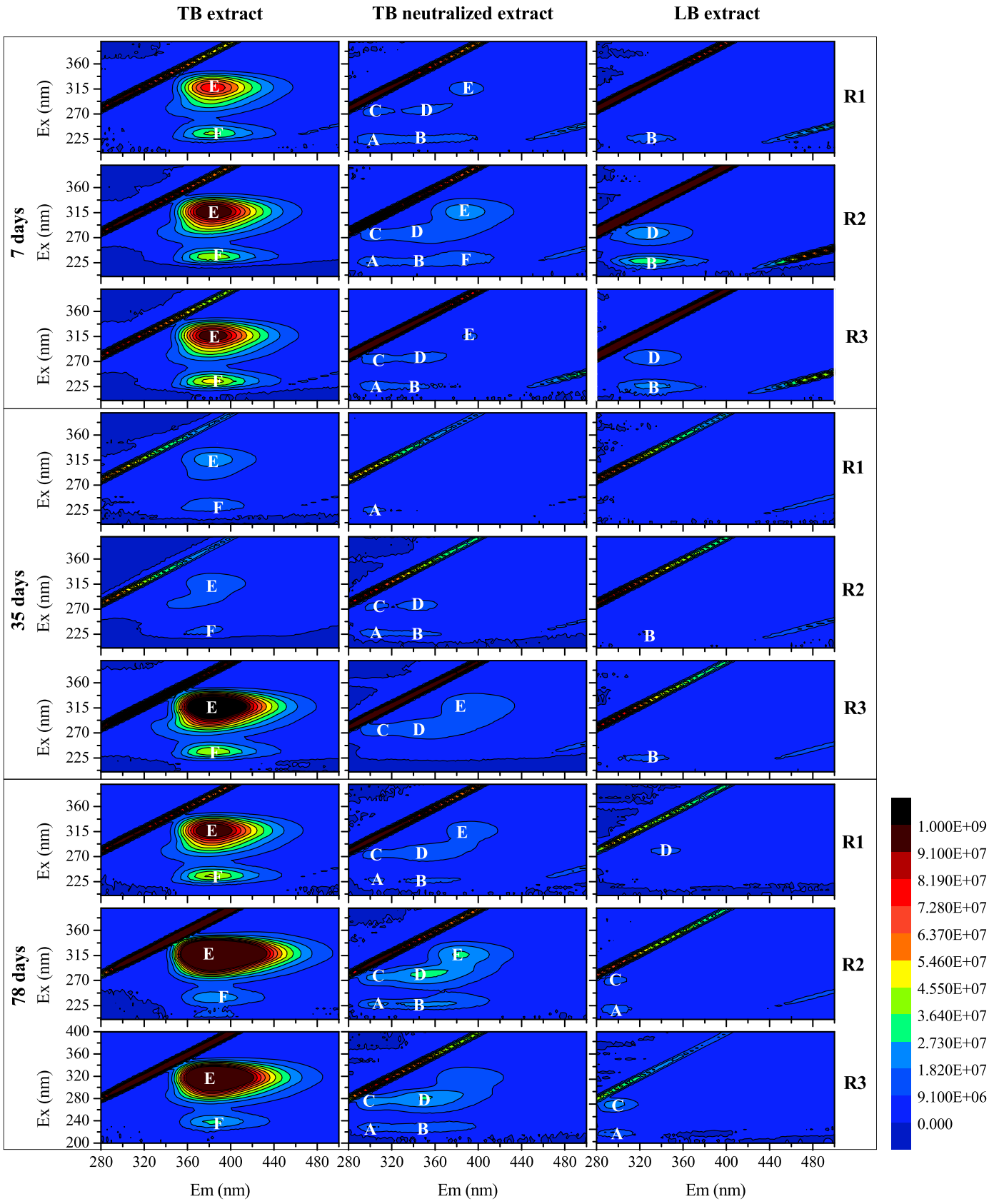

Fig. 6. Three-dimensional fluorescence excitation-emission matrix spectrum (3D-EEM) of EPS extracted from aerobic granules of each reactor on days 7, 35, and 70 of operation. **Note:** Tyrosine amino acid (A), Tryptophan amino acid (B), Tyrosine-containing proteins (C), Tryptophan-containing proteins (D), Polysaccharides (E), and Fulvic acid-like substances (F). R1 (no saline stress), R2 (constant stress), R3 (intermittent saline stress).

Table 2
Excitation/emission bands detected in 3D-EEM for EPS analysis [40,22,25].

Region	Ex/Em	Compounds
A	220–235/290–320	Tyrosine amino acid
B	220–235/330–360	Tryptophan amino acid
C	260–290/290–320	Tyrosine-containing proteins
D	280–290/330–360	Tryptophan-containing proteins
E	300–330/360–390	Polysaccharides
F	220–240/380–440	Fulvic acid-like substances
G	260–290/420–460	Polyaromatic-type humic acid
H	330–370/420–460	Humic acid-type polycarboxylate

between ALE data and SVI_{30} in R1. In R2 and R3, an inverse correlation ($r = -0.38$) is also verified, but it is weak. In general, ALE production is directly correlated with the good development of granules; however, other factors may interfere with its lower production, such as the F/M ratio and SRT, as previously observed [26]. For example, high salinity conditions negatively impacted the granule quality and ALE formation, as there was a reduction in the MM and GG blocks and subsequent reduction in the gel formation capacity (GG) and chain flexibility (MM), thus compromising granule structural stability [46].

On the other hand, according to Meng et al. [47], moderate levels of salinity induce the activation of the algC gene in *Pseudomonas*, resulting in the activation of the phosphomannomutase enzyme, which plays a crucial role in the alginate biosynthesis process. This group of microorganisms was found in the granule composition. They are reported to facilitate EPS production, justifying the interrelationship between EPS and ALE in reactors.

Like the production of EPS and ALE, the production of TRY did not show a significant difference among the reactors, considering the total dataset. The amino acid L-tryptophan is essential for humans, as well as for animals, plants, and some bacteria. It can be used in the food industry as preserves, in the pharmaceutical industry as antidepressant medications, and even in agriculture, being used in the treatment of seeds [20]. TRY synthesis occurs mainly through the biochemical route or biological fermentation, in which tryptophan can be produced directly or through

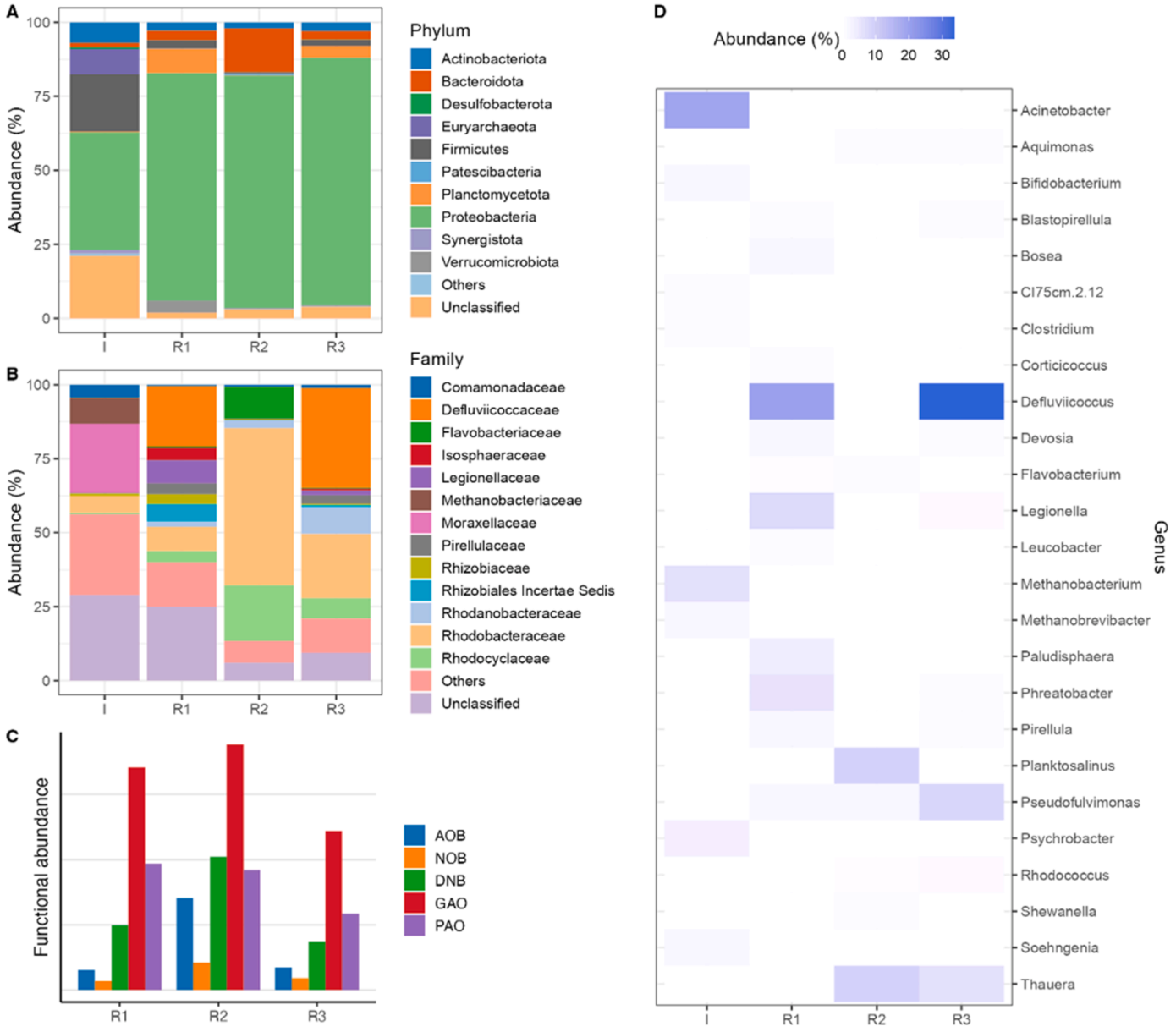

Fig. 7. Relative abundance of microbial clusters in inoculum sludge and AGS biomass. (a) phylum level; (b) family level; (c) distribution of functional groups; and (d) genus level.

enzymes. In this sense, the microbial groups in the granules are of fundamental importance for its production. Thus, salt stress can inhibit these microorganisms or have the opposite effect, depending on the microbial group. In the experiments, all three reactors exhibited a similar pattern, with an initial increase in TRY production attributed to the selection stress responsible for the initial granulation, followed by subsequent oscillations and a final increase.

As tryptophan is an amino acid that is present in proteins that form EPS, the general idea was that its recovery would directly correlate with EPS production. Thus, higher concentrations of tryptophan would correlate with an increase in EPS proteins. Therefore, it is possible to verify this effect through the salt stress caused by the alternation of cycles in R3. On the other hand, regarding EPS and TRY production rate, it was expected that R2 and R3 would have greater growth with the maturation process. However, this growth was only observed in R1, likely influenced by the F/M ratio.

3.5. Microbiological communities

At the end of the operation, microbial diversity was evaluated by phylum, family, and genus on samples collected from the inoculum sludge (I) and biomass extracted from reactors R1, R2, and R3 (Fig. 7).

The dominant phyla observed in the inoculum sludge comprised *Proteobactéria* (39.8%), *Firmicutes* (19.3%), *Euryarchaeota* (8.7%), *Actinobacteriota* (6.88%) and *Bacteroidota* (1.43%) (Fig. 7a). The *Proteobacteria* and *Bacteroidota* phyla were enriched with granulation, while the other phyla were inhibited. For instance, the *Euryarchaeota* and *Firmicutes* phyla were significantly reduced. *Euryarchaeota* microorganisms are highly sensitive to oxygen, and *Firmicutes* are typically found in anaerobic fermentation, both commonly present in extreme environments [56,66].

The literature has indicated that groups within the *Proteobacteria* phylum play a crucial role in sludge granulation and structural stability, commonly associated with EPS secretion for aerobic granule formation [37]. These microorganisms exhibited a relative abundance of 77.0%, 78.5%, and 83.6% in R1, R2, and R3, respectively, indicating the granulation level and reactor stability. Notably, R1, with 30% filamentous biomass, exhibited the lowest relative abundance of this group, contrasting with R3, which showed faster granulation and better stability throughout the operation.

The *Bacteroidetes* phylum, known for its halotolerant nature and prevalence in marine environments, is dominant in high-salinity activated sludge systems [56]. It exhibited a relative abundance of 14.8% in R2, likely due to the system's higher osmotic pressure.

The *Planctomycetota* phylum showed an abundance of 8.2% in R1, 0.13 in R2, and 3.9% in R3. These microorganisms are associated with organic matter oxidation. They are typically found in activated sludge biomass but are also commonly cited in AGS systems [25,56]. The higher abundance of *Planctomycetota* in R1 is justified by the greater presence of filamentous biomass in that system.

At the family level (Fig. 7b), in the inoculum sludge, groups with abundances greater than 5% were *Moraxellaceae* (23.4%), *Methanobacteriaceae* (8.7%), and *Rhodobacteraceae* (5,8%). While *Moraxellaceae* (< 0.04%) and *Methanobacteriaceae* (< 0.05%) were significantly reduced during the granulation process, the abundance of *Rhodobacteraceae* increased. This increase has been influenced by osmotic stress, as they exhibited values proportional to the imposed stress (8.2%, 53.2%, and 21.8%, respectively, in R1, R2, and R3).

Other predominant family groups with varying relative abundances include *Defluviicoccaceae* (20.4%, 0.1% and 33.7%), *Legionellaceae* (7.8%, 0.05% and 1.6%), *Rhodocyclaceae* (3.8%, 18.8% and 6.9%), *Flavobacteriaceae* (0.6%, 10.8% and 0.3%), *Rhodanobacteraceae* (1.8%, 2.6% and 9.0%), *Isosphaeraceae* (4.1%, 0.05% and 0.61%) and *Pirellulaceae* (3.6%, 0.04% and 2.9%) in R1, R2, and R3, respectively.

Han et al. [28] indicated that families such as *Rhodocyclaceae, Rhodobacteraceae,* and *Flavobacteriaceae* play essential roles in EPS production and granule development, stimulating the release of signaling molecules like cyclic guanosine monophosphate (c-di-GMP). *Flavobacteriaceae* members are known for their notable ability to secrete EPS, particularly PN-rich EPS, in marine environments. They are associated with ALE production, explaining the high PN and ALE production in R2 despite late granulation [14].

Among the most abundant genera (Fig. 7d) that were possible to be successfully classified were: *Acinetobacter* (19.5%) and *Methanobacterium* (6.7%) in the inoculum; *Defluviicoccus* (20.5%), *Legionella* (7.8%) and *Phreatobacter* (5.8%) in R1; *Planktosalinus* (9.2%) and *Thauera* (9.4%) in R2; and *Defluviicoccus* (33.7%), *Pseudofulvimonas* (8.5%) and *Thauera* (6.5%) in R3. *Legionella* [44] and *Phreatobacter* [70] are associated with nitrogen removal in granular systems and are also found in activated sludge and moving bed biofilm reactors.

Defluviicoccus microbes from the *Defluviicoccaceae* family are linked to denitrification and glycogen accumulation (DGAOs). He et al. [30] observed a reduction in these microbes at 0.5% saline concentrations, similar to what was seen in R2, indicating increased competitiveness and adaptive capabilities of PAOs/DPAOs. However, despite this group's inhibition at 5 g/L saline concentrations (R2), other GAOS/DGAOs from the *Rhodobacteraceae* family were enriched in R2.

Thauera, *Pseudofulvimonas* belonging to the *Proteobacteria* phylum, are known for their strong salt resistance, justifying their higher abundance in R2 and R3 [66]. *Planktosalinus* from the *Flavobacteriaceae*, present in greater abundance in R2, are halotolerant microorganisms and have been cited in other studies within saline concentrations of 10 g/L [25].

Thauera genus is also associated with EPS production, granule formation, and stability, as it can promote c-di-GMP production, which is crucial for the maturation of granules and the enduring system stability [37,65]. High correlations between c-di-GMP, EPS, and *Pseudofulvimonas* relative abundance were observed by Chen et al. [9], consistent with this study showing higher abundance of these groups in R2 and R3, where complete granulation was achieved. Other authors [71] suggested that quorum sensing stimulation (c-di-GMP) may lead to excessive ALE production. Therefore, low saline concentrations (0.5%) may enrich these groups and improve granulation and bioresource production.

Regarding specific microbial groups involved in carbon and nutrient removal (Fig. 7c), a higher abundance of AOB and NOB was observed in R2, possibly due to longer SRT (18±2 days). In R1 and R3, the SRT was 4 days (±2) at the end of operation, which may have caused a partial washout of these microorganisms, especially NOB. Furthermore, AOB dominance was observed in all systems.

NOB inhibition is reported to occur at low SRT and high temperatures, as in the SHARON process, leading to nitrite accumulation [31]. This happens because, at short SRTs, nitrite oxidizers can be washed out while ammonium oxidizers are retained in the reactor. At the normal wastewater treatment temperatures of temperate countries (5–20°C), NOB grow more rapidly than AOB, resulting in the complete oxidation of ammonium to nitrate; however, the reverse is true [31]. Zeng et al. [72] observed a decrease in NOB and an increase in AOB when the operational temperature was raised to 28ºC and a deterioration of nitritation (partial nitrification) when the temperature dropped to 18ºC. Therefore, in this study, with operational temperatures of 28ºC, the dominance of AOB can be justified. While a higher abundance of DNB relative to AOB and NOB was observed in all three systems, complete denitrification was not achieved (~65%), possibly due to a lack of biodegradable organic matter required for complete denitrification. The dominance of GAOs over PAOs in all three reactors indicates GAOs' advantage in substrate competition, justifying low phosphorus removal (~25%). Furthermore, when the microbial composition of the granules formed in each reactor is evaluated, GAOs and PAOs were more dominant, respectively, except for R2. In this latter reactor, DNB was dominant in relation to PAOs. However, as mentioned, some GAOs and PAOs can have denitrifying metabolism, in which denitrification efficiency will depend on carbon

availability.

4. Conclusion

EPS and ALE productions remained more stable in reactors with osmotic stress, proportional to improved biomass quality and sedimentation characteristics. Feeding subjected to intermittent saline stress proved to be a potential alternative for aerobic granule cultivation, showing fast granulation, stability in bioresource production, good carbon and nitrogen removal, and well-formed granules with good settleability characteristics.

The microbiological analysis revealed significant changes in microbial diversity, showing specific responses in each reactor and indicating adaptations to environmental factors. Genera such as *Thauera* and *Pseudofulvimonas*, associated with EPS production and granule maturation, exhibited higher abundance in reactors with complete granulation, suggesting a positive correlation between saline conditions (0.5%) and enhanced granulation and bioresource production.

These findings provide important insights for optimizing the performance of AGS systems in future applications on sewage treatment, leachate treatment, and saline wastewaters.

CRediT authorship contribution statement

Vicente Elício Porfiro Sales Gonçalves da Silva: Writing – original draft, Writing – review & editing. **Francisca Kamila Amancio Frutuoso:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **André Bezerra dos Santos:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Clara Bandeira de Carvalho:** Writing – review & editing, Writing – original draft, Investigation.

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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References

[1] APHA, Standard Methods for the Examination of Water and Wastewater, 2012, American Public Health Association, Washington, 2012.
[2] N.T. Bahgat, P. Wilfert, L. Korving, M. Van Loosdrecht, Integrated resource recovery from aerobic granular sludge plants, Water Res. 234 (2023) 119819, https://doi.org/10.1016/j.watres.2023.119819.
[3] A.R.M. Barros, S.L.S. Rollemberg, C.D.A. De Carvalho, I.H.H. Moura, P.I. M. Firmino, A.B. Dos Santos, Effect of calcium addition on the formation and maintenance of aerobic granular sludge (AGS) in simultaneous fill/draw mode sequencing batch reactors (SBRs), J. Environ. Manag. 255 (2020) 109850, https://doi.org/10.1016/j.jenvman.2019.109850.
[4] Bassin, J.P., Winkler, M.-K.H., Kleerebezem, R., Dezotti, M., van Loosdrecht, M.C. M., 2012. Improved phosphate removal by selective sludge discharge in aerobic granular.
[5] Z. Bin, C. Zhe, Q. Zhigang, J. Min, C. Zhiqiang, C. Zhaoli, L. Junwen, W. Xuan, W. Jingfeng, Dynamic and distribution of ammonia-oxidizing bacteria communities during sludge granulation in an anaerobic–aerobic sequencing batch reactor, Water Res. 45 (2011) 6207–6216, https://doi.org/10.1016/j.watres.2011.09.026.
[6] P. Carrera, T. Casero-Díaz, C.M. Castro-Barros, R. Méndez, A.Val Del Río, A. Mosquera-Corral, Features of aerobic granular sludge formation treating fluctuating industrial saline wastewater at pilot scale, J. Environ. Manag. 296 (2021) 113135, https://doi.org/10.1016/j.jenvman.2021.113135.
[7] M. Carvalheira, A. Oehmen, G. Carvalho, M. Eusébio, M.A.M. Reis, The impact of aeration on the competition between polyphosphate accumulating organisms and glycogen accumulating organisms, Water Res. 66 (2014) 296–307, https://doi.org/10.1016/j.watres.2014.08.033.
[8] H. Chen, L.-L. He, A.-N. Liu, Q. Guo, Z.-Z. Zhang, R.-C. Jin, Start-up of granule-based denitrifying reactors with multiple magnesium supplementation strategies, J. Environ. Manag. 155 (2015) 204–211, https://doi.org/10.1016/j.jenvman.2015.03.036.
[9] H. Chen, Y. Tian, Z. Hu, C. Wang, P. Xie, L. Chen, F. Yang, Y. Liang, C. Mu, C. Wei, Y.-P. Ting, G. Qiu, Y. Song, Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) mediated membrane fouling in membrane bioreactor, J. Membr. Sci. 646 (2022) 120224, https://doi.org/10.1016/j.memsci.2021.120224.
[10] J. Chen, Z. Yu, P. Cheng, Y. Tan, X. Fan, X. Xu, L. Zhu, Microbial synergy achieving simultaneous nitrification-denitrification (SND) in salt-tolerant aerobic granular sludge, J. Water Process Eng. 60 (2024) 105138, https://doi.org/10.1016/j.jwpe.2024.105138.
[11] W. Cheng, C. Ma, R. Bao, X. Yang, X. Zhou, Y. Liu, Q. Liu, A. Ruan, X. Li, Effect of influent ammonia nitrogen concentration on the phosphorus removal process in the aerobic granular sludge reactor, J. Environ. Chem. Eng. 11 (2023) 110476, https://doi.org/10.1016/j.jece.2023.110476.
[12] S.F. Corsino, R. Campo, G. Di Bella, M. Torregrossa, G. Viviani, Study of aerobic granular sludge stability in a continuous-flow membrane bioreactor, Bioresour. Technol. 200 (2016) 1055–1059, https://doi.org/10.1016/j.biortech.2015.10.065.
[13] S.F. Corsino, M. Capodici, M. Torregrossa, G. Viviani, Physical properties and Extracellular Polymeric Substances pattern of aerobic granular sludge treating hypersaline wastewater, Bioresour. Technol. 229 (2017) 152–159, https://doi.org/10.1016/j.biortech.2017.01.024 sludge reactors. Biotechnol. Bioeng. 109, 1919–1928. https://doi.org/10.1002/bit.24457.
[14] Y.-W. Cui, J.-L. Huang, F. Alam, Fast granulation of halophilic activated sludge treating low-strength organic saline wastewater via addition of divalent cations, Chemosphere 264 (2021) 128396, https://doi.org/10.1016/j.chemosphere.2020.128396.
[15] Y. Cui, H. Su, Yefei Chen, Yongbao Chen, Y. Peng, Mechanism of Activated Sludge Floc Disintegration Induced by Excess Addition of NaCl: Water, Clean. Soil Air Water 43 (2015) 1197–1206, https://doi.org/10.1002/clen.201400219.
[16] V.E.P.S.G. da Silva, S.L.S. Rollemberg, A.B. dos Santos, Impact of feeding strategy on the performance and operational stability of aerobic granular sludge treating high-strength ammonium concentrations, J. Water Process Eng. 44 (2021) 102378, https://doi.org/10.1016/j.jwpe.2021.102378.
[17] V.E.P.S.G. da Silva, S.L.S. Rollemberg, A.B. dos Santos, Step-feeding in aerobic/anoxic cycles enhanced the performance of aerobic granular sludge (AGS) systems treating effluents with low C:N ratios, Process Saf. Environ. Prot. 171 (2023) 1031–1042, https://doi.org/10.1016/j.psep.2022.10.004.
[18] V.E.P.S.G. da Silva, S.L.S. Rollemberg, S.G.S. Silva, T.F.C.V. Silva, V.J.P. Vilar, A. B. dos Santos, Evaluation of leachate impact on domestic sewage co-treatment in aerobic granular sludge systems, Chem. Eng. J. 470 (2023) 144178, https://doi.org/10.1016/j.cej.2023.144178.
[19] B.K. Das, D.N. Jha, S.K. Sahu, A.K. Yadav, R.K. Raman, M. Kartikeyan, Concept of Simple Correlation and Regression, Springer Nature, Singapore, 2022, https://doi.org/10.1007/978-981-19-4411-6_8.
[20] C.A. de Carvalho, A.F. Santos, T.J.T. Ferreira, V.N.S.A. Lira, A.R.M. Barros, A.B. dos Santos, Resource recovery in aerobic granular sludge systems: is it feasible or still a long way to go? Chemosphere 274 (2021) 12988, https://doi.org/10.1016/j.chemosphere.2021.129881.
[21] G. Di Bella, M. Torregrossa, Simultaneous nitrogen and organic carbon removal in aerobic granular sludge reactors operated with high dissolved oxygen concentration, Bioresour. Technol. 142 (2013) 706–713, https://doi.org/10.1016/j.biortech.2013.05.060.
[22] A.F. dos Santos, F.K.A. Frutuoso, C. de A. de Carvalho, V.N.S.A. Lira, A.R.M. Barros, A.B. dos Santos, Carbon source affects the resource recovery in aerobic granular sludge systems treating wastewater, Bioresour. Technol. 357 (2022) 127355, https://doi.org/10.1016/j.biortech.2022.127355.
[23] Michel DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, Fred Smith, Colorimetric Method for Determination of Sugars and Related Substances, Anal. Chem. 28 (1956) 350–356, https://doi.org/10.1021/ac60111a017.
[24] S. Felz, S. Al-Zuhairy, O.A. Aarstad, M.C.M. van Loosdrecht, Y.M. Lin, Extraction of Structural Extracellular Polymeric Substances from Aerobic Granular Sludge, JoVE (2016) 54534, https://doi.org/10.3791/54534.
[25] F.K.A. Frutuoso, A.F. dos Santos, L.L. da S. França, A.R.M. Barros, A.B. dos Santos, Influence of salt addition to stimulating biopolymers production in aerobic granular sludge systems, Chemosphere 311 (2023) 137006, https://doi.org/10.1016/j.chemosphere.2022.137006.
[26] F.K.A. Frutuoso, A.F. dos Santos, L.L. da S. França, A.R.M. Barros, A.B. dos Santos, Influence of operating regime on resource recovery in aerobic granulation systems under osmotic stress, Bioresour. Technol. 376 (2023) 128850, https://doi.org/10.1016/j.biortech.2023.128850.
[27] G. Guo, F. Tian, K. Ding, F. Yang, Y. Wang, C. Liu, C. Wang, Effect of salinity on removal performance of anaerobic membrane bioreactor treating azo dye

wastewater, Appl. Biochem. Biotechnol. 195 (2) (2022) 1589–1602, https://doi.org/10.1007/s12010-022-04223-w.
[28] F. Han, M. Zhang, Z. Liu, Y. Han, Q. Li, W. Zhou, Enhancing robustness of halophilic aerobic granule sludge by granular activated carbon at decreasing temperature, Chemosphere 292 (2022) 133507, https://doi.org/10.1016/j.chemosphere.2021.133507.
[29] Q. He, H. Wang, L. Chen, S. Gao, W. Zhang, J. Song, J. Yu, Elevated salinity deteriorated enhanced biological phosphorus removal in an aerobic granular sludge sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal, J. Hazard. Mater. 390 (2020) 121782, https://doi.org/10.1016/j.jhazmat.2019.121782.
[30] Q. He, H. Wang, L. Chen, S. Gao, W. Zhang, J. Song, J. Yu, Robustness of an aerobic granular sludge sequencing batch reactor for low strength and salinity wastewater treatment at ambient to winter temperatures, J. Hazard. Mater. 384 (2020) 121454, https://doi.org/10.1016/j.jhazmat.2019.121454.
[31] C. Hellinga, A. Schellen, J.W. Mulder, M.C.M. van Loosdrecht, J.J. Heijnen, The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water, Water Sci. Technol. 37 (9) (1998) 135–142.
[32] P.-N. Hong, R. Honda, M. Noguchi, T. Ito, Optimum selection of extraction methods of extracellular polymeric substances in activated sludge for effective extraction of the target components, Biochem. Eng. J. 127 (2017) 136–146, https://doi.org/10.1016/j.bej.2017.08.002.
[33] Kaumera, 2023 https://kaumera.com/english/kaumera/ Accessed 25 Set 2023.
[34] P. Kehrein, M. van Loosdrecht, P. Osseweijer, J. Posada, Exploring resource recovery potentials for the aerobic granular sludge process by mass and energy balances – energy, biopolymer and phosphorous recovery from municipal wastewater, Environ. Sci.: Water Res. Technol. 6 (2020) 2164–2179, https://doi.org/10.1039/D0EW00310G.
[35] M. Layer, M.G. Villodres, A. Hernandez, E. Reynaert, E. Morgenroth, N. Derlon, Limited simultaneous nitrification-denitrification (SND) in aerobic granular sludge systems treating municipal wastewater: Mechanisms and practical implications, Water Res. X 7 (2020) 100048, https://doi.org/10.1016/j.wroa.2020.100048.
[36] J. Li, J. Liu, D. Wang, T. Chen, T. Ma, Z. Wang, W. Zhuo, Accelerating Aerobic Sludge Granulation by Adding Dry Sewage Sludge Micropowder in Sequencing Batch Reactors, IJERPH 12 (2015) 10056–10065, https://doi.org/10.3390/ijerph120810056.
[37] J. Li, Z. Ma, M. Gao, Y. Wang, Z. Yang, H. Xu, X.-H. Wang, Enhanced aerobic granulation at low temperature by stepwise increasing of salinity, Sci. Total Environ. 722 (2020) 137660, https://doi.org/10.1016/j.scitotenv.2020.137660.
[38] Y. Lin, M. De Kreuk, M.C.M. Van Loosdrecht, A. Adin, Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant, Water Res. 44 (2010) 3355–3364, https://doi.org/10.1016/j.watres.2010.03.019.
[39] H. Lin, R. Ma, Y. Hu, J. Lin, S. Sun, J. Jiang, T. Li, Q. Liao, J. Luo, Reviewing bottlenecks in aerobic granular sludge technology: Slow granulation and low granular stability, Environ. Pollut. 263 (2020) 114638, https://doi.org/10.1016/j.envpol.2020.114638.
[40] Y.-Q. Liu, S. Cinquepalmi, Exploration of mechanisms for calcium phosphate precipitation and accumulation in nitrifying granules by investigating the size effects of granules, Water Res. 206 (2021) 117753, https://doi.org/10.1016/j.watres.2021.117753.
[41] X. Liu, H. Yang, K. Yang, Effect of different enrichment methods on the independent biological phosphorus removal performance after nitrogen removal using immobilized fillers, Biochem. Eng. J. 198 (2023) 109041, https://doi.org/10.1016/j.bej.2023.109041.
[42] OliverH. Lowry, NiraJ. Rosebrough, A.L. Farr, RoseJ. Randall, Protein measurement with the Folin Phenol reagent, J. Biol. Chem. 193 (1951) 265–275, https://doi.org/10.1016/S0021-9258(19)52451-6.
[43] C.-S. Lu, J.-M. Qiu, Y. Yang, Y. Hu, Y.-Y. Li, T. Kobayashi, Y.-J. Zhang, A review of anaerobic granulation under high-salinity conditions: Mechanisms, influencing factors and enhancement strategies, J. Water Process Eng. 55 (2023) 104227, https://doi.org/10.1016/j.jwpe.2023.104227.
[44] J. Luo, T. Hao, L. Wei, H.R. Mackey, Z. Lin, G.-H. Chen, Impact of influent COD/N ratio on disintegration of aerobic granular sludge, Water Res. 62 (2014) 127–135, https://doi.org/10.1016/j.watres.2014.05.037.
[45] W.H. Luo, H.V. Phan, M. Xie, F.I. Hai, W.E. Price, M. Elimelech, L.D. Nghiem, Osmotic versus conventional membrane bioreactors integrated with reverse osmosis for water reuse: biological stability, membrane fouling, and contaminant removal, Water Res. 109 (2017) 122–134, https://doi.org/10.1016/j.watres.2016.11.036.
[46] F. Meng, W. Huang, D. Liu, Y. Zhao, W. Huang, Z. Lei, Z. Zhang, Application of aerobic granules-continuous flow reactor for saline wastewater treatment: granular stability, lipid production and symbiotic relationship between bacteria and algae, Bioresour. Technol. 295 (2020) 122291, https://doi.org/10.1016/j.biortech.2019.122291.
[47] F. Meng, D. Liu, W. Huang, Z. Lei, Z. Zhang, Effect of salinity on granulation, performance and lipid accumulation of algal-bacterial granular sludge, Bioresour. Technol. Rep. 7 (2019) 100228.
[48] F. Motteran, E.L. Pereira, C.M.M. Campos, The behaviour of an anaerobic baffled reactor (ABR) as the first stage in the biological treatment of hog farming effluents, Braz. J. Chem. Eng. 30 (2013) 299–310.
[49] Y.V. Nancharaiah, G. Kiran Kumar Reddy, Aerobic granular sludge technology: Mechanisms of granulation and biotechnological applications, Bioresour. Technol. 247 (2018) 1128–1143, https://doi.org/10.1016/j.biortech.2017.09.131.
[50] W. Niu, J. Guo, J. Lian, Y. Song, C. Lu, H. Li, Y. Han, P. Yin, Rapid start-up of denitrifying granular sludge by dosing with semi-starvation fluctuation C/N ratio strategy, Bioresour. Technol. 241 (2017) 945–950, https://doi.org/10.1016/j.biortech.2017.05.206.
[51] X. Niu, X. Han, Y. Jin, J. Yue, J. Zhu, W. Xie, J. Yu, Aerobic granular sludge treating hypersaline wastewater: impact of pH on granulation and long-term operation at different organic loading rates, J. Environ. Manag. 330 (2023) 117164, https://doi.org/10.1016/j.jenvman.2022.117164.
[52] A. Panagopoulos, Study and evaluation of the characteristics of saline wastewater (brine) produced by desalination and industrial plants, Environ. Sci. Pollut. Res 29 (2022) 23736–23749, https://doi.org/10.1007/s11356-021-17694-x.
[53] D.G. Penagos, J.R. Victoria, M.V. Manrique, Formulation of a protocol to evaluate the aerobic granulation potential (AGP) of an inoculum, MethodsX 9 (2022) 101710, https://doi.org/10.1016/j.mex.2022.101710.
[54] M. Pronk, J.P. Bassin, M.K. de Kreuk, R. Kleerebezem, M.C. van Loosdrecht, Evaluating the main and side effects of high salinity on aerobic granular sludge, Appl. Microbiol. Biotechnol. 98 (2014) 1339–1348, https://doi.org/10.1007/s00253-013-4912-z.
[55] M. Pronk, A. Giesen, A. Thompson, S. Robertson, M. van Loosdrecht, Aerobic granular biomass technology: advancements in design, applications and further developments, Water Pract. Technol. 12 (2017) 987–996, https://doi.org/10.2166/wpt.2017.101.
[56] L. Quartaroli, C.M. Silva, L.C.F. Silva, H.S. Lima, S.O. de Paula, R.S. Dias, K. B. Carvalho, R.S. Souza, J.P. Bassin, C.C. da Silva, Effect of the gradual increase of salt on stability and microbial diversity of granular sludge and ammonia removal, J. Environ. Manag. 248 (2019) 109273, https://doi.org/10.1016/j.jenvman.2019.109273.
[57] S.L.S. Rollemberg, A.N.D. Barros, P.I.M. Firmino, A.B.D. Santos, Evaluation of sludge discharge methodologies in aerobic granular sludge reactors, Bioresour. Technol. Rep. 18 (2022) 101018, https://doi.org/10.1016/j.biteb.2022.101018.
[58] S.L.S. Rollemberg, A.F. dos Santos, T.J.T. Ferreira, P.I.M. Firmino, A.B. dos Santos, Evaluation of the production of alginate-like exopolysaccharides (ALE) and tryptophan in aerobic granular sludge systems, Bioprocess Biosyst. Eng. 44 (2020) 259–270, https://doi.org/10.1007/s00449-020-02439-w.
[59] C.M. Schambeck, B.S. Magnus, L.C.R. de Souza, W.R.M. Leite, N. Derlon, L. B. Guimarães, R.H.R. da Costa, Biopolymers recovery: dynamics and characterization of alginate-like exopolymers in an aerobic granular sludge system treating municipal wastewater without sludge inoculum, J. Environ. Manag. 263 (2020) 110394, https://doi.org/10.1016/j.jenvman.2020.110394.
[60] X. Shi, J. Li, X. Wang, X. Zhang, L. Tang, Effect of the gradual increase of Na2SO4 on performance and microbial diversity of aerobic granular sludge, J. Environ. Manag. 292 (2021) 112696, https://doi.org/10.1016/j.jenvman.2021.112696.
[61] Y. Sui, Y.W. Cui, J.L. Huang, M.J. Xu, Feast/famine ratio regulates the succession of heterotrophic nitrification-aerobic denitrification and autotrophic ammonia oxidizing bacteria in halophilic aerobic granular sludge treating saline wastewater, Bioresour. Technol. 393 (2024) 129995, https://doi.org/10.1016/j.biortech.2023.
[62] R. Tang, X. Han, Y. Jin, J. Yu, Do increased organic loading rates accelerate aerobic granulation in hypersaline environment? J. Environ. Chem. Eng. 10 (2022) 108775 https://doi.org/10.1016/j.jece.2022.108775.
[63] C. Tang, C. Li, Y. Jiang, Z. Hou, S. Wang, C. Wang, S. Chen, Insights into the rapid granulation for treating refractory wastewater under salt stress: variation of sludge property, treatment performance and microbial community, J. Water Process Eng. 53 (2023) 103744, https://doi.org/10.1016/j.jwpe.2023.103744.
[64] K. Van Leeuwen, E. De Vries, S. Koop, K. Roest, The Energy & Raw Materials Factory: Role and Potential Contribution to the Circular Economy of the Netherlands, Environ. Manag. 61 (2018) 786–795, https://doi.org/10.1007/s00267-018-0995-8.
[65] C. Wan, P. Zhang, D.-J. Lee, X. Yang, X. Liu, S. Sun, X. Pan, Disintegration of aerobic granules: Role of second messenger cyclic di-GMP, Bioresour. Technol. 146 (2013) 330–335, https://doi.org/10.1016/j.biortech.2013.07.073.
[66] H. Wang, L. Guo, X. Ren, M. Gao, C. Jin, Y. Zhao, J. Ji, Z. She, Enhanced aerobic granular sludge by static magnetic field to treat saline wastewater via simultaneous partial nitrification and denitrification (SPND) process, Bioresour. Technol. 350 (2022) 126891, https://doi.org/10.1016/j.biortech.2022.126891.
[67] M. Wang, J. He, X. Dong, J. Zhang, Effect of Salinity on Performance and Microbial Community during Granulation Process in a Sequencing Batch Reactor, Water 15 (22) (2023) 3961, https://doi.org/10.3390/w15223961.
[68] G. Wang, X. Huang, S. Wang, F. Yang, S. Sun, P. Yan, Y. Chen, F. Fang, J. Guo, Effect of food-to-microorganisms ratio on aerobic granular sludge settleability: Microbial community, potential roles and sequential responses of extracellular proteins and polysaccharides, J. Environ. Manag. 345 (2023) 118814, https://doi.org/10.1016/j.jenvman.2023.118814.
[69] Z. Wang, M.C.M. Van Loosdrecht, P.E. Saikaly, Gradual adaptation to salt and dissolved oxygen: Strategies to minimize adverse effect of salinity on aerobic granular sludge, Water Res. 124 (2017) 702–712, https://doi.org/10.1016/j.watres.2017.08.026.
[70] H. Wu, Q. Zhang, X. Chen, L. Wang, W. Luo, Z. Zhang, H. Liu, T. Zhao, Effect of HRT and BDPs types on nitrogen removal and microbial community of solid carbon source SND process treating low carbon/nitrogen domestic wastewater, J. Water Process Eng. 40 (2021) 101854, https://doi.org/10.1016/j.jwpe.2020.101854.

[71] Y.-C. Yang, X. Liu, C. Wan, S. Sun, D.-J. Lee, Accelerated aerobic granulation using alternating feed loadings: Alginate-like exopolysaccharides, Bioresour. Technol. 171 (2014) 360–366, https://doi.org/10.1016/j.biortech.2014.08.092.
[72] W. Zeng, B. Li, X. Wang, X. Bai, Y. Peng, Integration of denitrifying phosphorus removal via nitrite pathway, simultaneous nitritation–denitritation and anammox treating carbon-limited municipal sewage, Bioresour. Technol. 172 (2014) 356–364, https://doi.org/10.1016/j.biortech.2014.09.061.
[73] M. Zhang, T. Jiao, S. Chen, W. Zhou, A review of microbial nitrogen transformations and microbiome engineering for biological nitrogen removal under salinity stress, Chemosphere 341 (2023) 139949, https://doi.org/10.1016/j.chemosphere.2023.139949.