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VICENTE ELÍCIO PORFIRO SALES GONÇALVES DA SILVA

AEROBIC GRANULAR SLUDGE SYSTEM OPTIMIZATION STRATEGIES FOR LANDFILL LEACHATE TREATMENT AND CO-TREATMENT WITH DOMESTIC SEWAGE

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Thesis presented to the Graduate Program in Civil Engineering at the Federal University of Ceará, as a partial requirement for obtaining the degree in Civil Engineering. Area of concentration: Environmental Sanitation.

Advisor: Prof. André Bezerra dos Santos (Ph.D.)

Co-advisors: Dr. Vítor Jorge Pais Vilar (Ph.D.) and Prof. Silvio Luiz de Sousa Rollemberg (Ph.D.)

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Approved on: ___/___/______.

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To myself, for never giving up, and
that this serves as an inspiration
for my brother, Heitor Ruan

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“Last year I died, but this year I
don't die.”

(Belchior)

RESUMO

Embora a tecnologia de Lodo Granular Aeróbio (LGA) tenha sido bastante estudada e difundida para esgotos domésticos e outros efluentes mais complexos, a sua aplicação no tratamento de lixiviados de aterros sanitários ainda é bastante incipiente e apresenta alguns problemas como alta demanda temporal para granulação, perda de biomassa, grânulos com pequenas dimensões, desintegração de grânulos e acúmulo de nitrito. Diante disso, este trabalho buscou otimizar sistemas LGA para o tratamento de lixiviado ou co-tratamento com esgoto doméstico com o objetivo de minimizar ou eliminar esses problemas comumente reportados. Para isso, diversas estratégias operacionais combinando períodos óxicos (O), anóxicos (An), anaeróbios (A) e alimentação escalonada foram avaliadas a partir de efluentes sintéticos com relação C:N:P similares a de um lixiviado real. Após definição das melhores estratégias, o reator otimizado foi utilizado para o tratamento de lixiviado real. Na investigação inicial, o processo de granulação foi avaliado a partir de duas diluições de lixiviado real desde o inóculo: R1 (25%) e R2 (50%) em ciclos de 8 horas. Nos dois reatores o tempo para granulação foi elevado e houve baixa retenção de sólidos. Apesar dos grânulos produzidos serem pequenos (200 – 300 μm), no R2 as remoções de TN foram maiores, especialmente por apresentar matéria orgânica disponível por mais tempo durante o ciclo, já que neste reator a remoção de COD foi menor. Além das remoções de C, N e P nos dois reatores serem insuficientes, foi observado acúmulo de nitrito durante toda a operação, sendo necessária uma otimização para reduzir os principais problemas encontrados, que corroboraram com os problemas já reportados na literatura. Na segunda investigação, foi investigada a forma de alimentação dos sistemas RBS-LGA em ciclos de 12 horas de duração. As estratégias foram: alimentação anóxica/anaeróbia rápida (R1), lenta (R2) e escalonada (R3). O R3 apresentou a maior retenção de sólidos (3,4 g/L MLSS) e a melhor sedimentabilidade (SVI_{30} = 46,7 mL/g), enquanto que o R2 apresentou a pior concentração de biomassa (1,9 g/L MLSS) e a pior sedimentabilidade (SVI_{30} = 132,3 mL/g). Além disso, em comparação com a alimentação rápida (R1) e lenta (R3), este modo de operação escalonada promoveu as melhores remoções de fósforo total (PT, 53%) e nitrogênio total (NT, 92%), sem nenhum acúmulo de nitrito ou nitrato. As remoções de DQO foram muito semelhantes em R2 e R3, mas as remoções de NT e PT foram significativamente maiores em R3. Na terceira investigação, diferentes configurações de ciclo foram investigadas: A/O (R1 e R2), O/An com alimentação convencional e fase anóxica bem definida (R3), O/An com alimentação escalonada e fase anóxica bem definida (R4), O/An (R5 e R6). A alimentação escalonada continuou apresentando as menores perdas de biomassa e a melhor performance dos reatores em termos de remoção simultânea de carbono, fósforo e nitrogênio, com taxa de nitrificação de 99% sem acúmulos de nitrito. A intercalação de períodos *feast/famine* e anóxicos minimizaram o acúmulo de nitrito. Reatores do tipo O/An com fase anóxica bem definida apresentaram os maiores grânulos, com diâmetro em torno de 1mm. Nestes reatores, a presença de cocos em dominância de bacilos é claramente observada, bem como uma estrutura mais densa ao redor do grânulo, mantendo as comunidades microbianas mais agregadas. Em contraste, os grânulos dos reatores do tipo O/An sem fase anóxica bem definida (R5 e R6) foram caracterizados por apresentarem espaços vazios e com cavidades, que sugerem fragmentação interna. Foi observado, também que Planctomycetota foi o filo mais abundante nos reatores com fase anóxica bem definida e alimentação escalonada. Proteobacteria foi o filo mais abundante nos reatores com fase anaeróbia (R1 e R2) e no reator com alimentação rápida (R6). Na investigação final, o co-tratamento do lixiviado ocorreu gradativamente em reatores com alimentação escalonada (R1) e com alimentação convencional (R2), ambos com alternância de fases óxica/anóxica ao final do ciclo. Foi possível realizar o co-tratamento de esgoto doméstico com lixiviado em sistemas AGS. No entanto, a estratégia de alimentação, a configuração do reator e a suplementação de metanol desempenharam um papel importante na estabilidade do processo e nas remoções simultâneas de carbono, nitrogênio e fósforo. A alimentação escalonada produziu uma biomassa granular aeróbia mais compacta e resistente, resultando em uma melhor estabilidade operacional. Além disso, esta estratégia favoreceu a desnitrificação, especialmente durante a suplementação de metanol, minimizando um dos principais problemas reportados no co-tratamento de lixiviado em sistemas AGS. Como consequência, maiores remoções de nitrogênio total (TN) foram obtidas. Ao final do último período, no R1, as remoções de COD, TN e DOC foram de 93%, e a remoção de fósforo foi de 54%, atingindo valores maiores ou semelhantes a outras investigações de AGS com esgoto, lixiviado ou co-tratamento. Portanto, os resultados apresentados trazem uma boa perspectiva para o co-tratamento de esgoto doméstico com lixiviado e outros tipos de efluentes industriais.

Palavras-chave: Lixiviado de aterro sanitário; lodo Granular Aeróbio; reatores em batelada sequenciais; engenharia; microbiologia molecular.

ABSTRACT

Although the Aerobic Granular Sludge (AGS) technology has been extensively studied and disseminated for domestic sewage and other more complex effluents, its application in the treatment of leachate from sanitary landfills is still very incipient and presents some problems such as high temporal demand for granulation, biomass loss, granules with small dimensions, granule disintegration and nitrite accumulation. Therefore, this work sought to optimize AGS systems for leachate treatment and co-treatment with sewage to minimize or eliminate these commonly reported problems. For this, the impact of real leachate on the granule formation process and on the composition of the microbial structure in conventional AGS systems was initially evaluated. Subsequently, several operational strategies combining oxic (O), anoxic (An), anaerobic (A) periods and step-feeding were evaluated from synthetic effluents with a C:N:P ratio similar to that of a real leachate. After defining the best strategies, the optimized reactor was used for the treatment of real leachate. In the initial investigation, the granulation process was evaluated from two dilutions of real leachate from the inoculum: R1 (25%) and R2 (50%) in 8-hour cycles. In both reactors, the time for granulation was high and there was low retention of solids. Although the granules produced were small (200–300 μm), in R2 the TN removals were greater, especially because it had organic matter available for a longer period of time during the cycle, since in this reactor the COD removal was lower. In addition to the insufficient removal of C, N and P in the two reactors, nitrite accumulation was observed throughout the operation, requiring optimization to reduce the main problems encountered, which corroborate the problems already reported in the literature. In the second investigation, the way in which the AGS systems were fed in 12-hour cycles was investigated. The strategies were: fast (R1), slow (R2) and anoxic/anaerobic step-feeding (R3). R3 showed the highest solids retention (3.4 g/L MLSS) and the best settling (SVI_{30} = 46.7 mL/g), while R2 had the worst biomass concentration (1.9 g/L MLSS) and the worst settleability (SVI_{30} = 132.3 mL/g). In addition, compared to fast (R1) and slow (R3) feeding, this staggered mode of operation achieved the best removals of total phosphorus (TP, 53%) and total nitrogen (TN, 92%) without any accumulation of nitrite or nitrate. COD removals were very similar in R2 and R3, but TN and TP removals were significantly higher in R3. In the third investigation, different cycle configurations were investigated: A/O (R1 and R2), O/An with conventional power and well-defined anoxic phase (R3), O/An with step-feeding and well-defined anoxic phase (R4), O/An (R5 and R6). The step-feeding continued to show the lowest biomass losses and the best reactor performance in terms of simultaneous removal of carbon, phosphorus and nitrogen, with a nitrification rate of 99% without nitrite accumulation. Interleaving feast/famine and anoxic periods minimized nitrite accumulation. O/An type reactors with well-defined anoxic phase had the largest granules, with a diameter of around 1mm. In these reactors, the presence of bacillus-dominated coccus is clearly observed, as well as a denser structure around the granule, keeping the microbial communities more aggregated. In contrast, the granules of the O/An type reactors without a well-defined anoxic phase (R5 and R6) were characterized by having empty spaces and cavities, which suggest internal fragmentation. It was also observed that Planctomycetota was the most abundant phylum in reactors with well-defined anoxic phase and step-feeding. Proteobacteria was the most abundant phylum in the anaerobic phase reactors (R1 and R2) and in the fast-feeding reactor (R6). In the final investigation, the leachate co-treatment with sewage occurred gradually in reactors with step-feeding (R1) and with conventional feeding (R2), both with alternating oxic/anoxic phases at the end of the cycle. It was possible to accomplish the domestic sewage co-treatment with leachate in AGS systems. However, feeding strategy, reactor configuration, and methanol supplementation played an important role in process stability and simultaneous carbon, nitrogen, and phosphorus removals. Step-feeding produced an aerobic granular biomass more compact and resistant, resulting in a better operational stability. Moreover, this strategy favored denitrification, especially during methanol supplementation, minimizing one of the main problems reported regarding leachate co-treatment in AGS systems. As a result, higher total nitrogen (TN) removals were obtained. At the end of the last period, in R1, Chemical Oxygen Dissolved (COD), TN, and Dissolved Organic Carbon (DOC) removals were 93%, and phosphorus removal was 54%, reaching values higher or similar to other AGS investigations with sewage, leachate, or co-treatment. Therefore, the presented results bring a good perspective for domestic sewage co-treatment with leachate and other types of industrial wastewater.

Keywords: Sanitary Landfill Leachate; aerobic granular sludge; sequencing batch reactors; engineering; molecular microbiology.

LIST OF ABREVIATIONS AND ACRONYMS

AGS	Aerobic Granular Sludge
ACE	Abundance-Based Coverage Estimator
ANAMMOX	Anaerobic Ammonium Oxidation
AOB	Ammonia-Oxidizing Bacteria
AOP	Advanced Oxidation Processes
AOT	Advanced Oxidation Technology
ASV	Amplicon Sequencing Variant
BOD	Biochemical Oxygen Demand
CAS	Conventional Activated Sludge
CF	Coagulation/Flocculation
COD	Chemical Oxygen Demand
DGAOs	Denitrifying Glycogen-Accumulating Organisms
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
DPAOs	Denitrifying Phosphorus-Accumulating Organisms
EPS	Extracellular Polymeric Substances
EPS-LB	Loosely Bound
EPS-TB	Tightly Bound
FA	Free Ammonia
FAc	Fulvic Acids
FNA	Free Nitrous Acid
GAO	Glycogen Accumulating Organisms
HA	Humic Acids
LD	Leachate Dilution
LTP	Leachate Treatment Plant
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
MSW	Municipal Solid Waste
MWWTP	Municipal Wastewater Treatment Plants
NCBI	National Center for Biotechnology Information
NOB	Nitrite-Oxidizing Bacteria

OHO	Ordinary Heterotrophic Organisms
OS	Operating System
PAC	Powder Activated Carbon
PAO	Phosphorus-Accumulating Organisms
PFCs	Perfluorinated Compounds
PHAs	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PN	Protein
PS	Polysaccharide
SBR	Sequencing Batch Reactors
SEM	Scanning Electron Microscopy
SND	Simultaneous Nitrification and Denitrification
SNDPR	Simultaneous Nitrification, Denitrification, and Phosphorus Removal
SRT	Sludge Retention Time
SVI	Sludge Volume Index
T_f	Feeding Time
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
TP	Total Phosphorous
T_s	Settling Time
TSS	Total Suspended Solids
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids
WWTPs	Wastewater Treatment Plants

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SUMMARY

1 INTRODUCTION

Due to low costs and operational simplicity, the sanitary landfill has been disseminated worldwide as a very effective method for municipal solid waste management (Aziz, Rahmat and Alazaiza, 2022). However, among the main issues is the leachate, which is highly contaminated wastewater, composed of recalcitrant or refractory dissolved organic material, suspended solids, inorganic macronutrients, heavy metals and xenobiotic organic compounds (Chung *et al.*, 2015; Gomes *et al.*, 2019).

As the age of the landfill increases, the greater the difficulty in implementing the leachate treatment by the biological route, since microorganisms are inhibited due to the high concentrations of free ammonia, free nitrous acid and recalcitrant organic matter (Gao, *et al.*, 2021; Wei *et al.*, 2021). Thus, several strategies have been studied and adopted to increase treatment efficiency, from physical-chemical pre-treatment, co-treatment in domestic sewage plants and the use of other more efficient biological technologies, such as Aerobic Granular Sludge (Wei *et al.*, 2012; Ren *et al.*, 2017a; Bueno *et al.*, 2021; Chen *et al.* 2021).

The aerobic granular sludge (AGS) technology has specific characteristics that favor its implementation in both simple wastewater and more complex wastewater. One of these characteristics is the easy biomass separation from the treated effluent, which occurs in the same tank due to the high settling velocity of the compact granules formed, eliminating the use of clarification units and decreasing costs for sludge recirculation (Sarma and Tay, 2018). Furthermore, it is possible to simultaneously remove carbon, phosphorus and nitrogen due to the accentuated oxygen diffusion gradient from the surface towards granule core, where even in the oxic phase, anaerobic/anoxic metabolic processes can occur within the particles (Rollemberg *et al.*, 2018; Klein *et al.*, 2022).

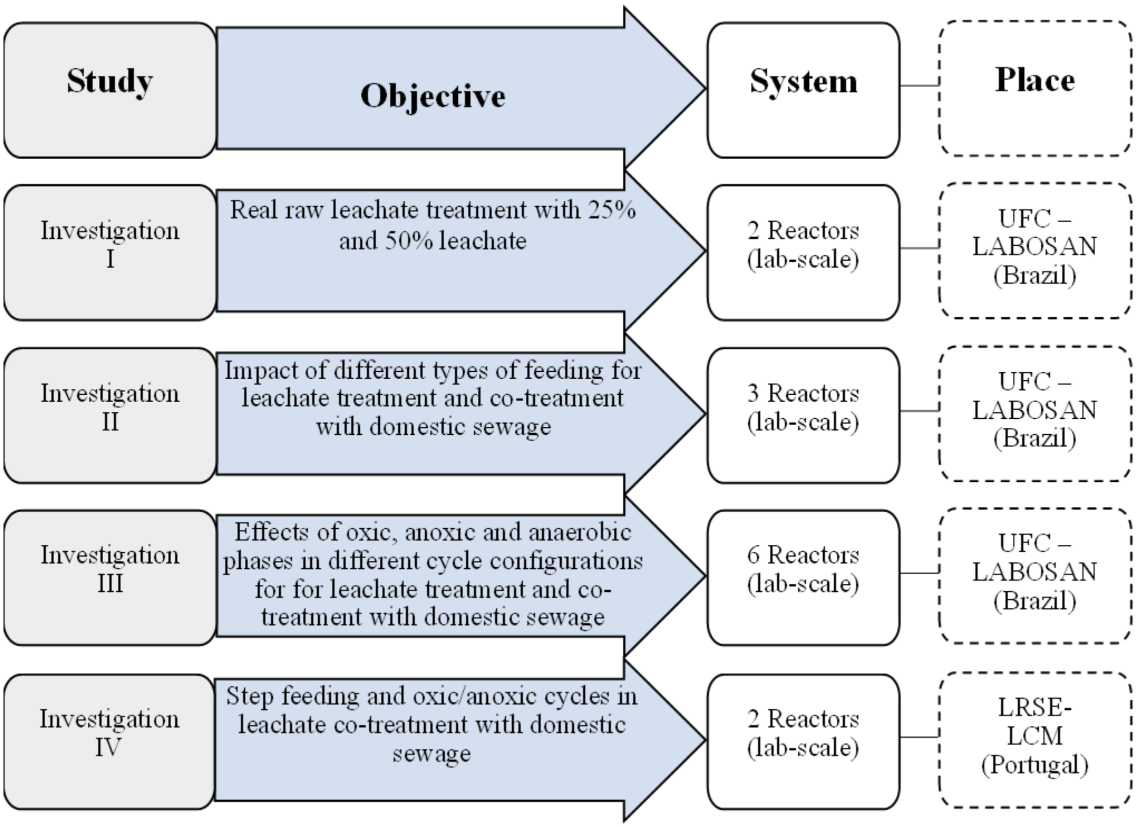
Until then, the AGS technology has been little studied in the treatment of leachate, generating different results and without a specific pattern. Wei *et al.* (2012), Bella & Torregrossa (2014) and Wei *et al.* (2021) investigated the application of physical-chemical pretreatment and optimizations in the aeration system. Ren *et al.* (2017a), Ren *et al.* (2017b), Ren, Ferraz, Yuan (2017b) and Wei *et al.* (2021) evaluated the effect of different leachate dilutions on the formation, stability and performance of granular systems. Ren, Ferraz, Yuan (2017a), Bueno *et al.* (2020) and Saxena *et al.* (2022) studied the co-treatment of leachate with domestic sewage. In all these investigations, Chemical Oxygen Demand (COD) removal ranged from 20 to 90%. Total Nitrogen (TN) removal was low and less than 50% (Bella & Torregrossa, 2014; Ren *et al.*, 2017a; Ren *et al.*, 2017b; Ren, Ferraz, Yuan, 2017a; Ren, Ferraz, Yuan, 2017b). Phosphorus removal, when it occurred, ranged from 34 to 64%. From these studies, it is possible to verify that the main common problems when using AGS technology to treat leachate were: biomass loss, long time to granulate, low granule growth and accumulation of NO_x compounds due to low denitrification efficiency. Thus, different operational strategies and cycle optimizations should be investigated to minimize these problems.

Feed form has been reported as one of the factors that affect selection and stabilities in granular systems (De Kreuk, Heijnen and van Loosdrecht, 2005; Hamza *et al.*, 2018), thus, optimizations in this operating phase have been studied and may be promising for high complexity effluents. Thus, step-feeding seems to be a good solution to increase the efficiency of nitrogen removal and favor granulation, because the feed is distributed throughout the cycle and thus, the toxic load is minimized, creating conditions for the denitrification occurs at low organic loads (Chen *et al.*, 2011. Wang *et al.*, 2012).

Furthermore, the intercalation of oxic and anoxic phases within the reaction cycle directly influences the selection pressure and favors granular stability and nitrogen and phosphorus removal (de Kreuk *et al.*, 2004; Kein *et al.*, 2022). In the anoxic phase, the absence of electron acceptors favors the growth of slow-growing microorganisms to the detriment of heterotrophic bacteria, metabolizing easily biodegradable compounds into storage polymers such as polyhydroxyalkanoates (PHAs), which increase the density of the biomass. In the subsequent oxic phase, these reactions are reversed, generating an equilibrium and strengthening of the granular biomass (Nancharaiah & Reddy, 2018; Klein *et al.*, 2022).

In this context, this work sought to optimize SBR-AGS reactors for leachate treatment and co-treatment with domestic sewage, regarding granule formation and stability, and simultaneous C, N and P removal capacity. The studies started with the granule formation and stability of the system during the treatment of a real raw leachate with 25% and 50% leachate only diluted in tap water. Soon after, synthetic effluent was used with a C:N:P ratio similar to that of a real leachate to obtain the best operational configuration for leachate treatment and co-treatment without NO_x accumulation, biomass loss and long times to reach granulation. After defining these best operating configurations, experimental tests were carried in leachate co-treatment with domestic sewage. The work was divided into four parts, as presented in Figure 1.

Figure 1 – Thesis general structure.



Source: Prepared by the author.

2 OBJECTIVE

2.1 General objective

To evaluate and optimize the granulation process, stability and performance of aerobic granular sludge (AGS) in reactors focusing on leachate treatment and co-treatment with domestic sewage.

2.2 Specific objectives

(i) To evaluate the possibility to treat in AGS systems real raw leachate with 25% and 50% leachate diluted in tap water (Chapter 5);

(ii) To analyze the leachate effect on the microbial community composition during the granulation process (Chapter 5);

(iii) To study the effect of feeding strategy on the formation, maintenance, stability and performance of aerobic granules (Chapter 6);

(iv) To analyze the impact of oxic, anoxic and anaerobic phases in different cycle configurations, on the physicochemical characteristics of granular biomass and on the performance of carbon, nitrogen and phosphorus removal (Chapter 7);

(v) To evaluate the effect of alternating oxic and anoxic phases on NO_x production and granular biomass stability from effluents with high C:N loads (Chapter 7);

(vi) To evaluate the leachate co-treatment with domestic wastewater in aerobic granular sludge reactors (Chapter 8);

(vii) To verify the advantages of a step-feeding during the simultaneous nitrification and denitrification process in leachate co-treatment with domestic sewage (Chapter 8);

(viii) To analyze the microbiological community dynamics and the physicochemical characteristics of aerobic granules previously formed in SBR-AGS reactors from the leachate co-treatment with domestic sewage (Chapter 8).

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3 LITERATURE REVIEW

Over the past century, with the fast population growth and rate of urbanization and industrialization, global waste generation has risen significantly to the point where it became one of the world's biggest challenges (Hoornweg *et al.*, 2013). In 2016, the municipal solid waste (MSW) production worldwide was about 2.01 billion tons per year, and it is estimated by 2050 that this value will reach 3.40 billion tons (Kaza *et al.*, 2018). Despite all the efforts decision-makers devoted to implementing management policies and raising awareness among the population towards more sustainable consumption habits, the increasing waste production will remain a barrier to sustainable development due to the risk it poses to the environment and public health (Environment Agency, 2007; European Parliament and Council of the European Union, 2008).

Among the different waste disposal methods, landfilling is still the most common worldwide, with around 40% of the total MSW (Kaza *et al.*, 2018), mostly due to its simplicity and relatively lower cost in some regions. Moreover, even if different techniques are employed, there will always be a certain amount of waste that will need to be landfilled, either because it is a by-product without any further use or is the only possible destination for such waste (McDougall *et al.*, 2001).

One of the consequences of landfilling is the generation of leachate - a high-strength liquid effluent containing a complex mixture of contaminants - that in the absence of proper treatment is a major pollution source, threatening the soil and water sources surrounding the landfill site (Christensen *et al.*, 2001; Environment Agency, 2003). Over the years, the leachate characteristics and the different biological and physicochemical treatment processes have been extensively researched and reviewed by several authors (Abbas *et al.*, 2009; Kjeldsen *et al.*, 2002; Lema *et al.*, 1988; Renou *et al.*, 2008; Wiszniowski *et al.*, 2006).

For over a century, biological wastewater treatment by conventional activated sludge (CAS) processes has been used especially due to its good cost-efficiency. However, they usually require high footprint and are very sensitive to abrupt variations of pollutants concentrations (Bengtsson *et al.*, 2019). When treating leachate biologically, some obstacles may impair their efficiency, like the presence of refractory organic matter, high concentrations of ammoniacal nitrogen (NH_3-N), heavy metals, and other toxic inhibitory substances (Renou *et al.*, 2008). For this reason, the development of a robust technology capable of attaining good treatment efficiencies even with highly contaminated wastewaters and complying with the discharge limits imposed by each country is essential.

Among the different biological processes, AGS has recently gained much interest in the wastewater treatment field for its unique characteristics that can overcome some of the barriers found in CAS treatment. AGS is a compact and cost-efficient technology characterized by a diverse microbial community capable of carrying out almost all biological conversions in a single system (Gao *et al.*, 2011). Its layered structure allows to, simultaneously, remove carbonaceous organic matter, nutrients (such as nitrogen and phosphorus species), metals, and even some aromatic compounds of difficult biodegradation along the different stages of the operation cycles (Gao *et al.*, 2011; Guo *et al.*, 2020; Sarvajith *et al.*, 2020). Also, granules with compact and denser structures result in faster settling, higher biomass concentration, and lower sludge volumes to be discarded (Guo *et al.*, 2020; Nancharaiah & Reddy, 2018).

Considering all its advantages, AGS has been extensively studied and successfully used in domestic wastewater treatment and has also shown promising results for industrial and other high-strength effluents (Abdullah *et al.*, 2011; Corsino *et al.*, 2018; Rosman *et al.*, 2014). For example, Rosman *et al.* (2014) reported removal efficiencies of 98.4% and 92.7% for chemical oxygen demand (COD) and ammonia, respectively, and 89.5% for total nitrogen in treating rubber wastewater by AGS. In another study reported by de Graaff *et al.* (2020), mature and stable granules were obtained in the treatment of seawater together with domestic sewage, achieving phosphorus removal values above 90%.

Nevertheless, even though AGS technology emerged just over two decades ago, there are several gaps to be filled regarding its application to high-strength wastewaters such as landfill leachates (Miao *et al.*, 2019; Rani *et al.*, 2020; Ren *et al.*, 2017b; Ren *et al.*, 2017c). This review aims to summarize the state-of-the-art and critically analyze AGS application for the treatment of high-load and recalcitrant wastewaters, focusing on organic matter and nitrogen species removals from landfill leachates. Hence, the main drawbacks and knowledge gaps concerning previous studies of leachate treatment by AGS will be addressed, and some prospects for future applications will also be presented.

3.1 Leachate generation and characteristics

3.1.1 Leachate generation

Due to population and economic growth, solid waste generation has exponentially increased, with an estimation to reach 2.2 billion tons by 2025, according to World Bank forecasts (Iskander *et al.*, 2018). Considering that solid waste disposal in landfills is the most common waste management strategy involving lower costs and low maintenance requirements, attention should be paid to leachate generation since a ton of waste can generate between 0.05 and 0.2 tons of leachate during the stabilization process at the landfill (Wang *et al.*, 2016).

Landfill leachate is a type of wastewater characterized by a high concentration of several pollutants, making this effluent a major threat to the environment and public health. Its generation is mostly related to the infiltration and percolation of water from precipitation and surface runoff through the landfill and to the moisture content of the waste, which by compression and biochemical reactions, will be released throughout time (Chelliapan *et al.*, 2020; Oller *et al.*, 2011).

3.1.2 Leachate composition

Leachate composition and pollutant load can fluctuate significantly over time. Nonetheless, four main groups of pollutants are often used to characterize leachates (Kjeldsen *et al.*, 2002): i) organic matter (biodegradable and refractory, like humic and fulvic acids), usually assessed in terms of COD, dissolved organic carbon (DOC) and 5-day biochemical oxygen demand (BOD_5), ii) inorganic compounds, such as nitrogen compounds (measured as total nitrogen-TN, nitrite-NO_2^-, nitrate-NO_3^- and ammonia-NH_4^+), phosphorus (P), chloride (Cl^-), sulfate (SO_4^{2-}) and some sulfide species, carbonate (CO_3^{2-}) and bicarbonate (HCO_3), iron (Fe^{2+} and Fe^{3+}), calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^+), sodium (Na^+), among others, iii) organic compounds, for instance, polyaromatic hydrocarbons (PAHs), perfluorinated compounds (PFCs), pharmaceuticals and pesticides, and iv) heavy metals. Besides determining these contaminants, other parameters can also be evaluated when characterizing leachates, such as color, turbidity, pH, conductivity, total and volatile suspended solids (TSS and VSS, respectively), among others.

Inside the landfill, the decomposition of organic matter generates a variety of compounds that will constitute the leachate composition. Humic substances are formed through complex chemical and biological reactions during the humification process and, due to the covalent bonds of the aliphatic and aromatic fractions, these substances are hydrophilic in the medium (Kjeldsen *et al.*, 2002; Iskander *et al.*, 2018). As a result of the diversity of precursors, types of waste, and environmental conditions, humic substances are macromolecules with very heterogeneous structures, often represented by humic and fulvic acids. In addition, the landfill age greatly influences their formation, with higher concentrations of humic acids being frequently found in older leachates compared to fulvic acids. Some authors also point out that humic substances are resistant to biological degradation, requiring more specific treatments since the multiple redox states increase their recalcitrance (Iskander *et al.*, 2018; Chelliapan *et al.*, 2020).

3.1.3 Impact of different factors in leachate characteristics

Many aspects can contribute to leachate's heterogeneity, such as: i) landfill age, ii) nature and composition of the waste deposited, iii) climate conditions and its seasonal variation (mean ambient temperature, precipitation, surface runoff, water permeation, and evaporation), and iv) landfill characteristics (topography, depth, temperature inside the waste cell, among others) (Bhalla *et al.*, 2013; Gao *et al.*, 2015; Renou *et al.*, 2008).

Since the beginning of its operation, a landfill goes through different stages (aerobic, acetogenic, methanogenic, and stabilization) as the deposited waste decomposes, leading to the formation of distinct types of leachates. Regarding the landfill age, the leachate produced can be categorized as young (<5 years), intermediate (5-10 years) and mature/stabilized (>10 years) (Kjeldsen *et al.*, 2002; Miao *et al.*, 2019; Renou *et al.*, 2008), and its characteristics vary throughout this time (Table 1).

At an early stage of the landfill (aerobic), the oxygen present in the waste interstices is consumed fast and is not renewed as more waste is deposited. The consequent absence of oxygen creates an anaerobic environment that favors the growth of specific microorganisms inside the waste cell, such as hydrolytic, fermentative, and acetogenic bacteria (Kjeldsen *et al.*, 2002).

Table 1 – Leachate composition based on landfill age (Gao *et al.*, 2015; Yadav & Dikshit, 2017; Tejera *et al.*, 2019; Meloni *et al.*, 2021; Mojiri *et al.*, 2021).

Parameter	Young	Intermediate	Old
Landfill age (years)	< 5	5 – 10	> 10
pH (Sørensen's scale)	< 6.5	6.5 – 7.5	> 7.5
COD (g/L)	> 10	5 – 10	< 5
BOD_5/COD	0.5 – 1.0	0.1 – 0.5	< 0.1
NH_4^+-N (mg/L)	< 400	400	> 400
Heavy metals (mg/L)	> 2	< 2	< 2

Organic species [a)]	80% VFA	5-30% VFA+HA+FAc	HA+FAc
TSS (mg/L)	> 1500	< 1000	< 1000
Landfill stage	Acetogenic	Transition	Methanogenic
Biodegradability	High	Medium	Low

[a)] Predominant organic compounds for each landfill stage.
COD – Chemical Oxygen Demand; BOD_5 – 5-day Biological Oxygen Demand; NH_4^+-N – Ammonium nitrogen; TSS – Total Suspended Solids; VFA ─ Volatile fatty acids; HA ─ Humic acids; FAc ─ Fulvic acids.
Source: Prepared by the author.

The hydrolysis of the organic matter and conversion of the resulting amino acids, monosaccharides, fatty acids, and other polymers into carboxylic acids, carbon dioxide (CO_2), and hydrogen (H_2) by fermentative and acetogenic bacteria is the first step of the waste degradation process (Kjeldsen *et al.*, 2002; Lema *et al.*, 1988). This phase generates a leachate with high concentrations of BOD and COD (mostly composed by volatile fat acids - VFAs), higher BOD/COD ratio (increasing leachate's biodegradability), and lower pH values due to the high concentrations of VFAs (Bohdziewicz and Kwarciak, 2008; Umar *et al.*, 2010; Wei *et al.*, 2012). Large alkalinity consumption would be necessary to neutralize the acid production and prevent pH drop. If the available alkalinity is not sufficient, the pH decrease may affect the activity of some microorganisms (Ren *et al.*, 2017b). Additionally, although heavy metals concentration in leachates is usually relatively low (Bueno *et al.*, 2020; Robinson, 1995), the lower pH values found in these early stages of the landfill increase their solubility into the leachate. Hence, higher concentrations of heavy metals may be reported for young leachates (Christensen *et al.*, 2001; Umar *et al.*, 2010).

Over the years, as the landfill matures and enters the methanogenic phase, the conversion of the reaction products from the previous stage into methane (CH_4) and CO_2 by methanogenic microorganisms increases considerably. The consumption of VFAs results in a rise in leachate pH values and a decrease in BOD and COD content, with lower BOD/COD ratios. When the landfill enters the stabilization phase, the CH_4 production rate reaches its maximum and stabilizes for several years, depending on the hydrolysis rate of the organic content present in the landfill (Kjeldsen *et al.*, 2002). Since methanogenic microorganisms predominate in this stage, the fraction of VFAs generated is quickly consumed. Thus, the remaining COD is mainly composed of refractory organic matter, like humic and fulvic acids, which present a great solubility in water (Kjeldsen *et al.*, 2002; Renou *et al.*, 2008; Umar *et al.*, 2010). The COD values usually vary between 500-4500 mg O_2/L, and the BOD/COD ratios are normally below 0.1, which is associated with the low biodegradability often found in mature leachates.

Furthermore, the heavy metals solubility in the leachate is reduced due to the higher pH found in older landfills, which allows the formation of metal precipitates, along with sorption processes on the colloidal matter surface, decreasing leachate toxicity (Iskander *et al.*, 2018; Rani *et al.*, 2020; Wiszniowski *et al.*, 2006). Concerning the nitrogen compounds, ammonia represents a considerable fraction of the total nitrogen present in leachate (Hamza *et al.*, 2019; Miao *et al.*, 2019). Ammonium nitrogen (NH_4^+-N) is mainly formed via hydrolysis and fermentation of the biodegradable organic matter during proteins degradation. Its concentration tends to increase with landfill age and be very stable under anaerobic conditions (Oliveira *et al.*, 2014; Umar *et al.*, 2010). Contrary to soluble organic substances, the release of nitrogen compounds into the leachate proceeds for an extended period, constituting a problem to biological systems due to its toxicity and inhibitory effect on certain microorganisms (Boonnorat *et al.*, 2018; Ren *et al.*, 2017b).

3.1.4 Microbial communities

In landfills, leachate treatment occurs through several microbial biodegradations and biotransformations of organic and inorganic molecules (Köchling *et al.*, 2015). Therefore, landfill leachate hosts a great diversity of microbial communities, reaching more than 100 different types of genetic sequences and more than 10,000 taxonomic units (Sogin *et al.* 2006; Meyer-Dombard and Malas, 2020), presenting a complex taxonomy that helps the biological treatment.

The most abundant taxonomic groups are microbial communities with individuals from the bacterial phyla Firmicutes, Proteobacteria, and Bacteroidetes. Populations of archaea, which typically consist of methanogenic species, are also found (Köchling *et al.*, 2015; Remmas *et al.*, 2017; Song *et al.*, 2015; Zhang *et al.* 2012). These same authors report that Firmicutes is the dominant phylum in all leachate types, given its dominance and known ability to break down a wide variety of frequently recalcitrant organic compounds. Within the Firmicutes, the dominant class is Clostridia (fermentative acetogens), composed mainly of genera such as Syntrophomonas, Sedimentibacter, Clostridium and Pelotomaculum (Remmas *et al.*, 2017; Meyer-Dombard and Malas, 2020). The growth of Sedimentibacter is supported by fermentation of pyruvate or amino acids, while Clostridium uses carbohydrates and/or proteins, depending on the species. The final fermentation products are VFAs (mainly acetate, propionate and butyrate) and, in the case of Clostridium, also short-chain alcohols and hydrogen. Syntrophomonas is usually found together with methanogenic archaea, with which it syntrophically degrades fatty acids. Song *et al.* (2015) also point out that Pseudomonas (known for degrading recalcitrant organic compounds) is the dominant group in the phylum Proteobacteria, reaching 92.4% of the total abundance.

Importantly, the decomposition stage and landfill age greatly affect the microbial community structure. As the landfill becomes older, biodiversity increases, presenting a diverse specialized bacterial community capable of degrading recalcitrant organic compounds and resisting the high concentrations of heavy metals accumulated in this effluent (Remmas *et al.*, 2017). With the increasing age of the landfill, the abundance and diversity of the phylum Firmicutes also increase. The abundance of the phylum Proteobacteria decreases, giving rise to the phylum Spirochaetes, which also becomes dominant in this type of leachate. The Bacteroidetes count does not show a linear trend over time (Köchling *et al.*, 2015).

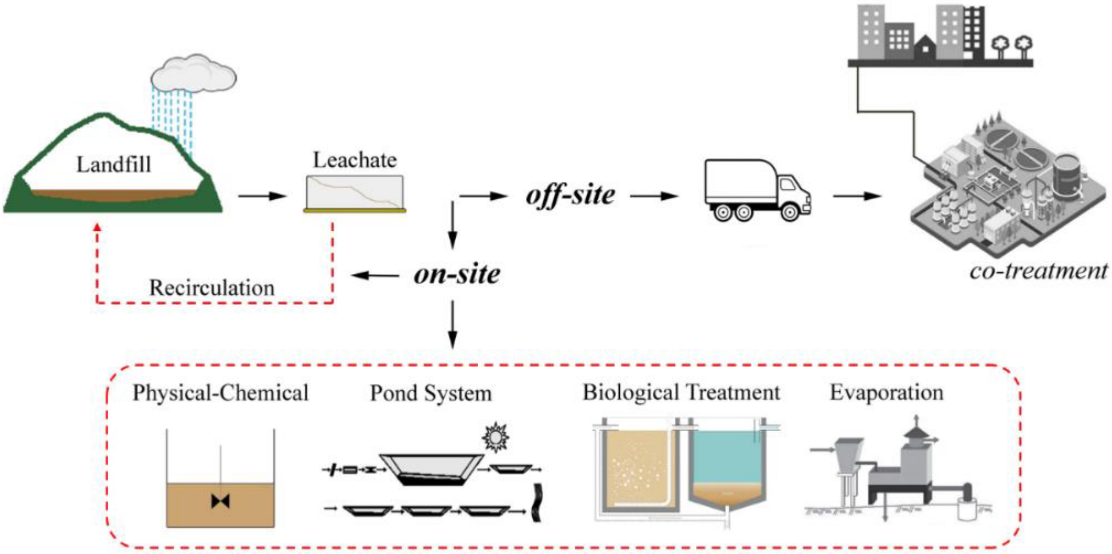
In particular, old landfill leachate can serve as a reservoir for isolating specialized degrading bacteria, which can be used in the bioremediation and bioaugmentation of toxic compounds accumulated in contaminated soils and aquatic environments. For example, strains of Pusillimonas were involved in the bioremediation of aged soils polluted with creosote (Lladó *et al.*, 2013; Remmas *et al.*, 2017).

3.2 Landfill leachate treatment

3.2.1 Selection of the treatment process

In virtue of leachate's high pollutant load, applying a treatment strategy before disposal is mandatory, which can either be performed externally (off-site treatment) or on the landfill site (on-site treatment) (Figure 2).

Figure 2 – Off-site and on-site leachate treatment processes.



Source: Prepared by the author.

For many years, the off-site co-treatment of leachate with municipal sewage in wastewater treatment plants (WWTPs) was very common due to its simplicity, smaller investment in structures, and lower operation/maintenance costs (Campos *et al.*, 2019; Renou *et al.*, 2008). In addition, this mixture reduces leachate toxicity through its dilution with domestic wastewater and may also increase biodegradability by balancing the carbon-nitrogen-phosphorus ratio (Dereli *et al.*, 2021; Ferraz *et al.*, 2016; Gao *et al.*, 2015). However, some disadvantages have contested this strategy's effectiveness, like the high loads of slowly biodegradable organic compounds, ammonium nitrogen, heavy metals, and other inhibitory substances to the biological processes of the municipal WWTPs, hindering the compliance with the discharge limits (Dereli *et al.*, 2021).

Nonetheless, for on-site treatment, the operation and maintenance of a Leachate Treatment Plant (LTP) on the landfill location is required, which can combine different physical, chemical, and biological processes (Campos *et al.*, 2019; Ferraz *et al.*, 2016; Yuan *et al.*, 2016). The variability in leachate composition with landfill aging, like the increase in ammonia content and lower COD (mainly refractory organics), not only increases leachate toxicity but also creates nutrient imbalances that can significantly impair its biological treatment, making the conventional treatment methods less efficient (Brennan *et al.*, 2017; Wu *et al.*, 2015). It has been reported that in high concentrations, free ammonia (FA) and free nitrous acid (FNA) can strongly inhibit the activity of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), reducing the effectiveness of the nitrification process (Ferraz *et al.*, 2016; Soliman and Eldyasti, 2018; Wang *et al.*, 2016; Yang *et al.*, 2004). Additionally, the lack of biodegradable organic carbon sources can also harm denitrification, which leads to lower nitrogen removal efficiencies (Miao *et al.*, 2014; Peng *et al.*, 2008). In such cases, the external addition of nutrients and readily biodegradable carbon sources to the biological process is often used to improve the carbon and nitrogen removal from leachate, consequently raising the treatment costs (Dereli *et al.*, 2021; Zhao *et al.*, 2012). Therefore, adding complementary treatment steps to remove recalcitrant compounds becomes necessary (Di Iaconi *et al.*, 2006).

3.2.2 Physicochemical processes

Leachate treatment by physicochemical processes is commonly used as a complementary step to biological treatment. Particularly in older leachates, where BOD_5/COD ratio is very low and refractory organics are predominant, the biological treatment is usually insufficient to achieve the desired characteristics for discharge. Thus, some physical and chemical processes can be included in the treatment strategy, either as a pre-treatment to eliminate target compounds that inhibit the following biological processes or as a final polishing step to remove the remaining recalcitrant organic matter and some toxic substances (Renou *et al.*, 2008).

Several researchers have studied in the past different physical and chemical processes, such as flotation (Palaniandy *et al.*, 2010; Zouboulis *et al.*, 2003), coagulation/flocculation (CF) (Amokrane *et al.*, 1997; Tatsi *et al.*, 2003; Zamora *et al.*, 2000), chemical precipitation (Altinbaş *et al.*, 2002; Calli *et al.*, 2005; Li *et al.*, 1999), chemical oxidation/advanced oxidation processes (AOPs) (Chen *et al.*, 2019; Oulego *et al.*, 2016; Silva *et al.*, 2016; Zhang *et al.*, 2005), adsorption onto activated carbon (Imai *et al.*, 1995; Kargi and Pamukoglu, 2003; Morawe *et al.*, 1995; Rodríguez *et al.*, 2004), ion exchange (Boyer *et al.*, 2011; Fernández *et al.*, 2005), and membrane filtration (Marttinen *et al.*, 2002; Pirbazari *et al.*, 1996). The combination of different methods (physicochemical and biological) has proven to be the most efficient way to remove both recalcitrant organic matter (shown by the low COD values after treatment) and ammonium nitrogen from stabilized leachates (Kurniawan *et al.*, 2006; Marttinen *et al.*, 2002). It is noteworthy that this choice must always consider the initial leachate characteristics.

However, the use of physicochemical processes can also have some disadvantages, like the higher costs associated with energy consumption and chemicals addition to the system, and the production of high volumes of sludge and subsequent need for its treatment/disposal. The limited applicability and chance of toxic by-products formation have also been reported as an inconvenience (Kurniawan *et al.*, 2006).

3.2.3 Biological processes

Conventional activated sludge systems are, amongst biological processes, the most commonly used in leachate treatment. As previously mentioned, these systems often come across a few obstacles related to recalcitrant organic matter and nutrients removal (Chys *et al.*, 2015). The presence of toxic aromatic compounds, large ammonium nitrogen concentrations, and high salinity environments not only can significantly inhibit AOBs and NOBs and compromise the nitrification process but may also negatively affect the biomass settling properties (Deng *et al.*, 2018; Park and Bae, 2009; Ramos *et al.*, 2015).

Additionally, the secondary clarifiers in CAS systems are often affected by sludge bulking due to the excessive growth of filamentous bacteria, resulting in poor settleability, low sludge compaction, and high concentrations of suspended solids in the treated effluent (Li *et al.*, 2011; Ren *et al.*, 2017b; Zou *et al.*, 2019). Sludge bulking can occur when the food to microorganisms (F/M) ratio and dissolved oxygen concentrations are low but may also be a consequence of high concentrations of sulfide, oils and greases, or when organic substrates are rapidly metabolized (Li *et al.*, 2011; Martins *et al.*, 2004; Zou *et al.*, 2019). Consequently, there will be a reduction in the abundance of slow-growing microorganisms, usually nitrifying or biodegrading microorganisms with low growth kinetics. Likewise, during the denitrification process, the conversion of the high nitrogen content (resulted from nitrite and/or nitrate accumulation) into gaseous nitrogen (N_2) can lead to sludge loss and formation of scum on the secondary clarifier surface and in the anoxic zones of the aeration tank (Zhang *et al.*, 2017).

Besides CAS systems, other technologies have been reported in the literature regarding biological leachate treatment, such as sequencing batch reactor (SBR) (Uygur and Kargı, 2004), membrane bioreactor (Xue *et al.*, 2015), moving bed biofilm reactor (Chen *et al.*, 2008), fluidized-bed biofilm reactor (Eldyasti *et al.*, 2010), rotating biological contactor (Castillo *et al.*, 2007), trickling filter (Matthews *et al.*, 2009), and also anaerobic systems like anaerobic filter (Wang and Banks, 2007), up-flow anaerobic sludge blanket reactor (Castillo *et al.*, 2007), among others. However, most of the difficulties noticed in CAS processes were also found in these systems (Deng *et al.*, 2018; Peng *et al.*, 2018).

As an alternative, AGS technology can tolerate high pollutant loads in the influent and still achieve COD, TN, and total phosphorus (P_{Total}) removals above 90% (Nancharaiah *et al.*, 2018). Most of the microbial groups in aerobic granules are resistant to the toxic compounds present in the leachate without compromising their performance and granules' stability (Ren *et *al.*, 2017a; Ren *et al.*, 2017c). In addition, when compared to CAS systems, AGS has lower associated costs (less 20-25% in operation and 23-40% in energy consumption) and lower footprint (50-75% lower) (Bay Area Clean Water Agencies, 2017; Bengtsson *et al.*, 2019). Therefore, AGS systems emerge as a promising technology to replace obsolete conventional biological systems. Despite being an excellent alternative for the treatment of domestic and industrial wastewaters, further investigations are required with landfill leachates, such as the need for dilution, physicochemical pre-treatment, external carbon source addition, different cycle times, among others. Additionally, pilot-scale studies for medium and long-term evaluation of leachate effect on the aerobic granule properties and efficiency of simultaneous removal of pollutants are needed.

3.3 Aerobic granular sludge technology in leachate treatment

Aerobic granules consist of dense spherically shaped aggregates of microorganisms bounded through physical, chemical, and biological phenomena (Liu and Tay, 2004). Their large size and compact structure provide exceptional settling abilities and great water-sludge separation, producing lower and very concentrated sludge volumes, thus eliminating the need for a secondary clarifier (Franca *et al.*, 2018). The main advantages of AGS compared to other biological systems are: i) retention of high biomass concentrations in the bioreactor, ii) presence of different redox microenvironments (anaerobic, anoxic, and aerobic regions) due to its layered structure; iii) possibility of controlling different metabolic reactions through the adjustment of dissolved oxygen concentrations, iv) metabolic cooperation between autotrophic and heterotrophic microorganisms, and v) capacity to withstand high influent loads and hydraulic shocks (Gao *et al.*, 2011; Nancharaiah & Reddy, 2018; Rosman *et al.*, 2014; Zheng *et al.*, 2020).

Several studies have shown that AGS is capable of treating high-strength effluents containing large concentrations of ammonia (Wei *et al.*, 2012), organic matter (Xiong *et al.*, 2020), phosphorus (de Graaff *et al.*, 2020), and even aromatic compounds (Ramos *et al.*, 2015), being an interesting biological alternative for leachate treatment (Ren, Ferraz and Yuan, 2017b). Even though AGS technology has been applied in the most diverse wastewater treatment fields, the number of studies using this technology in leachate treatment is relatively small. So far, advances in the application of AGS to treat leachate can be divided into three main phases: i) investigation of the need for pre-treatment and optimization of the AGS reactor aeration system (2012-2014), ii) study of different dilutions of leachate influent to the AGS and comparative analysis with activated sludge reactors (2014 to 2017), and iii) leachate co-treatment with domestic sewage (2017 to 2020).

Wei *et al.* (2012) carried out one of the first studies using aerobic granules to treat municipal landfill leachate, with and without pre-treatment for NH_4^+-N removal. The pre-treatment favored the granulation process since the high NH_4^+-N concentrations impaired nitrogen and COD removals. On the other hand, in work reported by Di Bella & Torregrossa (2014), the nitrogen removal was satisfactory without any pre-treatment due to the acclimatization period of the granules with leachate during its cultivation. This is very important for selecting specific/specialized microorganisms to degrade the compounds present. The progress on studies applying aerobic granular biomass in leachate treatment has allowed the comparison of performance with other systems, especially with CAS. The superiority of AGS over CAS was evidenced, both in COD and nitrogen removals, in addition to granules being less sensitive to high loads and toxicity (Ren *et al.*, 2017a; Ren *et al.*, 2017b; Ren *et al.*, 2018).

The third phase of studies evaluated the possibility of co-treating leachate with domestic wastewater, admitting leachate proportions between 20% to 60% (Ren, Ferraz and Yuan, 2017a). This mixture significantly benefited the carbon and nitrogen removals, but the efficiency decayed considerably for higher pollutants concentrations (Bueno *et al.*, 2020). The lack of acclimatization period or higher leachate ratios may decrease the Mixed Liquor Suspended Solids (MLSS) concentrations due to granules disintegration and biomass washout (Table 2). When the solids loss is superior to biomass growth, a decrease in MLSS concentration is observed, as previously reported (Bueno *et al.*, 2020; Di Bella & Torregrossa, 2014). Therefore, the composition of the leachate influent to the system has a significant impact on the granulation process.

Table 2 – Biomass retention in the reactor: effect of the granulation process and type of leachate treated in the concentration of mixed liquor suspended solids.

Leachate type	Granulation process	Initial MLSS [a] (mg/L)	Final MLSS [a] (mg/L)	References
Leachate pre-treated for NH_3-N removal	Leachate + PAC [b]	4000	3116	(Wei *et al.*, 2012)
Leachate without pre-treatment	Leachate + PAC [b]	4000	3083	
Real leachate diluted with tap water up to a COD of 9700 mg/L	Synthetic wastewater (with lower pollutant loads)	11000	< 5000	(Di Bella & Torregrossa, 2014)
Leachate diluted with synthetic wastewater up to a COD of 4500 mg/L	Synthetic wastewater (with lower pollutant loads)	11000	< 5000	
Synthetic old leachate [c]	Synthetic old leachate [c]	8070	8070	(Ren *et al.*, 2017a)
Leachate diluted with municipal wastewater (leachate volume ratio 10 – 40%)	Municipal wastewater	3215	6000	(Ren, Ferraz and Yuan, 2017a)
Leachate diluted with municipal wastewater (leachate volume ratio: 60%)	Municipal wastewater	6000	10000	
Leachate diluted with municipal wastewater (leachate volume ratio: 90%)	Municipal wastewater	10000	9000	
Leachate diluted with municipal wastewater (leachate volume ratio:10 – 65%)	Leachate (ratio: 10%) diluted with municipal wastewater	2591	6476	(Ren, Ferraz and Yuan, 2017b)
Leachate diluted with municipal wastewater (leachate volume ratio: 65 – 90%)	Leachate (ratio: 10%) diluted with municipal wastewater	6476	14533	
Leachate diluted with municipal wastewater (leachate volume ratio: 90 – 100%)	Leachate (ratio: 10%) diluted with municipal wastewater	14533	12707	
Leachate diluted with synthetic wastewater: 5%	Leachate + Synthetic wastewater (low pollutant load) [d]	3325	1525	(Bueno *et al.*, 2020)
Leachate diluted with synthetic wastewater: 10%	Leachate + Synthetic wastewater (low pollutant load) [d]	1525	2695	
Leachate diluted with synthetic wastewater: 20%	Leachate + Synthetic wastewater (low pollutant load) [d]	2695	2776	

[a] MLSS – Mixed Liquor Suspended Solids. Initial MLSS: value before granulation; Final MLSS: value after the leachate treatment by AGS.
[b] PAC – Powder Activated Carbon.
[c] Tannic acid used to simulate the refractory organic matter.
[d] Acclimatization period (40 days) only with synthetic wastewater.
Source: Prepared by the author.

Some parameters are essential to evaluate AGS reactor operation and control treatment efficiency, such as sludge retention time, dissolved oxygen concentrations, cycle duration, and settling phase (Table 3). They will influence the granules' formation, structure, stability, the bacteria distribution inside the granule, and their metabolic reactions (Franca *et al.*, 2018). Nevertheless, the use of SBR reactors to treat leachate with AGS technology still presents many gaps to be filled, requiring further studies.

Table 3 – Main operating parameters of AGS reactors reported on literature for leachate treatment (Bueno *et al.*, 2020; Di Bella & Torregrossa, 2014; Ren *et al.*, 2017c; Ren *et al.*, 2018; Wei *et al.*, 2012).

Parameters	Reference value
Leachate dilution	10 – 60%
Concentration of ammoniacal nitrogen	< 788 mg NH_3-N/L
Concentration of solids in the reactor	> 3 g TSS/L
Cycle time	12 – 24 h
SRT[a)]	< 30 d
Dissolved oxygen	2 – 4 mgO_2/L
Settling time	< 10 min
Volumetric exchange ratio	50 %
SVI_5/SVI_{30} [a)]	1.2 – 1.8

[a)] SRT – Sludge Retention Time; SVI_5 – Sludge Volume Index (after 5 min of settling); SVI_{30} – Sludge Volume Index (after 30 min of settling).
Source: Prepared by the author.

3.3.1 Formation and maintenance of the granules

One of the main differences between AGS and other types of biomasses is the higher extracellular polymeric substances (EPS) production (Nancharaiah & Reddy, 2018; Sun *et al.*, 2015). The formation of stable and mature granules will depend highly on the operating conditions and environmental stress they are subjected to (Franca *et al.*, 2018).

Aerobic granules are usually produced using activated sludge for the reactor startup. Upon the first contact with the leachate, the sludge in the AGS system starts to present a flocculating structure and dark brown color (Bueno *et al.*, 2020; Wei *et al.*, 2012). The time necessary for granulation to occur will depend on the influent characteristics. In other words, high carbon and nitrogen loads can significantly delay granule formation (Szabó *et al.*, 2016).

Bueno *et al.* (2020) reported granules formation in the first 40 days of reactor operation after the acclimatization period. During this period, the initial flocs in biomass went from a diameter of 10-95 µm to the formation of granules with a diameter of 382-421 µm in 45% of the biomass. At high loads, irregularities in the surface and structure of the granules are common, even after process stability. Thus, after the leachate incorporation, it has been observed that within 60 days, the biomass presented good aggregation, excellent settling properties, and a majority of irregular granules (Ren *et al.*, 2017b; Ren *et al.*, 2018; Wei *et al.*, 2012). The proportion of leachate diluted with domestic wastewater also influences the size of the granules, i.e., higher leachate ratios produce smaller granules and vice-versa (Bueno *et al.*, 2020; Ren *et al.*, 2017c).

Special attention should be paid between the 3rd and 4th months of operation. During this period, the selection pressure can still eliminate the sludge of worse quality that presents problems of settleability and difficulty to create anoxic/anaerobic zones inside the granule, causing TSS loss in the treated effluent. In addition, biomass disintegrates more easily, especially at concentrations approximately above 200 mg NH_3-N/L (Ren, Ferraz and Yuan, 2017a). However, even after observing that lower leachate concentrations favor granules' stability, Bueno *et al.* (2020) reported a significant TSS loss for the lowest leachate ratios (5 and 10%). Therefore, TSS loss during the granulation process may not depend on the leachate proportion used but instead be a consequence of the natural sludge selection occurring inside the reactors, where the less quality sludge is discarded (Ren *et al.*, 2017a; Ren *et al.*, 2017b).

Only after 90 days of reactor operation that 80-90% of the biomass was granular, with average sizes of 480-612 μm, showing good stability and without uncontrolled sludge losses (Bueno *et al.*, 2020; Ren *et al.*, 2018). After this period, the granules showed insignificant growth. However, controlling the organic load after the stabilization period is very important, as significant changes can affect granules' integrity (Di Bella & Torregrossa, 2014).

3.3.2 Sludge features in AGS systems for leachate treatment

When treating wastewater with high pollutant loads, it has been observed that parameters such as sludge volume index (SVI) and TSS present a slightly different behavior than expected for conventional loads, meaning that by increasing the COD and ammonia concentrations fed to the system, SVI tends to decrease (Abdullah *et al.*, 2011; de Kreuk & van Loosdrecht, 2004; Kocaturk and Erguder, 2015; Sarvajith *et al.*, 2020; Xiong *et al.*, 2020).

Some studies point out that the SVI_8/SVI_{30} or SVI_5/SVI_{30} ratio can be considered a good predictor of granulation in waters with high pollutant loads. In other words, a ratio: i) above 1.8 indicates the thickening of the sludge blanket, ii) between 1.2 and 1.8 indicates the predominance of aerobic granules in the biomass, and iii) closer to 1.0 shows that the sludge majority is constituted by granules (Corsino *et al.*, 2018; de Kreuk *et al.*, 2005; Hamza *et al.*, 2018; Kocaturk and Erguder, 2015; Ni and Yu, 2010; Schwarzenbeck *et al.*, 2004; Yilmaz *et al.*, 2008).

Of the few studies reported in the literature regarding leachate treatment by AGS, only some monitored SVI_{30} and SVI_5 after granulation and during the process. Therefore, it is not possible to establish a clear pattern regarding the contaminant loads and their effect on the SVI. However, there is a consensus that the settling velocity increases when the proportion of leachate incorporated into the system increases (Bueno *et al.*, 2020; Ren *et al.*, 2017b).

According to Ren *et al.* (2017b), SVIs lower than 50 are acceptable in treating effluents with high loads and guarantee a good sludge settling. In most works, granulation was obtained through low-load synthetic effluents or with low leachate proportions (Table 2). It appears that when the leachate is incorporated into the process or its proportion increases, the SVI decays (Table 4), possibly due to the granulation optimization or the frequent washouts and granules' disintegration.

In the reported studies, most MLSS are volatile and with very different concentrations (Table 4). Depending on the leachate proportion fed to the system, they may decrease (Bueno *et al.*, 2020; Di Bella & Torregrossa, 2014; Wei *et al.*, 2012) or increase with the increase in leachate ratio (Ren *et al.*, 2017b; Ren *et al.*, 2017c; 2018). In addition, very high MLSS concentrations (about 8 g/L) were achieved in some studies (Ren *et al.*, 2017c; 2018), while others did not surpass 4 g/L (Bueno *et al.*, 2020; Ren *et al.*, 2017b).

Solids loss from the mixed liquor is due to the increase in the NH_3-N load, which can negatively affect the denitrification and lead to biomass washout from the system (Bueno *et al.*, 2020; Di Bella & Torregrossa, 2014; Ren *et al.*, 2017b). Also, high leachate loads reduce cellular hydrophobicity and directly affect sludge aggregation, forming a flocculent sludge with a higher possibility of being discarded with the effluent (Ren *et al.*, 2017b; Ren *et al.*, 2017c).

Therefore, the inoculum quality is important in the recovery after loads shock. A good quality inoculum allows the system to stabilize quickly after the sludge loss period and favors high biomass growth and VSS concentrations (Ren, Ferraz and Yuan, 2017a). However, to minimize the excessive loss of solids, Bueno *et al.* (2020) suggested that some strategies can be used in these systems, such as incorporating a secondary settling tank after the AGS reactor.

In addition, high settling velocities in mixed liquor are crucial for increasing solids concentration at significant levels. Ren, Ferraz and Yuan (2017b) related the MLSS concentration to the sludge age and found that the older the sludge, the greater the MLSS concentration. However, if the sludge age is too high (for example, above 30 to 40 days), the MLSS concentration will decrease, although the concentration of VSS may still increase.

Table 4 – Physical parameters reported in the literature before and after adding leachate.

	TSS (mg/L)	VSS (%)	SVI_5 (mL/g)	SVI_{30} (mL/g)	TSS (mg/L)	VSS (mg/L)	SVI_5 (mL/g)	SVI_{30} (mL/g)	
5	5420	86	400	210	3325	2835	276	155	
10	3325	85	62	155	2695	2319	77	62	1
20	2695	86	36	77	2776	2085	36	30	
10 – 40	3215	85	45	40	6000	5300	20 – 25	15 – 20	
60	6000	88	20 – 25	15 – 20	9500	8800	20	15	2
90	9500	92	20	15	9900	7900	25	20 – 25	
10 – 65	6476	87	19	---	7500	5000	19	---	
65 – 90	7500	66	19	---	14533	8633	19	---	3
100	14533	59	19	---	12707	7878	19	---	

LD – Leachate dilution; TSS – Total Suspended Solids; VSS – Volatile Suspended Solids; SVI_5 – Sludge Volume Index (after 5 min of settling); SVI_{30} – Sludge Volume Index (after 30 min of settling). [1]Bueno *et al.*, 2020; [2]Ren, Ferraz and Yuan, 2017a; [3]Ren, Ferraz and Yuan 2017b.
Source: Prepared by the author.

3.3.3 Optimization of leachate treatment by AGS

3.3.3.1 Pre-treatment

As previously mentioned, despite presenting numerous advantages for leachate treatment, mainly regarding implementation and operational costs, the removal of nutrients (especially nitrogen) and recalcitrant compounds can be very low in biological systems. In turn, physicochemical processes have been widely used to reduce part of the influent nitrogen load from biological treatment systems or to remove recalcitrant compounds, although few studies refer to the removal of toxicity from the final effluent (Di Bella & Torregrossa, 2014; Oulego *et al.*, 2015; Queiroz *et al.*, 2011).

Previous works have used gradual dilutions, coagulants/flocculants, physical processes of separation by gravity, conventional fat removal processes, and even a previous biological treatment as leachate pre-treatment (Corsino *et al.*, 2017; Kocaturk and Erguder, 2015; Świątczak and Cydzik-Kwiatkowska, 2018). Wei *et al.* (2012) applied magnesium oxide and phosphoric acid coupling to struvite precipitation, resulting in a larger proportion of granules’ growth and higher efficiencies of simultaneous nitrification and denitrification (SND). Their main goal was to remove NH_3-N during the pre-treatment, especially FA that is considered toxic to the process. Although the retained sludge is conducive to soil fertilization, controlling its production and disposal is necessary. Attention should also be paid to the precipitant required dosage and the process sensitivity to pH (Kurniawan *et al.*, 2006).

Coagulation/flocculation satisfactorily reduces the levels of adsorbable halogenated organic compounds, suspended solids, heavy metals, polychlorinated biphenyls, and humic substances such as humic and fulvic acids. Organic colloidal compounds are thermodynamically stable, presenting negative surface charges. Thus, coagulation consists of particles destabilization by neutralizing surface electrical forces and reducing the repulsive forces between them, while flocculation aimed to increase the volume and density of the particles, which can be removed by settling or flotation (Miao *et al.*, 2019; Rani *et al.*, 2020; Yuan *et al.*, 2016).

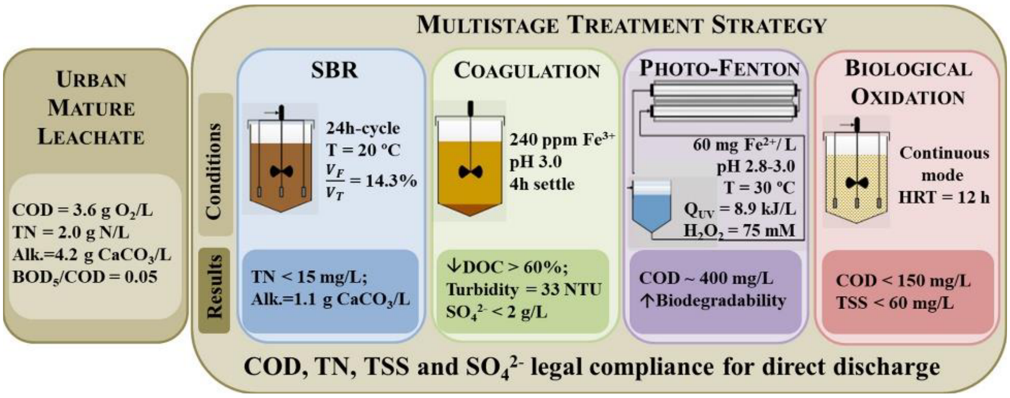
As the process efficiency depends on the molecular weight of the organic particles to be removed, CF has been more suitable for old landfills. The most frequently used coagulants are ferric chloride and aluminum sulfate due to their excellent cost-efficiency ratios. However, coagulants can reduce the final effluent quality by increasing the concentration of iron, aluminum, chloride, or sulfate (Rui *et al.*, 2012). Since there is no NH_3-N removal and leachate biodegradability is virtually unaffected, CF is commonly combined with other processes (Rui *et al.*, 2012; Torretta *et al.*, 2017).

In addition to CF and chemical precipitation, adsorption has been widely used when the goal is to remove recalcitrant and non-biodegradable organic compounds, with COD removal efficiencies over 90%. The most commonly used adsorbent is activated carbon, which efficiently removes carbon, metals, and other compounds but does not remove ammoniacal nitrogen. Still, activated carbon regeneration requires large energy consumption, which implies higher costs (Campos *et al.*, 2019; Li *et al.*, 2019; Miao *et al.*, 2019).

Alternatively to biological nitrification, ammonia stripping has been used for NH_3-N removal, eliminating volatile organic compounds as well. Mass transfer to the gas phase occurs for some constituents due to the large volume of air injected into the leachate, in which the increase in temperature and pH also favors the process (Gao *et al.*, 2015). Therefore, the exhaust gas must be properly controlled and undergo adequate treatment before being discharged into the atmosphere, thus avoiding air pollution and the release of toxic volatile compounds.

In general, besides favoring nitrification and biological denitrification, physicochemical pre-treatment makes the process more efficient and less toxic. However, due to the complex composition of the leachate, there is a need for combining different treatment strategies since there is not a single system that can remove all pollutants present in the leachate. Gomes *et al.* (2019) proposed a treatment in multiple stages combining biological processes with a physicochemical treatment and an advanced oxidation technology (AOT) (Figure 3). The first stage of the overall process took place in SBRs with 24-hour cycles so that TN and alkalinity reached values below 15 mg N/L and 1.1 g $CaCO_3$/L, respectively. In line with the multistage system reported by Silva *et al.* (2017), coagulation had the same effect by precipitating humic acids and removing colloidal and suspended material, increasing the photo-based post-treatment efficiency. Lastly, the final biological oxidation guaranteed compliance with the legal COD and TSS levels imposed by the legislation in force.

Figure 3 – Multistage treatment combining biological and physicochemical processes in the treatment of an urban mature leachate (Gomes, *et al.*, 2019).



Source: Prepared by the author.

Accordingly, due to the leachate refractory character and the high loads of organic matter and nitrogen, the integration of physicochemical processes with biological oxidation has proved to be an excellent alternative. The association of both types of technologies, in addition to reducing the concentration of organic and nitrogen species, also removes humic and fulvic acids, which present low removals in isolated biological processes. Moreover, the leachate pre-treatment in AGS systems should occur using coagulants that do not cause sudden changes in pH, such as aluminum-based coagulants (Rui *et al.*, 2012; Wiszniowski *et al.*, 2006). Nevertheless, there is still a lot to explore regarding combining AGS reactors with physicochemical systems, especially in pilot- or full-scale applications.

3.3.3.2 Post-treatment

When treating a young to intermediate leachate, the efficiency of the physicochemical treatments integrated with biological treatment has been satisfactory, minimizing the disadvantages of each process alone. However, with the aging of landfills, these conventional treatments (physical-chemical-biological) are not sufficient to achieve environmental compliance, so other approaches must be applied. AOTs have been proposed in recent years as an effective alternative for the oxidation of bio-refractory organic compounds from landfill leachate into biodegradable organic compounds or even its total mineralization into CO2, H2O, and inorganic compounds (Luo *et al.*, 2020).

These processes include: i) non-photochemical methods, such as ozonation (O_3), perozononation (O_3/H_2O_2), catalytic ozonation (O_3/catalyst), and Fenton processes (H_2O_2/Fe^{2+}); or ii) photochemical methods, for instance, O_3/UV, H_2O_2/UV, O_3/H_2O_2/UV, photo-Fenton (Fe^{2+}/H_2O_2/UV-Vis) and photocatalysis (UV/catalyst) (Costa *et al.*, 2019; Wiszniowski *et al.*, 2006). In addition, electrochemical methods can be used, such as the Fenton process combined with an electrochemically generated oxidizing agent and catalyst. However, AOTs are usually expensive processes, requiring high doses of oxidants and efficient control systems, and are energy demanding. In this regard, among the existing AOTs, Fenton- and ozone-based processes are the most used methods for leachate treatment (Bassam *et al.*, 2012; Leszczyński and Maria, 2018).

Septiariva *et al.* (2019) employed ozonation as a post-treatment of old leachate, increasing COD removals from 51% to 65% compared to the isolated biological process, while in young leachate, there was no significant difference. Mokhtarani *et al.* (2014) also evaluated the performance of an ozone post-treatment on a biologically pre-treated leachate featuring COD values between 0.5 and 1 g O_2/L and obtained COD removals of 56% (at pH 9, ozone dose of 0.4 g O_3/h and 60 min of reaction). Furthermore, Soubh and Mokhtarani (2016) studied the combination of O_3 and sodium persulfate as a post-treatment method, reaching 84% COD removal after 210 min of reaction in the optimal conditions (at pH 9, ozone dose of 0.79 g O_3/h and 4.5 g/L sodium persulfate). Besides, the combined process (O_3/persulfate) resulted in lower ozone consumption rates compared to ozonation alone (0.35 and 1.16 mg O_3/mg COD removed, respectively).

Mahdad *et al.* (2016) compared the post-treatment by the conventional Fenton and the photo-Fenton processes. The Fenton process removed 55.9% of COD and 65.7% of color, while the photo-Fenton treatment reduced 73.8% of COD and 83.6% of color and increased the effluent biodegradability. Notwithstanding, by allowing the process to occur close to neutrality, ozonation becomes a more promising post-treatment strategy for biological reactors, while Fenton processes require acidic pH values (Roy *et al.*, 2018; Wiszniowski *et al.*, 2006).

3.3.3.3 Cycle time

In AGS reactors, nitrogen and phosphorous removal mechanisms occur throughout the entire operating cycle. After the feeding stage, the reaction begins, and it can be: i) entirely aerobic, ii) aerobic and anaerobic, iii) aerobic and anoxic, or iv) other combinations. Most SBRs used for AGS cultivation in conventional loads operate with 4 to 12 hours cycle time. However, depending on the influent load, longer cycle times can favor the granulation process and improve AGS efficiency. Di Bella & Torregrossa (2014) indicated that for high-strength effluents such as leachate from landfills, the cycle time should not be lower than 12 hours. Other authors also reported that short cycles are not enough to achieve high removal efficiencies and favor the aerobic biomass characteristics in terms of EPS content, settling capacity, among others (Bueno *et al.*, 2020; Ren *et al.*, 2017a; Ren *et al.*, 2018; Wei *et al.*, 2012). Additionally, short cycles (for example, lower than 4 hours) can cause VSS loss through biomass washout but cycles greater than 24 hours are subject to the absence of nitrifying granulation (Nancharaiah & Reddy, 2018).

The alternation of aerobic and anaerobic conditions favors the growth of microorganisms beneficial to granulation (phosphorus-accumulating organisms-PAOs, glycogen accumulating organisms-GAOs, nitrifying bacteria) since the proliferation of heterotrophic microorganisms is suppressed by the lack of a soluble carbon source under aerobic conditions (Rollemberg *et al.*, 2018). However, since phosphorus concentration is not significant in the leachate, an extended anaerobic feeding may be enough to select PAOs and GAOs (Bueno *et al.*, 2020; Ren *et al.*, 2017b).

The selection of microorganisms in the system is also dependent on the settling time, which is a key factor in the formation of aerobic granules. The SBR operation with a short settling time (< 10 min) allows for a quick assortment of microbial aggregates by creating a high selective pressure, causing the washing of lighter microbial flocs and favoring granulation by creating a relatively high shear force (Nancharaiah & Reddy, 2018; Yuan *et al.*, 2019). All the studies applying AGS to treat leachate had a settling time of 5 minutes in an attempt to obtain a more stable and consistent granulation (Table 5).

3.3.3.4 Anaerobic and step-feeding

According to de Kreuk *et al.* (2005), in addition to organic load, shear force, selection pressure, substrate composition, among others, the feeding mode also affects the formation and stability of aerobic granules.

After pre-treatment, the anaerobic feeding is considered essential for the granules formed to remain stable and with good activity and for phosphorus removal by PAOs (de Kreuk & van Loosdrecht, 2004; Hamza *et al.*, 2018). During the anaerobic feeding period, all acetate is converted into internal storage polymers (e.g., polyhydroxybutyrate - PHB), and the phosphate is released into the liquid. Then, in the aerobic period, there is cell growth from the stored PHB and intracellular conversion of the phosphate available in the liquid phase into the polyphosphates. Thus, the selection of these organisms resulted in smooth, dense, and stable granules (de Kreuk & van Loosdrecht, 2004).

This strategy seems to be very well accepted and widespread when using AGS to treat leachate. Except for Di Bella & Torregrossa (2014), all other studies have addressed this form of feeding with at least 30 minutes of duration (Bueno *et al.*, 2020; Ren *et al.*, 2017a; Ren *et al.*, 2017b; Ren *et al.*, 2017c; 2018; Wei *et al.*, 2012). As a result, the smallest phosphorus removals were found in the work of Di Bella & Torregrossa (2014) (Table 5).

Less explored but with great potential to favor granulation at high loads, step-feeding is a strategy that can benefit conventional and autotrophic denitrification. It ensures greater stability to granules by reducing the influent load that can be toxic to microorganisms, distributing it throughout the cycle. Step-feeding associated with SBR affects the selection and growth of filamentous organisms, playing a critical role in granule structure and composition (McSwain *et al.*, 2004; Corsino *et al.*, 2016). Generally, floc-forming bacteria with relatively high substrate absorption kinetics have an advantage over filamentous bacteria if the feeding is distributed. It forces the bacteria to acquire and store substrate for maintenance and cell growth during periods of famine, favoring the selection and formation of aerobic granules. In addition, by allowing nitrification to occur with a lower organic load in the aerobic phase, this feeding mode accelerates the nitrification rate and saves the aeration consumption from oxidizing the influent organic matter (Chen *et al.*, 2011; Wang *et al.*, 2012).

3.3.3.5 Anaerobic/anoxic phase and intermittent aeration

Regarding the quality of the granules formed, both the type of cycle and the distribution of phases throughout the cycle are preponderant factors in the granulation process. In addition to an entirely aerobic reaction phase, various operational configurations, such as anaerobic-anoxic-aerobic, anaerobic-aerobic-anoxic, and aerobic-anoxic conditions, have been adopted for wastewater treatment (Nancharaiah & Reddy, 2018).

In the anaerobic/anoxic phase, nitrite and residual nitrate denitrification occur, in addition to EPS hydrolysis, fermentation, and VFA assimilation, with phosphate release. Being able to influence the granulation process, even in the anoxic phase, a low shear stress is imposed, different from what occurs in the aerobic phase when the granules are exposed to a greater shear stress caused by oxygen bubbles. Under aerobic conditions, a greater diversity of microorganisms starts to act in the granular biomass for simultaneous nitrification, denitrification, and phosphorus removal processes, such as AOB, NOB, common denitrifying heterotrophic microorganisms, PAOs, denitrifying PAOs (DPAOs), and denitrifying glycogen-accumulating organisms (DGAOs) (Nancharaiah & Reddy, 2018; Rollemberg *et al.*, 2018).

In order to favor DPAOs, which have a slower growth rate, the intercalation of anoxic and aerobic phases presents itself as a good strategy for high-strength effluents, and the exclusively anaerobic phase can be discarded since phosphorus concentrations are not high. Nancharaiah & Reddy (2018) suggest that integrating periods with high and low DO concentrations is necessary to achieve complete nitrogen removal. Zhang *et al.* (2014) point out that in anoxic phases, low phosphorus removal is due to the high presence of DGAOs, which compete directly with DPAOs.

In most studies using AGS to treat leachate, the anaerobic phase was non-existent, and when present, it was no longer than 90 minutes in long cycles (Table 5). However, there are no studies in which the anoxic phase has been used. Thus, the aerobic phase was an integral part of the reaction phase of AGS operating in SBR, and when operated in long cycles, it is expected that partial nitrification and complete denitrification can achieve higher rates, generating lower concentrations of toxic denitrification by-products and smaller accumulations of nitrate and nitrite.

3.3.4 Performance of AGS reactors in removing COD, Nitrogen, and Phosphorus from leachate

In AGS reactors, to simultaneously remove organic matter, nitrogen, and phosphorous species throughout the operational cycle, there must be a balanced oxygen supply to promote the nitrification without impacting the anoxic denitrification process or anaerobic phosphorus removal. Therefore, SBR with AGS can be adjusted to operate in cycle options: A/O (Anoxic, Oxic), A2/O (Anaerobic, Anoxic, Oxic), or A/O/A (Anaerobic, Oxic, Anoxic). The introduction of an anaerobic phase in the SBR operating cycle can reduce the aeration demand during the aerobic oxidation cycle (He *et al.*, 2018).

After feeding, the reaction phase known as feast begins, i.e., the period when the external substrate is readily available and in abundance. This substrate diffuses inside the granule completely. Part of the carbon is converted and stored aerobically, anaerobically, or under anoxic conditions by heterotrophic microorganisms in the form of intracellular polymers such as PHB. Oxygen penetration into the granule is smaller than that of organic carbon due to the rapid consumption by autotrophic and heterotrophic organisms in the granule's outer layer. In this way, DO is used mainly for nitrification, aerobic carbon conversion, and biomass growth. The autotrophic organisms in the outer layers of the granules convert ammonia into nitrogen oxides - NO_x (nitrite and nitrate) that diffuse towards the granule center and into the liquid phase. In the anaerobic zone, PHB is available to be used as a carbon source for the denitrification process (Nancharaiah & Reddy, 2018; Rollemberg *et al.*, 2018). Therefore, nitrogen removal occurs through the distribution of phases and inside the granules via SND process.

The famine period starts after the complete consumption of organic matter. Oxygen penetration into the granule will be greater since its concentration in the liquid medium will be higher, and only PHB will be available. The oxidation of the stored PHB will occur through NO_x production at a level that allows its introduction into the anaerobic zone, making it anoxic. This is due to high oxygen consumption through nitrifying autotrophic organisms. Thus, PAO and GAO will have access to oxygen, and DPAO and DGAO to NO_x (Nancharaiah & Reddy, 2018; Rollemberg *et al.*, 2018).

Phosphorus removal occurs by the accumulation of polyphosphate in PAO and DPAO, requiring an anaerobic condition to favor PAO development and ensure that it prevails over GAO since both compete for substrate during the feast, and for DPAO to prevail about DGAO, as they compete for NO_x during the famine (Nancharaiah & Reddy, 2018).

Some recent studies have shown that COD and nitrogen removal efficiencies in AGS systems depend on several aspects, such as: inoculum quality and cultivation strategy, system operation, mixing/equalization of leachate with sanitary sewage etc. (Bueno *et al.*, 2020). The efficiency of the main AGS systems on leachate treatment is shown in Table 5.

In the first studies using AGS technology to treat landfill leachate, Wei *et al.* (2012) and Di Bella & Torregrossa (2014) showed that the process efficiency depends on the influent load since by increasing influent ammonia concentration, the removal rates of ammonia itself, total nitrogen and COD tended to fall. Even so, the results indicated that AGS reactors easily achieved the removal of these compounds. However, it is important to note that leachate characteristics are key for system performance. For example, some studies have observed a low COD removal rate associated with a low leachate biodegradability, characteristic of old leachates (Di Bella & Torregrossa, 2014; Wei *et al.*, 2012).

Kocaturk and Erguder (2016) reported that the COD/ammonia ratio in the influent could influence the variety of dominant microorganisms in the granules, and the higher this ratio, the greater the COD removal. This was evidenced by Ren *et al.* (2017b), in which the COD removal efficiency decreased by 20% by reducing the COD/ammonia ratio in leachate from 5 to 1.5.

Yang *et al.* (2004) and Ren *et al.* (2017b) observed that free ammonia in the wastewater affects sludge and aerobic granules' aggregation and biomass washing by decreasing cellular hydrophobicity. In conventional biological systems (e.g., activated sludge), the inhibitory effects of free ammonia on the activity of nitrifying microorganisms were observed in concentrations above 10 mg/L for AOB and from 0.1-1.0 mg/L for NOB (Anthonisen *et al.*, 1976; Yang *et al.*, 2004; Zhou *et al.*, 2011). However, in AGS reactors, it has been observed that the total ammonia removal efficiency remains high and stable, even in high ammonia concentrations. Such a characteristic is due to the compact and unique structure of the AGS granules, preventing nitrifying microorganisms from having direct contact with these toxic compounds (Ren, Ferraz and Yuan, 2017a). In all cases, it is important to note that the low removal of TN can also be associated with the low availability of biodegradable COD, especially in old leachate, which decreases the carbon available to denitrifying microorganisms, thus hindering denitrification and SND (Ren, Ferraz and Yuan, 2017b).

Finally, Ren, Ferraz and Yuan (2017a) also found that phosphorus removal also decreases with the increase in leachate pollutants load. Muszyński & Miłobędzka (2015) point out that during high-load SBR cycles, it is expected that phosphorus removal will be opposite to nitrogen removal, possibly due to the competition for carbon between denitrifying heterotrophs, GAOs and PAOs. The excess of nitrate in the anaerobic phase also causes this competition (Ren, Ferraz and Yuan, 2017b). In addition, it has been reported that the high presence of ammonia also inhibits PAOs, even at concentrations below 1 mg/L (Saito *et al.*, 2004). Thus, low phosphorus removals have been observed (Bueno *et al.*, 2020; Ren *et al.*, 2017a; Ren *et al.*, 2017b; Ren *et al.*, 2018).

Table 5 – Efficiencies reported in the literature during leachate treatment with AGS technology.

												Final removals (%)					
COD	**TN**	**NH_3-N**	**P_{Total}**	**Flow rate (L/min)**	**Reactor type**	**Cycle (h)**	**F (min)**	**AN (min)**	**AER (min)**	**S (min)**	**D (min)**	**COD**	**TN**	**NH_3-N**	**P_{Total}**		
4298-5547	126-400	72-374	---	2	O	12	60	0	640	5	5	84	75	96	---	No	Wei *et al.* (2012)
4502-5992	691-1253	602-1168	---	2	O	12	60	0	640	5	5	83	35-58	39-77	---	Yes	
9738	3700	1960	---	---	O	24	5	0	1427	3	5	40-50	Low	40-60	---	Yes	Di Bella & Torregrossa (2014)
4560	1845	945	---	---	O	12	5	0	707	3	5	50-60	Low	20-50	---	Yes	
448-654	---	120-500	32.5	3	A2/O	8	30	90	345	5	12	66-73	39	61-95	34-54	No	Ren *et al.* (2017a)
1080	---	340	2-6	2-3	O	8	90	0	360	5	12	65	40	100	80	Yes	Ren, Ferraz and Yuan (2017a)
1194	---	580	4-6	2-3	O	8	90	0	360	5	12	43	25	100	40	Yes	
1539	---	900	5-6	2-3	O	8	90	0	360	5	12	20	< 10	91	40	Yes	
550-1000	---	130-785	3-6	1.5	A2/O	8	30	90	330	5	12	43-65	24-37	95-99	0	Yes	Ren, Ferraz and Yuan (2017b)
1000-1100	---	785-1085	3-6	1.5	A2/O	8	30	90	330	5	12	31-40	23-24	95-99	0	Yes	
1100-1200	---	1085-1209	3-6	1.5	A2/O	8	30	90	330	5	12	7-31	21-23	99	0	Yes	
650	114	88	13.1	1.5	O	8	45-60	0	425	5	5	87	99	99	36	No	Bueno *et al.* (2020)
863	187	136	15.2	1.5	O	8	45-60	0	425	5	5	89	99	99	42	No	
1421	304	281	17.5	1.5	O	8	45-60	0	425	5	5	88	98	99	45	No	

Source: Prepared by the author.

3.3.5 Main challenges in the application of AGS in the treatment of leachate

3.3.5.1 Nitrite accumulation

In the SND process, the critical step is to obtain stable nitrification. Nitrite accumulation has been widely reported in experiments with high ammonia loads, decreasing total nitrogen removal. Nitritation is affected by the control of the reaction conditions (in a macro perspective) or by the rapid growth of AOBs to the detriment of NOBs (Miao *et al.*, 2019; Poot *et al.*, 2016). Studies show that NOBs have a slower growth rate than AOBs, the latter with more oxygen affinity (Ma *et al.*, 2016; Regmi *et al.*, 2014). Therefore, low concentrations of dissolved oxygen prevent NOBs from developing. According to Ren *et al.* (2016) and Miao *et al.* (2019), partial nitrification depends on pH, temperature, and free ammonia at higher levels, intensifying AOBs activity.

Therefore, in leachate from old landfills, due to high concentrations of N-NH3, partial nitrification (nitritation) has been the most effective route (Miao *et al.*, 2014). Such a fact occurs since high levels of NH_3-N can increase the amount of free ammonia, generating a lot of NO_2^-N, which allows the availability of high concentrations of free nitrous acid. According to Chung *et al.* (2015), all microorganisms responsible for nitrification and denitrification are affected by FA and FNA, with NOBs being the most susceptible. Some strategies have been reported, and by inhibiting free ammonia, Wu *et al.* (2015) managed to obtain a stable nitrification by treating municipal sewage and leachate simultaneously.

As mentioned, pH control can be the key factor for good nitrification, as it varies considerably during the process (Miao *et al.*, 2019). When aeration begins, readily biodegradable organic matter is quickly degraded due to the high affinity of heterotrophic bacteria for oxygen. Subsequently to the COD consumption comes the conversion of ammonia to nitrite by autotrophic bacteria, in which the pH decreases as alkalinity is consumed. When this conversion ends, the pH rises again due to nitrite conversion into nitrate by the nitrite-oxidizing microorganisms, which do not require alkalinity consumption. Wang *et al.* (2013) point out that the pH is directly related to nitritation, conditioning the conversion of nitrite to nitrate. Thus, when adopting pH and DO control strategies, nitritation and denitritation efficiencies to over 90% were obtained (Chung *et al.*, 2015; Sun *et al.*, 2015; Chen *et al.*, 2016).

Therefore, since AOBs occupy the surface of the granules and NOBs the interior, NH3-N is immediately converted by AOBs (dominant group), and NOBs undergo two simultaneous inhibitions: by FA and FNA. In addition, the low DO concentration in the granule also influences NOB inhibition. All of this results in an accumulation of nitrite.

3.3.5.2 Biomass formation time

The high influent loads have significantly influenced the granulation process, especially organic matter and nitrogen. When working with landfill leachate, the required time to form a predominant granular biomass is relatively longer than in conventional effluents, such as domestic sewage. The granules' size is also smaller with uneven surfaces.

For example, Ren, Ferraz and Yuan (2017b) and Bueno *et al.* (2020) needed 70 to 90 days of operation to obtain a granular biomass with characteristics similar to those of Yu *et al.* (2014) and Corsino *et al.* (2016) with less than 40 days of operation in influents with low concentrations of organic matter and nitrogen. Di Bella & Torregrossa (2014) and Bueno *et al.* (2020) observed the formation of more resistant and stable aggregations with diameters of 261-621 μm, only after 60 days of operation.

Generally, in the first 40 days of operation with leachate, the sludge has a predominant filamentous structure with a dark brown flocculating structure, low diameter, and minor microbial aggregations (Bueno *et al.*, 2020; Ren *et al.*, 2017a). Due to these characteristics of the biomass, solids washout tends to occur more frequently, delaying the granulation process (TSS loss greater than biomass growth).

Ren *et al.* (2017a) had so much sludge loss that it was impracticable to measure AGS reactors' sludge age. Bueno *et al.* (2020) chose to recirculate daily the sludge lost to the reactor to favor the granulation process, thus suggesting a secondary clarifier after the AGS reactor for automatic and efficient recirculation. Initial granulation using synthetic effluents with lower loads to generate stable and mature granules that can withstand hydraulic shocks before leachate incorporation into the AGS reactors is also suggested (Di Bella & Torregrossa, 2014). Therefore, the treatment strategy must be adapted to minimize sludge loss and favor uniform granulation with large size granules.

3.3.5.3 Granule instability and disintegration problems

Recalcitrant wastewaters may cause inhibitory effects on AGS biomass, resulting in a low process yield (Li *et al.*, 2014; Zheng *et al.*, 2020). In wastewaters with high pollutant loads, especially landfill leachates, the main problems observed revolve around the disintegration and instability of granules. Several authors point out that these limitations are reflections of intracellular protein hydrolysis, as well as the destruction of EPS structure (PN proteins being essential for granules stability), anaerobic fermentation of dead and lysed cells in already stable granules, and degradation in the granule nucleus by the overgrowth of filamentous microorganisms at high rates of substrate transport, making them more floccular (Leal *et al.*, 2020; Long *et al.*, 2015; Lv *et al.*, 2014; Wagner *et al.*, 2015; Zhang *et al.*, 2015).

It is important to note that the granular structure instability can compromise the treatment process efficiency since disintegration increases the TSS in the effluent and worsen the biomass settling in the mixed liquor (Li *et al.*, 2014; Sarvajith *et al.*, 2020). Furthermore, this instability can reduce the pollutant removal rate.

High organic loads accelerated the granulation process. However, this biomass outbreak generated rapid granules disintegration due to its excessive growth and subsequent increase of dead cells in the center and methanogens in the granule core (Long *et al.*, 2015; Hamza *et al.*, 2018).

In studies with leachate, Ren *et al.* (2017a) and Ren, Ferraz and Yuan (2017b) found that granules formation occurred more slowly than the conventional one and that the granules formed were very unstable in load shocks. This profile was similar to other types of high-strength effluents. For example, Zhang *et al.* (2011) fed an AGS (SBR) with effluents from a petrochemical company and found that the granules' properties and performance declined with the shock.

Ren *et al.* (2017b) point out that in leachate, even after reaching operational stability, if there are significant changes in load, the granules disintegrate, and the denitrification process is compromised. Therefore, effluents with high concentrations of organic matter or enriched with nitrogen/phosphorus species, instead of the available substrate being transformed by anaerobic processes into storage constituents, this substrate is adsorbed on the granules surface, favoring the development of filamentous structures in the granules and making them more susceptible to breakage (Pronk *et al.*, 2015; Corsino *et al.*, 2018). Therefore, granular disintegration remains an unsolved problem with AGS technology, and it is necessary to develop an efficient strategy for the direct treatment of these wastewaters with high resistance components. Hamza *et al.*

(2018) propose selecting slow-growing organisms, such as phosphate-accumulating bacteria or glycogen, to maintain the granule stability upon high shock loads. In this regard, Wei *et al.* (2012) suggested that in leachate subjected to a physicochemical pre-treatment, the granules' disintegration was lower and the system achieved stability in time similar to the conventional low-load granulation.

López-Palau *et al.* (2012) found that an appropriate balance between the feast and famine phases must exist for the granules to be maintained in long-term operation. However, the duration of the substrate availability phase should not be greater than 25% of the total cycle period to guarantee the granule stability and an influent of adequate quality (Corsino *et al.*, 2018). In addition to these operational adjustments, one of the most recently used strategies that have achieved excellent results is the formation of granules with gradual increases in loads and the dilution of real high-strength effluents before being subjected to treatment with AGS (Hamza *et al.*, 2019; Leal *et al.*, 2020; Ren *et al.*, 2016; Xiong *et al.*, 2020).

3.4 Prospects for future work

The ability of aerobic granular sludge to withstand high carbon and nitrogen loads without significantly interfering in the removal efficiencies and biomass settling shows that this technology has lots of potential and offers an excellent alternative to the conventional biological treatments for landfill leachates.

Nonetheless, there is still a significant knowledge gap regarding the optimal operating conditions to maximize the AGS-SBR reactor's performance in leachate treatment without high costs, i.e., minimizing energy consumption and the addition of reagents. The reactor configuration (conventional SBR versus simultaneous fill/draw SBR), feeding mode (continuous or intermittent), the influence of feeding concentrations (dilution rates), and cycle time (longer operation cycles, increment of anoxic phases, among others) are some of the aspects that need further exploration to employ AGS technology for landfill leachate treatment.

It is worth noting that the variability of leachate composition with the landfill age may require different treatment strategies. Nitrite and nitrate accumulation and total nitrogen removal can represent a problem for these systems, especially for old landfill leachates. Thus, future work must fully understand the AGS system behavior when treating both young and mature leachates, including the need for: i) pre- and/or post-treatment, ii) leachate dilution, and iii) supplementation with external carbon/nutrients sources. Moreover, granules' formation and stability using high-strength wastewaters and/or leachate, rather than synthetic or domestic wastewater, must be evaluated.

Finally, it is extremely important to investigate the effect of such complex wastewater on the microbial populations involved and respective kinetics after long-term exposure.

4 MATERIAL AND METHODS

The current work was carried out in four main stages, developed at the Environmental Sanitation Laboratory (LABOSAN), Department of Hydraulic and Environmental Engineering (DEHA) of the Federal University of Ceará (UFC) and Laboratory of Separation and Reaction Engineering-Laboratory of Catalysis and Materials (LSRE-LCM), Department of Chemical Engineering (DEQ) of the Faculty of Engineering of the University of Porto (FEUP), Portugal.

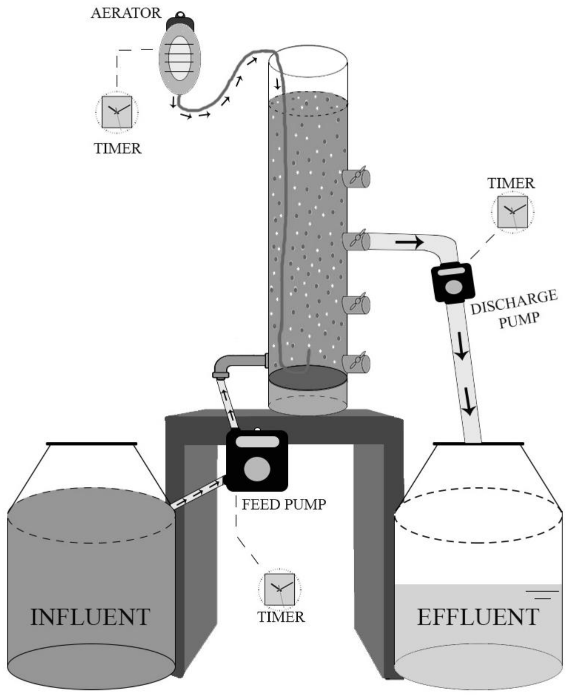
Thus, AGS systems were optimized and different configurations were evaluated from synthetic wastewater with a C:N:P ratio similar to that of a real leachate from an old landfill for later application with real leachate. In the first stage (Operating System I), the main objective was to evaluate the behavior of the biomass during the granulation process when adding leachate along with the inoculum sludge. As the results were not as expected, it was necessary to establish new operational configurations in order to optimize granule formation and reactor performance. Thus, the impact of different feeding methods (Second stage - Operating System II) and different combinations of oxic, anoxic and anaerobic periods (Third stage - Operating System III) were investigated. From the results obtained in these studies, it was possible to adopt the best strategies of AGS systems for the real leachate co-treatment with domestic sewage (Fourth stage - Operating System IV).

4.1 Operating System I (OS I)

Two sequencing batch reactors (SBR) were operated under the same conditions, changing only the influent leachate dilution factor, 25% in R1 and 50%, both raw and diluted with tap water. The working volume of each reactor was 7.6 L, with an internal diameter of 10 cm, height of 100 cm, and height-to-diameter ratio (H/D) of 10, presenting a volumetric exchange rate of 50% during all the experiment periods. Each cycle lasted 8 hours, and consisted of feeding (20-40 min), aerobic reaction (429-439 min), settling (20-10 min), and withdraw (1 min). During the aerobic phase, dissolved oxygen (DO) was injected from the bottom of the reactor (Yuting SUN air compressor, China) through porous fine bubble diffusers, satisfying a DO concentration between 3 and 6 mg/L. Systems' operation was controlled through synchronized timers (Figure 4).

Both reactors were operated at room temperature, and the leachate inside the reactors was around 28±2ºC. During the experiment, there was no sludge discharge, resulting in different sludge retention times (SRTs) in the systems due to biomass washing, which were not measured due to constant biomass washouts.

Figure 4 – Schematic of an experimental SBR-AGS system in this study.



Source: Prepared by the author.

The reactors operated for 95 days, divided into three periods. In the first part of the experiment (30 days), feeding time (T_f) and settling time (T_s) were 20 min (Period I). Subsequently, T_f was increased to 40 min, and the T_s reduced to 10 min (Period II). Finally, the DO rate was changed from 3 to 6 mg/L (Period III). Reducing T_s, in addition to favoring biomass selection, also increases efficiency and long-term stability, benefiting aerobic granulation (Rollemberg *et al.*, 2018). Furthermore, feeding mode, especially the prolonged anaerobic filling, is also one of the key factors in the granulation process, directly influencing granules' development and contaminant removal efficiency (De Kreuk, Heijnen and van Loosdrecht, 2005; Hamza *et al.*, 2018).

The AGS systems were inoculated with aerobic sludge from a sewage treatment plant (Fortaleza, Ceará, Brazil) (~4 g/L mixed liquor suspended solids - MLSS with 89% VSS/TSS and SVI_{30} of 189.5 mL/g). Feeding was carried out from the bottom of the reactor with leachate from the Municipal Sanitary Landfill (ASMOC, Ceará, Brazil). The leachate collected was free from any treatment at the landfill and had an average composition, as shown in Table 6.

Table 6 – Composition of the raw sanitary landfill leachate.

Parameter	Value
pH	7.5±0.5
Conductivity (µS/cm)	15330±182
BOD total (mg/L)	852±236
COD total (mg/L)	3743±453
NH_4^+-N (mg/L)	651±165
NO_2^--N (mg/L)	5±3
NO_3^--N (mg/L)	5±3
TKN (mg/L)	752±126
Total Phosphorous (mg/L)	73±12
Chloride (g/L)	8.5±0.5
Sulfate (mg/L)	43±33
Fluoride (mg/L)	30±9
Bromide (mg/L)	5±3
Sulfide (mg/L)	112±46

Source: Prepared by the author.

4.2 Operating System II (OS II)

The experiments were carried out in three identical SBRs inoculated with the same biomass and operated under the same conditions, changing only the feeding method. The reactors had 7.85 L, with working volume of 7.6 L, internal diameter of 10 cm, height of 100 cm, and height-to-diameter ratio (H/D) of 10, with a 50% exchange volume. The total experiment duration was 120 days for R1 and 134 days for R2 and R3, both divided into two periods by changing the settling time (T_s). In the first 40 days of operation, the T_s was 20 min (period I), being reduced to 10 min (period II) until the experiment completion. The reduction in T_s favors biomass selection and increases efficiency and long-term stability, benefiting aerobic granulation (Rollemberg *et al.*, 2018).

The duration of each cycle was 12 h, which consisted of feeding (20-40 min), aerobic reaction (659-679 min), settling (20-10 min), and withdraw (1 min). In the aerobic phase, the air was injected by porous fine bubble diffusers through the reactor bottom using an air compressor Yuting SUN, China, ensuring a dissolved oxygen (DO) concentration between 2 and 5 mg/L.

The feeding differentiation in the reactors followed the description below:

- R1: conventional feeding (plug flow) in anaerobic/anoxic phase lasting 20 min (fast);

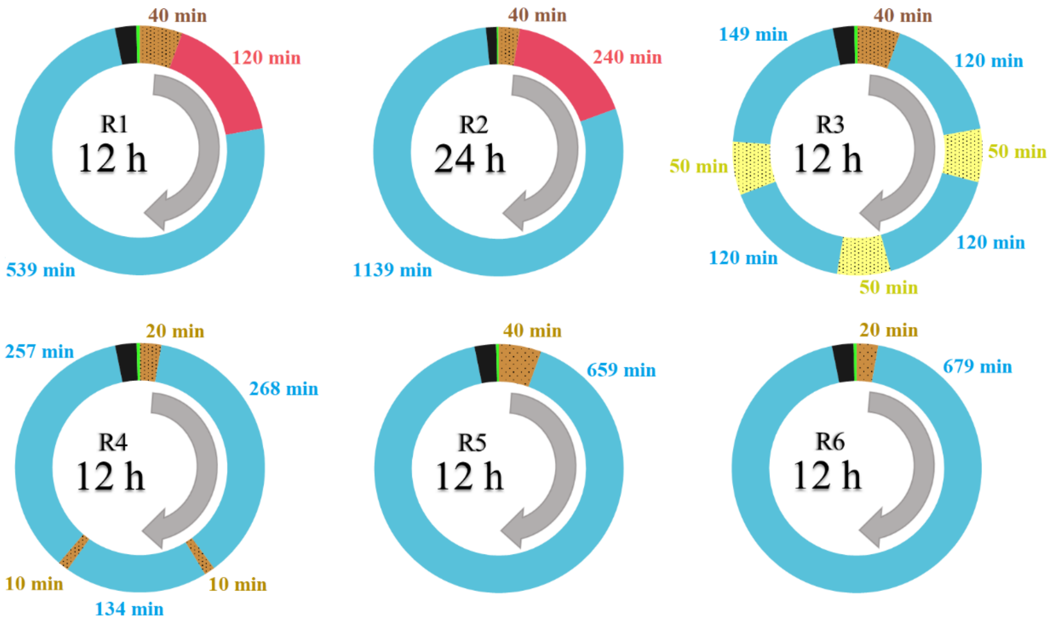
- R2: conventional feeding (plug flow) in anaerobic/anoxic phase lasting 40 min (slow);
- R3: step-feeding over the cycle in three moments, with 50% of the influent volume being introduced at the beginning of the cycle and the other half divided equally with 40 and 60% of the cycle.

4.3 Operating System III (OS III)

The experiments were carried out in six identical SBRs, inoculated with the same biomass, and operated under the same conditions. Each reactor had different phase distributions with a cycle duration of 12 and 24 hours (Figure 5). The insertion of anaerobic/anoxic phases ensures that nitrite and residual nitrate denitrification occur, in addition to EPS hydrolysis, fermentation, and VFA assimilation, with phosphate release. Under aerobic conditions, a greater diversity of microorganisms starts to act in the granular biomass for simultaneous nitrification, denitrification, and phosphorus removal processes, such as AOB, NOB, common denitrifying heterotrophic microorganisms, PAOs, denitrifying PAOs (DPAOs), and denitrifying glycogen-accumulating organisms (DGAOs) (Silva *et al.*, 2022). Therefore, these configurations were based on cases that have been successful in the literature for the treatment of other types of effluents (Nancharaiah & Reddy, 2018; Rollemberg *et al.*, 2018). Thus, the systems had the following characteristics:

- R1: 12-hour cycles with anaerobic phase followed by aerobic phase (A/O);
- R2: 24-hour cycles with anaerobic phase followed by aerobic phase (A/O);
- R3: 12-hour cycles with intercalation between anoxic and oxic phases, with 50 minutes of anoxic phase (dissolved oxygen < 0.5 mg/L) for every 120 min of aeration (O/An defined);
- R4: 12-hour cycles with anaerobic/anoxic feeding distributed along the cycle in three moments; 50% of the influent volume is introduced at the beginning, and the rest is divided equally at 40% and 60% of the cycle (O/An defined);
- R5: 12-hour cycles with 40 minutes of anaerobic/anoxic feeding followed only by an oxic reaction (O/An);
- R6: 12-hour cycles with 20 minutes of anaerobic/anoxic feeding followed only by an oxic reaction (O/An).

Figure 5 – Different phase configurations for the investigated reactors.



Colors: Brown – Anaerobic/Anoxic Feed; Red – Anaerobic phase; Blue – Aerobic phase; Yellow – Anoxic phase; Black – Settling time (20 – 10 min); Green – Withdraw (1 min).
Source: Prepared by the author.

The reactors had a working volume of 7.6 L, an internal diameter of 10 cm, a height of 100 cm, and a height to diameter (H/D) ratio of 10. The exchange volume applied to the reactors was 50%. In the aerobic phase, the air was injected by fine-bubble porous diffusers located at the reactor bottom through an air compressor (Yuting SUN, China), ensuring a dissolved oxygen (DO) concentration between 2 and 5 mg/L during this phase. The total experiment duration was 114 days for the six reactors, which were grouped into two periods according to the settling time (Ts). Initially, in the period I, the T_s was 20 minutes (40 days). Then, the T_s was reduced to 10 min (period II) until experiment completion.

In all reactors, on both OS II and OS III, the operation was automated through synchronized timers (Figure 4). The operation occurred at room temperature, and the wastewater inside the reactors was around 28±2 ºC. The reactors' sludge retention times (SRTs) reflected the biomass loss since no sludge removal occurred during the experiment. The systems were inoculated with aerobic sludge from a sewage treatment plant (Fortaleza, Ceará, Brazil) with approximately the same mixed liquor suspended solids concentration (MLSS ~3.4 g/L). The influent synthetic wastewater had a ratio of 50:10:1 C:N:P, close to the one found after the characterization of a real old leachate, which was collected from the municipal sanitary landfill (ASMOC, Ceará, Brazil) (Table 6). Thus, the influent was composed of 1000 mg chemical oxygen demand (COD)/L of sodium acetate as a carbon source, 200 mg/L of NH_4^+-N (from NH_4Cl) as nitrogen source, 20 mg/L of PO_4^{3-}-P (from KH_2PO_4) as phosphorus source, and 1 mL/L of trace elements solution as described by Rollemberg *et al.* (2019). pH was kept close to neutrality, being adjusted with sodium hydroxide.

4.4 Operating System IV (OS IV)

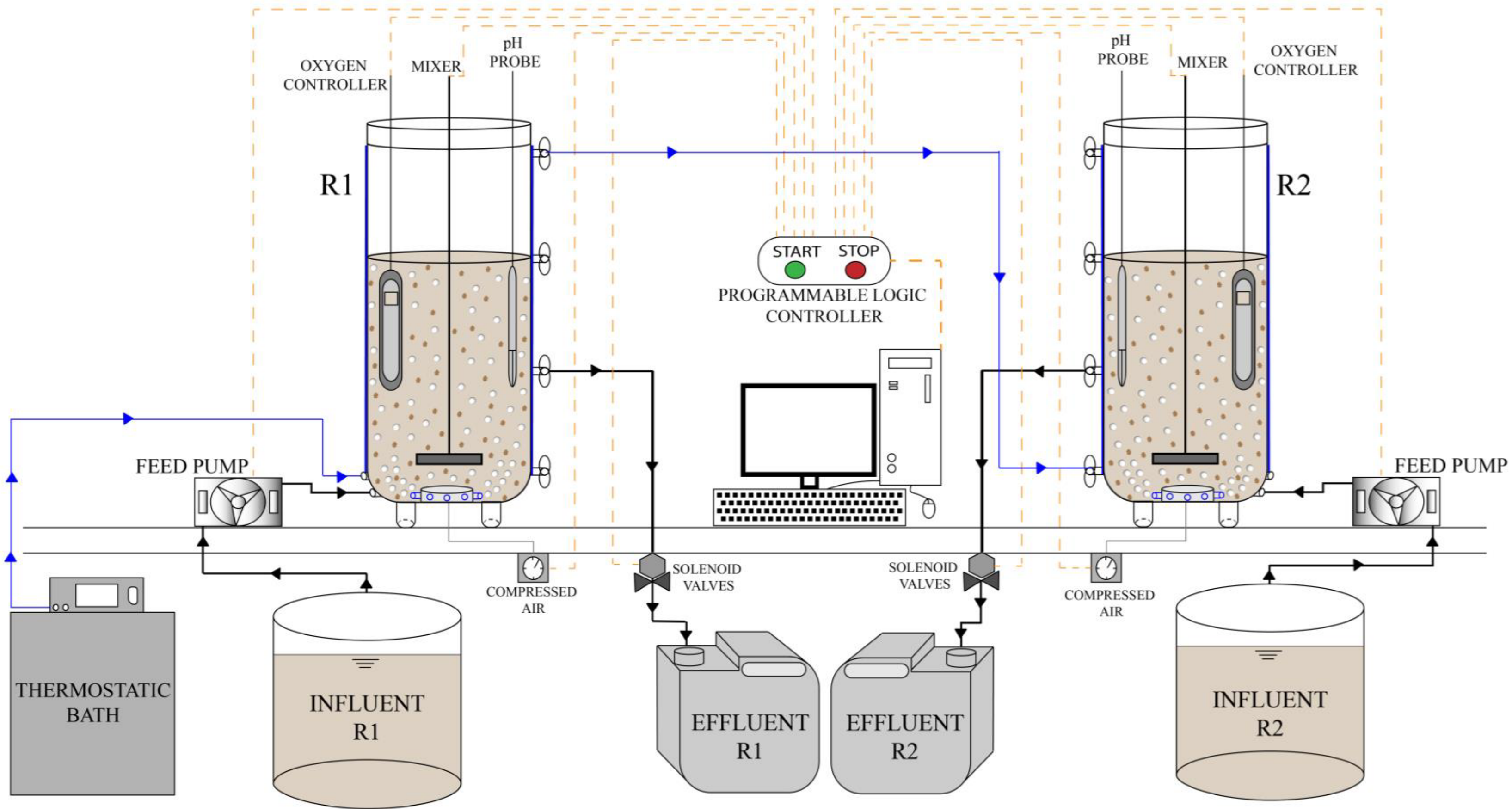
Two cylindrical double-walled acrylic reactors were operated in parallel as laboratory-scale SBR (Figure 6), with a total height of 70 cm and an internal diameter of 15 cm. The height of the liquid inside the reactor was 34 cm, providing a working volume of 6 L and a height-diameter (H/D) ratio of 2.3. There was temperature, dissolved oxygen and pH control. To control the temperature, a thermostatic bath with recirculation on the outer wall of the reactors was set to 22-25°C.

In each reactor, aeration was provided through 8 air bubble diffusers (porous stone, model AS-1) evenly distributed in the lower part of the reactor. To control the flow of air into the system, the long airline had pressure regulating valves, solenoid valves and rotameter. Inside the reactors there was an oxygen controller (HACH company, model sc200), which in the aerobic periods kept the DO between 2 - 4 mg/L. Also, in the bottom of the reactors, the influent was introduced in an upward flow through two analogous peristaltic pumps (Watson-Marlow, model 120S/DV).

During the anoxic and filling phase, two mechanical stirrers (VWR, model - VOS Power Control) positioned inside each reactor remained under agitation (50 min^{-1}). At the end of each cycle, with an exchange volume of 50%, the treated effluent was collected in two 30 L reservoirs, and discarded daily. In an automated way, each stage of the cycle and the activation of the equipment was regulated by a Programmable Logic Controller.

The experimental system was operated at the Laboratory of Separation and Reaction Processes - Laboratory of Catalysis and Materials, Department of Chemical Engineering of the Faculty of Engineering of the University of Porto, Portugal.

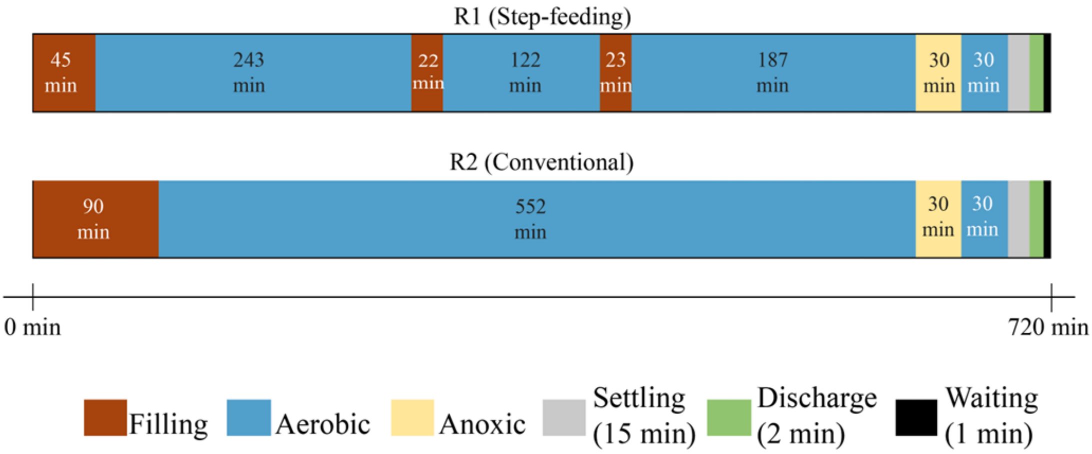
Figure 6 – Schematic of the experimental SBR-AGS system used this study.



Source: Prepared by the author.

The two reactors were operated under the same conditions, in 12-hour cycles and differing only in the feeding regime. In R1, the system operated with an anoxic step-feeding, where half of the feed (1.5L) was introduced at the beginning of the cycle, and the rest was divided and introduced equally (0.75L) 40 and 60% during the cycles. In R2, anoxic feeding was complete at the beginning of the cycle. After completion of feeding, in both reactors the reaction period was aerobic followed by 30 min of anoxic and 30 min of aerobic, respectively. Finally, there was a 15-minute settling, a 2-minute withdraw/discharge and a 1-minute rest time (Figure 7).

Figure 7 – Schematic summary of times and periods in each reactor.



Source: Prepared by the author.

The reactors were inoculated with 3L of Activated Sludge at 2.4 gVSS/L from a domestic wastewater treatment plant in Activated Sludge (Porto, Portugal), and operated for 138 uninterrupted days.

Low synthetic effluent carbon loads were defined for the initial granulation (Period I), being increased after biomass stabilization (Period II). After stabilization in a high synthetic load, leachate co-treatment was initiated, using the same synthetic effluent of the previous period and maintaining the same C, N, and P concentrations (Periods III, IV, and V). Biomass was considered granular when more than 80% of the reactor sludge had a diameter greater than 200 µm.

The amount of leachate received by an MWWTP usually corresponds to values below 10% of the average municipal wastewater influent flow, taking into account daily variations in both systems. Therefore, in this study, it was considered the co-treatment of 5% (Period III) and 10% (Period IV) leachate with the synthetic effluent. In Period V, methanol was added as an easily assimilated substrate source in an attempt to improve nutrients removal, thus increasing the influent load. This carbon source is pointed out as one of the best substrates to ensure good and stable granulation (Pronk *et al.*, 2015; Wang *et al.*, 2020). A summary of the experimental design is shown in Table 7.

Table 7 – Summary schematic of the investigation periods.

	Synthetic Sewage		**Leachate**		
	Period I Low concentration	**Period II** High concentration	**Period III** Co-treatment with 5% leachate	**Period IV** Co-treatment with 10% leachate	**Period V** Co-treatment with 10% leachate and methanol supplementation
Day	1 – 32	33 – 61	62 – 88	89 – 118	119 – 138
COD	300 mg/L (190 mg/L Acetate + 110 mg/L propionate)	1000 mg/L (600 mg/L Acetate + 400 mg/L propionate)	1040 mg/L (600 mg/L Acetate + 400 mg/L propionate + 40 mg/L leachate)	1080 mg/L (600 mg/L Acetate + 400 mg/L propionate + 80 mg/L leachate)	1580 mg/L (600 mg/L Acetate + 400 mg/L propionate and 500 mg/L methanol + 80 mg/L leachate)
NH_4^+-N	20 mg/L	220 mg/L	230 mg/L (220 mg/L synthetic sewage + 10 mg/L leachate)	240 mg/L (220 mg/L synthetic sewage + 20 mg/L leachate)	240 mg/L (220 mg/L synthetic sewage + 20 mg/L leachate)
PO_4^{3-}-P	6 mg/L	18 mg/L	18 mg/L (basically synthetic sewage)	18 mg/L (basically synthetic sewage)	18 mg/L (basically synthetic effluent)

Source: Prepared by the author.

The carbon source provided by the synthetic effluent was 100% volatile fatty acids (½ propionate and ½ acetate), while the nitrogen source was NH_4Cl and the phosphorus source was K_2HPO_4 and KH_2PO_4. The concentrations of these compounds were different, as shown in Table 7. In addition to them, the synthetic effluent from all periods was also composed of 70 mg/L $MgSO_4 \cdot 7H_2O$, 30 mg/L KCl, 60 mg/L $CaCl_2$ and 1 mL/L of trace elements prepared according to Vishniac and Santer (1957). Sodium bicarbonate was used to adjust the pH and keep it close to neutrality.

The intermediate leachate used in this work was collected at the outlet of a leachate treatment plant (LTP) located in a sanitary landfill in northern Portugal (Table 8). This landfill has been operating since 1999 and receives 450,000 tons annually, generating between 100 and 150 m^3 of leachate daily. In the co-treatment, the leachate was diluted with the same synthetic effluent from Period 2.

Table 8 – Composition of landfill leachate in Porto, Portugal

Parameter	Value
pH	8.3±0.8
Conductivity (µS/cm)	6387±239
COD total (mg/L)	847±43
NH_4^+-N (mg/L)	15±2
NO_2^--N (mg/L)	4±1
NO_3^--N(mg/L)	201±39
Chloride (mg/L)	1882±55
Sulfate(mg/L)	710±6
Phosphate (mg/L)	7±1
Calcium (mg/L)	109±1
Magnesium (mg/L)	66±3
Potassium (mg/L)	999±3
TSS (mg/L)	44±2
VSS (mg/L)	4±1

Source: Prepared by the author.

4.5 Analytical methods

For the analyses of COD, ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), phosphate (PO_4^{3-}-P), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), and sludge volumetric index (SVI), influent and effluent samples were collected two to three times a week.

COD, pH, NH_4^+-N, NO_2^--N, NO_3^--N, total phosphorous (TP), solids, and SVI in 10 and 30 min (SVI_{10} e SVI_{30}) were determined according to APHA (2012), while the DO was measured by a YSI 5000 model (YSI Incorporated, USA). Dissolved organic carbon (DOC) was determined by NDIR spectrometry in a TC-TOC-TN analyzer equipped with ASI-V autosampler (Shimadzu, model TOC-VCSN) after calibration with standard solutions of potassium hydrogen phthalate (total carbon) and a mixture of sodium hydrogen carbonate/sodium carbonate (inorganic carbon). DOC was given by the difference between TDC (Total Dissolved Carbon) and DIC (Dissolved Inorganic Carbon) (DOC=TDC-DIC). Total inorganic nitrogen (TNI) was considered as the sum of NH_4^+-N, NO_2^--N e NO_3^--N (Long *et al.*, 2014), except for OS IV, where total nitrogen was determined using a colorimetric kit, with spectrophotometer readings (Merck-Lange, Germany). To quantify the EPS (extracellular polymeric substances) content was used the modified heat extraction method proposed by Yang *et al.* (2014).

In OS I, II and III, protein (PN) and polysaccharide (PS) contents were performed, respectively, by the modified Lowry and sulfuric acid-phenol methods (Long *et al.*, 2014). The sum of PN and PS resulted in the total EPS. The extraction of EPS in OS IV was by the method of Li and Yang (2007), making it possible to determine the EPS that were on the surface of the granules (loosely bound, EPS-LB) and those that were closely bound inside the granules (tightly bound, EPS-TB). For polysaccharides quantification (PS) the phenol-sulfuric method was used (Dubois *et al.*, 1956). To quantify the proteins (PN), the Lowry method was followed, using bovine serum albumin as a standard (Lowry *et al.*, 1951).

The granule size distribution was performed by the sieve methodology described by Bin *et al.* (2011), using sieves with different mesh opening diameters. The average particle size was measured using Image-Pro Plus software from microscopic images to determine the granulation process and granules' stability. The reactor reaches the aerobic granulation stage only when more than 80% of the biomass has a diameter greater than 0.2 mm.

The processes that occurred during a cycle were evaluated to understand the SNDPR (simultaneous nitrification, denitrification, and phosphorus removal) mechanisms. The cycle tests were performed at the end of the experiment (period II in OS II and OS III) and at the end of each period (OS IV). Thus, samples along the 12 hours of cycle (distributed in 24-25 points) were also analyzed, at intervals of approximately 30 min. Each collected sample was filtered before being analyzed.

4.6 Granules morphology

The structure of the mature granules was analyzed by scanning electron microscopy (SEM) combined with spectrum energy dispersive X-rays (Inspect S50, FEI Company, USA). Granules were pretreated with fixing, washing, and lyophilized before the final procedure, according to the methodology described by Motteran *et al.* (2014).

4.7 Microbial community analysis

Analyzes to determine the composition of the microbiota were performed in the inoculum and at the end of each period in both reactors. All samples were collected during the aerobic phase (0.5 g fresh), in order to guarantee that all the biomass was homogenized in each reactor. Initially, DNA extraction was performed. For this, the V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) with primers (515 F-Y and 806R) (Parada *et al.*, 2015; Caporaso *et al.*, 2011). Each PCR reaction was performed in a volume of 30 µL where 2µL of genomic DNA (5 ng/µL), 0.75 µL of each primer (10 µM), 6.0 µL Go taq G2 HotStart Promega (5X) were added, 3.6 µL of $MgCl_2$ Promega (25 mM), 0.6 µL of Dntps Promega (10 mM), 0.2 µL of Promega Taq polymerase (5U/µL) and 16.1 µL of nuclease-free ultrapure water (Promega, Madison, WI, USA).

The reactions were incubated in the Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany) through DNA denaturation using the following temperatures and times: 95^o C, 3 min; 98^o C, 30 s (35 cycles); 55^o C, 30 s; 72^o C, 45 s; 72 ^{o} C, 5 min. Amplicons were checked on a 2% (w/v) agarose gel and purified with Ampure XP beads (Beckman Coulter, Inc., Brea, CA, USA). After purification, the amplicons were subjected to a new PCR for insertion of the Illumina sequencing adapters using 25.0 µL of 2X KAPA HiFi Ready Start Mix (Roche), 5 µL of each Nextera XT index (Illumina, San Diego, CA, USA), 5 µL of library and 10 µL of water. After indexing, libraries were purified using Ampure XP beads.

The concentration of each library was determined using a Qubit® 2.0 fluorimeter (Invitrogen, Carlsbad, CA, USA) and then normalized according to the protocol for preparing the sequencing library (Illumina, San Diego, CA, USA). Equimolar concentrations of each library were pooled in a single tube forming a pool that was diluted and denatured. Sequencing was performed using the Miseq V2-300 cycles reagent kit, according to the manufacturer's instructions (Illumina, 2013).

Sequencing reads were trimmed, assembled, and denoised using DADA2 package v1.20.0 (Callahan *et al.*, 2016). The resulting amplicon sequencing variants (ASVs) were taxonomically classified using the IDTAXA classifier (Murali, Bhargava & Wright, 2016), from DECIPHER package v2.20.0 (Wright, 2016), based on release 138 of SILVA rRNA database (Quast *et al.*, 2013).

The absolute abundances of the target enzymatic functions were predicted from taxonomic data with PICRUST2 (Douglas *et al.*, 2020). It is important to mention that PICRUST2 has limited credibility because it extracts functional information from metataxonomic data. For this reason, PICRUST2 results were considered only as an indication of the overall metabolic activity found within the samples and not as a detailed description. KEGG Orthology codes for the target functions were obtained by keyword-searching with KEEGREST engine v1.32.0 (Tenenbaum, 2021), resulting in the following codes: K10944, K10945, K10946, K10535, K05601, and K15864 for ammonia-oxidizing bacteria (AOB); K00370 and K00371 for nitrite-oxidizing bacteria (NOB); K20932, K20933, K20934, and K20935 for anaerobic ammonium oxidation (ANAMMOX); K20812, K00975, K00688, and K02438 for glycogen-accumulating organisms (GAO); K00937, and K22468 for phosphorus-accumulating organisms (PAO); and K00372, K00360, K00367, K00370, K00371, K00373, K00374, and K10534 for denitrifying bacteria (DNB). Bioconductor (Huber *et al.*, 2015) packages DADA2, DECIPHER, and KEGGREST were run in R, version 4.1.2.

The abundance-based coverage estimator (ACE), Chao1 index, Shannon index and Simpson index were calculated using the Phyloseq package. The raw sequences data obtained in this study have been deposited in the National Center for Biotechnology Information (NCBI) BioProject database, ID PRJNA836880.

4.8 Statistical methods and kinetic parameters

Statistical analyzes were performed with the Origin 2018 computer software applying the Mann-Whitney Rank Sum test to compare the performance of the reactors at a 95% confidence level, where the data groups were statistically different when $p <$ 0.05.

Biomass specific rates of nutrient removal (q_{COD}, q_{NOx}, q_{NH_4}, q_{TP}) were calculated based on Eq. (1).

$$q_{COD,NOx,NH_4,TP} = \frac{(C_i - C_f)}{V_R \times X_{VSS}} \times \frac{V_e}{t_c} \tag{1}$$

$q_{COD,\ NH_4,\ NOx\ TP}$ are the observed biomass specific rates of nutrient removal (COD, NH_4^+-N, NO_x-N or TP, in mg/d.gVSS);

C_i is the initial concentration (COD, NH_4^+-N, NO_x-N or TP, in mg/L); C_f is the final concentration (COD, NH_4^+-N, NO_x-N or TP, in mg/L); Vr is the useful volume of the reactor (L); Xssv is the concentration of volatile suspended solids in the reactor (gSSV/L); Ve is the effluent volume of a reactor operating cycle (L); and Tc is the time of one operating cycle of the reactor (d).

The full version of this paper is under review at theBioprocess and Biosystems Engineering.

5 AEROBIC GRANULATION PROCESS APPLIED TO LANDFILL LEACHATE TREATMENT

Aerobic granular sludge (AGS) process performance was evaluated for real leachate treatment, assessing the system capacity to form granules, biomass characteristics, and other engineering and microbiological aspects. Two sequencing batch reactors (SBR) were operated with leachate concentration of 25% (R1) and 50% (R2), with 8h cycle. The time required for granulation was greater than 80 days in both reactors, and solids loss was significant. The sedimentation rate was also outside the typical values for AGS reactors, with sludge volumetric index in 30 min (SVI_{30}) greater than 70 mL/g. Although the granules produced in R2 were more compact (200 μm), they were more resistant. Proteobacteria and Rhodobacteraceae were the phyla and the most abundant family in R2. The phylum Planctomycetota and the family Pirellulaceae were the most abundant in R1. Settling time reduction, feeding phase increase, and increased dissolved oxygen (DO) levels were fundamental strategies to improve reactors' performance and stability.

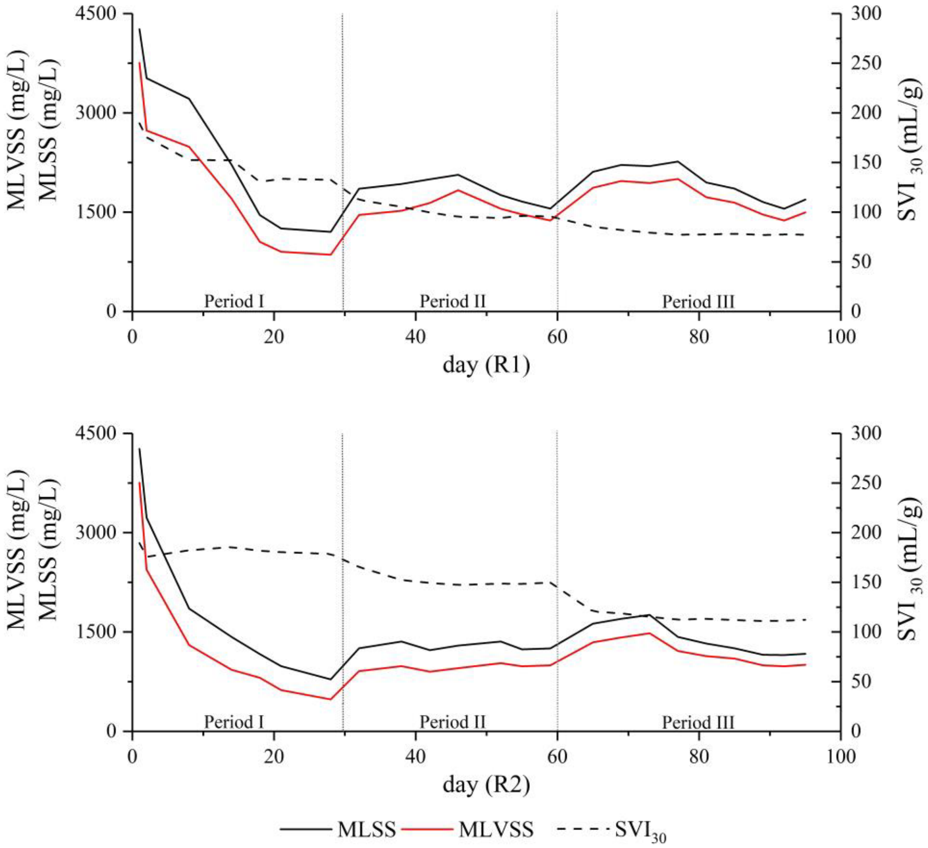
5.1 Granular biomass development

As previously mentioned, biomass was initially acclimatized for two days to minimize the hydraulic shock toxicity. Figure 8 shows the profile of solids and SVI_{30} throughout the AGS systems operation with 25% (R1) and 50% (R2).

The biomass concentration dropped significantly in both reactors after contact with leachate. During the period I, solids loss between reactors was statistically similar, suggesting that leachate toxicity reduces biomass retention. Throughout the strategies adopted, the two reactors showed significant differences. T_s reduction and the feeding time increase (Period II), and DO increase rate (Period III) favored biomass retention, reducing the effluent solids concentration. In the system with the highest leachate load (R2), the biomass retention was significantly lower both in period II ($p < 0.001$) and period III ($p = 0.001$).

It was also possible to verify that the final proportion of VSS in relation to TSS was 88% in R1 and 84% in R2. It was to be expected that R2 would have the lowest solids concentration since several studies indicate that higher leachate loads generate a more filamentous biomass, which is easily washed and consequently worsens sedimentation. Despite this, solids loss in R2 with 50% leachate was still lower than the values reported by Bueno *et al.* (2020) when using much lower concentrations of leachate (5%) and by Ren *et al.* (2017b) by increasing the influent ammonia concentration.

Figure 8 – Stability in terms of solids and SVI_{30} of AGS systems with 25% (R1) and 50% (R2) leachate with feeding and settling time of 20 min (Period I), feeding of 40 min and settling time of 10 min (Period II) and with increased DO concentration (Period III).



Source: Prepared by the author.

Still, a pattern behavior identified was that after the optimization stipulated in each period, there was an improvement in biomass development that worsened again until the beginning of the next optimization. Thus, this improvement and worsening peaks suggest that the raw leachate's toxicity does not support biomass growth, which always returns to the decay stage.

As for settling, the reactors had similar behavior, but the R2 presented higher SVI ($p < 0.001$). As with solids, the change in settling and feeding times and DO concentration favored settling. However, these optimizations were not enough to achieve the desirable SVI levels for AGS reactors (< 50 mL/g) since, at the experiment completion, R1 had an SVI_{30} of 79.1 mL/g and R2 of 114.1 mL/g. It was already expected that R2 would present this sedimentation outside the acceptable range, but R1 should have better sedimentation rates since it had less toxicity and less tendency to develop filaments.

The settling results with 50% real leachate alone (R2, tap water dilution) were similar to or better than those with lower concentrations of leachate diluted in synthetic wastewater (Bueno *et al.*, 2020; Saxena *et al.*, 2022). For instance, after the granulation phase, SVI_{30} of 210 and 155 mL/g were achieved for 5% and 10% leachate dilutions, respectively (Bueno *et al.*, 2020). Influent suspended solids (SS) impair granulation and produces poor-quality sludge (Pronk *et al.*, 2015). Settling time reduction contributed to this poor-quality sludge being washed. However, both systems showed considerably high SVI values for aerobic granulation reactors, implying that leachate made the granulation process difficult and impaired granule settling.

5.2 Granules' characteristics

Among the main problems reported in the literature when treating landfill leachate in AGS systems are the recurrent disintegration and the long time required for granulation (Ren, Ferraz and Yuan, 2017a; Bueno *et al.*, 2020). This investigation was no different (Figure 9). Although the SVI in 10 and 30 min ratio (SVI_{10}/SVI_{30}) remained between 1.0 and 1.5, which is referred to as a granulation stage (Kocaturk & Erguder, 2015; Hamza *et al.*, 2018), it took 81 and 89 days for the R1 and R2 systems, respectively, to meet the requirements to be considered granular.

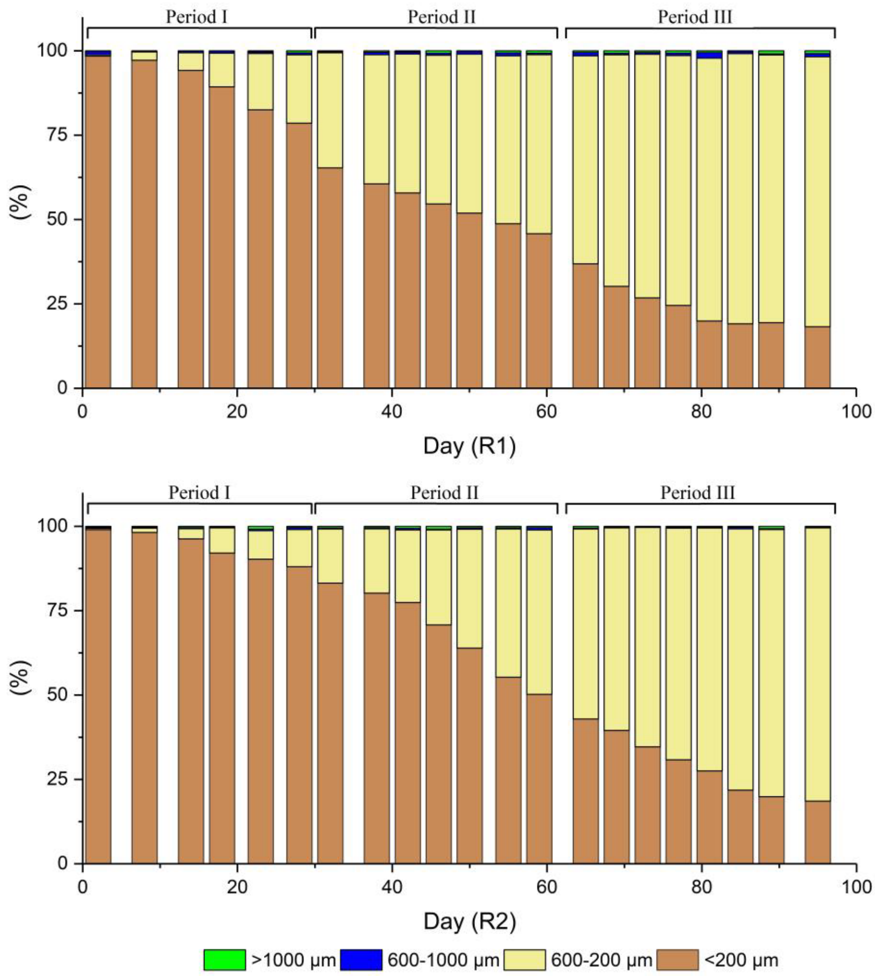
Concerning average granule diameters, although it is possible to find R1 values larger than R2, there were no statistically significant differences between them in periods I and II (p = 0.67) and III (p = 0.67). In periods I and II, both reactors presented granules with average diameters of 100 ± 100 µm, while in period III, the average diameters were 300 ± 100 µm (R1) and 200 ± 100 µm (R2).

As the reactors' granules were not large and the smallest sieve opening in the granulometry was 200 µm, it was not possible to verify the occurrence of disintegration episodes. In addition, it is important to emphasize once again that verifying complete biomass washouts in both systems was common, as it was necessary to return the biomass to the system manually. These washes were more frequent during the first two periods.

However, they did not stop happening during the last period.

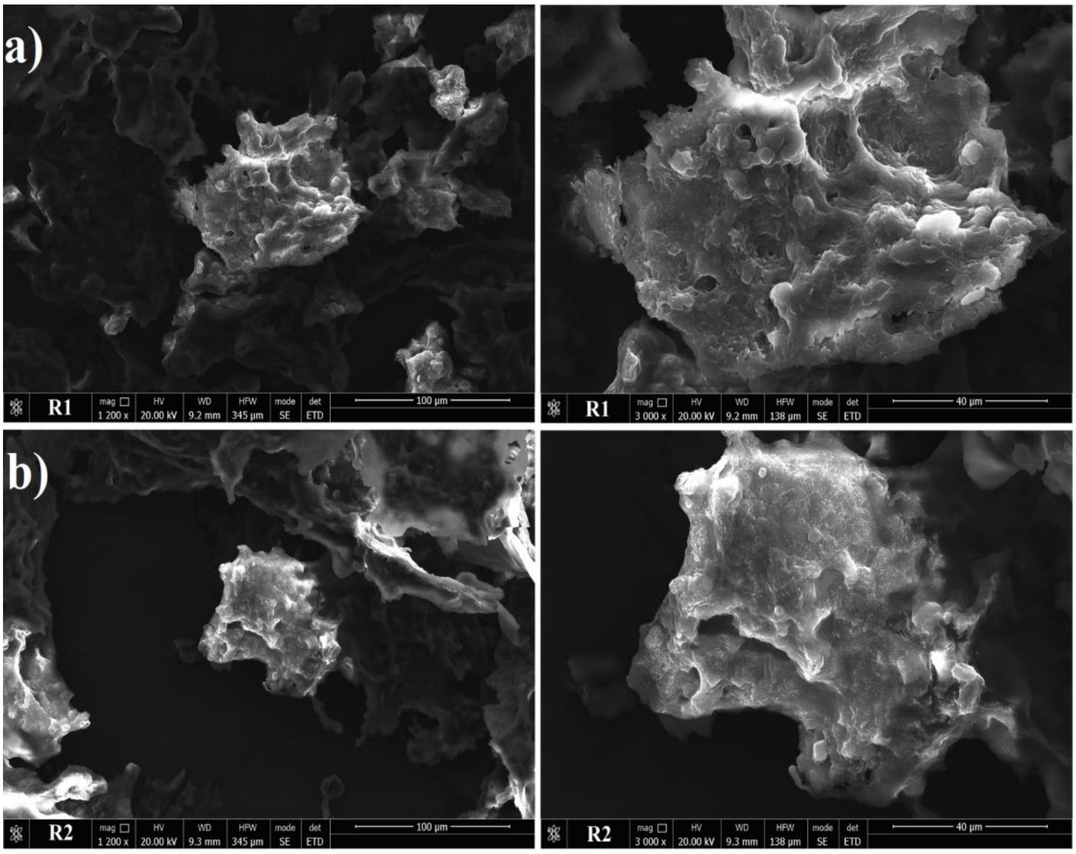
Microscopic analyses with a precision of 3000x were used to evaluate the granules' surface (Figure 10). Their structure was very similar, presenting an irregular surface with the development of ascending coccus. Despite this, it was possible to verify a strong microbial aggregation without the presence of exposed communities. In R2, the reactor with the highest leachate concentration, the presence of small internal structures that suggest fragmentation is evident. In this reactor, channels presence in the surface structure was also observed, contributing to the substrate permeability in the innermost layers of the granules.

Figure 9 - Granule size distribution (% mass) in AGS systems.



Source: Prepared by the author.

Figure 10 – Granule scanning electron micrograph of the reactors R1 (a) and R2 (b) at the end of period III accurate to 1200x (left side) and 3000x (right side).



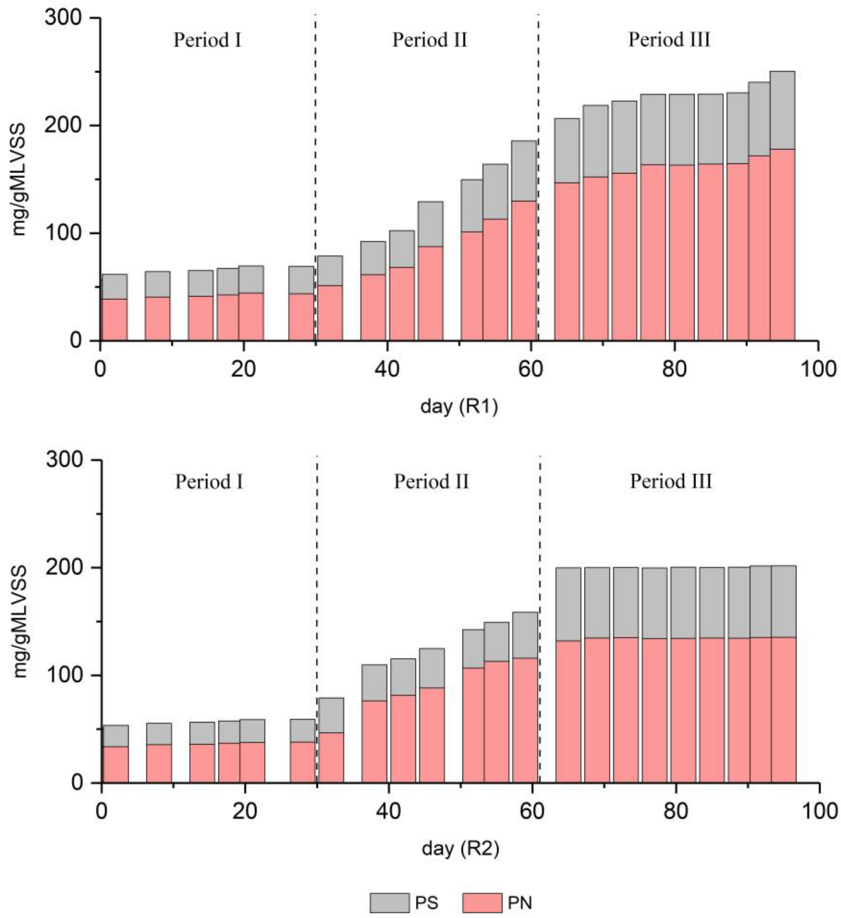
Source: Prepared by the author.

Thus, this microbial aggregation is directly influenced by EPS production, which play a key role in aggregation (Rollemberg *et al.*, 2018). In this regard, to complete the granular biomass profile, the protein (PN) and polysaccharide (PS) fractions were measured (Figure 11). Proteins production was greater than that of polysaccharides in both reactors and remained in the same proportions. This condition is AGS systems, as PN is responsible for granule aggregation, forming organic molecular bonds that are responsible for catalysis and degradation processes, while PS forms carbonaceous bonds that favor sedimentation, generating granule mechanical stabilization (Shi *et al.*, 2017; Rollemberg *et al.*, 2018).

During periods I and II, there were no significant differences between the reactors regarding total EPS production ($p = 0.76$ and $p = 0.94$, respectively), with the mean values in the period I being 64 ± 5 mg/gMLVSS (R1) and 56 ± 9 mg/gMLVSS (R2), while in period II were 129 ± 39 mg/gMLVSS (R1) and 129 ± 32 mg/gMLVSS (R2). In period III, when the DO concentration increased, R1 continued to produce EPS, and R2 remained stable. In R1, the mean production was 238 ± 25 mg/gMLVSS, significantly higher than the mean production of 198 ± 3 mg/gMLVSS in R2 ($p = 0.032$).

DO concentration and control are essential so that microbial communities can secrete EPS at satisfactory levels, favoring cell adhesion and generating the necessary stability for granule activity (Bella & Torregrossa, 2014; Adav *et al.*, 2010). As the EPS production was similar to the usual high production when simpler wastewaters are used, microscopic images revealed this strong aggregation on the granules' surface. Furthermore, despite their reduced diameters, the EPS production was expected to be high due to leachate toxicity since in environments with toxic substances, heavy metals, and severe conditions, microorganisms produce more EPS as a defense mechanism (Esparza-Soto & Westerhoff, 2003). Based on this principle, as in the last operation stages, R2 produced less EPS, it can be inferred that the granules produced were more resistant to leachate toxicity, not feeling the need to produce more EPS as a defense strategy.

Figure 11 – Average quantification of extracellular polymeric substances (EPS) in each AGS system.



Source: Prepared by the author.

5.3 Removal of carbon, nitrogen, and phosphorus in reactors

In each period, influent and effluent samples were analyzed in terms of C, N, and P to measure the reactors' efficiency (Table 9).

During periods I and III, organic matter removal was significantly higher in reactor R1, which had the lowest leachate concentration (p < 0.001 and p = 0.001, respectively). During period II, there were no statistical differences between the two reactors. In this study, chemical oxygen demand (COD) removal was very low, similar to experiments with 90% leachate (Ren, Ferraz and Yuan, 2017a; 2017b). That is, the readily available organic matter presented a recalcitrant character, hindering the assimilation conversions necessary for the biomass. A decrease in the COD removal by increasing the leachate influent organic matter load is also reported (Bella & Torregrossa, 2014).

As part of the organic matter in AGS systems is used for biomass growth and another part to sustain the denitrification process, the granules did not grow, and the nitrogen removal efficiency was impaired because the organic constituents are difficult to degrade. Thus, all nitrogen conversion processes were unsatisfactory, generating nitrite accumulation at concentrations above 60 mg/L in the last period.

Table 9 – Performance of AGS systems in terms of COD, nitrogen, and phosphorus.

Characteristics	Period I		Period II		Period III	
	R1	**R2**	**R1**	**R2**	**R1**	**R2**
COD_{inf} (mg/L)	935±254	1892±305	969±196	1938±375	996±236	1905±433
COD_{eff} (mg/L)	748±52	1625±53	755±59	1542±51	629±36	1415±42
COD removal (%)	19±3	13±2	21±3	18±5	36±3	24±4
NH_4^+-N_{inf} (mg/L)	166±38	374±49	183±42	367±35	165±31	384±49
NH_4^+-N_{eff} (mg/L)	125±8	215±8	109±7	200±4	81±7	165±5
NO_2^--N_{eff} (mg/L)	36±15	75±19	57±13	65±20	62±9	72±12
NO_3^--N_{eff} (mg/L)	6±3	25±8	12±3	39±7	7±2	30±9
NH_4^+-N removal (%)	35±6	41±9	37±5	42±4	48±5	56±6
TN removal (%)	9±6	27±4	20±8	30±6	21±10	33±9
PO_4^{3-}-P_{inf} (mg/L)	6±2	12±3	6±1	13±4	5±3	10±4
PO_4^{3-}-P_{eff} (mg/L)	5±1	11±2	5±2	11±1	4±2	9±2
P removal (%)	2±1	3±2	8±3	6±2	11±1	6±3

Source: Prepared by the author.

As R2 had the highest leachate concentration, it would consequently have a higher non-biodegradable organic matter concentration and lower COD removal. On the other hand, this remaining COD could sustain the denitrification process. Thus, R2 showed the highest removal of total nitrogen. Nitrification in the two reactors only showed a significant difference in period III ($p = 0.037$), in which the R2 efficiency was higher than R1. However, nitrite production was statistically similar between the reactors, except in period I. In R2, verifying a slight nitrate accumulation was also possible, greater than in R1 in all periods ($p < 0.001$). NO_x accumulation has been commonly reported as one of the main limitations of nitrogen removal upon aerobic granulation technology (Saxena *et al.*, 2022; Silva *et al.*, 2022). This accumulation may result from the high load of free ammonia and free nitrous acid available in the leachate, which can favor the nitritation route and negatively interfere with the microorganisms responsible for nitration and denitrification via nitrite or nitrate (Chung *et al.*, 2015). In any case, the nitrogen removal results of this study were similar to previous research (Bella & Torregrossa, 2014; Ren, Ferraz and Yuan, 2017b), in which TN removal was not greater than 30%, even at low leachate concentrations.

Phosphorus removal only showed significant differences in period III ($p = 0.02$), in which the DO concentration was higher. The lower leachate concentration (R1) was fundamental for the higher phosphorus removal, despite being much lower than those found elsewhere (Ren *et al.*, 2017a; 2017b; Ren, Ferraz and Yuan, 2017a; Bueno *et al.*, 2020), even with high leachate concentrations.

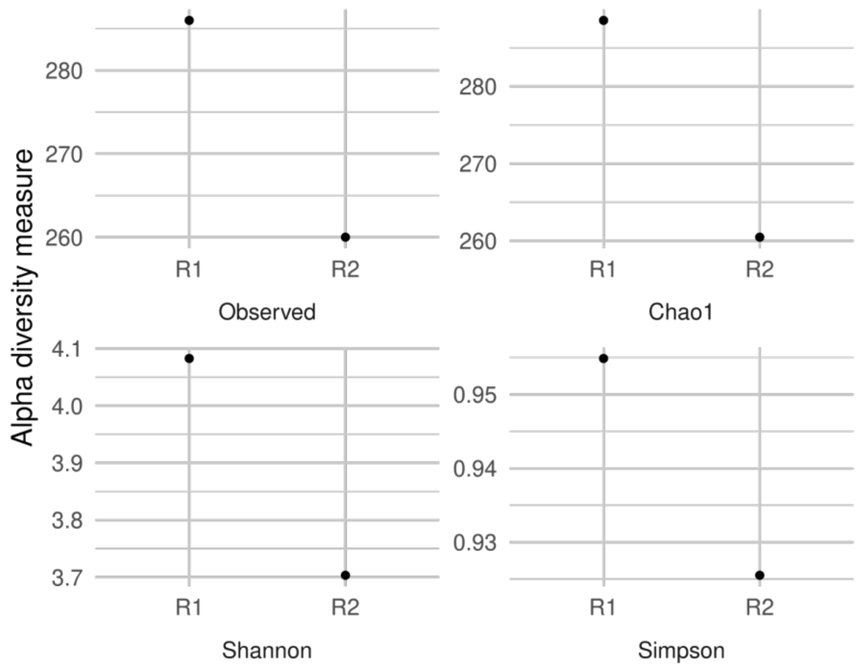
As the granules found in the above-mentioned literature were larger, and the granulation process was not exclusively with leachate, it was expected that organic matter, nitrogen, and phosphorus removals would be lower. Despite this, nitrogen removal was similar to the previous rates reported, even at influent leachate loads greater than those used in this work. In addition, the low removal of organic matter and phosphorus in R2 is a consequence of the toxicity conferred by the higher leachate influent load. In R1, it was expected that the removal of the contaminants would be greater since the influent leachate load is lower. This suggests that it is necessary to create a granulation optimization protocol that favors granules growth and reduces leachate toxicity so that, consequently, there is an improvement in the removal of other organic and phosphorous-based constituents.

5.4 Molecular biology

5.4.1 Taxonomic composition

The following alpha diversity indexes were used to determine the abundance and richness of the species: observed diversity, Chao1, Shannon, and Simpson (Figure 12). These four parameters had the same behavior and show that the abundance and richness of the species are inversely proportional to the influent leachate load. That is, the R1 reactor, which contains the highest leachate dilution, had the highest microbiological abundance and richness. Free ammonia and nitrous acid cause the microbiota's toxicity and limit its development, creating more severe environments as the dilution decreases (Ren, Ferraz and Yuan, 2017a; Wei *et al.*, 2021). Thus, the more leachate is incorporated into the system, the lower the probability of finding a great population diversity.

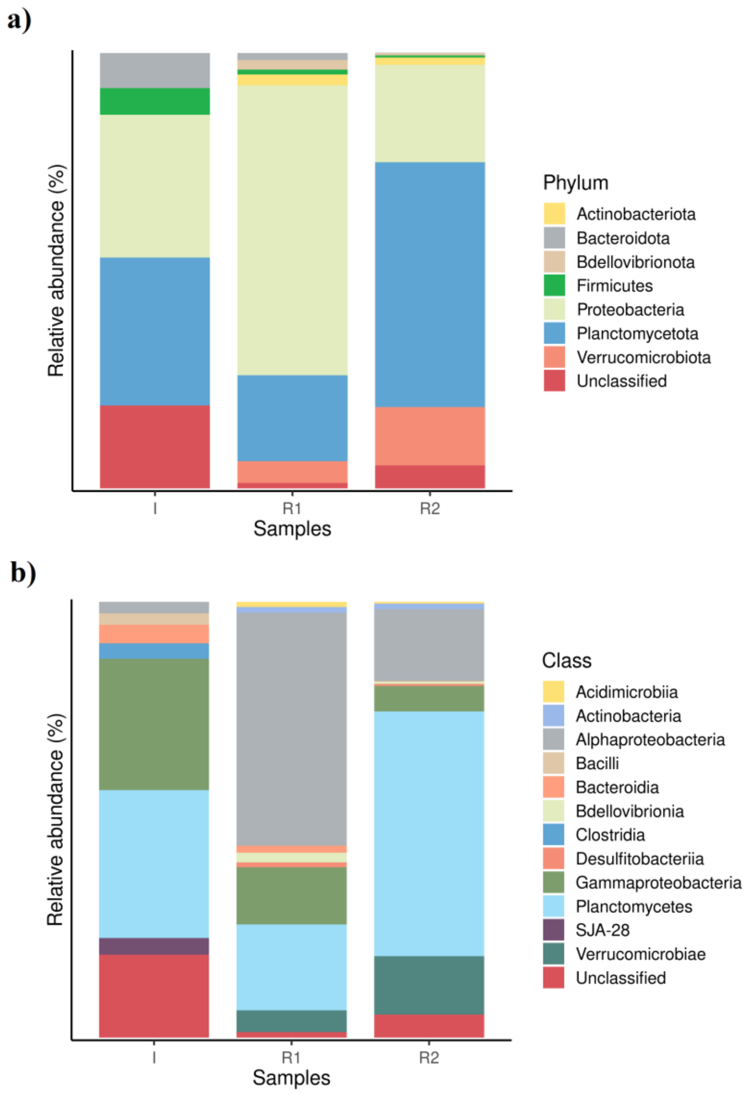
Figure 12 – Number of bacterial species and diversity indices in the two reactors and the inoculum (I).



Source: Prepared by the author.

From gene taxonomic analyses, it was possible to determine and analyze microbial communities at the phylum and class levels (Figure 13). The most abundant phyla in both reactors and in the inoculum were Planctomycetota and Proteobacteria. This result was consistent with previous experiments with leachate treatment in AGS systems, in which more than 80% of microbiota belonged to these two phyla (Saxena *et al.*, 2022), being reported as the main microorganisms involved in COD, NH_4^+-N, and PO_4^{3-}-P removals (Wang *et al.*, 2020).

Figure 13 – Bacterial community structure at the Phylum (a) and Class (b) level of the two reactors and the inoculum (I).



Source: Prepared by the author.

In the inoculum, these two phyla were in the same proportions. However, the leachate load significantly influenced the microbial ecology over time. In R1, the Proteobacteria abundance was much higher than that of Planctomycetota, while in R2, the opposite occurred. Thus, it is possible to infer that Proteobacteria were more sensitive to leachate toxicity since when increasing the influent leachate load (R2), this phylum was reduced, consequently favoring Planctomycetota and Verrucomicrobiota. The latter is reported as essential in organic components removal, so its development was necessary to increase organic removal in the systems (Li *et al.*, 2020). Furthermore, the presence of unclassified phyla was greater, suggesting that the leachate eliminated most of these phyla, which were probably more sensitive and did not resist the imposed selection pressure.

The same happened with Firmicutes and Bacteroidota. The presence of Bacteroidota favors SND in aerobic granules (Li *et al.*, 2020), and its small abundance is consistent with the low TN removals in both reactors.

At the class level, it is possible to verify that microorganisms of the phylum Proteobacteria (Alphaproteobacteria and Gammaproteobacteria) were present, and Gammaproteobacteria were more sensitive to the leachate. This type of effluent also favored the development of Planctomycetes and Verrucomicrobiae, which were more abundant at higher leachate concentrations. Furthermore, the presence of Acidimicrobiia indicates a slightly acidic environment and favors a stronger structuring, improving granules' stability (Li *et al.*, 2020).

5.4.2 Key functional groups

In the last stage of the microbiological analyses, the families found in the systems were categorized according to their functional type (Figure 14) and separated into ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), denitrifying bacteria (DNB), glycogen-accumulating organisms (GAOs), and phosphorus-accumulating organisms (PAOs).

The lowest abundances found were AOB and NOB. As the granules formed were small, these organisms that develop in the outermost layer were inhibited and possibly washed away. Slowly growing nitrifying bacteria improves biomass stability (Rollemberg *et al.*, 2018), reflecting this low abundance in instabilities throughout the operation. The increase in leachate concentration also influenced the microbial distribution, inhibiting NOB-type microorganisms, in which no families belonging to this group were found in R2.

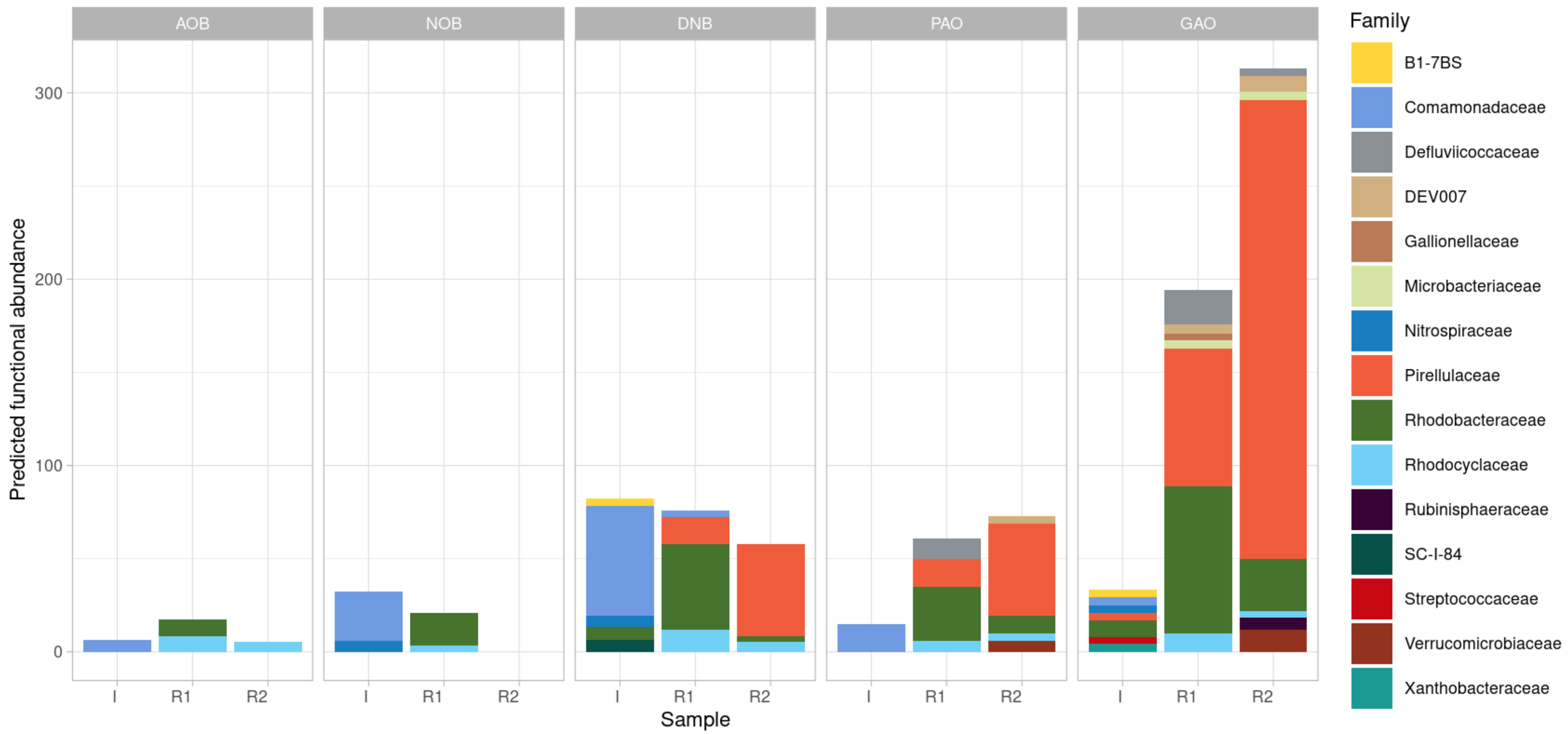
The two reactors showed similar abundances of DNB, with R1 represented mainly by Rhodobacteraceae and R2 by Pirellulaceae. Thus, the increase in leachate concentration favored Pirellulaceae and inhibited Rhodobacteraceae. This was observed in DNBs, as well as in PAOs and GAOs. Comamonadaceae was the most dominant DNB family in the inoculum and is essential in the nitrogen removal cycle (Fan *et al.*, 2018). However, they were inhibited in both reactors in the presence of leachate for requiring specific conditions. In general, great abundances and diversities of AOB, NOB, and DNB microorganisms were not found, which explains the low TN removals and NO_x accumulations.

Under conditions where the oxygen concentration is not zero, phosphate-accumulating microorganisms use polyhydroxyalkanoates (PHAs) to accumulate phosphorus. As organic matter is the energy and carbon source for many organisms in the microbial community, including PAOs, competition is inevitable. At the end of the experiment, it was possible to verify that the competition between PAOs and DNBs was not at considerable rates since the microorganisms of these two functional groups were practically at the same concentrations in both reactors. On the other hand, GAOs predominated in relation to PAOs. This fact may indicate that their competition was more intense and that the available organic matter benefited GAOs.

Microorganisms from the Pirellulaceae and Rhodobacteraceae families were the main PAOs and GAOs, respectively, found in the two reactors. In R1, they were practically in the same proportion. In R2, Pirellulaceae was much more abundant. These microorganisms prevent filamentous bacteria from developing in periods with excess oxygen by immediately consuming part of the available substrate (Rollemberg *et al.*, 2018). Therefore, the greater abundance of these families was not a cause for concern in this study, as they tended to be stable.

In general, the leachate load increase in R2 inhibited the development of important microorganisms for complete nitrogen removal, such as AOB and NOB, which was reflected in the low TN removal and incomplete denitrification values. The leachate load also influenced the profile of DNB, PAOs, and GAOs since Pirellulaceae was the most abundant family with higher leachate concentrations, suggesting that these microorganisms are less sensitive and more tolerant to the toxicity imposed by the leachate. In the reactor with the lowest leachate load, the Rhodobacteraceae family was the most abundant, both DNB and PAO, and GAO types.

Figure 14 – Functional taxonomic classification distribution at the experiment’s end in each reactor and in the inoculum (I).



Source: Prepared by the author.

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5.5 Strategic discussions

Considering that this investigation joins the small group of works that used the AGS technology to treat real leachate, some conceptions could be established and paradigms broken.

Apparently, an anaerobic phase becomes unnecessary concerning cycle distribution, unlike AGS systems for the treatment of simpler effluents, such as municipal wastewater, which is essential. For more complex effluents such as leachate, fermentation during this phase is insignificant and can select microorganisms of low energy value, such as PAOs. The cultivation of PAOs has been a widely used strategy in most granulation works, as they remove P and favor the formation and stability of aerobic granules by accumulating PHAs. However, as leachate has a low phosphorus concentration, other constituents' removals, such as nitrogen, COD, toxic compounds, among others, are more challenging.

GAOs have a higher growth rate than PAOs, being dominant at temperatures greater than 20°C, whose increased metabolism favors the stability and development of granules (Erdal, Erdal and Randall, 2003; Rollemberg *et al.*, 2018). That is, the temperature is a limiting factor for the growth of PAOs. In this sense, it is more advantageous to cultivate GAOs, which are similar to PAOs and favor mature granules' formation and stability but do not accumulate phosphorus. Like PAOs, GAOs also store volatile fatty acids (VFAs) in the form of polyhydroxyalkanoates (PHA) and use glycogen as an energy source. Furthermore, as both groups present denitrifying microorganisms (DPAOs and DGAOs), nitrogen removal would also be favored by the greater kinetics of GAOs.

The available organic matter in the leachate is insufficient to sustain complete nitrogen removal and to cultivate dense granules, requiring COD supplementation. This approach, in addition to favoring denitrification and reducing the accumulation of nitrite and nitrate in the effluent, also contributes to biomass growth. However, depending on the landfill age, the concentration required may be different, being higher in older leachates, which have a lower COD load. Therefore, organic matter supplementation is also the key to reducing the time required for granulation because a fraction will be used for granule development.

Special attention must be given to the granule size, as one of the problems frequently reported in investigations dealing with leachate in AGS systems is granule disintegration. This happens because the developed granules use only the organic matter available in the leachate, which is divided with the other processes, producing weaker granules. Furthermore, leachate toxicity plays a major role in this disintegration, which is minimized when dilutions and organic matter supplementation are used. The source of organic matter selected is expected to favor the development of more resistant granules, reducing the disintegration episodes, and also to favor specific microbial groups, such as GAOs and DGAOs.

It is important to note that the most common on a full scale is the simultaneous treatment of leachate and domestic effluents (co-treatment). As mentioned, this dilution also reduces leachate toxicity. In addition, this co-treatment is advantageous, as the daily sewage flow is much greater than the leachate flow. Thus, large leachate dilutions are acceptable (up to 20% leachate), eliminating the need to deal with the impacts of high leachate concentrations.

However, in Sewage Treatment Plants (STPs), there will be an increase in the concentration of phosphorus, slowly biodegradable organic compounds, ammoniacal nitrogen, heavy metals, and other substances that inhibit biological processes, making it more challenging to comply with the release limits. Thus, this dilution must be carried out most appropriately towards the ideal granule formation protocols, which must consider the mixture percentages, the cycle times, and the frequent alternation of aerobic and anoxic phases in the cycle.

Compared to the most used biological treatment system in the world, the Conventional Activated Sludge (CAS) system, granule disintegration can be a determining factor. In general, the resistance of the aerobic granules may be lower at high leachate concentrations than the activated sludge flocs resistance, but the recovery time is also shorter. Ren *et al.* (2017b) found that when free ammonia (FA) inhibited biomass, AGS systems could recover faster than CAS systems. Furthermore, FA concentration that inhibits microorganisms in AGS systems is twice as high as in CAS systems (Ren, Ferraz and Yuan, 2017b). Thus, when performing adequate dilutions in AGSs systems, resistance to toxicity will be amplified, and recovery will occur in shorter times.

Therefore, given the above, the AGS technology for leachate treatment, whether in the sanitary landfill or co-treatment with sewage, is feasible as long as all the aspects summarized here are considered.

5.6 Conclusion

The granulation process from real leachate required a high temporal demand and produced very small and unstable granules. By presenting a higher influent leachate load, the biomass in R2 was more susceptible to toxicity, which could have impaired granule development and reactor performance. On the other hand, R1 contradicted the initial hypotheses, presenting unsatisfactory results, being inferior and/or similar to R2 and those reported in the literature.

The two reactors showed settleability out of ideal and low solids retention. However, in R2, this retention was similar to works with lower leachate loads. This loss of solids in R2 was not enough to impair nitrogen removal, which, linked to the available organic load during the cycle due to the low COD removal, favored SND, which was higher than in R1 (with lower leachate load). Furthermore, EPS and microbiology results suggest that granules produced in R2 were more resistant to leachate toxicity.

Thus, the influent leachate concentration was also fundamental to differentiate the removal of C, N, and P and to shape the microbial profile. In both reactors, there were accumulations of NO_x, reflections of the inhibition of AOB, NOB, and DNB microorganisms by the increase in the leachate load.

Although the results were not desirable and the influent leachate concentration was high, they were similar to those obtained in studies with lower leachate loads diluted in domestic sewage and with previous granulation in the absence of raw leachate. In addition, settling time reduction and DO concentration increase were fundamental to increasing solids retention, favoring microbial aggregation, and improving reactor performance. Thus, new optimization strategies must be investigated for applying real leachate, focusing on protocols for granulation and cycle phases with oxygenation gradients to favor the development and stratification of oxygen inside the granules. A previous physical-chemical pre-treatment to reduce leachate toxicity and a COD supplementation by using a readily-available substrate are also recommended to be investigated.

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6 IMPACT OF FEEDING STRATEGY ON THE PERFORMANCE AND OPERATIONAL STABILITY OF AEROBIC GRANULAR SLUDGE TREATING HIGH-STRENGTH AMMONIUM CONCENTRATIONS

This investigation evaluated the impact of feeding strategy on the performance and operational stability of aerobic granular sludge (AGS) treating high-strength ammonium concentrations. Synthetic wastewater with characteristics closes to those found in sanitary landfill leachate was applied in sequential batch reactors (SBR) for biomass cultivation. In this sense, differing only in the feeding method, three identical 7.6 L (working volume) reactors were operated with the same total cycle time of 12 h duration. In R1 and R2, it was adopted feeding in the anaerobic period with a duration of 20 min (fast) and 40 min (slow), respectively. In R3, feeding was distributed throughout the cycle (step-feeding), half of which was introduced initially, and the other half divided equally with 40 and 60% of the cycle. Substrate distribution throughout the cycle (R3) minimized three of the biggest problems faced when treating leachate in AGS systems: granules' stability, biomass retention, and nitrite accumulation. Besides, compared to fast (R1) and slow (R3) feeding, this mode of operation obtained the best total phosphorus (TP, 53%) and total nitrogen (TN, 92%) removals, without any nitrite or nitrate accumulations. COD removals were very similar in R2 and R3, but TN and TP removals were significantly greater in R3. Therefore, the feeding method directly interferes with the performance, granules' characteristics, and system stability. The results obtained in this investigation can be used in future works applying the AGS technology for sanitary landfill leachate and other complex wastewaters treatment.

6.1 Start-up, formation, and stabilization of the granules

The three reactors were initially operated with the same sludge source, presenting about 3.4 g/L of MLSS, MLVSS/MLSS ratio of 88% and SVI_{30} of 190 mL/g. The evolution of these parameters throughout the experiment is shown in Figure 15. After start-up, the MLSS concentration gradually decreased in both R1 (20 min feeding) and R2 (40 min feeding). Settling time reduction was a key strategy for promoting granulation.

However, even after biomass stabilization, a constant sludge loss (washout) was observed in these reactors, which is very common in AGS systems operated with high-load wastewaters (Zhang, Zhang and Yang, 2015; Long *et al.*, 2015; Leal *et al.*, 2020). It may indicate the formation of a biomass with high growth of filamentous microorganisms at high rates of substrate transport, making them more flocculent.

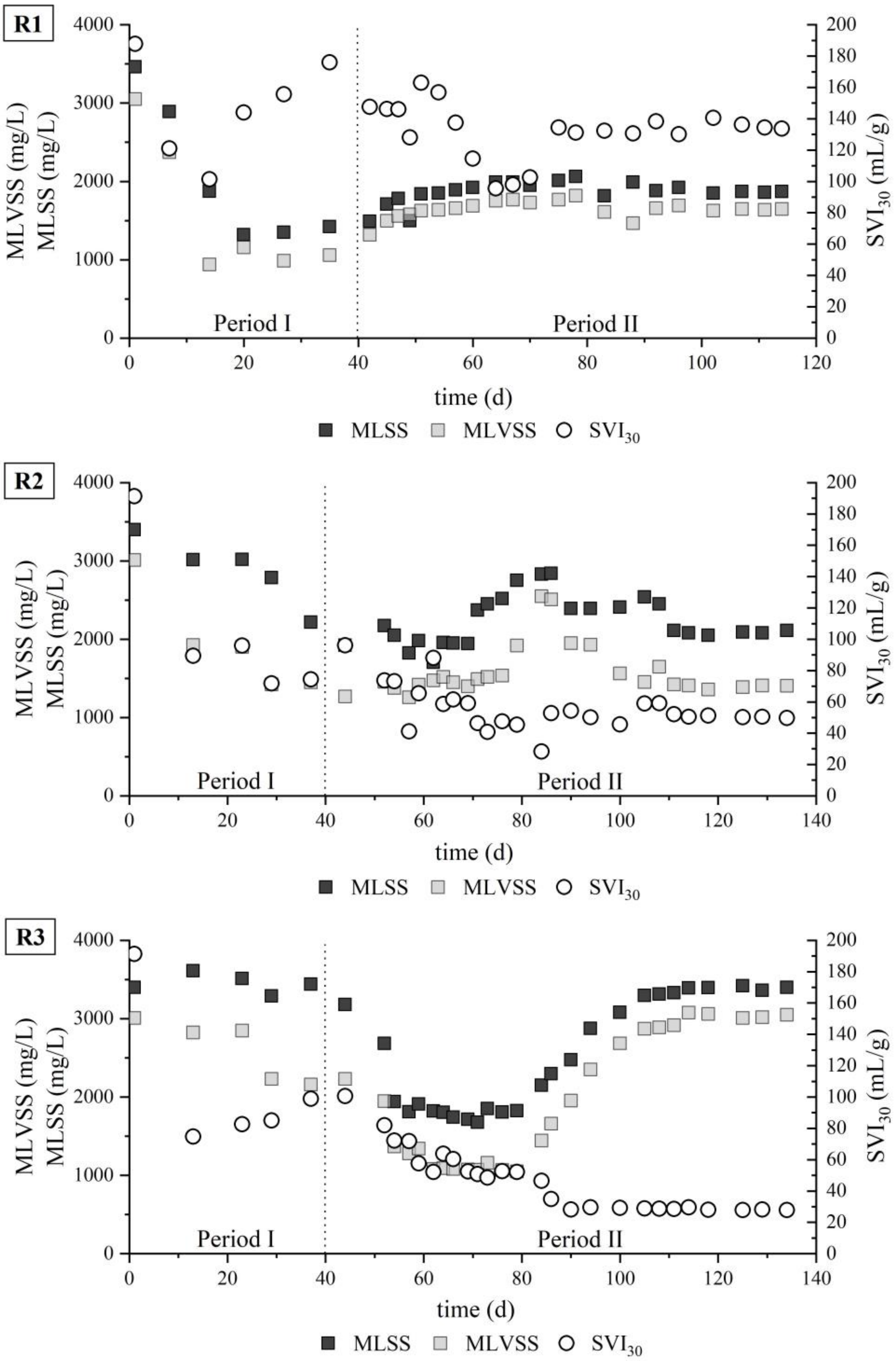
As the high influent organic load is not biodegradable, there will be impacts on the carbon supply for denitrification, which might also result in biomass washout (Bella & Torregrossa, 2014; Ren *et al.*, 2017b; Bueno *et al.*, 2020). Thus, an external addition of soluble COD becomes necessary for AGS cultivation when it is intended to treat effluents not favorable to slow-growing bacteria development. Besides, distributing the organic load throughout the cycle to reduce toxicity and favor denitrification seems to be an efficient strategy.

Disintegration of the granules was frequent in R2, and MLSS concentration was very unstable, failing to achieve a consistent regranulation. As previously reported for AGS systems treating leachate from sanitary landfills, granule disintegration also occurred after 50 days of operation and excessive biomass loss was found (Bueno *et al.*, 2020; Ren, Ferraz and Yuan, 2017a). These authors also pointed out that loads above 200 mg/L NH_3-N favored granules' disintegration.

Therefore, it becomes evident that the influent COD/N ratio is preponderant for the formation and maintenance of stable granules. When this ratio is high, there is a growth of filamentous microorganisms that can cause granule disintegration (Carrera *et al.*, 2004; Luo *et al.*, 2014). On the other hand, reducing this ratio generates great changes in the microbial community. It decreases the EPS content, impacting nitrification, and resistance, size, and settling capacity of the granules, and subsequent biomass loss.

Thus, the instability and disintegration of aerobic granules in high influent loads can be attributed to the increase in granule size due to the inability of carbon penetration, to the hydrolysis and protein degradation of the granule nucleus, and to the loss of microorganisms' ability to self-aggregate due to reduction of EPS protein content (Liu and Liu, 2006; Adav *et al.*, 2010).

Figure 15 – Stability in terms of SS, VSS, and SVI_{30} of AGS systems with fast feeding (20 min, R1), slow feeding (40 min, R2), and step-feeding (R3) for the settling times of 20 min (Period I) and 10 min (Period II).



Source: Prepared by the author.

In R3 (step-feeding), during period I (settling time of 20 minutes), despite MLSS concentration has increased, the SVI also increased, which indicates a sludge of low settleability, possibly dispersed or flocculent. After reducing the settling time, this poor-quality sludge was washed out. After 30 days of stability, there was again a biomass growth, significantly improving settleability and reaching a MLSS concentration similar to the inoculum. Therefore, as in the experiments by Wang *et al.* (2012) with two feeds throughout the cycle, the MLSS first decreased and then increased and stabilized. These results are also in line with those of Wei *et al.* (2012), treating leachate without dilution (3.2 g/L MLSS), and Bueno *et al.* (2020), with 5% leachate diluted in synthetic domestic sewage (3.3 g/L MLSS).

Therefore, R3 had greater solids retention (3.4 g/L MLSS), followed by R2 (2.1 g/L MLSS) and R1 (1.9 g/L MLSS). Also, at the end of the operation, MLVSS proportion in relation to MLSS was 90% in R3, 88% in R1, and 67% in R2. Retention of solids in AGS reactors has been one of the difficulties encountered when treating leachate, with controversial results and without a defined tendency. Wei *et al.* (2012) and Bella & Torregrossa (2014) obtained a decrease in MLSS concentration when they started with 4 and 11 g/L MLSS, respectively, and ended the experiments with 3 and 5 g/L MLSS. Ren, Ferraz and Yuan (2017a) and Ren *et al.* (2017b) practically achieved twice the initial MLSS concentration, while Ren *et al.* (2017a) did not obtain any change. Apparently, the only pattern found is that the higher the leachate concentration, the greater the solids loss, agreeing with some previous studies (Ren, Ferraz and Yuan, 2017b; Bueno *et al.*, 2020). Wang *et al.* (2012) point out that the MLSS maintenance is mainly linked to the inoculum quality, as the high concentration of inoculum sludge causes stronger and more frequent collisions and friction among microorganisms, resulting in the microbial self-aggregation improvement. Other causes are high influent carbon and nitrogen loads, system operation, and dilution factors.

In terms of settleability, in all reactors, the first falls in the SVIs were due to the initial biomass washout. However, except R1, the SVI improved a lot after the adaptation period, reaching a good stability. Thus, R3 had the best SVI_{30} result (< 30 mL/g), while R1 had the worst result with SVI_{30} greater than 120 mL/g. As in R1 the aeration phase was greater, it was expected to present better settleability, which did not occur. However, the SVI_{30} was greater than 160 mL/g during period I, being improved with settling time reduction (Period II).

Therefore, to improve the settleability in R1, lower settling time would be necessary to select the biomass better. In addition, between 60 and 70 days of operation, the sludge from R2 and R3 reached SVI_{30} < 60 mL/g, while in R1, it was above 100 mL/g. Therefore, the granulation process was better in R3 and R2, respectively, since normally mature granules have SVI_{30} between 30 and 80 mL/g (Derlon *et al.*, 2016). Even with higher carbon and nitrogen loads, the results for R3 were similar to those that adopted the same feeding configuration (SVI_{30} < 30 mL/g) (Wang *et al.*, 2012; Chen *et al.*, 2013) and those that used dilutions that varied between 10 and 100% (SVI_{30} < 25 mL/g) (Ren, Ferraz and Yuan, 2017a; 2017b).

These results showed that, compared with reactors with fast single feeding (R1) and slow single feeding (R2), the step-feeding distributed throughout the cycle is an excellent strategy to retain biomass and improve settleability. This configuration inhibits the excessive proliferation of fast-growing heterotrophic bacteria. Through the succession of feast/famine conditions, it promotes the development of granules of good settling with reinforced structure, contributing greatly to the system stability (Chen *et al.*, 2013; Corsino *et al.*, 2016). Besides, it has become evident that fast feeding imposes a strong selection pressure, making biomass retention and granulation difficult. As the COD is not readily oxidized in the anaerobic period and in the first hours of the aerobic reaction, ordinary heterotrophic organisms (OHO) begin to develop, mainly in filaments, being eliminated in the frequent washouts. Therefore, if a large part of the COD is not oxidized at the beginning of the cycle, problems with biomass may be more significant. As the organic matter present in the leachate is recalcitrant and of low biodegradability, a longer time is required for hydrolysis. Thus, an anaerobic feeding with a longer duration favors granulation, and studies with longer times than those used in this investigation are recommended. In the case of step-feeding, the COD toxicity is minimized as it is distributed throughout the cycle, favoring the development of beneficial microorganisms for the granulation without being eliminated since the washouts are much less frequent and biomass growth is greater than its loss.

6.2 Characteristics of the granules

The aerobic granules showed some different physical and chemical characteristics (Table 10). Therefore, how the reactors were fed affected granules' characteristics, probably due to the different microbial groups that were favored with each strategy adopted. In reactors R2 and R3, it can be seen that the values of SVI_5, SVI_{10}, and SVI_{30} became lower with the settling time decrease. This result demonstrates that biomass settleability has improved over time, being a typical evolution behavior from flocculent sludge to granular sludge. The opposite occurred with R1, in which SVI_5 and SVI_{10}

increased with reduced settling time, indicating poor settleability and filamentous biomass.

Table 10 – Granules' characteristics throughout the experimental periods for AGS systems with fast feeding (R1), slow feeding (R2) and step-feeding (R3).

Characteristics	Period I			Period II		
	R1	R2	R3	R1	R2	R3
SVI_{30} (mL/g)	139.6±29.2	83.0±11.7	85.3±10.0	132.3±17.4	55.8±14.5	46.7±19.9
SVI_{10} (mL/g)	156.1±31.4	105.2±21.0	99.4±8.9	172.6±35.5	59.6±15.2	49.8±23.4
SVI_5 (mL/g)	194.2±41.4	139.0±28.3	124.4±18.6	220.4±52.0	74.5±25.0	56.7±30.1
SVI_{10} / SVI_{30}	1.1±0.2	1.3±0.1	1.2±0.2	1.3±0.2	1.1±0.1	1.1±0.1
SVI_5 / SVI_{30}	1.4±0.3	1.7±0.2	1.5±0.3	1.7±0.3	1.3±0.2	1.2±0.2
Mean diameter (mm)	0.1±0.1	0.2±0.1	0.3±0.1	0.5±0.2	0.8±0.1	1.0±0.3
SRT (d)	-	5±3	6±4	-	11±4	11±5
PS (mg/g MLVSS)	141.1±108.5	46.1±9.2	46.7±2.1	54.9±12.5	50.7±14.2	60.1±8.7
PN (mg/g MLVSS)	385.3±203.7	217.4±17.2	182.8±11.7	285.0±58.9	236.0±23.0	233.6±25.6
PN/PS	4.1±2.7	4.8±0.6	3.9±0.3	5.2±0.5	4.9±1.1	3.9±0.4

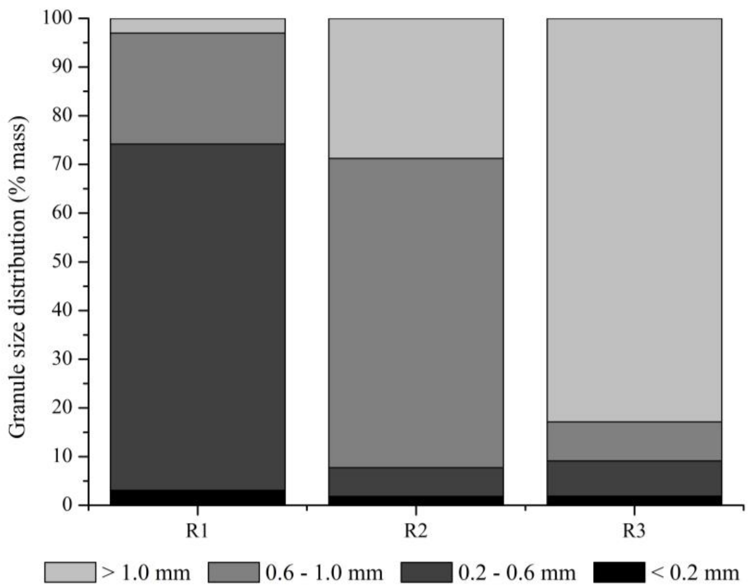
Source: Prepared by the author.

Several authors point out that the SVI_8/SVI_{30} or SVI_5/SVI_{30} ratios can be considered good predictors of granulation, meaning that a value closer to 1.0 indicates that the sludge consists mainly of mature granules (Liu *et al.*, 2010; Corsino *et al.*, 2018; Rollemberg *et al.*, 2019). For effluents with high loads, they observed that there is a predominance of aerobic granules when this ratio is between 1.2 and 1.8. Values above 1.8 characterize an AGS thickening. Thus, the results indicate that the granulation in R2 and R3 was better than in R1, whose SVI_5/SVI_{30} ratio of 1.7 ± 0.3 suggests biomass thickening. In addition, the higher the leachate proportion, the closer to 1.0 will be the SVI_5/SVI_{30} ratio (Ren *et al.*, 2017b; Ren, Ferraz and Yuan, 2017a; Bueno *et al.*, 2020). With this regard, R3 was the best strategy to achieve such a profile.

The literature also reports that the reactor is considered granular when more than 80% of the biomass has a diameter greater than 0.2 mm (Liu *et al.*, 2010). Therefore, the three reactors fit as aerobic granular systems since more than 80% of the granules are larger than 0.2 mm (Figure 16).

In R3, more than 80% of the granules were not only larger than 0.2 mm but larger than 1.0 mm, with an average diameter in period II of 1.0 mm and 1.3 mm at the end of the experiment. Thus, the average diameter of the granules in R3 after 134 days of operation was greater than those obtained in all existing AGS studies so far on leachate treatment: 0.36 - 0.60 mm (Wei *et al.*, 2012); 0.80-0.90 mm (Bella & Torregrossa, 2014); > 0.31 mm (Ren *et al.*, 2017a); 1.1 mm (Ren *et al.*, 2017b); 0.21-0.48 mm (Ren, Ferraz, Yuan, 2017a; Ren, Ferraz, Yuan, 2017b); 0.61 mm (Bueno *et al.*, 2020) (however, some granules with a diameter of 1.5 mm were observed). Furthermore, they were also superior to the granules reported in the studies by Wang *et al.* (2012) with feeding distributed in two stages of the cycle (~ 1.1 mm) and similar to those of Chen *et al.* (2013) with alternating feeding in 3 times (~ 1.3 mm).

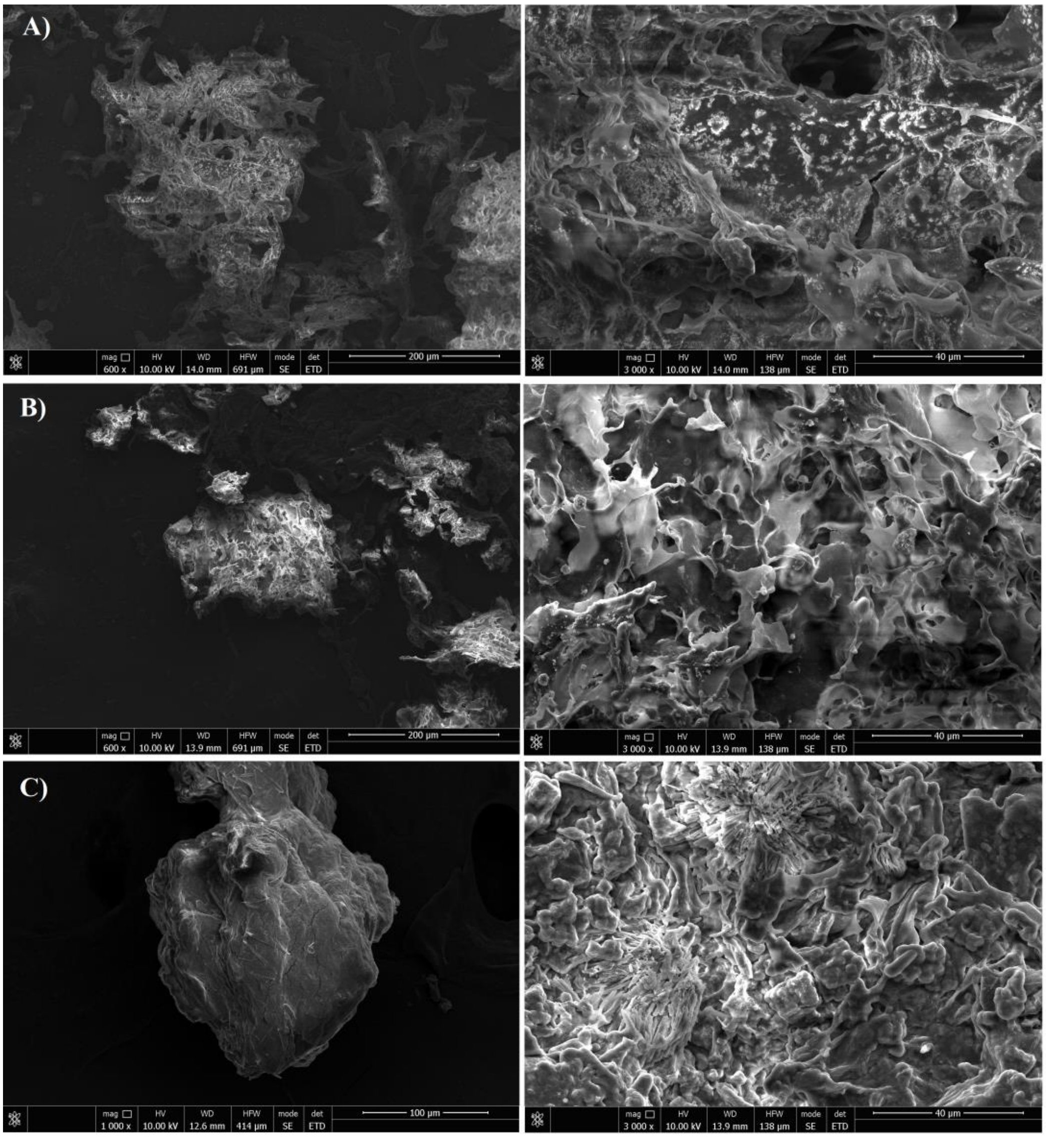
Figure 16 – Granule size distribution (% mass) at the end of period II for AGS systems with fast feeding (20 min, R1), slow feeding (40 min, R2), and step-feeding (R3).



Source: Prepared by the author.

Furthermore, as shown in Figure 17, only R3 presented a granule with a more stable and uniform surface, making it possible to verify the dominance of coccus over bacillus and filamentous bacteria. R1 and R2 did not exactly present a uniform granular structure, being observed a tangle of filaments. However, in R2, a more granular structure that tends to uniformity is verified, despite not showing dominance of coccus.

Figure 17 – Granule scanning electron micrograph of the reactors R1 (a), R2 (b) and R3 (c) at the end of period II.



Source: Prepared by the author.

Concerning EPS, these substances are biopolymers consisting of polysaccharides, proteins, and other substances, which play a fundamental role in the granules' structure, formation, and stability. In other words, they act as a "biological glue" in which PS and PN are responsible, respectively, for granule aggregation and mechanical stabilization (Rollemberg *et al.*, 2018).

As expected, R1 had a higher total EPS content, which agrees with Rusanowska *et al.* (2019), who reported that smaller granules have a higher amount of EPS. Besides, the longer aeration phase duration also influences the EPS content, confirming that EPS production is stimulated by the stress caused by the aeration condition (Rollemberg *et al.*, 2018). However, this high EPS production in R1 did not result in a better settling capacity of the granules.

The reported EPS results for R2 and R3 were lower than R1 and similar to each other, indicating a balance between EPS production and consumption. As is known, EPS production occurs mainly during the feast period, and its consumption occurs during the famine period. So, it was expected that in step-feeding, EPS production tended to balance, being lower than in the other reactors, since the operation produces successive periods of feast/famine distributed throughout the cycle.

In most studies, aerobic granules that are stable have a higher protein portion (PN) than polysaccharides (PS), being correlated to hydrophobicity. Therefore, because PN promotes AGS stability, PN/PS ratio is a way of characterizing its stability (Rollemberg *et al.*, 2018). Thus, the granules in R1 also showed better results (PN/PS = 5.2) than those in R2 (PN/PS = 4.9) and R3 (PN/PS = 3.9).

Retention of solids has generated inconsistent results among the leachate studies. It seems that EPS production does not follow a trend as well. For instance, PN/PS ratio was 4.8 (Wei *et al.*, 2012), while it did not exceed 0.6 in other studies (Ren *et al.*, 2017a; 2017b). EPS production is influenced by several factors such as aeration time, cycle time, shear stress, reactor settings, type of inoculum, amongst others. Therefore, the set of configurations adopted in this study favored EPS production and the PN/PS ratio, possibly improving granules' stability and structure.

6.3 Performance of the reactors during the granulation process

The performance of the reactors was evaluated in terms of COD, nitrogen, and phosphorus (Table 11). In all reactors, the COD removal was high, but nitrogen and phosphorus removals had different behaviors and were better with the settling time reduction.

Table 11 – COD, nitrogen, and phosphorous removals in AGS systems with fast feeding (20 min, R1), slow feeding (40 min, R2), and step-feeding (R3).

Parameters	Period I			Period II		
	R1	R2	R3	R1	R2	R3
COD T_{inf} (mg/L)	1029±44	1019±35	1019±34	1005±23	1022±38	1014±29
COD T_{eff} (mg/L)	695±57	239±54	176±37	180±37	143±31	91±18
COD S_{inf} (mg/L)	976±41	975±33	979±35	983±32	988±20	986±26
COD S_{eff} (mg/L)	639±58	152±35	108±40	160±31	42±15	34±13
COD T removal (%)	32±9	78±5	84±7	81±9	86±3	91±1
COD S removal (%)	31±9	85±3	89±5	81±10	95±5	97±1
NH_4^+-N_{inf} (mg/L)	196±4	194±4	193±5	198±7	197±3	198±2
NH_4^+-N_{eff} (mg/L)	97±11	54±16	47±15	15±8	2±1	1±1
NO_2^--N_{eff} (mg/L)	76±14	25±9	27±11	99±23	30±20	10±8
NO_3^--N_{eff} (mg/L)	2±1	4±2	10±4	4±3	9±7	4±3
NH_4^+ removal (%)	70±12	71±10	74±9	97±1	98±2	99±1
TN removal (%)	21±14	67±29	56±18	56±9	87±6	92±5
PO_4^{3-}-P_{inf} (mg/L)	20±1	21±1	20±1	20±1	20±1	20±1
PO_4^{3-}-P_{eff} (mg/L)	19±1	15±5	10±5	19±1	15±1	9±2
TP removal (%)	4±1	30±5	54±2	6±2	22±4	53±3

Source: Prepared by the author.

The fast anaerobic feeding (R1) showed total and soluble COD removals statistically different and lower than in R2 with slow anaerobic feeding ($p < 0.001$) and the one achieved in R3 with step-feeding ($p < 0.001$). R3 showed a greater and significantly different total COD removal compared to R2 ($p < 0.001$). However, there were no statistical differences between R2 and R3 regarding soluble COD removals (p = 0.604), which may once again emphasize that the constant washouts in R2 may have influenced the total effluent COD concentration.

Regarding total nitrogen (TN) removal, mean values above 50% were observed during the entire operation (except R1 in period I), and significant statistical differences between the three systems were found ($p < 0.001$). As the profile of nitrogenous fractions was different, it is worth mentioning that the removal mechanisms were also different. There was nitrite accumulation in R1 and R2, being significantly lower in R2 ($p = 0.004$). In R3, low NO_x concentrations were observed, resulting in higher TN removals (92%). TN removals in R3 were superior to the values of 75.4% (Wei *et al.*, 2012) and < 50% (Ren *et al.*; 2017a; Ren, Ferraz and Yuan, 2017a) reported with real sanitary leachate.

During the two periods, the nitrification process was observed in both R1 and R2 systems, with values greater than 70%, and increased when the settling time was reduced. However, in period II, it was possible to verify significant differences between R1 and R2 ($p < 0.001$) and between R2 and R3 ($p = 0.008$). Thus, the largest removal of ammonia occurred in R3 (99%), followed by R2 (98%) and R1 (97%).

As the MLVSS concentration decreased (R1 and R2) and remained unchanged (R3) after system stability, the demand for DO did not increase. Since the aeration flow rate was kept unchanged during the operation, nitrifying bacteria activity was not affected, favoring nitrification efficiency. In addition, in R2, during granules' disintegration and recurrent washouts, ammonia removal was reduced (although, on some days, ammonia removal was restored), possibly due to the loss of nitrifying bacteria that were present in the broken granules. When washouts occur at higher frequencies, the sludge age is reduced, and nitrification will be affected if the sludge age is too low. According to Rollemberg *et al.* (2018), several studies have shown that the sludge age is an important parameter for granules' stabilization and reactors' performance since it is directly related to the maintenance of slow-growing bacteria.

It has been reported that the step-feeding mode is effective for making good use of the influent carbon source, increasing the denitrification rate and TN removal (Chen *et al.*, 2011). Thus, nitrification occurs with a lower organic load in the aerobic phase, accelerating the nitrification rate and saving DO consumption to oxidize the influent organic matter. This feeding mode benefits ammonia-oxidizing bacteria (AOB) growth and inhibits nitrate-oxidizing bacteria (NOB), accelerating nitrite accumulation (Wang *et al.*, 2012; Chen *et al.*, 2013). However, in this study, no accumulation of nitrite was observed. It is important to note that in the later works, in addition to the low influent loads, the reaction phase was not totally aerobic, interspersed with anaerobic/anoxic phase, which may have contributed to failures in the simultaneous nitrification and denitrification (SND). Besides, in R3, there was no solids loss, favoring AOB and NOB maintenance in the system. The larger granules of R3 may also have favored SND since this process occurs mainly in granules of larger size, in which nitrification occurs in the outer layer and denitrification in the innermost layer (anoxic). As is known, the proportion of denitrified nitrate in relation to the nitrate produced increases with the average granule diameter, i.e., with a greater anoxic layer (Rollemberg *et al.*, 2019).

Concerning total phosphorus (TP) removals, the three systems showed significantly different values ($p < 0.001$) and were practically unchanged by decreasing the settling time. As expected, R1 had the lowest TP removals due to the rapid anaerobic feeding and the absence of anaerobic/anoxic phases during the cycle. R2 presented TP removals similar to traditional AGS cycles for low-load effluents and smaller than in R3. Probably, in R2, there may have been competition between phosphate accumulating organisms (PAOs) and denitrifying microorganisms, in which denitrifying ones may have been favored. Another probable cause is the presence of glycogen accumulating organisms (GAOs), which have a similar metabolism to PAOs but do not accumulate phosphorus. Besides, substrate complexity must be considered, which may not have favored phosphorus removals in both reactors.

The best TP removals were in R3 (53%), in which the three feast moments during the cycle favored the selection of PAOs. It is important to mention that from the 20th to the 35th, TP removals in R3 were greater than 80%; however, there was a reduction to stabilize then. Probably, the bacteria saturated, and the efficiency decreased, requiring sludge age control. Zhu *et al.* (2013) demonstrated that aerobic granule deterioration occurred more easily in AGS systems with high SRT of granular sludge, and an adequate selective sludge discharge favors process stability. Bassin *et al.* (2012) and Rollemberg *et al.* (2018) also suggest controlled sludge removal (bed or bottom) to remove these saturated bacteria and improve phosphorus removal. Also, TP removals in R3 were slightly higher than those by Bueno *et al.* (2020) when treating higher leachate concentrations. In both low and high leachate dilutions, Ren, Ferraz and Yuan *et al.* (2017b) did not obtain phosphorus removal, being sometimes even reported "negative" values.

Therefore, it was found that the anaerobic feeding with a longer duration had the best TN and TP removals (Table 12). For COD removals, feeding duration does not seem to interfere with efficiency. However, the influent load increase negatively impacts COD removal. However, the step-feeding investigated in this work showed a higher COD removal efficiency than all previous studies.

Except for Ren, Ferraz and Yuan (2017a), who used influent concentrations of phosphorus much lower than the current investigation, TP removal through step-feeding was also the highest observed. Regarding TN removal, step-feeding showed efficiency greater than 90%, also being better than the values reported elsewhere, likely because it provides carbon for denitrification to occur throughout the cycle.

Table 12 – Comparisons between the AGS systems in this study with related works.

Reference	Influent (mg/L)				Reactor			Removal (%)		
	COD	NH_4^+-N	C:N	TP	Type	Feed	Cycle (h)	COD	TN	TP
R1 (fast feeding)	1000	200	5	20	O/A	20	12	81	56	6
R2 (slow feeding)	1000	200	5	20	O/A	40	12	95	87	22
R3 (step-feeding)	1000	200	5	20	O/A	40	12	97	92	53
Wei *et al.* (2012)	4298-5547	72-374	18	-	O/A	60	12	84.4	75.4	-
Wei *et al.* (2012)	4502-5992	602-1168	5	-	O/A	60	12	82.8	35-58.1	-
Bella & Torregrossa (2014)	9738	1960	3	-	O	5	24	40-50	Low	-
Bella & Torregrossa (2014)	4560	945	2	-	O	5	12	50-60	Low	-
Ren *et al.* (2017a)*	448-654	120-500	2	32.5	A2O	30	8	66-73	39	34-54
Ren *et al.* (2017b)*	448-654	120-500	2	32.5	A2O	30	8	67-87	44-48	49
Ren, Ferraz and Yuan (2017a)	1080	340	-	2-6	O/A	90	8	65	40	80
Ren, Ferraz and Yuan (2017a)	1194	580	-	4-6	O/A	90	8	43	25	40
Ren, Ferraz and Yuan (2017a)	1539	900	-	5-6	O/A	90	8	20	<10	40
Ren, Ferraz and Yuan (2017b)	550-1000	130-785	-	3-6	A2O	30	8	43-65	24-37	0
Ren, Ferraz and Yuan (2017b)	1000-1100	785-1085	-	3-6	A2O	30	8	31-40	23-24	0
Ren, Ferraz and Yuan (2017b)	1100-1200	1085-1209	-	3-6	A2O	30	8	7-31	21-23	0
Bueno *et al.* (2020)	650	88	6	13.1	O/A	60	8	87	99	36
Bueno *et al.* (2020)	863	136	5	15.2	O/A	60	8	89	99	42
Bueno *et al.* (2020)	1421	281	5	17.5	O/A	60	8	88	98	45

O (Oxic), O/A (Oxic, Anoxic), A2/O (Anaerobic, Anoxic, Oxic). * studies with synthetic leachates.
Source: Prepared by the author.

Therefore, these COD, TN, and TP removal results clearly demonstrated that the step-feeding mode in AGS-SBR can be applied in high-load wastewater and/or with a C:N ratio similar to that of leachate, favoring granules' stability and treatment performance.

6.4 Cycle experiments

The simultaneous conversions and removals of organic, nitrogenous, and phosphorus constituents were investigated over a complete cycle (Figure 18). From oxygen analysis during the cycle, it was observed that the DO was between 2 and 4 mg/L during the first 4 h of aeration of R1 and, during the first 2 h of aeration of R2 and R3, being in all cases greater than 5 mg/L at the end of the aeration period. These times coincided with the famine period, i.e., when the available COD is in very low concentrations. Therefore, the famine period coincided with the DO increase since the microorganisms enter in the endogenous phase and require lower oxygen concentrations for their metabolism.

In R1, as soon as COD is practically consumed, nitrite begins to accumulate significantly, and the nitrate concentration increases slightly, simultaneously with ammonia oxidation. Although R2 had the same profile, nitrite accumulation was much lower. The low denitrification in R1 and R2 during the oxic period may be associated with the rapid carbon source consumption rate and the absence of an anoxic condition. Polyhydroxyalkanoates (PHA) accumulation during a short period of COD depletion in the aerobic phase may not be sufficient for the subsequent denitrification. In addition, the carbon source in the feeding was not fully utilized by denitrification due to microorganisms' growth and maintenance, which also led to incomplete denitrification and, therefore, decreasing TN removal.

In R3, ammonia was completely oxidized without significant nitrite and nitrate accumulations. Therefore, SND during the oxic period was the main mechanism of removing the nitrogen fractions. When complete nitrification occurred, there was still enough time in the oxic phase for the remaining nitrite to be converted to nitrate by NOBs since, in this reactor configuration, free ammonia did not cause toxicity to NOBs.

According to Wang *et al.* (2012), NOB was much more sensitive to FA than AOB. It is important to mention that heterotrophic denitrification can also occur using EPS as an electron donor during the starvation period. It is possible that at the end of the oxic phase, the extracellular content initially produced was used as an electron donor to remove nitrogenous fractions endogenously. In addition, from the data obtained, it is possible to point out that a fraction of the partial nitrification product was denitrified, and the remaining fraction underwent complete nitrification to be subsequently denitrified. Such results were similar to those of Chen *et al.* (2011), in which a step-feeding strategy created exclusive and ideal conditions for denitrification right after the total ammonia oxidation without relying solely on the anoxic zone within the granules.

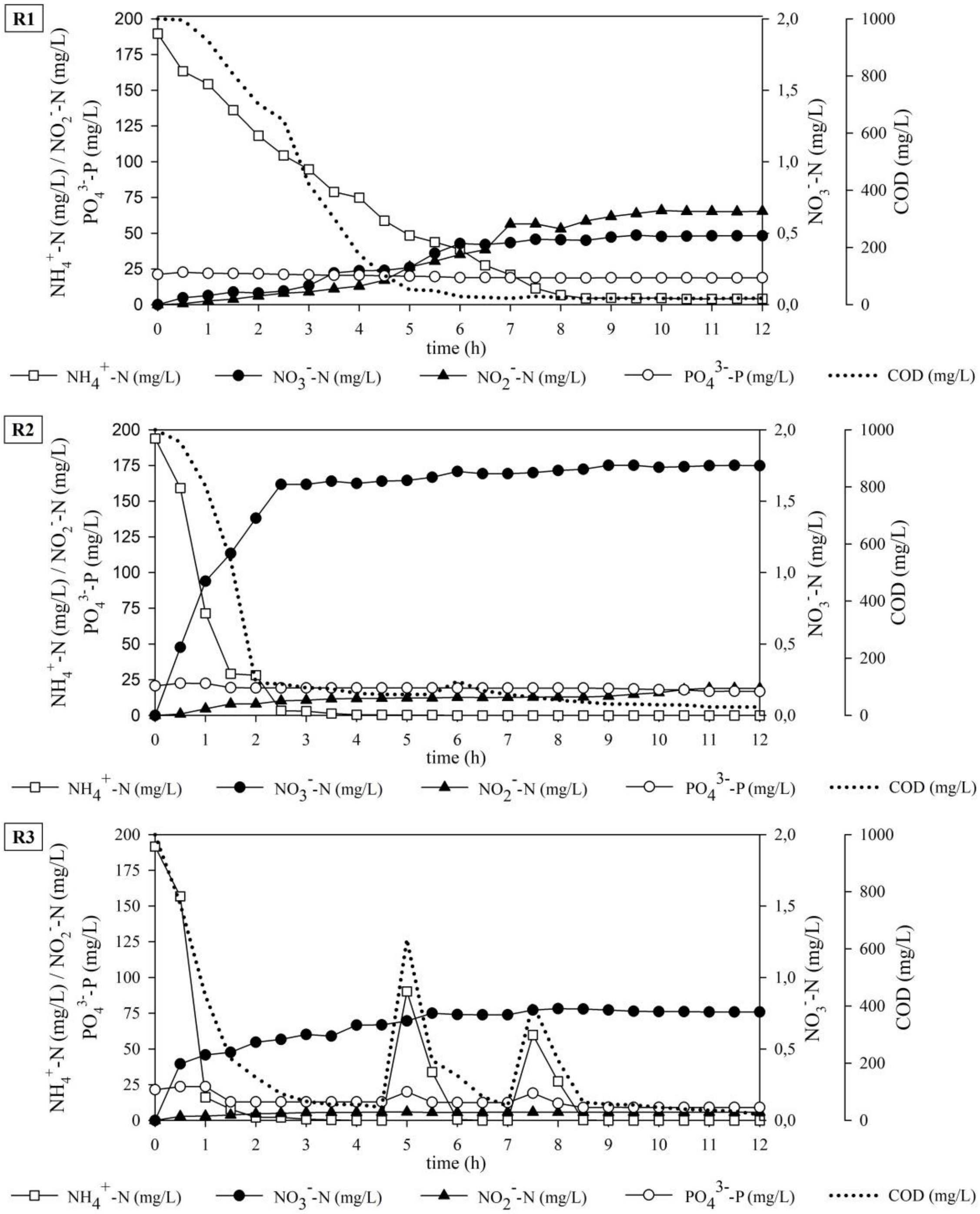
During the cycle, a low pH variation was also observed in R1 (7.0-7.1), and in R2 and R3 (7.1-7.9), probably due to the balance in alkalinity consumption and production during nitrification and denitrification, respectively (SND).

This profile is also in line with other investigations that have observed that step-feeding positively influences the distribution of the main functional groups of microorganisms, and the microbiota responsible for the denitrification process may change positively (Wang *et al.*, 2012; Chen *et al.*, 2013; Corsino *et al.*, 2016). Thus, microorganisms that remove phosphorus can also use the nitrogen products from nitrification as electron acceptors, which favors the high TP removal in R3 (He *et al.*, 2018).

Therefore, it is possible to verify that the removal of nitrogenous constituents may have occurred from different processes. The accumulated nitrogen fraction was immediately converted using the influent organic matter as an electron donor during the feeding period, performing exogenous denitrification. In the oxic periods, the SND process prevailed for ammonia conversion. During the step-feeding (R3), other nitrogenous fractions were removed endogenously, using the intracellular organic constituents as electron donors.

Therefore, the cycle that showed the best performance was the one used in R3, since, without affecting granules' settling and stability, the three feast periods interspersed with famine periods were sufficient for nitrification, facilitating denitrification due to the availability of COD in the feast periods distributed throughout the cycle and obtaining high phosphorus removals. This is excellent for treating more complex wastewater because the nitrification and denitrification processes generally depend on the amount of influent/available organic matter.

Figure 18 – Performance profile of AGS systems with fast feeding (20 min, R1), slow feeding (40 min, R2), and step-feeding (R3) distributed over a cycle.



Source: Prepared by the author.

6.5 Conclusions

Step-feeding (R3) minimized three great problems of leachate treatment in AGS systems: granules' stability, biomass retention, and nitrite accumulation. Compared to fast (R1) and slow (R2) feeding, step-feeding achieved the best TP (53%) and TN (92%)

removals. It also kept low carbon concentrations during the oxic period, which accelerated the ammonia conversion process, favored denitrification, and reduced the oxygen demand to remove organic matter. This is notable because the operation mode can reduce extra carbon addition for denitrification, expanding its practical application, especially for wastewater with high recalcitrant loads, such as sanitary landfill leachate.

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7 STEP-FEEDING IN AEROBIC/ANOXIC CYCLES ENHANCED THE PERFORMANCE OF AEROBIC GRANULAR SLUDGE (AGS) SYSTEMS TREATING EFFLUENTS WITH LOW C:N RATIOS

Aerobic granular sludge (AGS) systems treating effluents with a C:N:P ratio similar to real old landfill leachate were evaluated to reduce the main problems encountered in the treatment of leachate from AGS, such as nitrite accumulation, biomass loss, and granule disintegration. Therefore, six sequential batch reactors (SBR) were operated with different anaerobic (A), anoxic (An), and aerobic (O) configurations: A/O (R1 and R2), O/An with conventional feeding and well-defined anoxic phase (R3), O/An with step-feeding and well-defined anoxic phase (R4), O/An (R5 and R6). The O/An with step-feeding reactor (R4) had the highest biomass retention/settleability (SVI_{30} < 50 mL/g), the best nitrification rates (99%), and chemical oxygen demand (COD) (97%), total nitrogen (91%) and total phosphorous (55%) removals. Furthermore, there was no nitrite accumulation, and granules' disintegration were insignificant. The most abundant phylum in the reactors O/An was Planctomycetota, composed mainly of organisms from the Pirellulaceae and Legionellaceae families. In these reactors, the abundance of phosphorus-accumulating organisms (PAOs) and denitrifying bacteria was similar, while the abundance of glycogen-accumulating organisms was much higher than PAOs. Therefore, the type of cycle directly influences performance, granule characteristics, and system stability, being important for future investigations applying the AGS technology to leachate treatment.

7.1 Start-up, formation granules' stabilization

The six reactors were initially operated with inoculum with the same suspended solids concentration and SVI_{30} (~3.4 g/L MLSS with 88% MLVSS/MLSS and SVI_{30} of 190 mL/g). The parameters change throughout the experiment is shown in Figure 19.

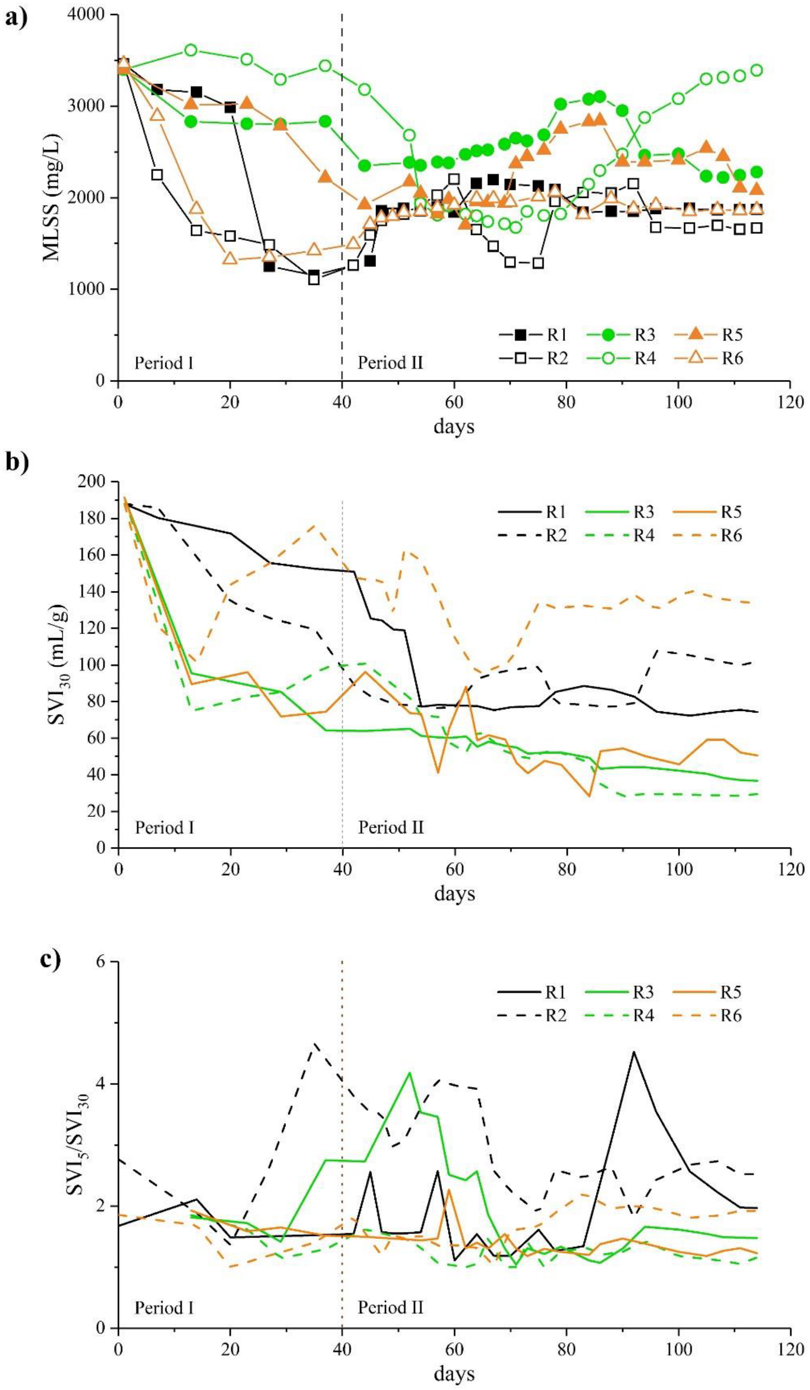
After start-up, except for R4, all reactors lost biomass, with a gradual decrease in MLSS and MLVSS concentrations. The greatest losses occurred in R1 and R6, which were the A/O-type reactors with 12-hour cycle and A/O with fast anaerobic feeding, respectively. R3 and R4, O/An-type reactors with well-defined phases, showed the lowest biomass losses, with lower washout and disintegration frequencies. Notably, R4 showed greater biomass growth, reaching inoculum levels. The reduction of settling time (Period II) was important to favor biomass stabilization in all reactors. In cycles with an anaerobic/anoxic reaction phase followed by an oxic phase, loss of solids was also reported when treating raw leachates (Ren *et al.*, 2017b).

In this sense, episodes of granule disintegration associated with constant washouts were more frequent in R2 and R5, contributing to the slower granulation process. The biomass grew and decayed successively in these two reactors, not achieving long-term stability. Due to the low wastewater biodegradability and concentrations above 200 mg/L NH_3-N, AGS systems submitted to complex effluents, such as landfill leachate, face recurrent washouts and disintegrations, indicating the formation of filamentous biomass and the granules returning to the flocculent stage (Ren *et al.*, 2017a; Leal *et al.*, 2020; Bueno *et al.*, 2020). Furthermore, in very long-lasting anaerobic phases, DO deficiency favors instability due to the high proliferation of filaments and pores clogging, instigating granules' breakage (Bella & Torregrossa, 2013).

Upon reaching biomass stability, the proportion of MLVSS in relation to MLSS was around 88% in R3, R4, and R6, 82% in R1 and R2, and 67% in R5. In terms of settleability, except for R6, SVI_{30} was very high in all reactors during the period I and decreased significantly with the settling time reduction. Such a fact indicates that initially, the biomass was formed by flocs of low settleability, and with this selection pressure, the poor-quality sludge was washed out, improving the AGS characteristics. In R6, even though the biomass was stable for most of the operation, SVI_{30} varied considerably and presented the worst SVI_{30} (> 120 mL/g), even at the experiment completion. Thus, not differing statistically from each other (p = 0.35), the O/An-type reactors (R3 and R4) presented the best SVI_{30} (< 40 mL/g) and mature granules, as normally mature granules have SVI_{30} between 30 and 80 mL/g (Derlon *et al.*, 2016).

Even with C and N loads superior to some previous investigations (Wang *et al.*, 2012; Chen *et al.*, 2013), the results for R4 were similar to those who adopted the same feeding configuration (SVI_{30} < 30 mL/g). They were also similar to the reactors that treated leachate in dilutions ranging from 10 to 100% with cycles of anaerobic/anoxic phases followed by an oxic reaction (SVI_{30} < 25 mL/g) (Ren *et al.*, 2017b).

Figure 19 – Evolution of stability of the six AGS systems in terms of a) MLSS, b) SVI_{30}, and c) SVI_5/SVI_{30} with different settling times: 20 min (Period I, 0-40 days) and 10 min (Period II, 41-114 days).



Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.
Source: Prepared by the author.

Despite favoring nutrient removal, very long anoxic and anaerobic phases, in addition to reducing the shear force, also form more unstable and fragile granules, with irregular and filamentous surfaces (Rollemberg *et al.*, 2020). For this reason, washouts and disintegration of granules are common, which can be minimized by interspersing these phases with oxic periods. This arrangement provides longer starvation periods with lower organic loads instigating competition between PAOs (or DPAOs) and GAOS (or DGAOs), favoring granules' stability and setting (Zhang *et al.*, 2014; Devlin & Oleszkiewicz, 2018).

Moreover, the step-feeding favored the AGS process even more. In addition to retaining more biomass, it also fostered and intensified its growth and significantly reduced fast-growing heterotrophic microorganisms' overgrowth (Chen *et al.*, 2013; Corsino *et al.*, 2016). Therefore, the alternation of feast/famine periods promotes the development of granules with excellent settling rates and more resistant structures that favor stability.

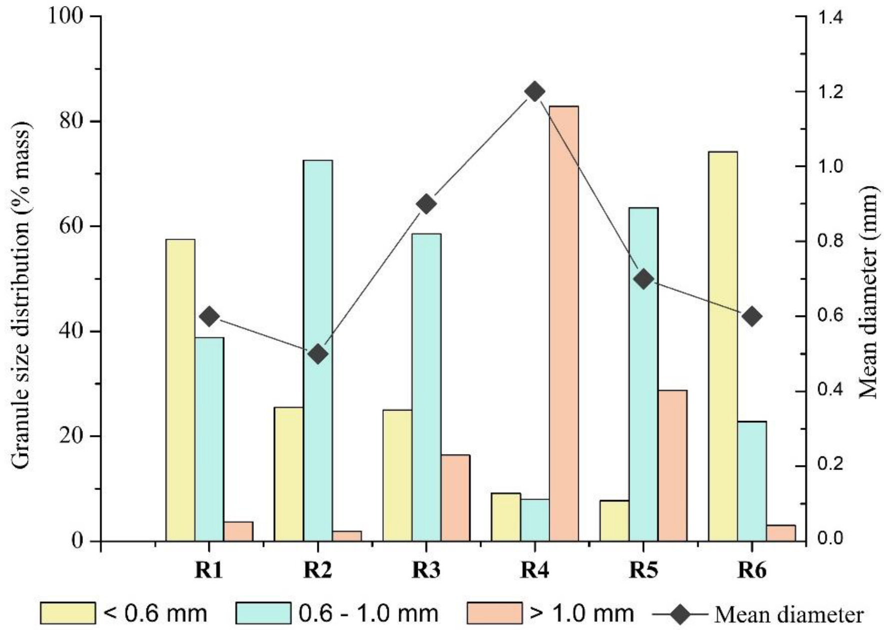
7.2 Granules' characteristics

AGS biomasses showed physicochemical characteristics with some similarities but mostly divergent. Therefore, the different granules' structures formed directly affected granules' characteristics, probably due to substrate gradients that influenced the prevalence of different microbiological niches. With the settling time reduction, there was a decrease in the SVI_{30} and SVI_5 values in all reactors, except in R6, which has increased (Figure 19). This reduction is typical of flocculent/filamentous sludge evolution to granular sludge, as it improves biomass settleability over time.

SVI_5/SVI_{30} ratios can indicate the granulation process. When this ratio is between 1.2 and 1.8 in effluents with high loads, it may indicate a predominance of mature granules (Liu *et al.*, 2010; Corsino *et al.*, 2018). Thus, there is evidence that the granulation in R3, R4, and R5 was better than in the other reactors, with R4 being statistically different and better than R3 (p < 0.001) and R5 (p = 0.021), and R3 significantly better than R5 (p = 0.024). Therefore, O/An-type reactors with well-defined anoxic phases seem to be the best strategy to grant such a profile by balancing anoxic and oxic periods, since the oxic periods regulate the granule structures and break the filamentous part created in the anoxic periods (Pishgar *et al.*, 2019).

According to Liu *et al.* (2010), complete granulation is considered when more than 80% of the biomass in the AGS system has a diameter greater than 0.2 mm. Therefore, at the end of period II, all reactors were classified as aerobic granular systems (Figure 20). As well as in settleability, the reactors R3, R4, and R5 were the ones that presented the best results in relation to granules size, being observed larger amounts of granules with sizes greater than 1.0 mm. In R4, the biomass diameter was predominantly greater than 1.0 mm. Thus, the granules' mean diameter in the O/An-type reactors after the operation was greater (R4) or very similar (R3) to those in the investigations conducted by Wei *et al.* (2012) (0.36 – 0.60 mm), Ren *et al.* (2017a) (0.21 – 0.48 mm) and Bueno *et al.* (2020) (0.612 mm) in O/An-type reactors, by Bella & Torregrossa (2014) (0.80 – 0.90 mm) in O-type reactors, and those by Ren *et al.* (2017a) (> 0.31 mm), Ren *et al.* (2017b) (1.1 mm), and Ren *et al.* (2017b) (0.21 – 0.48 mm) in A2O-type reactors, all treating leachates in AGS systems.

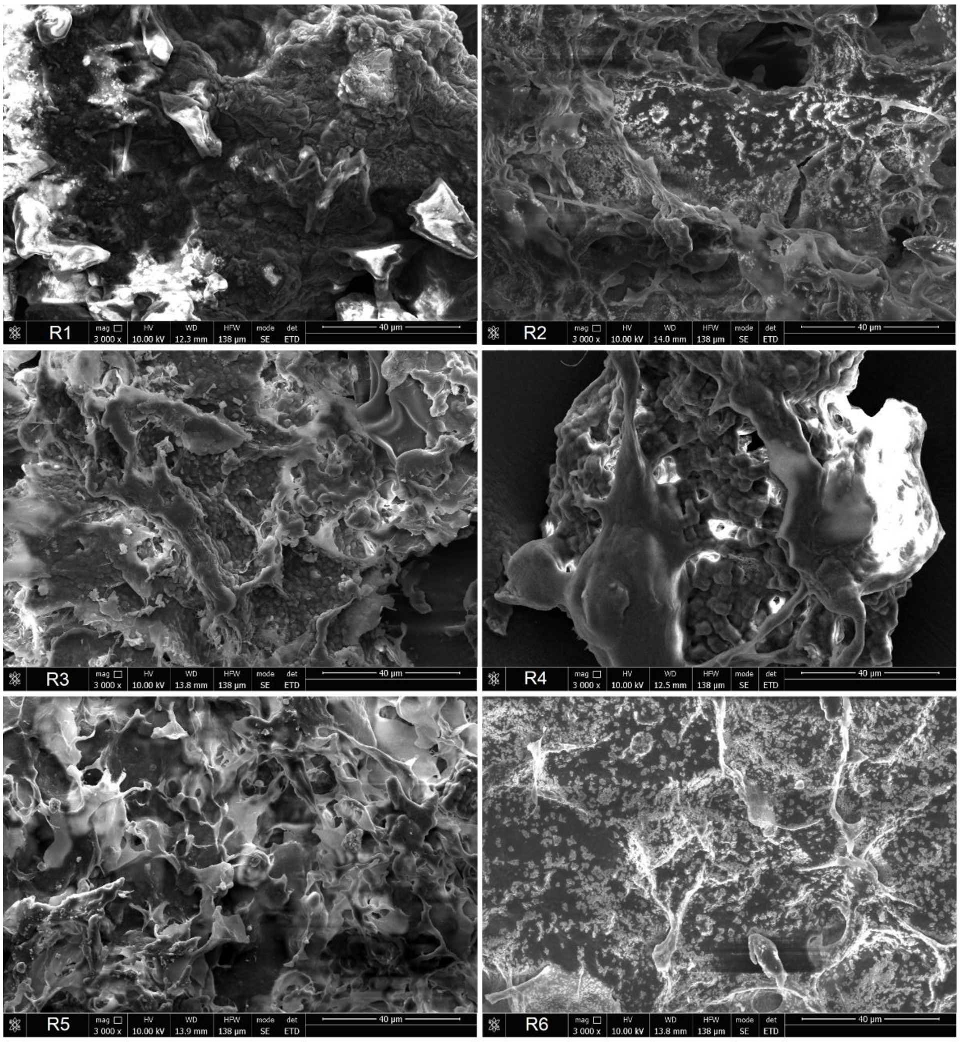
Figure 20 – Granule size distribution (% mass) at the end of period II (114 days of experiment) for each reactor.



Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.
Source: Prepared by the author.

Microscopy analyzes were used to the nearest 3000x to analyze the surface of the formed granules (Figure 21). It was observed that the granules of the A/O type reactors (R1 and R2) had very irregular, filamentous, and spongy structures. In the O/An type reactors with a well-defined anoxic phase (R3 and R4), the presence of coccus in bacillus dominance is clearly observed, with colonies of bacteria still exposed can be observed in R4 a larger dense structure around the granule, in which microbial communities tend to be more aggregated, presenting a larger microbial population. In contrast, the granules of the O/An type reactors without a well-defined anoxic phase (R5 and R6) were characterized by voids and cavities that suggest internal fragmentation, especially in the fast-feed reactor (R6).

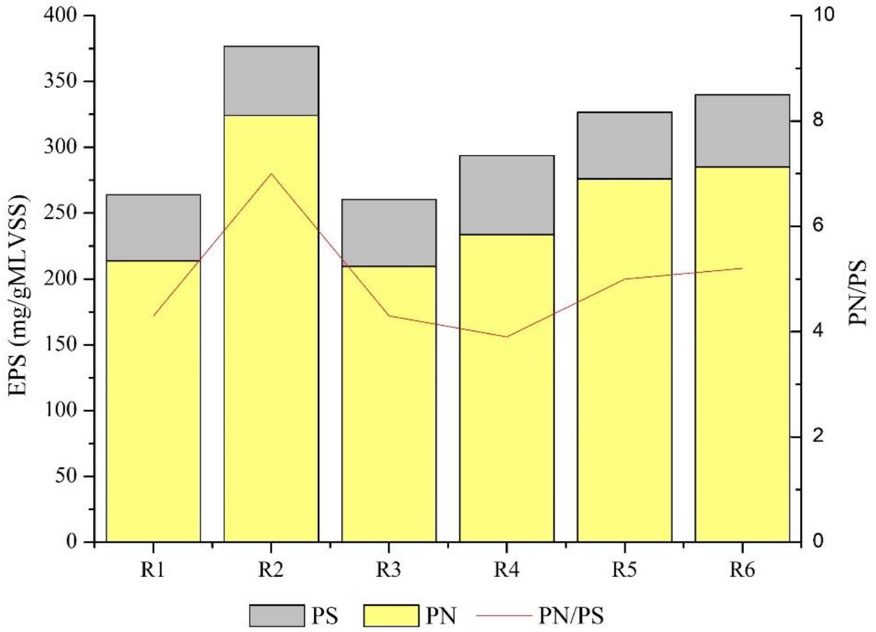
Figure 21 – Granule scanning electron micrograph of the six reactors at the end of period II to approx. 3000x



Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.
Source: Prepared by the author.

For granules' structure, formation and stability, the EPS role can still be evaluated (Figure 22). EPS are biopolymers composed of polysaccharides, proteins, and other substances, acting as a "biological glue". PS and PN are fractions responsible for granule aggregation and mechanical stabilization, respectively (Rollemberg *et al.*, 2018). For the A/O-type reactors (R1 and R2), the biomass had the highest EPS content, likely due to the smallest size of the granules (Rusanowska *et al.*, 2019). The highest EPS production was verified in R2 biomass (p = 0.041), likely because the oxic period was longer, and the shear force caused by air bubbles contributes to EPS production (Liu *et al.*, 2010).

Figure 22 – Average quantification of extracellular polymeric substances (EPS) in each AGS system during period II.



Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.
Source: Prepared by the author.

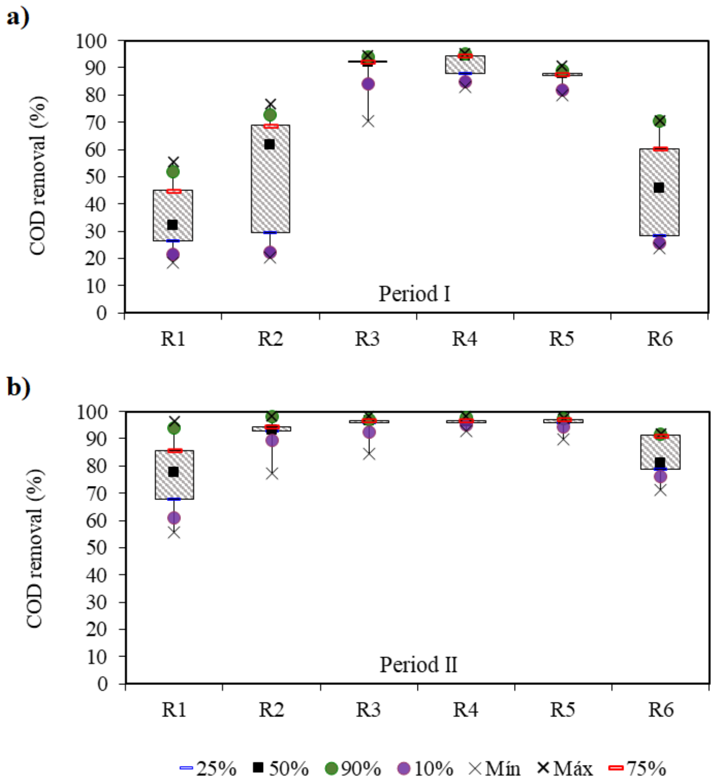
The O/An-type reactors had the lowest total EPS production, not significantly different from each other (p = 0.92), observing a balance between EPS production and consumption. As is known, short anoxic times interspersed with longer aerobic times favor the balance between production (during the feast phase) and consumption (during the famine phase) of EPS in O/An-type reactors (Bella & Torregrossa, 2013; Rollemberg *et al.*, 2020). So, it was expected that in these O/An-type reactors, especially in R4, the EPS production would tend to balance, being lower than in the other reactors since it produces successive periods of feast/famine distributed throughout the cycle.

In aerobic granules, the amount of PN is correlated with stability (Rollemberg *et al.*, 2018), in which the PN/PS ratio can be a good parameter to assess granules' stability. PN/PS ratios above 4.0 are reported to guarantee granules' stability in AGS systems treating leachate (Wei *et al.*, 2012). However, when this ratio is too high, the effect will be the opposite (Ren *et al.*, 2017a; Ren *et al.*, 2017b). Therefore, the O/An reactors also presented PN/PS ratios close to the ideal, confirming the stability observed during the operation, with no granules' washouts or recurrent disintegrations.

7.3 Reactor performance during the granulation process

Reactors' performance was evaluated in terms of COD, nitrogen, and phosphorus (Figures 23-26). The best removals of COD, total nitrogen (TN), ammonia, and phosphorus were in the O/An-type reactors (R3 and R4), which had a well-defined anoxic phase. The other reactors had different behaviors, and settling time reduction was key to improving the overall performance.

Figure 23 – Performance of AGS systems in terms of COD removal during different settling times: 20 min (a, 0-40 days) and 10 min (b, 41-114 days).



Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.
Source: Prepared by the author.

Among the investigated reactors, total COD removal was significantly higher in R4 and R2, O/An, and A/O-type reactors, respectively, which did not differ statistically from each other ($p = 0.61$) but differed from the other reactors ($p < 0.01$) (Figure 23). On the other hand, soluble COD removal was statistically better in the two O/An-type reactors and in R5, which showed no differences between R3 and R4 ($p = 0.47$), R4 and

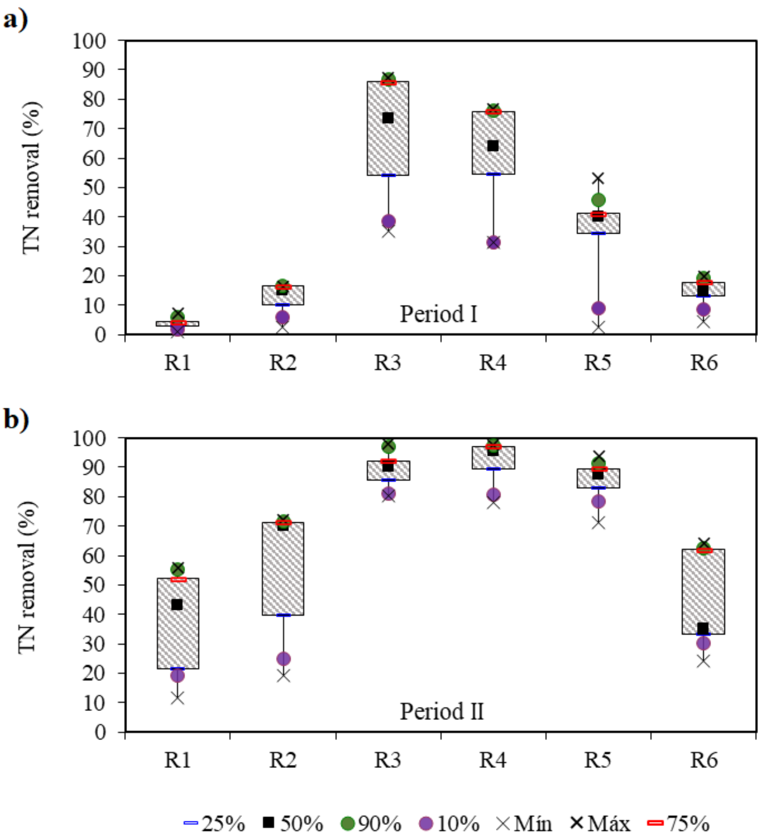
R5 ($p = 0.63$), and R3 and R5 ($p = 0.102$). Therefore, total and soluble COD removals were generally better in O/An-type reactors with well-defined phases than in other configurations, most likely due to better phase distribution during the cycle and the different layers existing in the structure of a stable granule.

The nitrification process was observed in both systems during the two periods, with values above 60% already in the period I, with no statistical differences found between any reactor ($p > 0.05$). With the settling time reduction (Period II), these values increased, and it was possible to identify statistical differences among all reactors ($p < 0.02$), except for R1 and R2, which did not differ from each other ($p = 0.33$). Thus, the greatest ammonia removal occurred in the O/An-type reactors (R4 and R3, respectively) (data not shown). As in R3 and R4, the MLVSS concentration varied very little after system stability, the DO demand did not increase, and the nitrifying bacteria activity was not affected since the aeration flow rate was kept unchanged during the operation, which favored the nitrification efficiency. Similar behavior was also observed in the O/An-type reactors without defined phases R5 and R6. However, granules' disintegration and the recurrent washouts reduced ammonia removal (although, after some days, the ammonia removal was restored), possibly due to the loss of nitrifying bacteria after granules' breakage and washout. When washouts occur at higher frequencies, it becomes impossible to control the sludge age (that is why this determination was not made in R1, R2, and R6), and, because it is very low, nitrification ends up being affected.

Concerning total nitrogen (TN) removal during the period I (Figure 24), reactors R1, R2, and R6 had the worst performances, and R3 and R5 the best ones, not differing from each other ($p = 0.71$). In period II, R4 was statistically higher than R3 and R5 ($p < 0.001$), which remained without significant differences between them ($p = 0.86$). As the profile of nitrogen fractions was different among the reactors (Figures 24 - 25), it is noteworthy that the removal mechanisms were also different. Only the well-defined O/An-type reactors had no nitrite accumulation, contributing to the high TN removals (Figure 24). They were also statistically similar ($p = 0.07$). A considerable accumulation of nitrite was observed in R1 and R6, being small in R2 and R5. Therefore, in reactors with very long anaerobic phases and high influent loads, the nitrification by-products accumulation is more notorious, as competition for organic material becomes evident between PAOs and denitrifying microorganisms (Bella & Torregrossa, 2013; Sarvajith, Reddy and Nancharaiah, 2020).

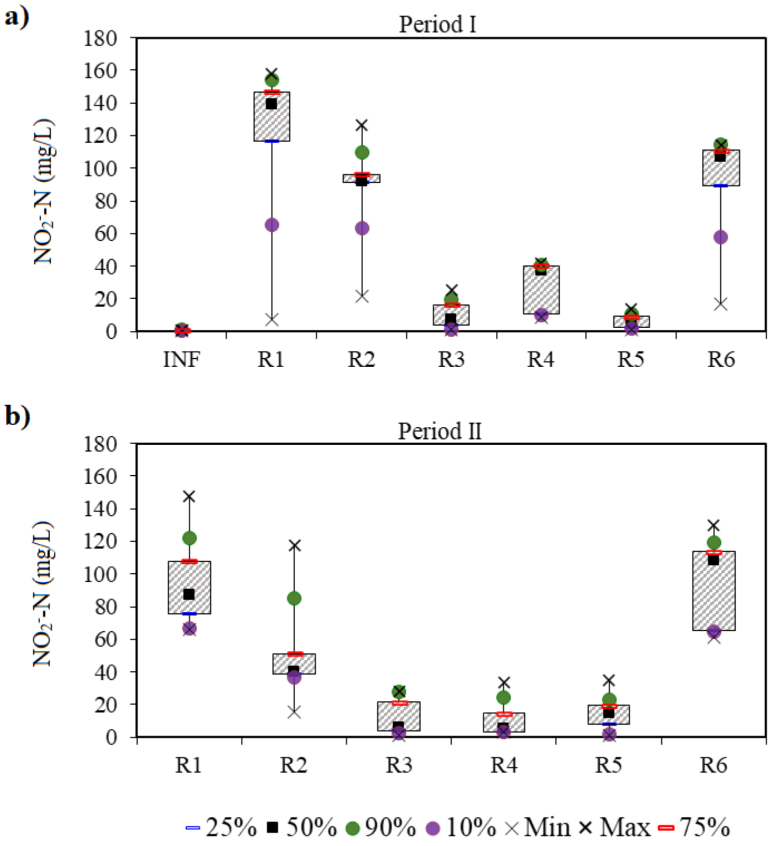
Even so, TN removals in the O/An reactors were superior to those in the leachate experiments by Ren *et al.* (2017a). They did not even achieve 50% removal with the anoxic phase incorporation in reactors that operated previously with full-oxic cycles (O). When the reactors presented well-defined anoxic phases (R3 and R4), they were also superior to those of Wei *et al.* (2012). The latter only achieved maximum removals of 75.4% even with ammonia pre-treatment in cycles without an anoxic phase.

Figure 24 – Performance of AGS systems in terms of TN removal during different settling times: 20 min (a, 0-40 days) and 10 min (b, 41-114 days).



Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.
Source: Prepared by the author.

Figure 25 – Evolutionary quantification of nitrite in AGS systems during different settling times: 20 min (a, 0-40 days) and 10 min (b, 41-114 days).

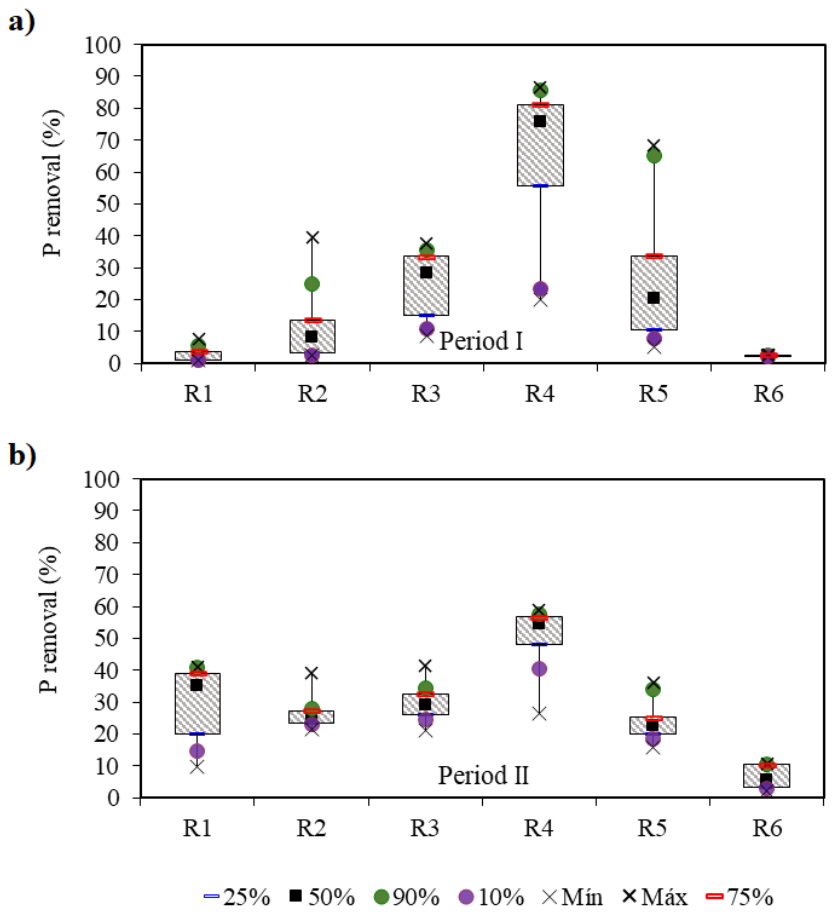


Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.
Source: Prepared by the author.

Therefore, the best TN removals were in the O/An-type reactors, especially in the step-feeding reactor. The anoxic phase increment selects specific microbiological functional groups, and the denitrification can also occur by DPAOs and DGAOs. Therefore, O/An-type reactors create more routes and conditions for heterotrophic denitrification to occur satisfactorily. Moreover, the residuals of nitrite and nitrate can also be used as electron acceptors by DPAOs. Feeding distributed throughout the cycle, in addition to selecting specific niches that favor nutrients removal, is also capable of providing carbon for longer periods, which supports denitrification.

Concerning phosphorus removal (Figure 26), the six systems showed different behaviors, and the reduction in settling time favored only the A/O-type reactors. As expected, R6 had the worst P removal rates due to the fast anaerobic feeding and lack of anaerobic/anoxic phases during the cycle. However, it was also expected that the A/O-type reactors R1 and R2 would present the best P removals due to the longer anaerobic period duration, which was not verified. Possibly due to the influent complexity, PAOs may have been disadvantaged in competitive situations. Thus, the greatest removals occurred in these O/An-type reactors, where R4 was significantly greater than R3 ($p < 0.001$).

Figure 26 – Performance of AGS systems in terms of P removal during different settling times: 20 min (a, 0-40 days) and 10 min (b, 41-114 days).



Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.
Source: Prepared by the author.

Therefore, the low overall removals probably occurred because the bacteria saturated and the efficiency stagnated, requiring a sludge age control. For AGS systems with high SRT, aerobic granule deterioration occurred more easily, and an adequate selective sludge discharge favors process stability (Zhu *et al.*, 2013). The selective sludge discharge (blanket or bottom sludges) is also suggested to remove these saturated bacteria and improve phosphorus removal (Bassin *et al.*, 2012; Rollemberg *et al.*, 2018).

Furthermore, phosphorus removals in these reactors with anoxic phases were greater than those removals reported by Ren *et al.* (2017b), which did not occur in reactors also with anoxic phases, treating both low and high leachate dilutions.

7.4 Cycle test

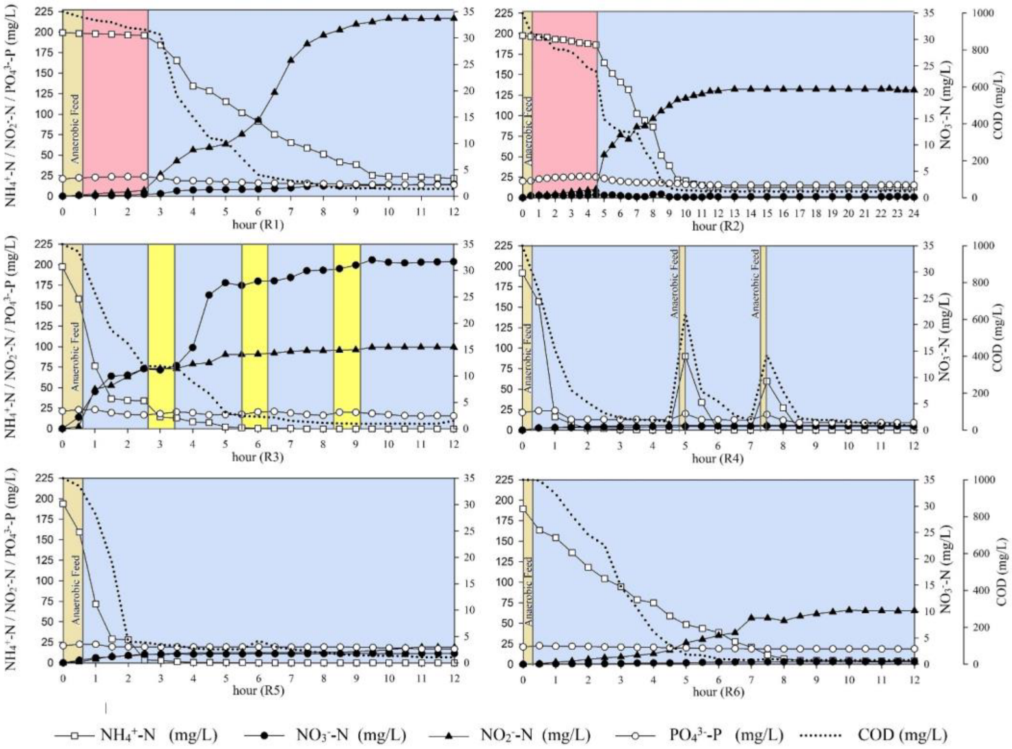
The simultaneous conversions and removals of organic, nitrogenous, and phosphorus constituents were investigated over a complete cycle (Figure 27). In this experiment, the pH variation was low, probably due to the SND process, which consumes (nitrification) and produces (denitrification) alkalinity.

During the cycle, it was observed that in the A/O-type reactors, DO was between 2 and 4 mg/L during the first hour of aeration after the anaerobic phase ended. In these reactors with an anaerobic phase, oxygen saturation occurred in the middle of the cycle, with six hours of operation, in which the DO consumption was lower and reached around 6 mg/L. In reactors with defined anoxic phases, R3 and R4, the drop in DO availability favored consumption equilibrium. Thus, in the following aerobic phase the DO levels were less than 5 mg/L. In both R5 and R6, DO was consumed quickly in the first two hours of the aerobic period, after which the DO was between 5 and 6 mg/L. These times coincided with the famine period, i.e., when the available COD is very low. Therefore, the famine period coincided with the DO increase, as microorganisms enter the endogenous phase and need lower oxygen concentrations for their metabolism.

In the A/O-type reactors, the COD fermentation was different due to the anaerobic phase duration. In R1, only 10% of the COD was consumed during the anaerobic phase, while in R2, almost 40% of the COD was already oxidized. At the end of the anaerobic period, it is observed that nitrite begins to accumulate gradually until the end of the oxic period, being greater in the reactor with a shorter cycle. This accumulation occurs as ammonia is oxidized, in which R1 and R2 were the ones that took the longest time for nitrification to occur. Furthermore, after the total COD consumption, the conversion of nitrogen fractions becomes stable.

In O/An-type reactors without well-defined anoxic phases, as soon as COD is practically consumed, nitrite starts to accumulate, being significantly more expressed in R6. Nitrate concentration also increases slightly, simultaneously with ammonia oxidation. In these reactors, as there is no anaerobic/anoxic phase and carbon is consumed quickly, denitrification reaches lower rates than the other systems. Therefore, PHA accumulation in the aerobic phase during a short period of COD depletion is not enough to sustain denitrification. Furthermore, the available carbon is also used for microorganisms' growth and development, leading to incomplete denitrification and partial nitrogen removal.

Figure 27 – Simultaneous nitrification and denitrification profiles and phosphorus removal in the investigated AGS systems, distributed over a cycle.



Colors: Brown – Anaerobic Feed; Red – Anaerobic phase; Blue – Aerobic phase; Yellow – Anoxic phase. R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.

Source: Prepared by the author.

In this sense, in the O/An-type reactors that had well-defined anoxic phases, especially the R4 in which the feed was distributed throughout the cycle, denitrification was not harmed due to the carbon availability throughout the cycle. The main nitrogen removal mechanism occurred likely via SND throughout the aerobic period, without significant accumulations of nitrite and nitrate. Additionally, denitrification may have occurred by another route, heterotrophically, using EPS as an electron donor during the famine period. In this case, the PS stored in the granules during the feast period is possibly used as an electron donor in endogenous denitrification at the end of the cycle. Also, when complete nitrification occurred in these reactors, there was still enough time in the oxic phase for the remaining nitrite to be converted to nitrate by nitrite-oxidizing bacteria (NOBs), as the toxicity effect of free ammonia on NOBs is lower. Furthermore, nitrite and nitrate can be used as electron acceptors by phosphorus-accumulating bacteria (DPAOs), which favored that the O/An-type reactors had the best phosphorus removals (He *et al.*, 2018).

Therefore, the removal of nitrogenous constituents occurred by different mechanisms depending on the phase of the cycle. In feeding, an exogenous denitrification occurred, in which the accumulated nitrogen fraction was converted through the use of organic matter as an electron donor. In the oxic phase, ammonia was converted through the process of simultaneous nitrification and denitrification (SND). In the anoxic phase, there was an endogenous denitrification to remove the other nitrogen fractions, also using organic constituents as electron donors.

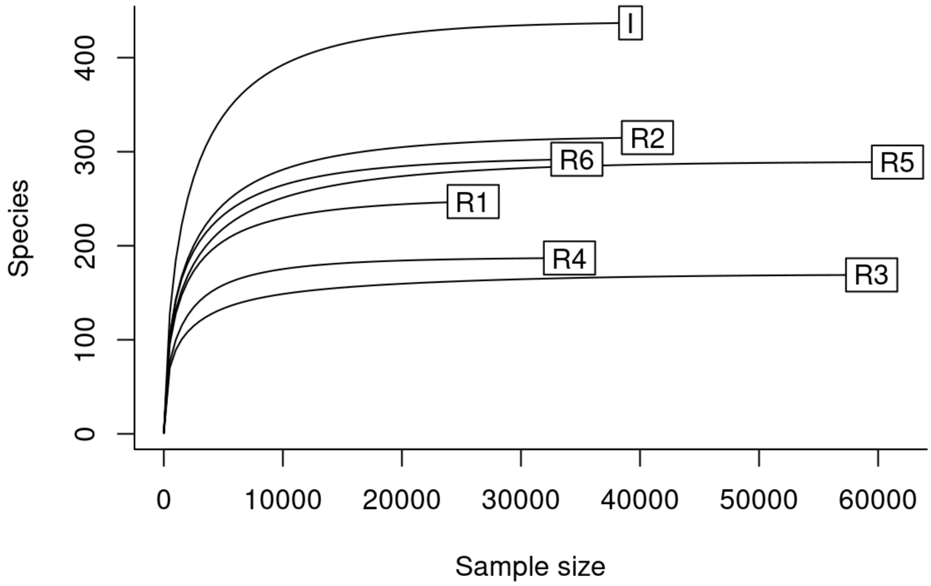
In general, it is possible to verify that in this study, anaerobic phases during the cycle do not significantly impact the removal of the constituents. It is also possible to verify that the organic load supplied throughout the cycle in the step-feeding reactor (R4) was sufficient to favor complete denitrification, without generating NO_x accumulations. So, investing in reactors with alternating anoxic and oxic phases seems to be the best strategy for this type of effluent.

7.5 Microbial community composition

7.5.1 General taxonomic populations

From the methanogenic analysis, it was possible to verify the existence of 39202 sequences in the inoculum, 25985 in R1, 40626 in R2, 59456 in R3, 34076 in R4, 61626 in R5, and 34693 in R6. Thus, the microbial communities for the different RBS configurations were analyzed by RNA sequencing. The rarefaction curve showed that the sequencing yielded sufficient depth for the analysis as all species counts reached a plateau as the sample sizes increased (Figure 28).

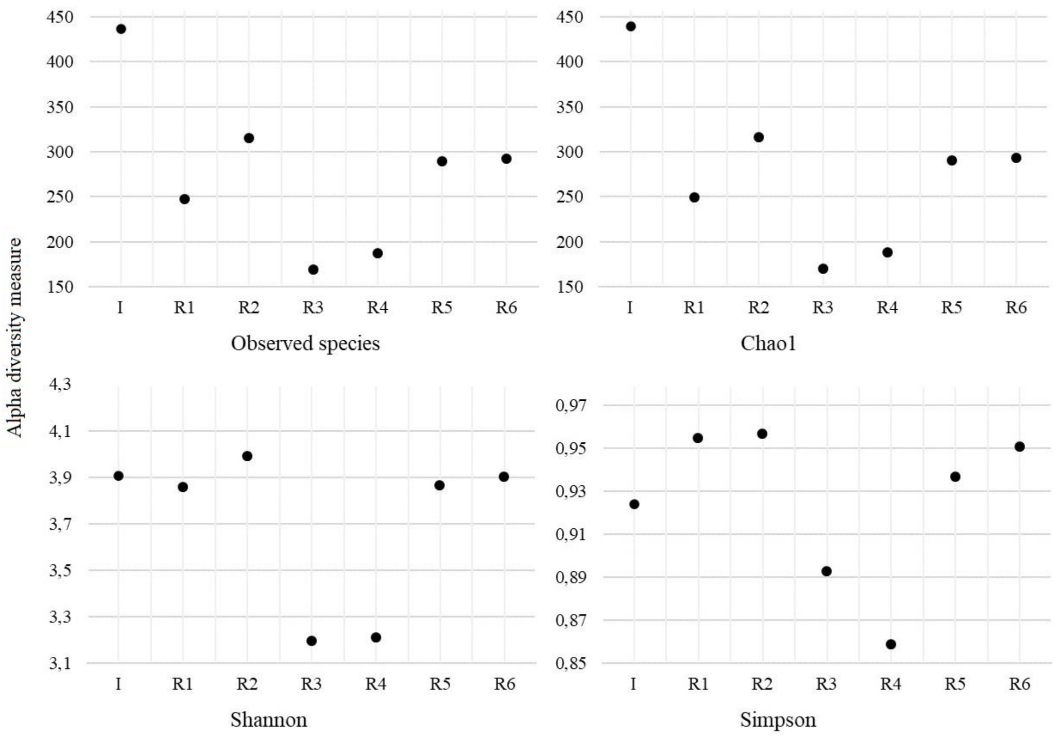
Figure 28 – Rarefaction curve of sequencing data.



Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.
Source: Prepared by the author.

Initially, alpha diversity (Figure 29) Chao1 was determined to report the abundance and richness of species and also to estimate the community's diversity through Shannon and Simpson indices. Through these results, it is possible to verify that the reactors with a well-defined anoxic phase (R3) and step-feeding (R4) impose a strong selection pressure, possibly greater than the other configurations since the richness and diversity were lower in these reactors. It was also observed that the inoculum presented higher diversity and richness, and it is possible to infer that AGS microbial communities are sensitive. Phase break and alternation within the reaction period are critical factors for this biomass selectivity. The sensitivity of microbial groups present in aerobic granular sludge is linked to selection pressure and other crucial factors, such as reactor configuration, operating conditions, and the inflowing nutrient load (Barros *et al.*, 2020; Huang *et al.*, 2021). It is also noticeable that the difference in feed rate (R5 and R6) is not sufficient for the richness and diversity of the bacterial species to be significantly affected, as there was enough similarity between these two reactors.

Figure 29 – Number of bacterial species and diversity indices in the 6 reactors and the inoculum (I).



Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.
Source: Prepared by the author.

Then, the microbial communities, employing a gene taxonomic analysis, were determined and analyzed at different levels, from phylum to class, family, and genus (Figure 30). From these assignments, it was reinforced that the selection pressure imposed by the different RBS configurations was sufficiently capable of modifying the microbial composition of the inoculum sludge. This is noticeable because bacteria belonging to the phylum Acidobacteriota and Bdellovibrionota were in higher abundance in the inoculum but were greatly reduced by the end of the experiments.

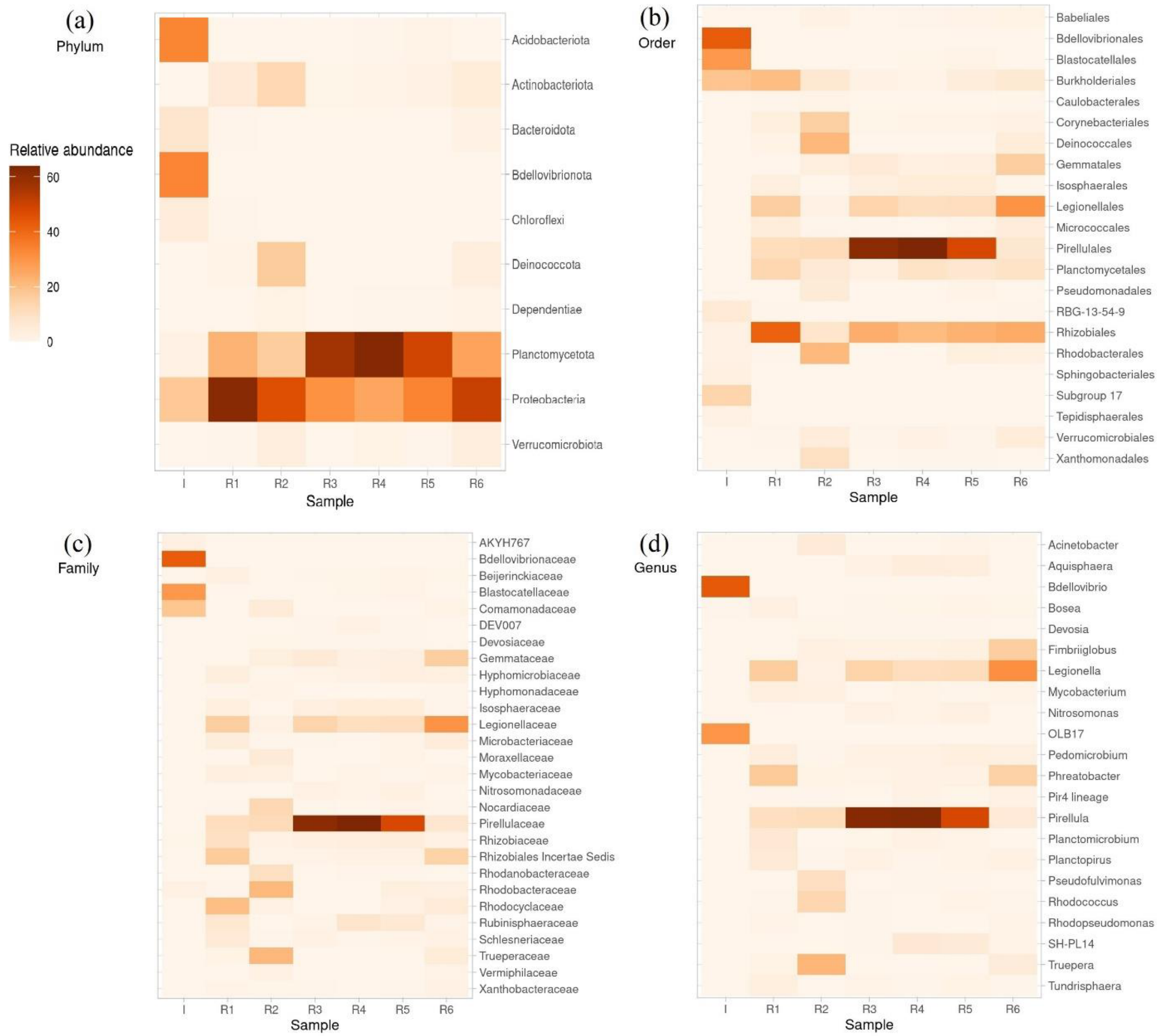
In the reactors with an anaerobic phase (R1 and R2) and fast feeding (R6), the phylum Proteobacteria was in higher abundance, followed by the phylum Planctomycetota. These two phyla are among the most dominant and commonly found in aerobic granular sludge (Fan *et al.*, 2018; Sarvajith, Reddy and Nancharaiah, 2020). Thus, organisms from the family Rhodocyclaceae and Rhodobacteraceae were dominant in R1 and R2, respectively, which are among the most abundant families in AGS experiments (Xia *et al.*, 2018; Zou *et al.*, 2019). After performing a complete separation of the granules formed in their SBRs, Sun *et al.* (2017) found that in the inner core of the granules, there was a dominance of Rhodocyclaceae, and in the outer spherical shell abundance of

Rhodobacteraceae could be found. This corroborates with the findings by Zou *et al.* (2019), who pointed out that as the granules became more mature, a significant increase in Rhodocyclaceae occurred, while Rhodobacteraceae decreased drastically. Probably, it is because Rhodocyclaceae is associated with the production of extracellular polymeric substances (EPS), playing an important role in the formation and stability of aerobic granules (Szabo *et al.*, 2016). In addition, disintegrations of granules can more easily wash out Rhodobacteraceae, as they are in the outer and more fragile layer of the granules.

In R2, an abundance of the phylum Deinococcota could also be verified, represented mainly by organisms of the family Trueperaceae, order Deinococcales. In R1 and R6, other organisms belonging to the phylum Proteobacteria were identified in significance, such as bacteria from the families Rhizobiales Incertae Sedis and Legionellaceae. In addition, in R6, the bacterial community was also formed in abundance by microorganisms from the family Gemmataceae, order Gemmatales, which are responsible for nitrification and, under specific conditions, can accumulate glycogen (Zeng *et al.*, 2022). Therefore, the presence of these microorganisms in R6 was mainly fundamental to maintaining nitrification, having the bias of not removing large concentrations of phosphorus.

An inversion occurred in the other reactors where the anoxic phase was well defined (R3 and R4) and the anoxic slow feeding (R5). The most dominant phylum in these reactors was the Planctomycetota, followed by the Proteobacteria, respectively represented mostly by organisms of the Pirellulaceae and Legionellaceae families, especially of the *Pirellulla* and *Legionella* genera. These three reactors showed quite similar microbial composition, although R5 showed lower quantitative dominance than R3 and R4. Planctomycetota is among the most abundant phyla in AGS reactors (Fan *et al.*, 2018). They are comparatively slow-growing organisms that require low carbon demand, favoring granulation (Rollemberg *et al.*, 2019). Furthermore, this phylum has species with anaerobic ammonium oxidation bacteria (ANAMMOX) metabolism (Mak *et al.*, 2018; Xia *et al.*, 2019). Therefore, Proteobacteria and Planctomycetota were the most abundant phyla in all reactors, as reported in the literature.

Figure 30 – Bacterial community structure at the Phylum (a), Order (b), Family (c), and genus (d) level of the six reactors and the inoculum (I).



Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.
Source: Prepared by the author.

7.5.2 Key functional groups

Finally, in parallel to the taxonomic characterization of the biological communities, a survey of specific groups of microorganisms responsible for C, N, and P removal was carried out (Figure 31). In this way, the families were divided into five main groups: AOB (ammonia-oxidizing bacteria), NOB (nitrite-oxidizing bacteria), DNB (denitrifying bacteria), GAOs (glycogen-accumulating organisms) and PAOs (polyphosphate-accumulating organisms).

The lowest ammonia removal by the nitrite pathway, which occurs through AOB bacteria, was in the reactor with a longer cycle duration and longer anaerobic phase (R2), being justified by the reduced number of AOB. In reactor R1, which had a similar configuration to R2, but with cycle time reduced by half, AOB bacteria of the Rhodocyclaceae family were found. This family was only found in R1 and is reported to grow in the core of aerobic granules. A possible reason is due to the frequent episodes of granule disintegration in R1, in which the microorganisms in the granules' outermost layers may have been discarded in the effluent after disintegration. In the other reactors, especially those with the highest nitrification efficiencies, R4 and R3, respectively, AOB belonging to the Nitrosomonadaceae family were dominant in the microbial community composition. In the studies by Winkler *et al.* (2013), microorganisms from this family were reported to be the dominant AOB, especially in high ammonia concentrations (Zhao *et al.*, 2013). Only R2 and inoculum showed significant abundances of bacteria belonging to the NOB class, suggesting that a partial nitritation occurred in the other reactors.

Concerning denitrification, all reactors showed similar abundances of DNB bacteria, with R4 having the highest abundance. This is in line with the fact that this reactor had the highest total nitrogen removal, with no nitrite or nitrate accumulations. However, with R3, reactor R4 had the lowest DNB diversity, mainly represented by Rhizobiaceae and Pirellulaceae. The latter was also dominant in R5 and is present in all reactors. Comamonadaceae and Blastocatellaceae were dominant DNB families in the inoculum, which were inhibited in the reactors. Comamonadaceae plays an important role in the nitrogen removal cycle. However, they require specific conditions that end up limiting their prevalence (Fan *et al.*, 2018; Li *et al.*, 2020).

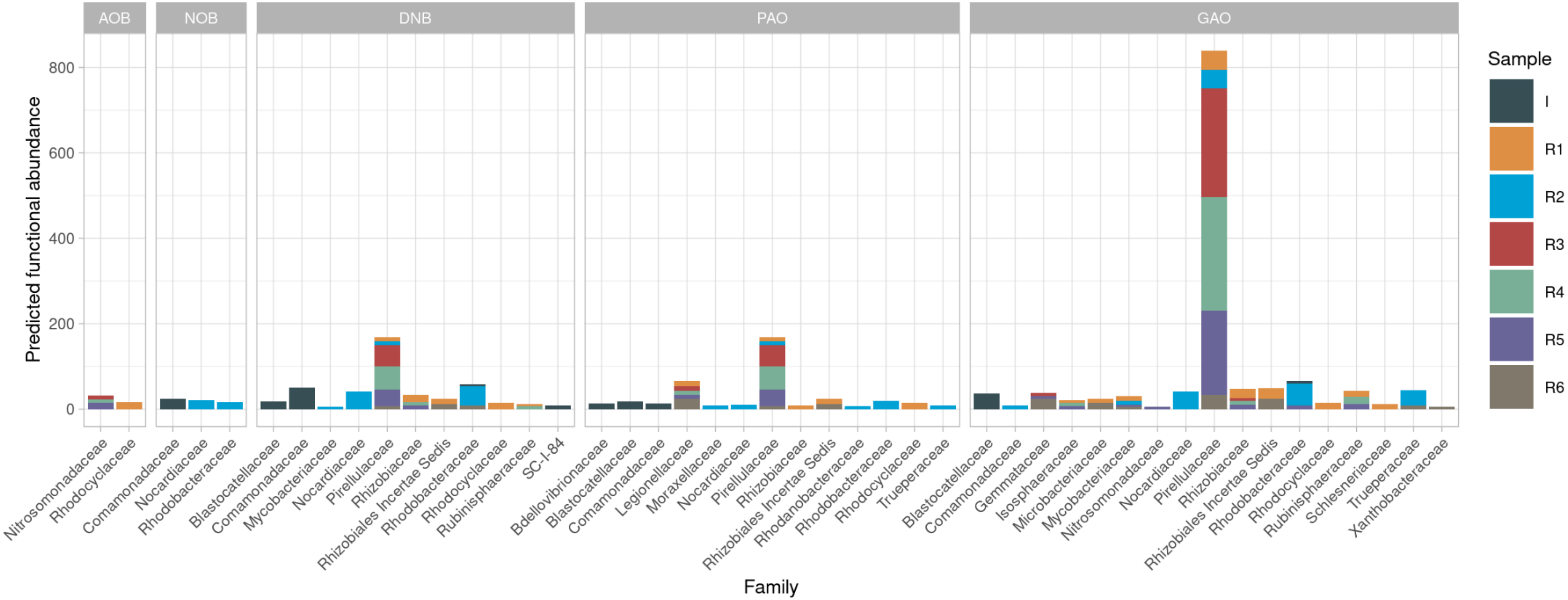
Although the reactors presented high abundances of DNB, compared to the presence of AOB and NOB, nitrite accumulation was noticeable (except for R3 and R4). Therefore, it can be inferred that denitrification did not occur due to the lack of substrate and excess of oxygen. In reactors R3 and R4, denitrification was favored by incrementing anoxic phases after oxygen saturation. These results were similar to those of Barros *et al.* (2020), who had high DNB abundances and low denitrification efficiency.

As for the organisms responsible for phosphorus removal, polyphosphate accumulators (PAOs) take up phosphorus under aerobic or anoxic conditions using energy from intracellular PHAs. They can compete with other microorganisms for the same organic substrate. In this study, except for the inoculum, the abundance of PAOs and DNBs was similar. In contrast, the abundance of GAOs was much higher than PAOs, suggesting that this latter competition was more significant and relevant than that between PAOs and DNBs.

P removal was expected to be lower, but it was not. GAOs may have been favored by influent organic matter and NO_x in the anaerobic/anoxic periods. The microorganisms of the Pirellulaceae family were the main PAOs and GAOs found in the six reactors. These functional groups are important for the stability of the granules because they consume part of the readily available substrate, making it unavailable for consumption by filamentous bacteria in the aeration period (Rollemberg *et al.*, 2018). The greatest diversity of these microorganisms was found in the reactors with anaerobic phase, and the greatest abundance was in the reactors with a well-defined anoxic phase. Legionellaceae was also a type of PAO found in dominance in the reactors, except in R6. It has been reported that Legionellaceae are classified as facultative intracellular bacteria with specific nutritional characteristics, capable of metabolizing existing phosphorus to obtain energy (Engleberg, 2009).

In general, denitrifying microorganisms and phosphate and glycogen accumulators belonging to Pirellulaceae were the most abundant family in reactors R3 and R4, which had higher removal rates of total nitrogen and phosphorus without NO_x accumulation. Reactors R1 and R2 with an anaerobic phase presented higher diversities of dominant DNB, PAOs, and GAOs. The reactor R5 with slow feeding showed similar composition to R3 and R4 but slightly higher diversity. Finally, in reactor R6 with fast feeding, low diversity and abundance of specific microorganisms were found.

Figure 31 – Functional distribution of taxonomic classification at the end of the experiment in each reactor and in the inoculum (I).



Note: R1, Anaerobic/Oxic with 12-hour cycles; R2, Anaerobic/Oxic with 24-hour cycles; R3, Oxic/Anoxic with well-defined anoxic phase; R4, Step-feeding; R5, Oxic/Anoxic with fast feeding; R6, Oxic/Anoxic with slow feeding.

Source: Prepared by the author.

7.6 Conclusion

Despite the proposed reactor optimizations, the time required for granulation was still long when compared to the use of domestic effluents. Nonetheless, through this investigation, it became evident that the type of cycle directly influences performance, granule characteristics, and system stability. Some significant considerations include:

- The O/An-type reactor with step-feeding (R4) had the highest biomass retention/settleability (SVI_{30} < 50 mL/g), the best nitrification rates (99%) and COD (97%), TN (91%) and TP (55%) removals.
- There was no nitrite accumulation, and granules' disintegration and washout episodes were insignificant in O/An-type reactors.
- A/O-type reactors and O/An-type reactors with fast feeding showed granules disintegration and recurrent washouts that impaired good nutrients removals.
- Proteobacteria was the most abundant phylum in the reactors with anaerobic phase (R1 and R2) and the reactor with fast feeding (R6).
- In the reactors with a well-defined anoxic phase and step-feeding (R3 and R4), Planctomycetota was the most abundant phylum. Since it has been reported in the literature that this phylum is composed of ANAMMOX bacteria, more detailed microbiological studies are needed.
- The competition between PAOs and GAOs was more significant and relevant than the competition between PAOs and DNBs.

Therefore, this study brings important findings in terms of engineering and microbiological aspects for future investigations applying the AGS technology to leachate treatment and other types of wastewaters. Investigations evaluating the effect of leachate dilutions, sludge age, selective sludge discharge, external electron donor addition to sustain denitrification and phosphorous removal, and reactor design modification to achieve a more stable operation are also recommended. In addition, a deeper bioinformatics/statistical analysis would reveal other important things concerning the microbial community.

A modified version is under preparation for submission.

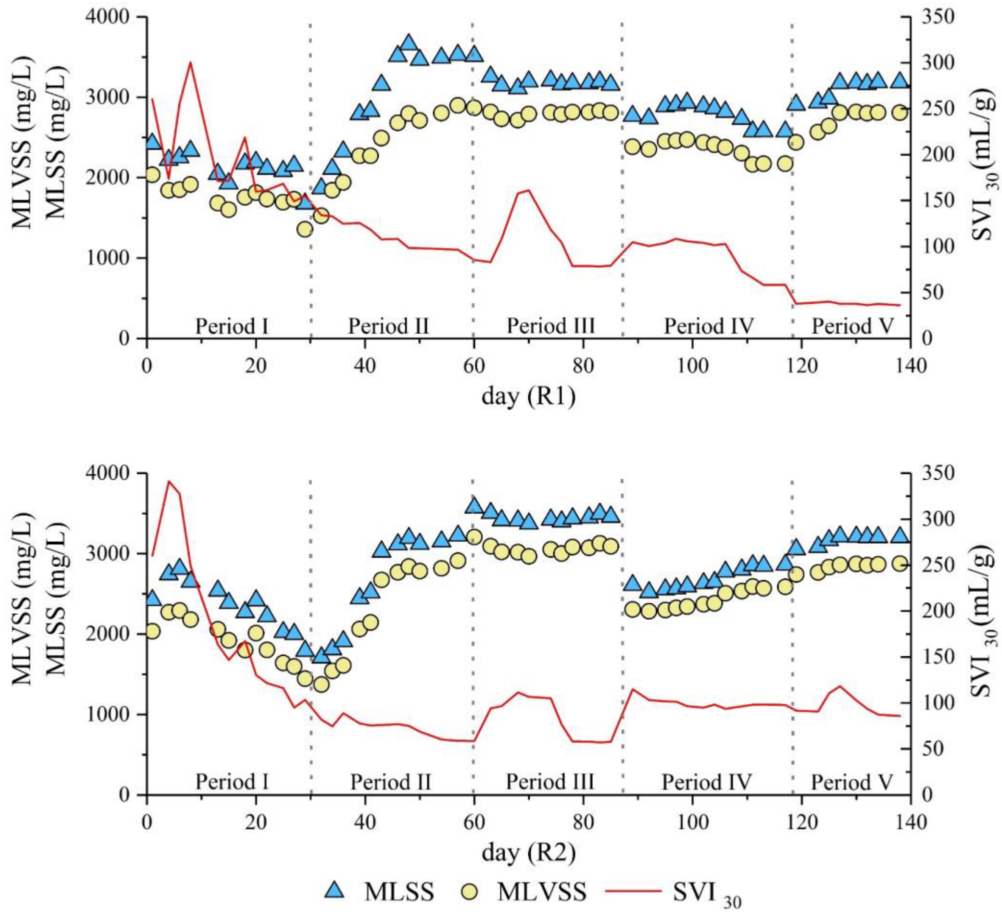
8 EVALUATION OF LEACHATE IMPACT ON DOMESTIC SEWAGE CO-TREATMENT IN AEROBIC GRANULAR SLUDGE SYSTEMS

Aerobic granular sludge (AGS) technology has been consolidated for sewage treatment. However, studies about the co-treatment of landfill leachate in municipal wastewater treatment plants (MWWTPs) with AGS systems are still incipient. This study aims to evaluate the engineering and microbiological aspects of leachate co-treatment with sewage in step-feeding (R1) and conventional (R2) AGS systems. Initially, during the first two stages, only synthetic sewage was fed to the AGS system with a gradual increase in the organic load for granules formation (Periods I and II). Subsequently, co-treatment with leachate pre-treated by coagulation-flocculation was carried out with 5% (Period III) and 10% (Period IV) concentrations. Finally, methanol supplementation was conducted as an attempt to improve nutrients removal performance (Period V). It was possible to accomplish the domestic sewage co-treatment with leachate in AGS systems. However, feeding strategy, reactor configuration, and methanol supplementation played an important role in process stability and simultaneous carbon, nitrogen, and phosphorus removals. Step-feeding produced an aerobic granular biomass more compact and resistant, resulting in a better operational stability. Moreover, this strategy favored denitrification, especially during methanol supplementation, minimizing one of the main problems reported regarding leachate co-treatment in AGS systems. As a result, higher total nitrogen (TN) removals were obtained. At the end of the last period, in R1, Chemical Oxygen Dissolved (COD), TN, and Dissolved Organic Carbon (DOC) removals were 93%, and phosphorus removal was 54%, reaching values higher or similar to other AGS investigations with sewage, leachate, or co-treatment. Therefore, the presented results bring a good perspective for domestic sewage co-treatment with leachate and other types of industrial wastewater.

8.1 AGS systems prior to leachate co-treatment

To generate granules with more stability for the co-treatment period, the influent organic load was gradually increased. Regarding solids concentration, total and volatile, a very similar behavior was observed between the two reactors, in which the selection pressure caused a reduction in the initial biomass (Figure 32). However, in the step-feeding reactor (R1), this reduction was significantly lower than in the conventional reactor (R2) ($p = 0.04$) in the Period I. A higher organic load during Period II favored biomass growth in both reactors, achieving concentrations higher than the inoculum. In this stage, the VSS percentage was significantly higher in R2 ($p < 0.001$).

Figure 32 – Stability regarding SS, VSS, and SVI_{30} of AGS systems with step-feeding (R1) and conventional configuration (R2).

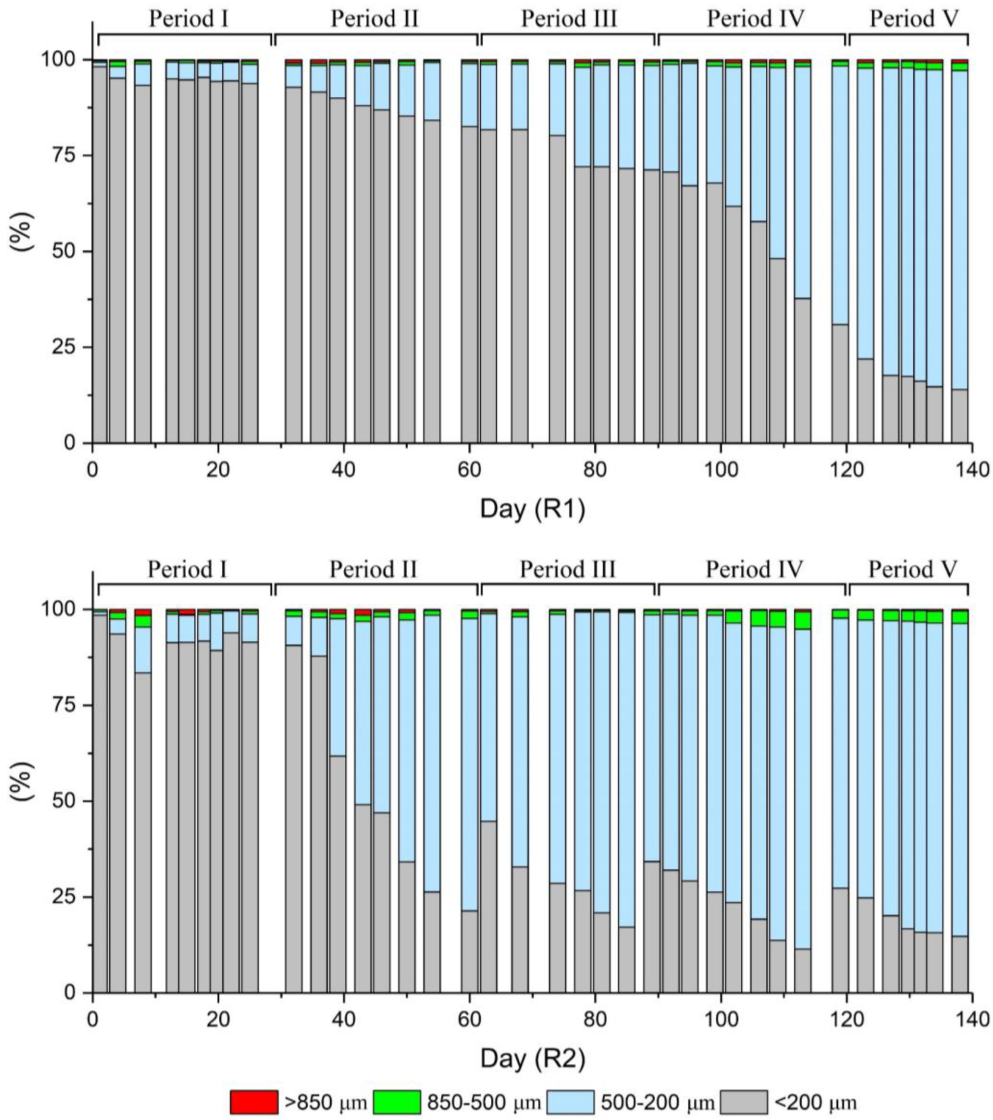


Source: Prepared by the author.

Settleability was also affected as a result of the adopted configuration. After increasing the organic load, R2 presented significantly better settling capacity than R1 ($p < 0.001$), despite the two reactors having SVI_{30} lower than 100 mL/g, a limit value commonly reported in granular biomass with high solids concentrations (Silva, Rollemberg and Santos, 2021). Thus, both systems showed good settleability after 60 days of start-up.

However, contrary to the initial hypothesis that the step-feeding would provide a faster granulation, the R1 took a longer time to be considered granular (Figure 33), i.e., when more than 80% of the biomass presented a diameter greater than 0.2 mm (Liu *et al.*, 2010). At the end of Period II, R2 presented granules with an average diameter of 0.4 mm, whose growth was stimulated by the influent organic load increase. The SRT was also significantly different between reactors. R1 showed a longer biomass retention time than R2 both in the Period I (p = 0.04) and Period II (p = 0.02) (Table 13).

Figure 33 – Granule size distribution (% mass) in AGS systems with step-feeding (R1) and conventional configuration (R2).



Source: Prepared by the author.

Table 13 – Granules' characteristics throughout the experimental periods for AGS systems with step-feeding (R1) and conventional configuration (R2).

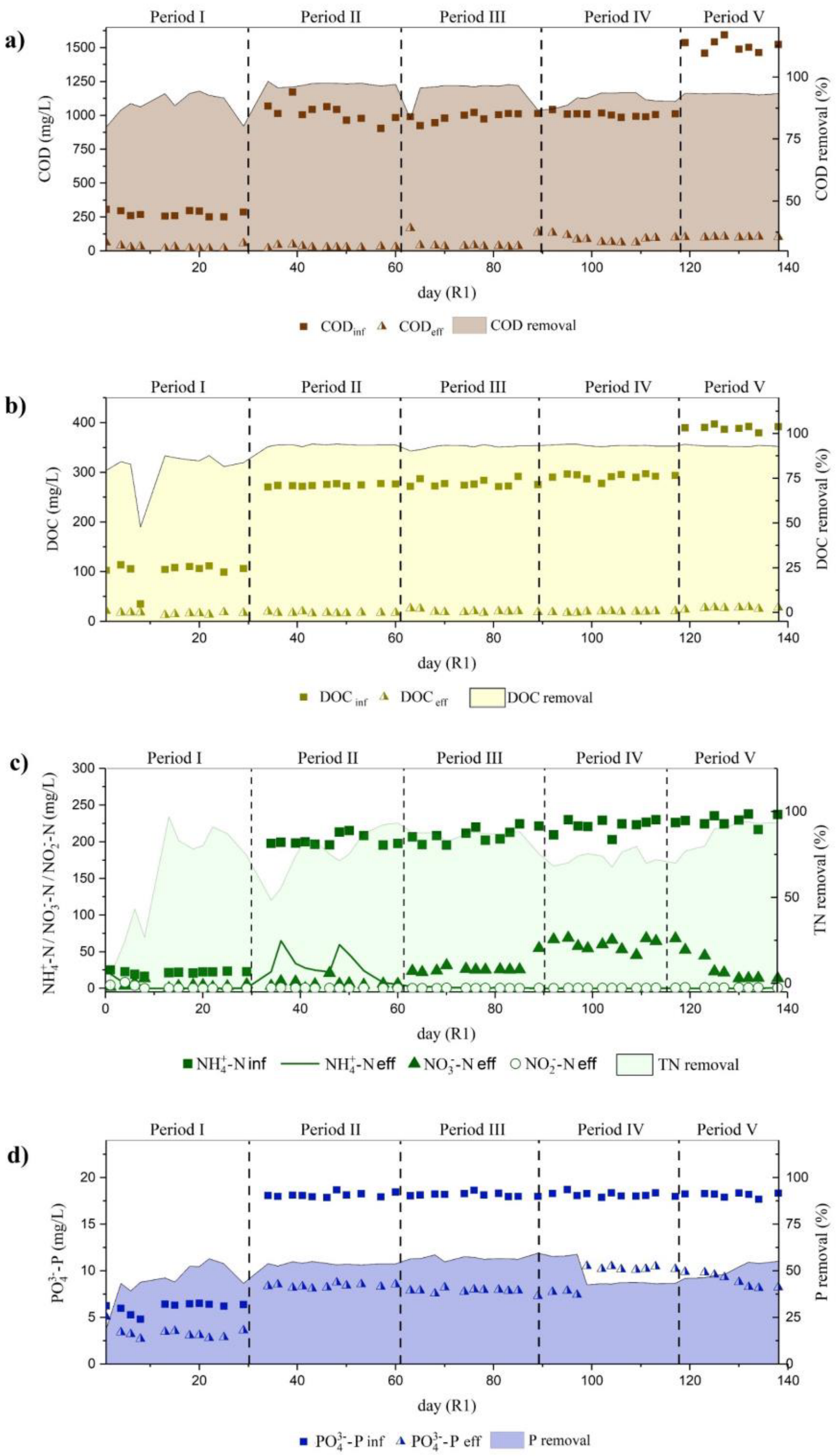
		SVI_{30} (mL/g)	**SVI_{10} (mL/g)**	**SVI_{50} (mL/g)**	**SVI_5/ SVI_{30}**	**Mean diameter (mm)**	**SRT (d)**
Period I	R1	182±35	291±62	396±37	2.3±0.3	0.1±0.1	28±11
	R2	177±72	254±86	317±63	2.0±0.4	0.1±0.1	25±9
Period II	R1	108±12	173±16	241±33	2.2±0.1	0.2±0.1	38±8
	R2	72±7	127±9	168±20	2.3±0.1	0.4±0.2	34±5
Period III	R1	105±25	169±37	202±50	2.0±0.2	0.2±0.1	31±4
	R2	82±20	142±42	159±54	1.9±0.2	0.5±0.2	30±5
Period IV	R1	91±18	136±35	180±58	1.9±0.3	0.4±0.1	27±7
	R2	100±4	146±4	207±20	2.1±0.1	0.5±0.1	25±6
Period V	R1	38±1	44±2	55±2	1.44±0.1	0.5±0.1	34±10
	R2	98±9	136±13	170±14	1.8±0.1	0.5±0.1	36±7

Source: Prepared by the author.

Frutuoso *et al.* (2023) also observed that conventional feeding promoted a faster granulation compared to step-feeding reactors, both treating saline wastewaters. Likely, selection pressure imposed by the feast/famine regime in conventional feeding reactors, i.e., rapid rate of carbon source consumption and long famine period, is established faster (Rollemberg *et al.*, 2018). Therefore, a longer famine period after the start-up is likely an important selection pressure for granulation to reach stability. However, as the system stabilizes, the granules have greater support capacity for shorter famine periods, as those established in step-feeding systems. Thus, feast/famine periods optimization during granulation and maturation is an important engineering aspect of AGS systems operation (Frutuoso *et al.*, 2023).

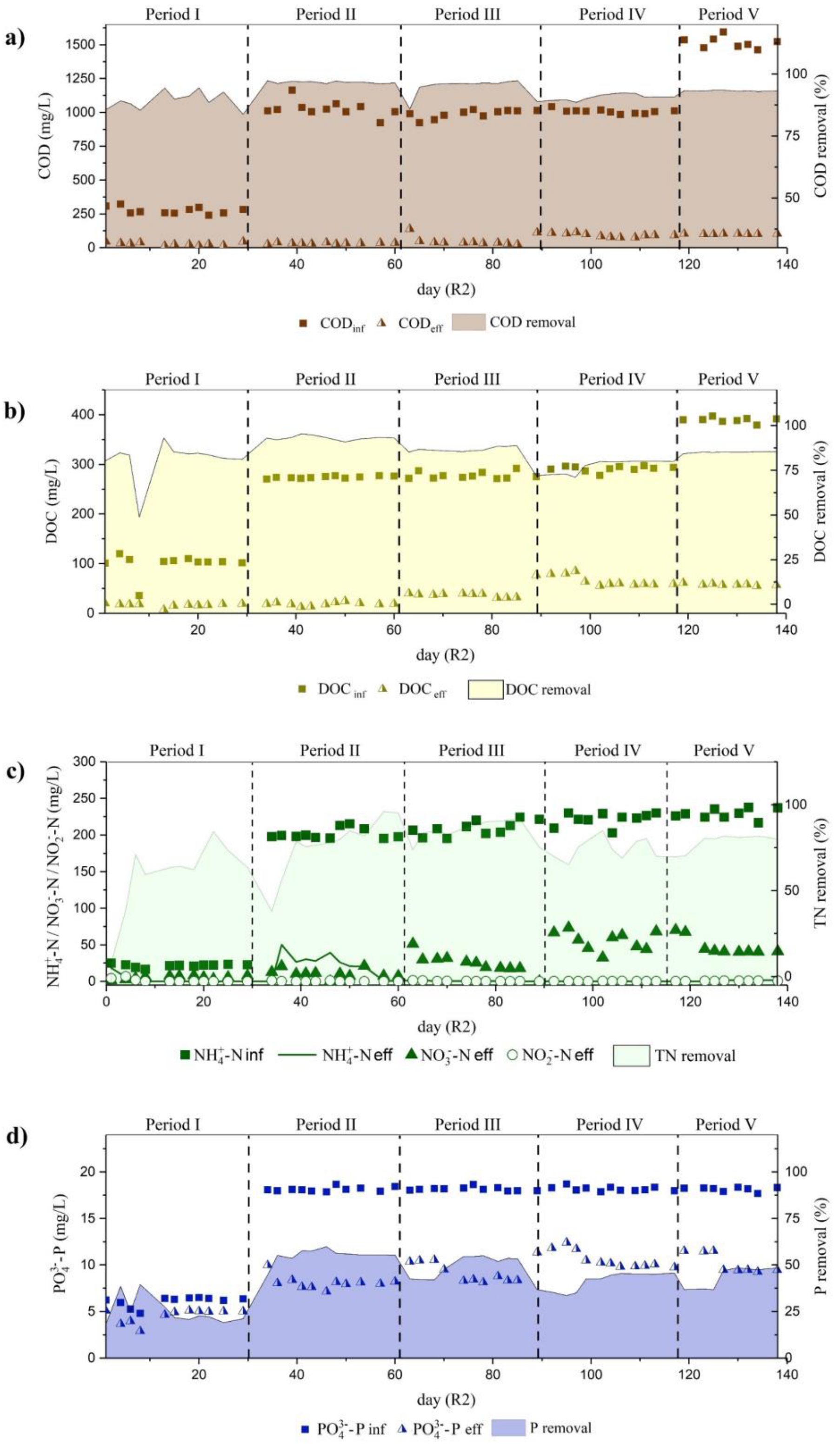
Regarding COD, DOC, and TN profiles, no significant removal efficiencies were observed for both reactors in Period I ($p = 0.95$; $p = 0.65$; $p = 0.29$) nor Period II ($p = 0.24$; $p = 0.13$; $p = 0.65$) (Figures 34 and 35). However, the removals were directly proportional to the organic load increase, being higher for the two reactors in Period II. It was observed that in the conventional reactor, there was a greater accumulation of nitrification by-products both in Period I ($p < 0.001$) and in Period II ($p = 0.02$), confirming the hypothesis that step-feeding reduces their accumulation, as found elsewhere (Wang *et al.*, 2012; Chen *et al.*, 2013). Similar results were reported by Rollemberg *et al.* (2019b) during the initial granulation phase operating with lower organic loads of synthetic effluent using acetate as the organic substrate. In addition, despite R1 showed greater phosphorus removal in Period I ($p = 0.001$) and almost the entire Period II, there was no statistical difference between the two reactors at the end of this stage ($p = 0.74$).

Figure 34 – Step-feeding reactor (R1) performance in terms of a) COD, b) DOC, c) nitrogen, and d) phosphorus removal over the 138 days of operation.



Source: Prepared by the author.

Figure 35 – Conventional reactor (R2) performance in terms of a) COD, b) DOC, c) nitrogen, and d) phosphorus removal over the 138 days of operation.



Source: Prepared by the author.

Thus, in general terms, the reactors presented very similar physical characteristics and performance at the end of the period with synthetic effluent. Despite producing only 20% of granules above 0.2mm, the step-feeding reactor retained more biomass and presented smaller accumulations of nitrogen removal by-products, favoring the development of more compact and resistant granules. On the other hand, the conventional feed reactor showed a lower temporal demand to be characterized as granular, presenting better settleability. Furthermore, it was not observed any washout and granules disintegration in R1, suggesting that this strategy is beneficial for biomass development. Santos *et al.* (2022) reported frequent disintegrations during the granulation process when treating domestic effluent in conventional reactors with acetate as organic substrate.

8.2 Leachate co-treatment in AGS systems

8.2.1 Effect on sludge settleability and stabilization

The co-treatment of synthetic sewage with 5% leachate (Period II) started after 62 days of operation, when both reactors reached stability. The reactors behaved differently regarding biomass retention (Figure 32). As R1 increased the volatile solids concentration, R2 remained stable. While R1 lost biomass, R2 increased. This loss in R1 is probably a result of the granulometry profile, as it presented very small granules with a tendency to instability (Table 13). Furthermore, during co-treatment with leachate, granular biomass suffers a hydraulic shock with a washout of many microbial communities, resulting in biomass loss and/or leading to worsen settling abilities (Bella & Torregrossa, 2014; Bueno *et al.*, 2020).

Although R2 did not lose biomass, settleability was affected immediately after starting the co-treatment with leachate, unlike R1, which did not have this initial shock and required a certain time to have a peak drop in settling quality. After 15-20 days of leachate addition, both systems improved settleability and stabilized, returning to the values at the end of the granulation phase with synthetic effluent. Saxena *et al.* (2022), when incorporating synthetic leachate in AGS systems, also observed a worsening in settleability during the first days of operation, being required around 20 days to recover the good settling properties and operation stability.

When the co-treatment occurred with 10% leachate (Period IV), the shock in R1 was lower than in R2, in which the solids concentration and settleability remained stable throughout the period. At the end of this period, on day 111, there was a small R1 biomass washing, which was fundamental to improving the settleability and was significantly smaller than R2 ($p = 0.03$). Although R2 had a worsening settleability peak due to increased leachate load, it remained stable throughout Period IV, using the available organic load for biomass growth. In some studies, part of the low biodegradability organic matter in the leachate was used as a substrate to increase the biomass concentration (Ren, Ferraz and Yuan, 2017b; Bueno *et al.*, 2020).

Thus, despite biomass loss with the increase in leachate load using the step-feeding reactor, this selection pressure was fundamental to reaching better settleability conditions. In addition, this reactor was more resistant to substrate change, as increasing the leachate concentration resulted in lower instabilities and biomass losses compared to the conventional reactor. That is, leachate co-treatment negatively influenced solid retention, especially for 10% leachate. The impact on settleability was greater in the conventional reactor, which worsened with leachate introduction (5%) and subsequent increase (10%), during co-treatment. The step-feeding reactor was able to maintain settleability at levels similar to the stages prior to leachate co-treatment and other investigations with AGS systems (Frutuoso *et al.*, 2023; Rollemberg *et al.*, 2020a).

8.2.2 Effect on granule size and composition

During the initial leachate co-treatment period, only R2 had more than 80% of particles with a diameter larger than 200 µm (Figure 33). It was expected to occur the disintegration of the already-formed granules, as a form of adaptation, as mentioned by some researchers (Bella & Torregrossa, 2014; Bueno *et al.*, 2020). However, this disintegration was observed only in R2, probably because it had a higher proportion of larger granules. In this reactor, for the periods of 5% (Period III) and 10% (Period IV) leachate co-treatment, there was a 20-25% reduction of granules larger than 200 µm. After this initial shock, the system recovered after 15-20 days. Furthermore, at the end of Period IV, episodes of granule disintegration were again observed in R2, suggesting that this reactor did not produce long-term resistant and stable granules.

In R1, despite granulation occurred more slowly, no granule disintegration was observed in any period during leachate co-treatment. Moreover, a continuous rise in the granule size was observed, indicating that the leachate co-treatment favored biomass aggregation. Apparently, step-feeding minimizes leachate toxicity by distributing it throughout the cycle, favoring granule development. Furthermore, in R1, there were more granules with a size greater than 850 µm than in R2, but fewer granules between 850-500 µm. As in R1, the granulation process with only domestic sewage was still slow, and leachate addition did not harm the process. On the contrary, co-treatment with leachate favored biomass development.

Notably, R2 presented average diameters higher than those of R1. During Period III, while R1 had granules with a size of 200 μm ± 100 μm, R2 had granules with a size of 500 μm ± 100 μm. In Period IV, R1 showed a more significant growth to 400 μm ± 100 μm, with R2 remaining as in Period III. The mean sizes found in these two periods were similar to those found elsewhere (Wei *et al.*, 2012; Ren, Ferraz, Yuan, 2017a; Ren, Ferraz, Yuan, 2017b; Ren *et al.*, 2017a) during leachate treatment in AGS reactors. In other words, this type of effluent is not expected to produce very large granules, commonly generated in treating domestic sewage or less complex wastewaters.

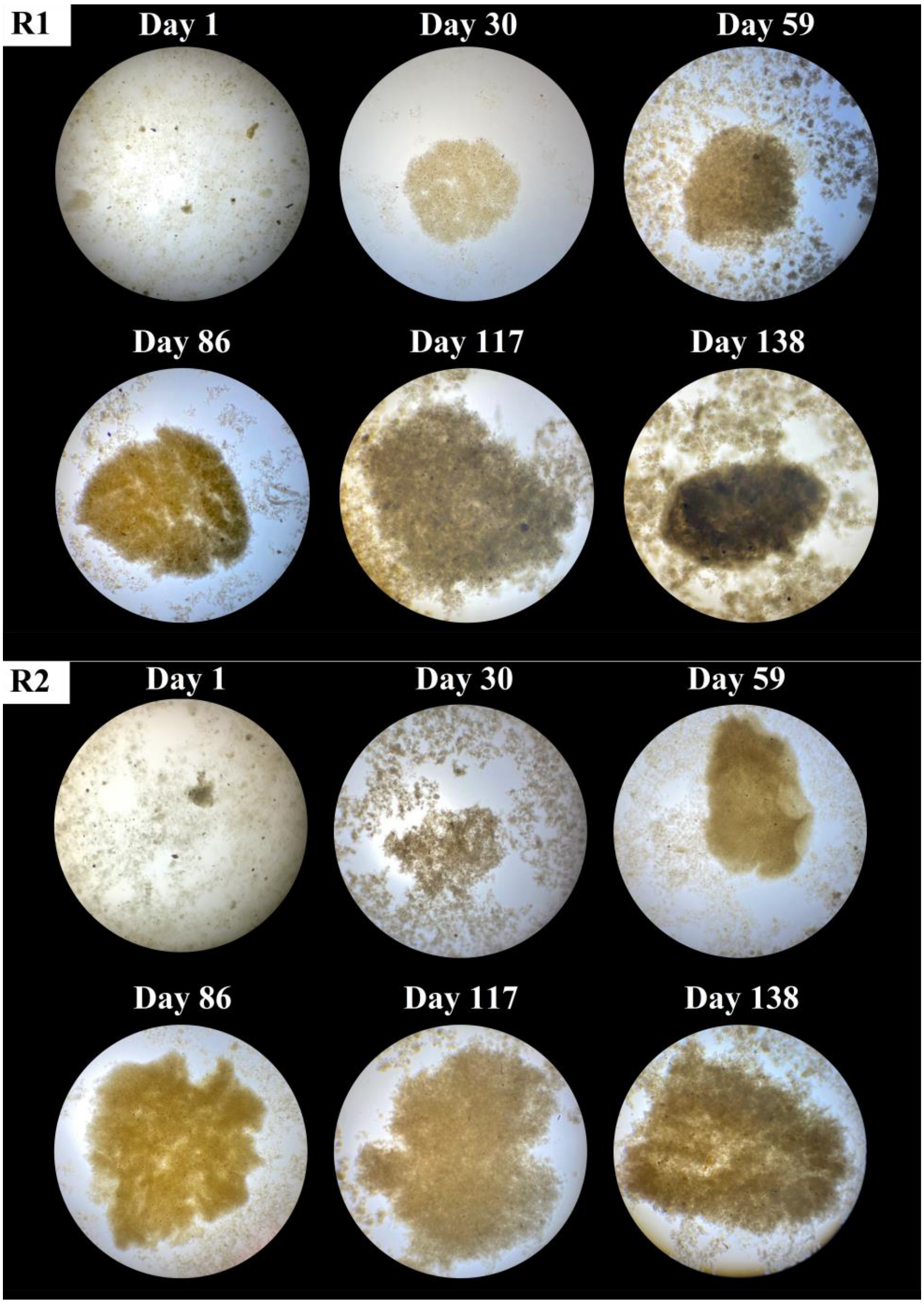
From a microscopic analysis throughout the experiment, it was possible to verify that the granules produced in R1, despite being small, had more microbial aggregates, making the granule more robust and compact (Figure 36). Furthermore, a more regular surface was observed, and, especially at the end of the operation, the internal zones resulting from the oxygen gradients are noticeable. In R2, in addition to being unable to verify these zones clearly, the presence of many pores is evident, contributing to the larger granule diameters.

Also, to evaluate the settleability and the microbial aggregates formation, the EPS were measured (Figure 37), as these substances play a key role in these characteristics, functioning as a "biological glue" (Rollemberg *et al.*, 2018). Therefore, to complete the granular biomass profile, the protein (PN) and polysaccharide (PS) fractions were measured both for the step-feeding (R1) and conventional feeding (R2) reactors.

Protein production was greater than polysaccharides in both reactors, which is desirable in biological reactors, as PN is responsible for aggregating granules and PS for mechanical stabilization (Moghaddam & Moghaddam, 2015; Rollemberg *et al.*, 2018). In the granulation period with synthetic effluent, the production of EPS and its fractions occurred similarly between the systems, being constant at low load (Period I) and increasing when the influent load was increased (Period II) until reaching stability at day 50-54. At the end of Period II, there were no statistical differences regarding EPS production in both reactors ($p = 0.60$).

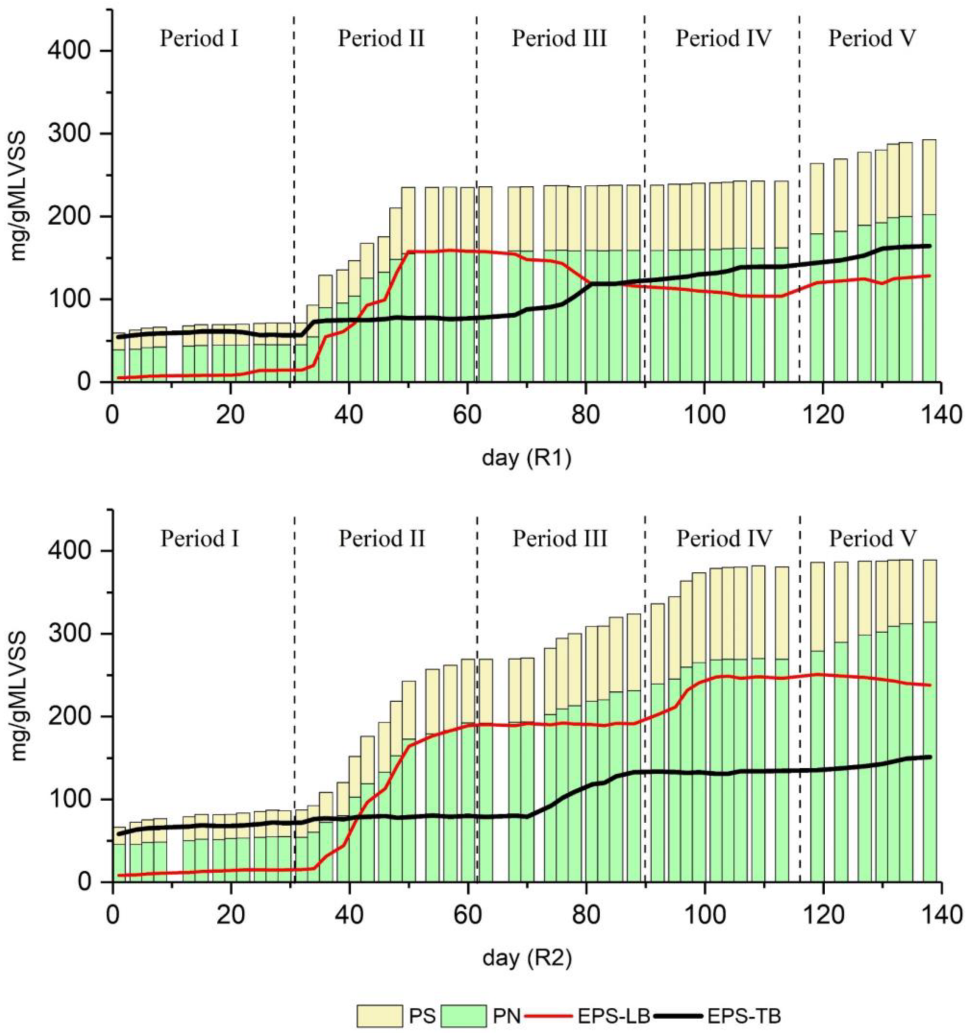
Leachate co-treatment in the step-feeding reactor R1 did not affect EPS production and its fractions, assuming mean values of 160 mg/gMLVSS for PN and 78 mg/gMLVSS for PS, both for 5% (Period III) and 10% (Period IV) leachate. This EPS production stability is consistent with the low granule growth during these two periods. Leachate addition did not hinder or favor EPS production. EPS values were similar to those produced by Frutuoso *et al.* (2023) using step-feeding reactors.

Figure 36 – Microscopic images of the medium granules formed over the 138 days of operation in AGS systems with step-feeding (R1) and conventional configuration (R2).



Source: Prepared by the author.

Figure 37 – Average quantification of extracellular polymeric substances (EPS) in each AGS system over the 138 days of operation in AGS systems with step-feeding (R1) and conventional configuration (R2).



Source: Prepared by the author.

In R2, the co-treatment with 5% leachate generated a slight drop in EPS production during the first days of operation, recovering again and continued to grow until its stabilization after 100 days of operation. The production growth was mostly from PN up to an average of 259 mg/gMLVSS, while PS maintained an average of 101 mg/gMLVSS at the end of Period IV. This constant low EPS production in R1 was in line with findings in conventional systems with synthetic domestic effluent in which the total EPS concentration was between 100 - 200 mg/g VSS (Rollemberg *et al.*, 2019a; Silva *et al.*, 2021). Leachate addition did not hinder or favor EPS production. On the other hand, in R2, leachate presence stimulated EPS production. Rollemberg *et al.* (2020a) reported total EPS concentrations above 500 mg/g VSS when using domestic sewage in a pilot-scale system.

As R2 had a longer uninterrupted aerobic period than R1, it was also to be expected a higher total EPS content, as their production is stimulated by the stress caused by the feast period, and the consumption occurs during the famine period. Thus, when producing successive periods of feast/famine distributed throughout the cycle, it was assumed that EPS production and consumption in the step-feeding reactor tended to balance, being lower than in the conventional reactor.

The EPS produced by aerobic granules have a dynamic double-layer structure, classified as EPS-LB (loosely bound) and EPS-TB (tightly bound), both of which can easily unite neighboring cells and favor granulation. In the outermost layer is the EPS-LB bond, which, as its name suggests, has a weaker bond that can be easily destroyed, unlike EPS-TB, that are consistently and stably bonded.

During the stage with synthetic effluent, the profile of these connections varied according to the influent load. In both reactors, when the influent load was low, the EPS-TB concentration was higher than that of EPS-LB, while in Period II, with a higher influent load, the behavior was the opposite, the EPS-TB remained stable, and the EPS-LB increased considerably until reaching values similar to PN concentrations. Large EPS-LB concentrations weaken the adhesion and microbial communities' aggregation capacity since this binding mainly comprises PS (Basuvaraj *et al.*, 2015). However, as PS concentrations were very low, it is believed that in this study, EPS-LB were formed mainly by organic acids, as already reported by Basuvaraj *et al.* (2015). Thus, from this profile analysis, it is possible to infer that the granules presented a low stability and that substrate shocks could trigger disintegration, as observed for R2 during the first days of operation with leachate co-treatment. During the co-treatment with 5% of leachate, EPS-LB concentration remained stable in R2, while EPS-TB increased. With 10% of leachate, the opposite occurred, EPS-LB increased, and EPS-TB remained stable. Despite EPS-TB growth, the EPS-LB connection was still superior to the EPS-TB. This also explains the worsening in R2 settleability since EPS-LB does not improve the sludge flocculation capacity (Yu, He and Shao, 2009).

In R1, during the co-treatment with 5% of leachate, EPS-LB production started to decrease, and EPS-TB increased until there was a constant and equal balance between the production of these two bonds. The co-treatment with 10% of leachate continued to favor EPS-TB growth and EPS-LB reduction. Therefore, the configuration adopted in R1 allowed the microbial communities to be more strongly aggregated in the granule core, producing more resistant granules and avoiding disintegration and washouts.

Therefore, the high EPS-TB production in R1 helped to avoid granule disintegration during the leachate co-treatment, resulting in stronger and more compact granules with good settleability characteristics. On the other hand, the high EPS-LB production in R2 created fragile zones in the granules that could not withstand the initial shock of leachate addition, generating disintegration and low settling velocity. In general, leachate co-treatment did not affect the profile of EPS-LB and EPS-TB in R2, maintaining the same behavior even when the influent concentration increased. In R1, leachate addition was crucial to change the EPS bonds characteristics, in which EPS-TB developed to the detriment of EPS-LB, making the granule stronger and more resistant.

8.2.3 Performance regarding simultaneous removal of C, N, and P

The COD removal showed very similar behavior in both systems, in which a decrease was verified for the periods of 5% and 10% leachate co-treatment (Figures 34 and 35). However, during co-treatment with 5% leachate, the removal grew again and stabilized at 95%, with no significant differences between the reactors ($p = 0.57$), generating an effluent with COD around 50 mg/L. During 10% leachate co-treatment, after the peak reduction, there was no increase in COD removal, stabilizing at 91%, also without statistical differences between R1 and R2 ($p = 0.92$), and with 90 mg/L of effluent COD. 5% leachate (Period III) did not affect the average COD removal in the reactors, but there was a decrease with 10% leachate (Period IV). The final COD values in the treated effluent were similar to work with domestic effluents in conventional reactors (Rollemberg *et al.*, 2019b)

The same behavior was observed for phosphorus removal, with statistical differences between the reactors both in Period III ($p < 0.001$) and in Period IV ($p = 0.04$). In R1, there was no drop in P removal during the co-treatment period with 5% leachate, although, after 100 days of operation, there was a decrease in P removal. This decay possibly occurred due to a saturation of phosphate and glycogen-accumulating microorganisms (Wei *et al.*, 2012; Bella & Torregrossa, 2014; Ren, Ferraz and Yuan, 2017b). In R2, in addition to the removal being lower than that of R1, it decreased from an average of 50 ± 5 % in Period III to 40 ± 4% at the end of Period IV. Leachate addition interfered with phosphorus removal in both reactors. In works where municipal wastewater was treated in AGS reactors, phosphorus removal was between 60 - 90% (Rollemberg *et al.*, 2020a).

In the step-feed reactor, the co-treatment did not change DOC removal, remaining around 93% (Figure 34). Only a slight decrease in DOC removal occurred in the first contact with the leachate, however, soon there was stabilization. In the conventional reactor, the co-treatment with 5% generated a significant decrease in DOC removal ($p=0.04$), which was even greater in the co-treatment with 10% leachate ($p<0.001$) (Figure 35).

There were no significant differences in TN removal between the two reactors in the two periods (p = 0.79; p = 0.75). However, R2 presented higher peaks of instability. During the co-treatment with 5% leachate, TN removal was around 87%, which dropped to 73% when the leachate concentration rose to 10%. Nitrification occurred immediately via nitrate, without nitrite accumulation, with a conversion of more than 99% of the influent ammonia. On the other hand, the denitrification was not complete, with nitrate accumulation, which increased as the influent leachate concentration also increased, but with no statistical difference. In Period IV, with 10% leachate co-treatment, the step-feeding reactor generated a lower nitrate concentration in the effluent (p = 0.06), 55 mg NO_3^--N/L, which can infer that this strategy in the long term and lower leachate dilutions stand out compared the conventional one. Nitrate accumulation between 50-100 mg NO_3^--N/L is also reported when treating leachate in AGS reactors (Wei *et al.*, 2012; Bella & Torregrossa, 2014; Ren *et al.*, 2017b). Therefore, leachate co-treatment with domestic sewage may impair TN removal, depending on the feeding strategy and reactor configuration adopted and carbon supplementation (as better explained in item 3.3.4).

Granule size was one of the limiting factors in denitrification since the zones created by the DO gradient were not well defined. These zones are of paramount importance for the SND process, as the DO and the soluble substrate available outside the granule diffuse into the aerobic zone and, depending on the concentration of DO, biodegradable organic matter, and ammonia, this DO can be consumed immediately inside the granule, making it unavailable in the inner layer. In this way, the nitrate produced in the aerobic zone would be diffused to the inner granule layer, causing denitrification. Therefore, an efficient strategy would be to promote a granule increase to favor SND. Despite the small granules, nitrate accumulation was lower in the step-feeding reactor, probably due to the supply of biodegradable organic matter throughout the cycle, which can sustain and favor denitrification.

Therefore, despite having similar efficiencies to the conventional reactor in removing the constituents, the step-feeding reactor managed to remove total organic carbon and phosphorus more efficiently. In addition, the readily available organic matter provided during the cycle favored a lower NO_x accumulation, which has been reported as one of the main problems of using AGS to treat leachate (Silva *et al.*, 2022).

8.2.4 Long-term effect and organic matter supplementation

In Period V, both systems were supplemented with 500 mg of methanol/L to provide more organic matter that could be used both for growth and constituents' removal. This supplementation showed significant improvement in the biomass characteristics and concerning reactors' performance, minimizing the negative interference of leachate co-treatment in all processes.

Biomass growth was reestablished (Figure 32), reaching similar concentrations of AGS systems that treated only domestic sewage (Rollemberg *et al.*, 2020b). Supplementation can be considered the key strategy for biomass retention, especially since the solids content dropped in the mixed liquor from co-treatment works where the leachate concentration increased without organic matter supplementation (Balla and Torregrossa, 2014; Bueno *et al.*, 2020). Settleability also changed, being beneficial in R1 (Table 13). In this reactor, there was a significant improvement in SVI_{30}, which was lower than 50 mL/g and statistically differing from R2 ($p < 0.001$). R2 had a worsening peak in settleability but soon improved and stabilized at values close to Period IV, between 75 and 100 mL/g.

Methanol supplementation had a more remarkable effect on R1 granule size. The amount of EPS also grew only in this reactor, both concerning proteins and polysaccharides and to EPS-TB and EPS-LB bonds, which maintained the same behavior as in the previous period, generating granules with aggregates more resistant due to the higher EPS-TB concentration (Figure 37). In R2, the total production of EPS remained constant, being possible to observe a reduction of PS, and subsequent growth of PN. In addition, EPS-LB binding was observed to have a slight drop, while EPS-TB increased. These changes favored its settleability control, preventing the biomass from disintegrating and being washed.

In Period V, the COD removal in the two reactors increased again, reaching an average of 93%, not differing statistically from each other ($p = 0.79$), but still producing an effluent with COD around 100 mg/L (Figures 34 and 35). Phosphorus removal was also favored, especially in R1, which rose from 48% to 54%. On the other hand, in R2, the impact was smaller, reaching an average of 44%. Phosphorus removal could have been better, but it was still superior to most investigations with leachate and sometimes in the same range of values of AGS systems treating only domestic sewage.

Due to the granule size increase by organic load supplementation, nitrogen removal improved greatly, especially in R1, corroborating the initial hypothesis that the organic load increase should favor biomass growth and denitrification. R1 and R2 showed a final total nitrogen removal of 93% and 81%, respectively.

Compared to other studies already carried out for leachate co-treatment in AGS systems, the step-feeding showed the best removal of organic matter and is among the lowest NO_x accumulations (Table 14).

Table 14 – Comparisons between the AGS systems in this study with related works.

Reference	Leachate % in the co-treatment	Influent (mg/L)			Removal (%)		
		COD	NH_4^+-N	TP	COD	TN	TP
R1 (step-feeding) *	5	1000	220	18	95	87	57
R2 (conventional) *	5	1000	220	18	95	86	50
R1 (step-feeding) *	10	1000	220	18	91	73	48
R2 (conventional) *	10	1000	220	18	91	74	40
R1 (step-feeding) *	10	1500	220	18	93	93	54
R2 (conventional) *	10	1500	220	18	93	81	44
Bella & Torregrossa (2014)	COD based	4560	945	-	50-60	Low	-
Bella & Torregrossa (2014)	COD based	9738	1960	-	40-50	Low	-
Ren, Ferraz and Yuan (2017a)	10-40	1080	340	2-6	65	40	80
Ren, Ferraz and Yuan (2017a)	60	1194	580	4-6	43	25	40
Ren, Ferraz and Yuan (2017a)	90	1539	900	5-6	20	<10	40
Ren, Ferraz and Yuan (2017b)	10-45	550-1000	130-785	3-6	43-65	24-37	0
Ren, Ferraz and Yuan (2017b)	45-65	1000-1100	785-1085	3-6	31-40	23-24	0
Ren, Ferraz and Yuan (2017b)	50-30	1100-1200	1085-1209	3-6	7-31	21-23	0
Bueno *et al.* (2020)	5	650	88	13.1	87	99	36
Bueno *et al.* (2020)	10	863	136	15.2	89	99	42
Bueno *et al.* (2020)	20	1421	281	17.5	88	98	45
Saxena *et al.* (2021)	20	848-906	8-13	75-77	62-65	-	56-64

* This study; COD based, dilution used by Bella and Torregrossa (2014) based on specific COD values determined by the authors; Source: Prepared by the author.

Except for the experiments conducted by Bueno *et al.* (2020), TN removal was superior to values reported elsewhere, especially in co-treatment with 10% leachate and methanol supplementation. Notwithstanding the slight NO_x accumulation, TN removal was still superior to pilot studies of AGS treating domestic sewage (Rollemberg *et al.*, 2020a). NO_x accumulation was also observed elsewhere (Bella and Torregrossa, 2014; Ren, Ferraz and Yuan, 2017a; Ren, Ferraz and Yuan, 2017a), although Wei *et al.* (2012) observed accumulation only in the leachate without pre-treatment.

The biggest problem in R1 was the lack of organic matter, which, when added, favored biomass growth and reduced nitrate accumulation, providing a greater denitrification efficiency. Therefore, the step-feeding reactor is a good strategy for domestic sewage co-treatment with complex effluents such as leachate, but only if it has enough organic load to sustain all the processes. Otherwise, the granules will be small, and the removals and conversions will be unsatisfactory.

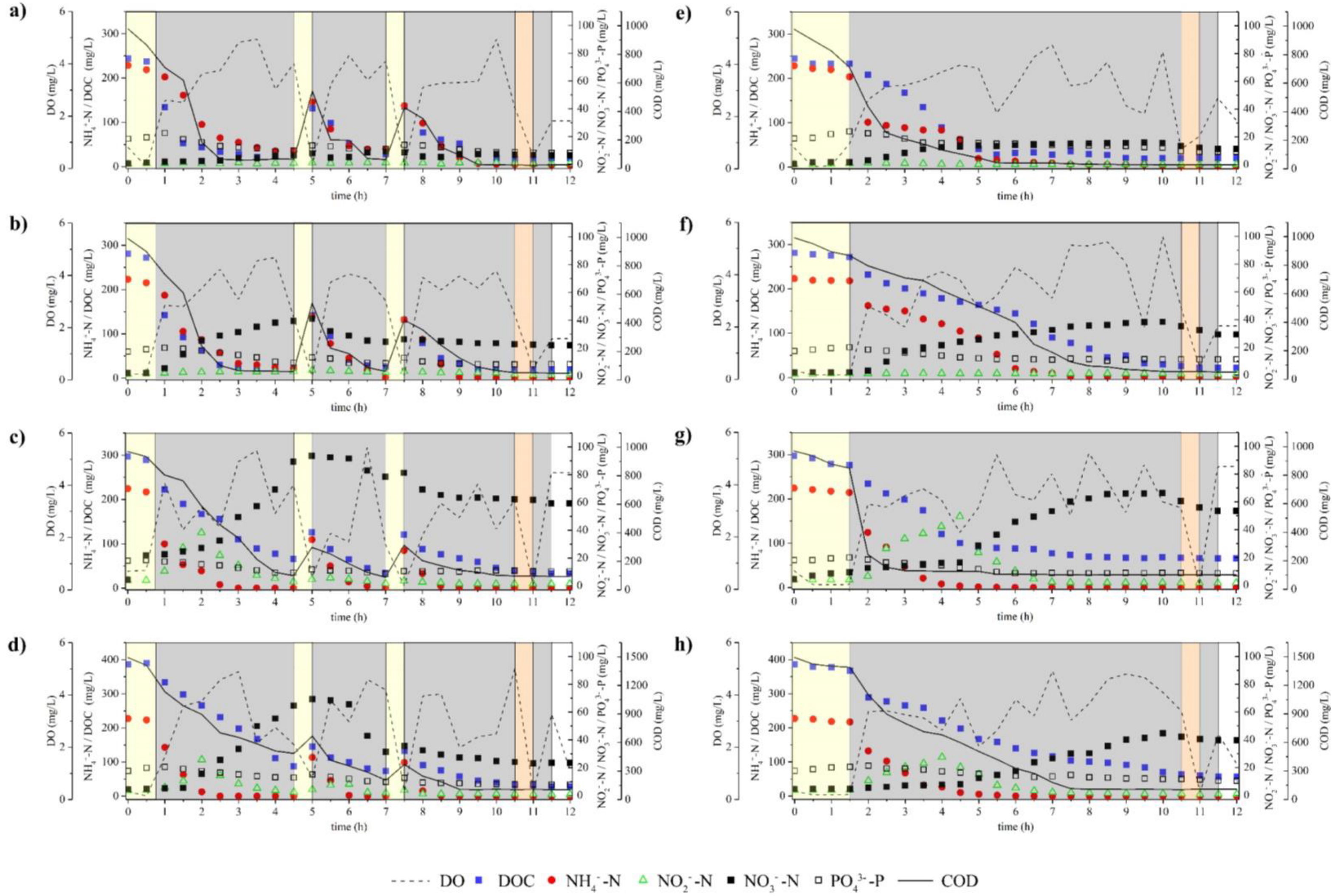
8.6 Cycle analysis and kinetic parameters

A full cycle analysis was performed to get insights into the simultaneous conversions and removals of organics, nitrogen, and phosphorus constituents, and the DO profile (Figure 38). DO was controlled during the operation, being defined a maximum value of 4 mg O_2/L during the aerobic stage. In both reactors, leachate co-treatment reduced the DO consumption, creating saturation peaks in shorter time intervals than the granulation profile. DO consumption was even lower in the last aerobic period during 10% leachate co-treatment with sewage.

In the step-feeding reactor, co-treatment with 10% leachate delayed the COD reduction in the first aerobic period. In comparison, this delay started in the co-treatment with 5% leachate using the conventional reactor. The DOC was converted immediately after feeding into both reactors.

Phosphorus removal showed a similar behavior with and without leachate co-treatment, in which PAOs and glycogen accumulating microorganisms (GAOs) performed slowly their functions throughout the cycle, releasing PO_4^{3-} during the filling phase and negligible changes in the COD profile. This suggests that carbon was used almost instantly by denitrifying organisms (DN) and PAOs. Subsequently, under aerobic conditions, the uptake of phosphate occurred throughout the entire phase.

Figure 38 – Performance profile of AGS systems with step-feeding (a-d, R1) and conventional configuration (e-h, R2) distributed over a cycle after synthetic effluent (a, R1; e, R2) and at the end of periods III (b, R1; f, R2), IV (c, R1; g, R2) and V (d, R1; h, R2).



Source: Prepared by the author.

In fact, complete SND was not achieved in this study, with nitrate accumulation in both reactors. SND can only be achieved when the DO saturation varies between 14 and 39% in AGS systems, with biomass presenting a diameter of 500 µm (Layer *et al.*, 2020). Thus, granules size and dissolved oxygen gradient can be attributed as the limiting factors which prevented the simultaneous reaction. As ammonia nitrogen was converted in the first hours of the cycle, NO_x, especially NO_2^-, was produced. The NO_2^- reduction was linked to the NO_3^- production, being able to verify the nitrate increase when the other nitrogen fractions were reduced.

In step-feeding R1, the filling throughout the cycle was fundamental to breaking the increase in nitrate production. In contrast, in the conventional R2, this break only occurred at the end of the cycle after the anoxic phase. This short anoxic phase favored denitrification, in which intracellular polyhydroxyalkanoate (PHA) may have been used as an electron donor to support denitrification in this phase. Importantly, the nitrate concentration at the cycle beginning was close to zero after dilution, indicating that residual nitrate was transformed into N_2 by denitrifiers during anoxic filling. In addition, heterotrophic endogenous denitrification in the famine period may have occurred, using the PS fraction of EPS (produced in the feast phase) as an electron donor (Rollemberg *et al.*, 2020b).

The biomass activity also showed differences between the investigated configurations (Tables 15 and 16).

In R1, the insertion of anoxic and aerobic phases at the end of the cycle did not bring noticeable improvements, and the nitrate, COD, and phosphorus consumption rates were practically null. Different from R2, which still showed slightly high biomass activity in these two final stages. Still, the nitrate production rate in R2 was lower than in R1. However, R1 had a higher nitrate consumption rate, resulting in better TN removal rates. In R1, nitrate consumption already started in the second aeration, while in R2, this consumption only became effective in the final anoxic and aerobic phases.

Table 15 – Kinetic parameters of biomass activity (q) in terms of consumption and production rates of organic, nitrate, and phosphorus compounds in reactor R1 after leachate addition.

Parameter	R1		
	Period III	Period IV	Period V
qCOD – Initial Feeding	0.032	0.016	0.029
qCOD – Aeration 1	0.300	0.324	0.330
qCOD – Feeding 2 (Addition)	0.171	0.091	0.070
qCOD – Aeration 2	0.171	0.097	0.166
qCOD – Final feeding (Addition)	0.128	0.103	0.060
qCOD – Aeration 3	0.134	0.099	0.094
qCOD – Anoxic	0.001	0.001	< 0.001
qCOD – Final aeration	0.001	0.001	< 0.001
qNO_3^--N – Initial Feeding	< 0.001	0.008	< 0.001
qNO_3^--N – Aeration 1 (Production)	0.014	0.031	0.021
qNO_3^--N – Feeding 2 (Addition)	0.001	0.002	0.002
qNO_3^--N – Aeration 2 (Consumption)	0.016	0.017	0.019
qNO_3^--N – Final feeding (Addition)	0.001	0.001	0.002
qNO_3^--N – Aeration 3 (Consumption)	0.012	0.014	0.015
qNO_3^--N – Anoxic (Consumption)	< 0.001	< 0.001	<0.001
qNO_3^- – Final aeration (Consumption)	< 0.001	0.001	< 0.001
qPO_4^{3-}-P – Aeration 1 (Capture)	0.004	0.004	0.003
qPO_4^{3-}-P – Aeration 2 (Capture)	0.002	0.002	0.002
qPO_4^{3-}-P – Final feeding (Addition)	0.002	0.001	0.001
qPO_4^{3-}-P – Anoxic (Release)	< 0.001	< 0.001	< 0.001
qPO_4^{3-}-P – Final aeration (Release)	< 0.001	< 0.001	< 0.001

Source: Prepared by the author.

Table 16 – Kinetic parameters of biomass activity (q) in terms of consumption and production rates of organic, nitrate, and phosphorus compounds in reactor R2 after leachate addition.

Parameter	R2		
	Period III	Period IV	Period V
qCOD – Feeding	0.040	0.047	0.036
qCOD – Initial aeration	0.264	0.289	0.297
qCOD – Anoxic	0.001	< 0.001	0.001
qCOD – Final aeration	0.001	< 0.001	< 0.001
qNO_3^--N – Feeding (Addition)	< 0.001	0.002	< 0.001
qNO_3^--N – Initial aeration (Production)	0.011	0.020	0.013
qNO_3^--N – Anoxic (Consumption)	0.010	0.010	0.011
qNO_3^- – Final aeration (Consumption)	0.009	0.008	0.008
qPO_4^{3-}-P – Feeding (Addition)	< 0.001	0.001	0.001
qPO_4^{3-}-P – Initial aeration (Capture)	0.001	0.001	0.003
qPO_4^{3-}-P – Anoxic (Release)	< 0.001	< 0.001	< 0.001
qPO_4^{3-}-P – Final aeration (Release)	< 0.001	< 0.001	< 0.001

Source: Prepared by the author.

In general, the periods of higher leachate concentration (10% leachate, Period IV) and methanol supplementation (Period V) increased the specific COD removal rate in the two reactors, reaching values higher than those reported by Xavier *et al.* (2021) when treating real domestic sewage in AGS reactors. Co-treatment with 10% leachate and methanol supplementation also increased the nitrate consumption rate, especially in R1. In the entire experiment, the phosphorus removal rate was very low in both reactors, being slightly higher when there was greater methanol supplementation.

Thus, from the cycle and kinetic tests, it is possible to verify that the step-feeding provides more favorable conditions for denitrification, especially when there is a high load of influent organic matter. This configuration also provided a considerable improvement in phosphorus and DOC removal.

8.7 Microbial community composition

8.7.1 Microbial community richness and diversity

From the metagenomic analysis, R1 presented an increase in the number of valid sequences until the co-treatment with 10% leachate (highest peak). However, when the co-treatment with 10% leachate and methanol supplementation occurred, a decay of around 50% was observed (Table 17). In R2, the largest sequence occurred with 5% leachate, which had a great reduction when the leachate concentration increased, growing again with methanol supplementation. From the rarefaction curve, it was possible to verify that the sequencing of the analyzed samples was adequate to analyze the microbial community structure because all species counts reached a plateau as sample sizes increased (Figure 39).

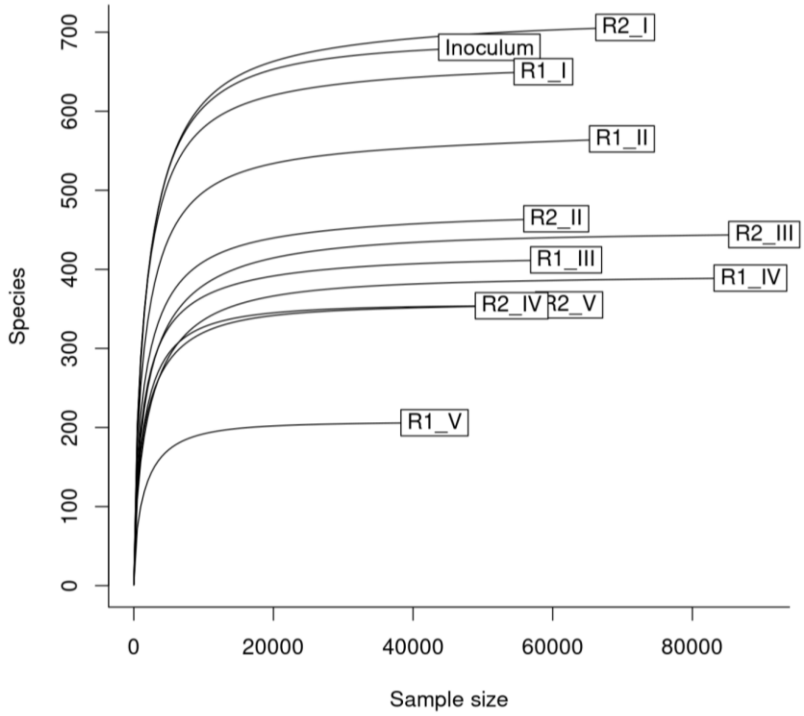
Alpha-diversity indices were used to determine the microbial richness and diversity (Table 17). The step-feeding reactor showed the highest microbial richness (Chao 1 and ACE indices) in almost all investigated strategies, except Period II, where the synthetic effluent load was increased. However, after adding leachate, the richness increased again in this reactor. That is, for the step-feeding reactor, leachate toxicity was not enough to reduce species richness, unlike the conventional reactor, which selected microorganisms that were more sensitive to toxicity.

Table 17 – The valid sequences and alpha-diversity indices of each sample.

Samples	Valid Sequences	Chao1	ACE	Shannon	Simpson
Inoculum	50926	687,32	685,38	5,01	0,98
R1_I	58667	713,29	712,65	5,30	0,99
R2_I	70378	652,89	654,13	5,39	0,99
R1_II	69912	466,62	468,05	4,83	0,98
R2_II	60502	600,00	572,73	4,98	0,98
R1_III	71918	448,67	446,00	4,36	0,97
R2_III	90295	414,33	414,61	4,57	0,98
R1_IV	88314	392,50	390,74	3,48	0,86
R2_IV	54125	354,60	354,68	4,13	0,96
R1_V	43075	362,00	357,03	4,10	0,95
R2_V	62344	206,60	206,62	2,35	0,64

Note: R1_I an R2_I, Reactor in Period I; R1_II and R2_II, Reactor in Period II; R1_III and R2_III, Reactor in Period III; R1_IV and R2_IV, Reactor in Period IV; R1_V and R2_V, Reactor in Period V. Source: Prepared by the author.

Figure 39 – Rarefaction curve of sequencing data.



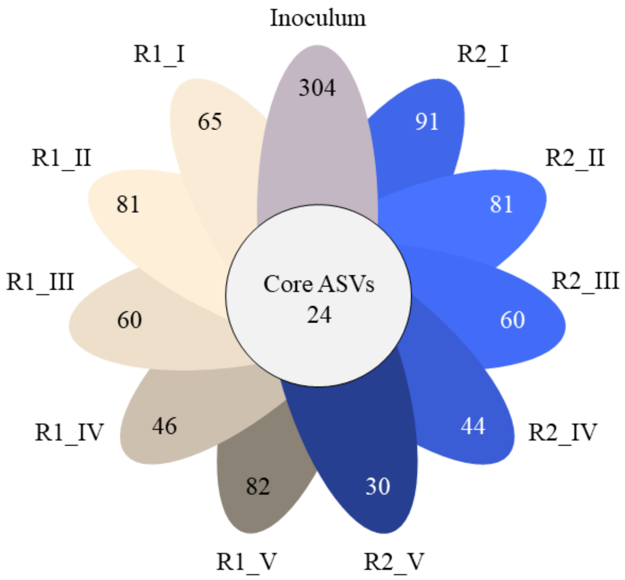
Note: R1_I an R2_I, Reactor in Period I; R1_II and R2_II, Reactor in Period II; R1_III and R2_III, Reactor in Period III; R1_IV and R2_IV, Reactor in Period IV; R1_V and R2_V, Reactor in Period V.
Source: Prepared by the author.

The scenario for microbiota diversity (Shannon and Simpson indices) was different. In the initial strategies (Periods I-III), the conventional reactor showed the greatest diversity, although the Simpson index suggests that this diversity was similar between the two reactors. For the co-treatment with 10% leachate (Period IV), diversity in R2 was much higher than in

R1. However, the increase in organic matter content in Period V was sufficient to increase diversity in R1 and reduce it in R2. Thus, it is possible to infer that the step-feeding reactor selected specific microorganisms and created conditions to maintain them. Furthermore, this reactor needs high concentrations of organic matter to create favorable environments for developing granules, increasing species diversity that can remove contaminants, corroborating the abovementioned conclusions.

From the flower diagram, it was possible to verify that only 24 amplicon sequencing variants (ASVs) (2.5%) were common between the reactors and the inoculum in all periods (Figure 40). Furthermore, the inoculum contained 304 unique ASVs, which were lost throughout the strategies. This result proves the high selection pressure imposed by the AGS system. In general, the number of unique ASVs decreased as the leachate concentration increased, indicating that the leachate imposes a complementary selection pressure on the system, eliminating more sensitive microorganisms. In addition, co-treatment with 10% leachate and methanol supplementation in the step-feeding reactor favored the growth of microorganisms unique to this system, which were possibly in a situation of latency. This development was favorable to the granulation process and the reactor performance since the best results were found in this period.

Figure 40 - Flower diagram to microbial diversity of granular sludge in R1 and R2.



Note: R1_I an R2_I, Reactor in Period I; R1_II and R2_II, Reactor in Period II; R1_III and R2_III, Reactor in Period III; R1_IV and R2_IV, Reactor in Period IV; R1_V and R2_V, Reactor in Period V.
Source: Prepared by the author.

8.7.2 Microbial community structure

From genetic taxonomic analyses, it was possible to determine and analyze the relative abundance of microbial communities at the level of phylum, class, order, and family (Figure 41). Proteobacteria and Planctomycetota, respectively, were the two most abundant phyla in both reactors, which were also the most dominant phyla in the aerobic granules produced by Saxena *et al.* (2022) when treating leachate. These phyla are composed of the main microorganisms that remove organic matter, nitrogen, and phosphorus (Wang *et al.*, 2020). Throughout the strategies, that is, from the gradual increase in the leachate concentration, the phylum that developed the most was Proteobacteria. Within this phylum, the abundances of Alphaproteobacteria and Gammaproteobacteria were different at higher leachate concentrations and varied according to reactor configuration. During the co-treatment with 5% leachate, their abundance was the same in both reactors. However, in the co-treatment with 10% leachate (Period IV) and the period with methanol supplementation (Period V), the Alphaproteobacteria class was more dominant in R1, while Gammaproteobacteria was dominant in R2.

Planctomycetota was present in all strategies but showed different behavior between reactors. In R1, this phylum had a growth peak during the co-treatment with 5% leachate. In R2, however, it was enough to reduce this phylum abundance. During the co-treatment with 10% leachate, both reactors dropped significantly. The same behavior was observed for Chloroflexi, whose abundance was compromised with a gradual reduction as the leachate load increased. That is, both Planctomycetota and Chloroflexi are more sensitive and less tolerant to the toxicity imposed by the leachate. Furthermore, it is also possible to infer that the staged-feeding reactor created conditions for decreasing toxicity events. However, this impact was inevitable when adding more leachate.

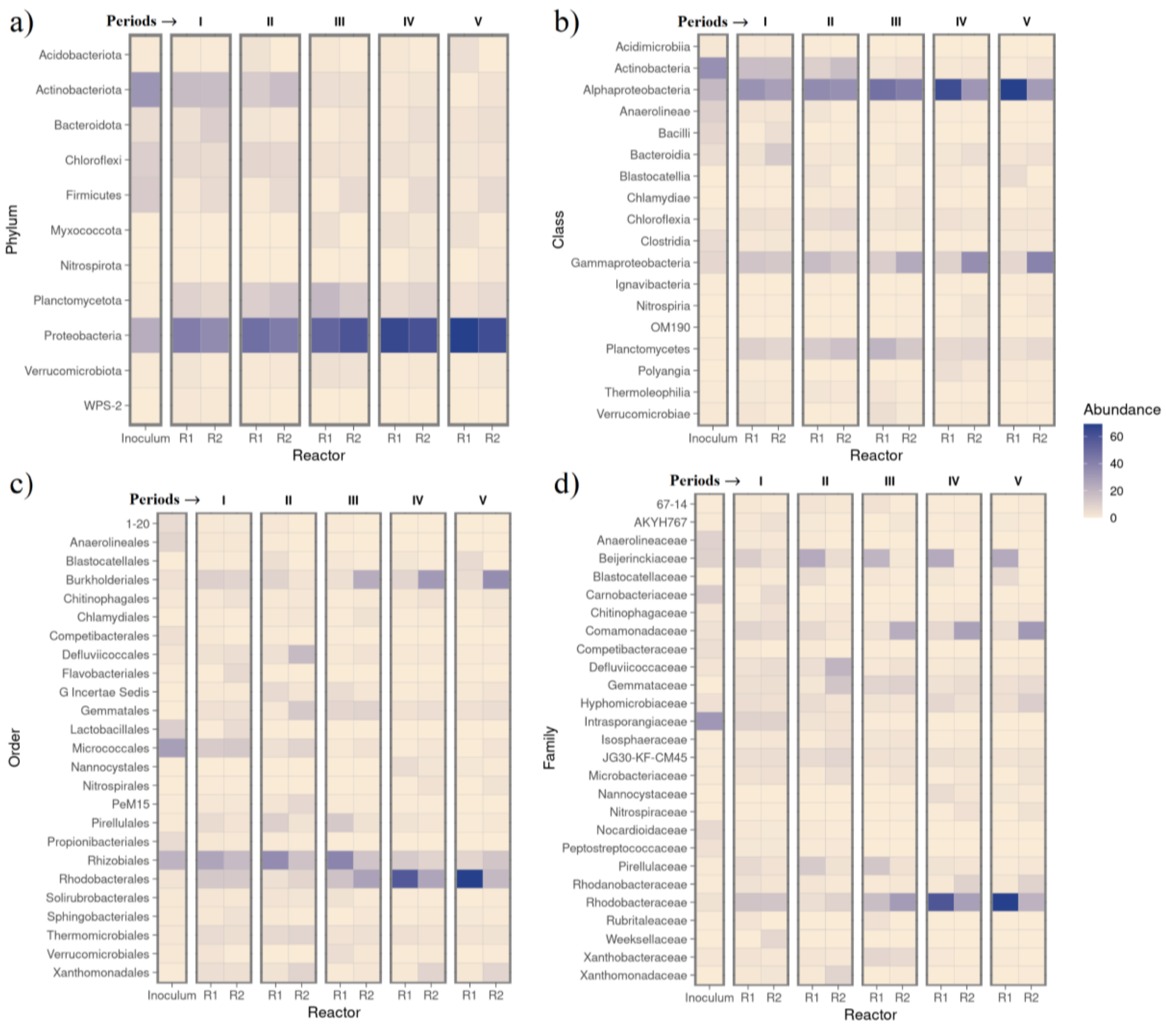
Another phylum with a representative highlight was Actinobacteriota, which is mostly responsible for the degradation of organic compounds (Zhang *et al.*, 2019). However, its abundance decreased during leachate co-treatment. In experiments with complex waters, Adler and Holliger (2020) also found the instability and sensitivity of this phylum to load shocks. They also verified that microorganisms belonging to the Proteobacteria phylum compete with microorganisms of Actinobacteriota. For this reason, in this study, one is in abundance to the detriment of the other.

In the step-feeding reactor, during the co-treatment with 10% leachate, a considerable increase of Myxococcota was observed. According to Xu *et al.* (2023), bacteria belonging to this phylum are responsible for significantly increasing EPS production. In fact, in this reactor, the amount of EPS increased as bacteria from the phylum Myxococcota became more abundant. So, likely there is competition between Firmicutes and Myxococcota (Purba *et al.*, 2023). For this reason, the two phyla were not found in significant abundance simultaneously in the same reactor. While R1 had a more significant presence of Myxococcota, in R2, it was possible to find Firmicutes.

During the co-treatment with 10% leachate (Period IV) and the subsequent methanol supplementation (Period V), there was also the microbial growth of the family Rhodobacteraceae (order Rhodobacterales) in the step-feeding reactor. However, the behavior was the opposite in the conventional reactor. These microorganisms are present in the outermost layers of the granules and are easier to wash off when disintegration occurs (Szabó *et al.*, 2016; Zou *et al.*, 2019). As granulation occurred later in R1, it was to be expected that these microorganisms would only develop when the granules reached larger sizes. In R2, granulation was faster, and disintegration was sometimes observed, so the concentration of these microorganisms decreased. In R1, there was also the development of Rhizobiales in all phases, represented mainly by the Beijerinckiaceae family. Organisms in this family are generally aerobic with the ability to fix nitrogen (Dasgupta, Clippeleir and Goel, 2019), contributing to better nitrogen removal results in this reactor than in the conventional reactor.

As in R1, the order of Rhodobacteraceae in R2 was the most significant. However, the behavior was the opposite of R1, decreasing with leachate increase. In R2, Burkholderiales was the next order that presented the most significant abundance, represented mainly by the Comamonadaceae family, and grew as the leachate concentration increased. This order is intrinsically linked to granule compaction (Wang *et al.*, 2019). Literature reports that the order of Burkholderiales is extremely diverse, including strict aerobic microorganisms and facultative anaerobes, which produce and accumulate polyhydroxybutyrate (PHB) (Dockx *et al.*, 2021; Hetz & Horn, 2021). These non-fermenting microorganisms reduce N_2O to N_2 and win the competition for acetate during denitrification. In addition, they are excellent EPS producers, contributing to the higher EPS production in R2 than in R1. In R2, verifying the abundance of Xanthomonadales in all periods with leachate was also possible. These microorganisms contribute to denitrification and are widely distributed in granules, despite creating weak aggregations (Świątczak & Cydzik-Kwiatkowska, 2017; Hetz & Horn, 2021). This fact corroborates the high concentrations of EPS-LB found in this reactor.

Figure 41 - Bacterial community structure at the Phylum (a), Class (b), Order (c), and Family (d) level of the two reactors and the inoculum in five periods (I-V).



Source: Prepared by the author.

8.7.3 Key functional groups

In the last stage of the microbiological characterization, simultaneously with the identification of the taxon, a survey was carried out of the 10 most representative families for C, N, and P removals (Figure 42), subdivided into five functional groups: AOB (ammonia-oxidizing bacteria), NOB (oxidizing bacteria of nitrite), DNB (denitrifying bacteria), GAOs (glycogen accumulating organisms) and PAOs (polyphosphate accumulating organisms).

As in both reactors, ammonia removal was complete, it was expected that the microbial community composition in terms of the AOB functional group would be the same in both R1 and R2. According to Wei *et al.* (2021), after specific AOBs establish themselves, their replacement by other microorganisms of the same function is much more difficult to occur, and thus the community structure is low. However, R1 showed a much higher dominant abundance than R2, with microorganisms of the Defluviicoccaceae family in periods I and III and with Rhodobacteraceae after 10% leachate addition and subsequent methanol supplementation. Defluviicoccaceae is a family reported to participate in nitrogen removal actively (Xu *et al.*, 2023), but because it is very sensitive, it was inhibited after increasing the leachate concentration. In R2, there was no significant dominance of AOBs microorganisms in most periods, with only Rhodobacteraceae appearing in the last period. As R2 presented greater diversity than R1 in periods I-IV and ammonia was removed with an efficiency of around 99%, likely several microorganisms performed this function and therefore did not appear statistically. In the experiments by Wei *et al.* (2012) also treating leachate, AOB were highly inhibited upon leachate addition. This makes the configuration investigated here advantageous, as there was no such inhibition.

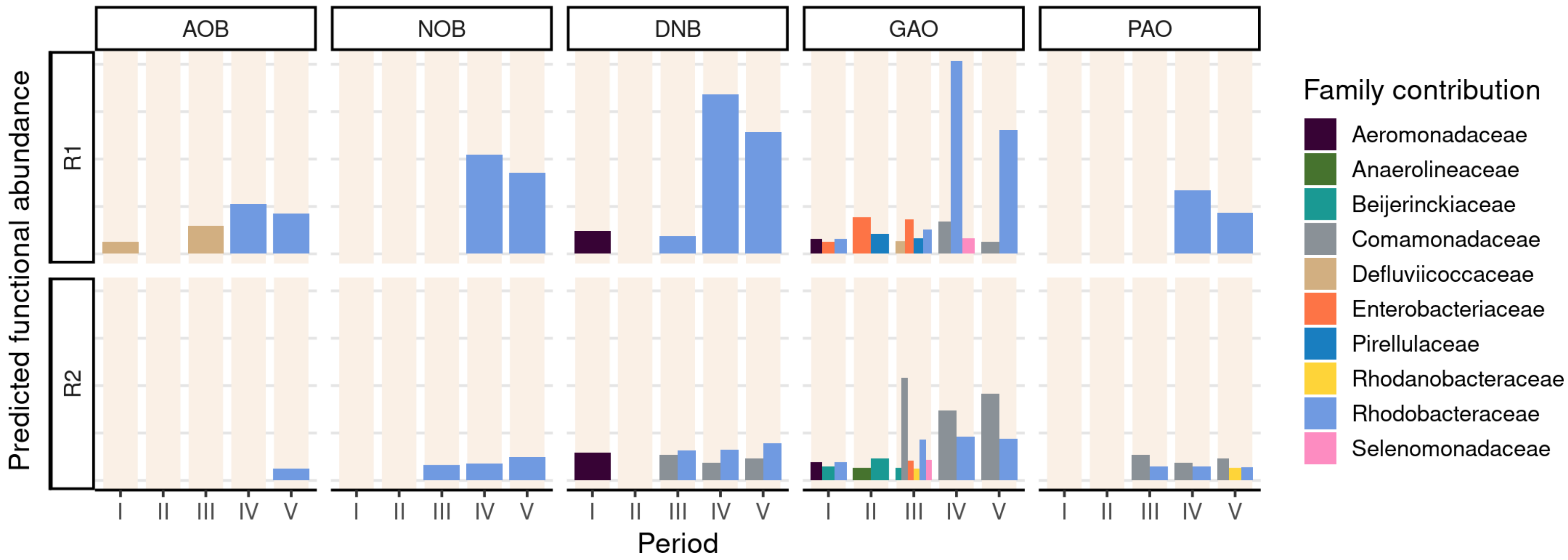
The oxidation of nitrite to nitrate also reached high rates, and as the NOB adapted to the leachate, the nitrification efficiency increased, resulting in higher nitrate production. In R1, NOB were dominant only during the co-treatment with 10% leachate, while this dominance already emerged with 5% leachate in R2. In both, the most representative family was also Rhodobacteraceae. Wei *et al.* (2021) reported that the initial high ammonia concentration inhibited NOBs, but the adapted microorganisms returned to participate in nitrogen removal actively.

For denitrification, at the beginning of the operation (Period I), the two reactors presented a similar abundance of Aeromonadaceae, decreasing over time. This family is very useful in the initial granulation process, as they easily adapt to different situations and substrates, harmoniously coexisting with different organisms (Wang *et al.*, 2019). However, as the granulation progresses, they are eliminated. As with the NOB, in R1, the only family with a representative dominance of the DNB group in the periods with leachate addition was Rhodobacteraceae. In R2, both Rhodobacteraceae and Comamonadaceae stood out. Although the reactors showed higher DNB abundances compared to the presence of AOB and NOB, denitrification could still be better.

In both reactors, the competition between PAOs and DNBs was also notorious. However, in R1, this competition was more relevant, in which DNB won. In R2, the competition was not as significant, and the two functional groups had similar abundances. During the Period I, in R1 and R2, there was a predominance of Aeromonadaceae of the GAOs type, which, like the DNBs of the same family, was inhibited in the following phases. In R1, there was also a predominance of Enterobacteriaceae, mainly in periods II and III, which were reduced during the co-treatment with 10% leachate. Meanwhile, in R2, Beijerinckiaceae developed in the periods with synthetic effluent, and the co-treatment with 5% leachate started to decrease until it had an insignificant abundance with 10% leachate. Both systems showed greater diversity in periods of co-treatment with 5% leachate compared to 10% leachate. After this latter period, Rhodobacteraceae and Comamonadaceae stood out with opposite behavior among the reactors. In R2, Comamonadaceae developed to the detriment of Rhodobacteraceae, while in R1, the opposite happened. Xu *et al.* (2018) verified that Comamonadaceae consume much acetate and do not play an essential role in removing nitrogen or phosphorus, directly influencing the reactor performance results.

Also, in both reactors, PAOs-type microorganisms only appeared significantly when leachate was added, especially during the co-treatment with 10% leachate in R1. In this reactor, Rhodobacteraceae remained the only dominant family, while in R2, in addition to Rhodobacteraceae, dominance was shared with Comamonadacea. Furthermore, when there was organic matter supplementation in R2, Rhodanobacteraceae also presented representative abundance. Rhodanobacteraceae is reported as a denitrifying PAO (DPAO) and an indicator of granule maturation and influencing the proteins' EPS increase (Iorhemen *et al.*, 2020; Hetz & Horn, 2021). In fact, in the last period, there was an increase in the portion of proteins in R2.

Figure 42 – Functional distribution of taxonomic classification at the end of the experiment in each reactor.



Source: Prepared by the author.

In general, Rhodobacteraceae was the family that most developed in the step-feeding reactor after adding leachate, regardless of the functional group, presenting microorganisms belonging to the five investigated groups. Rhodobacteraceae were also dominant in other AGS systems and have been reported as the main dominant microbes in the anaerobic process in a granule, having the ability to accumulate phosphorus during denitrification and simultaneously remove organic matter (Hamza *et al.*, 2018). In the conventional reactor, Rhodobacteraceae also developed significantly, but Commonadaceae was the one that grew the most in the classes of DNB, GAO, and PAOs.

8.8 Technological Implications

This investigation joins the small group of works that evaluated the viability of leachate co-treatment with domestic sewage using AGS systems. For simpler effluents, such as domestic sewage, AGS has been widely studied and has established itself as a very effective technology. However, there is still a lack of concepts for the co-treatment with more complex effluents, such as leachate. In this way, a deeper and more detailed study is necessary, mainly regarding optimizing cycle configurations.

Previous investigations (Silva, Rollemberg and Santos, 2021; Silva, Rollemberg and Santos, 2022) found that step-feeding enhanced the reactor performance and favored biomass development through organic load distribution throughout the cycle. However, these experiments were carried out with synthetic wastewater simulating a leachate composition. Some conceptions were confirmed for real leachate co-treatment using the step-feeding configuration, and others still need special attention, such as granulation time and granule size.

The granule size directly influences the contaminant removal processes, especially nitrogen. Thus, the smaller the granule, the more difficult it is to achieve high SND rates. Compact granules with smaller diameters are expected to be produced for complex effluents. However, the association of domestic sewage with a complex effluent (leachate) can minimize the toxicity impact on granule growth, if the organic load is sufficient to meet the microbiological requirements.

In this investigation, the step-feeding system granules only developed rapidly in the period of co-treatment with 10% leachate co-treatment and methanol supplementation. Thus, increasing the organic load was the key to improving all the biomass characteristics in the step-feeding reactor and its contaminant removal rates. After methanol supplementation, the step-feeding reactor showed removals of 93% for COD, TN and DOC, and 54% of phosphorus, which were higher than most of the literature findings with the same technology. Also, contrary to what has been reported in the literature, the strategies used in this investigation did not inhibit nitrifying microorganisms, and nitrogen removal was not impaired. Although the step-feeding biomass showed the lowest microbial diversity, it created more appropriate conditions for AOB, NOB, DNB, GAO, and PAO development.

Generally speaking, the results obtained support the hypothesis that the investigated optimizations will favor the full-scale leachate co-treatment from aerobic granular sludge systems. They also allow the evaluation of another technological route: replacing conventional activated sludge systems (CAS) in a multistage treatment system by AGS reactors. This substitution is expected to be sufficient to increase the rates of organic matter and total nitrogen removal, minimizing the by-products generated by SND.

Silva *et al.* (2017) proposed a sequential system with Advanced Oxidation Processes (AOP) polishing the CAS effluent to leachate treatment. In his system, removals of organic matter and TN content in the CAS were less than 25% and 37%, respectively, indicating the need for post-treatment. In this way, the co-treatment in step-feeding reactor investigated achieved better performances (even without organic matter supplementation), confirming that the co-treatment is a better strategy. In any case, new operational configurations with alternating oxic and anoxic phases must be studied further, and protocols developed to favor larger granule formation, in which the SND process can occur more efficiently. Tests with real domestic effluent are also encouraged.

8.9 Conclusions

It was possible to accomplish the domestic sewage co-treatment with leachate in AGS systems. However, feeding strategy, reactor configuration, and methanol supplementation played an important role in process stability and simultaneous C, N, and P removals.

Step-feeding produced an aerobic granular biomass more compact and resistant, resulting in a better operational stability. Moreover, this strategy favored denitrification, especially during methanol supplementation, minimizing one of the main problems reported regarding leachate co-treatment in AGS systems. As a result, higher total nitrogen removals were obtained. At the end of the last period, in R1, COD, TN, and DOC removals were 93%, and phosphorus removal was 54%, reaching values higher or similar to other AGS investigations with sewage, leachate, or co-treatment.

Therefore, the presented results bring a good perspective for domestic sewage co-treatment with leachate and other types of industrial wastewaters.

9 FINAL CONSIDERATIONS

Initially, when working with leachate in high concentration diluted only in water, it was possible to verify that the operational configurations of the AGS systems have a significant impact on the leachate treatment, even though the results were similar or better than previous investigations. Furthermore, although leachate concentration and granule size were inversely proportional, the more compact granules produced at higher concentrations were more resistant and the denitrification process achieved better rates than systems with lower leachate loads. Therefore, a careful optimization of operational parameters would favor the consolidation of technology in leachate treatment.

Thus, to investigate operational optimizations, subsequent works sought to evaluate the combination of different operational strategies from cycles with anaerobic, anoxic and/or oxic phases and different forms of feeding. As the main objective was now to define optimal configurations of the AGS systems for leachate treatment, the more control there was (especially of the influent constituents), the better the processes understanding to carry out adaptations. Thus, it was decided to use synthetic effluent in the same proportion of C:N:P as a real leachate.

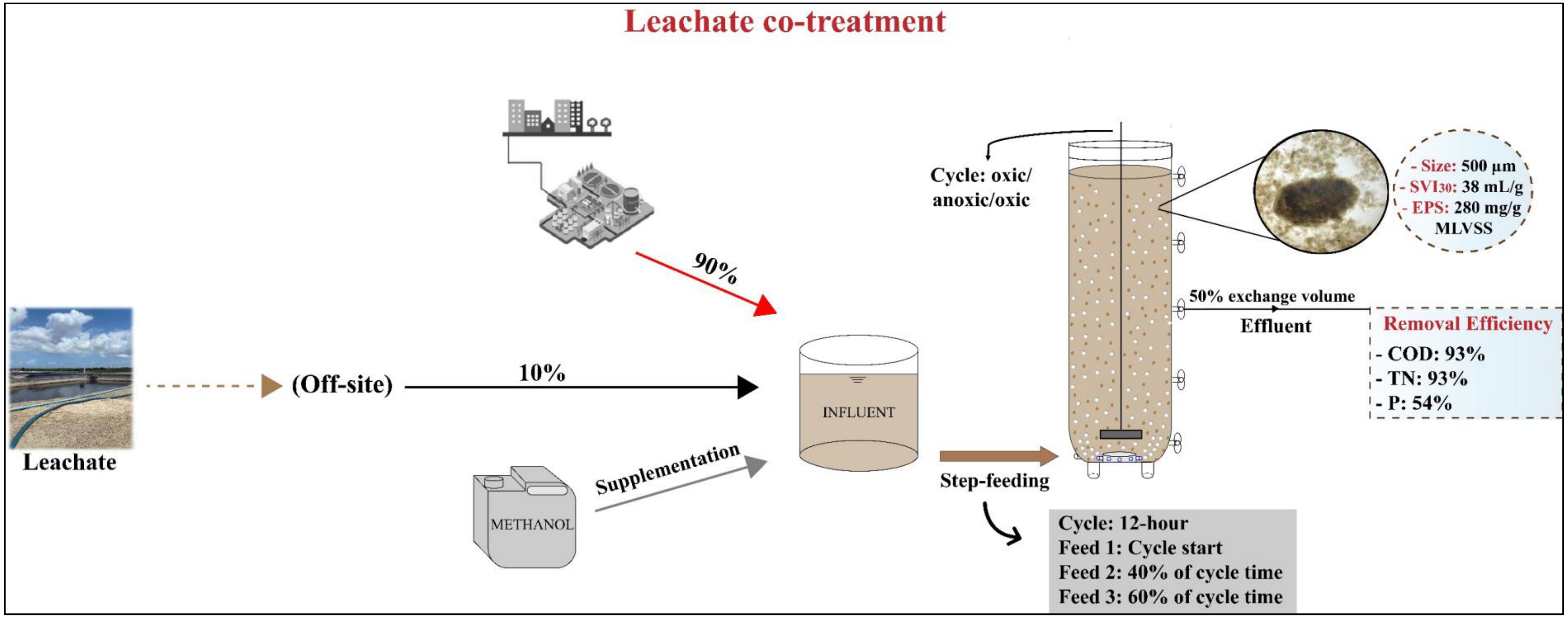
From the optimization studies, it was possible to verify that the insertion of anaerobic phases is dispensable for the treatment of this type of effluent, since fermentation is minimal and does not generate significant benefits to the process. It is also possible to infer that keeping GAOs in the system to the detriment of PAOs is more advantageous for treatments with leachate only whose phosphorus concentration is low, as they have better kinetic mechanisms and also favor granulation, produces EPS and denitrifies. In these investigations, the step-feeding and the intercalation of oxic and anoxic periods emerge as a key strategy for the granulation process, favoring microbial aggregation and creating much more compact and resistant granules. In this way, not only is biomass loss reduced but also granule disintegration, biomass washing, and nitrification and denitrification by-products accumulation, which has been proven in the application of co-treatment in the last stage of the work.

It was possible to accomplish the domestic sewage co-treatment with leachate in AGS systems (Figure 43). However, feeding strategy, reactor configuration, and methanol supplementation played an important role in process stability and simultaneous carbon, nitrogen, and phosphorus removals. Step-feeding produced an aerobic granular biomass more compact and resistant, resulting in a better operational stability. Moreover, this strategy favored denitrification, especially during methanol supplementation, minimizing one of the main problems reported regarding leachate co-treatment in AGS systems. As a result, higher total nitrogen (TN) removals were obtained. At the end of the last period, in R1, Chemical Oxygen Dissolved (COD), TN, and Dissolved Organic Carbon (DOC) removals were 93%, and phosphorus removal was 54%, reaching values higher or similar to other AGS investigations with sewage, leachate, or co-treatment. Therefore, the presented results bring a good perspective for domestic sewage co-treatment with leachate and other types of industrial wastewater.

Although the step-feeding and carbon supplementation strategy improved denitrification, this parameter still needs better optimization. Despite this, the results obtained were enough to support the leachate co-treatment and the hypothesis that the investigated optimizations will favor on-site leachate treatment in aerobic granular sludge systems, being able to replace activated sludge systems in multistage treatment system, like the chain investigated by Silva *et al.* (2017). In their work, the Conventional Activated Sludge system (CAS) was the first stage of the treatment and because it was considered insufficient in obtaining a final effluent within the release limits, other subsequent treatments were used. The reduction of organic matter content in the CAS was less than 25% both in terms of Dissolved Organic Carbon and COD. As the leachate is quite recalcitrant, presenting low biodegradability, Advanced Oxidative Processes (AOP) were used. Furthermore, the total nitrogen removal was in the order of 2-37%, producing large accumulations of nitrite. However, the strategy suggested in this investigation using the same leachate used by Silva *et al.* (2017), would be enough to increase the rates of organic matter and total nitrogen removal, minimizing the by-products generated by SND. That way, for the technology to be used in the landfill itself, a multistage system is presented as the best strategy, involving a physical-chemical pre-treatment and a post-treatment with advanced oxidative processes (Figure 44).

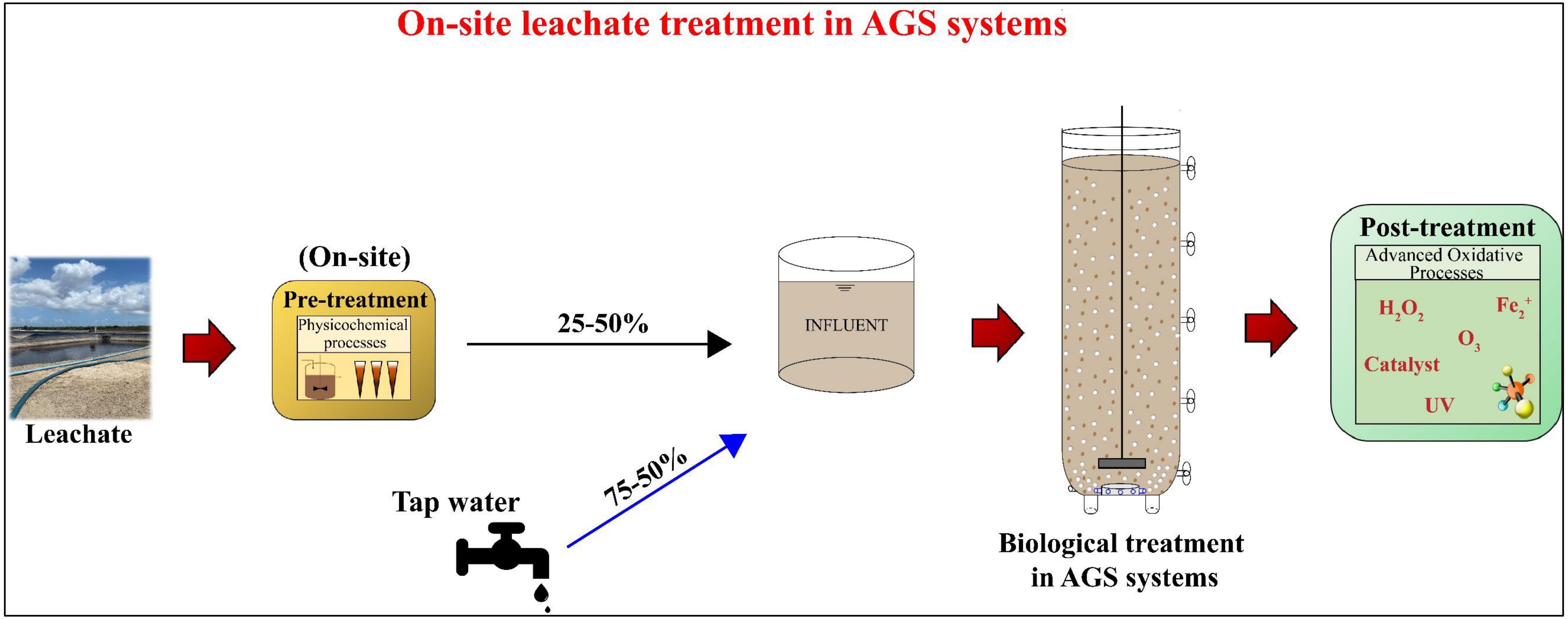
Therefore, in view of the results found and the discussions elucidated, leachate co-treatment in step-feed AGS systems at a 10% dilution is the best strategy, producing more resistant granules, with fast recovery capacity and that present better contaminant removals. As a form of biological treatment in the landfill itself, the AGSs systems can replace the traditional activated sludge systems, presenting several operational and performance advantages of the reactors. As an advantage, it is still possible to mention the smaller footprint and the lower financial costs.

Figure 43 – Graphical schematic representing the best settings found for leachate treatment.



Source: Prepared by the author.

Figure 44 – Proposition of multistage leachate treatment system with AGS technology.



Source: Prepared by the author.

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