

**AEROBIC GRANULATION PROCESS WITH
STABLY ENHANCED BIOLOGICAL PHOSPHORUS
REMOVAL UNDER APPLYING PHOSPHORUS
RICH-LIQUID EXTRACTION AT THE END OF
ANAEROBIC PHASE**

by

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ABSTRACT

As a newly established biological treatment technology, aerobic granulation offers notable advantages over conventional treatment processes, including superior settleability, higher removal under fluctuating organic loadings, reduced operational inputs, and minimized biosolids generation. Nevertheless, maintaining stable granule structure remains a major hurdle in granulation technology, with challenges arising from extended start-up periods, and the need for precise optimization of key operational factors such as influent characterises and distribution, cycle configuration, and feast/famine dynamics. Thus, the goal of these studies was to develop granular sequencing batch reactors suitable for achieving stabilized enhanced biological phosphorus removal via granular sludge cultivated previously under high organic loading during municipal wastewater treatment.

To achieve this goal, several specific objectives were addressed. Initially, stable mature aerobic granules were cultivated by treating high-strength, ammonium- and phosphorus-rich wastewater under limited dissolved oxygen, with granule stability, simultaneous nutrient-removal performance, and microbial community structure systematically assessed to guide subsequent optimization. Additionally, the feasibility of anaerobic step-feeding and reactor performance under elevated dissolved-oxygen optimization were evaluated to refine the operating strategy. Subsequently, the impact of imposing aerobic phosphorus starvation during municipal wastewater treatment, using granules pre-cultivated under high organic loading, was evaluated to stimulate PAO metabolism, accelerate aerobic phosphorus uptake, and deliver a more competitive, resilient enhanced biological phosphorus removal than conventional approaches.

Therefore, under extended operation, long-term simultaneous nutrient-removal performance, granule structural stability, and temporal shifts in microbial community composition were quantified to establish durability and mechanistic underpinnings of the proposed strategy. The synoptic results of this thesis showed that design and operational parameters for granular sequencing batch reactors can be effectively optimized through integrated strategies, including high-strength wastewater cultivation and aerobic phosphorus starvation, to achieve stably enhanced phosphorus removal during municipal wastewater treatment.

Contribution of Authors

The thesis is organized as a "sandwich" or grouped-manuscript thesis with chapters 3, 4, 5 and 6 containing material from journal articles that have been published and are awaiting publication. Following are the titles of the prepared research articles, the manuscript review status at the time of submission of this thesis, the authors' contributions, and relevant chapters of the thesis:

1. Chapter 3 includes the content published in the Processes journal manuscript titled “Feasibility study of applying anaerobic step-feeding mode for the treatment of high-strength wastewater in granular sequencing batch reactors (GSBRs)”.

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Author contributions:

Elsayed Mady (*Thesis author*): Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Methodology, Software, Writing original draft

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Dedication

Reaching this stage required hard work and perseverance.

*However, none of it would have been possible without the
unwavering love and support of my family. From the bottom of
my heart, thank you.*

This work is dedicated to:

My Mother, Amal Aly,

My Father, Mohamed Mady,

My Beloved Wife, Esraa Kamh,

My Sweet Sons, Marwan, Mohamed and Malek,

My Beloved Sisters, Asmaa and Aya,

And My Whole Family and Friends.

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NOTATIONS

AGS	Aerobic Granular Sludge
AOBs	Ammonia-Oxidizing Bacteria
Y_{obs}	Biomass synthesis yield
BNR	Biological Nutrient Removal
CAS	Conventional Activated Sludge
CFR	Continuous Flow Reactor
COD	Chemical Oxygen Demand
rbCOD	Readily Biodegradable Chemical Oxygen Demand
DO	Dissolved Oxygen
DPAOs	Denitrifying Polyphosphate-Accumulating Organism
EBPR	Enhanced Biological Phosphorus Removal
EPS	Extracellular Polymeric Substances
FA	Free Ammonia
F/M	Food to Microorganism Ratio
GAOs	Glycogen-Accumulating Organism
HRT	Hydraulic Retention Time
HNAD	Heterotrophic Nitrification-Aerobic Denitrification
HS	High-Strength Wastewater
LS	Low-Strength Wastewater
MBR	Membrane Bioreactor
MFC	Microbial Fuel Cell

MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
MS	Medium-Strength Wastewater
NOBs	Nitrite-Oxidizing Bacteria
OHO	Ordinary Heterotrophic Organisms
OLR	Organic Loading Rate
PAOs	Polyphosphate-Accumulating Organism
PHAs	Polyhydroxyalkanoates
SBR	Sequencing Batch Reactor
sCOD	Soluble Chemical Oxygen Demand
SND	Simultaneous Nitrification-Denitrification
SP	Soluble Phosphorus
SRT	Solids Retention Time
TAN	Total Ammoniacal Nitrogen
TN	Total Nitrogen
TP	Total Phosphorus
TSS	Total Suspended Solids
VSS	Volatile Suspended Solids
VER	Volume Exchange Ratio
WWTP	Wastewater Treatment Plant

CHAPTER 1: INTRODUCTION

1.1 Overview

Water is a critical resource underpinning human health, ecological integrity, and industrial development, serving essential roles in drinking, sanitation, and production processes. Its availability and quality directly influence the sustainability of ecosystems and socioeconomic progress. Recognizing its significance, the United Nations has declared access to safe and clean water a fundamental human right, formally enshrined in Sustainable Development Goal 6. This global mandate underscores the urgent need for advancing sustainable water management technologies, including innovative treatment approaches, to ensure long-term security of this finite resource.

Wastewater treatment plants (WWTPs) play a crucial role in managing ecosystem disturbance by effectively removing organic carbon, suspended solids, and nutrients (e.g., Nitrogen (N) and phosphorus (P)) from effluents before they are discharged into natural water bodies. Conventional biological processes, such as activated sludge (CAS), have been widely implemented in North America and Canada, providing a foundational approach to treating municipal wastewater. However, these systems often face significant challenges, including the need for improvements to meet increasingly stringent discharge limits, rising energy costs, substantial physical footprint, and high operational expenses (Adav et al., 2008 and Bengtsson et al., 2018). Consequently, there is a growing demand for upgrading these systems and innovative solutions that not only address these challenges but also enhance performance and sustainability as well as promote the recovery of valuable resources from wastewater.

Aerobic granular sludge (AGS) is an economically and environmentally promising replacement for CAS, for treatment of municipal and industrial wastewater, and its feasibility has been studied for implementation in the biological treatment process. AGS are compact biofilms formed by self-immobilized microbial cells during wastewater treatment in sequencing batch reactors (SBRs). These aerobic granules exhibit higher density and greater settling velocity, enabling them to separate more efficiently from treated effluent and improving overall process stability (Cassidy and Belia, 2005). These characteristics allow the wastewater treatment industry to minimize land requirements and capital investment, reducing construction costs for new treatment facilities by up to 75% (Winkler et al., 2012). By switching to aerobic granulation system, daily produced biomass will be decreased leading to reduction in the overall cost of up to 25% and energy requirement up to 30% (Sarma and Tay, 2018).

It is worth mentioning that AGS forms as compact microbial aggregates independent of support media, offering high biomass retention and stronger tolerance to shocks and toxicity than CAS (Liu et al., 2015). Aerobic granulation technology shows strong potential for the efficient simultaneous biological nutrient removal (BNR) across various wastewater types, such as low-strength municipal influents (Coma et al., 2012) and high-strength industrial wastewaters; such as, fish canning, textile, brewery, and landfill leachate (Figueroa et al., 2008, Muda et al., 2010, Corsino et al., 2017, Ren and Yuan, 2016). Nevertheless, the issue of granule disintegration remains a persistent and unresolved challenge in the application of AGS technology (Sarma et al., 2017).

Numerous studies have investigated AGS cultivation and stability in SBRs, identifying key operational and design parameters that govern the granulation process. These factors include the feast-famine conditions, anaerobic influent distribution, selective wasting (settling velocities as a selection pressure), superficial air velocities (i.e., hydrodynamic shear forces), influent characteristics, organic loading rates (OLRs), biomass characteristics, and production of extracellular polymeric substances (EPS), as well as other environmental conditions (e.g., DO, pH, and temperature) (Lashkarizadeh et al., 2016, Bassin et al., 2012, Isanta et al., 2015). Besides, studies suggest that granule stability and disintegration are influenced by factors such as the overgrowth of fast-growing or filamentous organisms (e.g., ordinary heterotrophic organisms) under high OLRs, anaerobic fermentation of lysed cells in matured granules, and deterioration in EPS composition, which is critical to AGS integrity (Liu and Tay, 2012, Lv et al., 2014, Long et al., 2015).

Enhanced biological P removal (EBPR) process offers a practical, cost-efficient, and eco-friendly alternative to traditional chemical methods for removing P in wastewater treatment. This process relies on polyphosphate-accumulating organisms (PAOs), which require cycling of anaerobic and aerobic conditions to promote their growth and perform luxury uptake by assimilating excess P under aerobic conditions, following its release during the preceding anaerobic phase (Cha et al., 2016). However, its performance is influenced by numerous factors, including temperature, pH, carbon source, C/P ratio, feeding strategy, sludge retention time, free ammonia concentration, and the presence of competing microorganisms (Song et al., 2009, Ersu et al., 2010, Bassin et al., 2012). AGS has shown significant potential for P removal, particularly through EBPR mechanisms,

where the recent studies demonstrate its effectiveness in achieving high P removal efficiencies were more than 90% (controlled conditions).

Recent research has concentrated on optimizing configurations for effective biological P removal, with the next critical step being the integration of P recovery measures (Chowdhury et al., 2017 and Wang et al., 2022). The increasing demand for P across different industries (e.g., fertilizers, textiles, rubber, leather, ceramics, and detergents) renders P recovery economically advantageous. Global mass balance analyses indicate that up to 20% of the world's P production, approximately 3 million tons yearly, is lost in anthropogenic waste (Cordell et al., 2009). Thus, efficient P removal is a win-win situation since recovering P from wastewater streams can decrease dependence on phosphate rock to fulfil P demand. In recent years, various alternative configurations and strategies have been developed for EBPR processes, aiming to improve P recovery from EBPR sludge by facilitating the precipitation of anaerobically released P as struvite, typically through pH adjustment using chemical additives (Cieslik and Konieczka, 2017). Baeza et al. (2017) designed the enhanced biological P removal and recovery (EBPR²) system, an innovative SBR cycle configuration that integrates an additional settling phase following the anaerobic mixing stage, establishing an anaerobic/settling/aerobic/settling sequence to facilitate P recovery. By extracting 4% of the anaerobic supernatant (i.e., P-rich liquid) for chemical precipitation, simulations showed that over 60% of influent P could be recovered while adhering to regulatory limits for organic matter, P, and N. EBPR² also demonstrated improved efficiency, operating with lower COD demands than conventional EBPR-based SBRs.

1.2 Need for Research

Although the various design and operational parameters that influence the aerobic granulation process, operational strategies must be implemented to mitigate common challenges reported in the literature, including biomass instability, prolonged start-up times with municipal wastewater treatment, nitrite accumulation, and inconsistent P removal performance. Among operational factors, feeding mode is key to determining the selection, development, and stability of aerobic granules (de Kreuk et al., 2005 and Hamza et al., 2018). Granular sequencing batch reactors (AGS-SBRs) are generally classified into two types: continuously aerated systems and those employing anaerobic influent distribution. Anaerobic filling in AGS-SBRs is widely recognized, with influent load and filling duration being critical determinants of overall process efficiency. Yet, the application of step-feeding in AGS systems has been minimally studied to date. Thus, the first part of this thesis focused on providing a fundamental understanding of the aerobic granules' stability (physical properties) and performance (simultaneous BNR) under applying anaerobic step-feeding strategy in the purpose of enhancing nitrate reduction performance.

The optimal OLR for AGS cultivation is strongly influenced by the carbon source, as substrate type directly governs granule structure and stability. Microbial metabolism depends on carbon as the primary electron donor, while nitrogen is essential for protein biosynthesis in microbial cells. Furthermore, influent characteristics and composition (i.e., COD/total nitrogen/total phosphorus) significantly affect the morphology, stability, and performance of AGS. Although aerobic granulation has been widely demonstrated under varying influent types and OLR conditions, research on AGS technology though long-term treatment of high-strength wastewater accompanied with elevated N and P loadings

remains limited. Additionally, the feasibility of increasing the actual DO levels that applied under such operating conditions remains underexplored. Therefore, the second part of this thesis was to addressing these gaps, where it investigated the aerobic granulation process using test SBRs seeded with flocculant sludge and treated high-strength synthetic influent (TCOD = 1400 mg/L) at DO content started with 1.5 mg/L and increased over the time and reached up to 8.5 mg/L through long-term operation extended for 18 months.

EBPR is the most widely applied and cost-effective method for P removal during the municipal wastewater treatment process (Bertanza et al., 2013). However, significant challenges affecting its performance, particularly when treating weak wastewater (low VFAs content), or due to the fluctuations in the microbial activity that causing maintaining consistently low P concentrations in the treated effluent remains highly challenging. Several studies have proposed modifications to EBPR processes, primarily aimed at enhancing P recovery through different bioreactor configurations (Wang et al., 2022, Baeza et al., 2017, Valverde-Pérez et al., 2015, Zou and Wang, 2016). In particular, model-based studies have suggested extracting part of the supernatant at the end of the anaerobic phase under no-mixing conditions, generating a P-rich liquid suitable for recovery processes. To date, few studies have examined the behavior of polyphosphate-accumulating organisms (PAOs) under aerobic P starvation conditions induced by anaerobic P-rich liquid extraction. Moreover, the long-term application of this extraction strategy in AGS-based systems treating municipal wastewater has not yet been explored. Therefore, the third part of this thesis addresses this knowledge gap by investigating the impact of aerobic P starvation on EBPR-SBRs using aerobic granules cultivated and matured under high organic loading and subsequently applied to low-strength influent. The results obtained

from this part emphasized understanding the changes in granule characteristics and performance under these operating conditions, highlighting aerobic P starvation as a sustainable long-term strategy to enhance P removal and ensure consistently low effluent concentrations.

Previous studies have emphasized that careful attention must be paid when applying P-rich liquid extraction in EBPR systems, to minimize the risk of sustained stress on PAOs bioactivity resulted from the aerobic P starvation mode (Bertanza et al., 2013 and Wang et al., 2022). Excessive application of aerobic P starvation may severely reduce and deplete intracellular P reserves due to limited aerobic P uptake. Such conditions can also compromise AGS stability, leading to gradual structural deterioration and potential disintegration over time. Despite these risks, this area remains largely underexplored, highlighting the need for further systematic investigation. Hence, in AGS-SBR systems coupled with P-rich liquid extraction, the daily extraction volume must be carefully optimized to balance P recovery with biological removal while ensuring long-term granule stability (Guisasola et al., 2019). Thus, the final part of this thesis was to experimentally evaluated two operational strategies, intermittent and continuous P-rich liquid extraction, to assess their long-term impacts on P removal efficiency and the physical stability of AGS during low-strength wastewater treatment.

1.3 Objectives of Research

To address the identified research gaps, the overarching theme of this doctoral study is to advance the application of AGS technology for stable EBPR. The specific objectives of this doctoral research are to:

- Explore the feasibility of applying an anaerobic step-feeding strategy for enhancing nitrate reduction in AGS-SBRs operated with cultivated aerobic granules treating high-strength wastewater containing elevated N and P loadings.
- Assess the AGS physical properties and evaluate its simultaneous BNR performance during long-term treatment of high-strength wastewater with elevated N and P loadings under limited DO (<1.5 mg/L), and investigate bacterial community profiling to elucidate microbial contributions to system performance, after that and under the same influent characteristics examine the effects of gradually increasing DO levels (up to 8.5 mg/L) on AGS stability and nutrient removal efficiencies.
- Investigate the impact of applying the aerobic P starvation strategy, induced by intermittent anaerobic P-rich liquid extraction, during long-term treatment of municipal wastewater in SBRs seeded with matured AGS (cultivated previously under high organic loading), on:
 - the overall stability of EBPR performance, PAOs metabolism, and the consistency of P content in the treated effluent,
 - long-term granules stability, EPS secretion, and the potential for granulation continuity and/or regrowth under these operating conditions,
 - P recovery potential from extracted P-rich liquids,
 - Microbial community dynamics and functional potential of AGS.
- Experimentally evaluate intermittent and continuous P-rich liquid extraction strategies, assessing their long-term effects on EBPR performance, AGS stability, and the bacterial community structure during low-strength wastewater treatment.

1.4 Original Contributions

This doctoral research makes several original contributions for the field of biological wastewater treatment and introduces extensive data on the application of AGS technology for enhanced simultaneous BNR, particularly under challenging operational conditions. The contributions extend beyond reactor-scale performance to include microbial ecology, process optimization, and resource recovery, thereby offering both scientific insights and practical implications for sustainable wastewater management:

- Providing new understanding of AGS formation and stability when exposed to wastewater with exceptionally high nutrients loadings. Unlike most previous studies limited to low-strength influents, this work demonstrated how flocculant sludge can be successfully transformed into stable granules under long-term stress conditions. Additionally, this study highlighted that step-feeding strategy must be applied with caution, since altering the feast/famine balance can compromise granule stability.
- Examining the impact of influent characteristics on aerobic granulation has provided critical insights into AGS behavior regarding BNR and granule stability. The findings enhance understanding of AGS application in treating nutrient-rich industrial and agricultural wastewaters, where CAS often fails. The research also revealed how gradually increasing DO levels affects simultaneous nutrient removal and granule stability. Oxygen was shown to be a strategic factor influencing EPS secretion, and granule density. These insights contribute to the broader goal of improving process reliability in full-scale treatment plants.
- Developing one of the first long-term demonstrations of applying intermittent anaerobic P-rich liquid extraction to promote aerobic P starvation in AGS systems

treating low-strength wastewater to enhance PAOs metabolism and accelerate P uptake behavior. Moreover, the effects of aerobic P starvation, implemented through the proposed operational strategy, on EPS secretion, granule stability, and temporal shifts in microbial community composition were systematically examined. In parallel, the resulting P-rich side streams were evaluated for their P recovery potential, offering a sustainable pathway (circular economy objectives).

- Conducted a comprehensive comparative analysis of the proposed aerobic P starvation strategy by applying both intermittent and continuous P-rich liquid extraction approaches. The study elucidated their long-term impacts on P removal efficiency, AGS structural integrity, and microbial community dynamics. By highlighting the operational trade-offs between these strategies, this research offers practical guidance for optimizing EBPR systems integrated with this approach, paving the way for future full-scale applications.

1.5 Structure of the Thesis

This thesis consists of seven chapters, as described below:

- **Chapter One** contains the introduction, problem definition/research significance, objectives, and contributions of the proposed research.
- **Chapter Two** provides a comprehensive literature review on aerobic granulation technology, covering fundamental theories of granule formation, key parameters for cultivation and maintenance, pollutant removal mechanisms, current and emerging applications, integration with advanced processes, and future challenges and prospects.

- **Chapter Three** presents the experimental study on AGS cultivation under high-strength wastewater with elevated ammonium and P loadings. It further evaluates the feasibility of applying an anaerobic step-feeding mode in granular sequencing batch reactors, focusing on system performance, granule stability, and nutrient removal efficiency, particularly its feasibility on enhancing nitrite/nitrate reduction.
- **Chapter Four** explores the maturation of AGS under the long-term treatment of high-strength influents with aforementioned characteristics (high ammonium and P loadings), focusing on simultaneous BNR using matured granules. It further analyzes the microbial community composition of matured AGS and evaluates reactor performance following operational optimization through enhanced DO levels.
- **Chapter Five** presents the results and discussion on the influence of aerobic P starvation in achieving stable EBPR performance using AGS cultivated under high organic loads during municipal wastewater treatment. It further evaluates simultaneous BNR performance, granule stability, and the dynamics of microbial community composition.
- **Chapter Six** describes the test results and discussion of applying aerobic P starvation strategy through two approached like intermittent and continuous P-rich liquid extractions at the end of anaerobic mixing stage during the treatment of low-strength wastewater.
- **Chapter Seven** provides a summary of the research findings, concluding remarks based on the testing and modeling results, and recommendations for future research.

CHAPTER 2: LITERATURE REVIEW

Granular sludge was first reported in 1980 in anaerobic sewage treatment systems (Lettinga et al., 1980), and nearly two decades later its presence under aerobic conditions was identified in a bubble-column lab-scale reactor (Morgenroth et al., 1997). Since then, AGS has attracted growing scientific and practical interest. Over the past two decades, research has demonstrated the versatility of AGS, not only in treating domestic and high-strength wastewaters but also in addressing broader environmental challenges. These include the bioremediation and biotransformation of toxic aromatic pollutants such as phenol, toluene, and pyridine, the treatment of industrial effluents from sectors like textiles, dairy, and brewing, the adsorption of heavy metals, and even the recovery of high value-added products. Liu and Tay (2004) described aerobic granules as densely packed microbial aggregates that can contain millions of microorganisms per gram of biomass. Within these aggregates, different bacterial groups perform specialized roles in degrading pollutants in wastewater, all without the need for support materials. AGS are characterized by their compact structure, excellent settling properties, and high biomass retention, which enable them to operate effectively under elevated organic loading rates (Zheng et al., 2006). AGS technology offers significant operational advantages, including 25% reduction in operating costs, 40% lower electricity consumption, and 75% decrease in space requirements (Adav et al., 2008, Bengtsson et al., 2019, Pronk et al., 2015). The comparison among AGS, anaerobic granules and CAS is shown in **Table 2.1**. When assessed against other compact treatment processes such as membrane bioreactors (MBRs), AGS shows an estimated 35–70% lower electricity demand (Bengtsson et al., 2019).

Within a single granule, oxygen penetration creates distinct layers that can range from aerobic to anoxic or even anaerobic zones, supporting the coexistence of diverse microbial populations. Because of this stratification, AGS has often been described as a “mini ecosystem” (Liu and Tay, 2004). The formation and stability of AGS are influenced by several operational factors (substrate composition, OLR, hydrodynamic shear forces, feast–famine conditions, feeding strategy), which will be discussed in details in the following sections. Although these parameters affect granule characteristics, only those that impose selective pressure on sludge particles appear to directly drive granule formation (Liu et al., 2005). Importantly, some studies have shown that while granulation can occur, long-term stability is not always guaranteed.

Table 2.1: Comparison of characteristics between CAS, anaerobic granular sludge and AGS (Bengtsson et al., 2019, Nancharaiah and Reddy, 2018, Rico et al., 2017).

Parameters	CAS	Anaerobic Granules	AGS
SHAPE	Irregular (filamentous),	Usually have spherical shapes	Usually have spherical shapes
Settling Velocity (m/h)	2 – 10	< 20	10 – 90
Size (mm)	< 0.2	0.2 – 2.0	0.2 – 5.0
Specific Gravity	0.997–1.01	1.10–1.15	1.010–1.017
Degree of compactness and density	Low	High	High
Layers	Aerobic	Facultative and Obligate Anaerobic	Aerobic, Anoxic, Anaerobic
Sludge Volume Index (SVI)	$SVI_5 \neq SVI_{30}$ (Higher Values)	$SVI_{30}/SVI_5 \geq 0.85$ (Moderate Values)	$SVI_5 = SVI_{30}$ (Lower Values)
Secretion of Extracellular Polymeric Substances	Low secretions	Middle secretions	High Secretions
Tolerance to toxic compounds	Lower tolerance	Lower tolerance	Higher Tolerance
Removal ability under shock and fluctuating organic loadings	Lower efficiency (poor removal)	Moderate efficiency (Removal maintain at stable level)	Higher efficiency (Removal maintain at stable level)

2.1. Theories of Granule Formation in Aerobic Granular Sludge

The transformation of flocculent sludge into compact, self-immobilized aerobic granular sludge is generally attributed to a combination of selective pressures and operational conditions (Hamza et al., 2022). However, the underlying mechanisms of AGS granulation remain only partially understood, as the process is governed by a complex interplay of physicochemical and microbial factors (Wilén et al., 2018). Despite extensive research, no consensus has been reached regarding a unified mechanism for granule formation. Several theoretical frameworks have been proposed to describe AGS granulation dynamics, notably the EPS hypothesis, the microbial self-aggregation (autocondensation) hypothesis, and the four-stage model introduced by Liu and Tay (2002) (as will be discussed below).

2.1.1. EPS Adhesion Theory

EPS is a structurally and functionally complex matrix primarily composed of polysaccharides (PS), proteins (PN), phospholipids, nucleic acids, and humic substances. Its production is influenced by multiple factors, including microbial secretion, cell lysis, and shedding, as well as environmental conditions such as nutrient availability and operational parameters. According to the EPS-based granulation hypothesis, EPS contributes significantly to microbial aggregation by neutralizing surface charges and reducing electrostatic repulsion between cells (Liu et al., 2004). EPS facilitates microbial clustering through adsorption and bridging, supported by various physical and chemical interactions, including hydrogen bonding, ionic bridges, and van der Waals forces (Basuvaraj et al., 2015). These interactions play a critical role in initiating and maintaining

the structural integrity and stability of granules during microbial proliferation. Operational strategies such as enhancing hydraulic shear stress, reducing settling time, and supplementing with divalent cations like Ca^{2+} and Mg^{2+} have been shown to stimulate EPS secretion and consequently promote AGS formation (Guo et al., 2022). A positive correlation between EPS production and sludge granulation has been reported by Liu et al. (2022), highlighting EPS as a key determinant in granule development.

2.1.2. Filamentous Bacteria Hypothesis

During start-up, the biomass was dominated by filamentous bacterial aggregates (pellets) that formed an initial scaffold. With continued hydrodynamic shear, surface filaments detached and the aggregates compacted. Subsequent internal lysis generated voids that enabled microcolony development; once dense and sufficiently large, these microcolonies settled and gradually matured into compact granules. This transformation suggests that the initial granulation process, mediated by filament-forming bacteria, is primarily governed by steric hindrance and mechanical entanglement, which contribute to forming a porous, high-surface-area network. Likely contributors include Actinobacterial filaments (e.g., *Microthrix parvicella*), Proteobacterial filaments (e.g., *Thiothrix*, *Pseudoxanthomonas*, *Flavobacterium*, *Acinetobacter*, *Luteimonas*), and Bacteroidetal filaments (e.g., *Haliscomenobacter*). Their long filaments provide steric bridging/entanglement, and EPS production cements particles, enhance capture of colloids—together supporting initial granule scaffolding. Such a structure is favorable for capturing suspended microorganisms and organic particles, promoting the formation of dense, well-settling granules (Liu and Tay, 2004 and Sharaf et al., 2019). Filamentous

bacteria also play a crucial scaffolding role by providing attachment sites for microbial colonization, growth, and reproduction. Supporting this hypothesis, Wu et al. (2018) observed that filamentous bacteria acted as structural backbones for granule morphology in MBR systems with internal circulation. However, it is important to note the dual role of filamentous bacteria; while limited filamentous growth supports granule formation and stability, excessive proliferation can destabilize the granular matrix, leading to structural weakening and disintegration. Moreover, in some systems, the scarcity of filamentous bacteria or their skeletal analogs can limit the applicability of this hypothesis, highlighting its system-specific relevance.

2.1.3. Four Stage Theory of Sludge Granulation

Liu and Tay's four-stage concept, as shown in **Figures 2.1** and **2.2**, can be refined to reflect current understanding that microbial aggregation can arise through multiple, context-dependent mechanisms (Liu and Tay, 2002 and Liu et al., 2023). Stage 1 (encounter and proximity): Cells are brought into contact by mixing, diffusion, and, in polymer-rich or viscous microenvironments, by physical confinement that limits motility (Staudinger et al., 2014 and Pabst et al., 2016). Stage 2 (reversible aggregation): Initial cell–cell joining can be mediated by surface appendages (e.g., fimbriae/pili) and adhesins, enabling both intra- and interspecific co-aggregation; however, in polymer-rich environments, reversible aggregation may also occur via depletion-type physicochemical forces that do not require classical biofilm functions (Secor et al., 2018). Stage 3 (matrix-mediated stabilization and maturation): Reversible clusters become stabilized through “bridging” interactions and matrix accumulation, where extracellular polymers and eDNA

contribute to cohesion and the recruitment/retention of additional cells, converting transient contacts into more persistent aggregates with emergent diffusion gradients (Borlee et al., 2010, Rybtke et al., 2015, Kragh et al., 2018). Stage 4 (selection and compaction): Hydrodynamic shear and short settling preferentially retain denser, fast-settling clusters, which further compact and differentiate into integrated aerobic granules (Liu and Tay, 2002 and Liu et al., 2023).

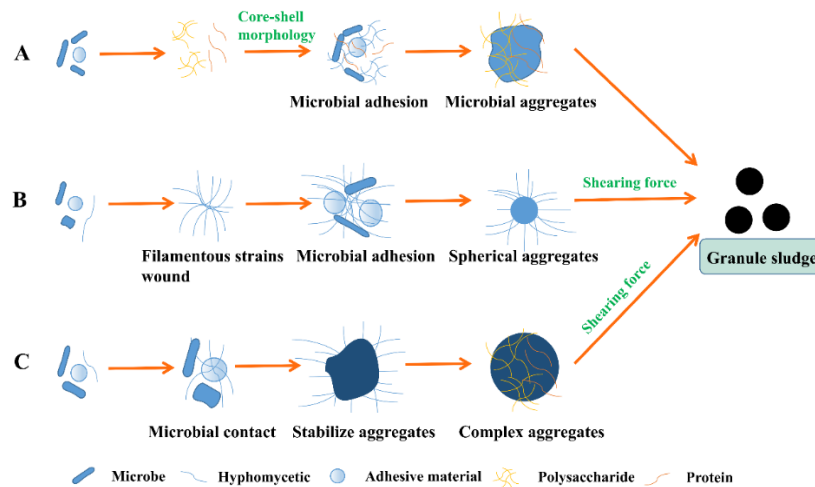


Figure 2.1. Schematic diagram of the theory of four-stage model shows the diverse pathways and mechanisms on AGS formation (source: Liu et al., 2023)

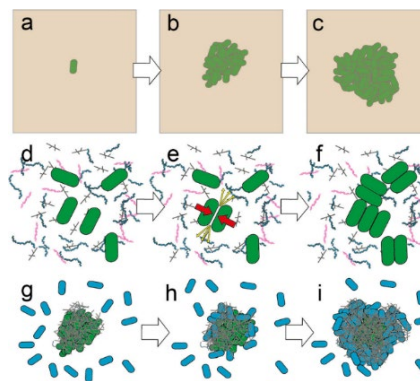


Figure 2.2. Mechanistic schematic outlining three competing hypotheses for aggregate formation (source: adapted from Kragh et al., 2023):

a–c Aggregate formation due to immobilization in viscous materials: (a) Single cell are immobilized in a viscous gel, (b) Over time the single cell proliferates to an immobilized microaggregate, (c) As the aggregate attains a certain size, its expansion becomes limited due to space restriction and/or resource stratification. d–f Depletion aggregation in polymer-rich environments: (d) Single bacterial cells found in polymer rich environment, (e) Cells in proximity of each other will attract due to entropy generated by osmotic imbalance (red arrows) as polymers move out from between the cells (blue arrows), (f) Depletion aggregation creates the distinct fence pattern aggregate formation. g–i Bridging aggregation: (g) Preformed aggregates (green cells) are in a biofilm state where they produce extracellular polymers, (h) Single cells (or other aggregates) are recruited to the aggregate, facilitated by the sticky polymers around the aggregates, (i) Over time the aggregates increase in size through growth, continuous recruitment, and incorporation of single cells and/or other aggregates.

2.1.4. Cell-surface appendages (pili/fimbriae) and aggregation

Beyond bulk EPS metrics, cell-surface appendages (pili and fimbriae) mediate the first stable contacts that nucleate aggregates. These nanofibers enable auto-aggregation (within species) and co-aggregation (between species) through specific adhesin–receptor interactions, thereby bridging cells and promoting micro-aggregate formation. Once initial attachments are established, EPS and extracellular DNA (eDNA) consolidate the contacts, transitioning aggregates from reversible to irreversible adhesion. In AGS, hydrodynamic selection (feast–famine, shear, short settling) then compacts and retains these pili-initiated clusters, while ongoing pili/fimbriae activity contributes to aggregate maintenance, surface

re-attachment, and resilience under shear. Therefore, pili/fimbriae and the matrix (EPS/eDNA) act synergistically: appendages start the ‘glue,’ the matrix locks it in (Nwoko and Okeke, 2021).

2.2. Physicochemical Properties of Aerobic Granular Sludge

2.2.1. Physical Characteristics

The compact and densely aggregated structure of AGS underpins many of its advantages over conventional activated sludge systems. AGS typically exhibits diameters ranging from 0.2 to 5 mm, with granules up to 23 mm observed under laboratory-scale sequencing batch reactor (SBR) conditions (Tay et al., 2001a,b, Zhu and Wilderer, 2003, Torregrossa et al., 2007). Toh et al. (2003) proposed that the observed increase in granule diameter may be attributed more to elevated inorganic content rather than a true increase in organic biomass or EPS accumulation. They further emphasized that granule growth beyond a critical size, approximately 4.0 mm in diameter, could negatively affect the mechanical strength and structural integrity of the granules. Excessive size may lead to internal diffusion limitations, reduced settling efficiency, and higher susceptibility to fragmentation. Based on their findings, optimal granule diameters in the range of 1.0–3.0 mm were recommended to ensure both process stability and cost-effective operation of aerobic granular sludge systems. This relatively large size, coupled with the granules' high density, contributes to their superior settling characteristics, often reflected in low sludge volume index (SVI) values between 20 and 80 mL/g (Su et al., 2012). Reported specific gravity values for AGS range from 1.004 to 1.1, significantly higher than those of conventional flocs, which generally fall between 1.002 and 1.006 (Su et al., 2012 and Li et al., 2009). These physical attributes not only facilitate rapid solid-liquid separation but also

enhance reactor performance and operational stability. AGS exhibits significantly higher specific gravity and settling velocities compared to CAS. Unlike the slow-settling, loosely structured flocs in CAS systems, AGS particles settle rapidly as discrete, dense granules. This fundamental difference necessitates a rethinking of traditional clarification strategies, as the physical behavior of AGS diverges markedly from the assumptions underlying conventional sludge separation processes.

Granule density plays a key role in determining structural integrity and settling behavior in AGS systems. Studies conducted in continuous flow reactors (CFRS) have reported aerobic granule densities ranging from 1052 to 1059 kg/m³ (Li et al., 2016), values slightly exceeding that of water. Notably, Li et al. (2016c) observed a significant decline in the density of large phosphorus (P)-removal granules (diameter > 0.6 mm) during the transition from SBR to CFRS, although the final density closely matched that of the seeded granules. These findings suggest that granule density can fluctuate during acclimation, with denser granules generally exhibiting enhanced mechanical stability and resistance to shear-induced disintegration (Corsino et al., 2016).

Although aerobic granules cultivated in both SBRs and CFRs exhibit similar structural integrity, a growing body of evidence suggests that granules formed under continuous operation possess enhanced mechanical strength. This trend is consistently observed in ultrasonic disruption tests, where granules developed in CFRs demonstrate prolonged resistance to disintegration compared to those from SBRs (Lee and Chen, 2015, Wan et al., 2014a,b). For instance, Wan et al. (2014c) reported that saline-preserved seed granules disintegrated after 15 min of ultrasonic treatment, while granules from SBRs and

CFRs endured for 20 and 25 min, respectively. Such findings indicate that continuous flow conditions may favor the development of more robust granular architecture. However, the influence of operational mode is not absolute. Li et al. (2016) observed comparable strength in phosphorus-removal granules cultivated under batch and continuous regimes, suggesting that factors beyond reactor configuration (e.g., microbial composition, EPS matrix quality, and operational shear) play critical roles in determining granule durability (Li et al., 2016). Therefore, while CFRs may enhance granule strength, a multifactorial approach is essential to fully understand and optimize granule stability in AGS systems.

2.2.2. Chemical Characteristics

2.2.2.1. Selective EPS secretion

Extracellular polymeric substances, microbiologically derived polymers essential for microbial aggregation, play a pivotal role in the formation and long-term structural stability of AGS. EPS serves as the foundational matrix that anchors individual cells and imparts mechanical integrity to biofilms and granules. Structurally, EPS exists in two main forms: cell-bound EPS, which remains attached to microbial surfaces, and soluble or free EPS, which is secreted into the surrounding medium. The functional versatility of EPS extends beyond structural support. It acts as a protective barrier, buffering microbial cells against environmental stressors, and serves as a reservoir of carbon and energy during periods of substrate limitation (Wang et al., 2006). Within AGS, gel-forming exopolysaccharides, have been identified as dominant structural components, contributing to the compact, gel-like architecture of mature granules (Seviour et al., 2012). These EPS components enhance microbial adhesion, reduce water loss from flocs, and facilitate

microbial self-immobilization, all of which are critical for granule development and resilience under fluctuating environmental and operational conditions. The widespread ability of microbes to secrete EPS underlines its evolutionary importance in promoting survival and enabling the formation of robust microbial aggregates.

Under conventional cultivation conditions, EPS production by microbial communities remains relatively low. However, elevated EPS synthesis is typically triggered in response to environmental stressors, which can be broadly categorized into two main mechanisms (Liu et al., 2004). First, shifts in environmental conditions may alter the microbial community composition, thereby increasing the prevalence of EPS-producing species. Second, these changes can affect the metabolic activity of existing microorganisms, inducing EPS production even without a shift in community structure. Operational strategies known to stimulate EPS secretion include the implementation of feast/famine regimes, commonly achieved using SBRs, and the application of selective pressures such as high hydrodynamic shear forces and short settling times (Lin et al., 2008). These conditions favor the enrichment of microorganisms with strong aggregation potential, promoting granule formation and structural stability in aerobic granular sludge systems.

The production, quantity, and biochemical composition of EPS in AGS are highly dependent on the microbial community structure, physiological state, and prevailing operational and environmental conditions, including feed characteristics (Tay et al., 2001). Several studies have confirmed that bacterial cells within granules are enveloped by an EPS matrix, which functions as a scaffold for microbial aggregation and structural

cohesion. Furthermore, EPS composition appears to be closely linked to cell surface properties. In particular, hydrophobic interactions, mediated by specific fibrillar appendages and surface-associated proteins, have been implicated in facilitating microbial aggregation and EPS enrichment (Singleton et al., 2001). This suggests a plausible correlation between increased cell surface hydrophobicity and enhanced EPS accumulation during granulation, reinforcing the importance of microbial surface characteristics in the granulation process. EPS in AGS are composed of a diverse array of macromolecules, primarily including polysaccharides, proteins, lipids, and nucleic, humic, and uronic acids (Adav et al., 2008). In addition to these polymers, EPS matrices also incorporate non-polymeric substituents such as acetyl, succinyl, pyruvyl, and various inorganic groups. Among these components, only polysaccharides are synthesized extracellularly, whereas proteins, lipids, and nucleic acids are produced intracellularly and subsequently excreted (Mahmoud et al., 2003). Proteins in the EPS can form complex structures through associations with lipids (lipoproteins) or covalent bonding with carbohydrates (glycoproteins), contributing to the structural and functional heterogeneity of the matrix (Czaczyk and Myszka, 2007). This heterogeneity gives rise to both hydrophilic and hydrophobic domains within the EPS, and the overall hydrophobicity of the matrix is influenced by the relative abundance of its components, particularly the ratio of proteins to polysaccharides (PN/PS). Such physicochemical characteristics of EPS play a crucial role in cell aggregation, granule cohesion, and microbial adhesion to surfaces.

Empirical evidence suggests that a decline in the PN/PS ratio within the EPS matrix is commonly associated with deteriorating granule structure (Zhang et al., 2011 and Corsino et al., 2015). This is likely due to the inherently lower resistance of polysaccharides

to biological and chemical degradation, given their relatively simple molecular configuration. As such, AGS with a low PN/PS ratio tend to exhibit weaker matrix integrity and reduced mechanical resilience. The composition of EPS has been shown to strongly influence the physicochemical characteristics of granular sludge. In an anaerobic sulfidogenic reactor, the PN/PS ratio exhibited a positive correlation with the ratio of loosely bound EPS to tightly bound EPS, suggesting a structural link between EPS stratification and biochemical makeup (Wang et al., 2017). A higher relative content of proteins, particularly in the loosely bound EPS fraction, was associated with increased sludge hydrophobicity, an indicator of enhanced microbial aggregation. Proteins are generally considered more influential than polysaccharides in establishing electrostatic interactions with divalent cations, owing to their abundance of negatively charged amino acid residues and their typically higher proportion in the EPS matrix. However, this shift in EPS composition was also accompanied by a reduction in mean particle size, implying that excessive protein accumulation may hinder granule enlargement. Unlike hydrophilic polysaccharides, proteins tend to exhibit hydrophobic characteristics, which significantly influence microbial aggregation and granule structure. Overall, increased PN levels within the EPS matrix appear to promote microbial adhesion and compact granule formation, likely due to the denser and more cohesive extracellular network.

AGS exhibits a highly porous structure, which facilitates effective mass transfer across the microorganism–EPS matrix. This porosity supports the establishment of internal concentration gradients, leading to the spatial stratification of aerobic, anoxic, and anaerobic zones within the granule. Consequently, functional microbial populations are distributed along these zones, enabling simultaneous nutrient removal through multiple

biological pathways. However, this advantageous structure can be compromised by operational or environmental factors. For instance, the excessive growth of filamentous bacteria on the granule surface, especially under aerobic conditions with abundant readily biodegradable substrates, can hinder oxygen and substrate diffusion into the granule core (Devlin et al., 2017). Similarly, accumulation of particulate organic matter on the granule surface may cause persistent substrate enrichment in outer layers, disrupting the internal redox gradient and promoting surface fouling (Wagner et al., 2015). Additionally, excessive EPS production in overloaded reactors can obstruct internal pore networks, limiting mass transfer and compromising granule stability (Corsino et al., 2016). These disruptions to porosity and internal transport mechanisms adversely affect AGS performance, as the accessibility of specialized microbial communities to essential substrates becomes increasingly restricted.

2.2.2.2. Cell surface hydrophobicity

Hydrophobicity and surface charge are fundamental physicochemical parameters influencing the structural integrity and formation dynamics of AGS. Throughout the granulation process, biomass evolution is typically accompanied by an increase in surface hydrophobicity, which enhances microbial aggregation through thermodynamically favorable interactions (Isanta et al., 2012). Specifically, the reduction in Gibbs surface-free energy associated with higher cell hydrophobicity facilitates spontaneous self-immobilization of microbial cells into compact granules (Liu and Tay, 2004). Such aggregation is not merely a passive consequence but is actively mediated by shifts in cell surface chemistry and EPS composition (Gao et al., 2011). Notably, an elevated PN/PS ratio within the EPS matrix has been associated with enhanced granule hydrophobicity,

which in turn strengthens cell-to-cell interactions (He et al., 2017). Conversely, increased polysaccharide content may attenuate surface charge negativity, thereby reducing electrostatic repulsion among bacterial cells and further promoting granulation. These findings underscore the complex and interdependent roles of EPS composition and surface energetics in the physicochemical maturation of AGS.

Granule formation in AGS systems is largely influenced by microbial aggregation processes similar to those observed in conventional activated sludge, but with additional complexities. Wan et al. (2015) highlighted the role of negatively charged bacteria in proximity to calcium phosphate precipitates, which stimulate exopolysaccharide production and promote aggregation. These interactions are governed not only by classical Derjaguin–Landau–Verwey–Overbeek (DLVO) theory but also by factors such as cation bridging, hydrophobic interactions, microbial surface charge, and surface tension in the aqueous phase (Lettinga et al., 1980 and Morgenroth et al., 1997). Substrate type also plays a critical role in determining granule structure. Muda et al. in 2011 found that complex carbohydrates promote stratified, layered granule morphologies due to multi-step microbial degradation, whereas simpler substrates like formate and acetate are associated with more uniform granule microstructures (Muda et al., 2011). Enhanced EPS production, often triggered by substrate complexity, has been linked to improved aggregation and long-term granule stability.

2.2.2.3. Redox gradient support

The layered structure of AGS allows chemical stratification (aerobic outer, anoxic middle, anaerobic core), enabling simultaneous biological nutrients removal (BNR). These

gradients are maintained by diffusional limitations and the chemical reactivity of the EPS and biomass. Cell-to-cell interactions are recognized as fundamental drivers in the structural development and functional organization of AGS. As illustrated in **Figure 2.3**, AGS exhibits a stratified architecture comprising distinct redox zones, each contributing to its overall treatment capacity. The outermost aerobic layer supports microbial populations responsible for the oxidation of organic matter and ammonia via aerobic respiration, with oxygen acting as the terminal electron acceptor (He et al., 2017). Beneath this lies the anoxic zone, characterized by limited oxygen diffusion. This microenvironment plays a pivotal role in facilitating denitrification and biological phosphorus removal, processes dependent on alternate electron acceptors such as nitrate and nitrite. At the granule core, oxygen and external electron acceptors are absent, but microbial activity persists via fermentation. Core fermenters hydrolyze and ferment particulate/soluble organics and endogenous biomass, producing VFAs, H_2 , and CO_2 , which diffuse outward to fuel PAO/DPAO metabolism and denitrification in the surrounding anoxic shell. Consequently, the granule exhibits steep redox gradients, from an aerobic exterior (oxidation, nitrification) to anoxic zones (denitrification) and an anaerobic core (fermentation/syntrophy), enabling spatially segregated pathways that together enhance simultaneous BNR removal. This multi-zonal structure results in steep gradients of electron donors and acceptors across the granule, enabling the spatial segregation of distinct metabolic pathways within a single microbial aggregate (Nancharaiah and Reddy, 2018). Such compartmentalization enhances process efficiency and supports simultaneous BNR.

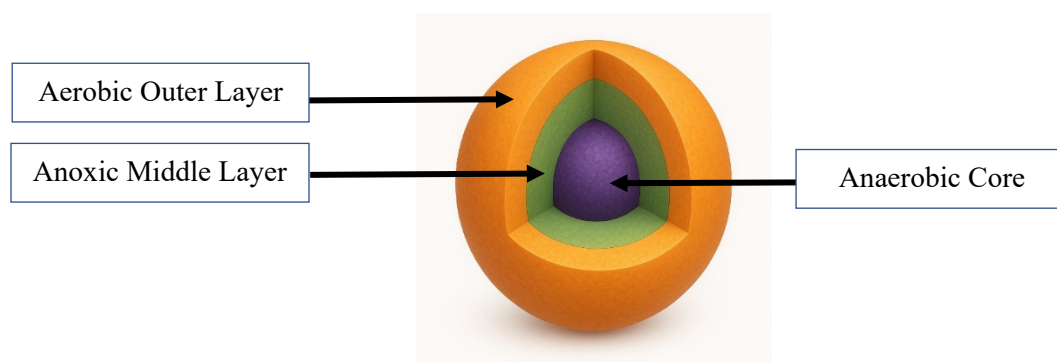


Figure 2.3: Schematic diagram of multilayered structure of AGS

2.2.2.4. Inorganic content

The presence of calcium-based precipitates, particularly calcium carbonates and calcium phosphates, has been widely documented within the cores of AGS. These precipitates are especially prominent in granules fed with acetate and are typically observed in granules ≥ 1.0 mm in diameter (Ren et al., 2008). Several studies have demonstrated that granules containing calcium precipitates tend to exhibit enhanced structural strength and larger size compared to those lacking calcium accumulation (Ren et al., 2008, Qin et al., 2004a,b). Notably, calcium appears to be the dominant metal component in AGS, with concentrations of other metals such as iron, magnesium, and aluminum remaining comparatively low and stable during the granulation process (Liu and Tay, 2004, Qin et al., 2004a,b). The progressive increase in calcium content has been correlated with improved granulation kinetics, reduced settling times and improved compactness.

However, calcium precipitation may also exert a negative impact on microbial activity, as evidenced by a decrease in the specific oxygen uptake rate (Qin et al., 2004a,b). Despite this, calcium-phosphate precipitation does not necessarily interfere with biological phosphorus removal. Angela et al. (2011) reported high concentrations of calcium and

phosphate in the inorganic cores of AGS developed under EBPR conditions. Further mineralogical analyses confirmed the formation of crystalline hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3(\text{OH})$) within the granules, suggesting that chemical P precipitation can coexist with biological uptake without compromising system performance.

2.3. Operating Conditions Control Strategy

In recent years, numerous studies have focused on advancing AGS technology. These advancements are expected to enhance the enrichment of diverse and functionally active microbial communities within the granules. Ongoing research continues to explore the influence of various wastewater types on the formation and structural development of AGS, particularly in relation to their multi-layered morphology. Key operational parameters that have been identified to affect granulation in SBRs include the composition and concentration of the influent substrate, hydraulic and organic loading conditions, settling time control, hydrodynamic shear forces, feeding strategy, cycle configuration, and sludge management, as detailed below.

2.3.1. Influent Characteristics (Substrate Composition and Concentration)

It is important to note that the optimal organic loading rate (OLR) for AGS formation is strongly influenced by the type of carbon source used. Substrate type plays a critical role in determining the structural stability of AGS. A wide range of substrates, including readily biodegradable compounds (e.g., acetate and ethanol), toxic compounds (e.g., phenol and pentachlorophenol), and complex wastewaters from municipal, dairy, and pharmaceutical sources, have been successfully applied in AGS systems (Tay et al., 2001, Beun et al., 1999, Jiang et al., 2002, Lan et al., 2005, Coma et al., 2012, Arrojo et al., 2004,

Inizan et al., 2005). Studies have shown that using glucose as the sole carbon source tends to promote the dominance of fast-growing heterotrophic bacteria, which significantly increases biomass concentration but also encourages the overgrowth of filamentous microorganisms, ultimately compromising granule integrity. In contrast, when acetate and succinate are provided as mixed carbon sources, AGS tends to be enriched with rod-shaped bacterial populations, resulting in granules with enhanced structural stability and improved denitrification performance (Yuan et al., 2019 and He et al., 2020). In addition to OLR, the type of carbon source has a direct impact on microbial growth, activity, and EPS production, all of which are important for forming and maintaining stable granules. Variations in carbon metabolism can lead to changes in both the yield and composition of EPS, a key determinant in microbial aggregation. Cerning et al. (1994) demonstrated that the type of carbon source influences the type of biopolymer produced; for instance, EPS synthesized from glucose differed significantly from that produced in lactose-based media. Moreover, granules cultivated on glucose have shown higher resistance to elevated OLRs compared to those formed using acetate. However, at low OLRs, glucose-fed granules tend to be loose and dominated by filamentous bacteria, while acetate-fed granules exhibit more compact morphology, smoother surfaces, and superior settling characteristics (Moy et al., 2002 and Gao et al., 2011).

AGS has demonstrated superior capabilities in degrading poorly biodegradable compounds, such as phenol, compared to conventional flocculent sludge (Liu and Tay, 2004). AGS can also develop in the presence of particulate organic matter, although granulation may be inhibited when the influent consists primarily of insoluble, particulate COD (Wagner et al., 2015). Pre-exposing such influent to an anaerobic phase has been

shown to enhance particulate solubilization, thereby improving its bioavailability to aerobic granules. Influent composition also strongly influences granule structure. For example, acetate promotes the formation of compact and well-structured granules (Liu and Tay, 2004), while glucose and other readily biodegradable substrates tend to encourage filamentous overgrowth (Liu and Liu, 2006). Similarly, particulate COD can lead to filamentous-dominated granules and may promote the formation of filamentous aerobic granules under certain conditions (Wagner et al., 2015).

The carbon source serves as the primary electron donor supplying energy for microbial metabolism, while the nitrogen source is essential for synthesizing proteins in microbial cells. As a result, C/N ratio is a crucial factor influencing microbial growth and activity. Numerous studies have confirmed that the C/N ratio significantly affects the structural morphology and stability of AGS. For example, Zhao et al. in 2012 cultivated AGS under various influent C/N ratios ranging from 100/5 to 100/50 (Zhao et al., 2012). They observed that at a C/N ratio of 100/10, the resulting granules exhibited a dense and compact structure, uniform particle size, excellent settling performance, and efficient simultaneous nitrification and denitrification (SND). In contrast, granules formed under lower C/N ratios tended to have larger particle sizes, looser structures, and weaker settling ability. Meanwhile, AGS formed at higher C/N ratios also exhibited dense structures; however, the dominance of smaller particles reduced settling efficiency. Similarly, Zhang et al. (2018) found that at C/N ratios of 5 and 15, the granules formed at a C/N of 5 demonstrated the most stable structure (Zhang et al., 2018). Although both extremely low and high C/N ratios appear unfavorable for developing structurally stable granules, AGS can still maintain good structural integrity and nitrogen removal capacity even under low

C/N conditions, such as those found in high-ammonia wastewater ($C/N < 1$) (Sarvajith et al., 2020). This resilience is attributed to the unique layered structure of AGS, which enables simultaneous nitrification and denitrification to occur within different regions of the granule, even in the presence of high dissolved oxygen concentrations. This characteristic of AGS makes it particularly promising for treating wastewaters with low C/N ratios and offers considerable advantages for energy-efficient nitrogen removal.

2.3.2. Influent Distribution

Aerobic granular sludge-based sequencing batch reactors (AGS-SBRs) systems can generally be classified based on their influent distribution strategies into two main types: (1) continuously-aerated distribution and (2) anaerobic influent distribution. The chosen method of influent introduction plays a significant role in shaping both the morphology and microbial composition of the resulting aerobic granules. Research has shown that applying a prolonged feeding period, up to 50 min, through a settled sludge bed is more favorable than a brief feeding time during the mixing phase (de Kreuk and van Loosdrecht, 2004). This approach offers two main advantages in full-scale applications: it simplifies SBR operational scheduling and supports the enrichment PAOs and GAOs by establishing alternating aerobic and anaerobic environments. Anaerobic influent distribution, in particular, promotes the diffusion of substrates deep into the granule, thereby favoring the growth of slower-growing microorganisms, which contributes to enhanced structural stability (Pronk et al., 2015). It should be noted that ‘selection of slow-growing communities’ refers to suppressing the uncontrolled surface proliferation of fast-growing floc-forming heterotrophs under feast conditions, rather than implying that slow growth

alone is sufficient to guarantee granulation. In addition, Slow-growing guilds can contribute to stability, but granulation additionally requires effective aggregation and retention mechanisms, including EPS-mediated cohesion, hydrodynamic shear, and diffusion-driven stratification.

For wastewater rich in easily biodegradable soluble substrates, anaerobic plug-flow feeding enhances AGS performance. In this mode, PAOs (e.g., *Acinetobacter*, *Microthrix*,) and GAOs (e.g., *Contendobacter*, *Defluviicoccus*) at or near the granule surface rapidly take up substrates and convert them to polyhydroxyalkanoates (PHAs)/glycogen, which are later oxidized during aerobic or anoxic phases at a slower rate (Thwaites et al., 2017). As anaerobic feeding progresses and the surface populations saturate, residual substrates diffuse inward, enabling uptake by inner-layer consortia. The outer aerobic shell supports nitrifiers (e.g., *Nitrosomonas*, *Nitrospira*), while anoxic layers facilitate denitrification and denitrifying P-uptake by taxa such as *Diaphorobacter*, *Hydrogenophaga*, *Rhodobacter*, *Ottowia*, *Thauera*, *Paracoccus*, *Denitratisoma*, and *Tetrasphaera*. In the anaerobic core, fermentative organisms (e.g., *Bacteroidota* and *Firmicutes*) generate VFAs and other intermediates that diffuse outward, sustaining PAO/DPAO activity.

On the other hand, applying an aerobic influent distribution strategy can introduce diffusion limitations, often leading to the development of a filamentous microbial layer at the granule surface. During the feast phase substrate consumption occurs rapidly but is typically restricted to the aerobic outer layer of the granules (Beun et al., 2002). This localized aerobic degradation, especially of septic substrates, is known to promote the proliferation of filamentous bacteria, which tend to accumulate on the granule surface and

impair structural integrity (Strom and Jenkins, 1984). The overgrowth of filamentous organisms negatively influences the settleability of aerobic granular sludge, subsequently reducing the quality of the treated effluent (Pronk et al., 2015). Excessive surface fouling by filamentous microorganisms has been reported particularly when the organic loading exceeds the microbial community's anaerobic uptake capacity, even in systems fed with pure acetate (Beun et al., 2002).

Particulate substrates play a significant role in influencing the formation and stability of AGS. Once granules are established, these particulate materials are primarily hydrolyzed at the granule surface (de Kreuk et al., 2010). The porous nature of AGS allows it to function as a physical filter, trapping particles that are too large to pass through its matrix. Under anaerobic influent distribution, hydrolysis of retained particulates generates soluble products that can be readily assimilated and stored as polymers by PAOs and GAOs, supporting granule development. However, depending on the rate of anaerobic hydrolysis and the concentration of particulate matter, a portion of the hydrolysis may occur aerobically. In such cases, the aerobic breakdown of particulates supplies a continuous source of biodegradable substrates (i.e., prolonged feast phase). These hydrolysis products are then predominantly consumed by heterotrophs at the granule surface, which can hinder substrate penetration into deeper granule layers (Mosquera-Corral et al., 2005). This diffusion limitation may lead to filamentous overgrowth, reduced granule cohesion, or increased suspended solids in the treated effluent.

Finally, when readily biodegradable substrates are gradually introduced into a mixed aerobic environment, significant substrate diffusion limitations may arise,

promoting the growth of filamentous microorganisms. This is because elevated concentrations of biodegradable organics in the bulk liquid during aeration favor the proliferation of aerobic heterotrophs. The resulting filamentous overgrowth can severely compromise the settling characteristics of AGS, thereby deteriorating effluent quality (Pronk et al., 2015 and Martins et al., 2011). To mitigate this, maximizing the uptake of biodegradable substrates under anaerobic conditions is essential. This strategy suppresses the dominance of fast-growing aerobic heterotrophs and facilitates the development of well-structured granules characterized by smooth surfaces and high compactness.

2.3.3. Organic Loading Conditions

OLR, defined as the amount of organic substrate (calculated from the product of influent flow and substrate concentration) per unit volume of the reactor, is a key factor influencing AGS formation. The food-to-microorganism (F/M) ratio refines this measure by relating the organic load to the concentration of active biomass, typically expressed as mixed liquor volatile suspended solids (MLVSS). In continuously aerated AGS systems, where aeration is maintained throughout the entire cycle including influent addition, both OLR and F/M have been shown to significantly impact granulation dynamics. Generally, AGS systems are capable of tolerating OLRs in the range of 2.5–15 kg COD/(m³·d) (Long et al., 2015). Elevated OLRs tend to accelerate granule formation but often lead to larger, loosely structured granules due to the dominance of rapidly growing heterotrophic bacteria. In contrast, lower OLRs favor the slower development of more compact stable granules.

While many studies on AGS development have focused on high-strength wastewater, the successful application of AGS technology to municipal and other low-

strength wastewaters has also been reported (Isanta et al., 2012 and Coma et al., 2012). Optimal OLRs for granulation vary depending on system configuration and influent characteristics. In systems fed with glucose-based substrates, a higher F/M ratio, up to 1.1 kg COD/(kg VSS·d), was shown to accelerate granulation and produce larger granules (Li et al., 2011). Some studies reported that granulation could not be achieved at OLRs below 2 kg COD/(m³·d) in continuously aerated SBRs (Liu and Tay, 2002). In contrast, other research demonstrated successful granule formation at lower OLRs of 1–1.7 kg COD/(m³·d), although nearly one year of operation was required to achieve mature granules under such conditions (Wang et al., 2009).

Both excessively low and highly fluctuating OLRs can negatively impact aerobic granule stability by promoting the growth of filamentous bacteria, which results in loose, porous granules and compromised system performance (Long et al., 2015). Conversely, sustained high OLRs, particularly above 6 kg COD/(m³·d), under aerobic feeding conditions may also favor filamentous growth, leading to granule instability and eventual disintegration during long-term operation (Zheng et al., 2006 and Liu and Tay, 2015). For instance, Liu et al. in 2015 reported that while AGS rapidly formed within 3 days under an OLR of 12 kg COD/(m³·d), the granules expanded and disintegrated after two weeks (Liu and Tay, 2015). However, when the OLR was reduced to 6 kg COD/(m³·d), stable granule structure was maintained over a prolonged period. This improved stability at moderate loading rates has been attributed to the enrichment of EPS-producing functional bacteria, particularly those contributing to the accumulation of tightly bound extracellular polymeric substances, which play a crucial role in maintaining granule cohesion and structural integrity (Chen et al., 2019).

2.3.4. Settling Time Control (Selective Washout)

Because heavier and larger bioparticles will settle faster than those that are less dense, the short settling time and quick discharge create a selection pressure that only allows the retention of biomass with good settling properties, i.e., biomass having a settling velocity less than a minimum value, namely (V_s) in **Eq. (1)**, will be washed out (**Figure 2.4**). Short settling time is widely recognized as one of the most critical selective pressures during the initial phase of aerobic granulation. This phenomenon will be referred to herein as “settling velocity-based selection pressure.” This selection pressure depends not only on the settling time, but also on the settling distance. Besides selecting for biomass with better settling properties, cell surface hydrophobicity and EPS production as a stress response are promoted and enhancing microbial aggregation and granule cohesion, as well (Gao et al., 2011 and Xiong and Liu, 2012). By limiting the duration available for biomass to settle, flocculent and slow-settling microorganisms are preferentially washed out, thereby enriching for denser and faster-settling microbial aggregates (Cassidy and Belia, 2005). This selective pressure reduces competition from floc-forming bacteria, facilitating the establishment of granule-forming populations (Adav et al., 2009). Such a trend suggests that selection pressure based on settling velocity is indeed the ultimate driving force for aerobic granulation in SBRs. The settling depth, however, is equally as important as the settling time (**Eq. (2.1)**), which explains how some specialized processes utilizing deep tanks, e.g., Nereda® (Pronk et al., 2015), allow for longer settling times.

$$(V_s) = \frac{\text{Settling distance } (L)}{\text{Settling time } (t)} \quad (\text{Equation 2.1})$$

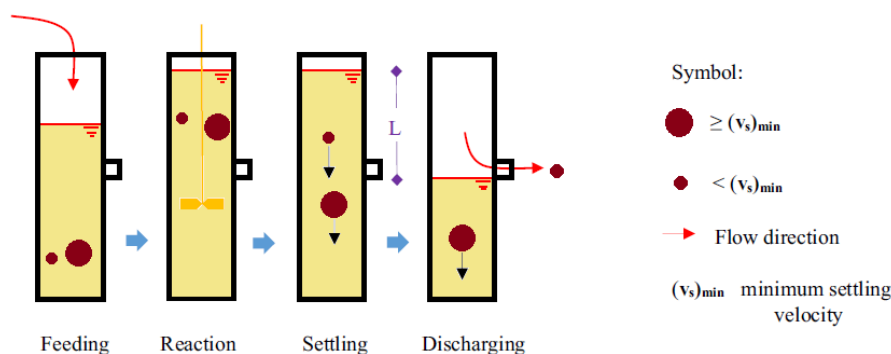


Figure 2.4: Biomass selection in AGS-SBRs based on minimum settling velocity threshold $(V_s)_{\min}$ (Source: Adapted from Kent et al., 2018)

2.3.5. Aeration and Shear Forces

Shear force, typically applied through aeration or mechanical mixing (is not common for AGS processes because of the fragile texture of the granules), serves as a critical selective pressure in promoting microbial aggregation and bio-granulation. It shapes the hydrodynamic flow patterns within the reactor, directly influencing granule morphology (Liu and Tay, 2002). High aeration rates generate circular flow patterns that encourage the formation of compact, spherical granules. In contrast, simultaneous use of aeration and mechanical mixing introduces irregular, multi-directional flow, which disrupts aggregation and results in loosely bound, irregularly shaped microbial structures.

Hydrodynamic shear forces, arising from friction between the bulk liquid and the surfaces of granules, are a critical operational parameter in AGS systems. During the initial stages of granulation, shear-induced hydraulic turbulence promotes microbial aggregation by enhancing cell-to-cell collisions. Although early studies emphasized the necessity of high shear for successful granulation, more recent evidence suggests that elevated shear is not always essential for granule formation. Instead, it plays a pivotal role in maintaining

granule stability, particularly under high organic loading conditions, by dislodging fast-growing heterotrophs and particulate matter from granule surfaces (Devlin et al., 2017). Conversely, in systems operating under low OLR, stable granulation has been achieved at superficial air velocities below 0.5 cm/s, implying that optimal shear intensity is context-dependent and influenced by system configuration and influent characteristics. More broadly, the superficial air velocity reported in AGS studies is frequently used as a hydrodynamic proxy for shear/mixing, rather than a direct design target for oxygen supply. Experiments have shown that upflow air velocity values above 2.5 cm/s favor the formation of dense, stable, and mature granules. Shear forces in SBRs are exerted by both bulk liquid movement (aeration and hydraulic flows) and inter-particle collisions (Liu and Tay, 2002). These forces stimulate EPS secretion, enhance cell surface hydrophobicity (Gao et al., 2011), and contribute to granule densification (Liu et al., 2005). However, insufficient shear, evidenced by superficial air velocities below 1–2.4 cm/s, has been associated with poor granule stability, prolonged feast phases, and oxygen diffusion limitations (Liu and Tay, 2002 and McSwain et al., 2004). Most of these findings originate from continuously aerated systems and may not fully translate to intermittently aerated or plug-flow configurations. Beyond structural implications, shear forces influence microbial stratification within granules. By selectively detaching loosely attached outer cells, shear helps regulate the spatial distribution of microbial populations, limiting the overgrowth of filamentous bacteria and supporting the structural integrity and functionality of mature AGS (Devlin et al., 2017). Overall, the benefits of applying high shear rates in AGS systems include: (1) accelerated granule formation, (2) improved granule stability, (3)

enhanced surface hydrophobicity, (4) suppression of filamentous bacteria and oversized granules, and (5) stimulation of EPS production.

2.3.6. Feeding Strategy

SBRs have been widely adopted in studies of AGS formation due to their operational flexibility, particularly their ability to alternate between feast and famine phases (Arrojo et al., 2004 and Cassidy and Belia, 2005). These alternating substrate conditions play a pivotal role in promoting bio-granulation by creating selective pressure that favors the enrichment of storage-capable microorganisms, such as PAOs and GAOs. These organisms enhance granulation by secreting EPS, which reduce cell surface hydrophobicity and promote microbial aggregation (Cassidy and Belia, 2005). Later research indicated that prolonged anaerobic feeding periods (e.g., 1 hour) are more advantageous, as they enrich for PAOs and GAOs, which contribute to the development of structurally stable and compact granules (de Kreuk et al., 2005). During anaerobic conditions, these accumulating bacteria convert readily biodegradable carbon sources into intracellular storage polymers such as glycogen and polyhydroxybutyrate (PHB), which are subsequently utilized during the aerobic famine phase (de Kreuk et al., 2005). This metabolic strategy not only limits the growth of fast-growing heterotrophs during the aerobic phase but also supports EPS production and granule consolidation (de Kreuk and van Loosdrecht, 2004). Overall, the careful control of feeding duration and feast–famine dynamics in SBRs is essential for guiding microbial selection and promoting robust AGS formation.

AGS-SBR systems are typically categorized based on influent distribution into either continuously-aerated or anaerobic feeding modes, both of which influence granule structure and microbial composition. Anaerobic influent distribution, particularly with extended feeding times (e.g., 50 min), has been shown to favor the selection of PAOs and GAOs, simplify operational scheduling, and enhance granule stability through gradual substrate diffusion across the granule depth (de Kreuk and van Loosdrecht, 2004). This approach promotes uniform biomass growth by encouraging storage polymer accumulation (e.g., PHA) in surface and inner-layer organisms, supporting slow-growing microbial communities essential for stable granulation (Pronk et al., 2015). Moreover, internal regions of the granule can utilize alternative electron acceptors like nitrate for polymer oxidation, improving simultaneous BNR (de Kreuk et al., 2005). Although strategies such as anaerobic–aerobic split feeding have enabled granulation from CAS inoculum, limitations in phosphorus removal may still arise depending on feed conditions and substrate availability (de Kreuk and van Loosdrecht, 2004).

In contrast to anaerobic feeding, aerated influent distribution can hinder AGS stability by creating diffusion limitations and promoting surface colonization by filamentous microorganisms, particularly during the feast phase when substrates are rapidly consumed at the granule surface (Beun et al., 2002). This aerobic consumption, especially of septic or easily biodegradable substrates, encourages filamentous growth, which negatively affects granule settleability and effluent quality (Pronk et al., 2015, Martins et al., 2011, Strom and Jenkins, 1984). High organic loading beyond the anaerobic uptake capacity further exacerbates surface fouling, even when pure substrates like acetate are used (Devlin et al., 2017).

Extended famine periods and lower OLR have been shown to improve granule stability under these conditions (Corsino et al., 2016 and 2017). Therefore, maximizing anaerobic substrate uptake is essential for minimizing filamentous proliferation and promoting compact, uniform granules with improved settling properties. Studies have shown that short famine periods can accelerate granule formation, but often at the expense of long-term stability (Liu and Tay, 2008). Extended famine periods may induce excessive EPS consumption, increase energy demands, and reduce reactor throughput (Wang et al., 2005). Liu and Tay (2008) reported that when the feast period accounted for 40% of the aeration time, granulation was accelerated, though granule stability was compromised. Overall, evidence indicates that maintaining a feast period $\leq 40\%$ of aeration time is critical for achieving granules with strong settling properties and long-term stability.

2.3.7. Cycle Configuration and Phasing

AGS-SBRs have shown strong adaptability in treating a wide range of wastewater types, including both low-strength municipal effluents and high-strength industrial discharges such as those from rubber manufacturing, landfill leachate, and dairy, brewery, or textile industries. Despite this versatility, the granulation process tends to be slower when treating low-strength wastewater, largely due to limited substrate availability. To enhance granule development under these conditions, it is beneficial to operate at a lower sludge loading rate and introduce a higher concentration of seed sludge at the start. Research by Seviour et al. (2011) indicated that operating with a 12-hour cycle led to granules averaging 0.75 mm in diameter, whereas Pronk et al. (2015) found that a shorter 6.5-hour cycle produced smaller granules around 0.2 mm. These outcomes suggest that

longer cycle durations are more conducive to granule formation in low-strength systems, while high-strength wastewater (i.e., enriched with ions such as calcium and magnesium) can support faster granulation, even under shorter cycles. The presence of these ions enhances EPS interactions, contributing to quicker and more stable granule maturation in high-strength wastewater scenarios (Chen et al., 2019).

Cycle time is an important operational parameter that can influence aerobic granule formation and stability, although it is not considered a primary determining factor (Liu et al., 2005). Shorter cycle times are often associated with enhanced granulation efficiency, as they enable higher organic loading rates and more frequent selection pressure. However, excessively long cycles may extend the starvation phase, resulting in excessive EPS consumption and ultimately granule disintegration. A balanced cycle duration, typically between 3 and 6 hours, is generally recommended to support microbial growth and biomass accumulation while minimizing the risk of biomass washout or destabilization (Zhu and Wilderer, 2003, Beun et al., 1999, de Kreuk et al., 2005). Despite the perceived benefits of shorter cycles, they may also introduce several drawbacks. Insufficient starvation periods can limit the selection of slow-growing, accumulating bacteria critical for long-term granule stability (de Kreuk and van Loosdrecht, 2004). Moreover, shorter cycles can lead to excessive biomass loss due to solids being discharged during effluent withdrawal, thereby reducing the SRT. Additionally, elevated OLR associated with rapid cycling may favor fast-growing heterotrophs, which can cause the formation of large, unstable granules with internal diffusion limitations (Zheng et al., 2006).

2.3.8. Sludge Management

The settling behavior of AGS is governed by fundamental physical parameters, including temperature, fluid viscosity, and granule morphology. Empirical studies have shown a marked dependence of settling velocity on granule size and ambient temperature: large granules (mean diameter 2.3 ± 0.5 mm) settled at rates of 84 m/h at 5 °C and 145 m/h at 40 °C, whereas smaller granules (1.5 ± 0.3 mm) exhibited slower velocities of 35 m/h and 63 m/h at the same respective temperatures (Winkler et al., 2012). These results underscore the superior settling kinetics of AGS compared to CAS, which is characterized by diffuse, flocculent structures and significantly lower settling velocities. The distinct particulate nature of AGS enables rapid, gravity-driven separation, thereby challenging the foundational assumptions of clarifier design in traditional systems. Additionally, compositional differences within AGS communities further influence physical behavior; granules enriched with PAOs exhibit higher densities and settle more efficiently than those dominated by GAOs, as observed in full-scale biomass characterizations (Chen et al., 2019). This differential settling performance not only highlights the critical role of microbial composition in granule hydrodynamics but also suggests opportunities for optimizing reactor design and sludge retention through targeted microbial selection.

2.4. Parameters for Aerobic Granular Sludge Cultivation and Maintenance

This section outlines the key parameters influencing the cultivation and long-term stability of AGS. These parameters are categorized into two levels: macroscale factors, which include reactor-related conditions (e.g., configuration and SRT) and reaction system parameters (inoculated sludge, DO, metal ions, pH, and temperature); and microscale factors, which focus on microbial community, PN/PS ratio, and granule size. Each of these

factors plays a critical role in shaping the formation, stability, and performance of AGS, as discussed in the following sections. **Table 2.2** summarizes the operational conditions and corresponding performance outcomes of AGS reactors reported in the literature.

Table 2.2: Operational conditions and results of different AGS systems reported in the literature (Adapted from Guzmán-Fierro et al., 2023)

Inf.	Reactor Configuration		Reaction Cycle	OLR	NLR	HRT	SRT	DO	T	COD Rem. (%)	N Rem. (%)	P Rem. (%)	AGS size (mm)	Ref.
	Vol.	H/D												
RWW	1.5	5.5	3-h cycles: 3 min of feeding, 171 min of aeration, 1 min of sedimentation and 5 min of discharge.	1.72	0.18	6	66	6.5 : 8.0	18	90	40		3.4	Figueroa et al., 2008
SWW	31.4	5.0	6-h cycles: 13 min of feeding, 325 min of aeration, 0 min of sedimentation and 12 min of discharge	0.69	0.04	13.3		2.0		87			2.35	Morgenroth et al., 1997
SWW	2.25	26.8	4-h cycles: 2 min of feeding, 237 min of aeration, 2 min of sedimentation and 1 min of discharge	7.5	0.14	6.75	3.4		20	100	4.38		3.3	Beun et al., 1999
RWW	1.8	4.5	3-h cycles: 5 min of feeding, 150 min of aeration, 15 min of sedimentation, 5 min of discharge and 5 min idle	3.6	0.24	6.0		4 : 7.6	27	98.4	92.7		2.0	Rosman et al., 2014

RWW	4.0	6.9	4-h cycles: 10 min of feeding, 185 min of aeration, 20 min of anaerobic phase, 15 min of sedimentation, 5 min of discharge and 5 min idle	9.0	1.8	0.8			27	74	73		4.1	Othman et al., 2013
RWW	1.75		4-h cycles: 5 min of feeding, 227 min of aeration, 1 min of sedimentation and 7 min of discharge	1.8	0.22	8.0	15.0	7.0	25	80	50		0.94	Carrera et al., 2019
RWW	7.0	5.71	6-h cycles: 13 min of feeding, 319 min of aeration, 18 min of sedimentation and 10 min of discharge	1.50	0.08	12.0		5.0 : 8.0		95.0	35.0		0.46	Chen et al., 2019
RWW	11.5	10.0	4-h cycles: 2 min of feeding, 90 min of anaerobic phase, 134 min of aeration, 20 min of sedimentation and 4 min of discharge	0.9	0.09	5.6	5.6	8.0	29	80.0	83.0		> 2.0	Alves et al., 2022
SWW	5.0	3.8	8-h cycles: 30 min of feeding, 90 min of anaerobic phase, 330 min of aeration, 5 min of sedimentation	1.22	0.20	16.0	54.0	2.0 : 3.0	20	52.0	99.0			Ren et al., 2017

			and 12 min of discharge											
SWW	4.0	8.0	12-h cycles: 40 min of anaerobic feeding, 676 min of aeration, 1.5 min of sedimentation and 2 min of discharge	0.8	0.06	48.0	30.0	7.50	20	87.0			1.5	Corsino et al., 2017

Inf.; Influent, **RWW**; Real Wastewater, **SWW**; Synthetic Wastewater, **Vol**; Volume (L), **OLR**; Organic loading rate (kg COD/m³ d), **NLR**; Nitrogen loading rate (kg N/m³ d), **HRT**; Hydraulic retention time (hour), **SRT**; Solid retention time (day), **DO**; Dissolved oxygen (mg O₂/L), **T**; Temperature (°C).

2.4.1. Macroscale Influencing Factors

2.4.1.1. Reactor configuration

SBR is widely regarded as the most effective configuration for AGS cultivation, primarily due to its fill-and-draw operational mode and the capacity to impose alternating feast and famine conditions within each cycle. One of the defining features of the SBR is its short settling time, which imposes strong selection pressure by preferentially retaining dense, fast-settling biomass, thereby promoting granule formation (Carrera et al., 2019). Another fundamental aspect is the presence of distinct substrate availability phases, particularly the extended aerobic starvation period, which can account for more than 75% of the total cycle time (Corsino et al., 2018). During this phase, microorganisms consume EPS, leading to a decrease in surface-negative charge and an increase in cell

hydrophobicity, both of which contribute to the aggregation of microbial cells and the formation of dense, stable granules (Nancharaiah and Sarvajith 2019).

Reactor configuration, particularly the height-to-diameter (H/D) ratio, has traditionally been considered influential in granulation (**Table 2.2**). Higher H/D ratios (>5) increase the vertical distance biomass must travel during settling, thereby enhancing selective retention of dense sludge with higher settling velocities (Liu and Tay, 2004). It should be noted that the very high H/D ratios commonly reported in the literature largely reflect lab- and pilot-scale column reactors, whereas full-scale reactor depths are constrained by civil/structural limits. Additionally, such configurations support the development of longer circular flow patterns during aeration, which are conducive to the formation of compact granules (Nancharaiah and Sarvajith 2019). High aspect ratios, typically in the range of 15–30, have conventionally been associated with improved granule ripening and surface density (Liang et al., 2019). However, recent findings challenge the necessity of high H/D ratios as a prerequisite for successful granulation. Hamiruddin and Awang (2021) confirmed that dense and structurally stable granules could form in SBRs with low H/D ratios under well-optimized operational conditions. Collectively, these studies suggest that while reactor configuration can influence granulation dynamics, it is not a determining factor when appropriate operational strategies are implemented.

The volume exchange ratio (VER), defined as the proportion of effluent withdrawn relative to the reactor's total working volume, plays a crucial role in promoting aerobic granulation by exerting selective pressure on biomass. Higher VERs (typically above 60%) favor the retention of bio-particles with superior settling velocities and higher EPS

production potential, both of which are essential for granule formation (Wang et al., 2006 and Qin et al., 2004b). When combined with short settling times, high VERs help eliminate slow-settling flocs, enriching the system with dense, fast-settling aggregates. Studies have shown that even under identical operational conditions and reactor configurations, increasing the VER alone can accelerate granule development. Wang et al. (2006) investigated four identical SBRs with VERs ranging from 20 - 80%, and found that granulation was significantly enhanced at higher VERs (60 and 80%), resulting in larger, more spherical granules with improved settling characteristics. In contrast, lower VERs (20 and 40%) led to incomplete granulation, producing a mixture of granules and suspended sludge.

2.4.1.2. Sludge retention time (SRT)

Sludge age is widely recognized as a fundamental parameter in wastewater treatment. During the start-up phase of AGS systems, SRT is typically variable due to intentional biomass washout imposed by reduced settling durations (Qin et al., 2004a,b). Once stable granulation is achieved and biomass concentrations in the effluent decline, SRT can be more precisely controlled. Beyond its operational relevance, SRT plays a critical role in shaping the microbial community within granules, influencing the balance between autotrophic and heterotrophic populations, as well as their nutrient removal capacities and resilience. Moreover, inappropriate SRT control may promote the overgrowth of filamentous organisms, thereby compromising granule integrity and long-term system stability (Liu and Liu, 2006). However, the role of sludge age in the formation of AGS remains a subject of debate, and several studies have reported that SRT is not a decisive factor in initiating granulation in SBRs.

For instance, Liu et al. in 2005 and Li et al. in 2008 demonstrated that SRTs ranging from 3 to 40 days did not significantly influence the onset of granulation (Liu et al., 2005 and Li et al., 2008). Instead, they emphasized that selection pressure based on settling velocity, rather than SRT, serves as the primary driving force behind aerobic granule formation. Similarly, Liu and Liu (2006) noted that in most AGS-SBR systems, SRT is not tightly controlled but is allowed to vary naturally with sludge settleability under applied selection pressures. Nevertheless, while SRT may not control granule formation, it plays a crucial role in maintaining granule stability during long-term operation (de Sousa Rollemberg et al., 2018). Zhu et al., (2013) reported that extended SRTs negatively impact granule integrity and emphasized the importance of selective sludge removal strategies to preserve granule structure. Therefore, while SRT may not initiate granulation, its appropriate control, particularly through strategic sludge wasting, remains essential for sustaining structural stability and preventing granule deterioration over time.

2.4.1.3. Inoculated sludge

While the concentration of inoculated sludge may not exert a dominant influence on AGS cultivation, given that successful granulation has been reported across a broad range of MLSS concentrations (1–20 g/L) and SVI values (7–220 mL/g) (Hamiruddin and Awang, 2021), the physicochemical and microbial characteristics of the seed sludge have been shown to play a far more decisive role in granule formation and stability. Specifically, properties such as settleability, surface charge, hydrophobicity, and EPS secretion capacity directly affect microbial aggregation and the early stages of granule development. Hydrophobic bacterial populations are particularly advantageous, as they promote stronger intercellular adhesion, leading to enhanced granulation compared to hydrophilic

communities. Therefore, seeding with sludge from EBPR systems or reactors enriched with EPS-producing and aggregating bacteria is often more conducive to initiating and sustaining granulation.

Lei et al. (2021) successfully cultivated saline AGS within 52 days by inoculating with anaerobic granular sludge, which transitioned into aerobic granules while maintaining morphological integrity. Moreover, Wang et al. (2018) demonstrated that the addition of stored granular sludge significantly reduced the time required for granulation relative to inoculation with flocculent sludge alone, enabling the formation of compact, well-settling granules in just 22 days. Similarly, Zou et al. (2019) reported that inoculating with intact granular sludge effectively suppressed sludge bulking and facilitated the formation of structurally robust AGS. Collectively, these findings underscore that while sludge concentration is a flexible parameter, the biological and physicochemical quality of the inoculum is a critical determinant of granulation success and reactor performance.

2.4.1.4. Dissolved oxygen (DO)

Although DO concentration is not considered a primary controlling factor for the initiation of aerobic granulation in SBRs, it plays a significant role in shaping microbial stratification and maintaining granule stability. Several studies have demonstrated successful granulation across a wide range of DO concentrations (0.7–8 mg/L), suggesting that DO alone does not dictate granule formation (Liu et al., 2005). However, DO levels strongly influence the structural integrity and microbial composition within granules. Low DO concentrations can promote the proliferation of filamentous bacteria and foster anaerobic conditions within the granule core, leading to gas production and subsequent

structural disintegration (Gao et al., 2011). Conversely, excessively high DO levels, particularly under prolonged aeration, can impose intense hydrodynamic shear forces, which have also been shown to cause granule breakage and instability (Yu et al., 2014).

Maintaining DO at approximately 40–50% of saturation has been identified as optimal for preserving granule stability and improving N removal performance, as lower DO levels enhance stratification and promote SND by supporting the coexistence of aerobic, anoxic, and anaerobic zones within the granule (de Kreuk et al., 2005, de Sousa Rollemberg et al., 2018, Metcalf & Eddy, 2014). It has been shown that oxygen penetration depth increases with bulk DO concentration, especially in granules of similar size, which can suppress the development of anoxic or anaerobic layers necessary for complete nutrient removal. Thus, while DO may not govern the granulation process itself, its regulation is essential for establishing favorable redox gradients, ensuring microbial diversity, and maintaining long-term structural stability of AGS.

As discussed in relation to microbial groups, DO concentration can be used as a selective factor to favor specific functional communities. However, determining an optimum DO concentration remains challenging, as it is strongly influenced by biomass concentration, granule size, substrate characteristics, and the applied OLRs. Current evidence suggests that stable granules are more likely to be maintained when DO concentrations exceed 50% of saturation. Nonetheless, further studies are needed to optimize this parameter, with the dual aim of ensuring long-term granule stability while simultaneously achieving energy savings and maximizing nitrogen removal.

2.4.1.5. Metal ions (divalent cation)

The interaction between metal cations and negatively charged sludge surfaces plays a crucial role in forming a stable three-dimensional matrix, which promotes and stabilizes the aerobic granulation process. Specifically, the presence of metal ions such as magnesium (Mg^{2+}), calcium (Ca^{2+}), and iron ($\text{Fe}^{2+}/\text{Fe}^{3+}$) in the influent has been shown to accelerate granule formation. These ions can precipitate within the sludge matrix, acting as nucleation centers that enhance granule structure and improve settling performance (Gao et al., 2011 and Xin et al., 2017). Several studies have demonstrated that precipitates of Mg^{2+} , Ca^{2+} , and $\text{Fe}^{2+}/\text{Fe}^{3+}$ enhance granule properties such as density, strength, and stability (Lee and Chen, 2015), although calcium precipitation itself is not strictly essential for granulation, as noted by (Zhou et al., 2013). It worth mentioning that the granules cultivated in the reactor with additional iron ions are larger and have a more tightly packed structure than those formed without iron ions. Ren et al. in 2018 investigated four SBRs to study the effect of adding Fe^{2+} , Fe^{3+} and Fe_3O_4 on the AGS formation (Ren et al., 2018).

The addition of divalent cations, particularly Ca^{2+} and Mg^{2+} , has been shown to significantly accelerate the formation of AGS. Specifically, supplementing the influent with 100 mg/L of calcium and 10 mg/L of magnesium can reduce the granulation time by approximately 50% (Li et al., 2009). Similarly, the bridging action of other divalent cations may influence the cohesion and long-term stability of aerobic granules by reinforcing the EPS network. The effect of Mg^{2+} on the granulation process was investigated and it was observed that the AGS formed by adding Mg^{2+} were denser, with better settleability and higher PS content, which showed that Mg^{2+} could enhance the cultivation of AGS in SBR.

However, increasing Ca^{2+} concentrations in the influent can influence the metabolic activity of PAOs. Barat et al. (2008) reported that elevated Ca^{2+} levels and the resulting precipitation of calcium phosphate reduced the P release-to-acetate uptake ratio, which in turn favored the proliferation of GAOs over PAOs, ultimately diminishing the efficiency of biological P removal. Therefore, the implications for P removal performance should be carefully evaluated prior to supplementing influent with divalent cations to enhance granulation. Additionally, the small size of AGS may hinder denitrification, as DO can penetrate deep into the biomass, preventing the development of an anoxic core required for denitrifying bacteria (Corsino et al., 2016). Nevertheless, denitrifiers have been observed within AGS and are considered beneficial in facilitating metal precipitation under high phosphate concentrations. This occurs by increasing the alkalinity within the granule core, thereby enhancing structural stability.

2.4.1.6. Other operating factors

Factually, some operating parameters such as pH, temperature, salinity, toxic compounds such as free ammonia are known as the influencing factors on granules stability will discussed in the following section.

pH plays a critical role in the formation and stability of AGS, as it influences not only microbial growth and metabolic activity but also the physicochemical characteristics of sludge and the composition of EPS (Bagheri et al., 2015 and Jiang et al., 2019). Fluctuations in pH can alter EPS structure and functionality, thereby impacting granule cohesion and pollutant removal efficiency. Jiang et al. (2019) reported that AGS maintained good structural integrity and settleability in weakly acidic (pH 5.5) to neutral conditions, whereas exposure to alkaline environments led to rapid and irreversible structural deterioration.

Similarly, extremely acidic conditions (pH 3.0) were shown to induce EPS hydrolysis, resulting in granule disintegration and system instability (Corsino et al., 2018). While some studies have suggested that acidic and neutral conditions support granulation (Rezasoltani et al., 2015), the microbial community shifts significantly with pH. Alkaline pH has been consistently associated with granule disintegration, increased effluent solids, and a reduction in granule size (Lashkarizadeh et al., 2016). These negative effects have been attributed to pH-induced alterations in EPS composition and chemical structure, underscoring the importance of maintaining near-neutral pH to ensure stable AGS.

Temperature is a key environmental factor influencing both the physicochemical and biological performance of AGS systems (**Table 2.2**). Beyond its well-documented impact on microbial growth kinetics, temperature also affects the physical behavior of granules, most notably their settling velocity, due to temperature-induced changes in the viscosity of the bulk liquid. Operating AGS systems at temperatures below 10 °C has been associated with excessive filamentous bacterial growth, which compromises granule integrity and leads to biomass washout. Conversely, temperatures above 30 °C may induce protein denaturation and enzyme inactivation, ultimately destabilizing the entire biological system (Muñoz-Palazon et al., 2020). These findings suggest that stable aerobic granulation under such low-temperature conditions is difficult to achieve, likely due to the significant reduction in microbial activity and slower EPS production, both of which are essential for granule formation and maturation. To expand the engineering applicability of AGS in extreme climates, including cold and tropical regions, some researchers have successfully acclimated AGS to low-temperature conditions (< 7 °C), achieving both effective pollutant

removal and stable granule structure under such challenging environments (Gonzalez-Martinez et al., 2017).

Salinity. Operational conditions such as fluctuating salinity levels can significantly influence the composition and gel-like structure of aerobic granules by altering microbial diversity and metabolic activity. The mechanical strength and long-term stability of granules have been shown to depend largely on the gel-forming properties of their EPS matrix. Although earlier studies suggested that salinity exerts a less significant influence on aerobic granule stability compared to factors such as pH, subsequent findings have demonstrated otherwise. Pronk et al. (2014) reported that elevated chloride concentrations ($20 \text{ g Cl}^- \text{ L}^{-1}$) led to a reduction in granule size and increased effluent turbidity, likely due to the weakening of the EPS matrix under saline conditions. Salinity has been shown to markedly affect the physical characteristics of AGS, particularly by altering the EPS composition. As salinity increases, the fraction of loosely bound EPS rises while the protein-rich EPS content declines, compromising the gel structure essential for granule cohesion. These structural disruptions become especially critical at salinity levels above 50 g NaCl L^{-1} , beyond which granule destabilization becomes irreversible.

AGS has demonstrated potential for treating various industrial wastewaters, including those from agro-food sectors such as fish-canning industries, which often produce high-salinity effluents, sometimes exceeding 150 g-NaCl/L . Effective biological treatment in such environments requires resilience to osmotic stress that can compromise microbial cell integrity. Notably, salt concentrations above 20 g-NaCl/L can inhibit nitrite-oxidizing bacteria (NOBs), leading to nitrite accumulation. Previous study demonstrated

the successful development of halotolerant AGS through a gradual increase in influent salinity (Corsino et al., 2016). This approach allowed for the stable operation of SND processes at salinity levels up to 50 g-NaCl/L, achieving TN removal efficiencies exceeding 95%, with effluent TN concentrations below 10 mg/L. However, when salinity exceeded 50 g-NaCl/L, nitrification efficiency significantly declined, likely due to impaired oxygen transfer in the salt-rich environment. Notably, organic matter removal remained unaffected by salinity and was instead influenced by the OLR.

Toxic compounds: free ammonia (fa) and free nitrous acid (FNA). In WWTPs, nitrogen is predominantly present in the form of ammonium (NH_4^+), originating primarily from the influent wastewater and from return liquors generated during the treatment of waste activated sludge via anaerobic digestion. Under alkaline conditions, a portion of total ammonia nitrogen (TAN: NH_4^+) exists in its un-ionized form as free ammonia (FA, NH_3), which can become more prevalent depending on pH and temperature, and considered as toxic compound. FA has been identified as a key factor influencing EPS secretion and granule stability (Zheng et al., 2013). Moreover, FA is recognized as a potent inhibitor of key microbial populations involved in wastewater treatment processes (Park et al., 2015). Its presence can significantly reduce the specific activity of functional microorganisms and may also alter the overall microbial community composition (Wang et al., 2017). Although the accumulation of FA is generally regarded as detrimental to conventional biological treatment processes in WWTPs, certain advanced treatment strategies have been developed to harness its effects for process enhancement (Wang et al., 2017 and Wang, 2017). These approaches leverage FA to promote sludge reduction and autotrophic nitrogen removal, offering potential benefits such as improved sludge minimization, increased bioenergy

recovery (e.g., methane production), and reduced requirements for both DO and external carbon sources during secondary treatment.

High FA concentrations tend to favor the proliferation of GAOs over PAOs, leading to a decline in biological phosphorus removal performance as PAOs are outcompeted and eventually eliminated (Zheng et al., 2013). Lochmatter and Holliger (2014) reported that maintaining a neutral pH is optimal for minimizing the start-up duration of AGS systems while preserving effective nutrient removal performance. Typically, an influent TAN concentration of around 30 mg-N/L at a pH range of 7 to 8 and a temperature of 20 °C can result in FA concentrations ranging from approximately 0.14 to 1.38 mg NH₃-N/L within the wastewater treatment system (Cervantes, 2009 and McCarty, 2018). It is important to consider that the proportion of FA is influenced by both pH and temperature (calculated by equation **Eq. 2.2**) (Anthonisen et al., 1976).

$$FA \text{ (mg/L)} = (17/14) \times \frac{TAN \times 10^{pH}}{\exp\left(\frac{6334}{273+T}\right) + 10^{pH}} \quad \text{(Equation 2.2)}$$

Free nitrous acid (FNA, HNO₂), which may form during acidification processes, has been identified as an inhibitory compound that can negatively affect bacterial metabolic activity essential for biological wastewater treatment (Zhou et al., 2011). High concentrations of FA and FNA, calculated by equation **Eq. 2.3** (Anthonisen et al., 1976), are known to inhibit nitrifying bacteria, often resulting in nitrification failure and nitrite accumulation (Liu et al., 2023). Studies have shown that granular sludge demonstrates greater resistance to such inhibitory compounds compared to floccular sludge, likely due to its compact structure, which limits the direct exposure of microorganisms to toxic

substances (Ren et al., 2017). Another study has shown that even a low FNA concentration of 1.2 µg/L at pH 6.5 can inhibit up to 88% of the activity of PAOs, highlighting its strong inhibitory effect (Jabari et al., 2016).

$$FNA \text{ (mg/L)} = (46/14) \times \frac{NO_2^-}{\exp\left(\frac{-2300}{273+T}\right) \times 10^{pH}} \quad \text{(Equation 2.3)}$$

2.4.2. Microscale Influencing Factors

2.4.2.1. Microbial community structure

The microbial composition of AGS is closely influenced by both the operational parameters of the reactor and the characteristics of the influent wastewater. Variations in HRT, substrate concentration, and temperature have all been shown to impact the diversity and distribution of microbial populations within the granules (Wan et al., 2014, Li et al., 2016, Reino et al., 2016). These findings align with earlier observations from SBRs, where microbial community structure was similarly found to depend on influent composition and system operating conditions (Liu et al., 2005, Gao et al., 2011). The addition of divalent cations, particularly Ca^{2+} and Mg^{2+} , has been shown to significantly accelerate the formation of AGS. Hao et al. (2016) investigated the biological impacts of common ions Ca^{2+} , Mg^{2+} , Cu^{2+} , Fe^{2+} , Zn^{2+} and K^+ on the microbial attachment of the AGS and the flocculent activated sludge in the biological wastewater treatment system.

Although AGS can develop under various operational conditions, studies indicate that AGS formed across different environments often shares similar dominant bacterial groups, while operational regime and substrate composition shift their relative abundance in ways that affect both granule morphology and treatment performance (Ou et al., 2018

and Sun et al., 2017). The predominant phyla commonly reported in AGS include *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Acidobacteria*, *Verrucomicrobia*, *Chloroflexi*, and *Saccharibacteria*. While the flow regime may not fundamentally change the overall community profile, it can alter the abundance of specific populations. For example, *Proteobacteria* and *Bacteroidetes* dominated AGS in a continuous-flow reactor, where *Proteobacteria* were linked to pollutant removal through enhanced EPS production, whereas *Actinobacteria* were associated with filamentous bulking (Geng et al., 2020). Similarly, *Bacteroidetes* and β -*Proteobacteria* were abundant in a pilot-scale SBR, with β -*Proteobacteria* contributing substantially to N and P removal (Xia et al., 2018). These taxonomic patterns are also relevant to EBPR guilds: PAO- and GAO-associated lineages are frequently embedded within *Proteobacteria* (and, depending on the system, *Actinobacteria*), so shifts at the class/phyla level may reflect changes in the functional balance between poly-P accumulation and carbon storage without P removal. In addition, filamentous organisms can act as structural scaffolds that support early aggregation, but excessive filament growth promotes bulking and weaker compaction, impairing long-term stability. Accordingly, the distribution of *Proteobacteria*, *Bacteroidetes*, and filament-associated groups across zoogloea-, filamentous-, and integrated-type AGS helps explain how microbial community structure governs granule integrity and treatment performance (Geng et al., 2020 and Xia et al., 2018).

Liu et al. (2025) recently provided a mechanistic synthesis that explicitly links AGS morphology to microbial community structure and function, addressing a key gap in earlier AGS reviews. They proposed a practical classification framework comprising four morphotypes; (a) Zoogloea-type (ZT-AGS), (b) filamentous bacteria-type (FBT-AGS), (c)

crystal nucleus-type (CNT-AGS), and (d) integrated-type (IT-AGS), and emphasized that each morphotype reflects distinct formation pathways and therefore tends to select for different dominant guilds and structural traits. Importantly, the review argues that AGS succession is shaped by a “core” set of genera that recur across systems while specialized taxa are differentially enriched depending on morphotype and operational context. From a community standpoint, this study highlighted that AGS communities consistently include major phyla such as *Proteobacteria* and *Bacteroidetes*, while functional groups supporting organic removal, nitrification/denitrification, and EBPR can be linked to recurrent genera. They report that *Thauera* is among the most frequently observed genera across compiled AGS datasets (appearing in 56.25% of samples), followed by *Flavobacterium*, *Zoogloea*, and *Nitrospira*, and interpret these high-frequency taxa as central contributors to EPS-associated aggregation and pollutant conversion. *Candidatus Accumulibacter* and *Rhodocyclaceae* are repeatedly associated with enhanced P removal, aligning community composition with EBPR function. This spatial stratification within granules enables redox and substrate gradients that can support coupled nutrient transformations, including simultaneous nitrification, denitrification, and P removal. In addition, they note that morphotype selection and community restructuring can be tuned by controllable drivers, particularly seed sludge, influent/feeding water, and temperature, reinforcing the idea that operational strategy may shift relative abundances and spatial partitioning of key populations even when broad phylum-level composition remains similar.

Short settling time is a widely applied selection pressure in AGS systems, with numerous studies confirming its effectiveness in accelerating granulation by promoting the washout of slow-settling flocs and enriching for dense, fast-settling microbial aggregates

(Jiang et al., 2002 and Hu et al., 2005). This initial biomass loss can stimulate EPS production by functional microbial strains, as competition from less-advantageous species is reduced. However, excessively short settling times may inadvertently remove key bacterial groups, such as PAOs, ammonium oxidizing bacteria (AOBs), and nitrite oxidizing bacteria (NOBs), before granule establishment, leading to reduced microbial diversity and impaired nutrient removal (Cassidy and Belia, 2005 and Weissbrodt et al., 2013). While short settling times effectively eliminate poorly-settling biomass, they also discharge the lighter biomass fraction with the effluent, which may lead to elevated effluent solids concentrations exceeding discharge standards (Qin et al., 2004).

Fluorescence in situ hybridization (FISH) analysis has shown that denser granules, typically located at the bottom of the sludge bed, are enriched with PAOs, whereas lighter granules near the surface are dominated by GAOs (Winkler et al., 2011). Selective removal of the surface layer, where GAOs prevail, has been demonstrated to significantly enhance P removal, increasing TP removal efficiency from 71% to nearly 100%. Moreover, separating biomass wasting from the effluent stream allows for better control of solids discharge, reducing effluent suspended solids concentrations to below 5 mg/L and improving overall treatment performance (Niermans et al., 2014).

Numerous studies have demonstrated that the substrate composition plays a pivotal role in shaping the ecological niche of AGS, leading to significant variability in microbial community structure and granule morphology (Liang et al., 2019). Specifically, glucose as a sole carbon source has been shown to favor the proliferation of GAOs, while simultaneously suppressing the growth of PAOs, thereby compromising phosphorus

removal efficiency. In contrast, He et al. (2020) reported that when sodium succinate and sodium acetate were supplied as mixed carbon sources, PAOs were successfully enriched as the dominant microbial group. This shift not only inhibited GAO proliferation but also enhanced the structural integrity and pollutant removal performance of AGS.

Heterotrophic nitrification and aerobic denitrification (HNAD) microorganisms constitute a broadly distributed and phylogenetically diverse functional guild, occurring across bacteria and fungi and spanning both Gram-positive and Gram-negative groups (Zhao et al., 2012 and Chen et al., 2016). Reported HNAD genera include *Acinetobacter*, *Bacillus*, *Cuprobacter*, *Halomonas*, *Klebsiella*, *Marinobacter*, *Pseudomonas*, and *Photobacterium*, isolated from various environments, reflecting wide environmental adaptability (Huang et al., 2020). A recent review (Song et al., 2021) also notes that some reported genera may be opportunistic/pathogenic (e.g., *Klebsiella*), whereas others may support water quality improvement (e.g., *Bacillus*), reinforcing the need to interpret community composition functionally rather than taxonomically (Wang et al., 2018).

2.4.2.2. Ratio of proteins to polysaccharides

Research on synthetic wastewater has shown that EPS are primarily produced during the feast phase of the SBR cycle, immediately after feeding when readily biodegradable COD is consumed. In contrast, significant EPS consumption occurs during the famine phase, when substrate concentrations are limited and microorganisms metabolize EPS as a carbon and energy source for endogenous respiration (Corsino et al., 2015). EPS play a central role in maintaining the stability of aerobic granules, acting as a “biological glue” that binds cells together and supports granule structure (Adav et al., 2008

and Wang et al., 2005). Beyond structural stability, EPS also contribute to the adsorption of metals and organic pollutants, and serve as an internal reservoir of carbon and electrons, particularly during famine conditions. Nancharaiah and Reddy (2018) further emphasized that granule stability depends strongly on the balance of PN/PS ratios within EPS, underlining their multifunctional importance in AGS systems.

Alginate (polysaccharides), along with a newly identified polysaccharide known as granulan, has been characterized as a dominant EPS component with strong gel-forming capabilities, particularly under acidic to neutral pH conditions (Seviour et al., 2012 and Lin et al., 2008). These biopolymers are believed to enhance the cohesion, mechanical strength, and resilience of granules, enabling them to withstand operational disturbances and hydraulic shear stress. Despite this, the respective contributions of PS and PN in EPS to AGS stability remain a subject of ongoing debate. Some researchers argue that PS promotes particle cohesion by enhancing microbial adhesion and biomass retention, thereby contributing to structural rigidity. Under this perspective, a lower PN/PS ratio ($\text{PN/PS} < 1$) is considered favorable for granule stability, especially under saline or stressful conditions.

PN is reported to increase hydrophobic interactions and modulate cell surface charge, thereby improving microbial aggregation and mechanical robustness (Wang et al., 2018). Wang et al. (2019) observed a substantial increase in PN secretion during the early granulation stages under fluctuating influent conditions, with the PN/PS ratio rising from 4.27 to 7.54, indicating an adaptive structural response. Similarly, an upward trend in PN/PS was observed during granule development and maturation, suggesting that PN contributes more significantly than PS to long-term granule stability and granulation

efficiency (Wang et al., 2018). Collectively, these findings reveal a complex and condition-dependent interplay between PN and PS in regulating AGS structural integrity.

2.4.2.3. Granule size

Table 2.2 summarizes the range of granule sizes reported under varying operational conditions across different AGS reactors in the literature. Oversized AGS particles pose significant challenges to system performance by impeding the diffusion of DO and nutrients into the granule interior. This limitation creates localized anaerobic conditions within the aerobic phase, which can promote the proliferation of filamentous bacteria, degrade settling properties, and compromise granule structural stability (Campo et al., 2022). Moreover, excessive anaerobic core volume in large granules has been associated with diminished microbial activity and a higher risk of granule disintegration. While larger granules can enhance SND by providing expanded anoxic zones beneath the aerobic layer (Xin et al., 2017), this benefit must be weighed against the risk of diffusion limitations and activity loss. Conversely, undersized granules often suffer from poor settleability and insufficient biomass retention, undermining reactor performance and process stability. These findings underscore the necessity of maintaining AGS particle size within an optimal range.

2.5. Spatiotemporal Distribution and Dynamics of Microbial Communities in AGS

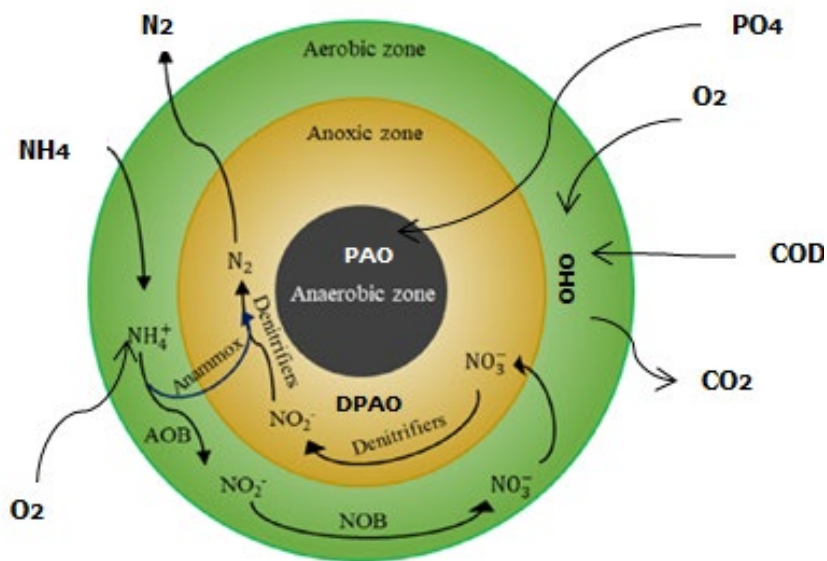


Figure 2.5: Simultaneous C, N, P removal pathways in AGS (Source: Adapted from Nancharaiah and Reddy, 2018)

AGS consists of self-aggregating microorganisms organized into multi-layered structures that exhibit distinct redox gradients due to radial variations in electron donors and acceptors. These gradients create aerobic, anoxic, and anaerobic microenvironments within the same granule, enabling the simultaneous removal of carbon, N, and P by diverse functional guilds, including PAOs, denitrifying PAOs (DPAOs), GAOs, AOBs, NOBs, and ordinary heterotrophic organisms (OHOs) (Campo et al., 2022). This stratification supports the coexistence of metabolically diverse populations, leading some authors to describe granules as “miniature ecosystems.” Microbial communities are essential for granule formation, stability, and nutrient removal performance (Geng et al., 2020). For example, specific PAOs and GAOs such as *Rhodobacteraceae* and *Competibacteraceae* have been shown to influence granule stability and EPS production. Additionally, *Proteobacteria* and

Chloroflexi have been identified as structural backbones that promote microbial aggregation and enhance granule formation (Shi et al., 2021).

Bernet and Spérandio in 2009 highlighted that, even under optimal cultivation conditions, nitrifying bacteria exhibit considerably slower growth compared to OHOs (Cervantes, 2009). Reported maximum specific growth rates range from 4 to 13.2 day⁻¹ for heterotrophs, while nitrifiers typically grow at only 0.62–0.92 day⁻¹. Among bacteria commonly found in granules, anaerobic ammonium oxidation (Anammox) bacteria display the slowest growth, with rates as low as 0.065 day⁻¹ (Isaka et al., 2006). Regarding to the selection of PAOs/DPAOs and GAOs/DGAOs, de Kreuk and van Loosdrecht in 2004 suggested that PAOs and GAOs contribute to the formation and stabilization of AGS (de Kreuk and van Loosdrecht, 2004). Consequently, the coexistence of slow-growing bacteria such as nitrifiers and Anammox, along with PAOs and GAOs, has been reported to play a beneficial role in promoting the cultivation of stable and robust granules. In addition, they confirmed that the presence of PAOs or GAOs supports the development of stable AGS. With respect to P removal, the key PAOs include *Accumulibacter* spp., *Rhodocyclus* spp., and *Enterobacter* spp. However, these organisms compete with GAOs, particularly those of the genus *Competibacter*, since both groups utilize volatile fatty acids (VFAs) and share similar metabolic pathways. Unlike PAOs, GAOs lack the ability to store P, which can reduce the overall efficiency of nutrient removal (Bassin et al., 2012).

It worth mentioning that DPAOs and denitrifying GAOs (DGAOs) are slow-growing microorganisms that play a role in achieving complete granulation. These organisms utilize internal storage polymers, such as PHAs, to reduce nitrite or nitrate, and

in the case of DPAOs, also contribute to phosphate uptake. DPAOs and DGAOs have commonly been associated with bacterial families including *Comamonadaceae*, *Sphingomonadaceae*, *Hyphomicrobiaceae*, *Rhodobacteriaceae*, *Xanthomonadaceae*, and *Rhodocyclaceae*, with representative genera such as *Thauera*, *Zoogloea*, *Meganema*, *Devosia*, and *Stenotrophomonas* (Weissbrodt et al., 2013). Moreover, members of the families *Rhodocyclaceae*, *Xanthomonadaceae*, and *Sphingomonadaceae* have also been linked to EPS production in AGS systems, further supporting stability (Szabó et al., 2017).

The main selection strategy for PAOs and GAOs involves a long anaerobic feeding period followed by an aerobic reaction phase, while DPAOs and DGAOs are favored when the subsequent phase is anoxic. In SBR operation, the intended anaerobic selector is achieved only when oxidized nitrogen ($\text{NO}_2^-/\text{NO}_3^-$) is minimized at the start of the feeding period. If NO_x is carried over from the previous aerobic phase, denitrification will occur first during feeding (i.e., anoxic conditions precede true anaerobic conditions), which can consume readily biodegradable COD and alter PAO–GAO competition. Therefore, controlling NO_x carryover (e.g., via cycle design and aeration/anoxic sequencing) is important when targeting PAO enrichment under anaerobic feeding.

OHOs generally grow more slowly on storage polymers such as PHA and glycogen than on readily biodegradable substrates. The application of a feast/famine regime in SBRs reduces the maximum growth rate of fast-growing OHOs, particularly during famine. Anaerobic uptake of easily biodegradable substrates prior to the aerobic or anoxic phase, along with substrate conversion by relatively slow-growing bacteria during these phases, is considered essential for stable granulation (Pronk et al., 2015). Operational parameters

(e.g., COD/N ratio and DO concentration) also influence microbial competition. Aeration is also a key factor: reductions in either aeration intensity or aeration duration suppressed GAOs growth while promoting PAOs enrichment, with GAOs abundance markedly decreasing when aeration time was reduced from 120 to 60 min (He et al., 2017).

Moving to the N removal and the selection of AOBs, NOBs and Anammox, Liu et al. (2004) examined the potential of enhancing aerobic granule stability by enriching slow-growing nitrifying bacteria. Their study showed that the dominance of nitrifiers within aerobic granules contributed to the relatively low overall growth rate of the biomass, which in turn supported structural stability. The findings also emphasized the importance of the COD/N ratio as a key operational parameter in promoting nitrifier selection. By controlling this ratio, the selective enrichment of slow-growing nitrifying bacteria was demonstrated to be an effective strategy for improving the long-term stability of aerobic granules. It is worth mentioning that several studies investigated the effect of the COD/N ratio on the abundance of AOBs and NOBs. In a related study, He et al. (2017) demonstrated that lowering superficial gas velocity from 0.17 to 0.11 and then to 0.04 cm/s enriched AOB populations while simultaneously inhibiting NOB accumulation, further confirming the importance of hydrodynamic conditions in shaping nitrifying communities. Szabó et al. (2017) identified *Brocadia* as a key family responsible for Anammox. Successful cultivation of Anammox bacteria at ambient temperatures requires maintaining a high SRT. Due to their inherently low biomass yield and slow growth rate, heterotrophic activity should be minimized to ensure that Anammox bacteria remain a dominant fraction of the microbial community in sludge. In AGS systems, competition for substrates plays a critical role in shaping microbial communities. Specifically, Anammox and NOBs compete for

nitrite, while AOBs and NOBs compete for oxygen. Low DO concentrations have been shown to favor AOBs over NOBs, thereby improving conditions for partial nitrification. Under oxygen-limited conditions, AOB, NOB, and Anammox tend to coexist within the same compact granule rather than growing in suspension.

Modern molecular microbiology provides essential tools to study AGS, including FISH, real-time quantitative polymerase chain reaction (qPCR), clonal library analysis, terminal restriction fragment length polymorphism (T-RFLP), high-throughput sequencing, and multi-omics approaches (Calderon-Franco et al., 2022 and Lv et al., 2014). Numerous studies have confirmed that microbial population dynamics are central to the granulation process under varying operational conditions. Lv et al. (2014) reported that the core of AGS was dominated by *Rhodocyclaceae*, whereas outer layers were enriched with *Flavobacteriaceae*, *Xanthomonadaceae*, *Rhodobacteraceae*, and *Microbacteriaceae*. Filamentous bacteria such as *Chloroflexi*, *Sphaerotilus natans*, *Thiothrix*, and *Meganema perideroedes* were also identified as structural contributors (Figuerola et al., 2015). Microbial populations varied among different AGS forms, yet similar dominant taxa often emerged under diverse growth conditions (Ou et al., 2018 and Sun et al., 2017). Functional gene analyses have demonstrated enhanced activity of genes related to nitrification (e.g., *amo*, *nxr*, *hao*), denitrification (e.g., *nir*, *nap*, *nor*), and polyphosphate accumulation (e.g., *ppk*, *ppk2*) in AGS.

The initial skeleton of aerobic granules is typically formed by *Rhodocyclus*, *Hyphomicrobiaceae*, and other rod-shaped or filamentous bacteria during sludge granulation in anaerobic–aerobic BPR systems, followed by colonization with globular

bacteria (Zhang et al., 2011). Zou et al. (2023) proposed a novel approach to simultaneously accelerate the granulation process and enhance the biological removal of the nitrogen forms through the addition of polyurethane sponges and periodic intensive aeration. Microbial analysis demonstrated that biofilm formation under these conditions enriched aerobic denitrifiers (e.g., *Dokdonella*, *Denitratisoma*), autotrophic denitrifiers (e.g., *Sulfuritalea*), and hydrolytic/fermentative bacteria (e.g., *Ferruginibacter*, *Opitutus*, *Lactobacillus*), collectively enhancing short-cut N removal (SND) and endogenous denitrification. Fra-Vazquez et al. (2016) investigated long-term (300-day) AGS treatment of pig manure in a pilot plant and reported that microbial diversity initially increased but subsequently decreased in response to fluctuating organic and N loads. Genera such as *Brachymonas*, *Zoogloea*, and *Thauera* were identified as structural bacteria capable of producing EPS, thereby maintaining granule stability. Functional contributions included partial nitrification by *Nitrosomonas*, denitrification by *Thauera* and *Zoogloea*, and organic matter oxidation primarily by *Comamonas*. He et al. (2020) further demonstrated that supplementing mixed carbon sources (sodium succinate and sodium acetate) promoted PAO dominance, effectively suppressing GAO enrichment and improving stability and treatment efficiency. In SBRs, bacterial and archaeal richness and bacterial diversity were higher, with five archaeal phyla identified (*Thaumarchaeota*, *Crenarchaeota*, *Euryarchaeota*, *Woesearchaeota*, and others). In continuous-flow systems, Chen et al. (2019) reported that at high OLRs, microbial community richness and diversity declined sharply during the first 40 days of granulation. Predominant families (*Flavobacteriaceae*, *Propionibacteriaceae*, *Burkholderiaceae*, *Rhodocyclaceae*, and *Chitinophagaceae*). Through these high-load

conditions, GAOs, EPS producers, and carbohydrate degraders, played critical roles in the cultivation of stable AGS.

Within AGS, microbial community dynamics are governed by strong spatiotemporal gradients in DO, substrates, and nitrogen intermediates, creating shifting ecological niches across the granule radius and throughout the cycle; in this context, HNAD microorganisms are particularly relevant because many can sustain nitrification and denitrification under aerobic conditions with strain-specific DO optima and, in some cases, broad oxygen adaptability according to a recent review (Song et al., 2021). In addition, this review further emphasizes that aggregation and retention (core features shaping spatial stratification in AGS) are tightly linked to EPS, which are produced under both aerobic and anaerobic conditions and comprise polysaccharides, proteins, nucleic acids, lipids, and humic acids; EPS quantity and composition modulate floc/granule structure and cell-surface properties (e.g., surface charge and hydrophobicity), thereby influencing microbial localization and persistence within aggregates (You et al., 2016). Several HNAD-associated strains exhibit strong self-aggregation and/or high EPS secretion (e.g., *Pseudomonas*), supporting the concept that EPS-mediated attachment and cell binding can reinforce biomass structuring and stability over time in reactor systems (Zhao et al., 2012). In parallel, the review indicates that mixed HNAD microflora can outperform single strains, particularly under low organic carbon, due to coexistence and cooperative interactions among organisms with complementary enzyme sets, which provides a mechanistic basis for phase-dependent functional shifts and community re-organization commonly observed in AGS operation (Liu et al., 2017, Yang et al., 2017, Zhang et al., 2019, Zhang et al., 2019).

Within granular systems, it should be noted that DO is not merely an operational variable but a key selector that shapes HNAD colonization and governs denitrification-route expression under nominally aerobic conditions. Importantly, nitrite reductase can be inhibited at high DO (Ka et al., 1997), creating a mechanistic basis for nitrite accumulation in oxygenated microzones and motivating why HNAD performance can vary across granule depth and over the aeration trajectory; for example, lowering DO improved nitrite removal by *Pseudomonas stutzeri* T13 (Xie et al., 2021). Additionally, it worth mentioning that “optimal” DO and substrate conditions are strain-specific and can differentially favor nitrification versus denitrification, as shown by *Acinetobacter* sp. ND7 where high DO favored ammonium removal while low DO favored nitrate removal (Ren et al., 2021). In parallel, carbon availability (C/N) acts as a dynamic ecological filter: the optimal C/N ratio for most HNADs is commonly reported between 8 and 15 (Huang and Tseng, 2001 and Rajta et al., 2020), yet pathway demands can diverge within a single organism (e.g., *Bacillus methylotrophicus* L7 requiring distinct C/N optima for heterotrophic nitrification versus aerobic denitrification) (Zhang et al., 2012), supporting a spatiotemporal interpretation in AGS where fluctuating substrate/oxygen fields continually reshape active HNAD subpopulations through the cycle.

2.6. Pollutant Removal Mechanisms of Aerobic Granular Sludge

Rapid economic development has intensified environmental pressures, particularly those related to water scarcity and pollution. Effective removal of nutrients such as carbon, N, and P from wastewater has therefore become an urgent global priority. AGS technology

offers a distinct advantage in this context, as its layered structure sustains diverse redox microenvironments that enable the simultaneous BNR removal within a single reactor.

2.6.1. Nitrogen Removal

Nitrogen is removed primarily through the biological processes of nitrification and denitrification, which occur under aerobic and hypoxic conditions and ultimately convert N compounds into gaseous N_2 released to the atmosphere (**Figure 2.6 [a]**). The unique structural properties of AGS allow these conditions to coexist within a single granule, where aerobic zones at the surface promote nitrification, while internal anoxic layers enable simultaneous denitrification (Bengtsson et al., 2019). This structural advantage makes AGS highly effective for practical n removal. Diverse microbial groups contribute to these processes, including AOBs, NOBs, denitrifiers, DPAOs, and anammox bacteria, highlighting the functional versatility of AGS systems (Zhang et al., 2022b).

2.6.1.1 N removal by short-cut nitrification and denitrification

Currently, most AGS research is conducted in SBRs, where SND represents the primary N removal pathway. Depending on the electron acceptor involved, SND in AGS can be categorized into full-course and short-cut pathways. Although bulk DO in SBR operation can be relatively high, AGS granules develop strong diffusion–reaction gradients. Oxygen is rapidly consumed in the outer aerobic layer, limiting oxygen penetration and creating low-DO/anoxic microzones in the granule interior. As a result, nitrification can proceed near the surface while denitrification occurs simultaneously in the anoxic core using influent or internally stored carbon. Short-cut SND, which uses NO_2^-

directly as the electron acceptor (**Figure 2.6**), offers clear advantages by reducing aeration and carbon demands, saving up to 25% of oxygen and 40% of external carbon requirements. More recently, Chen et al. (2022a) successfully cultivated AGS with strong settling performance in SBRs for the treatment of urban wastewater characterized by low strength and low C/N ratios, further demonstrating the adaptability of AGS for practical applications.

For example, Liu et al. (2022c) reported that applying a moderate magnetic field (50 mT) enhanced partial nitrification–denitrification and improved COD, $\text{NH}_4^+\text{-N}$, and TN removal relative to a control. However, while these results are informative at laboratory scale, magnetic-field application introduces additional equipment and energy requirements, and its economic feasibility at full scale remains uncertain. In contrast, operational control strategies that leverage existing instrumentation, such as aeration control using oxygen uptake rate and pH slope during SBR start-up, have demonstrated rapid maturation and high TN removal, and are therefore more directly implementable in practice (Dobbeleers et al., 2017).

2.6.1.2 N removal by anaerobic ammonium oxidation (Anammox)

Anammox has emerged in recent years as an innovative N removal technology that enables autotrophic conversion of ammonium and nitrite under strictly anaerobic or anoxic conditions. The anammox process critically depends on the establishment of stable short-cut nitrification as a prerequisite. It is particularly advantageous for treating inorganic, high-ammonia wastewater and low C/N organic wastewater, where conventional processes are less efficient. As a low-carbon biological pathway, anammox offers significant

potential for sustainable wastewater treatment applications (Zhang et al., 2022b). Its main advantages lie in high N removal efficiency combined with markedly lower energy and external carbon requirements, positioning anammox as a cost-effective and environmentally friendly alternative to traditional denitrification (Wang et al., 2022). The development of granular sludge has enabled the simultaneous realization of anammox and short-cut nitrification. Compared with conventional nitrification–denitrification, the partial nitrification–anammox (PN/A) process can reduce aeration demand by up to 60%, preserve organic carbon completely, and cut sludge production by 90% (Bagchi et al., 2012).

2.6.1.3 N removal by HNAD

Mechanistically, N removal under HNAD is best viewed as an integrated outcome of (i) heterotrophic nitrification, (ii) aerobic denitrification, and (iii) assimilation into biomass, with the relative contribution of each route varying by organism, DO, and carbon availability (**Figure 2.6 [b]**). In the commonly described coupled pathway, NH_4^+ is oxidized to NH_2OH by ammonia monooxygenase (AMO), NH_2OH is converted to NO_2^- by hydroxylamine oxidoreductase (HAO) and/or via a pyruvic oxime dioxygenase (POD)-mediated route, and NO_2^- can be further converted to NO_3^- (Duan et al., 2015 and Tsujino et al., 2017). Aerobic denitrification then proceeds through $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{N}_2$, supported by nitrate reductases (Nap/Nar/Nas) and nitrite reductases (NirK or NirS), with evidence that specific nitrate-reductase systems (e.g., Nap and/or Nas) can dominate under aerobic denitrification depending on the organism (Wehrfritz et al., 1993, Sparacino-Watkins et al., 2014, Zhang et al., 2020, Chen et al., 2021). An alternative hydroxylamine-reduction denitrification pathway ($\text{NH}_4^+ \rightarrow \text{NH}_2\text{OH} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$) is reported, supported by cases in

which nitrate/nitrite intermediates and their corresponding enzyme activities were negligible. This pattern suggests that certain HNAD microorganisms can channel NH_2OH more directly toward gaseous N products aerobically (Shoda, 2017).

A critical operational implication is the risk of incomplete denitrification and intermediate accumulation: missing genes/enzymes for Nir, Nor, and/or Nos (or enzyme impairment) can lead to $\text{NO}_2^-/\text{NO}_3^-$ build-up, and strains possessing HAO but lacking nitrite reductase may accumulate nitrite; furthermore, high DO combined with limited carbon supply can suppress nitrite reductase activity and promote nitrite accumulation (Duan et al., 2015, Wehrfritz et al., 1993, Xie et al., 2021). Finally, assimilation can represent a substantial fraction of apparent TN removal, with N incorporated into biomass via pathways involving glutamate dehydrogenase, glutamine synthetase, and glutamate synthase, and some organisms may exhibit incomplete “classical” nitrification/denitrification gene sets while relying strongly on assimilation-associated routes (Pal et al., 2015 and Jin et al., 2019); consistent with this, mixed HNAD microflora may enhance TN removal, especially in weak wastewater, through cooperative interactions among organisms expressing complementary N-metabolism enzymes (Zhang et al., 2019).

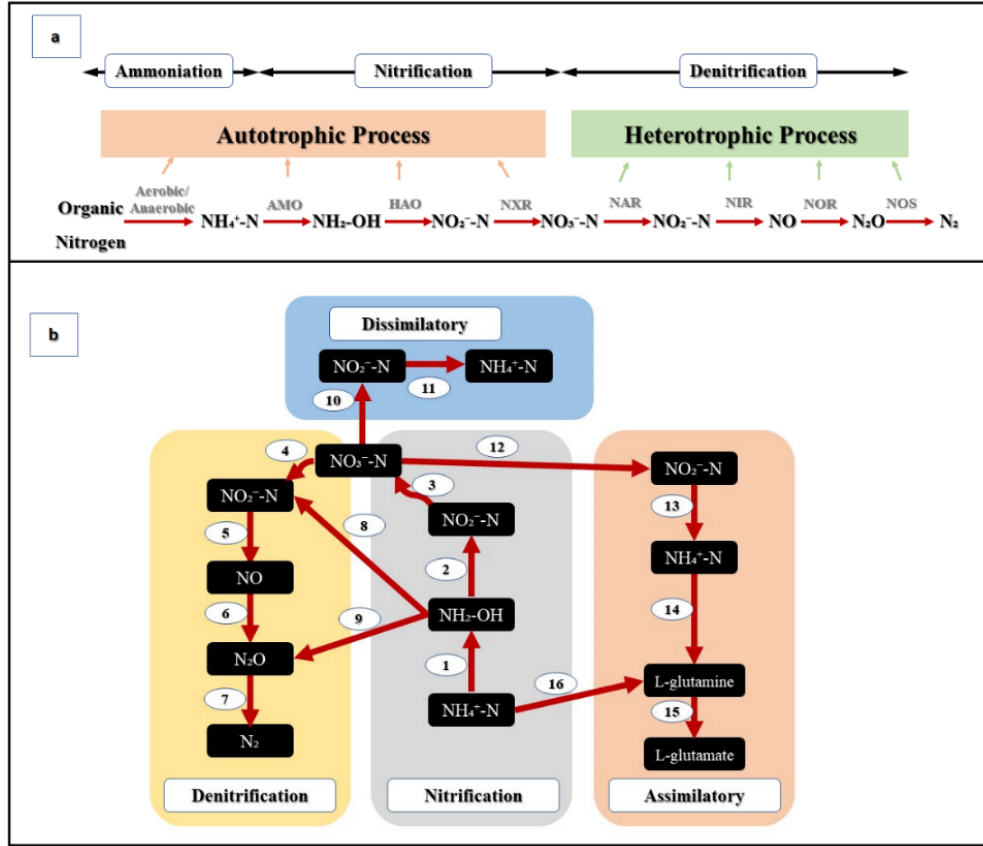


Figure 2.6: (a) Traditional N removal process, (b) N metabolism of HN-AD bacteria

(Source: Adapted from Li et al., 2024 and Fu et al., 2022):

- | | |
|--|---|
| (i) Shortcut NH_4^+ metabolic: 1→9→7. | (v) Aerobic NO_3^- denitrification: 4→5→6→7. |
| (ii) Incomplete NH_4^+ metabolic: 1→8→5→6→7. | (vi) NO_3^- incomplete denitrification: 12→13→14→15. |
| (iii) Complete NH_4^+ metabolic: 1→2→3→4→5→6→7. | (vii) Aerobic NO_3^- assimilation: 16→15. |
| (iv) Aerobic NH_4^+ nitrification: 1→2→3. | (viii) Assimilation/dissimilation branchpoints: 10→11. |

HNAD has been conceptualized as a coupled set of oxygen-tolerant nitrogen transformation pathways in which O_2 can serve as the terminal electron acceptor,

potentially increasing energy yield, supporting faster growth, and enhancing overall nitrogen removal. Reported observations include high NO_3^- removal with minimal NO_2^- accumulation and substantial NO_3^- to N_2 conversion supported by N-mass balances; several strains have also been shown to convert NH_4^+ to N_2 aerobically. As summarized in **Figure 2.6 (b)**, these routes can be grouped into (i) NH_4^+ nitrification, (ii) NO_3^- denitrification, (iii) incomplete NO_2^- denitrification, and (iv) aerobic NO_3^- assimilation/dissimilatory reduction (He et al., 2016, Yang et al., 2019, Lang et al., 2020, Kim et al., 2005).

Under HNAD conditions, nitrate reduction to N_2 can proceed despite the presence of oxygen, following the canonical denitrification sequence ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$) mediated by enzyme-catalyzed steps. However, pathway expression is strongly strain-dependent and is modulated by operational conditions, such that the extent of intermediate accumulation varies accordingly. Nitrite reduction is frequently the rate-limiting step, as nitrite reductase is often inhibited by elevated DO, leading to NO_2^- accumulation. Conversely, some HNAD strains maintain efficient NO_2^- conversion to gaseous products even under high DO, and NO_2^- may additionally be routed through coupled assimilation and denitrification. Evidence from isotope-based studies further suggests that N_2O can be reduced to N_2 aerobically (Song et al., 2021, Xie et al., 2021, Ren et al., 2021, Wan et al., 2011, Desloover et al., 2014).

Ammonium biotransformation pathway in HNADs. Conventional nitrification–denitrification relies on a compartmentalized redox sequence: ammonium is aerobically oxidized to NO_2^- by AOB and further to NO_3^- by NOB, followed by anoxic denitrification. In contrast, HNAD permits multiple aerobic NH_4^+ -conversion routes. A “classical”

pathway proceeds via detectable $\text{NO}_2^-/\text{NO}_3^-$ intermediates, whereas shortcut routes have been inferred using stable-isotope tracing and enzyme-inhibitor assays. Notably, some strains produce N_2O and N_2 from NH_4^+ without measurable $\text{NO}_2^-/\text{NO}_3^-$ accumulation, supporting an alternative hydroxylamine-mediated route ($\text{NH}_4^+ \rightarrow \text{NH}_2\text{OH} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$). In *Alcaligenes* sp. TB, inhibitor evidence indicates the coexistence of incomplete and complete NH_4^+ metabolic pathways, with co-occurring nitrogen species influencing route selection: $\text{NH}_4^+ + \text{NO}_2^-$ may promote shortcut conversion, whereas $\text{NH}_4^+ + \text{NO}_3^-$ may favor oxidation to nitrate prior to N_2 formation. The hydroxylamine route has been proposed as particularly relevant for high-strength N wastewaters due to its reduced pathway complexity (Wehrfritz et al., 1993, Jaroszynski and Oleszkiewicz, 2011, Zhao et al., 2010, Liu et al., 2019, Wei et al., 2021, Peng and Zhu, 2006).

Nitrate, nitrite, and nitrous oxide biotransformation pathways in HNADs. Aerobic NO_3^- denitrification in HNAD is typically represented as a stepwise reduction sequence ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$). The overall rate is frequently constrained at the nitrite-reduction step because nitrite reductase is often oxygen-sensitive, which can promote NO_2^- accumulation under high DO. Accordingly, lowering DO has been reported to improve NO_2^- removal for certain strains, whereas other HNAD organisms sustain efficient NO_2^- to N_2 conversion even at elevated DO. When NO_2^- is supplied as the primary substrate, “incomplete” routes have been proposed, including coupled assimilation and denitrification. Isotope-based evidence further indicates that N_2O can be reduced to N_2 under aerobic conditions when N_2O is the initial N source (Song et al., 2021, Xie et al., 2021, Ren et al., 2021, Wan et al., 2011, Desloover et al., 2014).

Assimilation and dissimilation of nitrogen in HNADs. Assimilatory uptake can represent a major N sink in HNAD systems by converting inorganic nitrogen into biomass-N, thereby directly supporting growth and cell synthesis. Reported case studies indicate that assimilation may dominate the N budget under specific substrate and oxygen regimes; for example, *Rhodococcus erythropolis* Y10 exhibited a very high assimilated fraction when NH_4^+ was the sole N source, and *Vibrio* sp. Y1-5 showed increased NO_3^- assimilation at higher DO. Multi-omics evidence can strengthen assimilation attribution: in *Klebsiella* sp. KSND, elevated transcription of NADP-glutamate dehydrogenase alongside the non-detection of glutamine synthetase is consistent with assimilation via an NADP-GDH route. In parallel, aerobic NO_3^- dissimilation to ammonium has been demonstrated using ^{15}N isotope tracing and appears feasible under both aerobic and anoxic conditions. Collectively, these observations support a framework in which HNAD performance reflects concurrent sinks and internal recycling loops rather than a single linear pathway (Jin et al., 2019, Ma et al., 2021, Li et al., 2017, Huang et al., 2020b, Yoon et al., 2015).

2.6.2. Phosphorus Removal

AGS has demonstrated excellent capability for P removal from wastewater (de Kreuk et al., 2005 and Yilmaz et al., 2007). Each granule exhibits a stratified structure consisting of an aerobic outer layer, a transitional anoxic zone, and an anaerobic core (Yilmaz et al., 2007), a configuration driven by oxygen diffusion limitations in large granules. This microenvironmental heterogeneity, coupled with sequencing batch operation, enables highly effective P removal. The underlying mechanisms include (i) enhanced biological P removal (EBPR) and (ii) P precipitation within the granular matrix

(Mañas et al., 2011, Jiang et al., 2002, Gao et al., 2011, Adav et al., 2009, Henriët et al., 2016, Filali et al., 2012, Sarma and Tay, 2018). Previous studies have shown that EBPR accounts for the majority of P removal in AGS systems, typically ranging between 55% and 73.7% of the TP (Mañas et al., 2011 and Huang et al., 2015). In EBPR, PAOs play a central role by hydrolyzing intracellular polyphosphates to release energy under anaerobic conditions, followed by excessive P uptake under aerobic conditions, with net removal achieved through sludge discharge. The efficiency of this process is influenced by multiple operational and environmental factors, including temperature, pH, salinity, wastewater composition, organic loading, SBR cycle configuration, and aeration strategy.

2.7. Current and Potential Applications of Aerobic Granular Sludge

AGS has been proposed as a promising alternative to CAS for municipal and industrial wastewater treatment. Despite its advantages, full-scale implementation faces persistent challenges. First, complete conversion of activated sludge into stable granules is not achievable, as the process selectively retains dense biomass with superior settling properties, leading to low SVI. Strategies to accelerate and enhance granulation efficiency therefore remain a key research priority. In addition, AGS cultivation is predominantly performed in SBRs, whereas most existing treatment plants operate with continuous-flow configurations, creating significant barriers for large-scale integration.

Heavy metal discharges are strictly regulated due to their toxicity, persistence, and tendency to biomagnify through plants and animals. Aerobic granulation has emerged as a promising technology for removing metals such as nickel, cadmium, copper, and zinc from wastewater (Sarma et al., 2017). Studies using SEM–EDX demonstrated that heavy metal

biosorption by AGS occurs not only on the granule surface but also within the core (Ahn and Hong, 2015). The process is strongly influenced by functional groups in the granules, such as carboxylate, alcohol, and ether groups, that serve as binding sites for metal ions. Furthermore, the porous structure of AGS facilitates diffusion and transport of metals into deeper layers, enhancing overall uptake capacity. These properties highlight AGS as an effective and robust biosorbent for heavy metal removal, combining structural complexity with functional group diversity to improve treatment efficiency.

Recent studies further examined Ni^{2+} effects on SND, revealing nitrification inhibition at 1–10 mg/L (Feng et al., 2017). They concluded that N removal efficiency under Ni^{2+} stress depended on both the internal microenvironment and microbial community, with aerobic–anoxic zone ratios explaining variations in SND performance. Zheng et al. (2015) reported that Zn^{2+} concentrations ≥ 10 mg/L reduced nutrient removal efficiency, with COD and ammonium removal declining as concentrations increased. In addition, Zn^{2+} was shown to influence EPS production, altering the morphological structure of aerobic granules while simultaneously enhancing tolerance to toxicity. This protective effect was linked to interactions between Zn^{2+} and EPS, which increased the PS/PN ratio.

Copper (II) removal by AGS has been extensively studied. Zheng et al. (2010) reported that Cu^{2+} concentrations of 0.5–3.0 mg/L promoted stable MLSS, improved settleability, and reduced SVI, indicating favorable granule performance. However, excessive Cu^{2+} disrupted microbial metabolism and impaired genetic processes. At concentrations exceeding 50 mg/L, filamentous bacteria dominated the granules, likely reflecting elevated EPS C/N ratios as a protective response to stress. Similarly, when Cu^{2+}

exceeded 10 mg/L, internal filamentous bacteria caused granules to loosen, further compromising stability. These findings suggest that excessive Cu^{2+} concentrations induce structural and functional instability, underscoring the importance of maintaining controlled metal levels in wastewater treatment.

Chromium in wastewater typically occurs as Cr(III) or Cr(VI), originating from anthropogenic activities such as mining, electroplating, pigment production, and metal finishing. Notably, Cr(III) was detected on granule surfaces, indicating in-situ reduction of Cr(VI) to Cr(III) during adsorption (Wang et al., 2014b). Fang et al. (2015) reported that $\text{Cr(VI)} \geq 0.5$ mg/L severely inhibited P removal due to reduced PAO abundance, while GAOs and other microorganisms increased. A similar trend was observed for N removal, as ammonium oxidation, nitrite oxidation, and nitrate reduction rates all declined with higher Cr(VI) concentrations.

2.8. Future Prospects and Challenges of Aerobic Granular Sludge Technology

Over the past two decades, research has established the principal drivers of aerobic granulation and stable operation, including selective pressure through short settling and biomass retention, feast–famine substrate regimes, hydrodynamic shear, and EPS-mediated aggregation, along with the roles of microbial community structure and intracellular storage in shaping granule architecture and function. Remaining challenges are therefore less about identifying the existence of these mechanisms and more about developing predictive, transferable design rules that reliably link operating conditions to start-up time, granule size distribution, and long-term stability under full-scale disturbances. Granule formation can still require extended start-up periods, and long-term stability can be

compromised by disintegration pathways that are not yet predictable or consistently preventable across sites. Despite these challenges, AGS offers clear engineering advantages relative to CAS, including compact biomass with excellent settling, high-rate BNR potential, and demonstrated capacity to treat complex and inhibitory wastewaters when robust granule structure is maintained.

One major challenge in AGS processes is reducing start-up time. Some researchers have seeded reactors with anaerobic granules to accelerate formation, but these often disintegrate under aerobic conditions (Huang et al., 2005 and Muda et al., 2010). Others attempted seeding with mixtures of crushed aerobic granules and floccular sludge (Pijuan et al., 2011), yet this approach requires large amounts of crushed granules, which are rarely available in well-operated reactors. Moreover, reliance on preexisting granules makes this strategy impractical for industrial-scale applications, especially in regions where AGS technology is not yet established.

Alginate has been proposed as an immobilization technique for various granular cell types (Lee and Mooney, 2012 and Ladnorg et al., 2019). Its key structural component, alginate-like exopolysaccharides (ALE), provides granule stability and integrity (Ladnorg et al., 2019). Biosorption studies have further demonstrated that EPS in AGS exhibits sorption capacities over ten times higher than conventional biosorbents (Liu et al., 2015b), underscoring their significant potential for biotechnological and environmental applications. The long maturation time of AGS has been proposed to be addressed by immobilizing activated sludge with high BNR capacity in commercial alginate to form artificial granules (Smidsrød and Skjåk-Bræk, 1990). However, this approach has not yet

proven effective for aerobic sludge granulation. For example, encapsulating cultivated bacteria from AGS in sodium alginate negatively impacted granulation (Zhang et al., 2020). This limitation may stem from the fact that cultured bacteria do not represent the full aerobic sludge consortium, as some cannot be cultivated or may be outcompeted under fixed culture conditions.

Consequently, extending alginate-based artificial granulation to AGS requires further effort, though its application has been limited by the high cost of alginate and calcium chloride for urban wastewater treatment. Nevertheless, given AGS's ability to remove non-biodegradable pollutants such as xenobiotics, metals, and polyvalent cations, processes already associated with high operational costs, the production of “artificial granules” from activated sludge through alginate immobilization remains promising. Industrial wastewaters, such as landfill leachate, often contain high calcium ion concentrations (10–7200 mg/L (Kjeldsen et al., 2002)), which naturally support granulation since Ca^{2+} cross-links alginate polymers. Moreover, integrating autoinducer application to maintain metabolic activity with alginate immobilization to reinforce structural integrity could offer a synergistic strategy. Such combined approaches may significantly advance AGS cultivation, ensuring both functional robustness and physical stability for practical wastewater treatment applications.

In line with the principles of the circular economy, wastewater perception has shifted from being solely an environmental and public health concern to also serving as a source of value-added products. Resource recovery establishes a cyclical link among water, nutrients, and energy, offering pathways to improve sustainability. Currently, sludge

disposal accounts for nearly 50% of total wastewater treatment costs, yet this burden could be reduced if biomaterials recovered from waste sludge were reused (Lin et al., 2015). There remains strong research potential in elucidating the underlying mechanisms of enhanced pollutant removal, identifying and regulating functional genes, and advancing the recovery of high value-added biomaterials (EPS, sodium ALE, PHA, and P) within AGS-based systems. These recovered products have applications across agriculture, construction, and chemical industries (Hamza et al., 2022, Chen et al., 2022, Tavares Ferreira et al., 2021). Therefore, recent studies have focused on enhancing the yield, quality, and practical application of such biomaterials from AGS, highlighting its potential as a sustainable platform for both wastewater treatment and resource recovery.

2.8.1. EPS Production and Recovery

Recently, AGS has attracted interest as a potential source of recoverable EPS (Campo et al., 2022 and de Carvalho et al., 2021). Reported AGS sludge yields can reach 0.12–0.14 kg TSS·kg⁻¹ COD, with EPS constituting a substantial fraction of the dry mass (Feng et al., 2019). This is consistent with emerging commercial developments in granular-sludge biopolymer recovery (e.g., Kaumera), which are positioned not only around product valorization but also around reducing sludge processing/disposal burdens. Methods are generally grouped as physical, chemical, or biological. Physical approaches (e.g., centrifugation, ultrasound, heating) preserve cell integrity but yield limited EPS, while chemical methods (e.g., chelators, cation resins, acidic or basic agents) achieve higher dissolution. Biological approaches rely on enzymatic hydrolysis to selectively break matrix bonds (Feng et al., 2021).

Chemical extraction generally yields higher EPS recovery from AGS because structural EPS is chemically dissolved, allowing greater release (Tan et al., 2021). However, the composition and concentration of EPS are strongly influenced by extraction and purification strategies, including preparation, enrichment, and recovery steps (Feng et al., 2021). Alkaline conditions facilitate EPS release by enhancing the solubilization of negatively charged components, breaking disulfide bonds in proteins, and removing hydrolyzed sugars via β -elimination, thereby dissolving the hydrogel matrix (Tan et al., 2021). Similarly, alkali treatment and cation exchange resins disrupt ionic interactions within biopolymers, while aldehyde reagents (e.g., formamide, formaldehyde) minimize cell lysis and stabilize cells during extraction. These aldehyde methods are often combined with alkaline treatments to improve yield while preserving cell integrity. Despite advances, EPS extraction methods remain uncommon at full scale.

2.8.2. PHAs Production

PHAs are biopolymers synthesized by diverse microorganisms as intracellular reserves under nutrient-limiting conditions (Mannina et al., 2020). They are biodegradable and hold promise as sustainable alternatives to conventional plastics; however, large-scale commercialization remains constrained by high production costs. These costs are largely due to three factors: (1) the requirement for sterile cultivation of pure or engineered strains at industrial scale, (2) reliance on expensive carbon substrates (about 40% of production costs), and (3) the expense of extraction and purification (Kourmentza et al., 2017 and Samorì et al., 2015). To overcome these challenges, previous study proposed using mixed microbial cultures under optimized conditions of temperature, concentration, and contact

time, achieving 74% recovery and 100% purity (Mannina et al., 2020). This approach not only reduces costs but also integrates directly with the treatment of organic waste and influents biologically, offering both environmental and economic advantages.

The ability of AGS to retain high biomass levels provides a competitive edge for PHA production. Since all treatment processes occur within one treatment unit (a single reactor), both land footprint and operational costs can be reduced if similar levels of PHA accumulation are maintained in the sludge system (Tavares Ferreira et al., 2021). The extraction processes of PHA typically involves two stages: cell digestion followed by solvent extraction (Tan et al., 2014). Samorì et al. (2015) reported that the initial treatment with sodium hypochlorite, combined with dimethyl carbonate extraction, achieved the highest recovery rate up to 82%, while also mitigating adverse impacts associated with solvent dissolution.

2.8.4. Phosphorus Recovery

Well-developed, full-scale P recovery trains typically combine biological P release (to concentrate soluble orthophosphate) with controlled precipitation/crystallization (to recover a fertilizer-grade mineral). WASSTRIP and PhoStrip (**Figure 2.7**) are established sludge-fermentation “P-conversion” processes that exploit PAOs’ ability to hydrolyze and release stored poly-P anaerobically in exchange for a carbon substrate. They retain (EBPR) sludge in an anaerobic “stripper” tank and may add acetate or divert influent to stimulate poly-P release, with PhoStrip reported to release up to 67% of P from activated and EBPR sludge (van Loosdrecht et al., 1997). Practical constraints are noted, including the need for substantial chemicals for neutralization where stripper alkalinity/pH is high (van

Loosdrecht et al., 1997 and Wang et al., 2005), and the fact that these approaches are most applicable to polyphosphate-rich EBPR sludges and are not designed to solubilize other non-reactive P fractions (Kroiss et al., 2011).

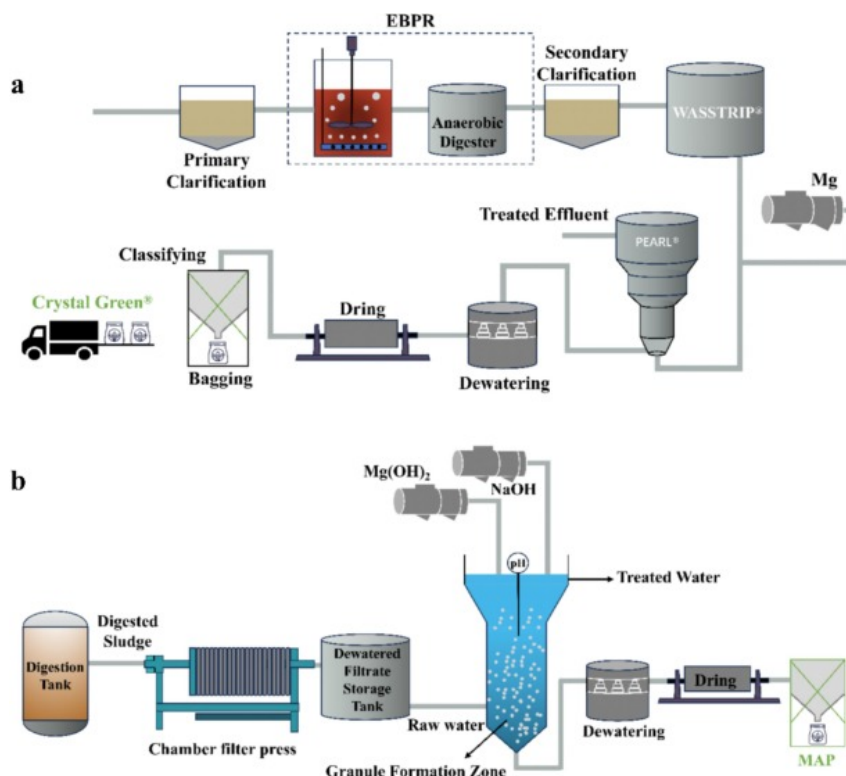


Figure 2.7: Two processes for P recovery from digested sludge liquor: (a) The Ostara Pearl® process and (b) The Phosnix process (source: adapted from Guo, Y. et al. (2026))

After generating a P-rich sidestream (e.g., stripper supernatant or dewatering liquor), many plants apply struvite crystallization ($MgNH_4PO_4 \cdot 6H_2O$) as a selective recovery route that produces a slow-release fertilizer and reduces uncontrolled scaling risk (Schröder et al., 2010). Struvite precipitation underpins multiple commercial systems (Figure 2.7), including PHOSNIX, Rem-Nut, and Ostara processes (Schröder et al., 2010 and USEPA, 2010), while calcium phosphate products (e.g., hydroxyapatite) are also cited

as alternatives that can substitute for mined phosphate rock in fertilizer manufacturing (Morse et al., 1998). The operational motivation is also practical: when P is released during digestion, spontaneous struvite can form and scale pipes/pumps, creating maintenance burdens (Le Corre et al., 2009, Ohlinger et al., 1998, Martí et al., 2008), so upstream P capture can be advantageous. A widely cited example is the Netherlands “nutrition factory” concept, where WASSTRIP is used to separate P from excess sludge to form a P-rich supernatant prior to digestion, and Pearl (Ostara) treats high-P/high-N sidestreams to recover >90% of P in the supernatant (Zhu et al., 2021).

P recovery from AGS was first reported by Yilmaz et al. (2007). Subsequent studies showed that in sludge highly enriched in PAOs (e.g., 60–90% PAOs) obtained using synthetic feed containing acetate/propionate, P stored as polyphosphate can represent 15–20% of PAO cell dry weight. However, in EBPR sludges treating domestic wastewater, PAO abundance is typically lower (e.g., *Accumulibacter* often represents 5–20% of the bacterial community), and the bulk sludge P content is correspondingly lower but can still reach 5–7% (dry weight) compared with 1–2% in non-EBPR sludges. Therefore, surplus biosolids discharged from AGS/EBPR systems can still represent a meaningful concentrated P stream for downstream release and recovery (Yuan et al., 2012). This makes the surplus sludge discharged from AGS systems a valuable source for P recovery.

The feasibility of recovering P from P-rich AGS obtained over the treatment process of the wastewater has been demonstrated, where phosphorus can be recovered from the granules through several approaches: (i) Post-crystallization chemical precipitation, (ii) direct application of sludge to soil, (iii) recovery using biosorbents derived from natural

polymers, and (iv) incineration with subsequent recovery from sludge ash (Tavares Ferreira et al., 2021). Among these, struvite precipitation has been widely recognized as an effective method for recovering P from wastewater (Parsons and Smith, 2008). For example, Lu et al. (2016) proposed a synchronous nitrification–denitrification P removal process that eliminated the need for excess sludge production. In this system, 93.19% of influent P was released as orthophosphate into the liquid phase, enabling efficient recovery through struvite precipitation.

2.9. Concluding Summary and Engineering Implications

Over the past two decades, AGS has emerged as a compact, high-rate alternative to CAS, enabling simultaneous BNR in a single reactor; granulation is now understood as a stepwise process in which pili/adhesin-mediated co-aggregation and early EPS/eDNA stabilization progress to dense micro-aggregates shaped by hydrodynamic shear and selective washout under feast–famine regimes, creating stratified microenvironments that support complementary microbial guilds whose balance governs granule compactness, cohesion, and treatment performance, while key operating levers directly link microbial competition to granule structure (size, density, EPS chemistry) and long-term stability. The main forward challenge is transferring these SBR-derived selection principles to full-scale, especially continuous-flow plants where weaker cycling signals and different hydrodynamics can erode selection pressure and promote surface feast, filament enrichment, and destabilization; practical solutions include incorporating anaerobic/selective zones, applying pulsed or step-feed strategies to sharpen substrate gradients, using selective retention (e.g., hydrocyclones or size/density-based wasting) to

retain mature granules and remove fines, tuning mixing/airlift intensity and inlet geometry to balance shear with breakage risk, and implementing online DO/ORP control with soft sensors to identify feast–famine endpoints and stabilize redox stratification under variable loads.

Targeted knowledge gaps now limit confident design rules for plant-ready AGS in municipal applications, particularly for (i) cultivating and sustaining stable, rapidly settling granules under high organic loading as a practical route to mainstream deployment, and (ii) defining operational frameworks that translate this robustness to low-strength municipal influents while preserving structural integrity and treatment reliability. The near-term trajectory is therefore twofold: (1) leverage AGS rapid settling and selective retention to test aerobic P-starvation as an intentional selection pressure in the mainstream by implementing P-rich liquid extraction, and quantify its ability to deliver stable EBPR without granule destabilization; and (2) operationalize the extracted P-rich sidestream as a resource, establishing realistic pathways and performance envelopes for P recovery that can be integrated with AGS-based municipal wastewater treatment.

CHAPTER 3: FEASIBILITY STUDY OF APPLYING ANAEROBIC STEP-FEEDING MODE FOR THE TREATMENT OF HIGH- STRENGTH WASTEWATER IN GRANULAR SEQUENCING BATCH REACTORS (GSBRs)

To the best of our knowledge, currently, there is limited information on the effect of applying anaerobic step feeding strategy on matured AGS cultivated and matured under the effect of treatment of high-organic loads in GSBRs systems at the same operational conditions. Thus, this chapter focuses on investigating the potential enhancement in the substrate and nutrients removal performance of large AGS after changing feeding strategy of these GSBRs from fast single feeding mode followed by anaerobic mixing to step-feeding distributed throughout the anaerobic stage, when applied under the same previous operational conditions for treatment of high-strength wastewater.

3.1. Introduction

AGS is an economically and environmentally promising replacement for CAS, for purification of different types of wastewaters (e.g., municipal and heavily loaded industrial wastewater) (Nancharaiah and Reddy, 2018). Layered structure of AGS is known for high removal performance of different nutrients via multiple biological processes (e.g., nitrification and denitrification) occurring in a single tank (Gao et al., 2011). AGS system has biofilms composed of auto-immobilized cells that are generated during treatment in a SBR, where the clarifier is eliminated, and a settling phase is included in the reactor schedule. Thus, a return activated sludge line is not applicable, depending on the amount

of biomass retained in the reactor after decanting. By switching to aerobic granulation system, daily produced biomass is also decreased leading to reduction in the overall cost of up to 25% and energy requirement up to 30% (Sarma and Tay, 2018a). Since the inception of AGS in the late 1990s as an innovative technology (Sarma and Tay, 2018a), it has been applied successfully for the treatment of different influents; however, there are some unresolved problems such as granule disintegration, long period of granule formation when using sewage with high organic loads, and nitrite accumulation (Sarma et al., 2017).

The literature review findings show the cultivation of AGS in SBR by investigating the impact of various operational parameters that can control the granulation process (i.e., formation and stability), such as alternating feast and famine conditions, reactor configuration (H/D ratio, HRT, and VER), hydrodynamic shear forces, influent characteristics (COD:TN:TP ratio), and influent distribution (influence the morphology and microbial community of AGS), selective wasting (in most GSBRS, settling time is the selection pressure for granulation process), organic loading rates, biomass characteristics, and other environmental factors (e.g., dissolved oxygen, pH, and temperature).

Different studies have reported varying conditions for the development of aerobic granulation process. For example, R.A. Hamza et al. (2018) investigated the long-term stability of AGS treating high organic loads through a semi-pilot scale SBR and found that F/M ratio has played a key role in the formation and stability of granules (Hamza et al., 2018). At F/M ratios between 0.5 and 1.4 gCOD/gVSS·d, stable granules with excellent settleability were maintained, while fluffy granules were caused when the F/M value exceeded 2.2 gCOD/gVSS·d. Another study demonstrated that utilizing longer HRT caused

a reduction in the applied OLR, which was found to be more effective for granulation process by providing longer starvation time (Corsino et al., 2017).

Stable AGS was achieved successfully in a previous study by applying low hydrodynamic shear (0.4 cm/s) (Henriet et al., 2016). In addition, successful removal of the fast-growing organisms (e.g., ordinary heterotrophic organisms (OHO)) from granules' surface has been achieved by applying higher hydrodynamic shear (Show et al., 2012 and Devlin et al., 2017). A lot of challenges are related to an efficient and continuous application of the obtained mature granules in the treatment processes. For example, causes of granule instability have also been studied and linked to a range of factors such as: proliferation of filamentous bacteria at high OLR (Show et al., 2012 and Devlin et al., 2017), as well as decrease and destruction in the structure of EPS, with the decrease in protein-to-carbohydrate ratio (proteins play an essential role in granule stability) (Long et al., 2015a,b and Kang and Yuan, 2017). EPS in the aerobic granulation process, acts similarly to a 'glue' to induce biofilm formation among the microorganisms, where EPS are synthesized as a derivative of organic compounds, such as proteins, polysaccharides, lipids, glycoproteins, nucleic acids (Tay et al., 2001 and Rollemberg et al., 2018). It depends on several factors, including aeration time, cycle duration, superficial air velocity, reactor conditions, and inoculum sludge that affect EPS production and composition.

Feeding mode is one of the key factors in the selection, formation, and stability of AGS. GSBRS are categorized into two types: continuously aerated, and anaerobic influent distribution. Anaerobic filling in GSBRS has been widely accepted, in which the duration and influent load are considered efficiency determining factors. A few previous studies

have demonstrated that step-feeding can be an efficient strategy to remove organic matter and nutrients simultaneously, especially with great potential to favor granulation in effluents with high OLRs (e.g., sanitary landfill leachate and high-strength industrial discharges). For example, another previous study has evaluated the effect of filling mode on the performance and granules' operational stability to treat high-strength ammonium concentrations through three SBRs: R1, fast feeding in the anaerobic period (20 min); R2, slow feeding in the anaerobic period (40 min); and R3, distribution of feeding throughout the cycle (da Silva et al., 2021). Results of step-feeding in R3 were the best, where the problems such as granules stability, biomass retention, and nitrite accumulation were minimized while treating leachate, as well as higher nutrients removal efficiencies were achieved compared to R1 and R2. In addition, the results from this study showed the positive impact of applying the strategy of step-feeding in R3 compared to R1 and R2, where the approach of step-feeding distributed throughout the cycle of operation was an excellent method to develop the sedimentability of biomass. Moreover, this strategy enhanced the proliferation of slow-growing organisms through the prevention of growing ordinary and fast-growing heterotrophic bacteria (da Silva et al., 2021).

The effect of different feeding strategies on the proliferation dynamics of PAOs and P removal has also been investigated (Iorhemen et al., 2022). Herein, three feeding strategies (e.g., anaerobic slow feeding, pulse feeding followed by anaerobic mixing, and pulse feeding) were applied through GSBRS. The obtained results show that feeding strategies had no impact on organic and ammonia removal and did not alter the microbial community. However, P removal performance has been significantly influenced. Reactors that have anaerobic slow feeding and pulse feeding followed by anaerobic mixing

demonstrated high P recovery from wasted sludge; therefore, active PAOs can be accumulated, and GAOs can be significantly inhibited within the bioreactor leading to high specific P uptake and specific P release rates.

3.2. Material and Methods

3.2.1. Experimental Set-Up

The experiments were conducted through two SBRs named R1 and R2 (cylindrical acrylic reactors) seeded with the same biomass (R1 and R2 in operation are shown in **Figure A1—Appendix A**). The reactors had a 6.0 L capacity, with working volume of 5.0 L, internal diameter of 12 cm, and H/D ratio of 3.7. VER of 60% was ensured through discharging the top 3 L of supernatant in 10 min following a 10 min of settling period. Reactors were operated with 8 h cycles daily including 10 min feeding, 95 min of anaerobic reaction, 350 min of aeration phase, and 20 min of settling and discharging, as well as 5 min of an idle stage before starting a new cycle. Pumping influents and discharging effluents was performed by peristaltic pumps. Treatment cycles were automated using outlet timers (Traceable Digital Outlet Controller, Fisher Scientific, CA).

Nitrogen gas was used during the anaerobic phase and fed from the bottom of reactors to create anoxic environment (DO levels were ≤ 0.1 mg/L during the total period of anaerobic mixing), and to avoid biomass sedimentation since complete mixing using nitrogen gas enhances the contact between cells and nutrients and improves removal efficiency. Moreover, during the aerobic phase, fine air bubbles were introduced from the bottom of the reactors (DO levels were between 1.5–2.0 mg/L). Both nitrogen gas and air were introduced at the same level with a flow rate of 2.92 L/min resulting in superficial air

velocity of 0.43 cm/s to induce hydrodynamic shear to achieve compact granules with desirable surface properties (Devlin et al., 2017, Long et al., 2015a,b, Kang and Yuan, 2017). The following schematic diagram (**Figure 3.1**) describes the GSBRSs used in this study.

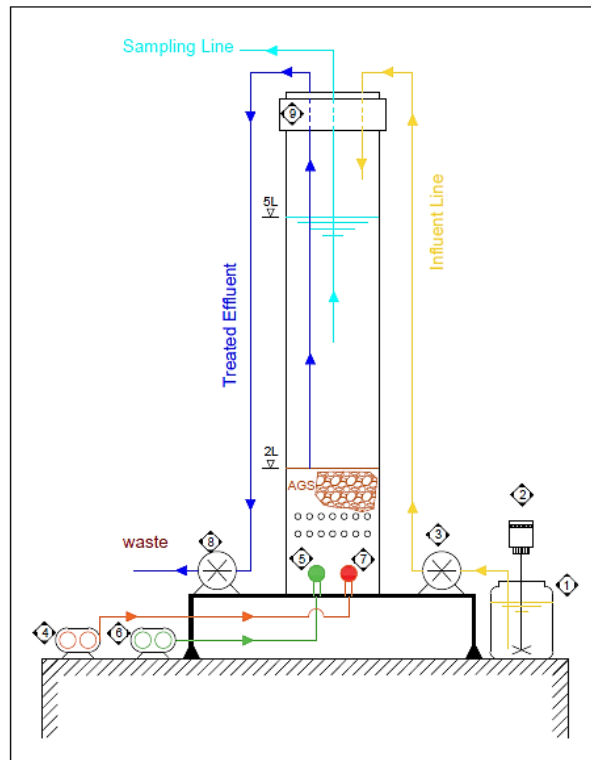


Figure 3.1: Schematic of the GSBRS in this study; (1) feeding tank; (2) mixer; (3) feeding pump; (4) nitrogen pump; (5) nitrogen diffuser; (6) air pump; (7) air diffuser; (8) decant pump; and (9) reactor's cap

The total experiment duration for reactors was 360 days to treat high-strength synthetic wastewater (**Table 3.1**) under the same operational conditions: (a) 125 days for stage I (Cultivation stage: formation of AGS) after inoculation with the same sludge for R1 and R2, (b) 175 days for stage II (Maturation stage: operation under the same conditions), and (c) 60 days for stage III (R1 continued working under the same operational conditions

of stage II, while R2 operated through new anaerobic step-feeding strategy for 60 days). Acclimation period for the first one was two weeks of adaptation after inoculation, while the second period was 10 days after changing filling mode of R2.

The feeding mode of R2 only was changed from fast single feeding mode followed by anaerobic mixing to anaerobic step-feeding strategy, while R1 operated with the same previous filling mode. As mentioned previously, the discharge is 3 L/cycle, and anaerobic stage is performed in 95 min for each cycle. Therefore, a liter is pumped to R2 at the beginning, and another liter is added after 30 min of anaerobic mixing, and the third liter is added after another 30 min. It is important to mention that 10 days after changing the filling mode of R2 was taken as an adaptation period before performing the required measurements of different parameters during this stage.

Table 3.1: Operation stages for R1 and R2

Period (Start and End Days)		Conditions of GSBRS
Stage I Cultivation	125 days Day 1 to 125	- Two weeks for acclimation of microorganisms after inoculation - 75 days as startup period for R1 and R2 - Cultivation of large AGS were achieved by the day 125 (size ≥ 2 mm) - Observation of physical properties (size, sludge volume indexes (SVI₅ and SVI₃₀)), and performance (nutrients removal).
Stage II Maturation	175 days Day 126 to 300	- Reactors are operated under the same conditions as stage I - Maturation of the granules were achieved by day 175 (size ≥ 3 mm) - Observation of physical properties (size, SVI₅, and SVI₃₀), and performance (nutrients removal), as well as monitoring stability of matured granules.
Stage III Operation under Step- feeding	60 days Day 301 to 360	- R2 only changed to anaerobic step-feeding, then 10 days for adaptation - R1 has been operated under the same conditions of stages I and II - Observation of physical properties (size, SVI₅, and SVI₃₀), and performance (nutrients removal), as well as monitoring stability of matured granules.

The start-up of GSBRS with respect to both physical properties and substrate/nutrient removal is described above. Furthermore, the settling parameters $SVI_{30} < 90 \text{ mL/g}$ and $SVI_{30}/SVI_5 \text{ ratio} > 0.7$, the size fraction $d > 0.5 \text{ mm}$ constituting at least 50% of total suspended solids (TSS), granule appearance based on microscopic images, and stable substrate and nutrient removal are provided in appendix A.

3.2.2. Sludge Source and Synthetic Wastewater Composition

The same volume (2 L) of return activated sludge (RAS) was used to seed both reactors, which was obtained from the West End Water Pollution Control Centre in Winnipeg, MB, Canada. The mixed liquor suspended solids (MLSS) of the inoculated RAS was measured as 8.1 g/L (average of triplicates samples). It is important to mention that particulate matter was not used in the substrate of the influent since it has been demonstrated to affect surface properties of granules and would therefore affect the results. During the entire operational period, GSBRS were treating high-strength synthetic wastewater containing an average of $1250 \pm 70 \text{ mg/L}$ of sCOD, $165 \pm 36 \text{ mg/L}$ of TN, and $54 \pm 11 \text{ mg/L}$ of TP.

The synthetic municipal wastewater feed recipe was based on a previous study (Kang and Yuan, 2017), which used high-strength synthetic wastewater; therefore, the influent synthetic wastewater comprised 1250 mg/L of yeast extract, 33 mg/L of ammonium chloride, and 9 mg/L of dipotassium phosphate. Other components such as $MgSO_4$ (5.8 mg/L), $CaCl_2$ (12 mg/L) and mineral solution (0.3 mg/L) containing 0.15 g/L H_3BO_3 , 0.03 g/L $CuSO_4 \cdot 5H_2O$, 0.03 g/L KI, 0.12 g/L $MnCl_2 \cdot 4H_2O$, 0.06 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.12 g/L $ZnSO_4 \cdot 7H_2O$, 0.15 g/L $CoCl_2 \cdot 2H_2O$, and 1.5g/L $FeCl_3 \cdot 6H_2O$

were also added. Ethylenediaminetetraacetic acid (EDTA: 3 mg/L) is used to prevent the precipitation of salts, such as calcium and orthophosphate ($\text{PO}_4^{3-}\text{-P}$) present in the media. Sodium bicarbonate ($12\text{g NaHCO}_3/\text{g NH}_4^+\text{-N}$) was added to the influent each cycle during the entire experimental duration to achieve sufficient alkalinity ($7.14\text{ g CaCO}_3/\text{g NH}_4^+\text{-N}$) and subsequently achieve complete nitrification (Kang and Yuan, 2017).

3.2.3. Analytical Methods

The dissolved oxygen and pH were measured near the center of the GSBs using a DO probe and a pH probe. The concentrations of MLSS, MLVSS, total suspended solids of the effluent (TSS_{eff}), chemical oxygen demand of the influent and effluent (sCOD_{inf} , sCOD_{eff}) and Sludge Volume Index after 5 and 30 min (SVI_5 and SVI_{30}) of settling were measured according to standard methods (APHA, AWWA and WEF, 2012). Concentrations of phosphorous in form of $\text{PO}_4^{3-}\text{-P}$ and nitrogen in forms of ammonium, nitrite, and nitrate for the influent and effluent were measured via a flow injection analyzer (Lachat Quick Chem 8500, HACH, CA). Kinetic analysis was performed during operational cycles through the measurements for N and P using filtered samples (soluble concentration) through medium porosity Q5 filter paper (Fisher Scientific, CA).

Granules size was measured to estimate the development of the granulation process and granules' stability through microscopic images in SteREO Discovery (Axio Vert.A1, ZEISS). Moreover, surface morphology is investigated using the microscopic analyses on weekly to bi-weekly basis. In this study, we established that the mature aerobic granulation has been achieved (i.e., start of stage II) only when more than 80% of the biomass in R1 and R2 had a diameter greater than 0.2 mm (de Kreuk et al., 2007), and both of sludge

volume indices (SVI₅ and SVI₃₀) are lower than 50 mL/g. It is worth mentioning that the cycle tests were conducted more than three times weekly during the whole period of the experiment (i.e., stage I, II, and III) in order to investigate the performance of GSBRS and understand the mechanisms of simultaneous nitrification, denitrification, and P removal.

Analysis of the EPS content of the AGS has been performed according to (Adav et al., 2008). For the extraction, low frequency ultrasound at 120 W for 5 min in an ice bath (before adding chemicals) extraction was applied for 10 mL of mixed liquor samples. Pre-treatment for the mixed liquor samples using 0.06 mL formaldehyde was conducted prior to storing these samples for 1 h at 4 °C. For the next step, 4 mL of NaOH is added, and stored for 3 h at 4 °C. EPS content was physically extracted at 10,000 rpm for 20 min at the end. Following the extraction, the supernatant for all samples was filtered through 0.2 µm filters to collect soluble fractions. The extracted samples were stored at -20 °C in aliquots until analyses were performed.

3.3. Results and Discussion

Firstly, there was a strong convergence in the results (as shown in **Table 3.2**) such as physical properties (size, SVI₅, and SVI₃₀), and performance (i.e., removal of organics and nutrients) of R1 and R2 over a period of 300 days of operation after inoculation (through cultivation and maturation of AGS until changing the filling mode of R2 on day 301). This was due to both GSBRS worked under the same operating conditions (e.g., reactors configuration, design parameters, influent characteristics, seeding sludge, etc.).

The mean value of the measured pH through the anaerobic phase for both reactors during cultivation period (first 125 days after inoculation) was about 8.9, and it decreased through the aerobic phase to 8.2. During the maturation stage (day 126 to 300), these values were further decreased to 8.3 and 7.9 through the anaerobic and aerobic phases each cycle. It is worth mentioning that DO concentrations have been measured during the entire period of operation for both reactors in order to continuously monitor the aerobic and anaerobic conditions during operational cycles. DO concentrations during anaerobic phase did not exceed 0.1 mg/L, while it ranged between 1.5–2.0 mg/L during the aerobic phase. It is important to mention that the experiment was conducted at room temperature (18 ± 2 °C).

Table 3.2: Removal efficiencies of sCOD, NH_4^+ -N, and PO_4^{3-} -P in R1 and R2

Characteristics	Stage I		Stage II		Stage III	
	R1	R2	R1	R2	R1	R2
sCOD _{inf} (mg/L)	1250 ± 70 (COD = 1392 ± 44)					
sCOD _{eff} (mg/L)	85.2 ± 15	74.8 ± 11	68 ± 16	70 ± 15	60 ± 10	64 ± 11
sCOD removal (%)	93.2 ± 1.3	94 ± 0.8	94.6 ± 1.1	93.8 ± 1.4	95.4 ± 1.2	94.5 ± 1.0
NH_4^+ -N _{inf} (mg/L)	115.2 ± 4.6					
NH_4^+ -N _{eff} (mg/L)	46.1 ± 8	41.5 ± 9	20.43 ± 7.2	18.83 ± 5.7	12.42 ± 3.6	24.27 ± 6.5
NH_4^+ -N removal (%)	55.2 ± 2	55.3 ± 7	82.2 ± 6.2	83.6 ± 5	89.2 ± 1.2	79 ± 1.6
NO_2^+ -N _{eff} (mg/L)	27.9 ± 25	25.2 ± 22	14.8 ± 8.1	13.3 ± 9.6	0.67 ± 1.0	10.6 ± 1.3
NO_3^+ -N _{eff} (mg/L)	1.6 ± 1.8	1.9 ± 2.55	27.2 ± 12.1	25.43 ± 14.6	0.88 ± 1.3	47.2 ± 2.1
PO_4^{3-} -P _{inf} (mg/L)	17.02 ± 0.9					
PO_4^{3-} -P _{eff} (mg/L)	9.68 ± 2.3	9.8 ± 1.7	4.13 ± 0.8	4.3 ± 0.1	1.39 ± 0.13	9.23 ± 1.4
PO_4^{3-} -P removal (%)	42.8 ± 5.9	42 ± 7.2	75.6 ± 5.9	74.5 ± 9.6	92.1 ± 1.2	46.43 ± 2.7

3.3.1. Granulation Processes

The seed sludge was dark brown in colour and had MLSS, mixed liquor volatile suspended solids (MLVSS), and SVI_{30} of 8100 mg/L, 6955 mg/L, and 128 mL/g, respectively (average of triplicates samples), as well as a mean particle size of 115 μm . The two SBRs were initially operated with the same volume (2 L). During the acclimation period (15 days), the MLSS concentrations were gradually reduced in GSBs accompanied with higher TSS_{eff} (974 ± 122 mg/L). The reason for this reduction is due to the impact of applying short sedimentation phase (10 min) during the cycle operation, which is considered as the selection pressure and a key strategy for developing aerobic granulation in order to wash out the poor settling biomass. However, after adaptation period and even after biomass stabilization, higher amounts of solids existed in the effluents. In other words, constant washout sludge was observed in R1 and R2. This is consistent with previous studies of AGS systems applied to treat high-strength wastewaters (Leal et al., 2020 and Leal et al., 2020). Factually, 75 days were estimated as a startup period for both reactors.

Biomass characteristics (MLSS, SVI_5 , and SVI_{30}) for R1 and R2 are presented in **Figure 3.2** and **Table 3.3**. As mentioned, MLSS concentrations continued to decrease in both reactors from the first day of operation until day 50. During the same stage, in terms of sedimentability and according to previous studies, various researchers demonstrated that the SVI_{30}/SVI_{10} or SVI_{30}/SVI_5 ratios can be considered good indicators of granulation, meaning that a value closer to 1.0 indicates that the sludge consists mainly of mature granules (Zhang et al., 2015, de Kreuk and van Loosdrecht, 2004, Rollemberg et al., 2019, Corsino et al., 2018). It took about 63 days for flocculent sludge (SVI_{30} more than 125 mL/g and SVI_{30}/SVI_5 ratio lower than 0.4) to convert to small aerobic granules, as shown

in **Figure 3.2** and **Figure 3.3** (SVI_{30} is 85 mL/g and SVI_{30}/SVI_5 ratio is 0.52 for R1, while SVI_{30} is 93 mL/g and SVI_{30}/SVI_5 ratio is 0.56 for R2). Measurements of the size of AGS that were performed at day 50 were exceeded by 0.5 mm for R1 and R2, meeting the requirement for granular sludge. By the end of cultivation stage (day 125), biomass concentrations (MLSS) increased because of the granules' superior settleability and high biomass density and reached 10 g/L and 11.8 g/L for R1 and R2, respectively, which is consistent with previous studies (de Kreuk and van Loosdrecht, 2004). Moreover, large AGS have been achieved in both GSBRS $SVI_{30} = 48.2$ mL/g and SVI_{30}/SVI_5 ratio = 0.78 for R1, and $SVI_{30} = 58.5$ mL/g and SVI_{30}/SVI_5 ratio = 0.73 for R2. While granules' size reached more than 2.22 mm and 2.13 mm for R1 and R2, respectively, by day 125.

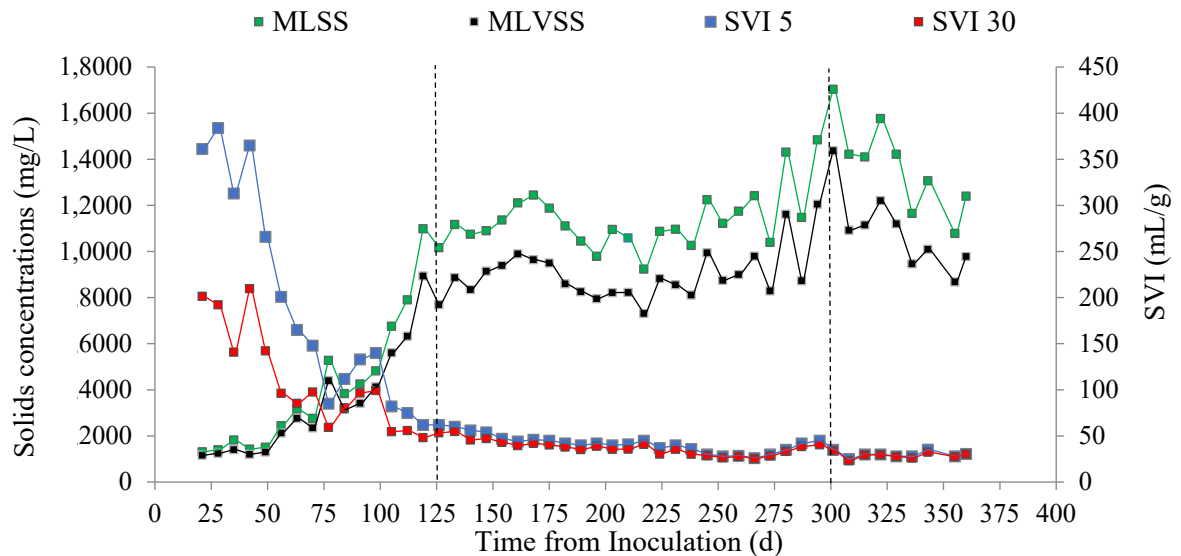


Figure 3.2: Stability and characteristics of AGS over the entire operational period for R1.

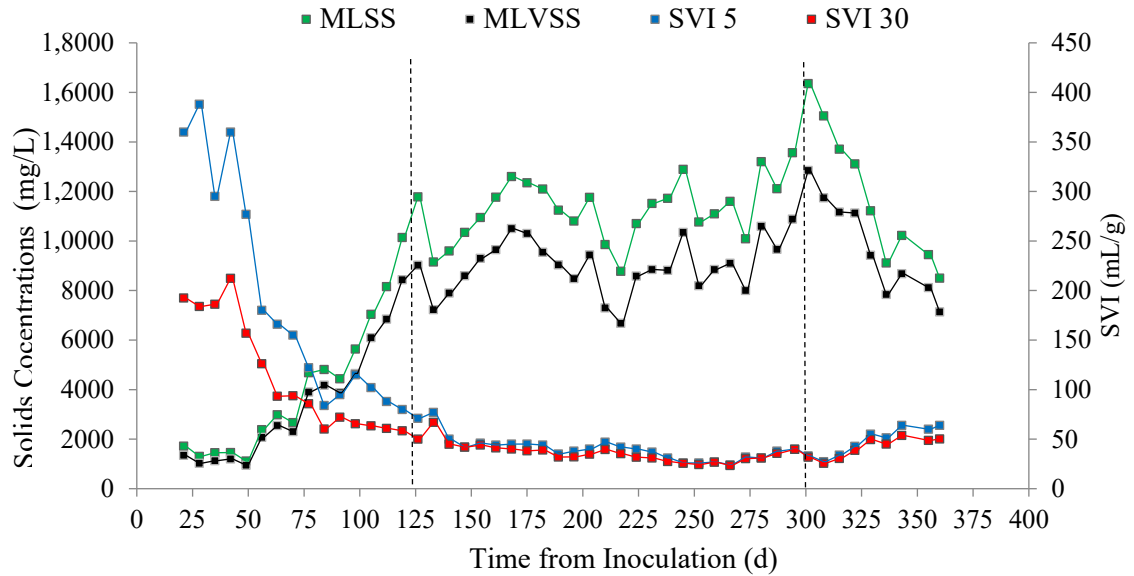


Figure 3.3: Stability and characteristics of AGS over the entire operational period for R2.

The biomass concentrations for R1 and R2 were 10164 ± 111 mg/L and 9156 ± 157 mg/L, respectively, on day 125 (end of cultivation stage). During the following period (day 126–300), biomass accumulation continued and directly increased biomass concentrations for GSBRS. **Table 3.3** demonstrates the average MLSS concentrations during the whole maturation period. MLSS and MLVSS of both reactors are shown in **Figure 3.2** and **Figure 3.3**. The rise in biomass concentrations were achieved during the same period on day 294, where the highest MLSS was 14844 ± 78 mg/L for R1, and 13564 ± 128 mg/L for R2. This is a direct result from continued biomass accumulation. In this context, there were fluctuations in the TSS_{eff} for R1 and R2 which can be attributed to applying shorter settling time (10 min) during the operational cycle (i.e., discharging of poor settling biomass selection pressure). The average concentrations of TSS_{eff} for R1 and R2 during cultivation period (1–125 day) ranged between 175 and 190 mg/L, respectively (except the first 42 days after inoculation since there were high concentrations of TSS_{eff} and VSS_{eff} of reactors

due to discharging the poor settling flocs). In addition, based on the concentration of TSS_{eff} and MLSS, the average SRT was calculated during the cultivation period (day 15–125) of 19 ± 1 day and 18 ± 1 day for R1 and R2, respectively, while average SRT during the maturation stage (day 126–300) was 30 days for R1 and R2 (**Table 3.3**).

Table 3.3: AGS characteristics (mean values) throughout the operational period for R1 and R2

Characteristics	Stage I		Stage II		Stage III	
	R1	R2	R1	R2	R1	R2
SVI ₅ (mL/g)	62 ± 4.8 at day 120	80 ± 8.8 at day 120	40.85 ± 4.5	39.2 ± 6.2	29.34 ± 1.5	49.91 ± 5.8
SVI ₃₀ (mL/g)	48.2 ± 12.1 at day 120	58.5 ± 10.8 at day 120	36.62 ± 3.8	35.56 ± 2.1	28.27 ± 1	42.77 ± 6
Mean diameter (mm)	2.22 ± 0.13 day 120	2.02 ± 0.2 day 120	3.02 ± 0.01 at day 300	3.08 ± 0.6 at day 300	3.13 ± 0.08 at day 360	2.94 ± 0.11 at day 360
EPS content (mg/gVSS)	130.78 ± 41.6	141.62 ± 90.1	203.7 ± 23.1	207.9 ± 35.3	228.6 ± 11.4	194.6 ± 15.6
F/M ratio (gCOD/gVSS·d)	0.437 ± 0.77	0.398 ± 0.28	0.24 ± 0.1	0.24 ± 0.06	0.196 ± 0.04	0.22 ± 0.07
OLR (Kg COD·m ⁻³ ·d ⁻¹)	2.16 ± 0.25					
MLSS (mg/L)	6265 ± 2523	6415 ± 2136	11348 ± 1260	11295 ± 1219	13695 ± 1964	10860 ± 2808
TSS_{eff}^* (mg/L)	175 ± 22	189 ± 13	200 ± 18	204 ± 15	188 ± 11	244 ± 26
HRT (h)	13.33					
Y_{obs}^{**}	0.113	0.123	0.129		0.115	0.156
SRT (d)	19 ± 1	18 ± 1	30 ± 2	31 ± 2	29 ± 1	24 ± 1

* TSS_{eff} was calculated as an average value only during the stable performance (steady state)

** The observed biomass yield (Y_{obs}) was calculated as an average value during the last months of operation through each stage by dividing the net volatile solids in total biomass (ΔVSS) by the net COD consumption (ΔCOD).

It has been demonstrated through several studies that higher F/M ratios favor formation and development of the AGS because of the enhanced EPS production (Kang and Yuan, 2017). Therefore, it should be noted that through the first stage (cultivation stage—day 125) and under continuous treatment of high organic loads (2.25 Kg COD/m³·d), the gradual increase in the biomass concentrations (MLSS and MLVSS) might be the reason for the delay in the formation of large granules. F/M ratio decreased from 1.71±0.11 gCOD/gVSS·d for R1 and 1.35±0.18 gCOD/gVSS·d for R2 (at the beginning of cultivation period) to 0.29±0.1 gCOD/gVSS·d for R1 and 0.19±0.12 gCOD/gVSS·d for R2 (day 125). Consequently, this led to lower production of EPS compared to the following stage (maturation) as shown in **Table 3.3** and **Figure 3.4 (a, b)**.

Regarding EPS, these components are biopolymers consisting of polysaccharides, proteins, and other substances, which play a crucial role in the granules' structure, production, and stability. Several studies have shown that stable AGS have higher PN structures than PS, and PN/PS ratio is a way of characterizing its stability (Rollemberg et al., 2018). PN/PS ratio higher than 25 (Kang and Yuan, 2017), as well as lower values of 4.8 (Wei et al., 2014) and 0.6 (Ren et al., 2017a,b) have been reported. The concentrations of EPS are presented in **Figure 3.4 (a, b)**. In the current study, EPS has been estimated as a combination of PN and PS. EPS ranged between 36±1.1 mg/gVSS (PN/PS ratio of 1.11) for R1 and 31±0.8 mg/gVSS (PN/PS ratio of 1.38) for R2 on day 28 and increased to 153.3±4.8 mg/gVSS (PN/PS ratio of 4.3) for R1 and 172.7±2.1 mg/gVSS (PN/PS ratio of 7.2) for R2 on day 120. Thus, the granules' size (**Figure 3.4 (a, b)**) increased during the same stage and reached 2.22±0.13 mm for R1 and 2.13±0.13 mm for R2. During the maturation period, there was a strong correlation of the average concentrations of EPS for

both reactors. EPS for R1 and R2 were 203.7 mg/gVSS (PN/PS ratio of 9.17) and 207.9 mg/gVSS (PN/PS ratio of 9.51). Accordingly, the granules' size (**Figure 3.4 (a, b)**) was increased to about 3.02 ± 0.09 mm for R1 and 3.08 ± 0.06 mm for R2, respectively.

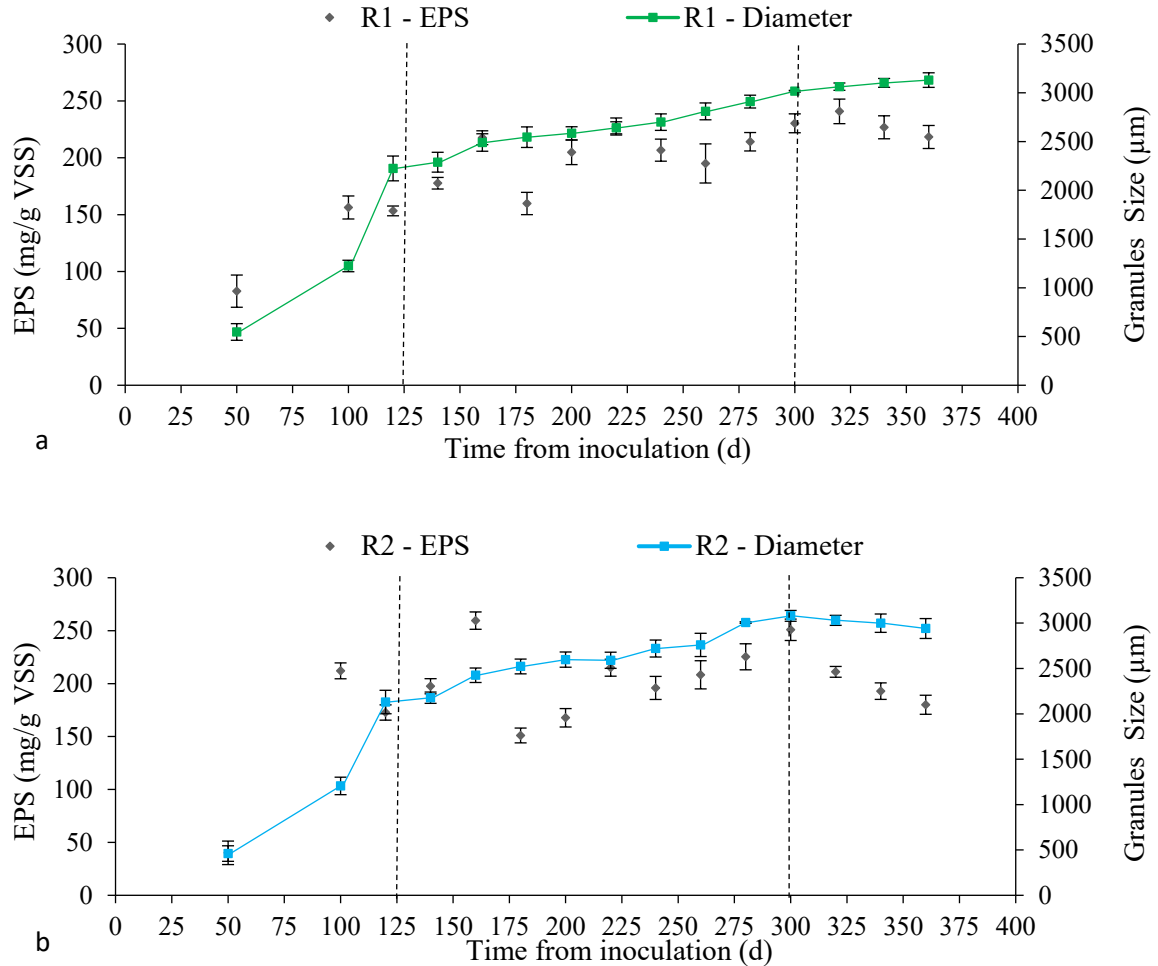


Figure 3.4: EPS measurements (protein + carbohydrate) over the entire operational period (360 days) of (a) R1 and (b) R2

3.3.2. Performance of the Reactors

There was a strong correlation of the reactors' performance in terms of sCOD, $\text{NH}_4\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, and $\text{PO}_4^{3-}\text{-P}$ concentrations between R1 and R2 through cultivation and maturation stages (day 1–300). These results were expected because of the similar

conditions of operation and same influent characteristics. Moreover, both reactors had good COD removal efficiency, but nitrogen and phosphorus removals were different and improved over time as mature granules were formed.

Both reactors exhibited high sCOD degradation, during the entire operational period (360 days: before and after changing filling mode for R2). R1 and R2 had mean removal efficiencies of $91.5 \pm 2\%$ and $91.2 \pm 1\%$, respectively, during 360 operation days. This suggests that there was no effect of the feeding mode on the removal of organics. The results of sCOD removal efficiencies showed in this study are consistent with previous AGS systems that used both municipal and industrial wastewaters (Kang and Yuan, 2017 and França et al., 2018). It should be noted that during the first stage (day 1–125: cultivation), R1 and R2 had mean sCOD concentrations in the effluent of 127.7 ± 24 mg/L and 115.4 ± 25 mg/L, respectively. While during maturation period as well as after changing the feeding mode of R2 (day 126–360), the mean sCOD concentrations in the effluent of R1 and R2 were estimated at 93 ± 14 mg/L and 91 ± 12 mg/L, respectively.

3.3.2.1. Ammonium removal performance

The average influent concentrations of ammonium were about 115.2 ± 4.6 mg/L (**Table 3.2**). During the first 27 days and after two weeks of adaptation post inoculation (day 42), there were higher concentrations of ammonium in the effluents in both reactors. The mean value of $\text{NH}_4\text{-N}$ removal efficiency was about 30% for GSBRS until day 42: $\text{NH}_4\text{-N}$ concentrations in the effluents of R1 and R2 were found between 73 and 86 mg/L. After this point, an efficient ammonium oxidation was observed with $\text{NH}_4\text{-N}$ removal efficiency of 51% and 55.9% for R1 and R2 at day 49, respectively. After that, a continuous

gradual decrease in the $\text{NH}_4\text{-N}$ concentrations of the effluents was observed until the end of the cultivation stage, where the mean values of $70.8 \pm 1.5\%$ and $69.6 \pm 2.1\%$ for R1 and R2 were found, respectively. Only nitritation ($\text{NH}_4\text{-N}$ converted to $\text{NO}_2\text{-N}$) was observed, while full-nitrification ([a] $\text{NH}_4\text{-N}$ oxidized to $\text{NO}_2\text{-N}$ then [b] $\text{NO}_2\text{-N}$ oxidized to $\text{NO}_3\text{-N}$) was not observed during this stage. This was indicated by the highest effluent concentrations of $\text{NO}_2\text{-N}$ accompanied with lowest $\text{NO}_3\text{-N}$ concentrations. At the end of this stage (day 120), effluent concentrations of $\text{NO}_2\text{-N}$ were 62.3 mg/L and 58.4 mg/L for R1 and R2, while for $\text{NO}_3\text{-N}$ concentrations were 4.4 mg/L and 7.3 mg/L for R1 and R2, respectively.

The potential reasons for this behavior might be due to the type of the inoculated sludge that used for both reactors at the beginning of operational period. Moreover, the lower sludge age that estimated for GSBRS during the first stage based on the MLSS and TSS_{eff} concentrations have played a direct role in the difficulty of the proliferation of slow-growing organisms (e.g., ammonia oxidizing bacteria (AOBs), and nitrite oxidizing bacteria (NOBs)). SRT for R1 and R2 have been estimated by 19 and 18 days, respectively. In addition, applying higher OLR ($2.25 \text{ Kg COD/m}^3\cdot\text{d}$) might be considered one of the potential reasons caused these low efficiencies towards the $\text{NH}_4\text{-N}$ removal through increasing the possibility of fast-growing, heterotrophic bacteria growing on granule's surfaces. Besides, applying higher influent $\text{NH}_4\text{-N}$ concentrations ($120 \pm 3.4 \text{ mg/L}$) was one of the reasons of lower performance of ammonium removal. Previous study (Kang and Yuan, 2017), has demonstrated that an improvement through the nitrification process has been achieved through a reduction in influent $\text{NH}_4\text{-N}$ from 140 to 80 mg/L. This enhancement in the nitrification process for GSBRS has been confirmed through a gradual

decrease in the effluent $\text{NO}_2\text{-N}$ to less than 1 mg/L accompanied with gradual increase in $\text{NO}_3\text{-N}$ from 35 to 48 mg-N/L.

During the maturation stage (day 126–300), $\text{NH}_4\text{-N}$ removal efficiencies for R1 and R2 have gradually increased due to accumulation growth of nitrifiers (AOB and NOB) as well as the average SRT during this stage increased and reached 30 and 31 days for both R1 and R2. The average $\text{NH}_4\text{-N}$ concentrations in the effluent of R1 and R2 were estimated at 20.4 ± 1.4 mg/L and 18.8 ± 2.2 mg/L, respectively. Basically, the performance of GSBRS in $\text{NH}_4\text{-N}$ removal through maturation period can be discussed considering the following three phases:

- (a) The commencement of full nitrification, and gradual enhancement in ammonium oxidation was observed between days 130 and 178. Reduction in effluent concentrations of $\text{NH}_4\text{-N}$ from 24.8 ± 1.7 mg/L and 27.7 ± 1.6 mg/L to 16.7 ± 2.4 mg/L and 15.5 ± 3.1 mg/L for R1 and R2, respectively, was estimated, and these reductions were accompanied with the reduction in $\text{NO}_2\text{-N}$ concentrations with simultaneous increase in $\text{NO}_3\text{-N}$ concentrations in the effluent. By day 178, effluent concentrations of $\text{NO}_2\text{-N}$ decreased and reached 16.7 ± 3 mg/L and 19.1 ± 1.2 mg/L for R1 and R2, respectively, while effluent concentrations of $\text{NO}_3\text{-N}$ increased to 45 ± 9 mg/L and 49.4 ± 12 mg/L for R1 and R2, respectively.
- (b) From day 179 to 222, active and efficient full nitrification of the oxidized ammonium is observed in addition to the continuous reduction in effluent concentrations of $\text{NO}_2\text{-N}$, which have been decreased to 7.9 ± 1.2 mg/L and 10.4 ± 0.3 mg/L for R1 and R2, respectively. In addition, there was a reduction in the effluent

concentrations of $\text{NO}_3\text{-N}$ during the same period, and these concentrations reached about 18 ± 0.7 mg/L and 22.4 ± 1.8 mg/L for R1 and R2, respectively. It is worth mentioning that the decreasing in nitrate concentrations accompanied to the reduction in nitrite was due to the action of SND, which will provide an important benefit to the GSBs system by preventing the accumulation of nitrite and nitrate. The potential reasons for this can be characterized by the compact layer structure of the mature granules and the size of these AGS that can extend beyond 2.6 ± 0.06 mm for R1 and R2 (**Figure A2c,d: Appendix A**).

- (c) Both reactors exhibited a good tendency to perform SND process for the rest of this stage, between days 223 and 300, (which was confirmed by the results of the kinetic tests as shown in **Figure 3.5** and **Figure 3.6**) through noticeable reduction in the effluent concentrations of $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$. However, the effluent concentrations of $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ for reactors reached more than 6 mg/L and 10 mg/L, respectively. In addition, it should be noted that the removal efficiency of $\text{NH}_4\text{-N}$ by the end of the maturation stage (day 300) was estimated for R1 and R2 as $85.5 \pm 1.8\%$ and $87.1 \pm 1.6\%$, respectively, ($\text{NH}_4\text{-N}$ concentrations in the effluents ranged between 15 and 20 mg/L).

Accordingly, and based on the levels of nitrite and nitrate in the effluent, a new operational stage (stage III) was planned to investigate the possibility of the enhancement of denitrification process for reactors and minimizing both of nitrite and nitrate accumulation. Thus, feeding strategy was changed to anaerobic step-feeding for R2 only to investigate its influence and feasibility on the enhancement of the denitrifiers performance, while R1 was operated under the same conditions during the previous 300

days. In addition, and because of the low efficiency of P removal for GSBRs (75% as shown in the following section), studying the impact of changing filling mode on the activity of PAOs will be investigated. Results for GSBRs are discussed below.

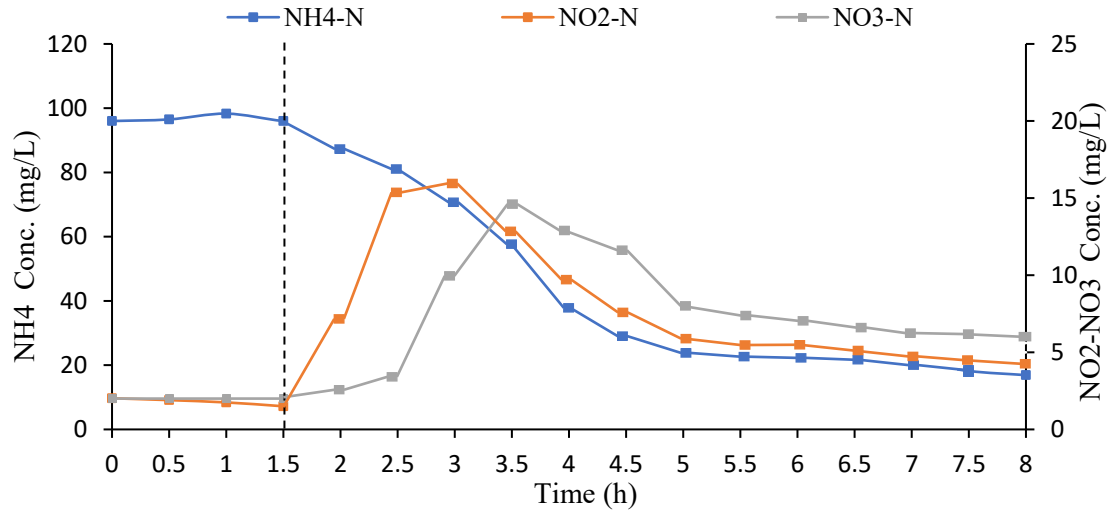


Figure 3.5: Performance profile of nitrogen distributed over a typical cycle during stage II (days 223 - 300) by R1

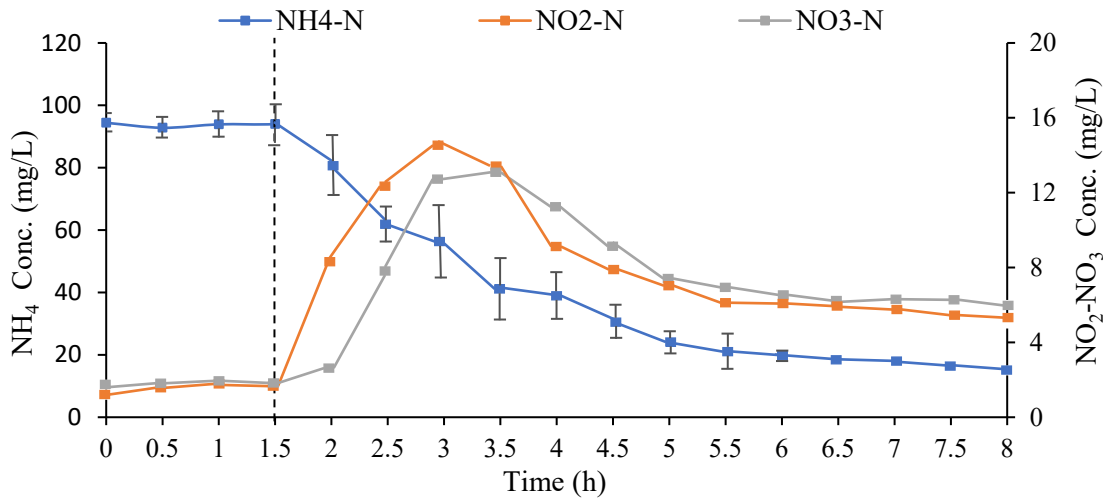


Figure 3.6: Performance profile of nitrogen distributed over a typical cycle during stage II (days 223 - 300) by R2

3.3.2.2. Phosphorus removal performance

The average influent concentration of $\text{PO}_4\text{-P}$ was 17.02 ± 0.9 mg/L (**Table 3.2**). Until the stage of cultivation (day 125), lower efficiencies of P removal for GSBRS were observed. The mean P removal efficiencies for R1 and R2 were $42.8 \pm 5.9\%$ and $42 \pm 7.2\%$, respectively. The potential reasons for these lower efficiencies can be insufficient carbon source ($\text{sCOD} = 1250 \pm 43$ mg/L) for PAOs in the influents (COD:TN:TP ratio is 25:3:1). Thus, there will be a competition of carbon uptake during anaerobic stage (1.5 h) in the operational cycle (8 h) between these PAOs and other denitrifiers. However, higher efficiencies of phosphorus removal have been observed for R1 and R2. For instance, P removal efficiency for R1 and R2 were estimated at $75 \pm 1.4\%$ and $77.2 \pm 0.9\%$ at day 98.

The seed sludge used in the reactors played a role in the enhancement of P removal after inoculation in GSBRS and especially during the initial days in cultivation stage (i.e., before formation of large granules) because it was RAS from a BNR wastewater treatment plant. Kinetic tests shown in **Figure 3.7** and **Figure 3.8** demonstrated relatively lower rates of the anaerobic P release (3.17 mg-P/gVSS and 2.96 mg-P/gVSS, for R1 and R2, respectively) and lower aerobic P uptake (0.68 mg-P/gVSS and 0.77 mg-P/gVSS, for R1 and R2, respectively) during the cultivation stage. P content in the biomass at the end of an operational cycle was observed at lower concentrations of 72 mg-P/gVSS as a mean value during the first 125 days of operation. The lower P removal in both reactors implies that the layered structure of the formed AGS (with an aerobic outer layer and an anaerobic core) at this stage (size of granules was about 2 mm) was insufficient to allow anaerobic P release and efficient P uptake. Low P removal was previously reported in GSBRS systems (Iorhemen et al., 2019). Comparable results were found by Iorhemen, O.T et al. (2022),

where three GSBs were operated for 84 days to treat an influent with COD:TN:TP ratio of 100:5:1. The results showed that the P removal efficiency was about $55 \pm 11\%$ for the GSBs that used pulse feeding without anaerobic mixing.

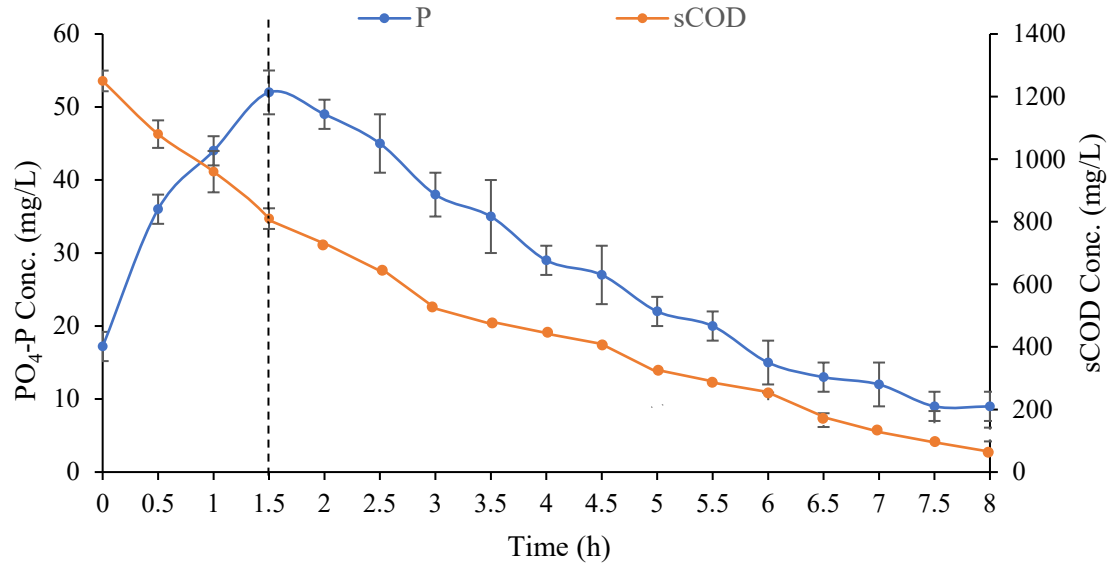


Figure 3.7: Phosphorus and sCOD removal through a typical cycle (8 h) during stage I (day 1 - 125) in R1

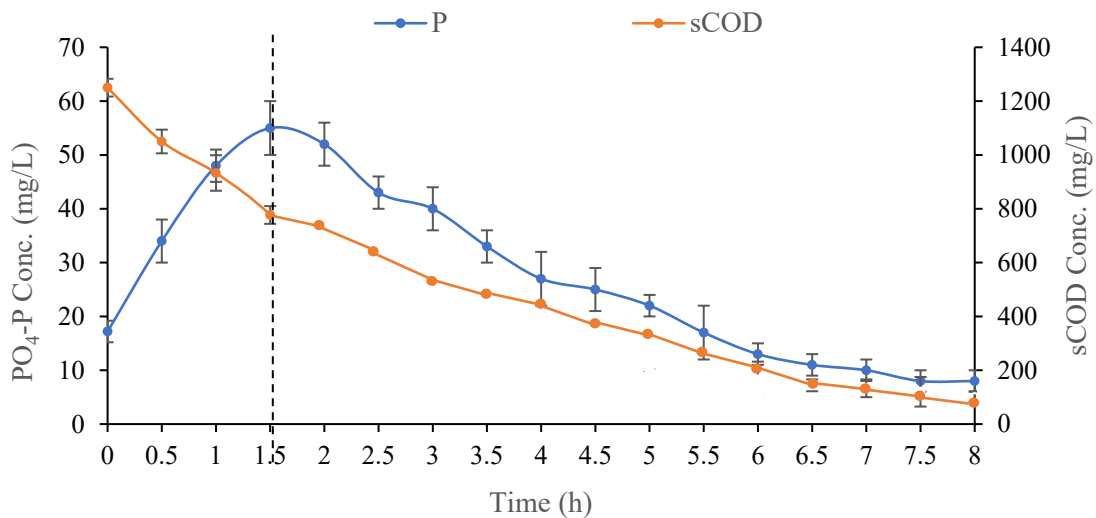


Figure 3.8: Phosphorus and sCOD removal through a typical cycle (8 h) during stage I (day 1 - 125) in R2

Till day 175 of operation (maturation stage), and under the same influent characteristics (high OLR and COD:TN:TP) and same operational design parameters (time of cycle's stages, HRT, superficial air velocity), relatively higher P removal efficiencies for R1 and R2 were observed compared to the previous cultivation stage. Variation in the P removal of R1 and R2 was observed, and the mean P removal efficiencies for R1 and R2 were $75.6 \pm 5.9\%$ and $74.5 \pm 9.6\%$, respectively. In addition, the variance in the removal efficiency of $\text{PO}_4\text{-P}$ concentrations for the GSBRSs was observed and concentrations as high as 7.6 mg/L and 7 mg/L were recorded for R1 and R2, respectively, at the beginning of maturation period (day 140). Moreover, after day 220 until the end of this period (day 300), P concentrations in the effluents of both reactors were between 2–3 mg/L.

As mentioned previously, the size of the aerobic formed granules in both reactors increased from 2.1 mm (by the end of cultivation stage) to more than 3 mm during this stage. Consequently, the mechanisms for P removal in these GSBRSs systems have improved during this stage due to the impact of EBPR, which depends on the enrichment of PAOs in the GSBRSs, as well as P precipitation inside the granular matrix (Gao et al., 2011, Henriot et al., 2016, Cassidy and Belia, 2005, Mañas et al., 2011, Filali et al., 2012, Sarma and Tay, 2018b, Purba et al., 2020). A slight improvement in PAOs activity has been confirmed during the maturation stage and according to the frequent kinetic analysis (**Figures 3.7 – 3.10**), it was found that influent sCOD concentrations have been degraded during anaerobic phase (95 min) by about 60% for both GSBRSs compared to 46 % for 125 days of the previous stage (mean values for the initial 175 days of operation). Therefore, this led to slight increase of aerobic P uptake rates (0.85 mg-P/gVSS and 0.93 mg-P/gVSS for R1 and R2, respectively), however no significant changes in anaerobic P release were

observed during the maturation stage, which was confirmed by the results of the kinetic tests as shown in **Figure 3.9** and **Figure 3.10**. In addition, the highest P removal efficiency for reactors was observed, where for R1 it was about $95.1 \pm 1.5\%$ at day 238, and $97.6 \pm 0.8\%$ for R2. Additionally, high P content in the biomass at the end of a completed operational cycle was observed (more than 20–50 mg-P/gVSS, which was reported for OHO (Metcalf & Eddy, 2014)) and reached about 0.21 g-P/gVSS, on day 234.

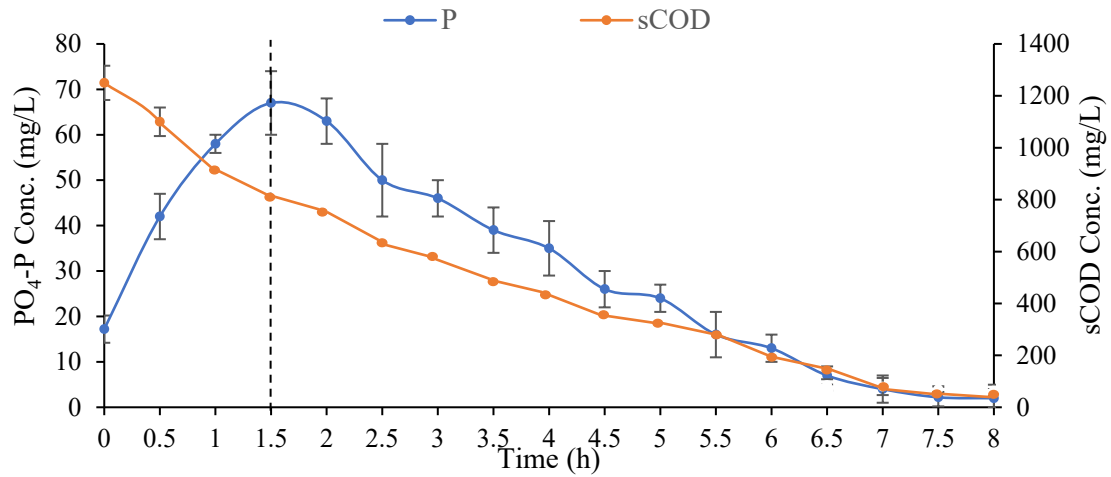


Figure 3.9: Phosphorus and sCOD removal through a typical cycle (8 h) during stage II (day 126 - 300) in R1

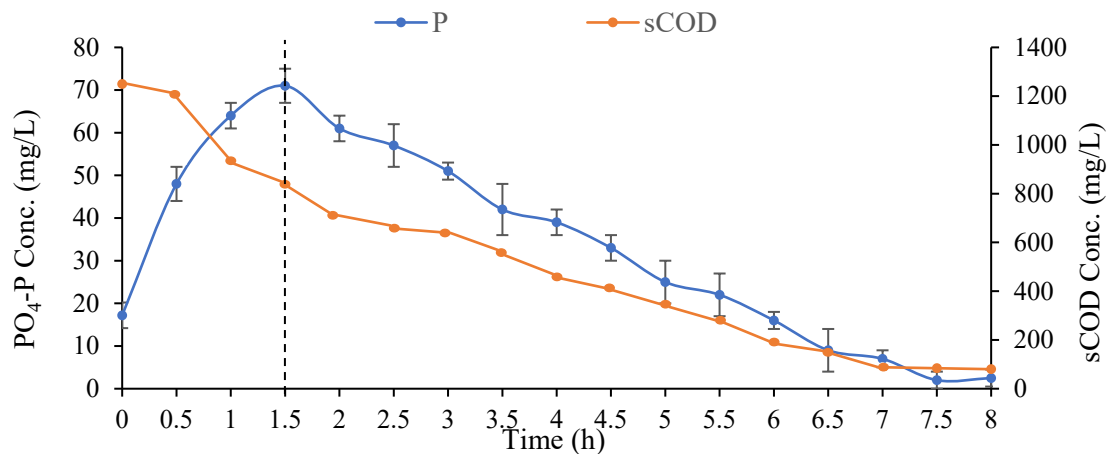


Figure 3.10: Phosphorus and sCOD removal through a typical cycle (8 h) during stage II (day 126 - 300) in R2

3.3.2.3. Influence of anaerobic step-feeding

Improving the performance of these GSBs was the primary objective after finishing 300 days of operation under the effect of high-strength wastewater (microscopic images of the matured AGS through these two stages are shown in **the Appendix A, Figures A2 and A3**). Therefore, a third operational stage of 60 days was proposed to investigate the influence of changing the filling strategy on the reactors' performance. In addition, the microscopic images of the aerobic granules for both reactors during the last stage are shown in **Figure A4 (Appendix A)**. The feeding mode of R2 was changed from fast single feeding mode followed by anaerobic mixing to anaerobic step-feeding strategy, while R1 has been operated with the same previous filling mode.

The results demonstrated that changing filling strategy did not influence the removal efficiency of organics. R1 and R2 had similar mean organic removal efficiencies during this stage (60 days) of $94.55 \pm 1.4\%$, and $92.6 \pm 2.3\%$, respectively. These results are consistent with previous studies of AGS systems that investigated the effect of feeding strategy on the removal efficiencies of organic substrate (da Silva et al., 2021 and Iorhemen et al., 2022).

It should be noted that, after switching to the anaerobic step-feeding mode, anaerobic organic consumption in R2 decreased relative to the control (**Figures 3.11 and 3.12**), which disrupted the intended feast–famine regime and prolonged feast conditions. This shift in carbon availability promoted structural deficiencies in the granules (progressive disintegration and size reduction, poorer settleability, and elevated effluent TSS), particularly under the applied high OLR where filamentous outgrowth can be

stimulated by aerobically hydrolyzed organic matter. Although literature indicates that well-defined famine/starvation periods tend to stabilize AGS by selecting for slow-growing organisms, the extended feast conditions in Stage 3 reduced this stabilizing selective pressure. Consistent with this, the observed biomass yield in R2 increased during the final two months ($Y_{obs} = 0.123 \rightarrow 0.135 \rightarrow 0.156$ gVSS/gCOD), indicating higher biomass production; however, because biomass retention was governed by settling-based selection (10-min settling with washout of poorly settling solids), the increased fraction of disintegrated/flocculent biomass was preferentially removed, resulting in the marked MLSS decline (**Figures 3.2 - 3.4**).

The measured values of EPS and size of AGS, as demonstrated in **Figures 3.4 (a,b)**, show rapid disintegration of the mature granules in R2. Moreover, at the end of this stage (day 360), high portion of granules had become fluffy and viscous flocs (**Figure A6 - Appendix A**). Consequently, lower physical properties were observed for R2 by the end of the current period (day 360) as shown in **Figure 3.2**, where SVI_{30} increased from 31.8 mL/g (end of maturation stage) to 50.2 mL/g and also SVI_{30}/SVI_5 ratio decreased from 0.96 (end of maturation stage) to 0.77 (**Figures A4 and A6 - Appendix A**). Furthermore, granules' size in the same reactor decreased from 3.08 ± 0.06 mm (end of maturation stage) to 2.94 ± 0.11 mm (**Figure A4 - Appendix A**). This deterioration of the mature AGS in R2 can be considered a direct result for the notable reduction in the EPS concentrations. EPS concentrations decreased from 250.82 mg/L (end of maturation stage) to 180 mg/L by day 360, as well as the reduction in PN/PS was noted during the third stage (e.g., $PN/PS = 9.1$ at day 360). In contrast, the performance of R1 as well as the physical properties had the same trend during the initial 175 days of operation during maturation, where the EPS

concentrations were higher than those of R2. Thus, larger AGS volume have been achieved in R1, where $SVI_{30} = 27.5 \text{ mL/g}$ and SVI_{30}/SVI_5 ratio = 0.98, while granules' size became greater than $3.13 \pm 0.07 \text{ mm}$ (**Figure A3 - Appendix A**).

In addition, biomass concentrations in R2 have decreased in the 60 days of operation after changing feeding mode, where MLSS concentrations were about 8502.3 mg/L. It is important to mention that TSS_{eff} for R2 indicated a deterioration and disintegration of the granules, which was comparable with these concentrations in R1 (**Figure A7 - Appendix A**). The mean value of TSS_{eff} for R2 increased during this period to 244 mg/L, while for R1, TSS_{eff} was much lower than R2 at 188 mg/L.

Accordingly, and after the evident deterioration of granules in R2 during the third stage of operation, it was expected that the performance of R2 in terms of P and ammonium removal will be lower than the performance of R1 (as shown in **Figures 3.11** and **3.12** that show the kinetic analysis during the third stage). For the performance of R1 in terms of P removal (**Figure 3.11**), the average $PO_4\text{-P}$ concentrations in the effluent were about $1.38 \pm 0.5 \text{ mg/L}$. In contrast to the P removal of R2, the average $PO_4\text{-P}$ concentrations were about $9.22 \pm 1.3 \text{ mg/L}$. Deterioration had occurred in PAOs activity for R2, during the last stage after changing the filling mode, and according to the frequent kinetic analysis (**Figure 3.12**), it was found that influent sCOD concentrations have been degraded during 1.5 h anaerobic phase by about 45% for R2 compared to 60% for the previous stage, while for R1 it was about 65% during the same period (stage III). This indicated that the anaerobic mixing time was not sufficient to provide enough degradation of sCOD during the same phase (i.e., anaerobic mixing). In other words, sCOD was not readily oxidized during the

anaerobic phase and in the first period of the aeration phase, OHO began to develop, mainly in filaments, at expense of the growth of other slow-growing organisms (PAOs, GAOs, nitrifiers, and denitrifiers). Accordingly, granules stability has been deteriorated, and the performance of R2 for the biological nutrient removal has been dropped. Anaerobic P release and aerobic P uptake was about 1.55 and 0.26 mg-P/gVSS, respectively, for R2, while R1 had 3.88 and 0.72 mg-P/gVSS, respectively, during the third stage.

Moreover, performance of ammonium removal in R2 based on kinetic tests is demonstrated in **Figure 3.14** for the entire 60 days of the third stage. Due to the similarity and the disintegration of granules occurring in R2, it was expected that performance of R2 in terms of ammonium removal will be minimized compared to R1. The average $\text{NH}_4\text{-N}$ concentrations in the effluent of R2 have been estimated at 24.3 ± 6.3 mg/L. In addition, a significant rise in the concentrations of nitrate, as well as slight increase in nitrite concentrations during aerobic phase was noticed, which led to nitrite/nitrate accumulation. Effluent concentrations of nitrate were between 46 and 50 mg/L, while it was 10 mg/L for nitrite. For R1 (**Figure 3.13**), the average $\text{NH}_4\text{-N}$ concentrations in the effluent were about 12.41 ± 3.3 mg/L.

The granules' disintegration in R2 after changing filling mode and recurrent washouts because of applying short settling time (10 min) had resulted in a loss of nitrifying bacteria (AOBs and NOBs) as well as some PAOs that were present in the broken granules. In addition, when washouts occur at higher frequencies during these 60 days of operation, SRT is reduced (mean SRT for R2 was estimated at 24 days during this period), therefore the performance of ammonium (i.e., SND) and P removal was negatively affected due to

the reduction in sludge age (compared to SRT for R1 which was about 39 ± 1 days). This further substantiates the key role of the STR in granules' stabilization and reactors' performance since it is directly related to the accumulation and growth of these slow-growing bacteria, which is also confirmed in other studies (Rollemberg et al., 2018). Overall, the performance of R1 was continuing in the same trend of enhancement along with physical properties of AGS. However, the slow rate of this improvement of R1 might be due to the high strength of influent wastewater characteristics (high influent concentrations of COD, $\text{NH}_4\text{-N}$ and $\text{PO}_4\text{-P}$) and composition (i.e., COD:TN:TP ratio).

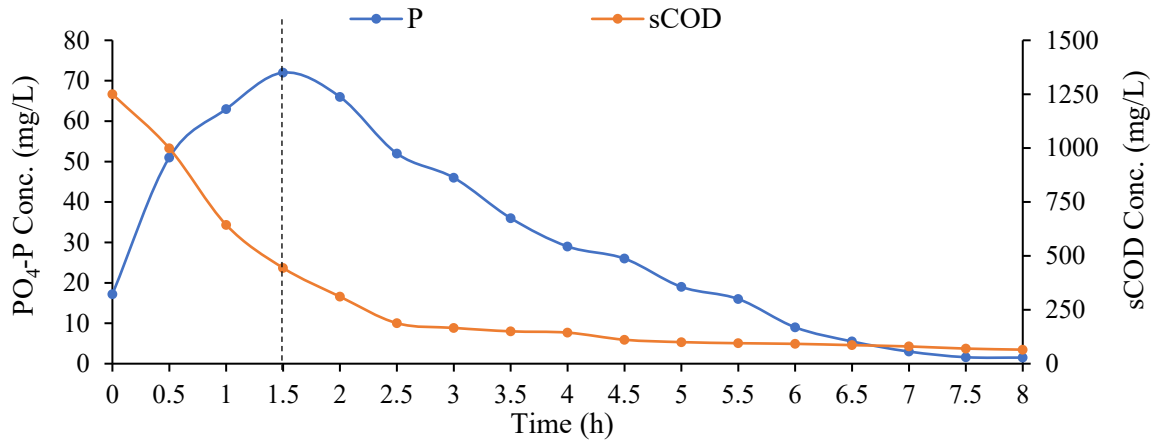


Figure 3.11: SP and sCOD removal through a typical cycle (8 h) (days 300- 360) in R1

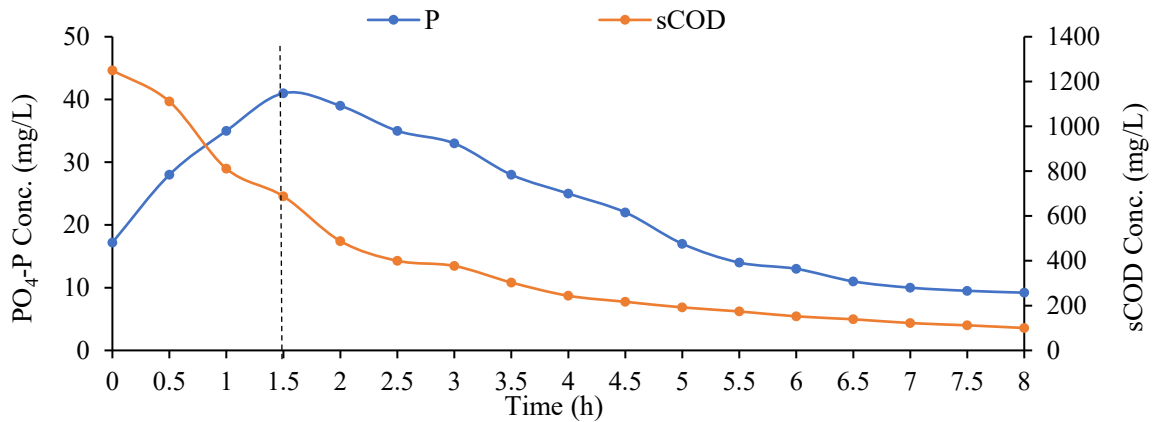


Figure 3.12: SP and sCOD removal through a typical cycle (8 h) (days 300- 360) in R2

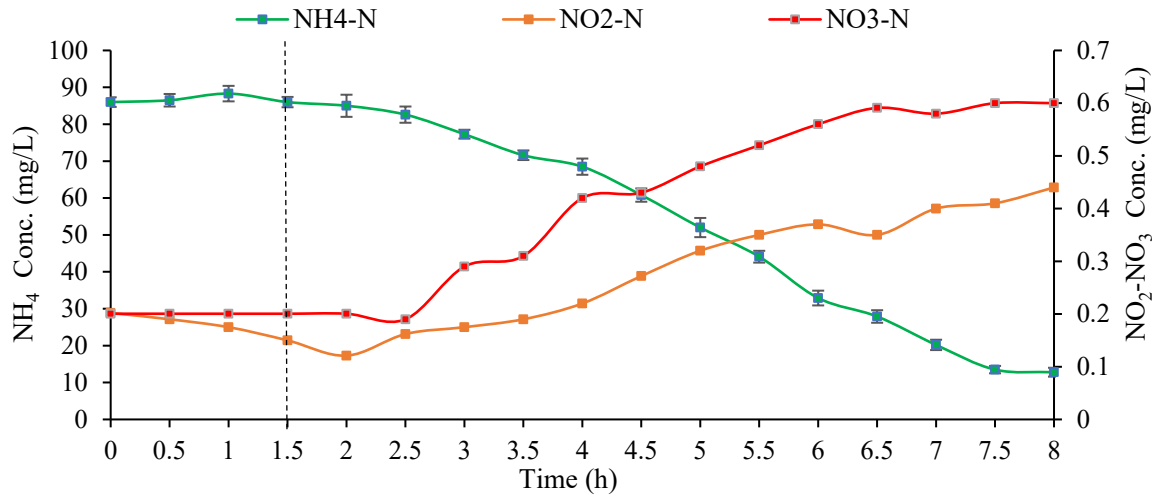


Figure 3.13: Performance profile of N distributed over a typical cycle (days 300 - 360)
by R1

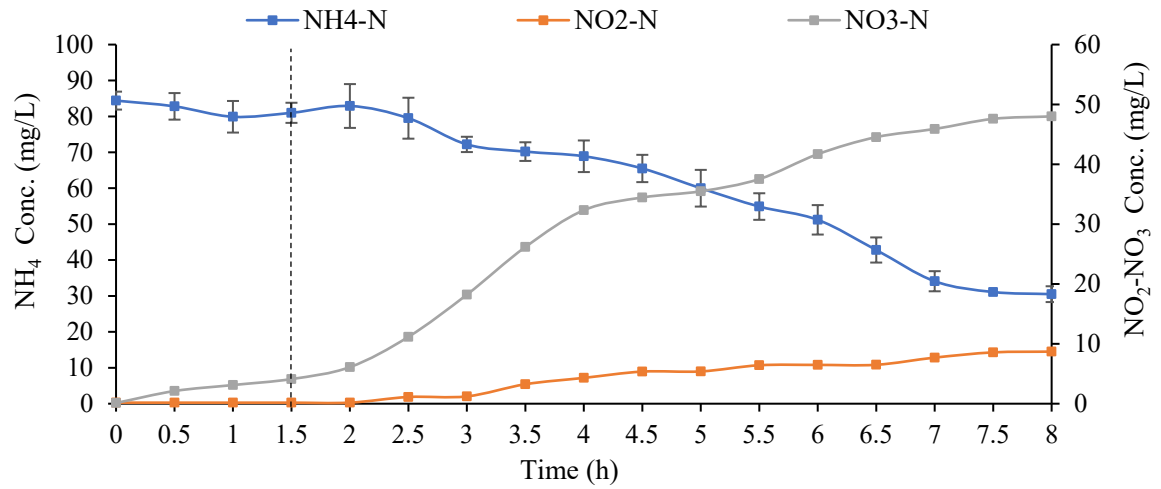


Figure 3.14: Performance profile of N distributed over a typical cycle (days 300 - 360)
by R2

CHAPTER 4: SIMULTANEOUS BIOLOGICAL NUTRIENTS REMOVAL FROM WASTEWATER WITH HIGH AMMONIUM AND PHOSPHORUS LOADING USING AEROBIC GRANULAR SLUDGE

Only a limited number of research investigations have focused on the efficient simultaneous removal of nutrients from low COD/total nitrogen/total phosphorus wastewaters through the aerobic granulation technology, especially at long solid retention time. In this context, this chapter focused for the first time a long-term AGS-SBRs fed with a high-strength synthetic wastewater with high ammonium and P concentrations to evaluate the start-up of the aerobic granulation process and concurrent removal performance of biological nutrients occurring simultaneously during the treatment of high-strength wastewater with elevated N and P loadings. The microbial community composition of matured granules and the impact of long-term maturation of the formed granules under the same operating conditions on the enhancement of the biological nutrient removal are also investigated. Especial attention is given to the influence of increasing the applied DO levels on the stability of granules, ammonium oxidation rate, as well as P release and uptake rates in the purpose of comparing between the feasibility (performance of AGS-SBR) of using matured granules and applying high DO concentrations under the treatment of these influents.

4.1. Introduction

The discharge of diverse wastewater streams, including agricultural, industrial, and untreated domestic wastewater into water bodies poses a significant environmental challenge. While many municipal WWTPs have undergone considerable improvement and upgrades to meet discharge limits and prevent pollution, treated effluents from some plants may still contain high levels of nutrients, such as N and P, with the potential to induce eutrophication in lakes and rivers and disrupt ecosystems. An effective management of nutrient-rich effluents is crucial to prevent surface water pollution and compromise the quality of drinking water sources (Cai et al., 2013). Adopting advanced strategies for wastewater treatment is imperative on a global scale to address this concern. For more than a century, activated sludge processes have been developed and used with success in many applications for wastewater treatment. However, to overcome the limits of conventional activated sludge processes, more compact and efficient treatment technologies have been developed over the past decades for nutrient removal, and among the most attractive are membrane bioreactors (MBRs), moving bed biofilm reactors (MBBR), and AGS.

AGS has emerged as an effective solution for wastewater treatment and nutrient removal, outperforming the conventional activated sludge processes. It has the potential to effectively eliminate various organic materials and nutrients from diverse wastewater sources including low-strength municipal wastewater (Coma et al., 2012 and Lashkarizadeh et al., 2015) high-strength industrial effluents (e.g., fish canning (Figueroa et al., 2008), textile (Muda et al., 2010), brewery (Corsino et al., 2017), palm oil mill (Abdullah et al., 2011), piggery (Kishida et al., 2009)) as well as landfill leachate (Ren and Yuan, 2016). AGS has also been investigated successfully in the last decades for the

degradation of phenolic compounds (Jemaat et al., 2014 and Liu et al., 2014). AGS consist of spherical aggregates of microorganisms which do not rely on any media for attachment, offering a compact microbial structure, with high biomass retention, tolerance to toxicity and resistance to shock loading faster and more effective than conventional activated sludge (Tay et al., 2001, Liu and Tay, 2004, Adav et al., 2008, Liu et al., 2015). Several studies have explored the cultivation process of AGS as well as granules' stability using SBRs and have highlighted various operational and design factors that influence the granulation process. These factors include the feast-famine conditions, anaerobic influent distribution, selective wasting (settling velocities as a selection pressure), hydrodynamic shear forces caused by superficial air velocities, influent characteristics (COD/TN/TP ratio), OLRs, biomass characteristics, and production of EPS. Additionally, maintaining optimal levels DO, pH, and temperature is crucial for the AGS process (Lashkarizadeh et al., 2016, Bassin et al., 2012, Isanta et al., 2015). Furthermore, researches indicate that factors such as the proliferation of fast-growing organisms or filamentous organisms (OHOs) at high OLRs, anaerobic fermentation of dead and lysed cells in matured granules (i.e., large sized), and deterioration in the composition of EPS (which plays a fundamental role in the stability of AGS) are contributors to both the stability and disintegration of granules (de Kreuk et al., 2005, Liu and Tay, 2012, Lv et al., 2014, Long et al., 2015).

The biological treatment of high-strength $\text{NH}_4^+\text{-N}$ influent wastewater using AGS has been explored in various studies, with a focus on addressing operational challenges such as the deterioration of physical characteristics (i.e., granule disintegration), prolonged granule formation periods with high OLRs in sewage, and $\text{NO}_2^-\text{-N}$ or $\text{NO}_3^-\text{-N}$ accumulations (Sarma and Tay, 2018 and Sarma et al., 2017). Over the past two decades,

extensive efforts have been dedicated to advancing autotrophic N removal processes. The autotrophic biological nitrification process involves the conversion of $\text{NH}_4^+\text{-N}$ to $\text{NO}_2^-\text{-N}$ by slow-growing AOBs, followed by the oxidation of $\text{NO}_2^-\text{-N}$ to $\text{NO}_3^-\text{-N}$ by NOBs. Subsequently, the heterotrophic denitrification process can be achieved, reducing $\text{NO}_3^-\text{-N}$ to nitrogen gas by denitrifiers, with nitrite, nitric oxide, and nitrous oxide as intermediates. The SND process requires the coexistence of autotrophs nitrifying bacteria and denitrifiers. With its compact and multilayered structure, different redox conditions are created in the AGS process, allowing the occurrence of SND even in aerated conditions. In AGS, nitrification occurs on the surface of the granules, while denitrification occurs in the core (i.e., anoxic environment) (Nancharaiah et al., 2016). However, the performance of the SND process, specifically TN removal, strongly relies on the availability of DO which should be high enough to sustain nitrification on the surface of granules yet low enough to prevent oxygen penetration into the deeper anoxic layers where denitrification occurs (Third et al., 2003). In more recent studies, it was reported that SND performed well at high levels of DO (up to 8–9 mg/L) during the treatment process of synthetic wastewater (Layer et al., 2020). However, little information was available on how the treatment of low COD/TN influents under limited applied DO (1.5 – 2 mg/L) during aerobic mixing stage affects the behaviour of SND process.

In fact, recent studies have focused on HNAD as a promising approach for nitrogen removal, as it allows for simultaneous nitrification and denitrification processes in aerobic environments (Khanichaidecha et al., 2018). The aerobic denitrifying bacteria involved in HNAD processes exhibit favorable characteristics such as high growth rates and strong oxygen tolerance (Rout et al., 2017). HNAD microorganisms thrive at low DO levels (< 3

mg/L) but have demonstrated resilience to various environmental stress conditions including salinity, the presence of heavy metal ions and antibiotics, high $\text{NH}_4^+\text{-N}$ concentration and low temperature (Rout et al., 2018), therefore emerging as a promising alternative for N removal during the biological treatment process. For instance, *flavobacterium* sp. has been identified as having the necessary enzymes to perform an HNAD process in AGS-SBRs (Su et al., 2015). Some strains of *Flavobacterium* and *Diaphorobacter* bacteria are able to carry out SND in pure cultures aerobically (Xi et al., 2022 and Pishgar et al., 2019). These genera rely on an organic or oxidizable carbon source as an electron donor for optimal growth to complete the denitrification process aerobically. However, their effective contribution as HNAD bacteria in mixed cultures has yet to be fully established, and till now there is limited information supporting the N removal by HNAD bacteria at various C/N ratios (Castignetti and Hollocher, 1984). Nevertheless, high influents $\text{NH}_4\text{-N}$ loadings (high concentrations of TAN) and its byproducts such as FA, which are crucial components in various BNR processes including HNAD, could potentially affect the treatment efficiency, particularly in alkaline environments (Khardenavis et al., 2007). Moreover, FA has been identified as a potent inhibitor for certain crucial microorganisms that are responsible for biological treatment generally and HNAD process particularly (Tan et al., 2020 and Liu et al., 2019). Moreover, FA has been identified as a potent inhibitor for certain crucial microorganisms that are responsible for biological treatment generally and HNAD process particularly (Park et al., 2014 and Yenigün and Demirel, 2013).

4.2. Material and Methods

4.2.1. Experimental Set-Up (Lab-scale AGS-SBR)

The current study was conducted with two sequencing batch reactors, R1 (experimental reactor) and R2 (control reactor), consisted of an acrylic cylinder with an internal diameter of 5 inch and a height of 18 inch. Each reactor has a total volume of 6.0 L and a working volume of 5 L (**Figure 4.1**). At the end of each treatment cycle, 3 L of supernatant is withdrawn from the reactors as treated effluent, and an additional 3 L of fresh influents is pumped at the start of the following new treatment cycle. A new batch of 18 L of synthetic wastewater was prepared every 2 days and stored at 4 °C. Approximately 3.5 h before the beginning of each cycle, 3 L of this prepared synthetic wastewater was pumped out from the cold chamber to 6 L tank equipped with a stir bar and placed on a magnetic stir plate, serving as the feeding source for each new treatment cycle, keeping a temperature range of 20-22°C (**Figure 4.1**).

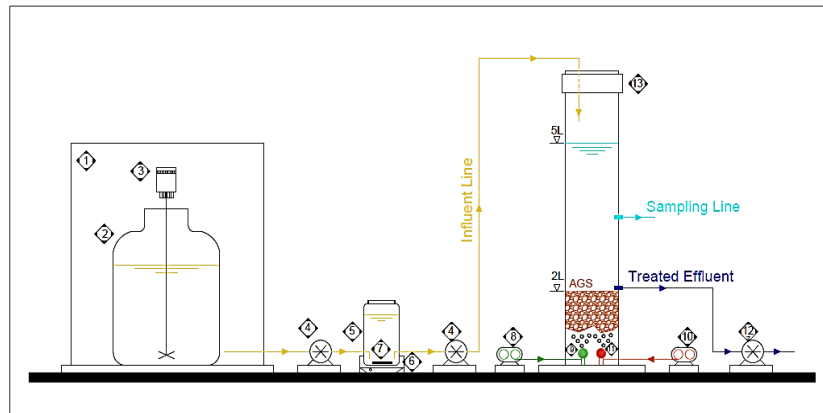


Figure. 4.1. Schematic diagram of the granular sludge SBR in this study;

(1) fridge (4°C); (2) 20 L storage tank (fresh feeding); (3) mixer; (4) feeding pumps; (5) 3 L feeding tank (room temperature - each cycle); (6) magnetic stir plate; (7) stir bar; (8) air compressor; (9) air diffuser; (10) nitrogen pump; (11) nitrogen diffuser; (12) decant pump; and (13) reactor's cap.

A volume exchange ratio of about 60% was applied during the operation by discharging the top 3 L of supernatant over 10 min. The reactors were operated with a HRT of 13.33 h, and the total time of the treatment cycle was 8 h including 10 min for influent feeding, 95 min for the anaerobic mixing phase, and 350 min for the aeration reaction followed by 20 min for both settling and discharging periods. In addition, it should be noted that biomass was only wasted by effluent solids through decant stage (i.e., SRT of biomass was not controlled) (Mady et al., 2022, Derlon et al., 2016, Ren et al., 2017). A 5-min idle zone was observed before starting each new cycle. Peristaltic pumps were used for influents feeding and effluents discharge, and outlet timers were used to automate the treatment cycles (Traceable Digital Outlet Controller, Fisher Scientific, CA).

4.2.2. Inoculum, Influent Composition and Operational Conditions

In this study, R1 and R2 were up-flow seeded with 2 L of RAS collected from the West End Water Pollution Control Centre, a full-scale wastewater treatment plant in Winnipeg, MB, Canada. The measured suspended solids concentration of the RAS inoculum (reported here as MLSS-equivalent) was 8100 mg/L (average of triplicate samples).

A synthetic municipal wastewater feed recipe, with COD:TN:TP ratio of 100:14:5, was used, and the synthetic wastewater characteristics is detailed in **Table 4.1** (Madeira et al., 2023 and Pongthornpruek, 2017). EDTA was used to prevent salts' precipitation. Additionally, sodium bicarbonate was introduced into the influent during each cycle to maintain sufficient alkalinity (7.14 g CaCO₃/g NH₄⁺-N), facilitate complete nitrification and ensure that the pH remained above 7.5s.

Aeration was provided by magnetic piston pumps at a controlled rate of 2.92 L/min through two, 3 cm diameter spherical stone diffusers located on the SBRs' bottom, resulting in a superficial air velocity of 0.43 cm/s, and DO concentrations maintained at 1.5–2 mg/L. Accordingly, mechanical mixing in this configuration of SBRs was not applicable during the anaerobic mixing phase and might not ensure anoxic conditions. Thus, nitrogen gas was introduced from the bottom by another 3 cm diameter spherical stone diffuser. Pumping nitrogen gas provided complete mixing and maintaining DO levels ≤ 0.1 mg/L (i.e., establish an anoxic environment) and also prevented biomass sedimentation. This approach improves the removal efficiency by promoting contact between cells and nutrients due to enhanced mixing (Mady et al., 2022).

Table 4.1: Composition of the synthetic wastewater and wastewater characteristics

Parameter	Value	Characteristics	Value
Yeast extract, mg/L	1250		
NH ₄ Cl, mg/L	33	sCOD, mg/L	1250±70
K ₂ HPO ₄ ·3H ₂ O, mg/L	9		
MgSO ₄ , mg/L	5.8	TN, mg/L	165±36
CaCl ₂ , mg/L	12		
EDTA, mg/L	3	TP, mg/L	54±11
Mineral solution, mL/L:	0.3		
- H ₃ BO ₃ , g/L	15	HRT, h	13.33
- CuSO ₄ ·5H ₂ O, g/L	0.15	OLR,	
- KI, g/L	0.03	(kgCOD/m ³ ·d)	2.16±0.25
- MnCl ₂ ·4H ₂ O, g/L	0.03		
- Na ₂ MoO ₄ ·2H ₂ O, g/L	0.12		
- ZnSO ₄ ·7H ₂ O, g/L	0.06	pH* (Average)	8.22±0.15
- CoCl ₂ ·2H ₂ O, g/L	0.12		
- FeCl ₃ ·6H ₂ O, g/L	1.5		

*pH estimated by 8.9 (during the anoxic reaction phase) and 8.2 (during the aerobic mixing phase) through the first 4 months (i.e., cultivation period), and during the maturation period (day 120 till the end of experiment) pH decreased and reached 8.1 (during the anoxic reaction phase) and 7.7 (during the aerobic mixing phase).

4.2.3. Analytical Procedures and Measurements

Samples were collected regularly from both influent and effluent and filtered via medium porosity Q5 filter paper (0.45 μm) for the physico-chemical analyses (Fisher Scientific, CA). Standard methods were applied to measure the concentrations of MLSS, MLVSS, TSS_{eff} , and sCOD_{inf} , sCOD_{eff} (APHA/AWWA/WEF, 2012). The concentrations of dissolved phosphate (soluble P (SP): $\text{PO}_4^{3-}\text{-P}$) and nitrogen in the forms of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ for the influent and effluent were measured via a flow injection analyzer (Lachat Quick Chem 8500, HACH, CA). Probes of DO and pH have been used to measure their levels near the center of the reactor. Concentration of FA was estimated by the following equation (Eq. 4.1) (Anthonisen et al., 1976):

$$FA \text{ (mg/L)} = 17/14 \times \frac{TAN \times 10^{pH}}{e^{(6334/273+T)} + 10^{pH}} \quad \text{(Equation 4.1)}$$

The biomass of granular sludge was characterized in terms of particle size, SVI_{15} and SVI_{30} , and EPS. Microscopic images from SteREO Discovery were used to determine the progress of the granulation process and assess the stability of the granules by measuring their size (Axio Vert.A1, ZEISS). Besides, the surface characteristics are examined through microscopic analysis on a weekly or bi-weekly schedule. The EPS contents, represented by the combination of the biomass concentrations of protein and carbohydrate (Jafari Kang and Yuan, 2017) were extracted from AGS and analyzed according to (Adav et al., 2008).

Extraction of EPS content has been described in the previous chapter (Chapter 3: 3.2.3. Analytical Methods).

For microbial community investigation, two samples of AGS (R1 and R2) were collected (50 mL MLSS) on day 420 (end of maturation period), then centrifuged at 4000 rpm for 15 min, and stored at -18 °C. The samples were sent the following day to Integrated Microbiome Resource (IMR), Dalhousie University (Halifax NS, Canada), where after DNA extraction, 16 S-rRNA amplicon sequencing was performed to identify the bacterial species in the samples representing AGS following the protocols reported by IMR (Comeau et al., 2017). It is worth mentioning that the microbial analysis uses the new Microbiome Helper pipeline of IMR (GitHub, 2024), based on QIIME2, and so uses Amplicon Sequence Variants (ASVs) created with Deblur or DADA2 instead of the older OUT (operational taxonomic units) methods.

4.3. Results and Discussion

In this study, a dark brown sludge with MLVSS/MLSS ratio of about 0.86, SVI₃₀ 128 mL/g, and a mean particle size of 0.1 mm (average of triplicates samples) was inoculated. After 15 days acclimation period of the microorganisms, the biomass characteristics (MLSS, MLVSS), physical properties (size, SVI), and the reactor's performance for substrate and nutrients removal were monitored. The evaluation of the granules' stability has been performed based on the measurements of granules' size and the analysis of EPS concentrations and settleability properties.

The results presented in Chapters 3 and 4 are derived from the same long-term experimental program using the same two systems. Chapter 3 reports the initial baseline

operating period (300 days) during which both reactors were run under identical conditions. A temporary step-feeding strategy was then applied for approximately two months and was subsequently discontinued due to granule deterioration; both reactors were returned to the baseline pulse-feeding/aerobic-mixing mode and operated for an additional month to allow granule recovery. The biomass from both reactors was then homogenized (mixed and re-split) to re-establish comparable granules, after which the reactors resumed operation under the Chapter 4 conditions for an additional 258 days. Accordingly, the “558 days” referenced in this chapter represents the combined duration of comparable baseline operation (300 days from Chapter 3 + 258 days from Chapter 4), excluding the intervening ~3-month step-feeding and recovery period.

A strong agreement (over 96 %) was observed between R1 and R2 (**Figure B1 – Appendix B**) throughout the entire operational period (558 days). Consequently, all results represent the average of replicates from the two identical AGS-SBRs with the standard error of the mean (SEM). However, the results of removal efficiencies of the substrates and nutrients are individually reported for each reactor. The start-up duration has been estimated to be 75 days considering both physical properties and substrate/nutrient removal, which has been evaluated for 75 days.

4.3.1. Formation of AGS and Biomass Characteristics

The following chart (**Figure 4.2**) shows the granules' size distribution over the entire period of operation.

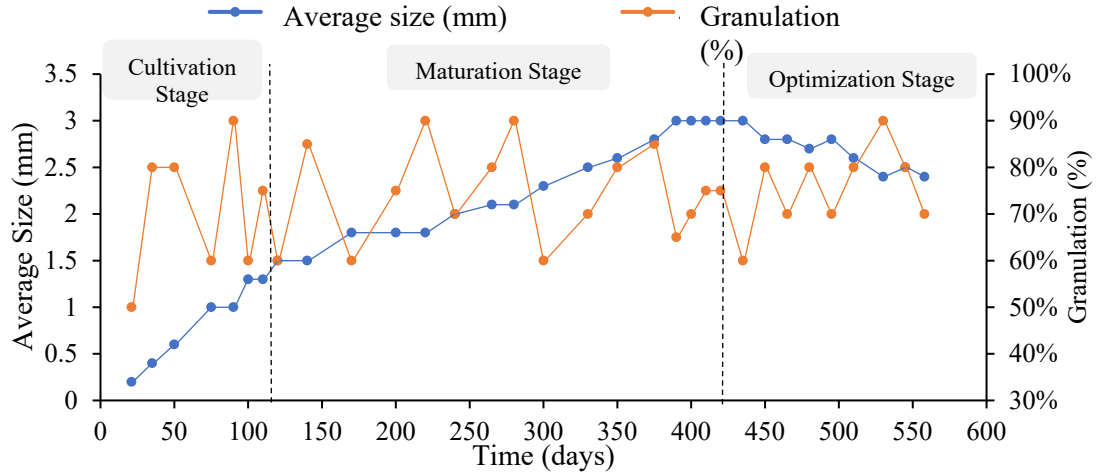


Figure. 4.2. Granules size distribution (%) over entire operational period for AGS-SBRs

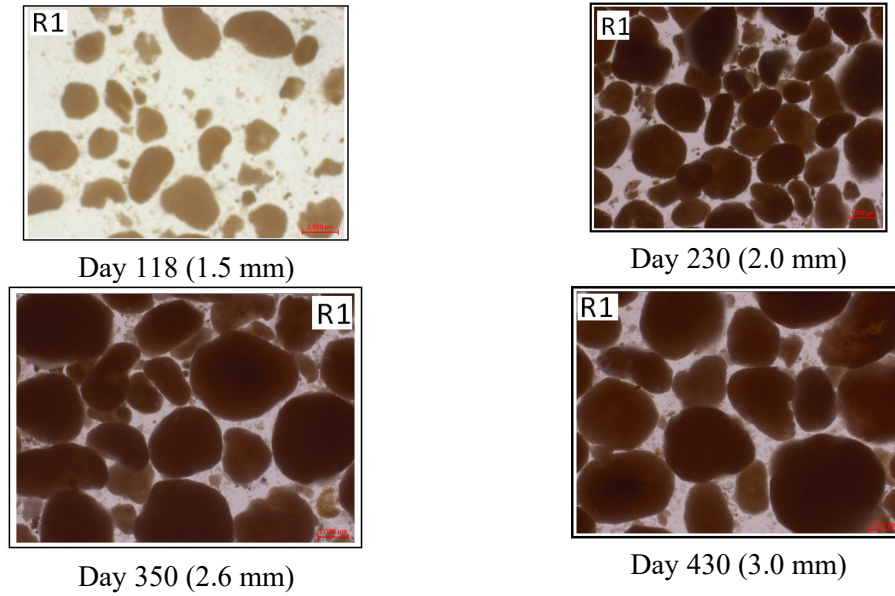


Figure. 4.3. Continuous development in the granules' size over the maturation stage (R1)

During the first month following the adaptation period, the MLSS concentrations significantly decreased to 1.65 ± 0.5 g/L by day 42, accompanied by higher TSS content in the effluents ($2.32 \pm 0.9 - 0.53 \pm 0.2$ g/L) as illustrated in **Figure 4.4**. This reduction of biomass concentrations resulted in the discharge of biomass with poor settling (initial

washout of flocculent biomass) and F/M jumped to 0.6 ± 0.02 gCOD/gVSS.d. Subsequently, there was a gradual and slight increase of MLSS till day 70. While a high OLR can accelerate the aerobic granulation process (Jafari Kang and Yuan, 2017), the prolonged start-up period was attributed to the low COD/TN/TP characteristics of the influent and extended HRT which lowered the applied OLR. Although aerobic granulation has been used in various influents treatment, the formation of AGS with stable nutrient removal can take an extended period. For instance, Schwarzenbeck et al. (2004) required 147 days to achieve full granulation for carbon removal from malting wastewater (Schwarzenbeck et al., 2004). The continual sludge loss (washout) was a result of settling velocity-based selection pressure after applying a short sedimentation phase (10 min) during the cycle operation, which is considered as a key strategy for aerobic granulation development, as heavier and larger bioparticles settle faster, leading to the washout of less dense and poor-settling biomass.

Over the first 100 days of operation following the inoculation, AGS were successfully formed with a size > 1.2 mm as shown in **Figure 4.3**. The application of a high OLR (2.16 kgCOD/m³·d) during the cultivation period accelerated the formation of stable AGS with particle size > 0.65 mm after 2 months from the inoculation. Furthermore, in the last 40 days of the cultivation period (day 75–118), there was a significant increase in MLSS concentrations, rising from 3.1 ± 0.22 to 10.7 ± 0.4 g/L (day 110) as depicted in **Figure 4.4(a)**. This increase was accompanied by 180 ± 18 mg/L TSS in the treated effluent. In the operational design of both reactors there was no dedicated sludge wasting stage (i.e., biomass was only wasted during the decant stage), thus, the sludge age was evaluated based only on the solids concentrations existing in the treated effluent. At the end of the

cultivation stage (day 90–118), there was a stability in the characteristics of the formed granules, with AGS achieving SRT of 18 ± 1 days. Additionally, F/M ratio was estimated at $0.44 \text{ gCOD/gVSS}\cdot\text{d}$. The results confirmed the impact of selective pressure (i.e., 10 min settling time) and applied hydrodynamic shear (resulted from superficial upflow air velocity of 0.43 cm/s) in the process of aerobic granulation of activated sludge.

Over the following 10 months (maturation period; day 118–422), there was a fluctuation in the MLSS concentration ($10 \pm 0.6 - 12.3 \pm 0.25 \text{ g/L}$), while the effluent TSS varied from 57 ± 13 to $285 \pm 40 \text{ mg/L}$ (**Figure. 4.4-b**), and the average SRT reached 38 ± 7 days from day 330 to day 422. The increase of the MLSS led to a continuous reduction of applied F/M ratio to $0.26 \pm 0.01 \text{ gCOD/gVSS}\cdot\text{d}$ by the end of the period. These results align with a previous study (Li et al., 2011), which suggested an F/M ratio lower than $0.3 \text{ gCOD/gVSS}\cdot\text{d}$ for the stabilization of formed AGS. However, the rise of SRT during the maturation period caused a slight decrease in the MLVSS/MLSS ratio from 0.73 to 0.71 in both reactors. Furthermore, it is noteworthy that AGS production rates during the second half of the maturation period were lower than those in the first half of this phase (**Figure. 4.4**). This was primarily attributed to higher sludge ages in SBRs, leading microorganisms to demand more energy for their maintenance within these systems. Thus, biomass decay processes become more important, resulting in a reduction of net biomass synthesis, and considered as a practical approach for minimizing sludge production.

Large-sized matured granules with a diameter $> 3 \text{ mm}$ (**Figure 4.2 and Figure 4.3**) with stable physical properties (SVI_{30} and $\text{SVI}_{30}/\text{SVI}_5$ of 28 mL/g and 95%) were formed within the 10 month of maturation period, **Figure 4.4 (a)**. Introducing calcium and

magnesium ions in the synthetic influents over the long-term operation might enhance the formation of compact structure and maturation process of AGS through their precipitates on AGS surface, which can bind with other proteins to enhance adherence of bacteria to the surface as demonstrated in previous studies (Khan et al., 2020). However, these findings are consistent with the analysis of EPS concentrations, highlighting the crucial role of EPS biopolymers components (e.g., polysaccharides, proteins, and other substances) in the aerobic granulation process by enhancing stability (i.e., granules' structure, production, and stability).

It has been demonstrated from the previous studies that stable aerobic granules have higher PN structures than PS, and PN/PS ratio is a way of characterizing its stability, and various PS/PN (e.g., 4.8 and up to 25) ratios have been reported (Wei et al., 2012, Jafari Kang and Yuan, 2017, Khan et al., 2020). In this work, EPS content has been estimated as a combination of PN and PS, and is presented in **Table 4.2**. There was a significant increase in EPS content during the cultivation period, rising from 80 ± 5.1 mg/gVSS on day 50 to 153.3 ± 10.8 mg/gVSS on day 100. Subsequently, although there was some variation in EPS concentrations, there was an overall increasing trend. Moreover, during the second half of the maturation period (days 268–422), EPS concentrations as high as 234 ± 8.5 mg/gVSS were observed, confirming the development of the matured granules.

Table 4.2. EPS measurements (PN + PS) and PN/PS ratio over the entire operational period of AGS-SBRs

		R1		R2	
		EPS (mg/gVSS)	PN/PS ratio	EPS (mg/gVSS)	PN/PS ratio
Cultivation	Day 28	36 ± 1.1	1.11	31 ± 0.8	1.38
	Day 50	80 ± 5.1	3.0	99 ± 3.1	4.2
	Day 100	153 ± 10.8	4.3	172.7 ± 2.1	7.2
Maturation	Day 170	177 ± 6.4	6.8	173 ± 11	7.5
	Day 215	205 ± 11.2	9.0	182 ± 14	8.8
	Day 260	195 ± 8	9.4	220 ± 3.8	10.8
	Day 300	225 ± 7.8	10.1	230 ± 12	10.5
	Day 370	237 ± 5	11.2	244 ± 8.8	10.7
	Day 420	234 ± 8.5	10.5	258.3 ± 10	11.3
Optimization Stage	Day 440	228.2 ± 11	11.0	237.2 ± 8	10.5
	Day 480	216 ± 12	10.4	222 ± 10	9.0
	Day 502	198.3 ± 6	9.0	191 ± 13	8.0
	Day 522	200 ± 9	9.3	196±24	8.4
	Day 538	172 ± 16	6.5	185 ± 18	7.8
	Day 558	158 ± 19	5.0	162 ± 13	6.5

However, because settling-based selection was applied, episodic increases in effluent TSS were observed, reaching 626 ± 122 , 844 ± 87 , 582 ± 57 , and 422 ± 91 mg/L on days 192, 277, 312, and 333, respectively. During the same days, significant reductions in MLSS concentrations were recorded; however, biomass stabilization occurred rapidly (the following day). This could be attributed to the accumulation of biomass exceeding the capacity of the AGS-SBRs, leading to poor effluent quality due to the presence of granules. Despite the extended SRT applied during the maturation period (38 ± 7 d), there was an

increase in VSS/TSS ratio from 74 ± 4 % up to 87 ± 3 % (**Figure 4.4-b**), and the aerobic granules appeared compact and stable (**Figure B2 – Appendix B**). This confirmed the significant role of EPS production and the preferential selection of slow growing microorganisms during this period.

It is worth mentioning that the impact of this maturation period of AGS (days 118 - 422), has been directly linked to the significant stabilization in biomass concentrations (MLSS and MLVSS were increased) and settleability (SVI₅ and SVI₃₀) as shown in **Figure 4.4 (a)**. This resulted in a significant raise of the sludge age, as mentioned previously. Furthermore, results from microscopic analysis shown in **Figure 4.3** indicated that AGS were uniform and had a compact structure with a smooth surface. It should be noted that, because no intentional sludge wasting was applied in these AGS-SBRs, the calculated SRT is governed primarily by solids losses in the treated effluent rather than by the absolute MLSS/MLVSS concentration. As granules matured and settleability improved, TSS_{eff} decreased, reducing biomass washout and thereby increasing the estimated SRT. In parallel, the biomass content is also controlled by net biomass production, which declined over time as reflected by the measured variation in observed yield (Y_{obs}): ~0.14 in early maturation (days 120–150), 0.122 in mid-maturation (days 250–300), and ~0.11 in the final maturation window (days 360–422). This progressive reduction in Y_{obs}, together with endogenous respiration, provides a consistent explanation for why MLSS/MLVSS could decrease while SRT increased.

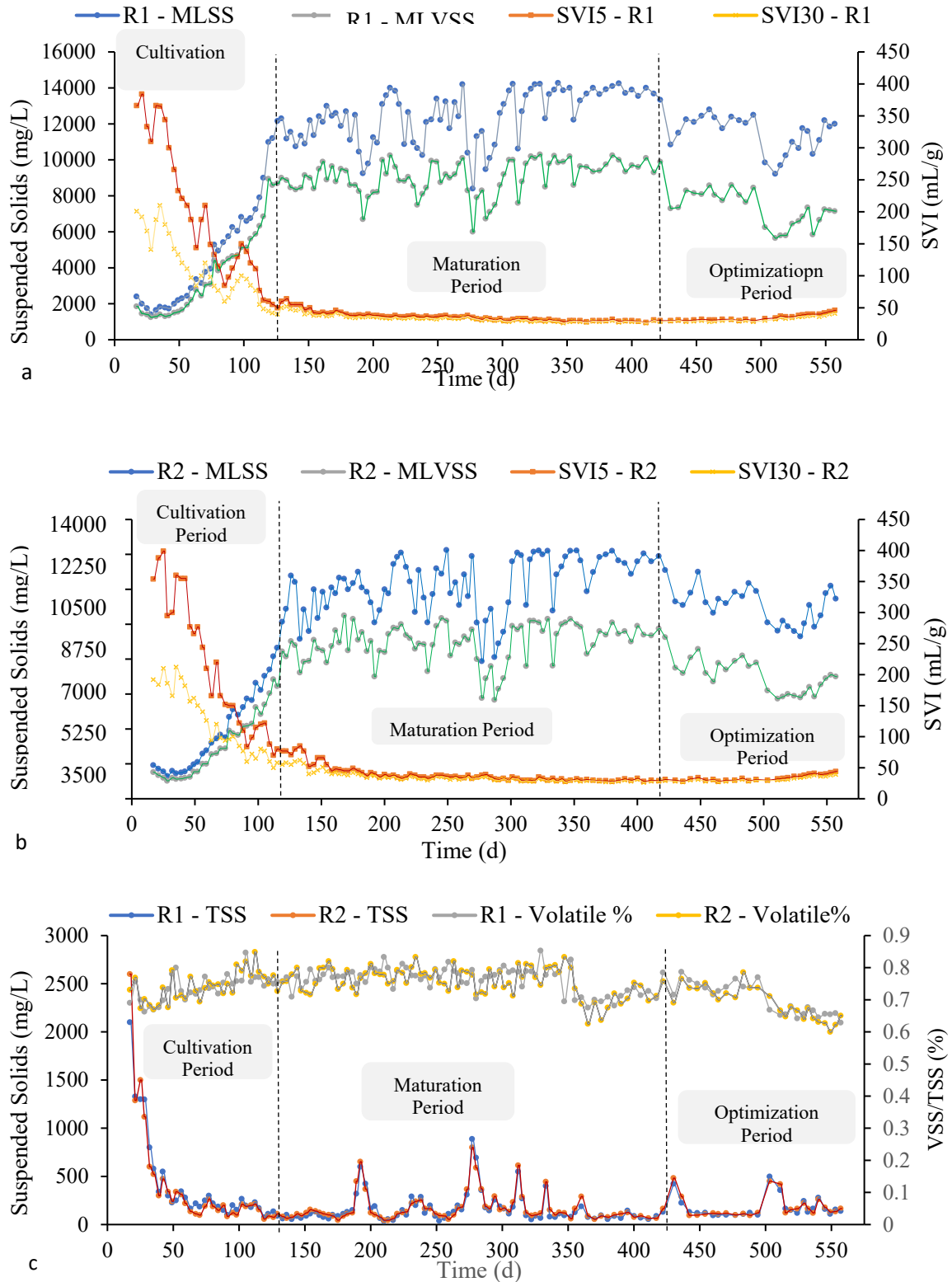


Figure 4.4. (a) MLSS, MLVSS, SVI₅, SVI₃₀ for R1; **(b)** MLSS, MLVSS, SVI₅, SVI₃₀ for R2; **(c)** Effluent concentrations of suspended solids (TSS and VSS) for both reactors

4.3.2. Long-term Performances of AGS-SBRs

4.3.2.1. Organic substrate removal

During the first 4 weeks following the inoculation, treated effluents sCOD contents were not stable and rose to 188 and 215 mg/L for R1 and R2, respectively. After day 35, the average sCOD decreased to 121 ± 6 and 115 ± 3 mg/L achieving sCOD removal efficiency $\geq 90\%$. Until day 118 (end of cultivation period), the average sCOD removal efficiency ranged from 91% to 93.5% for both reactors, attributed to the substrate ability to diffuse into the small-sized granules, enhancing the sCOD removal efficiency. During the maturation period, the increase in granules' size and SRT did not induce a significant variation in sCOD removal performance for AGS-SBRs, with mean removal efficiencies reaching $94.2 \pm 2\%$ between days 330 and 422. However, the anaerobic consumption of COD significantly increased during the maturation period (55-62%) compared to cultivation period ($\leq 45\%$). This confirmed that most COD were removed anaerobically by slow growing microorganisms such as PAOs and/or their competitors (i.e., GAOs), as well as denitrifiers and/or other bacteria responsible for performing HNAD, which favored growing under applied long sludge ages. It is reported that an influent COD/P ratio in the range 10–20 gCOD/gP (current study) is favorable for PAOs growth, whereas ratios higher than 50 gCOD/gP promotes GAOs growth (Lopez-Vazquez et al., 2009). This could be the main reason for the significant enhancement of P removal performance for R1 and R2 during maturation period as will be illustrated in the following sections.

4.3.2.2. Nitrogen removal performance

The effect of a very low sCOD/TN ratio (7.7) was explored to assess the feasibility of treating high-strength ammonium (TAN = 118 ± 1.7 mg-N/L) and high-strength

wastewater using AGS technology. **Figure 4.5 (a, b)** illustrates the profile of nitrogen removal efficiencies throughout the entire experimental period (evolution in time of nitrogen forms; $\text{NH}_4\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$ over 558 days).

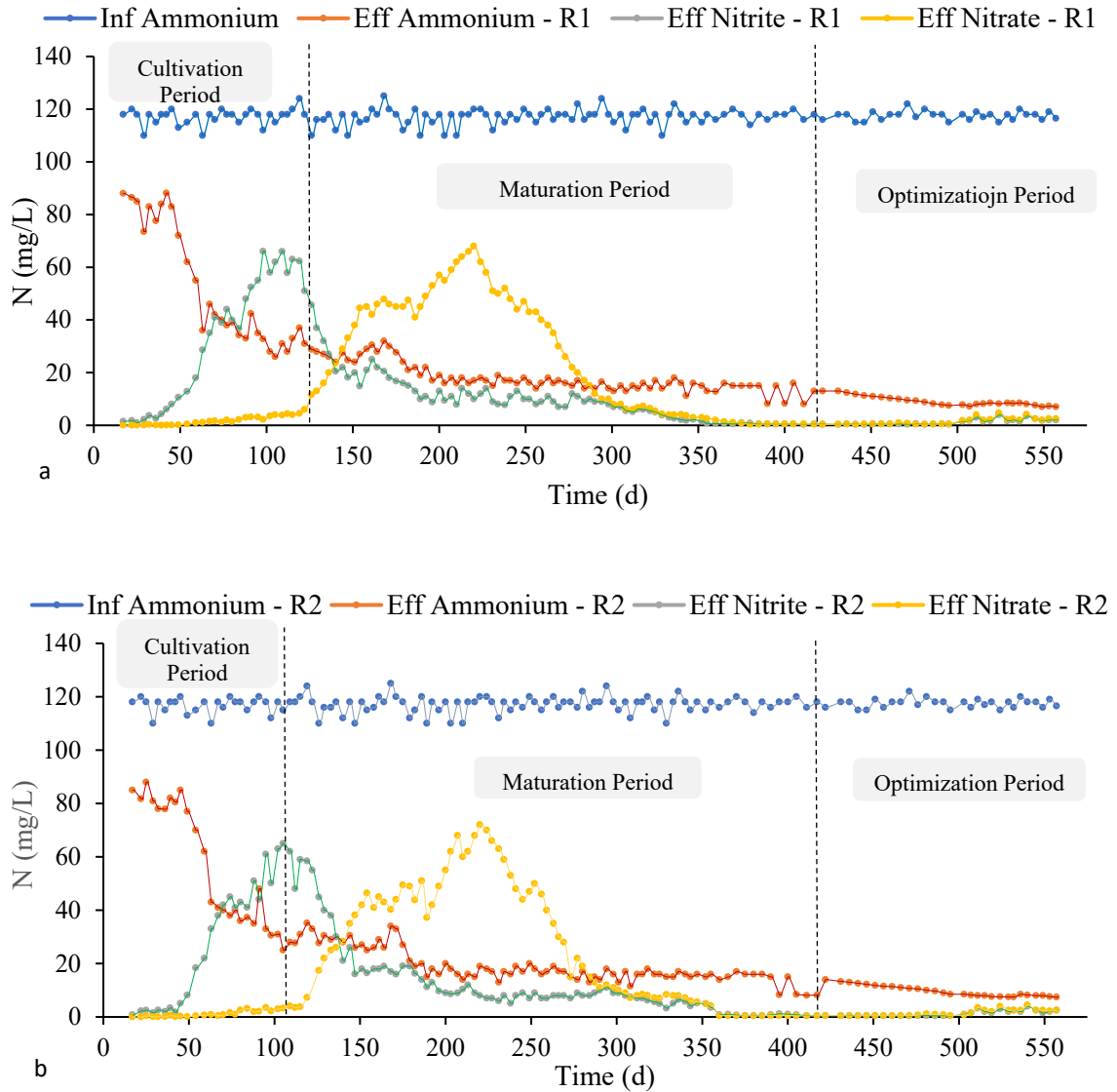


Figure 4.5: Nitrogen removal performance (TAN, Nitrite, and Nitrate) over the entire operational period for; (a) R1, and (b) R2.

During the cultivation phase until the end of the second month of operation (day 58), higher effluent concentrations of ammonium were observed (effluent TAN up to 80 mg-N/L) with a mean TAN removal efficiency of 30% (**Figure 4.5-a**) for both reactors. Only nitritation (first step of nitrification process) has been detected through high levels of NO₂-N (up to 22±3.5 mg-N/L) accompanied with very low NO₃-N concentrations (1.8±0.6 mg-N/L) in the treated effluent for both systems. This was attributed to the short settling time, which led to the discharge of poor settling sludge (TSS_{eff} over 420±152 mg/L; **Figure 4.4-a**). Thus, lower biomass concentrations in AGS-SBRs were observed (mean MLSS = 1820±530 mg/L). Under these short sludge ages (4–6 d) there is a challenging proliferation of slow-growing organisms (AOBs and NOBs) responsible for effective ammonia removal. Additionally, the high OLR of 2.25 Kg COD/m³·d resulted in the selection of fast-growing, heterotrophic bacteria rather than slow-growing nitrifying bacteria.

During the second half of the cultivation period (days 59 - 118), and after the stabilization of biomass in SBRs (day 75), the TAN removal efficiency consistently ranged between 68% and 71% (over 35 mg NH₄-N/L effluent) as depicted in **Figure 4.5**. However, TN removal efficiency was approximately 31±1.2 % due to the accumulation of nitrite and nitrate in the treated effluent, which resulted in a nitrification efficiency of up to 5±0.3 % during the same period. The bioactivity of nitrifying microorganisms was around 1.1 mg-NH₄/g-MLVSS·h. Despite the increase of SRT to 18 days, resulting in the selection of nitrifiers through an adequate level of growth of AOBs, the proliferation of NOBs was not enough to achieve full nitrification. Thus, the ammonium oxidation process was mainly performed by nitritation indicated by the high effluent concentrations of NO₂-N (62.3±17.1 and 55.3±12 mg-N/L for R1 and R2, respectively), accompanied with low NO₃-N

concentrations (4.4 ± 2.5 and 7.1 ± 5.1 mg-N/L for R1 and R2, respectively) as shown in **Figure 4.5**. Probably due to the higher sensibility of NOBs than AOBs to high FA levels (≥ 10 mg/L), resulting from treating high-strength ammonium influents under limited DO (≤ 2 mg/L). These results are consistent with a previous study that achieved better TAN removal by reducing the influent $\text{NH}_4\text{-N}$ content from 140 to 80 mg/L (Jemaat et al., 2014). Also, a low bioactivity of denitrifiers was observed based on the accumulation of $\text{NO}_2\text{-N}$ and the low anaerobic COD consumption (40.7 - 44.2%). Thus, considering the high-strength wastewater with elevated N and P loadings, the obtained results are noteworthy, compared with the reference literature (Derlon et al., 2016, Campo et al., 2020).

There was a significant increase on the performance of nitrification for both reactors from 5 ± 0.3 % at the end of cultivation period (day 118) to over 49 ± 2 % at the end of the first 2 months of the maturation period (day 178). This resulted in a significant reduction of the $\text{NO}_2\text{-N}$ concentrations and an increase of $\text{NO}_3\text{-N}$, indicating a starting of complete process of nitrification. In day 178, the effluent showed 25.8 ± 3.6 , 27 ± 2.1 mg $\text{NH}_4\text{-N/L}$, (TAN removal efficiency 81 ± 2.1 %), accompanied with 16.7/45 and 19/49 mg-N/L of $\text{NO}_2/\text{NO}_3\text{-N}$ concentrations for R1 and R2, respectively. Accordingly, such modest efficiency of full nitrification resulted in a significant TN removal performance of 43 ± 2.5 %. From day 179 to 209, there was a fluctuation in NH_4 removal performance (**figure 4.5**), while the effluent content of $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ maintained the same trend. At day 210, $\text{NO}_2/\text{NO}_3\text{-N}$ concentrations in the treated effluent were 8/68 and 9.1/72 mg-N/L for R1 and R2, respectively, while TAN dropped to 14 ± 1.3 mg-N/L. These results indicated that the full nitrification process (68 ± 2.2 %) has been developed (**Figure 4.5-a**), confirming the proliferation of NOBs in AGS-SBRs. NOBs were indeed a part of microbial structure in

AGS, yet their activity appeared to be partially subdued, possibly as a consequence of the effect of reduced DO concentrations (≤ 2 mg/L) during the aeration zone of the treatment cycle where NOBs may be outcompeted by AOBs, PAOs/GAOs and OHOs. In addition, NOB activity can be limited by operating at low oxygen availability relative to ammonium, expressed as a DO/TAN (DO-to-NH₄-N) concentration ratio (0.09–0.21 gO₂/gNH₄-N), as suggested in the literature (Campo et al., 2020 and Bartrolí et al., 2010).

Between days 211 and 300, the TAN removal efficiency increased reaching an average of 88 ± 0.8 % for R1 and 84 ± 2.1 % for R2, with achieving denitrification process performance about 87 ± 1.2 % at day 300 (R1 and R2). Nonetheless, the AGS performance on TN removal by day 300 reached only 75 ± 1.1 % (R1 and R2). An enhancement in ammonium oxidation rate of 1.4 ± 0.12 mg-NH₄/g-MLVSS·h was observed in day 300. The reactors' performance was accompanied by an increase of granules' size (2.4 mm) and EPS content (over 230 ± 12 mg/gVSS). No significant changes were observed in the nitrite concentrations in the effluent during this period (**Figure 4.5**). During the last four months of the maturation period (days 300 - 422), AGS-SBRs achieved stable performance in the TAN removal ($87 \pm 0.6\%$ for R1 – $89 \pm 1.4\%$ for R2), with a maximum ammonium oxidation rate that has been recorded of 1.72 ± 0.14 mg-NH₄/g-MLVSS·h (day 405). The increase in the granules' size along with the formation of compact structure enhanced the capability of denitrification process (up to 98%) by eliminating the nitrite/nitrate accumulation after the treatment cycle (i.e., achieving SND), especially during the last three months in this period. Effluent concentrations of nitrite/nitrate ranged from 2.2 and 5 mg-N/L, respectively, between days 301 and 325. Furthermore, with continuous maturation under the same operating conditions, matured AGS demonstrated highly efficient performance in SND ($\geq$

99% for both reactors), achieving low nitrite/nitrate content ($\leq 0.5 \pm 0.3$ mg-N/L) between days 330 and 422. Accordingly, a significant enhancement of TN removal performance (82 ± 0.7 %) was recorded for R1 and R2 by the end of maturation stage (day 422). The kinetic studies performed during these four months of operation are reported in **Figure 4.6**, showing a stable performance of the bioactivity of nitrifiers with constant effluent concentrations of $\text{NH}_4\text{-N}$ (about 13 ± 0.8 mg/L), low $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ content. It is worth mentioning that $\text{NH}_4\text{-N}$ removal efficiency over 90% was achieved only in few days (days 390, 400, 411 for R1 and days 395, 405, 411, 417 for R2) as shown in **Figure 4.5**.

Overall, the increase of granules' size and applied SRT during the maturation period had a positive impact on the selection of nitrifiers and denitrifiers. The results showed an enhancement of the denitrification process, with SND performed at AGS size higher than 2.6 mm between days 330 - 422. Thus, obtaining matured granules was effective to protect both nitrifying and denitrifiers from inhibition factors (e.g., FA) via their large size and compact structure (however it was not sufficient to achieve complete oxidation of the influent $\text{NH}_4\text{-N}$). This was accompanied by a significant high consumption of organic matter during the anaerobic phase (58%), confirming the rbCOD uptake by denitrifying organisms. The performance of nitrite/nitrate reduction process registered in the present study is comparable with other literature results for low COD/TN wastewater treatment with aerobic granulation technology (Derlon et al., 2016 and Campo et al., 2020).

4.3.2.3. Phosphorus removal

The reduction of P content in AGS-SBRs effluent over time is presented in **Figure 4.6 (a, b)**. P removal was not observed until day 40 after inoculation, mainly due to the

type of seeded sludge at the beginning of the operation and fluctuations of P removal performance were observed until day 75. At day 118 (cultivation period), the effluent SP content dropped to around 9.6 ± 0.7 mg/L (42 - 44% P removal efficiency) for both reactors (**Figure 4.6**) and the rates of anaerobic P-release and aerobic P-uptake was estimated around 3.3 and 0.8 mg-P/g-MLVSS·h. Despite the availability of sCOD during the anaerobic feeding phase (1250 ± 70 mg-COD/L) these incoming influents could not provide a sufficient carbon source (COD/TP was 20), resulting in biological competition for carbon uptake between PAOs and their competitors (GAOs and denitrifiers). Additionally, after start-up period (days 77 - 118), anaerobic sCOD removal for both reactors did not exceed $38.2 \pm 2.5\%$ (i.e., extended feast conditions), indicating proliferation of fast-growing microorganisms. These results show that, the structure of the cultivated granules (1.2 mm on day 118) was not enough to provide sufficient P-release and P-uptake rates, and this can be explained by blockage of the pores on the granules' surface which obstructed by an overabundance of EPS and the growth of filamentous microorganisms. However, efficient P removal was only achieved after 300 days of maturation, and the effluent SP content dropped to 1.5 ± 0.2 mg-P/L by day 422 (**Figure 4.6**).

P removal efficiency gradually improved and reached $75 \pm 4.2\%$ on day 210, but no significant increase was observed from day 210 to 266 (**Figure 4.6-a**), despite AGS growing to over 2.0 mm. This could be attributed to the long-term treatment of high-strength ammonium influents (COD/TN is 100/14) under limited DO conditions, where the resulted FA (more than 10 mg/L based on **Eq. 4.1**) can prevent the biological removal of phosphate. As reported in previous studies, under this condition the diversity and

abundance of PAOs are minimized and inhibited in the systems exposed to FA over 10 mg NH₃/L, affecting the P removal efficiency (Cao et al., 2017).

Fluctuations were observed on the P removal efficiency between days 267 and 330, where over 88% removal was achieved in some days (**Figure 4.6**) and P-release and P-uptake rates reached 4.1 ± 0.27 and 0.63 ± 0.1 mg-P/g-MLVSS·h, respectively. Interestingly, during the last three months of the maturation period, the increasing in the granules' size had a direct effect on the P removal performance. The average diameter of AGS in both reactors increased from 2.6 mm at day 330 to 3.0 mm at day 422. AGS-SBRs achieved P removal up to 92%, accompanied by 60% of anaerobic sCOD removal performance (**Figure 4.8; a, c**). Throughout the study, the COD/TP ratio relatively constant at 20, likely favoring the PAOs activity as the influent sCOD content was degraded by about 60% during the anaerobic phase. These results are consistent with previous studies, which reported that influent COD/TP ratios between 10–20 gCOD/gP are favorable to the growth of PAOs compared to their competitors (Hamza et al., 2018). This confirmed that maturation process helped on enhancing P removal performance significantly through converting the cultivated granules (fragile AGS) into stable matured AGS with a large and multilayered structure (i.e., compact AGS) which help the granule itself to be a favorable environment that allows the selection (coexistence and proliferation) of slow-growing bacteria (e.g., PAOs, DPAOs).

By the end of the maturation period, P content in the settled biomass (matured AGS) at the end of a typical treatment cycle has been estimated of 0.21 g-P/gVSS, exceeding the 50 mg-P/gVSS reported in the literature for the P content in sludge caused mainly by ordinary heterotrophic organisms (Tchobanoglous and Eddy, 2014). Thus, this content

indicated that P removal performance primarily was primarily attributed to the enhanced biological phosphorus removal through various microorganisms of PAOs and/or DPAOs.

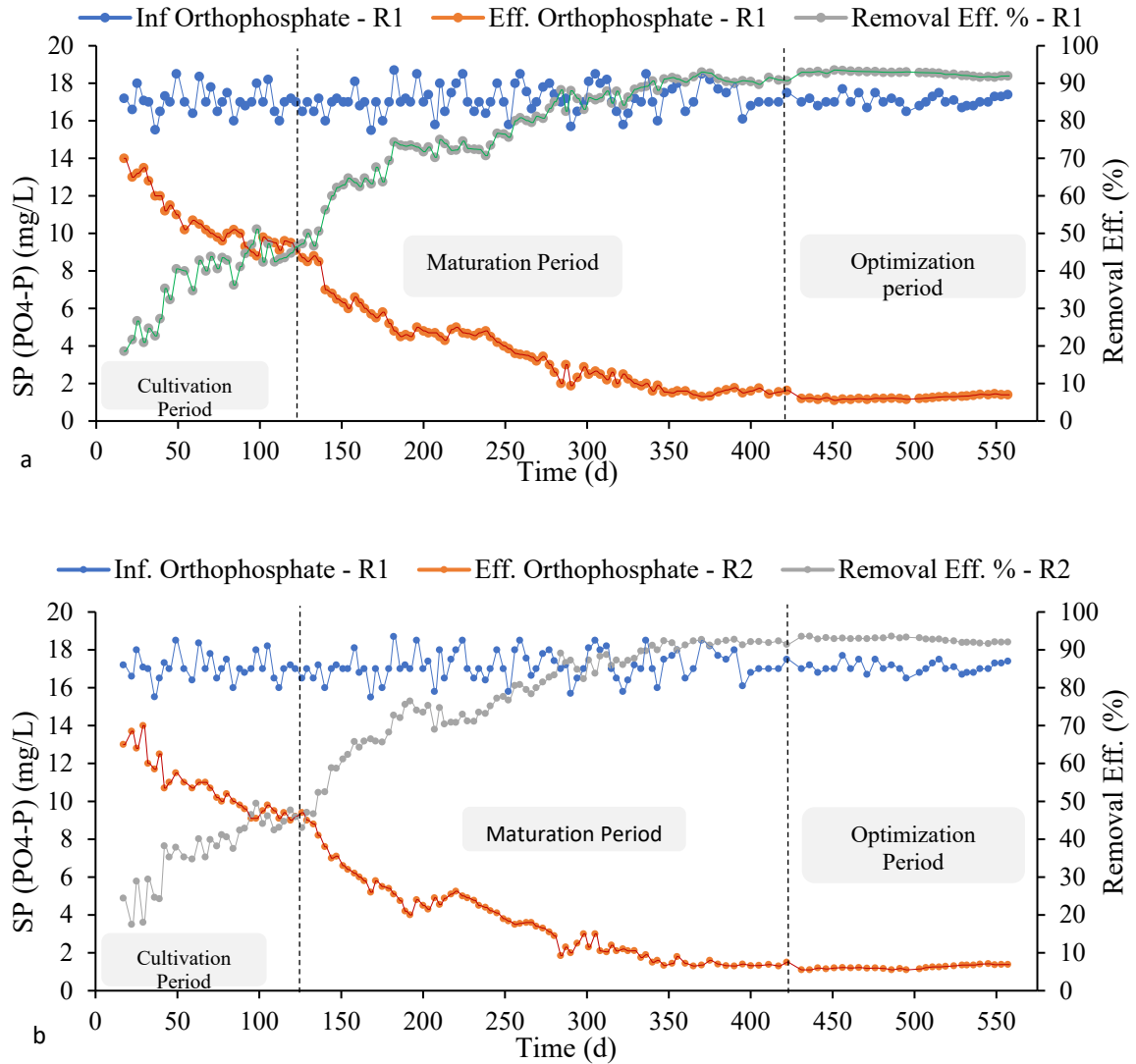


Figure 4.6: Evaluation of the P-removal performance over the entire operational period for; (a) R1, and (b) R2.

4.3.3. Microbial Community in Matured Granules for AGS-SBRs

The microbial community composition of matured granules by the end of 14 months of operation following the inoculation (cultivation and maturation period) was analyzed and reported in this section. The taxonomic affiliation of the dominant bacteria at

class, order, family, and genus levels, with the relative abundance in the matured AGS (day 422) is illustrated in **Figure 4.7 (a, b, c and d)** and **Table 4.3**. 16S rRNA gene sequencing of the microbial consortium of aerobic granules has been classified into 30 bacterial phyla, 60 classes, 117 orders, 186 families, 315 genera and 469 identified species. These taxonomic results were comparable to those found in a previous study (Fall et al., 2022), correlating the composition of the microbial community in AGS-SBR to the removal performance of C and N over more than 3 years of operation.

The results showed no significant differences between samples from R1 and R2 at the phylum level (**Figure B3, Microbial community fingerprinting – Appendix B**). *Proteobacteria* (34.2% - 39.9%), *Firmicutes* (28.5% - 32.9%), and *Bacteroidota* (25.2% - 11.6%) were the top three predominant phyla in all the samples, followed by *Actinobacteriota* (7.21% - 6.14%), *Synergisota* (1.25% - 4.73%), *Verrucomicrobiota* (1.12% - 1.56%). The results show that the long-term treatment of high-strength wastewater (1250±70 mg COD/L) with elevated NH₄-N (118±1.7 mg/L) and TP (54±11 mg/L) loadings under limited DO levels (≤ 2 mg/L) had a significant impact on microbial community composition, favoring the growth of *Proteobacteria*, *Firmicutes* and *Bacteroidota* as top abundant genera. *Proteobacteria* are the most common bacteria in WWTPs and are involved in biological N removal processes (Ding et al., 2019). After achieving compact granules (3.0 mm) by day 425 of operation, the transfer of oxygen within the AGS structure was limited, thus promoting the growth of *Firmicutes* (primarily composed of denitrifying bacteria).

Gammaproteobacteria (24.4% - 31.1%), *Clostridia* (23.6% - 28.6%), *Bacteroidia* (24.6% - 11%), *Alphaproteobacteria* (9.8% - 8.7%), and *Actinobacteria* (7% - 6.1%) were the predominant classes of the AGS in both reactors as shown in **Figure 4.7 (a)**. *Bacilli*, *Synergistia*, *Verrucomicrobiae*, *Negativicutes* BRs were between 1% and 4%. Unexpectedly, the analysis of microbial structure demonstrated that *Betaproteobacteria* (order: *Burkholderiales*; family: *Comamonadaceae*) were only between 3.6% and 9.0% for R1 and R2. This contrasts with results in various literature that considered *Betaproteobacteria* as the most abundant class in municipal WWTPs worldwide, largely responsible for organic and nutrients removal (Isazadeh et al., 2016 and Cydzik-Kwiatkowska and Zielińska, 2016).

The dominant order in the AGS for both reactors was *Peptostreptococcales-Tissierellales* (17.8% - 19.9%) which belongs to *Clostridia* (*Firmicutes*) class (**Figure 4.7; b**), while *Comamonadaceae* (10.3% - 8.7%) was the dominant family (**Figure 4.7; c**). It is noteworthy that these bacteria (especially *Comamonadaceae*) in the AGS for both SBRs that treating high-strength ammonium influents (118 ± 1.7 mg-N/L) could convert carbon sources into storage matter, which provides carbon and energy source in the aerobic famine phase (350 min), performing simultaneous removal of carbon and nitrogen. *Rhodobacteraceae* (5.4% - 5.5%) (aerobic bacterium) and *Rhodocyclaceae* bacteria (1.0% - 0.7%), (along with family *Flavobacteriaceae*) have played a major role in EPS secretion and facilitate the granulation process during 10 months of maturation period. Both *Comamonadaceae* and *Rhodocyclaceae* were reported in the literature to positively involve in denitrification process with positive impact on nitrate removal (Xiong et al., 2020). In addition, the relative abundance of *Xanthomonadaceae* in AGS was between 6.4% and

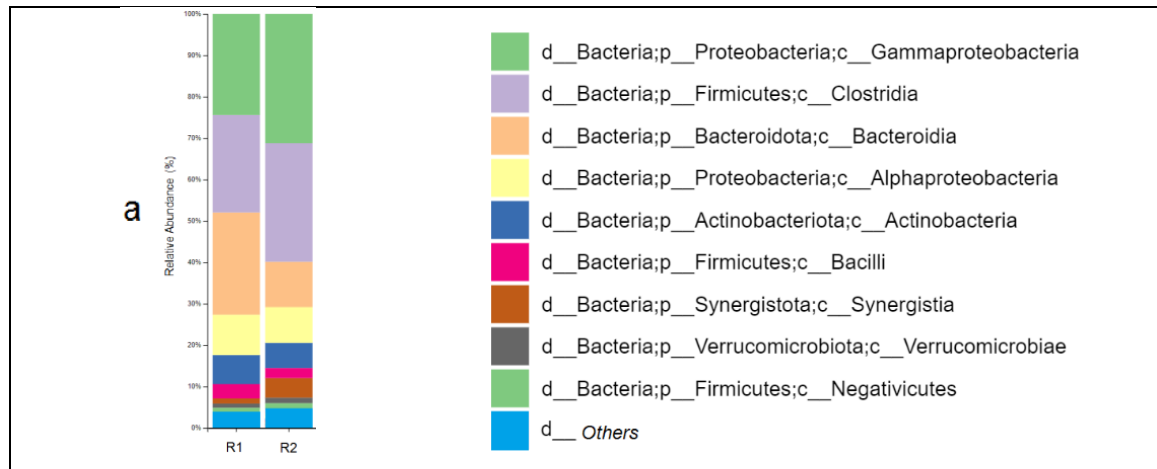
4.8%, and affected positively in enhancing granular stability and used as a core part of AGS.

Compared with the composition of microbial structure at the phylum and class levels, the compositions at genus level showed significant differences. **Table (4.3)** illustrates a listing of the top 27 bacterial genera, which represent about 88% of the total abundance for AGS-SBRs. *Leadbetterella* was the dominant genera in AGS was reported to play an important role in the aerobic granulation process (i.e., formation and stability) irrespective of the type of wastewater being used as substrate (Abdullah et al., 2013). *Thiothrix* sp. was reported to behave like PAOs with the ability to fix atmospheric carbon dioxide for P removal and use an intracellular sulphur pool as a source of energy (mixotrophic metabolism) (Rubio-Rincón et al., 2017). The other three dominant bacteria, *Acetoanaerobium*, *Proteocatella*, *Proteiniclasticum*, were reported in the literature as anaerobic acetogenic bacteria, with the ability to perform carbohydrates fermentation to produce acetate as well as other volatile acids (Supaphol et al., 2011). *Pseudoxanthomonas* has been reported as important for the formation and stabilization of the aerobic granules (Wan et al., 2014). In addition, these bacteria were responsible for removing P, where it has been reported in a previous study (Wang et al., 2018) that they could perform denitrification (preferably using nitrite) associated with P removal.

Among the dominant discriminating genera in the AGS-SBRs were *Rhodobacter*, *Diaphorobacter*, *Hydrogenophaga*, and *Niabella*, which are considered the main candidates potentially responsible for HNAD as demonstrated in the previous studies (Third et al., 2003, Yin et al., 2019, Bucci et al., 2021, Wang et al., 2019). These facultative

hydrophilic autotrophic bacteria are capable of anaerobic nitrate respiration and denitrification, and their abundance may enhance SND in AGS. Furthermore, *Ottowia* sp. was found in AGS-SBRs, facultative anaerobic bacteria that can use NO₂-N and NO₃-N as electron acceptors for denitrification, and its presence in the microbial consortia is assumed to be responsible for the high TN removal efficiency. *Ottowia* sp., as functional denitrifying bacteria, is related to carbon and nitrogen removal, and has showed an upward trend during the aerobic granulation process, effectively decomposing organic substances (He et al., 2017 and Xu et al., 2021).

Microbial analysis showed that there were other putative EPS bacterial groups which have the ability to secrete EPS, and might have become the main contributors to the stability of AGS for both reactors over 10 months of maturation period, such as *Pseudoxanthomonas*, *Flavobacterium* and *Acinetobacter*. Additionally, several bacterial genera found in AGS-SBRs, including, *Rhodobacter* and *Luteimonas*, have been reported to be capable of promoting cohesion and granulation (Xiong et al., 2020).



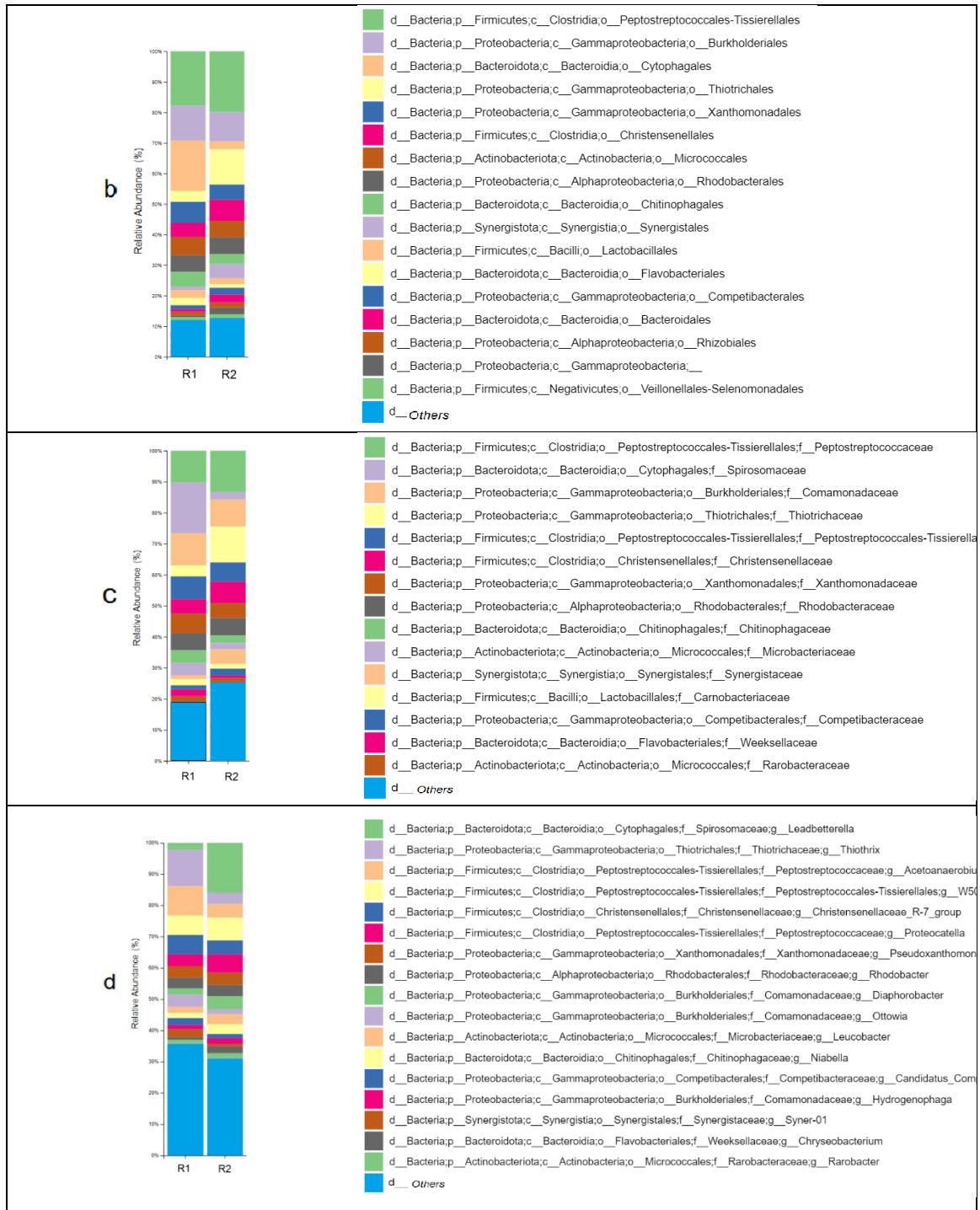


Figure 4.7: Distribution of dominant microbial diversity (relative abundance > 1%) at;
(a) Class, (b) Order, (c) Family, and (d) Genus levels in R1 and R2

Table 4.3. Dominant bacterial genera of the AGS for both reactors*

Rank #	Canonical genus name	Relative abundance** (%)	Rank #	Canonical genus name	Relative abundance** (%)
1	<i>Leadbetterella</i> (p_Bacteroidota;c_Bacteroidia)	13.7% - 16%	15	<i>Candidatus_Competibacter</i> (p_Proteobacteria;c_Gammaproteobacteria)	1.7 – 2 %
2	<i>Thiothrix</i> (p_Proteobacteria;c_Gammaproteobacteria)	11.5% - 8%	16	<i>Comamonas</i> (p_Proteobacteria;c_Gammaproteobacteria)	1.5 – 2 %
3	<i>Acetoanaerobium</i> (p_Firmicutes;c_Clostridia)	9.4% - 4.4%	17	<i>Hydrogenophaga</i> (p_Proteobacteria;c_Gammaproteobacteria)	1.5 – 1.9 %
4	<i>W5053 (Tissierellales;g)</i> (p_Firmicutes;c_Clostridia)	6.2% - 7.3%	18	<i>Luteimonas</i> (p_Proteobacteria;c_Gammaproteobacteria)	1.3 – 1.9 %
5	<i>R-7_group (Christensenellaceae)</i> (p_Firmicutes;c_Clostridia)	6.2% - 4.4%	19	<i>Neomegalonema</i> (p_Proteobacteria;c_Alphaproteobacteria)	1 – 1.8 %
6	<i>Proteocatella</i> (p_Firmicutes;c_Clostridia)	3.8% - 5.8%	20	<i>Rarobacter</i> (p_Actinobacteriota;c_Actinobacteria)	1.2 – 1.6 %
7	<i>Pseudoxanthomonas</i> (p_Proteobacteria;c_Gammaproteobacteria)	3.7% - 4.0%	21	<i>Thauera</i> (p_Proteobacteria;c_Gammaproteobacteria)	0.8 – 1.3 %
8	<i>Rhodobacter</i> (p_Proteobacteria;c_Alphaproteobacteria)	3.4% - 3.5%	22	<i>Gemmobacter</i> (p_Proteobacteria;c_Alphaproteobacteria)	1 – 1.2 %
9	<i>Diaphorobacter</i> (p_Proteobacteria;c_Gammaproteobacteria)	2.2% - 4.1%	23	<i>Tetrasphaera</i> (p_Actinobacteriota;c_Actinobacteria)	0.7 – 1 %
10	<i>Ottowia</i> (p_Proteobacteria;c_Gammaproteobacteria)	3.2% - 1.8%	24	<i>Lentimicrobium</i> (p_Bacteroidota;c_Bacteroidia)	1.1 – 1.2 %
11	<i>Leucobacter</i> (p_Actinobacteriota;c_Actinobacteria)	1.9% - 3.0%	25	<i>Candidatus_Accumulibacter</i> (p_Proteobacteria;c_Gammaproteobacteria)	0.6 – 0.9 %
12	<i>Niabella</i> (p_Bacteroidota;c_Bacteroidia)	1.7% - 2.8%	26	<i>Acinetobacter</i> (p_Proteobacteria;c_Gammaproteobacteria)	0.5 – 0.9 %
13	<i>Syner-01</i> (p_Synergistota;c_Synergistia)	2.2% - 1.9%	27	<i>Nitrosomonas</i> (p_Proteobacteria;c_Gammaproteobacteria)	0.5 – 0.9 %
14	<i>Chryseobacterium</i> (p_Bacteroidota;c_Bacteroidia)	1.4% - 1.9%	28	<i>Others</i>	< 0.9 %

* The top 27 genera represent 88.6% for R1, and 87.8% for R2

** Relative abundance (%) of each genus in R1 and R2.

*** Relative abundance of "Others" is less than 1.0% for both reactors including;

(i) Group (A) 0.3% - 0.9%: OLB8 (Saprospiraceae;g), Uncultured (Rikenellaceae;g), Uncultured (Prolixibacteraceae;g), Roseomonas, Enterococcus, Carnobacterium, Propioniceella, Paracoccus, Ferruginibacter, Luteolibacter, Microbacterium, Bosea, Ignavibacterium Acinetobacter, Shinella, Propionivibrio, Persicitalea, Sandaracinobacter, Brachymonas, Nitrosomonas.

(ii) Group (B) less than 0.3%: about 270 genera (some of them are represented in Table B1.

Microbial community fingerprinting – Appendix B).

4.3.4. Cycle Experiments in the Maturation Period

Figure 4.8 illustrates the simultaneous conversions and removals of organic substrate and P, nitrogenous constituents over the typical treatment cycle between days 330 – 422, where large sized matured granules were present. During the maturation period, the mean $\text{NH}_4\text{-N}$ removal efficiency for R1 and R2 were $88\pm1.7\%$ and $86\pm1.2\%$, respectively (**Figure 4.5; a, b**). **Figure 4.8 (b, d)** shows that the treatment cycle started with 118 ± 1.7 mg $\text{NH}_4\text{-N/L}$. During 5 h through the aeration phase (5.83 h), about 104 ± 2.2 mg $\text{NH}_4\text{-N/L}$ for R1 and 105 ± 1.5 mg $\text{NH}_4\text{-N/L}$ for R2 were oxidized to nitrite/nitrate, which were finally reduced to N_2 . There were almost no $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ detected in the treated effluent ($\leq 0.5\pm0.3$ mg-N/L due to high SND performance), resulting in 82% TN removal, with $\text{NH}_4\text{-N}$ oxidation rate of 1.86 ± 0.11 mg- $\text{NH}_4\text{/g-MLVSS}\cdot\text{h}$ between days 400 and 422.

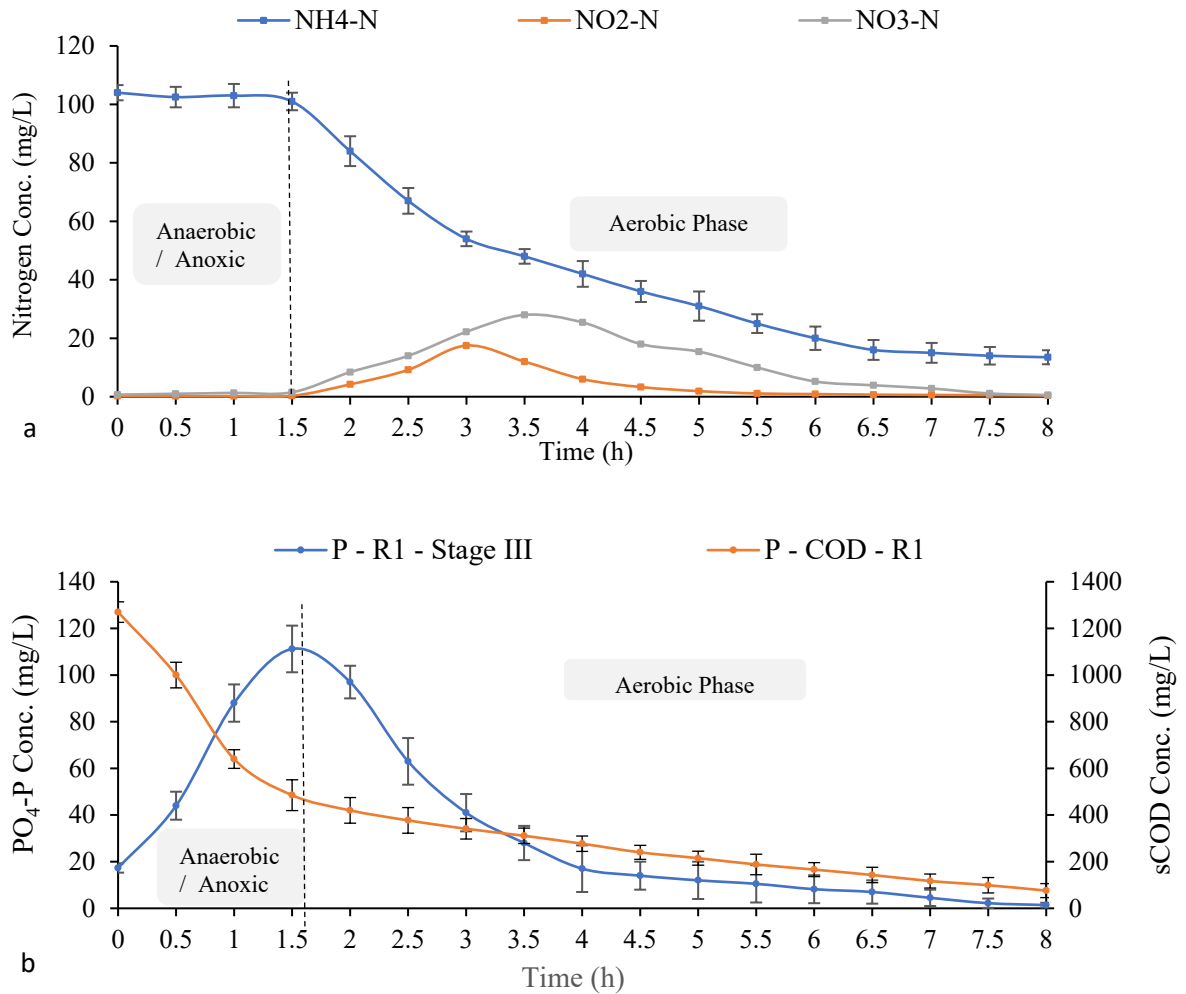
These results agreed with previous studies, demonstrating effective SND with macro-size granules due to the coexistence of aerobic and anoxic layers, but not efficient with too small granules. It can also be affected by various factors such as DO, electron acceptor availability, and microbial activity (Coma et al., 2012 and Nancharaiah and Reddy, 2018). In addition, although relatively high FA concentrations (10 mg/L) were created during the treatment cycle, stable TAN removal efficiency followed by stable effective SND performance was detected toward the maturation period. This can be attributed to the compact and unique structure of the matured granules. Furthermore, this improvement in the N removal performance was confirmed by the relative abundance of nitrifying and denitrifying bacteria (e.g., *Rhodobacter*, *Diaphorobacter*, *Hydrogenophaga*, *Ottowia*, and *Niabella*) based on the microbial sequencing analysis performed at the end of the maturation period (day 425), and discussed in the previous section. It should be noted

that during the last three months of the maturation period (days 330 – 422), a relative reduction of pH has been recorded in both reactors (anaerobic phase: 7.8 ± 0.2 ; aerobic 7.3 ± 0.1) compared to the cultivation period and the first months of operation in the maturation period, probably because of the balance between alkalinity consumption and production achieved during nitrification and denitrification (SND).

With an initial of $\text{PO}_4^{3-}\text{-P}$ content of 17 ± 0.8 mg-P/L, the effluent $\text{PO}_4^{3-}\text{-P}$ dropped between 1.33 and 1.68 mg-P/L during the last three months of the maturation period as shown in **Figure 4.8 (a, c)**. The anaerobic removal of organic substrate also increased, achieving 60% to 61.4% of sCOD removal, while the remaining sCOD (480 ± 42 mg-COD/L) was utilized aerobically very fast (famine conditions). sCOD concentrations decreased below 80 mg-COD/L after 2 h under aerobic mixing. This significant anaerobic consumption of sCOD could be due to the successful growth of slow growing microorganisms (e.g., PAOs, GAOs, DPAOs and DGAOs). Based on the kinetic tests (**Figure 4.8a,c**), biological P removal improved during maturation. Community profiling showed enrichment of *Thiothrix* (11.5%) and *Pseudoxanthomonas* (4%), which have been reported in the literature as putative PAO-like and putative DPAO/DPR-associated taxa, respectively (Rubio-Rincón et al., 2017, Alves et al., 2022, Wang et al., 2018). However, definitive functional assignment cannot be made from 16S genus-level data alone.

Future work should validate these putative functional assignments using targeted functional evidence (e.g., poly-P staining and/or FISH/qPCR of PAO/DPAO markers, complemented by metagenomic/functional gene analyses), since genus-level 16S data alone cannot confirm PAO/DPAO activity. The mean value of anaerobic P-release and

aerobic P-uptake rates during the same period was estimated at 4.2 ± 0.11 and 0.5 ± 0.1 mg-P/g-MLVSS·h, achieving a mean P removal efficiency of 90.2 ± 1.1 % for R1 and 89.5 ± 0.7 % for R2. In addition, the effect of long-term maturation under continuous hydraulic shear and short settling time (10 min) have played a significant role in facilitating EPS secretions (EPS content of 258 mg/gVSS) by various microorganisms such as *Rhodobacteraceae* (5.5%) and putative EPS bacterial groups (*Pseudoxanthomonas*, *Flavobacterium* and *Acinetobacter*). This directly helped in promoting granulation and the enrichment of slow-growing bacteria (e.g., PAOs, DPAOs) in the AGS and maintained its physical properties (large stable AGS), as reported in the literature (Cheng et al., 2023).



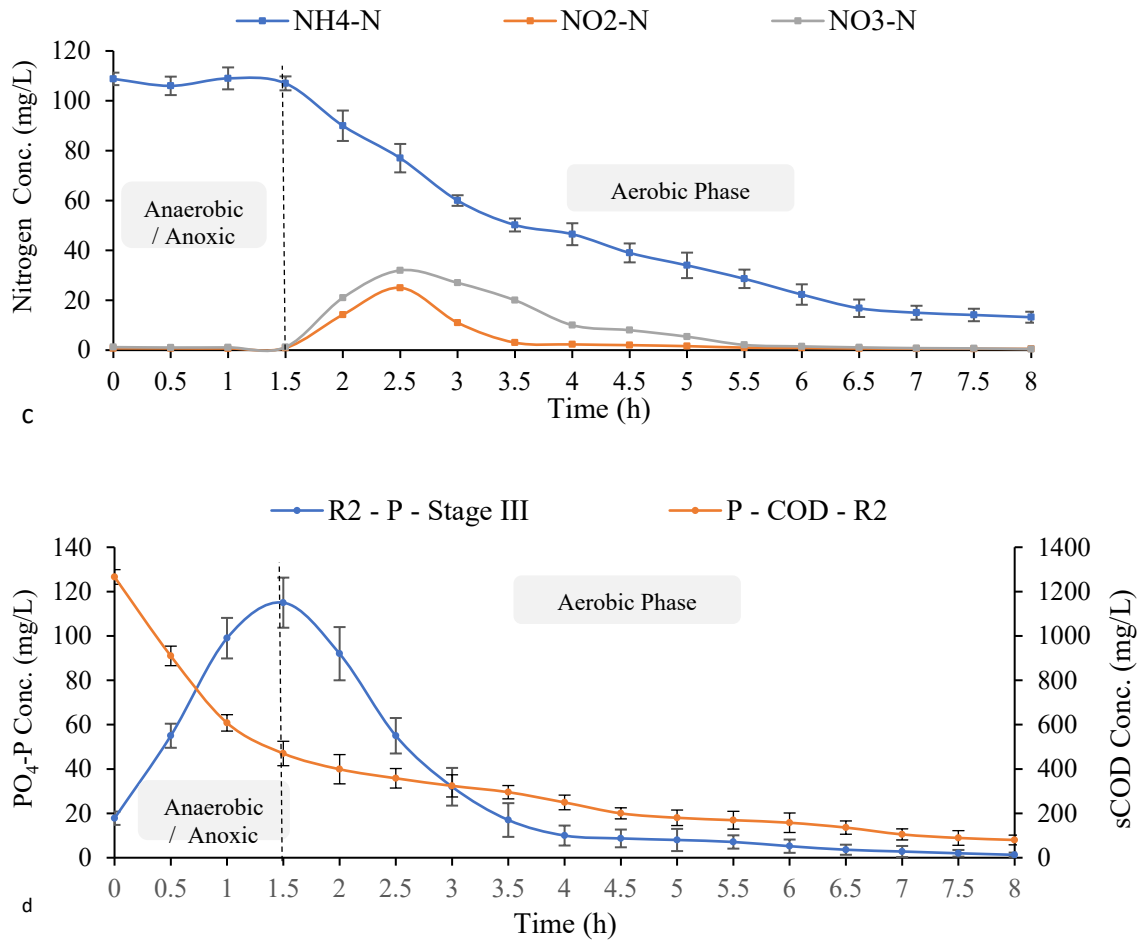
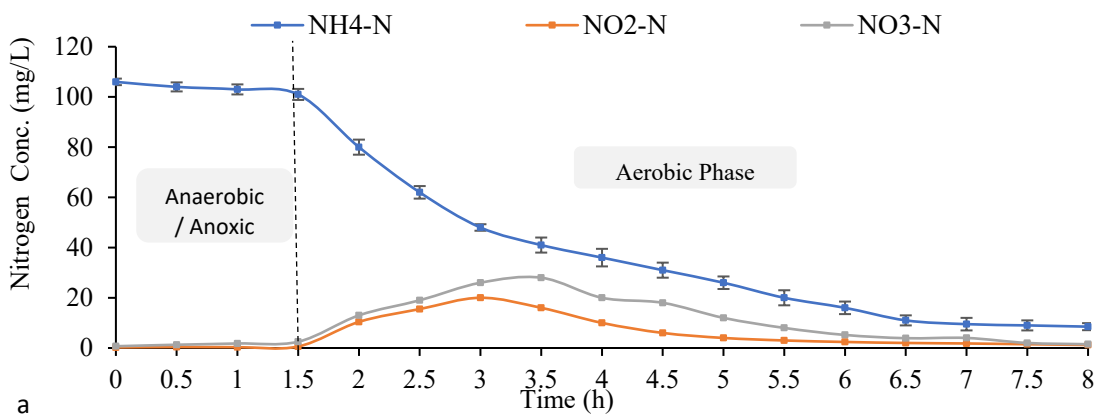


Figure 4.8: Performance profiles of AGS-SBRs distributed over a typical treatment cycle (8 h) from days 330-422; (a) N constituents and (b) sCOD and P constituents for R1, (c) N constituents and (d) sCOD and P constituents for R2.

4.3.5. Optimization of AGS-SBRs

By the end of the maturation period, large stabled granules were successfully obtained, displaying relatively high simultaneous BNR efficiency of 94/82/92% for R1 and 93/83/91% for R2 (days 370 - 422). Furthermore, the formation of settled layer of biomass (0.3 L) was observed at the bottom of both SBRs during the aerobic phase (**Figure B4 – Appendix B**). This can be attributed to: (a) the applied superficial air velocity (0.43 cm/s)

resulted from air pumps (2.92 L/min) became insufficient to provide the required mixing conditions to large matured granules, (b) the biomass concentrations in each reactor increased incredibly over 14 g MLSS/L (**Figure 4.3; a, b**), (c) no sludge wasting phase, so all suspended solids were discharged during the decant stage. Thus, under low bulk DO, mature AGS likely DO gradients that can support SND; the observed high denitrification/SND efficiencies are consistent with this behavior. Nevertheless, intra-granular DO/anoxic zones and HNAD activity were not directly measured in this study, so this interpretation should be considered a hypothesis requiring targeted validation. After that, an optimization was conducted for AGS-SBRs over four months, during which improvements in biomass mixing conditions and DO levels were implemented by introducing an additional air pump. The operational adjustments aimed to prevent sedimentation during aerobic mixing phase, and raise the applied DO up to 8.5 mg/L through two phases; (i) Stage I (days 423 - 495): DO 5 - 6 mg/L; both air pumps (i.e., old and additional pump) were operated with the air flow rate (3.73 L min^{-1}), and superficial air velocity (0.55 cm/s), (ii) Stage II (days 496 - 558): DO 6.5 – 8.5 mg/L; both air pumps were operated with new air flow rate (4.8 L/min), and superficial air velocity of (0.71 cm/s). An adaptation period of 7 days was observed for both SBRs at the beginning of each stage.



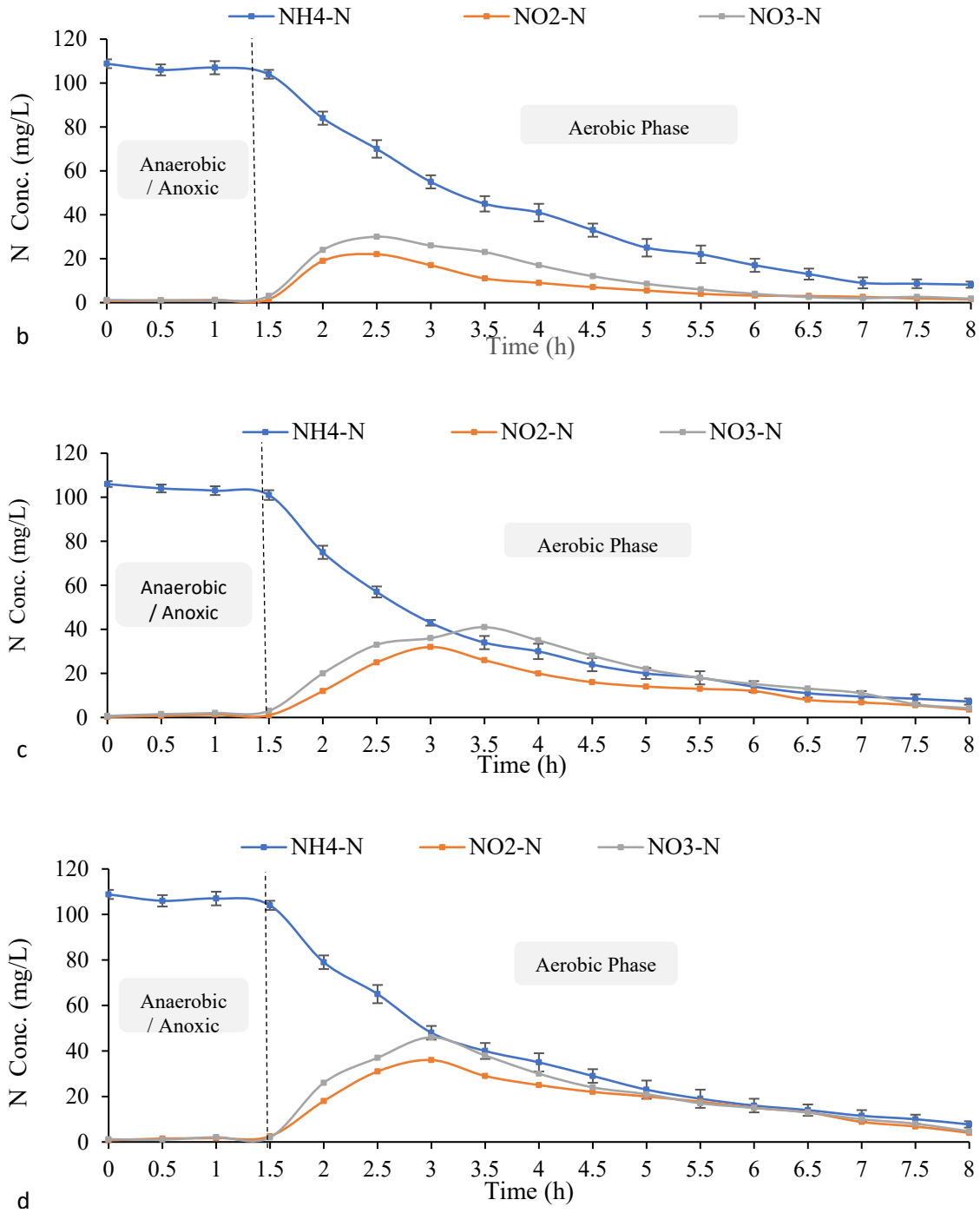


Figure 4.9: Changes in the concentrations of nitrogenous constituents distributed over a typical treatment cycle during the optimization period (days 423–558); (a), (b) R1 and R2 during stage I (days 423–495), (c), (d) R1 and R2 during stage II (days 496–558).

Following the aforementioned optimization, the TSS_{eff} content increased, reaching 452 ± 20 mg/L on day 424 in both SBRs, then stabilized between 95-122 mg/L (**Figure 4.4-c**), slightly higher than TSS_{eff} concentrations during maturation period. During stage II, high TSS_{eff} up to 474 ± 33 , 357 ± 42 , 244 ± 18 , 279 ± 22 mg/L on days 490, 498, 516, and 527, respectively. Stable TSS_{eff} between 118-165 mg/L (**Figure 4.4-c**) were achieved in stage II, 39% higher than the concentrations during the maturation period (TSS_{eff} 88-112 mg/L). The poor effluent quality observed during stage II is attributed to the application of superficial air velocity of 0.71 cm/s resulted from introducing high air flow rate of 4.8 L/min. Moreover, a gradual decrease in both MLSS and MLVSS were observed (**Figure 4.4; a, b**) until the end of both stages (day 558). The mean MLSS and MLVSS concentrations were 11.9 ± 0.6 and 7.9 ± 1.3 g/L during stage I and 10.7 ± 0.8 and 6.4 ± 1.1 g/L during stage II. MLVSS/MLSS ratio decreased from 0.71 (days 430-424) to 0.69 during stage I and to 0.66 during stage II. This decrease is mainly due to the accumulation of fixed non-volatile matter (e.g., $CaCO_3$ that was introduced with the influents (synthetic wastewater)), through the long-term operation (with long SRT) which quickly settled at the bottom of SBRs. This trend indicates an increase in inert/fixed solids relative to volatile biomass, consistent with long-term retention of inorganic material ($CaCO_3$ precipitation/carryover) under the long SRT operating conditions. Consequently, the average SRT has been reduced from 48 ± 7 days (end of maturation period: day 422) to 41 ± 2 days during stage I and to 33 ± 3 days during stage II.

The results of stage I, as shown in **Figure 4.9 (a and b)**, confirmed the key role of increasing DO levels during the aeration phase on the development of NH_4 -N oxidation. Effluent NH_4 -N concentrations were between 7.5 and 8.8 mg-N/L after 12 days of

increasing DO until the end (days 435-488). In addition, $\text{NH}_4\text{-N}$ oxidation of 90% was achieved through the total aerobic duration (315 min of 350 min) compared to 5 h only during the maturation period (TAN removal performance was 89% on day 422). $\text{NH}_4\text{-N}$ oxidation rates reached 2.19 ± 0.14 mg- NH_4 /g-MLVSS·h. Accordingly, the removal efficiencies of $\text{NH}_4\text{-N}$ for both reactors raised to 92.4 ± 1.1 % during stage I. These findings confirmed that DO concentrations could influence nitrification, because both heterotrophs and autotrophs are available on the surface layers of AGS, and heterotrophs were the majority in most layers, competing with autotrophic bacteria (nitrifiers) for DO and space.

However, high DO levels of 6.5–8.5 mg/L through the rest of optimization period (stage II: days 496-558) did not alter the nitrification process significantly as shown in **Figure 4.9 (c and d)**. Effluent $\text{NH}_4\text{-N}$ contents were between 7 and 8.5 mg-N/L through the last month of operation (days 530-558). However, this improvement in TAN removal performance was accompanied by a slight deterioration of the SND process (96% on day 558) based on the increase in the effluent $\text{NO}_2/\text{NO}_3\text{-N}$ concentrations for AGS-SBRs. This was probably because high DO have a negative effect on the growth of HNAD and other aerobic denitrifiers and might reduce their bioactivity as well as applying high DO (up to 8.5 mg/L) might be toxic to anaerobic denitrifiers. The effluent $\text{NO}_2/\text{NO}_3\text{-N}$ concentrations were between 1.5 and 2.5 mg-N/L, and the overall denitrification performance was not affected significantly (97% on day 558) by these high DO levels (6.5–8.5 mg/L). This can be explained by two reasons: (a) the presence of numerous aerobic denitrifying and HNAD bacteria (e.g., *Diaphorobacter*, *Hydrogenophaga*, and *Niabella*) with high relative abundance, so increasing DO during aerobic phase did not affect them, (b) SND performance was probably attributable to the compact large and stable matured granules.

Moreover, there was a slight improvement in the reactors' performance of P removal during stage I of the optimization period (days 423-495: DO of 5-6 mg/L). The mean of SP removal efficiency was as high as 93.1%, and P-release and P-uptake rates reached 4.6 ± 0.22 and 0.7 ± 0.1 mg-P/g-MLVSS·h, respectively. After that, SP removal efficiency reduced to 90.1 ± 0.2 % and P-uptake rates minimized to 0.4 ± 0.1 mg-P/g-MLVSS·h during stage II (days 530-558). These results can be attributed to the negative impact of high DO concentrations (8.5 mg/L) on PAOs/DPAOs, where applying high DO resulted in shifting the composition of microbial community by decreasing the abundance of PAOs/DPAOs and increasing the population of GAOs/DGAOs as reported in the literature (Bi et al., 2023 and Izadi and Eldyasti, 2021). This is confirmed by the anaerobic COD consumption, which was not affected during stage I and was around 62.6 ± 0.3 %, while decreasing to 59.8 ± 0.3 % between days 496 and 558 (stage II).

A reduction in EPS in both SBRs slightly deteriorated granule physical properties, as confirmed by SVI_5 and SVI_{30} (**Figure 4.4; a, b**). EPS secretions dropped from 233.8 ± 8.5 (by the end of maturation period) to 216 ± 12 (days 455–488; stage I) and to 196 ± 24 mg/gVSS (days 489–522; stage II). In addition, a significant reduction in EPS has been recorded after applying high DO between 7.5–8.5 mg/L, where EPS content was 158 ± 19 mg/gVSS (days 530–558). Therefore, applying high DO level, directly affected the bioactivity of microorganisms responsible for EPS secretions (*Rhodobacteraceae*, *Pseudoxanthomonas*, *Flavobacterium* and *Acinetobacter*) as discussed in the previous section and supported by findings in the literature (Xiong et al., 2020 and Cheng et al., 2023). This deterioration in EPS content was also confirmed by the poor quality in the treated effluent due to high concentrations of suspended solids (**Figure 4.4; a, b**).

CHAPTER 5: FEASIBILITY OF AEROBIC PHOSPHORUS STARVATION TO ENHANCE THE EBPR PERFORMANCE OF AEROBIC GRANULES CULTIVATED AT HIGH ORGANIC LOADS DURING MUNICIPAL WASTEWATER TREATMENT

This study investigated aerobic P starvation as a sustainable long-term strategy to enhance P removal, ensuring consistently low effluent concentrations in municipal wastewater treatment, while supporting the optimization of EBPR–AGS systems to prevent failures linked to microbial activity fluctuations. The primary objectives of this study were to assess how the separation approach affected mature AGS cultivated under high organic load during long-term LS wastewater treatment, and how it influenced bacterial metabolism to accelerate aerobic P uptake rates. Additionally, microbial community shifts were examined to further understand the impact of this strategy on bacterial structural development. This study also evaluated the potential of reusing extracted P-rich liquid as a side-stream P-enriched source, leveraging high orthophosphate concentrations for effective chemical precipitation and P recovery.

5.1. Introduction

P discharge from wastewater treatment plants without effective removal threatens ecosystems by causing eutrophication and disrupting natural nutrient cycles. EBPR provides a feasible, cost-effective, and environmentally friendly option to conventional chemical approaches for eliminating P in wastewater treatment. This process depends on PAOs, which necessitate alternating anaerobic and aerobic conditions to promote their

growth and achieve luxury uptake by assimilating excess P under aerobic conditions, after its release during the preceding anaerobic phase (Cha et al., 2016). Without electron acceptors, PAOs can utilize stored energy in the form of poly-P and glycogen, to support the assimilation and storage of VFAs as PHAs intracellularly. Moreover, during the same anaerobic conditions, P within PAOs is released into the liquid phase, resulting in an elevated in P concentration (i.e., indicating great potential for P-recovery). Subsequently, in aerobic conditions, PAOs oxidize these stored compounds while simultaneously accumulating P from the liquid (Cha et al., 2016 and Metcalf & Eddy et al., 2014). The performance of the EBPR process is governed by numerous factors, including carbon source, C/P ratio, feeding strategy, SRT, FA and FNA concentration, and the presence of competing microorganisms (Saito et al., 2004, Song et al., 2009, Bassin et al., 2012). Major stability challenges affecting EBPR performance, particularly when treating municipal wastewater, can be mitigated by supplementing VFAs produced via the fermentation of readily biodegradable organic matter in the wastewater, fermented primary sludge reintroduced into the anaerobic phase, or exogenous sources (Barnard and Abraham, 2006). Nevertheless, maintaining consistently low P levels in the treated effluent remains highly challenging, largely due to fluctuations in the microbial activity (Bertanza et al., 2013).

Recent studies have focused on optimizing configurations for effective biological P removal, with the next critical step being the integration of P recovery measures (Chowdhury et al., 2017 and Wang et al., 2022). The growing demand for P across multiple sectors (e.g., fertilizers, textiles, etc.) renders P recovery financially attractive. Global mass balance analyses showed that nearly 20% of worldwide P production, about 3 million tons annually, is lost in anthropogenic waste (Cordell et al., 2009). Therefore, efficient P

removal offers dual benefits, as recovering P from wastewater reduces reliance on phosphate rock to meet global demand. In recent years, several alternative EBPR configurations and strategies have been developed to enhance P recovery by precipitating anaerobically released P as struvite, typically achieved through pH adjustment using chemical additives (Cieslik and Konieczka, 2017). In a model-based study, Baeza et al. (2017) developed the EBPR² system, a SBR cycle adding a settling phase after anaerobic mixing, yielding an anaerobic/settling/aerobic/settling sequence to enable P recovery. Extracting 4 % of the anaerobic supernatant (P-rich liquid) for chemical precipitation, simulations showed > 60 % of influent P could be recovered while meeting regulatory limits for organic matter, P, and N. EBPR² also operated with lower COD demands than conventional EBPR-SBRs.

Since its development in the 1990s, AGS has emerged as a promising alternative to CAS owing to its compact structure, high nutrient removal efficiency, and capacity to simultaneously eliminate organic matter and nutrients within a single reactor through dense microbial aggregates (Giesen et al., 2013 and Thwaites et al., 2018). Numerous studies have examined AGS cultivation and stability, highlighting key operational and design parameters influencing granulation, including feast–famine regimes, anaerobic influent distribution, selective biomass wasting based on settling velocity, hydrodynamic shear forces, influent composition, OLRs, and EPS production. AGS offers substantial advantages over the CAS system, achieving energy savings of 50 % and reducing spatial requirements up to 75 % (Ashish et al., 2018). The stratified oxygen profile across AGS establishes three functional zones: an outer aerobic region, an intermediate oxygen-limited layer, and an anoxic or anaerobic core, each supporting specialized microbial activities

(Castellanos et al., 2021 and Nancharaiah and Reddy, 2018). Previous studies have shown that P removal in AGS systems is facilitated by both biological uptake through EBPR pathways and inorganic precipitation processes occurring within the granule structure (Mañas et al., 2011, Filali et al., 2012, Henriët et al., 2016, Sarma and Tay, 2018, Purba et al., 2020). Besides, the recent studies have highlighted the potential for resource recovery from AGS (Song et al., 2009). It is worth mentioning that aerobic granulation technology shows strong potential for the efficient simultaneous nutrient removal across various wastewater types, such as low-strength (LS) domestic influents (Lashkarizadeh et al., 2015) and high-strength (HS) wastewaters (Ren and Yuan, 2016).

5.2. Material and Methods

5.2.1. Mother SBRs (cultivation and maturation of AGS)

Two 5.0 L identical GSBRS were run in continuous operation for 18 months ahead of this study to cultivate large-sized AGS through the treatment of HS wastewater with an average composition of TCOD=1400±52, TN=140±19, and TP=42±9 mg/L. The reactors were inoculated with 2.0 L of return activated sludge (VER=60 %), comprising flocculent biomass sourced from a full-scale WWTP (West End Water Pollution Control Centre, Winnipeg, MB, Canada) as previously described (Mady et al., 2022). The GSBRS operated on an 8-hour cycle with a hydraulic retention time of 13.3 h, consisting of 10 min feeding, 95 min anaerobic mixing under nitrogen gas, 350 min aeration, 10 min settling, 10 min effluent discharge, and 5 min idle. This airflow rate (3.73 L/min; superficial air velocity 0.55 cm/s) was adopted from the aeration-optimization described in Chapter 4 to improve

aerobic mixing and maintain DO around 5.0 mg/L, which alleviated low-DO limitations (<1.5 mg/L) and enhanced nitrification (Mady et al., 2024).

5.2.2. Experimental Design (Setup of the test GSBRS)

5.2.2.1. Inoculum, setup and influent wastewater composition

Matured AGS were directly taken out from the mother reactors, then were combined and evenly distributed into five smaller reactors, each with a working volume of 2.0 L (inner diameter of 10 cm). Four GSBRS (R1-R4) were designated as test reactors for applying aerobic P starvation by extracting part of P-rich liquid at the end of the anaerobic phase, while the fifth reactor served as a control system (i.e., without P-rich liquid extraction). The seeding process for all GSBRS involved introducing 100 g (wet weight) of AGS (at the end of the aerobic mixing stage) after centrifugation, which was suspended in 0.8 L of supernatant and supplemented with 1.2 L of wastewater. Similar to the mother reactors, all GSBRS operated with the same VER, treating HS influents, and followed the same operational sequence described previously for 290 days as operating stage 1. Biomass wastage was limited to solids discharged with the effluent during the decant phase (i.e., SRT was not actively controlled). Additionally, peristaltic pumps facilitated influent feeding and effluent discharge, with cycle automation achieved through Traceable Digital Outlet Controllers (Fisher Scientific, CA). All GSBRS utilized the same aeration system and maintained DO levels comparable to those in the mother reactors. Nitrogen gas was supplied through a 3 cm spherical stone diffuser to achieve thorough mixing, maintain DO concentrations below 0.1 mg/L to sustain an anoxic environment, prevent biomass settling, and enhance nutrient–biomass interactions for improved removal performance.

After that, all GSBRS were operated for 55 days treating medium-strength (MS) wastewater as a transitional phase, before switching to LS wastewater treatment (**Table 5.1**) as the operating stage 2 in this study. In this study, different strengths of the synthetic wastewater feed were prepared based on a previous study (Kang and Yuan, 2017) for all GSBRS. The synthetic wastewater composition was strategically adjusted by modifying the ratios of yeast extract, ammonium chloride, and dipotassium phosphate to achieve controlled variations in sCOD, TN, and TP (**Table 5.1**). Additional components included 5.8mg/L MgSO_4 , 12mg/L CaCl_2 , and 0.3 mg/L mineral solution comprising 0.15g/L H_3BO_3 , 0.03g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.03g/L KI , 0.12g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.06g/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.12g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15g/L $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, and 1.5g/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. Furthermore, 3 mg/L of ethylenediaminetetraacetic acid was added to prevent precipitation of salts (Ca^{2+} and $\text{PO}_4^{3-}\text{-P}$) present in the medium. Sodium bicarbonate (12 g NaHCO_3 per g $\text{NH}_4^+\text{-N}$) was dosed in the influent at each cycle throughout the experiment to ensure sufficient alkalinity (7.14 g $\text{CaCO}_3/\text{g NH}_4^+\text{-N}$) to support complete nitrification. Synthetic wastewater was prepared every two days and stored at 4 °C.

Table 5.1: Influent characteristics and operation parameters *

Parameter/Component (mg/L)	HS Wastewater (Operating Stage 1)	MS Wastewater (Transitioning stage)	LS Wastewater (Operating Stage 2)
Yeast extract	1250	670	380
NH_4Cl	33	55	29
$\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$	125	15	17
TCOD	1400	750	425
sCOD	1250	670	380
TN	140	85	47.5
TAN	117	65	35
TP	42	12	8
Soluble P (SP)	20	5.5	3.72

* pH in this study was around 8.1 (anoxic reaction phase) and 7.8 (aerobic mixing stage).

5.2.2.2. Extraction of P-rich liquid and operational conditions

The operational configuration of the first treatment cycle in all test GSBRs was modified by introducing an additional settling stage after the anaerobic phase, resulting in the following sequence: pulse feeding (10 min), anaerobic mixing (80 min), settling (15 min), aerobic mixing (350 min), final settling (10 min), decant (10 min), and idle (5 min). During the operating stage 1, this study initially assessed the impact of intermittently extracting varying volumes of P-rich liquid in the test GSBRs, aiming to evaluate the feasibility and optimize the extraction strategy for enhanced P removal efficiency and granule stability. To maintain a constant water level in the test GSBRs following P-rich liquid extraction, an equivalent volume of wastewater was introduced at the start of the aeration phase, ensuring a fixed VER throughout operation, which was performed through different scenarios; (a) adding fresh influent (scenario 1), and (b) reusing the treated effluent from the previous treatment cycle (scenario 2). The test GSBRs were operating during this study to examine three different extraction ratios of 5, 10, and 14 % (100, 200, and 280 mL, respectively), as shown in **Figure 5.1**. The volume of P-rich liquid was extracted during the final 4 min of the additional settling stage, at flow rates of 25, 50, and 65 mL/min for the extraction ratios of 5, 10, and 14 %, respectively.

To mitigate operational challenges associated with this separation approach, specific measures were implemented. Direct extraction of P-rich liquid from the reactor bottom under static conditions was deliberately avoided to prevent potential disturbances to sludge properties. Instead, extraction was performed from the middle of the upper liquid

phase using a porous stone separator (**Fig. C1 – Appendix C**), reducing the risk of biomass disruption. Moreover, continuous P starvation was avoided to minimize the risk of sustained stress on PAOs bioactivity (Wang et al., 2022). Accordingly, an alternating extraction strategy, comprising one extraction cycle followed by two recovery cycles without extraction, was employed to balance P removal performance with the long-term stability of ASG and preservation of PAOs bioactivity.

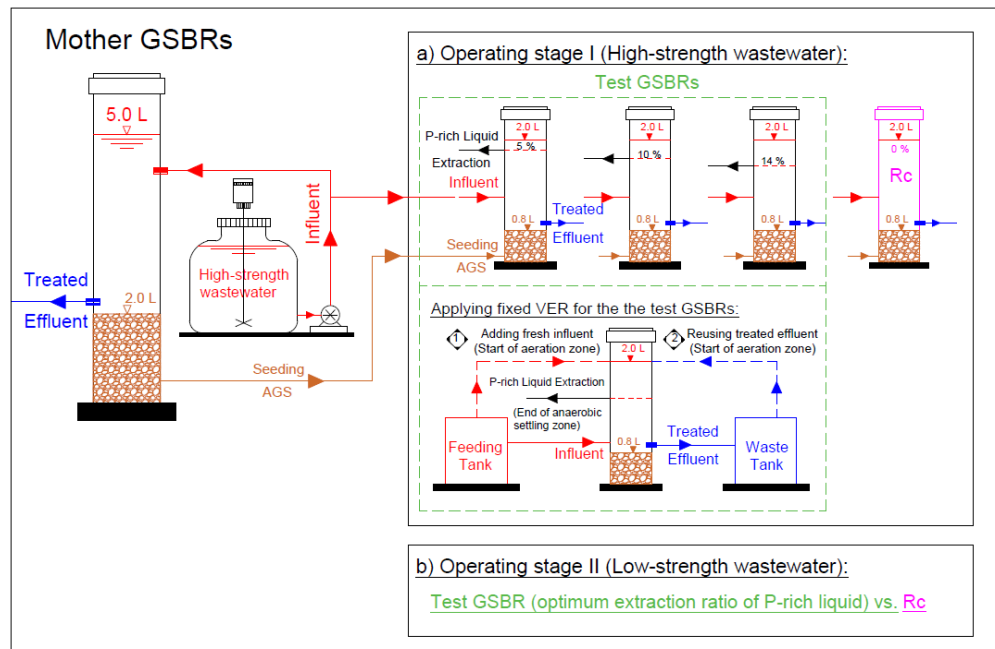


Figure 5.1: Schematic diagram of GSBRs in this study: Mother GSBRs (source of AGS), test GSBRs (various extraction ratios of P-rich liquid), and Rc.

5.2.3. Sampling and Analytical Procedures

5.2.3.1. Wastewater analyses

A 14-day acclimation phase was implemented following the initiation of P-rich liquid extraction across all test GSBRs. Influent and effluent samples were collected daily throughout the experiment to evaluate treatment performance and filtered with medium

porosity Q5 filter paper (0.45 μm ; Fisher Scientific, Canada) prior to analysis. Standard analytical methods (APHA/AWWA/WEF, 2012) were employed to quantify MLSS, MLVSS, TSS_{eff} , and sCOD. N forms and $\text{PO}_4^{3-}\text{-P}$ were measured using a flow injection analyzer (Lachat Quick Chem 8500, HACH, Canada). DO and pH were continuously monitored using calibrated electrodes positioned in the reactor center. AGS characterization and stability included SVI_5 , SVI_{30} , and morphological evaluation, with particle size measurements (Axio Vert.A1 microscope (ZEISS)) were conducted.

EPS content, comprising both PN and PS fractions, were extracted from AGS following the protocol of Adav et al. (2008). Briefly, 10 mL of mixed liquor was pre-treated with 0.06 mL formaldehyde and incubated at 4 °C for 1 h, followed by ultrasonication (120 W, 5 min) in an ice bath. 4 mL of NaOH was added, and the mixture was stored at 4 °C for 3 h before centrifugation at 10,000 rpm (20 min). The supernatant was filtered through 0.2 μm membranes to isolate soluble EPS, were determined by the phenol-sulfuric acid method and the modified Lowry assay kit (Thermo Fisher Scientific, USA).

Extracted P-rich liquid samples were immediately filtered (0.22 μm) and stored at -18 °C after pre-dilution to prevent precipitation prior to freezing. P recovery from the P-rich liquid was assessed via chemical precipitation, specifically through struvite crystallization (Le Corre et al., 2009 and Amann et al., 2018). For each batch experiment, Mg^{2+} were added at defined molar Mg:P ratios to induce struvite precipitation. A baseline pH of 9 was established via 20 min of CO_2 stripping with aeration (eliminating pH adjustment). Higher pH conditions (9.2 and 9.5) were subsequently evaluated through NaOH addition to investigate the effect of further alkalinity enhancement.

Only polyhydroxybutyrate, polyhydroxyvalerate and polyhydroxyhexanoate were analyzed, and their sum was used to represent the PHAs concentration of the AGS (Braunegg et al., 1978). Cellular mcl-PHAs content was quantified following the acid-catalyzed methanolysis method described by Brandl et al. (1988). Briefly, 5 mg of dried biomass was transferred to 10 mL reaction vials, and PHAs was converted to methyl esters. The same protocol was applied for octanoic acid analysis in the culture medium, substituting 1 mL of thawed, vortexed culture supernatant (brought to room temperature) as the sample matrix in each reaction vial. Quantification of cellular mcl-PHAs and octanoic acid was performed using an Agilent 7890A gas chromatograph equipped with a split/splitless inlet (operated in split mode, split ratio 10:1), a DB-23 capillary column, and a flame ionization detector, as detailed in Fu et al. (2014) and Blunt et al. (2018).

5.2.3.2. Performance indicators

In the EBPR processes, P release rates were considered as an effective indicator, which calculated by linear regression over the 1.5-hour anaerobic phase, while total P uptake rates were determined by linear regression during the aerobic phase (Zhang et al., 2011). In addition to the anaerobic P release rates, the molar P_{rel}/C_{upt} ratio, which indicates the efficiency of PAOs in converting the carbon substrate (C_{upt} ; Carbon uptake) into intracellular poly-P storage (P_{rel} ; P released) (Acevedo et al., 2012 and Wang et al., 2022). Furthermore, specific removal efficiency (SRE) was applied as a quantitative performance metric, calculated according to **Equation 5.1**. This parameter normalizes net P removal efficiency to the biomass concentration, enabling a direct assessment of specific activity of PAOs within the reactor biomass. The mass of P removed per cycle was quantified as the

net P removal (mg P/L), and P the percentage of influent P removed per treatment cycle which represented the P removal efficiency. Additionally, the recovery efficiency represents the proportion of P recovered in the P-rich liquid, expressed as a percentage of the influent P concentration and indicating the maximum potential for struvite recovery. This study assessed the PHAs content of the biomass (%PHAs, mg PHAs/mg MLSS, **Equation 5.2**) and the specific PHAs production rate (q_{PHA} , mg COD-PHAs/mg COD-X·h, **Equation 5.3**) as key process responses (Ahmadi et al., 2020).

$$SRE = \frac{P_{Inf} - P_{Eff}}{MLVSS \times V_{SBR}} \quad (\text{Equation 5.1})$$

$$\%PHAs = \frac{PHAs_e - PHAs_0}{MLSS} \times 100 \quad (\text{Equation 5.2})$$

$$q_{PHA} = \frac{PHAs_e - PHAs_0}{Xa \cdot t} \quad (\text{Equation 5.3})$$

Where, $PHAs_0$: PHAs concentration at the beginning of the feast phase; $PHAs_e$: PHAs concentration at the end of feast phase; Xa : average activated biomass concentration (the difference between MLVSS and PHAs storage); t : the length of feast phase in SBR.

The observed sludge yield (Y_{obs}) was calculated by dividing the net volatile solids in total biomass (ΔVSS) by the net COD consumption (ΔCOD) (**Equation 5.4**) (Cheng et al., 2024).

$$Y_{obs} = \Delta VSS / \Delta COD = \frac{MLVSS_{t2} + \Delta t \times \Sigma Q \times VSS_{eff} - MLVSS_{t1}}{\Delta t \times \Sigma Q \times (COD_{inf} - COD_{eff})} \quad (\text{Equation 5.4})$$

Where, $MLVSS_{t1}$ and $MLVSS_{t2}$: the VSS in the SBR at the beginning and at the end of the experiment phase; Q : the volume of daily influent; VSS_{eff} : the VSS in the effluent; Δt : the duration time; COD_{inf} and COD_{eff} : the COD in the influent and effluent, respectively.

5.2.4. DNA Extraction and Bacterial Community Analyses

To gain deeper insight into the microbial dynamics underpinning system performance, granular sludge samples were systematically collected from both the control and test reactors (R1–R4) throughout the entire operational period, which included phases treating HS and LS influents. This approach ensured a comprehensive understanding of microbial shifts under varying operational conditions. DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN, Venlo, Netherlands), following the manufacturer's protocol to ensure consistency and reliability in downstream analysis. Extracted community DNA was stored at -80°C until sequencing analysis. The V3–V4 regions of the 16S rRNA gene were sequenced on an Illumina MiSeq (2×300 bp paired-end) at the Integrated Microbiome Resource (IMR) (Halifax, NS, CA). The specific PCR primer set included a forward primer 341F (5'-CCTACGGGNGGCWGCAG- 3') and a reverse primer 805R (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013). The detailed protocol for 16S rRNA amplicon sequencing followed the method described and reported by the IMR's publication (Comeau et al., 2017). Community profiling and functional abundance prediction were assessed using QIIME2 workflow and PICRUST2.

5.2.5. Data Analyses and Visualization

Statistical analysis of data across different extracted ratios of P-rich liquid was performed using a one-way analysis of variance (ANOVA) test. This analysis aimed to evaluate variations in granule characteristics and treatment performance under the different aerobic P starvation scenarios. A significance level (P-value) < 0.05 was considered statistically significant. All results represent the average of replicates from the two identical

GSBRs with the standard error of the mean (SEM). However, the results of removal efficiencies of the substrates and nutrients are individually reported for each reactor. Alpha and beta diversity analyses were provided by IMR. The outputs from QIIME2 and PICRUST2 were visualized in RStudio.

5.3. Results and Discussion

Mature granules (>2.5 mm) with stable settling properties (e.g., $SVI_{30} \leq 28 \pm 5$ mL/g) were harvested in the mother GSBRs and used as a seeding biomass for all GSBRs (**Fig. C2 – Appendix C**). The MLSS concentration was maintained at 11 ± 0.4 g/L with a MLVSS/MLSS ratio about 0.74, as previously reported (Le Corre et al., 2009). By the end of operation period (> 400 d), mother GSBRs achieving BNR efficiencies of COD, TAN, and SP are 94, 90, and 89 %, respectively, with $SRT \geq 50$ days. Building on this stable baseline, the primary aim of the test GSBR phase was to critically optimize aerobic P-starvation operation under HS influent conditions identical to those of the mother GSBRs. Specifically, different P-rich liquid extraction ratios were systematically evaluated to identify an optimal withdrawal volume that could strengthen P removal performance without compromising granule integrity and settling stability. As noted earlier, two operational scenarios were implemented, starting with a fixed VER to characterize reactor response under well-controlled conditions, as discussed in the following sections.

5.3.1. Operating stage 1 (HS influent): Optimizing the extraction strategies

In R1 and R2, a small fraction of the working volume (5%) was extracted to test whether limited P-rich liquid withdrawal could trigger aerobic P starvation without

compromising operational stability. Both reactors were evaluated under two scenarios: in R1, 100 mL of fresh influent was added at the start of the aeration phase, whereas in R2, 100 mL of treated effluent from the preceding cycle was reused to maintain a fixed VER in both systems. R1 showed a clear and persistent deterioration in performance, with sCOD/TAN/SP removal efficiencies declining to 84/59/66% (**Figure 5.2**).

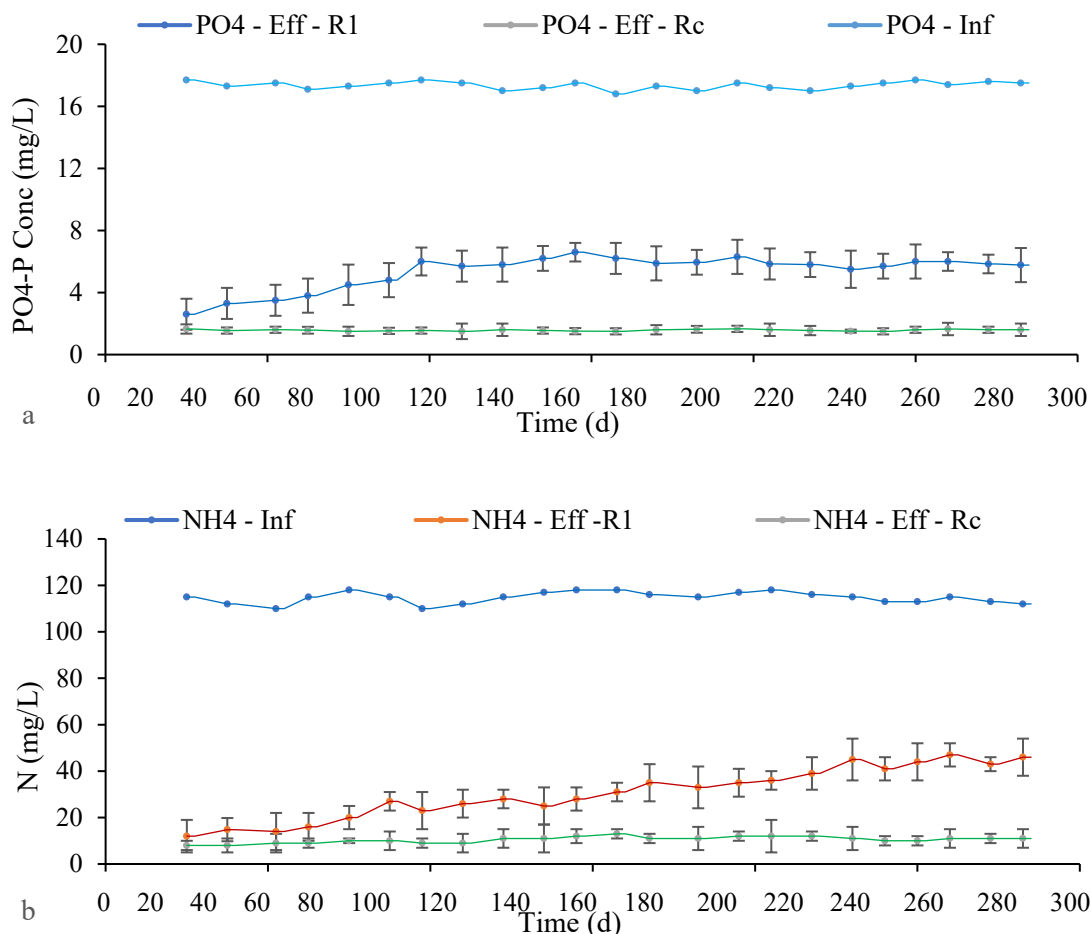


Figure 5.2: Low PO₄-P (a) and NH₄ (b) removal performance in R1 through scenario 1 over 290 days of operation (HS influents)

Moreover, granule integrity declined substantially, evidenced by marked size reduction (≤ 0.5 mm), increased SVI₃₀ (≥ 88 mL/g), and sustained MLSS washout (≤ 1.7

g/L), resulting in an SRT of <11 d during days 250–290. Collectively, these indicators point to a shift toward prolonged feast conditions, likely initiated by adding fresh influent immediately after extraction. This substrate shock created a clear mismatch between carbon availability and microbial acclimation, weakening the selection pressure needed to maintain granule structure and retain slow-growing functional guilds (e.g., PAOs and nitrifiers). Accordingly, these results demonstrate that even limited P-rich liquid extraction, if poorly aligned with operational timing and substrate management, can critically undermine system performance. On the other hand, the results of R2 showed that this GSBP performed comparably to R_c throughout the entire operational period (**Figure 5.3** and **5.4**) without any deterioration. No significant differences (P-value > 0.05) were observed between R2 and R_c during the operating stage 1 in terms of granule physical properties, biomass characteristics, EPS secretions, or overall BNR performance. By the end of the operating stage 1, the actual SRT in R2 and R_c was 43 and 47 days, respectively. Accordingly, the second scenario adopted an alternative method to maintain the fixed VER.

It should be noted that replacing the extracted P-rich liquid with fresh influent inherently increases the net nutrients mass loading aerobically, and this effect becomes more pronounced at higher extraction ratios (e.g., 10–14%). Under this make-up mode, the additional substrate supplies prolonged “feast” conditions and reduced the intended selective pressure (i.e., aerobic P starvation mode). In addition, increased aerobic P loading counteracts the targeted aerobic P-starvation selection. For these reasons, no further optimization using higher extraction ratios was pursued under the fresh-influent make-up scenario; instead, treated-effluent reuse was applied as the make-up source to minimize incremental aerobic nutrient loading, strengthen aerobic P-starvation selection.

This strategy ensured hydraulic stability while reinforcing the aerobic P starvation conditions central to this study. Importantly, the limited phosphate content in the recycled effluent minimized aerobic nutrient loading, thereby sustaining the intended metabolic pressure on PAOs. In contrast, introducing fresh influent would have elevated aerobic P concentrations, counteracting the study's objective. Notably, J. Wang et al. (2022) demonstrated improved P removal in GSBs through sporadic P-rich liquid extraction with fresh influent addition to maintain fixed VER; however, their investigation was restricted to a short-term duration of 10 days and did not address long-term granule stability. To examine the influence of varying P-rich liquid extraction volumes under HS influent, the other two GSBs were operated in parallel with different extraction ratios (R3, 10%; R4, 14%). In each case, extraction was performed during the first cycle only. The subsequent performance of R3, and R4 was systematically evaluated in terms of nutrient removal efficiency and compared to Rc (**Figure 5.2 – 5.7**).

Initially (days 1–107), R4 showed a marked MLSS decline accompanied by poor effluent quality; in the later months, TSS_{eff} increased to as high as 165 mg/L, remaining substantially higher than Rc (90–125 mg/L). This was mainly due to the high P-rich liquid extraction volume of 280 mL applied, which likely disrupted sludge stability (settlement behavior), leading to greater solids washout. R4 exhibited BNR performance comparable to Rc during the first month; however, it subsequently declined, with removals of $\geq 95\%$ sCOD, 81% TAN, and 83% SP (**Figures 5.3 and 5.4**). Moreover, a gradual significant decline in MLSS, accompanied by an increase in the MLVSS/MLSS ratio, was observed.

By the end of operation, R4 exhibited average MLSS and MLVSS/MLSS values of 6.31 ± 0.4 g/L and 0.86, respectively, while Rc maintained higher values up to 10.4 ± 0.3 g/L and 0.72. This shift likely reflected progressive reduction in inorganic P precipitates within the AGS caused by excessive P-rich liquid extraction, reducing granule density and aligning with the observed deterioration in effluent quality. Granule size progressively declined to 1.0 mm, while SVI_{30} increased to 66 mL/g. Despite using a porous stone separator and an 11-min settling period prior to extraction, the withdrawn P-rich liquid still contained residual suspended solids (up to 35 mg/L), indicating non-negligible solids carryover during extraction. In parallel, the sludge age in R4 fell to < 21 days by day 290.

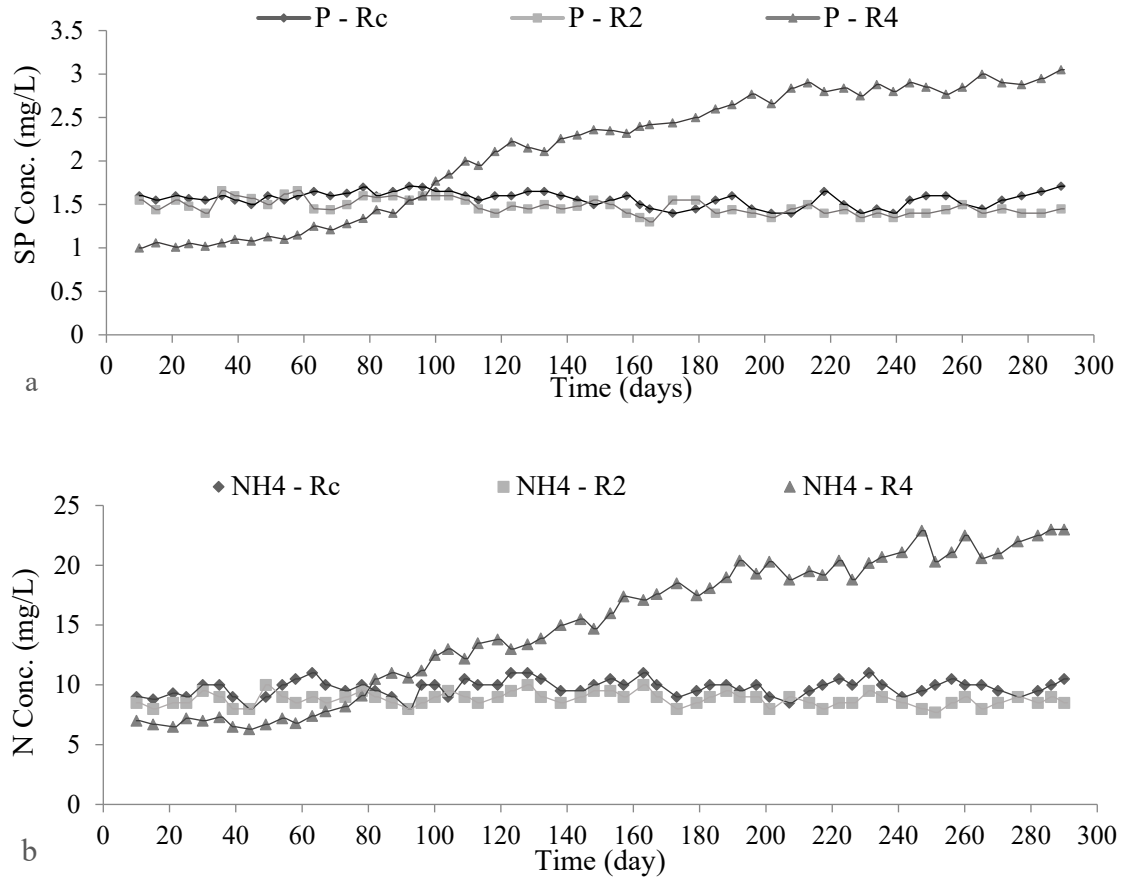
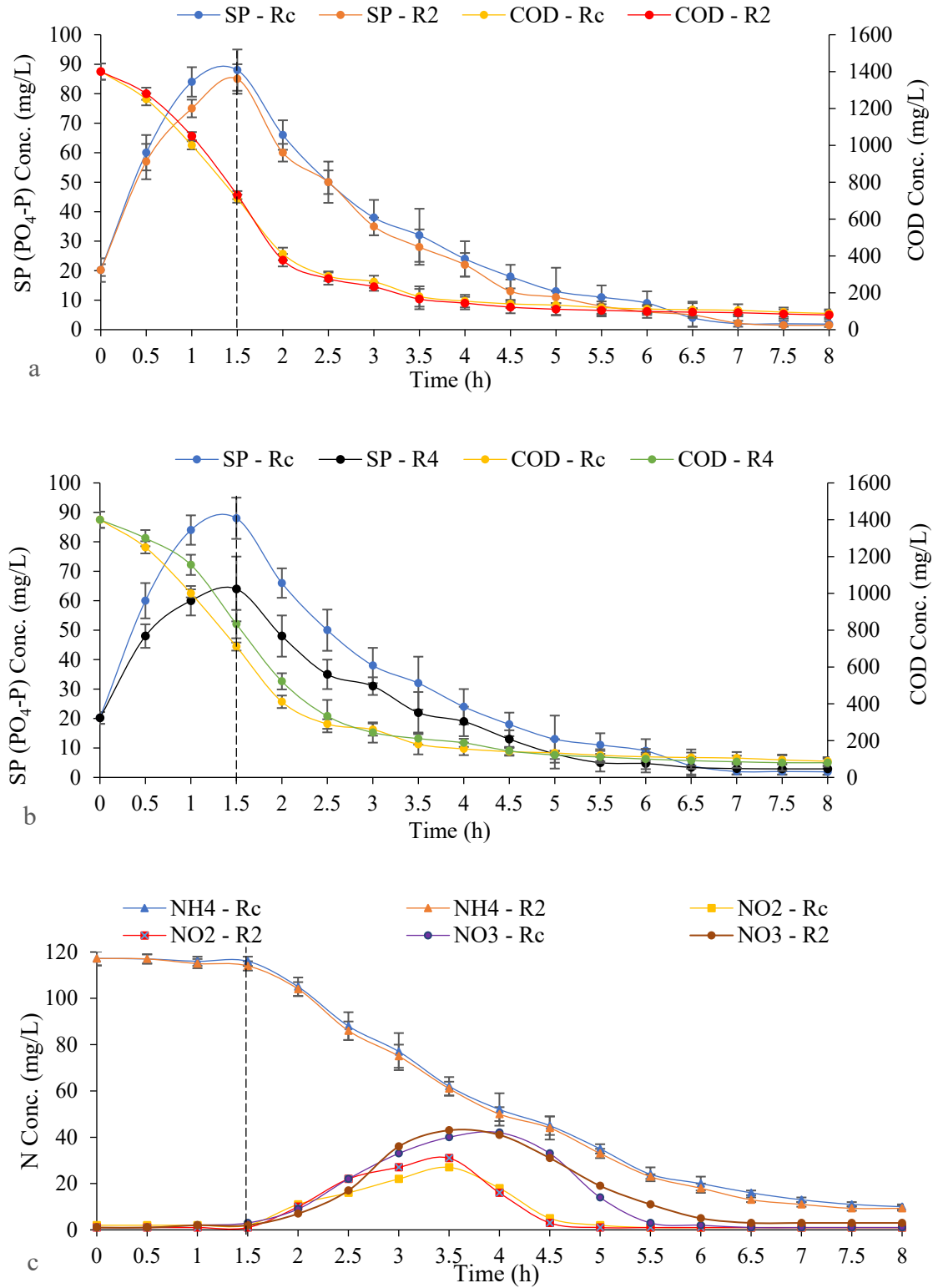


Figure 5.3: $PO_4\text{-P}$ (a) and NH_4 (b) removal performance in R2 and R4 (scenario 2) compared with Rc over 290 days of operation (HS influents)



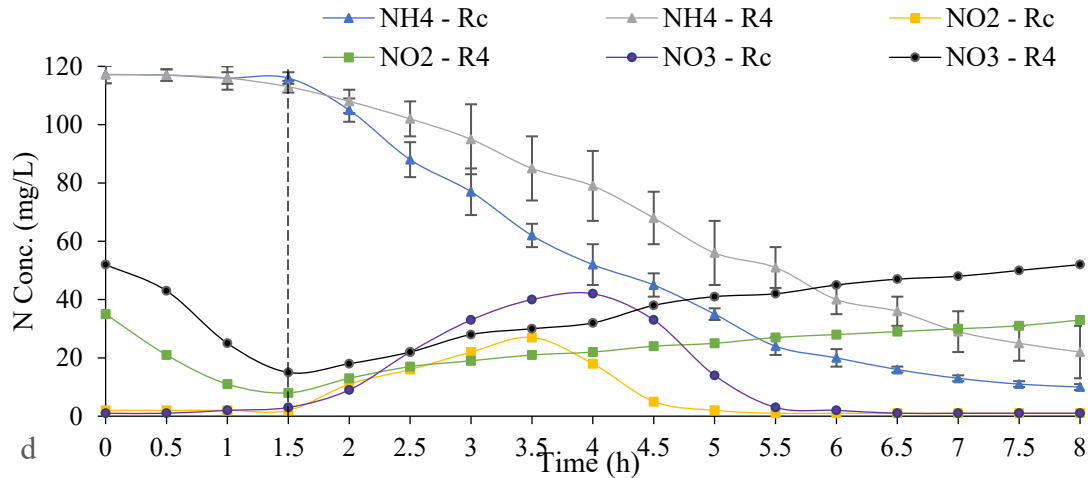


Figure 5.4: Profiles of soluble P, COD and N-forms for R2 (a and c), R4 (b and d), compared with Rc during the typical treatment cycle (last 2 months of operation)

Sustained application of high P-rich liquid extraction appeared to impair the reactor's SND capability, with effluent nitrite and nitrate concentrations averaging 25 ± 2.2 and 30 ± 4 mg/L. In contrast, R4 showed an initial improvement in P removal (**Figure 5.3-a**) through the first three months, reducing effluent $\text{PO}_4^{3-}\text{-P}$ to 1 mg/L (Rc; 1.6 ± 0.10 mg/L). After this point, effluent P increased steadily and stayed high till the end of operation (days 220 - 290), and the mean $\text{PO}_4^{3-}\text{-P}$ removal was 85%. This performance decline was further exacerbated by settling-velocity selection pressure, which favored washout of poorly settling biomass. Under these conditions, the proliferation of nitrifiers and PAOs became increasingly challenged. The high OLR of $2.25 \text{ kg COD/m}^3 \cdot \text{d}$ applied likely selected for fast-growing heterotrophs over these slow-growing microorganisms, which may explain the persistently high sCOD removal despite compromised N and P removal performance.

As shown in **Figure 5.4-a**, P-release rate in R4 averaged $3.3 \pm 0.14 \text{ mg-P/g-MLVSS} \cdot \text{h}$, significantly lower than Rc ($4.6 \pm 0.1 \text{ mg-P/g-MLVSS} \cdot \text{h}$). Such declining

indicated deteriorated EBPR performance, likely because PAOs could not sustain activity under prolonged aerobic P starvation. Rapid depletion of intracellular poly-P reserves ultimately compromised their long-term adaptive capacity. These results align with previous studies (Acevedo et al., 2015 and Baeza et al., 2017), which suggested that the deterioration in P removal is likely attributable to the severity of the extraction strategy, which imposed excessive stress on PAOs. P removal declining was associated with a marked reduction in the metabolic PAOs activity, as indicated by lower anaerobic P-release rates, an established proxy for PAOs bioactivity (Wang et al., 2022, Zhang et al., 2011).

To assess sludge yield performance across the four GSBs, the observed biomass yield was calculated as the ratio of net biomass production to COD consumed over a defined period (**Eq. 5.4**). In R1, Y_{obs} was maintained at approximately 0.10 g VSS/g COD (mean) during the first two months; however, it decreased markedly over time and reached 0.06 g VSS/g COD between days 255 and 290. This reduction is primarily attributed to deterioration of granule properties (e.g., reduced size and impaired settling), resulting in increased washout (selective wasting), as reflected by the decline in SRT. In R2 and Rc, Y_{obs} remained relatively stable throughout operating stage 1, with both systems exhibiting values around 0.08 g VSS/g COD. These findings are consistent with previous studies indicating that lower Y_{obs} is often associated with extended sludge ages (Amanatidou et al., 2015 and Cheng et al., 2024). Moreover, the larger size and compact structure of mature granules likely promoted persistent diffusion-limited anoxic/anaerobic zones, which can enhance endogenous respiration, EPS turnover, and in situ fermentation of decay products, thereby contributing to reduced net sludge production in AGS systems.

In contrast, R4 exhibited the highest Y_{obs} among the GSBRS (approximately 0.113 g VSS/g COD during the last two months). This behavior is attributed to the operational conditions in R4, including the higher P-rich liquid extraction ratio and the higher F/M ratio relative to R_c (1.05 vs. 0.75 g COD/g VSS·d, respectively), which are known to increase Y_{obs} due to higher substrate availability per unit biomass and reduced relative maintenance demand (Amanatidou et al., 2015). Overall, the long-term P removal performance was notably impacted by applying the extraction of high volumes of P-rich liquids. These findings highlight the adverse effects of excessive P-rich liquid extraction on both PAOs functionality and overall P removal efficiency.

Among the three GSBRS operated under scenario 2, R3 (10% P-rich liquid extraction) exhibited the most stable long-term performance when treating HS wastewater, maintaining superior granule properties and consistently high P removal efficiency. Statistical analysis (ANOVA) confirmed that variations in granule characteristics and P removal under different aerobic P starvation strategies were significant ($p < 0.05$), with R3 consistently yielding the best results. The following section provides a detailed discussion of R3's performance throughout HS wastewater treatment.

5.3.2. The optimum extraction regime during stage 1: Results of R3

5.3.2.1. Granule stability, biomass characteristics and treated effluent quality

Over the first six months, high TSS_{eff} concentrations were observed up to 180 mg/L, and eventually stabilizing at 117 ± 11 mg/L between days 200 and 290. Notably, improved effluent quality was consistently achieved during the subsequent recovery cycles, with TSS_{eff} concentrations reduced by 14%. These findings highlight the important stabilizing

role of the recovery phases in mitigating solids washout, with the gradual improvement in effluent quality further reflecting the system's resilience under scenario 2, in alignment with previously reported trends in the literature (Guisasola et al., 2019). A progressive reduction in biomass content was observed (**Figure 5.5-a**), where MLSS remained relatively stable at 7.6 ± 0.1 g/L until day 165. After this point, there was a slight increase in MLSS (8.6 ± 0.31 g/L) through the last three months, associated with the SRT of 39 days. Notably, this early-phase decrease in biomass was associated with reduced EPS secretion, which collectively contributed to a decline in granule size and structural integrity.

By day 165, R3 exhibited a marked reduction in EPS to 86 ± 8 mg/gVSS and in PN/PS to 8.4 ± 0.6 (**Figure 5.5-c**). This suggested that the application of aerobic P starvation led to a reduction in mineral precipitates, evidenced by an increased MLVSS/MLSS, which in turn contributed to lower granule density. This was accompanied by a significant reduction in granule size to 1.75 ± 0.08 mm. In the meantime, SVI_{30} in R3 increased to 45 ± 3 mL/g, while R_c maintained a granule size of 2.66 mm, with superior settling characteristics ($SVI_{30} \leq 29$ mL/g) on day 165 (**Figure 5.5-b**). Although this temporary deterioration, these conditions appeared to support long-term structural stability by enhancing nutrient diffusion into the granule matrix. Successful regrowth occurred under the applied conditions, with granules gradually adapting and increasing their EPS secretion over time. Between days 166 and 290, steady improvements were observed in terms of EPS, PN/PS, and size, reached 151 ± 8 mg/gVSS, 10.9 ± 0.6 , and 2.07 ± 0.08 mm. Settleability also improved ($SVI_{30} \leq 34$ mL/g) approaching the performance of R_c . This trend was accompanied by a gradual increase in sludge yields; 0.072, 0.075, 0.087, and 0.091 gVSS/gCOD during days 180–194, 212–226, 250–262, and 276–290, respectively.

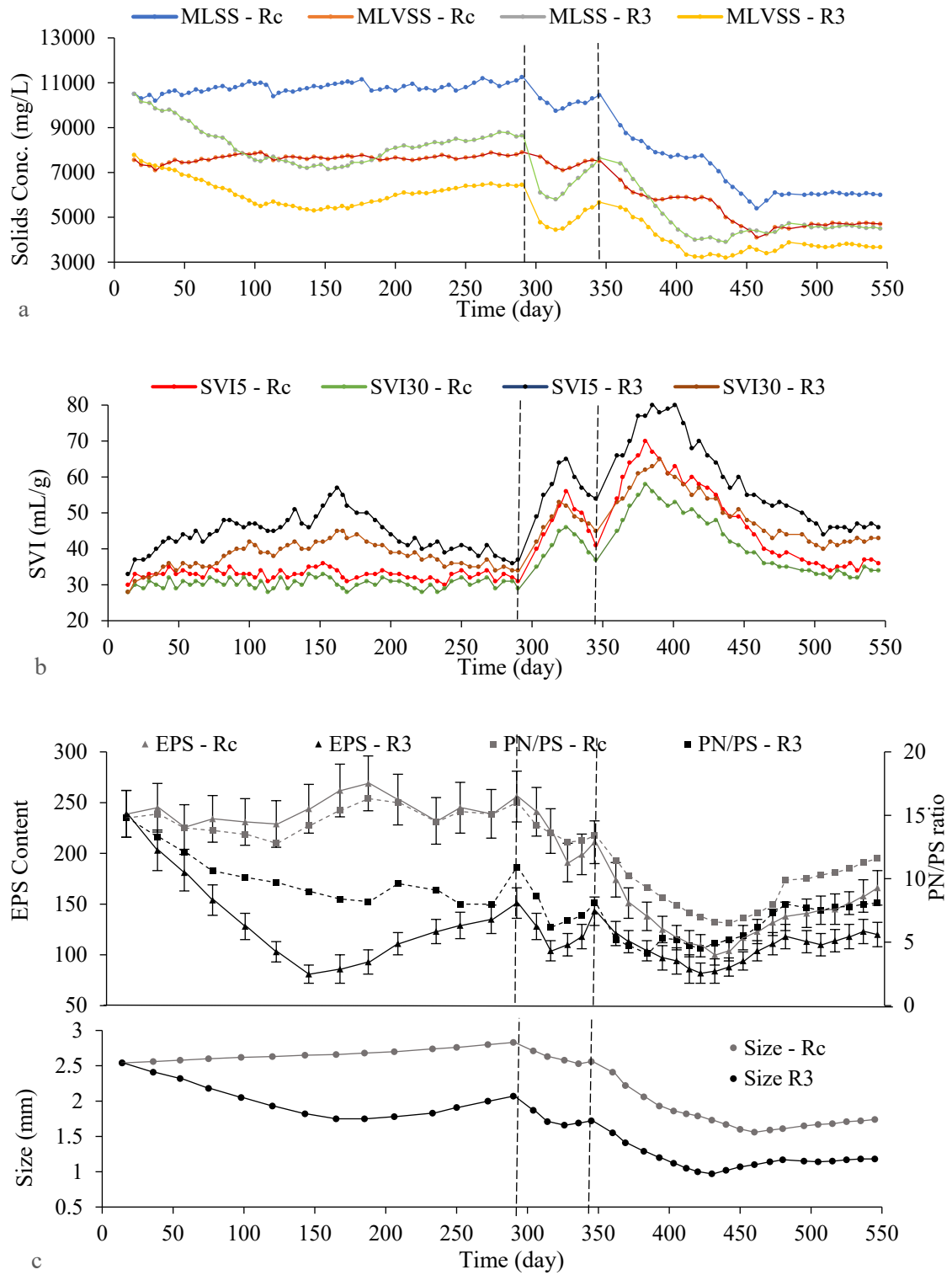


Figure 5.5: Biomass concentrations (a), physical properties (b) and EPS content, and granules size (c) for R3 and Rc (scenario 2) over 290 days of operation (HS influents)

5.3.2.2. P removal performance

The long-term profile of SP concentrations in the treated effluent from Rc and R3 during stage 1 is illustrated in **Figure 5.6-a**. Both systems received influent with $\text{PO}_4^{3-}\text{-P}$ levels of 20.7 ± 0.8 mg/L. Notably, during the entire period of operating stage 1, R3 consistently maintained low and stable effluent P concentrations, ranging between 0.42 and 0.55 mg/L, with occasional peaks reaching up to 0.64 mg/L on certain days. These results confirm the effectiveness of coupling intermittent P-rich liquid extraction with AGS technology in enhancing long-term P removal, highlighting the value of this proposed approach as an efficient operational strategy for achieving stable low effluent P.

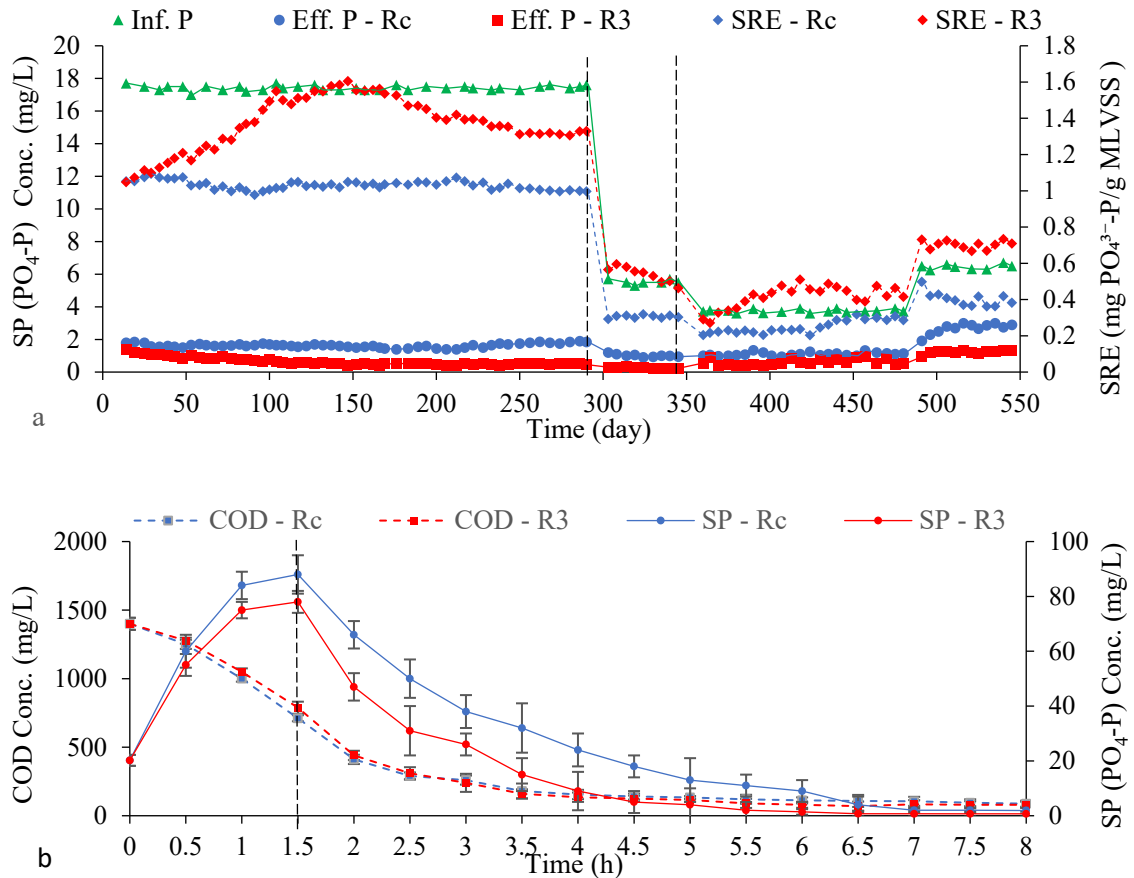


Figure 5.6: $\text{PO}_4\text{-P}$ removal performance in R3 compared with Rc (a) and profiles of $\text{PO}_4\text{-P}$ and sCOD through the typical treatment cycle during stage 1 (HS influents) (b)

Based on the results of the kinetic tests between days 235 and 290 presented in **Figure 5.6-b**, a slight decline in PAO activity was observed in R3 compared to Rc, where the mean value of P-release rates in R3 reached 3.76 ± 0.08 mg-P/g-MLVSS·h. Additionally, the molar P_{rel}/C_{upt} ratio was slightly lower in R3 (0.031) compared to Rc (0.044), further indicating reduced PAOs metabolic activity under the imposed conditions. The P_{rel}/C_{upt} values in this study were lower than those reported previously (Acevedo et al., 2012 and Wang et al., 2022), likely due to two factors: (i) the seeding sludge was not highly enriched with PAOs, unlike in earlier studies; and (ii) yeast extract, a more complex carbon source than acetate, was used. In contrast, acetate as the sole substrate strongly favors PAOs and is typically consumed almost completely anaerobically in prior studies.

However, it is worth mentioning that a modest recovery in PAO bioactivity was evident during the following recovery cycles within each treatment day in R3. This was demonstrated by notable increases (P-value < 0.05) in the anaerobic P-release rates across the third follow-up cycle (**Table C1** in the **Appendix C**), suggesting that intermittent separation and the inclusion of recovery phases helped mitigate the impact of the initial aerobic P starvation condition. These findings highlighted the effectiveness of the proposed operational strategy in R3, which applied a moderate P-rich liquid extraction volume (200 mL) from the mid-depth rather than the bottom. This approach likely avoided over-extraction of P-rich zones that could intensify aerobic starvation, thereby preserving PAO activity and sustaining long-term P removal. Such operational adjustments appear critical in enabling gradual adaptation of PAOs to the extraction regime, supporting the stability and resilience of EBPR over extended operation (Wang et al., 2022).

Despite the slight reduction in PAOs bioactivity anaerobically, AGS in R3 exhibited markedly enhanced the aerobic P uptake rates (**Figure 5.6-b**) during the last two months of operation between days 230 and 290. P uptake rate in R3 reached 0.97 ± 0.05 mg-P/g-MLVSS·h, nearly double that of Rc (0.50 ± 0.02 mg-P/g-MLVSS·h). This accelerated P removal highlights the effectiveness of applying aerobic P starvation strategy intermittently in promoting a highly responsive PAOs community. Additionally, in R3 the SRE (key indicator of P removal activity) increased significantly from 1.06 to 1.63 mg PO_4^{3-} -P/g MLVSS by day 145. Thereafter, a slight but steady decline was observed during the second half of the operational period, with SRE averaging 1.35 mg PO_4^{3-} -P/g MLVSS (**Figure 5.6-a**). In contrast, Rc exhibited a relatively constant SRE throughout the operating stage 1, maintaining values around 1 mg PO_4^{3-} -P/g MLVSS. Despite moderate reductions in biomass concentration and granule density, P removal performance in R3 remained resilient and gradually improved. The enhanced SRE highlights the system's adaptive capacity under operational stress and confirms the effectiveness of the proposed strategy in sustaining and optimizing EBPR.

Furthermore, the observed enhancement in aerobic P removal following P-rich liquid extraction can be attributed to the sustained intracellular P starvation experienced by PAOs. Under these conditions, the insufficient availability of external phosphate limits poly-P replenishment, thereby allowing stored PHAs, accumulated in the prior anaerobic phase, to remain available for cellular maintenance and energy. This metabolic shift promotes efficient P uptake once phosphate becomes accessible, as supported by Lv et al. (2014). Consistent with this mechanism, R3 demonstrated markedly improved P removal, with P removal efficiency of 97.6 ± 0.2 % (89.6 ± 0.3 % in Rc). These results indicated that

the intermittent P-rich liquid extraction strategy effectively maintained a physiologically active PAO population, supporting stable long-term P removal under HS influents. It should be noted that previous studies have shown PAOs are highly sensitive to FNA, with up to 88% inhibition at concentrations as low as 1.2 µg/L (Jabari et al., 2016). Thus, the improved P removal in R3 reflected the mitigation effect by the proposed operational strategy which likely reduced FNA inhibition. In contrast, Rc experienced more pronounced deterioration, potentially due to prolonged exposure to higher FNA levels, though this explanation requires further validation.

The P mass balance was analyzed to further understand the EBPR process in both systems (**Figure 5.7**) over the final 40 days of the operating stage 1 (days 251 and 290), during stable AGS performance and properties, shedding light on the mechanisms involved and understand the biological P removal process under applying aerobic P starvation conditions intermittently. As shown in **Table 5.2**, the overall P-mass balance showed that influent TP had three different fates (**Figure 5.7**): TP in the P-rich liquid extracted (1st cycle only), P content in the added volume of treated effluent (beginning of the aeration phase of the 1st cycle only), TP in effluent liquid and solids, and the remaining TP fraction was calculated as the unaccounted mass (i.e., Loss of TP) required to close the balance (e.g., part of the biomass). TP input in Rc was solely from the influent (6221 ± 33 mgP), while R3 received an additional 8 ± 0.5 mgP from the treated effluent added to maintain a fixed VER.

Between days 251 and 290, total phosphorus content in the reactor biomass was monitored frequently in Rc and R3, with mean values of 0.2034 and 0.184 mg P/mg MLSS, respectively. R3 exhibited a substantial net P recovery via P-rich liquid extraction

(893±12 mgP), demonstrating the effectiveness of the aerobic P-starvation strategy in mobilizing excess P for potential recovery. Additionally, Rc released 8.2% of its total input P in the treated effluent, while R3 discharged only 5.7%, indicating enhanced P removal efficiency due to the proposed strategy (intermittent aerobic P-starvation strategy).

Furthermore, the P content accumulated in AGS (mg P/g MLSS) in both systems was incorporated within the overall TP loss term. Additionally, because the reused treated effluent experienced a long settling time (2 h) prior to transfer between the third-cycle decant and the first-cycle extraction step, suspended-solids carryover at the start of aeration was negligible and was assumed not to influence the balance. However, future work should include P fractionation (TP, organic P, inorganic P, nonapatite inorganic P, and apatite P) to differentiate Bio-P (biomass-associated P, including intracellular poly-P and cellular organic P) from Mineral-P (inorganic precipitates/mineral-bound P) within the granules. Overall, these findings confirm that the moderate P-rich liquid extraction approach in R3 contributed effectively to P recovery while maintaining low effluent P concentrations. This underscores the operational advantage of integrating aerobic P starvation via intermittent separation to enhance biological P removal and facilitate downstream P recovery.

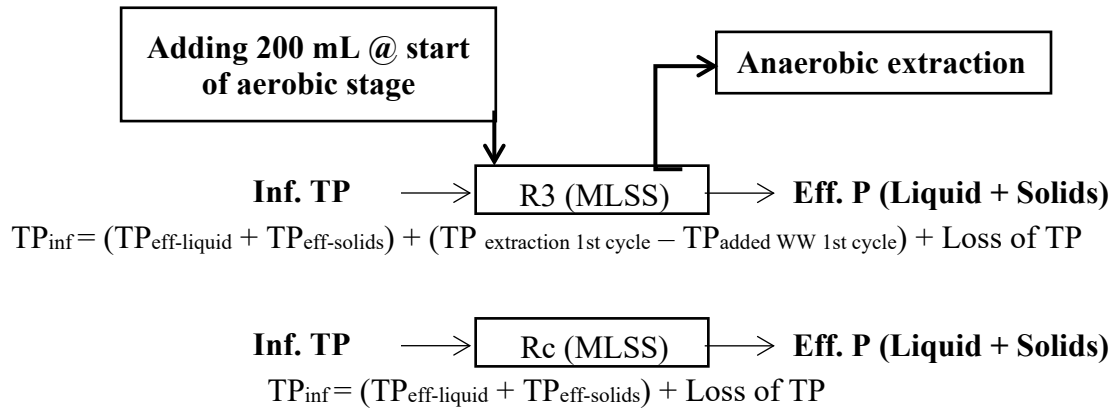


Figure 5.7: Fate of influent P for (a) R3 and (b) and Rc.

Table 5.2: P mass balance for R3 and Rc during the last 40 days through the operating stage 1 (HS influents) and another 20 days through the operating stage 2 (LS influents)

	Operating Stages	HS influents		LS influents	
	Total operation period of P-mass balance analysis	40 days (Days 251 and 290)		20 days (Days 461 and 480)	
	P-Source	R3 (120 cycle)	Rc (120 cycle)	R3 (60 cycle)	Rc (60 cycle)
Input P (mgP)	Inf. P	6221 ± 33		632 ± 11	
	P in the added treated effluent* (Fixed VER)	8 ± 0.5 (40 cycles only)	-	3 ± 0.7 (20 cycles only)	-
Output P (mgP)	P in the P-rich liquid extracted	893 ± 12 (40 cycles only)	-	98 ± 3.3 (20 cycles only)	-
	P content in the effluent liquid	355 ± 13	494 ± 11	28 ± 1	94 ± 2
	P content in the effluent solids**	3164 ± 55	3380 ± 64	503 ± 10	530 ± 9
Loss of TP (mgP)		1817 ± 23 (29.2%)	2347 ± 33 (37.73%)	6 ± 1 (1.0%)	8 ± 1 (1.27%)

* P content in the solids with added WW (start of aerobic phase) is negligible

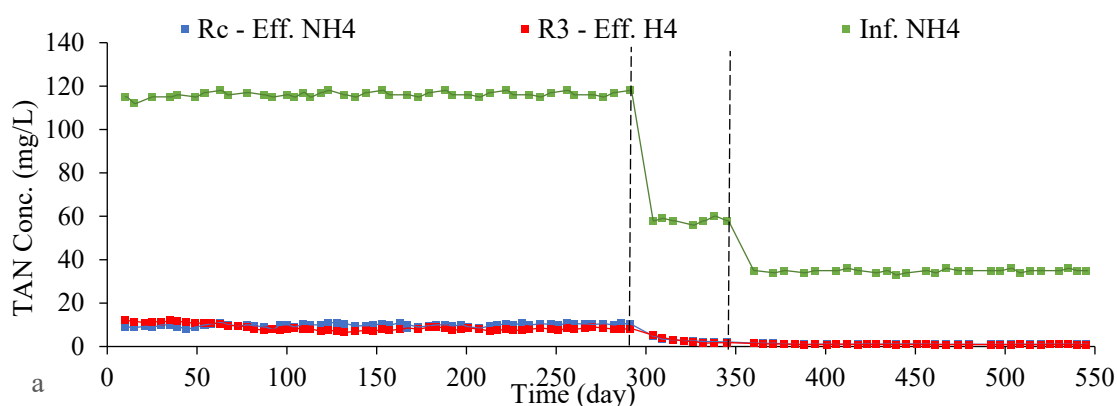
** P – Eff solids = Avg P content in AGS × Avg TSS_{eff} × 1.2L × N cycles

5.3.2.3. Organic and ammonium removal performance

During the operating stage 1, R3 demonstrated stable sCOD removal performance, and the effluent sCOD content was around 70 mg/L (Rc; 85 mg/L). It is worth mentioning that the proposed strategy in R3 effectively reduced the actual aerobic organic loading (14%), resulted in a slight improvement in sCOD removal efficiency compared with Rc.

Overall, NH₄⁺-N removal performance in R3 slightly lagged behind that of Rc, particularly during the first two months of operation (**Figure 5.8-a**). R3 exhibited elevated effluent ammonium concentrations (11.2±1.1 mg/L), alongside nitrate levels averaging

5.2±0.4 mg/L, indicating a temporary decline in SND performance. This initial deterioration may be attributed to the intermittent extraction which could temporarily alter granule structure/density during the initial period (adaptation stage), change oxygen penetration and internal redox zonation, and reduce the stable anoxic niches needed for SND. It can also transiently shift competition for oxygen and space among heterotrophs, PAOs/DPAOs, and nitrifiers, delaying the re-establishment of balanced nitrification–denitrification until the community adapts. However, as operation progressed, granule stability improved through successful reggranulation (**Figure 5.5; b,c**), enabling microbial communities to gradually adapt to the imposed conditions. R3 recovered its N removal efficiency, and by the final 60 days of operation, effluent $\text{NH}_4^+\text{-N}$ concentrations decreased to 8.3±0.5 mg/L ($\text{NO}_2^-/\text{NO}_3^-\text{-N} < 1.5$ mg/L). Meanwhile, the treated effluent from Rc exhibited 10±0.6 mg $\text{NH}_4^+\text{-N/L}$ ($\text{NO}_2^-/\text{NO}_3^-\text{-N} < 1$ mg/L) throughout the entire operational period. Besides the detrimental influence of high OLRs during the treatment of HS wastewater on the proliferation of nitrifiers, the consistently high TN load of 0.57 ± 0.11 KgN/m³·d may have exceeded the nitrification capacity, potentially due to the inhibitory effects of elevated FA levels associated with the influent which reached up to 10 mg $\text{NH}_3\text{-N/L}$, which could plausibly contribute to nitrification inhibition (Ren et al., 2017).



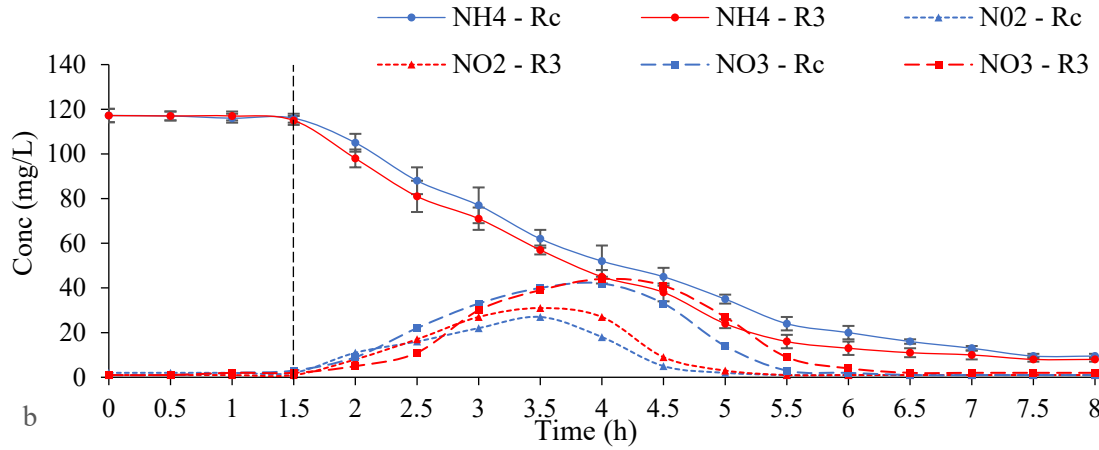


Figure 5.8: NH₄-N removal performance in R3 compared with Rc (a) and profiles of NH₄, NO₂, and NO₃ through the typical treatment cycle during stage 1 (HS influents) (b)

Operational parameters and organic/nutrient removal efficiencies of Rc and R1-R4 during the last month in operating stage 1 (HS influent) are summarized in **Table 5.3**.

Table 5.3: Summary of granules physical properties and BNR performance of GSBRS through the last month (days 255 - 290) during the operating stage 1 (HS conditions)

Characteristics	Control SBR Rc	Test Reactors			
		R1	R2	R3	R4
P-rich liquid extraction	No extraction	5% (100 mL from one cycle only daily)	5% (100 mL from one cycle only daily)	10% (200 mL from one cycle only daily)	14% (280 mL from one cycle only daily)
Operating scenario* (fixed VER)	-	Scenario 1	Scenario 2	Scenario 2	Scenario 2
MLSS (g/L)	10.4	1.77	10.1	8.6	6.31
MLVSS/MLSS (%)	72	69	74	80	85
Granules' size (mm)	2.7	< 0.5	2.4	2.07	1.2
SVI ₃₀ (mL/gm)	< 29	>88	< 32	< 38	66
TSS _{eff} (mg/L)	90 – 115	80 – 90	95 – 125	115 – 130	120 – 145

SRT (days)	50	11	48	39	21
Biomass yield (Y_{obs})	0.082	0.06	0.073	0.086	0.112
sCOD removal (%)	95	88	96	97	96
NH_4^+ -N removal (%)	92	57	92	93	81
SP removal (%)	91	66	92	98	82

* Operating scenario 1 and 2 by adding same volume of fresh influent and reusing treated effluent, respectively, at the beginning of the aeration phase to fix VER

5.3.3. Operating Stage 2: LS Municipal Wastewater Treatment

Before initiating the second stage to investigate the feasibility of the proposed approach under the treatment of LS wastewater, R3 and Rc were operated between days 291 and 345 under the treatment of MS influents ($TCOD = 750 \pm 11$ mg/L) as a transitional phase. Firstly, there was a negative impact that was recorded in R3 and Rc shortly from the first day of operation. This deterioration temporarily affected aerobic granules during the first month (day 324). Additionally, this was characterized by a decline in biomass concentrations associated with poor effluent quality, elevated SVI_{30} values, and reduced granule size (**Fig. C3 – Appendix C**). This could be attributed by the reduction in the applied OLR to 1.35 kg COD/ $m^3 \cdot d$.

Nevertheless, the system exhibited notable resilience, with granule stability recovering during the last three weeks of the operation as the biomass adapted to the lower influent sCOD content. At the end of this stage, the MLSS, EPS, granules size, and SRT for R3 and Rc were measured as 7.6 g/L, 143 mg/gVSS, 1.72 mm, 33 days, and 10.4 g/L, 211 mg/gVSS, 2.56 mm, 41 days, respectively. R3 and Rc achieving simultaneous sCOD/ NH_4^+ -N/ PO_4^{3-} -P removal efficiencies of 97/96/96 %, and 96/94/83 %, respectively.

5.3.3.1. Response of LS municipal influent transition on biomass quantity and quality

During operating stage 2, LS wastewater treatment in R3 and Rc resulted in a marked decrease in OLR ($0.684 \text{ kg COD/m}^3 \cdot \text{d}$) and in the F/M ($0.1 \text{ g COD/g MLVSS} \cdot \text{d}$). AGS physical properties deteriorated rapidly within two months (**Figure 5.5; a,b**), with MLSS and granule size declining by day 420 to $4.0 \pm 0.14 \text{ g/L}$ and $1.0 \pm 0.11 \text{ mm}$ in R3, and $7.7 \pm 0.28 \text{ g/L}$ and $1.79 \pm 0.13 \text{ mm}$ in Rc. Additionally, R3 exhibited poor effluent quality, with TSS_{eff} concentrations reaching as high as $215 \pm 18 \text{ mg/L}$, during the same period of operation. These observations are consistent with earlier studies (Kang and Yuan, 2017 and Hamza et al., 2018) that reported granule disintegration under low F/M ratios. The decline in MLSS was primarily attributed to selective biomass wasting based on settling velocity. The more pronounced deterioration in R3 likely resulted from P-rich liquid extraction, which limited mineral precipitate accumulation and reduced granule density and integrity, as reflected by a higher MLVSS/MLSS (0.82) compared to Rc (0.77).

Between days 421–480, R3 exhibited granule stabilization with an F/M ratio increase to $0.193 \text{ g COD/g MLVSS} \cdot \text{d}$, supporting biomass regrowth and successful regranulation, with granule size $\geq 1.17 \text{ mm}$ and MLSS $4.7 \pm 0.15 \text{ g/L}$, while Rc experienced prolonged granule disintegration (**Figure 5.5-a**). Despite the increasing in F/M to $0.17 \text{ g COD/g MLVSS} \cdot \text{d}$ by day 440, granule size and MLSS continued declining until stabilization at day 460, then reaching $1.61 \pm 0.05 \text{ mm}$ and $6.0 \pm 0.14 \text{ g/L}$, respectively, at day 480. Despite the observed reduction in granule size in R3 and Rc, the systems maintained favorable settleability (**Figure 5.5-b,c**), with SVI_{30} remaining $\leq 44 \text{ mL/g}$ in R3 and $\leq 35 \text{ mL/g}$ in Rc. These findings underscored the feasibility of implementing AGS technology for municipal wastewater treatment, particularly when granules are first

matured during HS influent treatment, probably resulting in enhancing long-term structural resilience under subsequent LS loading. The average SRT was 22 days in R3 and 34 days in Rc (day 480). These results align with Jafari Kang and Yuan (2017), who reported reversible AGS disintegration under low F/M once conditions were restored, and highlighted that reduced OLR impairs EPS production, underscoring the essential role of PN and PS in granule integrity.

In R3, the transition to LS wastewater initially led to a reduction in EPS secretion. However, following successful re-granulation between days 451 and 480, EPS levels recovered significantly, reaching 118 ± 7 mg/gVSS (PN/PS ratio = 8), comparable to or even exceeding the values observed during the earlier transitional phase (**Figure 5.5-b**). Notably, this enhancement was not mirrored by a similar increase in granule size, which remained lower than the initial values. This limitation can be attributed to the reduced OLR applied during this phase, a factor previously reported to constrain granule growth (Tay et al., 2004 and Kang and Yuan, 2017). In contrast, EPS secretions are influenced more by operational conditions such as hydrodynamic shear forces, selective washout, and the feast/famine balance. The enhanced EPS production in R3 is likely a response to intensified starvation conditions, promoted by the extended aeration period (350 min) and reusing of treated effluent at the start of the aerobic phase after P-rich liquid extraction. This strategy helped in sustaining proper feast/famine dynamics (prolonged the famine phase by reducing the aerobic nutrient loadings), and stabilizing granule structure by reinforcing microbial aggregation, as well.

These findings are consistent with previous studies (Lin et al., 2008, Tay et al., 2001, Liu and Tay, 2007), which showed that the duration of the starvation period plays a pivotal role in stimulating EPS secretion. Meanwhile, Rc exhibited a similar declining trend in EPS dynamics (**Figure 5.5-b**) till day 450, then stabilizing around 138 ± 11 mg/gVSS by day 480. However, re-granulation in Rc occurred more slowly, likely due to the persistence of large, compact granules formed during stage 1. This contrast further highlights the advantages of the P-rich liquid extraction strategy applied in R3, which not only facilitated better nutrient diffusion into the granules but also promoted a more adaptive and responsive microbial environment under LS operational conditions as well as the enrichment of EPS-producing, slow-growing microbes (**5.3.4. Bacterial community structure of granules**).

In addition, the results indicated no significant deterioration in the settling properties of either SBR, as shown in **Figure 5.5 (b)**. Despite the observed reduction in granule size in R3 and Rc, the systems maintained favorable settleability, with $SVI_5/SVI_{30} > 0.9$ and SVI_{30} values remaining ≤ 44 mL/g in R3 and ≤ 35 mL/g in Rc. These findings underscore the feasibility of implementing AGS technology for municipal wastewater treatment, particularly when granules are first cultivated and matured under HS influent conditions, probably resulting in enhancing long-term structural resilience and settling performance under subsequent LS loading. The following table (**Table 5.4**) summarizes the granules physical properties (e.g., biomass characteristics and settling properties), effluent quality, and BNR efficiencies.

Table 5.4: Summary of granules physical properties of Rc and R3 through the last two months during the operating stage 2 under the LS conditions (days 421 - 480)

Characteristics	Control SBR (Rc)	Test SBR (R3)
P-rich liquid extraction	No extraction	200 mL (10%) from one cycle daily
MLSS (g/L)	6.0 ± 0.2	4.7 ± 0.4
MLVSS/MLSS (%)	75	83
Granules' size (mm)	1.61	1.17
SVI ₃₀ (mL/gm)	< 35	< 44
TSS _{eff} (mg/L)	75 – 90	85 – 105
SRT (days)	38	25
Biomass yield (Y_{obs})	0.178	0.191
SRE (mg PO ₄ ³⁻ -P/g MLVSS)	0.28	0.53
sCOD removal (%)	≥ 98	≥ 99
TAN removal (%)	≥ 97	≥ 97
SP removal (%)	66	≥ 91

5.3.3.2. Impact of P-rich liquid extraction on the BNR treatment performance of granules

Throughout the operating stage 2, sCOD in both SBR effluents remained consistently low, averaging below 7 ± 2 mg/L with removal efficiencies $\geq 98\%$. However, R3 demonstrated a slight enhancement attributed to the addition of treated effluent at the start of the aerobic phase, which reduced the actual aerobic sCOD loading by approximately 16% (**Appendix C**). Accordingly, this allowing R3 to achieve its organic removal target more rapidly than Rc (**Figure 5.9-b**). On the other hand, anaerobic sCOD consumption in R3 was slightly lower ($46 \pm 1.2\%$) than in Rc ($50 \pm 2\%$). This difference may indicate a modest reduction in anaerobic carbon uptake by PAOs/DPAOs in R3, potentially due to the P-rich liquid extraction strategy affecting intracellular poly-P reserves and, consequently, the extent of anaerobic substrate assimilation.

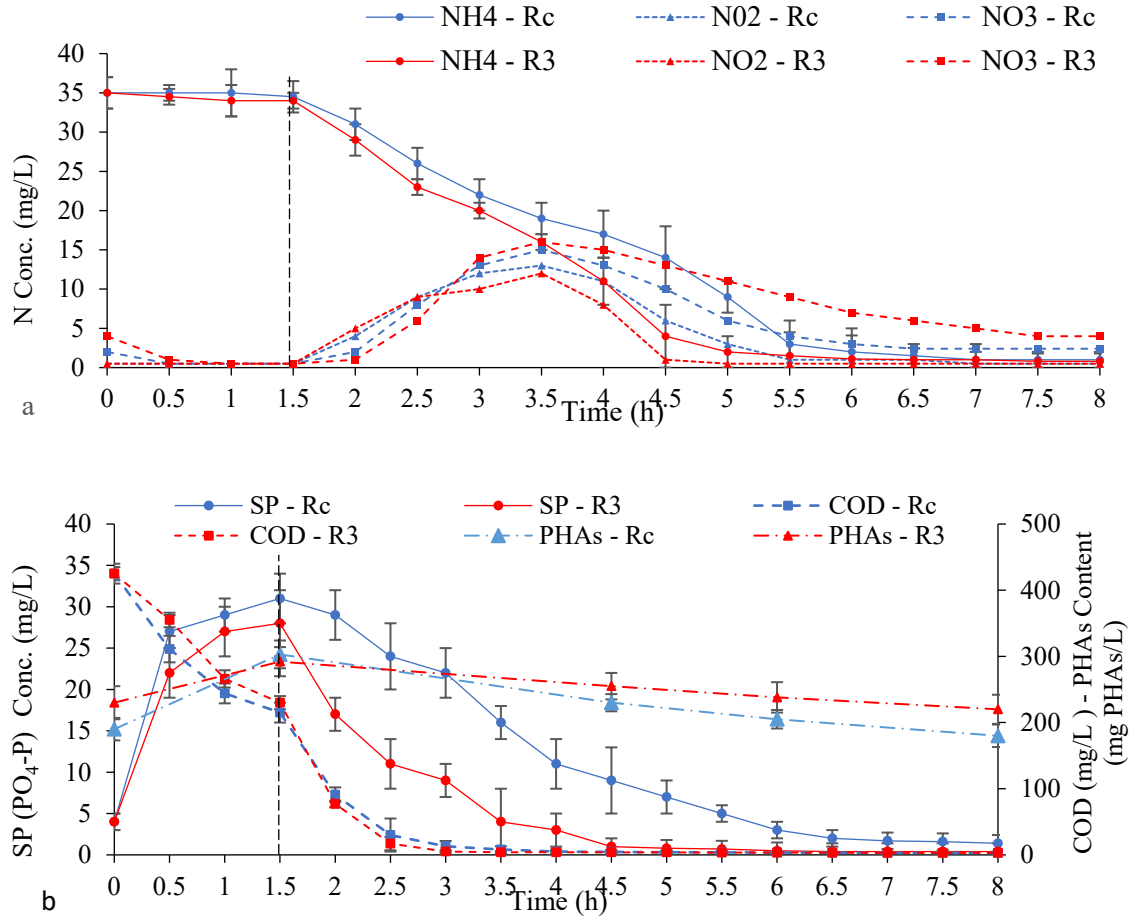


Figure 5.9: Profiles of N-forms (a) and PO₄-P, sCOD and PHAs (b) in R3 compared with Rc through the typical treatment cycle during the treatment of LS influents

Nitrification performance improved significantly in R3 and Rc, primarily due to the reduction in influent ammonium concentrations to 35 ± 2 mg/L. Effluent NH_4^+ -N levels in R3 and Rc had decreased to below 1 mg/L, with average removal efficiencies exceeding 97.6 ± 0.4 % between days 451 and 480 (**Figure 5.9-a**). These results are consistent with prior studies reporting enhanced nitrification when influent NH_4^+ -N concentrations were reduced (Kang and Yuan, 2017). Denitrification remained stable during the first month in R3 but declined thereafter due to the rising nitrate levels by day 450. These findings align

with earlier observations of lower anaerobic sCOD consumption in R3 (**Figure 5.9-b**). It should be noted that through between days 455 - 480, granules in both SBRs demonstrated clear adaptation to the limited sCOD availability, particularly as their physical properties began to stabilize, leading to successful regrowth. Moreover, this improvement might be linked to the proliferation of HNAD bacteria, which were identified as part of the microbial community structure in the AGS of the mother GSBR used in this study, as demonstrated in our previous work (Mady et al., 2024). These species exhibited relatively high abundance and likely played a significant role in supporting simultaneous N removal. Notably, this recovery coincided with improved denitrification performance (**Figure 5.9-a**), where nitrate concentrations declined to below 2.4 mg/L in Rc and 4 mg/L in R3 (nitrite remained < 1.0 mg/L in both systems). Consequently, SND efficiency increased, reaching 91 ± 0.9 % in Rc and 87 ± 0.4 % in R3 by day 480.

During operating stage 2, both systems were fed with LS wastewater containing 3.72 ± 0.14 mg- PO_4^{3-} /L. R3 responded promptly and favorably to aerobic P starvation, achieving high P removal efficiency (up to 85 %) during the first 3 months, with effluent SP concentrations averaging 0.6 ± 0.08 mg/L. In contrast, Rc maintained a lower removal efficiency of 73 ± 1.6 %. The highest aerobic P-uptake rates were observed averaging 1.02 ± 0.04 mg-P/g-MLVSS·h in R3 and 0.74 ± 0.03 mg-P/g-MLVSS·h in Rc, highlighting achieving enhanced P removal performance under the applied operational conditions. It should be noted that the increased nitrate formation resulted in transition from anaerobic to anoxic environment in R3, which likely intensified competition between PAOs and denitrifiers (limited rbCOD available) and affected the overall P removal performance during this stage. However, despite this potential competitive limitation, the net EBPR

outcome in R3 was governed by the sustained increase in PAO aerobic uptake capacity under the proposed strategy, which outweighed the adverse effect of elevated NO_3^- -N during this stage. Despite these challenges, PAOs in R3 gradually adapted to LS conditions, with aerobic P uptake rate increasing until day 480, reaching 1.33 ± 0.11 mg-P/g-MLVSS·h, nearly twice that of Rc (**Figure 5.9-b**), demonstrating enhanced biological activity under the proposed strategy. Furthermore, R3 demonstrated accelerated aerobic P uptake during this operating stage, removing most PO_4^{3-} -P within 3.5 h, significantly faster than the control GSBP which required >5 h (**Figure 5.8-b**). Consequently, such rapid P removal behavior reflected accelerated P removal in the GSBPs, demonstrating a more competitive performance, particularly during the treatment of LS domestic wastewater, compared to conventional EBPR systems, consistent with previous findings (Barat et al., 2006, Zhang et al., 2015, Wang et al., 2022). These results further suggested that PAOs remained metabolically active despite extended exposure to low ambient P, likely sustained by their internally stored reserves, including PHAs, poly-P, and glycogen. Such conditions, limited extracellular phosphate availability aerobically, thereby maintaining selective pressure for efficient and rapid P uptake. This outcome reinforces the potential of intermittent P-starvation regimes to support stable and resilient EBPR performance over time.

It is worth noting that the sustained and enhanced P removal observed in R3 was primarily achieved during the treatment cycle involving P-rich liquid extraction, while a slight reduction in performance was noted during the subsequent recovery cycles. Aerobic P uptake rates in R3 during the 2nd and 3rd cycles were 1.26 ± 0.08 and 1.16 ± 0.1 mg-P/g-MLVSS·h. The stability in enhanced P removal performance may be attributed to the ability of PAOs to retain high PHAs levels under aerobic P starvation conditions, coupled

with their reliance on intracellular reserves such as poly-P and glycogen for effective functioning, as reported in previous studies (Lopez et al., 2006). Consequently, PHAs were likely preserved during the anaerobic phase of the recovery cycles and efficiently utilized during the subsequent aerobic phase, supporting sustained and rapid P uptake.

This dynamic explained the robust and reproducible P removal performance observed in R3 throughout daily operations, and also supported effective P uptake once $\text{PO}_4^{3-}\text{-P}$ was reintroduced. This translated into significantly lower effluent P concentrations in R3 (0.32 - 0.63 mg $\text{PO}_4^{3-}\text{-P/L}$) compared to Rc (1.23 - 1.62 mg $\text{PO}_4^{3-}\text{-P/L}$), reaffirming the robustness of the proposed strategy under reduced nutrient loading until day 480. Despite the overall effectiveness of the applied strategy, R3 exhibited intermittent fluctuations in effluent P concentrations, where elevated values (e.g., 0.77–0.95 mg/L) were recorded on days 364, 413, 430, 440, 452, 457, and 470 (**Figure 5.6-b**). These temporary spikes may indicate transitional microbial adaptation or granule instability under LS loading, underscoring the need for fine-tuning and further optimization of the proposed strategy (Section 3.3) to ensure consistent and reliable EBPR performance. Moreover, granules in R3 achieved an average SRE (**Figure 5.6-b**) of 0.53 ± 0.11 mg $\text{PO}_4^{3-}\text{-P/g}$ MLVSS, nearly double Rc (0.28 ± 0.03 mg $\text{PO}_4^{3-}\text{-P/g}$ MLVSS), confirming enhanced removal capacity under the proposed strategy.

As shown in **Table 5.2**, a 20-day P mass balance (days 461–481) further demonstrated the selectivity of the applied operational strategy. Despite the significantly lower influent P load (632 ± 11 mgP), R3 achieved a notable P recovery efficiency of approximately 16% (98 mgP) and maintained minimal soluble P losses (28 ± 1 mgP). In

contrast, Rc released nearly threefold more soluble P (94 ± 2 mgP) with no P recovery. It should be noted that during days 461–480, mean biomass P content was 0.081 mg P/mg MLSS in R3, compared with 0.066 mg P/mg MLSS in Rc. In addition to confirming the effectiveness of the proposed strategy in sustaining EBPR performance, the recovered P fraction in R3 presents a valuable feedstock for downstream P recovery applications (**Section 5.3.5**). Moreover, P accumulation in biomass was slightly lower in R3 than Rc, consistent with trends during HS treatment and reflecting reduced mineral precipitation due to P-rich liquid extraction. Collectively, these findings underscore the robustness of intermittent P-rich liquid extraction under nutrient-limited conditions, highlighting its scalability and dual benefit in enhancing biological P removal and enabling resource recovery in domestic wastewater systems.

However, a slight decline in PAO activity was detected in R3, as evidenced by the kinetic test results in **Figure 5.9 (b)**, which depict $\text{PO}_4^{3-}\text{-P}$ and sCOD concentration profiles over a typical treatment cycle (days 451–480). This reduction in PAO activity was reflected in lower anaerobic P-release rates in R3 (2.41 ± 0.08 mg-P/g-MLVSS·h) compared to Rc (2.7 ± 0.07 mg-P/g-MLVSS·h), alongside a concurrent decrease in the molar $P_{\text{rel}}/C_{\text{upt}}$ ratio in R3. Comprehensive data for these parameters (P-release, -uptake rates, molar $P_{\text{rel}}/C_{\text{upt}}$ ratio, %PHAs, and q_{PHA}) throughout all treatment cycles are presented in **Table C2** of the **Appendix C**. This could be attributed by the limited carbon availability or uptake efficiency during the anoxic phase. However, the intermittent nature of extraction through the effective role of the recovery cycles, appeared to create a balanced P starvation environment that was sufficient to stimulate enhanced P uptake while avoiding excessive stress on PAOs. This adaptation was essential for sustaining robust and efficient P removal,

highlighting the system's resilience and the effectiveness of the intermittent extraction strategy in maintaining EBPR stability under modified aerobic conditions.

These findings highlight two key outcomes. First, the cultivation and maturation of AGS under HS influent conditions provided a structural and functional advantage when transitioning to municipal wastewater treatment. This pre-adaptation helped preserve granule integrity and maintain acceptable physical properties, while supporting stable simultaneous BNR performance. Second, this approach mitigated many common operational challenges reported in the literature, such as prolonged start-up times, granular disintegration, limited nutrient removal efficiencies, and constrained microbial diversity, typically encountered when AGS is applied directly to LS influent systems (Liu and Tay, 2004, Zheng et al., 2006, Adav et al., 2008, Muda et al., 2010, Lee et al., 2010, Peyong et al., 2012, Adav et al., 2010). In addition, although the application of intermittent P-rich liquid extraction resulted in a reduction in granule size due to decreased P mineral precipitation, the smaller granules in R3 demonstrated sustained and improved EBPR performance over the long term compared to Rc, both during HS and municipal wastewater treatment. These findings are consistent with previous studies suggesting that P removal in AGS systems is not solely reliant on intragranular chemical precipitation but is primarily driven by EBPR, which has been shown to account for approximately 55–73.7 % of total P removal in such systems according to the literature (Mañas et al., 2011, Filali et al., 2012, Henriët et al., 2016, Sarma and Tay, 2018, Purba et al., 2020, Huang et al., 2015).

Between days 451 and 480, the intracellular PHAs profiles were assessed (**Figure 5.9-b**). Results revealed a marked improvement in PHAs accumulation in R3 compared to

Rc, with average values of 1.65 ± 0.02 and 1.31 ± 0.05 % (mg PHAs/mg MLSS) in R3 and Rc, respectively. Likewise, q_{PHA} was higher in R3 (0.014 mg COD-PHAs/mg COD-X·h) than in Rc (0.012 mg COD-PHAs/mg COD-X·h). These findings highlighted a notable increase in the PHAs accumulation capacity of granules in R3, sustained over the entire operational period. This enhancement suggested that the residual PO_4^{3-} concentration was insufficient to fully support PHAs metabolism aerobically, as demonstrated in a previous study (Ren et al., 2017), thereby validating the effectiveness of the imposed aerobic P-starvation regime. As a result, the microbial community in R3 exhibited stronger metabolic activity, likely stimulated by the improved feast/famine dynamics. Therefore, enhanced intracellular PHAs storage played a key role in maintaining PAOs functionality, which supports the long-term EBPR stability under nutrient-limited, LS wastewater conditions.

Overall, extraction of P-rich liquid during the first cycle promoted substantial intracellular PHA retention, enabling PAOs to achieve rapid and efficient P uptake in the subsequent aerobic phase. However, extended aeration accelerated PHA depletion, compromising PAO metabolic functionality over time. Preserving PHAs within PAOs is thus critical to maintaining consistent EBPR performance, especially during recovery cycles. These findings highlight the importance of reducing aeration duration in GSBs, not only to sustain enhanced P removal in the initial extraction cycle but also to ensure sufficient PHAs availability and PAO activity across consecutive non-extraction cycles.

Similar to the P mass balance, N mass balance was evaluated for Rc and R3 during the same steady-state periods under HS treatment stage (days 251–290) and LS stage (days 461–480) to clarify whether P-rich liquid extraction altered N pathways. However, because

the reactors were operated as open SBRs, gaseous denitrification products were not directly quantified, which limits full resolution of N losses and indicates that future studies should use closed bioreactors to measure N_2O , NO , and N_2 . Despite this limitation, the balance developed from frequent measurements of biomass N content, influent and effluent TN, and cycle-based NH_4^+ , NO_2^- , and NO_3^- profiles was sufficient to show that the proposed extraction approach did not induce any significant change in overall N fate relative to Rc, as summarized in **Table 5.5**.

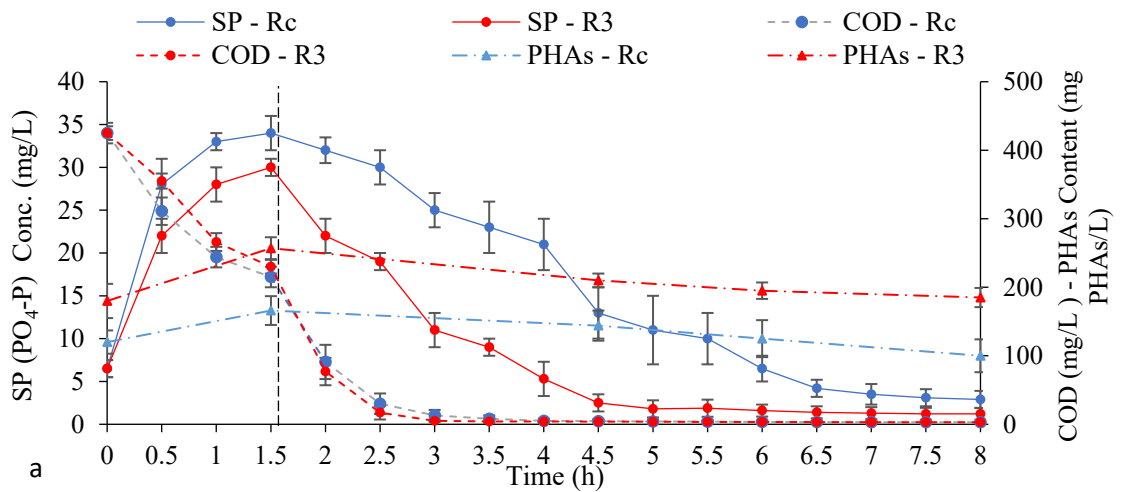
As shown in **Table 5.5**, N removal in both reactors was governed mainly by retention of TN in the effluent, while the remaining fraction was attributed to denitrification-related N loss. Across both operating stages, Rc and R3 exhibited comparable N removal performance, with only minor differences that did not suggest any systematic effect of P-rich liquid extraction on overall N fate.

Table 5.5: N mass balance for R3 and Rc during the operating stage 1 and stage 2.

	HS influents 40 days (Days 251 and 290)		LS influents 20 days (Days 461 and 480)	
	R3 (120 cycle)	Rc (120 cycle)	R3 (60 cycle)	Rc (60 cycle)
Influent TN (mg N)	20200 ± 140		3450 ± 20	
Effluent TN (mg N)	7066 ± 40	7020 ± 48	1170 ± 10	1220 ± 12
Nitrate denitrified + N Gases + Loss of TN (mg N)	13134 ± 90	13180 ± 74	2280 ± 21	2230 ± 18

5.3.3.3. LS influent with elevated P loadings: performance limits and operational trade-offs

In this phase of operation, between days 481 and 545, during the LS wastewater treatment (similar sCOD of 380 ± 20 mg/L), the influent soluble P concentration was intentionally increased for R3 and Rc to 6.5 ± 0.07 mg/L to impose a more challenging loading condition. This adjustment was designed to critically evaluate the feasibility and adaptability of the proposed separation strategy in R3 under elevated P stress. In addition, reducing the aeration duration may help prevent PHAs depletion, thereby safeguarding PAOs functionality and reinforcing enhanced EBPR performance across all daily treatment cycles. However, it should be noted that performing daily aeration reduction (i.e., all cycles), aimed at promoting aerobic PHA accumulation, should be performed carefully since the excessive reduction may potentially lead to EBPR failure. Under such conditions, PAOs could increasingly rely on their intracellular poly-P reserves (important energy source for PAOs) during extended aerobic starvation, risking poly-P depletion, which can weaken PAO activity, promote GAO proliferation, and intensify competition for carbon sources, ultimately compromising EBPR performance (Zhang et al., 2011, Oehmen et al., 2006, López-Vázquez et al., 2007, 2009).



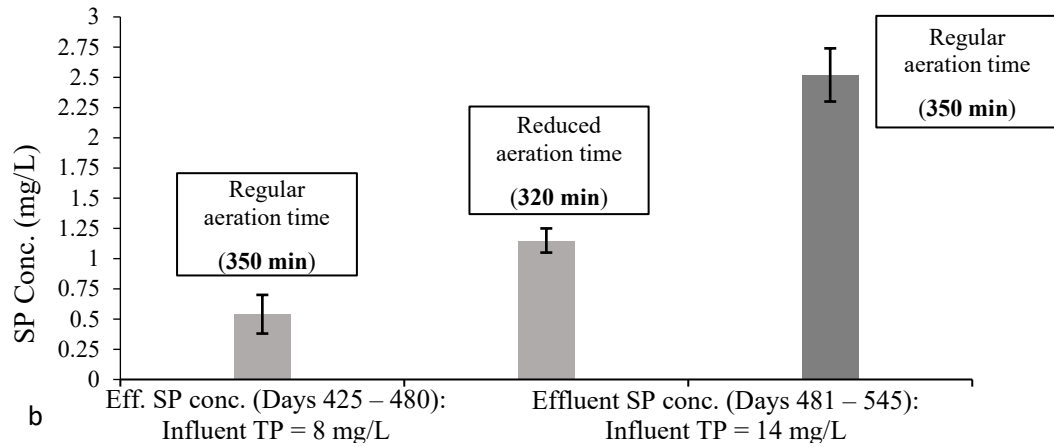


Figure 5.10: Treatment of LS wastewater (Influent SP = 6.5 mg/L): (a) Results of kinetic tests (SP and PHAs profiles) between days 520 - 545, (b) SP levels in the effluent for R3 before and after air adjustment compared with Rc over more than 9 weeks of operation.

Accordingly, during this operational phase, the aeration period in R3 was reduced from 350 to 325 minutes (i.e., final sedimentation extended to 35 min) to maximize the preservation of intracellular PHA reserves, thereby supporting long-term EBPR stability and efficiency, particularly during recovery cycles without P-rich liquid extraction. In the meantime, Rc continued under its original operational configuration, without any operational changes. It should be noted that based on the rapid P removal behavior observed in R3, where most aerobic P uptake occurred within approximately 210 min, as mentioned previously, therefore, it was expected that the reduced aeration duration was sufficient to meet the required mixing time. Importantly, this adjustment aimed to strike a balance between preserving PHAs accumulated after P-rich liquid extraction and preventing excessive poly-P depletion. As reported by Acevedo et al. (2012), low poly-P levels can trigger a metabolic shift from PAOs to GAOs, potentially compromising EBPR performance. Although a few previous studies have addressed this separation approach,

most primarily focused on enhancing P recovery (Guisasola et al., 2019). Only limited research has evaluated its long-term application or investigated the intracellular dynamics of PHAs storage in PAOs and strategies to preserve these reserves to support PAO functionality under aerobic P starvation (Wang et al., 2022 and Guisasola et al., 2019). This section presents a targeted evaluation of the reduced aeration phase under lowered COD/P conditions as an operational refinement for systems operating with intermittent aerobic P starvation, aiming to sustain enhanced EBPR performance over extended periods while reinforcing the physiological resilience of the microbial community. The outcomes of this phase provide valuable insights for practical applications in domestic wastewater treatment.

No significant differences (P-value >0.05) were observed in biomass concentrations or the physical characteristics of the granules in either system before and during this operational phase, likely due to the stable applied OLRs and F/M ratios, as demonstrated through the previous studies (Tay et al., 2004 and Kang and Yuan, 2017). Despite higher influent P levels, R3 consistently outperformed Rc in SP removal, achieving 81.2 ± 0.08 % efficiency with effluent concentrations of 1.15 - 1.43 mg/L, compared to 57.1 ± 0.33 % (average) and 2.5 - 3.0 mg/L, respectively, through the control system (**Figure 5.10 (a, b)**). In addition to reducing daily aeration periods, determining the optimal extraction ratio of P-rich liquid under varying P loadings in municipal wastewater EBPR systems warrants further investigation. For example, while a higher extraction ratio may achieve high P removals ($>90\%$) when treating organic-deficient wastewater with elevated P loadings, such conditions can also accelerate PHA metabolism, leading to faster depletion of intracellular PHA reserves in PAOs. Over the long term, this could compromise EBPR

stability despite the initial performance gains. As shown in **Figure 5.10-b**, both the average SP concentration and its variability in the treated effluent of R3 were notably reduced between days 481–545 compared to the preceding two months (days 425–480). This simultaneous decrease in mean concentration and variability suggests that the reduced daily aeration periods in systems coupled with P-rich liquid extraction, enhanced process stability and predictability, thereby lowering the risk of effluent quality excursions.

Additionally, no significant differences ($P\text{-value} > 0.05$) were observed in sCOD or N removal in R3 and Rc after the operational changes applied between days 481 and 545, compared to their performance during LS influent treatment prior between days 425 and 480. Moreover, although an anoxic period of 35 min was created at the end of each cycle in R3, comprising 25 min of aeration shut-off followed by 10 min of sedimentation, no improvement in nitrite or nitrate reduction was observed. Nitrite and nitrate concentrations remained below 1 and 2.5 mg/L, respectively. This outcome can be attributed to the lack of available carbon at the end of the aeration phase (**Figure 5.10-a**).

It is important to note that the reduction in P removal during the current operation phase in both systems, compared with their performance previously, was primarily due to the higher influent P applied. This resulted in lower C/P ratios, placing both GSBs under carbon-limited conditions and increasing the likelihood of intensified competition between PAOs and their competitors. These findings are consistent with previous studies reporting that TP removal efficiency can be negatively affected by PAO–GAO competition, with low C/P ratios favoring GAOs enrichment (Oehmen et al., 2007 and Wang et al., 2015).

A significant enhancement (P-value < 0.05) in P release rates was recorded in R3, reaching 2.8 ± 0.04 mg-P/g-MLVSS·h, underlining the improved physiological activity of PAOs during this phase (**Figure 5.10-a**). In contrast, Rc exhibited no significant differences in P release performance (P-value > 0.05), with release rates averaging 2.64 ± 0.10 mg-P/g-MLVSS·h. Interestingly, during the aeration phase, R3 exhibited a modest yet consistent improvement in P uptake rates, averaging 1.77 ± 0.12 mg-P/g-MLVSS·h during the first cycle, and no significant differences (P-value > 0.05) were observed in the subsequent recovery cycles. These results (**Table C3 – Appendix C**) highlight the positive impact of reducing daily aeration periods in R3, even under high P loadings, and underscore the sustained metabolic activity of PAOs throughout daily operations. Furthermore, these results reinforce the effectiveness of the shortened aeration phase in preserving intracellular PHAs generated during the initial anaerobic period, thereby sustaining high PAO functionality in subsequent cycles. R3 also maintained rapid P uptake kinetics, achieving near-complete P removal within the first 3 h of the aerobic phase. In contrast, Rc showed limited P removal performance, with P uptake rates of 0.91 ± 0.07 mg-P/g-MLVSS·h and extended P removal over the entire aeration period.

By the end of operation, R3 displayed a higher PHAs content by the end of the typical treatment cycle compared with its value before decreasing the aeration period as expected (**Figure 5.9-b** and **5.10-a**). PHAs analyses extended between days 520–545 demonstrated a substantial increase in average PHAs accumulation to 2.11 ± 0.07 % (mg PHAs/mg MLSS) and a corresponding rise in the q_{PHA} to 0.0185 mg COD-PHAs/mg COD-X·h. Elevated PHAs levels and q_{PHA} values remained consistent across subsequent recovery cycles with no significant variation (P > 0.05). These

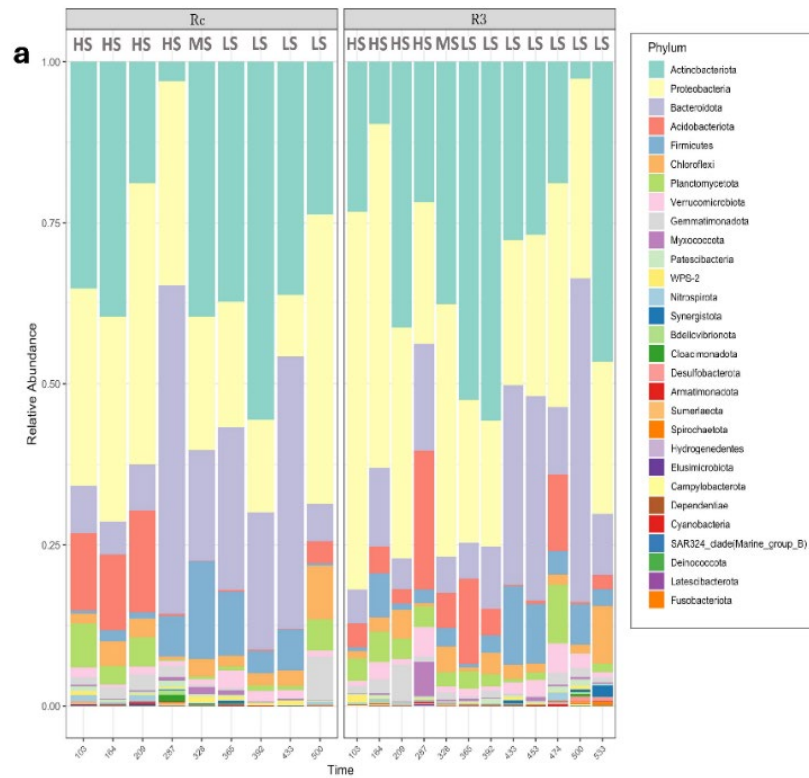
findings confirmed the ability of PAOs to retain high levels of PHAs, accumulated during the first cycle, particularly after the aeration duration was reduced. This retention likely contributed to the consistent operating conditions observed during subsequent recovery cycles, effectively replicating the aerobic P-starvation environment. The limited availability of residual PO_4^{3-} released during the anaerobic phase, relative to the high intracellular PHAs content, suggests that PAOs prioritized the sequential utilization of poly-P and glycogen for maintenance energy, rather than immediately consuming PHAs. This metabolic preference is further supported by the lower P accumulation in the biomass of R3 compared to Rc, as shown in the P mass balance analysis (**Table 5.2**). It is worth noting that these observations align with the findings of Lopez et al. (2006), who reported that poly-P and glycogen are sequentially utilized to meet cellular maintenance energy demands under prolonged anaerobic starvation. The measured P uptake rates and PHAs accumulation indicated that the shorten aeration time led to more controlled and reliable operation. Collectively, these observations suggested that boosting PHAs storage, achieved by shortening the aeration phase, may have provided the granules in R3 with an energy reserve that helped sustain fast, stable P removal in the ensuing cycles, although this causal link warrants further verification.

5.3.4. Microbial Community Dynamics and Functional Potential of Aerobic Granules

5.3.4.1. Microbial community shifts across stages

The bacterial community structure of AGS exhibited distinct shifts at both phylum and genus levels, reflecting the influence of operational conditions, particularly the transition from HS to LS wastewater and the aerobic P starvation strategy (**Figure 5.11**).

During HS wastewater treatment, Proteobacteria ($34.44 \pm 6.19\%$) and Bacteroidota ($17.67 \pm 22.24\%$) dominated in Rc, consistent with their known tolerance for high organic loads (Thomas et al., 2022). Although some variation was present, fluctuations at the phylum level in R3 were broadly comparable to those in Rc. Statistical tests for stability were not performed due to limited sample size; therefore, interpretations are based on descriptive observations of the available data. Specifically, R3 exhibited a notable increase in Acidobacteria (from 3.76 to 21.59%) and a decrease in Proteobacteria (from 58.65 to 21.96%) compared to Rc (**Figure 5.11-a**). Acidobacteria, typically associated with low-nutrient or acidic environments (Kielak et al., 2016), may have been favored by localized microenvironmental changes in granules, despite the reactor's near-neutral pH (7.8–8.1). The community patterns suggest that fluctuations at the phylum level occurred even under differing operational conditions, including aerobic P starvation and effluent recycling.



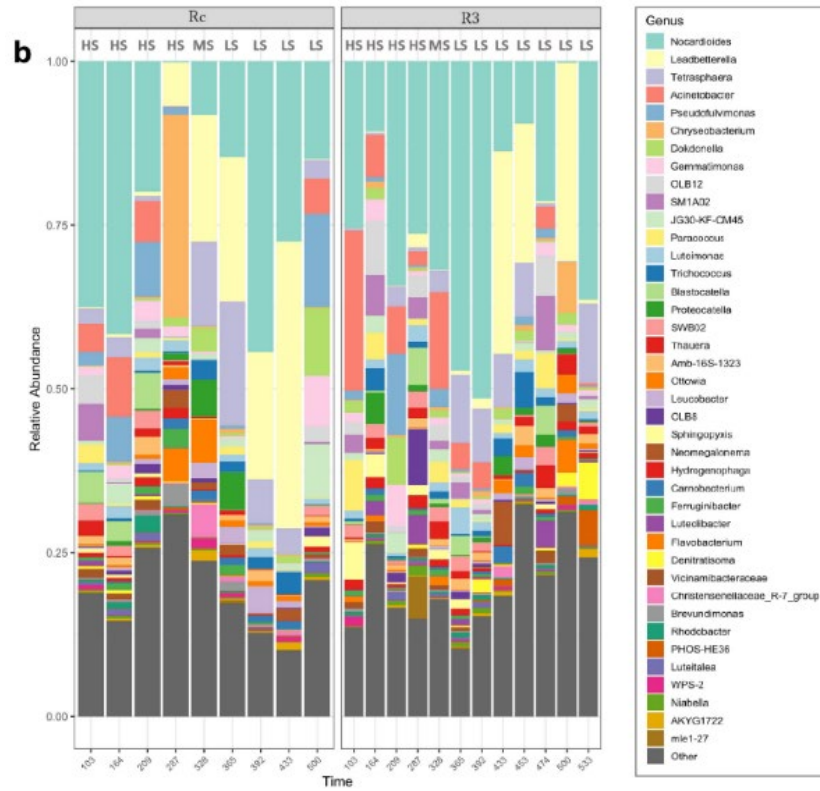


Figure 5.11: Distribution of dominant microbial diversity (relative abundance) at; (a) Phylum, and (b) Genus levels in Rc and R3

When transitioned to LS wastewater (days 345–545), granule disintegration occurred in both reactors starting around day 350, followed by recovery after day 450 (**Figure 5.5-b**). During this period, Acidobacteria abundance sharply declined in both systems (**Figure 5.11-a**), likely due to their reduced fitness after loss of favorable microenvironments due to disintegration. Concurrently, Proteobacteria, Actinobacteria, Bacteroidota, and Chloroflexi became dominant (**Figure 5.11-a**), all of which are recognized for their roles in biofilm and granule formation (You et al., 2025 and Zhang et al., 2025). At the genus level, *Leadbetterella* (Bacteroidota) emerged as a dominant taxon in both R3 and Rc following LS wastewater application (**Figure 5.11-b**). Bacteroidota's

role in granulation is well-documented (Zhang et al., 2025), and *Leadbetterella*'s prominence likely contributed to reggranulation post-disintegration, as supported by prior AGS studies (Mady et al., 2024). The decline in Proteobacteria, particularly *Nocardioides*, which excels at degrading HS wastewater (Ma et al., 2023), coincided with Bacteroidota's rise, reflecting competitive dynamics driven by reduced organic loading.

Diversity analyses provided deeper insights into these microbial dynamics (**Figure 5.12**). The Shannon diversity index, a measure of species richness and evenness, mirrored operational shifts and granule properties (**Figure 5.12-d**). During granule disintegration (days 350–450), the Shannon index decreased, reflecting reduced microbial diversity. Following granule recovery (after day 450), diversity improved, with R3 generally maintaining higher values (5.43 ± 0.57) than Rc (5.01 ± 0.82) under LS conditions. These suggests that R3's microbial community diversity was less perturbed by the shift to LS wastewater, likely due to the stabilizing effect of effluent reuse.

Principal coordinate analysis (PCoA) based on Bray–Curtis, weighted UniFrac, and Jaccard metrics provided additional insight into community dynamics (**Figure 5.12(a–c)**). Bray–Curtis reflects differences in relative abundances, weighted UniFrac incorporates phylogenetic relatedness and abundance, and Jaccard considers only species presence or absence. Across all metrics, Rc showed a stronger separation than R3, indicating greater sensitivity to feed changes. In Rc, a distinct shift in community composition followed the LS transition (**Figure 5.12-a**), whereas R3 exhibited greater community plasticity, defined as the ability of microbial communities to adapt their structure and function in response to changing environmental conditions, resulting in more

gradual, diffuse shifts in Bray–Curtis and UniFrac spaces. Jaccard-based PCoA also revealed two well-separated clusters in Rc corresponding to HS and LS phases, while R3 formed overlapping clusters, suggesting continuity in species turnover (**Figure 5.12-c**). This plasticity in R3 likely resulted from effluent reuse, which reintroduced residual taxa and buffered the nutrient regime, enabling smoother adaptation to operational changes.

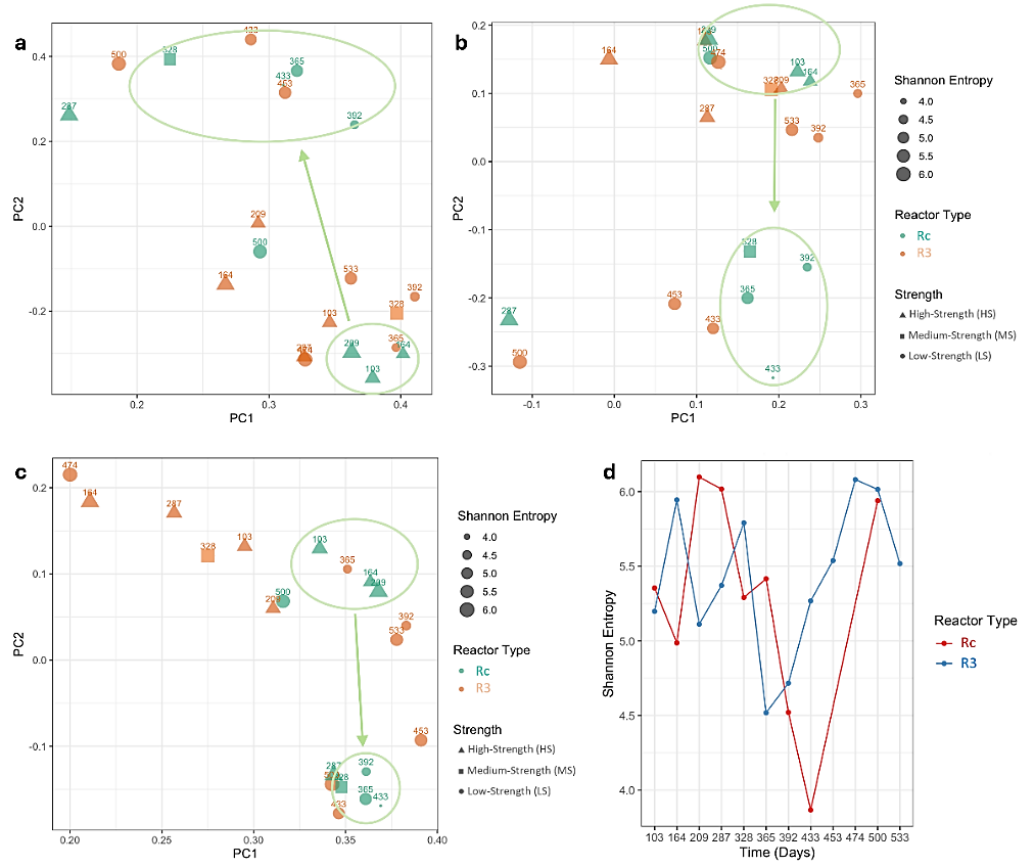


Figure 5.12: Microbial community dynamics in AGS for both systems: (a–c) Principal coordinate analysis based on (a) Bray-Curtis, (b) weighted UniFrac, and (c) Jaccard distances, illustrating microbial community shifts. (d) Shannon diversity index

5.3.4.2. Functional potential of the reactors

The enhanced nutrient removal in R3, particularly for phosphorus, was associated with a higher relative abundance of PAOs. Taxa such as *Tetrasphaera*, *Thauera*, *Acinetobacter*, *Paracoccus*, *Pseudoxanthomonas*, *PHOS-HE36*, *Acidovorax*, *Gemmatimonas*, and *Microthrix* were more abundant in R3 ($15.23 \pm 5.72\%$) than in Rc ($12.48 \pm 4.54\%$) (**Fig. C4 – Appendix C**). These PAOs, known for their roles in EBPR (Zhang et al., 2025, Wang et al., 2018a, Medhi and Thakur, 2018, Sun et al., 2021, Kawakoshi et al., 2012, Nielsen et al., 2002), likely drove R3's superior P uptake rates (**Section 5.3.2.2**).

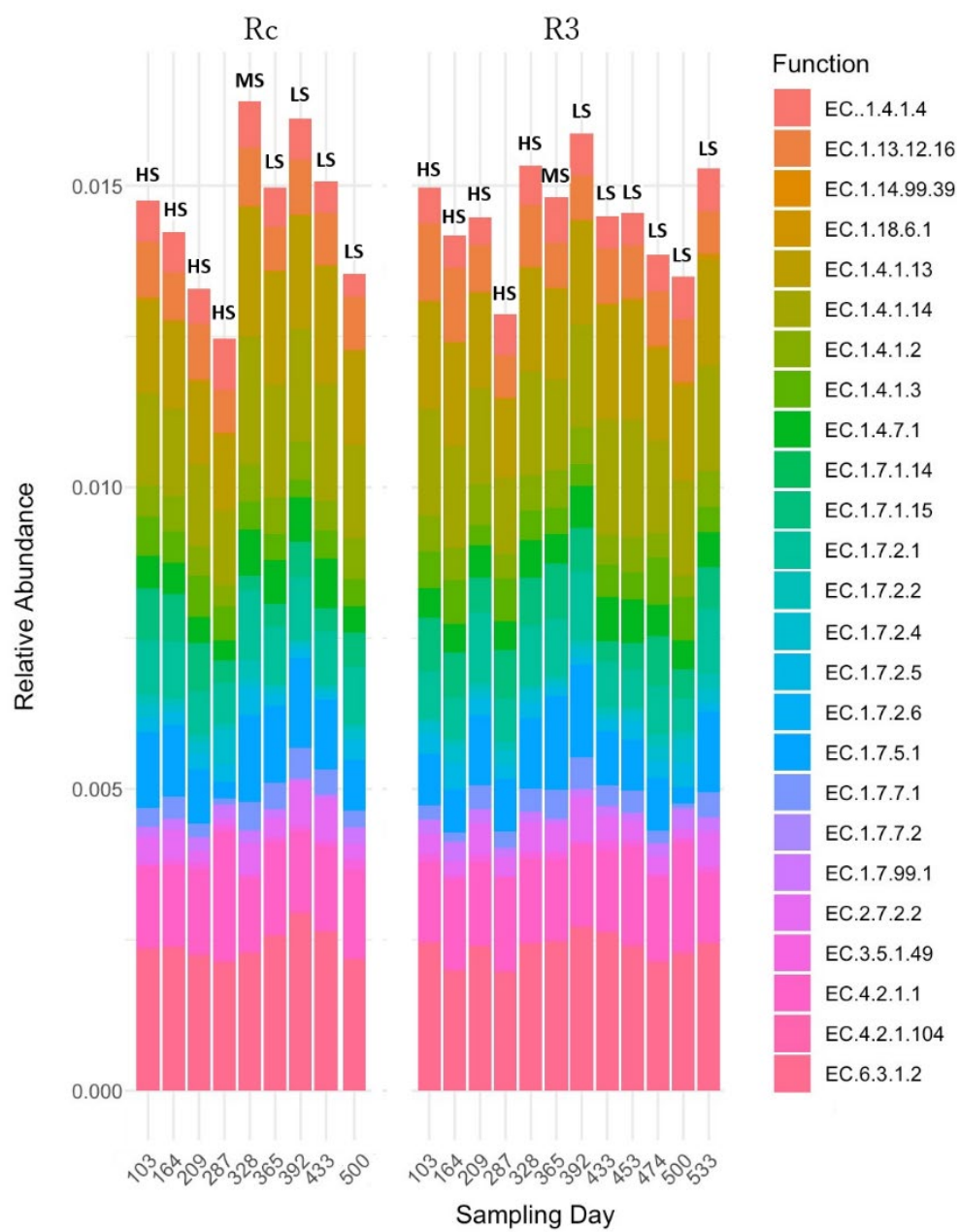
Notably, *Tetrasphaera* and *Thauera* are key PAOs in EBPR systems, with *Thauera* also contributing to denitrification (Sun et al., 2021). *Candidatus Accumulibacter*, a frequently identified PAO, was absent from the reactors, suggesting that HS wastewater may inhibit its proliferation. However, overall P removal may still remain high (up to 97.5% efficiency) if other PAOs like *Tetrasphaera*, which utilize more complex carbon sources, compensate for the reduced *Accumulibacter* activity (Feng et al., 2022).

Figure 5.12 should be interpreted as a comparison of the PICRUSt2-inferred functional potential for EBPR-relevant phosphorus cycling across sampling days in Rc and R3. Each stacked bar represents a sampling day, where the total bar height reflects the predicted cumulative relative abundance of the selected P-related functions, and each colored segment indicates the contribution of an individual gene/function (e.g., *pho* regulon associated with P-stress sensing/regulation, *pit*/PHO84 for phosphate transport, and *ppk/ppx/ppnP/ppgK* for poly-P synthesis and turnover). The close similarity in the stacked profiles between reactors and across time indicates apparent functional redundancy,

meaning that both communities retained comparable genetic capacity for phosphate transport and poly-P metabolism even when taxonomic composition shifted.

Importantly, this functional consistency was observed despite pronounced fluctuations in microbial diversity, emphasizing that reactor performance cannot be inferred from taxonomy alone. Instead, the results highlight the importance of physiological traits and in situ activity (e.g., carbon storage capacity such as PHA accumulation, effective P uptake kinetics, and denitrification/SND capability) in governing EBPR stability and nutrient removal, even when the specific taxa contributing to these functions vary over time.

Metabolic profiles, inferred from community composition (**Figure 5.13**) using PICRUST2 (Douglas et al., 2020), showed no stark differences between reactors, suggesting functional redundancy across microbial taxa. However, R3's greater community plasticity enabled it to refresh key populations, such as PAOs, sustaining robust nutrient removal. For instance, higher PHA accumulation in R3 (1.65 compared with 1.31% in Rc; **Section 5.3.2.3**) indicates enhanced carbon storage, critical for PAO activity under P-limited conditions. This metabolic advantage, likely driven by intermittent P starvation and effluent reuse, supported faster P uptake (1.33 mg-P/g-MLVSS·h in R3, while 0.74 mg-P/g-MLVSS·h in Rc) and maintained EBPR stability. These findings underscore the aerobic P starvation strategy's role in fostering a resilient microbial community, capable of adapting to fluctuating wastewater conditions while enhancing nutrient removal efficiency.



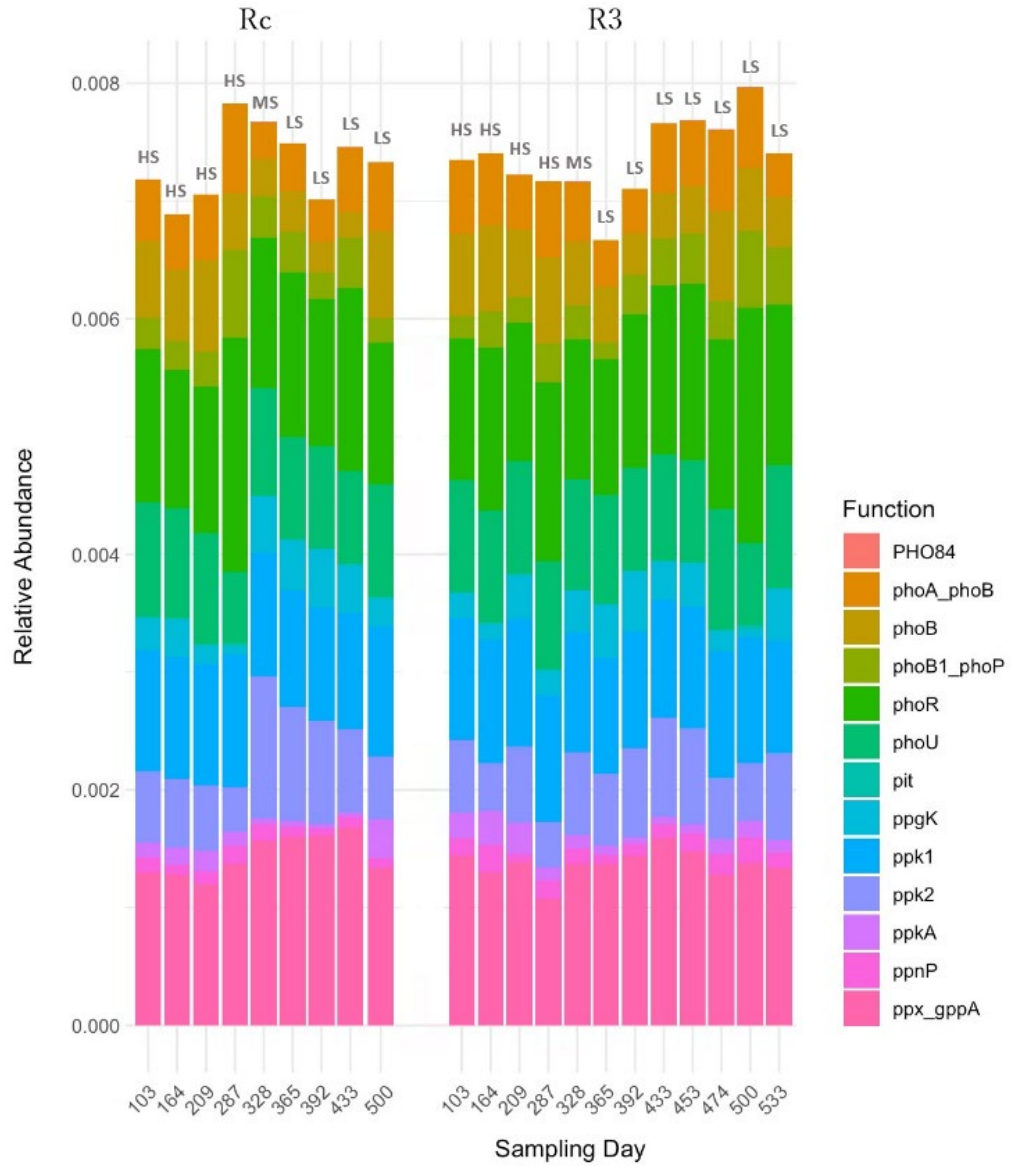


Figure 5.13: Metabolic profiles of aerobic granular sludge in reactors R3 and Rc under HS and LS wastewater conditions: (a) N and (b) P metabolism

5.3.5. P-rich liquid characteristics and phosphorus recovery potential

The primary aim of this study was to optimize supernatant extraction under no-mixing conditions at the end of the anaerobic stage, focusing on P-removal performance and granule stability, with a secondary objective of evaluating the potential of extracted P-

rich liquid as a resource for P recovery. Thus, the P-rich liquid samples collected during LS influent treatment were subsequently analyzed for their recovery potential. The anaerobic phase preserved most influent ammonium while releasing P, producing a nutrient-rich supernatant highly suitable for struvite-based recovery (**Fig. C5. – Appendix C**). In batch experiments, the extracted P-rich liquid samples typically contained 26–32 mg PO_4^{3-} -P/L and 37 ± 3.4 mg NH_4^+ -N/L. Four Mg:P molar ratios (1.0, 1.3, 1.6, and 2.0) were investigated to evaluate the efficiency of P recovery via magnesium-induced struvite precipitation (**Figure 5.14**). P recovery was successfully achieved through magnesium-induced struvite precipitation, with efficiencies exceeding $68 \pm 2.1\%$ at a Mg:P molar ratio of 2 and pH 9, conditions recognized as optimal for struvite crystallization.

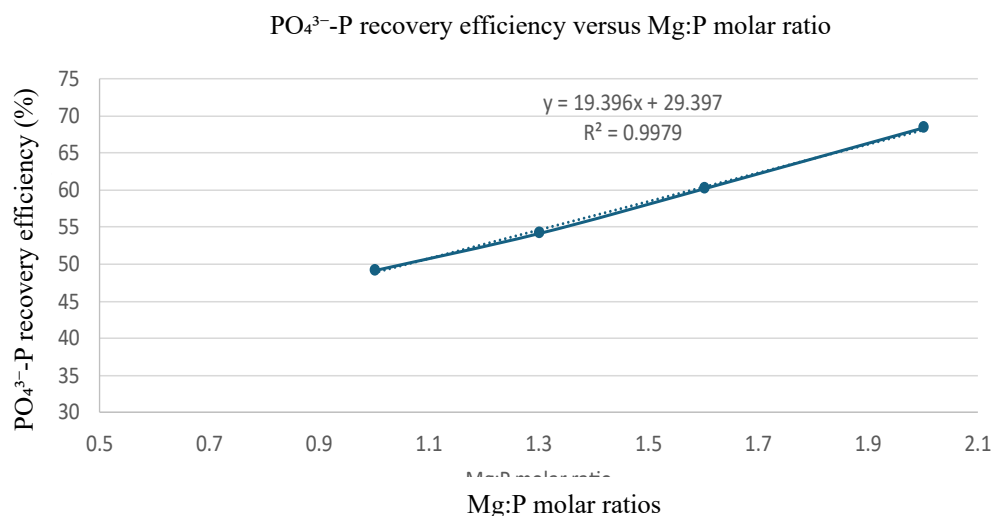


Figure 5.14. Relationship between P recovery efficiency from the P-rich liquids extracted from R1 and the Mg:P molar ratios. The P recovery experiments lasted for 2 h.

Notably, this pH was reached naturally after 20 min of CO_2 stripping with aeration, without external alkaline dosing and thereby reducing operational costs. As shown in

Figure 5.14 strong linear correlation ($R^2 = 0.998$) between the Mg:P molar ratio and P recovery efficiency was observed, confirming the predictability and controllability of the process under the tested conditions. Collectively, these results highlight not only the operational simplicity and cost-effectiveness of this recovery method but also its potential for seamless integration with AGS-based EBPR systems, offering a practical pathway for sustainable P recycling.

5.3.6. Practical implications, optimization windows, and scale-up considerations

Aerobic P starvation induced by intermittent P-rich liquid extraction has strong practical value as a selective pressure to accelerate aerobic P-uptake kinetics and maintain stable, low effluent $\text{PO}_4^{3-}\text{-P}$ under variable influent P loadings (seasonal shifts, municipal variability, and episodic P spikes). In practice, the strategy can be used as an operational lever to strengthen EBPR “polishing” without chemical dosing, because short, targeted starvation periods can reinforce PAO selection and bioactivity and improve process resilience during changing nutrient loads.

At the same time, the approach is sensitive to extraction severity; excessive or continuous extraction should be avoided to prevent gradual depletion of PAO intracellular reserves (notably poly-P) and loss of EBPR stability. Scale-up therefore requires safeguards in the extraction step: adequate settling before extraction, a controlled extraction duration and flow rate that does not disturb the sludge bed, and high-reliability, well-calibrated separation/pumping equipment to minimize unintended solids withdrawal with the extracted P-rich liquid and preserve biomass concentration over time. Full-scale implementation is most feasible as a side-stream EBPR control step integrated with existing

solids–liquid separation infrastructure (e.g., secondary clarifiers, selector zones, or dedicated side-stream tanks), with extraction setpoints tied to routine plant signals (influent/effluent $\text{PO}_4\text{-P}$, COD:P ratio, solids loading/effluent TSS) and clear rules to reduce or suspend extraction during low-carbon periods or when solids losses increase. Beyond improving mainline effluent quality, the concentrated P-rich sidestream also supports P recovery (e.g., struvite crystallization), enabling a combined compliance and resource-recovery upgrade; successful adoption depends on commissioning with a conservative extraction window, validating under seasonal variability, and periodically re-optimizing extraction volume as influent characteristics change.

CHAPTER 6: PHOSPHORUS-RICH LIQUID EXTRACTION FROM AEROBIC GRANULAR SLUDGE FOR ENHANCED PHOSPHORUS REMOVAL IN MUNICIPAL WASTEWATER SBRs

EBPR is a widely applied and cost-effective strategy for P removal from wastewater. However, maintaining consistently low effluent P levels remains challenging due to process fluctuations. This study evaluates two aerobic P starvation-based operational strategies, intermittent and continuous extraction of P-rich liquid at the end of the anaerobic phase, using AGS, to enhance EBPR performance in municipal wastewater treatment while preserving granule stability. Three SBRs were operated for 127 days with an 8-hour cycle: R1 with intermittent P-rich liquid extraction, R2 with continuous extraction, and a control (Rc). Results showed R1 had the highest P-uptake rate ($1.42 \text{ mg P/g MLVSS}\cdot\text{h}$), outperforming R2 and Rc (0.86 and $0.71 \text{ mg P/g MLVSS}\cdot\text{h}$). While the extraction strategies affected granule stability, R1 successfully reggranulated during the latter half of operation, whereas R2 granules continued to disintegrate. Additionally, R1 achieved removal efficiencies of 99% sCOD, 92% ammonium, and 90% orthophosphate, outperforming R2 and Rc. The P-rich liquid extracted from R1 demonstrated high potential for P recovery via struvite precipitation. Sludge samples from R1 at the end of operation had the highest bacterial richness and diversity. Overall, intermittent aerobic P starvation effectively safeguarded granules structural integrity while enhancing EBPR sustainability under municipal wastewater conditions. This approach achieved consistently low effluent P and

opened pathways for resource recovery, underscoring its value for future circular wastewater treatment strategies.

6.1. Introduction

P is an essential nutrient for plant growth and global food production, yet it is a non-renewable resource primarily derived from finite phosphate rock reserves. The steadily growing demand for P has raised long-standing concerns about the depletion of global P reserves (Reijnders, 2014). With P availability expected to decline sharply in the coming decades, the potential impact on global food security is significant. At the same time, wastewater generated from human activities represents a largely untapped resource, offering the potential to recover 15-20% of the global P demand through innovative treatment technologies (Yuan et al., 2012). While recent decades have advanced the optimization of biological P removal, the pressing next frontier is to seamlessly integrate efficient P recovery into these systems to ensure long-term sustainability and resource security (Chowdhury et al., 2017, Cieřlik and Konieczka, 2017, Mehta et al., 2015). Furthermore, recovering P from municipal wastewater provides a strategic pathway to conserve this critical resource while enabling sustainable fertilizer production and supporting long-term agricultural and phosphorus management (Ribarova et al., 2017 and Liu et al., 2023).

Releasing P from WWTPs without proper removal threatens the environment, leading to eutrophication and disrupting natural nutrient cycles. Recent studies have focused on optimizing biological P removal, with the next key step being the implementation of P recovery strategies (Yuan et al., 2012, Chowdhury et al., 2017, Cieřlik and Konieczka, 2017, Ribarova et al., 2017). EBPR systems provide a cost-effective and

sustainable alternative to chemical treatment in wastewater processes. This method relies on PAOs, which release P under anaerobic conditions and subsequently take up excess P from wastewater under aerobic conditions, a phenomenon known as luxury uptake. Under anaerobic conditions, without an electron acceptor (e.g., oxygen or nitrate), PAOs utilize stored polyphosphate and glycogen for energy, causing P release into the liquid phase. In the subsequent aerobic phase, PAOs consume stored compounds while reabsorbing P, promoting their growth and enabling PHAs accumulation through anaerobic-aerobic cycling (Cha et al., 2016 and Metcalf & Eddy et al., 2014).

In recent decades, AGS has emerged as a promising alternative to CAS due to its compact structure, resilience, and high BNR efficiency, where it forms dense microbial aggregates that enable the simultaneous removal of organic matter and nutrients within a single reactor, enhancing treatment efficiency (Thwaites et al., 2018). Compared to the AS system, aerobic granulation technology reduces operational costs by 20–25%, lowers electricity consumption by 23–40%, and decreases space requirements by 50–75% (Nereda, 2017 and Bengtsson et al., 2019). Additionally, when compared to other compact treatment options like membrane bioreactors, the AGS process consumes approximately 35–70% less electricity, therefore the benefits of operational simplicity, process flexibility, and cost-effectiveness have significantly driven the broader implementation of AGS technology in domestic WWTPs (Bengtsson et al., 2019). Oxygen gradients within the granule create distinct microenvironments, fostering microbial diversity. Typically, AGS forms three layers: an outer aerobic zone, a middle layer with low oxygen, and an anoxic or anaerobic core, enabling simultaneous biological processes (Nancharaiah and Reddy, 2018). Previous studies have revealed that there are two main mechanisms for P removal

in the treatment systems using aerobic granulation technology; (a) EBPR and (b) P precipitation within the granular sludge matrix (Mañas et al., 2011, Filali et al., 2012, Henriët et al., 2016, Sarma and Tay, 2018). Aerobic granulation technology enables efficient simultaneous BNR across wastewater types, from low-strength municipal influents to high-strength wastewaters and landfill leachate (Lashkarizadeh et al., 2015, Ren and Yuan, 2016, Mady et al., 2024).

Several studies have highlighted stability challenges in EBPR performance, especially in treating low-strength municipal wastewater with limited VFAs. This issue can be addressed by introducing additional VFAs through internal fermentation, fermented sludge, or external sources like acetate (Bassin et al., 2012 and Barnard and Abraham, 2006). However, maintaining low and stable P levels (<1 mg/L) remains difficult due to microbial activity fluctuations, often requiring chemical phosphate precipitation for consistent P removal (Gebremariam et al., 2011). Although the recent modifications which have been proposed to EBPR systems, most of them focused on enhancing P recovery, particularly through struvite precipitation from anaerobically released P. For example, Baeza et al. (2017) introduced the EBPR2 system, an SBR configuration with an anaerobic/settling/aerobic/settling sequence, allowing for P-rich liquid extraction (4%) for chemical precipitation. Simulations showed over 60% P recovery while meeting regulatory limits, with lower COD demands than conventional EBPR-based SBRs. Shi et al. (2012) developed the anaerobic-anoxic-nitrifying induced crystallization (A2N-IC) process, an adaptation of the anaerobic/anoxic/aerobic (A2O) system, which combines A2N with chemical P precipitation from a side stream after the anaerobic stage. This recovered P as

hydroxyapatite, reducing the plant's need for conventional P removal by chemically extracting some influent P from the mainstream.

Enhancing the rate of P removal offers a competitive advantage over conventional EBPR by stimulating PAO metabolism and increasing aerobic P uptake. One promising approach is to impose aerobic P starvation by partially extracting P-rich liquid at the end of the anaerobic phase. However, systematic studies on such P starvation strategies via selective liquid extraction remain insufficiently studied and largely unexplored, particularly regarding their long-term effects on AGS stability and function in municipal wastewater treatment, as most modifications of EBPR-based systems have primarily targeted P recovery rather than process stability (Guisasola et al., 2019, He et al., 2023, Fan et al., 2026, Zhang et al., 2022). The dense structure of mature granules is well suited to this approach, as they facilitate rapid phase separation and direct extraction of P-rich liquid under static anaerobic conditions. A previous study demonstrated that P-rich liquid extraction from bacterial and algal–bacterial AGS-SBRs could reduce effluent P to <0.05 mg P/L while maintaining $>99\%$ sCOD and NH_4^+ -N removal, however the work was limited to a short 10-day operation (40 cycles) and did not evaluate granule stability, EPS dynamics, or long-term applicability (Wang et al., 2022). In particular, the feasibility of using AGS cultivated under high organic loading for P-rich liquid extraction during subsequent municipal wastewater treatment, and the comparative performance of intermittent versus continuous extraction regimes at similar daily extraction ratios, remain unexplored. Therefore, this study aims to (i) apply aerobic P starvation via P-rich liquid extraction to AGS previously cultivated with high-strength wastewater and assess its effectiveness for P removal in municipal wastewater treatment, (ii) compare intermittent

and continuous extraction strategies in terms of EBPR performance, granule stability, regranulation, and EPS secretion over 127 days of operation, and (iii) evaluate the potential of the extracted P-rich liquid for P recovery and its implications for AGS-based EBPR process design.

6.2. Material and Methods

6.2.1. Experimental Cultivation of AGS in the Mother SBRs

Mature AGS used in this chapter was cultivated in the mother AGS-SBRs as previously described in Section 5.2.1. Mother SBRs (cultivation and maturation of AGS) (Chapter 5). Briefly, the mother SBRs (5.0 L working volume) were operated long-term under high OLR to produce stable granules (>2.5 mm). Prior to initiating the current study, the reactors were operated for two months under medium-strength wastewater (TCOD = 750 ± 25 mg/L) as a transitional phase without P-rich liquid extraction before switching to low-strength municipal influent.

6.2.2. Lab-Scale Test Reactors

6.2.2.1 Experimental setup and influent composition

Before commencing the current study, 1.6 L of AGS from one of the mother reactors was pooled and evenly distributed into two smaller test SBRs (R1 and R2; 800 mL each - **Figure 6.1** and **6.2**), each operating with a working volume of 2 L and retained the same VER of 60% as the mother reactors (i.e., 1.2 L of low-strength influent; actual OLR = 0.684 ± 0.06 kg COD/m³·d). R1 and R2 also operated under the same configuration and sequence of operational stages as the mother reactors, as detailed in the previous section

(2.1. Experimental Cultivation of AGS in the Mother SBRs). **Appendix D (Fig. D1)** presents images of the mother SBR and test AGS-SBRs during operation. Biomass wastage was limited to effluent solids during the decant stage (i.e., SRT was not controlled). Peristaltic pumps were used for influent feeding and effluent discharge, while cycle automation was controlled via Traceable Digital Outlet Controllers (Fisher Scientific, CA). As in the mother reactors, the test SBRs employed the same aeration technique and maintained a similar DO levels. Nitrogen gas was introduced through a 3 cm spherical stone diffuser to ensure complete mixing, maintain DO levels below 0.1 mg/L for an anoxic environment, prevent biomass sedimentation, and enhance removal efficiency by improving cell-nutrient interaction (**Figure 6.1**).

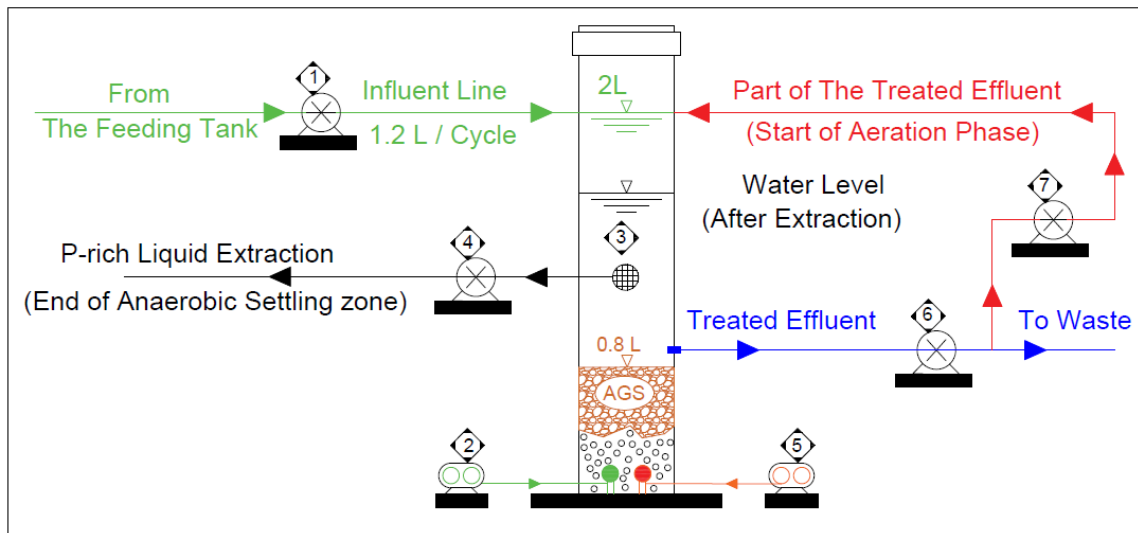


Figure. 6.1: The experimental set-up: (a) Schematic diagram of the test SBRs in this study; (1) feeding pump; (2) nitrogen pump; (3) porous stone; (4) extraction pump; (5) air compressor; (6) decant pump; and (7) returning feeding pump

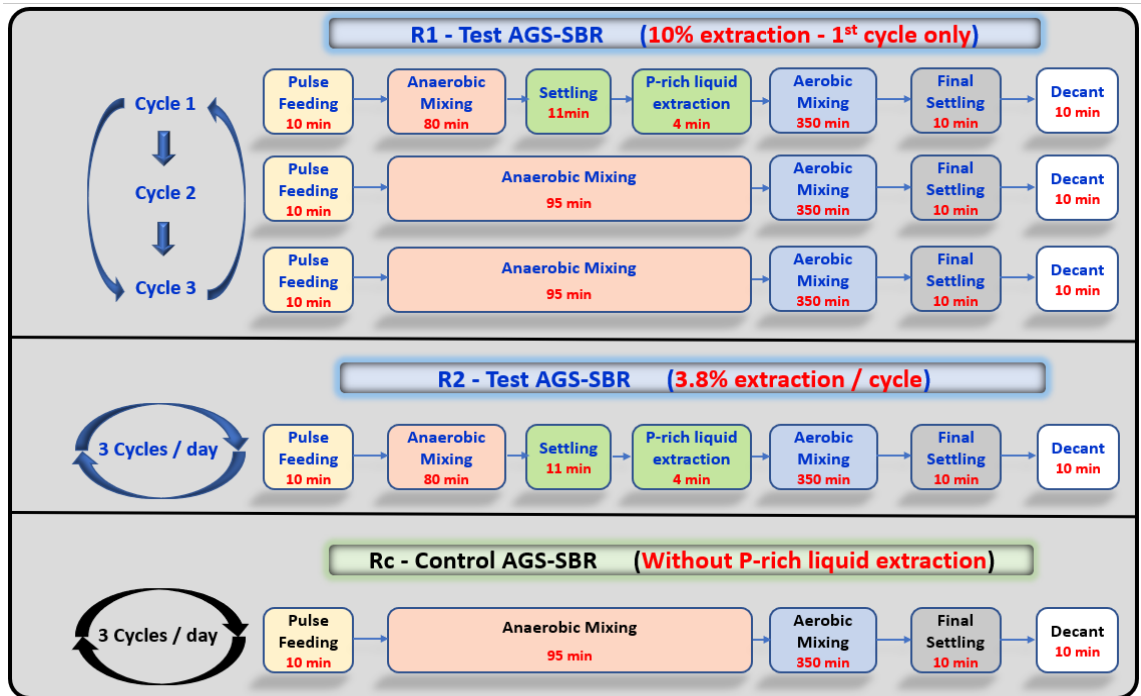


Figure. 6.2: Schematic diagram illustrating the operational cycles and the different P-liquid extraction strategies applied in R1 and R2

After that, all AGS-SBRs were operated for two months treating medium-strength wastewater as a transitional phase, before starting the current study (low-strength influents treatment). The synthetic wastewater composition was strategically adjusted by modifying the ratios of yeast extract, ammonium chloride, and dipotassium phosphate to achieve controlled variations in TCOD, sCOD, TN, TAN, TP, and Soluble P (SP), as shown in **Table 6.1** based on a previous study (Kang and Yuan, 2017). Additional components included 5.8 mg/L MgSO_4 , 12 mg/L CaCl_2 , and 0.3 mg/L mineral solution comprising 0.15 g/L H_3BO_3 , 0.03 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.03 g/L KI, 0.12 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.06 g/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.12 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g/L $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, and 1.5 g/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$.

Table 6.1: Influent characteristics and operation parameters

Parameter/Component (mg/L)	Low-strength Wastewater
Yeast extract	380
NH ₄ Cl	29
K ₂ HPO ₄ ·3H ₂ O	17
TCOD	425
sCOD	380
TN	47.5
TAN	35
TP	8
Soluble P (SP)	3.72
Hydraulic retention time (hour)	13.33
pH	8.1 (anoxic reaction phase) 7.8 (aerobic reaction phase)

Furthermore, 3 mg/L of ethylenediaminetetraacetic acid was added to prevent precipitation of salts (e.g., Ca²⁺ and PO₄³⁻-P) present in the medium. Sodium bicarbonate (12 g NaHCO₃ per g NH₄⁺-N) was dosed in the influent at each cycle throughout the experiment to ensure sufficient alkalinity (7.14 g CaCO₃ per g NH₄⁺-N) to support complete nitrification. Synthetic wastewater was prepared every two days and stored at 4 °C. Before each reactor cycle, 6.0 L of feed was distributed into five mixing tanks, equipped with magnetic stirrers to ensure thorough homogenization prior to feeding at room temperature.

6.2.2.2 P-rich liquid extraction

As shown in **Figure 6.1 (b)**, the 8-hour treatment cycle for R1 and R2 was modified by introducing an additional settling stage after the anaerobic mixing phase, resulting in the following sequence: pulse feeding (10 min) / anaerobic mixing (85 min) / settling (15

min) / aerobic mixing (350 min) / final settling (5 min) / decant (12 min) / idle (3 min). Additionally, while the same configuration was applied to Rc, including the new settling stage before the aerobic mixing phase, P-rich liquid extraction was not implemented.

Based on experimental investigations conducted using a series of AGS-SBRs operated under different intermittent P-rich liquid extraction ratios (5%, 10%, and 14%), to be further elaborated in our forthcoming studies, the 10% extraction ratio yielded the most favorable outcomes, achieving superior phosphorus removal efficiency and enhanced granule stability. In R1, 10 % of the total reactor volume (200 mL) of P-rich liquid was extracted only during the first treatment cycle, whereas R2 operated with continuous P-rich extraction of 76 mL/cycle, resulting in a daily extraction of 11.4 % (228 mL) throughout the entire operational period (**Figure 6.1-b**). To achieve this, the study adopted strategic solutions to address some operational challenges associated with implementing this separation approach in AGS-SBRs. For instance, direct extraction of P-rich liquid from the bottom of AGS-SBRs under no mixing conditions was avoided to prevent disruptions to sludge characteristics (e.g., MLSS and SRTs). Instead, P-rich liquid was extracted from the upper liquid phase, at the middle point in the liquid above the settled AGS, using a porous stone separator as shown in **Figure 6.1 (Fig. D2; Appendix D)**. Additionally, continuous P starvation mode for all treatment cycles could cause potential stress through affecting PAOs bioactive as reported in the literature (Zhang et al., 2022). Thus, by alternating one cycle with P-rich extraction and two recovery cycles without extraction, this operating strategy was proposed in this study to effectively to potentially reserve the long-term stability of mature granules and PAOs bioactivity. In addition, P-rich liquid was extracted at the last 4 min in the additional settling stage, through a flow rate of 50 mL/min for R1

and 19 mL/min for R2. In order to maintain the original water level inside the SBRs, adding same volume of treated wastewater from the previous treatment cycle at the beginning of the aeration mixing stage was performed, thus fixed VER was applied during the operation.

6.2.3. Sampling, Analytical Methods and Process Parameters Studied

A 14-day adaptation period was implemented after introducing P-rich extraction in the test SBRs. Throughout the experiment, influent and effluent samples were routinely collected daily for physico-chemical analysis to evaluate various removal performance parameters. Samples were collected and filtered using medium-porosity Q5 filter paper (0.45 μm , Fisher Scientific, CA). Standard analytical methods were employed to measure MLSS, MLVSS, TSS_{eff} , and sCOD (APHA/AWWA/WEF, 2012). N forms ($\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, $\text{NO}_3^-\text{-N}$) and SP (orthophosphate: $\text{PO}_4^{3-}\text{-P}$) were measured using a flow injection analyzer (Lachat Quick Chem 8500, HACH, CA). Additionally, DO and pH levels were monitored using advanced electrodes positioned at the reactor's central zone. Biomass characterization of granular sludge involved assessing SVI_5 and SVI_{30} , and EPS secretion, while microscopic imaging (SteREO Discovery) monitored granulation and granule stability, with size measurements performed using the Axio Vert.A1 microscope (ZEISS) and weekly surface examinations conducted to track structural and morphological changes.

In this study, the SRE was used as a performance indicator for the operation of the test SBRs, and was estimated by the following equation (eq. 6.1). This parameter quantifies the system's net P removal efficiency relative to the biomass concentration (MLVSS) within the reactor, providing insight into the PAOs activity in the sludge. It should be noted

that all measurements were performed in triplicate, and data are presented as mean $\pm$ standard error of the mean (SEM).

$$SRE = \frac{P_{Inf} - P_{Eff}}{MLVSS \times V_{SBR}} \quad \text{(Equation 6.1)}$$

The EPS content (protein (PN) + carbohydrate (PS)) was extracted as outlined in (Adav et al., 2008), and as explained in the previous chapters. PN and PS contents were subsequently quantified using the phenol–sulphuric acid method (Ren, 2015), and the modified Lowry assay (Thermo Fisher Scientific, USA), respectively.

In this study, P recovery from P-rich liquid was investigated using chemical precipitation, specifically through struvite formation, a widely recognized and favorable technique due to its simultaneous removal of ammonium and the added benefit of producing struvite, a reusable and eco-friendly fertilizer (Le Corre et al., 2009). Notably, struvite recovery from municipal wastewater has already been successfully commercialized in several countries, highlighting its practical relevance (Le Corre et al., 2009 and Amann et al., 2018). To carry out the recovery tests, P-rich liquids were partially extracted at the end of settling zone following the anaerobic phase, diluted to prevent premature precipitation, and then stored in a refrigerator for later use. In each batch experiment, the collected P-rich liquid was supplemented with ions Mg^{+2} at varying molar ratios (Mg:P) to induce struvite formation under controlled pH conditions.

6.2.4. DNA Extraction and Bacterial Community Analyses

To gain deeper insight into the contribution of microbial composition to system performance, AGS samples were systematically collected from all reactors (Rc, R1, and

R2) at the conclusion of the operational period (day 127). Total community DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN, Venlo, Netherlands), following the manufacturer's protocol to ensure consistency and reliability in subsequent analyses. The DNA extraction procedure has been described in the previous chapter (Chapter 5: 5.2.4. DNA Extraction and Bacterial Community Analyses).

6.3. Results and Discussion

It should be noted that the seeding AGS (cultivated in mother SBRs) had stable properties with SVI_{30} of 28 ± 5 mL/g ($SVI_5/SVI_{30} \geq 0.95$) and granules' size ≥ 2.5 mm (average of triplicates samples) as shown in **Figure 6.3** and **Fig. D3 (Appendix D)**. Additionally, EPS content was achieved 239 ± 14 mg/gVSS (PN/PS ratio = 14.8). For the BNR performance, mother SBRs achieved simultaneous removal efficiencies for COD, NH_4^+-N , $PO_4^{3-}-P$ of 95 ± 0.5 %, 92.4 ± 1.1 %, and 90.1 ± 0.4 %, respectively, with SRT estimated of 55 ± 2 days. After this point, granules in the mother SBRs face an initial deterioration coincided with the feeding with medium-strength influents which could be attributed to the reduced OLR (1.35 ± 0.1 kg COD/m³·d) in this transitional stage. However, within 3 weeks, AGS-SBRs regained stability, with granules reaching a size of 2.1 mm ($SVI_{30} \leq 36$ mL/g), an MLSS concentration of 8.6 ± 0.2 g/L, and applied SRT of 46 ± 2 days. In summary, AGS exhibited a rapid adaptation to the reduction in influent sCOD. AGS-SBRs were successfully established and initiated with low-strength wastewater, and their performance were subsequently evaluated.

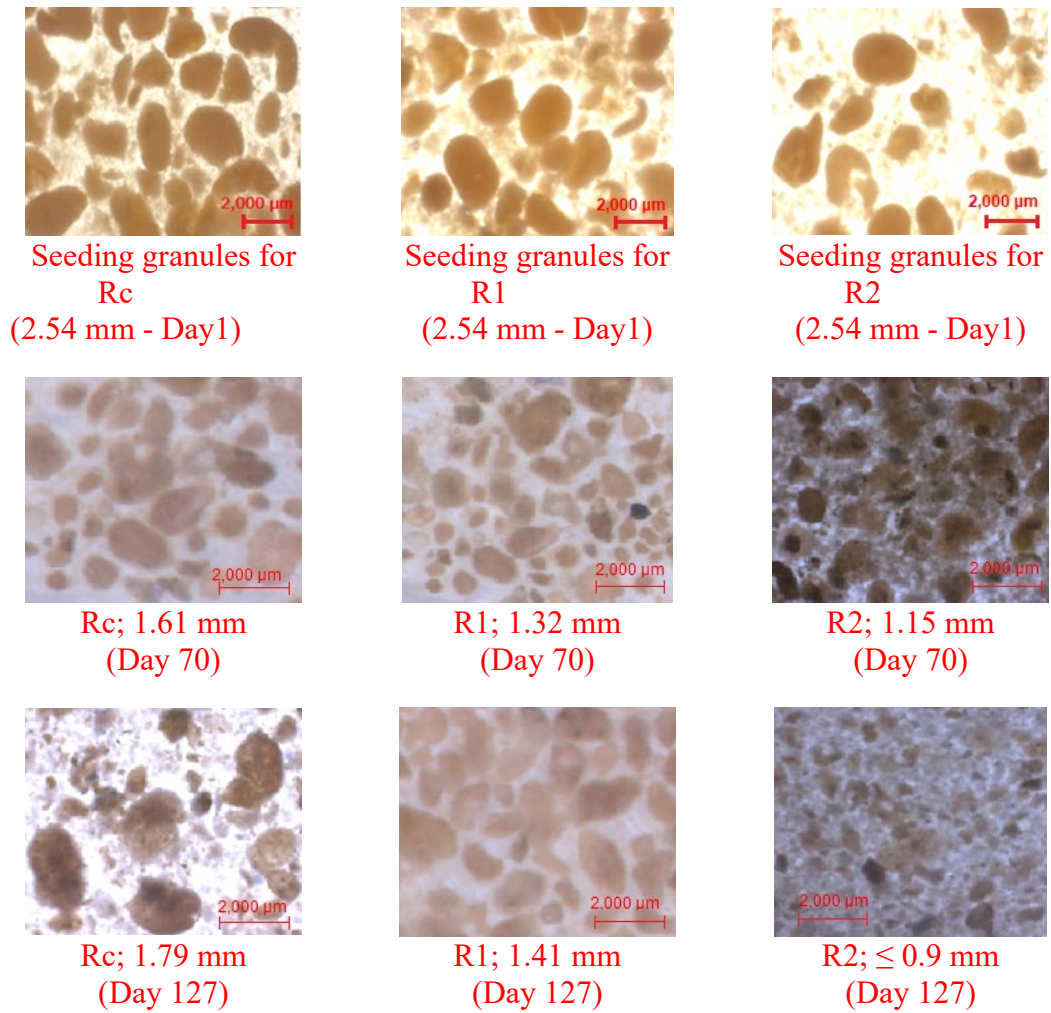


Figure 6.3. Variation in granule size in Rc, R1, and R2 over time (microscopic images between days 1 and 127)

6.3.1. Granule Properties

Both test SBRs showed a similar trend of MLSS reduction (**Figure 6.4 (a)**). By day 42, MLSS in R1 and R2 had decreased to 5.7 ± 0.2 g/L and 5.1 ± 0.11 g/L, respectively, accompanied with poor effluent quality ($\text{TSS}_{\text{eff}} \geq 110$ mg/L - **Figure 6.4-b**). Subsequently, MLSS in R2 continued to decline and stabilized at 3.5 g/L during the last month (days 100-127), resulting in an actual $\text{SRT} \leq 20$ days by the end of the experiment. In contrast,

biomass in R1 decreased until day 82, reaching 4.1 ± 0.21 g/L. Afterwards, a gradual increase was observed, with MLSS concentrations up to 5.2 g/L by day 127, and the actual SRT of 30 ± 1 days. During the last 50 days of operation, TSS_{eff} concentrations in R2 were ≥ 90 mg/L, higher than in R1 (70 mg/L). A modest enhancement in effluent quality occurred during the recovery cycles in R1, as the TSS_{eff} decreased by approximately 14% compared with the extraction cycle, indicating improved solids retention following the cessation of P-rich liquid extraction.

TSS concentrations in the extracted P-rich liquid from R1 averaged 22 ± 6 mg/L (**Figure 6.4(c)**). Conversely, TSS levels in R2 exhibited a continuous increase over time, despite the porous stone separator (**Fig. D2; Appendix D**) as shown in **Figure 6.4(c)**, achieving 38 ± 4 mg/L by day 127. These observations suggest that intermittent P-rich liquid extraction was more effective in preserving biomass concentration and granule structure integrity than continuous extraction, highlighting the stabilizing effect of recovery phases. Additionally, higher MLVSS/MLSS ratios were observed in the test AGS-SBRs (0.81 in R1 and 0.86 in R2) than Rc (0.77). It should be noted that application of the P-rich liquid extraction strategy enhanced MLVSS retention and mitigated mineral P accumulation within the granules, as reflected by increased MLVSS/MLSS ratios. However, this effect was more pronounced under continuous extraction conditions. R2 exhibited higher MLVSS/MLSS ratios, indicating lower inorganic content, the prolonged continuous extraction ultimately led to compromised granule stability (continuous disintegration). In a previous EBPR study focused on P recovery, Guisasola et al. (2019) reported that P-rich liquid extraction enhances MLVSS retention by decreasing mineral P accumulation and reducing granule density. Accordingly, the comparable results between R1 and Rc

emphasize the critical role of recovery cycles in preserving structural integrity under the intermittent extraction strategy.

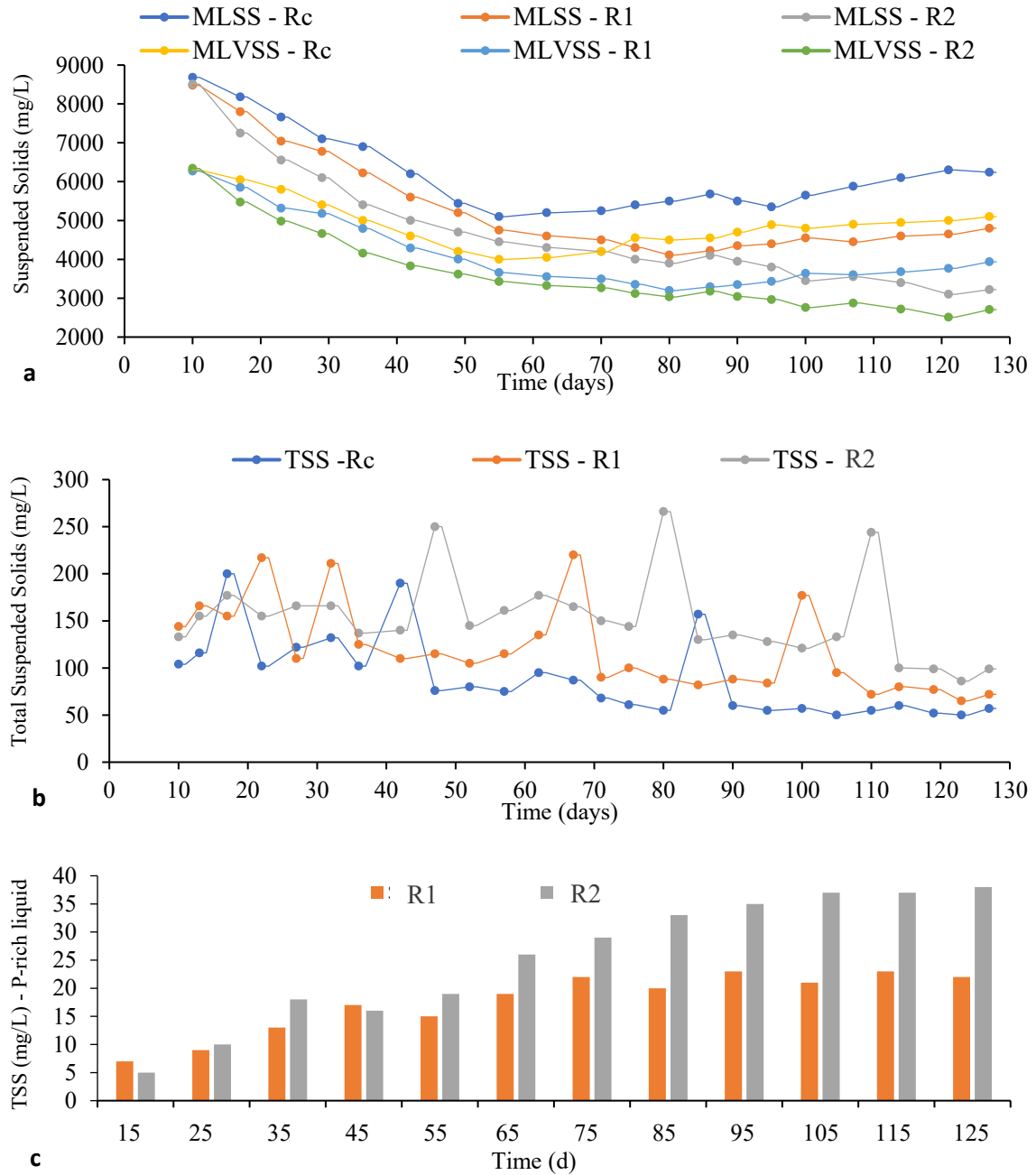
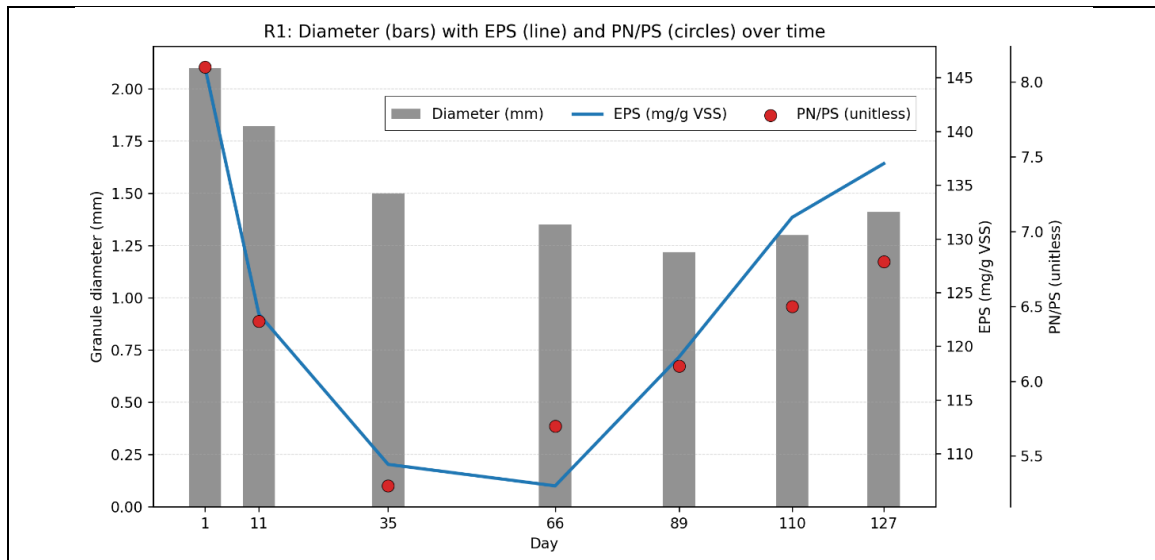


Figure 6.4: Solids content in R1, R2 and Rc over the entire operational period: (a) MLSS and MLVSS concentrations, (b) TSS_{eff}, and (c) TSS concentrations in the extracted P-rich liquid from R1 and R2

After feeding with low-strength wastewater, the OLR decreased to 0.684 kg COD/m³·d and the F/M ratio declined from 0.21 to 0.17 gCOD/gVSS·d, and a significant reduction in granule size was detected in the test SBRs compared with Rc (**Figure 6.5**). AGS disintegration in all SBRs was in agreement with the reduction in EPS secretions and reduced protein dominance (low PN/PS ratio). In Rc, MLSS decreased gradually through the first two months of operation (5.1 g/L - day 66), which was associated with a reduced size and EPS content (**Figure 6.5(c)**). Thereafter, no further reduction was observed, and the AGS remained stable, as indicated by the increase in EPS secretion and the PN/PS ratio. As expected, a higher F/M ratio developed due to the applied selective pressure, which promoted the washout of poorly settling biomass. This process supported successful regranulation (i.e., enhance EPS production and PN/PS ratio as shown in **Figure 6.5(c)**, and the biomass continued to grow, reaching 6.3 g/L by day 127 (**Figure 6.4-a**).



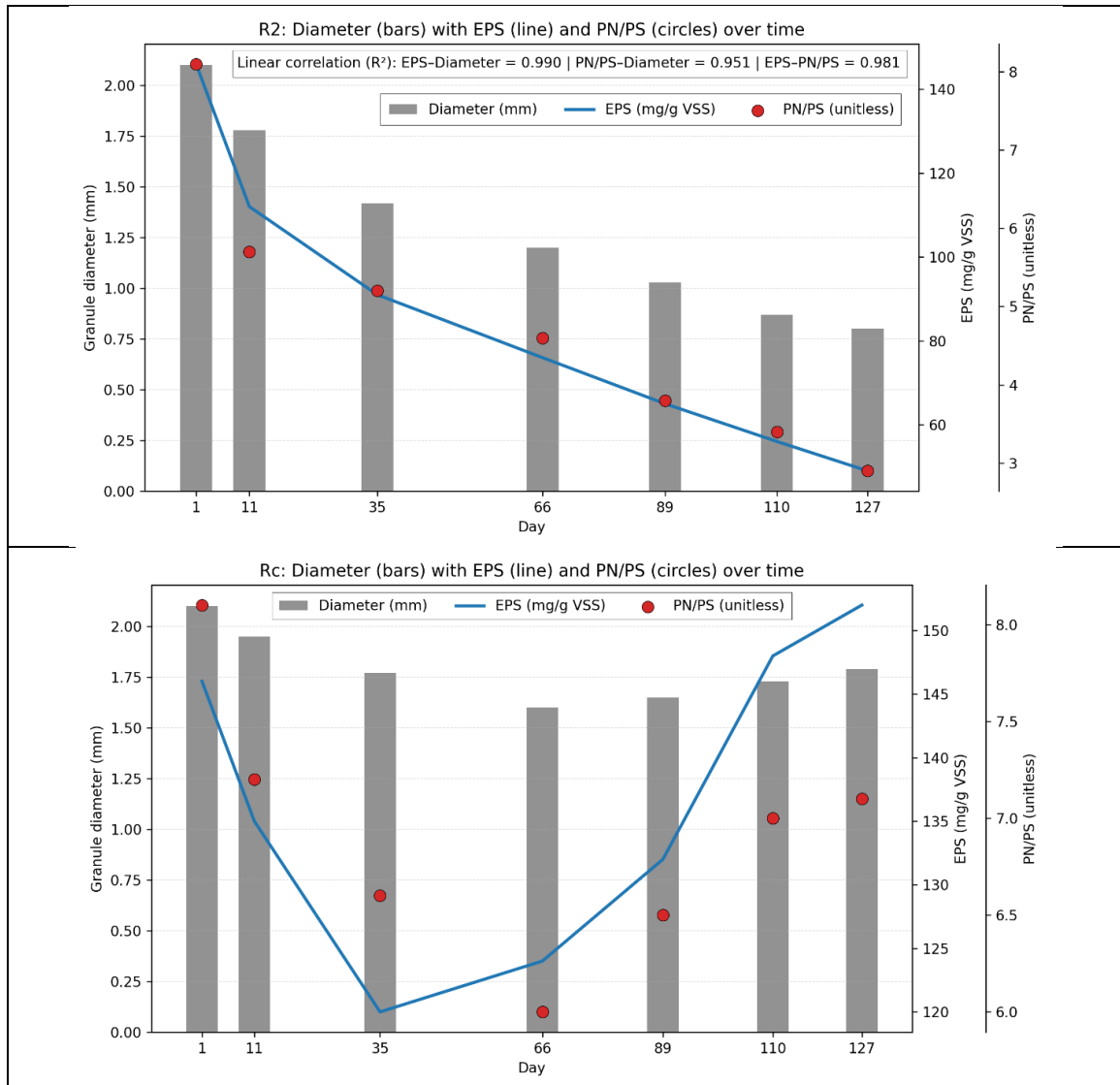


Figure 6.5: Temporal profiles of granule diameter (bars) and EPS (lines) and PN/PS (circles) in (a) R1, (b) R2, and (c) Rc (days 1, 11, 35, 66, 89, 110, 127)

Although the intermittent aerobic P starvation in R1 reduced mineral precipitates and consequently lowered granule density, it also facilitated improved nutrient diffusion within the granule matrix (**Figure 6.5(a)**), thereby enhancing granule stability during operation, consistent with previous findings (Lee et al., 2010). Consequently, successful

regranulation was observed in R1, as illustrated in **Figure 6.3**. Similarly, granules in Rc successfully re-granulated following initial deterioration caused by the transition from high-strength influent during the cultivation in the mother SBR to low-strength influent in the current study, which can be attributed to the increase in the actual applied F/M ratio. In R1, EPS levels increased from 119 ± 10 mg/gVSS (corresponding to a granule size of 1.22 mm on day 89) to 137 ± 10 mg/gVSS (1.41 mm on day 127) (R^2 (EPS - Diameter) = 0.639). Similarly, in Rc (**Figure 6.5(c)**), EPS rose from 124 ± 11 mg/gVSS (1.6 mm on day 35) to 152 ± 12 mg/gVSS (1.79 mm on day 127) (R^2 (EPS - Diameter) = 0.711). This enhancement in EPS for R1 and Rc was accompanied with increasing in protein dominance (high PN/PS ratios) (R^2 (EPS – PN/PS) = 0.908 for R1, and R^2 (EPS – PN/PS) = 0.674 for Rc). In contrast, the most pronounced and sustained reduction was observed in R2. This size loss was accompanied by concurrent declines in EPS and PN/PS (R^2 (EPS – Diameter) = 0.997, and R^2 (PN/PS - Diameter) = 0.984), suggesting that structural destabilization (smaller granules) coincided with depletion of the EPS matrix (and reduced protein dominance) under continuous extraction (R^2 (PN/PS - EPS) = 0.989).

EPS content during the final six weeks of operation exceeded earlier levels; however, granule size remained below initial values, likely constrained by the lower actual applied OLR. These results are consistent with previous studies identifying applying selective pressure and acceptable hydrodynamic shear forces promote EPS secretion and elevated PN/PS ratios in AGS systems (Kang and Yuan, 2017). In contrast, continuous extraction in R2 caused a sustained reduction in EPS production (coincided with PN/PS ratios), which negatively impacted granule size over time (**Figure 6.5(b)**).

These results highlight two key insights: (a) AGS cultivated under high-strength wastewater exhibits resilience to lower OLR and nutrient levels, enabling successful reggranulation under low-strength influent, as demonstrated in Rc; and (b) recovery cycles are crucial for preserving AGS stability, as observed in R1. Visual evidence of AGS deterioration under continuous extraction is provided in **Figure D4 (a–c) in the Appendix D**, which illustrates the decline in granule integrity.

6.3.2. P Removal Performance and Recovery Feasibility from P-rich Liquid

The treatment of low-strength wastewater in this study adversely affected the P removal performance of aerobic granules compared with previous phases operated with medium- and high-strength influents, likely due to the limited availability of sCOD. In Rc, effluent SP concentrations remained relatively stable between 1.2 and 1.4 mg/L (**Figure 6.6**), corresponding to removal efficiency of $65 \pm 1.5\%$. During the first two months, effluent SP concentrations in the test SBRs remained relatively stable at 0.42–0.54 mg/L (removal efficiencies $\geq 88\%$). This similarity can be attributed to the comparable total volumes of P-rich liquid extracted daily (R1: 200 mL; R2: 224 mL). During this period, both systems benefited from the aerobic P starvation conditions established through this separation approach, resulting in high P removal efficiencies in both reactors. However, a divergent trend emerged over the following 10 weeks. In R2, effluent SP concentrations displayed a fluctuating increase, reaching 0.8 ± 0.14 mg/L (removal efficiency of 78% - day 127), while R1 exhibited a gradual improvement, maintaining low effluent SP concentrations of 0.37 ± 0.10 mg/L (up to 90% removal).

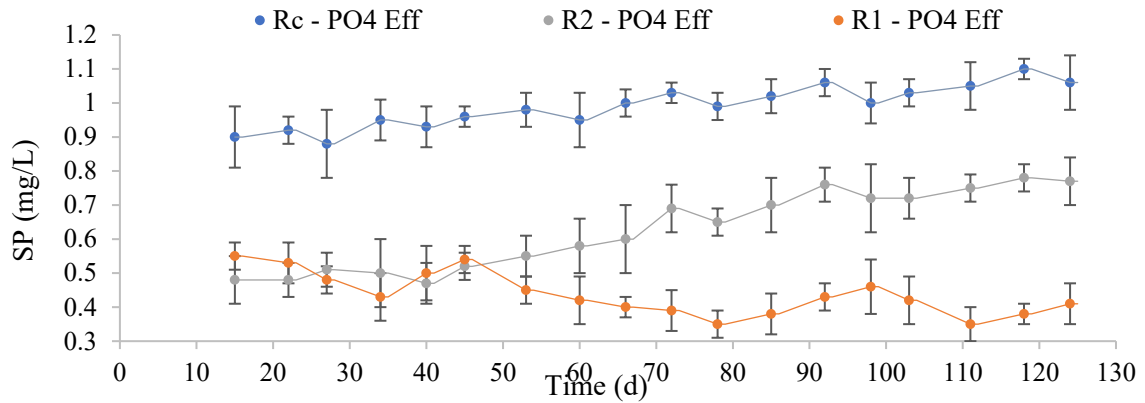


Figure 6.6: Effluent SP profiles ($\text{PO}_4^{3-}\text{-P}$ concentrations) for R1, R2 and Rc over the entire operational period.

Additionally, **Figure 6.7** shows the SRE (i.e., the net mg-P removed per gram of MLVSS) for all AGS-SBRs. In Rc, SRE remained relatively constant around 0.28 mg $\text{PO}_4^{3-}\text{-P/g}$ MLVSS. In contrast, SRE in R1 increased from 0.32 mg $\text{PO}_4^{3-}\text{-P/g}$ MLVSS in the first six weeks to 0.45 mg $\text{PO}_4^{3-}\text{-P/g}$ MLVSS in the final month, indicating enhanced PAOs performance despite a lower biomass concentration than Rc. Interestingly, R2 exhibited a continuous increase in SRE, reaching 0.48 mg $\text{PO}_4^{3-}\text{-P/g}$ MLVSS by day 127. Consequently, these results underscored the crucial role of the proposed operating strategy in enhancing P removal under municipal wastewater conditions via aerobic P-starvation, despite deterioration in granule physical properties.

These outcomes are consistent with previous studies showing that EBPR, rather than chemical precipitation, predominates in AGS-SBRs, accounting for 55–73.7% of TP removal (Ren and Yuan, 2016, Mady et al., 2024, Bassin et al., 2012, Barnard and Abraham, 2006, Gebremariam et al., 2011, Acevedo et al., 2012), confirming the long-term biological effectiveness of the strategy. Collectively, the effluent $\text{PO}_4^{3-}\text{-P}$ and SRE trends

show that aerobic P-starvation can significantly intensify biological P removal under low-strength municipal wastewater, with R1 outperforming both Rc and R2. These results critically demonstrate that properly controlled extraction regimes, rather than conventional AGS operation alone, govern long-term EBPR robustness and efficiency.

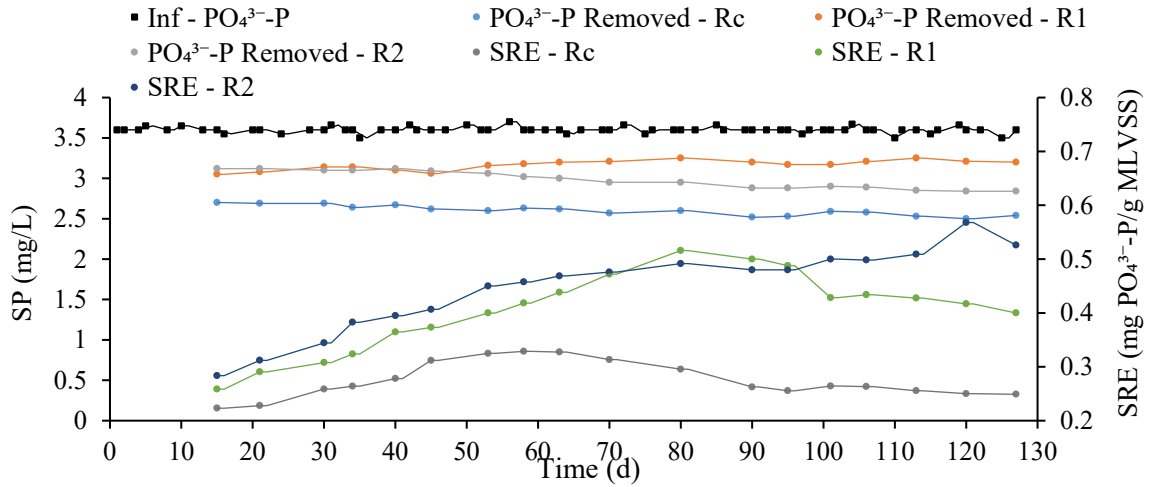


Figure 6.7: Profiles of influent SP ($\text{PO}_4^{3-}\text{-P} = 3.6 \pm 0.3 \text{ mg/L}$), SP removed and SRE for R1, R2 and Rc.

This divergence can be linked to the continuity in extraction of anaerobic supernatant in R2 during all treatment cycles, which may have disrupted granules structural integrity and altered their composition ultimately impairing their function and stability. Conversely, applying recovery cycles through the intermittent extraction strategy in R1 appeared to preserve granule structure and function, supporting sustained long term P removal. As discussed in Section 3.1 (Granule Properties), continuous extraction probably reduced mineral P precipitates within the granules, lowering granule density. This effect is further supported by the higher MLVSS/MLSS ratios observed in R2 compared with R1. Thus, while both extraction strategies initially enhanced P removal through aerobic P starvation, prolonged continuous extraction in R2 ultimately compromised granule stability

and P removal performance. J. Wang et al. (2022) emphasized that implementing additional recovery cycles is beneficial for maintaining the long-term stability of AGS systems when coupled with this separation technique.

Kinetic analyses during the final month (**Figure 6.8**) showed a slight decline in PAO activity in the test SBRs compared with Rc, as indicated by the anaerobic P release rates. Rc exhibited a rate of 2.83 ± 0.19 mg $\text{PO}_4^{3-}\text{-P/g MLVSS}\cdot\text{h}$, whereas R1 and R2 showed slightly lower rates of 2.52 ± 0.11 and 2.30 ± 0.10 mg $\text{PO}_4^{3-}\text{-P/g MLVSS}\cdot\text{h}$, respectively. This decline was further supported by the lower molar P/C ratios in R1 (0.042) and R2 (0.036) compared with Rc (0.051), as shown in **Table 6.2**, where Polyphosphate accumulation in PAOs is typically inferred from the molar ratio of P released to carbon consumed anaerobically (Acevedo et al., 2012). Despite this, R1 showed a higher aerobic P uptake rate (0.804 mg $\text{PO}_4^{3-}\text{-P/g MLVSS}\cdot\text{h}$) than Rc (0.301 mg $\text{PO}_4^{3-}\text{-P/g MLVSS}\cdot\text{h}$). R2 also exhibited an improved rate (0.448 ± 0.082 mg $\text{PO}_4^{3-}\text{-P/g MLVSS}\cdot\text{h}$), though lower than R1, indicating the negative impact of continuous P-rich liquid extraction.

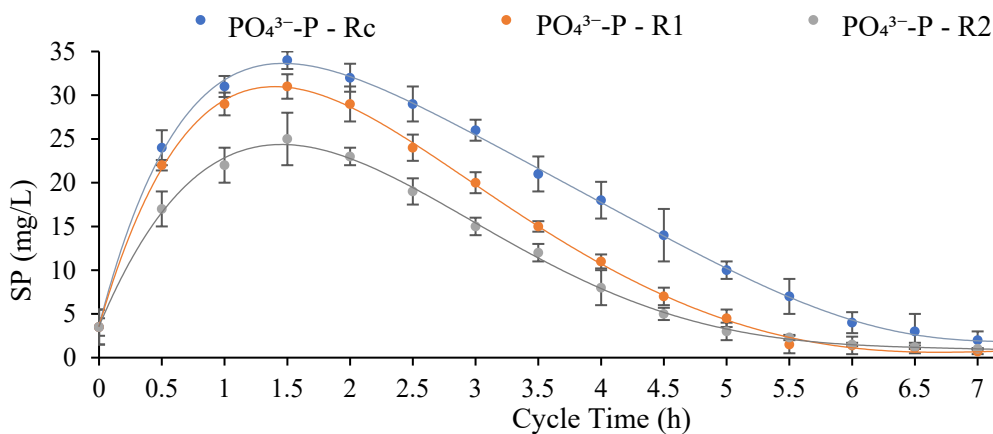


Figure 6.8: SP profiles through the typical treatment cycle for R1, R2 and Rc (kinetic tests on days 95, 101, 107, 114, 120 and 127)

Table 6.2: P-release, uptake rates, and molar P/C ratio for R1, R2 and Rc (days 100 - 127)

	R1			R2	Rc
	1 st cycle (10% extraction)	2 nd cycle (No extraction)	3 rd cycle (No extraction)	(Continuous extraction)	(Without extraction)
P release rate (mg-P/g-MLVSS·h)	2.52 ± 0.11	2.57 ± 0.13	2.6 ± 0.1	2.3 ± 0.1	2.83 ± 0.19
molar P/C ratio	0.042	0.042	0.043	0.036	0.051
P uptake rate* (mg-P/g-MLVSS·h)	0.804 ± 0.093	0.773 ± 0.064	0.711 ± 0.082	0.448 ± 0.082	0.301 ± 0.041

* P uptake rates nearly complete within 4.0 h in R1 and R2, compared to more than 5.5 h in Rc during 1st cycle.

Overall, the enhanced P uptake in both test SBRs highlighted the positive effect of the aerobic P-starvation strategy. Additionally, both test SBRs achieved nearly complete P removal within 3 h, whereas Rc required over 5 h, indicating a competitive advantage over conventional EBPR systems, as reported previously (Wang et al., 2022, Barat et al., 2006, Zhang et al., 2015). Notably, P-release rates in R1 increased slightly during recovery cycles (Table 6.2), indicating that this operational strategy maintained PAO bioactivity, while P uptake rates remained approximately consistent, reinforcing the role of recovery phases in maintaining overall system performance. The proposed strategy maintained low extracellular P levels during the aerobic phase, reinforcing selective pressure for efficient P uptake. These conditions indicate that PAOs remained metabolically active under extended P limitation by utilizing intracellular reserves (e.g., PHAs, poly-P, and glycogen).

The enhanced aerobic P uptake following P-rich liquid extraction was attributed to sustained intracellular P starvation, which limited Polyphosphate replenishment but promoted the utilization of the intracellular reserves for energy, consistent with Lv et al., (2014). Overall, this kinetics demonstrated that aerobic P-starvation can intensify aerobic P uptake and markedly accelerate P removal, even under slightly diminished anaerobic P-release capacity. The results further showed that only regimes incorporating recovery cycles preserve PAO activity and secure these kinetic gains, whereas prolonged continuous extraction progressively undermines both stability and P removal performance.

It should be noted that a fixed VER was maintained by replacing the extracted P-rich liquid with an equivalent volume of treated effluent rather than fresh influent, to avoid prolonged feast conditions that could disturb the feast–famine regime. Such imbalances are known to suppress PAO enrichment and EPS synthesis, thereby compromising granule cohesion and long-term AGS stability (Mady et al., 2023, Huang et al., 2015, Corsino et al., 2017). Consequently, this approach was adopted to preserve granule stability and maintain balanced feast–famine dynamics, while simultaneously reinforcing the aerobic P starvation by reducing P loading during the aerobic phase (up to 14% in R1 during the first cycle only, and 7% in R2). While J. Wang et al. (2022) demonstrated improved P removal in AGS-SBRs using sporadic P-rich liquid extraction, their study was limited to only 40 treatment cycles over 10 days and did not address the long-term impacts on granule stability. Moreover, although their objective was to induce aerobic P starvation, they did not verify this via fixed VER with effluent reuse at the start of the aerobic phase, a critical step incorporated and validated in our investigation.

As shown in **Table 6.3**, P mass balance calculations were performed for AGS-DBRs over the final 20 days of operation. All AGS-SBRs received a similar influent P load of 756 ± 13 mg-P. Besides, R1 and R2 received additional P loading through the treated wastewater reused at the start of the aeration stage. The total P content in the extracted P-rich liquid represented approximately 12–15% of the total influent P, reflecting the intentional use of relatively low-strength extraction to avoid strongly inhibiting PAO activity. Continuous P-rich liquid extraction in R2 resulted in less P content through the extracted volumes (72 mg from 60 cycles), supporting the observation that continuous extraction lowered the released P concentration by suppressing of PAO bioactivity.

Table 6.3: P mass balance for R1, R2 and Rc between days 107 and 127

	P-Source (81 cycle)	Rc	R1	R2
Input P (mgP)	Inf. P	840±14		
	P in the added treated effluent* (Fixed VER)	-	4±0.3 (27 cycles only)	5±0.5 (81 cycles)
Output P (mgP)	P in the P-rich liquid extracted	-	129 ± 2 (27 cycles only)	93±4 (81 cycles)
	P content in the effluent liquid	125±3	37±2	86±3
	P content in the effluent solids**	697±9	664±12	641±10
Loss of TP (mgP)		18±2 (2.14%)	14±1 (1.7%)	25±3 (3%)

* P content in the solids with added WW (start of aerobic phase) is negligible

** P – Eff solids = Avg P content in AGS × Avg TSS_{eff} × 1.2L × N cycles

These findings are consistent with the decline in P-release rates observed in the kinetic profiles (**Figure 6.8**). Rc exhibited the highest net P accumulation in AGS, while

both test reactors showed reduced intracellular P storage, especially in R2, indicating reduced polyphosphate accumulation under extended aerobic P starvation. Consequently, the separation strategy weakened PAO activity to some extent under continuous extraction, while intermittent extraction in R1 maintained a more balanced performance, allowing both extracellular P release and intracellular storage. These results are in line with Acevedo et al. (2015), who reported that while high-concentration P recovery was achievable from a single batch (with added COD), continuous extraction led to EBPR failure and microbial destabilization (Acevedo et al., 2015). Despite effectively lowering soluble P in the treated effluent, a substantial fraction of P remained in the solid phase, suggesting that future optimization should integrate solid-phase P harvesting to improve overall P recovery efficiency. Collectively, P mass balancing shows that low-intensity, intermittent extraction can recover dissolved P while continuous extraction disproportionately suppresses PAO-mediated polyphosphate accumulation and intracellular storage.

Based on the previous results, which indicated the feasibility of sporadic P-rich liquid extraction compared to continuous extraction, the P recovery potential from P-rich liquids was evaluated for R1. Under anaerobic conditions preserve most influent ammonium was preserved while P was released, generating a nutrient-rich supernatant for struvite-based recovery. During the anaerobic mixing phase, P-rich liquids from R1 typically containing up to 32 mg $\text{PO}_4^{3-}\text{-P/L}$ and 39 mg $\text{NH}_4^+\text{-N mg/L}$ in each batch P recovery test. P recovery was successfully achieved through chemical precipitation using Mg^{+2} for struvite formation with an optimal P recovery efficiency exceeding 64% at a Mg:P molar ratio of 2 and pH 9, consistent with theoretical optima. Notably, this pH was attained without alkali addition, as natural carbon dioxide stripping via aeration raised pH to 9

within 20 minutes, demonstrating a cost-effective and environmentally sustainable pathway for struvite formation and reduced chemical demand (Chrispim et al., 2019). It should be noted that P recovery efficiency further increased with sodium hydroxide addition, reaching approximately 72.5% at pH 9.2 and up to 84% at pH 9.5. However, extending the settling time to 66 hours did not yield further improvements in P recovery efficiency (**Table D1 – Appendix D**). Moreover, the relationship between P recovery efficiency and the Mg:P molar ratio showed a remarkably strong linear correlation, with a coefficient of determination ($R^2 = 0.998$). These findings highlight the feasibility of integrating low-energy, chemically optimized struvite recovery with AGS-based wastewater treatment systems to enhance nutrient recycling.

6.3.3. sCOD and Ammonium Removal Performance

Throughout operation, R1 effluent consistently exhibited slightly lower sCOD concentrations than Rc (likely due to the dilution from treated effluent addition at the start of the aeration phase (fixed VER), which reduced aerobic sCOD loading in the test AGS-SBRs), and R2, as well, Overall COD removal in R2 was comparable to R1 and Rc, with no meaningful deterioration over the study period; all reactors achieved $\geq 98\%$ sCOD removal, with treated effluent sCOD ≤ 10 mg /L. Additionally, the marked decrease in actual SRTs in R2 (23 ± 2 days) may have negatively impacted denitrifying populations. By the end of the study, sCOD concentrations at the end of the anaerobic stage were 182.5 ± 10 mg/L in R1 (52% removal), 209.1 ± 13 mg/L in R2 (45%), and 167.4 ± 8 mg/L in Rc (56%). These findings confirm that COD removal remained largely stable under both intermittent and continuous P-rich liquid extraction regimes.

Figure 6.9 (a, b) present the N species profiles (NH_4^+ , NO_2^- , and NO_3^-) in R1 and R2 throughout the entire experimental period. The control reactor demonstrated stable and efficient N removal via SND, achieving a nitrification efficiency of $95 \pm 0.22\%$, with effluent $\text{NH}_4^+\text{-N}$ of 1.3 ± 0.3 mg/L. SND performance in Rc remained high at $94.8 \pm 0.4\%$, with $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ levels consistently below 0.8 ± 0.21 and 1 ± 0.3 mg/L, respectively, over the 127-day period. In R1, ammonium removal remained comparable to Rc until day 92, with a removal efficiency of $97 \pm 0.11\%$ and $\text{NH}_4^+\text{-N}$ concentrations around 1.25 ± 0.19 mg/L (**Figure 6.9 (a)**). A slight increase in $\text{NH}_4^+\text{-N}$ was observed during the final month, reaching 1.5 ± 0.21 mg/L, corresponding to a decrease in nitrification efficiency to $96 \pm 0.4\%$. Despite this, nitrite was not detected during the last month, and nitrate concentrations remained below 2 mg/L throughout the operation of R1.

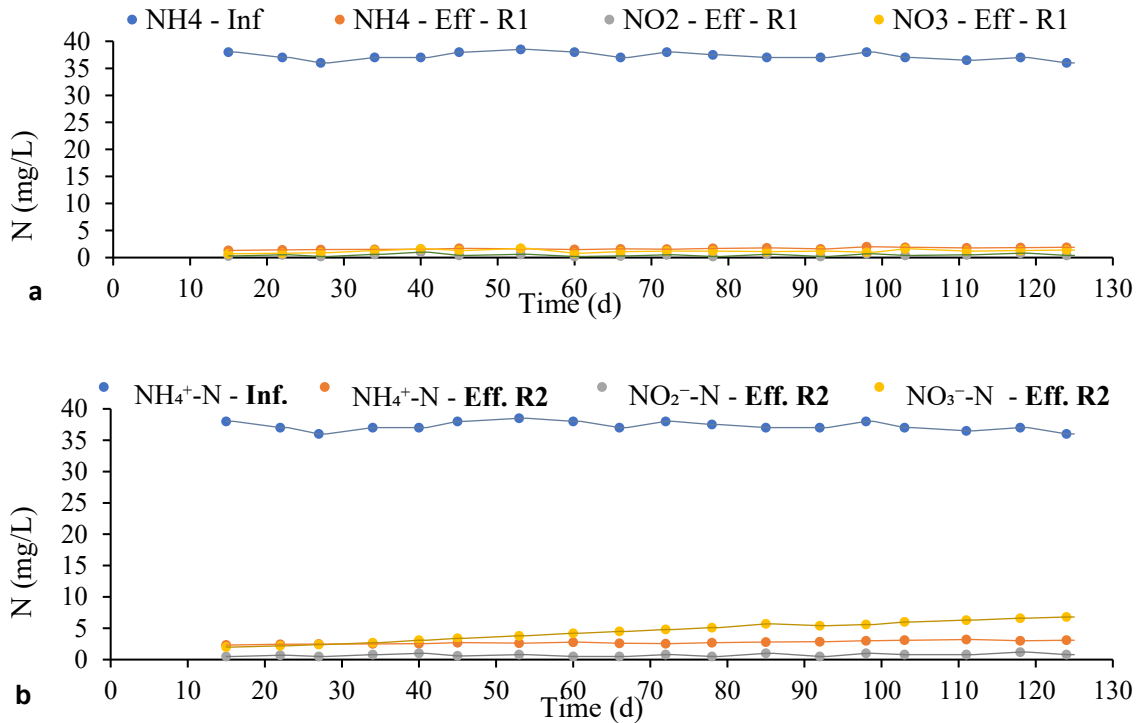


Figure 6.9: Profiles of NH_4^+ , NO_2^- , and $\text{NO}_3^-\text{-N}$ (removal performance) through the entire operational period for R1 (a) and R2 (b) (Influent $\text{NH}_4^+\text{-N}$ = 35 ± 2 mg/L)

These findings align with the slight decline in anaerobic sCOD consumption observed in R1 compared to Rc, possibly affecting denitrifier activity, yet AGS in R1 still maintained satisfactory SND performance ($94.1 \pm 0.31\%$), emphasizing the potential of cultivating compact, mature granules from high-strength wastewater for application in low-strength influent treatment. A slight reduction in $\text{NH}_4^+\text{-N}$ removal was observed in R2 during the final month of operation, with nitrification efficiency decreasing to $84.1 \pm 1.7\%$ and average effluent $\text{NH}_4^+\text{-N}$ concentrations reaching 3.3 ± 0.51 mg/L (**Figure 6.9-b**). Throughout the entire experimental period, $\text{NO}_2^-\text{-N}$ levels in R2 remained below 1.2 mg/L, while $\text{NO}_3^-\text{-N}$ concentrations in the treated effluent increased steadily, reaching 6.8 ± 1.1 mg/L by day 127. This trend indicates a gradual deterioration in the denitrification process, with overall performance declining to $72.1 \pm 1.5\%$. This reduction aligns with previously observed increases in sCOD concentrations at the end of the anaerobic phase, suggesting impaired the activity of denitrifiers (e.g., denitrifying PAOs).

The deterioration in denitrification performance in R2 coincided with a measurable reduction in anaerobic sCOD consumption based on the cycle-kinetic analysis, indicating lower carbon uptake/storage availability to support subsequent anoxic reduction. In parallel, continuous granule disintegration and biomass disruption (evidenced by elevated TSS in the extracted P-rich liquid and TSS_{eff}) likely increased washout of denitrifying biomass under the settling-velocity selection pressure and reduced diffusion-limited anoxic microenvironments within the granule matrix, thereby weakening SND/denitrification. In contrast, the use of sporadic extraction in R1 appeared to support microbial stability, as reflected in improved performance and higher SRT (30 ± 1 days). The inclusion of recovery cycles under aerobic P starvation conditions may have helped preserve the structure and

activity of slow-growing functional groups within the AGS. This observation is consistent with previous reports recommending limited removal of anaerobic supernatant in EBPR systems to avoid detrimental impacts on microbial community performance, particularly for slow-growing bacteria (Huang et al., 2015, Guisasola et al., 2019).

It worth mentioning that these results were in agreement with N mass balance with conducted between days 100 and 127. As shown in **Table 6.4**, Rc and R1 exhibited comparable N removal performance, with similar effluent TN and denitrification-related N loss, indicating that intermittent P-rich liquid extraction did not adversely affect overall nitrogen fate. In contrast, R2 showed slightly higher effluent TN, suggesting that continuous extraction may have imposed a modest negative effect on N removal efficiency relative to Rc and R1.

Table 6.4: N mass balance for Rc, R1, and R2 between days 100 and 127.

	LS influents		
	27 days (Days 100 and 127) – 81 cycles		
	Rc (No extraction)	R1 (Intermittent extraction)	R2 (Continuous extraction)
Influent TN (mg N)	4660 ± 28		
Effluent TN (mg N)	1630 ± 11	1680 ± 11	1920 ± 12
Nitrate denitrified + N Gases + Loss of TN (mg N)	3030 ± 20	2980 ± 18	2740 ± 20

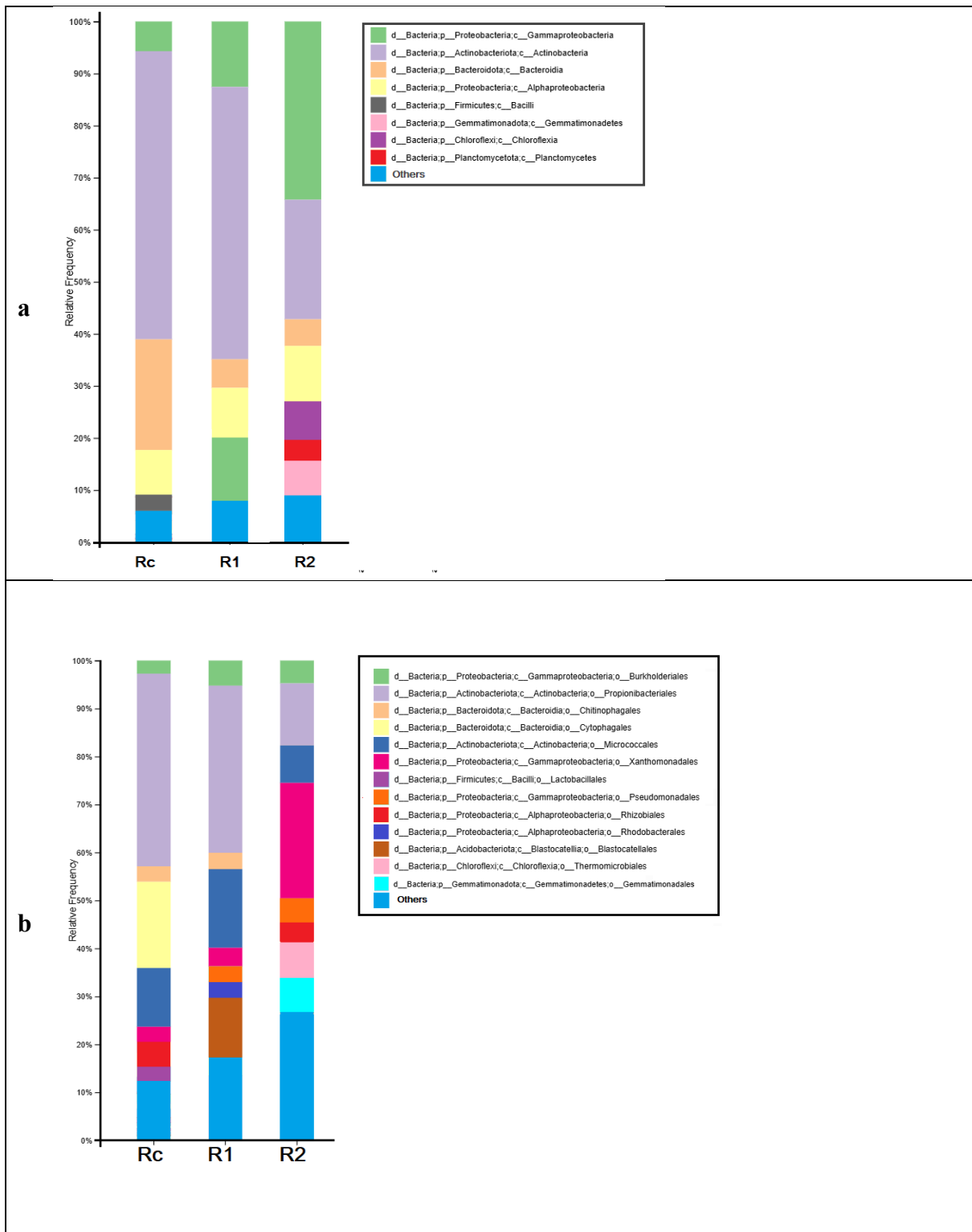
The following table (**Table 6.5**) summarizes the granules physical properties (e.g., biomass characteristics and settling properties), effluent quality, and BNR efficiencies.

Table 6.5: Summary of granules physical properties of R1, R2, and Rc (days 100 - 127)

Characteristics	Control SBR Rc	Test SBR R1	Test SBR R2
P-rich liquid extraction	No extraction	200 mL (10%) from one cycle only daily	76 mL/cycle (11.4% daily)
MLSS (g/L)	5.8 ± 0.2	4.8 ± 0.12	3.5 ± 0.22
MLVSS/MLSS (%)	76	83	87
Granules' size (mm)	1.79	1.41	< 0.9
SVI ₃₀ (mL/gm)	≤ 32	≤ 40	52
TSS _{eff} (mg/L)	70 – 80	80 – 95	100 – 115
SRT (days)	42	29	19
Biomass yield (Y _{obs})	0.16	0.194	0.219
SRE (mg PO ₄ ³⁻ -P/g MLVSS)	0.28	0.45	0.48
sCOD removal (%)	≥ 98	≥ 99	≥ 99
TAN removal (%)	≥ 97	≥ 96	84
SP removal (%)	65	≥ 90	≥ 78

6.3.4. Microbial community Structure

At the conclusion of the operational period (day 127), the microbial community composition of aerobic granules from all AGS-SBRs was comprehensively analyzed. The dominant bacterial groups were taxonomically classified at the class, order, family, and genus levels, with their relative abundances illustrated in **Figure 6.10 (a–c)** and detailed in **Table. D2 (Appendix D)**. High-throughput 16S rRNA gene sequencing revealed a diverse microbial consortium, comprising 34 phyla, 81 classes, 192 orders, 342 families, 645 genera, and 645 identified species, highlighting the complexity and richness of the bacterial communities within the mature granules.



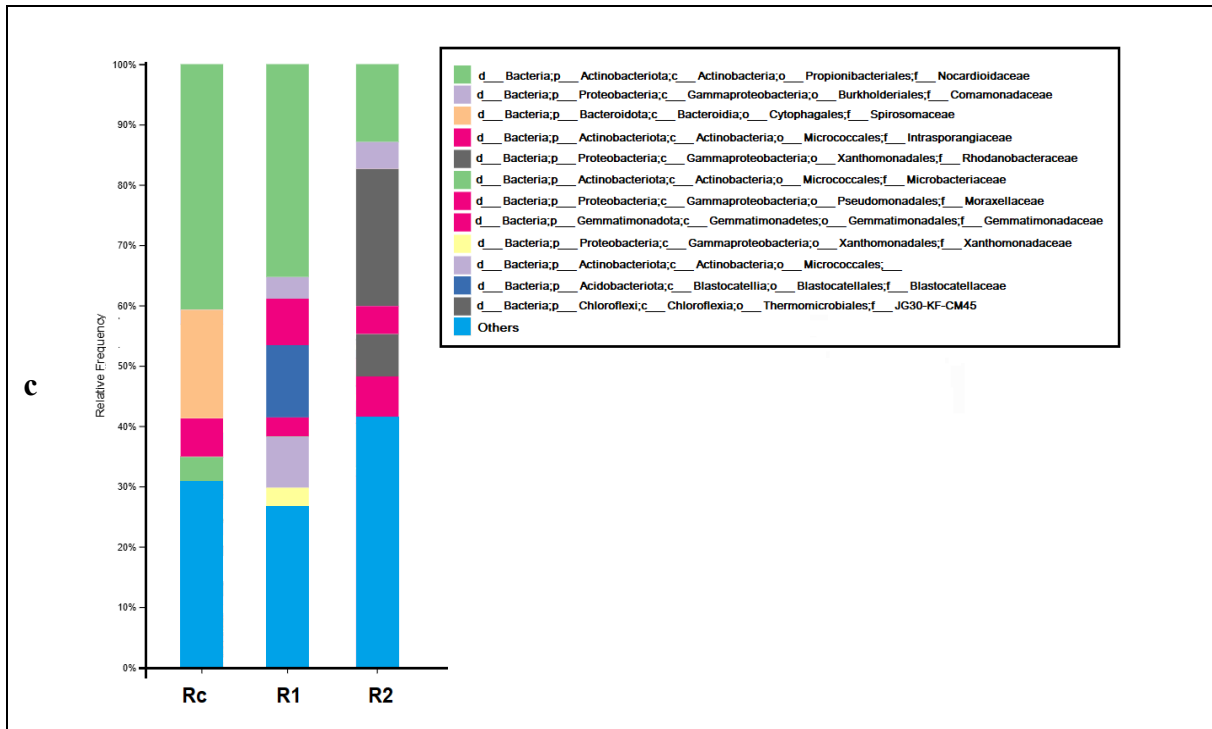


Figure 6.10: Distribution of dominant microbial diversity (relative abundance > 3 %) at; (a) Class, (b) Order, and (c) Family levels in Rc, R1 and R2

The results revealed no significant differences in microbial composition at the phylum level between the test AGS-SBRs where, the three most dominant phyla were *Actinobacteriota* (R1: 52.5%, R2: 23.8%), *Proteobacteria* (R1: 22.2%, R2: 44.9%), and *Acidobacteriota* (R1: 13.2%, R2: 3.5%). Additional predominant phyla included *Bacteroidota* (R1: 5.6%, R2: 5.7%), *Chloroflexi* (R1: 1.0%, R2: 8.3%), and *Gemmatimonadota* (R1: 0.5%, R2: 6.8%). The control reactor displayed a broadly similar microbial profile, dominated by *Actinobacteriota* (55.6%), *Bacteroidota* (21.4%), and *Proteobacteria* (14.3%). *Proteobacteria* are among the most dominant bacterial groups in WWTPs and play a key role in facilitating simultaneous BNR processes (Ding et al., 2019, Ye and Zhang, 2013, Isazadeh et al., 2016, Cydzik-Kwiatkowska and Zielińska, 2016).

Proteobacteria, *Actinobacteria*, *Bacteroidota*, and *Chloroflexi* emerged as the dominant phyla, all of which are well known for their crucial contributions to granule formation and structural stability (Mady et al., 2024). The relative abundance of *Firmicutes* in the R1 and R2 reached < 1%, compared to > 4% in Rc, which could be attributed to the reduction in granule size in the test SBRs resulted from the application of aerobic P starvation via P-rich liquid extraction (3.1. *Granule Properties*). These findings align with a previous study (Shi et al., 2021), which suggested that *Firmicutes* are predominantly denitrifying bacteria, and their enrichment is favored in larger granules where oxygen transfer is more limited.

The dominant bacterial classes in the AGS of both test reactors (**Figure 6.10-a**) were *Actinobacteria* (R1: 52.3%, R2: 23%), *Gammaproteobacteria* (R1: 12.6%, R2: 6.8%), *Alphaproteobacteria* (R1: 9.6%, R2: 10.7%), and *Bacteroidia* (5.5% in both R1 and R2). In addition, *Blastocatellia* (12.3%) and *Phycisphaerae* (2.0%) were among the next most abundant classes in R1, whereas *Chloroflexia* (7.5%), *Gemmatimonadetes* (6.7%), and *Planctomycetes* (4.1%) were more prominent in R2. Rc was dominated by *Actinobacteria* (55.3%), *Bacteroidia* (21.3%), *Alphaproteobacteria* (8.6%), *Gammaproteobacteria* (5.7%), and *Bacilli* (3.2%). Notably, despite the granule disintegration observed in test SBRs, this condition appeared to enhance nutrient diffusion within their granules, as evidenced by the higher bacterial diversity compared to Rc (**Figure 6.10-b**).

The dominant bacterial orders varied significantly between AGS-SBRs, reflecting the impact of the operating strategies related to aerobic P starvation and effluent recycling on microbial community structure. In R1, the microbial community was primarily

composed of *Propionibacteriales* (35.4%) and *Micrococcales* (16.4%) from the *Actinobacteria* class, along with *Blastocatellales* (12.3%) from *Blastocatellia*, and several *Gammaproteobacteria* orders including *Burkholderiales* (5.5%), *Xanthomonadales* (4%), and *Pseudomonadales* (3%). Orders from *Alphaproteobacteria*, namely *Rhodobacterales* and *Rhizobiales* (3%), were also present. R2 showed a broader and more evenly distributed community, with *Xanthomonadales* (24%) dominating, followed by *Pseudomonadales* and *Burkholderiales* (5%) from *Gammaproteobacteria*. The presence of *Propionibacteriales* (13%) and *Micrococcales* (8%) from *Actinobacteria*, along with *Chitinophagales*, *Cytophagales*, and *Corynebacteriales* (2.5%), further reflected the shift in community structure. Additionally, orders such as *Thermomicrobiales* (7.5%, *Chloroflexia*), *Gemmatimonadales* (7%, *Gemmatimonadetes*), and *Planctomycetales* (2.5%, *Planctomycetes*) were enriched under continuous extraction conditions. Conversely, the control reactor maintained a more limited diversity, dominated by *Propionibacteriales* (41%) and *Micrococcales* (12.5%) from *Actinobacteria*, *Cytophagales* (18%) and *Chitinophagales* (3%) from *Bacteroidia*, as well as *Xanthomonadales* and *Burkholderiales* (3%) from *Gammaproteobacteria*. These variations in the test AGS-SBRs probably resulted from effluent reuse (fixed VER), which reintroduced residual taxa and buffered the nutrient regime, enabling smoother adaptation to operational changes.

At the family level, notable differences in microbial composition were observed across the AGS-SBRs. R1 was mainly enriched with *Nocardioidaceae* (35.3%), *Blastocatellaceae* (12.5%), and *Intrasporangiaceae* (8%), whereas R2 was characterized by a more diverse community, dominated by *Rhodanobacteraceae* (23%), followed by *Nocardioidaceae* (13%) and *Gemmatimonadaceae* (7%). The control reactor showed a

distinct profile, with *Nocardioidaceae* (26%), *Spirosomaceae* (18%), and *Intrasporangiaceae* (6.5%) as the most abundant families. At the genus level, R1 featured *Nocardioides* (35.28%), *Blastocatellaceae* (10.1%), *Micrococcales* (8.4%), *Tetrasphaera* (7.78%), *Acinetobacter* and *Luteimonas* (> 3%). In R2, diversity was more pronounced as shown in **Table. D2 (Appendix D)**, *Nocardioides* (12.9%), *Pseudofulvimonas* (12.3%), *Dokdonella* (8.9%), *JG30-KF-CM45* (7.1%), *Gemmatimonas* (6.7%), *Acinetobacter* (4.6%), *Comamonadaceae* (3.21%), *Dermatophilaceae* and *Tetrasphaera* (2.5%). In contrast, Rc was dominated by *Nocardioides* 40.7%, *Leadbetterella* 18.8%, *Tetrasphaera* 6.2%, *Leucobacter* 3.8%.

Tetrasphaera, a polyphosphate-accumulating genus within the phylum *Actinobacteria*, is frequently found in biological treatment systems treating complex wastewaters and is distinguished by its ability to fermentatively utilize a wider range of substrates, including sugars, amino acids, and polysaccharides, compared to *Candidatus Accumulibacter* (Stokholm-Bjerregaard et al., 2017). Some *Tetrasphaera*-related PAOs have also been shown to hydrolyze starch, highlighting their capacity to utilize polysaccharides in EBPR systems (Ortega et al., 2022). A high relative abundance of *Tetrasphaera* has been consistently reported in AGS systems, reinforcing their key functional role under complex substrate conditions (Ortega et al., 2022 and Ali et al., 2019). In this study, *Tetrasphaera* abundance in R1 remained relatively stable and higher than Rc, suggesting that the intermittent P-rich liquid extraction supported their persistence through enhancing better nutrient diffusion into granules matrix in R1 compared with the persistence of dense compact granules in Rc. Conversely, the pronounced decline observed in R2 suggests that continuous aerobic phosphorus starvation destabilized the biomass,

leading to elevated solids washout in the extracted liquid and consequently compromising the long-term viability of *Tetrasphaera*. It worth mentioning that, *Candidatus Accumulibacter* was not detected in AGS-SBRs, indicating that the HS wastewater used for cultivating the seed AGS in the mother reactors may have hindered its proliferation. Nevertheless, effective P removal was sustained, likely due to functional compensation by other putative PAOs (*Thauera*, *Acinetobacter*, *Paracoccus*, *Pseudoxanthomonas*, *Acidovorax*, *Gemmatimonas*, *Microthrix*), which possess the ability to utilize more complex substrates (Wang et al., 2018a, Medhi and Thakur, 2018, Sun et al., 2021, Kawakoshi et al., 2012).

Microbial analysis also identified the presence of other putative EPS-producing genera, such as *Acinetobacter* and *Luteimonas*, which are recognized for secreting EPS that enhance microbial cohesion and promote granule formation (Xiong et al., 2020). These taxa likely contributed to AGS stability, particularly in Rc and R1. Notably, *Leadbetterella* exhibited the highest abundance in Rc, consistent with previous reports highlighting its essential role in granule formation and stability regardless of wastewater type (Abdullah et al., 2013). Its reduced abundance in R1 and R2 suggests that P-rich liquid extraction may have disrupted the enrichment of such supportive bacteria. Furthermore, continuous aerobic P starvation in R2 resulted in a marked decline of *Nocardioides* (*Nocardioidaceae*), with relative abundance dropping to 12.9% compared to 35.3% in R1 and 40.7% in Rc. This reduction implies that prolonged P limitation may negatively influence the growth and competitive advantage of this genus, potentially compromising granule structural integrity.

Despite the high N removal performance observed in Rc and R1 (consistent with SND behavior), the relative abundance of canonical nitrifiers was low in these reactors.

For example, *Nitrosomonas* accounted for only 1.7% and 2.3% of the community in Rc and R1, respectively, and was even lower in R2 (<1%). This pattern suggests that the intermittent P-rich liquid extraction applied in R1 may have improved mass transfer within granules, potentially by reducing mineral P precipitates and increasing internal nutrient diffusion, relative to the more compact granule structure in Rc. In contrast, the continuous extraction regime likely imposed stronger operational stress, promoting biomass disruption and solids loss, which may have limited the proliferation of slow-growing nitrifiers. In addition to canonical nitrifiers, several putative HNAD-associated genera (*Rhodobacter*, *Diaphorobacter*, *Hydrogenophaga*, *Ottowia*, *Niabella*) were detected, consistent with the microbial composition established during granule cultivation and maturation in the mother SBRs (previous chapters) used to seed the present study.

3.5. Operational Insights and Future Recommendations for Full-Scale Application

Practically, the findings indicate that intermittent, rather than continuous, P-rich liquid extraction is a more robust strategy for AGS-SBRs treating municipal wastewater, because it improves P removal, preserves granule stability, and promotes granule regrowth during long-term operation. For full-scale design and operation, an intermittent extraction regime, involving P-rich liquid extraction at the end of the anaerobic phase under non-mixed conditions once daily, can serve as an initial guideline for process control in engineered systems. This configuration maintained high simultaneous BNR efficiencies and high P uptake rates. Furthermore, the P-rich side-stream generated can be sent to a struvite crystallization unit, enabling integrated P recovery and supporting circular, resource-oriented municipal wastewater treatment.

Aerobic P starvation should be examined in SBRs seeded with flocculent sludge to evaluate its effects on AGS start-up, performance, and stability under LS influent conditions. Such studies are essential to clarify impacts on granulation, possible risks of instability or undesirable microbial shifts, and to support efficient AGS design for municipal wastewater treatment applications. Additionally, optimal P-rich liquid extraction ratios under varying P loadings, particularly at low C/P, should be determined carefully. Although higher ratios may initially improve P removal, they may also accelerate PHAs depletion in PAOs (weaken EBPR stability). Systematic evaluation is therefore required to establish guidelines that balance process efficiency and operational resilience. Aeration conditions should be optimized in such systems to limit PHAs consumption (sustain PAOs activity), though other operational factors, cycle length, and feast/famine balance, must also be considered for long-term stability and resilience.

CHAPTER 7: SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

7.1. Summary

Wastewater discharges release organic carbon and nutrients such as N and P, driving eutrophication and ecosystem degradation. To address this, treatment plants must achieve stricter nutrient removal targets. Conventional processes like activated sludge are widely applied but face major limitations. While carbon removal is relatively simple, N and P removal remains complex, requiring multiple treatment steps. These challenges highlight the urgent need for advanced, integrated technologies capable of achieving high-efficiency nutrient removal in a single step while reducing costs, energy use, greenhouse gas emissions, and the overall environmental footprint, ensuring more sustainable wastewater management. AGS has emerged as a promising established and sustainable alternative, offering compact structure and efficient simultaneous BNR across diverse wastewater types. However, there are persistent and unresolved challenges in the application of the aerobic granulation technology included long-start up periods and granule disintegration.

Feeding mode is a critical factor influencing the selection, formation, and stability of AGS. Anaerobic filling in AGS-SBRs has been widely adopted, with influent load and duration recognized as key determinants of process efficiency. Previous studies have shown that anaerobic slow feeding or pulse feeding, followed by anaerobic mixing, can favor the accumulation of slow-growing organisms. However, limited information is available regarding the impact of feeding mode changes on AGS-SBRs. Therefore, the first part of this thesis examined the effect of applying an anaerobic step-feeding strategy on

matured AGS in SBRs treating HS wastewater with elevated ammonium and P loadings. This stage aimed to evaluate potential improvements in simultaneous BNR of large-sized granules and to explore changes in granule stability when shifting from a single fast feeding mode with anaerobic mixing to distributed step-feeding throughout the anaerobic stage.

The second part of this thesis conducted the first long-term evaluation of AGS-SBRs treating HS wastewater with elevated ammonium and P concentrations, focusing on simultaneous BNR performance, granule physicochemical characteristics alongside the microbial community composition. It further examined the effects of prolonged granule maturation and increasing DO levels (up to 8.5 mg/L) on EPS, physical integrity, and nutrient removal dynamics (e.g., ammonium oxidation and P release/uptake rates), enabling a comparison between the feasibility of using large-sized matured AGS and applying high DO conditions.

To date, few studies have examined the behavior of PAOs under aerobic P starvation induced by anaerobic P-rich liquid extraction, and its long-term application in AGS systems remains largely unexplored. Building on matured aerobic granules cultivated under high organic loadings in the previous stage, the third part of this thesis investigated the effects of aerobic P starvation on EBPR-SBRs treating municipal wastewater. This work focused on assessing key changes in granule characteristics and simultaneous BNR performance, particularly P uptake behavior, after reconfiguring AGS-SBR operations to apply this strategy under long-term conditions.

Little is known about the implications of applying continuous aerobic P starvation conditions to AGS, emphasizing the need for systematic investigation. Excessive P

starvation aerobically may deplete intracellular P reserves, compromising granule stability and ultimately leading to deterioration. In AGS-SBR systems coupled with P-rich liquid extraction, optimizing the daily extraction volume is therefore essential to balance P recovery, biological removal, and granule stability. Accordingly, the final part of this thesis experimentally evaluated two operational strategies, intermittent and continuous P-rich liquid extraction, to assess their impacts on P removal efficiency and AGS physical stability during low-strength wastewater treatment.

7.2. Conclusions

7.2.1. Feasibility of Anaerobic Step-Feeding for Treating HS Wastewater in Granular SBRs (Chapter 3)

- AGS were successfully cultivated (>2.1 mm) and matured (>3 mm) under HS wastewater with limited substrate (COD:TN:TP = 100:12:4). However, P removal remained low due to competition between PAOs and denitrifiers for rbCOD during the anoxic phase, alongside long start-up periods and nitrite/nitrate accumulation.
- Application of anaerobic step-feeding aimed to reduce PAO–denitrifier competition and enhance reactor performance. Unexpectedly, this strategy caused granule deterioration, reduced EPS (PN/PS ratio), and led to declines in P, ammonium, and sCOD removal efficiencies, indicating insufficient anaerobic mixing duration.

7.2.2. Simultaneous BNR from High N and P Wastewater Using AGS: Effects of Gradually Increased DO as an Optimization Strategy (Chapter 4)

- This study demonstrated that aerobic granulation can be successfully cultivated and matured when treating influent with low COD/TN/TP ratios, achieving stable AGS

with acceptable BNR. Under favorable conditions, AGS is a viable technology for high-strength ammonium wastewater treatment.

- Operating under limited DO conditions (1.5–2.0 mg/L) promoted the enrichment of HNAD bacteria as dominant microbial populations within the mature AGS. This microbial shift enhanced SND by leveraging internal anoxic zones, ultimately improving overall N removal performance.
- Extended operation with large, matured granules highlighted the importance of adjusting operating conditions, particularly increasing superficial air velocities, to prevent anoxic zones at the reactor bottom during aeration mixing, thereby improving simultaneous BNR.
- Operating at high DO levels (5–6 mg/L) and under optimal mixing enhanced nitrification by increasing ammonium oxidation rates; however, DO above 8.5 mg/L reduced EPS secretions, leading to granule deterioration, disintegration, and poor effluent quality.

7.2.3. Feasibility of Aerobic P Starvation to Stabilize EBPR in AGS Treating Municipal Wastewater (Chapter 5)

- Applying the proposed strategy of aerobic P starvation condition via sporadic extraction of LS P-rich liquid (end of the anaerobic phase, under no-mixing conditions), consistently achieved high P removal during long-term SBRs operation. Using AGS cultivated under high organic loading proved effective in sustaining EBPR performance under aerobic P starvation during municipal wastewater treatment.

- Maintaining a fixed volumetric exchange ratio in the SBRs through effluent reuse after extraction preserved starvation pressure and the feast/famine balance, which protected granule structure and prevented disintegration.
- Although the proposed operating strategy initially caused temporary deterioration in AGS physical properties (lower granules density), the analysis revealed that these conditions ultimately promoted long-term structural stability. This was attributed to improved nutrient diffusion within the granule matrix, which supported successful regrowth and restored aerobic granule integrity.
- Metagenomic analysis revealed that operational modifications induced distinct microbial community shifts, with the test reactors showing greater adaptability. Stable diversity and enrichment of PAOs supported enhanced functional resilience.
- These microbial adaptations directly contributed to improved nutrient removal across transitions from HS to LS wastewater, emphasizing the importance of operational strategies in sustaining robust EBPR performance under variable environments.
- Measurements of intracellular PHAs indicated that prolonged aeration led to accelerated reserve depletion and diminished PAOs activity. However, optimizing the SBRs by reducing daily aeration duration effectively preserved sufficient PHAs availability, which in turn enhanced PAOs performance and P removal efficiency.

7.2.4. Comparative Impacts of Intermittent and Continuous P-Rich Liquid Extraction on EBPR Performance and AGS Stability (Chapter 6)

- This study demonstrated that enhanced P removal in AGS-SBRs treating LS wastewater can be achieved through aerobic P starvation, implemented via two distinct strategies: intermittent and continuous P-rich liquid extraction at the end of

the anaerobic phase. Despite initial declines in granule density and stability due to reduced mineral P precipitates, gradual adaptation occurred, most notably under intermittent extraction, leading to regrowth and long-term stability.

- Both extraction strategies promoted rapid P uptake behavior, outperforming conventional EBPR systems. Intermittent extraction, achieved >90% P removal efficiency. P balance analysis revealed that sporadic extraction produced P-rich liquids with higher P concentrations, enabling over 68% recovery through struvite precipitation without chemical alkalinity adjustment, positioning this strategy as a sustainable option for integrated P removal and recovery.
- Microbial analysis indicated that P-rich liquid extraction influenced bacterial community structure and selection, with potential implications for functional performance and granule stability. These findings confirm the promise of this integrated strategy for sustainable P removal and recovery in AGS systems.

7.3. Recommendations

- In AGS-SBR systems, extended anaerobic mixing following step-feeding may enhance PAOs and denitrifiers activity, improving P removal and preventing nitrite/nitrate build-up. Thus, future studies should evaluate this approach under wastewater with lower COD:TN:TP ratios and focus on optimizing simultaneous nitrogen removal.
- Following the successful maturation of AGS-based systems, it is essential to optimize DO levels and mixing regimes to create favorable conditions for nitrifying bacteria and reduce competitive pressure in aerobic zones. Strategic control of these parameters can enhance nitrification efficiency without compromising granule integrity. However, further research is warranted to develop cost-effective and operationally stable AGS

systems capable of treating wastewater with high ammonium and P loadings. In particular, a deeper understanding of DO gradients within the granule matrix under varying aeration strategies is needed to support long-term nutrient removal performance and granule sustainability.

- Investigate aerobic P starvation in SBRs seeded with flocculant sludge to assess its effects on AGS start-up, structural stability, and nutrient removal performance, thereby providing guidance for reliable full-scale application under low-strength influent conditions. In addition, incorporate microbial profiling during and after granulation to monitor bacterial community shifts, track the washout of initial populations, and identify the emergence of new functional groups critical for process stability.
- Optimize SBR operational parameters under P-rich liquid extraction by systematically evaluating factors such as cycle length, feast/famine balance, aeration strategy, and PHA dynamics to improve biological P removal during low-strength influent treatment.
- Future research should evaluate AGS systems coupled with aerobic P starvation strategies under varying influent C/P, with emphasis on intracellular polymer dynamics of PAOs and GAOs. Such investigations will not only reveal critical metabolic shifts but also provide practical insights to improve EBPR modeling, guiding the design and operation of full-scale WWTPs for more resilient and sustainable nutrient removal.

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APPENDIX A: SUPPLEMENTAL RESULTS FOR CHAPTER 3



Figure A.1: GSBRS (R1 and R2) in operation.

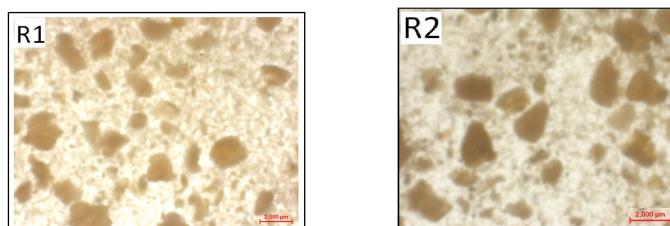


Figure A.2 (a): Microscopic images of the formed granules in both reactors on day 50 (during of the cultivation stage).

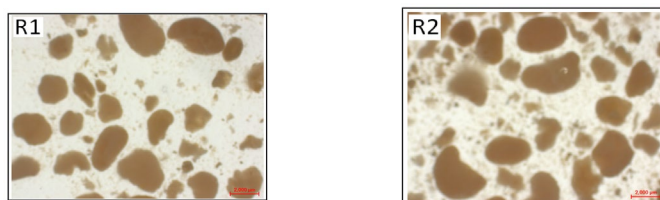


Figure A.2 (b): Microscopic images of the formed granules in both reactors on day 120 (end of the cultivation stage).

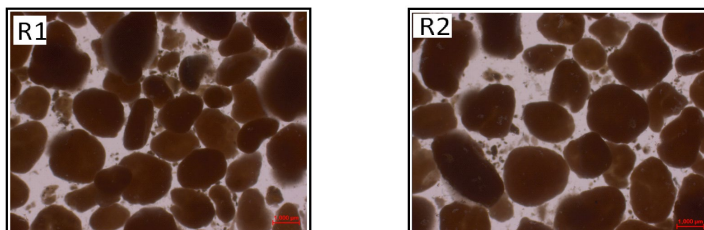


Figure A2.c. Microscopic images of the formed granules in both reactors on day 210 (during the maturation stage)

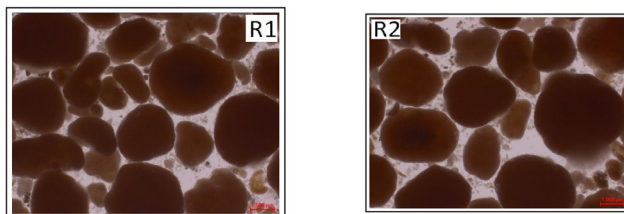


Figure A2.d. Microscopic images of the AGS in both reactors on day 300 (end of the maturation stage)

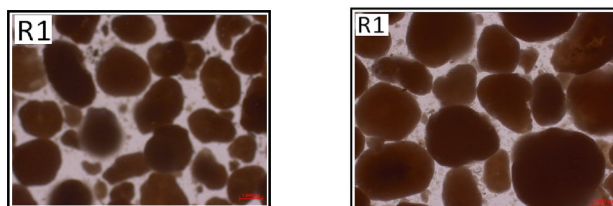


Figure A3. Microscopic images of the AGS in R1 during stage III (day 301 -360)

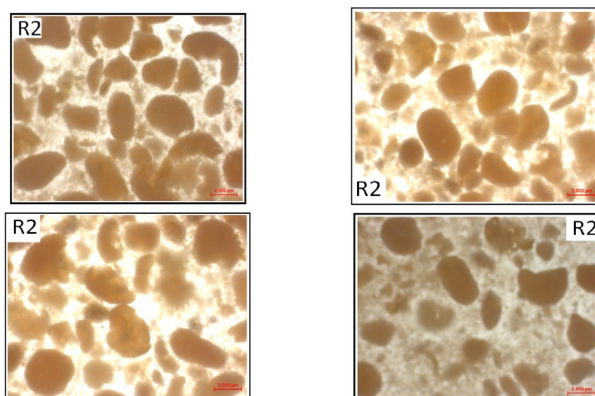


Figure A4. Microscopic images of AGS in R2 after applying anaerobic step-feeding mode (during stage III; day 301 until 360)\

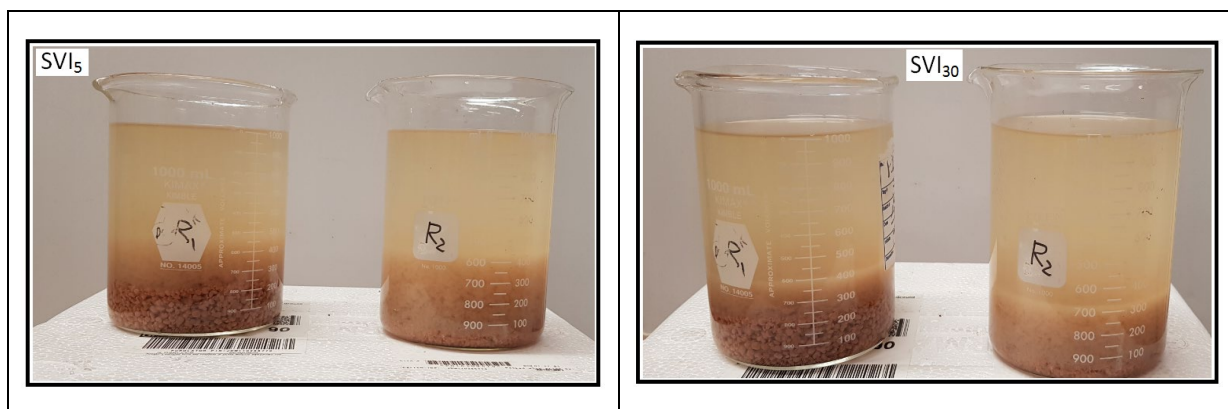


Figure A5. Measurements of SVI₅ and SVI₃₀ for both reactors in day 352 (deterioration of the AGS in R2 after applying anaerobic step-feeding strategy).

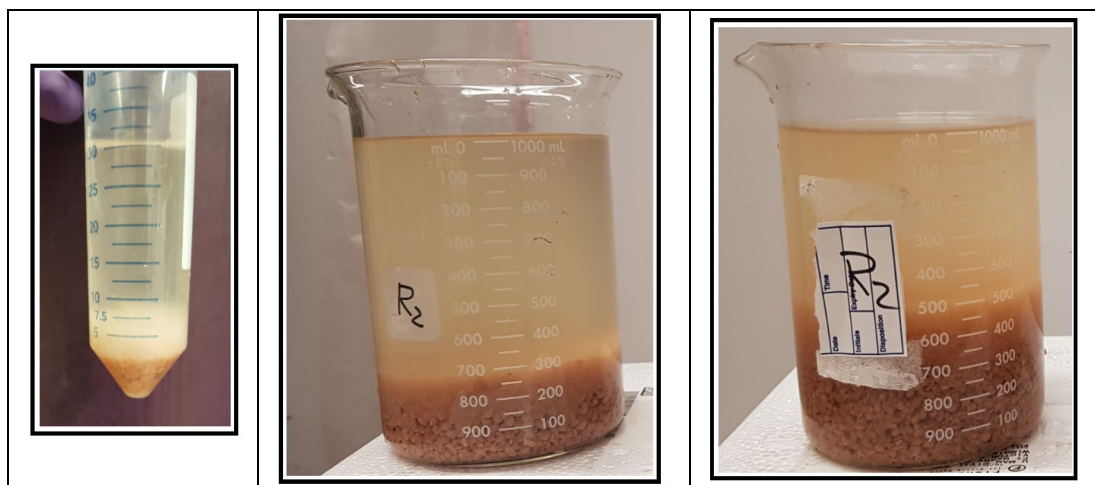


Figure A6. AGS in R2 rapidly lost its structural integrity, after changing the filling mode to anaerobic step-feeding, resulting in loose and fluffy aggregates

During stage III, after change feeding mode for R2, results showed a disintegration of the AGS in R2 which caused higher content of TSS concentrations in its effluents compared to R1:

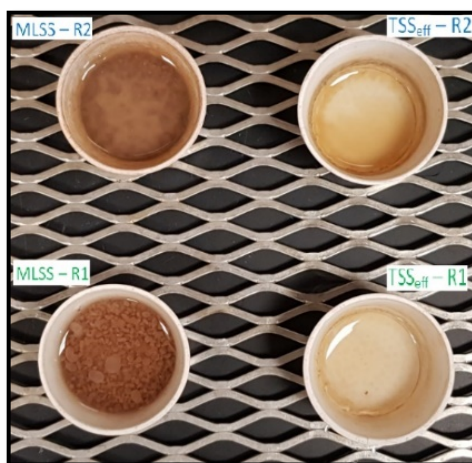


Figure A7. Measurement of the solid content: biomass samples (MLSS) and TSS concentrations for R1 and R2

APPENDIX B: SUPPLEMENTAL RESULTS FOR CHAPTER 4



Figure B1. AGS-SBRs (R1 and R2) in operation

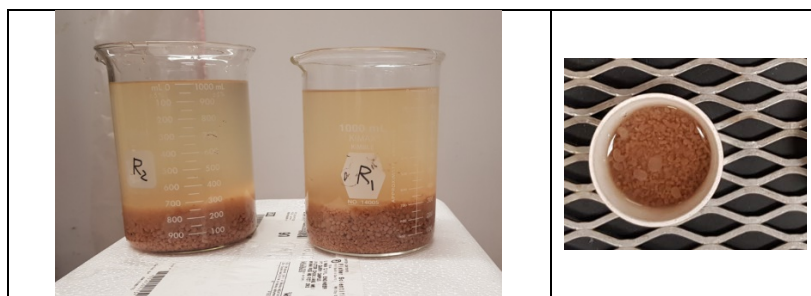


Figure B2. Formation of large sized and compact granules in both reactors during maturation period (days 118 - 422) - Measurements of SVI₅, SVI₃₀, MLSS and MLVSS

Microbial community fingerprinting (Matured AGS – day 420)

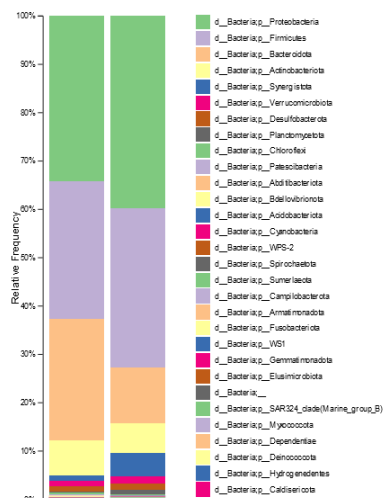


Figure B3. Microbial community characterization for matured AGS in R1 and R2 (Phyla level)

Table B1: Bacterial genera of the AGS for both reactors wastewater
(Relative abundance 0.1 – 0.3%)

No.	Genus Name	%
1	Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;__	0.19
2	Bacteria;p__Chloroflexi;c__Anaerolineae;o__SBR1031;f__A4b;g__A4b	0.17
3	Bacteria;p__Synergistota;c__Synergistia;o__Synergistales;f__Synergistaceae;g__Lactivibrio	0.20
4	Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Aeromonadaceae;g__Aeromonas	0.15
5	Bacteria;p__Firmicutes;c__Clostridia;o__Peptococcales;f__Peptococcaceae;g__uncultured	0.21
6	Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Propionibacteriales;f__Propionibacteriaceae;g__Luteococcus	0.14
7	Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Pseudorhodobacter	0.25
8	Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Mesorhizobium	0.20
9	Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Aquamicrobium	0.10
10	Bacteria;p__Firmicutes;c__Clostridia;o__Peptostreptococcales-Tissierellales;f__Anaerovoracaceae;g__[Eubacterium]_brachy_group	0.10
11	Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingopyxis	0.14
12	Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Brachymonas	0.27
13	Bacteria;p__Planctomycetota;c__Planctomycetes;o__Isosphaerales;f__Isosphaeraceae;g__Paludisphaera	0.14
14	Bacteria;p__Acidobacteriota;c__Holophagae;o__Subgroup_7;f__Subgroup_7;g__Subgroup_7	0.10
15	Bacteria;p__Bacteroidota;c__Bacteroidia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Pedobacter	0.30
16	Bacteria;p__Planctomycetota;c__Planctomycetes;o__Isosphaerales;f__Isosphaeraceae;g__Paludisphaera	0.28
17	Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Hyphomicrobium	0.28
18	Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Falsirhodobacter	0.11
19	Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Dysgonomonadaceae;g__Proteiniphilum	0.21
20	Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Brachymonas	0.24

Settled layer of the matured AGS at the bottom of AGS-SBRs

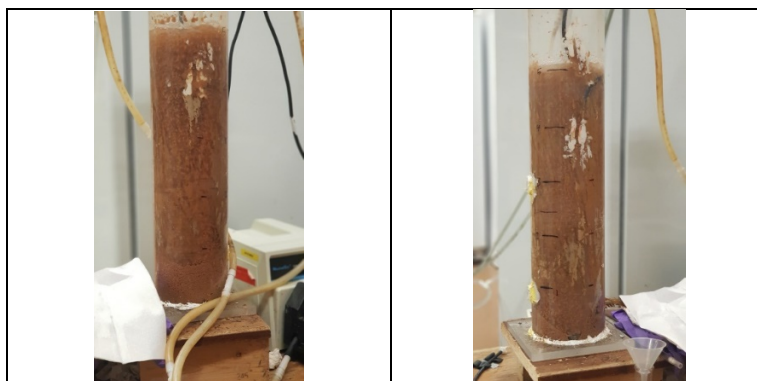


Figure B4. Formation of settled layer of AGS at the bottom of AGS-SBRs (before the optimization stages by increasing applied DO)

APPENDIX C: SUPPLEMENTAL RESULTS FOR CHAPTER 5

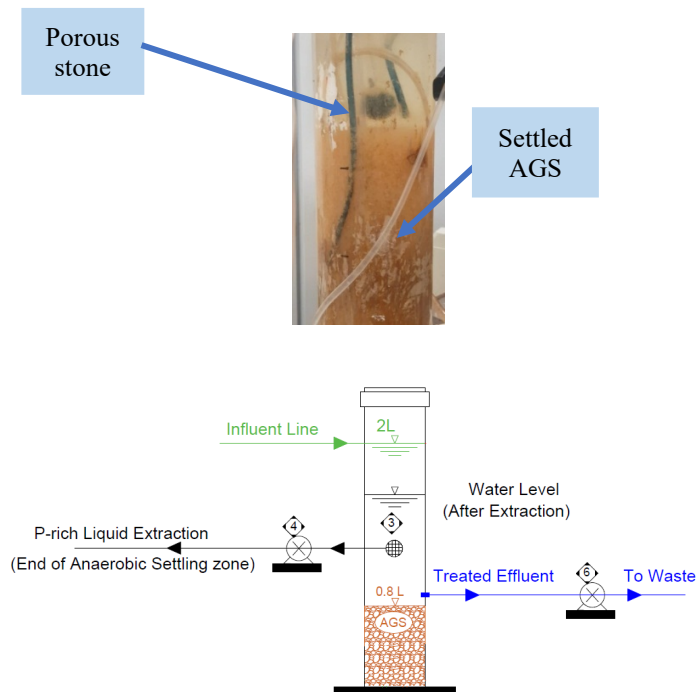


Figure C.1: Porous stone in the middle point in the liquid phase inside the reactor to extract the supernatant liquid.

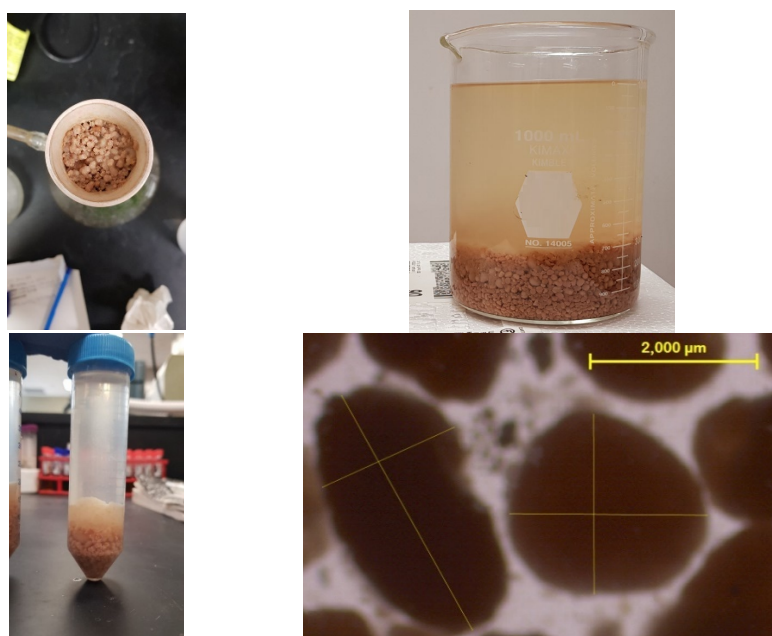


Figure C.2: Cultivation of large sized AGS (≥ 2.5 mm) through the mother SBR under during the treatment of high-strength wastewater

The transitional operating stage: Treatment of Medium-strength wastewater

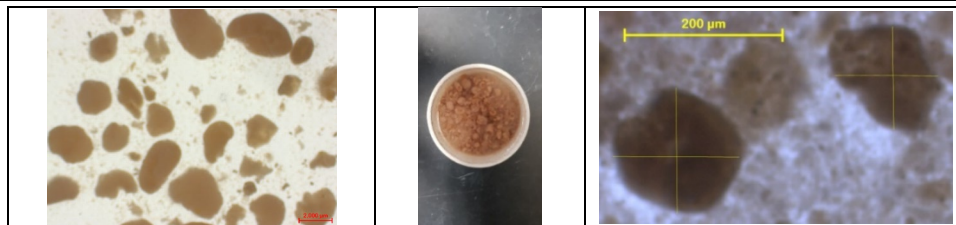


Figure C.3: Initial deterioration in granules physical properties through R3 during the treatment of medium-strength wastewater

Functional potential of the reactors

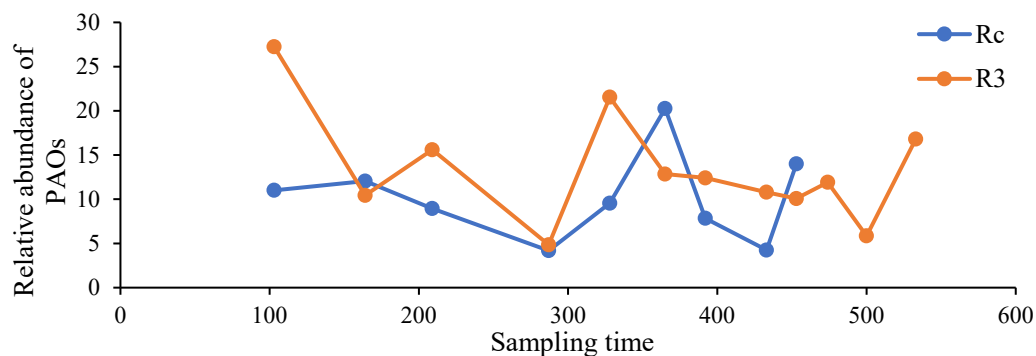
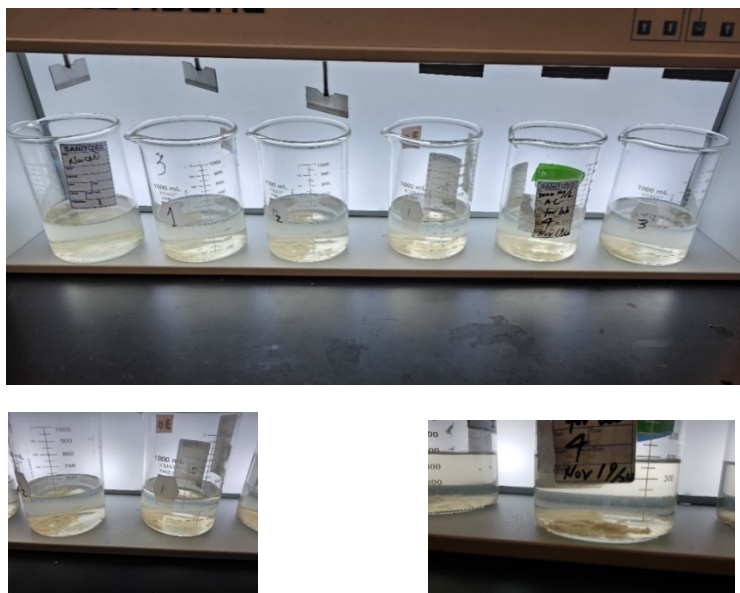


Figure C.4: Enhanced P removal in R3, correlated with a higher relative abundance of putative PAOs than in Rc

P recovery from the extracted P-rich liquid from R3 under the treatment of LS wastewater (operating stages 2)



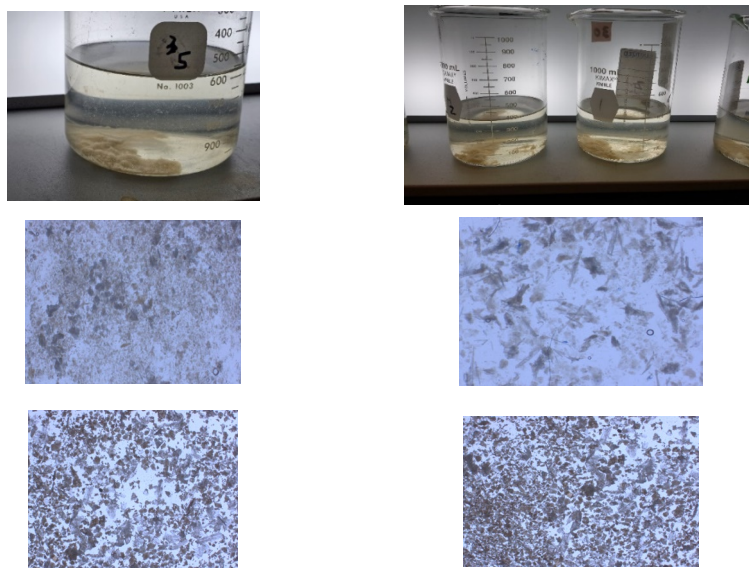


Figure C5.a. P recovery potentials from the P-rich liquids extracted from R3 through chemical precipitation as struvite (using magnesium ions)

Table C1: P-release, uptake rates, and P_{rel}/C_{upt} for R3 and Rc (HS wastewater treatment)

	R3			Rc (Without extraction)
	1 st cycle (10% extraction)	2 nd cycle (No extraction)	3 rd cycle (No extraction)	
P release rate [*] (mg-P/g-MLVSS·h)	3.76 ± 0.2	3.79 ± 0.15	3.84 ± 0.19	4.6 ± 0.10
molar P_{rel}/C_{upt} ratio [*]	0.031 ± 0.01	0.031 ± 0.01	0.032 ± 0.01	0.044 ± 0.01
P uptake rate ^{**} (mg-P/g-MLVSS·h)	0.97 ± 0.05	0.91 ± 0.08	0.89 ± 0.04	0.50 ± 0.02

^{*} R3; a deterioration in P reserves and PAOs bioactivity (reduced P release rate and drop in the molar P_{rel}/C_{upt} ratio).

^{**} P uptake rates nearly complete within 4 h in R3, compared to ≥ 5 h in Rc during 1st cycle (i.e., faster P removal behavior).

Table C2: P-release, uptake rates, and P_{rel}/C_{upt} for R3 and Rc (LS wastewater treatment)

	R3			Rc (Without extraction)
	1 st cycle (10% extraction)	2 nd cycle (No extraction)	3 rd cycle (No extraction)	
P release rate (mg-P/g-MLVSS·h)	2.41 ± 0.08	2.48 ± 0.10	2.51 ± 0.08	2.72 ± 0.07
molar P_{rel}/C_{upt} ratio	0.042 ± 0.01	0.042 ± 0.01	0.043 ± 0.01	0.051 ± 0.01
P uptake rate [*] (mg-P/g-MLVSS·h)	1.33 ± 0.11	1.28 ± 0.08	1.25 ± 0.10	0.74 ± 0.03
%PHAs ^{**} content (mg PHAs/mg MLSS)	1.56 ± 0.02			2.02 ± 0.05
q_p ^{***} (mg COD-PHA/mg COD-X·h)	0.0123			0.017

^{*} P uptake rates nearly complete within 3.5 h in R3, compared to ≥ 5 h in Rc.

^{**} PHAs reached 292.1 ± 7.8 mg/L at the end of the anaerobic zone in R3, and 303.7 ± 10.1 mg/L in Rc.

^{***} Slight PHAs consumption rate occurred in R3 (220 mg/L end of aeration) compared with Rc (180 mg/L end of aeration).

APPENDIX D: SUPPLEMENTAL RESULTS FOR CHAPTER 6

Test AGS-SBRs in operation (R1 and R2):

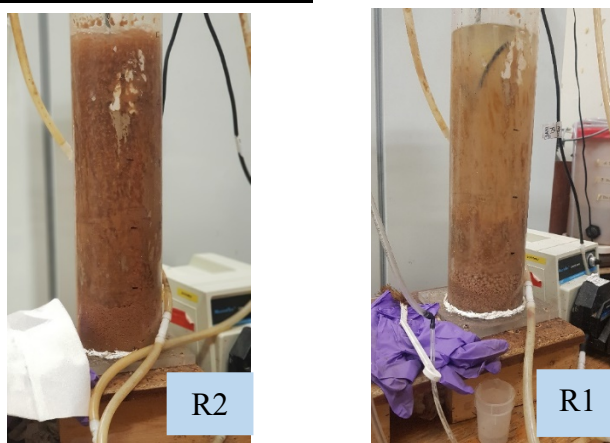


Figure D1. The test SBRs: R1 (10% Sporadic P-rich liquid extraction through the 1st cycle only), and R2 (Continuous P-rich liquid extraction - 76 mL/cycle (11.4%/day))

The mother SBR (matured AGS) during the treatment of high-strength wastewater:



Figure D2. Cultivation of large sized AGS (≥ 2.5 mm) through the mother SBR under during the treatment of high-strength wastewater

Continuous P-rich liquid extraction in R2: Deterioration in AGS physical properties



Figure D3.a. Deterioration in the physical properties via continuous P-rich liquid extraction (R2) (significant reduction in granules' size)

	
Figure D3.b. Deterioration in the physical and settling properties in R2 (continuous extraction) compared with R1 (intermittent extraction)	Figure D3.c. Granules disintegration after applying continuous extraction (R2) compared with intermittent extraction (R1)

P recovery from the extracted P-rich liquid: R1 (sporadic extraction)

Table D1. P recovery efficiencies through different operational conditions for pH and settling time from the extracted P-rich liquids (R1 - sporadic extraction)

pH Conditions	P recovery eff. (%)		
	Settling times		
	T = 0 h	T = 20 h	T = 66 h
<u>Without adjustment</u>	<u>64.0</u>	65.6	29.5
9.2	72.5	74.6	55.8
<u>9.5</u>	<u>83.9</u>	84.7	68.3

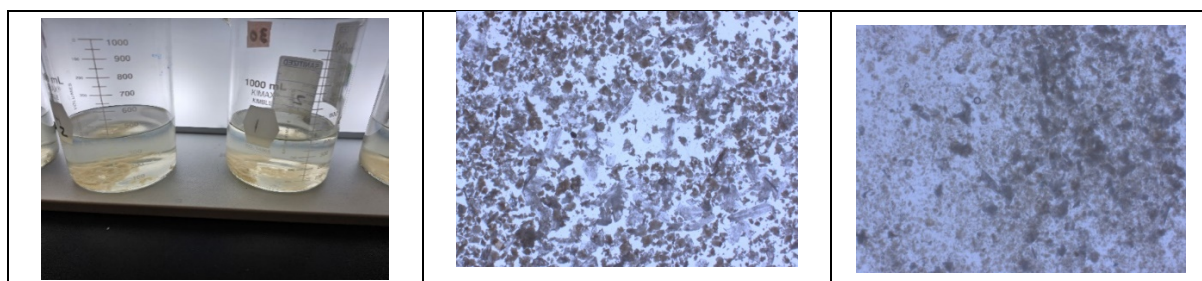


Figure D5. P recovery from the extracted P-rich liquids using magnesium ions (chemical precipitation) at varying molar ratios (Mg:P) to induce struvite formation