Investigating the Impact of Anaerobic Retention Time and Phosphorus-to-Carbon Influent Ratio on Phosphorus Accumulating Organisms' Kinetics

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Abstract

Existing phosphorus removal technologies face challenges in achieving recent, more stringent regulatory discharge limits on effluent phosphorus levels, necessitating further process optimizations. While anaerobic-phase metabolic activities drive Enhanced Biological Phosphorus Removal (EBPR)'s core mechanism, the design of the aerobic phase dominates the system sizing. This thesis aims to investigate the influence of anaerobic retention time and phosphorus loading on the EBPR's performance. EBPR performance in bench-scale reactors was compared under baseline conditions and test conditions, which include varying Phosphorus-to-Carbon (P/C = 3:80, 1:80 and 1:40) ratios and anaerobic retention times (60, 90, 120 minutes). The polyphosphate content, release/uptake rates, and maintenance energy demands are evaluated to analyze performance. Polyphosphate content in the biomass was shown to influence P/C ratios (R^2= 0.673), directly impacting phosphorus release and uptake rates. Meanwhile, reducing the retention time from 90 mins to 60 mins preserved the specific release rates but significantly lowered uptake and removal efficiency. Extending the anaerobic phase to 120 minutes decreased release rates without affecting uptake. Secondary phosphorus release, indicative of maintenance energy demands, varied across anaerobic retention times. This variation highlights how PAOs adapt polyphosphate hydrolysis rates to sustain baseline metabolism during shortened or extended anaerobic phases, beyond initial substrate-driven release. Results confirm polyphosphate reserves in biomass govern release/uptake dynamics which varies with anaerobic retention time.

Dedication

To the Palestinian struggle and resilience…

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List of Acronyms

WWTP	Water Treatment Plant
DO	Dissolved Oxygen
WW	Waste Water
EBPR	Enhanced Biological Phosphorus Removal
HRT	Hydraulic Retention Time
SBR	Sequencing Batch Reactor
WRRF	Water Recovery Facility
PAOs	Phosphorus Accumulating Organisms
poly-P	Polyphosphates
COD	Chemical Oxygen Demand
BOD	Biochemical Oxygen Demand
TOC	Total Organic Carbon
OUR	Oxygen Uptake Rates
TCOD	Total Chemical Oxygen Demand
bCOD	Biodegradable Chemical Oxygen Demand
nbCOD	Non-Biodegradable Chemical Oxygen Demand
sbCOD	Slowly Biodegradable Chemical Oxygen Demand
pCOD	Particulate Chemical Oxygen Demand
TP	Total Phosphorus
OP	Orthophosphate
TKN	The Total Kjeldahl Nitrogen

OHOs	Ordinary Heterotrophic Organisms
VFAs	Volatile Fatty Acids
RAS	Return Activated Sludge
MLSS	Mixed Liquor Suspended Solids
SRT	Solids Retention Time
PHAs	Polyhydroxyalkonates
GAOs	Glycogen Accumulating Organisms
EPS	Extracellular Polymeric Substances
TB-EPS	Tightly Bound EPS
LB-EPS	Loosely Bound EPS
PN/PS	Proteins To Polysaccharides
ATP	Adenosine Triphosphate
AMP	Adenosine Monophosphate
PMF	Proton Motive Force
EM	Embden Meyerhof
ED	Entner Doudoroff
NADH	Nicotinamide Adenine Dinucleotide
PHB	Poly-B-Hydroxybutyrate
WAS	Waste Activated Sludge
TSS	Total Suspended Solids
VSS	Volatile Suspended Soild
VS	Volatile Soilds
PLR	Phosphorus Loading Rate

HAc/P	Acetate To Phosphorus ratio
poly-P/MLVSS	The Polyphosphate Content Per Active Biomass Ratio
ISS	Inorganic Suspended Solids
PHV	Polyhydroxyvalerate
PH_2MV	Poly-beta-hydroxy-2-methylvalerate
3HV	3-hydroxyvalerate

Chapter One: Introduction

1.1 Background

Eutrophication is a critical global water quality challenge in lakes, rivers, and coastal waters, primarily caused by excessive nitrogen and phosphorus introduction to the environment from anthropogenic sources. Key sources include agricultural runoff combined with fertilizers, livestock manure, municipal sewage, and industrial effluents, entering the ecosystems via points sources as WWTP discharges and non-point sources as agricultural and urban runoff. This phenomenon triggers the overproduction of algae and aquatic plants, leading to harmful algal blooms and cyanobacteria. It also creates hypoxic “dead zones” by depleting the dissolved oxygen (DO), causing change on the long term in aquatic ecosystem structures and function (e.g. biodiversity loss) (Smith & Schindler, 2009).

Phosphorus introduction to waterbodies is caused by natural and anthropogenic activities. Without human-related activities, soil and rock erosion introduce phosphate into waterways, where its concentration depends on the type and quantity of rock in the area. On the other hand, agricultural and forestry runoff carrying excessive amounts of fertilizers and manure, urban expansion, municipal WW discharge, and industrial emissions contribute to the increase of phosphorus concentration in water and soil (ECCC, 2017).

The key approaches for phosphorus removal are physical, chemical and biological processes, or a combination of them. Enhanced Biological Phosphorus Removal (EBPR) has been extensively researched and implemented in full scale treatment units. EBPR,

considered as highly performative biological configuration, assimilates phosphorus in range of 0.06 to 0.15 mgP per mgVSS. Meanwhile at the conventional activated sludge, the heterotrophic bacteria remove the phosphorus, ranging around 0.02 mg P/mg VSS (Petriglieri et al., 2022).

Enhanced biological phosphorus removal (EBPR) is an engineered water resource recovery facility (WRRF) process configuration that can produce effluent P < 0.5 mg/L (Coats et al., 2018). The removal and treatment of phosphorus in wastewater streams have shifted from a localized pollution removal or control objective to a global strategic view in the light of circular economy. Phosphorus is a finite and nonrenewable resource essential component in fertilizers and essential for all forms of life; However, the increased discharge of phosphorus into water surfaces is the primary driver of eutrophication. Its recovery (e.g. Struvite) can transform it from a pollutant into valuable resource (Coats et al., 2011). EBPR process relies on selectively enriching a group of heterotrophic microorganisms known as phosphorus accumulating organisms (PAOs) in an alternating anaerobic/aerobic phase to store phosphorus as polyphosphates (poly-P) allowing its physical removal via sludge waste (Maurer, 1995).

The adoption of EBPR within a Sequencing Batch Reactor (SBR) configuration offers distinct advantages for modern WRRFs. The SBR is an activated sludge system where biological treatment and clarification occur within a single vessel through a time-bounded sequence of phases, this allows for precise control over alternating anaerobic and aerobic conditions required for PAOs enrichment. The metabolic shifts necessary for phosphorus removal and recovery are induced by integrating an anaerobic selector non aerated, mixed zone prior to aerobic reaction phase (Yeoman et al., 1988).

1.2 Research gap and Objectives

As phosphorus effluent limits grows stricter globally, chemical and physical treatments escalate in cost due to coagulant demands and hazardous sludge management, although offering compact footprints and reliable performance (Zhang et al., 2024). In contrast, EBPR provides a sustainable alternative but remains sensitive to influent variability (e.g. peak flowrates) and operational parameters, pushing the technology to be vulnerable to performance instability (Brown et al., 2022). Primary EBPR costs stem from aeration energy and strict process control for larger reactor volumes. Optimization frameworks increasingly prioritize land area as a core design objective alongside cost and carbon emissions, recognizing that treatment infrastructure demands substantial space which often constrains facility's location, treatment configuration, future expansion, and retrofitting (Becattini et al., 2022). Smaller footprints directly reduce hydraulic retention time (HRT) by limiting the reactor's basin volumes relative to fixed influent flows, compromising the biological process kinetics and removal performance. Moreover, wet weather causes high flow peaks where it drastically decreases HRT and dilutes the available carbon in the influent, causing EBPR failure.

HRT is a critical operational parameter influencing EBPR performance, microbial community structure, and process stability. Across literature, the applied overall HRT for SBR mode generally ranges from 4 to 12 hours with peak efficiencies observed within this range, with most systems operating in the 6 – 10 hours range. High rate SBR systems operating from (3.7 – 4.8 hours) have been successfully applied, however, excessively short HRT can cause a washout of key organisms (Roots et al., 2020). Membrane SBR systems showed high phosphorus removal at HRTs as low as 6 hours, with performance

improving as HRT decreased from 24 to 6 h (Xu et al., 2014). Conventional SBRs typically employ HRTs of 6 – 8 hours to optimize nutrient removal while ensuring process stability (Yea et al., 2022). On the other hand, extended HRTs between 12 to 18 hours are usually applied for in case of low strength or complex wastewater or start-up period (Yea et al., 2022).

The knowledge gaps related to anaerobic retention time persist, largely due to the overshadowing focus of research on overall EBPR design and aerobic zone performance. There is insufficient understanding of anaerobic zone in EBPR systems and its influence on the overall performance is yet unrecognized in the engineering practices. This thesis's goal is to systematically understand the metabolic and microbial respond under different anaerobic retention times. The thesis's main objectives are as follows:

1) Developing a comprehensive understanding of P/C influent ratio on EBPR performance by operating different phosphorus influent concentrations with constant carbon concentration.
2) Evaluating the role of polyphosphate content in anaerobic and aerobic kinetics.
3) Investigating the impact of anaerobic retention time on the hydrolysis kinetics and overall phosphorus removal by shortening and extending the anaerobic retention time.

1.3 Thesis layout

The thesis starts with Chapter One: Introduction, which introduces the research background, motivation, and the critical role of EBPR in sustainable phosphorus removal and recovery from wastewater.

Chapter Two: Literature Review presents a comprehensive literature review on phosphorus removal strategies, encompassing physico-chemical and biological methods, and evaluated their practical applicability in treatment systems. It delves into EBPR fundamentals, explaining the microbial communities, particularly phosphorus accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs), and their metabolic pathways for phosphorus release and uptake. The review further examines key operational parameters influencing EBPR efficacy, such as phase durations, carbon type, carbon-to-phosphorus (C/P) influent ratios, dissolved oxygen (DO) levels, and pH, while highlighting optimized conditions for enhance, stable performance.

Chapter Three: Materials and Methods details the laboratory-scale EBPR reactor setup, configuration, and operational conditions, including baseline and variable conditions tailored to the study's objectives. Analytical techniques for monitoring phosphorus profiles, microbial activity, and kinetics are described alongside calculation methodologies for performance metrics like removal efficiency and kinetic rates.

Chapter Four: Results and Discussion reports empirical findings on phosphorus removal dynamics and EBPR kinetics under varying influent phosphorus load rates, which altered the C/P ratio to optimize performance at short total HRTs. Following baseline optimization, the effects of reducing and extending the anaerobic retention times on PAO hydrolysis kinetics were investigated. Subsequent trials assessed reduced carbon source availability, demonstrating impacts on overall process stability.

Chapter Five: Conclusion and Future Directions synthesizes key findings, discusses broader implications for EBPR's basin scalability, addresses study limitations,

and proposes future research directions, including raw wastewater validation and advanced microbial analysis.

Chapter Two: Literature Review

2.1 Wastewater Characteristics

Wastewater is collected from various sources such as municipal, industrial, commercial, etc., and directed to WWTP, resulting in a complex influent with numerous constituents. The influent consists of organic materials, nutrients, inorganic materials, pathogens, and metals. The main function of WWTP is to remove the organic materials, nutrients, and pathogens to ensure safe disposal to water bodies. The influent characterization influences the WWTP process design and configuration, and its performance.

This thesis focuses on the Enhanced Biological Phosphorus Removal (EBPR) configuration to improve the phosphorus removal and optimize the operational conditions. The removal process depends mainly on various factors including but not limited to carbon availability, carbon type, and operational conditions and is easily inhibited by the presence of nitrogen. Thus, this section is dedicated to introducing the different characterizations of organic material, nitrogen, and phosphorus found in the wastewater influent.

2.1.1 Organic Matter

Due to the complexity of the organic materials present in the influent, the amount of oxygen required to oxidize the organics is used for quantification such as chemical oxygen demand (COD) or biochemical oxygen demand (BOD) which indicates the amount of dissolved oxygen (DO) needed for microorganisms to consume organic matter. Other methods of organic matter quantification are total organic carbon (TOC) or

measuring the oxygen uptake rates (OUR) (Metcalf & Eddy, 2014). However, COD is widely conducted for its simple usage and accuracy to access the WW treatment removal efficiency. Total COD (TCOD) is classified into two categories based on the biodegradability of the organic matter: biodegradable (bCOD) and non-biodegradable (nbCOD). These categories are additionally classified based on their solubility where the soluble fraction of the biodegradable COD is named readily biodegradable COD (rbCOD), while the slowly biodegradable particulate is sbCOD. The same categorization is applied on the nonbiodegradable COD for soluble (nbsCOD) and the particulate (nbpCOD) (Henze et al., 2008). The soluble COD (sCOD) main characterization is the ability to go through the filter of 0.45 μm diameter, the filtered COD is divided into particulate COD (pCOD) and colloidal COD (cCOD). It is worth mentioning that the sbCOD requires further breakdown via hydrolysis to be oxidized in a simpler organic form, following different hydrolysis kinematics based on the carbon chain type (Von Sperling, 2008). Flocculation and settling is needed for nbpCOD removal, while the nbsCOD is present in the effluent as it requires long residence time to form flocs and settle (Metcalf & Eddy, 2014). For the purpose of this thesis, synthetic wastewater was used as influent in the form of rbCOD where the selection rationale was explained in section 2.3.

2.1.2 Nutrients

Nutrients are essential for microorganisms and plants growth, acting as biostimulants. Aside from trace elements such as iron and sulfur, the main important nutrients are nitrogen and phosphorus. Phosphorus has a key role in aquatic plants and algae growth and overall productivity in freshwater environments, and nitrogen as in

ammonia and nitrates accelerates the process in poor nutrient rivers and coastal regions (ECCC, 2017).

Phosphorus is broadly classified as particulate and dissolved, subclassified into reactive and nonreactive in each fraction. Reactive phosphorus is the phosphorus form that does not require hydrolysis or oxidative digestion for colorimetric tests, while the nonreactive form does. The nonreactive forms are divided into acid hydrolysable or digestible. The dissolved forms of phosphorus consist of reactive orthophosphates, non-reactive polyphosphate, and nonreactive organic phosphate (Metcalf & Eddy, 2014). The loosely attached or adsorbed phosphates to precipitates are also considered as orthophosphates. The orthophosphates in the form of (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, H_3PO_4, etc..) are readily consumed by microbial metabolism without any further hydrolysis. Meanwhile, polyphosphates need hydrolysis to transform into orthophosphate form in aqueous solution as they are complex molecules of phosphorus and oxygen atoms, and in some cases hydrogen (Henze et al., 2008). Total phosphorus (TP) is used for to quantify the phosphorus in the WW influent, where orthophosphate (OP) contributes up to 90% of the total TP in the influent and the remaining portion consists of biodegradable and non-biodegradable organic phosphorus. Therefore, TP can be used interchangeably with OP to determine the removal efficiency and P concentration in WWTP. Phosphate (PO_4) is the abandoned form of phosphorus in biological organisms as it is used for cellular energy, DNA formation, and cell membrane (Metcalf & Eddy, 2014).

Meanwhile, nitrogen availability in wastewater treatment is essential for biological processes, as it is the building block for protein synthesis. The main sources of nitrogen in the water bodies are from the nitrogenous compounds coming from plants and animals,

atmospheric nitrogen, and mineral deposits of sodium nitrate (Orhon, 2015). The common nitrogen species of interest in WW are organic nitrogen, ammonia (NH_3), ammonium (NH_4), nitrite (NO_2), and nitrate (NO_3). The soluble and particulate nitrogen fractions in the influent are essential indicators to determine the WW treatability. Organic nitrogen is composed of variant compounds that could be soluble or particulate, such as amino acids, protein, and amino sugars. These compounds are readily transformed into ammonia via biomass processing in the aquatic and soil environment. Urea is also found in the WW influent and converts to ammonium carbonate (Rittmann & McCarty, 2001). Total nitrogen consists of organic nitrogen, ammonia, nitrite, and nitrate. The total Kjeldahl nitrogen (TKN) measures the total of NH_3 and organic nitrogen in the influent wastewater to account for the hydrolysis of organic nitrogen by the microorganisms giving NH_3 (Henze et al., 2008). It is worth noting that the existence of ammonium ion (NH_4^+) and ammonia gas (NH_3) depends mainly on the pH of the aqueous solution as shown below in the equilibrium equation (Metcalf & Eddy, 2014). The inhibitory effect of nitrogen species on phosphorus removal process was discussed in section 2.3.3.

2.2 Phosphorus removal techniques

Eutrophication and the excessive introduction of phosphorus into water bodies were the driving forces behind developing phosphorus removal technologies in the 1950s. Chemical precipitation was used as an initial removal method and remains to be the dominant approach till today. However, technologies such as biological phosphorus removal and crystallization have become well established and have reached the commercialization stage. Advanced chemical precipitation methods for enhanced nutrient removal have progressed beyond the pilot stage, while several other related

technologies are in different phases of development or under investigation (Morse et al., 1998).

2.2.1 Physico-Chemical Treatment

2.2.1.1 Coagulation

Chemical precipitation is fundamentally a physico-chemical process that involves adding divalent or trivalent metal salt to wastewater to react with dissolved phosphorus in order to form insoluble metal phosphates (Yeoman et al., 1988). These precipitates are then removed through sedimentation. The most commonly used metals for this process are iron and aluminum, typically introduced in the form of chlorides or sulfates; however, iron salts are generally preferred due to their lower cost (Wilfert et al., 2015). Alternatively, lime can be used to precipitate phosphorus as calcium phosphate. Anionic polymers may also be added to enhance flocculation and settling efficiency to improve solid-liquid separation (Morse et al., 1998). However, the iron phosphate presence in the biosolids decreases soluble phosphorus concentration and its overall availability in the treatment plant, especially if added in the primary clarification (Brandt et al., 2004). Generally, coagulant dosing in the treatment plant is carried out before the primary clarifier but may also be added directly to the aeration tank and prior to the secondary clarifier or applied before both the primary and secondary clarifiers where the Return Activated Sludge (RAS) is directed to the primary clarifier instead of the aeration tank (Yeoman et al., 1988). Other treatment plants may adopt the scheme of adding a mixer prior to the secondary sedimentation unit. The main drawbacks of chemical treatment are high sensitivity for pH change, increase of hazardous sludge production, and the presence of bicarbonate ions (HCO^{3-}) that compete with PO_4^{3-} over the coagulant, causing an increase of the chemical

dose requirements (Yeoman et al., 1988). Chemical sludge is directed to landfills, preventing the ability to recover materials as methane gas from the anaerobic digester or minerals, leading to a higher carbon footprint by 13% compared to biological treatments (Coats, Watkins, & Kranenburg, 2011). Yet, the soluble nonreactive phosphorus remains in the effluent due to its difficulty to remove by chemical and biological removal processes (Metcalf & Eddy, 2014).

2.2.1.2 Crystallization

Crystallization is a solid-liquid separation approach which is considered as an effective technique for phosphorus recovery in different aqueous phases. The dissolved phosphorus in the form of phosphate anions (solute) is converted into a solid crystalline phase via precipitation by using phosphorus containing salts (Hiroyuki & Katsutoshi, 2019). Governed by their solubility, seed crystals are introduced to the reactor, and the deposition occurs on the surface in a pH level that prevents new crystal production (Lu et al., 2017). The salts used in crystallization are calcium and magnesium based, which the selection between them depends on the relative concentration of phosphorus, ammonia, and potassium in the wastewater (Lu et al., 2017). The applicable crystallization technique for phosphorus removal is Drowning-out crystallization, where it achieves high product yield at ambient temperature. This technique relies on extracting the water from the salt solution or decreasing the salt solubility in water by adding organic solvent because of the insoluble chemical properties of phosphate in alcohol. The inorganic phosphate salt crystallizes from the oversaturated solution, then removed by filtration process. The solvent is recycled later via low pressure distillation column and re-fed to the

crystallization reaction. Inside the reactor, strong mixing is preferred to ensure efficient contact time between the water molecules and organic solvent (Bian et al., 2011).

2.2.1.3. Adsorption

Chemical adsorption is increasingly recognized as a promising approach for phosphorus removal due to its selectivity, low cost, simple design, and minimal by-product. This method not only provides phosphorus capture but also enables its subsequent desorption from the adsorbent bed, creating opportunities for material regeneration and recovery from wastewater (Ramasahayam et al., 2014). Adsorption is a process where atoms, ions, or molecules adhere to the sorbent solid surface from the bulk fluid by using chemical bonds or physical forces (Hussain & Mitra, 2012). The interaction happens at the adsorbent interface, creating a dimensional form. Meanwhile, desorption is the reverse process where the adsorbate (pollutant) is released back to the bulk fluid. The equilibrium between adsorption and desorption depends on the temperature, the sorbent saturation levels, surface area, and charge (Usman et al., 2022). The adsorbent material for phosphorus removal varies from metal oxides and hydroxides, furnace slag, fly ash, modified clay, activated carbon, and nanomaterials such as aluminum-modified biochar, etc. (Ramasahayam et al., 2014). Sorbent material made of recycled material from waste is extensively researched for lower carbon footprint and production cost.

Phosphate adsorption mechanisms are commonly investigated using electron microscopy and other characterization techniques to examine the morphology of both the adsorbent and adsorbate before and after adsorption. Additionally, FT-IR spectroscopy and related analytical methods are employed to assess how adsorption performance is

enhanced by analyzing the functional groups present on the adsorbent surface (Du et al., 2022). Overall, the dominant mechanisms governing phosphate adsorption are associated with the material's specific surface area, the nature of its functional groups, and physio-chemical interactions explained by the Lewis acid–base concept (Issaian & Reyes Cuellar, 2020). Activated carbon can also adsorb phosphate through Lewis acid–base interactions. In acidic conditions, phosphate ions act as weak bases and donate electrons, whereas in alkaline environments, they behave as weak acids and accept electrons. These properties enable electron donor–acceptor interactions between phosphate ions and the surface elements of activated carbon adsorbents (Usman et al., 2022)

2.2.2 Biological Phosphorus Removal

In full-scale WWTP, nutrients are typically removed by different integrated biological and environmental processes where the WW is subjected to anaerobic, aerobic, and anoxic conditions to encourage microbial activities such as phosphorus uptake and release, nitrification, and denitrification. Applying specific environmental conditions promotes the growth of microbial communities performing certain activities such as COD removal and nitrification in aerobic conditions and denitrification in anoxic phase. Phosphorus removal with >90% removal was first noticed in 1974 at a four-stage nitrogen removal system (Barnard, 2006). The required conditions for EBPR were better understood after implementing specific process configuration that applied sequential operational steps. The EBPR process depends on the enrichment and selection of polyphosphate-accumulating organisms (PAOs) that can store orthophosphate in excess of their growth requirements. The presence of readily biodegradable organic carbon

(rbCOD) and sufficient phosphorus concentration in the WW influent and applying sequential anaerobic and aerobic zone configuration ensures a successful EBPR system (Santos et al., 2020). However, achieving simultaneous nitrogen and phosphorus removal is more complex than simply adding an anaerobic zone to promote PAO growth. In many systems, nitrification and denitrification processes can negatively impact EBPR performance due to the presence of nitrate and nitrite introduced through internal recycling streams. When these electron acceptors enter the anaerobic zone, they disrupt the strictly anaerobic conditions required for PAOs, potentially promoting the growth of ordinary heterotrophic organisms (OHOs), which can outcompete PAOs and ultimately lead to process failure (Zheng et al., 2014).

The anaerobic degradation of complex organic carbon into the end products (methane and carbon dioxide) consists of multistep processes that involve four main stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The acidogenic phase includes hydrolysis, which includes the complex organic polymers breakdown into simpler soluble compounds. Then, these compounds are fermented into intermediate products, mainly short chain volatile fatty acids (VFAs) in the acidogenic phase. Subsequently, the VFAs and other intermediate products are converted into acetate, hydrogen, and carbon dioxide in acetogenesis phase. Finally, at the methanogenesis phase, the substrates are followed with methane and carbon dioxide transformation (Coats et al., 2018). Sufficient VFA concentration is essential to achieve consistent low phosphorus concentrations in the effluent. Acetate is commonly used as a substrate for the EBPR process. However, a mixture of VFAs, especially mixed with propionate, has shown process stability and performance enhancement (Shen et al., 2017). Influent

streams in WWTPs are often low in VFAs, necessitating either the external supplementation with commercially available VFAs or strategy implementation to boost VFA concentrations such as primary sludge fermentation (López-Vázquez et al., 2008a).

Biological phosphorus removal systems are generally classified into three categories: side-stream, main-stream, and cycling processes. A defining characteristic of side-stream configurations is that the return activated sludge (RAS) is only subjected to anaerobic treatment for additional supply of VFA (D. Wang et al., 2019). In contrast, main-stream systems expose the entire mixed liquor flow to anaerobic conditions to facilitate phosphorus removal. By diverting some or all of the RAS to an anaerobic side stream reactor, volatile fatty acids (VFAs) can be generated through hydrolysis and fermentation prior to re-introduction into the mainstream process. This approach helps mitigate VFA limitations in the influent and supports more stable phosphorus removal. This strategy underpins the Side-Stream EBPR (S2EBPR) process, in which a portion or total of the RAS or mixed liquor suspended solids (MLSS) is subjected to anaerobic fermentation (Wang et al., 2019). The fermented liquor is then recirculated to the main treatment line. Compared to conventional EBPR systems, S2EBPR offers several advantages, including reduced chemical demand, a smaller footprint, fewer odor issues, improved availability of carbon for denitrification, and compatibility with existing configurations without depending on influent carbon sources (Sabba et al., 2023).

For cycling systems, Sequencing Batch Reactors (SBRs) operate by utilizing multiple treatment stages occur sequentially within a single tank. A key advantage of SBR technology is that it does not require a RAS system, as both operation and settling take place in the same reactor. The process typically follows a series of defined phases: fill,

react, settle, decant, and idle. Sludge wasting is regulated to control the solids retention time (SRT), and in most SBR configurations, it is carried out during the reaction phase to ensure consistent and uniform sludge discharge (D. Wang et al., 2008).

2.3 EBPR Fundamentals

EBPR is a water resource recovery facility (WRRF) that enables the recovery of phosphorus from wastewater. The term WRRF has been recently recognized as a sustainable and resource focused which WW is perceived as valuable sources of various products, including biofuels, biopolymers, fertilizers, and other chemicals. Various technologies and configurations have been developed or revisited to improve economic performance through enhancing energy and recovery efficiencies, supporting their shift into WRRFs (Regmi et al., 2019).

2.3.1 Microscopic Level

Microbial storage provides microorganisms with a competitive advantage over those lacking capability, as it helps in substrate utilization during famine conditions. Specific microbial groups in the activated sludge were widely reported to store rbCOD intracellularly as insoluble polymers. The microbial storage was observed to be alternating in varying conditions between feast and famine cycles under different redox environments (Erdal et al., 2008). The stored products consist of polyhydroxyalkonates (PHAs), glycogen, and lipids which are characterized by high molecular weight (Metcalf & Eddy, 2014). It was suggested that these types of microorganisms store the mentioned products inside cytoplasm to decrease the accumulating osmotic pressure over the cell membrane during the substrate abundance as OHO’s ability for substrate acquisition

diminished (Carvalheira et al., 2014). Others suggested that it is necessary for microorganisms to balance between the elevated substrate transfer rate and decreased utilization rate by converting and storing the substrate (Zhang et al., 2024). Additionally, the absence of electron acceptor and nutrients availability pushes the microbes for intercellular storage to ensure stable growth during anaerobic/aerobic conditions (Q. Zhou et al., 2023).

2.3.1.1 PAOs identification

Polyphosphate-accumulating organism (PAO) is a gram-positive bacterium of polyphosphate storage granules making them Neisser-positive with size of 0.5 μm. The first identified PAO, *Acinetobacter*, was introduced over 30 years ago using culture-dependent techniques (Günther et al., 2009). Fuhs & Chen (1975) were the first to successfully isolate this strain, and for decades, it was widely believed to be solely responsible for phosphorus removal. With FISH probes development, *β-Proteobacteria* and *Actinobacteria* were detected to contribute majorly to P-removal compared to *Acinetobactor*. Additional evidence using respiratory quinones analysis, fluorescent antibody application, and 16s-rRNA technique indicated negative predominance of Acinetobactor in EBPR as assumed for decades (Seviour et al., 2003). Meanwhile, *Actinobacteria* exhibited different metabolic behavior from the PAOs' suggested pathway due to its lack of VFA uptake ability, despite their presence in EBPR system (Kong et al., 2005). Further studies have indicated the presence of *Planctomycetes and Bacteroides* alongside *β-Proteobacteria* and *Actinobacteria.* Other microorganisms such as *Mirolunatus Phosphovorus, Lampropedia, and Tetrasphaera* were identified as potential PAOs but proved to be incapable of PHA formation. *Tetrasphaera* strain displayed its

ability of denitrification, where all isolated clades reduce nitrate to nitric acid; however, only two clades can further reduce nitric acid to nitrous oxide (Marques et al., 2018). *Tetrasphaera* fermentation capability of complex organic molecules (acetic acid, sugars, etc..) supplies VFA requirements for other PAOs in anaerobic phase (Ruiz-Haddad et al., 2024). However, under anaerobic conditions, all *Tetrasphaera* clades lack the ability of PHA generation storage but can produce and store glycogen and release phosphate. Then, *Tetrasphaera* utilizes the stored glycogen to use the energy in polyphosphate production (Kristiansen et al., 2013).

Other PAOs species and the famous one is *Candidatus Accumulibacter* which belongs to *β-Proteobacteria* class of *Rhodocyclacea* family and divides into two major groups type I and type II (Izadi et al., 2020). Skennerton et al. (2015) identified ten different *Accumulibacter* genomes, hypothesizing that clades are favored according to different operational conditions due to the variations of nitrogen metabolic pathways; however, all clades exhibit identical carbon and phosphorus metabolism. The EBPR process contains a diverse community of microorganisms, with some dominant strains, such as *Dechloromonas β-Proteobacteria, Acinetobacter δ-Proteobacteria*, and *Candidatus Accumulibacter*, functioning with mutual interdependence with other communities (Izadi et al., 2021a).

2.3.1.2 GAOs identification

The presence of microorganisms of cocci shape in glucose or acetate feeding system was observed as they removed COD without contributing to phosphate removal. Mino et al. (1995) introduced Glycogen Accumulating Organisms (GAOs) that demonstrated similar metabolism with PAOs with the key difference of the energy source

type as GAOs assimilate internally stored glycogen as a reducing power. The other distinction is that PAOs are Neisser positive both within and outside of cell walls and polyphosphate granules, whereas GAOs are solely Neisser positive on cell walls. Since GAOs store PHA in the anaerobic phase so that it can oxidize in the aerobic phase for the generation and storage of glycogen, they compete with PAOs on VFA consumption. *Alphaproteobacteria, Betaproteobacteria*, and *Gammaproteobacteria* were culture isolated and assumed as potential GAOs, however, none of the mentioned microbial groups were found to dominate in either laboratory or full-scale systems experiencing performance deterioration (Liu et al., 1996). *Candidatus Competibacter Phosphatis* and *Competibacteraceae* family were identified and classified under *Gammaproteobacteria.* Other competitions with PAOs on the carbon source uptake were identified such as organisms belonging to *Sphingomonas, D. Vanus,*and *Micropruina (Nielsen et al., 2019).*

2.3.1.3 Extracellular polymeric substances (EPS)

Extracellular polymeric substances (EPS) refer to complex external microbial secretions, consisting of a wide range of macromolecules with proteins and polysaccharides as the main components. The main function of EPS remains unclear, but it provides carbon and energy in case of starvation conditions and facilitates floc adsorption. Additionally, it protects the cells from harsh environments such as the presence of toxic substances or dewatering conditions. EPS serves as an intermediate medium for immobilized exoenzymes used in hydrolysis (Long et al., 2021). The main characterization of EPS is high adsorptive capacity due to the presence of various functional groups, their hydrophobic nature exhibited in some parts of EPS, and their high protein content. EPS are known to electrostatically bind with positive ions as they are

negatively charged. EPS are generally categorized into tightly bound EPS (TB-EPS) and loosely bound EPS (LB-EPS) (Long et al., 2017). External factors such as the reactor's operational conditions, nutrients content, sludge age, and microbial community impact the EPS quantity and composition. Furthermore, the proteins to polysaccharides (PN/PS) ratio can significantly impact EPS adsorption ability as it is considered as an indicator for EPS quality as well as the ratio between TB-EPS and LB-EPS (TB/LB) (Zhao et al., 2019).

Recently, research focused on the TB-EPS role not only as an intermediate in phosphorus transfer between the bulk liquid and the cells but also in their ability to accumulate and store poly-P and orthophosphate as they hold a substantial fraction of sludge phosphorus (Li & Xi, 2019). It was also suggested that poly-P hydrolysis occurred intracellularly and extracellularly, driving orthophosphate accumulation in cells and EPS. Aerobically, EPS drives the required energy for poly-P regeneration from intercellular PHA oxidation (Long et al., 2021). Almost 30% of orthophosphate at the end of aerobic phase in a granular system was observed to be stored in EPS with similar fractions of K^+, Mg^{2+}, and Ca^{2+}(R. Wang et al., 2014). Furthermore, around 85% of total phosphorus in the biofilm SBR was accumulated in EPS, where the PAOs cell provided the main energy sources and drivers, but EPS played the main role in absorption and release (Y. Chen et al., 2022). The main edge of EPS is the presence of abundant functional groups such as carboxyl, phosphate, amino, and hydroxyl, giving strong phosphate adsorption/complexing capability (Huang et al., 2022).

2.3.2 Metabolism

In anaerobic conditions, polyphosphate serves as internal energy source that contributes to adenosine triphosphate (ATP) generation through direct catalysis of

polyphosphate kinase. It transfers the phosphate group to adenosine diphosphate (ADP) to have ATP through polyphosphate degradation as ATP regenerates by the coupled action of adenosine monophosphate (AMP), phosphotransferase and adenylate kinase, finally the ATP synthesis is achieved by the arising proton motive force (pmf) (Müller et al., 2019). The pmf is a chemiosmotic gradient that occurs due to the efflux of uncharged metal-phosphate complexes and associated protons at the cytoplasmic membrane of the microorganism cell wall (Le et al., 2021). The produced ATP per released phosphate is estimated to be approximately 0.33 because of polyphosphate hydrolysis. The main drivers of the pmf are the electrical potential produced from the internal and external charge difference at the cell membrane, and the pH gradient caused by the proton released during the ATP synthesis and the cytoplasm alkalinity (Comeau et al., 1986). Additionally, metal release is accompanied by orthophosphate release, where potassium (K^+) and magnesium (Mg^{2+}) were observed to be released at 1 mg per 3 mg of released phosphorus. The released phosphate from the cell consists of metal-phosphate complex, predominantly with Mg^{2+} and K^+, and symports with hydrogen ions across the cell membrane by secondary phosphorus transport system, leading to an increase of bulk concentration of Mg^{2+} and K^+. Based on stoichiometry, the ratio of K^+:P is 0.22 mol:mol and 0.32 mol:mol for Mg^{2+} :P ratio (Coats et al., 2011) .

Furthermore, PAOs anaerobic metabolism undergoes the tricarboxylic acid cycle (TCA) pathway or the glycolysis pathway as a reducing power depending on external and internal factors such as VFA and glycogen availability (Thomson et al., 2025). Lanham et al. (2013) suggested that full scale systems exhibiting glycolysis activities demonstrated more efficient phosphorus removal over the TCA cycle. Energy generation pathway by

transforming glycogen to pyruvate follows Embden Meyerhof (EM) or Entner Doudoroff (ED) pathways. For PAOs, glycolysis usually undergoes the EM pathway to break down glucose to produce 2 ATP, 2 Nicotinamide Adenine Dinucleotide (NADH), and 2 pyruvates to be further converted to acetyl-CoA in the aerobic phase.

The main energy consuming activities the PAOs undertake are the substrate diffusion into the cell, cellular maintenance, and the synthesis of polyhydroxyalkanoates (PHAs). PAOs require ATP and reducing power to assimilate rbCOD, generate and intracellularly store PHA as an energy-rich source by reducing the glycogen via glycolysis or a partial TCA cycle through glyoxylate pathway. The complete TCA cycle generates reduced flavin adenine dinucleotide ($FADH_2$); however, the cycle does not proceed further in the anaerobic phase due to the absence of a terminal electron acceptor (Ruiz-Haddad et al., 2024). The bulk phosphorus concentration increases over time due to the accumulation of released phosphate from the intracellular polyphosphate. Meanwhile, GAOs exhibit similar metabolism to PAOs which internal glycogen hydrolysis in anaerobic zone takes place for energy production and power reduction to uptake the VFAs and convert them to PHAs. The deterioration performance of EBPR due to the inefficient phosphorus removal is linked to GAOs assimilation for substrate, leading to competition between PAOs and GAOs on the VFAs (Acevedo et al., 2012a).

In aerobic phase, PAOs metabolize the internal PHAs in the absence of an external carbon source to restore glycogen reserve, uptake the aqueous orthophosphate for polyphosphate generation, and support cell growth and maintenance. The aerobic phase consists of six reactions: 1) PHA degradation, 2) glycogen replenishment, 3) phosphate transfer, 4) cell synthesis, 5) cell maintenance, and 6) phosphorylation (Izadi

et al., 2021a). The phosphorus uptake under aerobic conditions is highly dependent on phosphorus release rate and PHA production in the anaerobic phase as insufficient bulk concentration of phosphorus hinders the polyphosphate generation in aerobic phase. Additionally, lower polyphosphate in the anerobic phase leads to a decreased PHA generation due to lack of energy. The phosphorylation follows the PHA degradation which the reaction efficiency is controlled by the amount of generated energy in form of ATP per the amount of NADH utilized (Vargas et al., 2009). The produced ATP is used in cell growth of an estimated 1.5 mol of ATP per Carbon mole of biomass. The phosphate transport is coupled with the energy produced by proton intake and the following release due to the PHA oxidation across the cell membrane. Accordingly, the amount of adsorbed phosphate into the cell membrane is directly linked to NADH consumption (Smolders et al., 1994). On the contrary, GAOs follow a similar metabolic pathway but fail to store orthophosphate as polyphosphate, hence not contributing to the phosphorus removal. Although the literature emphasizes GAOs kinetic advantage in the anaerobic phase, the aerobic conditions play a critical role in PAOs and GAOs competition (Erdal et al., 2008).

The type of electron acceptor characterizes the metabolic phase into aerobic phase with presence of oxygen or anoxic phase with the nitrate and nitrite presence. In the anoxic zone, PAOs are able to utilize substrate using nitrite and nitrate as electron acceptors instead of oxygen, calling the microorganism denitrifying PAOs (DPAOs) (S. Li et al., 2023). Nevertheless, a subset of PAOs can utilize oxidized nitrogen as an electron acceptor, pushing the other fraction that is unable to do so to release phosphate and uptake carbon (Sun et al., 2015). Therefore, the total phosphorus removal under anoxic conditions depends mainly on the relative abundance of DPAOs and their metabolic

activity. More recently, PAOs were classified into two main types: Type I and Type II with clades of IA-E and IIA-G for the two types, respectively. Clade IA is recognized for its ability to highly reduce nitrate due to the presence of *nar* gene, while clade IIA uses solely oxygen as electron acceptor (Kapagiannidis et al., 2013a). Additionally, a representative of clade IC, *Candidatus Accumulibacter delftensis*, demonstrated phosphate uptake only under aerobic conditions. For the nitrite as a terminal electron acceptor, research focused on the inhibitory role of nitrite instead of its potential for energy production (Vargas et al., 2011). Metagenomic studies observed that *Accumulibacter* clade IIA possesses nitrite reduction pathway gene, suggesting that only DPAOs use necessary enzymes to nitrate and nitrite as electron acceptors, while other PAOs are restricted to aerobic metabolism (Zeng et al., 2016).

2.3.3 Operational Conditions

EBPR process implements sequentially anaerobic and aerobic phases on the sludge to favor the growing conditions of Phosphorus Accumulating Organisms (PAOs) over the heterotrophic microorganisms. The removal takes place by uptaking the phosphorus for PAOs metabolic activities, then, the enriched phosphorus sludge is wasted. During the anaerobic phase, PAOs consume VFA produced mainly from the fermentation of sludge microorganisms or the influent's readily biodegradable organic materials. The main competitive edge for PAOs growth in anaerobic phase is utilizing the substrate to produce and store intracellular energy-rich polymers by oxidizing their stored polyphosphates, leading to orthophosphate release (L. Chen et al., 2022). The absence of electron acceptors as oxygen prevents the heterotrophic bacteria from substrate consumption. In the aerobic phase, the stored polymers are metabolized for cell

maintenance and growth, and glycogen production. The released energy from oxidation is used to produce new polyphosphate bonds for cellular storage by uptaking the released orthophosphate from the anaerobic phase. The biomass absorbs higher phosphate levels from the liquid than being released in the anaerobic phase, causing a net phosphorus removal in the system (Metcalf & Eddy, 2014).

2.3.3.1 Carbon source Type and loading

The carbon source type (e.g. acetate, propionate) feeding the system impacts directly the anaerobic and aerobic metabolism of PAOs and GAOs. Generally, the most common VFA in domestic wastewater are acetic and propionic acids. The rbCOD shortage in the influent causes EBPR instability and failure, which is considered the main barrier to achieving stable PAO performance. Domestic WW consists of 5-25% of VFAs and 40-75% of BODs, depending on the organic content found in the wastewater source (Miao et al., 2018). Acetate as a carbon source is intensively used in most EBPR focused research, limiting the understanding of propionic acid impact on PAOs and GAOs (Zhang et al., 2007).

Under anaerobic conditions, the total PHA production per unit of utilized carbon and the composition of stored PHA products and the PHA fraction in the microorganism differ depending on the type of carbon source. The PHA products are classified into poly-β-hydroxybutyrate (PHB), PHV and PH_2MV (Zhang et al., 2025a). Evidence observed that *Accumulibacter*-PAO has the ability to utilize acetate and propionate at similar efficiency and kinetic rate at a higher pH value around 8.1 (C. Zhang et al., 2007). Other evidence reported that propionic acid produced PHV which is less effective in EBPR process. Yet, Oehmen et al. (2005) observed that *Accumulibacter*-PAOs exhibited higher activity

utilizing propionate compared to the acetate-fed system, as the P-release per C uptake rate ratio was 0.41 for acetate and 0.58 for propionate. However, acetate has a higher substrate uptake ratio than propionate, with values of 0.023 mmol-C/g-VSS and 0.017 mmol-C/g-VSS, respectively (Pijuan, Baeza, et al., 2004).

Meanwhile, *Competibacter*-GAO competes with *Accumulibacter*-PAO on acetate, as it displayed a similar uptake rate; however, the propionate uptake rate becomes negligible. It is worth mentioning that *Alphaproteobacteria*-GAO demonstrates different patterns from *Competibacter*-GAO, as their uptake rate declines by 50% for acetate, yet it becomes similar to *Accumulibacter*-PAO's propionate uptake rate. *Alphaproteobacteria*-GAO are favored in propionic acid-fed systems over *Competibacter*-GAO (Majed & Gu, 2019).

Other VFA with more than three carbons (e.g. butyrate and valerate) were investigated as they are present in municipal wastewater with low concentrations as 3.5-6.7% of total VFA (Yun et al., 2013). Butyrate was investigated as the sole source of external carbon. Phosphorus removal efficiency experienced no change with values of 96-99%; however, a microbial shift was observed from *Accumulibacter*-PAO of 37.4% abundance in acetate-fed system to *Rhodocylaceae*-PAO of 14.5% abundance (L. Wang et al., 2021). The P-release/C-uptake ratio of Butyrate (0.22 mol-P/mol-C) is lower than the values of acetate of 0.32 mol-P/mol-C and propionate of 0.27 mol-P/mol-C due to the slower rate of butyrate consumption with a substrate uptake rate of 0.004 mmol-C/g-VSS (Pijuan, Baeza, et al., 2004). Other research studied non-VFA options to demonstrate the PAOs versatility metabolism, such as glucose, glycerol, and alcohol. Although glucose can be utilized by most microorganisms as rbCOD, it was suggested that PAOs are

unable to digest glucose without prior hydrolysis. On the other hand, GAOs utilize glucose to store intercellular glycogen without contributing to phosphorus removal in EBPR (Majed & Gu, 2019). Glucose was observed to exhibit a lower P-release/C-uptake ratio compared to acetate and propionate of value 0.053 mol-P/mol-C (Pijuan, Baeza, et al., 2004). For relatively longer SRT, PAOs abundance in the biomass decreased 27% compared to GAOs increased activity and proliferation when using glucose as a carbon source (Zengin et al., 2010). Meanwhile, methanol has been studied as an alternative for external carbon source supplementary in WRRF due to its affordability and high biodegradability compared to VFAs. However, it resulted in poor anaerobic P-release (7.2 mg/L) compared to acetate (17.0 mg/L) and propionate (18.2 mg/L) (Puig et al., 2008). However, ethanol has demonstrated stable and high phosphorus removal (~99.9%) with P-release/C-uptake ratio of 0.41 compared to 0.50 value of acetate (Puig et al., 2007).

Carbon to phosphorus (C/P) ratio in the influent has been observed to influence the performance and stability of EBPR process. Available rbCOD in the influent must meet the minimum stoichiometric requirements, typically ranging between 10-20 mg-C/mg-P to maintain consistent and effective phosphorus removal in EBPR (Nadeem et al., 2022). Yet, higher rbCOD/P ratio between 40 to 50 mg-C/mg-P observed to associate with GAOs abundance. The recommended ratio of rbCOD/P ranges between 15 to 25 mg-C/mg-P (Majed & Gu, 2020a). Ma et al. (2005) reported a linear increase in phosphorus removal efficiency with rising rbCOD/P ratios up to 32 mg COD/mg P, beyond which efficiency declined within the 40-50 range. In anaerobic phase, higher carbon loading increased intercellular PHA concentrations (Coats et al., 2021); however, the increase in carbon concentration created phosphorus-limiting conditions, where the remaining COD was not

assimilated at the end of the anaerobic phase. Excessive residual carbon in aerobic phase leads to less PHA oxidized, and the biomass utilizes the excessive resource for cell growth and maintenance, causing a lower phosphorus removal rate (Izadi et al., 2021c).

Conversely, polyphosphate (poly-P) capacity-limiting conditions prevailed when bulk phosphorus limitation was absent under excessive carbon (Yagci et al., 2003). Excessive influent phosphorus concentration (low rbCOD/P ratio) was observed to increase biomass poly-P content and contribute to higher effluent phosphorus concentration (Schuler & Jenkins, 2003a; Welles et al., 2017). The increased poly-P content in the biomass positively influences the carbon uptake and phosphorus release rates up to a threshold of 0.2 mg P/mg VSS, beyond which these kinetics declined (Welles et al., 2017).

Overall, selecting appropriate Carbon sources relies on the system performance, financial feasibility, and the response of microbial community which yields various outcomes because of process configuration and operational conditions (Aghilinasrollahabadi et al., 2024).

2.3.3.2 pH

pH is another essential operational parameter impacting the EBPR process. pH impacts the P-release per VFA uptake ratio and polyphosphate intracellular consumption in anaerobic phase. Proton and ion are generated as the VFA is diffused in the cell wall, causing a proton motive force (pmf). Thus, pH increase causes higher phosphorus release as the ion-proton equilibrium is disturbed (Izadi et al., 2020). PAOs are pushed to

break polyphosphate bonds by releasing orthophosphate to achieve stabilization. Evidence showed the PAOs competitive edge over GAOs when pH values were above 7.25, reaching maximum acetate uptake rates at a pH value of 8 with a decline in glycogen degradation (Schuler & Jenkins, 2002). It is worth mentioning that the PAOs are sensitive to pH in aerobic conditions, while GAOs are tolerant to the change (C. Zhang et al., 2007).

Furthermore, pH influences the rate of substrate consumption as Zhang et al. (2007) demonstrated in their research on the impact of pH on the PAOs and GAOs while using different carbon sources. The enhancement of propionic acid uptake was achieved in high pH values where the anaerobic contact time significantly decreased from 120 minutes to 30 minutes to fully utilize the supplied substrate when the pH was shifted from 6.4 to 8 as the average uptake rate increased by 95.6% for PAOs.

However, Filipe et al. (2001) observed that pH increase had a neutral impact on the anaerobic contact time for acetate uptake for PAOs but negatively influenced the GAOs as the required energy for acetate transport increased when their saturation was reached. Zhang et al. (2007) confirmed that the acetic acid uptake reached 80% in the first 20 minutes of the anaerobic cycles at different pH values for PAOs systems. In GAOs systems, the acetic and propionic uptake rates are inversely impacted by the pH change under anaerobic conditions as the amount of utilized glycogen and PHA accumulation per acetate uptake increase when the external pH decreases (Filipe et al., 2001a)

2.3.3.3 Contact Time

Conventional BNR systems generally consist of anaerobic zone to cultivate PAOs with typical HRT of 0.5 to 2 hours, anoxic zone for denitrification with HRT ranging from 1 to 4 hours, and aerobic zone for nitrification and phosphorus uptake with 4 to 12 hours

(Zhang et al., 2016). Anaerobic contact time is a critical factor for phosphorus removal where PAOs store the PHA as energy reserves for future use in the aerobic phase. Short anaerobic HRT (~ 0.5 hours) leads to developing anoxic conditions where the phosphorus uptake in aerobic zone will be hindered due to insufficient energy reserves (Izadi et al., 2020). Additionally, shorter anaerobic HRT (< 1 hour) in full-scale facilities provides insufficient time for complete carbon utilization, causing aerobic phosphorus release due to the presence of residual COD. The aerobic phosphorus release forces the operators to increase aerobic HRT to avoid high effluent concentrations (He et al., 2008). Anaerobic HRTs ranging 1 to 1.5 hours were investigated, and no EBPR deterioration observed where the phosphorus release rate decreases at longer anaerobic HRT. The P-release/VFA uptake ratio reached around 1.27 – 1.40 at 1 h HRT, where it significantly decreased to 0.55 in the 1.5 h HRT system (Izadi et al., 2021c).

The common risk associated with prolonged anaerobic HRT is VFA depletion which can trigger a phenomenon called “secondary phosphorus release” (Wouters-Wasiak et al., 1996). It was observed that bulk phosphorus concentration during the anaerobic phase increases specifically after rbCOD depletion where PAOs continue hydrolyzing polyphosphate bonds. Consequently, the imbalance between the hydrolyzed polyphosphate and stored VFA in form of PHA causes reduced overall phosphorus removal due to the insufficient PHA to be oxidized in the aerobic phase (Coats, et al., 2011). Additionally, Coats et al. (2021) observed anaerobic P release without PHA synthesis, suggesting that some of the VFAs are metabolized via TCA cycle to generate NADPH for energy production, cellular maintenance, and to support PHA synthesis, and not directly channeled to PHA synthesis only. The biomass mainly undertakes internal

reactions called endogenous processes during starvation conditions when the external supplied substrate depletes. The endogenous process consists of various mechanisms such as cell maintenance, cell lysis, and regeneration (Lobos et al., 2005). For the anaerobic phase, PAOs undergo maintenance metabolism by degrading polyphosphate under starvation conditions. However, biomass decay via cell lysis and subsequent oxidation takes place in the aerobic phase with a decay rate of 0.15/d as PAOs lose their ability for maintenance due to the absence of PHAs, causing phosphorus release (Lopez et al., 2006a). However, Lu et al. (2007) investigated the starvation impact on PAOs during anaerobic, anoxic, and aerobic conditions and observed the decay rate in the aerobic starvation conditions to be 0.03/d at 22˚C. Under both anaerobic and anoxic conditions, polyphosphate and glycogen were the main energy sources for maintenance till glycogen reached critically low levels. For aerobic conditions, PAOs depend solely on glycogen in the absence of PHA till full consumption but do not utilize polyphosphate for energy production as happening in anaerobic and anoxic conditions (Lu et al., 2007).

Nevertheless, complete phosphorus removal in a longer anaerobic HRT of 3 hours was observed, where the removal took a shorter time, suggesting that the aerobic kinetics were enhanced (Coats et al., 2011). Other literature reported deteriorated phosphorus removal when the anaerobic retention time was extended from 2 hours to 2.5 hours as the *Accumulibacter*-PAO population decreased, where the anaerobic retention time shapes the microbial population (Y. Wang et al., 2013).

Furthermore, research investigated the impact of introducing intermittent anaerobic stage in the aerobic phases to simultaneously enhance nitrogen and phosphorus removal. By introducing two stages of 50 minutes of anaerobic phase in a

200-minute aerobic stage, VFA were fully consumed compared to continuous aeration system, where residual VFA were found at the start of the aerobic phase in the continuous system. Additionally, the PHA generation per VFA uptake ratio was observed to be lower for the continuous reactor (1.18) compared to the intermittent reactor (1.43), consequently, the phosphorus removal efficiency was higher in the intermittent system by 8% (Izadi et al., 2021b).

2.3.3.4 Dissolved Oxygen (DO) Levels

Phosphorus removal is accomplished in the aerobic phase where the net total of influent phosphorus and released phosphorus in the anaerobic phase is taken up. In the aerobic phase, the PAOs utilize the phosphorus by forming polyphosphate bonds with the energy released from oxidizing PHA and carbon (Acevedo et al., 2012). Phosphorus in the bulk liquid is also consumed for cell synthesis and maintenance. However, EBPR performance is unsustainable due to the unpredictable instability and unreliability of PAOs, causing violation of discharge regulations. External factors such as heavy rainfall, a spike of nitrate concentration in the anaerobic zone, or excessive aeration of the biomass contributed to unstable performance or system failure (López-Vázquez et al., 2008). Excessive aeration drives up operational cost and energy consumption, substantially amplifying the overall carbon footprint of EBPR systems. In conventional WWTPs, 45-75% of the operational cost is due to aeration (Rosso et al., 2008). Meanwhile, excessive aeration challenges the sludge to settle well, opposing the main characteristics of PAOs presence with densely settling flocs (Bruce, 2015). Thus, investigating the external factors is necessary to lower the phosphorus levels introduced

to the water bodies and enhance the cost-effectiveness of EBPR compared to other phosphorus removal technologies.

The competition between PAOs and GAOs arises when the DO concentration in the system increases due to excessive aeration. GAOs compete with PAOs on the availability of carbon sources as they exhibit similar metabolism due to its proliferation under sequential anaerobic and aerobic conditions. Furthermore, in over-aerated zone, the PHA stored content is depleted leading to minimum phosphorus uptake (Brdjanovic et al., 1998). The incomplete phosphorus uptake in the aerobic zone negatively impacts the PAOs ability to consume acetate in the next anaerobic cycle because of the decline in polyphosphate regeneration that is used as an energy source to assimilate acetate and form and store PHA (Guisasola et al., 2004). Thus, GAOs overcompete with the PAOs in the biomass by uptaking the unconsumed substrate.

On the other hand, insufficient DO concentration hinders the metabolization process of PHA products and subsequently the polyphosphate regeneration as the released energy from the oxidation is limited (Metcalf & Eddy, 2014). Lower DO concentrations between 0.1 – 0.6 mgO_2/L caused incomplete P-uptake and glycogen consumption to compensate for the limited oxidation energy in the system. Glycogen utilization was associated with the cell's necessity of maintenance under challenging oxygen conditions (Carvalheira et al., 2014). Typically, PHA degradation provides the required energy for cell maintenance during the aerobic phase after the completion of phosphorus uptake and glycogen stabilization (Carvalheira et al., 2014). However, EBPR operation with low DO levels of 0.8 mgO_2/L was successfully achieved, as the PAOs have the edge in lower DO levels (Izadi et al., 2021c).

The abundance of GAOs was observed at higher DO levels ranging from 5 to 8 mgO_2/L, while the PAOs dominated at lower DO levels of 2.5 – 3.0 mgO_2/L, accompanied by high phosphorus removal. According to Izadi et al. (2021), at a DO level of 4 mgO_2/L, the P-release per VFA uptake ratio decreased while the glycogen concentration increased to 5.3 mmol/gVSS and glycogen degradation rate surged from 0.78 to 1.1 mmol/gVSS, suggesting high GAO population as the glycogen is the main reducing power. Carvalheira et al. (2014) investigated the aeration impact on the PAOs and GAOs fractions in the biomass, where *Accumulibacter*-PAOs population increased from 71 to 90% when the DO concentration was lowered from 8 to 2 mgO_2/L, while *Competibacter*-GAOs population was reduced from 20% to <1%. In the anaerobic phase, the P-release per VFA uptake ratio increased significantly when the system was operated at 2 mgO_2/L and the PHA production rate improved by 72% at the end of the phase (Carvalheira et al., 2014).

2.3.3.5 Solid Retention Time (SRT)

Phosphorus removal in EBPR process depends mainly on absorbing the aqueous phosphorus in the bulk liquid into the sludge, and the removal happens when the sludge is discharged. During discharge process, SRT is an essential operational parameter to estimate the amount of sludge withdrawal, yet its impact on the phosphorus removal performance is contradictory. Initially, long SRT was presumed to give PAOs an advantage to dominate because of the lower decay rate that PAOs exhibit compared to GAOs and OHOs. However, recent evidence indicated lower biomass yield for PAOs and reported high competition between GAOs and filamentous bacteria causing sludge bulking (Li et al., 2008; Zhu et al., 2013).

Recently, research investigated the impact of the short SRT on phosphorus removal when Roots et al. (2020) shortened the SRT to 1.8 days and achieved 40% phosphorus removal. The removal is suggested to be attributed to assimilation due to the higher solid generation rate and lower endogenous decay rate, leading to increased removal efficiency. Yet, the ratio between the assimilated phosphorus and produced solids exceeds the theoretical values ranging from 0.015 to 0.027 mg-P/mg-VSS (Truong, 2021). The minimum SRT to avoid PAOs washout was indicated by Valverde-Pérez et al. (2016) to be 3 days, as the system exhibited 66% phosphorus removal, yet the removal efficiency increased by 23% when the SRT increased to 3.5 days. Furthermore, AlSayed et al. (2024) observed no EBPR pattern of P-release in anaerobic phase and P-uptake in aerobic phase at short SRT (< 1 day) although the lab scaled system achieved 63% of overall removal. However, EBPR activities were only induced when the anaerobic retention time in the secondary clarifiers was extended despite the short SRT value ~ 0.56 day (AlSayed et al., 2024).

2.3.3.6 Nitrate and nitrite presence as inhibition factors

Most conventional EBPR configurations include pre-anoxic denitrification, which causes residual nitrate concentration in the effluent and subsequently remains in the RAS stream (Zheng et al., 2014). The presence of nitrate, introduced by RAS, in the anaerobic zone compromises EBPR performance by shifting anaerobic conditions to anoxic. Elevated RAS nitrate concentration is one of the most frequently reported EBPR failure causes (Guerrero et al., 2012). Although nitrate has been researched as an alternative electron acceptor utilized by DPAOs to reduce oxygen requirements compared to the conventional configuration, evidence indicates that the presence of nitrate inhibits

phosphorus release in anaerobic conditions. The inhibition may be triggered by OHOs' increased activity in consuming the substrate and competing with PAOs due to the presence of electron acceptor. Another explanation is that P-uptake happens simultaneously with P-release as the electron acceptor and donor are available in the same phase (Guo et al., 2018). One of the solutions addressing the issue is positioning pre-anoxic zone upstream of the EBPR anaerobic phase, as adopted in the Johannesburg Process and the Westbank Process. Denitrification in the mentioned configuration is governed by endogenous decay or by raw wastewater or fermented primary solids liquor introduction. Nitrite is also considered as an inhibitor for phosphorus removal in EBPR process. Nitrite serves as an intermediate compound formed during nitrification and denitrification, that can be later oxidized to nitrate. The accumulation of nitrite with the free nitrous acid causes GAOs to compete with PAOs, disrupting the phosphorus removal efficiency (Izadi et al., 2020).

Chapter Three: Materials and Methods

3.1 System configuration

The used experimental setup was Sequential Batch Reactor (SBR) system which consisted of two bioreactors of 2 liters, four peristaltic pumps where each two were for influent and effluent, two air pumps, two air flow control, two magnetic mixing plates, timers to operate the cycles, DO sensors to monitor the DO concentration, and pH sensor, 20 L feeding tank, and effluent tank with volume 20 L as well.

The influent was pumped through a fixed tube at the top of the reactor, meanwhile the discharge tube for the effluent was placed at 1600 ml level from the top. It is worth mentioning that the total and active volumes of both reactors was 2000 ml. Wastage volume was 400 ml and took place at the end of the settling phase for 15 minutes. Peristaltic pumps were used to maintain 26.7 ml/min of influent and effluent flow rates. Furthermore, air pumps supplied air to the reactors through air diffusers to generate small to medium-sized air bubbles which were placed at the reactor's bottom to ensure saturation while mixing. The air pump was connected with tubes and air flow controller to reach and maintain DO concentration inside the bioreactor. The DO probe placed at reactor's mid-level was used to calibrate the air flow controller to supply the air at certain concentrations and to monitor the DO levels during the experiment. Figure 3-1 demonstrates the schematic diagram of the set up.

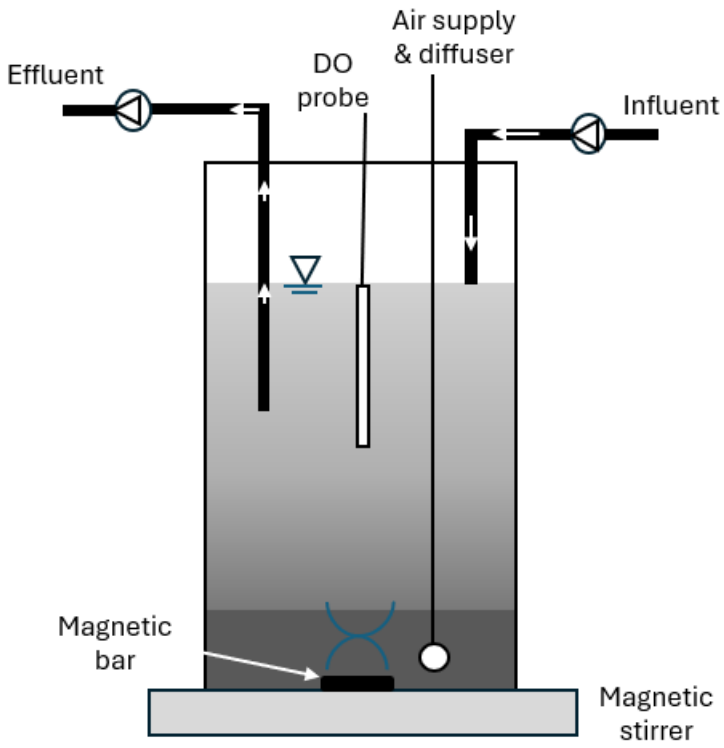

Figure 3-1: Schematic diagram of the SBR configuration

SBR reactors were operated with 6-hour cycle with four cycles per day. The cycle is comprised of feeding, anaerobic phase, aerobic phase, settling, and decanting. The duration of anaerobic and aerobic phases was variable based on this thesis objective; however, the overall cycle duration was maintained at 6 hours total. The following figure shows the duration of each phase in the cycle for different cases. A control device was programmed with the cycle schedules and connected to the influent and effluent pumps, stirrers, and air pumps to implement the different phases of the cycle through on-off switching.

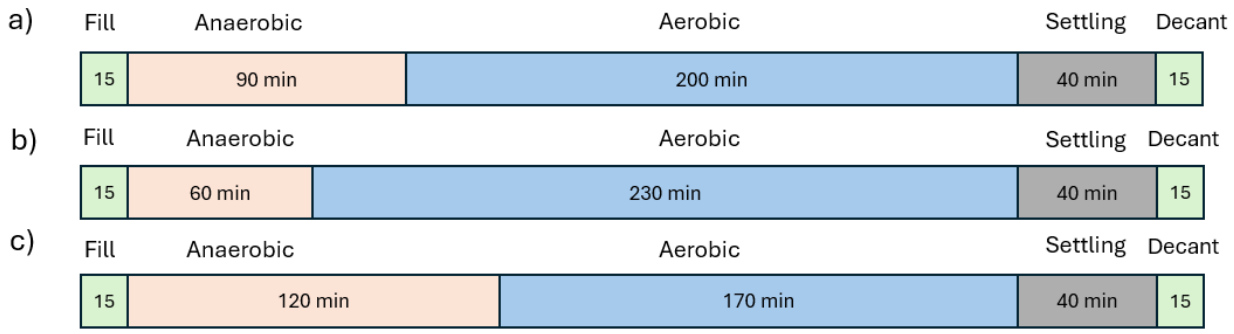

Figure 3-2: SBR cycle phase breakdown for different experimental scenarios to achieve the thesis objectives

Continuous mixing was applied during the feeding, anaerobic, and aerobic phases only to distribute the substrate evenly and ensure uniform concentrations of substrate and oxygen inside the reactor. A magnetic stir bars were used for mixing around 300 – 350 rpm. There was no mixing during the settling and decanting to retain the biomass inside the reactor and minimize its washout, meaning that the effluent discharge composed of the supernatant.

3.2 Operational parameters

Operational conditions such as temperature and pH were uncontrolled but monitored where the temperature was kept around room temperature of 23.3 ± 0.3˚C and pH was neutral (7.1± 0.2). It is worth mentioning that the temperature experienced minimal fluctuations due to the laboratory's centrally regulated HVAC system. The solid retention time (SRT) was controlled by allowing the biomass to settle down and discharging the supernatant where it was regulated around >10 days. Other parameters such as HRT and DO were controlled as discussed as the following.

3.2.1 Hydraulic Retention Time (HRT) – parameter control

The flowrate of the effluent and influent, cycle duration and volume exchange ratio controlled the values of hydraulic retention time (HRT), where the volume exchange ratio was consistently 20%. The system's configuration was sequential batch reactor, operating with different phases during a cycle. However, the duration of anaerobic and aerobic phases was changed to evaluate the impact of anaerobic HRT on the removal performance. The system's HRT and the anaerobic and aerobic HRTs were calculated using **equation 3-2**.

$$HRT = \frac{V_{reactor}}{Q_{inflow}} \qquad \textit{Equation 3-1}$$

$$HRT_{anaerobic\ or\ aerobic} = HRT\,\frac{t_{ana\ or\ aero}}{t_{cycle}} \qquad \textit{Equation 3-2}$$

$\boldsymbol{Q}: the\ daily\ flow, \boldsymbol{V}: active\ volume, \boldsymbol{t_{cycle}}: cycle\ duration\ excluding\ settling\ and\ decant$

In this thesis, the HRT conditions were considered as 90 mins of the anaerobic phase and 200 mins of aerobic phase which was adopted from Izadi et al. (2021) and referred as baseline HRT conditions. The baseline HRT operational condition was applied in "**Phase One**" and "**Phase Two**" to evaluate the impact of the influent phosphorus concentration on the EBPR performance. Then, the operational conditions, which referred to "**Phase three**", were changed to decrease the anaerobic phase duration to be 60 mins and increased the aerobic phase duration by 30 mins to become 230 mins to maintain the cycle duration of 6 hours. The other operational conditions "**Phase four**" were extending the anaerobic phase duration to be 120 mins and shortening the aerobic phase

to be 170 mins. Table 3-1 demonstrates the different duration phases with their corresponding HRT.

Table 3-1: The anaerobic and aerobic durations with the equivalent HRT

	Anaerobic duration	**Aerobic duration**	**Cycle time (t_c)**	**HRT_{ana}**	**HRT_{aero}**	**HRT_{ana}/HRT_{aero}**
				(hours)		
Phase 2 (Baseline)	1.5	3.33	5.08	0.37	0.82	0.71
Phase 3	1	3.83		0.25	0.94	0.26
Phase 4	2	2.83		0.49	0.70	0.71

3.2.2 Dissolved Oxygen (DO) – parameter control

Aeration was only supplied in the aerobic phase as the DO concentration started to drop in the settling, decanting, and filling phase to reach 0.07 mgO_2/L in the anaerobic phase. During the aeration phase, the DO concentration started to increase till it reached a constant value at equilibrium state. The DO concentration was controlled by air flow valves and flowmeter. During all phases of the experiment, the DO concentration was operated above 2.50 mgO_2/L and maintained under 4.00 mgO_2/L to ensure PAOs favoring conditions (H. Li & Chen, 2011).

3.2.3 P/C influent ratio – Parameter Control

The phosphorus concentration in the influent was also subjected to changes in the baseline scenario exclusively. In phase 1, the phosphorus concentration was kept around

12.88 ± 3.10 mg P/L where P/C ratio was around 3:80; Then, it was lowered to approximately 1:80 by decreasing the influent phosphorus concentration to 6.80 ± 0.50 mg P/L in phase 2. The P/C ratio of 1:80 was carried to phase 3 and phase 4. After comparing the EBPR performance for phase 2, 3, and 4, the P/C ratio was increased in phase 5 by lowering the COD concentration to be 191 ± 28 mg/L and maintaining the influent phosphorus concentration at 6.80 ± 0.50 mg P/L which resulted in the P/C ratio to be 1:40 mg P/ mg COD. The P/C influent ratio was also calculated in terms of COD as carbon metric as seen in **equation 3-3**.

$$P/C\ ratio\ = \frac{P_{influent}}{TCOD_{influent}} \qquad \textit{Equation 3-3}$$

3.2.4 Summary of Operational Parameters:

Table 3-2 summarizes the operational parameters for both reactors in the five phases (Phases 1-5). The experiment was designed to evaluate the reactors' performance and removal efficiencies under varying HRT and influent concentrations. Phase 1 and 2 utilized two SBRs referred to as R1 and R2, then the bioreactors were split to be used in phase 3, 4 and 5. The table covers different anaerobic and aerobic HRT conditions yet maintaining the total HRT. Furthermore, the ratio of anaerobic to aerobic duration HRT_{ana}/HRT_{aero} was also included. The table also shows the varying influent characteristics for phosphorus and COD concentration.

Evaluating the impact of P/C ratio on EBPR was achieved by applying increased P_{inf} concentration in phase 1 then reducing it in phase 2 by creating polyphosphate capacity-limiting conditions. Then, the anaerobic retention times in phases 3 and 4 were

shortened and extended, respectively, to understand the influence of the contact time on anaerobic hydrolysis kinetics and overall performance. Finally, phase 5 experienced lower COD influent concentration and applied to the R1 operating at HRT_{ana} = 60 mins and R2 operating at HRT_{ana} = 120 mins to investigate the potential in optimizing EBPR performance.

Table 3-2: Summary of Operational Parameters

Phase no.	Reactor	Notation	HRT_{ana}	HRT_{aero}	HRT_{ana}/HRT_{aero}	P_{inf}	$TCOD_{inf}$
			(Minutes)			mg/L	
Phase 1	R1	SBR11	90	200	0.45	15	400
	R2	SBR12	90	200	0.45	15	400
Phase 2	R1	SBR21	90	200	0.45	5	400
	R2	SBR22	90	200	0.45	5	400
Phase 3	R1	SBR31	60	230	0.26	5	400
Phase 4	R2	SBR42	120	170	0.71	5	400
Phase 5	R1	SBR51	60	230	0.26	5	200
	R2	SBR52	120	170	0.71	5	200

3.3 Cultivation and influent characteristics

Waste activated sludge (WAS) from Humber wastewater treatment plant in Ontario, Canada was used to seed the reactors. The EBPR conditions of anaerobic and aerobic phases were applied to enrich the sludge with PAOs. After the system reached performance stability by observing the releasing phosphorus in the anaerobic phase and uptaking it in the aerobic phase, data were considered after SRT of 7 days which agrees with the literature (Izadi et al., 2020).

The system was supplied by synthetic wastewater which was composed of organic material, nutrients and trace metals. Concentrated stocks of organics, different nutrients, and trace solutions were prepared and stored at 4˚C which further diluted with the deionized water to reach the influent concentrations as stated in Table 3-3.

Table 3-3: Influent wastewater composition and concentration

Components	Concentration (mg/L)
Stock Solution	
Na-acetate.3H_2O	80,000
NH_4Cl	5600
$K_2HPO_4.3H_2O$	3100
Trace Solution	
$FeCl_3.6H_2O$	1500
H_3BO_3	150
$CuSO_4.5H_2O$	30
KI	180
$MnCl_2.4H_2O$	120
$NaMoO_4.2H_2O$	60
$ZnSO_4.7H_2O$	120
$CoCl_2.6H_2O$	150
EDTA	10,000
Influent Characterization	
COD	400 – 200
NH_3 – N	25
PO_4 – P	5-15

The feeding tank was filled with deionized water and spiked with the stock solutions to reach the targeted influent concentrations. The used equation to calculate the needed amount from the stock solution to add in the feeding tank is as follows.

$$C_{stock}V_{stock} = C_{target}V_{tank} \quad \text{Equation 3-4}$$

$$\boldsymbol{C_{stock}}: stock\ concentration, \boldsymbol{V_{stock}}: stock\ volume,$$

$$\boldsymbol{C_{target}}: Feed\ Concentration, \boldsymbol{V_{tank}}: Tank\ Volume$$

The chosen carbon source for the whole experiment was acetate as it provides the highest substrate uptake rate as discussed in Chapter two. The equivalent COD from the acetate was calculated based on the redox reaction below where sodium acetate trihydrate dissolves into acetate and sodium ions. Then, the needed amount of acetate to reach the targeted COD concentration in the feed was calculated. The stock solution was prepared to be ×200 concentrated on the amount needed per filling. To maintain the targeted concentration in the feed to supply nutrients, the number of free ions of the used stock solution was calculated in order to prepare the stock solutions. Furthermore, chemical analysis for COD, phosphorus, and ammonia were conducted to determine the actual concentration values.

$$CH_3COONa.3H_2O_{(s)} \rightarrow CHCOO^-_{(aq)} + Na^+_{(aq)} \quad \text{Equation 3-5}$$

$$2CHCOO^-_{(aq)} + 2.5\ O_2 \rightarrow 2CO_2 + H_2O \quad \text{Equation 3-6}$$

$$COD_{eq} = \frac{(\#of\ moles \times\ molecular\ weight)_{O_2}}{(\#\ of\ moles \times\ molecular\ weight)_{CHCOO^-}} = 0.70\ \ gCOD\ /\ gCHCOO^-$$

3.4 System operation, maintenance and sampling

Two identical systems, as shown in Figure 3-3, were running at the same time, supported by one air pump with a splitter and different calibrated influent and effluent

pumps feeding from a feed tank and discharging to a waste tank. Each peristaltic pump was manually calibrated by measuring the amount of pumped liquid at certain amount of time, 15 minutes in our case. The peristaltic pump's head is translated into rpm values because it functions as positive displacement through compressing and releasing flexible tubing in a circular motion. The pump's head is impacted by the pump's location relative to the reactor (potential head), the tube configuration whether there is a bent angle or not, and the tube's length. The tubes were regularly cleaned to remove any growing biofilm and avoid partial clogging to ensure that the targeted volume was consistently pumped. The used pump model was Longerpump attached with pump head model YZ1515X with compatible pumping tube of size #18.

Figure 3-3: Two identical bioreactors with total of four used pumps

The two systems were connected to a control device timed with identical cycle schedule with the same phase durations. The control device switched on or off the peristaltic pumps, air pump, and the stirrer. The stirrer started to work at the beginning of the cycle to mix the settled sludge with the influent during the feeding phase and kept working during the reaction time in the anaerobic and aerobic phase. For the air pump, it

was programmed to work only at the beginning of the aerobic phase and shut down when the settling phase started to avoid any biomass suspension and to deplete the oxygen concentration to reach almost zero for the anaerobic phase in the next cycle.

As mentioned before, the feed was prepared by diluting the stock solutions of carbon, phosphorus and ammonia, where a feeding tank of volume 20L was filled with deionized water. Before filling the tank, it was disinfected by adding diluted chlorine disinfectant with tap water to avoid any microbial growth in the tank that may flourish in the presence of carbon and nutrients and alter their concentrations. Trace solution was added with ratio of 1 ml trace solution per 1 liter of filled volume. After filling the feeding tank with synthetic wastewater, a rigorous mixing was applied to ensure uniform concentration, then a random grab sample was taken to be analyzed to check if the different feed concentrations match the target. Additionally, the systems' tubes were regularly disinfected by dismantling them from the bioreactors and pumping diluted chlorine disinfectant solution to avoid algae and biofilm growth. Furthermore, the bioreactors were covered by aluminum sheets to prevent algae growth and dominance in the sludge or to be attached to the walls. Another maintenance procedure was scrapping the bioreactor walls especially with high SRT values, due to the accompanied increase level of filamentous bacteria in the sludge. The probes and air diffusers were consistently cleaned to prevent biofilm growth and ensure that the probe readings were accurate, and the diffusers were unclogged to deliver the targeted oxygen concentration.

Samples were periodically taken to monitor the systems' performance. Sample collection plan differed from one phase to another as demonstrated in Table 3-4, but the common collection point was collecting fresh effluent from the bioreactor at the end of the

settling phase or at the start of the decant phase. All samples were randomly grabbed from the bioreactor with volume of 25 ml for the effluent samples and 14 ml for the anaerobic phase sample. In phase 1, one sample was collected at the end of anaerobic phase, and one after the settling phase started by 10-15 minutes to represent the effluent. Then, after applying baseline and experimental conditions in phase 2, 3, and 4, three sampling points in the anaerobic phase were collected. The samples were stored in a refrigerator at 4˚C to be further processed and analyzed.

Table 3-4: Sampling frequency and time from the start of the anaerobic phase

	Experiment Scenario		
	Phase 2	Phase 3	Phase 4
Sample No./frequency per cycle	Collection time (mins)		
1	30	20	40
2	60	40	80
3	90	60	120

3.5 Analytical methods

All collected samples from the two bioreactors and the effluent were consistently analyzed to measure total phosphorus (TP), soluble COD (sCOD), total COD (tCOD), total suspended solids (TSS) and volatile suspended solids (VSS). Samples from the feed were regularly analyzed to determine the concentration of COD and TP, hence no further preparation for the sample to measure COD as the used carbon source was sCOD. Ammonia was occasionally measured in the feed to monitor its concentration. For TP and ammonia, HACH testing kits and HACH DR3900 spectrophotometer (HACH, Loveland,

CO, USA) were used for analysis and measurement. Furthermore, Total solids (TS) and volatile solids (VS) were measured for the inoculum from Hamber WWTP before seeding the two bioreactors. All samples were vigorously mixed immediately before analysis to ensure homogenous concentration for accurate and representative results.

3.5.1 TSS and VSS

The total suspended solids analysis characterizes all the unevaporated organic and inorganic suspended solids having a diameter >1.5 µm. First, a filter and aluminum plate were put in 550˚C furnace for five minutes to eliminate any organic residual for accurate measurements, then were weighted. Volume of 10 ml from the sample was added and filtered using filter paper. Samples collected from the anaerobic phase were × 10 diluted by adding 1 ml of the sample to 9 ml of deionized water, however effluent samples were not diluted. The aluminum plate and filter with the trapped particles were dried in 105˚C oven for two hours and weighed afterwards. The TSS value was calculated using **equation 3-7**. For VSS analysis, the aluminum plate and filter with the sample were further dried at 550˚C for 20 minutes to evaporate all the organic constituents, then weighed and calculated by **equation 3-8**.

$$TSS = \frac{W_2 - W_1}{V} \qquad \textit{Equation 3-7}$$

$$VSS = \frac{W_3 - W_2}{V} \qquad \textit{Equation 3-8}$$

$\boldsymbol{W_1}: weight\ with\ no\ sample\ after\ 550℃, \boldsymbol{W_2}: weight\ with\ sample\ after\ 105℃$

$\boldsymbol{W_3}: weight\ with\ sample\ after\ 550℃, \boldsymbol{V}: added\ sample\ volume$

3.5.2 Total phosphorus (TP)

High range molybdovanadate TP HACH reagent set of range 1.0 - 100.0 mgPO^{-}_{4}/L was used to measure TP concentration of the feed, anaerobic, and effluent samples. First, the sample was filtered by using a syringe and filter of 0.45 µm, then 5 ml was added to the test tube. Potassium persulfate Powder Pillows were added, and the test tube was vigorously mixed and added to the HACH DRB200 digital digester at 150˚C for 30 minutes. After the test tube cooled down, 2 ml of Sodium hydroxide solution (1.54 N) and 0.5 ml of Molybdovanadate Reagent were added. The spectrophotometer was used to measure TP concentration after 7 minutes of adding the reagents.

3.5.3 Total and soluble COD

High range COD digestion vials from VWR Chemicals were used to measure the TCOD and sCOD. The only difference between TCOD and sCOD analysis was filtering the sample using 0.45 µm filter and a syringe for sCOD, and 2 ml was added to the test tube afterwards. After vigorously mixing, the test tube was added to the digester at 150˚C for 120 minutes. Then, the concentration reading was taken by the spectrophotometer.

3.5.4 Ammonia

Salicylate Ammonia reagent HACH test kit of range 0.4 – 50.0 mg NH_3/L was used to measure the ammonia concentration for the stock solution and feed. The sample first was filtered by a syringe and 0.45 µm filter, then 0.1 ml was added to the test tube. Ammonia cyanurate powder and sequentially ammonia salicylate powder were added to the test tube. The readings were taken after 20 minutes by the spectrophotometer.

3.6 Further Calculations

Various parameters were computed using the results from the periodic sample analysis to evaluate and monitor the system performance. The relevant parameters such as mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were used to characterize the reactor's biomass. System's characterization parameters as SRT, HRT, carbon and nutrient removal efficiencies, loading rates, and P release and uptake rates were calculated.

SRT was calculated using **equation 3-9** where the solid concentration (X) was determined by TSS values. The only wastage happening in the sequential batch system is the discharged effluent. The effluent solid concentration ($X_{effluent}$) was determined by averaging the TSS values through daily effluent collection. The $X_{reactor}$ was represented by the sample points collected from the reactor during mixing.

$$SRT = \frac{X_{reactor} \times V_{reactor}}{X_{effluent} \times Q_{effluent}} \qquad \textit{Equation 3-9}$$

$$SRT_{ana} = SRT \frac{t_{ana}}{t_c} \qquad \textit{Equation 3-10}$$

$$\boldsymbol{Q_{effluent}}: effluent\ flow\ rate, \boldsymbol{X}: solids\ concentration\ as\ TSS, \boldsymbol{V}: active\ volume$$

The production rate, as shown in **equation 3-11**, was computed using the TCOD value from the effluent and the reactor's VSS change during an interval step of one day, assuming that the biomass in the reactor represented all the particulates. Meanwhile, the substrate utilization rate was also calculated as the mass flowrate change of COD from

the influent TCOD and sCOD from the filtered effluent. Then, the observed yield (Y_{obs}) was calculated using the **equation 3-13** by computing the solid production rate and the substrate utilization rate.

$$P = TCOD_{eff} \times Q_{eff} + (\frac{(X_{r,t1} - X_{r,t2}) \times V_{reactor}}{\Delta t} \quad \textit{Equation 3-11}$$

$$U = (TCOD \times Q)_{influent} - (sCOD \times Q)_{effluent} \quad \textit{Equation 3-12}$$

$$Y_{obs} = \frac{P\ (M\ COD/T)}{U\ (M\ COD\ /T)} \quad \textit{Equation 3-13}$$

$\boldsymbol{P}: solid\ production\ rate, \boldsymbol{U}: substrate\ utilization\ rate, \Delta \boldsymbol{t}: time\ interval\ difference$

The substrate uptake rate was calculated to assess the metabolic activities, using **equation 3-14**, where the PAOs biomass concentration (X_{PAO}) was assumed to be all the active biomass measured by VSS analysis which represents 90% of the VSS value as the synthetic wastewater was used (Metcalf & Eddy, 2014). Furthermore, $P_{release}$ rate in the anaerobic phase and the P_{uptake} rate in the aerobic phase were computed using **equation 3-15** and **equation 3-16**, respectively, by calculating the phosphorus concentration change during an interval of time. For $P_{release}$ rate, the chosen time interval for the anaerobic phase was third of the anaerobic duration where samples were collected as demonstrated in Table 3-3 and analyzed to get TP concentration. Meanwhile, the total aerobic phase duration was considered in **equation 3-16** to calculate the change of phosphorus concentration from the TP concentration at the end of anaerobic phase and in the effluent. The value of specific phosphate release rate ($q_{p\text{-}release}$) and specific

phosphate uptake rate (q $_{p\text{-}uptake}$) were determined by solving **equation 3-15** and **equation 3-16**, respectively.

$$r_{substrate\ uptake} = \frac{d[S_{COD}]}{dt} \qquad \textit{Equation 3-14}$$

$$r_{P-release} = \frac{d[S_P]}{dt} = q_{P-release} X_{PAO} \qquad \textit{Equation 3-15}$$

$$r_{P-uptake} = -\frac{d[S_p]}{dt} = q_{P-uptake}\, X_{PAO} \qquad \textit{Equation 3-16}$$

$\boldsymbol{q_{s,max}}: maximum\ specific\ substrate\ rate, \boldsymbol{S}: substrate\ concentration, \boldsymbol{K_s}: half\ saturation\ constant$

$\boldsymbol{q_{P-release}}: specific\ phosphate\ release\ rate, \boldsymbol{q_{P-uptake}}: specific\ phosphate\ release\ rate$

The Polyphosphate concentration in PAOs was calculated by **equation 3-17** which was developed by Brdjanovic et al. (1998) with the assumption of no chemical precipitation and the solid of the effluent were negligible. The ratio of non-poly-P phosphorus per VSS (f $_{P,b\ VSS}$) was estimated to be equivalent to P-content of non-EBPR biomass equals to 0.052 mg P/mg VSS.

$$Poly - P = (TSS - VSS) - \frac{5}{95} \times VSS \qquad \textit{Equation 3-17}$$

$\boldsymbol{Poly - P}: polyphosphate\ concentration, \boldsymbol{P}: TP\ concentration, \mu: microbal\ growth\ rate,$

To understand the system's removal efficiency, $COD_{removal}$ (%) was calculated as influent mass flowrate percentage in **equation 3-18**, and $P_{removal}$ (%) was computed as the overall removal percentage from the influent as shown in **equation 3-19**. Additionally, the P removal through non-EBPR biomass assimilation was calculated using **equation**

3-20, where it considered the biomass yield of ordinary heterotrophs (Y_{hetro}) and the endogenous decay rate (b), and the biodegradable fraction of new biomass (f_d).

$$COD_{removal}\,\% = \frac{(TCOD \times Q)_{inf} - (TCOD \times Q)_{eff}}{(TCOD \times Q)_{inf}} \qquad \textit{Equation 3-18}$$

$$P_{removal}\% = \frac{(P \times Q)_{inf} - (P \times Q)_{eff}}{(P \times Q)_{inf}} \qquad \textit{Equation 3-19}$$

$$P_{assimilation} = \frac{0.0267 \times Y_{OHO}(1 + (1 - f_d)b \times SRT)(TCOD_{inf} - sCOD_{eff})}{1 + b \times SRT}$$

Equation 3-20

$\boldsymbol{Y_{OHO}}$: $OHO\ Biomass\ yield$, $\boldsymbol{f_d}$: New biomass biodegradable fraction

$\boldsymbol{b}$: Endogenous decay rate, $\mathbf{P}_{\boldsymbol{assimilation}}$: $assimilated\ phosphorus\ concentration$

3.7 Statistical Analysis

For this thesis, SigmaPlot 12.5 (Systat Software Inc., Bangalore, India) was used for all statistical analyses. Among these studies were the determination of means, standard deviations, and the Pearson correlation coefficient (R). Additionally, the data were compared using t-test and the confidence interval was computed using 0.05 p-value.

Chapter Four: Results and Discussion

4.1 The impact of Phosphorus Loading on EBPR performance

Two lab-scale SBRs were operated for four six-hour (4 x 6 hr) cycles per day applying an anaerobic-aerobic pattern to ensure the dominance of PAOs. For the five phases, SBR1 and SBR2 were kept at a total HRT of 4.8 h (HRT_{ana} = 90 min, HRT_{aero} = 200 min) with a SRT of 11.8 days, without considering the potential loss of solids during regular cleaning. In phase 1, the two systems were fed with relatively high phosphorus concentration (12.88 ± 3.10 mg P/L), operating with phosphorus loading rate (PLR) of 8.59 mg P/L/h and acetate to phosphorus (HAc/P) influent ratio of 32.38 ± 6.00 mg COD/mg P. The EBPR performance was unstable in SBR11 and SBR12 as seen in Figure 4-1 where the overall P-removal efficiency for SBR11 and SBR12 were 28.33 ± 11.10 % and 20.95 ± 9.21 %. The EBPR's deterioration was coupled with low phosphorus release and uptake where the specific $P_{release}$ rates in SBR11 and SBR12, respectively, were 2.18 ± 0.37 and 2.73 ± 0.73 mg P/g VSS/h and the specific P_{uptake} rates were 0.74 ± 0.65 and 0.75 ± 0.61 mg P/g VSS/h.

In phase 2, the influent phosphorus ($P_{influent}$) concentration was lowered to 6.80 ± 0.50 mg P/L, operating with a PLR of 4.51 mg P/ L/h and HAc/P influent ratio of 56.80 ± 5.16 mg COD/ mg P. The total specific $P_{release}$ rates for SBR21 and SBR22 were 4.83 ± 1.03 and 4.87 ± 1.34 mg P/g VSS/h, respectively. The specific $P_{release}$ rates were higher in phase 2 by 121.6% and 78.4% for SBR21 and SBR22, respectively. Meanwhile the total specific P_{uptake} rates were 2.76 ± 0.61 and 2.95 ± 0.68 mg P/g VSS/h resulting in an

overall P-removal of 79.67 ± 10.30% and 85.75 ± 9.39% for SBR21 and SBR22, respectively.

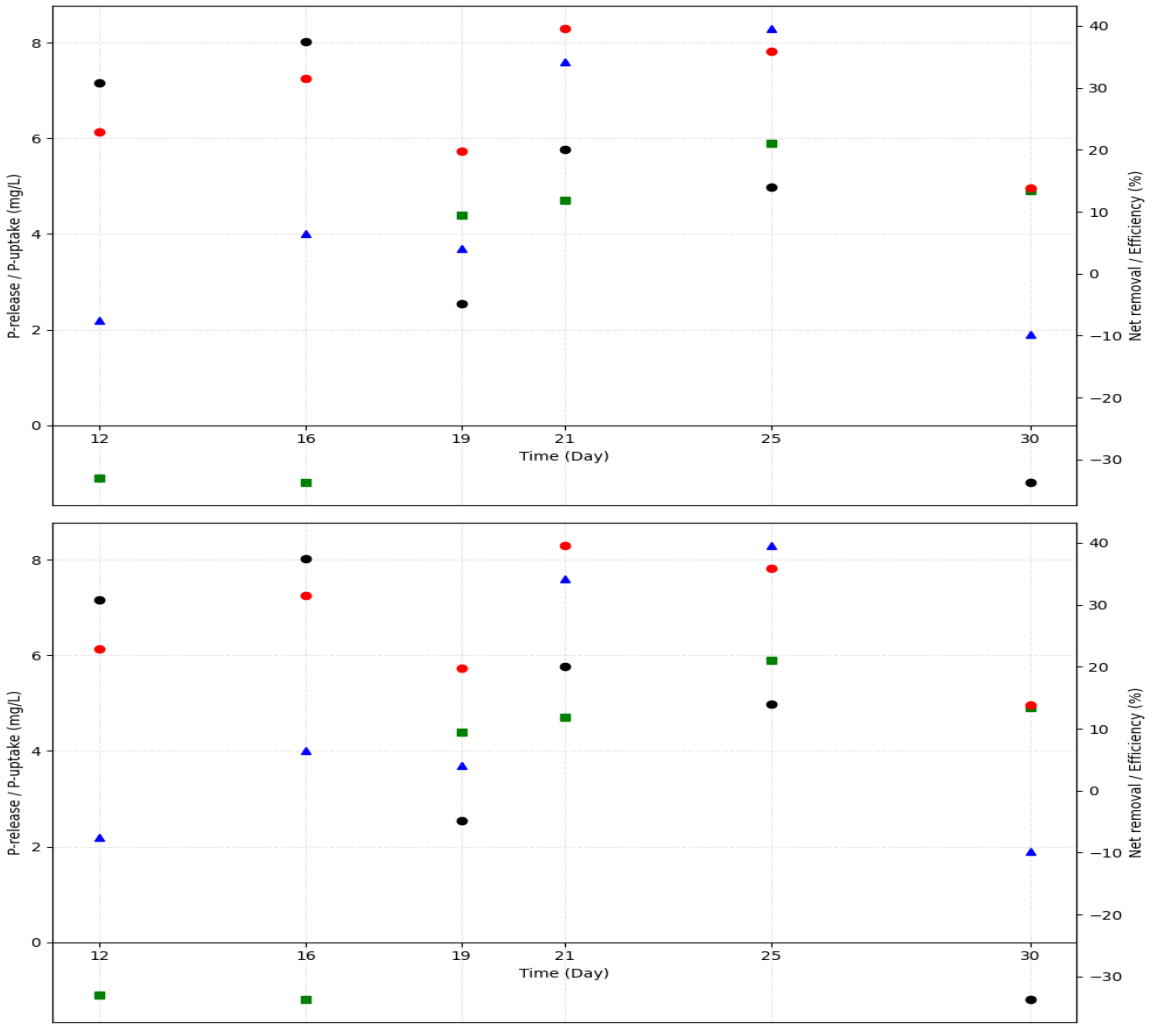

Figure 4-1:EBPR performance profile over the experiment run for a) SBR1 and b) SBR 2 for total P-removal (● black), P-uptake (Δ blue), P-release (□ green), and P-removal efficiency (● red)

Phase 1 exhibited poor P-removal efficiency which dramatically increased when the PLR was reduced by 47.5% in phase 2, despite operating the system on the C/P influent ratio recommended by Aghilinasrollahabadi et al. (2024) and Barnard (2006),. Lower PLR values shifted the system towards phosphate limiting conditions where the available carbon was stored as PHA products in the anaerobic phase and later oxidized in the aerobic phase to uptake the available P. In contrast, increasing the PLR value made

phosphorus capacity a limiting factor, implying different stoichiometry for carbon uptake and $P_{release}$ and consequently for P_{uptake} rates. The biomass phosphorus content influenced the stoichiometry of phosphorus release to acetate uptake (P_{rel}/HAc_{up}) ratio where it was lower from the reported values in literature as the polyphosphate content per active biomass ratio (poly-P/MLVSS) increased (Schuler & Jenkins, 2003a; Welles et al., 2017). Additionally, PHV and PHB production per acetate uptake ratios (PHV/HAc and PHB/HAc), and PHV/PHB production rate ratio were noted in literature to decrease when the poly-P/MLVSS increased (Welles et al., 2017). This finding was also observed when the PHA production per HAc uptake ratios was reduced while increasing the influent phosphorus concentration (Yagci et al., 2003). Consequently, lower PHA levels in the aerobic phase limit the phosphorus uptake (P_{uptake}) and glycogen regeneration, decreasing the reducing power reserves available for the subsequent anaerobic phase. As a result, anaerobic kinetics are impaired due to insufficient glycogen to support VFA uptake and PHA synthesis (Brdjanovic et al., 1998; Welles et al., 2017).

The inorganic suspended solids (ISS) ratio to the TSS is considered as an indicator to the sludge's phosphorus content; a higher ISS/TSS ratio corresponds to a greater phosphorus concentration stored in the biomass (Carvalheira et al., 2014; Izadi et al., 2021c; Petriglieri et al., 2022). As reported by Welles et al. (2017), the poly-P/MLVSS ratio increases when the ISS/TSS ratio is increased by elevating the influent phosphorus concentration. This aligns with our findings ($R^2 = 0.673$) which show that Poly-P/MLVSS ratio was higher in phase 1 than in phase 2 as the $P_{influent}$ increased, as shown in Figure 4-2a. Additionally, the lower Poly-P content was accompanied by the increase of $P_{release}$ Figure 4-2b and P_{uptake} Figure 4-2c suggesting an increased P_{rel}/HAc_{up} ratio. The

relationship between Poly-P content and $P_{release}$ as shown in Fig 2b indicates an inhibitory effect on the anaerobic phase. Meanwhile in the aerobic phase, Poly-P content inversely impacted the P_{uptake} (Figure 4-2c); the uptake rate decreased when the Poly-P increased, indicating lowered stoichiometry of PHA oxidation which caused higher $P_{effluent}$ concentration. As indicated by Brdhanovic et al. (1998), the P_{uptake} depends not only on the biomass PHA pool but also on the maximum Poly-P storage capacity. Consistently, P_{uptake} did not respond directly to the changes of influent P/C ratio (Figure 4-2d). As described by the literature, PHA is the sole source of reducing power and carbon for rebuilding Poly-P bonds by consuming phosphorus from bulk liquid, whereas glycogen is used for cellular maintenance and growth (Metcalf & Eddy, 2014). Therefore, the increase in P_{uptake} suggests the elevation of PHA accumulation during the anaerobic phase.

Theoretically, the EBPR performance mainly depends on the amount of VFAs utilized in the anaerobic phase in relation to the energy availability which translates to the polyphosphate reserve in the system. Thus, P_{rel}/HAc_{up} ratio is considered an indicator of GAO presence and hence EBPR success. In phase 1, SBR11 and SBR12 exhibited extremely low P_{rel}/HAc_{up} ratio of values 0.014 and 0.022 mmol P/mmol C, respectively. These values fall within the typical range reported for glycogen accumulating metabolism (GAM) acetate-fed systems (0.010 – 0.020 mmol P/mmol C), indicating that GAOs were present and likely dominant in the biomass (Filipe et al., 2001a; Oehmen et al., 2005a). The high poly-P content in Phase 1 (0.282 ± 0.012 mg P/mg VSS) suggests the presence of PAOs, yet with minimal PAM activity (H. Li & Chen, 2011; Liu et al., 1997). Later, the P_{rel}/HAc_{up} ratio in Phase 2 was enhanced to reach 0.053 and 0.049 mmol P/mmol C for SBR21 and SBR22, respectively. The P_{rel}/HAc_{up} values in the two phases were roughly

an order of magnitude lower than the typical values reported for PAO anaerobic metabolism, which generally vary between 0.27 to 0.75 mmol P/mmol C (Aghilinasrollahabadi et al., 2024).

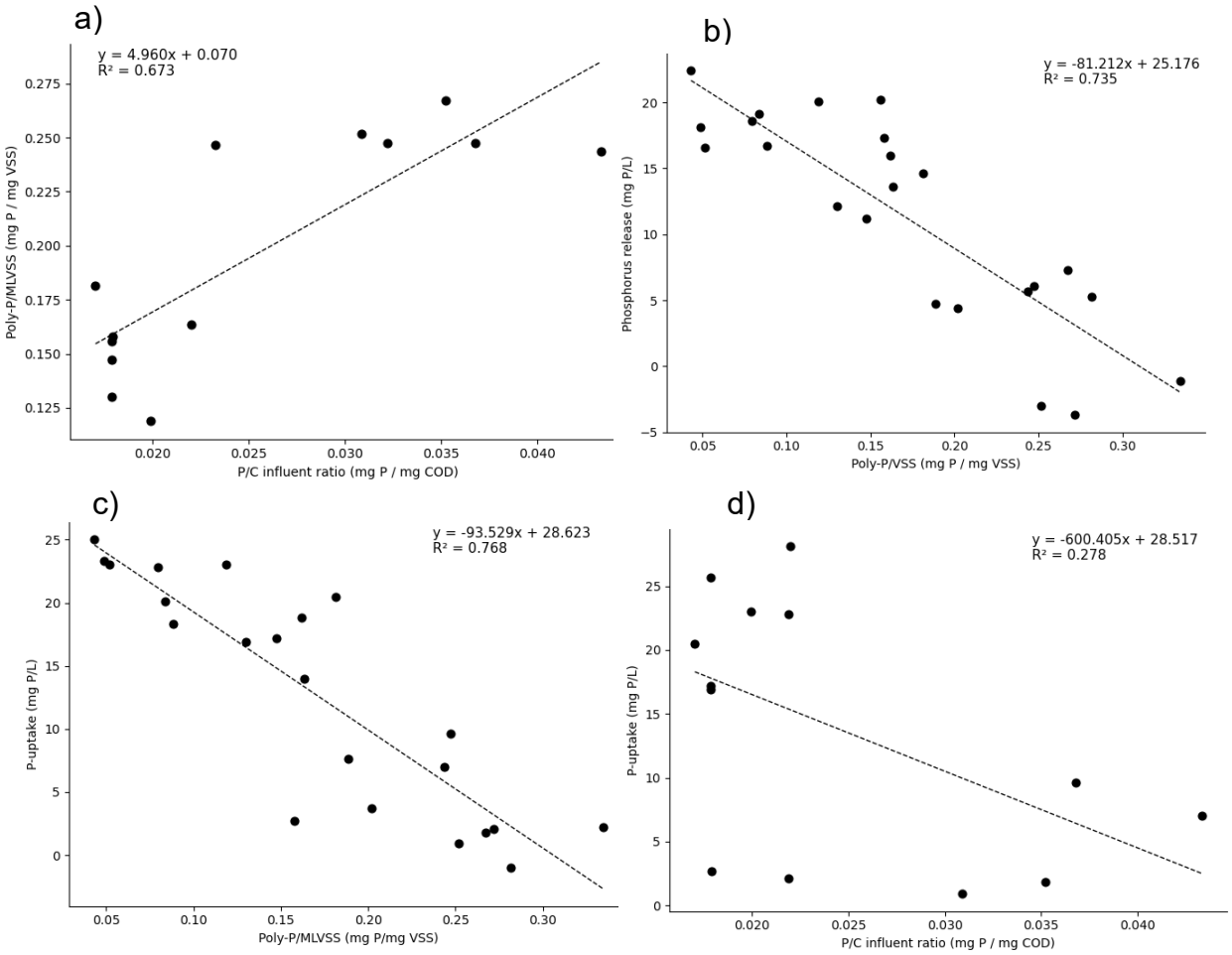

Figure 4-2: a) poly-P biomass content as a function of influent P/C ratio, b) phosphorus release as a function of the poly-P content, c) phosphorus uptake as a function of the poly-P content, and d) phosphorus uptake as a function of P/C influent ratio

In phase 1, the system's sCOD removal in SBR11 during the anaerobic phase fluctuated between 53.55% to 91.45%, while SBR12's sCOD removal performance varied between 40.36% and 90.61% causing EBPR instability as the effluent phosphorus concentration exceeded the influent concentration as shown in Figure 4-1. On the contrary, on day 11 and 15 of the experiment, 9.46% and 28.00% of the influent

phosphorus were consumed at the end of anaerobic phase in SBR11 and SBR12, respectively, suggesting the presence of electron acceptors in the anaerobic phase causing phosphorus uptake; this may be a result of the over saturation of oxygen in the sludgedue to poor DO control. However, the overall sCOD removal percentages during stable operation were 90.8 ± 1.8% and 92.8 ± 4.1 % for SBR11 and SBR12, respectively. The high HAc uptake coupled with the low $P_{release}$ and P_{uptake} suggests GAM activity where the carbon cycling from HAc uptake and transformation to PHA in the anaerobic phase, then glycogen and CO_2 production took place. The deterioration of EBPR performance may be linked to the proliferation of GAOs in the system; however, this can only be confirmed through additional analyses of the microbial community structure. The competition over the acetate uptake between the PAOs and GAOs hinders the PHA production and accumulation in the anaerobic phase and consequently lowers the P_{uptake} (Lanham et al., 2013; Stokholm-Bjerregaard et al., 2017; Yuan et al., 2020). The low observed $P_{release}$ rate indicates that PAOs were primarily hydrolyzing poly-P to meet maintenance energy demands, rather than to support HAc uptake and PHA synthesis. Under these conditions, a small fraction of the hydrolyzed poly-P was restored during the aerobic rate, which is reflected in the very low P_{uptake}. The imbalance between the anaerobically hydrolyzed poly-P and the aerobically resynthesized poly-P caused depletion of the poly-P pool over time; the poly-P/MLVSS ratio decreased by 0.175 mg P/mg VSS/h in SBR11 and 0.0148 mg P/mg VSS/h in SBR12. This corresponded with an increase in the $P_{release}$ and P_{uptake} rates, but still exhibited poor P-removal efficiency.

Table 4-1: Kinetic and performance parameters for Phase 1 and Phase 2

Parameters	Units	Phase 1		Phase 2	
		SBR11	SBR12	SBR21	SBR22
P/C ratio	mg P/mg C	0.043 ± 0.017		0.019 ± 0.0018	
P sludge loading[a]	mg P/ mg VSS/d	0.160	0.135	0.057	0.061
MLVSS	mg VSS/L	1541 ± 184	1824 ± 432	2267 ± 435	2138 ± 322
Poly-P/MLVSS	mg P/mg VSS	0.272 ± 0.07	0.282 ± 0.012	0.091 ± 0.05	0.144 ± 0.03
P_{rel}/HAc_{up} [a]	mg P/mg COD	0.014	0.022	0.047	0.052
μ release	mg P/mg VSS/h	2.18 ± 0.37	2.73 ± 0.73	4.83 ± 1.03	4.87 ± 1.34
μ uptake	mg P/mg VSS/h	0.74 ± 0.65	0.75 ± 0.61	2.76 ± 0.61	2.95 ± 0.68
P effluent	mg P/L	12.62 ± 3.8	12.29 ± 3.9	2.37 ± 0.91	1.37 ± 0.95
P-removal	%	28.33 ± 11.1	20.95 ± 9.21	79.67 ± 10.30	85.75 ± 9.39

a represented by average values

In phase 2, sCOD removal was 89.54 ± 2.75% for SBR21 and 88.35 ± 1.69% for SBR22, a slight decrease compared to Phase 1 yet with increased $P_{release}$ and P_{uptake} rates. The imbalance between the release and the uptake created a variation in the poly-P content where SBR22's poly-P content is consumed, decreasing from 0.11 mg P/mg VSS to reach 0.088 mg P/mg VSS. Then, the release and the uptake rates increased by 20.51% and 18.86%, respectively, to increase the poly-P content to 0.18 mg P/mg VSS. This suggests that the poly-P content capacity creates a driving force to release and uptake phosphorus. A similar pattern was observed in SBR21 with a more varied profile

of poly-P content associated with increased $P_{release}$ and P_{uptake} rates to compensate the depleted pool.

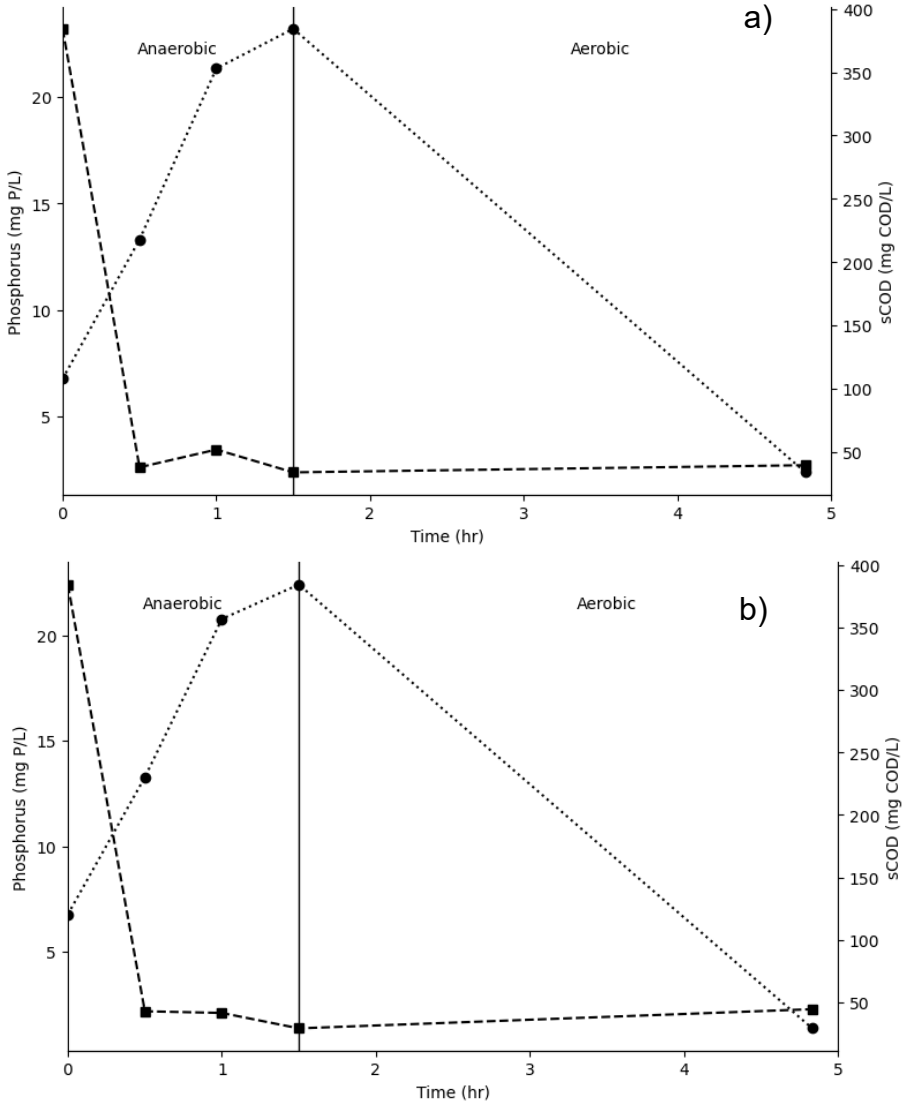

Figure 4-3: Phosphorus profile throughout the anaerobic and aerobic phases; a) SBR21, and b) SBR22

To investigate the PAOs dynamic kinetics in the anaerobic phase, both reactors in Phase 2 were monitored with a time step (Δt) of 30 mins from the beginning of the anaerobic phase. Although the overall specific phosphorus release rate was almost constant, the released phosphorus concentration profile varied as seen in Table 4-2. For SBR21, the initial released phosphorus concentration (ΔP_{30}) was 8.56 ± 0.12 mg P/L then

dropped to 2.60 mg P/L during the first 30 mins which subsequently impacted the rate of released phosphorus in the remaining time. It is worth mentioning that 96% of the phosphorus released was released in the first 60 minutes. For SBR22, the initial release rate was lower than observed in SBR21 where ΔP_{30} was 5.57 ± 0.42 mg P/L, while ΔP_{60} was 10.40 ± 1.45 mg P/L and 98% of phosphorus release was within 1 h of the anaerobic phase. During the first 30 mins of the anaerobic phase as seen in Figure 4-3, 90.10 ± 4.70% of the sCOD was consumed in SBR21, while 88.80 ± 6.00% was consumed in SBR22. Similar sCOD utilization in the first 20 to 30 minutes of anaerobic reaction was also observed by Filipe et al. (2001) and Coats et al. (2011). Although $P_{release}$ and VFA uptake metabolisms are theoretically and intrinsically linked, the $P_{release}$ rate in SBR22 was concentrated in the second time interval (from 30 minutes to 60 minutes), suggesting a different and delayed biochemical response. It was also noted in both reactors that there was a continuous $P_{release}$ after the sCOD was consumed and remained almost constant especially in the last 30 minutes as seen in Figure 4-3. The total specific $P_{release}$ rates after the sCOD consumption were 6.26 ± 1.46 and 4.15 ± 0.16 mg P/g VSS/h for SBR21 and SBR22, respectively, indicating secondary phosphorus release. The high secondary release rates suggest that the PAOs are altering how they manage the intracellular phosphorus reserve, assuming that the maintenance coefficient of the microorganisms is approximately constant under defined environmental conditions. Additionally, the elevated $P_{release}$ indicate the presence of high poly-P content in the sludge. Although the P_{rel}/HAc_{up} values in both reactors were slightly above the GAM kinetic range, the P-removal efficiency increased to reach approximately 80%, implying a strong shift towards PAM activity. It would appear that PAO metabolism tends to hydrolyze more poly-P to

compensate for the low internal carbon storage. It was reported in the literature that high $P_{release}$ is usually associated with low glycogen degradation due to the reduced glycogen reserve (Welles et al., 2017). However, PAOs must be capable of utilizing the acetate while maintaining the redox balance in the cell stable; the redox stability largely depends on the maintenance of glycogen reserves. Thus, the decrease in glycogen reserves forces the PAOs to shift their metabolism pathway to the TCA cycle and the glyoxylate pathway to compensate for the loss in reducing power, which is required to maintain the generation of necessary ATP for maintenance and survival (Y. Zhou et al., 2009).

Table 4-2: Release rates at different time points for Phase 2 and their overall uptake rates

Reactor	$\mu_{rel\ at\ t = 30\ min}$	$\mu_{rel\ at\ t = 60\ min}$	$\mu_{rel\ at\ t = 90\ min}$	$\mu_{overall\ release}$	$\mu_{overall\ uptake}$
	mg P/g VSS/h				
SBR21	5.76	7.08	1.65	5.16	2.75
SBR22	6.079	7.01	3.23	5.74	2.95

4.2 Changing the Anaerobic Retention Time

4.2.1 Process performance in 60 mins anaerobic-HRT

In phase 3, the reactors' anaerobic and aerobic contact time were changed to test the HRT impact on the $P_{release}$, P_{uptake} and overall removal efficiency. SBR21's anaerobic contact time was shortened from 90 mins to 60 mins and the aerobic contact time was 230 mins with the ratio of anaerobic to aerobic HRT (HRT_{ana}:HRT_{aero}) of 0.26, denoted as $SBR31_{ana=60}$. The system was operated for four six-hour cycles a day at a total HRT of 4.8 hr and SRT of 11.8 d, with an influent phosphorus concentration was 6.37 ± 1.39 mg P/L and influent HAc:P ratio of 65.82 ± 14.48 mg COD/mg P. The P-removal performance

for $SBR31_{ana=60}$ was 32.71 ± 9.68%. The specific release and uptake rates of phosphorus were 4.68 ± 1.58 and 1.62 ± 0.44 mg P/g VSS/h, respectively. When the anaerobic HRT was reduced to 60 mins, there was no statistically significant change (p-value = 0.56) in the overall $P_{release}$ rate compared to an anaerobic HRT of 90 mins. This result indicates that the anaerobic contact time can be reduced to 60 mins without adversely impacting the $P_{release}$ rate.

Investigating the anaerobic phase more closely reveals that specific phosphorus release rates during the initial 20-30 minutes for $HRT_{ana=60}$ was higher than $HRT_{ana=90}$, indicating increased metabolic rates for acetate uptake and PHA synthesis. Secondary $P_{release}$ occurred after sCOD consumption reached 87.7 ± 3.45% within the first 20 minutes (with the exception of day 37 where the primary reaction took place within 40 minutes). Yet, the secondary $P_{release}$ rate (4.47 mg P/g VSS/h) was lower than observed in $HRT_{ana=90}$ (7.08 mg P/g VSS/h) after one hour of reaction time as seen in Figure 4-4 between the phosphorus profile in the two phases. This observation suggests reduced PAO maintenance requirements under lower anaerobic time, leading to less poly-P hydrolysis and consequently a higher poly-P/MLVSS ratio of 0.162 ± 0.032 mg P/mg VSS. On the other hand, the P_{rel}/HAc_{up} ratio for $SBR31_{ana=60}$ was reduced to half; 0.023 mg P/mg COD compared to SBR21 (0.049 mg P/mg COD). This value still falls under the range of GAM metabolism, suggesting GAO presence, especially considering that the overall $P_{release}$ was not negatively impacted by shortening the anaerobic duration.

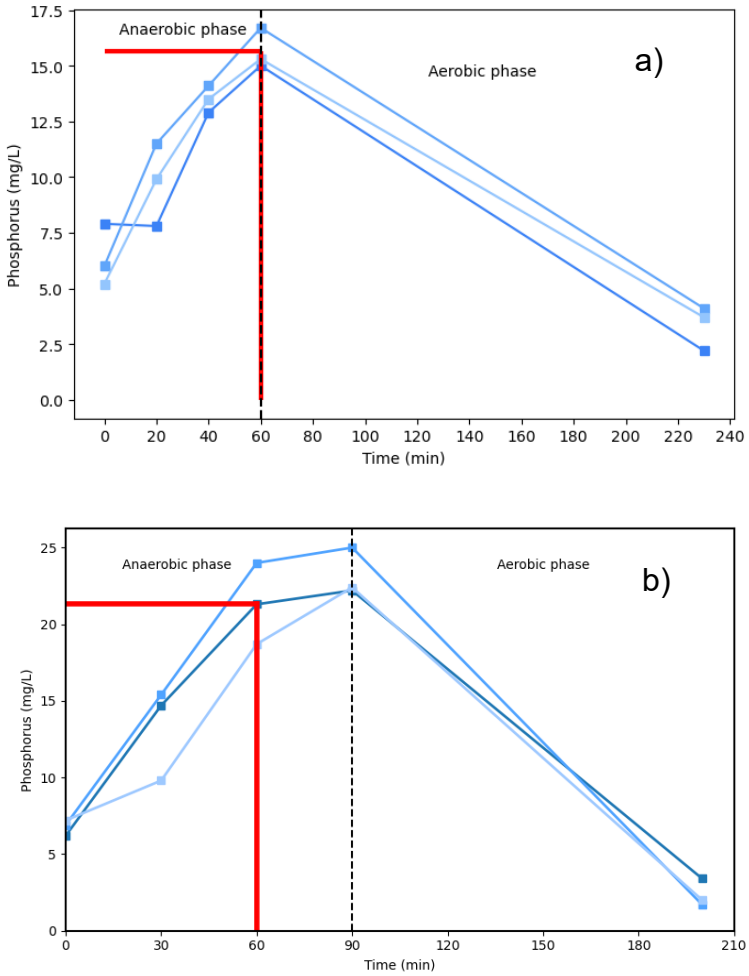

Figure 4-4: Phosphorus profile during anaerobic and aerobic phases for a) SBR31, and b) SBR21

The overall average secondary $P_{release}$ in $SBR31_{ana=60}$ was 4.47 ± 1.46 mg P/ g VSS/L which was lower than $SBR21_{ana=90}$ of 6.26 ± 1.46 mg P/ g VSS/L as seen in Figure 4-5a. Although excess $P_{release}$ with no equivalent PHA synthesis adversely affects subsequent aerobic uptake, the uptake rate in $SBR31_{ana=60}$ was lower by 41.30% compared to $SBR21_{ana=90}$, despite optimal conditions (pH 7.3± 0.2 and DO levels > 2 mg O_2/L) (Izadi et al., 2020). The insufficient PHA limits energy availability for poly-P regeneration and P_{uptake}, resulting in higher $P_{effluent}$ concentration (Barnard & Abraham, 2006). Additionally, the increase in HRT_{aero} decreases the P_{uptake} due to the rapid PHA depletion (Brdjanovic et al., 1998). That implies that the dynamics of PAOs have changed

to adapt to lower poly-P synthesis in the aerobic phase and maintain the poly-P reserves in the anaerobic phase, which was noted in the secondary $P_{release}$ reduction.

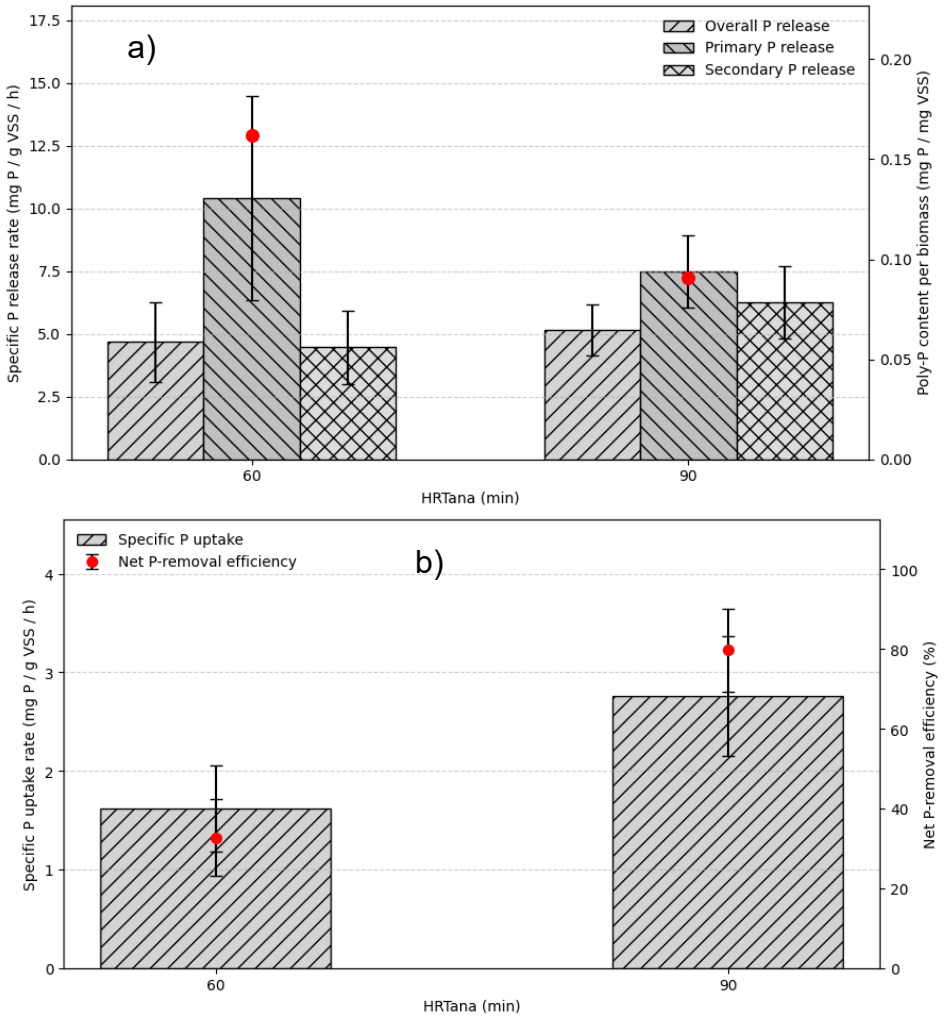

Figure 4-5: Kinetics of HRTana at 60 and 90 mins, where a) the overall specific P release, including primary and secondary releases with the average poly-P content per active biomass, and b) the overall specific uptake rate and the net P-removal %

Although the primary $P_{release}$ rates were elevated during the anaerobic phase, implying higher PHA production capacity during the aerobic phase, the uptake rate was statistically lower than expected (p-value = 0.017). This deterioration may stem from aerobic phosphorus release in prolonged HRT_{aero}, which elevates the phosphorus concentration in bulk liquid; however, measuring the phosphorus concentration profile

throughout the aerobic phase is required to confirm this hypothesis. This phenomenon aligns with extended HRT_{aero}, where secondary $P_{release}$ occurs at the end of the phase to meet the energy requirements for maintenance after PHA and glycogen reach their minimum values (Carvalheira et al., 2014b; Izadi et al., 2021c). It is worth noting that the residual sCOD of the anaerobic phase contributed minimally to the aerobic phosphorus release as the effluent sCOD exhibited no change from the residual sCOD. An exception to that is observed on day 38, where effluent sCOD decreased by 20 mg COD/L, likely due to maintenance and growth demands (You et al., 2004).

Another possible reason for the decline of the P_{uptake} rate is that PAOs have reached their maximum poly-P storage capacity within the cells. This saturation may have limited their ability to utilize the stored PHA and regenerate poly-P as the saturated poly-P reserves were the limiting factor in the aerobic phase (Brdjanovic et al., 1998). The reduced anaerobic contact time may have forced the PAOs to manage their reserves differently which restricted the extent to which PAOs needed to regulate and hydrolyze their excess poly-P content. The lower secondary $P_{release}$ rates may have pushed the PAOs to maintain higher poly-P reserves which was observed in the increased poly-P content per MLVSS ratio.

During the aerobic phase, the PAOs were possibly unable to fully utilize the stored PHA for P_{uptake} ; the poly-P regeneration is a constraint leading to lower PHA oxidation. Instead, the excess PHA carbon was potentially redirected towards replenishing glycogen stores (Metcalf & Eddy, 2014). This metabolic shift favors GAOs over PAOs, since GAOs rely exclusively on glycogen for energy and reducing power under anaerobic conditions, and the enhanced availability of glycogen was advantageous to GAO proliferation.

Investigating the validity of this hypothesis requires monitoring of PHA degradation and glycogen generation in the aerobic phase and is left for future work.

4.2.2 Process performance in 120 mins anaerobic-HRT

Moving to phase 4, SBR22's anaerobic HRT was increased to reach 120 minutes, and the aerobic phase HRT was reduced to 170 minutes with a HRT_{ana}:HRT_{aero} ratio of 0.70, and the reactor is denoted as $SBR42_{ana=120}$. All operational conditions like total HRT and SRT and the influent characteristics remained the same. The overall P removal efficiency was 31.34 ± 7.50% with specific $P_{release}$ rate of 3.21± 0.67 mg P/g VSS/h and specific P_{uptake} rate of 2.41 ± 0.51 mg P/g VSS/h. There was a statistically significant decrease in the overall specific $P_{release}$ rate (p-value = 0.03) when the HRT_{ana} increased to 120 minutes compared to a HRT_{ana} of 90 minutes. Although it was suggested that the increase of HRT_{ana} would enhance the fermentation process and subsequently the acetate utilization, the primary specific $P_{release}$ (coupled with acetate utilization) in $SBR42_{ana=120}$ showed no statistically significant difference (p-value = 0.62) compared to $SBR22_{ana=90}$ (C. Zhang et al., 2025b). Additionally, the literature reported that the increase of HRT_{ana} would increase the secondary $P_{release}$ rates due to the imposed starvation conditions after the VFA depletion (Brown et al., 2011; Pang et al., 2019; Y. Wang et al., 2009).

Yet the specific secondary $P_{release}$ rate in $SBR42_{ana=120}$ showed a stastically significant decrease compared to that observed in $SBR22_{ana=90}$ (p-value = 0.042) as shown in Figure 4-6a. The unexpected decrease in the secondary $P_{release}$ rate suggests that either the energy requirement of PAO maintenance changed or an alternative reducing power equivalent than poly-P was being utilized. Lopez et al. (2006)

hypothesized and later confirmed by Lu et al. (2007) that PAOs exhibited the same maintenance pathway as GAOs under anaerobic starvation where glycogen was utilized to support PAO maintenance. This results in less poly-P hydrolysis, hence helping PAOs to maintain their poly-P reserves. The alternative maintenance pathway involves a fermentation process centered on 3-hydroxyvalerate (3HV) production. The pathway integrates glycolysis, converting glycogen to pyruvate, which later feeds into the propionate-succinate fermentation cycle, a branch of the TCA cycle. This cycle converts pyruvate into acetyl-CoA and propionyl-CoA which contribute to 3HV synthesis, which falls under PHA products. These biochemical transformations help sustain the intracellular redox balance during anaerobic conditions. It was also reported that PAOs exhibit a decreased ratio of PHB to 3HV, indicating a metabolic shift in carbon storage products towards 3HV-enriched PHAs under anaerobic starvation conditions (Lu et al., 2007b; Y. Zhou et al., 2008). Taken together, these observations imply that extending anaerobic duration enhances the fermentation pathways within PAOs. This metabolic adjustment reduces secondary $P_{release}$ rate by decreasing poly-P hydrolysis, ultimately helping PAOs preserve their pool during extended anaerobic starvation periods.

In the aerobic phase, the specific P_{uptake} rate in $SBR42_{ana=120}$ was not changed in a statistically significant way compared to $SBR22_{ana=90}$ (p value = 0.29), suggesting that shortening the aerobic phase did not impact the removal rate negatively. Alternatively, the unchanged specific P_{uptake} rate could be attributed to the lower phosphorus concentration at the end of the anaerobic phase due to the lower secondary $P_{release}$ rate which balanced the PHA utilization with P consumption.

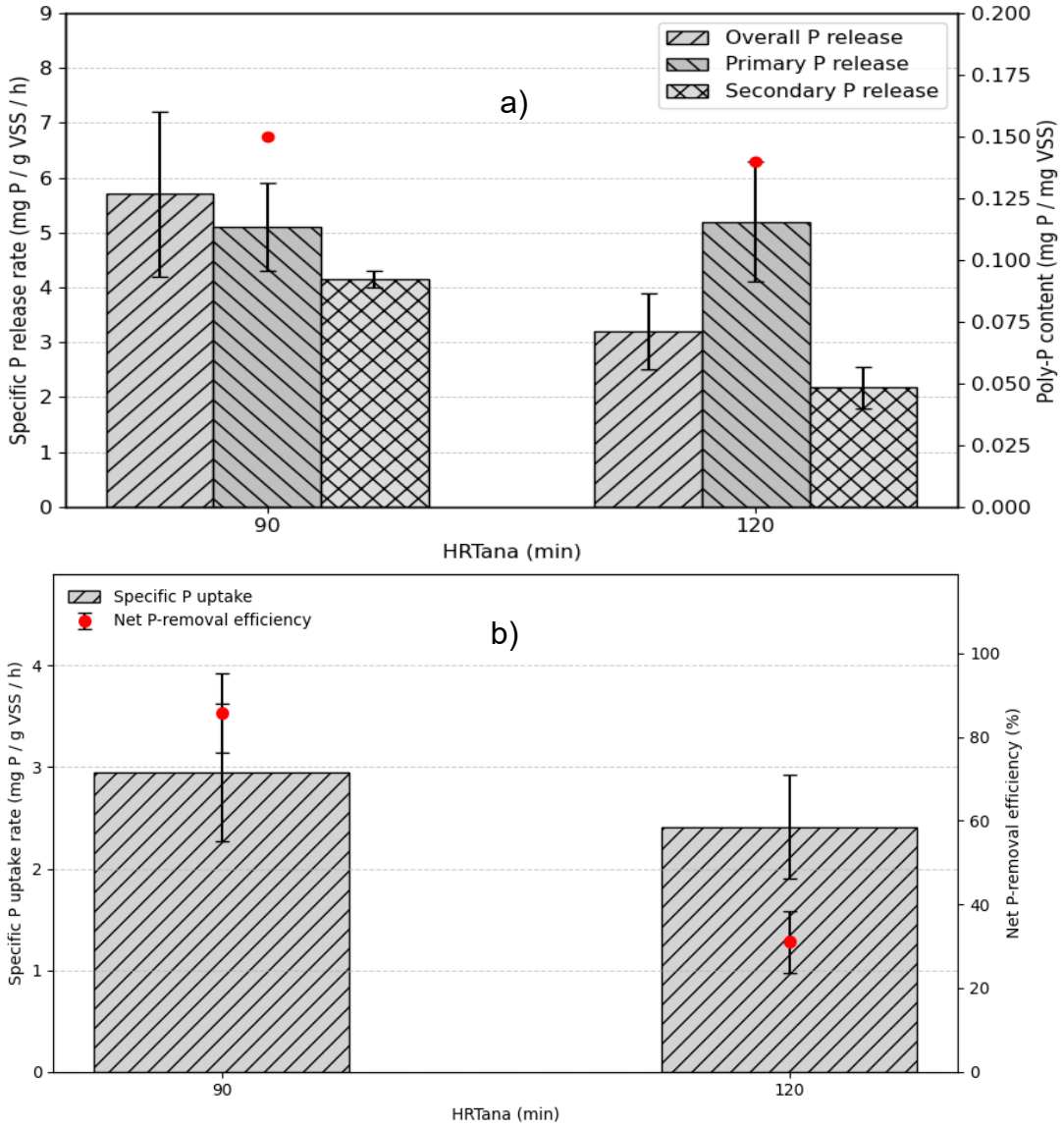

Figure 4-6: Kinetics of HRTana at 90 and 120 mins, where a) the overall specific P release, including primary and secondary releases with the average poly-P content per active biomass, and b) the overall specific uptake rate and the net P-removal

The deteriorated EBPR performance (~ 30% P removal) despite the unchanged specific P_{uptake} rates can be attributed to the anaerobic kinetics. The P_{rel}/HAc_{up} in $SBR42_{ana=120}$ decreased by 52% to reach 0.027 mg P/mg COD, indicating a shift towards GAM kinetics. The increase of GAO abundance is reportedly associated with longer HRT_{ana} (Coats et al., 2021; Y. Wang et al., 2013). Extending the HRT_{ana} gives a

competitive advantage to GAOs over PAOs primarily because of the PAOs' reduced ability to hydrolyze and expend poly-P over prolonged HRT_{ana} which limits their specific HAc uptake. Consequently, GAOs assimilate the available acetate because their metabolism relies solely on glycogen reserves, further enhancing their proliferation (Carvalheira et al., 2014). It is also worth mentioning that the specific HAc uptake rate of PAOs under prolonged starvation periods deteriorates faster than the GAOs' (Y. Wang et al., 2013). GAOs possess the ability to consume acetate with minimal glycogen degradation under limited substrate and glycogen availability; however, PAOs require poly-P hydrolysis to utilize the same acetate quantity consumed by GAOs. The acetate competition results in faster depletion of poly-P compared to the GAOs' stored glycogen consumption (Vargas et al., 2013). Therefore, short-term starvation favors GAOs over PAOs. Another reason for GAO proliferation and survival is their ability to uptake acetate and convert it to PHAs while PAOs showed a significant reduction in PHA synthesis under similar starvation conditions (Vargas et al., 2013). This was also observed to result in a higher anaerobic decay rate of PAOs compared to GAOs (Carvalheira et al., 2014).

Table 4-3: Kinetics parameter and removal efficiencies of Phase 2, 3 and 4

Parameter	Units	SBR21 ana=90 min	SBR31 ana=60 min	SBR22 ana=90 min	SBR42 ana=120 min
MLVSS	mg VSS/L	2267 ± 435	1989 ± 530	2138 ± 322	1860 ± 523
poly-P per biomass	mg P/mg VSS	0.091	0.162	0.155	0.144
COD removal	%	89.54 ± 2.75	87.70 ± 3.45	88.35 ± 1.69	88.53 ± 9.98
Overall release	mg P/g VSS/h	5.16 ± 1.01	4.68 ± 1.58	5.75 ± 1.49	3.21 ± 0.67
primary release	mg P/g VSS/h	7.49 ± 1.44	10.4 ± 4.07	5.2 ± 0.8	5.18 ± 1.14
secondary release	mg P/g VSS/h	6.26 ± 1.46	4.47 ± 1.46	4.15 ± 0.16	2.17 ± 0.37
Overall uptake	mg P/g VSS/h	2.76 ± 0.61	1.62 ± 0.44	2.95 ± 0.68	2.41 ± 0.51
P eff	mg P/L	2.37 ± 0.91	3.33 ± 1.00	1.37 ± 0.95	3.56 ± 1.04
P-removal	%	79.67 ± 10.3	32.71 ± 9.68	85.75 ± 9.39	31.00 ± 7.50

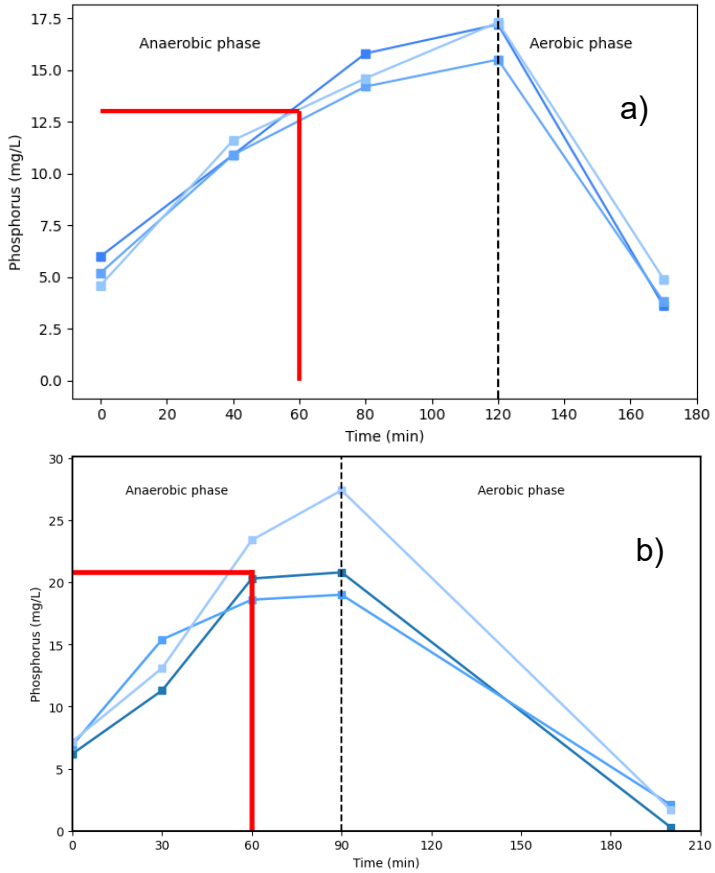

Figure 4-7: Phosphorus profile during anaerobic and aerobic phases for a) SBR42 and b) SBR22

4.2.3 COD residual and removal efficiency

The COD removal efficiency in reactors (SBR21 and SBR31) and (SBR22 and SBR42) were calculated and compared. Ma et al. (2005) observed no statistical change in COD removal efficiency when the P/C ratio was changed. Looking closer into the COD profile, at $HRT_{ana= 60\ min}$, the COD consumption was not significantly impacted (p-value = 0.48) by lowering the influent value in the first 20 mins where it was utilized by 89.0 ± 1.4% in SBR21 and 85.8 ± 3.11% in SBR31. However, the residual COD concentration at the end of the anaerobic phase in both reactors showed no statistical difference (p-value = 0.13) with mean values of 63 mg/L in SBR21 and 36.0 mg/L in SBR31. This absence of a significant difference is unexpected, as lowering the influent COD should theoretically reduce the residual COD (You et al., 2004). This may be explained by the increase of cell lysis in SBR31 where the MLVSS was reduced by 17.8% in SBR31. Meanwhile, during the HRT_{ana} = 120 min, the residual COD was significantly different (p-value = 0.038) with mean values of 60.0 mg/L in SBR22 and 19.7 mg/L in SBR32.

Literature reports the ability of PAOs to release phosphorus aerobically in the presence of carbon source accompanied with PHA generation (Pijuan et al., 2005; You et al., 2004). However, the release rate was lower than the usual EBPR configuration of sequential anaerobic and aerobic phases due to the competition of other microbial organisms over the carbon, especially in the presence of an electron acceptor (Pijuan et al., 2005). Consequently, the P_{uptake} rate was negatively impacted due to the increased phosphorus concentration in the aerobic phase and the competition over carbon sources and energy to regenerate the poly-P. In our experiments, the effluent COD exhibited a variable trend between decreasing (aerobic utilization) and increasing (cell lysis) with the average

aerobic COD removal being 98.5% lower than anaerobic rates. This minimal aerobic COD consumption likely supports maintenance metabolism of GAOs and OHOs rather than PAOs.

4.2.4 P uptake correlation with P release

The relationship between $P_{release}$ and P_{uptake} was established to be linear with a slope approximately slightly higher than one (Figure 4-8). This reflects the effective EBPR functionality which depends on higher P_{uptake} to remove the anaerobically released P to achieve net removal. In ASM2d, this stoichiometric ratio is assumed to be unity, a value empirically confirmed by Coats et al. (2021) with a strong fit (R^2 = 0.923). In contrast, it was observed that there was a higher stoichiometric ratio of 1.25 between total $P_{release}$ and P_{uptake} concentrations, with a somewhat worse fit (R^2 = 0.771). When examining the ratio between specific rates of $P_{release}$ and P_{uptake} , the value approached unity (1.025) but exhibited even lower model confidence (R^2 = 0.519). These deviations suggest that our systems exhibited inefficiencies in phosphorus cycling under our experimental conditions.

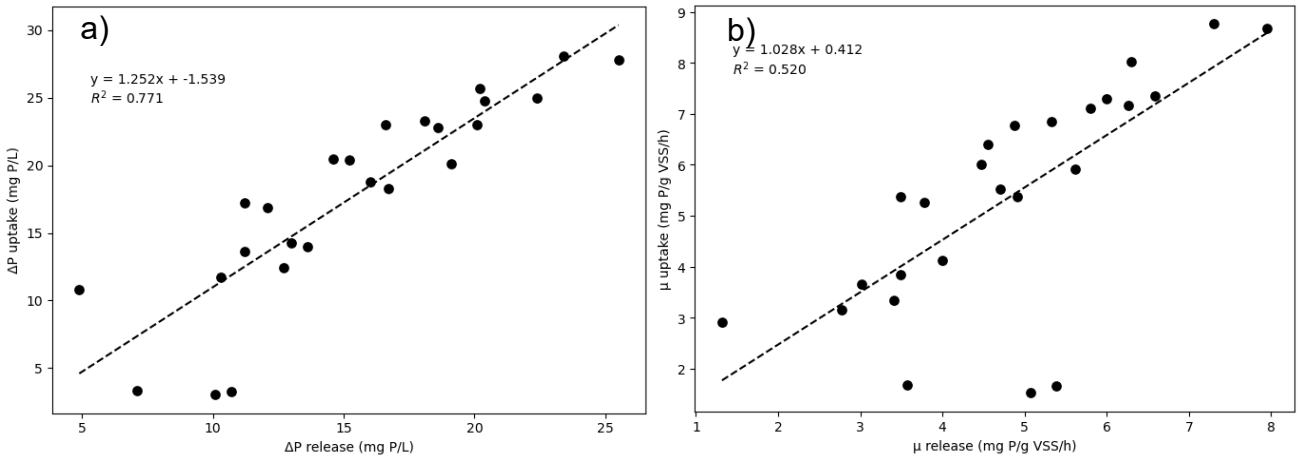

Figure 4-8: Linear regression as a) Phosphorus uptake concentration (∆P uptake) as a function of released phosphorus concentration (∆P release), and b) specific phosphorus uptake rate (μ uptake) as a function of specific phosphorus release rate (μ release).

Previous works suggest that the y-intercept of the linear relationship between $P_{release}$ and P_{uptake} concentrations on the P_{uptake} axis represents the baseline phosphorus metabolic requirements of the sludge for cellular functions, independent of the anaerobically released phosphorus (Wentzel et al., 1985). The elevated slope observed in Figure 4-8 suggests that a larger fraction of phosphorus was assimilated aerobically for maintenance and growth rather than achieving net removal. The steep slope suggests that the system's overall P removal stoichiometry was compromised by these increased endogenous requirements although PAOs maintained their uptake capacity per unit of anaerobic release.

For the relationship between the specific rates of release and uptake, the slope approached unity (1.025) but exhibited substantially lower model confidence. The shift toward 1:1 ratio with reduced goodness of fit implies that the specific uptake rates are not solely governed by corresponding release rates. Instead, they may appear to be influenced by additional factors such as total sludge mass concentration, poly-P content, or varying PAO/GAO population dynamics within the biomass.

The P assimilation by OHOs to maintain their metabolic activities (i.e. maintenance and growth) were calculated for the above phases. The assimilated P in all phases was almost constant with value of 2.45 ± 0.14 mg P/L as the P assimilation is impacted by the SRT and the influent COD (Stricker et al., 2010). The P assimilation contributed to the total P removal by 10.93% in phase 2, while it increased to 24.3% in phase 3, and 20.3% in phase 4, reflecting deteriorated EBPR in phases 3 and 4 that may be caused by a shift toward ordinary biomass growth.

4.3 Lowering the C/P ratio

In phase 5, the influent COD was lowered to half the baseline value for both $SBR31_{ana=60}$ and $SBR42_{ana=120}$, denoted as $SBR51_{ana=60}$ and $SBR52_{ana=120}$. Thus, both systems were operating on lower HAc:P influent ratio of 40.23 ± 5.54 mg COD/mg P and other operational conditions from phases 3 and 4 were kept the same, including the HRT_{ana} and HRT_{aero}. The overall performance enhanced to reach 87.18 ± 6.35% and 100% for $SBR51_{ana=60}$ and $SBR52_{ana=120}$, respectively.

4.3.1 Process performance in 60 mins anaerobic HRT

For SBR51, the overall specific $P_{release}$ was 3.69 ± 0.87 mg P/g VSS/h with no statistically significant change (p value = 0.37) from the $SBR31_{ana=60}$. The specific primary $P_{release}$ rate decreased to 5.25 mg P/g VSS/h which occurred when 85.81± 1.63% of the acetate was consumed, whereas the specific secondary $P_{release}$ rate exhibited minimal changes, reaching 5.05 mg P/g VSS/h. The decrease of the primary $P_{release}$ rate from 10.40 to 5.25 mg P/g VSS/h was associated with the decrease of acetate availability in the influent, making the carbon the limiting factor. Although PAOs exhibit a higher acetate uptake rate than GAOs which gives them the advantage at lower influent COD (Oehmen et al., 2005). Yet, it seems that GAOs and other OHOs competed with PAOs which led to a lower primary $P_{release}$ rate. The lower primary $P_{release}$ rate indicates lower PHA synthesis and poly-P hydrolysis.

The secondary $P_{release}$ rate was more or less unchanged, suggesting that it was not impacted by the change in the influent COD concentration and rather by the amount of poly-P content in PAOs. Due to the limited poly-P hydrolysis during the VFA uptake and

PHA synthesis, the trend of the secondary $P_{release}$ rate by PAOs was maintained to manage their poly-P content. It is worth noting that the poly-P per MLVSS ratio in the beginning of the phase dropped to 0.011 mg P/mg VSS and increased on the second day to reach 0.194 mg P/mg VSS. The overall P removal efficiency was reduced to 33% then increased to 87% when the poly-P ratio increased. Yet, the primary $P_{release}$ rate increased at the lower poly-P ratio by 27% and the secondary $P_{release}$ rate was markedly reduced by 58.6% due to the lower poly-P presence in PAOs. The P_{uptake} rate did not deteriorate as much as the removal efficiency; removal efficiency decreased by 16% only. This could be associated with the increase of PHA synthesis in the anaerobic phase as the primary $P_{release}$ rate increased, leading to higher PHA oxidation and poly-P replenishment to increase their pool.

The enhancement of overall P removal was accompanied by an increase of P_{rel}/HAc_{up} ratio of 0.056 mg P/mg COD. The increased anaerobic metabolism may be explained by a slight shift of microbial populations from GAOs to PAOs; however, it still represents GAM activity. Literature reports a decrease in GAO dominance when the influent COD was reduced (Majed & Gu, 2020b). The PAM metabolism flourishes over GAM under lower acetate availability in case of sufficient $P_{influent}$; PAM utilizes energy by ATP consumption or PMF establishment via poly-P hydrolysis to uptake the acetate while GAM utilizes minimal ATP or none for acetate transport, leading to lower acetate uptake (Schuler & Jenkins, 2003b). Additionally, PHA production increases at lower influent COD as PAOs tend to shift their metabolism to consume more internal glycogen along with poly-P hydrolysis (Kapagiannidis et al., 2013). Therefore, the $P_{release}$ rate decreases, leading to an apparent decrease in P_{rel}/HAc_{up} ratio.

Regarding the P_{uptake} rates, there was no statistically significant difference between the two phases (p-value = 0.77) where P_{uptake} rate in $SBR51_{ana=60}$ was 1.47 ± 0.26 mg P/g VSS/h. That suggests that the uptake rate was not impacted by lowering the influent COD and the PHA oxidation rate may have been similar in both cases. The $P_{uptake}/P_{release}$ ratio increased as the $P_{release}$ rate decreased, leading to higher removal efficiency. The released secondary P (no equivalent PHA synthesis) was also removed during the aerobic phase.

4.3.2 Process performance in 120 mins anaerobic HRT

After lowering the influent COD, $SBR52_{ana=120}$ exhibited a complete P-removal from the effluent with overall specific $P_{release}$ rate was 4.15 mg P/g VSS/h and specific P_{uptake} rate of 4.15 mg P/g VSS/h. The specific $P_{release}$ rate increased as the secondary $P_{release}$ rate increased by 80.50% compared to $SBR42_{ana=120}$. The significant increase could be associated with the change of maintenance metabolism due to the decrease in acetate availability combined with prolonged anaerobic reaction. The primary $P_{release}$ rate was reduced by 46% reaching 2.79 mg P/g VSS/h which was expected due to the lower HAc concentration. As the poly-P hydrolysis during HAc uptake was lowered, PAOs managed their poly-P content through hydrolyzing extra poly-P without the presence of a carbon source to maintain their pool around 0.130 mg P/mg VSS. Additionally, the primary $P_{release}$ rate compared to $SBR42_{ana=120}$ was lower, suggesting either the presence of another energy source other than poly-P or microbial competition over the carbon source.

The overall P_{uptake} rate was almost doubled compared to SBR42, providing complete P-removal. The complete P-removal suggests that PHA accumulation was enhanced by a combined effect of lowering the influent COD and increasing the anaerobic

reaction time. For similar reasons, oxidation in anaerobic and aerobic phases was also enhance. The higher HAc uptake kinetics of PAOs likely underwent a stoichiometric shift to optimize their metabolism for increased PHA storage efficiency under limited carbon availability. PAOs may have effectively coupled poly-P hydrolysis for ATP generation alongside elevated glycogen degradation to generate reducing power, favoring carbon diversion toward PHA synthesis in PAOs rather than GAOs (Kapagiannidis et al., 2013). This metabolic shift of favoring higher glycogen degradation per acetate would yield maximized PHA synthesis per acetate uptake stoichiometry while preserving the poly-P reserves, though direct quantification of glycogen depletion per acetate remains essential to confirm this hypothesis. It is worth mentioning that the ability of PAOs to degrade more glycogen through glycolysis and TCA cycle pathways mimicking GAO metabolism under challenging conditions was demonstrated when the poly-P content reached its minimal values and when prolonged starvation conditions were applied (Acevedo et al., 2012; Lopez et al., 2006; Y. Zhou et al., 2009).

Furthermore, the extended HRT_{ana} may have ensured complete VFA conversion to PHA reserves, eliminating substrate competition and preventing excessive glycogen depletion that could favor GAO growth. Although the biomass was reported to shift towards GAO dominance during prolonged anaerobic contact time due to their ability to maintain lower glycogen degradation under starvation conditions, P-removal efficiency and elevated P_{rel}/HAc_{up} ratio suggest a shift of PAOs from endogenous metabolism to EBPR activity (Coats et al., 2021). The preserved poly-P from hydrolysis during VFA uptake and PHA accumulation may have helped PAOs survive the extended starvation

conditions by supplying the needed ATP for maintenance requirements, which may explain the 80% increase in secondary $P_{release}$ rates when the influent COD was changed.

The observed doubling of P_{uptake} capacity may stem from extensive aerobic PHA oxidation that provided more energy toward assimilating phosphorus from the bulk liquid. This would help in reestablishing the intracellular poly-P pools extensively after significant depletion, implying that the poly-P content is a strong driver for P_{uptake}. Furthermore, glycogen replenishment may have been substantially reduced compared to the other phases, as GAOs could have exhibited lower glycogen degradation during the anaerobic phase due to the limited VFA availability and decreased competition over HAc uptake (Kapagiannidis et al., 2013b). With GAOs being less active in mobilizing glycogen, a greater portion of aerobic PHA oxidation was directed toward the energy-intensive phosphorus assimilation rather than glycogen replenishment. Additionally, the shift on relying on energy sources coming from poly-P hydrolysis during the starvation period rather than incorporating higher glycogen degradation to meet the maintenance requirements could have conserved the intracellular glycogen content in PAOs (Lopez et al., 2006b; Lu et al., 2007b). This suggests that a metabolic shift in endogenous activity occurred when the TCA cycle was not active during the anaerobic phase due to the energy availability from the poly-P hydrolysis. This metabolic prioritization enhanced the P_{uptake} efficiency, and channeled excess reducing power and ATP from PHA oxidation into poly-P reformation and phosphorus assimilation from the bulk liquid, thereby enhancing the overall P-removal capacity.

4.4 Operational Conditions Applicability

Such performance in phase 1 at lower total HRT was reported in a full scale EBPR system under wet conditions where the total HRT was reduced to operate at 6.4 ± 0.3 h due to the increased inflows (Brown et al., 2022). The performance of the decreased HRT system experienced a P-removal rate of 20.79 ± 7.80 % as the system failed to achieve EBPR at HRTs lower than 4 h with low specific $P_{release}$ and P_{uptake} rates of 1.90 ± 0.35 and 0.7 ± 0.12 mg P/g VSS/h, respectively. Also, Coats et al. (2009) observed high specific $P_{release}$ rate of 3.90 mg P/g VSS/h and a lower P_{uptake} rate of 1.00 mg P/g VSS/h when operating a full scale EBPR at a total HRT of 6.8 h. However, the poor performance may have been associated with GAO presence due to the decrease in pH value (6.4 – 6.8) causing a disruption to the PMF, the driving force of poly-P hydrolysis in the anaerobic phase (Filipe et al., 2001). Usually, the deterioration of EBPR efficiency is attributed to GAO presence although GAOs' role in EBPR failure may have been overstated in lab scale research. Their proliferation could have happened after EBPR failure, acting as opportunists rather than competitors (Tu & Schuler, 2013). Yet, their presence in rich PAO EBPR system (29% of the microbial population) which exhibited high $P_{release}/HAc_{up}$ ratios impacted the P_{uptake} rate significantly compared to $P_{release}$ rates (He et al., 2008).

However, our present research operating at total HRT of 4.8 h showed significant P-removal efficiency (80 %) in phase 2 when the poly-P content was lowered by 48 - 66% due to the P_{inf} concentration reduction. Schuler and Jenkins (2003a) observed that, under excess influent phosphorus, $P_{effluent}$ concentrations remained high while the biomass P-content increased from 0.018 to 0.160 mg P/mg TSS, indicating phosphorus overloading rather than complete removal. Nevertheless, the increase in poly-P content from 0.015 to

0.2 mg P/mg VSS was accompanied by a rise in the P_{rel}/HAc_{up} from 0.029 to 0.75 mg P/mg COD. These trends are also consistent with Welles et al. (2017), who reported an upper limit for poly-P storage capacity of 0.2 mg P/mg VSS. The phosphorus removal efficiency increases linearly as the influent C/P ratio increases up to a threshold beyond which the removal efficiency starts to decline where Ma et al. (2005) observed enhanced phosphorus removal when C/P ratio increased from 23 to 32 mg COD/mg P. However, reducing P_{inf} to further increase the C/P ratio to 45 then 55 mg COD/mg P caused a reduction in removal efficiency as phosphorus sludge loading increased. Consistent with this trend, Ni et al. (2021) reported a roughly 25% removal efficiency drop when the influent phosphorus concentration increased.

Previous work has reported that the P_{rel}/HAc_{up} ratio significantly increased when the Poly-P content increased from 0 to 0.26 mg P/mg VSS but declined when Poly-P exceeded this level (Welles et al., 2017). Conversely, Poly-P content as low as 0.01 mg P/mg VSS has been shown to cause a significant reduction in the P_{rel}/HAc_{up} ratio (Acevedo et al., 2012). In our research, the P_{rel}/HAc_{up} ratio increased in Phase 2 compared to Phase 1, yet the value is lower than PAM kinetics reported in the literature (Acevedo et al., 2012; Aghilinasrollahabadi et al., 2024). Gu et al. (2008) suggested that the $P_{effluent}$, total P-removal and overall system stability are not dependent on high PAO abundance and were not impacted by the increasing the GAO fraction; the system exhibiting the lowest P_{rel}/HAc_{up} ratio showed the lowest average $P_{effluent}$ and highest stability.

From phase 2 to 5, the secondary $P_{release}$ rates were notably high with low P_{rel}/HAc_{up} ratios lying in the GAM kinetics regime. Secondary $P_{release}$ rates usually occur

in lower rates (0.2 – 0.4 mg P/g VSS/h) when VFAs are depleted completely in the system as the used energy from the release directed towards maintenance (Wouters-Wasiak et al., 1996). However, Pijuan et al. (2004) noted a high secondary P release of 7.44 and 6.51 mg P/g VSS/h when the system was fed solely with acetate and propionate and was associated with maintenance requirements. The maintenance parameter for anaerobic metabolism was also evaluated by Smolders et al. (1995) to be 2.42 mg P/ g VSS/h. Bond et al. (1999) demonstrated that raising intracellular pH can result in significant secondary $P_{release}$ rates in neutral and slightly alkaline conditions where poly-P hydrolysis happened to regulate the intracellular pH, yet this study was operating in optimized pH recommended by Filipe et al. (2001b). It is worth mentioning that the secondary $P_{release}$ rates were observed to be decoupled from the influent COD when reduced in phase 5. Generally, the secondary release was demonstrated in literature to be triggered by pH shifts, environmental stress, endogenous decay, VFA depletion, and extracellular poly-P turnover (Bond et al., 1999; Feng et al., 2020; X.-Y. Long et al., 2021a; Tian et al., 2022).

Reducing the HRT_{ana} from 90 mins to 60 mins had no statistically significant impact on overall specific $P_{release}$ rates, with decreased secondary $P_{release}$ rates. Consistent with Coats et al. (2011), where a decrease in HRT_{ana} from 2 h to 1 h resulted in most of the phosphorus release ocurring within the first 30 minutes at HRT_{ana} = 1 h, after which the phosphorus concentration remained almost constant. The lack of impact in decreasing anaerobic time can be attributed to the rapid VFA uptake that typically takes place within the first 20 minutes of the anaerobic phase, as previously reported by Filipe et al. (2001a). Additionally, He et al. (2007) observed while surveying five full scale EBPR facilities that applying HRT_{ana} = 45 mins caused aerobic $P_{release}$ due to the presence of

residual sCOD and was completely depleted after 30 mins from the aerobic phase. While increasing the HRT_{ana} in phase 4 to 120 mins caused a statistically significant decrease the overall specific $P_{release}$ rates, with notable decrease in secondary $P_{release}$ rates. In contrast, Coats et al. (2021) demonstrated that increasing the HRT_{ana} from 1 h to 4 h has enhanced the $P_{release}$ rate and PHA generation, suggesting more fermentation, yet the increase did not impact the $P_{effluent}$. However, the P_{uptake} rate was statistically unchanged by increasing HRT_{ana} and decreasing the HRT_{aero}. This aligns with Coats et al. (2011)'s findings that longer anaerobic phases improved the P-uptake kinetics, enabling a complete phosphorus removal within the first 30 minutes, compared to slower rates observed when the sludge was exposed to a HRT_{ana} of 1 h.

The P_{rel}/HAc_{up} ratios in all phases were either falling under GAM kinetics or slightly exceeding it but still an order of magnitude lower than PAM kinetics, suggesting the presence of GAOs (Acevedo et al., 2012). The P-removal of systems exhibiting high-abundance of GAOs and PAOs was reported to be governed by EPS mediated adsorption and desorption, driven by genes linked to EPS secretion and cellular PAO and GAO metabolism (Y. Zhang et al., 2025). Literature also suggests the role of GAOs in producing more EPS in stressed conditions, giving them advantage over PAOs and contributing to P-removal through EPS phosphorus storage rather than their inability to store poly-P themselves (Meng et al., 2023; Y. Zhang et al., 2025). Furthermore, SBR systems operating at C/P ratio of 40 mg COD/mg P achieved 75.9% P-removal efficiency with *Competibacter*-GAO representing 24.9% and Defluviicoccus representing 16.0% of the microbial population with the absence of *Accumulibacter* -PAO and *Tetrasphaera* – PAO

(Song et al., 2022). Thus, the pattern of EBPR and $P_{release}$ and P_{uptake} stoichiometry cannot reliably indicate PAO enrichment.

In Phase 5, Both reactors responded positively when the influent COD was reduced to reach C/P ratio of 40 mg P/mg COD and achieved a high P-removal efficiency. Literature reported that increasing the C/P by increasing the COD pushed the GAOs to flourish over the excess carbon which created phosphorus limiting conditions (Majed & Gu, 2019, 2020a). It was also noted that the P-removal was enhanced linearly by increasing the C/P to 32 mg COD/ mg P and the system achieved high P-removal (90-98%) going beyond this point till 50 mg COD/mg P (Ma et al., 2005). Xing et al. (2013) also investigated the impact of carbon concentrations by feeding 200 and 400 mg COD/L of acetate. The peak $P_{release}$ concentration was noted to increase when the HAc concentration decreased, contradicting our findings. However, the glycogen and PHB generation kinetics were enhanced at lower HAc.

Chapter Five: Conclusion and Future Directions

EBPR process provides an efficient, sustainable, and cost-effective alternative to chemical and physical phosphorus removal methods in wastewater treatment. Despite its advantages, EBPR systems are subject to performance failures that elevate phosphorus concentrations, prompting operators to supplement with chemical precipitation to meet discharge limits. Addressing these challenges requires a deeper understanding of microbial community dynamics, particularly PAOs and their metabolic pathways, as well as operational and design parameters optimization to enhance P-removal, recovery, and long-term stability. EBPR undergoes sequentially through anaerobic then aerobic phases, during which PAOs hydrolyze intracellular polyphosphate to uptake VFA and store PHAs, followed by phosphorus uptake under aerobic conditions. This thesis builds on prior research by evaluating EBPR performance and removal efficiencies under varying anaerobic contact times, incorporating optimized conditions such as influent characteristics, DO levels, temperature, and pH derived from literature to inform practical advancements.

Existing wastewater treatment facilities frequently experience EBPR deterioration following wet weather events, as peak flows reduce basin HRT, disrupting the sensitive balance required for PAOs dominance. EBPR's sensitivity from diluted VFA concentrations, increased COD, and temperature drops often resulting in elevated effluent phosphorus levels that require emergency chemical dosing. Moreover, many plants face spatial constraints that are needed for basin expansions, forcing operators to lower design HRTs and compromise EBPR stability, with effluent violations in biological

nutrient removal (BNR) facilities reported in up to 30-50% of U.S. facilities during high-flow periods. (U.S. Environmental Protection Agency, 2022).

Design guidelines and research commonly recommend total HRTs of 6-12 hours for EBPR in SBR configurations, with 12-hour HRT most prevalent in literature. Where design codes for nutrient removal in SBR mode recommend the cycle times to range between 9 and 24 hours, incorporating an anoxic phase for denitrification (NEWIPCC, 2005). However, most lab-scale studies employ 6–8-hour cycles focused exclusively on phosphorus removal, utilizing anaerobic-aerobic phasing without anoxic periods. Each SBR cycle comprises five sequential phases: fill where substrate addition without mixing to minimize oxygen intrusion, anaerobic which represents 15-25% of the total cycle where VFA uptake and PHA storage occur via poly-P hydrolysis, aerobic (50-60%) where P-uptake and PHA oxidation for poly-P regeneration take place, settle for biomass clarification, and supernatant decant.

This thesis investigated the effects of varying anaerobic retention times on a lab-scale EBPR system with SBR configuration, operating at the lower end of the recommended total HRTs. The baseline employed 90 min anaerobic and 200 min aerobic phases; influent C/P were first optimized for stable performance, followed by evaluating shorter and longer anaerobic durations to study the impacts on carbon uptake, phosphorus release, subsequent uptake, and overall removal efficiency.

5.1 Research Implications

Initially, the baseline scenario examined the impact of influent P/C ratios to stabilize and optimize EBPR performance. Results revealed a strong correlation (R^2 = 0.673)

between the P/C and poly-P content, with elevated poly-P exerting significant influence on anaerobic $P_{release}$ rates (R^2 = 0.735) and aerobic P $_{uptake}$ rates (R^2= 0.786). While prior studies have addressed the impact of VFA concentration variations by either creating phosphorus limiting conditions or carbon limiting conditions, the consequences of excessive phosphorus loading in which results in poly-P overload and $P_{release}$ and P $_{uptake}$ stoichiometry shift remain underexplored in the literature.

Moving forward to the thesis's main objective, anaerobic retention time was reduced from 90-min baseline to 60 mins with the aerobic retention time extended by 30 minutes to maintain constant total HRT. The overall specific phosphorus release experienced no statistical change (p-value > 0.05). However, detailed phosphorus profiling across the anaerobic phase revealed distinct kinetic division: primary release which directly linked to VFA uptake and PHA synthesis has increased under shortened anaerobic retention time, while secondary release that indicates PAO's maintenance demands under post-VFA conditions, decreased significantly (p-value = 0.013). On the other hand, the aerobic kinetics significantly deteriorated, and the P-removal was negatively impacted by reaching average removal efficiency of 32% despite consistent COD removal (88-90%).

Moreover, the anaerobic retention time increased to 120 min and the aerobic retention time decreased to 170 mins. The overall specific phosphorus release was significantly reduced when the anaerobic retention time extended. The primary phosphorus release was statistically maintained as it occurred efficiently within the first 30-40 mins by capturing 85% of sCOD. Literature reported that the anaerobic retention time increase would increase the secondary phosphorus release to provide energy to

meet the maintenance requirements, yet the secondary release in our experiment was reduced to half, suggesting a metabolic pathway shift under prolonged anaerobic starvation conditions.

Impact of increasing the P/C ratio, achieved by reducing the influent COD, was also evaluated under both shorter (60 mins) and longer (120 mins) anaerobic retention times. This COD reduction induced carbon-limiting conditions, previously shown to favor PAOs over GAOs. Overall P-removal efficiency improved dramatically, approaching complete removal. While total specific phosphorus release and uptake rates declined due to significantly reduced primary release from VFA scarcity, secondary release remained unaffected, indicating that maintenance demands depend more on retention time than carbon availability.

5.2 Research Limitations

However, this study has certain limitations that future research could address to provide a more comprehensive understanding of anaerobic retention time's on the EBPR process:

- No direct Metabolic analysis to assess PHA, glycogen, and poly-P content as they were inferred from the $P_{release}$ and P_{uptake} stoichiometry and ISS/TSS ratio.
- Community Analysis (qPCR/FISH) was needed to determine the microbial population and PAOs and GAOs quantification to estimate their role and response when various operational conditions were applied.
- Lack of sampling points in the aerobic phase to construct phosphorus and carbon profile to check for aerobic phosphorus release, especially in the presence of residual sCOD.

- Synthetic wastewater was used instead of real wastewater, where only acetate was used. Real wastewater usually contains a variety of VFA where it impacts the microbial community and process stability and enhancement (Shen et al., 2017).
- The absence of low influent COD testing on the baseline phase (90 min anaerobic, 200 min aerobic), precluding direct comparison between phosphorus-capacity-limiting (high P/C) and carbon-limiting (low C/P) conditions across all HRT_{ana} scenarios.

5.3 Research Contributions

This thesis addresses gaps in phase optimization in lab-scale SBRs under acetate-fed conditions, where it contributes to the following:

- It provided stoichiometric insights on the impact of P/C overload where excessive influent phosphorus concentrations suppressed Prel/HAc ratio to GAM levels, despite high poly-P content with strong correlations between the poly-P content with $P_{release}$ and P_{uptake}.
- It demonstrated the role of secondary phosphorus release in regulating the poly-P content in PAOs and how the anaerobic retention times influenced the secondary release, suggesting different metabolic pathways to provide required energy for maintenance.
- It developed an integrative operational strategy which resulted in acceptable biological P-removal under GAM activity.

5.4 Future Directions:

Based on this thesis's findings on anaerobic retention time and P/C stoichiometry and its limitations, future work should tackle microbial validation and kinetic modeling. Future directions should include the following:

- PAO/GAO microbial community profiling should be conducted and tracking the role of TB-EBS role in P-removal and quantify its contribution, especially in GAO dominated systems, is essential.
- Higher HRT_{ana}:HRT_{aero} ratio over 0.35 should be studied to understand the anaerobic phase influence on aerobic kinetics.
- The impact of poly-P content saturation on glycogen and PHA consumption and regeneration should be investigated in anaerobic and aerobic phases.

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