Experimental Study and Mathematical Modeling of Enhanced Biological Phosphorus Removal Based on Aeration Effects: Operational and Metabolic Insights

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Abstract

Enhanced Biological Phosphorus Removal (EBPR), as a promising technology, has been implemented in many wastewater treatment plants (WWTP) worldwide, with high efficiency in phosphorus removal performance. In a well-operated EBPR, lower operational cost, reduced sludge production, and lower environmental impacts are achievable. Yet, with the proven capability of EBPR in efficient phosphorus removal, disturbance and periods of unexplained insufficient phosphorus removal have been detected in real WWTP in different cases due to loss of PAO biomass under presumed favorable conditions for EBPR. These complications may lead to process upset, system failure, and violation of discharge regulations. Disruption in process performance may originate from several external factors such as heavy rainfall, excessive nitrate loading to the anaerobic reactor, excessive aeration of activated sludge, or it may be a result of PAOs competition with other groups of microorganisms such as glycogen accumulating organisms (GAO). Therefore, the key in reaching low P-effluent levels is to optimize the operation and minimize the effect of inefficient factors. This Ph.D. study has focused on aeration as a crucial operational factor in the EBPR process in sequential batch reactor (SBR) systems. EBPR aerobic P-uptake, anaerobic P-release, and carbon storage of phosphorus accumulating organisms (PAOs) are closely related to oxygen mass transfer. The study is oriented to different aspects of aeration, addressing aeration concentration (dissolved oxygen (DO) concentration), aeration duration (aerobic hydraulic retention time (HRT)), and aeration pattern (continuous/intermittent). The performance of EBPR in SBRs under various aeration strategies was investigated for different DO concentrations (0.4-4 mg/L), HRT (120-320 minute), and aeration patterns of continuous and intermittent (25 to 50 minute on/off intermittent aeration/non-aeration intervals).

Moreover, this study investigated the effect of reaching micro-aeration with adaptation strategies on EBPR performance. The development of steady and instant-DO reduction in different aeration strategies was studied in batch tests with enriched PAOs at different DO levels. Subsequently, comparative modeling using calibrated BioWin® software was implemented for SBRs to predict the nutrient removal performance by changing DO concentration and the aerobic-HRT and understanding the effect of parameters on treatment performance to improve operation and control.

Dedication

To Mom and Dad,

All I ever am is because of you

Acknowledgement

Throughout the writing of this dissertation I have received a great deal of support and assistance.

I would first like to thank my supervisor, Professor Ahmed Eldyasti, whose expertise was invaluable in formulating the research questions and methodology. Your insightful feedback pushed me to sharpen my thinking and brought my work to a higher level. And during the most difficult times when writing this thesis, you gave me the moral support and the opportunities I needed to move on. I would also like to thank my committee members, Dr. Usman Khan and Dr. Magdalena Krol, for their valuable guidance throughout my studies. You provided me with the tools that I needed to choose the right direction and successfully complete my dissertation.

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Table of Contents

List of Tables

List of Figures

List of Acronyms

ACS	Acetyl-CoA Synthetase
AD	Anaerobic Digestion
ADP	Adenosine Diphosphate
ALM	Aqueous Liquid Module
AND	Alternating Nitrification Denitrification
ANNAMOX	Anaerobic Ammonium Oxidation
ANOVA	Analysis Of Variance
AOB	Ammonia Oxidizing Bacteria
APC	Activated Sludge Clarifier
APE	Average Percentage Error
AS	Activated Sludge
ASM	Activated Sludge Model
ATP	Adenosine Triphosphate
BCFS	Biological-Chemical Phosphorus And Nitrogen Removal Configuration
BNR	Biological Nutrient Removal
BOD	Biochemical Oxygen Demand
BPR	Biological Phosphorus Removal
COD	Chemical Oxygen Demand
DBDNF	Deep Bed Denitrification Filter
DGAO	Denitrifying Glycogen Accumulating Organisms
DNA	Deoxyribonucleic Acid
DNB	Denitrifying Bacteria
DO	Dissolved Oxygen
DPAO	Denitrifying Phosphorus Accumulating Organisms
EBPR	Enhanced Biological Phosphorus Removal
ED	Entner Doudoroff
EDTA	Ethylenediaminetetraacetic Acid
EM	Embden-Meyerhof
EMP	Embden-Meyerhoff-Parnas
FADH	Flavin Adenine Dinucleotide
FISH	Fluorescence In Situ Hybridization
FNA	Free Nitrous Acid
GAM	Glycogen Accumulating Metabolism
GAO	Glycogen Accumulating Organisms
GC	Gas Chromatography
HPLC	High-Performance Liquid Chromatography
HRT	Hydraulic Retention Time

JHB	Johannesburg
MBR	Membrane Bioreactor
MLE	Modified Ludzack-Ettinger
MLSS	Mixed Liquor Suspended Solids
N	Nitrogen
N2OR	Nitrous Oxide Reductase
NADH	Nicotinamide Adenine Dinucleotide
NAR	Nitrate Reductase
NIR	Nitrite Reductase
NOB	Nitrite Oxidizing Bacteria
NSE	Nash–Sutcliffe Efficiency
OHO	Ordinary Heterotrophic Organisms
ORP	Oxidation Reduction Potential
P	Phosphorus
PAO	Phosphorus Accumulating Organisms
PCR	Polymerase Chain Reaction
PH2MV	Poly-Beta-Hydroxy-2-Methylvalerate
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHMB	Poly-3-Hydroxy-2-Methylbutanoic Acid
PHMV	Poly-3-Hydroxy-2-Methylvaleric Acid
PHV	Polyhydroxyvalerate
PTA	Phosphotransacetylase
PUR	Phosphate Uptake Rate
RAS	Returned Activated Sludge
RNA	Ribonucleic Acid
S2EBPR	Side-Stream Enhanced Biological Phosphorus Removal
SBR	Sequencing Batch Reactor
SCFA	Short Chain Fatty Acids
SCOD	Soluble Chemical Oxygen Demand
SCVFA	Short Chain Volatile Fatty Acids
SMP	Soluble Microbial Products
SND	Simultaneous Nitrification Denitrification
SRT	Solid Retention Time
SSE	Sum Of Square Errors
SVI	Sludge Volume Index
TCA	Tricarboxylic Acid Cycle
TCOD	Total Chemical Oxygen Demand
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen

TOC	Total Organic Carbon
TP	Total Phosphorus
TS	Total Solids
TSS	Total Suspended Solids
VFA	Volatile Fatty Acids
VIP	Virginia Initiative Plant
VS	Volatile Solid
VSS	Volatile Suspended Solid
WW	Wastewater
WWTP	Wastewater Treatment Plant

1 Background

As the critical nutrients in wastewater, phosphorus (P) and nitrogen (N) are discharged into receiving waters in high concentrations, causing eutrophication. Thus, there is a developing worldwide awareness for nutrient control with strict regulations, resulting in substantial adjustments and advancements in wastewater treatment plants (WWTPs). Enhanced biological phosphorus removal (EBPR) is one of the most promising technologies as an economical and environmentally sustainable technique for removing phosphorus from wastewater (WW). However, with the high capacity of EBPR, insufficient P-removal is a significant yet common issue of many full-scale wastewater treatment plants (WWTP) due to misinterpreted environmental parameters and microbial disturbance. This Ph.D. study has focused on the EBPR process in SBR-mode reactor systems. The study is oriented to different aspects of aeration, addressing aeration concentration (dissolved oxygen concentration), aeration duration (aerobic hydraulic retention time), and aeration pattern (continuous or intermittent).

Fundamentals and process operation of EBPR process (Chapter 2)

Enhanced biological phosphorus removal (EBPR), which is accomplished through sludge recirculation between anaerobic and aerobic conditions, is broadly implemented as a eutrophication control in wastewater treatment plants (WWTP) [1][2]. EBPR, as one of the most economical and environmentally sustainable processes for phosphorus removal, relies on the enrichment of phosphorus accumulating organisms (PAOs) through alternating anaerobic and aerobic/anoxic phases [3]. Therefore, the EBPR process depends on the growth and flourishment of PAOs. Thus identifying and understanding the factors that influence EBPR performance should

be highly considered in implementing EBPR configuration in WWTPs. This chapter introduces detailed background information on the EBPR process and the purpose of this Ph.D. research.

Overview of biochemical diversity and metabolic modeling of EBPR (Chapter 3)

EBPR, as a commonly accepted process for sustainable biological phosphorus removal, is employed to WWTPs by recirculation of sludge through anaerobic and aerobic/anoxic phases. Enrichment of PAOs is essential in reaching high performance; however, in different cases, the proliferation of glycogen accumulating organisms (GAOs) results in microbial competition for the limited carbon substrate available. Therefore, understanding microbial metabolism and performance will help to improve phosphorus removal efficiency. In this chapter, the metabolic pathway of PAOs and GAOs under anaerobic and aerobic/anoxic conditions was reviewed based on various substrates. This work summarized the present knowledge on biochemical pathways of the EBPR process and contemplated the research gaps with a pressing need for extensive focus.

Scope of Thesis (Chapter 4)

Knowledge on the overall energetics and oxygen requirements provides valuable information on whether EBPR contributes to aeration and energy-saving accompanied by high-quality effluent production. This chapter briefly explains and summarized the aeration effect on EBPR performance based on various studies. Moreover, the knowledge gaps regarding aeration strategies and conditions have been pointed out, followed by the objective of this research based on the multiple systems applied in experimental plans.

Impact of aeration on EBPR performance and PAO diversity (Chapters 5 to 7)

To reach a well-operated EBPR system with lower operational cost, reduced sludge production, and lower environmental impacts while encountering less unexplained disruption due to loss of PAO biomass, optimizing the influence of any influential factors such as aeration may significantly reduce the effluent P-level entering waterbodies.

In **Chapter 5**, the effect of DO concentration in a range of 0.8 to 4 mg/L was investigated to determine EBPR performance in terms of P-removal, aerobic kinetics, and energetic costs. Moreover, an aerobic HRT of 120 to 200 minutes as an impactful operational factor in controlling EBPR was investigated. In addition, the effect of the aeration factor on anaerobic conditions has been examined in terms of storage and degradation of internal reserves.

In **Chapter 6**, the aeration pattern and the aeration duration have been taken into account with a constant aerobic/anoxic duration ratio to assess the effect of oxygen availability on the biological nutrient removal system. Furthermore, to evaluate synchronous nitrification, denitrification, and P-removal feasibility, P and N profile performance, aerobic kinetics, and bacterial structure, potential drawbacks, and improvement alternatives were inspected and clarified in case of aerobic and anoxic phases and possible occurred processes. In addition, nutrient removal was evaluated by the contribution of the critical functional microbial groups through microbial analysis for identification of relative abundance on phylum, class, and genus levels for different EBPR samples.

In **Chapter 7**, the effect of DO concentration in a range of 0.4 to 2 mg/L with continuous aeration is specifically investigated on phosphorus removal by understanding the various microbial communities for achieving high P-removal. Moreover, the effect of steady and instant DO reduction in different aeration strategies was studied in batch tests with enriched PAOs at different

DO levels. Recent studies have established an achievable nutrient removal process at low-DO levels with an adaptation period of activated sludge to lower oxygen rates. Aeration reduction was performed in an instant and step-wise manner, evaluating the adaptation of microbial community to aeration change and minimal oxygen availability.

Implementation of mathematical modeling for Micro-aerated EBPR (Chapter 8)

In **Chapter 8**, the results from the experiments were implemented for mathematical modeling. The primary goal was to develop a simulation model as an evaluation and comparison tool for micro-aerated SBRs performance. Moreover, the simulation model was aimed to predict the nutrient removal performance by changing dissolved oxygen (DO) concentration and the aerobic hydraulic retention time (HRT) and understanding the effect of parameters including DO concentration and HRT on treatment performance to improve operation and control.

Overall, the stepwise research demonstrated:

- The significance of aeration as a crucial operational factor on EBPR performance in particular at the aerobic stage
- The importance of understanding various aspects of aeration, including concentration, duration, and pattern, and its direct effect on EBPR process performance
- The role of population dynamics, specifically PAOs structure and abundance in different aeration conditions and high acclimatization of PAOs in the system by a selection of balanced aeration strategy
- Explanation on practical issues due to inefficient aeration concentration and aerobic retention time as well as insufficient aeration/non-aeration intervals, which causes severe upsets to EBPR process

2 General Introduction on Design, Operation and Technology Configurations for Enhanced Biological Phosphorus Removal (EBPR) Process

Adapted from:

Parnian Izadi, Parin Izadi, and A. Eldyasti, "Design, operation and technology configurations for enhanced biological phosphorus removal (EBPR) process: a review," Reviews in Environmental Science and Bio/Technology, vol. 19, no. 3. Springer Netherlands, 2020.

Preface:

This chapter of the thesis is a comprehensive review of the Design, Operation, and Technology Configurations EBPR process, published by the author, Parnian Izadi.

2.1 Abstract

Phosphorus as a fundamental element for the growth and metabolism of living organisms, yet problematic to water quality, is an irreplaceable component. Application of Enhanced biological phosphorus removal (EBPR) technology in wastewater treatment plants (WWTP) offers Phosphorus removal and recovery in addition to potential eutrophication prevention. This process is dependable on the enrichment of phosphorus accumulating organisms (PAO) in activated sludge to gather a significant amount of poly-phosphate inside their cell interior to enhance phosphorus removal. Yet, inadequate removal performance in pilot and full-scale systems raises the need for optimization in the operation and design of functional configuration. In addition to applying advancement strategies, minimizing the growth of undesirable microorganisms through cost-effective phosphorus removal and potential P-recovery and sustainability is of interest. Primary research has undoubtedly advanced the insight into this area of investigation.

Notwithstanding, there are still numerous unresolved issues to be undertaken. This comprehensive review paper aims to revisit the current knowledge and fundamental understanding of microbiology and biochemical transformations in the EBPR process. In view of application and

structure, EBPR design and operation considerations and process configurations are critically reviewed. This comprehensive review hopes to touch on the critical operation points to help understand the overall EBPR process and further provide insights on future work on EBPR process developments.

Keywords: Phosphorus, PAO, EBPR, WWTP, process configuration, operational factors

2.2 Introduction

Eutrophication, the overgrowth of algae and dead zones in coastal marine ecosystems [4], ammonia toxicity, nitrate accumulation, and limited water resources urge the need for a proper nutrient removal and management strategy to preserve water quality. Excessive nutrients are problematic to living organisms' health, harming the human quality of life [5]. Phosphorus, as a critical nutrient for the growth of microorganisms and a fundamental element for producing essential organic molecules such as adenosine triphosphate (ATP), originates the adverse effects on living organism communities [6]. This element promotes eutrophication due to its anthropogenic activity in aquatic ecosystems [7]. Significant sources of phosphorus enter freshwater from agricultural drainage, livestock discharge, and wastewater effluents [8].

Many case studies have shown worldwide challenges. Mississippi River watershed, which is among the world’s top ten rivers [9] regarding length, is a well-known example of change in the landscape caused by high nutrient loads due to human activity [10]. The Black Sea is known to be severely impacted by human activity since the 1970s [11]. One of the significant effects of eutrophication has been the abundance of gelatinous organisms in the Black Sea, which are the cause of a decline in the health levels of animals. Lake Erie, one of the warmest, shallowest, and

biologically productive lakes in North America are highly jeopardized by harmful algal bloom and low dissolved oxygen in the central basin.

In most cases, mitigation strategies were developed to reduce the P-loading; however, the conditions may never fully recover due to the nonlinear response of coastal ecosystems to nutrient loading [12]. Either recycling nutrients causes this resistance from the seafloor or biological, atmospheric nitrogen fixation by cyanobacteria that sustains eutrophication [13]. Indeed, advanced removal processes are crucial in the case of prohibition and preservation of current waterbodies.

The development of technologies for removing phosphorus as a justification for excess-P environmental damage offers advantages such as recycling and phosphorus sustainability [14]. Major treatment approaches lie in Physico-chemical removal, biological removal, or a combination of both processes with the most widespread use [8] [15]. Currently, many treatment plants comprise chemical precipitation using alum or lime as either the sole or integrated P-removal method [16]. As the biological removal method, enhanced biological phosphorus removal (EBPR) has grown from an incidental remark to a well-structured application through years by extensive research and full-scale works [17].

Biological removal takes place by phosphorus uptake in higher amounts needed for bacterial metabolic requirements [8]. EBPR is applicable by sequentially recirculating sludge through anaerobic and aerobic conditions [6] linked with anaerobic influent wastewater addition. The accumulated P is wasted through the P-rich sludge wastage procedure [18]. This process is dependable on the enrichment of phosphorus accumulating organisms (PAO) in activated sludge [19] to accumulate a significant amount of poly-phosphate inside their cell interior for enhancement of BPR [20]. The EBPR performance for higher PAO enrichment depends on influent composition, carbon source, and operational conditions [21].

Notwithstanding, there is microbial competition between PAOs and other microorganisms with similar metabolism and biochemical transformations known as glycogen accumulating organisms (GAO). GAO correspondingly is capable of proliferation in sequential anaerobic, aerobic phase without assisting in the P-removal process [6]. Therefore, in EBPR systems and full-scale plants, optimization in operation and design of functional configuration and applying advancement strategies will minimize the growth of undesirable microorganisms through cost-effective phosphorus removal. The development of this system offers phosphorus removal from wastewater along with potential P recovery and sustainability [14].

In this review paper, the potential superiority of the EBPR process over chemical precipitation will be thoroughly addressed. The review aims to briefly discuss the microbiology structure and mechanism of abundant microorganisms in biological phosphorus removal and to comprehensively review the EBPR design and operation and process configurations and technologies in full-scale plants to evaluate their applicability for a sustainable P-removal and potentially P recovery.

2.3 Phosphorus removal methods

Phosphorus removal technologies initially constituted in the 1960s with increased attention to eutrophication and high phosphorus levels in surface waters. By the 1970s, various process configurations were developed to remove phosphorus from wastewater [14]. Phosphorus in wastewater can be incorporated into biological solids or removed by Physico-chemical treatment or a combination of both methods [22]. In recent years, much work has been done to enhance P-removal from wastewater.

Various chemical treatments such as absorptive media, chemical dosing, and ion exchange are applicable in the P-removal process, where solids with different metal and phosphate concentrations are established [23]. The most widely used chemical method is precipitation by adding divalent or trivalent metal salt to wastewater, with a removal efficiency of up to 90% causing precipitation of insoluble metal phosphate [14]. This method is relatively easy to apply to treatment plants since it only requires a chemical holding tank and sufficient sludge settling time. In addition, the flexibility of chemical precipitation is of interest, as it can be applied at any stage of the treatment process. Although chemical precipitation by addition of chemicals such as iron (Fe) and aluminum (Al) or lime with dosing up to 95% negatively impacts the forthcoming sludge handling and treatment process by generating less biogas and methane in an anaerobic digestion system in comparison to not-chemically treated sludge [24]. Moreover, phosphorus with strong bonding with ions is unavailable for recovery due to the complex bonding matrix [25]. Therefore, there has been a rising interest in other potential alternative technologies suggesting valuable recycle products along with high removal efficiencies [14].

Biological P-removal method effectiveness in terms of environment and cost can prevail the chemical treatment; however, most WWTPs still incorporate chemical precipitation with biological methods [16] as a supplement to control P-discharge from WWTPs effluent. However, literature findings confirm that EBPR economic benefits may fade away if chemical addition causes inhibition in the biological removal of phosphorus mechanism [26] [27]. Thus, enhancing biological processes to reach high removal efficiencies or intermittent chemical addition is highly advised for a consistent P-removal approach in WWTPs [28][27].

Algal hybrid treatment is a possible novel nutrient removal system in which phosphorus controls the algal growth as a critical nutrient for their proliferation. As shown in Figure 2-1**,** under specific

P-limited conditions, higher phosphorus levels are stored as poly-phosphate granules in microalgae cultures comprising *Chlorella*, *Scenedesmus*, and *Spirulina* [29] [30]. Thus, algal systems offer potential high nutrient removal, with low operational cost, no carbon requirement, and avoidance of sludge handling problems [29]. However, this technology is yet in its early stages of identification and demonstration, particularly for implementation at pilot and full-scale systems [7]. The summary of removal methods is indicated in Figure 2-1. Biological phosphorus removal as the environmentally friendly and sustainable method in this research is the main focus [31] [14].

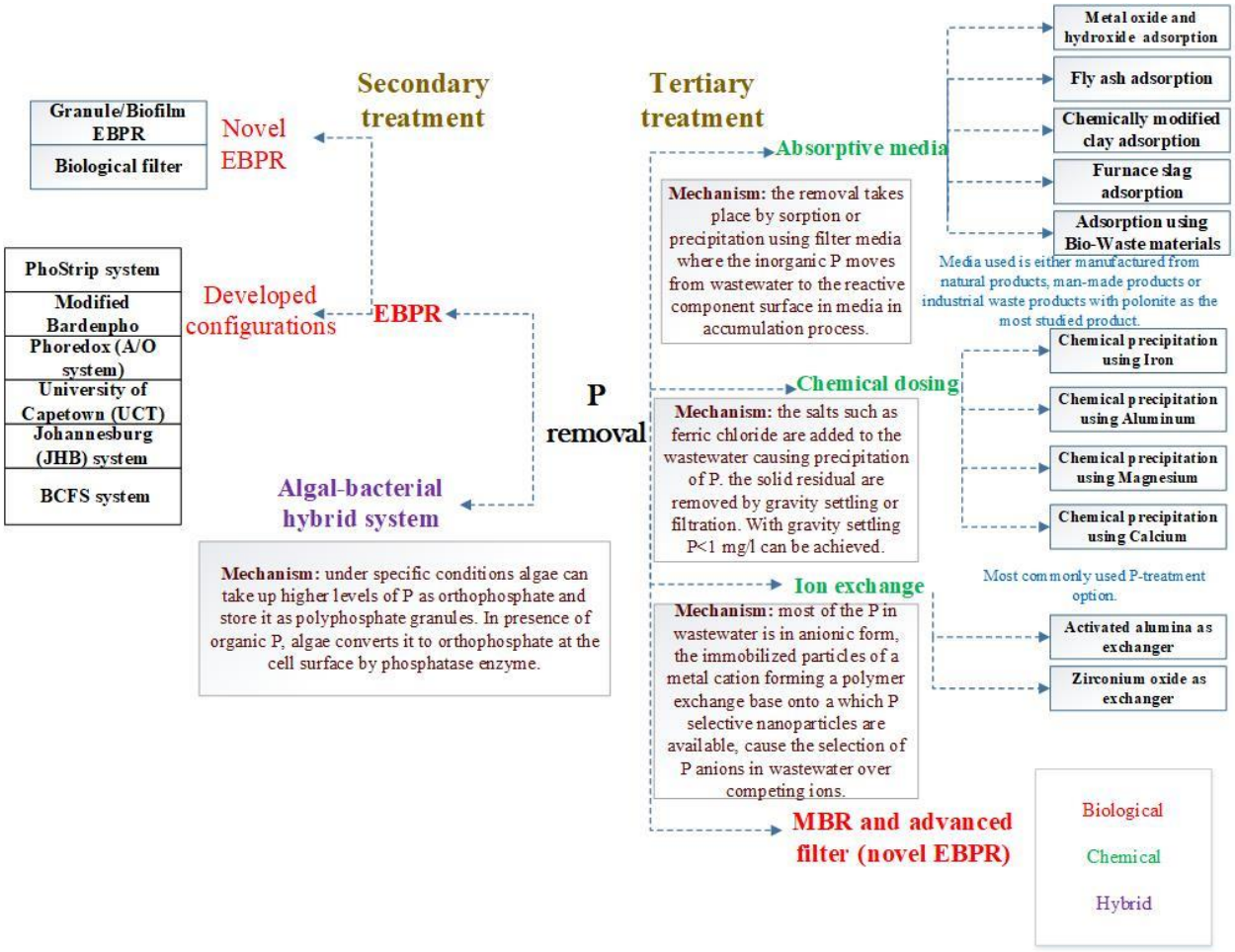

Figure 2-1 Phosphorus removal methods applied in wastewater treatment plants including biological, chemical, and combination of both methods

2.4 Fundamentals of EBPR

In typical aerobic activated sludge, phosphorus commonly biologically assimilated is roughly 0.02 mgP/mgVSS and the expected phosphorus removal is approximately 15 to 25%, while in enhanced biological phosphorus removal (EBPR) systems, the assimilated P-value increases to 0.06 to 0.15 mgP/mgVSS with an adaption of operation and design [17]. EBPR favors the P-recovery process in wastewater treatment since phosphorus is captured in a suitable form and biological sludge is more bioavailable [22]. At the same time, it requires more complicated process configurations and operation establishment [14]. PAO in high population provides a reliable EBPR system. The growth of these microorganisms relies on conditions that favor them while having disadvantages for other groups of microorganisms regarding food accessibility [32]. PAOs have a 13% lower yield due to energy-wasting metabolism than ordinary heterotrophic bacteria, increasing the urge to provide specific conditions for their growth and proliferation. Providing an anaerobic phase before secondary treatment, where no electron acceptor is present, increases PAO potential growth in the following anoxic and aerobic steps. The rapid substrate uptake under anaerobic conditions is the primary path for survival among other microorganisms [33][34].

In anaerobic conditions, as indicated in Figure 2-2, PAOs do not grow but convert the simple organic materials such as volatile fatty acids (VFAs), mainly produced by fermenting microorganisms, to energy-rich polymers known as polyhydroxyalkanoates (PHA) with the most common sorts of polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV). The energy required for VFA uptake and PHA generation is produced from hydrolyzing the phosphodiester bonds of stored polyphosphates [35] and release of phosphate molecules resulting in an increase in phosphate concentrations in the anaerobic stage. PHA formation requires energy for 1- active transport of VFAs across cell membrane 2- production of coenzyme A from VFAs and 3-

nicotinamide adenine dinucleotide (NADH) production as reducing power for PHA production [17]. Energy is produced in oxidation-reduction reactions, where adenosine diphosphate (ADP) is converted to ATP by capturing 7.4 kcal/mole in the phosphate bond. With the energy utilization of cells, ATP is converted to ADP with P-release [22]. Once PAO takes up the VFA, the Acetyl-CoA is activated.

Consequently, two acetyl-CoA are summated into acetoacetyl-CoA, which is subsequently reduced to 3-hydroxybutyryl-CoA followed by polymerization to PHB [36][6]. However, there has been a controversy on the origin of reduction equivalent necessary for PHA production with initially two biochemical models proposed for reducing equivalent generation in anaerobic conditions. Comeau et al. [37] and Wentzel et al. [38] accounted tricarboxylic acid cycle (TCA) for reducing power generation, while Mino et al. [39] suggested glycolysis via Embden-Meyerhoff-Parnas (EMP) pathway responsible for reducing power generation without any cooperation of TCA cycle [40][6]. Notwithstanding, cyclic experiments indicated the insufficiency of the glycogen degradation pathway to produce the amount of NADH to balance the biochemistry of the EBPR process, suggesting the functionality of the TCA cycle [41]. Therefore, studies have suggested the possibility of both mechanisms' existence in contribution to the total process [42].

In EBPR systems, under anaerobic conditions, GAO's have a similar metabolism to PAOs with a significant difference in their energy source as GAO cannot store polyphosphate. Therefore, glycogen as the primary energy production source is consumed in higher amounts, resulting in more reducing equivalent for redox balance maintenance of cells. In addition, the difference in fractions of polymers produced has been indicated between GAOs and PAOs due to diversity in

intermediate production and conversion rates through the glycolysis pathway and TCA cycle [41][6].

In the aerobic zone, sequential reactions occur, classified into energy generating and energy-consuming reactions. The energy-generating reactions are PHB catabolism and oxidative phosphorylation, while the energy-consuming reactions are biomass production, Poly-P synthesis, and glycogen synthesis [43]. Catabolism of the solely available substrate (PHA) is responsible for the growth of PAOs and, eventually, the growth rate of biomass and cell maintenance. Moreover, external phosphate uptake and polyphosphate generation are dependent on PHA degradation as energy and carbon source, decreasing by PHA consumption whereby complete exhaustion of PHA, the P-uptake process is disrupted [44]. In aerobic conditions, the phosphate level decreases in bulk liquid and increases in biomass as poly-phosphorus with as high as 15 % of the dry weight [45][46]. It is essential to mention that higher phosphate is absorbed in the aerobic phase rather than P-released in the anaerobic period, leading to a P-concentration decrease in wastewater (Figure 2-2) [34].

The metabolic model of EBPR, shown in Figure 2-2, can potentially be established under denitrifying conditions instead of the aerobic phase, where the main difference is in the P/NADH ratio (δ). The aerobic coefficient for electron transport phosphorylation is 5/4 times higher than the anoxic coefficient as a consequence of available electron per mole of electron acceptor for oxygen (4 electrons) in comparison to nitrate (5 electrons) [47].

GAO with a practically similar aerobic/anoxic metabolism is passive in polyphosphate synthesis but only utilizes polymer degradation as a source of energy and carbon for biomass growth and glycogen regeneration along with cell maintenance [48][6]. Therefore, implementing proper

operational conditions for the proliferation of PAOs for outcompeting GAOs decreases the chance of EBPR deterioration [49][6]. In an EBPR process with adequate performance, PAOs comprise approximately 40% of the active organisms present, with the ability to remove 10-12 mgP/L per 500 mgCOD/L of influent [17] by exploiting the potential of PAOs to store phosphate as intracellular storage in the excess amount required for metabolism [16].

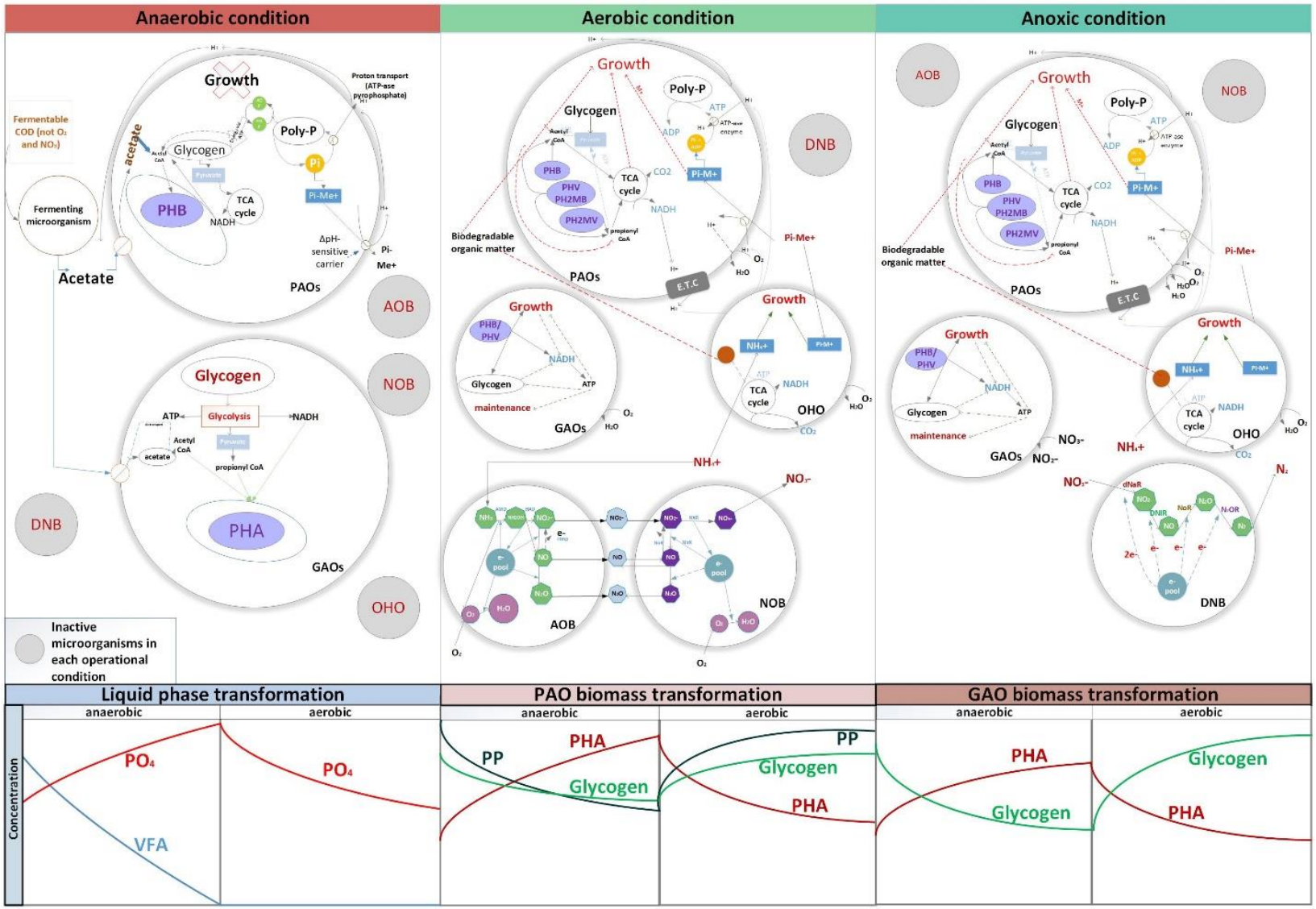

Figure 2-2 Microbial metabolism and phase transformations in anaerobic, aerobic, and anoxic operational conditions for active phosphorus accumulating and non-phosphorus accumulating microorganisms in EBPR system

2.4.1 The microbiology of EBPR

Although the EBPR system has been established in full-scale for many years, microbiological and biochemical sectors lack fundamental understandings. The predominance of PAOs in EBPR demonstrates their importance in phosphorus removal mechanism; however, GAO dominating in EBPR causing internal disturbance to the process operation raises the need for extensive research in microbiology of both microbial groups [39]. (A summary of identified PAOs is available in **Appendices A, Table 10.1**).

2.4.1.1 PAOs identification and microbiological aspects

The initial observations of PAO characteristics indicated very densely, good settling flocs in activated sludge and non-motile rods or cocci, gram-positive bacteria with Neisser positive granules existing in clusters with a size of typically more significant than 0.5 μm [50]. These bacteria are relatively slow-growing with at least one week of cultivation time [39].

The first PAO (*Acinetobacter)* was proposed over 30 years ago through a culture-dependent technique. The first attempt by Fuhs et al. [51] in isolating PAOs resulted in the identification of *Acinetobacter* in *Ɣ-Proteobacteria* as the primarily responsible microorganism for P-removal in the EBPR system. This belief went on for decades, and little effort was made for further identification [46]. After that, with rising technologies such as FISH probes, minimal contribution in P-removal was detected from *Acinetobacter* compared to *β-Proteobacteria* and *Actinobacteria* [52][46][6]. Various experimental evidence such as fluorescent antibody technique, respiratory quinones analysis, and 16s-rRNA application, in addition to pure culture studies, indicated evidence against *Acinetobacter* predominance in the EBPR process. Studies have shown the presence of *Actinobacteria* in the EBPR process; however, their identification as PAOs requires

further study since these microorganisms lack VFA uptake ability. Intracellular storage of *Actinobacteria* differs from PHA, resulting in different behavior towards biochemical models proposed for other PAOs [53][6]. Recent molecular techniques have revealed the diversity of bacterial species in EBPR sludge, including *Proteobacteria*, gram-positive high G+C *Actinobacteria, Planctomycetes,* and *Bacteroides*. In further studies, [54][55][56], microorganisms such as *Mirolunatus Phosphovorus, Lampropedia,* and *Tetrasphaera* were isolated as potential PAOs; however, additional research proved their difference with PAO characteristics [55][6]. *Candidatus Accumulibacter,* one of the most studied PAO, has been resolved to strain level by polyphosphate kinase gene (ppk1) sequencing. This genus belongs to the family *Rhodocyclacea* of the class *β-Proteobacteria* that has still not been isolated as pure culture [34]. Two major groups of *Accumulibacter,* type I and type II, with different divisions were demonstrated. Skennerton et al., specified ten *Accumulibacter* genomes as follows: BA-93 and UW-2 from IA clade, BA-91, SK-01, and SK-02 from IIC clade, UW-1 from IIA clade, BA-93 from IC clade, SK-11, SK-12, and BA-94 from IIF clade. In this study, it was hypothesized that operational condition variation may favor different *Accumulibacter* clades due to metabolism differences. All clades share identical carbon and phosphorus metabolism pathways that are essential for PAOs, however, differences are considerable in nitrogen metabolism and carbon sources [57].

A novel Strain *Tetrasphaera* as a potential PAO is capable of fully performing the physiological EBPR process. At the same time, other groups of PAOs are known to function by mutual interdependence with other communities. This genus is also known for its denitrifying capabilities [58]. All four metagenomic isolated clades of *Tetrasphaera* can reduce nitrate to nitric acid, though only two can minimize nitric acid to nitrous oxide [59]. *Tetrasphaera,* with the fermentation of complex organic molecules such as amino acids and sugars, is capable of sequentially storing

energy and carbon source; however, approximately all members are incapable of PHA formation yet, store glycogen. Fraction of *Tetrasphaera* produces VFA in anaerobic condition and provide VFA requirement of other PAOs [60]. *Tetrasphaera*, unlike other PAO groups, synthesizes glycogen and releases phosphate under anaerobic conditions followed by utilization of glycogen as an energy source for generating polyphosphate, which induces its versatility in terms of ecophysiology, comparing to other microorganisms [61].

Altogether, the most common PAOs are *Candidatus Accumulibacter Phosphatis β-Proteobacteria, Acinetobacter δ-Proteobacteria, Dechloromonas β-Proteobacteria,* and *Actinobacteria*. Conventionally it was believed only one single microorganism is responsible for the EBPR process; however, the EBPR community is genuinely diverse composing of few dominant bacterial strains.

Data available on the phylogeny of the EBPR community proposes a very high diversity in the population of PAOs. Different studies have indicated sequences that are similar but different from each other [62][46]. With challenges in identifying the PAOs, the *Accumulibacter Phosphatis* or *Rhodocyclus*-related bacteria were placed in the phosphorus-removing community. Carbon fixation potential, with high-affinity transporters for phosphorus, forms *Accumulibacter* suitable for EBPR systems [57]. Studies [63][64] positively confirm that these groups correspond to PAO characteristic of poly-P and PHA cycle of release and uptake in anaerobic/aerobic sequence. Further work on WWTP confirmed the high contribution of *Accumulibacter Phosphatis* in EBPR processes [6]. **Appendix** (A. Table 10.1) summarizes a few studies on the population composition of EBPR communities and the PAO identified.

2.4.1.2 GAO identification and microbiological aspects

Earlier studies have observed the presence of cocci-shaped organisms arranged in a tetrad (not always the case) in systems fed with glucose or acetate, which participate in COD removal without any phosphate release in the system [65][6]. In 1995, Mino et al. [66] proposed GAO (glycogen accumulating organisms) for these microorganisms. The metabolism of PAOs and GAOs is considerably the same, with a difference that GAO provides energy and reducing power from internally stored glycogen without disturbing the redox balance of the cell. PAOs and GAOs are morphologically different, where GAOs Neisser straining is positive on cell walls, however for PAOs, Neisser is strongly positive inside the cell for granules. PAOs and GAOs with almost similar functional pathways and variation in energy generation source [67] contrarily affect the EBPR process, where GAOs limit VFA source for PAOs, causing failure in the P-removal process [67]. GAOs, are known to anaerobically assimilate carbon sources to produce PHA, where eventually it will be utilized in the aerobic phase for glycogen formation [68][46]. However, GAOs metabolism is more complex and less efficient than PAOs, resulting in faster carbon source (acetate) uptake by PAOs [39].

Culture-dependent method of isolation identified phylogenetically diverse bacteria belonging to *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria,* and *Actinobacteria* as potential GAOs, yet none of these groups of microorganisms dominated in a lab or full-scale deteriorated systems. Culture-dependent methods identified GAO phenotype *Candidatus Competibacter Phosphatis* and *Competibacteraceae* family belonging to *Gammaproteobacteria* to disrupt EBPR systems potentially. Moreover, *Sphingomonas*-related organisms and microorganisms related to *D. Vanus* and *Defluviicoccus* belonging to *Alphaproteobacteria* class, the actinobacterial genus *Micropruina,* and the betaproteobacterial genera *Propionivibrio* [69] have shown potential carbon

source uptake and active competition with PAOs [70]. However, further investigation and research is necessary for the determination and confirmation of their role in the EBPR system

2.5 EBPR design and operation considerations

EBPR process depends on the growth and flourishment of PAOs by the alternation of anaerobic and aerobic or anoxic conditions; therefore, identifying and understanding the factors that influence EBPR performance should be highly considered before implementing EBPR configuration [71].

2.5.1 Influent wastewater characteristics and VFA source

PAOs anaerobically require substrate mainly as VFAs to release phosphorus into liquid-phase along with polymer storage within cells. There is a consideration that in the case of municipal wastewaters containing simple carbohydrates, amino acids, and alcohols other than VFAs, non-PAO heterotrophs ferment these substrates to simple volatile acids [72] [73] [74]. Sufficient VFA content is essential for high phosphorus removal, which relying on fermentation of rbCOD in the anaerobic zone in WWTPs may not be efficient [75].

In case of insufficient carbon availability in raw wastewater as substrate, there is a requirement for additional external carbon sources, mainly in VFAs such as acetate and propionate. All other VFAs such as butyrate, valerate, iso-valerate, and lactate needs to be further fermented to become effective [60]. VFAs have shown the most effective results in nutrient removal; however, the addition is costly, increasing the carbon footprint [76]. Moreover, the Consumption of VFAs by GAOs with no contribution to P-removal has an inhibition effect on the EBPR process. Therefore, the selection process of carbon source depends on the economic cost of external source and PAOs performance under adding conditions. Different substrates have been examined for the EBPR process, causing various inhibition, enhancement, or negligible influence [73].

As proposed by many research [77] [78][79], particular carbon sources and VFA composition, such as acetate, have shown a relatively stable EBPR performance with consideration of other operational factors such as pH and temperature [80]. However, it creates conditions favorable to GAOs and disadvantageous to PAOs where *Competibacter*, as a culture of GAOs dominates in these processes while EBPR subjected to propionate has shown high P-removal efficiency [81]. In propionate as the primary substrate, *Competibacter* is incapable of proliferation while *Alphaproteobacterial* GAOs mainly dominate. Although based on investigations, PAOs have the higher capability in utilizing propionate in anaerobic conditions. Moreover, PAOs ability to take up various substrates and high total PHA yield contribute to their diversity and abundance compared to GAOs, which mainly have specific carbon source preferences, rather than mixed VFA substrate [77] [82].

Apart from VFAs, other carbon sources such as glucose and glycerol have been used as potential substrates in various experiments [83]. However, there is a controversy on the direct impact of glucose on PAOs metabolism. A generally accepted concept proposed the inability of PAOs to utilize glucose unless hydrolyzed. At the same time, GAOs take up glucose for glycogen accumulation without release and uptake of phosphorus through anaerobic and aerobic phases, respectively [84] [85]. On the other hand, Tracy and Flammino et al. [86] indicated anaerobic P-release by glycogen storage in PAOs. A study by Nakamura et al. [87] supported this theory by reporting *Microlunatus Phosphovorus* as PAO culture capable of glucose or peptone uptake. In most studies with successful EBPR process using glucose as substrate, PHAs storage and linkage between glucose concentration decrease and P-release were not detected in anaerobic conditions [88]. With a further need for evaluation, there is a possibility of PHA replacement with glycogen as the energy provider in PAOs metabolism, fed with glucose [80]. In the case of glycerol,

supplementation of a VFA-enriched supernatant via co-fermentation with waste-activated sludge had rather more promising results compared to the direct application of glycerol as the sole carbon source [89].

In addition to carbon source addition, on-site primary-sludge fermentation is a considerable method for increasing VFA content without higher cost; however, there are several disadvantages such as high ammonium and phosphate release along with VFA production, which affects the main-stream process as well as costly and ineffectiveness of VFA separation process from fermented sludge [76]. According to literature, acetic acid is the leading organic composition available in fermentation liquid of organics due to its easy bio-recovery, which is responsible for nutrient removal [90].

As a whole, in choosing the suited carbon source regarding system performance, cost, feasibility, and microbial community, different results have been obtained based on process configuration and operational factors [80].

2.5.2 Presence of nitrate and nitrite in the anaerobic zone

Nitrite is an intermediate product of nitrification and denitrification. Due to its accumulation properties in coexistence with its protonated form, free nitrous acid (FNA) will generate a harmful competition for PAOs with GAOs [28] to the extent that microbial population shifts causing an unfavorable situation P-removal. Consequently, aerobic-PAO is more affected by nitrite presence than anoxic-PAO due to less tolerance to nitrite inhibition [91]. There is a wide range of results and studies reporting the effect of nitrite on PAOs and EBPR process in contradiction to each other, part of them arguing very low tolerance of PAOs to nitrite concentrations as low as 3 mg N/L [92] [93]. In contrast, others have reported no inhibition on P-uptake in nitrite levels of 115 mgN/L [94]. In addition, the disagreement of results may be related to inconsideration of FNA

effect on growth and energy generation of microbial community [93]. At the same time, only nitrite toxicity has been investigated against bacteria growth and respiration process. Moreover, it has been reported that increasing nitrite/FNA ratio tends to stimulate nitrous oxide (N_2O) production as a greenhouse gas, where FNA reaction with N_2O reductase results in N_2O accumulation [95]. However, the N_2O accumulation only takes place by an imbalance in the reduction activity of enzymes, including nitrous oxide reductase (N_2OR), nitrate reductase (NAR), and nitrite reductase (NIR) [96].

Therefore, most research on nitrite has focused on its inhibitory factors rather than the potential of serving as electron acceptor [97], while studies investigating P-removal by operating anaerobic/anoxic/aerobic performed at initial nitrite concentrations as high as 20 mgN/L as an electron acceptor in anoxic conditions [98].

Most of the research on nitrate presence in the EBPR system has conducted nitrate as an electron acceptor for denitrifying phosphorus removal resulting in lower sludge production and oxygen requirement than the conventional system [97]. However, the presence of nitrate has been reported to inhibit P-release at the level of adenylate kinase [99] in the anaerobic conditions, which may be caused by either i) increased activity of ordinary heterotrophs in nitrate or nitrite reduction, consuming the available COD for PAOs as electron donor which lessens the PAOs growth, ii) simultaneous P-release and P-uptake due to presence of electron acceptor and electron donor and iii) inhibitory effect on EBPR activity wherein a study conducted by Guerrero et al. on A2/O process showed activity inhibition is the most plausible hypothesis regarding low P-removal [100].

The metabolism of denitrifying polyphosphate accumulating organisms (DPAOs) is yet not entirely known. Several studies have suggested the presence of two types of DPAOs, one rod morpho-type able of utilizing nitrate and nitrate as electron acceptor (Nitrate-DPAO) where the

other linked to coccus morpho-type only is capable of using nitrite (Nitrite-DPAO) [101]. The latter lacks the gene responsible for reducing nitrate to nitrite while it contained genes for denitrification of nitrite [102]. Flower et al. [103] demonstrated the presence of different clades of *Accumulibacter Phosphatis* as the putative PAO depending on the primary available electron acceptor. By developing FISH probes for PAO I and II, the study concluded the capability of PAO I in utilizing nitrate while PAO II solely utilizes nitrite. Instead of using oxygen in the aerobic phase, DPAO is capable of coupling nitrate and nitrite reduction to P-uptake [104].

Further investigations in metagenomics revealed the inability of the majority of type II subgroups to use nitrate. Only clade IIF containing nitrate reductase and nitrite reductase could take up nitrate as an electron acceptor, while other clades only have periplasmic nitrate reductase whose role is unclear [105] [106]. Nitrite-based P-removal accompanied development strategies for stable performance. The lowest nitrous gas emission can establish an innovative cost and energy-saving P-removal with approximately 25 and 40% less oxidation cost and carbon consumption than nitrate-fed systems [95].

2.5.3 Anaerobic and Anoxic contact-time

Hydraulic retention time (HRT) as a critical operational parameter for biological nutrient removal (BNR) influences the reliability and stability of systems, which directly affects the infrastructure, design, and operating cost of WWTPs. This operational factor determines the substrate loading, biomass and pollutant contact time, and process performance [107]. HRT may also influence the intracellular polymeric storage production that initiates carbon capture and energy recovery of PAOs [108]. The literature proposes an anaerobic HRT of 0.5 to 2 hours for P-release, anoxic HRT of 1 to 4 hours for denitrification, and aerobic HRT of 4 to 12 hours for simultaneous nitrification and P-uptake. [109]. Due to differences in anaerobic needs of nitrogen and phosphorus removal,

various HRTs are required for an efficient BNR process. As a critical factor on P-removal, Anaerobic HRT serves as a proper condition for energy reserve generation since sufficient time is needed for complete metabolism of carbon and polyphosphate and depletion of VFAs [110]. Short anaerobic HRT creates anoxic conditions with low levels of PHA generation, leading to the insufficient energy source for aerobic P-uptake. Long HRTs, decrease the PHA reserves to almost complete depletion, resulting in secondary phosphorus release [111]. The higher the anaerobic VFA availability, the less anaerobic HRT is required. Moreover, very long anaerobic HRT has a detrimental effect on nitrogen removal due to limiting COD requirements for denitrification. In the case of anoxic HRT, both nitrogen and phosphorus removal is affected by the duration. The short anoxic phase results in incomplete denitrification; increasing the HRT improves nitrogen removal, but excessive anoxic conditions deteriorate EBPR performance. Therefore, the optimum range of HRT differs between nitrogen and phosphorus removal processes. Efficient N-removal occurs at low anaerobic HRT and high anoxic HRT, while the P-removal process is highly efficient in high anaerobic HRT and low anoxic HRT [111]. While, the effect of extended anaerobic contact-time along with the extent of the maximum duration of efficient anaerobic condition is not very well-understood, Barnard et al. [112] has proposed excessive anaerobic condition with no VFA uptake as secondary release since, this amount of P-released is not associated to stored PHA.

It has been observed that even with complete exhaustion of rbCOD, microbes tend to anaerobically hydrolyze polyphosphate, causing an imbalance in the amount of P-released and the PHA generated from VFA storage [110]. Therefore, the inefficient energy source is available in the subsequent aerobic phase for P-uptake [113].

2.5.4 pH control in anaerobic and aerobic zones

pH with an essential role in the EBPR process affects the P-release/VFA uptake ratio and intracellular polyphosphate degradation in anaerobic conditions [114]. Increasing the pH results in higher P-release due to higher energy requirement for acetate transformation and subsequent higher poly-phosphate degradation [115]. In anaerobic metabolism of PAO, by VFA uptake through cells, ion and proton are generated, creating proton motive force imbalance. To reach stabilization, PAO hydrolyzes polyphosphate and release phosphate into the bulk liquid. Increasing the pH further decreases proton motive force leading to higher P-release rates [116] [40]. Anaerobic metabolism of PAOs and GAOs is dependent on pH values. By increasing pH from 6.5 to 8, P-release in PAOs increased. Based on the maximum acetate uptake rate in both microbial groups, it is suggested that PAOs have a competitive advantage over GAOs in pH levels higher than 7.25 since manipulating pH into elevated levels adversely affects GAOs anaerobic substrate uptake [116] [117]. The most increased anaerobic activity of PAOs is reached at this level of pH without any further incline. In higher pH levels, GAOs have a slower rate of VFA uptake comparing to PAOs. In comparison, GAOs are the dominant group in lower pH levels in acetate assimilation, resulting in a clear microbial shift from PAOs to GAOs and EBPR failure [118]. Unlike GAOs, PAO's PHA synthesis and glycogen degradation are dependent on pH variation. In the subsequent aerobic phase, due to the complexity of metabolism of PAOs as the only sensitive bacterial group to pH, more data on PHA and glycogen profile is required [117]. Studies have shown that values greater than seven should be maintained [119].

However, in WWTPs, controlling the pH at a certain level is not efficient. Therefore Zhang et al. [120] investigated the optimal initial pH range for high EBPR performance. In short-term and long-term A/O operation, a range of 6.4-7.2 and 7.6-8 was proposed for high P-removal [121].

Monitoring the pH through anaerobic and aerobic phases indicates a slight increase in the anaerobic phase due to P-release and potential denitrification. In the subsequent aerobic phase, due to carbon stripping and phosphorus uptake, a significant increase in pH occurs, followed by a decrease in value after complete P-removal due to the CO_2 stripping and nitrification process [122].

2.5.5 Microorganism growth control

High-performance EBPR is reliable on the metabolism of PAOs in sequential steps of carbon source and electron acceptor availability. PAOs in biomass anaerobically deplete organic matter in wastewater into PHA storage with P- release into sludge. In the subsequent aerobic phase, PAOs utilize PHA as an energy source to take up phosphorus from WW and for their growth. P-removal takes place by sludge discharge with high P-content. In the discharge process, SRT needs to be taken into account since sludge withdrawal shortens the SRT of the process. In simultaneous N and P-removal systems, considering the slow-growth of nitrifiers, the shortening retention time will decrease nitrogen removal efficiency [123].

SRT as an operational parameter effective on P-removal performance yet creates several contradictory viewpoints on its exact influence [38][124][125]. Literature has addressed long SRT as a positive impact on PAO dominance in EBPR systems due to lower PAO decay rate comparing to other microorganisms, however recent studies demonstrated a decrease in biomass yield [126], GAOs high competition with PAOs in addition to sludge bulking in long SRTs [127]. Moreover, Li et al. indicated an increase in lag-time and startup period and a secondary anaerobic P-release by increasing SRT in the EBPR system [127]. On the other hand, short SRT improves PAO's growth, sludge settling properties, and phosphorus-rich biomass production [128]. Several studies have demonstrated the negligible effect of SRT change on the final phosphorus removal; however it may affect the P-content of biomass [125]. In addition, evaluation of SRT as a sole parameter is

complicated since several factors including food to microorganism (F/M) ratio, mixed liquid suspended solids (MLSS) concentration, hydraulic retention time (HRT), and biomass yield are effective on EBPR [129]. In general, from a practitioner's perspective, a lower and more stable effluent P level is achieved in SRT lower than ten days in the EBPR process [130].

2.6 EBPR configurations

Due to water shortage and stringent limitations for effluent release, there is an urgent need to upgrade WWTPs consisting of nitrification, denitrification, and phosphorus removal [131]. EBPR process relies on the growth and selection of PAOs capable of storing orthophosphate above the required amount for growth. A successful EBPR process is dependent on the presence of readily biodegradable organic carbon and phosphorus, an anaerobic zone before the aerobic zone, and a sufficient amount of nutrients [32]. Initially, phosphorus reduction was observed in a 4-stage nitrogen removal process with higher than 90% removal by Barnard in 1974. By changing the sequential steps in the specific configuration, low phosphorus removal was achieved, leading to an understanding of the necessary conditions of EBPR [75]. In real WWTPs, there is a combined process of nitrogen and phosphorus removal. The WW undergoes different environmental conditions, including anaerobic, aerobic, and anoxic zones, to promote biological and microbiological techniques such as phosphorus release and uptake, nitrification, and denitrification. Exclusively, specific conditions favor each process; the aerobic condition favors COD removal and nitrification while denitrification takes place in an anoxic zone. In phosphorus removal, all conditions are essential for microbial metabolism of PAOs and complete phosphorus removal [132]. Simultaneous nitrogen and phosphorus removal are not as straightforward as adding an anaerobic zone in favor of PAOs growth [133]. In most systems, nitrification and denitrification may cause a detrimental impact on EBPR due to the availability of nitrite and nitrate

in external recycling, which enters the anaerobic zone, leading to process failure. Anaerobic availability of electron acceptors such as nitrate and nitrite potentially sparks OHO growth and PAOs out-competition [131]. As shown in Figure 2-3, various biological phosphorus removal processes are well-understood and developed, and utilized on a pilot scale. In most WWTPs, the selection of the proper configuration is not as apparent due to many effective variables on process performance. This section comprised of a comprehensive review on different configurations of EBPR process.

All biological phosphorus removal systems fall into three categories of side-stream, main-stream, and cycling processes. The common feature of all side-stream configurations is the only treatment of return sludge anaerobically. At the same time, in main-stream methods, all the mixed liquor flow goes through the anaerobic conditions for phosphorus removal [134] [135] [32].

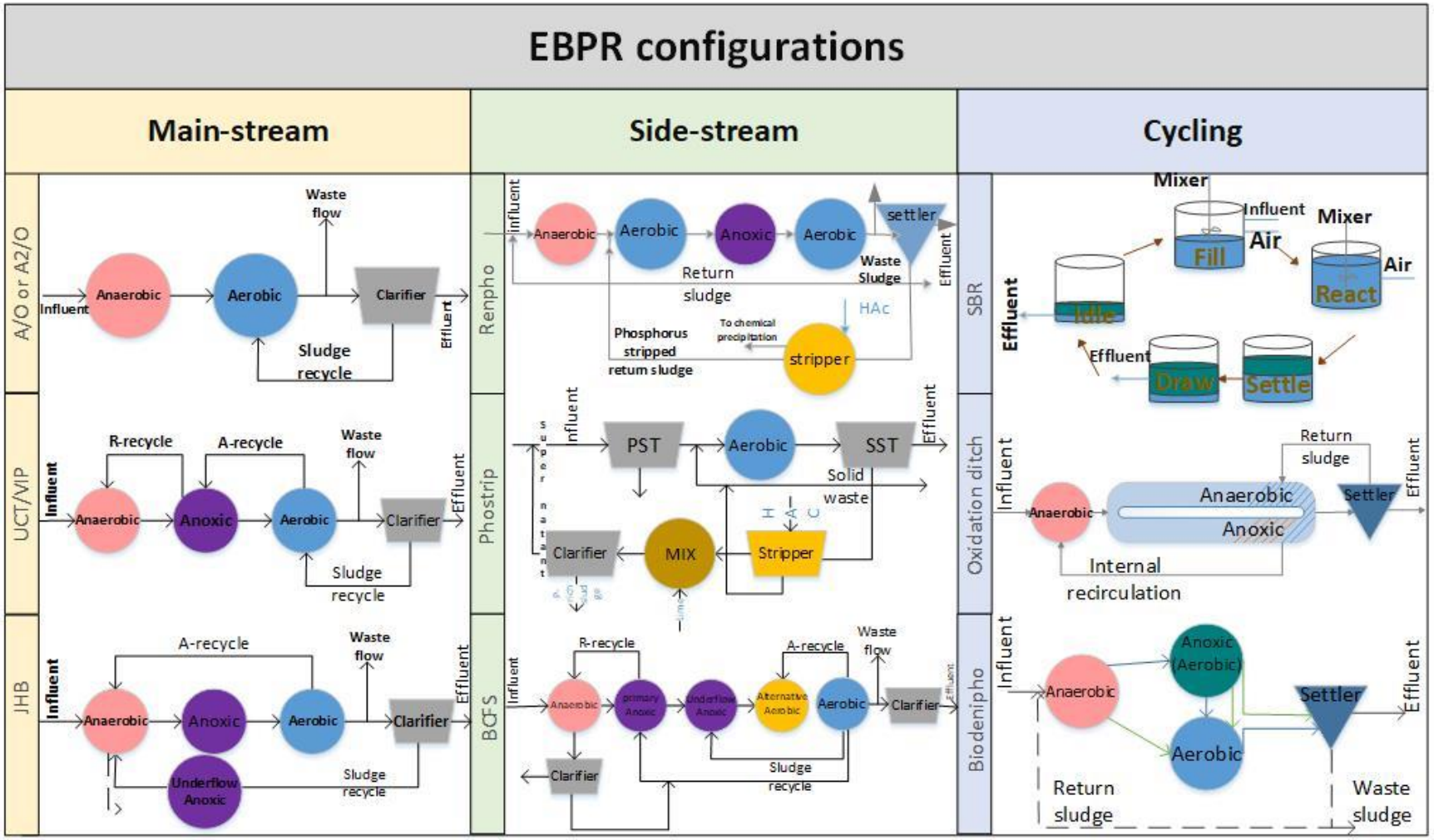

Figure 2-3 Main-stream, side-stream and cycling configurations of Enhanced Biological Phosphorus Removal process applied in WWTPs

2.6.1 Main-stream processes

The anaerobic microbial decomposition of complex organic matter to the end products methane and carbon dioxide is a multistep process comprising hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The anaerobic conversion is often thought to consist of two phases, the acidogenic and the methanogenic phases. The acidogenic step comprises hydrolysis and acidogenesis, the hydrolysis of complex organic molecules to simpler organic compounds, followed by the fermentation of these compounds into fermentation products often dominated by short-chain volatile fatty acids (VFAs). The methanogenic phase comprises acetogenesis and methanogenesis, resulting in converting VFA and other intermediary products to the end products methane and carbon dioxide.

For consistent low P-effluent, adequate quantity of VFAs are required for the series of biochemical reactions necessary for maximal P-removal to take placeS. Although acetate is the model substrate for EBPR [51], a blend of VFAs—specifically propionate—is more favorable for enhancing and stabilizing EBPR. For WRRFs receiving wastewater streams low in VFAs, some form of primary solids fermentation can be implemented, or supplementation with purchased VFAs may be considered. However, synthetically derived VFAs increase treatment costs considerably while concurrently increasing the WRRF carbon footprint. A potential alternative source of carbon that is readily available for VFA production through fermentation within a WRRF is mixed liquor. Specifically, the return activated sludge (RAS) could serve as a co-substrate to potentially generate additional carbon and thus potentially enhance EBPR; VFAs would be generated via side stream fermentation (i.e., inserting an anaerobic zone to ferment some or all of the RAS before re-introduction into the mainstream EBPR system). In addition to ensuring available VFAs, the RAS

nitrate concentration should be minimized to sustain EBPR. Most conventional EBPR process configurations, which incorporate pre-anoxic denitrification [22], result in residual, parasitic nitrate in the effluent and thus in the RAS stream. Excess RAS nitrate introduced in the anaerobic zone of the EBPR system can induce process failure (i.e., anaerobic conditions becoming anoxic); indeed, excess RAS nitrate is commonly a cause of EBPR failure [136]. Process configurations aimed at ameliorating RAS nitrate— namely the Johannesburg Process and the Westbank Process— incorporate a pre-anoxic zone up a gradient of the EBPR anaerobic zone, where denitrification is achieved either through endogenous decay or through the addition of raw wastewater or primary solids fermenter liquor. Alternately, it has been suggested that RAS nitrate reduction could potentially be integrated with and achieved in concert with VFA production through a side stream fermenter configuration. Conceptually RAS fermentation would seemingly be beneficial to EBPR-coupled VFA production and RAS denitrification. However, there are some potential concerns with adopting such a process configuration. RAS is less readily fermentable; thus, VFA production could be limited or could require excessive retention times (i.e., excess WRRF tankage) to achieve desired productivity.

Regarding process performance, anaerobic secondary P release is a concern. Exposure of EBPR biomass to anaerobic conditions in the absence of VFAs—or under conditions that might facilitate MLSS fermentation [137]—can induce hydrolysis of poly-P stores metabolically delinked from EBPR metabolisms. Another potential concern relates to use of glycogen reserves for denitrification of RAS prior to introduction into the anaerobic zone; while research has shown that some glycogen use for post-anoxic denitrification will not impair EBPR, excess glycogen utilization could impair process performance. Finally, GAOs also store glycogen, and RAS denitrification will certainly utilize glycogen as the electron donor (if an exogenous carbon

substrate is not added; PHA reserves are typically fully depleted in the EBPR process aerobically/anoxically); as such, it is possible that side-stream RAS denitrification might enhance this microbial population putatively detrimental to EBPR by creating a condition for denitrifying GAOs to become competitive. Ultimately, limited peer-reviewed data is available on the effects of RAS fermentation on VFA production, nitrate concentrations, PAO/GAO microbiology, and overall EBPR performance.

In the fermentation process, the critical parameter is: what portion of volatile suspended solids (VSS) is fermented? The amount of fermentable scVFA per g of VSS varies with temperature, the flatness of the sewer system, and the amount of fermentation taking place in the sewer. Numbers as low as 13% (Minneapolis-St. Paul, Minn.) and 28% (Westbank, B.C.) and as high as 40% of VSS in primary sludge (Kalispell, Mont.) being fermented to scVFA have been cited [138].

Carbon, especially rapidly degradable COD (rdCOD), is the electron donor required for denitrification and enhanced biological phosphorus removal (EBPR). When wastewater is carbon-deficient, methanol is used for denitrification. Methanol has a lower cost but requires the development of slow-growing specialized methanol utilizing biomass and thus cannot be used on an “as needed” basis. Nevertheless, methanol is the most popular substrate in denitrification. Ethanol would be a preferred source for denitrification; some full-scale research in Sweden has shown the nitrate utilization rates (NUR) to be in the order of 15 mg NO_3–N/g VSS·h versus methanol-NUR of 4 to 5 mg NO_3–N/g VSS·h [139]. New York City and the Washington Area Sewer Authority are considering using ethanol during winter in the hope of achieving higher denitrification rates. Trials with Winnipeg-North centrate denitrification using industrial wastes have demonstrated that potato processing wastewater accelerated denitrification to 12 mg NO_3–N/g VSS·h, which was higher than methanol at ten and lower than ethanol at 17 mg NO_3–N/g

VSS·h [140]. Methanol and similar compounds are not suitable for phosphorus removal. Sugar wastes have been added to primary sludge fermenters, which generate a combination of acetic and propionic acid. Adding acetate only, or sugar wastes, directly to the anaerobic zone may create conditions that encourage the growth of glycogen accumulating organisms (GAO) which successfully compete with phosphorus accumulating organisms (PAO) for the volatile fatty acids. Adding sugar waste to the fermenter will produce the proportion of VFA compounds optimal for phosphorus removal by PAO. A case in point is the New Eagle's Point BNR plant for South Washington County, Minn., where molasses was added to the fermenter, when needed, to improve the EBPR [138]. A variety of novel auxiliary carbon sources are used, of which fermentation of mixed liquor is perhaps the most interesting. First demonstrated incidentally in Barnard's original pilot work in 1974, it is an excellent way of producing the required VFA without an additional carbon load to the plant. Typically, some of the biomass is extracted from the anaerobic zone, fermented, and returned to the same anaerobic zone. In Western Canada, the U.S.A., throughout the U.K., and northern EU countries such as Poland and Germany, fermentation of primary sludge became standard practice, greatly enhancing plant performance. An excellent case study of fermentate benefits is provided by Calgary's 500 ML/d Bonnybrook (Westbank/JHB mode) plant where the new, fully separate, train (plant C: ADWF 100 ML/d) is equipped with 2-stage fermenters feeding short-chain volatile fatty acids (scVFA) to the anaerobic zone. Plant C operates in parallel with two separate trains (older plants, also in Westbank mode) operating without the benefit of scVFA. The fermenter connected plant C has worked, in the words of the operator, "flawlessly" for 10 years compared to the other two trains which had phosphorus removal and TN removal problems (P. Do, Senior Process Engineer, Bonnybrook Plant, Calgary, Alberta, pers. comm.). A one-stage static fermenter (as opposed to the two-stage fermenter-thickener

combination used in Calgary, Alberta, and in Saskatoon, Saskatchewan) consists of a gravity picket fence thickener, typically with 4 to 8-d SRT and 16 to 24-h HRT, and appears to be the most successful. Edmonton's 310 ML/d Gold Bar plant is built on a very tight site and retrofitted to a 4-pass BNR plant operating in a WestBank or step-feed mode. Excellent experience with a static fermenter led to the current construction of three more fermenters. The other Edmonton plant, the 70 ML/d Alberta Capital Region plant, was recently retrofitted to BNR and equipped with static fermenters (G. Stevens, EarthTech, Kelowna, B.C., pers. comm.). Production of VFAs or rbCOD from primary sludge fermentation to improve biological nutrient removal is now an established practice (e.g., in North America, South Africa) [141]. There are various primary sludge fermentation process configurations, e.g., activated primary clarifiers (APCs), elutriation sludge thickeners, continuous flow sludge fermentation, batch sludge fermentation, and sludge fermentation with recuperation thickening. Construction of primary sludge fermenters can be costly, but performance is considered reliable. Fermentation of return or waste-activated sludge to produce carbon to enhance biological nutrient removal has recently attracted increased attention. There are experiments in producing rbCOD from return or waste-activated sludge by fermentation. A review indicates that VFAs obtained by fermentation are a more sustainable carbon source than external sources in terms of cost comparison [142]. Fermentation of return or wasted sludge is in some ways more complex than primary sludge fermentation because it involves the decay processes of biomass within the mixed liquor, with associated release of phosphorus and nitrogen. One important concern, particularly in colder climates, is the effect of maintaining a higher anaerobic mass fraction on nitrification.

Side-stream EBPR (S2EBPR) process with addition of a side-stream anaerobic reactor for recycle of a portion or all of the return activated sludge (RAS) or mixed liquor suspended solids (MLSS),

undergoes hydrolysis and fermentation. The overflow from this reactor is directed back to the main-stream process. The S2EBPR process requires fewer chemicals comparing to conventional EBPR process with a smaller footprint, fewer odors, additional carbon availability for denitrification, and implementation to existing EBPR configurations without reliance on influent carbon [143] [144].

2.6.1.1 A/O or A2/O

This configuration, first proposed by Barnard et al. and later patented under the name of A/O, is based on the addition of anaerobic phase before aerobic phase as indicated in Figure 2-2, which allows PAO selection over other microorganisms without nitrogen removal while introducing an anoxic zone between anaerobic and aerobic phase results in simultaneous nitrogen and phosphorus removal. In this configuration, due to the removal of nitrogen in the anoxic zone, there will be low levels of nitrate returned to the anaerobic phase, contrary to the A/O configuration. On the other hand, the presence of nitrate and nitrite may also result in ordinary heterotroph organism's activity and shortage of available COD for PAO growth [145]. Various studies have been conducted on the anaerobic-aerobic activated sludge process with different substrates. For example, studies proposed PHB accumulation in anaerobic conditions, in which with NADH as reducing power, acetyl-CoA is converted to PHB [146].

By applying the A2/O configuration, adequate SRT levels, wasting rates, and sludge loading should be maintained to avoid secondary phosphorus release [32]. A2/O system is the most commonly used process for BNR systems where denitrifiers and PAOs are responsible for nitrogen and phosphorus removal, respectively, require organic matter as a carbon source. Insufficient COD levels will decrease denitrification capacity, where COD will be taken up by PAOs in the anaerobic phase making it unavailable for denitrifiers in the following anoxic zone. Occasionally, the

unstable performance of conventional A2/O in biological nutrient removal is observed due to inaccurate operational conditions and environmental factors [147]. Furthermore, the A2/O configuration may still be severely restricted due to nitrate recycling even with an additional anoxic phase compared to the A/O process. This phenomenon results in excessive energy consumption and dissolved oxygen concentration in the anoxic phase, negatively influencing denitrification [148].

In the case of nitrogen removal in the system, the A2/O process is modified to a 5-stage Bardenpho process by adding an anoxic and re-aeration reactor since complete denitrification is not possible and oxidized nitrogen will partially enter the anaerobic phase by external recycling [131]. Zeng et al. studied the addition of pre-anoxic zone and carbon source addition to the A2/O system. Denitrifying phosphorus removal by DPAOs took place using nitrate or nitrite as electron acceptors, resulting in simultaneous nitrogen and phosphorus removal and higher energy savings, lower COD requirement, and lower sludge production [147]. Kuba et al. has indicated 50% lower COD requirement and sludge production and 30% lower oxygen consumption with the same levels of nutrient removal for DPAOs by comparing the stoichiometry and kinetics [149]. In denitrifying phosphorus removal, the C/N ratio is critical for TN and TP removal efficiencies. Maximum TN and TP removal are obtained by maintaining the C/N balance between 5 and 7.1 [150]. Apart from sufficient COD, the difference in external carbon sources alters the denitrification rate. By studying different types of carbon sources on the A2/O process for simultaneous nitrogen and phosphorus removal, Wu et al. indicated acetate and nitrate and propionate and oxygen as carbon sources and electron acceptors, respectively, as more effective.

Primarily, propionate as a more suitable carbon source results in less mass transfer and higher phosphorus removal [151]. However, the concentration of electron acceptors available in the

system is critical for efficient phosphorus removal. In a study by Peng et al., excessive aeration was applied to the A2/O process with DO levels as high as 2.3-5.28 mg/L at the end of the aerobic zone, which led to deterioration in P removal efficiency due to PHB source exhaustion in excessive aeration causing low P uptake in the aerobic zone [152]. Moreover, more recent studies have focused on enhancements of A/O configuration. Tsuneda et al. studied a novel configuration of anaerobic/aerobic/anoxic proposed for simultaneous phosphorus and nitrogen removal, resulting in a more significant anoxic/aerobic phosphate uptake rate than conventional processes [153]. Kerrn-Jespersen et al. experimented on a fixed-film reactor with an alternating anaerobic/anoxic sequence with nitrate as the oxidizing agent for phosphorus removal. In the anaerobic zone, 0.52 mg Phosphate released/ mg of acetate taken up was achieved, while in the following anoxic phase, 2 mg of phosphate was taken up per each mg of nitrate reduced [154].

2.6.1.2 University of Cape Town (UCT)/ modified UCT/ VIP

In UCT, as the development of A2/O configuration, returned activated sludge (RAS) is returned to an anoxic zone instead of the anaerobic zone (Figure 2-3), preventing consumption of readily biodegradable COD (rbCOD) for denitrification process, which results in PHA storage by PAOs. For further enhancement, two anoxic zones were supplemented to A2/O configuration in modified UCT for minimizing nitrate in the anaerobic stage, where nitrate is recycled back to the second anoxic zone, and mixed liquor is returned from the first anoxic zone to the anaerobic zone [22] [155].

The higher the phosphorus released in anaerobic conditions, the higher the phosphorus concentration taken up in the consequential aerobic phase. The anaerobic selector in the UCT process releases phosphorus, takes part in phosphorus removal, and facilitates the forming of large flocs for improvement of settle-ability [156]. This configuration results in high nitrogen removal

with complete nitrate removal in the anoxic phase and efficient time for PHA storage in the anaerobic step [32]. These processes demonstrated desirable results in removal efficiencies with sufficient organic carbon sources and a legitimate C/N ratio.

With the stringent limitations of effluent qualities, many existing processes have been optimized and modified. The modified UCT process, with the advantages of fully maintaining an anaerobic environment due to internal recycle sub-system, will result in the minimal inhibitory effect of nitrate and nitrite on anaerobic phosphorus release, anoxic denitrifying phosphorus removal, and lower organic substrate requirements. This method also leads to high performance and energy saving due to sludge recycle minimization and primary sedimentation exclusion [156]. Moreover, implementing aerated-anoxic zones within the UCT reactor makes further contributions for lower operational costs and slightly higher nutrient removal [157]. However, there are drawbacks to achieving high removal efficiencies for low C/N and C/P ratio wastewaters in this configuration, and operation is complex due to two internal recycle streams [158]. Ge et al. studied the combination of UCT and step-feed A/O process for higher nitrogen and phosphorus removal. During the experiment, almost complete nitrification was achieved. With an inflow distribution of 40:30:30% for COD, TN, and TP, the highest removal efficiencies of 89%, 88%, and 93% were reached for COD, TN, and TP, respectively [159].

A study investigated modified UCT with an initial anaerobic selector, followed by three identical tanks comprising an anoxic/oxic tank for denitrification and nitrification, respectively, accompanied by step-feeding. The experiments showed the highest removal efficiencies in 9 hour HRT and feed distribution of 60:25:15% in the anaerobic selector, second and third anoxic tank, achieving 94% of COD removal, 93% of phosphorus removal, and 83% of nitrogen removal as TN [156]. Furthermore, reconfiguring the modified UCT process with step feeding could further

lead to DPAO enrichment and improvement in anaerobic phosphorus release and anoxic phosphorus uptake [159]. As another variation of UCT, VIP is operated with a similar configuration with a slight difference in mixed liquor recycle location and shorter sludge age of 5 to 10 days [160].

2.6.1.3 Johannesburg configuration (JHB)

As a modification of UCT, as shown in Figure 2-3, in JHB configuration, a single anoxic reactor is located on the RAS line for minimization of nitrate recycle to anaerobic zone. The organic electron donor is either from influent, external carbon addition, or internal carbon storage [76]. The reactor is more compact with no dilution effect on the RAS line, although the denitrification capacity is restricted due to COD scarcity [32]. JHB configuration mainly lacks the capability of complete denitrification, having lower rates than the UCT process resulting in lower nitrogen removal efficiency [158] [160]. Denitrification in the anoxic zone in this configuration takes place by endogenous respiration, where nitrate reduction is dependent on endogenous respiration rate, sludge thickening degree, and retention time. Studies have shown JHB capability of removing approximately 10 mg/L of nitrate in the anoxic zone with a varying retention time of 0.5 to 2 hours. The second anoxic zone must be mindfully performed and only utilized for raw sewage fed to the system [161]. In this configuration, with a TN/COD ratio of 0.07 mgN/mgCOD, nitrate will be entirely removed in underflow recycle with recycling ratios of 1:1. By increasing the TN/COD ratio to 0.1 mgN/mgCOD, with recycling ratios of 0.5 to 0.7, all nitrate will be fully removed in the recycle flow [162]. In experiments carried out by Burke et al., with no nitrate generated, the A/O system had a very high potential in phosphorus removal due to high anaerobic mass fraction; however, once nitrate was introduced, this configuration lost its ability for P removal. By applying

JHB configuration with 3 day SRT, nitrate was removed in the anoxic zone, yet less P removal was achieved compared to A/O configuration due to less anaerobic mass fraction [162].

2.6.2 Sidestream processes

2.6.2.1 Phostrip

Phostrip was introduced as a side-stream biological phosphorus removal in the early 1970s. The first investigation on P-uptake, demonstrating phosphorus removal, was completed by Levin and Shapiro [17]. The sequential phenomena of phosphorus release and uptake in anaerobic and aerobic conditions resulted in patenting the first commercial P-removal system, namely Phostrip [163]. Phostrip controls the microorganisms available in wastewater-activated sludge to take up phosphorus in aerated phase and release phosphate further when sludge is settled in anaerobic conditions [134].

This configuration is effective in chemical P-removal as a separate side-stream process that minimally affects the mainstream and activated sludge from chemical sludge [164]. As a side-stream hybrid configuration, it is a combination of chemical and biological removal processes. In this configuration, an anaerobic stripper tank in the side-stream is used for phosphate release. In the phostrip process (Figure 2-3), presenting a high fraction of RAS to carbon source in limited volumetric flow creates a high P-release in side-stream liquor, allowing efficient chemical precipitation of phosphorus in wastewater [164]. After supernatant removal, RAS is recycled back to the aeration basin. The effluent is chemically treated with lime, and chemical sludge is precipitated while the remaining liquid heads back to the system [32]. Since this configuration has wastage both from the waste-activated sludge and the supernatant, phosphorus removal points are two points.

Combining chemical and biological phosphate removal processes has the beneficial effect of being highly selective, where bacteria have a noticeably high affinity for phosphate. In these configurations, deficient phosphate concentrations below 0.1 mgP/L can easily be achieved by an overdose of chemicals [165]. Szpyrkowicz et al. studied the addition of a phostrip configuration to an existing plant with simultaneous nitrification and denitrification in the aerobic basin, leading to a 7 mg P/gMLSS.day removal. After start-up, the start of the dephosphatation process in phostrip configuration took approximately two cycles of anaerobic and aerobic stages, carried out by species already present in the biomass. In this study, researchers further investigated cutting lime addition to aerobic basins and indicated that the chemical process does not detrimentally affect the enzymatic system for biological phosphorus removal [166]. Furthermore, by adding an anoxic tank before the aerobic basin, in PhoStrip process, biological nitrogen removal is achievable [158].

The first full-scale phostrip process was developed in Seneca Falls, New York, in 1973 with a production of effluent with less than one mg/L of total phosphorus. This plant reduced the cost of chemical usage by 90% compared to conventional chemical procedures. A number of other plants were conjointly modified for adding the capability of phosphorus removal, such as Reno-Sparks joint water pollution control plant in Nevada in 1974. This configuration was favored over conventional chemical methods by the flexibility of design, minimal modification requirement, high operating cost savings up to 6 times compared to the conventional method, higher stability, and 40% lower SVI of sludge [167]. In comparing several phosphorus removal processes, Lee et al. [168] indicated that the release and uptake of phosphorus in phostrip configuration are not affected by the COD/TP ratio; however, very low ratios will inhibit the process. This study showed that with the same SRT for both A/O and phostrip configuration, the phosphorus content is lower in the phostrip process due to continuous P-removal of the P-stripping tank supernatant that

decreases the phosphorus load of aeration basin sludge [168]. Phostrip systems are mainly utilized for phosphorus removal. By operating the aeration tank at a complete nitrification condition, the nitrate recycled back would inhibit the phosphorus removal process. In a study by Kim et al. [169], the addition of a denitrification tank before aeration tank for overcoming phostrip limitation and reaching simultaneous nitrogen and phosphorus removal experimented. For achieving a high nitrogen and phosphorus removal, maximum TKN loading of 0.04 kgTKN/kg MLSS.d in influent, COD/TKN ratio of 5.1 to 5.9, and phosphorus loading rate of lower than 0.008 kgT-P/kgMLSS.d in aeration tank were recommended. With the development of new configurations, phostrip has not been frequently utilized due to chemical addition, inefficiency, and low P levels in the stripper tank [170], since the 1970's. However, recently, there is a rising interest in phostrip development for P-recovery of valuable products such as struvite [164] from the sludge settler supernatant [171]. Due to the abundance of PAOs in activated sludge due to anaerobic conditions in RAS lines, there is an opportunity to develop a phostrip on non-EBPR systems for low effluent phosphate concentrations [164].

2.6.2.2 Biological-chemical phosphorus and nitrogen removal configuration (BCFS)

The combination of chemical and biological P-removal with in-line P-stripping and off-line precipitation is called BCFS-process (Figure 2-3). This process is a configuration of modified UCT where a chemical step is added in the sludge line that provides further phosphorus removal by chemical addition, generating a highly selective system. Since the chemical tank is separately located in the sludge line, sludge from the tank is not returned to the mainstream, preventing an inert material return to the activated sludge basin [32]. In this system, for achieving low effluent concentrations of both nitrogen and phosphorus, SRT is preferred; however, it may disadvantage the Bio-P removal by lowering biomass production. Phosphorus that cannot be removed

biologically will be precipitated chemically. Availability of COD for denitrification is beneficial by utilizing denitrifying PAOs (DPAOs) to allow P and N removal with lower COD requirements [172]. DPAO proliferation in UCT-type setup is achieved by sludge recycling through anaerobic and anoxic conditions with long SRT. High biomass contact time is feasible in a sludge thickener that gathers P-rich supernatant from the anaerobic stage accompanied by iron chloride for adjusting the flow as required for phosphorus removal. BCFS was developed for extensive usage of DPAOs, which can reduce sludge generation by 30% due to their lower cell yield [173].

In the 1990s, through a project, all WWTPs of Waterboard, Netherlands, were upgraded to minimize chemical costs, integrate nitrification, denitrification, and biological phosphorus removal, and lower energy costs. Holten WWTP was chosen as a pilot project and was upgraded to a BCFS process by adding a plug flow anaerobic reactor to the two fully-aerated plug-flow activated sludge tanks. After upgrading, the WWTP indicated very stable P-removal and nitrification with low SVI. The results of this pilot project were employed for upgrading other WWTPs to BCFS processes for achieving high denitrifying P-removal capacity [174]. Furthermore, a dynamic activated sludge model for biological phosphorus and nitrogen removal was developed to inspect the metabolic modeling approach. Subsequently, a simulation model was performed on Hardenberg WWTP, one of the seven WWTPs upgraded to BCFS-process in Waterboard, Netherlands, to evaluate the metabolic model on long-term population dynamics. In addition, the simulation aided in evaluating PAO kinetics and glycogen formation rate calibration as a critical process parameter [175]. On further projects, a WWTP was planned on a modified UCT-type process (BCFS) in the Netherlands. A model was structured to justify the optimal COD/P ratio, COD/N ratio, energy consumption, and greenhouse gas emission concerns. The

modeling results demonstrated a COD saving for methane production due to the contribution of denitrifying PAOs in N-removal.

The DPAOs approximately saved up to 53 to 59% of COD for nitrogen removal, leading to a 154-271% methane production increase and a 104 to 119% energy consumption reduction [176]. In a study by Barat et al. [177], a new approach to phosphorus recovery has been studied other than achieving phosphate effluent standards. With evaluations on steady-state simulation, they indicated the potential of BCFS for phosphate recovery; however, there needs to be further study on the nitrification process under limiting phosphate concentrations. Approximately 60% of influent load can be recovered by adopting simple changes to the process [177]. Phosphate recovery from P and N-removal systems recycles limited phosphorus sources and improves COD saving and effluent quality, and improves sludge settling properties [158]. A study by Hao et al. performed modeling on BCFS system for justifying recovery of phosphorus as struvite, COD saving in methane production, and greenhouse gas emission prevention. They investigated the optimal stripping recovery for P recovery and detected that exceeding limits for stripping flowrate would increase the cost and negatively affect P- removal [178].

2.6.3 Cycling systems

2.6.3.1 Sequencing batch reactors (SBR)

In SBR reactors series of time-steps are attained in a single reactor for the treatment of wastewater. One particular aspect of SBR is its independence of the RAS system since aeration and settling occur in the same tank. As shown in Figure 2-3, SBRs mainly include different sequential operating steps consisting of fill, react, settle, decant, and idle. For control of SRT, sludge wasting is determined. In most SBR reactors, sludge wasting occurs during the reaction phase for a uniform discharge [22]. The use of this reactor for nutrient removal has been reported since the 1980s with

the ability to remove nitrogen and phosphorus with high efficiency. In the 1990s, the arising concerns on nutrient discharges resulted in modifying existing SBRs, which were conventionally used for BOD and TSS removal in wastewater rather than biological nutrient removal [179]. In an early study by Chang et al., pH and oxidation-reduction potential (ORP) were considered process parameters to adjust the cyclic duration to achieve high removal efficiencies. By changing the SRT to 15 days, initially, high removal efficiencies were achieved; however, this time duration was found to be excessive for the treatment process. By decreasing SRT to 10 days and shortening reaction phase time with 6-hour cyclic duration, higher nutrient removal efficiencies of 98% as well as lower energy consumption were accomplished [180]. Early studies' assumption was on PAOs inability to function under nitrate availability as an electron acceptor, while further investigation revealed high phosphorus uptake under anoxic phase by DPAOs.

Therefore, anoxic phase addition to SBR reactors has been extensively experimented with ever since. The feasibility of nutrient removal in a single sludge SBR reactor by adding an anoxic phase in the middle of the aerobic step was studied by Lee et al. [181] with an 8-hour cycle duration. This modification increased the denitrifying population by 53%, resulting in 88% and 100% of nitrogen and phosphorus removal, respectively. Several studies additionally showed simultaneous N and P-removal in SBR reactors with energy requirement and COD demand reduction, demonstrating the capability of both nitrite and oxygen utilization by DPAOs [182] [153]. With modifications on SBR and its high ability in nutrient removal, treatment of wastewaters with N, P, and organic matter content such as piggery wastewater. Obaja et al. experimented with an 8-hour cycle SBR reactor for simultaneous nitrification, denitrification, and phosphorus removal on the supernatant of piggery waste. With an initial N and P concentrations of 1500 mg/L NH^{+}-N and 144 mg/L PO_4^{3-}-P, 99.7% and 97.3% nitrogen and phosphorus removal were achieved [183].

With improved insight on microbiology of PAOs, studies focused on developing PAO-rich EBPR systems. Pijuan et al. [184] studied *Accumulibacter Phosphatis* in propionate-fed SBR with 6-hour cycles. Microbial analysis was performed by fluorescence in situ hybridization (FISH), where 55% of the PAOs in the reactor appeared to be *Accumulibacter* with very little signs of *Competibacter* (GAO) presence, showing GAO inability to compete in propionate-fed systems. Focusing on microbial communities' analysis in the activated sludge process for identifying the microorganisms responsible for P-removal, Jeon et al. experimented on acetate-fed anaerobic/aerobic SBR with 8-hour cycles. Different approaches, including Quinone profile, slot hybridization, and 16S rRNA gene sequencing, showed that the most abundant species belonged to members of the Proteobacteria β-subclass, related to the Rhodocyclus-like group likely to be responsible for EBPR [185].

Recent studies have focused on overcoming the issues regarding SBR reactors. One of them is limiting substrate in nutrient removal systems. The denitrification process inhibits EBPR by consuming the substrate through denitrifiers before their availability for PAOs, resulting in lower P-release in the anaerobic phase. Therefore, there has been a focus on the addition of carriers or granules to SBR reactors. The biofilm produces an anaerobic, anoxic, and anaerobic zone by mass transfer, which favors simultaneous nitrification and denitrification. Yang et al. investigated a sequencing batch moving bed membrane bioreactor. The average 93.5% and 82.6% COD and total nitrogen (TN) removal efficiency was achieved, and with anaerobic and aerobic phase lengths of 2 hours each, optimal total phosphorus (TP) removal of 84.1% was reached [186]. In addition to studies on integrating moving bed media with SBR reactors, few studies have focused on fixed-bed SBR reactors. Supplementing plastic media, fixed on the bottom of the SBR reactor, was

investigated by Rahimi et al., reaching higher COD and nutrient removal than conventional SBR reactor with 25-30% lower sludge production [187].

One other investigation is on understanding the effect of low dissolved oxygen concentration on SBR reactors. Excessive aeration as an external disturbance leads to high dissolved oxygen concentrations causing competition between PAOs and GAOs [188]. Experiments on very low DO levels as low as 0.15-0.45 mg/L for simultaneous nitrogen and phosphorus removal have achieved a range of 60 to 99% of P-removal [189]. However, still, there is a lack of synergic understanding on effective factors with microbial shift and metabolic pathway on phosphorus release and uptake mechanism, internal storage, consumption functions, and cyclic performance.

2.6.3.2 Oxidation ditch design

Originally, oxidation ditch was used for only organic removal, although to date, these systems operate for nutrient removal as well [190]. As an endless loop activated sludge reactor (Figure 2-3), this process has been critical in developing clarifier-less activated sludge systems. Horizontal axis paddles and rotating brushes are utilized in the reactor for mixing and aeration, where a clarifier is provided by separating a zone with baffles inside the oxidation ditch. For P-removal, an anaerobic reactor has to be placed before the oxidation ditch system [191]. These reactors are mainly accepted in small communities because of their advantages, such as easy operation, effluent quality stability, and low sludge production. In addition, the aeration mode and intense recirculation occurring in oxidation ditch reactors toughen the identifying of anoxic and aerobic phases, behaving differently from conventional nutrient removal systems [192].

Evidence shows efficient P-removal in oxidation ditch-type reactors despite the complete absence of an anaerobic zone. With an increase in nutrient discharge limits, there has to be a solution for existing oxidation ditches to be modified for nutrient removal. Therefore, there is a requirement

for increasing the understanding of process feasibility and EBPR potential. Datta et al. investigated four full-scale oxidation ditches for EBPR performance. The experiment showed all plants indicated EBPR phosphorus removal capability with P-release and uptake rates comparable to conventional EBPR systems [193]. Peng et al. experimented on a pilot scale anaerobic-anoxic oxidation ditch process configuration to characterize simultaneous nitrification, denitrification and biological phosphorus removal and understand the excessive aeration damage and recovery options. Results showed that the oxidation ditch process is applicable for achieving high phosphorus and nitrogen removal as high as 85 and 80%, respectively. Excessive aeration results in deterioration of N-removal down to 40% [194].

2.6.3.3 Biodenipho process

Biodenipho system consists of two alternating oxidation ditches between aerobic and anoxic conditions for simultaneous nitrogen and phosphorus removal, as indicated in Figure 2-3. In this process, the flow from the anaerobic reactor is fed to an anoxic zone for supplementing organic matter through the nitrate reduction process [8]. In each phase, the wastewater flows through anaerobic, anoxic, and aerobic reactors with a 2–hour length between aerobic/anoxic shifts. In this process, phosphorus is removed with the excess sludge taken from the aerobic reactor [195]. Due to the semi-batch manner of this reactor, there are interactions between nitrate and organic substrate. For example, in an investigation by Meinhold et al., in a Biodenipho process, P-uptake took place in the presence of nitrate followed by a partial P-release by nitrate exhaustion [196]. In addition, a full-scale biodenipho plant showed high P-release with the accumulation of acetate, propionate, and lactate in an anaerobic reactor [197].

2.7 Full-scale biological phosphorus removal plants with different EBPR configurations

The initial development of the biological nutrient removal process and the high cost of physical-chemical treatments triggered the proliferation of biological processes for nitrogen and phosphorus removal in the early seventies [138]. The first Full-scale EBPR system was developed at Seneca Falls in New York in July 1973. A PhoStrip process was installed for biological Phosphorus removal accompanied by chemical precipitation in a mixing tank [198]. With a variation in North American and European countries' approaches towards WWTP effluent quality, considering the plant capacity, different WWTP design and control processes are of interest. In Europe, the most prevailing nutrient removal processes consist of sequential anaerobic, anoxic, and aerobic zones, including modified bardenpho process, JHB besides biodenitro, and biodenipho systems with continuous alternating flow. Most WWTPs for EBPR systems combine a 3-stage anaerobic/anoxic/aerobic process with RAS pre-denitrification similar to Westbank, which was initially developed in Canada, or utilize either phoredox or A/O process configuration with alternating SND system. There has been upgrading of processes taking place in WWTPs either to convert outdated WWTPs with only COD and nitrogen removal into systems with EBPR or to increase the efficiency of nutrient removal by acidogenic fermentation of primary sludge for higher VFA content in anaerobic zone and increase savings regarding chemical precipitation [199]. The latter upgrade is known as side-stream EBPR (S2EBPR) as a new alternative to address the usual challenges of the EBPR process [144]. More than 80 full-scale WWTPs implementing S2EBPR are successfully operating to tackle weak wastewater influent and improve process stability [143].

Retrofitting process with a current control structure design should take place by considering the phosphorus concentrations and biomass recycle stream [199]. In countries with cold winters, methanol addition and chemical precipitation may be integrated for denitrification and phosphorus

removal, respectively. Novel technologies such as nitrification trickling filter on top of activated sludge reactor, moving bed bioreactors (MBBR), IFAS and EBPR process accompanied with fermenter thickener has been used to achieve high levels of nutrient removal. Due to less stringent regulations in North American countries, several plants yet rely on chemical treatments rather than biological processes for P removal, which can easily be converted to phoredox (A/O) process by partitioning of aeration stage due to short SRTs [75]. However, for reaching a low phosphorus concentration lower than 0.1 mg/L, chemical treatment processes such as coagulation, flocculation, and filtration are needed as a polishing step [75]. In the last decade, there has been an increase in accomplishing nutrient removal in a one-stage activated sludge process with chemical precipitation; however, in western parts of Canada, complete biological nutrient removal as Westbank configuration is working in full-scale.

Moreover, similar configurations such as JHB and JHB/Westbank processes are more frequently implemented as an effective nutrient removal system [138]. Recent applications focus more on integrating processes to achieve consistent nutrient removal by overcoming limiting factors such as high nutrient load, low temperatures, scarcity of carbon source, and clarifier overload. Various investigations take place on the most widely used configurations for combined nutrient removal with minimal annual operating cost [132]. There are various reports on high simultaneous phosphorus and nitrogen removal in UCT systems and different modifications of the MBRs with advantages of higher effluent quality and smaller footprint. Membrane technologies are used instead of sand filters for effluent filtration since it reduces TSS of effluents by maintaining the same nutrient effluents levels similar to media filters [138]. Granular sludge as development has been applied on a commercialized scale reaching up to 87% P-removal.

2.8 Conclusion and Future potentials

Nowadays, most of the phosphorus in use is attained from mining, accompanied mainly by sulfuric acid, nitrogen, and potassium. This source of phosphorus is rapidly utilized, leading to resource depletion in the next 50 to 100 years. Without any considerations, phosphorus scarcity is one of the most significant challenges of the 21st century [200]. While there have been actions in managing the resource shortage complications by efficient resource handling, prevention of over-fertilization, and introducing alternative potentials, there is a need for more investment, economically and scientifically, to reach higher levels of P-availability [201]. With EBPR in WWTPs, most phosphorus is concentrated in sludge and chemically precipitated for incorporation into fertilizers, not reaching a stage for mainstream usage [200]. Moreover, the worldwide awareness of the adverse effects of excessive phosphorus utilization on plants and animals, prevention of eutrophication, and overgrowth of toxic blue-green algae, ultimately raise higher regulations on P-concentrations and more efficient P-removal [6]. Although there has been extensive research with valuable improvement in these areas for removing and recovering phosphorus, there are still numerous unresolved issues to solve.

3 Biochemical Diversity and Metabolic Modeling of EBPR Process Under Specific Environmental Conditions and Carbon Source Availability

Adapted from:

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Preface:

This chapter of the thesis is a comprehensive review of Biochemical Diversity and Metabolic Modeling of EBPR, published by Parnian Izadi.

3.1 Abstract

Enhanced biological phosphorus removal (EBPR) is one of the most promising technologies as an economical and environmentally sustainable technique for removing phosphorus from wastewater (WW). However, with the high capacity of EBPR, insufficient P-removal is a significant yet common issue of many full-scale wastewater treatment plants (WWTP) due to misinterpreted environmental and microbial disturbance. The optimal operational conditions, environmental changes, and microbial population interaction are efficiently predicted by developing a rather extensive understanding of biochemical pathways and metabolic models governing the anaerobic and aerobic/anoxic processes.

Therefore, this paper critically reviews the current knowledge on biochemical pathways and metabolic models of phosphorus accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) as the most abundant microbial populations in the EBPR process with an insight on the effect of available carbon source types in WW on phosphorus removal performance. Moreover, this paper critically assesses the gaps and potential future research in the metabolic modeling area. With all the developments in the EBPR process in the past few decades, there is still a lack of knowledge in this critical sector. This paper hopes to touch on this problem by

gathering the existing knowledge and providing further insights on the future work on chemical transformations and metabolic strategies in different conditions to benefit the quantitative model and WWTP designs.

Keywords Metabolic model; PAOs; GAOs; polyhydroxyalkanoate; EBPR

3.2 Introduction

Enhanced biological phosphorus removal (EBPR), as one of the most economical and environmentally sustainable processes for phosphorus removal, relies on the enrichment of phosphorus accumulating organisms (PAOs) through alternating anaerobic and aerobic/anoxic phases [3]. In contrary to most microorganisms, PAOs take up volatile fatty acids (VFA) anaerobically and store them as carbon polymers named polyhydroxyalkanoate (PHA). Hydrolysis of intracellularly stored polyphosphate and P-release outside the cell provides part of the energy for these reactions. Moreover, glycogen is utilized for reducing power generation. In the following aerobic/anoxic phase, in the absence of a carbon source, PHAs are used as an energy and carbon source by PAOs for P-uptake and storage, glycogen replenishment, and cell growth [6]. The net P-removal occurs by higher aerobic Poly-P formation as is hydrolyzed anaerobically and high P-content sludge withdrawal. In these conditions, bacteria capable of storing substances as energy and carbon sources and balanced growth are mainly selected over the microorganisms that increase in the presence of carbon sources [202].

Anaerobic carbon uptake, polymer storage, and anaerobic-aerobic/anoxic cycling of poly-phosphate are contemplated as the selective assets of PAOs against other microorganisms; however other organisms capable of anaerobically storing VFAs, vigorously challenge this projection by causing EBPR failure [203]. Concurrently, glycogen accumulating organism (GAO) has the potency to increase the EBPR system, competing with PAOs for the available carbon

source without contributing to the P-removal process [3]. Under anaerobic conditions contrary to PAOs, GAOs only degrade glycogen as an energy production path without storing poly-phosphate. Therefore, with a sole dependence on glycogen as an energy source, the glycogen consumption and PHA production per VFA uptake is higher with the presence of GAOs, resulting in lower P/HAc ratios [204]. In the following aerobic phase with electron acceptor availability, GAOs use PHA storage only for glycogen replenishment [205]. Correspondingly, higher carbon sources and chemical additives are required to reach high P-removal efficiency reaching a total-P concentration lower than 1 mg/L [1], which increases sludge handling and operational costs.

The competition between PAOs and GAOs is complicated due to various biochemical processes such as accumulation, reproduction, carbon source uptake, and biomass yield that takes place in these microorganisms [206]. Improving the understanding of stoichiometry and kinetics describing the biochemical conversions of chemical compounds, including VFA uptake procedure in anaerobic phase for both PAOs and GAOs, substantially increases the knowledge on optimum operational conditions for PAOs abundance, and it assists in the identification of parameters that only negatively impacts the GAOs anaerobic performance [48][206]. Metabolic modeling using biochemical transformation characterization. Based on microorganisms' metabolism and anticipating bacterial activity, it can assist with helpful information for optimization and design purposes [136]. However, in the EBPR process, PAOs and GAOs with complex metabolisms demand sophisticated metabolic models compared to conventional activated sludge models. Notwithstanding, metabolic-based models have been successfully introduced that highly evaluate the population dynamics of PAOs and GAOs and understand the effective parameters on microorganism interaction [1].

Metabolic models are established based on intracellular biochemical interactions, which benefit in clarifying biological systems and removal processes. Moreover, these models are beneficial in facilitating the calibration procedure to minimize the operational factors by expressing interrelated complex processes as one single function, increasing the consistency and reliability of the model at the same time [1]. However, a comprehensive study has been done on PAOs and GAOs metabolic modeling considering different carbon sources as substrate, yet, there are various inconsistencies from a biochemical point of view. Therefore, a comprehensive review of biochemical model aspects of EBPR will clarify the questionable issues and clarifies the uncertainties for future research.

In the present paper, the metabolic models of PAOs and GAOs in acetate, propionate, and glucose-fed systems in EBPR are addressed in detail. Moreover, this review paper aims to illustrate the process modeling in the EBPR system by summarizing the findings on biochemical pathways and metabolic models and discussing the following required steps to further improve the EBPR process's understanding.

3.3 Environmental conditions for EBPR process

3.3.1 Anaerobic Chemical Transformations

The principal role of the anaerobic conditions is to provide favorable carbon sources for PAOs and prepare PAOs to outcompete other heterotrophic bacteria [165]. Under anaerobic conditions, polyphosphate is used as a source for energy production in the form of adenosine triphosphate (ATP) in 3 mechanisms of 1) direct catalysis by polyphosphate kinase 2) regeneration of ATP by the combined action of polyphosphate: adenosine monophosphate (AMP) and adenylate kinase resulting in polyphosphate degradation and 3) production of ATP through the generation of proton motive force due to efflux of uncharged metal-phosphate complex and proton [207], [208] with a concurrent release of orthophosphate, potassium (K) and magnesium (Mg) [209]. Most studies

have shown a 1:3 release ratio for K: P and Mg: P [210]. The proton motive force as a chemiosmotic gradient over bacterial cytoplasmic membrane contains two main components: 1) electrical potential from net negative charge produced from the charge difference of cytoplasmic cell membrane side and outside. 2) pH gradient due to bacterial cytoplasm alkalinity [211]. Fundamental electrochemical gradient due to release of intracellular ion concentration and poly-P degradation produces energy source for cells with approximately 0.33 ATP production by each released phosphate [212]. However, studies propose that glycogen catabolism also provides ATP for PHA generation [46]. In addition, there are several discrepancies on the suggested ATP generation per P-mol hydrolyzed.

Glycogen conversion to pyruvate as another energy-producing pathway is carried out through Embden-Meyerhof (EM) pathway or the Entner Doudoroff (ED) pathway. The ED produces an extra mole of ATP in comparison to EM. Based on studies, the main pathway utilized by PAOs for glycolysis is the ED pathway [213],[44], [214], where two ATP are produced through glycogen conversion to pyruvate, which is further transformed to Acetyl-CoA or Propionyl-CoA [209]. However, several findings have indicated the dependence of PAO anaerobic metabolism on external and internal factors in utilizing the TCA cycle or glycolysis pathway as a source for reducing equivalent generation [215]. In an EBPR system, PAOs must be prepared to take up either oxidized or reduced organic substances without disrupting the redox balance in the cell. Redox balance in PAOs is dependent on the maintenance of stored glycogen. There are two main pathways of conversion when PAOs are fed with various carbon sources, which are glycogen conversion to Acetyl-CoA and CO_2 with reducing power production or glycogen conversion to Propionyl-CoA through succinate–propionate pathway with reducing power consumption, where glycogen is acting as redox balance regulator through cell functions [39].

The energy-consuming processes in the anaerobic phase are 1) external carbon source transportation into the cell interior, 2) PHA production, and 3) cell maintenance. PAOs take up readily biodegradable carbon sources from glucose to short-chain fatty acids (SCFA) and store it as PHA inside their cell interior by using ATP and reducing power which is generated from the TCA cycle via glyoxylate pathway by polyphosphate decomposition or through glycogen degradation [165] since the complete TCA cycle produces reduced flavin adenine dinucleotide (FADH). Based on studies, both pathways are active in the anaerobic metabolism of PAOs, where extra reducing power is produced with an increase in PAOs dominance [216]. Therefore, anaerobic bulk phosphorus concentration increases with time [217]. GAOs with similar metabolism hydrolyze internal glycogen under anaerobic conditions for energy and reducing power production for taking up VFA and the subsequent transformation of fatty acids to PHAs [3]. The studies reporting the loss of P-removing activity in the EBPR process have proposed anaerobic organic substrate assimilation by GAOs by using aerobically synthesized glycogen as an energy source. This matter decreases the total organic carbon (TOC) availability for P-removal activity [218].

3.3.2 Anaerobic Metabolic Models in Acetate-fed system

Several studies have focused on the biochemical model of the EBPR process with acetate as the sole carbon source by assuming only a single PAO population performing P-release, PHA production, and regeneration mainly in the form of PHB [46]. The PHB-like polymer includes two monomeric units of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) [37]. When the EBPR system is mainly fed with acetate, the 3HB unit is the largest in the polymer composition known as PHB [40]. However, *Periera et al*. [219] conducted a study in which multiple experiments resulted in the production of 3HB-co-3HV co-polymers due to glycogen biosynthesis by acetate consumption as an early study *Hesselmann et al.* proposed that [209], by acetate uptake, Acetyl-CoA activation is either formed by Acetyl-CoA synthetase (ACS) which requires two ATP or by

the simultaneous activity of acetate kinase (AK) and phosphotransacetylase (PTA) which consumes 1 ATP.

The activity of the AK enzyme is only observed in high acetate concentration systems; however, in most EBPRs, the active transport of acetate takes place by activation of the ACS enzyme [209]. Although this proposal on uptake of acetate through cell assimilation rather than active transport is not following EBPR biochemistry and bioenergetics since passive diffusion does not require energy. Moreover, the importance of AK for the uptake process has been denied while other models were based on this enzyme rather than Acetyl-CoA synthetase [46]. The reducing power for PHB production is either generated through the TCA cycle or glycolysis pathway, which oxidizes acetate into CO_2 and produces reducing power in the form of NADH.

Based on previous studies and research [146], [220], [41], depending on redox balance, the glycolysis pathway cannot individually fulfill the reducing power requirements. Therefore, acetate metabolizing through the TCA cycle generates a small fraction of approximately 30% [39]. The type of substrate used in the EBPR process directly influences the relative polyphosphate concentration needed for substrate uptake. Acetate as a substrate had the highest ATP requirement and lowest growth yield in anaerobic conditions, resulting in a high polyphosphate measure which achieves the high performance of EBPR [213]. Based on various studies on anaerobic metabolic models on PAOs, the anaerobic P-release/ acetate uptake ratio was not constant, affected by the pH of mixed liquor [40]. Increasing the pH resulted in increased phosphate release due to more energy requirement for acetate uptake in higher pH levels. However, in a study conducted by *Fleit et al.* [210], it was proposed that anaerobic phosphate release results from EBPR sludge acidification inside the cells, originating from passive diffusion of unconnected VFA over bacterial membrane [207].

The metabolism of GAOs is very similar to PAOs, with the only difference where GAOs utilize glycogen as the sole energy source rather than polyphosphate for acetate uptake. The glycogen accumulating metabolism (GAM) includes glycogen degradation for ATP supplementation for anaerobic Acetyl-CoA synthesis, acetate transport in excess amounts only for NADH production, without any polyphosphate decomposition. In the case of GAM, for maintaining the reducing equivalent balance, along with PHB, PHV, poly-3-hydroxy-2-methylbutanoic acid (PHMB), and poly-3-hydroxy-2-methylvaleric acid (PHMV) are produced, which either consume or don't change the reducing equivalents [221]. As specified in literature, acetate uptake distinction between PAOs and GAOs is dependent on factors including pH, temperature, and sludge age [206]. A comparison of acetate uptake rate for PAOs and GAOs indicated a higher influence of pH levels for GAOs, resulting in a slight energetic benefit that offsets the PAOs higher growth [222]. Overall, in an EBPR with active glycogen and phosphate accumulating metabolism, higher P-removal follows a higher P-release/TSS ratio, P-release/acetate-uptake, and PHV/PHA lower glycogen-degradation/acetate-uptake [221].

3.3.2.1 Anaerobic metabolic model of PAOs in Acetate-fed system

In the metabolic model offered by *Smolders et al.* In the anaerobic stage, the metabolic stoichiometry model is based on the TCA cycle or glycogen pathway, as shown in Figure 3-1**.** The model consists of reactions of acetate uptake, degradation of Poly-P, and reducing power production [223]. The acetate uptake stage is dependent on pH levels (α_1) [40]. The processes in this stage are divided in three steps of 1) The acetate uptake and polymeric substance storage, 2) poly-phosphate conversion to phosphate, and 3) reduction equivalent production. Since the acetate uptake and phosphate release are coupled, the ratio is expected to be constant. However, there is a variation of 0.25 to 0.75 P-mol/C-mol reported in the literature due to the dependency on pH. Although, the variation substantially differs from the model's explanation. The presence of other

groups of bacteria utilizing substrate without P-release occurrence is another reason behind this matter [224]. Based on stoichiometric reactions in the acetate-fed system, PHB/acetate, glycogen/acetate, and CO_2/acetate ratio are independent of pH variation. In contrast, phosphorus/acetate ratio is pH-dependent and conditional to pH ranges [40].

A basic model to indicate the influence of the pH difference and the proton motive force was proposed by *Kashket et al.* [225], shown in Equation 3-1 to 3-4,

$$PMF = \Delta\Psi + 2.3RT(pH_{in} - pH_{out}) \quad \textit{Equation 3-1}$$

Where, PMF= proton motive force, kJ

ΔΨ= membrane potential;

R= ideal gas constant=8.314 J/mol k and

T= temperature, K [223].

Transfer of cells grown at pH of 7 to lower pH levels will increase the proton motive force which is shown as ΔPMF_0:

$$\Delta PMF_0 = K(7 - pH_{out}) \quad \textit{Equation 3-2}$$

Where K is a constant, cells require energy ($ENERGY_{PMF}$) to bring back the proton motive force to the reference level:

$$\Delta PMF_0 + ENERGY_{PMF} = \Delta PMF/Hac \quad \textit{Equation 3-3}$$

Where ΔPMF/Hac is the decrease in the proton motive force from the uptake of 1 mol of acetic acid.

With the following Equation (Equation 3-4), the ratio of phosphorus to acetate relation to pH can be calculated [223]:

$$\frac{P}{Hac} = \left[\frac{\frac{0.5\Delta PMF}{Hac} - 3.5k}{\eta.(-\Delta G^0_{ATP})} + 0.25\right] + \left[\frac{0.5k}{\eta(-\Delta G^0_{ATP})}\right] \times \left(\frac{P-mol}{C-mol}\right)$$ *Equation 3-4*

Where ΔG^0_{atp} is the standard free energy change for hydrolysis of ATP=-50 kJ/mol;

η is the efficiency of membrane processes, and k is constant. Although the Mino model shown in Figure 3-1-2 is highly efficient in acetate-fed systems for PAOs in anaerobic conditions, the partial reducing power production through the TCA cycle cannot be neglected since the reducing power produced by the glycolysis pathway is not sufficient for PHA production, indicating a 30% PHA production under anaerobic TCA cycle [39].

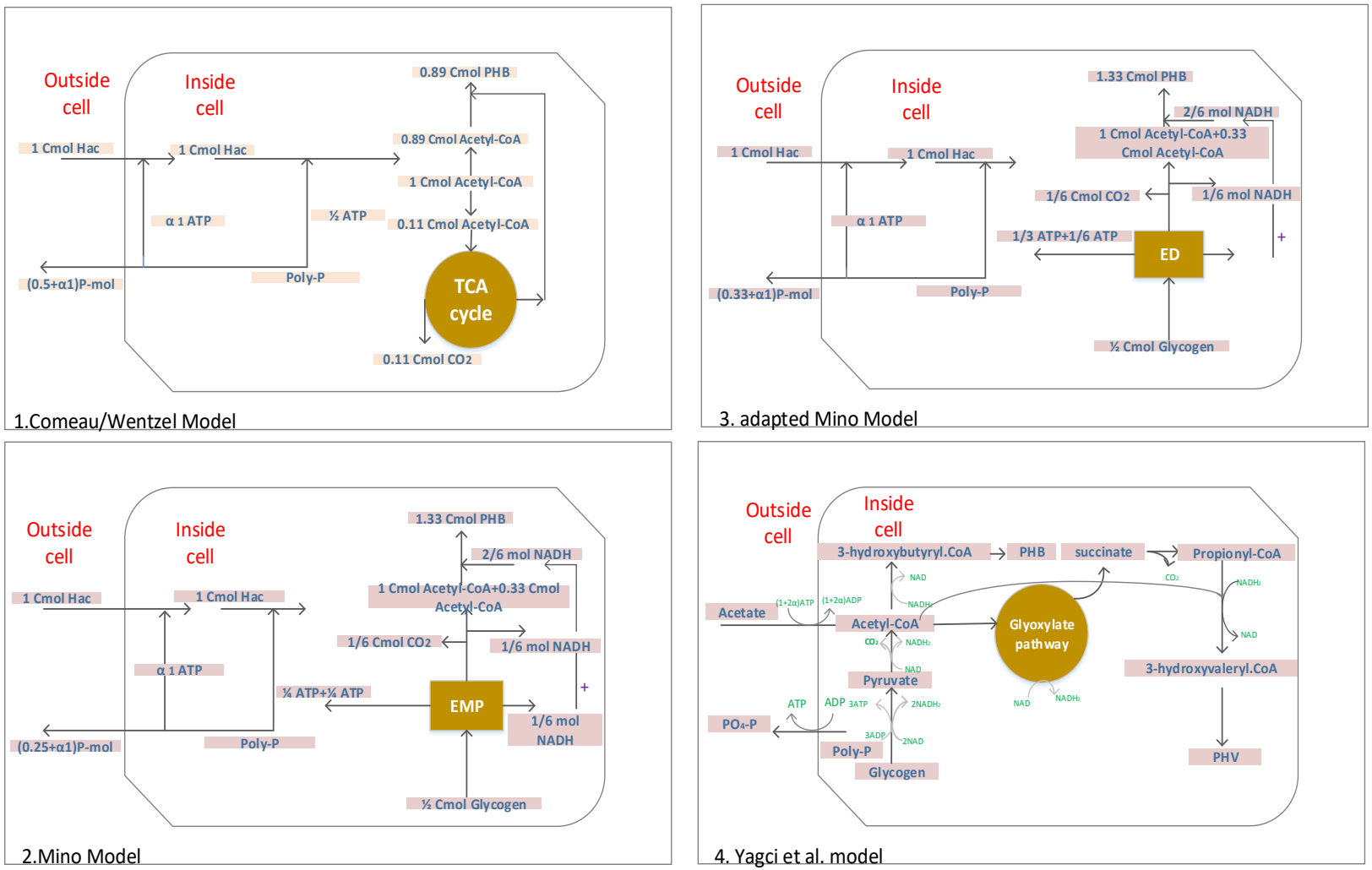


Figure 3-1 schematic anaerobic metabolic model 1. TCA cycle 2. Glycogen, Embden-Mayerhof-Parnas (EMP) pathway 3. Glycogen Embden-Doudoroff (ED) pathway as a source of reduction equivalent 4.combination of both pathways

In further studies by *Yagci et al.* [224], the anaerobic metabolism of GAOs and PAOs has shown in Figure 3-1-4 were known to be similar, both taking up acetate and storing it as PHA with a difference that PAOs provide energy from both Poly-P degradation and glycolysis while GAOs only depend on glycogen degradation. In this metabolic model, one mole of acetate and an energy source is utilized to activate Acetyl-CoA. In PAOs, since there is a need to reduce power for PHB storage, pyruvate goes through further oxidation and produces Acetyl-CoA. Propionyl-CoA production should be available since 3HV formed from an Acetyl-CoA, and a Propionyl-CoA alongside PHB are present as PHAs. Thus, there should be pathways available for Propionyl-CoA production. The main difference between GAOs and PAOs in anaerobic conditions is in the Propionyl-CoA production pathway. There are two pathways proposed for pyruvate conversion to Propionyl-CoA: the succinate-propionate pathway and acrylic acid pathway. Since the compound in the succinate-propionate pathway is metabolized by PAOs, this method is more likely to occur. Acetyl-CoA and Propionyl-CoA are utilized to produce 3 hydroxybutyrate and 3HV, which will be further polymerized into PHB and PHV. In early studies, PHB was the only type of polymer formed in acetate-fed models in anaerobic conditions for PAOs; however, results of further studies contradicted this hypothesis.

The proposed metabolic model by *Yagci et al.* further proposes a glycogen utilization/acetate uptake ratio higher than 0.17 in favor of GAOs. However, this ratio is not constant for PAOs since they can partially produce reducing power through PHV conversion from acetate in the glyoxylate pathway. Finally, the phosphate released/acetate uptake ratio is indicated in the following stoichiometry (Equation 3-5) [224]:

$$\frac{P}{HAc} = f_{PAO}((1 + 2\alpha_{PAO}) - \frac{3}{6+3F_{GLX}})$$ *Equation 3-5*

Where f_{GLX} is the fraction of Acetyl-CoA conversion to Propionyl-CoA, f_{PAO} is the fraction of PAOs and α_{PAO} is the energy requirement for acetate transport in PAOs.

3.3.2.2 Anaerobic metabolic model of GAOs in Acetate-fed system

GAOs and PAOs, as different organisms, have very similar metabolic behaviors [226]. Since in Poly-P content limitation conditions, PAOs can shift to GAOs metabolism [204] steadily. The energy for GAO activation and substrate uptake partially comes from glycogen utilization, and the rest of the energy is provided via the EMP pathway. The stoichiometry reactions of GAOs in anaerobic conditions include a significant amount of glycogen consumption during acetate uptake, accompanied by an increase in PHA content in cells [48] without any poly-P degradation. This matter demands the production of additional NADH during glycolysis for redox balance maintenance in the cell [224]. The energy requirement for acetate transport in GAOs (α_{GAO}) and Propionyl-CoA accumulation are pH dependent, increasing with higher pH levels, which demands extra glycogen degradation [227]. By pH increase the glycogen consumption increases which requires more reducing power to be utilized in propionate-succinate pathway. As shown in overall reaction (Equation 3-6) on a C-mmol basis, there is no P-release from the cells [48] [228].

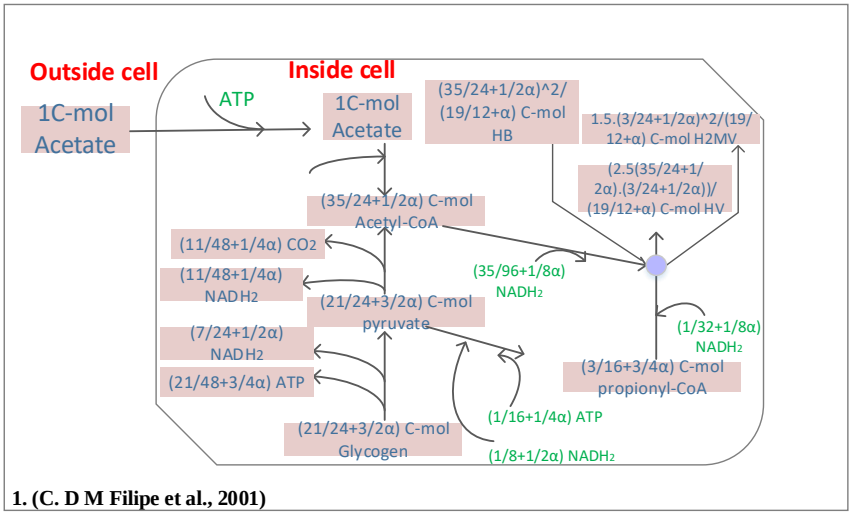
$$Acetate + \left(\frac{21}{24} + \frac{3}{2}\alpha_{GAO}\right) Glycogen \rightarrow \frac{(\frac{35}{24}+\frac{1}{2}\alpha_{GAO})}{(\frac{19}{12}+\alpha_{GAO})} PHB + \frac{2.5(\frac{35}{24}+\frac{1}{2}\alpha_{GAO})(\frac{3}{24}+\frac{1}{2}\alpha_{GAO})}{(\frac{19}{12}+\alpha_{GAO})} PHV + \frac{1.5(\frac{3}{23}+\frac{1}{2}\alpha_{GAO})}{(\frac{19}{12}+\frac{1}{2}\alpha_{GAO})} PH2MV + (\frac{11}{48} + \frac{1}{4}\alpha_{GAO})CO_2$$ *Equation 3-6*

where the specific acetate uptake is indicated in Equation 3-7, being initially constant and dropping by the end of tests:

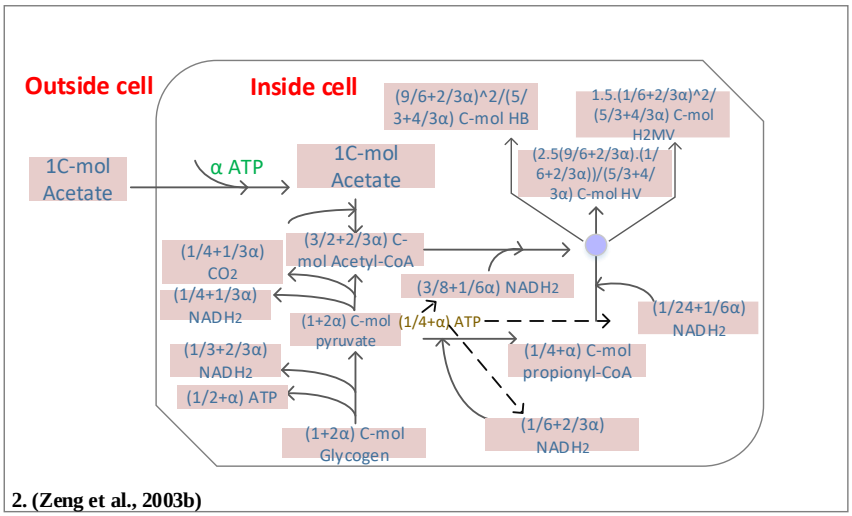
$$r_{acetate}^{GAO} = (q_{GAO}^{max} - q_{glycogen} \cdot \frac{1}{f_{glycogen}})(\frac{C_{acetate}}{C_{acetate}+0.001})C_x$$ *Equation 3-7*

Where $r_{acetate}^{GAO}$ is the rate of acetate uptake (C-mmol/h), q_{GAO}^{max} is the maximum specific rate of acetate uptake (C-mmol/Cmmol. h), $q_{glycogen}$ is a constant to describe for specific acetate uptake rate with the glycogen content [C-mmol/(C-mmol. h)], $f_{glycogen} = \frac{C_{glycogen}}{C_x}$ is the glycogen content of the biomass (Cmmol/ C-mmol), $C_{acetate}$ is the acetate concentration (Cmmol/ L), and C_x is the biomass concentration (C-mmol/L) [48].

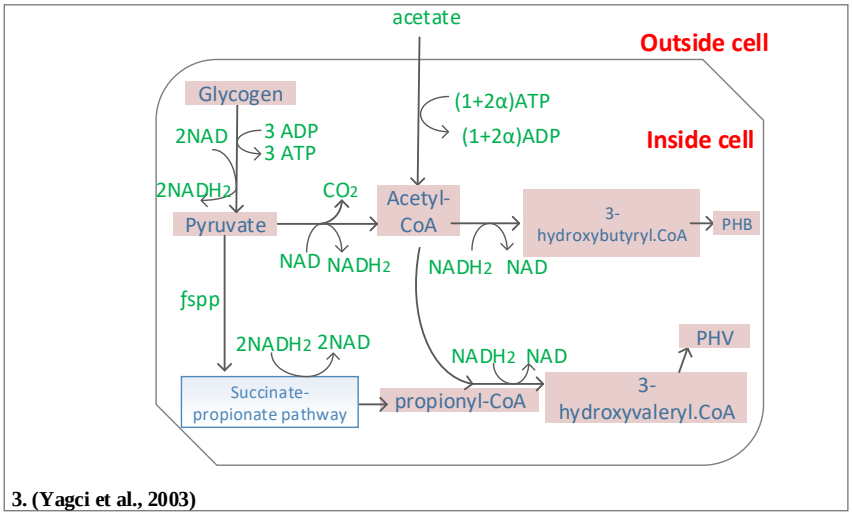
The schematic representation of the metabolic model of GAOs is represented in Figure 3-2.1 in an anaerobic condition for acetate uptake [48]. The model for anaerobic GAOs with adjusted stoichiometric coefficients [229] is indicated in Figure 3-2.2.



1. (C. D M Filipe et al., 2001)



2. (Zeng et al., 2003b)



3. (Yagci et al., 2003)

Figure 3-2 metabolism of GAOs in anaerobic condition for acetate uptake based on different energy and reducing power production pathways

According to the metabolic model proposed by Yagci et al [224], indicated in Figure 3-2.3, GAOs produce energy required for substrate uptake and activation and reduce power source for PHA generation by glycogen degradation. In this modeling approach, it has been proposed that GAOs produce the excess $NADH_2$ requirement through the succinate-propionate pathway in addition to PHB synthesis. Therefore, a certain amount of pyruvate (f_{spp}) will be transformed to Propionyl-CoA via glycogen consumption, while the rest is converted to Acetyl-CoA by oxidative decarboxylation. The Propionyl-CoA and Acetyl-CoA are then stored as PHB and PHV inside the cell, yielding to overall reaction in Equation 3-8 [224]:

$$acetate + \left(\frac{1}{3} + \frac{2}{3}\alpha_{GAO}\right) glycogen \rightarrow \frac{2}{3} PHB + \left(\frac{1}{6} + \frac{2}{3}\alpha_{GAO}\right) PHV + (\frac{1}{2} + \frac{2}{3}\alpha_{GAO}) CO_2 \quad \textit{Equation 3-8}$$

3.3.3 Anaerobic Metabolic Model in Propionate-fed systems

Many studies have proposed the favor of propionate as a prevalent carbon source for PAOs rather than acetate due to observation of high P-removal efficiency in full-scale EBPR fed with high propionate content [6], [230], [231], [184]. These studies have suggested a clear strategy for GAO growth control in EBPR systems [232]. Moreover, a study by *Oehmen et al.* indicated the ability of acetate-enriched PAOs to immediately taking up propionate as a carbon source; however, GAOs were unable to metabolize propionate [232]. The higher EBPR performance in propionate-fed systems is linked to the slow rate of propionate uptake of *Competibacter* GAO, which is the most studied GAO in EBPR systems, and also less competition for acetate uptake compared to propionate uptake (only *Alphaproteobacteria*-GAO) [6][36]. On the other hand, various studies have hypothesized the more effective EBPR system with 2-5-carbon VFAs instead of propionic acid [233], [234], [235]. This was related to the biochemical pathway of PHA production from VFA consumption and the corresponding NADH balance. These studies proposed relatively higher P-removal with VFAs resulting in net NADH consumption compared to VFAs resulting in positive

NADH balance (propionic acid) related to the oxidation state of the uptake carbon source. It is proposed that the availability of reducing power in a more reduced form inhibited the metabolic activity and stopped reducing power regeneration and PHA synthesis in anaerobic conditions [38]. In these studies, lower phosphorus release and uptake and lower phosphorus-release/VFA uptake were observed compared to acetate-fed systems. Nonetheless, long-term studies have been conducted with propionate as the primary carbon source, without any lack of polymer acclimation [236]. This indicates a significant difference between long-term experiments with acclimation and short-term studies without acclimation in propionate-fed systems [217].

3.3.3.1 Anaerobic metabolic model of PAOs in Propionate-fed systems

In the anaerobic stage of the process, the propionate is converted to Propionyl-CoA with an energy source, as shown in Figure 3-3.1. The energy required for taking up the propionate is produced from hydrolyzation of Poly-P, which depends on pH range [40]. Due to more evidence for glycogen degradation through ED, this pathway is used in the model. Depending on the type of binding occurring, random or selective, different types of PHA may be produced. For example, there were unexpected binding results in PHB, PHV, Poly-B-hydroxy-2-methylbutyrate (PH2MB), and Poly-B-hydroxymethylvalerate (PH2MV) production. However, there are no PHB produced in selective binding while different PHV and PH2MV are formed [232] [77].

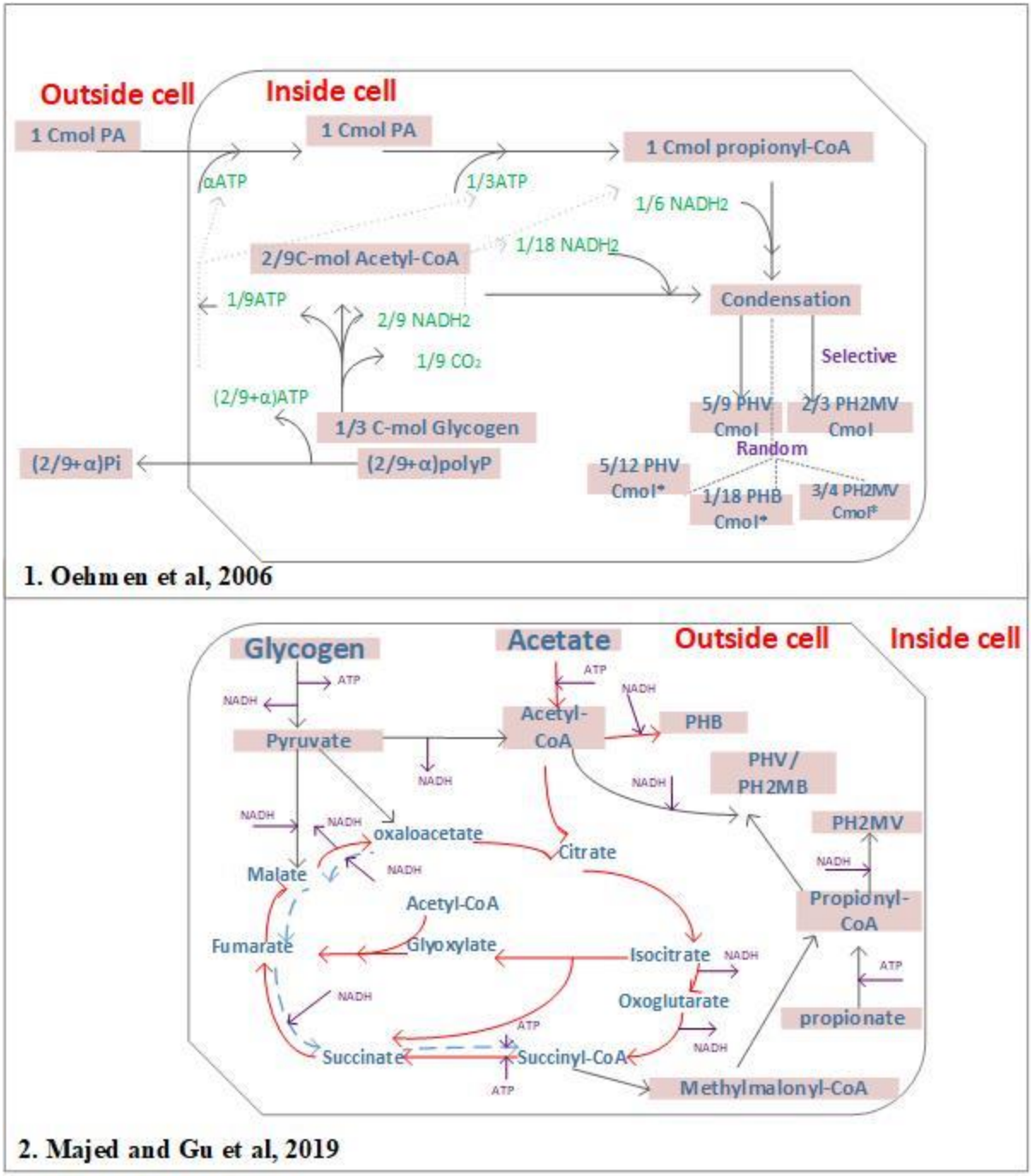


Figure 3-3.1. Anaerobic metabolism of PAOs with propionate as substrate 2. anaerobic biochemical pathway for PAOs with possible reducing power production pathways of glycolysis (blue line), partial or full TCA cycle (red line), and succinate-propionate pathway (dotted blue line)

Yet, there are uncertainties in PAOs reducing power generation pathway, which eventually impacts PHA formation and stoichiometric ratios. Recent studies agree on partial TCA cycle (also

known as succinate-Propionyl-CoA pathway) and reverse TCA cycle or glyoxylate shunt besides glycolysis pathway for reducing power production. The quantity of reducing power produced from each responsible pathway is dependent on the phylogeny of PAOs and external nutrient availability. In a study conducted by [237], shown in Figure 3-3.2. In P-limiting conditions, the energy and reducing power production was mostly reliable on glycolysis rather than the TCA cycle. This condition also stimulated PHV production rather than PHB since PHV is mainly formed through the glycolysis pathway combined with reverse TCA cycle with glyoxylate shunt. At the same time, PHB generation is dependent on both the TCA cycle of the glycolysis pathway or a combination of both. High PHV content in PAOs due to change in operational conditions in this study is against the previous findings on the association of high PHV concentrations to an abundance of GAOs [237].

3.3.3.2 Anaerobic metabolic model of GAOs in Propionate-fed systems

Putative GAOs from the *Gammaproteobacteria* class called *Candidatus Competibacter Phosphatis* consume acetate as a carbon source and very slowly take up propionate, resulting in outcompeting by PAOs. Although other groups of GAOs, namely from *Alphaproteobacteria* class and members of orders *Sphingomonadales* and *Rhodospirillales*, demonstrated to be capable of propionate uptake [205].

In GAOs anaerobic metabolism, propionate is taken up through energy generation by glycogen hydrolysis. Pyruvate produced from glycolysis is partly converted to Acetyl-CoA, and the remainder is converted to Propionyl-CoA through EM pathway. Then PHA is produced by activating Acetyl-CoA and Propionyl-CoA [205] (**Appendices**, **B , Fig 10.1**). Finally, Acetyl-CoA and Propionyl-CoA are produced from pyruvate through oxidative decarboxylation and propionate succinate pathway, respectively.

A study conducted by [67] suggests the potential of PAOs (i.e., *Accumulibacter*) in taking up acetate and propionate at a similar rate. At the same time, strains of GAOs such as *Competibacter* are less potent for propionate uptake. Changing carbon sources from acetate to propionate PAOs requires acclimation time for P-uptake because of slower PHA accumulation with propionate as the primary carbon source compared to acetate. GAOs belonging to *Alphaproteobacteria* closely related to *Defluvicoccus Vanus* and *Sphingomonas* [77], [78] have shown slower response to change in carbon source from acetate to propionate, which can be used as a controlling factor for their growth in the EBPR system. However, the advantage of PAOs over GAOs in propionate uptake is not an assurance for their dominance in the EBPR system since various system failures have been reported with propionate feeding [238].

3.3.4 Anaerobic Metabolic Model of Glucose-fed systems

It is mainly believed that the EBPR process only performs well under the SCFA-fed procedure; however, studies have indicated a wide range of organic matters anaerobically utilized by PAOs. Glucose, a carbon source metabolized by almost all microorganisms, was believed in early research to deteriorate EBPR performance [239] and induce GAOs metabolism. Incapacity of anaerobic acidogenesis with glucose as the sole carbon source and the inability for glucose fermentation were ideas behind EBPR failure [240]. However, some studies observed a good EBPR performance in the case of the glucose-fed process. In an experiment by *Zengin et al.* [241] conducted on glucose-fed EBPR, lactic acid bacteria were responsible for lactic acid production used by PAOs and GAOs as carbon sources. Although with a good initial P-removal, the EBPR performance was negligible and deteriorated after approximately 30 days of the experiment due to the proliferation of GAOs. This finding is mutual between various studies [242]. In another experimental study, unlike the previously mentioned study, the rapid uptake of glucose by PAOs showed a rapid buildup of reserves without any need for glucose fermentation [243]. With a need

for further investigation, there is a possibility for PHA replacement with glycogen as an energy and carbon source for P-uptake [80]. *Wang et al.* further indicated that [243], much more energy was derived from hydrolysis of polyphosphate in acetate-fed system comparing to glucose-fed EBPR, which results in higher anaerobic P-release. Therefore, these results indicated more substantial potential in poly-phosphate hydrolysis and re-synthesis and higher PHA accumulation as critical anaerobic metabolic activities for the acetate-fed system than the glucose-fed process [243]. Some researchers also discovered that efficient EBPR could be achieved with glucose as a subordinate substrate [28]. Conclusively, there is a contradiction in the literature on the effect of glucose as a carbon source on the EBPR process, where several studies claimed deterioration of the EBPR process. In contrast, others have shown a stable EBPR performance without the proliferation of GAOs [241].

3.3.4.1 Anaerobic metabolic model of PAOs in Glucose-fed systems

In a study conducted by *Jeon et al.* [244], glucose was used as the only carbon substrate. The observations showed a rapid glycogen accumulation by a decrease in glucose and an increase in lactate concentration. In contrast, the release of ortho-phosphate and PHA accumulation was not related to glucose concentration but relative to total organic carbon. Except for PHAs, the study suggested converting glucose to other storage compounds, including lactate polymer. Therefore, the glycogen accumulation corresponded with lactic acid generation but not phosphate release, as shown in Figure 3-4.1.

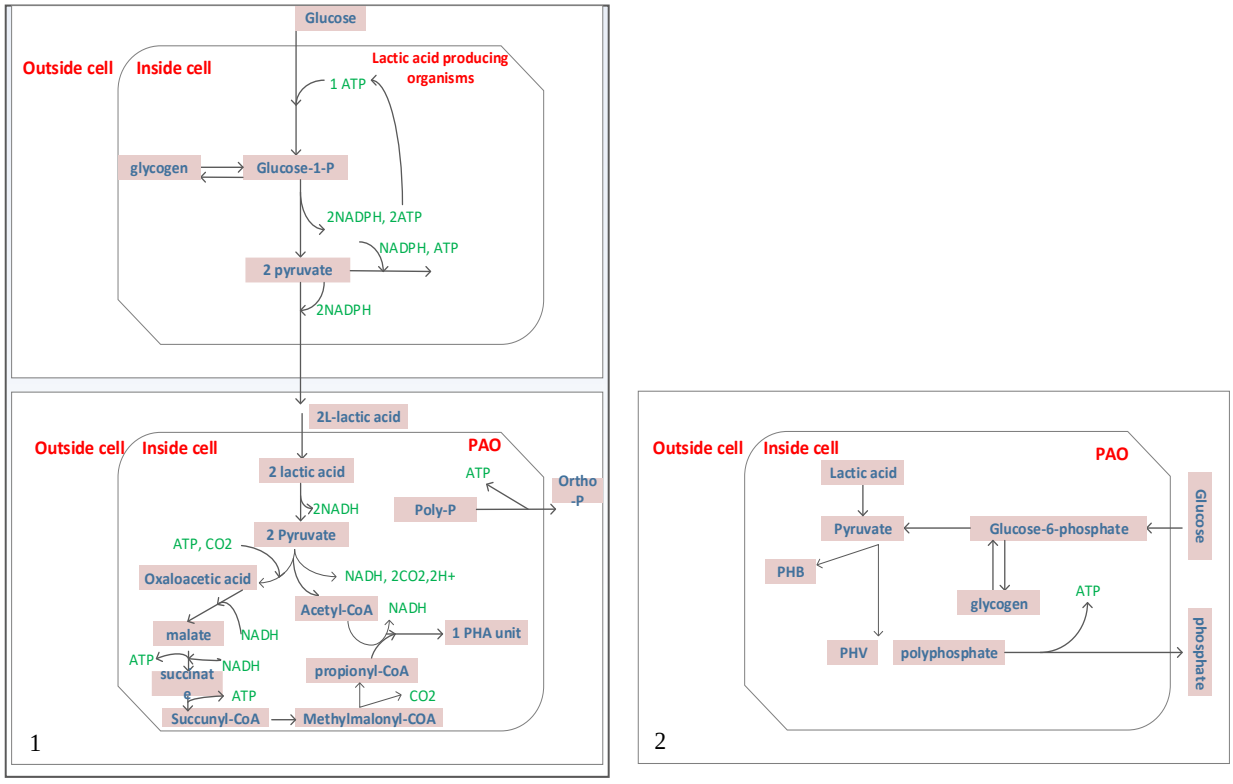


Figure 3-4 Proposed anaerobic metabolic pathway for PAOs in glycogen-fed system 2. hypothesized anaerobic metabolic pathway for PAOs in glucose-fed system

In this proposed model [244], two groups of microorganisms are involved in biological P-removal, lactic acid-producing organisms (LPO) and PAOs, where LPOs produce lactic acid by glucose accumulation. The ATP requirement for glycogen storage is generated through the glycolysis of glucose. PAOs produce PHA by conversion of lactic acid accompanied by poly-phosphate degradation and P-release.

Further investigation proposed a more comprehensive model by Wang et al. [245], available in Figure 3-4.2, wherein anaerobic phase the glucose uptake was explained by active transport of this compound through cytoplasmic membrane followed by conversion of phosphorylated glucose to glycogen. Therefore, the glycogen content in PAOs increased, but it observed a decrease due to glycogen degradation for PHA generation.

Energy (ATP) required for the mentioned reactions is provided by poly-phosphate degradation. Moreover, lactic acid was also detected as an end product of glycolysis short after glucose addition. The metabolic reactions are shown in Table 3.1. However, both PHB and PHV levels increased during anaerobic conditions, but since 80% of PHA accumulation was in the form of PHV, for simplifying the metabolic model, PHV has been accounted for as the only PHA accumulated in PAOs.

Table 3.1 metabolic reaction in anaerobic condition for PAOs in the glucose-fed system by[245]

Reactions in anaerobic condition	***equations***
Glucose transport Equation 3-9	$\frac{1}{6}ATP + CH_2O \rightarrow CH_{\frac{13}{6}}O(PO_3)_{\frac{1}{6}} + \frac{1}{6}ADP$
Glycogen accumulation Equation 3-10	$CH_{\frac{13}{6}}O(PO_3)_{\frac{1}{6}} + \frac{1}{6}ATP + \frac{1}{6}H_2O \rightarrow CH_{\frac{5}{3}}O_{\frac{5}{6}} + \frac{1}{6}ADP + 1/3H_3PO_4$
Glycogen degradation Equation 3-11	$CH_{5/3}O_{5/6} + 1/6H_3PO_4 \rightarrow CH_{13/6}O(PO_3)_{\frac{1}{6}}$
Polyphosphate hydrolysis Equation 3-12	$HPO_3 + H_2O \rightarrow H_3PO_4$
Glycolysis (ED pathway) Equation 3-13	$CH_{\frac{13}{6}}O(PO_3)_{\frac{1}{6}} + \frac{1}{3}ADP + \frac{1}{6H_3PO_4} + \frac{1}{3NAD} \rightarrow CH_{\frac{4}{3}} + \frac{1}{3}ATP + \frac{1}{3}NADH_2 + \frac{1}{6}H_2O$
PHV synthesis Equation 3-14	$1.2CH_{4/3} + \frac{2}{5}NADH_2 + \frac{1}{5ATP} \rightarrow CH_{\frac{8}{5}}O_{\frac{2}{5}} + \frac{1}{5}H_2O + \frac{2}{5}NAD + \frac{1}{5}CO_2 + \frac{1}{5}ADP + \frac{1}{5}H_3PO_4$

Wang et al. [239] predicted the anaerobic metabolic activities by proposing a biochemical model that utilized an external carbon source (glucose) to produce an internal carbon source in both forms

of PHB and PHV and no anaerobic biomass growth. The major anaerobic reactions are available in Table 3.2.

Table 3.2 anaerobic metabolic reactions for PAOs in the glucose-fed system by [239]

Anaerobic reactions	***Equations***
Glycogen uptake Equation 3-15	$ATP + glucose \rightarrow glucose - 6 - ATP + ADP$
Glycogen accumulation Equation 3-16	$glucose - 6 - phosphate + ATP + (C_6H_{10}O_5)_n + H_2O \rightarrow (C_6H_{10}O_5)_{n+1} + ADP + 2H_3PO_4$
Glycogen degradation Equation 3-17	$(C_6H_{10}O_5)_n + H_3PO_4 \rightarrow glucose - 6 - phosphate + (C_6H_{10}O_5)_{n-1}$
Poly-P hydrolysis and ATP production Equation 3-18	$(polyphosphate)_n + H_2O \rightarrow (polyphosphate)_{n-1} + H_3PO_4$ 1 mol of polyphosphate degradation is equivalent to 1.29 mol of ATP production
Glycolysis (ED pathway) Equation 3-19	$glucose - 6 - phosphate + 2ADP + H_3PO_4 + 2NAD \rightarrow 2CH_3COCOOH + 2ATP + 2(NADH + H^+) + H_2O$
Glycolysis (EMP pathway) Equation 3-20	$glucose - 6 - phosphate + 3ADP + 2H_3PO_4 + 2NAD \rightarrow 2CH_3COCOOH + 3ATP + 2NADH_2 + 2H_2O$
Lactic acid conversion Equation 3-21	$CH_3COCOOH + NADH_2 \leftrightarrow CH_3CHOHCOOH + NAD$
PHB formation Equation 3-22	$2CH_3COCOOH + NAD + (C_4H_6O_2)_n \rightarrow (C_4H_6O_2)_{n+1} + 2CO_2 + NADH_2$
PHV formation Equation 3-23	$2CH_3COCOOH + 2NADH_2 + ATP + (C_5H_9O_2)_n \rightarrow (C_5H_9O_2)_{n+1} + H_2O + 2NAD + CO_2 + ADP + H_3PO_4$ PHV production requires two precursors in equal amount: Acetyl-CoA and Propionyl-CoA

In this study, the metabolic reactions for anaerobic conditions are suggested based on two major principles: 1) the need for redox balance in bacteria 2) microorganisms mainly take part in reactions purposefully [239].

3.3.5 Aerobic/Anoxic Chemical Transformations

In the presence of electron acceptor in the aerobic/anoxic phase, the anaerobically stored PHA is used for glycogen replenishment, PAOs growth, and polyphosphate recovery, leading to a decrease in PHA levels soluble phosphate accompanied by an increase in glycogen and polyphosphate storage. Therefore, polyphosphate bio-integration occurs by energy production through aerobic

catabolism with PHA as the major carbon source [236]. While in GAOs, the energy is utilized only for glycogen replenishment and cell growth. With a cyclic increase and decrease in glycogen and Poly-P concentration in PAOs cells, energy is wasted, resulting in a 13% lower yield for PAOs than other heterotrophic bacteria. However, in sequential anaerobic and aerobic/anoxic conditions, PAOs are the dominant population due to their ability to grow aerobically without exogenous carbon and energy sources [46]. The type of carbon source indirectly influences the PAOs and GAOs aerobic metabolism since the maximum aerobic yield is dependent on the anaerobic PHA fraction storage [246].

PAOs are capable of using nitrate and nitrite as electron acceptors in the absence of oxygen. These bacteria are called denitrifying PAOs (DPAOs), which can significantly save in COD consumption with anaerobic PHA storage [247] [248]. The sole variation from dissolved oxygen utilization is in the terminal electron acceptor of the electron transport chain for energy production [211]. However, 40% lower energy production efficiency is achieved through nitrate than oxygen, resulting in a 20% decrease in cell yield values in anoxic conditions than the aerobic process [149], where it originates from the difference in oxidative phosphorylation between oxygen and oxidized nitrogen [249]. Although Poly-P production requires a limited amount of energy compared to growth and PHA production, the overall P-removal efficiency is highly comparable to the aerobic process [39]. However, it is proposed that since only a fraction of PAOs can utilize oxidized nitrogen as an electron acceptor, the PAOs lacking this ability will release phosphate as they are accumulating carbon sources. Accordingly, the net anoxic P-uptake depends on each PAO group's mass and activity [211]. Studies [250], [251], [252] have focused on comprehending PAOs and DPAOs as different organisms or one population with varying levels of denitrifying activities. Late studies [253],[103], [254] categorize PAOs as two types (I and II), each type consisting of different

clades (IA-E, IIA-G). Clade IA is known to have high nitrate reduction ability, yet; clade IIA is limited to reduce oxygen as an electron acceptor due to lack of *nar* gene for respiratory nitrate reductase [247] and *Ca. Accumulibacter Delftensis*, clade IC has not shown considerable anoxic P-uptake on nitrate [255]. In the case of nitrite, there is more research on the inhibitory effect than the potential use as an electron acceptor. However, further metagenomics studies revealed that *Accumulibacter* clade IIA, which lacks the gene for encodification of the respiratory nitrate reductase, encodes the nitrite denitrification pathway [256]. Therefore, although all clades obtain enzymes for aerobic metabolism, only DPAOs hold enzymes for using nitrate and nitrite as electron acceptors [247]. The effective factor in the competition between PAOs clades is mainly phosphate availability, which results in each clade's utilization of different metabolic pathways. Mainly, Clade type I dominates in high biomass P-content while clade type II is abundant on low biomass P-content [204].

3.3.5.1 Aerobic metabolic model in Acetate-fed systems

Under aerobic conditions, in the absence of an external carbon source, phosphate accumulating metabolism includes internal PHA consumption, glycogen pool restoration, growth, and orthophosphate recovery as Poly-P. While glycogen accumulating metabolism lacks the Poly-P storage [257]. Therefore, PHA contributes to glycogen replenishment in PAOs and GAOs as a substantial measure of carbon skeleton [236]. Contrarily to the literature proposal in GAOs leverage in anaerobic acetate uptake due to kinetic advantage and its prevalence in acetate-fed systems, *Whang et al.* [206] stressed the importance of aerobic condition in competition between PAOs and GAOs. Therefore, the PAOs and GAOs population estimation is a practical matter [258]. Furthermore, in the case of PAOs the extent of aerobic P-uptake correlates with the degree of anaerobic phosphate release [211].

Moreover, the ATP-produced/NADH-oxidized, ATP requirement for orthophosphate, and ATP requirement for anabolic processes of biomass formation coefficients determine the extent of aerobic metabolism and growth [249]. The experiment conducted, despite the advantage of GAOs in anaerobic acetate uptake, PAOs were able to outcompete GAOs by growing more biomass from stored PHA rather than GAOs. Therefore, GAOs may produce more PHA in anaerobic conditions, but less biomass production leads to their eventual failure in competing with PAOs.

3.3.6 Aerobic/Anoxic metabolic model of PAOs in Acetate-fed system

In the aerobic stage of the process, the energy required for growth and glycogen production roots to phosphorylation either by oxygen or nitrate. There are six main reactions involved in the aerobic/anoxic phase, starting with PHB degradation, phosphorylation, biomass synthesis, phosphate transport and polyphosphate storage, glycogen replenishment, and cell maintenance. Either oxidative phosphorylation follows the first reaction in aerobic conditions or the electron transport phosphorylation in anoxic conditions [47]. The δ and δ_N indicate the efficiency of the phosphorylation, demonstrating the amount of energy produced (as ATP) per NADH oxidized. These metabolic ratios have an impact on all aerobic yields [246]. There is biomass growth taking place in this condition. The required ATP for biomass growth is shown by K, determined as the polymerization coefficient with an expected value of 1.5 mol ATP/C-mol biomass. The required energy is generated through proton import and subsequent export by oxidation of reducing power over the cell membrane through phosphate transport. Therefore, based on the NADH consumption, a specific amount of phosphate is transported across the cell membrane (ε) [43]. Moreover, maintenance processes (m_{ATP}) of cells require energy as well. δ (P/O ratio), ε (transport coefficient) and K can be calculated following the relations:

$$1\frac{1}{Y_{opp}^{max}}M_{pp} = 1.125\frac{\frac{\delta}{\varepsilon}+\alpha_3}{2.25\delta+0.5}M_{pp} \quad \textit{Equation 3-24}$$

Where M_{pp} is the mass of polyphosphate accumulation and α_3 is the ATP required for poly-P synthesis.

$$K = -\frac{(2.25\delta+0.5)r_0+\left(0.154+1.125m_{\frac{atp}{\mu}}\right)r_x+1.125\left(\frac{\delta}{\varepsilon}+\alpha_3\right)r_{pp}+1.188r_{gl}}{1.125r_x} \quad \textit{Equation 3-25}$$

Where r_0, r_x , r_{pp} and r_{gl} are oxygen consumption, biomass production, poly-P accumulation and glycogen production, respectively.

In this stage, the transportation of phosphate through the cell membrane requires energy provided from taking in the protons exported in the NADH oxidation process. Finally, in the aerobic/anoxic stage, the glycogen utilized in the anaerobic zone is reproduced through the glyoxylate cycle, as shown in **Appendices C, Fig 10.2** [43].

Murnleitner et al. [47], [149] proposed an integrated metabolic model describing phosphorus removal under aerobic and denitrifying conditions, with the same biochemical reactions only with different electron acceptors. The aerobic/denitrifying phase is a six-stage process with four identical stages of polyphosphate formation, growth and maintenance, glycogen formation, and PHB degradation in the TCA cycle. The reactions are dependent on membrane processes; therefore, they differ for different electron acceptors. Where $\delta_\circ$ and δ_n are the required amount of ATP produced per NADH with oxygen and nitrate as electron acceptor respectively.

Without any substrate limitations, the PHB consumption, polyphosphate storage, glycogen formation, and biomass formation are as follows:

$$r_{phb} = K_{phb} * (f_{phb})^{\frac{2}{3}} * C_x \quad \textit{Equation 3-26}$$

$$r_{pp} = K_{pp} * \frac{1}{f_{phb}} * C_x \quad \textit{Equation 3-27}$$

$$r_{gly} = K_{gly} * \frac{(f_{phb})^{\frac{2}{3}}}{f_{gly}} * C_x$$ Equation 3-28

Where K is the kinetic constants, and 2/3 indicates the PHB granule in the cell-specific surface area.

The proposed kinetic model for the PAOs by *Filipe et al*. is available in Table 3.3 [223], a developed form previously introduced by *Smolders et al.* [44].

Table 3.3 kinetic model for PAOs in anaerobic and aerobic stages

Process	***Kinetic expressions***
Anaerobic phase	
Anaerobic uptake of S_A (Equation 3-29)	$q_A(\frac{S_A}{S_A + K_A})(glycogen\ switch)(poly - P\ switch)(\frac{K_{O_2}^{swith}}{K_{O_2}^{swith} + S_{O_2}})X_{PAO}$
Anaerobic maintenance (Equation 3-30)	$m_{ana}\left(\frac{K_{O_2}^{swith}}{K_{O_2}^{swith} + S_{O_2}}\right)X_{PAO}$
Aerobic phase	
Aerobic growth based on PHA (Equation 3-31)	$\widehat{\mu_{PAO}}.(\frac{X_{PHA}}{X_{PAO}})(\frac{S_{O_2}}{K_{O_2}^{swith} + S_{O_2}})X_{PAO}$
Poly-P storage (Equation 3-32)	$q_{pp}(\frac{S_{PO4}}{S_{PO4} + K_{PO4}})(\frac{S_{O_2}}{K_{O_2}^{swith} + S_{O_2}})(\frac{\frac{X_{PHA}}{X_{PAO}}}{\frac{X_{PHA}}{X_{PAO}} + K_{PHA}})(\frac{K_{PP}^{MAX} - \frac{X_{pp}}{X_{PAO}}}{1.05K_{PP}^{MAX} - \frac{X_{pp}}{X_{PAO}}})X_{PAO}$
Glycogen synthesis (Equation 3-33)	$q_{GL}(\frac{S_{O_2}}{K_{O_2}^{swith} + S_{O_2}})(\frac{X_{PHA}}{X_{PAO}})(\frac{\frac{X_{GL}}{X_{PAO}} + K_{GL}}{\frac{X_{GL}}{X_{PAO}}})X_{PAO}$
Aerobic maintenance (Equation 3-34)	$m_{aer}(\frac{S_{O_2}}{K_{O_2}^{swith} + S_{O_2}})X_{PAO}$

As shown in the above stoichiometry, acetate is almost used as the exclusive carbon source since it is the most abundant source of VFA in wastewater; however, propionate is available in sufficient

amount along with acetate. Propionate seems to offer advantages to PAOs over their competitors, the GAOs, where GAOs imply to have no ability in metabolizing propionate anaerobically [259].

3.3.6.1 Aerobic/Anoxic metabolic model of GAOs in Acetate-fed systems

The reactions in the aerobic phase for GAOs include PHA catabolism, glycogen production, biomass growth, oxidative phosphorylation, and maintenance. Where in the beginning, PHA is hydrolyzed to Acetyl-CoA and Propionyl-CoA before other reactions. The reactions following PHA catabolism (Acetyl-CoA catabolism and Propionyl-CoA catabolism) represent glycogen production from PHA (glycogen production from Acetyl-CoA* and glycogen production from Propionyl-CoA*), GAO biomass synthesis from PHA (biomass synthesis from Acetyl-CoA* and biomass synthesis from Propionyl-CoA*), oxidative phosphorylation, and maintenance followed by the overall stoichiometry [229]. In the aerobic stage, when all the substrate has been consumed, the stored polymers (PHA), act as buffers for the substrate that has been taken up but has not been used for growth. Therefore, the development of GAOs continues to occur with an almost similar or moderately lower rate without external substrate supplementation [260]. In the case of GAOs, the ATP/NADH ratio (δ) is difficult to determine due to its cross-correlation with the maintenance coefficient and ATP requirement for biosynthesis. However, an estimated value of 1.6 to 1.85 has been determined in the literature for δ of GAOs [202].

3.3.7 Aerobic Metabolic Model in Propionate-fed systems

The aerobic metabolic reactions are mainly categorized as energy-generating, including PHB catabolism and oxidative phosphorylation, and energy-consuming with three reactions of biomass production, polyphosphate synthesis, and glycogen replenishment [43]. These reactions simultaneously take place in aerobic conditions with no clear preference in their respect of occurrence [249]. Due to the high abundance of acetate in full-scale WWTPs. At the same time, propionate is typically prevalent, specifically in pre-fermentation [136]. Differences in phenotypic

characteristics are proposed in aerobic metabolism with propionate as a carbon source comparing to acetate-fed systems due to dominancy of different microbial strains of PAOs resulting in alteration in metabolic behavior [136]. However, in general, in this stage, cell growth, polyphosphate biosynthesis, and glycogen replenishment are driven by the energy produced from aerobic catabolism, in which PHA is the main carbon source [236]. The metabolism-based models, predicting the PAOs and GAOs activity in the EBPR process, mainly have hypothesized the advantage of PAOs over GAOs in propionate-fed systems [136]. In the case of electron acceptor, studies have proposed the higher feasibility of nitrate-based configuration with the propionate-fed system than the acetate-fed process. Feeding with acetate as the main substrate in anaerobic-anoxic conditions, resulting in an accelerated P-removal loss due to glycogen limitation and nitrite inhibition [256].

3.3.7.1 Aerobic metabolic model of PAOs in Propionate-fed system

In the aerobic phase, the PHA stored is utilized for phosphate uptake, glycogen synthesis, and maintenance in the cell. The metabolic model of the aerobic stage with propionate as the carbon source is described thoroughly by *Oehmen et al.* as shown in **Appendices, C, Fig 10.2** [259]. In the first step, the PHAs, including PHB, PHV, and PH2MV are used to produce Acetyl-CoA and Propionyl-CoA. Then the activated Acetyl-CoA and Propionyl-CoA are converted for producing ATP and reducing power. Consequently, the phosphate is taken up through the cell membrane and stored as polyphosphate utilizing an energy source. Next, the glycogen synthesis and biomass growth take place by Acetyl-CoA and Propionyl-CoA reactions. The overall reaction is:

$$PHA + \left(\frac{0.21}{1.27}\lambda + \frac{0.21}{1.06}\beta\right) NH_3 + \left(\frac{1.7}{1.27}\lambda + \frac{1.38}{1.06}\beta\right) ATP + \left(\frac{0.69}{1.27}\lambda + \frac{0.55}{1.06\beta}\right) H_2O \rightarrow$$
$$\left(\frac{1}{1.27}\lambda + \frac{1}{1.06}\beta\right) CH_{2.17}O_{0.84}N_{0.21}P_{0.015} + \left(\frac{0.89}{1.27}\lambda + \frac{0.68}{1.06}\beta\right) NADH_2 + \left(\frac{0.27}{1.27}\lambda + \frac{0.06}{1.06}\beta\right) CO_2$$

Equation 3-35

The ATP required in aerobic reactions is provided by conversion of reducing power in oxidative phosphorylation. The summary of propionate-fed aerobic metabolism of PAOs is available in **Appendices, C, Fig 10.2**.

3.3.7.2 Aerobic metabolic model of GAOs in Propionate-fed systems

Aerobic metabolic reactions follow the steps mentioned in the aerobic stage in acetate-fed systems for GAOs, since carbon source changes only affect the anaerobic fractions of Acetyl-CoA and Propionyl-CoA [205].

Studies are evaluating whether GAOs and PAOs are the same microorganisms performing differently under various conditions. In a limited phosphorus condition, with low GAO existence, *Schuler et al.* [261] suggested that the PAOs were going through the glycogen accumulating metabolism; however, no proof was offered. Later on, a study by *Zhou et al*. [2] demonstrated that in a P-deficient environment with very low phosphorus release, PAOs would still take up phosphorus. Therefore, the conversion from phosphate accumulating metabolism to GAM by PAOs has been confirmed [262]. With very limited GAOs in the system measured by Fluorescence in Situ Hybridization (FISH), it was proposed that the PAOs were the main group using glycogen as the energy source. Recent studies have indicated the shift of PAOs and GAOs phenotype and metabolic pathway under different conditions showing their metabolic flexibility [237].

On the other hand, culture-independent methods have been identifying PAOs and GAOs in EBPR systems. *Gammaproteobacteria* has been recognized as one containing many putative GAOs, where *Candidatus Competibacter Phosphatis* was identified as a GAO capable of consuming acetate and propionate; however, the slow uptake-rate of propionate of these GAOs, results in out-competition by PAOs. Studies have also indicated the capability of *Defluvicoccus Vanus* in consumption of propionate in high-rate, as a member of *Alphaproteobacteria* as tetrad-forming

organisms (TFOs) [205]. TFOs mainly proliferate in lab-based systems with synthetic carbon source addition [263].

3.3.8 Aerobic Metabolic Model in Glucose-fed systems

With all the contradiction on the effect of glucose on the EBPR process, this organic carbon source is the foremost reason for EBPR failure due to enrichment of GAOs as unwanted microorganisms in the P-removal process. These microorganisms accumulate glycogen without polyphosphate storage under aerobic conditions [240]. However, recent studies have conducted that the *Actinobacterial* PAOs are closely related to genus *Tetrasphaera* as one of the putative PAOs known, which is distinctive from *Accumulibacter*, utilizes glucose and amino acids, synthesizes glycogen by polyphosphate degradation in the anaerobic phase. Similarly, a study by [264], indicated the capability of *Tetrasphaera*-related organisms in anaerobically taking up glucose, glutamic acid, and casamino acid. At the same time, *Accumulibacter* showed negative or very low signals in taking up these carbon sources. Thus, aerobically the stored glycogen is degraded for cell growth, P-uptake, and polyphosphate formation [264]. Furthermore, using Raman spectroscopy technology for in-situ intracellular compound quantification, a higher abundancy of *Tetrasphaera* was indicated than *Accumulibacter* in full-scale WWTPs in Denmark [265]. This finding was justified by a survey of 32 full-scale EBPR plants in 12 countries with a higher abundance of *Tetrasphaera* in most plants [266]. Yet, there is still a lack of knowledge regarding this specific genus's intracellular compounds and metabolic pathways [267]. Moreover, *Microlunatus Phosphovorus*, another isolated PAO candidate, has shown P-removal activity in glucose-fed systems [268].

3.3.8.1 Aerobic metabolic model of PAOs in Glucose-fed systems

In the study conducted by *Jeon et al.* [244], as shown in Figure 3-5.1, in EBPR system with glucose feeding, in the aerobic phase, with a constant glycogen concentration, phosphate was taken up with

a decrease in PHA as an energy source for polyphosphate synthesis to almost complete disappearance.

In another study conducted by *Wang et al.* [245], as shown in Figure 3-5.2, in the aerobic phase of the glucose-fed EBPR system, PHV as the primary stored energy and carbon source was oxidized entirely to provide energy for all metabolic reactions.

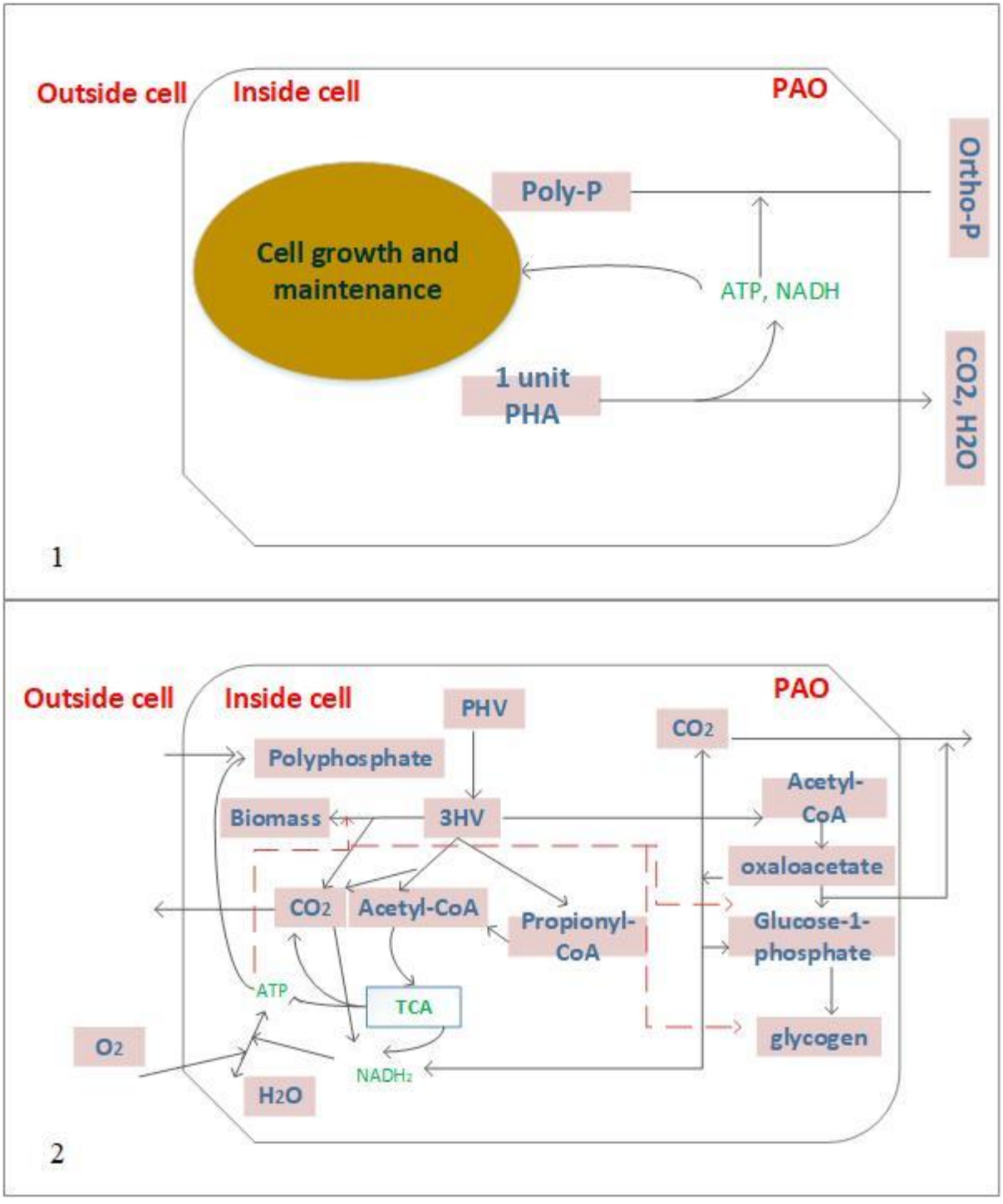


Figure 3-5 proposed aerobic metabolic model for PAOs in glucose-fed systems 2. hypothesized aerobic metabolic pathway for PAOs in glycogen-fed systems

In the PHV oxidation pathway, various intermediates are produced consisting of Acetyl-CoA and Propionyl-CoA production by depolymerization of 3-hydroxyvalerate, pyruvate production

through methylcitrate cycle from Propionyl-CoA and further Acetyl-CoA production by pyruvate conversion in TCA cycle which finally results in NADH production as the final product as shown in Table 3.4. ATP is produced through the phosphorylation process. Moreover, growth and cell synthesis occur in aerobic conditions, where PHV is the main carbon and energy source. With the energy produced from PHV oxidation and reducing power, PAOs can also take up phosphate as a negatively charged ion to synthesize polyphosphate. In contrast to the previously mentioned study, glycogen content increased in aerobic conditions by PHV conversion through the glyoxylate pathway [245]. There is still research to be done in this area for a far better understanding of the effect of glycogen on the EBPR process. The summary of metabolic equations for PAO and GAO microorganisms for various substrates mentioned are available in **Appendices, D, Table 10.2, 10.3, and 10.4**.

Table 3.4 metabolic reactions in aerobic phase for PAOs in glucose fed systems by [245]

Reactions in aerobic condition	***equations***
PHV oxidation Equation 3-36	$CH_{8/5}O_{2/5} + \frac{12}{5}NAD + \frac{6}{5}H_2O + \frac{2}{5}H_3PO_4^{in} + \frac{2}{5}ADP \rightarrow \frac{12}{5}NADH_2 + CO_2 + \frac{2}{5}ATP$
Oxidative phosphorylation Equation 3-37	$NADH_2 + 0.5O_2 + 1.85ADP + \frac{1}{85}H_3PO_4^{in} \rightarrow NAD + 2.85H_2O + 1.85ATP$
Active biomass synthesis from PHV Equation 3-38	$1.27CH_{8/5}O_{2/5} + 0.2NH_3 + \left(2.012 + \frac{\mu_{atp}}{\mu}\right)H_2O + 0.8055NAD + \left(1.5 + \frac{\mu_{atp}}{\mu}\right)ATP \rightarrow CH_{2.09}O_{0.54}N_{0.2}P_{0.015} + 0.27CO_2 + 0.8055NADH_2 + \left(1.5 + \frac{\mu_{atp}}{\mu}\right)ADP + \left(1.485 + \frac{\mu_{atp}}{\mu}\right)H_3PO_4^{in}$
Phosphate transport into biomass Equation 3-39	$H_3PO_4^{out} + \frac{1}{7}NADH_2 + \frac{1}{14}O_2 \rightarrow H_3PO_4^{in} + \frac{1}{7}H_2O + \frac{1}{7}NAD$
Polyphosphate synthesis Equation 3-40	$H_3PO_4^{in} + ATP \rightarrow 2HPO_3 + H_2O + ADP$
Glycogen synthesis from PHV Equation 3-41	$\frac{5}{3}CH_{8/5}O_{2/5} + 2NAD + \frac{7}{3}H_2O + \frac{5}{6}ATP \rightarrow CH_{8/5}O_{5/6} + 2NADH_2 + \frac{2}{3}CO_2 + \frac{5}{6}H_3PO_4^{in} + \frac{5}{6}ADP$

3.3.9 PAOs and GAOs metabolism with real wastewater feed

The type of carbon source plays a vital role in the viability and stability of EBPR as one of the main differences between lab-scale and full-scale systems [269]. As mentioned earlier, in each of the studies, the proposed metabolic model highly correlated with the experimental results; however, the results were generated from lab-scale systems with simple model carbon sources under optimum or constant operational conditions. Moreover, the models only indicate a single anaerobic/aerobic cycle for a single cell [236]. Yet, investigations on full-scale EBPR systems are scarce comparing to lab-scale experiments. Therefore this projects the various upcoming

challenges on handling the number of parameters in the complex and dynamic process [215] since single-substrate studies don't provide a proper image of the feeding system in real WWTPs [270].

Moreover, even with a hint on various substrates, cycles, and environmental factors, the implication of factors such as the effect of additional exogenous and endogenous nutrients on polymeric substance storage and the nature and composition of the organic substrate is lacking in the emerging outlook of EBPR [236]. Therefore, more effort is needed in the metabolic model accounting for full-scale systems containing real sludge in operating plants [271]. However, recent studies have shown exceptional improvement in predictive models to enhance WWTP performance. For example, in a study conducted by [272], two models (META-ASM and ASM-inCTRL) with a difference in their bio-kinetic models were tested to predict EBPR instability in full-scale, long-term operation. By mitigating these strategies, it was found that the META-ASM model is a powerful tool to predict process upset, evaluate new process design, and optimize performance [272].

As vital for PAOs metabolism, the presence of carbon source in WW is imperative for efficient EBPR process [273]. The type of carbon source highly determines the EBPR performance, although the real wastewater consists of various components. It is sensible to assume that except for VFAs and *Candidatus Accumulibacter*, other carbon sources and PAOs drive the P-removal process in full-scale systems [269]. In activated sludge systems, except for a range of low molecular weight substrates including VFAs such as acetate, propionate, butyrate, and valerate, there is a diverse range of other substrates available [242], such as non-VFA organics that some may be taken up and metabolized by PAOs and GAOs, [80]. One of the main operational factors besides carbon composition is the carbon to phosphorus ratio (C:P), which correlates with EBPR performance and stability [274]. Mainly in the case of domestic strength wastewater, in the EBPR

process, effective P-removal occurs at a C:P ratio of 7:1 to 10:1 with a COD of approximately 400 mg/L and acetate as the sole carbon source. EBPR subjected to propionate as the sole carbon source shall have an effective P-removal using a COD: P ratio of 15:1 and COD of 800 mg/L for domestic wastewater [275]. Studies on real domestic wastewaters have shown a wide range of propionic/acetic acid ratios in warmer climates (full-scale Bardenpho plant, Florida) without any pre-fermenters a value of 0.28 to 0.75 has been achieved. In comparison, in colder climates (Canada and Australia) in the presence of pre-fermenters, this value ranged from 0.41 to 0.82 in WWTPs [78]. Notwithstanding, the effect of simultaneous acetate and propionate presence in various ratios (75-25, 50-50% acetate-propionate) on the microorganism's metabolism has not been studied in detail in WWTPs. However, it may be utilized as a useful measure for GAOs minimization [276]. The presence of putative GAOs, including the genome of Propionivibrio, along with those of the Competibacteraceae and Defluviicoccus in simultaneous VFA-fed systems, directs the research to control methods GAO abundance in full-scale EBPR WWTPs [277].

The most widely studied substrate except for VFAs is glucose, where there are many uncertainties regarding the glucose fermentation process, PHA production, glycogen hydrolysis, and generation. Moreover, the effect of various compositions of readily biodegradable substrates in WWTPs such as sugars and amino acids on PAO/GAO competition has not been well studied [243]. Furthermore, there is limited knowledge on the abundance of these compounds in wastewater systems [6] which requires further exploration. Although the proportion of proteins in most real wastewater cases is approximately 50%, amino acids could be a potentially available carbon source for EBPR [278]. *Randall et al.* have reported EBPR failure with amino acid addition as substrate. Although *Ubukata* [279] proposed P-removal in the EBPR process fed with casamino acid and

glutamic acid as amino acid derivatives, the PHA formation, and NADH production were not further studied to justify the findings with EBPR redox balance [235].

Further studies have focused on various amino acids such as asparate, glutamate, casamino, and glutamic acids for process optimization [280] [264]. In the case of substrate mixtures, studies have reported contradictory results on uptake rates with a combination of acetate, carbohydrates, and starch, indicating either no difference or rather lower P-uptake in comparison with EBPRs associated with sole-substrate feeding [270]. Except for diversity in organic substrates, other elements such as heavy metals [281], dissolved oxygen concentration, temperature, organic loading, pH and sludge age affect the biochemical pathways of PAOs and GAOs and their relative abundance in the EBPR system.

3.3.10 Discussion and future research requirements

The development of conceptual and mathematical models with the incorporation of microbiological tools assists in a deeper understanding of processes [282]. These models can be used to analyze lab and full-scale data as, in many cases, the experimental stoichiometry data corresponds with theoretical model predictions. Notwithstanding, despite the number of studies on the metabolic modeling of GAOs and PAOs with proposed biochemical pathways, deep knowledge is lacking as a useful method for controlling microbial competition [6]. Biochemical models fail to account for experiments on the EBPR process. Moreover, developing the connection between EBPR performance activity and the relative microbial population community structure and monitoring other key populations such as GAOs in the case of the biological P-removal process is quite challenging [283].

Despite researchers' substantial contribution to discovering the true process for PAOs and GAOs mainly in anaerobic reducing power production sources, after all, a generally approved model is

not available [216]. Uncertainties in the source of reducing power generation dependent on the phylogeny of PAOs and availability of nutrients would impact the stoichiometric ratios accounting for type and amount of PHA production by carbon source uptake [237]. The exact role of each pathway, including glycolysis and the TCA cycle, remains unresolved. In addition, controversies in stoichiometry and kinetic rates are mainly explained based on microbial composition differences, operational conditions, and metabolism contrast/flexibility of abundant microorganisms [284]; however, lack in characterizing the microbial population, in particular, the presence of GAOs in PAOs biochemical study, is a major limitation in accordingly interpreting the results [6]. Moreover, diminishing process upset is mainly a challenging procedure due to the lag-time between process upset and detectable higher P-levels in the effluent. WWTP mainly revisits chemical treatment to reach effluent limits [272].

The review of metabolic pathways proposed by various studies on GAOs and PAOs raises the need for understanding the metabolism utilized by different microbial groups in the EBPR process and whether PAO/GAOs are capable of alternating between different biochemical models in case of environmental and operational changes [6]. Furthermore, existing biochemical models cannot cover all experimental data, such as reported EBPR processes without anaerobic PHA storage or lack of Poly-P degradation and glycogen replenishment for PHA production.

Inadequacy of a biochemical pathway that can be interpreted to any condition with consideration of the structure and function of the active microbial population is recognizable; however, the models yet only simulate putative known PAOs and GAOs as pure cultures under specific conditions in EBPR systems [46] without even taking into consideration of intrinsic variabilities in “physiological and morphological properties” of different PAO clades [204]. Different clades tend to have different morphology, stoichiometry, and kinetic characteristics, which leads to

different stress tolerance and metabolic conversions under certain conditions [284]. Therefore, study on the metabolism of each clade is important since the large differences in the kinetics rates of clades make it impossible to consider PAO clades as one organism regarding the modeling concept.

For a more efficient EBPR process performance, there is a rising need to extensively understand the microbial population dynamics by improving the metabolic model studies to predict the effect of operational conditions, environmental changes, and microbial population interactions on microbial proliferation and complex community functions in addition, to enhance anticipation capabilities of the model to reach a more stable, efficient and effective EBPR.

Only on the assumption of obtaining a well-established biochemical metabolic model that accounts for different environmental conditions and microbial populations, cost-effective ways to minimize GAOs abundance and increase P-removal efficiency in pilot plants and full-scale systems can be implemented [6].

Recent studies with a modeling approach have proposed ASM (ASM2 and ASM2d) models based on biochemical transformation in activated sludge. These models may be used as references for biological nutrient removal modeling in activated sludge systems that describe the EBPR system along with COD, nitrogen removal, oxygen requirement, and sludge generation [285]. In this approach, substrate, energy, and reducing power balances in biochemical transformations are used to calculate yield coefficients in PAOs and GAOs. The kinetic parameters are the only calibration-required parameters, which highly contribute to the consistency and reliability of the model by reducing calibration demand. Yet, the current ASMs used in EBPR systems require substantial stoichiometric and kinetic parameters to adjust the lack of understanding in details and dynamics of the process as a generalized EBPR model. Moreover, a key concern in most applied models is

the lack of identification in the calibration process for bio-kinetics, resulting in kinetic parameter diversity over literature studies [286]. Combination of EBPR models with the heterotrophic, autotrophic, and hydrolytic process and incorporating operational conditions such as pH, temperature, substrate, oxygen concentration, electron acceptor, and metabolic shifts deriving microbial population dynamics in models are promising approaches to achieve an adequate model with the prediction of long-term EBPR performance, that can be a valuable tool in research and engineering applications [287].

3.3.11 Conclusion

This paper summarized the present knowledge on biochemical pathways of the EBPR process and contemplated the research gaps with a pressing need for extensive focus. Current chemical transformation models in anaerobic and aerobic/anoxic conditions are mainly limited to simple VFAs and, in some cases, non-VFA organics such as glucose. Moreover, the present models only simulate putative known PAOs and GAOs as pure cultures under specific conditions in EBPR systems. Moreover, there is a lack of general metabolic chemical transformations that can be interpreted to any state with consideration of the structure and function of the active microbial population. Advances in metabolic modeling can be highly useful in understanding the complex microbial interactions and for dynamic population predictions. Moreover, this knowledge helps design and operate activated sludge systems, stimulate the microbial population among PAOs, and minimize GAOs abundance for higher stability and reliability of EBPR and increase in phosphorus removal efficiency. As microbes are the main drivers of treatment processes, reaching microbiologically developed processes by enhancing metabolic modeling highly elevates treatment systems since WWTPs stand on the structure and function of microbial communities.

4 Scope of Thesis: Aeration as a factor affecting EBPR performance

Making DO transfer from the gas phase to the liquid phase is an energy-intensive process in the WWTP and crucial for the biological process to operate adequately. Aeration systems transfer oxygen into the liquid media by each of two, shearing the liquid surface with a mixer or turbine or releasing air through macroscopic orifices or porous materials [288]. Oxygen acts as an electron acceptor when organic carbon and nitrogen in the form of ammonium are oxidized [289]. Oxygen provided through aeration provides enough DO for BOD removal and nitrification by aerobic organisms in activated sludge, where the nitrification capacity is dependent on the aeration concentration and volume. Other effective factors on nitrification such as solids retention time (SRT), pH, the temperature might ultimately impact DO control [289]. The biological phosphorus removal for high-quality effluent production is achieved through the EBPR process in series of anaerobic/anoxic and aerobic reactors, where biomass recycling takes place between reactors in a complex manner. The energy gradient in an EBPR process is rather different from an aerobic activated sludge system, where rapid ORP and pH fluctuation affects the respiratory electron transport and cell metabolism energetics. Understanding the overall energetics and oxygen requirements provides useful knowledge on whether EBPR contributes to aeration and energy-saving accompanied by high-quality effluent production [290].

In EBPR, with an effect on oxygen dynamics, aerobic P-uptake, anaerobic P-release, and carbon storage of PAOs are closely related to oxygen mass transfer. Literature proposed the coexistence of PAOs and GAOs in the EBPR system [291]. Therefore, controlling the competition between microbial groups is necessary to improve the oxygen utilization for biological P-removal. On the other hand, the inflow of an electron acceptor such as DO in the anaerobic or settling phase inhibits the anaerobic P-release and production of storage pools. Therefore, with aeration as an effective

factor, inconsistent results have been achieved for successful EBPR operation. On the other hand, aeration is responsible for 50 to 75% of plant power usage. On the whole, controlling aeration as a costly process and optimizing DO level for high-efficiency P-removal is required for minimizing energy consumption and reaching stringent discharge limits [292].

With aeration as an effective factor, inconsistent results have been achieved regarding optimum aeration for successful EBPR operation. In the case of DO concentration and aerobic HRT, there is a requirement for a further synergic understanding of the effect of aeration level, aerobic duration, microbial shift, and metabolic pathway on phosphorus release and uptake mechanism, internal storage, and consumption functions cyclic performance. Having outlined the importance of aeration in the EBPR system, consideration of the synergic effect of intermittent aeration on simultaneous N and P- removal performance, microbial community structure, bacterial abundance is essential. Moreover, there is insufficient information on EBPR performance and PAOs abundance under fast and slow transition from high to low DO concentration conditions due to the complex microbial community structure with different microbial groups containing diverse metabolic abilities.

This thesis focuses on the effect of Aeration as a critical operational factor on EBPR performance. In addition, it examines the potential of different aeration strategies to reach maximum efficiency of Phosphorus removal. The purpose of this work was:

1. To investigate the effect of DO concentration, aerobic HRT, and aeration pattern on EBPR performance and compare SBR-mode reactors operated at particular aeration scenarios. Different DO concentrations of 0.4 mg/L to 4 mg/L, aerobic HRT of 120 minutes to 320 minutes, and aeration patterns were investigated. Moreover, the negative impact of excessive aeration on phosphorus removal was also examined.

2. To study the EBPR performance of PAOs under distinctive aeration conditions and evaluate the system's recovery after the disruption.
3. To evaluate the feasibility of low DO levels to improve EBPR efficiency in a gradual DO decrease condition when receiving acetate-based synthetic wastewater. In addition, the proliferation of PAOs and GAOs and their contribution to phosphorus removal were inspected.
4. To investigate by model simulation how aeration can be controlled in an optimal manner using BioWin® Software. This includes an explanation of the non-linear dynamic model and the performed simulation.

5 Understanding microbial shift of Enhanced Biological Phosphorus Removal process (EBPR) under different Dissolved Oxygen (DO) concentrations and Hydraulic Retention Time (HRTs)

Adapted from:

Parnian Izadi, Parin Izadi, A. Eldyasti, "understanding microbial shift of Enhanced Biological Phosphorus Removal process (EBPR) under different Dissolved Oxygen (DO) concentrations and Hydraulic Retention Time (HRTs)", Biochemical Engineering Journal, Volume 166, 2021.

Preface:

This chapter of the thesis is an original work based on the experimental apparatus and data, published by the author, Parnian Izadi.

Author statement:

Parnian Izadi: Conceptualization (Lead), Methodology (Lead), Validation (Lead), Investigation (lead), Resources (lead), Writing- original draft (lead), Writing-review and editing (equal), Visualization (lead)

Parin Izadi: Conceptualization (supporting), Methodology (supporting), Investigation (supporting), Writing- original draft (supporting), Writing-review and editing (equal)

Ahmed Eldyasti: Conceptualization (supporting), Methodology (supporting), Investigation (supporting), Writing-review and editing (Lead), Supervision (Lead), Project Administration (Lead), Funding Acquisition (Lead)

5.1 Abstract

Enhanced biological phosphorus removal effectiveness is specifically conditional to systems ecology and microbial community structure. Understanding process operation aids in comprehending microbial communities and their underlying mechanisms to enhance process control and troubleshooting. The performance of biological phosphorus removal in sequential batch reactors under various aeration strategies was investigated for different dissolved oxygen (DO) concentrations and hydraulic retention times (HRT). The results showed that oxygen

concentration as an operational factor highly influences phosphorus removal performance and phosphorus accumulating organism's (PAO) dominance where high P-removal is achieved at lower DO levels. This study showed that 0.8 mg/L DO concentration could achieve successful biological P-removal with higher than 90% removal efficiency due to a shift in bacterial population towards PAOs.

Further investigation on high aerobic HRT retention time showed a decline in PAOs population and increased glycogen accumulating organism's activity. In addition, a combination of high HRT and high DO level indicated very limited P-removal and further process failure with no anaerobic P-release. Therefore, controlling DO at low levels and aerobic HRT retention time at optimal duration promotes biological phosphorus removal and lower operational and aeration costs.

Keywords: EBPR; phosphorus release and uptake; dissolved oxygen; PAO; GAO; phosphorus removal

5.2 Introduction

Maintaining background levels of phosphorus (P) concentration in water bodies, as an essential factor affecting water quality, minimizes the detrimental effects on animals and plants [293]. Enhanced Biological Phosphorus Removal (EBPR) as a biological activated sludge process without the addition of chemicals operates in sequential anaerobic and aerobic stages [33]. It effectively removes phosphorus to control the overgrowth of algae in waters for eutrophication inhibition in waterways [294]. The alternative anaerobic and aerobic phases stimulate the ability of a group of microorganisms called phosphorus accumulating organisms (PAO) to "luxuriously" uptake phosphorus in higher amounts required for bacterial metabolism [8]. Bacteria group consisting of *Acinetobacter, Pseudomonas, Candidatus Accumulibacter Phosphatis,* and many other communities designate PAOs as the dominant species in activated sludge for phosphorus

removal [33]. The PAOs, anaerobically consume volatile fatty acids (VFAs) and subsequently store them as polyhydroxyalkanoates (PHA), mainly including polyhydroxybutyrate (PHB), by utilizing energy in the form of adenosine triphosphate (ATP) and reducing power provided by internally stored polyphosphate and glycogen cleavage which leads to P_i release into the bulk liquid [295][271]. Aerobically, alongside cell synthesis, maintenance, and glycogen replenishment, the P-accumulating microorganisms store phosphate as polyphosphate using stored PHB as energy and carbon source, contributing to phosphorus removal from bulk liquid [294]. P-removal is accomplished when higher phosphorus is taken up aerobically than the influent phosphorus and the phosphorus anaerobically released jointly [295]. Thus, the P-rich sludge wastage contributes to net P-removal in the activated sludge system [18]. PAOs, as a group of microorganisms that take in high levels of phosphorus, tend to utilize stored compounds internally, to sustain the conditions where electron acceptor and electron donor are not available simultaneously [57]. This exclusive metabolic system, provides PAOs superior dominance in EBPR process [296].

In a well-operated EBPR, lower operational cost, reduced sludge production, and lower environmental impacts are achievable. However, at the same time, it can encounter unpredictable and occasionally unexplained instability and unreliability due to loss of PAO biomass [294][271]. These complications may lead to process upset, system failure, and violation of discharge regulations. Instability in process performance may be originated from several external factors such as heavy rainfall, excessive nitrate loading to the anaerobic reactor, or excessive aeration of activated sludge, or it may be a result of PAOs competition with other groups of microorganisms such as glycogen accumulating organisms (GAOs) [297][6]. Therefore, enhancing the process by

diminishing the influence of any factors may significantly lower the effluent P-level entering waterbodies.

Excessive aeration as an external disturbance leading to high dissolved oxygen (DO) concentrations causes competition between PAOs and GAOs. GAO overgrowth, as a cause of low P-removal efficiencies, proliferate under anaerobic/aerobic sequential steps. With the phosphorus removal aspect, GAOs don't contribute to P-removal processes and anaerobically utilize available carbon sources [188], hydrolyzing glycogen as the sole energy and carbon source for VFA uptake [77]. Inefficient aeration in the EBPR process may also alter internal storage pools, leading to overconsumption of PHB. Therefore, in EBPR systems, high phosphorus release takes place instantly after the presence of organic substrate, although limited PHB content leads to minimal P-uptake. This effect decreases the phosphorus removal efficiency by negatively influencing the aerobic phosphate uptake rate [295].

Furthermore, wastewater treatment plants (WWTP), to reach safe discharge levels for wastewaters (WWs), utilize high levels of energy during operation [298]. Therefore, improving process sustainability by enhancing energetic efficiency is imperative for decreasing greenhouse gas production and subsequent operational costs. As an operational factor highly impacting WWTPs, Aeration is responsible for 45 to 75% of the energetic cost. Therefore, minimizing the aeration in processes such as EBPR economically improves the WWTP [188].

Previous studies have achieved successful EBPR operation with DO levels lower than 0.8 mg/L [299],[182], where it was found that DO concentration will affect the quantity of GAOs in WWTP sludge. High DO levels as high as 5 mg/L increased GAOs abundance while a DO range of 2.5 to 3 mg/L correlated with high phosphorus removal and PAOs dominance [292]. Furthermore, experiments on very low DO levels as low as 0.15-0.45 mg/L for simultaneous nitrogen and

phosphorus removal have achieved a range of 60 to 99% of P-removal [189],[300]. *Brdjanovic et al.* [295] focused on evaluating the aeration duration effect on the EBPR process. The experiment results confirmed deterioration of biological P-removal efficiency due to slow reduction of PHB by Poly-P. *Carvalheira et al.* [188] investigated the effect of aeration on competition between GAOs and PAOs, experimenting on both DO concentration levels and aerobic HRT retention time. The results of the study revealed higher PAO concentration in low DO levels and aerobic HRT retention time. Although with all the investigations on aeration for the EBPR process, there is a lack of synergic understanding on the effect of aeration level, aerobic duration, microbial shift, and metabolic pathway on phosphorus release and uptake mechanism, internal storage, and consumption functions and cyclic performance.

In this study, the effect of DO concentration in a range of 0.8 to 4 mg/L and aerobic HRT retention time in a range of 120 to 200 minutes as one of the main operational factors in controlling the EBPR process was investigated to determine the EBPR performance in terms of anaerobic and aerobic phosphorus profile, P-removal, aerobic kinetics and feasibility in addition to comparison of system operation at different aeration strategies. In addition, the effect of the aeration factor on anaerobic conditions has been investigated in terms of storage and degradation of internal reserves. Moreover, microbial analysis was performed to identify the relative abundance of EBPR samples on phylum, class, and genus levels.

5.3 Materials and methods

5.3.1 Reactor setup and operation

Four sequencing batch reactors (SBR) with 5 L working volume, as shown in Figure 5-1 with an internal diameter of 25 cm, were operated for the EBPR process. Returned activated sludge (RAS) from the Humber treatment plant in Ontario, Canada, was enriched in all anaerobic-aerobic SBRs.

The reactors were maintained at 21±1°C and operated with a cycle time of 4 or 6 hours. The 4-hour cycle consisted of 15 min feeding, 60 min anaerobic, 120 min aerobic, 30 min settling, and 15 min decanting. The 6-hour cycle consisted of 15 min feeding, 90 min anaerobic, 200 min aerobic followed by 40 min settling, and 15 min decanting. One to two-liter of synthetic wastewater comprising acetate, phosphate, and other required nutrients was pumped into the reactors during the feeding phase with an overall hydraulic retention time (HRT) of 10-15 hours and a solid retention time (SRT) of approximately 25 days. In the aerobic phase, the air was provided with an online DO detector from the bottom of the reactors to keep the DO level in the proper range for each process stage (0.8 to 4 mg/L). Reactors were operated with a control processing system for initiating lower DO levels. pH, ORP, and oxygen concentration were monitored online through a control processing system. The mixing speed was controlled at 50 to 100 rpm depending on the DO concentration, reducing the rate with each DO surpass a deviation of ±0.5 mg/L. During the anaerobic and aerobic phases, reactors were constantly mixed; no mixing occurred in settling and decanting phases. Wastewater from the sequential anaerobic and aerobic phase pays in the settling phase. The initial chemical oxygen demand was in the range of 300-350 mg/L containing acetate as the sole carbon source fed to the system. pH was not controlled, but real-time monitored through the experiment. The SBR reactors were routinely monitored through regular sampling for VFA, PHB, and glycogen through each cycle. Chemical analyses such as total suspended solids (TSS) and volatile suspended solids (VSS) were measured on samples from the end of the aerobic phase.

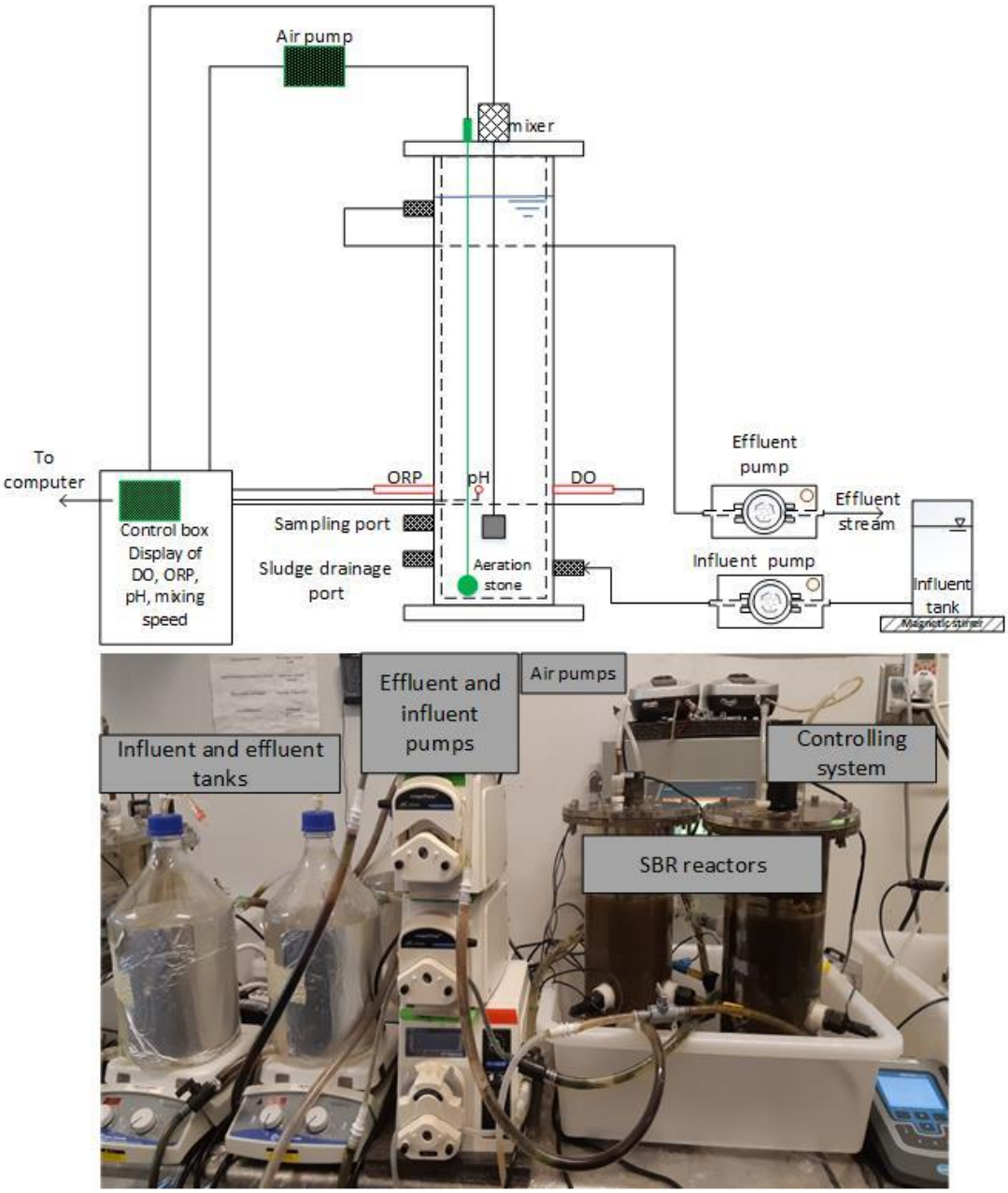


Figure 5-1 Schematic diagram of the experimental EBPR process SBR-mode reactor for EBPR process experimentation under anaerobic and aerobic conditions

5.3.1.1 Synthetic feed composition

The synthetic wastewater used for this study approximately contained (per liter): 0.85g $NaAc.3H_2O$ (400 mg COD/L) as carbon source, 107mg NH_4C1 (28mg N/L), 75.5mg $NaH_2PO_4.2H_2O$ (15mg P/L), 90mg $MgSO_4.7H_2O$, 14mg $CaCl_2.2H_2O$, 1mg yeast extract, and 0.3

mL nutrient solution. The concentrated nutrient solution contained, per liter: 1.5g $FeCl_3.6H_2O$, 0.15g H_3BO_3, 0.03g $CuSO_4.5H_2O$, 0.18g KI, 0.12g $MnC1_2.4H_2O$, 0.06g $Na_2MoO_4.2H_2O$, 0.12g $ZnSO_4.7H_2O$, 0.15g $CoCl_2.6H_2O$, and 10g EDTA. The pH of feed was adjusted at 7.5±0.1.

5.3.1.2 Analytical method

Total solids (TS), volatile solids (VS), TSS, VSS, total chemical oxygen demand (TCOD), and soluble chemical oxygen demand (SCOD) were determined using the standard methods (APHA 2005). pH was determined using a digital pH meter (hach HQ440d multi). Phosphate, nitrate, nitrite, and ammonia were simultaneously determined through ion chromatography by Thermo Scientific™ Dionex™ Integrion™ HPIC™ system. In advance of all IC measurements, samples were properly diluted with deionized water and passed through a membrane filter (0.45 µm). For PAO enrichment confirmation and evaluation in the reactors, initially, samples were analyzed for particle size distribution using an aqueous liquid module (ALM) in LS 13 320 Particle Sizing Analyzer, later on, samples were analyzed using PCR (RNA was extracted, amplified using PCR then the products were subjected to gel electrophoresis to confirm the existence of PAOs). Total RNA was refined from the bacterial lysate with the support of the QIAGEN RNeasy Mini Kit. The extracted RNA of the samples was treated following the QIAGEN One-Step RT-PCR Kit protocol. The successful amplification of the desired bacterial RNA confirms the presence of the investigated bacterial family. The volatile fatty acid was assayed by an SRI gas chromatography (GC) equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). PHB was extracted from cellular biomass and quantified with gas chromatography using the following procedure. Primarily, 10-15 mg of lyophilized biomass were collected, and 2 mL of acidified methanol (3% sulphuric acid), as well as 2 mL of chloroform, were added in a glass vial. After gentle mixing, the cocktail was heated at 100 ∘C for

3.5 hours, cooled down to room temperature afterward. 1 mL of deionized water was added later on, and the mixture was vortexed for 1 minute and then left until phase separation was achieved. The lower organic phase was tested for PHB quantification using SRI gas chromatography equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). The temperature program was 1 min 80 ∘C, 10 ∘C min-1, 180 ∘C for 4 min. Results were compared with standard curves obtained using PHB standards (Sigma Aldrich). Benzoic acid was used as an internal standard to increase accuracy. Intracellular glycogen is determined via digestion and hydrolysis of glucose. Glucose will be analyzed enzymatically (Sigma–Aldrich PGO enzyme kit, Saint Louis, Missouri, USA). Sample absorbance is measured at 425 nm using a Spectronic spectrophotometer.

5.3.1.3 Statistical analysis

The t-test (for two groups of samples), F-test for equal variances (for two groups of samples), and One-way ANOVA analysis were applied with Matlab® Statistics and Machine Learning Toolbox™ (Matlab R2015a, MathWorks, USA) to evaluate the significance of differences in efficiency, performance and fit between models and to study the effect of oxygen concentration on P-removal efficiency. In the SBRs with different operations, correlation among operational and environmental parameters was evaluated to assess statistical relationship between aeration changes and performance and removal efficiencies.

5.4 Results and discussion

5.4.1 Process performance

5.4.1.1 DO levels in 120 min aerobic-HRT retention time

Reactors were routinely monitored with continuous aeration in the reaction phase with DO levels controlled at 4 mg/L and 2 mg/L for $SBR_{4\ mg/L\text{-}120\ min}$ and $SBR_{2\ mg/L\text{-}120\ min,}$ respectively, through 67

days of the experiment. Subsequently, in the next step for verifying the effect of DO, the concentration was lowered to 0.8 mg/L ($SBR_{0.8\ mg/L\text{-}120\ min}$). DO levels were held constant for all reactors; however, pH wasn't controlled, but real-time monitored during each cycle. Previous studies suggest that higher pH is more beneficial to PAOs as it improves the phosphate release in the anaerobic stage cause of higher VFA uptake [301] [222]. On day 15 of the experiment, as shown in Figure 5-2, in the fill/anaerobic phase, the addition of synthetic feed decreased the pH to neutral levels in $SBR_{0.8\ mg/L\text{-}120\ min}$ and $SBR_{2\ mg/L\text{-}120\ min}$.

In contrast, in the following aeration phase, the pH gradually increased to approximately 8.23 mainly due to CO_2 stripping [302], followed by a slight decrease to 8 by the end of aerobic period. $SBR_{4\ mg/L\text{-}120\ min}$ had relatively the same pH profile with slightly lower pH increase in aeration period. Overall, pH variation indicated minimal difference in case of different DO concentrations, suggesting that aeration level has no clear impact on pH variation.

In the settling and draw phase, DO concentration was comprised of oxygen in the aqueous and settled phases in settled sludge. DO levels in settled sludge drastically decreased in settling and decanting phase to 0.5, 0, and 0 mg/L in $SBR_{4\ mg/L\text{-}120\ min}$, $SBR_{2\ mg/L\text{-}120\ min,}$ and $SBR_{0.8\ mg/L\text{-}120\ min}$ respectively, while for the aqueous form is maintained in the same level as an aerobic phase with a maximum 0.5 mg/L decrease in concentration. Yet, $SBR_{4\ mg/L\text{-}120\ min}$ DO sludge concentration decreased by 3.5 mg/L in the settling and draw phase, 0.5 mg/L residual DO was recirculated to the anaerobic phase, causing interruption to COD uptake.

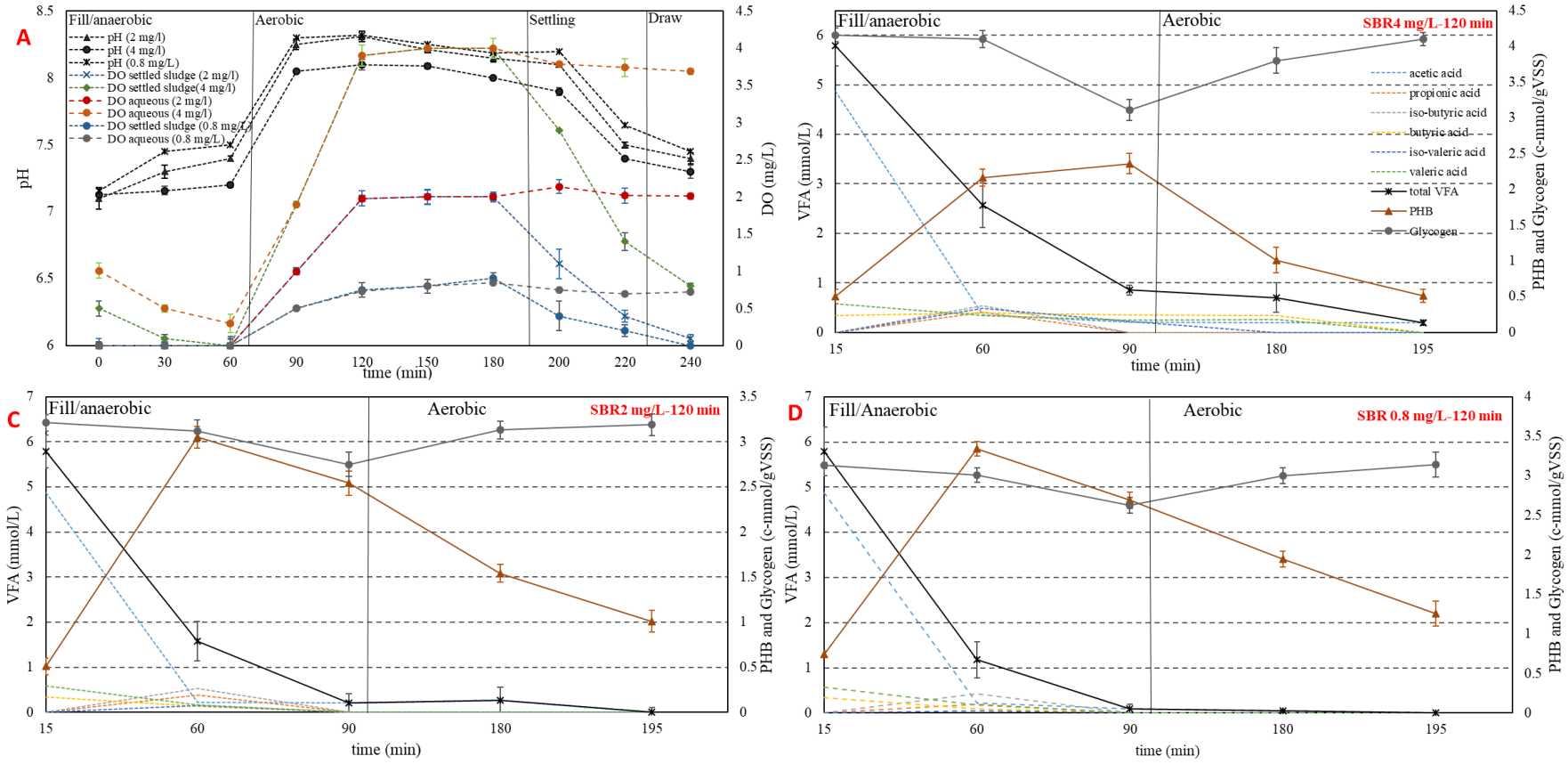


Figure 5-2 A. DO and pH profile in 4-hour cycle SBR-mode reactors with two different dissolved oxygen levels of 0.8, 2 and 4 mg/L, B, C, D. VFA and PHB transformation profile in EBPR reactors at different DO levels (0.8, 2, 4 mg/L) through each cycle

As shown in Figure 5-2, rapid VFA reduction in the anaerobic phase resulted in high P-release and PHB generation and glycogen degradation. In the following aerobic stage, higher P-uptake than the amount released was observed, coupled with a decrease in accumulated PHB concentration and regeneration of glycogen as expected [303]. This behavior is similar to previous PAO-rich processes under other researchers' sequential anaerobic/aerobic stages studies [40] [184]. However, it was observed that by the end of the anaerobic phase, $SBR_{0.8\ mg/L\text{-}120\ min}$ and $SBR_{2\ mg/L\text{-}120\ min}$ almost consumed all available VFAs, while $SBR_{4\ mg/L\text{-}120\ min}$ had residual VFA in the bulk liquid. Considering the metabolic pathway of PAOs investigated by *Smolders et al*. [43][44], for anaerobic VFA transportation inside the cell, conversion of VFAs to acetyl-CoA and subsequent production of PHAs from acetyl-CoA, ATP, and nicotinamide adenine dinucleotide ($NADH_2$) are required for energy and redox balance purposes. In the A/O system, the sources of $NADH_2$ and ATP are tricarboxylic acid cycle (TCA)/glycogen degradation and Poly-phosphate breakdown, respectively. In $SBR_{4\ mg/L\text{-}120\ min}$ with lower VFA uptake, a slightly lower PHB production to VFA uptake rate was achieved [303]. The P-removal process is highly dependent on the transformation of PHAs and glycogen intracellularly, where it influences anaerobic P-release and aerobic P-uptake. In a well-operated EBPR, rapid acetate uptake with anaerobic P-release and PHB accumulation simultaneously occurs with a slight glycogen decrease. However, except for PAOs, GAOs metabolism and activity are closely linked with PHA and glycogen transformation. Glycogen concentration and transformation during a cycle of $SBR_{4\ mg/L\text{-}120\ min}$ and $SBR_{0.8\ mg/L\text{-}120\ min}$ on day 20 of experiment with 3.21 mmol/gVSS and 3.13 mmol/gVSS and 0.46 mmol/gVSS and 0.5 mmol/gVSS decrease in aerobic phase implied high PAO activity. However, 4.16 mmol/gVSS of glycogen concentration and 1.05 mmol/gVSS decrease in aeration period suggested an increase in the GAOs activity for $SBR_{4\ mg/L\text{-}120\ min}$ with 4 mg/L DO concentration.

The literature proposed that the GAOs consume approximately twice as much glycogen as PAOs during the acetate uptake process. Therefore, to uphold a sufficient amount of glycogen for acetate uptake, more glycogen restoration is needed compared to PAOs during aeration [117], confirming the microbial shift towards GAOs in $SBR_{4\ mg/L\text{-}120\ min}$.

With polyphosphate hydrolysis taking place, soluble Ortho-phosphate concentration increased, resulting in anaerobic P-release in bulk liquid and subsequent aerobic P-uptake due to PHA oxidization and degradation well as glycogen replenishment for different, DO concentrations of 4 mg/L, 2 mg/Land 0.8 mg/L [20]. As indicated in Figure 5-2, at high DO levels, lower phosphorus uptake in the aerobic phase was observed, reducing the ability of PAOs to compete in the system. With a decrease in uptake of phosphate, less acetate is taken up in the following anaerobic phase. Therefore, a lower P-uptake diminishes the aerobic regeneration of poly-phosphate, which will be used as an energy source in the anaerobic zone. In this condition, GAOs will consume the acetate not be taken up by PAOs, resulting in an increase in mass of GAOs in the system and a deteriorating P-removal process [117].

Increasing the DO levels attributed to a decrease in P-release and P-uptake in the sequential anaerobic and aerobic stages by 38% and 35%, respectively. By decreasing the DO levels from 4 mg/L down to 2 mg/L and consequently to 0.8 mg/L, the P-release to VFA uptake ratio substantially increased. This 41% increase is associated with the higher P-release in the anaerobic phase. VSS/TSS ratio profile decreased with DO concentration decrease, resulting in higher inorganic polyphosphate content in the lower DO level sludge. As shown in Figure 5-3, a high COD consumption was attributed to MLSS increase in the anaerobic stage from 3715 to 3900 mg/L in 2 mg/L DO concentration. However, for $SBR_{4\ mg/L\text{-}120\ min}$, the COD consumption was fairly lower,

where abundant carbon sources in the aerobic stage restrained P-uptake, decreasing the observed Poly-P content in the sludge.

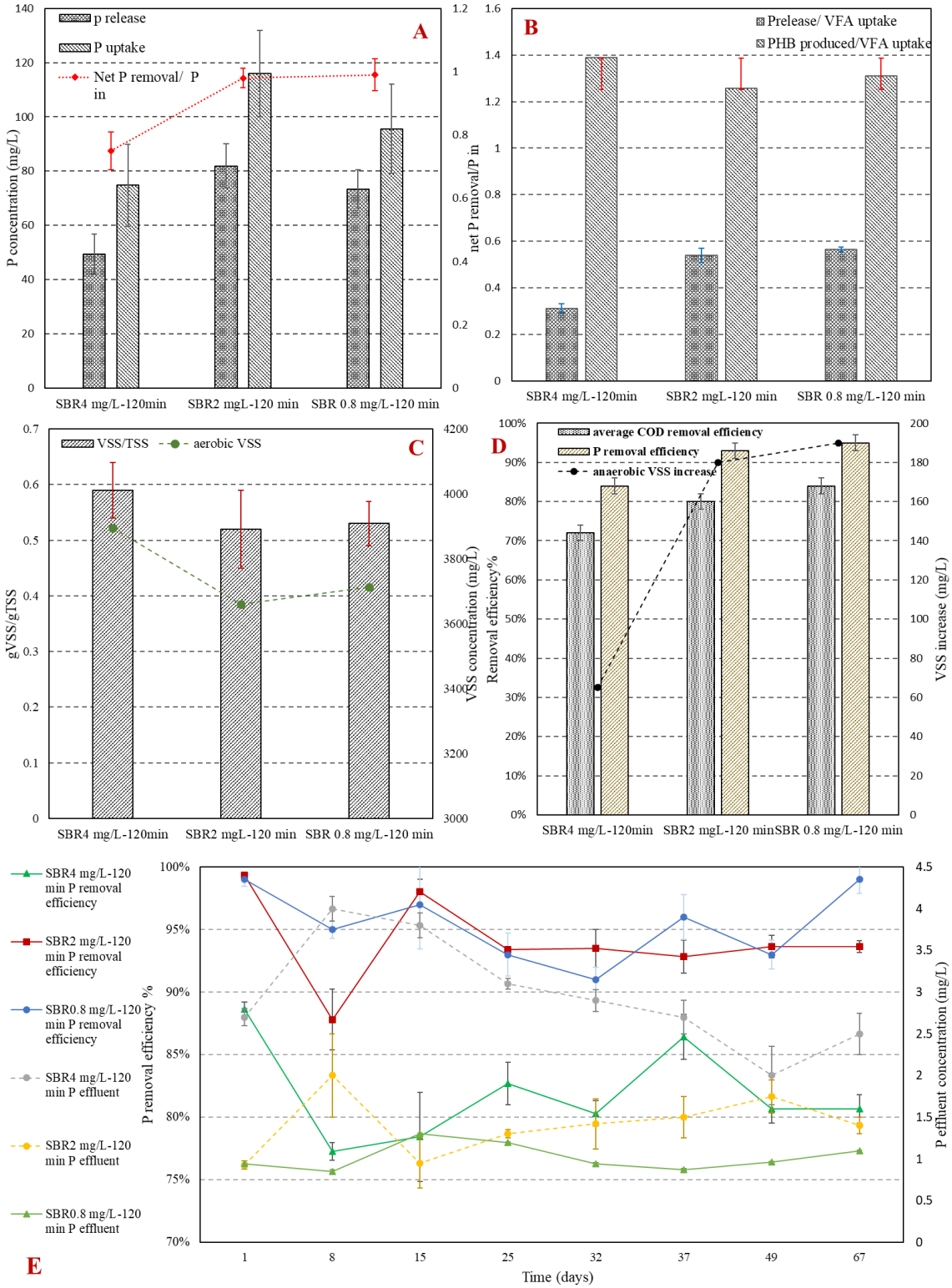


Figure 5-3 at 4-hour cycle duration: A) P-profile for SBR 4mg/L-120 min, SBR 2 mg/L-120 min and SBR 0.8 mg/L-120 min B) P-release and PHB production per VFA uptake ratio in different SBRs C) VSS/SS ratio and VSS concentration in different DO concentrations, D) COD and P-removal efficiency in SBR reactors E) effluent P concentration and P-removal efficiency for SBR reactors

Figure 5-3 shows the P-removal efficiency and P-effluent concentration for different DO levels during 67 days of the experiment. Reactors faced fluctuations in the first 7 days of the investigation, with poor performance on day 8. The P-effluent concentration of 4, 2, and 0.8 mg/L was monitored on day 8, resulting in lower P-removal. After a week of operation, a series of reactors reached a stable phosphorus removal with an average of 2.8, 1.54 and 1.02 mg/L of phosphorus in the effluent with 82%, 93%, and 95% P-removal efficiency, respectively. As observed in Figure 5-3, lower DO levels caused an increase in anaerobic phosphorus release and subsequent phosphorus uptake. In lower DO levels, higher phosphorus release correlated to higher P-uptake. By conducting a significance of difference test on the P-removal efficiencies for all three scenarios, a *p*-value lower than 0.05 indicated a significant difference between the results, showing a considerable enhancement with lower DO level. (A list of measured data is available in **Appendices, E, Table 10.5**).

5.4.1.2 DO levels in 200 min aerobic-HRT retention time

The cyclic period in all SBRs was changed to reach a 6-hour cycle. The DO was kept constant for the reactors with 4 mg/L in $SBR_{4\ mg/L\text{-}200\ min}$ and 2 mg/L in $SBR_{2\ mg/L\text{-}200\ min}$. Subsequently, in the next step for verifying the effect of DO, the concentration was lowered to 0.8 mg/L ($SBR_{0.8\ mg/L\text{-}200\ min}$). In this stage of the experiment, at the beginning of the anaerobic stage, the pH of all reactors was roughly 7.4 to 7.6, which slightly decreased to lower levels with an increase in the anaerobic time duration to 6.8 to 7. In the aerobic stage, the pH increased drastically in the first 60 minutes and gradually decreased to lower levels (7.3) by the end of aerobic duration. Table 5.1 Stoichiometric and removal efficiency comparison for 3 DO levels in 6 6-hour cycle presents the production and uptake ratios and removal efficiencies on day 10 of the experiment. P-concentration in the SBRs varied with different DO levels. The extent of P-release increased by 42% and 44%, decreasing the DO

levels to 2 mg/L and 0.8 mg/L, respectively, reaching an average 62 mg/L of P-release. With respect, P-removal in the following aerobic phase increased, reaching 85% and 90% of P-removal efficiency for 2 mg/L and 0.8 mg/L of DO. However, the total effluent P-concentration for $SBR_{4\ mg/L\text{-}200\ min}$, was an average of 5.5 mg/L with a gradual increase after day 10, reaching up to 12 mg/L of phosphorus on day 28. With an increase in the DO level to 4 mg/L the biological phosphorus removal steadily declined to 61% with low P-release and P-uptake in the sequential anaerobic/aerobic stages. This phenomenon can be attributed to a decrease in PAOs fraction in the microorganisms and a rapid shift towards GAOs. The cause of such lower P-release and P-uptake may be due to excessive aeration in $SBR_{4\ mg/L\text{-}200\ min}$. By conducting a significance of difference test on the P-removal efficiencies, a *p*-value lower than 0.05 indicated a significant difference between the results. This showed a considerable enhancement in EBPR performance with decreasing the DO concentration at high aerobic HRT.

Previous studies have reported that the dominant PHA class that accumulates in the anaerobic stage in acetate-fed reactors is PHB [302]. For $SBR_{4\ mg/L\text{-}200\ min}$ as shown in Table 5.1 on day 10 of the experiment, there was lower PHB production per VFA uptake, causing a decline in anaerobic phosphorus release and subsequent phosphorus uptake in the aerobic phase. Extending the anaerobic duration, the intracellular enzyme activities and intermediate metabolite production are negatively impacted, resulting in lower poly-phosphorus levels and P-removal performance [28]. Moreover, there is a possibility of the scarcity of COD for PHA synthesis due to the organic utilization of ordinary denitrifiers at the beginning of the extended anaerobic stage [304]. The limiting carbon source availability for PAOs increases the sensitivity of the P-removal process [305]. As stated by *Brdjanovic et al.* [295], over-aeration gradually depletes PHB storage, leading to low aerobic P-uptake. Therefore, with limited P-uptake in the aerobic phase, many anaerobic

released phosphorus will be left, not handled due to PHB scarcity. $SBR_{2\ mg/L\text{-}200\ min}$ had higher rates in correspondence with higher PHB synthesis and glycogen degradation in lower DO levels. Since the reducing power for PHB generation is produced by glycogen degradation, higher PHB synthesis results in higher glycogen degradation [39]. With a higher external carbon source available in $SBR_{4\ mg/L\text{-}200\ min}$ at day 10 in the aerobic stage, less PHB was oxidized and used for cell growth; therefore, less phosphorus uptake resulted in lower P-removal. Glycogen is the key storage in GAOs as energy and reducing power source required for anaerobic uptake of VFAs [39]. The decrease in P-release/VFA uptake for 4 mg/L DO reactors as well as an increase in glycogen concentration and glycogen degradation rate up to 5.3 mmol/gVSS and 1.1 mmol/gVSS respectively, in these systems, as shown in Figure 5-4, indicates high GAO population in high aeration rate. At a low aeration rate of 2 mg/L there was a slight increase in glycogen content, suggesting a shift towards GAOs. However, for 0.8 mg/L DO concentration, the glycogen degradation rate was maintained at the same level of 0.78 mmol/gVSS through the experimental duration. Looking into correlation between DO decrease and glycogen concentration, a strong positive correlation coefficient of 0.8 indicated a strong relationship between aeration and internal metabolism of PAOs. (A list of measured data is available in **Appendices, E, Table 10.6**).

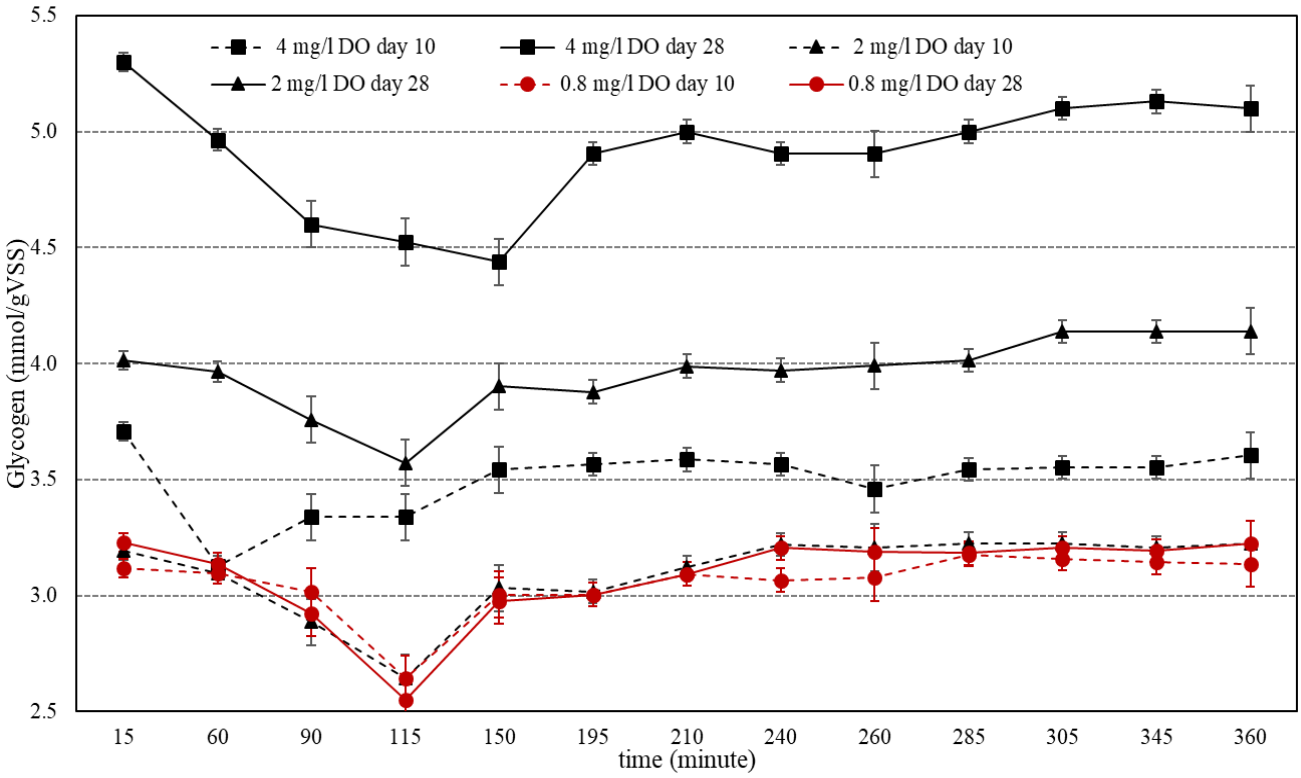


Figure 5-4 Variation of glycogen concentration in a typical 6-hour cycle EBPR process for 3 DO levels of 0.8, 2, and 4 mg/L on days 10 and 28 of the experiment

Moreover, the lower VSS/TSS ratio in the lower DO levels also indicates a higher concentration of stored phosphorus in the sludge, which shows the higher activity of PAOs, resulting in higher P-removal. Therefore, as shown in Table 5.1**,** COD removal efficiency was slightly improved with a decrease in DO concentration, while P-removal efficiency was strongly dependent on the level of aeration.

Table 5.1 Stoichiometric and removal efficiency comparison for 3 DO levels in 6 6-hour cycle

	PHB produced/VFA uptake	**P release/VFA uptake**	**VSS/TSS ratio**	**P removal efficiency %**	**COD removal efficiency%**
SBR_4 mg/L-200 min	0.9±0.05	0.315±0.03	0.73±0.05	61±5	81±2
SBR_2 mg/L-200 min	1.12±0.03	0.564±0.03	0.54±0.04	85±4	84±3
$SBR_{0.8}$ mg/L-200 min	1.15±0.02	0.55±0.04	0.53±0.05	90±6	91±2

All the findings indicate GAOs existence in high DO concentration levels. As proposed [306], anaerobic PAOs and GAOs activity evaluation was indicated for enhanced understanding by assuming that acetate consumption and glycogen degradation takes place by PAOs and GAOs, acetate uptake for PAOs and GAOs as a and b respectively is presented as Equation 5-1 and 5-2:

$$a = \frac{(1+2\alpha_{GAO})HAc - Gly}{0.5+2\alpha_{GAO}} \qquad \textit{Equation 5-1}$$

$$b = \frac{Gly - 0.5HAc}{0.5+2\alpha_{GAO}} \qquad \textit{Equation 5-2}$$

α_{GAO} indicating the required energy for transportation 1c-mol of acetate across the GAO cell membrane depends on pH. For pH of 7.3, it is approximately 0.075 molATP/c-mol HAc with *a* and *b* results; PHB and PHV concentration is measured as shown in Equations 5-3 and 5-4.

$$PHB = 1.33a + \frac{(\frac{9}{6}+(\frac{2\alpha}{3}))^2}{\frac{5}{3}+\frac{4\alpha}{3}}b \qquad \textit{Equation 5-3}$$

$$PHV = \frac{2.5*(\frac{9}{6}+(\frac{2\alpha}{3}))(\frac{1}{6}+(\frac{2\alpha}{3}))}{\frac{5}{3}+\frac{4\alpha}{3}}b \qquad \textit{Equation 55-4}$$

As proposed by smolders et al. [40], anaerobic P-released is calculated depending on pH value as equation 5-3 to 5.

$$P - release = (0.19 * 7.3 - 0.85) * a \qquad \textit{Equation 5-5}$$

The results in Table 5.2 prove microorganism shift towards GAOs in high aeration level with a GAO acetate uptake up to 4.5 mmol/L; however, this parameter was as low as 0.05 mmol/L for 0.8 mg/L DO concentration. The PHB production and P-release for the low DO levels match the

anaerobic PAO model prediction by *smolders et al.* (9.08 mmol/L and 3.28 mmol, respectively). Moreover, as indicated in the literature, as the main PHA produced in the acetate-fed system is PHB, it is in line with the findings with only 0.02 PHV mmol/L in 0.8 mg/L DO concentration. The PAO model predicted no PHV production with acetate as the sole carbon source [182].

Table 5.2 Degradation and production for anaerobic phosphorus and anaerobic carbon compounds for 3 different DO levels in 6-hour cycle

	4 mg/L DO	**2 mg/L DO**	**0.8 mg/L DO**
acetate consumption mmol/L	4.69	6.32	6.54
glycogen degradation mmol/L	4.5	3.7	3.28
PHB production mmol/L	6.57	8.31	8.7
PHV production mmol/L	1.52	0.38	0.02
P release mmol	0.82	2.86	3.43

5.4.1.3 Aeration duration levels in different DO concentration degrees

Aeration duration is an essential factor in the energetic aeration input [188]. Extended aerobic HRT retention time was examined on 4, and 2 mg/L DO levels to investigate the impact of aeration duration on EBPR performance. Literature suggests short cyclic periods in SBR mode reactors tend to resist process failure and maintain biomass activity. By reducing the HRT, the F/M ratio increases, elevating the biological treatment capacity [28]. Increasing the aeration period from 120 to 200 minutes in 4 mg/L DO concertation was monitored in all SBRs over a 30-day experiment. Increasing the reaction time clearly resulted in lower sequential P-release and uptake with a decline in P-removal efficiency.

Moreover, a slight secondary release of phosphorus was observed due to a complete depletion of PHB at the end of the aeration period. With the secondary release of phosphorus into bulk liquid, the effluent resulted in higher P-concentrations. In addition, lower concentrations of Poly-P inside cells corresponded to less VFA uptake in the subsequent anaerobic stage.

In 2 mg/L DO concentration, the cycle duration was initially prolonged from 4 hours to 6 hours by increasing the settling period from 30 minutes to 150 minutes, maintaining the same HRT for anaerobic and aerobic phases. Reactors instantly reacted to the change. With the prolonged settling phase, a high secondary phosphorus release occurred, which was suspected to be related to the operational condition of long settling. Due to a high secondary phosphorus release, carbon source uptake in the anaerobic phase highly deteriorated. Availability of carbon source in aerobic stage inhibited the phosphorus uptake process, resulting in low removal efficiencies. In the subsequent experiment, by maintaining the cycle duration at 6 hours, anaerobic and aerobic HRT retention time was increased from 60 to 90 minutes and from 120 minutes to 200 minutes and lowered the settling phase time to 40 minutes. Compared to the long settling experiment, this process favored in terms of P-removal and COD consumption. Concerning shorter HRT at low DO levels, the P-removal was maintained at a relatively similar level; moreover, the long aerobic HRT did not highly affect the biological phosphorus removal. By increasing the aeration duration in low DO concentrations, the phosphorus release and uptake profile decreased to slightly lower than low aerobic HRTs retention times; however, the total removal efficiencies were maintained at high levels.

Except for extending the aerobic period in the experiments, the anaerobic retention time has also been prolonged. The extended anaerobic phase directly influences the microbial community structure and biochemical transformations in the EBPR process. With excessive anaerobic periods,

the PHA synthesis and glycogen degradation highly decreases. With lower Poly-P pools and glycogen contents in the subsequent anaerobic phases, the PAOs utilize PHA in substrate-level phosphorylation instead of oxidative phosphorylation in the TCA cycle to maintain the required energy in the extended period anaerobic endogenous period [304]. This may have also resulted in secondary P-release in the anaerobic phase not accompanied by VFA uptake. Therefore, in the consequent aerobic phase due to imbalance of anaerobic VFA uptake and P-release, there is a scarcity of PHB as a carbon source to take up the extra P-released in the system, resulting in lower P-removal efficiency [304]. Lowering the DO concentration to 0.8 mg/L inhibited the excessive aeration followed by complete exhaustion of PHB storage in the aeration phase. In high DO levels due to extreme usage of internal sources, scarcity of internal carbon source, and energy reserves, PAOs tend to release phosphorus for recovering their polymeric inventory. This phenomenon results in secondary phosphorus release. Due to the nonexistence of PHB sources in correlation with secondary P-release, this extra-P is not taken up by PAOs increasing the P-content in the effluent.

The experiments conducted that P-removal efficiency depended on aerobic period/ anaerobic period ratio (Ae/AN). With an increase in Ae/AN ratio, P-removal efficiency decreased in different DO concentrations. A comparably shorter anaerobic retention time is obtained by increasing the ratio from 2 to 2.2 in cycles 4 to 6 hours. Short anaerobic contact-time results in a decline in P-release and uptake, reaching 19% and 6% lower P-removal efficiency for 4 mg/L and 2 mg/L DO levels. Therefore, simultaneously, excessive aeration and inadequate Ae/AN ratio highly impacted BPR performance in 4 mg/L DO concentration. The experiment with a 90-minute anaerobic phase and 200-minute aeration period at a DO level of 4 mg/L, faced secondary P-release in the first 10 days of the experiment, showing high P-release with limited P-uptake in the following aerobic

phase. However, the P-release decreased gradually to a very limited amount in the remaining experimental period, indicating the loss of active PAOs in biomass. The correlation study indicated a strong relationship between increase in aeration and P-removal in which a correlation coefficient of -0.89 indicated a rather strong negative relationship.

5.4.1.4 EBPR Community population

The bacterial structure and the relative abundance of EBPR samples with different DO concentrations were shown in Figure 5-5-1, 2, and 3 on phylum, class, and genus level. According to the taxonomic assignments, there is a very diverse abundance of samples. *Proteobacteria, Bacteriodetes,* and *Firmicutes* are the most abundant species in all samples, accounting for 96, 91, and 92% respectively in the 4, 2, and 0.8 mg/L DO concentrations. Further analysis based on top genera in both samples present in Figure 5-5-3 showed larger variation than class and species. The shift of the dominant genus was indicated by operating different DO levels from 0.8 to 4 mg/L. The *Gamma-Proteobacteria* subclass comprises very limited abundance in all cases. As presented in the literature, there are high levels of doubt on the importance of *Acinetobacter* spp as a genus of *Gamma-Proteobacteria* sub-class. In the studies conducted, in 4 mg/L DO concentration, there was no *Acinetobacter* present. However, by decreasing the DO to 2 mg/L, the abundance increased to a very small percentage (1.1%) of members of this genus. In 0.8 mg/L, DO concentration is further increased to 2.1% of total abundance. There is still controversy on the effect of this very small percentage of Acinetobacter on EBPR performance. It is suggested that even a very limited percentage of total microorganisms present a million cells per gram that can accumulate poly-phosphate. However, with less than 10% of the total bacteria, it is improbable for *Acinetobacter* to be presented as PAO [307][308][46].

Many studies have reported high levels of *β-Proteobacteria* and *Actinobacteria* as dominant bacterial groups in EBPR systems. However, in *Actinobacteria*, the dominance depends on reactor configuration [46], increasing to high levels in continuous mode to the only detectable amount in SBR reactors. In this study, remarkable differences were detected in different DO levels at β-Proteobacteria with a shift from 0.08% to 19.9% and 22.6% by decreasing the DO from 4 mg/L to 2 mg/L 0.8 mg/L, respectively. The use of acetate as the sole carbon source in substrate greatly assisted in *β-Proteobacteria* abundance [63]. A limited amount of Actinobacteria was detected in DO levels lower than 2 mg/L (0.76 to 0.84%) with no indication in 4 mg/L DO concentration. This is in line with [63] study, where it was found in acetate-fed SBRs, *β-Proteobacteria* dominated while very few *Actinobacteria* were present.

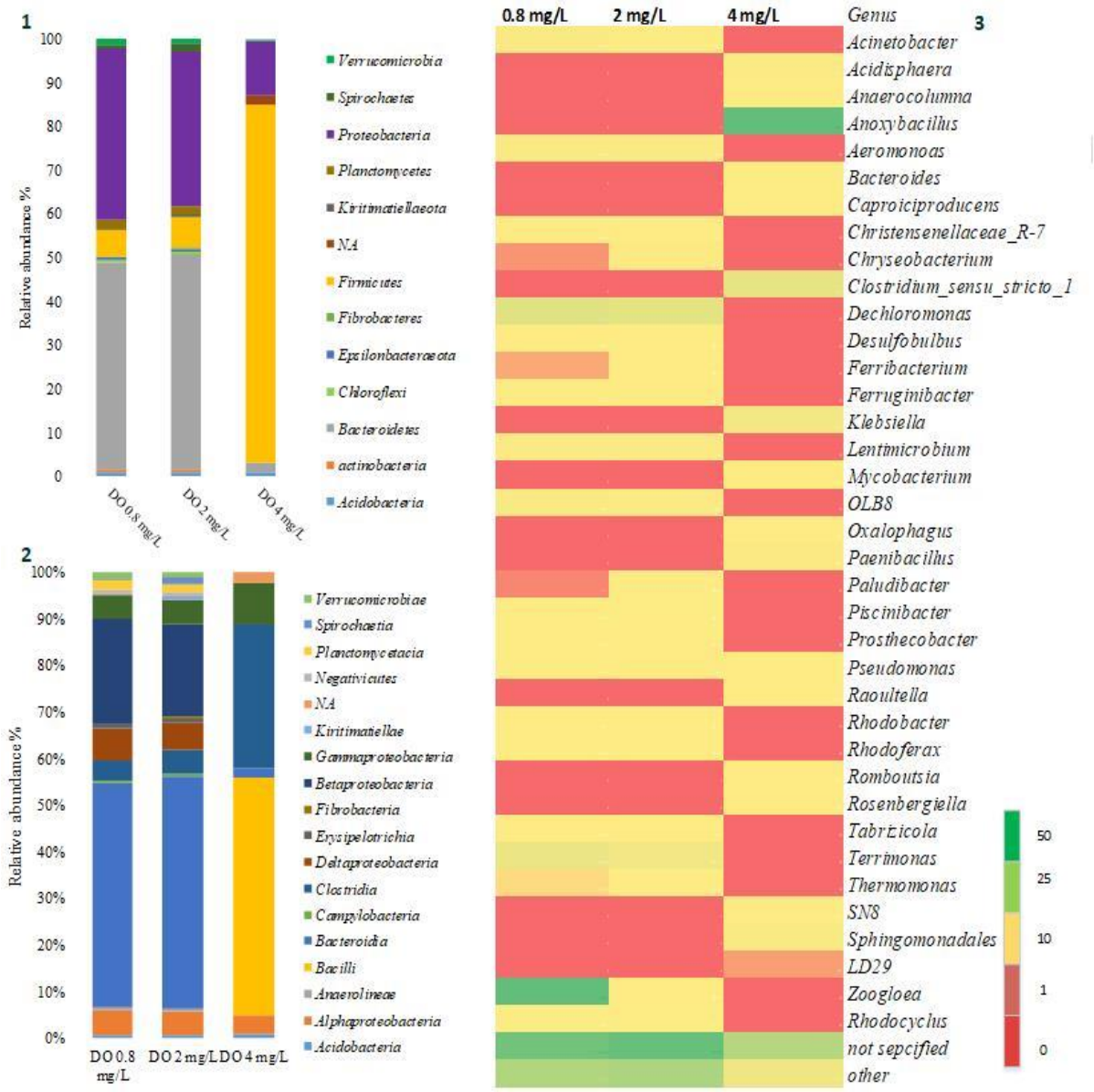


Figure 5-5 Microbial population dynamics and microorganisms abundance of SBRs in different DO levels of 0.8, 2 and 4 mg/L at 1.phylum 2.class and 3. genus levels

Overall, the bacterial community drastically changed by the difference in DO levels on genus level, as shown in Figure 5-5, which showed larger differences between reactors comparing to phylum and class level. Unlike evidence of a high population of *Chloroflexi* in EBPR systems, in this study, less than 1% of this filamentous group of bacteria was present, regardless of DO concentration. Previous research has indicated *Chloroflexi* as an important bacterial phylum, including genera having a role in carbohydrate and cellular material biodegradation and nitrification and denitrification [309]. However, a low abundance of this specific phylum was observed in this study with limited nitrogen removal. The results also showed *Rhodocyclus* bacteria in acetate-fed SBRs in low DO levels of 2 and 0.8 mg/L. In addition, the representatives of these specific groups were more prominent in the case of 0.8 mg/L of DO concentration. Previous studies have proposed a direct correlation between EBPR performance regarding P-removal and *Rhodocyclus*-related microorganisms from a subclass of *β-Proteobacteria* as contributors to P-removal in EBPR [310][64][46]. The *Rhodocyclus* group also contains the genus zoogloea, a heterogeneous bacterium [62], important in the P-removal process. Experiments on low DO (2 and 0.8 mg/L) indicated this genus as a phototrophic genus of *Rhodocyclus*.

Moreover, species of this genus are present in the α-*Proteobacteria* and *Burkholderia* family under the *β-Proteobacteria* sub-class. SBRs with 2 and 0.8 mg/L DO concentration consisted of 4.12 and 4.48% of *Burkholderia* family and 5.1 and 5.5% *α-Proteobacteria* sub-class, respectively. Moreover, other genera such as *Pseudomonas spp*., *Dechloromonas* and *Aeromonoas* can accumulate Poly-P in the anaerobic phase, resulting in P-removal.

In literature, *γ-Proteobacteria* and α-*Proteobacteria* are suggested to contain genus that behaves as GAOs, which could synthesize PHA but not aerobically store poly-P [311][312][46]. However,

in this study, the microbial analysis indicates a relatively 40% higher γ-Proteobacteria in 4 mg/L DO concentration than 2 and 0.8 mg/L DO level. Furthermore, *Sphingomonadales,* a genus related to α-*Proteobacteria,* has approximately two times higher abundance in 4 mg/L DO concentration than low DO levels, which is identified for belonging to GAOs, resulting in poor phosphorus removal performance in lab-scale systems [312][6].

5.5 Conclusion

As an operational factor, DO concentration highly influences EBPR performance and PAOs dominance where high P-removal is achieved at lower DO levels. This study showed that the 0.8 mg/L DO concentration could achieve successful biological P-removal with approximately 90% removal efficiency due to a shift in the bacterial population towards PAOs. Further investigation on high aerobic HRT shows a decline in PAOs population and an increase in GAOs activity. In addition, a combination of high HRT and high DO levels showed minimal P-removal and further process failure with no anaerobic P-release. Therefore, controlling DO at low levels and aerobic HRT retention time at optimal duration promotes EBPR accompanied by lower operational and aeration costs.

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6 Enhancement of Simultaneous Nitrogen and Phosphorus Removal Using Intermittent Aeration Mechanism

Adapted from:

Parnian Izadi, Parin Izadi, A. Eldyasti, "Enhancement of Simultaneous Nitrogen and Phosphorus Removal Using Intermittent Aeration Mechanism", Journal of Environmental Sciences, Volume 166, 2021.

Preface:

This chapter of the thesis is an original work based on the experimental apparatus and data published by the author, Parnian Izadi.

Author statement:

Parnian Izadi: Conceptualization (Lead), Methodology (Lead), Validation (Lead), Investigation (lead), Resources (lead), Writing- original draft (lead), Writing-review and editing (equal), Visualization (lead)

Parin Izadi: Conceptualization (supporting), Methodology (supporting), Investigation (supporting), Writing- original draft (supporting), Writing-review and editing (equal)

Ahmed Eldyasti: Conceptualization (supporting), Methodology (supporting), Investigation (supporting), Writing-review and editing (Lead), Supervision (Lead), Project Administration (Lead), Funding Acquisition (Lead)

6.1 Abstract

Biological nutrient removal grows into a complicated scenario due to the microbial consortium shift and kinetic competition between phosphorus (P)-accumulating and nitrogen (N)-removing microorganisms. This study tested three sequential batch reactors with constant operational conditions except aeration patterns at 6-hour cycle periods. Intermittent aeration was applied to develop a robust nutrient removal system to achieve high energy saving and removal efficiency. The results showed higher correspondence of P-uptake, polymeric substance synthesis, and glycogen degradation in intermittent-aeration with longer interval periods than continuous aeration. Increasing the intermittent-aeration duration from 25 to 50 minutes, resulting in higher

process performance where the system exhibited approximately 30% higher nutrient removal. This study indicated that nutrient removal strongly depends on reaction phase configuration representing the importance of aeration pattern. The microbial community examined the variation in abundance of bacterial groups in suspended sludge. The 50-minute intermittent aeration favored the growth of P-accumulating organisms and nitrogen removal microbial groups, indicating the complications of nutrient removal systems. The successful intermittently aerated process with high capability of simple implementation to conventional systems by elemental retrofitting is applicable for upgrading wastewater treatment plants. With aeration as a major operational cost, this process is a promising approach to potentially remove nutrients in high competence, in distinction to optimizing cost-efficacy of the system.

Keywords: EBPR, nutrient removal, intermittent aeration, dissolved oxygen, PAO, polyhydroxyalkanoate

6.2 Introduction

There is a developing worldwide awareness for nutrient control with strict regulations that have resulted in substantial adjustments and advancements in wastewater treatment plants (WWTPs) [6]. Biological nutrient removal processes comprising anaerobic, aerobic and anoxic compartments by sludge recirculation are used to remove organic matters and nutrients from wastewater [313]. Commonly, this sequence is required for the growth and proliferation of phosphorus accumulating organisms (PAOs) that are responsible for Phosphorus (P) removal, along with nitrogen removing microorganisms for sequential nitrification and denitrification processes [129]. Understanding the mechanism of the Enhanced Biological Phosphorus Removal (EBPR) process grows into a complicated scenario in interaction with simultaneous nitrogen (N) removal. The EBPR process is mainly based on the PAOs releasing phosphorus during the anaerobic phase and then taking up more phosphorus and transforming it into polyphosphate during the aerobic phase. The aerobic energy is generated from aerobic/anoxic heterotrophic oxidation of anaerobically stored polymeric substances (polyhydroxyalkanoates (PHAs)). While nitrification takes place by oxidization of ammonia into nitrate/nitrite in the presence of electron acceptor, indicating the presence of various microbial groups (Ammonia oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB)) in the complex nutrient removal process. Providing anoxic conditions in alteration with anaerobic phase tend to increase the occurrence of denitrifying PAOs (DPAOs) beside denitrifying bacteria (DNB), capable of utilizing nitrate/nitrite as an electron acceptor for simultaneous denitrification and phosphorus uptake, causing less evident anaerobic phosphorus release [314][315].

However, mainly in conventional systems, including SBR reactors, the biological nitrogen removal occurs by pre-denitrification in the fill/anaerobic phase. Therefore, denitrifiers take up the

available readily biodegradable COD, resulting in lower substrate availability for PAOs, increased microbial competition, and higher operational cost [316], [317]. Therefore, the stability and reliability of EBPR may be problematic due to external disturbance of excessive nitrate loading to the anaerobic phase. In addition, aeration as a crucial factor for nutrient removal and odor control is accompanied by high equipment cost and energy consumption [318]. Intermittently aerating the system in the aerobic phase, on the other hand, allows simultaneous nitrification, denitrification, and P-removal with lower aeration rate requirement in a single reactor. Therefore, an Intermittent-aerated SBR reactor decreases the readily biodegradable chemical oxygen demand (rbCOD) requirement and microorganism competition. In the aeration phase, ammonium-nitrogen is oxidized to nitrate/nitrite in high DO levels by aerobic nitrifiers. In the following low DO stages, anoxic denitrifiers reduce nitrate/nitrite to N_2 gas. Therefore, minimum nitrogen removal occurs in the fill/anaerobic phase, allowing PAOs to internally store the carbon source as PHA [316]. Concurrently, PAOs (DPAOs) utilize the available DO or nitrate in the aeration period to take up phosphorus from wastewater [292].

Moreover, applying anoxic phases, with a possibility of DPAOs co-action, saves 40% in organic carbon and 25% in oxygen requirement, along with reduced sludge production [319]. Conceivably, intermittent aeration in an SBR reactor reduces energy consumption while maintaining or rather enhancing effluent compliance [320].

However, to achieve the highest prosperity of intermittent aeration and minimum energy requirement, a comprehensive investigation on phosphorus and nitrogen conversions in sequential anaerobic/aerobic stages is required to develop a well-operated process. Having outlined the intermittent aerated nitrogen removal systems in literature, most studies [321][129][316][322][314][323] have limited consideration on the synergic effect of intermittent

aeration on simultaneous N and P- removal performance, microbial community structure, bacterial abundance and shifts in a single reactor. In studies on intermittent aerated SBRs for nitrogen removal [324][325][326][327], approximately 90% of total nitrogen removal efficiency was achieved with an anoxic to aerobic duration ratio of 2 (4h/2h) and 1(3h/3h), while limiting the ratio to 0.5 resulted in a decrease of efficiency down to 70%. Although, not only the ratio but the reaction duration coordinates nitrogen removal. An anoxic to the aerobic ratio of 1 but 30 minutes of aeration showed only 35% of total nitrogen (TN) removal due to incomplete nitrification. Very few studies have focused on the effect of aeration pattern with various high/low DO durational sets on biological phosphorus removal, PAOs/DPAOs mechanism and microbial performance, metabolic models accompanied with nitrification and denitrification processes. In particular, in a study by *Lu et al*. [328], intermittent aerobic-anaerobic strategy indicated a slower aerobic PAO decay, glycogen, and poly-phosphorus consumption than anaerobic and anoxic storage. Yet, there was a need to evaluate nitrogen removal and linking the P and N-profile performance combined. A recent study investigated a combination of intermittent aeration and granulation on nitritation sludge for simultaneous nitrogen and phosphorus removal. The results indicated a high P and N-removal efficiency and lower energy consumption. Correlation of high nutrient removal with an abundance of related phosphorus and nitrogen removing organisms was detected [329]. The monitoring and determination of microbial community structure more effectively declare the system performance and nutrient removal mechanism.

Furthermore, it assists in perceiving the bacterial community dynamics and characterization during the nutrient removal operation [330]. Yet, limited studies systematically evaluated the relative abundance of key functional groups in a variation of aeration patterns as the alternating sole operating factor for nutrient removal processes. Therefore, the objective of this study was to create

favorable conditions in single-sludge simultaneous nitrification, denitrification (SND)/P-removal process to facilitate aerobic and anaerobic microorganism growth and metabolism in absence and presence electron accepters by applying intermittent aeration and non-aeration time periods without adding considerable operational complexity. Furthermore, the aeration pattern and the aeration duration have been taken into account with a constant aerobic/anoxic duration ratio to assess the effect of oxygen availability on the biological nutrient removal system.

In this study, the performance of three SBRs was examined at different aeration patterns of: continuous ($EBPR_{CONT}$), 50 and 25 minutes on/off intermittent aeration/non-aeration intervals ($EBPR_{INT}$) at constant DO concentration of 2 mg/L and 200-minute aerobic hydraulic retention time (HRT). Synchronous nitrification, denitrification, and P-removal feasibility evaluation, P and N profile performance, aerobic kinetics, and bacterial structure were conducted. Potential drawbacks and improvement alternatives were inspected and clarified in the case of aerobic and anoxic phases and possible occurred processes. In addition, nutrient removal was evaluated by the contribution of the key functional microbial groups through microbial analysis for identification of relative abundance on phylum, class, and genus levels for different EBPR samples.

6.3 Material and methods

6.3.1 SBR operation

Three sequencing batch reactors ($EBPR_{CONT}$, $EBPR_{INT-50}$, $EBPR_{INT-25}$) with 5 L working volume and internal diameter of 25 cm indicated in Figure 6-1-a were operated for the EBPR process. The reactors were seeded with returned activated sludge (RAS) from the Humber treatment plant in Ontario, Canada. The reactors were maintained at 21±1°C and operated with a cycle time of 6 hours. The 6-hour cycle in $EBPR_{CONT}$ consisted of 15 min feeding, 90 min anaerobic, 200 min reaction (aerobic) followed by 40 min settling, and 15 min decanting. For $EBPR_{INT-50}$ and $EBPR_{INT-25,}$ the 200 minutes of reaction phase was divided by 50 minutes and 25 minutes of

aeration and non-aeration phases, respectively, as shown in Figure 6-1-b. one to two-liter of synthetic wastewater was pumped into the reactors during the feeding phase with a hydraulic retention time (HRT) of 10-15 hours and a solid retention time (SRT) of approximately 25 days. The substrate COD concentration for each SBR was in the range of 300-350 mg/L containing acetate as the sole carbon source fed to the system. During the aerobic period, the air pumped was regulated by online dissolved oxygen (DO) detector from the bottom of the reactors to keep the DO level in the proper range for each stage of the process. A control processing system was used for setting up a constant DO level of 2 mg/L throughout the experiment. pH, ORP, and oxygen concentration were monitored online by the control processing device. The mixing speed was controlled in a range of 50 to 100 rpm for maintaining the DO concentration, reducing the speed with each DO outstrip with a deviation of ±0.5 mg/L. The SBRs were constantly mixed during the anaerobic and aerobic phases. At the same time, no mixing took place in settling and decanting phases, where the wastewater from sequential anaerobic and aerobic phases precipitated out in the settling phase. The reactors were routinely monitored through regular sampling for VFA, polyhydroxybutyrate (PHB), and glycogen through each cycle. Chemical analyses such as total suspended solids (TSS) and volatile suspended solids (VSS) were measured on samples from the end of the aerobic phase.

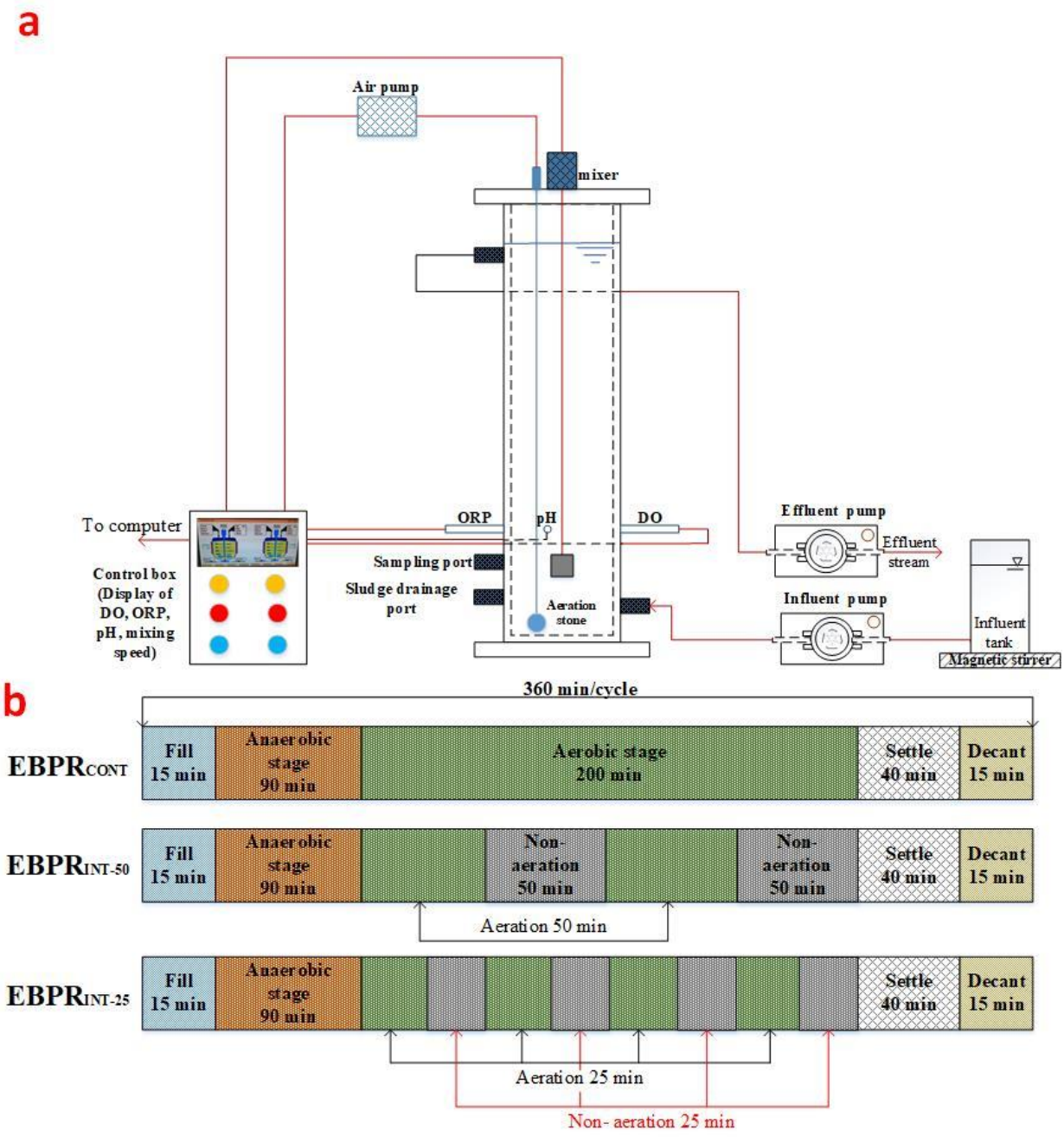

Figure 6-1 a-Schematic diagram of SBR-mode reactor for EBPR experimentation b-The cyclic periods of SBRs for EBPR process for continuous and intermittent aerated reactors

6.3.2 Synthetic feed composition

The synthetic wastewater used for this study to stimulate the High-P influent wastewater, approximately contained (per liter): 0.85 g NaAc.3H_2O (400 mg COD/L) as carbon source, 107

mg NH_4C1 (28 mgN/L), 75.5 mg $NaH_2PO_4.2H_2O$ (15-20 mgP/L), 90 mg $MgSO_4.7H_2O$, 14 mg $CaCl_2.2H_2O$, 1 mg yeast extract, and 0.3 mL nutrient solution. The concentrated nutrient solution contained, per liter: 1.5 g $FeCl_3.6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4.5H_2O$, 0.18 g KI, 0.12 g $MnC1_2.4H_2O$, 0.06 g $Na_2MoO_4.2H_2O$, 0.12 g $ZnSO_4.7H_2O$, 0.15 g $CoCl_2.6H_2O$, and 10 g EDTA as used by [40]. The pH of the feed was adjusted at 7.5±0.1. After the synthetic wastewater preparation, including the above components, the solution was maintained at 4°C, before injection to reactors with an average COD, TN, and TP of 400, 28, and 15 mg/L, respectively. The feeding took place from the bottom of the reactors for a uniform distribution of feed.

6.3.3 Analytical method

Standard methods (APHA 2005) were used to determine Total solids (TS), volatile solids (VS), TSS, VSS, total chemical oxygen demand (TCOD), and soluble chemical oxygen demand (SCOD). pH was measured by a digital pH meter (hach HQ440d multi). Phosphate, nitrate, nitrite and ammonia were simultaneously determined through ion chromatography by Thermo Scientific™ Dionex™ Integrion™ HPIC™ system. In advance of all IC measurements, samples were properly diluted with deionized water and passed through a membrane filter (0.45 µm). To confirm PAO enrichment and evaluation in the reactors, samples were initially analyzed for particle size distribution using an aqueous liquid module (ALM) in LS 13 320 Particle Sizing Analyzer; later on, samples were analyzed using PCR. Earth Microbiome Project benchmarked protocols (http://www.earthmicrobiome.org/emp-standard-protocols/) were used for DNA extraction and amplification. Mechanical and enzymatic lysis along with phenol-chloroform extraction and clean-up was performed using MoBio PowerMag soil DNA isolation kit for DNA extraction, where the primers used included 515FB (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806RB (5′- GGACTACNVGGGTWTCTAAT-3′). Standard gel extraction kits were utilized for

purifying the final product after amplification, polymerization, and separation (Qiagen, Netherland). Accordingly, the product was quantified with the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher). The resulting PCR products were sequenced using the Illumina MiSeq personal sequencer (Illumina Incorporated, San Diego CA) at the McMaster Genomics Facility, Ontario, Canada. The volatile fatty acid was assayed by an SRI gas chromatography (GC) equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). PHB was extracted from cellular biomass and quantified with gas chromatography using the following procedure. Primarily, 10-15 mg of lyophilized biomass were collected, and 2 mL of acidified methanol (3% sulphuric acid), as well as 2 mL of chloroform, were added in a glass vial. After gentle mixing, the cocktail was heated at 100 ∘C for 3.5 hours, cooled down to room temperature afterward. 1 mL of deionized water was added later on, and the mixture was vortexed for 1 minute and allowed phase separation to occur. The lower organic phase was tested for PHB quantification using SRI gas chromatography equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). The temperature program was 1 min 80 ∘C, 10 ∘C min^{-1}, 180 ∘C for 4 min. Results were compared with standard curves obtained using PHB standards (Sigma Aldrich). Benzoic acid was used as an internal standard to increase accuracy. Intracellular glycogen was determined via digestion and hydrolysis to glucose, where glucose is analyzed enzymatically (Sigma–Aldrich PGO enzyme kit, Saint Louis, Missouri, USA). Sample absorbance is measured at 425 nm using a Spectronic spectrophotometer.

6.3.4 Mass balances analysis

In this study, nitrogen removal was primarily associated with three denitrification pathways, microbial assimilation and nitrogen loss in the SBR reactors. Based on the following equations, the mass balance was analyzed for all three systems:

$$N\ removal = N\ denitrification + Nmicrobial\ assimilation + N\ nitrogen\ loss \quad \textit{Equation 6-1}$$

$$N\ removal = (N_{inf} - N_{eff})/N_{inf} \quad \textit{Equation 6-2}$$

$$N\ denitrification = (N_{A-inf} - N_{A-eff})(R + r + 1)/N_{inf} \quad \textit{Equation 6-3}$$

$$N\ microbial\ assimilation = (VSS_{waste} * f_{\frac{VSS}{SS}} * V_{waste} * f_{\frac{N}{biomass}})/(N_{inf} * Q) \quad \textit{Equation 6-4}$$

$$N\ nitrogen\ loss = N\ removal - N\ denitrification - N\ microbial\ assimilation \quad \textit{Equation 6-5}$$

Where N removal is the total nitrogen removal, N_{inf} and N_{eff} are the influent and effluent total nitrogen concentrations (mg/L), respectively, $N_{A\text{-}inf}$ and $N_{A\text{-}eff}$ are the total nitrogen concentrations in influent and effluent of reaction phases (mg/L), R and r are nitrate recycle ratio and sludge return ratio, VSS is the waste sludge concentration (mg/L), $f_{N/VSS}$ is nitrogen fraction in sludge with a 0.1 mgN/mgVSS assumption and $f_{VSS/SS}$ is VSS/MLSS, and Q is inflow rate (L/day) [317].

6.3.5 Statistical analysis

The t-test (for two groups of samples), F-test for equal variances (for two groups of samples), and One-way ANOVA analysis were applied with Matlab® Statistics and Machine Learning Toolbox™ (Matlab R2015a, MathWorks, USA) to evaluate the significance of differences in efficiency, performance and fit between models and to study the effect of oxygen concentration on P-removal efficiency. In the SBRs with different operations, correlation among operational and environmental parameters was evaluated to assess statistical relationship between aeration changes and performance and removal efficiencies.

6.4 Result and discussion

6.4.1 Removal of COD and Nitrogen

The performance of the three reactors in nutrient removal with the synthetic substrate is given in Table 6.1. Mainly intermittent strategies tend to decrease PAOs aerobic decay rate, glycogen, and Poly-P usage rate leading to long-term storage of EBPR sludge [331]. In the case of nitrogen removal, intermittent aeration has been reported to enhance simultaneous nitrification and denitrification (SND), increase efficiency in organic carbon utilization for denitrification and improve denitrifiers abundance in the system [332]. Yet, as shown in the table, $EBPR_{INT-25}$, despite its intermittent aeration, projected a lower removal performance in the case of nitrogen and phosphorus compared to a continuous-aerated reactor. At 25 minute on/off intervals, the effluent contained high nutrients not reaching the emission standards. Due to invaded anaerobic conditions and availability of electron acceptors in this stage in $EBPR_{INT-25}$, processes requiring oxygen, including carbonaceous oxidation, nitrification, and enhanced biological phosphorus uptake, occurred. This led to the inefficient anaerobic performance of PAOs. Furthermore, due to high dissolved oxygen concentration, the remaining NO_2-N and NO_3-N from the preceding cycle were not removed by means of denitrification, resulting in an accumulation of nitrate/nitrate in the system.

Table 6.1 process performance for EBPRcont., EBPRINT-50 and EBPRINT-25

	$EBPR_{CONT.}$		**$EBPR_{INT-50}$**		**$EBPR_{INT-25}$**	
	Effluent concentration	Removal efficiency	Effluent concentration	Removal efficiency	Effluent concentration	Removal efficiency
pH	7.51	-	8.27	-	7.86	-
COD (mg/L)	45±10.7	84%±3%	31.2±4.3	91%±1%	33.7±3.3	90%±1%
TN (mg/L)	8.1±0.5	71%±3%	5.4±0.6	81%±4%	14.01±0.3	50%±2%
Ortho-P (mg/L)	2.55±0.5	83%±3%	1.35±0.3	91%±3%	5.55±1.2	63%±8%
NH_4^+-N (mg/L)	2.1±0.7	93%±2%	1.8±0.5	93%±1%	2.32±0.4	92%±3%
VSS (mg/L)	45±2	-	40±4	-	51±3	-

The SBRs were operated for 50 days at an influent COD concentration between 340 to 370 mg/L with a rather stable COD removal. The COD removal mainly occurred in the anaerobic phase wherein $EBPR_{INT-50,}$ COD concentration at the end of the anaerobic phase mainly correlated with the effluent COD. The COD concentration in the effluent was an average of 45, 31, and 33 mg/L with COD removal efficiency of 84%, 91%, and 90% for $EBPR_{CONT}$, $EBPR_{INT-50,}$ and $EBPR_{INT-25}$, respectively. Although a high and stable COD removal indicated an acceptable organic removal performance in all reactors, a rather higher COD removal in intermittent reactors might be due to the efficient use of organic matters and carbon sources for denitrification and phosphorus release. In alternating aerobic/anoxic stages, the anaerobic COD is sufficiently utilized and stored as an intracellular carbon source [314] [333]. All systems' observed yield (Yobs) was estimated based

on a solid and COD mass balance by integrating total biomass wastage, and COD removed as given in Equation 6-6.

$$Y_{obs}=\frac{\Sigma V_{waste}.VSS_{waste}}{\Sigma Q.\left(SCOD_{inf}-SCOD_{eff}\right).t}$$ *Equation 6-6*

Where V_{waste} is the total volume of waste sludge as well as sample collection volume (L); VSS_{waste} is the total volatile suspended solids concentration of wasted sludge (mg/L); $sCOD_{inf}$ and $sCOD_{eff}$ are the influent and effluent sCOD concentrations (mg/L); t is the time intervals between sample collection and analysis (day). Net microbial growth rate affects the Y_{obs}, where rapid cell growth requires less energy for maintenance and a higher energy portion for growth, indicating Y_{obs} as an essential constituent on the magnitude of microorganism production influenced by various factors. A higher yield coefficient is associated with a more efficient substrate uptake for higher energy production, maximum biomass synthesis, and lower portion of maintenance [334]. Moreover, adenosine triphosphate (ATP) synthesis in anaerobic metabolism indicates the magnitude of Y_{obs}. ATP as an important element in catabolism (substrate oxidation process) and anabolism (biomass synthesis) reactions are mainly used as an energy source in lack of oxygen or substrate (anaerobic and anoxic conditions) and is rebuilt aerobically with microbial growth [335]. Therefore, higher production of ATP as an energy source in microorganism metabolism pathways results in higher yield coefficients [336]. The Y_{obs} were estimated at 0.19, 0.18, and 0.26 gVSS/gsCOD for $EBPR_{CONT}$, $EBPR_{iNT-25,}$ and $EBPR_{INT-50}$, respectively. The estimated yields were lower than the typical biomass yield reported in the literature of 0.3-0.6 gVSS/gCOD in $EBPR_{CONT}$ and $EBPR_{iNT-25}$. This matter may be associated with rather long SRT and high biomass concentration in anaerobic conditions [313].

For reaching a high nitrogen removal, balancing the aerobic and anoxic duration for complete SND is suggested. Insufficient aeration period restricts complete ammonia oxidation and decreases

denitrification efficiency [332]. NH_4^+-N removal remained stable at higher than 90% with a loading rate of 30 g NH_4^+-N /Lday for continuous and $EBPR_{INT-50}$. With intermittent aeration, $EBPR_{INT-50}$ reached a rather stable ammonia effluent concentration of 1.8 mg/L considering the 25-30 mg/L of average ammonia influent concentration. On the other hand, $EBPR_{INT-25}$ showed very low TN removal, leaving <10 mg/L of nitrogen in WW effluent. Based on the nitrogen mass balance analysis from Equations 6-1 to 6-5, only 37.5% and 40% of TN removal in $EBPR_{CONT}$ and $EBPR_{INT-25}$ accounted by denitrifying phosphorus removal pathway ($N_{denitrification}$), respectively. However, by increasing the interval duration to 50 minutes in $EBPR_{INT-50}$, $N_{denitrification}$ reached 54% of the total nitrogen removal. This indicates higher DPAO abundance, denitrifying performance, and simultaneous nitrification and denitrification (SND) in longer duration intervals resulted in lower nitrogen loss and higher TN removal efficiency. A high abundance of DPAOs and aerobic PAOs decreases the COD requirement compared to aerobic systems, with savings in aeration [337][6]. In addition, approximately 5 to 18% of TN removal contributed to nitrogen assimilation by biomass with the feasibility of SND at different levels in all reactors. A phase study of a typical operational cycle at different aeration patterns is shown in Figure 6-2. In $EBPR_{INT-50}$, NH_4-N concentration drastically decreased in the second aeration period and reached 1.4 mg/L by the end of the cycle. A correlation coefficient of 0.81 indicated a strong relationship between intermittent interval duration and TN removal, in which higher interval durations resulted in higher removal efficiencies. Ammonia removal, on the other hand, did not show any specific correlation (correlation coefficient of 0.23) with increase of interval durations. NO_3-N and NO_2-N concentrations started to rise from the first aeration period, with a lower detection of NO_2-N than NO_3-N.

By applying a controlling system, DO surpassing 2 mg/L in the 50-minute intermittent reactor through aeration period was negligible. Moreover, due to DO drop in non-aeration periods to almost anaerobic conditions, nitrification was unable to occur, which enhanced denitrification process, while the high DO phases fostered nitrification. On the other hand, in $EBPR_{INT-25}$ system, during the first aeration period, the DO increased to 2 mg/L and remained in high concentrations in the following non-aeration phase. Therefore, due to oxygen availability, post-denitrification in non-aeration phases was not as effective. Studies have further suggested that oxygen is not rapidly consumed in lack of carbon source [338]. In other words, time is required to change the aerobic phase to anoxic phase when there is a limited simple organic substrate (VFA) available. Therefore, in this case, 25 minutes appears to be insufficient for phase shift. When the 25-minute aeration/non-aeration was applied to the reaction phase, a rather significant and rapid NH4-N reduction took place in the first three aeration periods equal to 81% ammonia removal. However, there was very limited ammonia removal in the fourth aeration phase, leaving 2.7 mg/L in the effluent. This matter resulted in an upward trend in NO_3-N concentration during each cycle, causing 4.12 mg/L of nitrate recycled into the next cycle. Because of the remaining nitrate from previous cycles, pre-denitrification took place in the anaerobic zone.

Considering the SND efficiency equation: $\eta_{SND} = N_i - (N_e + N_{biomass})/N_i$ Equation 6-7

where:

N_i is the nitrogen fed to the reactor, N_e is the nitrogen in the effluent, and $N_{biomass}$ is the nitrogen in the withdrawn sludge. It was found that SND efficiency for $EBPR_{INT-25}$ only reached 49.3% while $EBPR_{CONT}$ and $EBPR_{INT-50}$ achieved 70.3% and 81%, respectively. This indicates a high nitrate/nitrite availability in effluent due to nitrification followed by incomplete denitrification with 25-minute time intervals, indicating that short aeration may hamper complete denitrification

in the following non-aeration period. Besides, pH values for intermittent reactors with an inclination to increase due to air stripping support the complete nitrification process. The nitrification performance was determined by ammonium uptake rate (AUR) measurement according to the method described by *Lee et al.* [339]. The AUR was determined 8.84 and 12.27 mg NH_4-N/L.h for two aerobic stages of $EBPR_{INT-50}$ and an average of 14.4 mgNH_4-N/L.h for four aeration periods in $EBPR_{INT-25,}$ with the fourth aeration period having the lowest rate. It should be noted that the AUR of both intermittent aerated systems was consistently greater than a continuous aerated system with 6.9 mg NH_4-N/L.h of removal rate. Generally, intermittent aeration by providing an alternating sequence of aerobic and anoxic conditions within the same tank increases the possibility of SND and luxury phosphorus uptake [340]. Results of studies conducted on alternating aerobic/anoxic treatment processes [341] [342] have further justified the capability of high nitrogen removal by reducing nitrate to nitrogen gas during the denitrification process.

However, during the non-aeration periods, due to incomplete denitrification in $EBPR_{INT-25}$, the nitrate production rate only reached 5.26 mgNO_3-N/L.h that is 48% lower than $EBPR_{INT-50}$, indicating 25 minute non-aerated periods are insufficient for complete denitrification to take place. In SBR reactors, there shall be coordination between aeration duration and reaction cycle in intermittent aeration to minimize operational period and energy requirement. Short aeration duration and excessive cycles resulted in high energy input and increased effluent TN concentration [332]. By implying the calculations proposed by *Lee et al.* and *Dytczak et al.* [343],[339], $EBPR_{INT-50}$ nitrification rates were 9% and 10% higher than those in $EBPR_{INT-25}$ and $EBPR_{CONT,}$ respectively. The higher nitrification rate in 50-minute intermittent conditions is likely related to the effect of the feast (anaerobic/anoxic)/famine (aerobic). It generates a distinctive position for bacterial groups with anaerobic organic carbon uptake capability, such as PAOs.

Inefficient external substrate feast/famine, bacteria rapidly take up and store substrate as internal storage compounds in the feast mode and utilize the stored slowly biodegradable polymers to gain more balanced growth and preserve energy sources for nitrification and denitrification means [344] [345].

Moreover, although ammonia is present in anoxic conditions, autotrophic microorganisms are neither capable of taking up ammonia due to oxygen scarcity nor storing it as an energy source. This increases the possibility of higher stress and damage to biomass and bacterial metabolism during the anoxic phase. This matter increases the nitrate requirement due to the additional substrate needed for the repair process. Therefore, more oxidation of ammonia to nitrite and conversion of nitrite to nitrate takes place to fulfill the nitrate requirement for repair purposes resulting in an average higher nitrification rate in $EBPR_{INT-50}$. In addition, the COD is reduced in the anoxic phase, which decreases the competition with heterotrophs for ammonia uptake. These circumstances of intermittent aeration in 50-min intervals lead to a high nitrification rate. However, short aerobic/anoxic intervals in $EBPR_{INT-25}$ resulted in incomplete conversion of nitrate/nitrite to nitrogen gas in anoxic periods, leading to average lower nitrification rates in aeration phases [339]. Therefore, nitrate was the dominant compound of effluent nitrogen in case of 25-minute intermittent aeration. Correspondingly, in an intermittent aerated activated sludge system, sufficient aeration is required for complete nitrification. A controlled non-aeration stage is essential for complete denitrification; thus, DO concentration and aeration duration concurrently affect nitrogen removal [332].

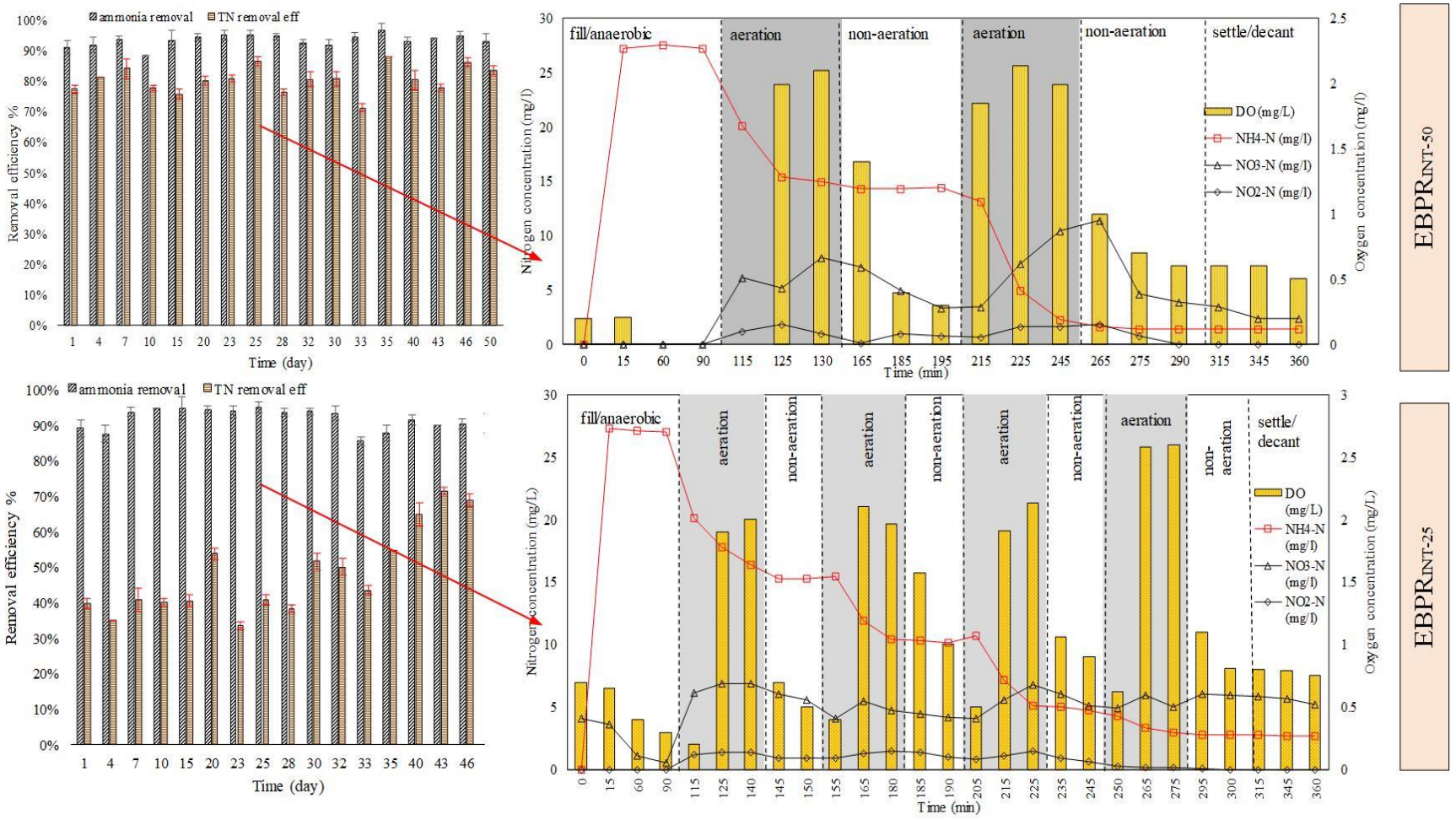

Figure 6-2 Ammonia and total nitrogen removal efficiencies and NH_4-N, NO_3-N, NO_2-N and DO profiles during a cycle of aeration/non-aeration phases at day 28 for: a.$EBPR_{INT-50}$ b.$EBPR_{INT-25}$

6.4.2 Evaluation of Phosphorus removal performance in $EBPR_{CONT}$ and $EBPR_{IA}$

All the results in this study were collected from SBR reactors at steady-state conditions. For optimum performance, a stable NH_4^+-N removal along with biological phosphorus removal is desired. As normally expected, the VFAs were taken up in the fill/anaerobic phase simultaneously with phosphorus release, PHB production, and glycogen degradation. However, as shown in Figure 6-3**,** it was observed that by the end of the anaerobic phase, $EBPR_{INT-50}$ consumed almost all VFA, while residual VFA were available in $EBPR_{CONT}$ and $EBPR_{INT-25}$. Availability of nitrate and oxygen in low levels in this phase limits the PAOs performance regarding concurrent anaerobic VFA-uptake and P-release. According to the metabolic model proposed by *Smolders et al.* [40], the process of VFA uptake and its conversion to acetyl-CoA requires energy in the form of ATP, while the following PHA production requires $NADH_2$ for redox balance purposes, with two sources of $NADH_2$ production; tricarboxylic acid cycle (TCA) and glycogen degradation [303]. On day 28 of the experiment, the PHB production/VFA uptake ratio was detected in the SBRs with a value of 1.18, 1.04, and 1.43 for $EBPR_{CONT}$, $EBPR_{INT-25,}$ and $EBPR_{INT-50,}$ respectively. In $EBPR_{INT-25}$ with lower VFA uptake, slightly lower PHB production and PHB production/VFA uptake ratio was achieved. Lower production ratios in 25-minute intermittent intervals imply that the majority of the energy requirement for PHA production was achieved through polyphosphate hydrolysis. Therefore, a lower P-release was observed.

As featured, by maintaining the DO level at 2 mg/L, the anaerobic PHB production decreased in $EBPR_{INT-25}$ compared to $EBPR_{INT-50}$. Due to electron acceptor availability in the anaerobic period of $EBPR_{INT-25}$ and potential competition of other microorganisms, such as denitrifiers, PAOs stored fewer polymeric substances. Additionally, residual VFA presence in the initial aeration period

caused low PHB consumption in the reaction phase. However, with VFA total uptake by the second aerobic stage, PHB degradation drastically increased as fuel for microbial growth and phosphorus removal.

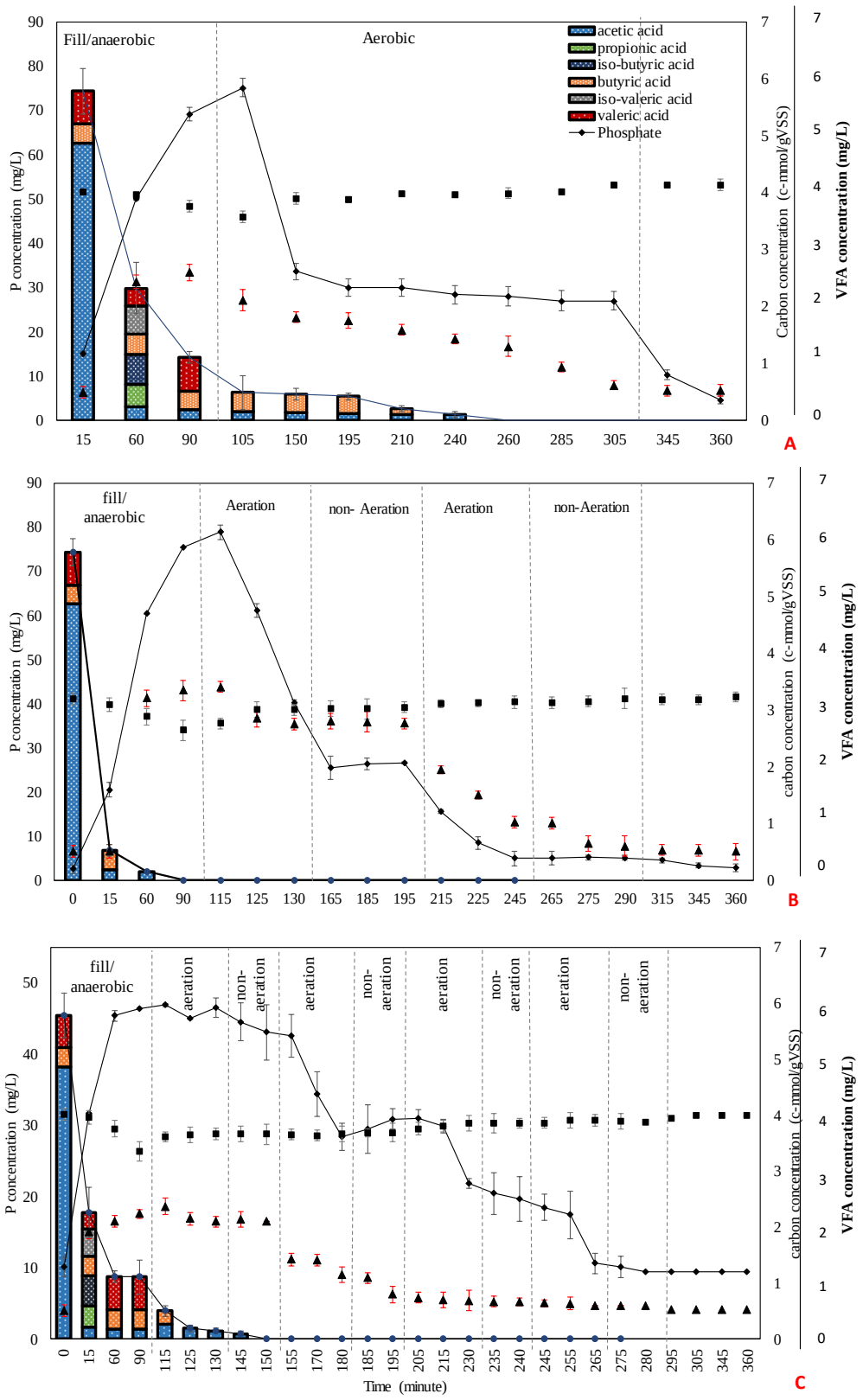

Figure 6-3 PHB, VFA transformation, carbon and phosphorus concentration in a cyclic periods of aeration and non-aeration phases A. $EBPR_{CONT}$ B.$EBPR_{INT-50}$ and C.$EBPR_{INT-25}$

Through the course of 50 days, the phosphate removal efficiencies were 83%±3%, 91%±3% and 63%±8% for EBPRCONT, EBPRINT-50 and EBPRINT-25, respectively, which showed approximately 25% higher performance with 50 minutes of intermittent intervals rather than 25-minutes. By looking into significance if difference between the P-removal results, a *p*-value of lower than 0.05 showed a statistically higher phosphorus removal with 50-minute aeration/non-aeration phases. Since the aeration/non-aeration phases occurred in the same SBR reactor, for a better understanding, the temporal P-profile and carbon sources with time were investigated, as shown in Figure 6-3. In this study, with constant 2 mg/L of DO concentration in aeration phases, 25-minute aeration/non-aeration appeared to be insufficient to remove phosphorus from synthetic wastewater biologically. The primary factors affecting the P-removal performance were insufficient reaction time, variation in DO concentration, and carbon source. Yet, the effect of residual NO_3^--N from previous cycle on DO concentration and P-release of PAOs are considerable. Therefore, an optimal NO_3^--N:PO_4^3-P and N/COD ratio were found to facilitate in flourishment of phosphorus-removing organisms [346]. A TP and TN removal of 50% and 54% in EBPR$_{INT-25}$ showed an apparent negative effect of imbalance ratio on process performance. In a study [347], an increase of N/COD ratio higher than 0.31 negatively impacted TP removal by dropping to 49.8%. However, Liu et al. experimented using a novel Modified Ludzack-Ettinger (MLE) process with the implementation of intermittent aeration. They reached 76% nitrogen removal at 0.62 N/COD ratio at low temperatures and high TP removal. Yet, the high P-removal was temporary in MLE configuration as in longer operational time; the PAOs could potentially be washed out through sludge wasting [348]. In this study, by maintaining N/COD ratio at approximately 0.07-0.1, the major impact in EBPR$_{INT-25}$ was the nitrate/phosphate ratio imbalance. Considering the EBPR$_{INT-25,}$ VFAs were partially taken up in the anaerobic phase due to inefficient PAO

performance, caused by anaerobic nitrate availability, disrupted PHB production, glycogen consumption, and phosphorus release. In the subsequent reaction phase with alternative aerobic/anoxic phases, PHB was consumed, and glycogen was regenerated. However, there was a gradual P-uptake in the first aeration period with only 6% phosphorus removal. As generally known, nitrate availability in the anaerobic phase inhibits P-removal and PHA storage. Three hypotheses were proposed by literature on the negative influence of nitrate in the EBPR process: 1. EBPR activity inhibition by nitrate where inhibits the anaerobic P-release. 2. Concurrent P-release and P uptake due to the availability of electron donor (substrate) and electron acceptor (nitrate) in conjunction with anaerobic conditions. 3. Nitrate presence may further trigger the ordinary heterotrophic bacteria (OHO) performance in nitrogen reduction using the available COD for PAOs growth [145]. While on the other hand, several studies have demonstrated the capability of DPAOs in achieving nitrogen and phosphorus removal [349]. Condition of anaerobic/anoxic/aerobic systems requires a proper PAO population, capable of coexisting with denitrifiers for carbon source and nitrate availability without inhibition [28], which lacked $EBPR_{INT\text{-}25}$. Yet, with inefficient denitrifying phosphorus organism performance, since DPAOs possess nitrate reduction ability, the inhibitory effect was considerably lower on P-release. Therefore, despite nitrate accumulation, the phosphorus removal was not deteriorated but was reduced by 25%. (A list of measured data is available in **Appendices, F, Table 10.7**).

6.4.3 Potential complications associated with Intermittent aeration

Simultaneous removal of phosphorus and nitrate occurs by two different pathways: 1. internal PHB utilized for P-uptake and denitrifiers for nitrate conversion to N_2 gas, or 2. internal PHB as carbon source and nitrate as electron acceptor utilized by DPAOs for phosphate uptake in lack of oxygen [340]. Studies have suggested DPAOs capability to take up phosphorus with nitrate as the electron

acceptor for PHB oxidation under anoxic conditions. Therefore, the intermittent aerated systems with anaerobic/aerobic/anoxic zones have a higher phosphate uptake rate (PUR) than the continuous aerated reactor. With 50-minute intermittent intervals, during the non-aeration phases (anoxic phase) with limited available short-chain acids, DPAOs degrade PHB as an energy source and utilize nitrate as an electron acceptor, resulting in rapid simultaneous denitrification and P-uptake [314]. DPAOs are beneficial for simultaneous nitrogen and phosphorus removal by means of lower carbon and aeration demand. However, relatively low denitrifying P-uptake suggests the dominance of aerobic PAO metabolism rather than the DPAOs. Results from intermittently aerated reactors propose that longer interval periods may select more DPAO activity [350]. In the case of phosphorus removal, in this study, even the limited phosphate uptake in anoxic phases of $EBPR_{INT-50}$ did not adversely affect total phosphorus removal due to long enough aerated stage for completion of P-removal.

With an influent phosphate-concentration of 15-20 mg/L and a consequent anaerobic P-release, the majority of phosphorus was removed in the subsequent first 50-minute aeration with 68% of initial P-removal. The concentration of phosphate initially increased by a very limited measure during the following non-aeration (anoxic) phase, yet, the P-uptake continued to occur. The uptake rate observed during the aerobic phase were higher than anoxic phase due to substrate scarcity. Higher P-uptake in $EBPR_{INT-50}$ is in correspondence with higher PHB synthesis and glycogen degradation, comparing to continuous aerated reactor. Glycogen as the reducing power source for anaerobic VFA uptake and the key energy source of GAOs, decreased in anaerobic phase in $EBPR_{INT-50}$ with a degradation rate of 0.6 C-mmol/gVSS, which is in the range reported for PAOs metabolism. In case of nitrogen removal, $EBPR_{INT-50}$, showed a rather higher TN removal compared to continuous and shorter intermittent aeration duration, yet, it didn't reach its highest

removal potential. The length of each anoxic phase in each cycle is impacted by the DO concentration in the aerobic phase which reduces the actual anoxic duration due to oxygen availability. As shown in Figure 6-2, potential higher nitrogen removal wasn't reached with 50 minute intervals, due to availability of residual DO in anoxic phases.

Slow decrease of DO concentration, consequently affects the denitrification rate and overall performance of nitrogen removal in the intermittent aeration. A proposed solution for gaining high nitrogen removal, is to apply lower DO concentrations combined with a balanced intermittent aerated mode to SBR system [332]. Since DO concentration in the aerobic phase directly affects the actual anoxic duration in the intermittent aeration process. Based on a study conducted by [351], in constant high DO aeration without control, issues regarding N-removal were observed due to the availability of ammonium in the aeration phase. However, high orthophosphate removal was observed, with 88% removal efficiency. By controlling the DO concentration in the same continuous aeration pattern, N-removal increased from 60% to 65%. The study's implementation of alternating high and low DO periods remarkably improved denitrification and increased N removal by 10%. Yet, approximately 25% of denitrification took place in the feeding phase, which depends on the NO_x^- concentration by end of cycle. The results from this study agreed with a study by [352] with an increase of 15% in N-removal by switching from continuous SND to alternating nitrification denitrification (AND), however phosphorus was not focused for treatment.

Lower phosphate uptake by decreasing the intervals to 25 minutes confirmed the insufficient process duration in which considerable concentrations of organic compounds remained under anoxic conditions. In this case, the DPAOs activity significantly decreases, and phosphate release occurs by PAOs while ordinary heterotrophs are responsible for the denitrification process. As shown in Figure 6-4, in one cycle, high nitrate concentration in the anaerobic phase and residual

VFA presence in the reactions phase results in less PHB oxidization for cell growth and phosphorus release along with gradual nitrate and substrate consumption after reduction of nitrate to a certain level in anoxic phase [353]. A glycogen concentration of 4.01 c-mmol/gVSS in $EBPR_{INT-25}$, with a slight increase in glycogen content, suggests a shift towards GAOs. In $EBPR_{INT-25}$, lower P-removal efficiency was due to the loss of P and N-removal activity by incomplete phosphorus uptake and denitrification process. This phenomenon caused high nitrate concentration and phosphorus accumulation of bacteria with high PHA content to move into the settling phase. While in the settling phase, nitrogen gas is produced by the possibility of either using the stored PHA in PAOs as the electron donor for denitrification or nitrogen removal by DPAOs without the presence of soluble COD Endogenous nitrification may conjointly occur in this phase. This phenomenon called rising sludge due to gas production intensifies the biomass floatation, followed by biomass loss in the decant phase. As a result, PAOs as a critical part of biomass are washed away, decreasing nutrient removal efficiency [354]. Rising sludge was avoided in $EBPR_{INT-50}$ with limited polymeric substance storage at the end of aerobic phases due to high P-removal efficiency. This indicates the importance of settling performance as an integrated section of nutrient removal in SBR systems. From the literature, it is concluded that the highest nutrient removal is achieved when nitrification, denitrification, and P-removal, as the three major processes are all active in the reaction (aerobic/anoxic) period. Yet, the rates of each process and their advantage over one another in the aerated and non-aerated phases indicate the process performance efficiency [351].

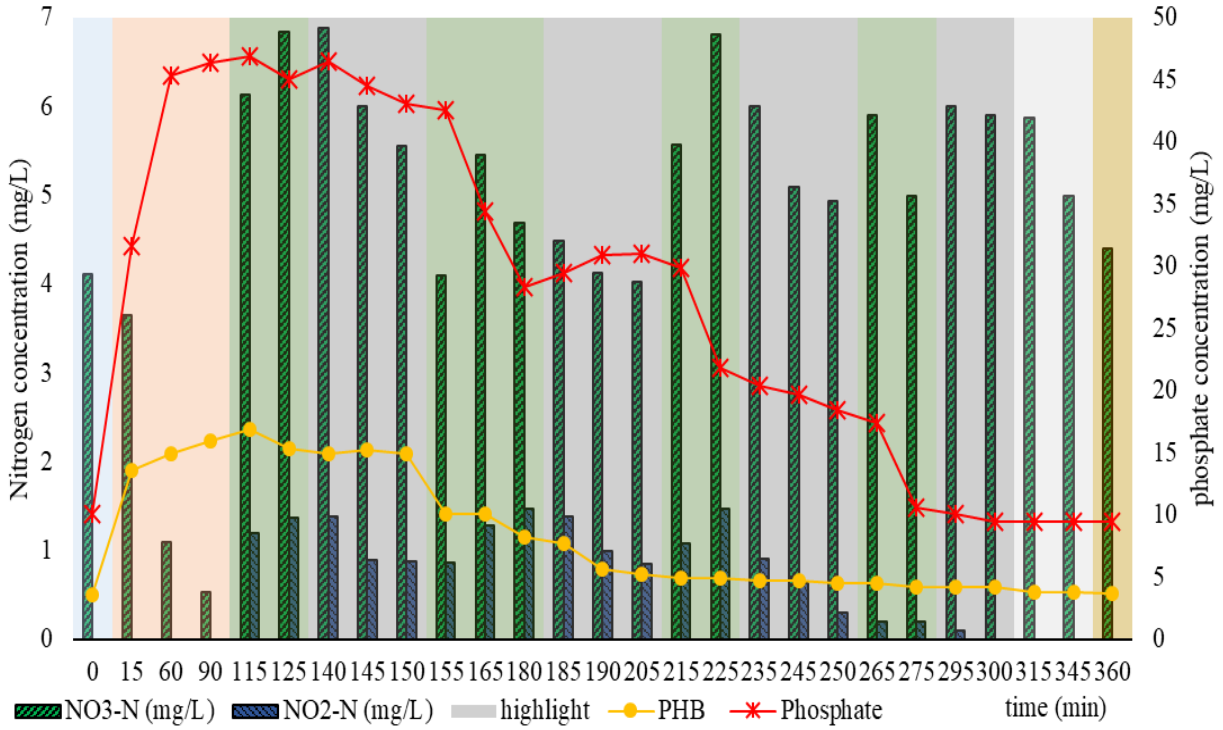

Figure 6-4 Nitrate, Nitrite and phosphate and PHB cyclic period profile and changes in EBPR process with 25 minutes aeration/non-aeration intervals

6.4.4 Mechanism of functional species for nutrient removal

In this study, a sequential batch mode reactor for biological phosphorus removal coupled with simultaneous nitrification and denitrification under intermittent aeration was investigated. The diversity of active populations of microorganisms, as shown in Figure 6-5 were effective in the nutrient removal process. The phosphorus removal took place with the cooperation of PAOs and DPAOs in the intermittent aerobic and anoxic phases. DPAOs, Denitrifying GAOs (DGAOs), AOB, NOB, DNB, and ordinary heterotrophic bacteria (OHO) participated in nitrogen removal through simultaneous nitrification and denitrification, cell growth, and electron acceptor requirements [330]. The phylogenic classification of functional groups at the genus level results consist of PAOs, DPAOs, GAOs, AOB, NOB, and DNB. *Nitrosphira* and *Nitrosomonas* were the abundant NOB, and AOB identified, respectively, mainly responsible for ammonia removal in the

reactors. *Candidatus-Accumulibacter* and *Aeromonas* were identified as the enriched PAOs for the EBPR process in the acetate-fed system, with 4.5 and 6.1% increase by mode change to EBPRINT-25 $EBPR_{INT-50}$ respectively, compared to $EBPR_{CONT}$.

Moreover, DPAOs, *Pseudomonas,* and *Dechloromonas* in genus level were identified with an 8 to 12 % high abundance in all reactors. Despite the potential activity of DPAOs, P-uptake in anoxic phases of $EBPR_{INT-25}$ was considered negligible. This is associated with nitrate competition between conventional denitrifiers and DPAOs [313]. As indicated, the higher PAO and DPAO abundance were not in accordance with higher TP removal performance, mainly in $EBPR_{INT-25}$, due to a 5% increase in GAOs availability. Competition between PAOs and GAOs, as one of the main challenges in biological P-removal, enhances the competition for available short-chain fatty acids. However, DNB, GAOs, PAOs, and DPAOs all compete for VFAs. Besides PAO-GAO competition, nitrate availability in the anaerobic zone in high concentrations in $EBPR_{INT-25}$, triggered higher competition for VFA consumption between DNB and PAOs. However, there was a 6% increase in DNB population by aeration mode change from continuous to intermittent; due to the accumulation of NO_3-N in 25-minute intervals, nitrogen removal failed to reach higher levels similar to $EBPR_{INT-50}$ system.

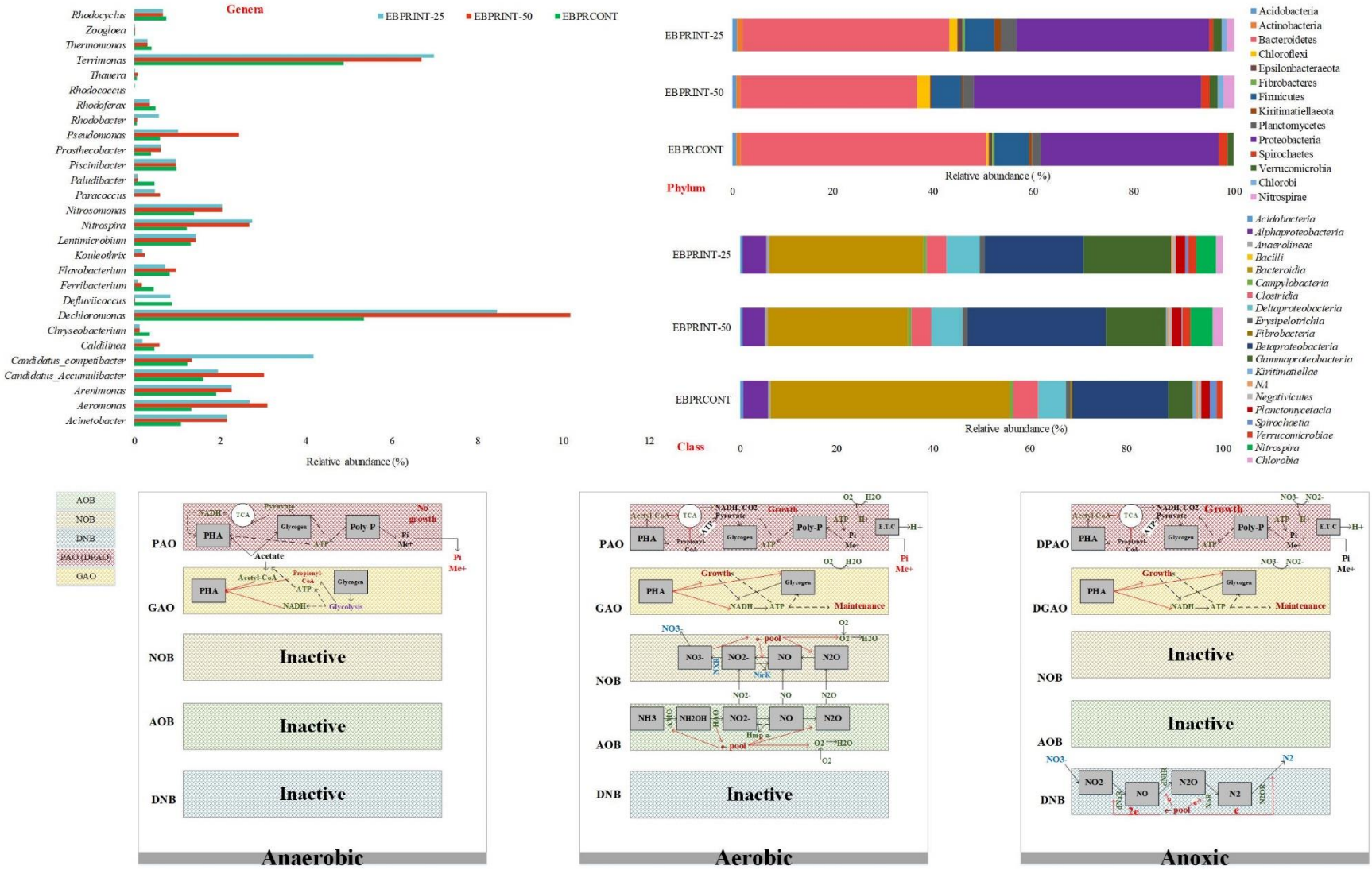

Figure 6-5 Microbial population dynamics in different aeration patterns at phylum, class and genus levels and Conceptual model of active and inactive microorganisms in EBPR process in anaerobic, aerobic and anoxic conditions

6.4.5 Bacterial community population in EBPR

The bacterial structure and the relative abundance of EBPR samples with different aeration patterns are shown in Figure 6-5 on phylum, class, and genus levels, respectively. According to the taxonomic assignments, the microbial variation descended as the aeration pattern was changed to intermittent intervals with 50-minute periods, suggesting the enrichment of phosphorus removing organisms. *Proteobacteria, Bacteroidetes,* and *Firmicutes* are the most abundant species in all samples, accounting for 91.2%, 86.6%, and 85.6%, respectively in $EBPPR_{CONT}$, $EBPR_{INT\text{-}50,}$ and $EBPR_{INT\text{-}25}$, while different compositions and distribution of communities were exhibited in the SBR reactors. As a classified phylum in intermittent aerated reactors, the High Proteobacteria population contributes to nutrient removal performance, as most PAOs and denitrifiers are classified under this species. Concurrently, *Bacteroidetes* and *Firmicutes* contribute to denitrification where a few DPAOs belong to the former phylum [349]. Previous studies have reported the importance of *Chlorobi, Spirochaetae,* and *Chloroflexi* phyla in simultaneous nitrification and denitrification by biodegradation of carbohydrates and cellular materials [309]. Summing up to 5.34% in $EBPR_{INT\text{-}50}$, these phyla enhanced organic compound degradation and nitrogen removal [355]. Moreover, *Nitrospirae* accounting for 2.4% and approximately 3% of *Planctomycetes* of phylum in $EBPR_{INT\text{-}50}$ are responsible for autotrophic metabolism, nitrite oxidation, and nitrification. On the other hand, $EBPR_{CONT}$ and $EBPR_{INT\text{-}25}$ consisted of only 1 to 3% of *Planctomycetes* and *Nitrospirae*, indicating less nitrification capability.

The predominant classes identified in SBRs were *α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria*, *Actinobacteria*, *Clostridia,* and *Bacteroidia*. Change in aeration mode from continuous to intermittent affected the dominant bacterial classes of *Bacteroidia* (49.6%), *β-*

Proteobacteria (19.9%) and *γ-Proteobacteria* (5.1%) from 49.6%, 19.9% and 5.1% to 29.1%, 28.6% and 12.4% due to variation in oxygen concentration from a constant value in $EBPR_{CONT}$ to intermittent aerobic/anoxic phases. Distinguish differences were observed at class level, shifting towards *β-Proteobacteria, γ-Proteobacteria,* and *Δ-Proteobacteria* from 30% in continuous aeration to 47.5% in $EBPR_{INT-50}$. At the same time, a decrease in *α-Proteobacteria* was indicated. According to *Li et al.* findings, *β-Proteobacteria* is more robust to operational changes and adaptable than *α-Proteobacteria* [330]. Further analysis based on top genera presented in Figure 6-5 showed larger variation comparing to class and phyla. Six dominant genera consisting of *Candidatus Accumulibacter phosphatis, Dechloromonas, Acinetobacter, Terrimonas, Rhodocyclus,* and *Aeromonas* were found as phosphorus removing microorganisms in this study. *Nitrosomonas* was available as a classified AOB with approximately doubled in abundance with intermittent aeration. Moreover, a similar abundance of *Nitrospira* as NOB coincided with stable nitritation in aerobic/anoxic intervals. The denitrifying bacteria population was diverse in all samples, including various denitrifying bacterial groups and genera such as *Zoogloea, Rhodocyclaceae,* and *Thauera*. Moreover, DNB content increased with implementing intermittent aerobic/anoxic phases and providing the enrichment of these groups of bacteria under oxygen-limited conditions, including *Rhodobacter, Terrimonas, Aeromonas,* and *Azoarcus* [355]. The presence of *Kouleothrix* (0.1 to 0.3%) and *Caldilinea* (0.2 to 0.6%) additionally is associated with high denitrification in intermittent reactors, where the latter appears to be also effective in phosphorus removal [346]. The results showed the presence of *Rhodocyclus* bacteria in acetate-fed SBRs at different levels. $EBPR_{CONT}$, $EBPR_{INT-50,}$ and $EBPR_{INT-25}$ consisted of approximately 1-2% *Rhodocyclus sp*. [356]. Previous studies have reported the dominancy of *Rhodocyclus sp.* from *β-Proteobacteria* in nitrite-fed reactors as electron acceptors [357]. Moreover, a study by

Chung et al. [340], on characteristics of DPAOs in anaerobic-intermittently aerated processes, conducted the presentation of *Rhodocyclus*-like organisms in a significant portion of the microbial population, which plays an important role in simultaneous phosphate uptake and denitrification in anoxic conditions. Therefore, the genera *Accumulibacter* and *Dechloromonas* belonging to the Rhodocyclaceae and Zoogloea are possibly denitrifying PAOs with widely apparent relations to nitrate removal. At the same time, *Acinetobacter,* a known PAO, has a negligible effect on the denitrification process, as stated in EBPR acclimation [346][358]. Principally, cooperation of *Zoogloea* and *Dechloromonas* for denitrification, dephosphorization, and nutrient removal capacity enhancement in addition to endogenous denitrification by *Zoogloea*, correlates with direct consumption of nitrate and phosphate in *Candidatus Accumulibacter*. In this study, the dominant PAOs identified were *Candidatus Accumulibacter phosphatis* and *Aeromonas,* while *Pseudomonas* and *Dechloromonas* were the main DPAO identified in denitrification and P-removal in intermittent aerated systems. At the same time, other available genera in lower abundance, including *Thauera* and *Zoogloea,* have the potential ability of simultaneous denitrification and phosphorus removal [358]. *Candidatus Accumulibacter,* a well-known PAO in lab-scale processes, existed with 5% abundance in EBPRINT-50, while they were hardly detected in a continuously aerated reactor. In the intermittently aerated reactors with prolonging of non-aeration time by 25 minutes, the abundance of DPAOs increased due to elongation of the anaerobic environment. *Pseudomonas* as the dominant denitrifying PAO genera changed with aeration pattern, increasing from 0.6% in $EBPR_{CONT}$ to 2.5% in $EBPR_{INT-50}$, performing biological phosphorus removal with nitrate/nitrite as electron acceptor, which potentially could compete with denitrifying bacteria [309]. *Dechloromonas* and *Paracoccus,* known as DPAO, capable of degradation of organics in the presence of nitrate and nitrite as an electron acceptor, occupied

11.2% and 0.59%, respectively, in $EBPR_{INT-50}$. $EBPR_{INT-50,}$ with the highest abundance of these genera, was greatly able to assimilate acetate, produce and store PHA and accumulate polyphosphate compared to the other two SBRs. This is in accordance with lower DPAO abundance in the continuously aerated reactor.

In all SBRs, PAOs and GAOs coexisted and assisted in nutrient removal. Likewise, the lower GAO genera abundance was closely related to longer aeration/non-aeration periods. DGAOs under anoxic conditions produce high nitrite accumulation [359], as observed in $EBPR_{INT-25}$, where these microorganisms reduce nitrate to nitrite under anoxic conditions, causing partial denitrification to occur. *Candidatus-Competibacter* (1 to 5%) and *Defluviicoccus* (0.1 to 0.9%) were identified as GAOs [360] with 4.2% and 0.9% in $EBPR_{INT-25}$, while in $EBPR_{INT-50}$ the abundance of *Candidatus-Competibacter* decreased to 1.3%, and *Defluviicoccus* dropped to a nearly undetectable level. These genera were detected with an increase of 2.9% in population by decreasing the intermittent aeration periods from 50 minutes to 25 minutes, according to higher anaerobic glycogen consumption, lower process performance, and limited phosphorus removal. Yet, PAO-GAO competition may reach a stable status where low P and N-effluent levels are achieved [361]. $EBPR_{INT-25}$ GAO's propagation over PAOs did not entirely deteriorate phosphorus removal but considerably decreased the efficiency.

6.5 Conclusion

Nutrient removal strongly depended on reaction phase configuration representing the importance of aeration pattern. The results indicated:

- For reaching a high N-removal, balancing the aerobic and anoxic duration for complete SND is suggested.

- A proposed solution for gaining high N-removal is to apply lower DO concentrations combined with a balanced intermittent aerated mode to the SBR system, therefore DO level and aeration duration concurrently affect N-removal.
- The primary factors affecting the P-removal performance were known to be insufficient reaction time, variation in DO concentration, and carbon source.
- In SBR reactors, for minimizing operational period and energy requirement, coordination between aeration duration and reaction cycle in intermittent aeration is needed. However, short aeration duration and too many cycles resulted in high energy input and increased effluent TN and TP concentration.
- The highest nutrient removal is achieved when nitrification, denitrification, and P-removal, as the three major processes are all active in the reaction (aerobic/anoxic) period. Yet, their process rates and advantage over one another in the aerated/non-aerated phases specify the process performance efficiency.

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7 Development of Phosphorus removal under stepwise low-aeration adaptation in EBPR process

Adapted from:

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Preface:

This chapter of the thesis is an original work based on the experimental apparatus and data, published by the author, Parnian Izadi.

Author statement:

Parnian Izadi: Conceptualization (Lead), Methodology (Lead), Validation (Lead), Investigation (lead), Resources (lead), Writing- original draft (lead), Writing-review and editing (equal), Visualization (lead)

Parin Izadi: Conceptualization (supporting), Methodology (supporting), Investigation (supporting), Writing- original draft (supporting), Writing-review and editing (equal)

Ahmed Eldyasti: Conceptualization (supporting), Methodology (supporting), Investigation (supporting), Writing-review and editing (Lead), Supervision (Lead), Project Administration (Lead), Funding Acquisition (Lead)

7.1 Abstract

Recent research has shown the adaptability of BNR systems to very low level dissolved oxygen (DO) concentration, mainly focusing on the nitrification ability that maintains the nitrogen oxidation process even at very low DO levels. Although step-wise aeration decrease on Enhanced Biological Phosphorus Removal (EBPR) is not fully comprehended. This study investigated the effect of reaching micro-aeration with adaptation strategies on EBPR performance. A step-wise oxygen concentration decrease, arriving at an average aeration level of 0.4 mgO_2/L was evaluated, with an outcome of approximately 90% phosphorus removal efficiency. Compared with different aeration modes, the highest phosphorus (P)-removal efficiency, P-release, and lowest effluent phosphorus was achieved in a gradual DO decrease strategy.

On the other hand, an instant decrease in aeration from stable EBPR process from 2 mgO_2/L to 0.4 mgO_2/L adversely impacted P-removal by decreasing the efficiency to an average 60% and deteriorating the phosphorus removal microbial consortium. Performance comparison between the instant and gradual DO-decrease strategies justified the importance of microbial adaptation to minimal oxygen availability for high process performance. Therefore, this study proposes a potential aeration mode, which contributes to reducing energy consumption in BNR systems through wastewater treatment.

Keywords: EBPR; Phosphorus; dissolved oxygen; PAO; GAO; aeration

7.2 Introduction

Phosphorus accumulating organisms (PAOs) are the main organisms available in Enhanced Biological Phosphorus Removal (EBPR) for eliminating phosphorus (P) to avoid eutrophication in aquatic systems. EBPR is achievable by sludge recirculation through anaerobic and aerobic/anoxic conditions [6]. Operating in this sequence promotes PAOs to sequester volatile fatty acids (VFA) and store polymeric substances, polyhydroxyalkanoates (PHA), in the anaerobic phase, where internal polyphosphate is hydrolyzed as an energy source, resulting in the release of orthophosphate. This leaves almost no available VFA for ordinary heterotrophic organisms in the aerobic phase [227]. Therefore, in aerobic conditions, PAOs growth and flourishment occur on PHA degradation and P-uptake, increasing the sludge P-content [224]. As a result, PAOs consume excessive phosphorus than their normal metabolic requirement [362]. The EBPR process is operated in an oxygen-limited stressing condition for PAOs high performance in activated sludge; however, with any further stress factors, PAOs efficacy may drop [363], providing the growth of PAOs competitors as glycogen accumulating organisms (GAO). Minimizing the competitor's abundance in the EBPR system enhances oxygen utilization for the P-removal process with

reduced effluent-P [188]. Microorganisms, including PAOs, mainly promote steady-state conditions, although external disturbance such as excessive aeration may cause P-removal deterioration and failure along with impaired denitrification process [351]. Therefore, reaching a steady-state condition in highly dynamic wastewater treatment plants (WWTP) is rarely achievable [363].

Traditional high-rate biological nitrogen and phosphorus removal and organic matter oxidation are mainly performed under considerable aeration levels for a secure high nutrient removal efficiency [364]. Although, improving the energy efficiency of the EBPR process by lowering the energy contribution and consumption factors such as aeration requirements shall be considered for sustainability enhancement and process stability [188]. Aeration energy is mainly accountable for more than 30% to 60% of total energy consumption; therefore, aeration control and optimizing the air delivery to the aerobic phase is critical for achieving stringent discharge limits and high energy efficiency [289] [364]. In WWTPs, the aeration requirement relies on actual oxygen demand and oxygen transfer efficiency, where the former is demonstrated by oxidized contaminant and produced biomass extent. At the same time, the latter is relatable to aeration devices, dissolved oxygen (DO), and mixed liquor suspended solid concentration (MLSS). Decreasing the DO concentration by 1.5 mg/L from 2 mg/L to 0.5 mg/L, a total 16% and 10% increase are achievable for overall oxygen transfer efficiency and total energy saving of WWTP, respectfully [365].

From an operational point of view, lower DO levels improve nutrient removal besides decreasing the oxygen requirement for a more cost-effective WWTP [188]; however, several studies have reported low level DO conditions leading to potential nitrification and denitrification separation, lack of COD availability, and secondary phosphorus release [351] indicating a scarcity of information on EBPR process performance. Moreover, by decreasing the aeration rate, aeration

duration is mainly increased to ensure high removal performance. Yet, an incline in aeration time may lead to lower pollutant removal capability in anoxic and anaerobic phases by functional microorganisms [366]. Recent studies have established an achievable nutrient removal process at low-DO levels with an adaptation period of activated sludge to lower oxygen rates, specifically in the case of nitrification, since nitrifiers are less competitive in DO usage comparing to heterotrophic bacteria. However, by slowly reducing the DO levels, the nitrification rate was not impacted. Despite the importance of investigating nitrogen transformation in sequential anaerobic/aerobic/anoxic zones, understanding phosphorus profile change and PAOs behavior is necessary to reduce aeration concentration [300]. Research on molecular ecology has shown a diverse microbial community, phylogenetically and morphologically, with various metabolism competence in the EBPR process, influenced by operational factors such as DO concentration [362]. Although research has demonstrated the lower oxygen affinity of GAOs comparing to PAOs, which allows higher PAO activity in low-DO levels [188] [364]. Yet, limited knowledge and studies are available for P-removal under low-DO conditions and the required adaptation period. Thus, there is insufficient information on EBPR performance and PAOs abundance under fast and slow transition from high to low DO concentration conditions due to the complex microbial community structure with different microbial groups containing diverse metabolic abilities [362].

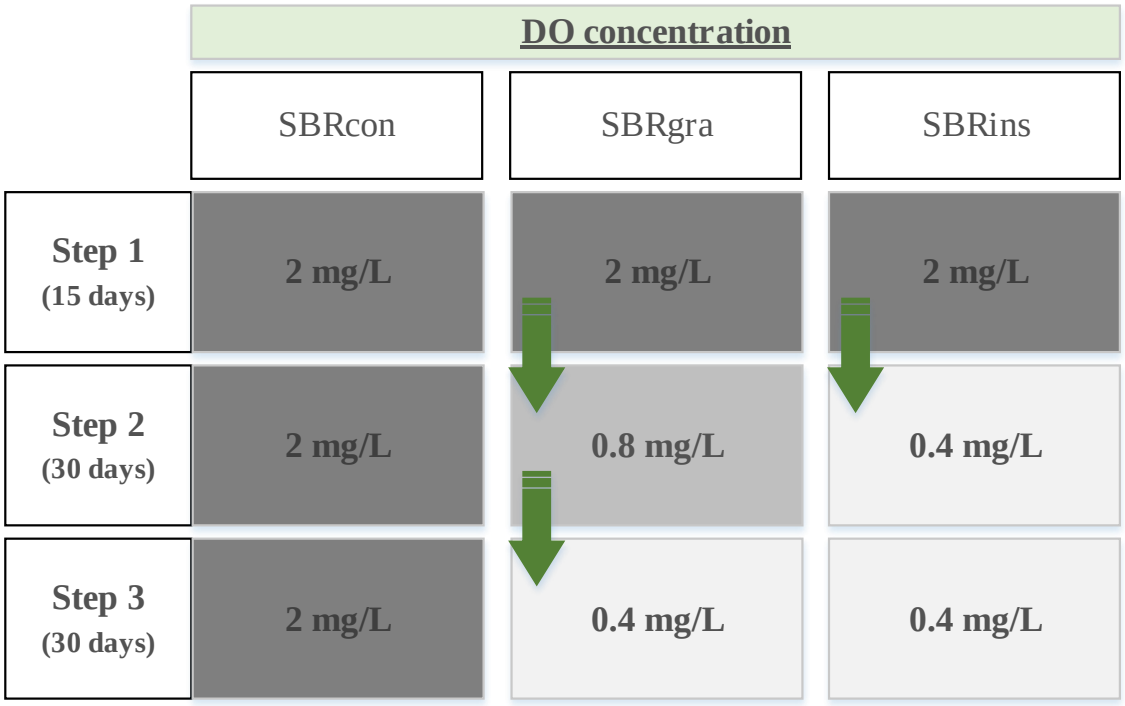
In this study, the effect of DO concentration in a range of 0.4 to 2 mg/L with continuous aeration is specifically investigated on Phosphorus removal by understanding the various microbial communities for achieving high P-removal. Moreover, the effect of steady DO reduction in different aeration strategies was studied in batch tests with enriched PAOs at different DO levels. Another primary aspect of this paper is the statistical confirmation of the relationship between DO

level reduction and EBPR performance. Aeration reduction was performed in an instant and step-wise manner, evaluating the adaptation of microbial community to aeration change and minimal oxygen availability.

7.3 Materials and methods

7.3.1 Reactor setup and operation

In this research, three sequential batch reactors (SBR) with a working volume of 5 liters and an internal diameter of 25 cm were operated at ambient temperature. All three reactors were inoculated with returned activated sludge (RAS) from the Humber treatment plant, Ontario, Canada. All operating systems were set up with a cycle time of 8 hours, and an initial DO concentration of 2 mg/L. The 8-hour cycle consisted of 15 min feeding, 90 min anaerobic, 320 min reaction followed by 40 min settling, and 15 min decanting. Three steps of the experiment were conducted, as indicated in Figure 7-1. One liter of synthetic wastewater comprising acetate, phosphate, and other required nutrients was pumped into the reactors during the feeding phase with a hydraulic retention time (HRT) of 10-15 hours and a solid retention time (SRT) of approximately 25 days. The synthetic wastewater used for this study approximately contained (per liter): 0.85 g $NaAc.3H_2O$ (400 mg COD/L) as carbon source, 107 mg NH_4C1 (28 mg N/L), 75.5 mg $NaH_2PO_4.2H_2O$ (15 mg P/L), 90 mg $MgSO_4.7H_2O$, 14 mg $CaCl_2.2H_2O$, 1 mg yeast extract, and 0.3 mL nutrient solution. The concentrated nutrient solution contained, per liter: 1.5 g $FeCl_3.6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4.5H_2O$, 0.18 g KI, 0.12 g $MnC1_2.4H_2O$, 0.06 g $Na_2MoO_4.2H_2O$, 0.12 g $ZnSO_4.7H_2O$, 0.15 g $CoCl_2.6H_2O$, and 10 g EDTA. The pH of the feed was adjusted at 7.5±0.1. The initial chemical oxygen demand was in the range of 300-400 mg/L containing acetate as the sole carbon source fed to the system.



	VFA	Influent P (mg/L)	Influent COD (mg/L)	P/C ratio	Total cycle (min)	anaerobic phase (min)	aerobic phase (min)	DO change
SBR_{con}	HAc	15	350	0.043	480	115	320	constant
SBR_{gra}	HAc	15	350	0.043	480	115	320	gradual
SBR_{ins}	HAc	15	350	0.043	480	115	320	instant

Figure 7-1 Experimental layout and operational factors of SBRcon, SBRgra, and SBRins in steps 1 to 3 of EBPR experimentation

DO concentration of 2 mg/L was chosen as the conventional oxygen availability, and 0.8 mg/L and 0.4 mg/L were chosen as the low DO, and extremely low DO levels for aeration. Reactors initially went through a start-up and acclimatization period in which they were operated at 2 mg/L of DO concentration and stable feeding to reach reliable EBPR performance before moving into micro-aeration strategy experimentation. In the aerobic phase, the air was provided with an online air pump aerator (EcoPlus Eco Air Commercial Air Pump 1 - 18 W - 793 gph) connected to the bottom of the reactors. DO was measured in real-time using a probe (Hach LDO10101 IntelliCAL LDO Lab Probe, 1 m cable) connected to a DO controller. The DO was constantly monitored and controlled by the Controlling panel to maintain the DO level in the acceptable range for each step

of the process (0.4 to 2 mg/L). pH, ORP, and oxygen concentration were monitored online through a control processing system. The mixing speed was controlled at 50 to 100 rpm depending on the DO concentration, reducing the speed with each DO surpass a deviation of ±0.5 mg/L. During the anaerobic and aerobic phases, reactors were constantly mixed; no mixing occurred in settling and decanting phases. Wastewater from the sequential anaerobic and aerobic phase settles in the settling phase. To eliminate the nitrification process in the anaerobic stage, nitrogen gas was inserted to remove any residual oxygen available. Yet, with nitrification not of interest, by any signs of nitrate and nitrite production in the reactors, nitrification inhibitor, Allyl-Nthiourea: ATU was added to the substrate to avoid any consumption of oxygen by NOBs and AOBs in aerobic EBPR and to prevent nitrite/nitrate accumulation. The SBRs were routinely monitored through regular sampling for nutrients, VFA, PHA, and glycogen through each cycle. Chemical analysis such as chemical oxygen demand (COD), total suspended solids (TSS), and volatile suspended solids (VSS) was measured on samples from the end of the aerobic phase.

7.3.2 Batch experiments at different DO levels

Besides the SBR cyclic operation at DO concentrations of 2, 0.8, and 0.4 mg/L, four aerobic batch tests were performed to determine the effect of different DO concentrations on nutrient removal and the effect of adaptation strategy on EBPR performance with acetate addition in the previous anaerobic phase. After reaching pseudo-steady-state conditions in all systems, batch experiments were performed.

All batch tests were implemented in 500 mL amber batch reactors with 450 mL of effective volume and 50 mL of headspace. All reactors were supplied with an online DO and pH meter, mixed with a magnetic stirrer, and were securely sealed. For all the batch tests, the sludge was taken from the end of the aeration phase, and the temperature was adjusted to the target study temperature. Any

interference with PAO, including the presence of nitrate and nitrite in aerobic sludge samples, should be avoided. Therefore, sludge samples may have required a short pretreatment step for troubleshooting. With any indication of nitrate, the sludge was transferred to an airtight batch bioreactor. It was gently mixed under non-aerated conditions until the nitrate or nitrite concentration dropped to below detection level. Once no nitrate was available, then the batch tests would start. Before testing, the sludge was maintained at the aerobic condition with gentle mixing and aeration for the proper acclimatization for batch reactor conditions. The tests included an execution of an anaerobic test with a continuous N_2 gas supplement for avoiding any gas intrusion, immediately followed by an aerobic batch test with sparge of air to reach the desired DO concentration. After complete execution, the remainder of the sludge was returned to SBR reactors. The samples were collected based on determining the specific kinetic rates and stoichiometric ratios. The kinetic and stoichiometric parameters specified the maximum acetate uptake rate, maximum specific anaerobic phosphate release rate, P/HAc ratio, and aerobic P-uptake rate. Samples were further analyzed for PHA, glycogen, nutrients, TSS, VSS, and microbial community quantification.

7.3.3 Analytical method

Total solids (TS), volatile solids (VS), TSS, VSS, total chemical oxygen demand (TCOD), and soluble chemical oxygen demand (SCOD) were determined by standard methods (APHA 2005). pH was measured by a digital pH meter (hach HQ440d multi). Ion chromatography was utilized to simultaneously determine phosphate, nitrate, nitrite, and ammonia through Thermo Scientific™ Dionex™ Integrion™ HPIC™ system. Samples were properly diluted with deionized water and passed through a membrane filter (0.45 μm) for all IC measurements. To confirm PAO enrichment and evaluation in the reactors, samples were initially analyzed for particle size distribution using

an aqueous liquid module (ALM) in LS 13 320 Particle Sizing Analyzer; later on, samples were analyzed using PCR. Earth Microbiome Project benchmarked protocols (http://www.earthmicrobiome.org/emp-standard-protocols/) were used for DNA extraction and amplification. Mechanical and enzymatic lysis along with phenol-chloroform extraction and clean-up was performed using MoBio PowerMag soil DNA isolation kit for DNA extraction, where the primers used included 515FB (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806RB (5′-GGACTACNVGGGTWTCTAAT-3′). Standard gel extraction kits were utilized for purifying the final product after amplification, polymerization, and separation (Qiagen, Netherland). Accordingly, the product was quantified with the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher). The resulting PCR products were sequenced using the Illumina MiSeq personal sequencer (Illumina Incorporated, San Diego CA) at the McMaster Genomics Facility, Ontario, Canada. The volatile fatty acid was assayed by an SRI gas chromatography (GC) equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). PHB was extracted from cellular biomass and quantified with gas chromatography using the following procedure. Primarily, 10-15 mg of lyophilized biomass were collected, and 2 mL of acidified methanol (3% sulphuric acid), as well as 2 mL of chloroform, were added in a glass vial. After gentle mixing, the cocktail was heated at 100 ∘C for 3.5 hours, cooled down to room temperature afterward. 1 mL of deionized water was added later on, and the mixture was vortexed for 1 minute and then left until phase separation was achieved. The lower organic phase was tested for PHB quantification using SRI gas chromatography equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). The temperature program was as follows: 1 min 80 ∘C, 10 ∘C min 1, 180 ∘C for 4 min. Results were compared with standard curves obtained using PHB standards (Sigma

Aldrich). Benzoic acid was used as an internal standard to increase accuracy. Intracellular glycogen is determined via digestion and hydrolysis to glucose. Glucose will be analyzed enzymatically (Sigma–Aldrich PGO enzyme kit, Saint Louis, Missouri, USA). Sample absorbance is measured at 425 nm using a Spectronic spectrophotometer.

7.3.4 Statistical analysis

A variety of both parametric and non-parametric statistical tests were performed on the normalized data. For the statistical analysis, the t-test (for two groups of samples), F-test for equal variances (for two groups of samples), and One-way ANOVA analysis were applied with Matlab® Statistics and Machine Learning Toolbox™ (Matlab R2015a, MathWorks, USA) to evaluate the significance of differences in efficiency, performance and fit between models and to study the effect of oxygen concentration on P-removal efficiency, P-effluent concentration and PAO abundance, the pattern of changes and their significance difference. In the SBR with a rather strong performance, correlation among operational and environmental parameters was evaluated. Regression analyses were performed to determine the quantitative relationships that form the best models to relate key variables to DO levels in the system—the parameters comprised of the operational data and the results from EBPR lab experiments. Significance was accepted at $p \leq 0.05$ level.

7.4 Results and discussion

7.4.1 EBPR performance at different DO levels and Aeration strategies

The three SBRs were routinely monitored for characterizing the performance of the reactor at different DO levels with continuous aeration in aerobic conditions. In step 1, all reactors were initially operated with 2 mg/L of DO to reach a stable performance. In the second step, SBR_{con} maintained the same condition. The P-removal of SBR_{con} with a rather constant DO concentration

was not significantly affected, with an approximately constant mean of 78%, while for SBR_{gra} and SBR_{ins} the DO was lowered to 0.8 and 0.4 mg/L, respectively.

pH levels were controlled but not kept constant through the experiment by monitoring the pH and DO concentration during cyclic periods. An average increase of 0.7 in pH levels was observed in all DO levels at the initial aerobic phase, followed by a slight decrease by the end of aeration. Variation in DO levels minimally affected the pH alteration, showing that DO has no noticeable effect on pH variation. All reactors had a primary decrease in pH levels anaerobically followed by an initial aerobic rise due to CO_2 stripping. SBR_{gra} had higher pH levels on average, with the highest point attained to 7.65, indicating an advantage towards PAO's anaerobic VFA uptake and P-release at elevated pH levels, which were in line with the findings of *Liu et al*. that reported the benefit of an alkaline environment on anaerobic P-release [367]. In all SBRs, the DO was controlled at the desired level in the aerobic phase. The DO comprised the aqueous and settled phase. The aqueous form maintained the same level in the following settling and decanting phase, while the settled DO drastically decreased in the subsequent periods. Although at 2 mg/L of DO concentration, residual DO was pumped back to the anaerobic phase, which disrupted anaerobic P-release and VFA uptake. In the case of 0.8 mg/L reactor, after complete aerobic removal of phosphorus, the DO concentration gradually rose.

Based on the available data, the phosphorus profile is strongly dependent on oxygen concentration. In all three reactors in step 2, anaerobic P-release and aerobic P-uptake were observed. However, the quantity of phosphorus in both phases decreased by increasing the aeration rate. All reactors showed P-removal with a variation in the efficiencies. The highest phosphorus removal efficiency of 90% was obtained at a DO concentration of 0.8 mg/L, in addition to a high level of phosphorus release and uptake. Although lower DO concentration commonly shows higher EBPR

performance, in 0.4 mg/L reactor, lower performance was achieved due to a rapid decrease in DO concentration and limited time for adaptation of biomass to the operational factors. As expected in EBPR processes, VFAs were taken up in anaerobic conditions and stored as PHA accompanied by glycogen degradation and phosphorus release. The quick VFA uptake is in consolidation with anaerobic phosphorus release. In aerobic conditions, phosphorus uptake in a greater amount than P-release is coupled with PHA hydrolysis and glycogen reproduction. During 30 days in step 2 of the experiment, all SBRs demonstrated VFA consumption, followed by polyphosphate hydrolysis, which remarkably increases the phosphorus level in the bulk liquid. As a crucial step in EBPR, the anaerobic P-release provides energy for PHA formation by degradation of polyphosphate. As a linear polymer, Poly-phosphorus is formed of high-energy phosphoanhydride bonded phosphorus residues connected, where EBPR metabolic capability is highly dependent on Poly-P function [362]. Therefore, this step is considered critical since it directly affects the aerobic phosphorus uptake and removal efficiency. Based on the stoichiometric measurements, the PHA production/VFA uptake ratio in SBR_{con} was lower than SBR_{gra}. The SBR_{gra} had the highest P-release/P-uptake and PHA synthesis/degradation, although the glycogen alteration was quite comparable to other reactors. In consideration of metabolic conversions, the energy source in the form of adenosine triphosphate (ATP) is required for VFA uptake and Acetyl-CoA production. In contrast, the following PHA production fraction requires reducing power $NADH_2$ for redox balance purposes. In an A/O process, both glycogen degradation and Poly-P hydrolysis are responsible for energy and reducing power production for PHA generation [303].

As shown in Figure 7-2, SBR_{con} with constant DO level, in step 2 of the experiment, maintained the P-release observed during the first step with an average P-release of 50 mg/L. A rather gradual P-release increase for SBR_{gra} in step 2 was indicated, reaching an average 65 mg/L of anaerobic P-

release. In this reactor (SBR_{gra}), a steady decrease in the DO concentration from 2 mg/L to 0.8 mg/L aided in remaining stable during this period and constructively impacted the system's performance. The phosphorus release of SBR_{ins} was considerably lower than SBR_{gra}, with approximately 18 mg/L lower P-release in 30 days. In the following aeration phase, P-uptake remained stable with an average 4 mg/L of P-effluent in SBRcon. At the same time, in SBRgra, which was characterized by a steady decrease in the DO concentration, P-uptake increased through a gradual DO decrease from 2 mg/L to 0.8 mg/L reaching an average > 1.5 mg/L of phosphorus in the effluent. During this phase, SBRins was highly sensitive to any disruption in the experiment as it reached considerably lower P-uptake and an average of 8 mg/L of P-effluent. This reactor faced noticeable fluctuations, including major disruptions in P-profile, mainly due to the lack of adaptation to extremely low DO levels.

With less energy produced by polyphosphate degradation, lower PHB concentration was available in SBR_{ins} with an average 2.2 C-mmol/gVSS in comparison to 3 C-mmol/gVSS for SBR_{gra}. In the aerobic phase with 0.8 mg/L of DO concentration, high PHB content contributed as an energy and carbon source for P-uptake, microorganism growth, and glycogen replenishment, reaching an average of 90% phosphorus removal. However, the P-effluent and P-uptake rate in SBR_{ins} confirmed low P-removal efficiency of 59% due to a shortage of PHB as the energy provider and insufficient oxygen as electron acceptor. In the absence of an electron acceptor, the PAO assimilates acetate to synthesize PHA, mainly in the form of PHB. The PHB-like polymer includes two monomeric units of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). When the EBPR system is mainly fed with acetate, the 3HB unit is the most abundant in the polymer composition known as PHB (Smolders et al., 1994). For justification of this matter PHV was measured based on Zeng et al. study, in which PHB and PHV concentration is measured by assuming that acetate

consumption and glycogen degradation takes place by PAO and GAO, acetate uptake for PAO and GAO as a and b, respectively. Where a and b are presented in Equations 7-1 and 7-2:

$$a = \frac{(1+2\alpha_{GAO})HAc - Gly}{0.5+2\alpha_{GAO}} \quad \textit{Equation 7-1}$$

$$b = \frac{Gly - 0.5HAc}{0.5+2\alpha_{GAO}} \quad \textit{Equation 7-2}$$

Where αGAO is the required energy for transportation of 1c-mol of acetate across the GAO cell membrane depends on pH. In pH of 7.3, αGAO is approximately 0.075 molATP/c-mol HAc, PHB and PHV concentration is measured as shown in Equations 7-3 and 7-4.

$$PHB = 1.33a + \frac{(\frac{9}{6}+(\frac{2\alpha}{3}))^2}{\frac{5}{3}+\frac{4\alpha}{3}} b \quad \textit{Equation 7-3}$$

$$PHV = \frac{2.5*(\frac{9}{6}+(\frac{2\alpha}{3}))(\frac{1}{6}+(\frac{2\alpha}{3}))}{\frac{5}{3}+\frac{4\alpha}{3}} b \quad \textit{Equation 7-4}$$

The calculations for PHB were in line with the measured results obtained from the experiment, indicating 8.31, 7.93, and 8.7 mmol/L of PHB for SBRcon, SBRins, and SBRgra, respectively. However, the results for PHV were rather negligible, standing at 0.031, 0.015, and 0.02 mmol/L for SBRcon, SBRins, and SBRgra in respect. In the conventional activated sludge treatment process, P-removal occurs through cell synthesis and maintenance linked to microbial growth, where 10 to 30% removal can be achieved [368]. Therefore, in SBR_{ins}, considering the P-removal associated with microbial growth, only an excess 29 to 49% P-removal was achieved by the enriched PAOs. A clear poor P-removal efficiency of below 60% during day 15 to 45 of the experiment with a rapid DO decrease in SBR_{ins}, suggested that the EBPR community seek longer adaptation time to fully acclimatize to grow into the functionally applicable process in extreme low-DO levels. These results also proved the adaptability of biological phosphorus removal to step-wise oxygen rate decrease in a well-controlled EBPR system. Higher phosphorus removal efficiency in SBR_{gra}, compared to SBR_{con} and SBR_{ins}, points out the importance of step-wise DO

decrease to reach phosphorus removal. Adaptation to low DO concentrations was reflected in high aerobic phosphorus uptake. Moreover, lower VSS/TSS ratios in SBR_{gra} than SBRcon demonstrate the inorganic polyphosphate content increase in the sludge at lower DO levels. The lower rate of VSS/TSS and high P-accumulated biomass indicate the bacterial community's dominancy and their effective impact on reactor operation.

Therefore, this mode of aeration, consisting of step-wise DO decrease, results in efficient P-removal with micro-aeration. Yet, despite the reports in the literature [351], the lowest P-removal efficiency and P-release was observed in the extremely controlled low DO concentration and not at high DO levels, due to the lack of adjustment of EBPR microbial community.

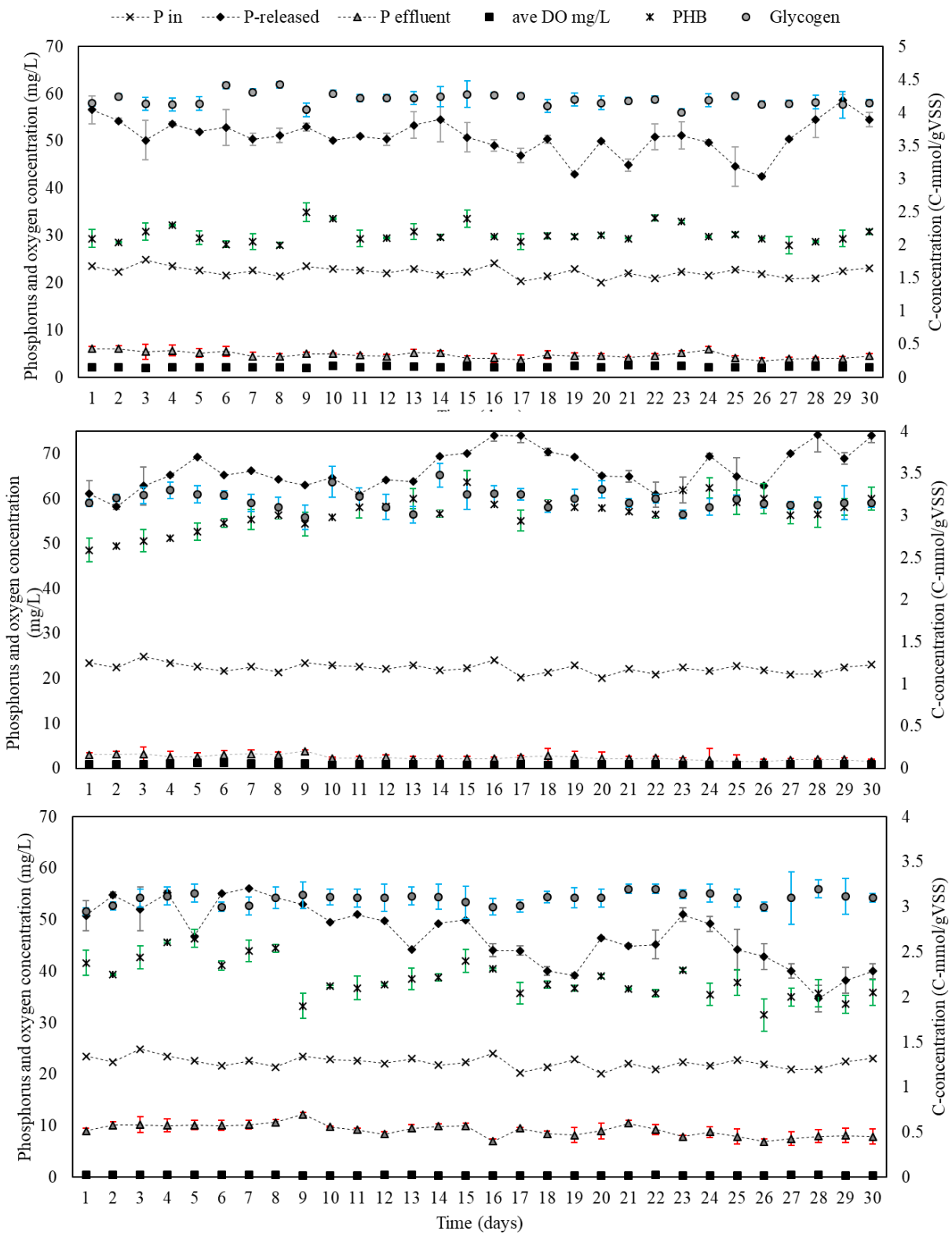


Figure 7-2 PHB, glycogen, influent phosphorus, effluent phosphorus, P-release and oxygen concentration change in A.SBRcon B.SBRgra C.SBRins in step 2

In the following third step of operation, SBR_{con} and SBR_{ins} maintained the same condition while the DO concentration in SBR_{gra} was decreased to 0.4 mg/L. This step was operated to evaluate the effect of instant and gradual oxygen concentration decrease to a very low level. Variation of DO, $P_{release}$, P_{ieff}, and P-removal have been indicated in Figure 7-3 during the step-3 SBR operation. To control DO after step 1 for SBRs, the aeration rate was decreased and controlled by a controlling panel to maintain the level of aeration at its set point. As shown in Figure 7-3, average DO concentrations in step 1 in SBR_{gra} and SBR_{ins} were 2.1±0.14 and 2.13±0.15 mg/L, wherein step 2 it decreased to 0.9±0.11 and 0.41±0.04 mg/L, respectively. While SBR_{ins} maintained its DO level at approximately 0.4 mg/L, in step 3, SBR_{gra} reached an average DO concentration of 0.43±0.04 mg/L. As one of the major obstacles, the adaptability of PAO metabolism to gradual DO decrease was investigated. With a step-wise oxygen reduction, PAOs balanced themselves with limited oxygen concentration. Even though SBR_{con} with a stable 2 mg/L DO concentration through the experiment has complied the effluent phosphorus concentration of lower than 3 mg/L, decreasing the aeration rate resulting in lower cost is a reason to motivate the utilization of low-aeration configuration. In the second operational step with a steady DO decrease in SBR_{gra}, P-removal remained stable during this period with an effluent concentration of 2 mg/L, corresponding to an average 90±2% P-removal efficiency. Therefore, gradually decreasing DO from 2 mg/L to 0.8 mg/L in SBR_{gra}, resulted in a slow increase of 7% in P_{ian} (anaerobic P-concentration).

In comparison, P_{ieff} remained stable, and two mg/L of phosphorus in the effluent was achieved. With further step-wise DO decrease in step 3, at days 47 to 51, P-removal was sensitive to any disruptions in operational performance. However, the system recovered to reach a P-removal efficiency of 87%±2%. The VFA, PHB, and glycogen profile maintained their stability in step 2

at 0.8 mg/L, yet a few fluctuations were observable at the start of this phase. The P-removal mean in SBR_{gra} underwent a noteworthy increase from 77% to 89% in step 2, while a subtler change was observed when reaching step 3.

In contrast to a balanced EBPR process with minimum disruption to process performance, abrupt low-DO concentration resulted in a jump of P_{ieff} concentration to higher than 8 mg/L. During steps 2 and 3 of operation (60 days) with a constant DO concentration of average 0.41±0.03 mg/L, severe disruptions were followed by a decrease in P-removal efficiency. The P-removal was low during step 2 (59%±5%), yet no recovery and adaptation took place after these operational upsets, maintaining an average 58%±4% P-removal efficiency in step 3. Looking into statistical difference in P-removal efficiencies of SBR_{con}, $SBR_{gra,}$ and SBR_{ins} in all three steps of the experiment (Figure 7-4), the comparison in step 1 shows the minimal difference between the data range of all reactors with almost identical mean. A *p*-value of 0.7629 exceeding 0.05 further justifies that P-removal efficiencies between reactors in stage 1 did not exhibit a significant difference. However, in stage 2 significant difference was found in P-removal efficiencies as the *p*-value did not exceed 0.05, clearly indicating a higher average P-removal with a gradual DO decrease from 2 mg/L to 0.8 mg/L. Similar to step 2, at step 3, intense differences were observed regarding P-removal efficiencies with superiority of gradual DO decrease to instant DO decrease to 0.4 mg/L.

The experimental results indicated a significant difference in removal efficiencies of different scenarios, yet statistical procedures were used to determine and justify the statistical significance of the measurements. Mean values were calculated for P-removal efficiencies in all stages for all SBRs. Of the three SBRs, SBR_{ins}, showed a statistically significant reduction during treatments by a decrease in mean value from 77% to 58%. These uprising interruptions may be due to the incapability of bacterial consortium adjustment to the new operational conditions applied to the

system. The metabolism requires building up and activation for out-competing other heterotrophic bacteria. A long operation at low DO concentration justified the eradication of P-removal ability, which shows the struggle of the bacterial community in acclimatizing with the sudden extreme change.

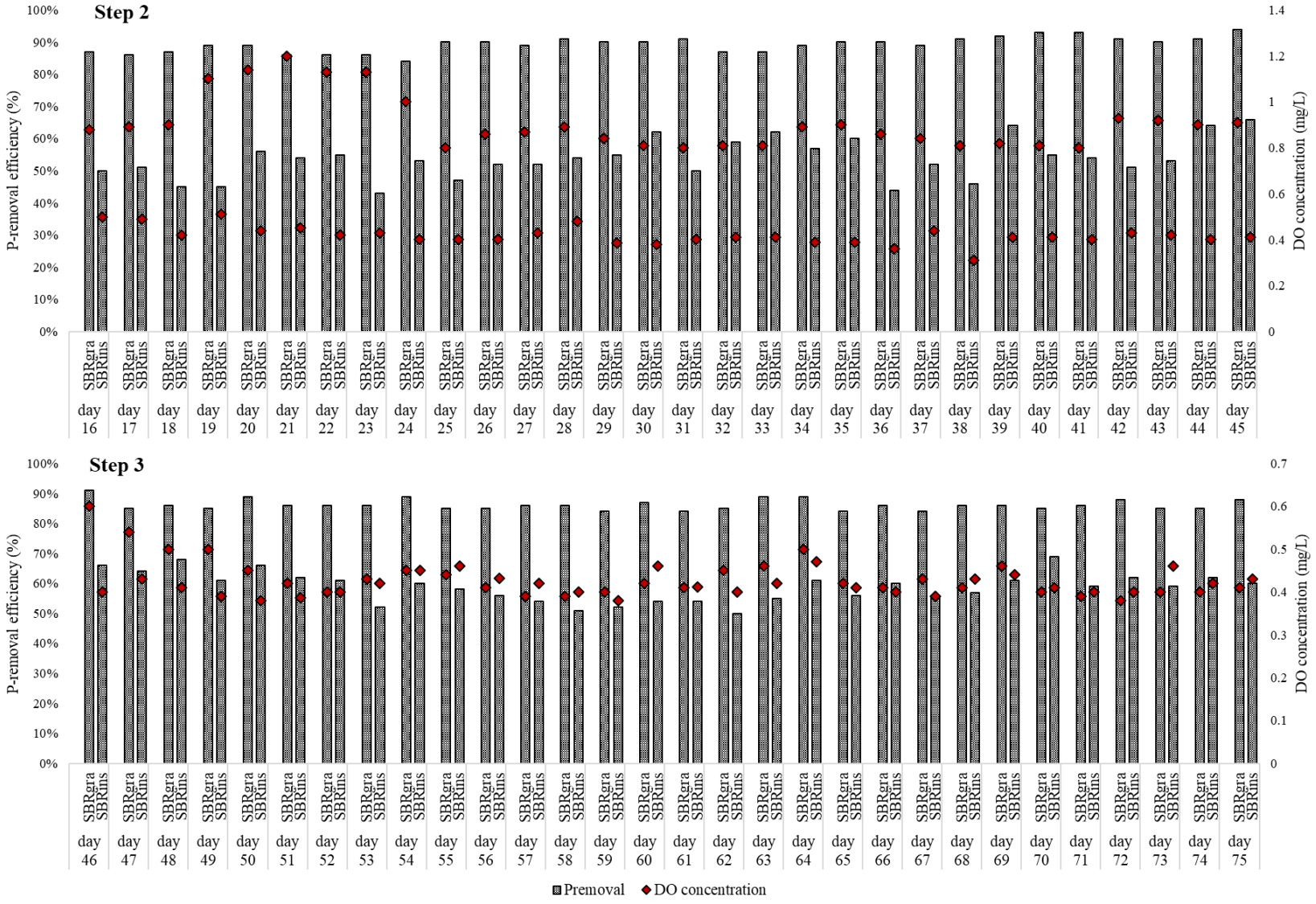


Figure 7-3 DO concentration change and phosphorus removal performance under different aeration strategies for SBR_{gra} and SBR_{ins} in step 2 and step 3

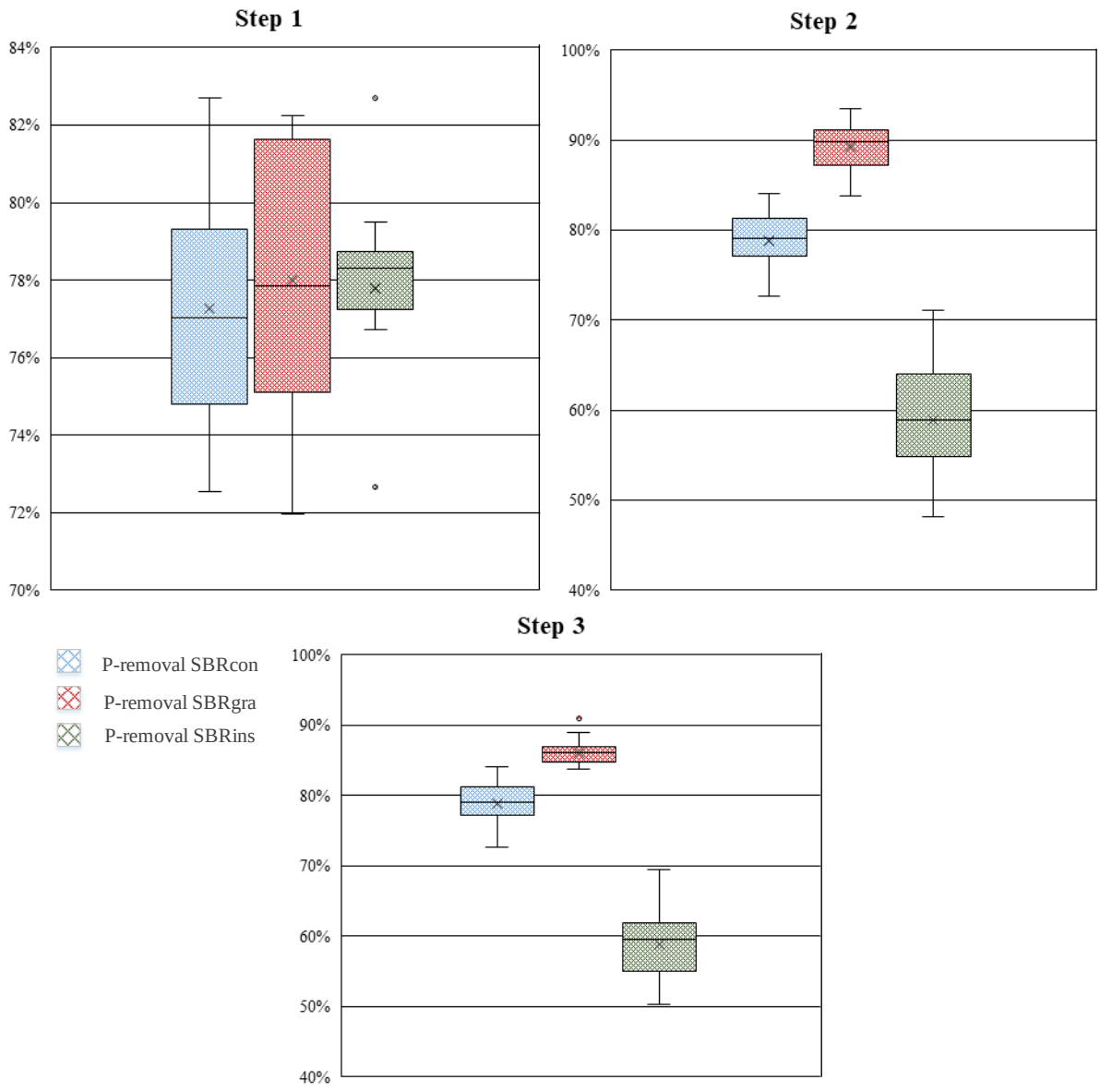


Figure 7-4 significance testing and statistical difference in P-removal efficiency of SBRcon, SBRgra and SBRins at three different steps

7.4.2 Gradual and instant effect of aeration rate decrease on process performance and population dynamics

Achieving a stable low-DO P-removal is of interest since decreasing the oxygen concentration will increase oxygen transfer and eventually decrease total energy saving. It will also increase sludge production and decrease actual oxygen demand and operational DO requirements [365]. Recent experiments have demonstrated reaching efficient and stable low DO phosphorus removal, yet the adaptation of microbial community to the new condition is infrequently studied. However, there is an understanding of the need for the phosphorus accumulating organism's adaptability to low DO conditions. Then, EBPR reaches the required P-removal capacity after prolonged exposure of the culture to a slowly reduced DO in a lab-scale system [369]. The result of these studies suggests PAOs high affinity for oxygen being responsible for P-uptake and overall P-removal at low-DO conditions [370]. Keene et al. [369] studied stable and efficient BNR with low DO conditions and suggested slow step-wise oxygen reduction as a novel strategy to reach high-rate low-DO BNR from conventional high-rate high-DO BNR. An estimated 25% of aeration energy saving is achievable through this conversion. This study pointed out that adaptation to less DO concentration effectively impacted the P-uptake rate compared to conventional processes. Mainly the COD uptake is insensitive to DO change; however, a rather lower COD removal efficiency was indicated by increasing the DO level due to the lower ability of anaerobic carbon-source uptake and subsequent P-release. Moreover, it has been discovered that P-uptake is negatively impacted by excess COD under aerobic conditions, which may lead to P-release rather than uptake.

Anaerobic organic carbon consumption (COD_{con}) in this experiment with a focus on phosphorus removal consists of limited COD consumption for exogenous denitrification. Mainly, the COD is consumed to store intracellular carbon source (COD_{intra}) by PAO and GAO. Based on the equations proposed by [40][371], the proportion of PAO ($P_{pao,an}$) contributed in COD_{intra} in SBR_{gra} reached 0.34 molP/molC, which is lower than the reported PAO model value of 0.5 molP/molC, showing that PAO consumed a part of the CODintra for PHB generation. While higher PHB production/COD_{intra} and Glycogen generation/COD_{intra} than PAO model values (1.33 molC/molC and 0.5 molC/molC) and lower than GAO model value (1.86 molC/molC, 1.12 molC/molC) indicated contribution of both PAO and GAO in intracellular carbon storage with superiority of PAO metabolism. PAO and GAO presence as a combined microbial population in the system could potentially utilize all the available influent COD for carbon storage, which results in no COD oxidization or wastage in aerobic phase [371]. $P_{pao,an}$ in SBR_{ins} was 0.2 molP/molC, which is considerably lower than the PAO model value of 0.5 molP/molC. However, Values of 1.24 molC/molC and 0.39 molC/molC of PHB production/COD_{intra} and Glycogen generation/$COD_{intra,}$ respectively, in SBR_{ins} did not indicate a higher propensity towards GAO in the mutual contribution of PAO and GAO in intracellular C-storage. Thus, in SBR_{ins}, considerably lower anaerobic intracellular carbon uptake and storage was mainly due to phosphorus and glycogen accumulating organisms decay. On the other hand, lower activity of PAO and GAO, may trigger the activity of ordinary heterotrophs which use COD as electron donor, reducing available COD for PAO growth [100].

The microbial community variation in all SBRs indicated PAO and GAO's co-exist in the system, as shown in Figure 7-5. In the first step, with constant 2 mg/L DO concentration in all SBRs, PAO

and GAO accounted for approximately 13% of the total biomass. With the high activity of PAO, a rather promising P-removal was achieved in this step. In step 2, PAO was highly enriched in SBR_{gra}, reaching 20% of the total biomass on day 45, while GAO retained an average low level of 5%. This confirms the findings in literature where generally, reduction of aeration concentration could inhibit GAO growth while stimulating the PAO flourishment [309]. On days 20 to 45, in step 2, PAO accounted for 7% of the average total biomass in $SBR_{ins,}$ with a slight increase in GAO up to 6%. Thus, the microbial distribution is in accordance with the carbon storage characteristics wherein SBR_{ins}, PAO, and GAO proportion wasn't considerable for high PHB production and glycogen generation. In step 3, in SBR_{ins} the population of PAO and GAO continued decreasing to 10% of total biomass. While SBR_{gra} maintained its performance by reaching a stable enriched PAO and GAO coexistence.

To better understand the driving factors of EBPR in various aeration strategies, microbiological aspects, including population abundance and PAO and GAO dynamics, shall be further examined. The main functional groups involved in phosphorus removal were PAO and GAO. *Candidatus Accumulibacter, Tetrasphaera, Dechloromonas, Rhodocyclus,* and *Acinetobacter,* the main species, were detected in the SBRs at different steps of the experiment (Figure 7-5). The total abundance of PAO with a gradual decrease in SBR_{gra} increased from 8.1% to 18.5%, indicating the enhanced biological phosphorus removal performance. *Dechloromonas* and *Candidatus Accumulibacter* were relatively abundant during good phosphorus removal as some of the main drivers of EBPR performance. Nevertheless, *Rhodocyclus* and *Acinetobacter* positively correlated with P-removal suggesting their involvement as phosphorus accumulating organisms. Conversely, the GAO, consisting of gammaproteobacterial *Candidatus Competibacter* and alphaproteobacterial *Defluviicoccus,* maintained an almost same population of 5.1% in gradual

DO decrease. Apart from a gradual aeration decrease, in SBR_{ins} with an instant oxygen concentration drop, PAO showed higher sensitivity towards limited oxygen availability. *Candidatus Accumulibacter* and *Tetrasphaera* decreased from 2.5% and 1.35% to 1.25% and 0.5% respectively. Farther, *Acinetobacter* dropped to nearly undetectable by the end of step 3. Despite the importance of this genus in the EBPR system, they may have been outcompeted from the SBR by the aeration strategy applied.

Regarding the GAO, the abundance of *Competibacter* and *Defluviicoccus* in SBR_{ins} increased from 4.5% in step 1 to 6.6% in step 2, whereas a gradual decrease to 5.4% was indicated by the end of step 3. Thereby, GAO was less sensitive to DO change in the system, yet, unfavorable conditions inhibited their performance as strong competitors over PAO. Therefore, the activity of the EBPR process in SBR_{ins} was disturbed when the system experienced instant oxygen concentration change with drastically drop of PAO abundance and decrease in GAO population. In addition, results from EBPR activity in SBR_{gra} indicated an increase in the population fraction of certain phosphorus accumulating organisms. Correlation among these microorganism populations, operational factors, and the significant level of each correlation (p) was calculated. The significant contribution of *Accumulibacter Phosphatis* and *Acinetobacter* to P-removal is confirmed considering a significant correlation (p=0.037 and p=0.06 respectively) of EBPR performance with microbial activity. Moreover, *Dechloromonas* had highly significant correlations with DO concentration (p=0.012) and P-removal efficiency (p=0.004). Strong positive correlations between PAOs abundance frequency and P-removal efficiency (0.8-0.98) supported the observed high EBPR activity. However, *Tetrasphaera* indicated a non-significant correlation with DO concentration and P-removal efficiency (p=0.517, p=0.166), suggesting less insensitivity of *Tetrasphaera* abundance to aeration concentration. *Acinetobacter* also showed a rather weak correlation with P-

removal efficiency in transition from step 2 to step 3, while a sharp increase from step 1 to 2 indicated a strong positive correlation. The relationships obtained justify that the significant phosphorus-removing organism's growth drove by gradual DO decrease represented a favorable condition for higher P-removal efficiency. (A list of measured data is available in **Appendices, G, Table 10.8**).

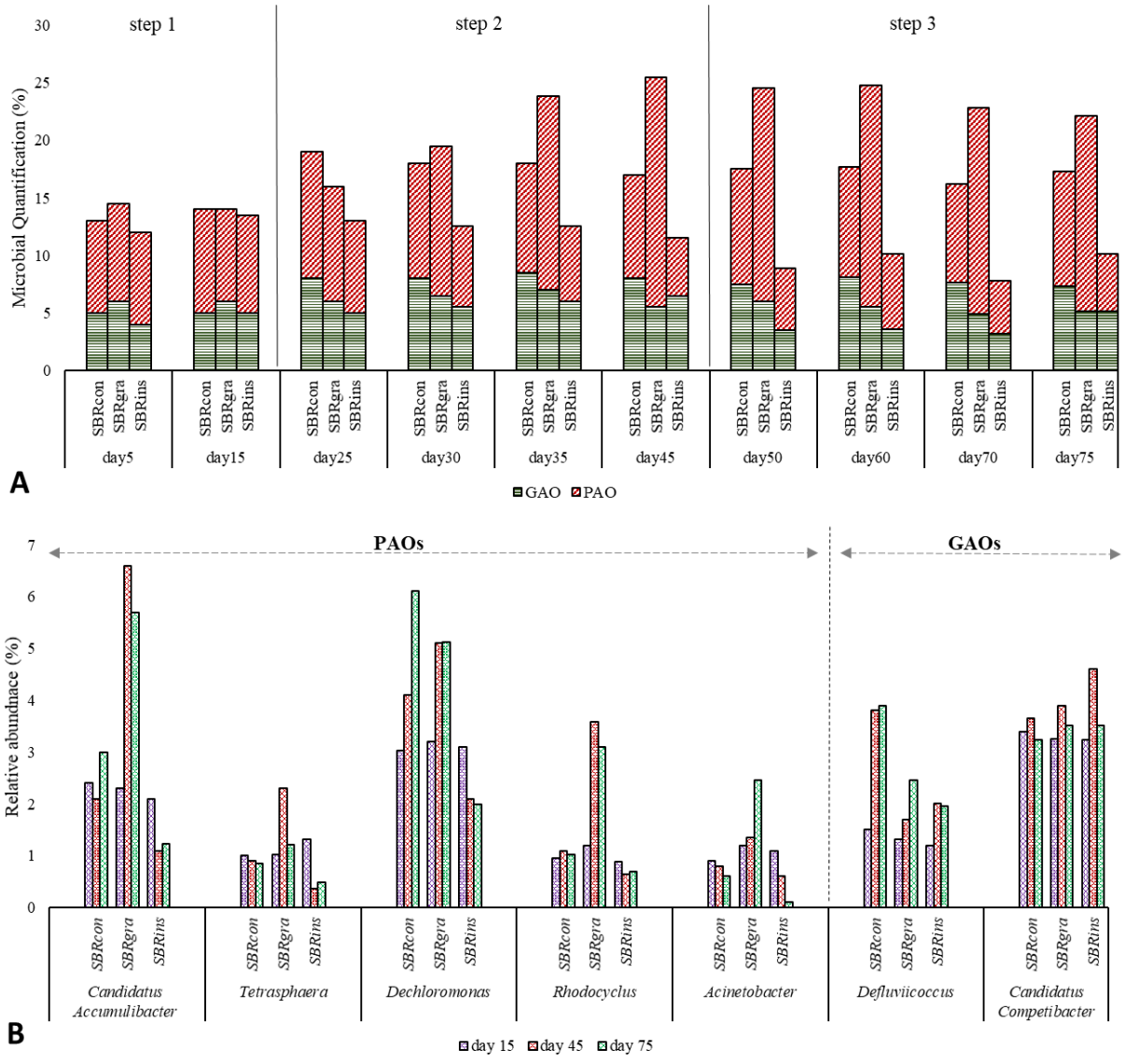


Figure 7-5 Microbial quantification of PAOs and GAOs and relative abundance of putative PAOs in all three steps of an experiment for all three SBRs

7.4.3 Batch EBPR tests with different DO concentrations

To further investigate the process performance of SBRs under a variation of DO concentrations and adaptation strategies, batch EBPR tests were operated. In these batch series, three DO concentrations of 2, 0.8, and 0.4 mg/L were tested from step 2. Furthermore, the EBPR batch test was conducted on both instant and gradual aeration decrease to extreme-low DO conditions. The EBPR activity during the batch tests (Table 7.1) was comparable to the results collected from the experimental SBR reactors. The anaerobic stoichiometry, anaerobic and aerobic kinetic rates were tested to evaluate the potential for P-release, P-uptake, P-removal, and biomass growth rate. Moreover, a kinetic analysis was performed to evaluate the effect of DO concentration and adaptability on P-uptake.

Table 7.1 stoichiometry and kinetic parameters of three SBRs

conversion	symbol	Unit	Control	Gradual	Gradual	Instant
			SBR_{con} 2 mg/L	SBR_{gra} 0.8 mg/L	SBR_{gra} 0.4 mg/L	SBR_{ins} 0.4 mg/L
anaerobic stoichiometry						
net P-released to Ac uptake ratio	Yac-PO_4-P,An	Pmol/Cmol	0.265	0.330	0.318	0.253
PHB production to acetate uptake ratio	Yac-PHA,An	Cmol/Cmol	0.775	0.997	0.837	0.737
glycogen consumption to Ac uptake ratio	Ygly/Ac, An	Cmol/Cmol	0.480	0.478	0.364	0.604
anaerobic kinetic rates						
maximum volumetric Ac uptake rate	rAc, An	Cmmol/L.h	4.808	4.840	4.840	4.408
maximum specific Ac uptake rate	qAc,An	Cmmol/Cmol.h	0.065	0.067	0.065	0.060
maximum volumetric PO_4 release rate	rpp-PO_4,An	Pmmol/L.h	1.644	1.725	1.709	1.562
maximum specific PO_4 release rate	qpp-PO_4,An	Pmmol/Cmmol.h	0.022	0.014	0.008	0.023
secondary anaerobic PO_4 release rate	rpp-PO_4-sec,An	Pmmol/L.h	0.127	0.060	0.057	0.043
anaerobic maintenance coefficient	mpp-PO_4,An	P-mol/Cmol.h	0.0017	0.0008	0.0021	0.0006
aerobic kinetic rates						
maximum volumetric PO_4 uptake rate	rPO_4-pp,Ox	Pmmol/L.h	1.560	1.988	1.466	0.846
maximum specific PO_4 uptake rate	qPO_4-pp,Ox	Pmol/Cmol.h	0.021	0.027	0.020	0.011
volumetric aerobic NH_4 consumption rate	rNH_4-bio,Ox	Nmol/L.h	0.024	0.036	0.026	0.019
maximum specific biomass growth rate	qPAO,Ox	Cmol/Cmol.h	0.0017	0.0024	0.0018	0.0013

The P-release/Acetate uptake ratio as an anaerobic stoichiometric parameter is impacted by various factors, including pH, oxygen concentration, and carbon source. This parameter is an indicator for PAOs and GAOs relative activities and abundance [144]. The profiles of P-rel/HAc indicate that each culture has a different P/HAc ratio. This is in accordance with findings in the literature, showing elemental variation in the anaerobic stoichiometry of different PAO clades [372]. In SBRs, the P-rel/HAc ratio varied between 0.2 molP/molC to 0.35 molP/molC which is in accordance with the reported range of 0.01 to 0.93 molP/molC in EBPR systems. As literature suggested, in an acetate-fed system, the ratio reaches 0.5 to 0.75 molP/molC. Yet, due to the presence of GAOs that assimilate acetate anaerobically without participation in Poly-P generation, the ratio reached a maximum of 0.35 molP/molC. A higher ratio of 0.33 and 0.318 in step and 3 in SBRgra suggests a low GAO to PAO abundance ratio, while a lower ratio of 0.223 in SBRins indicates a rather considerable GAO abundance compared to PAOs population. Glycogen consumption to acetate uptake ratio demonstrates the energy and reducing power pathways by either glycolysis or TCA cycle in anaerobic metabolism.

Regarding polymeric substance synthesis, ATP and NADH are required in PAO and GAO metabolism. However, GAOs rely on the glycolysis pathway as the main energy source Therefore, a higher glycogen/acetate uptake rate is observed compared to PAOs. Categorically, GAOs solely utilize the glycolysis pathway to supply ATP and NADH. While PAOs use glycolysis for ATP and NADH production to reduce acetate to PHB, additional ATP is produced through Poly-P conversion. Additionally, several PAO clades with more flexible metabolism use a combination of PAO and GAO pathways [372]. The SBRs exhibited an average of 0.4 of Gly/HAc. Underbalanced ATP and NADH presence, the theoretical values of Gly/HAc in acetate-fed systems are 1.1 and 0.5 for GAOs and PAOs, respectively. The results obtained suggest an

association of the glycolysis pathway in PAOs metabolism along with the presence of GAOs. However, with indicating low GAO abundance in SBR systems, the Gly/HAc ratio is mainly involved in the glycolysis pathway of PAOs. As indicated in Table 7.1, by a gradual decrease of DO to 0.4 mg/L in SBR_{gra}, the Gly/HAc reached a low level of 0.3, suggesting a change of energy source supply pathway from glycolysis to TCA cycle utilization for NADH generation. With a theoretical PHA/HAc ratio of 0.6 to 2.1 molC/molC reported, an average of 0.8 molC/molC for all SBRs indicates whether heterotrophic bacteria use acetate without any PHB storage or *Tetrasphaera* in the system. This group of PAOs tends to anaerobically take up acetate and aerobically store Poly-P without any PHB storage. The higher PHB/HAc ratio of 1 molC/molC in SBR_{gra} is attributed to the higher involvement of PAOs in EBPR performance. The results obtained from the microbial analysis and batch tests provide the evidence that under similar operating conditions such as carbon source, pH, P/C ratio in the same SBR system, change in oxygen concentration results in different dominancy of PAO clades that affects the anaerobic stoichiometry specifically the anaerobic P-release rate [372].

The aerobic kinetic parameters were estimated to describe the aerobic metabolism of microorganisms. As shown in the table, in an instant DO decrease at 0.4 mg/L, with an incomplete P-uptake, the $r_{PO4\text{-}PP,\ OX}$ was 42% lower than gradual DO decrease at 0.4 mg/L, suggesting that a favor for EBPR process at low DO concentration with gradual decrease. As shown in the table, there were considerable fluctuations in the anaerobic secondary phosphorus release rate and maintenance coefficient. Although the batch test for SBR_{con} showed high anaerobic phosphorus release and aerobic phosphorus uptake rates, high rates of secondary phosphorus release were observed, leading to lower P-removal efficiencies than SBR_{gra}. The gradual DO decrease strategy's

higher P-uptake ratio and biomass growth rate suggest an optimized reactor performance with higher PAO enrichment.

7.5 Conclusion

1. The phosphorus profile in the EBPR system is strongly dependent on oxygen concentration. Therefore, enhanced phosphorus-removing microorganism growth due to an efficient aeration strategy could take full advantage of the carbon source for nutrient removal.
2. Although lower DO concentration commonly shows higher EBPR performance, lower performance was achieved with a rapid decrease in DO concentration from 2 mg/L to 0.4 mg/L and limited time for biomass adaptation to the operational factors.
3. A steady decrease in the DO concentration from 2 mg/L to 0.8 mg/L followed by a decrease to 0.4 mg/L in a stable EBPR system aided in remaining stable and constructively impacted the system's performance with a P-removal efficiency of 90%.
4. The gradual DO decrease promoted anaerobic PAO metabolism by a considerable increase in net P-released to acetate uptake ratio, PHB production to acetate uptake ratio, and a decrease in glycogen consumption to acetate uptake ratio. Thus, higher PAO abundance driven by gradual oxygen concentration decrease illustrated the higher degree of phosphorus removal.

7.6 Acknowledgement

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7.7 Funding

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8 Development of dynamic BioWin® model simulation for SBR mode EBPR system with micro-aeration strategies

Preface:

This chapter of the thesis is the author's original work based on the experimental apparatus and data. The author, Parnian Izadi, is responsible for all major areas of concept formation, data collection and analysis, and the majority of the manuscript composition.

8.1 Abstract

An effective way to predict biological treatment and effluent quality is to apply mathematical modeling. However, model calibration of sensitive parameters is required for reaching the high accuracy of the simulation. Steady operational data from four parallel sequential batch reactors (SBR) consisting of anaerobic and aerobic phases, throughout a biological nutrient removal process at four and six-hour cyclic period, dissolved oxygen concentration of 0.8 and 0.4 mg/L was used to calibrate and compare the simulation model in BioWin®. Carbonaceous and nutrient fractions and kinetic parameters of biomass were used to carry out the calibration process. The developed BioWin® model could predict effluent characteristics, including total phosphorus, phosphate, nitrate, and nitrite concentration, with an accuracy higher than 80%. The results from this study indicate that a feasible way of monitoring treatment performance in SBR-EBPR treating high concentration phosphorus is to use BioWin® modeling with the incorporation of sensitivity analysis, calibration, and validation steps.

8.2 Introduction

Recognition of eutrophication as an environmentally destructive phenomenon has guided the initiation of comprehensive projects for reducing the leading contributor, Phosphorus (P), from effluents of wastewater treatment plants (WWTP). Resolving the contamination and preventing the introduction of nutrients from entering waterbodies justify implementing P-removing technologies [18]. Most treatment approaches rely on Physico-chemical, biological, or a

combination of both strategies [374]. Enhanced biological phosphorus removal (EBPR) is widely applied in WWTPs as an efficient and sustainable biological pathway that is dependable on the enrichment of select microorganisms, phosphorus accumulating organisms (PAO) in activated sludge systems [28]. EBPR as a modified activated sludge system with anaerobic/aerobic unit processes, can remove phosphorus in the liquid phase by converting it to poly-phosphate in the sludge phase [375]. Anaerobically, PAOs take up available carbon sources such as volatile fatty acids (VFA) and store them as polymeric substances, polyhydroxyalkanoates (PHA), with the mainly intracellular Poly-P hydrolysis and partially from glycolysis pathway. While reducing equivalents are yielded through glycogen degradation. Under the aerobic phase, with the availability of electron acceptors and PHA as energy sources, excess P-uptake, Poly-P formation, cellular growth, and glycogen replenishment occur. P-removal is achieved by higher aerobic P-uptake rather than anaerobic P-release due to biomass growth and through Poly-P enriched sludge wastage [28].

EBPR, as a designed and implemented system to WWTPs, can reach very low phosphorus concentration levels in the effluent, with significantly lower operating cost, sludge production, and chemical requirement. Moreover, most existing conventional systems can conveniently be retrofitted for EBPR to remove phosphorus efficiently. Yet, EBPR, as a complex process that is not well understood, similar to all biological processes with non-linear dynamics and large uncertainty [376], is prone to unpredictable failures due to microbial activity loss, which necessitates the introduction of chemical treatment to meet regulatory limits. Moreover, the process performance is prominently sensitive to various factors such as wastewater characteristics, temperature, oxygen concentration, and other operational conditions [376]. Notwithstanding, in

most cases, troubleshooting in both plants and lab-scale processes for failure and inconsistent results can be very time-consuming and challenging [375] [18].

Moreover, the considerable resource has been already supplied to WWTPs infrastructure, including the EBPR unit. At the same time, there is a need to upgrade existing plants to increase nutrient efficiency and loading with maximizing usage to reach more stringent effluent demand [377]. Therefore, studies have focused on numerical simulation methods for water quality prediction and operational effects under various treatment cases to improve technical design and optimization with a limited time frame, lower cost, and risk involved [378].

Mathematical modeling with a service, advice, and analysis role [377] has turned into a practical and potent practice through years of development to support implementing various strategies, evaluating discharge standards, and predicting actual process efficiency [379] with quite less time and cost investment. Two main modeling concepts of activated sludge model (ASM) and metabolic models or a combination of both are currently in use. An activated sludge dynamic model is indicated as a fundamental structure for plant design and management, with calibration procedure as its main impediment [380]. Further, computer software was promoted to simulate the diverse treatment processes [378]. Regarding engineering purposes, attaining a balance between a simplified and complicated mechanistic approach is critical [381]. One-dimensional completely dynamic and steady-state models are used in WWTPs simulation such as stratified dynamic modeling in AQUASIM software or ASM models by International Water Association (IWA) available at software packages including Simba® (Ifak GmbH, Magdeburg, Germany), ASIM® (EAWAG, Switzerland), EFOR® (DHI Inc., Denmark), GPS-X® (Hydromantis Inc., Hamilton, ON) and BioWin® (Envirosim Associates Ltd., Burlington, ON) [381]. A well-known modeling approach for EBPR systems combined with chemical oxygen demand and nitrogen removal,

oxygen requirement, and sludge generation is BioWin® biological model as a state-of-the-art reference for BNR modeling [287] due to its high flexibility and user-friendly environment. BioWin® as a WW model simulator is a powerful tool used by various water authorities, municipalities, and manufacturers to provide beneficial insights on applied processes to WWTPs. As a robust asset for selecting optimal treatments, reducing cost and energy consumption, as wll as for analysis and prediction purposes, BioWin® includes many process modules to configure treatment processes [382] in one single model to reduce the complexity of WWTP modeling.

In a study conducted by *Vitanza et al.* [380], a homemade developed ASM model was calibrated based on data gathered from three WWTPs and implemented to BioWin® software, where the simulation results and the BioWin® prediction was in agreement with the historical data obtained from WWTPs. Which indicated the ability of BioWin® in predicting nitrification and denitrification performance and effluent COD and ammonia profile. The advantage of BioWin® in predicting the effluent values and its adaptability even with the change in effluent quality limitations and regulations was demonstrated in this study. Considering the prediction capability of BioWin® software, *Dorofeev et al.* conducted a study on the comparison of mathematical modeling and experimental data in the ANNAMOX process. The mathematical modeling and experimentation results are highly correlated, which validates the reliability of BioWin® software as a prediction tool for ANNAMOX microbial consortium behavioral shift [383]. Consistent results were also obtained between measured and model values after calibration in a dynamic simulation study on advanced nitrate-nitrogen removal in a deep-bed denitrification filter (DBDNF) [378].

Contrary to previous studies mentioned, *Oleyiblo et al.* investigated improvement strategies in the performance of WWTPs. By calibrating the BioWin® software based on WW characteristics and

evaluating various strategies, BioWin® successfully concluded in a possibly effective way to waste sludge from existing units (aeration tank and secondary clarifier) rather than an increased cost constructing new units, including oxidation ditch or equalization tank [377]. Moreover, in a study by *Lei et al.*, the effectiveness of adding units regarding the performance and cost in Avondale WWTP expansion project was evaluated through BioWin® modeling and simulation. Alternative options of adding influent equalization basins and primary clarifiers, expansion to the aeration basins and secondary clarifiers, and conversion of existing activated sludge process to membrane bioreactors were evaluated. Eventually the model indicated the addition of equalization tank as the most cost-effective option with high effluent quality [384]. Yet, based on comprehensive literature review and research, there is a lack of software designs for modeling biological nutrient removal in sequential batch reactor (SBR), where there are very few models available to accurately predict biological phosphorus removal in a micro-aeration SBR system.

Therefore, in this study the effect of DO concentration in two micro-levels of 0.4 and 0.8 mg/L with continuous aeration at aerobic HRT of 120 and 200 minutes is investigated by understanding the various microbial communities for achieving high P-removal. Moreover, EBPR performance in terms of anaerobic and aerobic phosphorus profile, P-removal, and aerobic kinetics were evaluated. Based on the data collection process from experimental plan, BioWin® process model of micro-aerated SBR was developed and calibrated. The primary goal of this study was to develop a simulation model as an evaluation and comparison tool for micro-aerated SBR performance. Moreover, following the development and calibration of the BioWin® model multiple aeration strategies (experiments) were evaluated, using the calibrated model, including modification in dissolved oxygen (DO) concentration and the aerobic hydraulic retention time (HRT). This study aimed to develop a primary simulation model as a start point for future studies on micro-aerated

SBR-EBPR for prospective evaluation of treatment performance, aeration demand, energy and chemical costs through modification and enhancement of the current model.

8.3 Material and methods

8.3.1 Experimental setup

Experiments were conducted in bench-scale sequential batch reactors (SBR) with anaerobic-feeding/aerobic/settling/decanting phases with a total operating volume of 5 L and an internal diameter of 25 cm. The SBRs were operated at ambient temperature, inoculated with returned activated sludge (RAS) from the Humber Treatment Plant, Ontario, Canada. The operating systems were set up with cyclic periods of 4 and 6 hours. The 4-hour cycle consisted of 15 min feeding, 60 min anaerobic, 120 min aerobic followed by 30 min settling, and 15 min decanting. The 6-hour cycle consisted of 15 min feeding, 90 min anaerobic, 200 min aerobic followed by 40 min settling and 15 min decanting as shown in Figure 8-1. Air was introduced using an on-line DO detector from the lower section of the reactors to keep the DO levels in the proper range of each phase of the experiment. The reactors were initially operated at 2 mg/L of oxygen concentration to achieve acclimation of the microorganisms. The mixing speed was controlled at 50 to 100 rpm, based on the DO concentration, adjusting the speed with each DO variation with a deviation of ±0.2 mg/L. The mixing profile consisted of constant mixing in settling and decanting phases and no mixing in anaerobic and aerobic phases. Two DO concentrations of 0.8 mg/L and 0.4 mg/L were experimented, to evaluate micro-aeration in SBR. ORP, pH, and DO concentration were consistently monitored through controlling system.

The synthetic wastewater comprising acetate, phosphate, and required nutrients was fed into the bioreactors during the anaerobic-feeding phase. The synthetic wastewater used for this study approximately contained (per liter): 0.85 g $NaAc.3H_2O$ (400 mg COD/L) as carbon source, 107

mg NH_4C1 (28 mg N/L), 75.5 mg $NaH_2PO_4.2H_2O$ (15 mg P/L), 90 mg $MgSO_4.7H_2O$, 14 mg $CaCl_2.2H_2O$, 1 mg yeast extract, and 0.3 mL nutrient solution. The concentrated nutrient solution contained, per liter: 1.5 g $FeCl_3.6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4.5H_2O$, 0.18 g KI, 0.12 g $MnC1_2.4H_2O$, 0.06 g $Na_2MoO_4.2H_2O$, 0.12 g $ZnSO_4.7H_2O$, 0.15 g $CoCl_2.6H_2O$, and 10 g EDTA. The pH of the feed was adjusted at 7.5±0.1. The initial chemical oxygen demand was in the range of 300-400 mg/L containing acetate as the sole carbon source fed to the system. The SBR reactors were routinely monitored through regular sampling for VFA, PHA, and glycogen through each cycle. Chemical analyses such as total suspended solids (TSS) and volatile suspended solids (VSS) were measured on samples from the end of the aerobic phase.

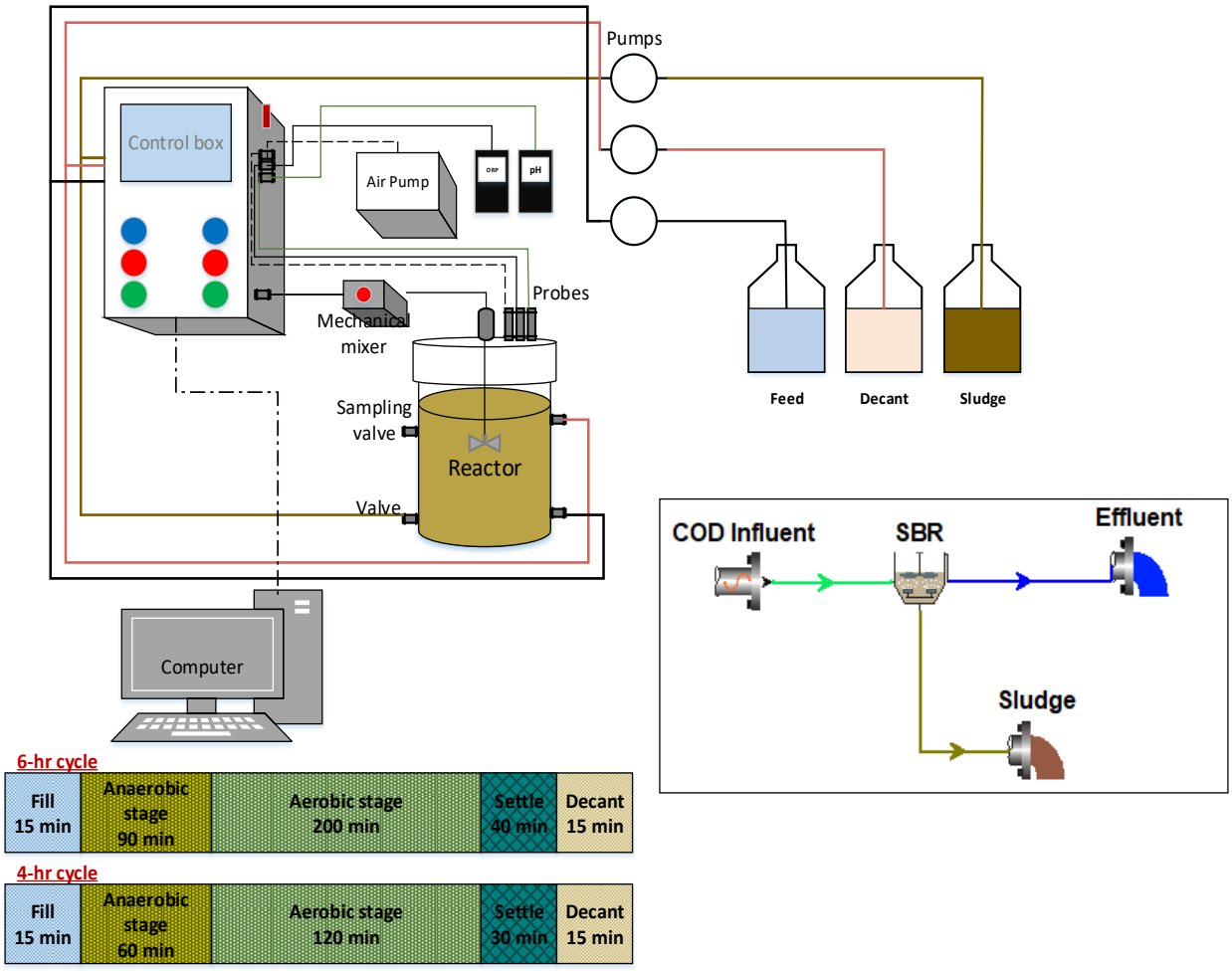

Figure 8-1 schematic diagram, 4 hour and 6 hour cyclic periods and BioWin® flow diagram of SBR-mode reactors for EBPR experimentation

8.3.2 Analytical methods

Total solids (TS), volatile solids (VS), TSS, VSS, total chemical oxygen demand (TCOD), and soluble chemical oxygen demand (SCOD) were determined using the standard methods (APHA 2005). pH was determined using a digital pH meter (hach HQ440d multi). Phosphate, nitrate, nitrite, and ammonia were simultaneously determined through ion chromatography by Thermo Scientific™ Dionex™ Integrion™ HPIC™ system. In advance of all IC measurements, samples were properly diluted with deionized water and passed through a membrane filter (0.45 µm). For PAO enrichment confirmation and evaluation in the reactors, initially, samples were analyzed for

particle size distribution using an aqueous liquid module (ALM) in LS 13 320 Particle Sizing Analyzer, later on, samples were analyzed using PCR (RNA was extracted, amplified using PCR then the products were subjected to gel electrophoresis to confirm the existence of PAOs). Total RNA was refined from the bacterial lysate with the support of the QIAGEN RNeasy Mini Kit. The extracted RNA of the samples was treated following the QIAGEN One-Step RT-PCR Kit protocol. The successful amplification of the desired bacterial RNA confirms the presence of the investigated bacterial family. The volatile fatty acid was assayed by an SRI gas chromatography (GC) equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). PHB was extracted from cellular biomass and quantified with gas chromatography using the following procedure. Primarily, 10-15 mg of lyophilized biomass were collected, and 2 mL of acidified methanol (3% sulphuric acid), as well as 2 mL of chloroform, were added in a glass vial. After gentle mixing, the cocktail was heated at 100 ∘C for 3.5 hours, cooled down to room temperature afterward. 1 mL of deionized water was added later on, and the mixture was vortexed for 1 minute and then left until phase separation was achieved. The lower organic phase was tested for PHB quantification using SRI gas chromatography equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). The temperature program was 1 min 80 ∘C, 10 ∘C min-1, 180 ∘C for 4 min. Results were compared with standard curves obtained using PHB standards (Sigma Aldrich). Benzoic acid was used as an internal standard to increase accuracy. Intracellular glycogen is determined via digestion and hydrolysis of glucose. Glucose will be analyzed enzymatically (Sigma–Aldrich PGO enzyme kit, Saint Louis, Missouri, USA). Sample absorbance is measured at 425 nm using a Spectronic spectrophotometer.

8.3.3 Modeling and Simulation

8.3.3.1 General model development

The biological WW process was carried out in BioWin® V.4.1 (Envirosim Associates Ltd., Burlington, ON), where the experimental results were calibrated and modeled using the software. BioWin® uses the integrated activated sludge/anaerobic digestion (AS/AD) model, considered as the general model [386]. When developing a BioWin® model, key parameters and input values are required as inputs. The important parameters and values are influent components, the physical design and arrangement (reactor size, configuration and aeration conditions) and kinetic and stoichiometric parameters. In BioWin® modeling, the influent components demonstrate WW characteristics, whereas the kinetic and stoichiometric parameters are for calibration and evaluation purposes.

In the general BioWin® model, 21 fractions were specified as compositions of the influent wastewater. The fractions consist of ammonium, particulate organic nitrogen, soluble non-biodegradable TKN, phosphate, nitrogen-to-COD ratio, phosphorus-to-COD ratio, and fractions for autochthonous biomass [385]. In addition, the characteristics indicated in BioWin® are COD based, where it calculated TSS, VSS, and BOD by the specification of un-biodegradable particulate and non-colloidal slowly biodegradable fractions.

To model the EBPR process, in BioWin®, an SBR-mode reactor was used, a single-zone SBR in which the feed is introduced into the zone where the decanters are located. The cycle time, fill/react time, decant duration, aeration pattern, and sludge wastage information were specified. Physical information and flow distribution details including flowrate, dimensions of SBRs and bioreactor inputs were used as the physical layout of the treatment system.

BioWin® with an influent specifier, determines the WW characteristics through input measurements for inflow, COD, BOD, ammonia, oxidized nitrogen, TKN, orthophosphate, TP, alkalinity, VSS, TSS, acetate and pH. The various fractions for carbonaceous and nutrients of the feed were estimated based on the actual concentrations from the synthetic wastewater and were used as inputs for the model. After inputting the values, carbonaceous and nutrient fractions were estimated to match the measured values. The various carbonaceous and nutrient fractions based on the wastewater influent are listed in Table 8.1**,** which provides information on the fractions with default and changed values. The simulations were performed with the assumption of constant process temperature of 20°C. Autotrophic and heterotrophic kinetic parameters were initially set to default values. As SBR-mode reactors are never at steady-state, the system was simulated as dynamic.

Table 8.1 Default and assumed carbonaceous and nutrient fractions in synthetic wastewater

Element name	*Unit*	*Default*	*Influent*
Fbs - Readily biodegradable (including Acetate)	**[gCOD/g COD]**	0.16	0.21
Fac - Acetate	**[gCOD/g COD]**	0.15	0.46
Fxsp - Non-colloidal slowly biodegradable	**[gCOD/g COD]**	0.75	0.75
Fus - Un-biodegradable soluble	**[gCOD/g COD]**	0.05	0.05
Fup - Un-biodegradable particulate	**[gCOD/g COD]**	0.13	0.13
Fna - Ammonia	**[gNH3-N/gTKN]**	0.66	0.68
Fnox - Particulate organic nitrogen	**[gN/g N]**	0.5	0.5
Fnus - Soluble unbiodegradable TKN	**[gN/gN]**	0.02	0.02
FupN - N:COD ratio for unbiodegradable part. COD	**[gN/gCOD]**	0.035	0.035
Fpo4 - Phosphate	**[gPO4-P/gTP]**	0.5	0.74
FupP - P:COD ratio for un-biodegradable part. COD	**[gP/gCOD]**	0.011	0.011
FZbh - OHO COD fraction	**[gCOD/gCOD]**	0.02	0.02
FZbm - Methylotroph COD fraction	**[gCOD/gCOD]**	1.000E-4	1.000E-4
FZaob - AOB COD fraction	**[gCOD/gCOD]**	1.000E-4	1.000E-4
FZnob - NOB COD fraction	**[gCOD/gCOD]**	1.000E-4	1.000E-4
FZaao - AAO COD fraction	**[gCOD/gCOD]**	1.000E-4	1.000E-4
FZbp - PAO COD fraction	**[gCOD/gCOD]**	1.000E-4	1.000E-4
FZbpa - Propionic acetogens COD fraction	**[gCOD/gCOD]**	1.000E-4	1.000E-4
FZbam - Acetoclastic methanogens COD fraction	**[gCOD/gCOD]**	1.000E-4	1.000E-4
FZbhm - H2-utilizing methanogens COD fraction	**[gCOD/gCOD]**	1.000E-4	1.000E-4
FZe - Endogenous products COD fraction	**[gCOD/gCOD]**	0	0

8.3.3.2 Model calibration, validation and sensitivity analysis

After investigation on the BioWin® model inputs, a list of needed parameters for calibration of model was developed. Almost all parameters required for calibration including COD, VSS, acetate concentration, BOD, pH, TKN were routinely analyzed and measured during the experimentation process. However, there were some missing analyses needed for calibration, in which default values of BioWin® were used (including CBOD, and FCOD). The data were organized based on the 60 days of experimentation. The initial 10 days of experiment was used for model development which was also accurate for WW characteristics. Once calibrated to the data from days 10 to 40, the model was simulated using the data from days 40 to 60, to determine the accuracy of the model.

Following the plant configuration setup in simulator and assumption of typical influent WW components and default kinetic and stoichiometry; physical values, unit dimensions, influent flows, temperature and DO values were specified. After layout of WW characteristics and fractions, during the calibration procedure, effluent solid concentration was checked and adjusted if necessary. If the values for solids including TSS and VSS did not match the measured data, the stoichiometric parameters for OHOs and PAOs were modified to match. Ammonia profile and concentration was checked in the SBR reactor and effluent, if in need of adjustment, iterative optimization of specific growth rate of AOBs were conducted. Eventually orthophosphate and TP were checked both in SBR reactor and effluent at different stages. If P-release and P-uptake profile were not available, the DO concentration, VFA availability and nitrate loading were reviewed. Model parameter adjustment is required to reach high effluent quality in EBPR system.

Wastewater treatment models have been developed to predict various biological processes including EBPR system, yet limited information on EBPR mechanism prevents the development of models. Therefore, the application of EBPR models are highly dependable on the determined

parameters and characteristics based on the experimental measurements. These parameters are mainly site specific and cannot be applied for other systems with different physiological states. Since not all parameters could be measured, they have to be estimated for model utilization. The quantification of parameter effect can be done through determining the sensitivity of model output to different input values. The values with the highest impact on the effluent concentration are selected as the calibration variable parameters. In this study, since the results were used for evaluation of phosphorus removal treatment system, then relative sensitivity of model output of TP was considered to be of greatest importance and was used to determine the key growth parameters. Based on the variation on kinetic and stoichiometric parameters, upper and lower limits were provided for sensitivity analysis to investigate the effect on effluent TP concentration. The sensitive parameters were further analyzed through identifying the range of each value, comparison of parameter ranges with literature and selection of parameter values to design SBR-EBPR in BioWin®. With applying sensitivity analysis, the most critical EBPR parameters enabled EBPR in BioWin® simulation.

8.3.3.3 Statistical analysis

For the statistical analysis, excel sheet and Matlab® Statistics and Machine Learning Toolbox™ (Matlab R2015a, MathWorks, USA) were employed. The t-test (for two groups of samples), and One-way ANOVA analysis were applied with to evaluate the significance of differences in efficiency, performance and fit between models and to study the effect of oxygen concentration on P-removal efficiency, P-effluent concentration and the pattern of changes and their significance difference. Average percentage error (APE) was calculated as a measure of the accuracy of the model. It was calculated as the sum of the absolute difference of experimental and predicted values divided by the experimental values, averaged over the number of data points. NSE was performed to evaluate the model performance. NSE is calculated as one minus the ratio of error variance of

the model values divided by the variance of the experimental data. Coefficient of determination (R^2) was also calculated as a measure of fit that indicated the variation of modeled values explained by the experimental data. In the SBRs correlation among operational and environmental parameters was evaluated. Correlation coefficient was indicated and the linear relationship of experimental and model values were evaluated.

8.4 Results and discussion

8.4.1 Process configuration and Total performance

Each SBR was controlled and monitored at the specific DO concentration of 0.4 mg/L and 0.8 mg/L to determine the performance of continuous micro-aeration at aerobic stages. As expected in the EBPR process, VFAs were taken up anaerobically in each reactor, with simultaneous PHB production and glycogen degradation. Anaerobic P-release was observed in all aeration strategies, yet the final anaerobic P-concentration vary due to DO-level/duration impact. Aerobically, PHB degradation, glycogen replenishment, and P-uptake take place. Lowering the aeration duration and increasing the micro-aeration from 0.4 mg/L to 0.8 mg/L increased P-release and uptake quantity, while less impact was observed in the case of P-removal efficiency. Figure 8-2, depicts the experimental profiles for some significant measurements, including PHB, glycogen, VFA, and phosphorus obtained from cycles of the SBR-mode reactors. As indicated in Figure 8-2.B, in SBR0.8-120 with the highest P-release/uptake profile, during the 75-minute fill/anaerobic mixed react period, P-levels increased from 0.5 mmol/L to 2.96 mmol/L and COD decreased from 350 mg/L down to 53 mg/L. At the end of the aerobic phase, the P-levels were lower than the detection level, while the COD level was in a range of 20-25 mg/L. By increasing the aerobic HRT to 200 minutes at the same DO levels, the anaerobic P- levels increased to 3.06 mg/L with a gradual increase in P-release rate. SBR0.4-200 with only 1.85 mmol/L of P-release, followed by a

relatively lower P-uptake description with Residual-P by the end of the aerobic phase. As shown in Figure 8-2, the amount of PHB stored in the sludge was higher in the anaerobic/aerobic cycles of 75-min/120-min at 0.8 mg/L of DO concentration, with a PHB-production/VFA-uptake ratio of 0.92. Yet as shown in the figure, SBR0.4-120 and SBR0.8-200 produced relatively similar quantities of PHB anaerobically. However, due to residual VFA availability in SBR0.8-200 by the end of the anaerobic period, a higher PHB-production/VFA-uptake ratio was established. Considering the P-release/VFA uptake and PHB-production/VFA uptake ratios in all SBRs, the higher PHB storage did not linearly correspond to higher P-release in all systems. Although, SBR0.8-200 had the highest PHB production and P-release rate. Glycogen storage profile remained mainly consistent, yet a higher increase/decrease rates in anaerobic/aerobic phases of SBR0.4-120 indicated a higher reducing power and energy requirement as the TCA cycle might have less efficiency in covering the reducing power/energy demand in comparison to other scenarios. Glycogen consumption/VFA uptake rates of 0.2-0.3 C-mmol/C-mmol were indicated in all SBRs, which illustrates the similar glycogen involvement in the systems. Glycogen consumption/VFA uptake rate in the range of 0.2 to 0.3 C-mmol/C-mmol and P-release/VFA uptake ratio of 0.8-0.9 P-mmol/C-mmol are in accordance with higher phosphorus accumulating microorganism's abundance and lower GAO population in the process.

The P-release to VFA uptake ratio is an adequate demonstration of EBPR activity. The P-release/VFA-uptake ratios calculated were in a range of 0.5-0.7 P-mol/C-mol in all SBRs. This ratio has stabilized at approximately 0.65 P-mol/C-mol in SBR0.8-200, while in the case of other scenarios, an approximate 0.53 P-mol/C-mol, interpreted a rather lower P-release, normalized by the amount of C-source uptake. SBR0.8-120 has shown the highest net P-release (mmol). However, by considering the amount of VFA uptake, it can be depicted that it released more

phosphorus anaerobically with the same VFA uptake quantity SBR0.8-200. When the aeration duration increased, the P-uptake behavior was slightly affected mainly in lower DO levels, where the P-content of influent increased to considerable amounts. The maximum volumetric P-uptake rate of 0.65 P-mmol/L.h in SBR0.4-320 justifies the lower performance comparing to an average of 0.9 P-mmol/L.h in other applied strategies.

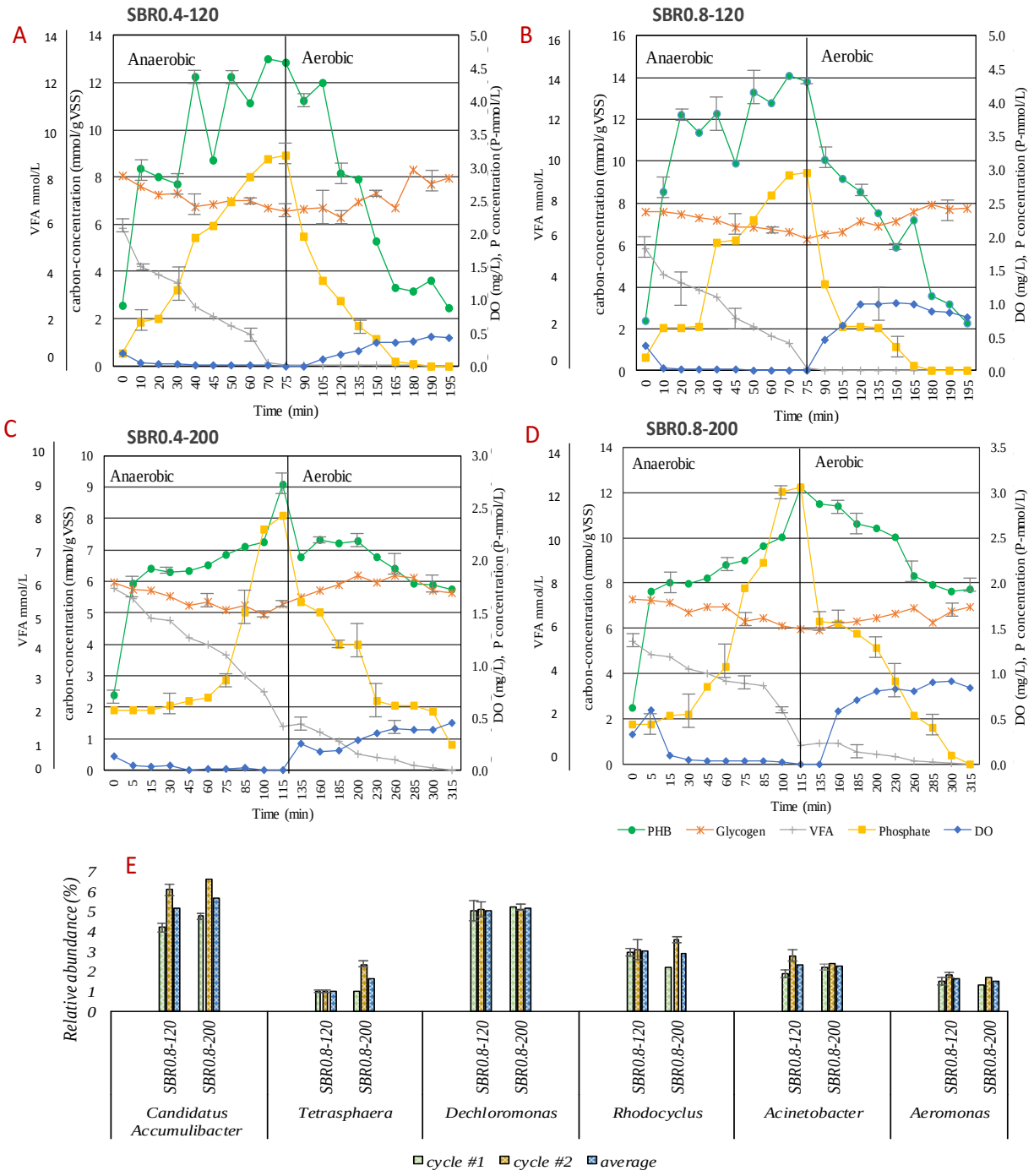

Figure 8-2 A,B,C,D. PHB, glycogen, VFA, phosphorus, and oxygen concentration cyclic profile. E. Microbial quantification and relative abundance of putative PAOs in 0.8 mg/L DO concentration

For a better understanding of the advanced performance of P-removal, analysis was done on a cyclic period for SBR0.8-120 and SBR0.8-200. The microbial community variation and activities

of functional microorganisms in the P-removal and intracellular carbon transformation were established based on the microbiology analysis and stoichiometry methodology. In these EBPR-SBRs, *Proteobacteria, Firmicutes,* and *Bacteriodetes* were the predominant phyla accounting for approximately 91% of the total phyla. Generally, *Firmicutes* and *Bacteriodetes* are observed for organic material degradation, hydrolysis, and acidification. Moreover, the literature suggests the ability of *Firmicutes* in degrading organic compounds to short-chain fatty acids (SCVFA), which provides further available carbon sources for P-removal. *As the predominant phylum in nutrient removal systems, Proteobacteria* accounted for 39.2% and 35.4% of SBR0.8-200 and SBR0.8-120, respectively. The main functional groups accounted for P-removal, PAOs, and DPAOs were detected as *Candidatus Accumulibacter, Tetrasphaera, Dechloromonas, Rhodocyclus, Acinetobacter,* and *Aeromonas* as the top genera. In contrast, other genera, including *Thauera* and *Zoogloea,* were detected as possible nutrient-removing organisms. The total possible abundance of P-removing organisms in SBR0.8-200 and SBR0.8-120 were 29.6% and 22.6%. *Dechloromonas* and *Candidatus Accumulibacter* with an average abundance of 8.1% and 8.7% were detected as critical P-removing genera. *Rhodocyclus* and *Acinetobacter* with relatively lower abundance in a range of 2-3.5%, yet positively correlated with efficient P-removal performance. As shown in Figure 8-2, at constant 0.8 mg/L of DO concentration, increasing the HRT by 80 minutes gradually increased the P-removing abundance by 3%, where the process performance was slightly improved.

More than 87% of the SCOD was removed in all configurations, leaving 20 to 50 mg/L of SCOD with an average of 25 mg/L in the final effluent. A well-balanced EBPR process and a low-variation of SCOD in the feed resulted in a stable final effluent SCOD concentration. Final effluent phosphorus concentrations were as low as 1.1±0.3 mg/L and 1.2±0.4 mg/L in SBR0.8-200 and

SBR0.8-120, yet by decreasing the oxygen concentration to 0.4 mg/L, the P-effluent concentration jumped to an average of 3.1±0.5 mg/L. In P-removal, SBR0.8-200 gave the highest removal at approximately 92% than all the other configurations. By maintaining a consistent DO level at 0.8 mg/L, the balanced HRT in the anaerobic and aerobic zone with AN/AE ratio of 0.57 suggests an improvement in P-release and P-uptake to higher P-removal. In the light of the observations for all four configurations, SBR0.8-200 was selected for further statistical investigation and was exploited by operational changes for increased performance.

8.4.2 Solid, nutrient and aeration calibration

The calibration process consists of effluent solid and nutrient as well as aeration calibration. Once the primary influent values were optimized and carbonaceous and nutrient fractions were adjusted, calibration took place to compare measured and model values. when calibrating the solids, the OHO aerobic yield of 0.66 was adjusted to 0.74. In case of nitrogen, nitrification performance was analyzed. Initially, the same global AOB growth rate was used in case of all SBR reactors, however with limited nitrogen removal in the experiments and a focus of P-removal, this value was considered inappropriate. Iterative analysis was performed on AOB growth rate until the effluent ammonia and nitrification process were adjusted and optimized parameter was achieved. To improve calibration, in all SBRs, SOB maximum growth rate was adjusted to 0.73 1/d. This value is lower than the typical AOB growth rate (0.9 1/d). It is assumed that the value is in line with limited nitrification achieved at average SRT in experimental systems. The NOB growth rate was also adjusted to 1.2 1/d from a default value of 0.7, due to higher concentration of nitrate comparing to nitrite in the effluent and a possible match of the measured concentration with model values for oxidized nitrogen.

With calibration of solids and nitrification, performance of phosphorus removal was then evaluated. Looking into the primary results, achieving the measured P-removal with the default values was not possible. Therefore, the PAO maximum growth rate was adjusted based on iterative process to reach high prediction of TP and orthophosphate effluent concentration to measured data. The PAO growth rate was adjusted from 0.95 1/d to 1.5 1/d for all SBRs, based on the treatment performance attained in the system.

For the final step of calibration, the aeration demand and parameters were checked. The aeration parameters available in BioWin® including Henry's law constant, mass transfer and diffuser were not adjusted and were kept at default values. only the DO input values for each zone were updated to match the sequential anaerobic and micro-aerobic phases (0.4 and 0.8 mg/L) of SBR-mode reactors.

8.4.3 Sensitivity analysis

The lab-scale SBR model was established on BioWin® software and was calibrated to the actual results reached in the experiments. The input of the model included the influent (synthetic wastewater) characteristics and the operational conditions, including DO concentration, cyclic periods, and retention times. The model output included the effluent characteristics as well as the flow and aeration profile of SBR. Sensitivity analysis was performed to determine the key parameters.

Regarding the simulation parameters to which TP was extremely sensitive and could not maintain EBPR performance, were considered critical. As expected EBPR performance was not affected by the autotrophic growth parameters, showing a relative sensitivity of zero. While phosphorus concentration was sensitive to non-PAO and PAO heterotrophic parameters. In the case of PAOs, various parameters are of importance. P/Ac release ratio and yield of low PP are two of the most

important stoichiometric coefficients. The P/Ac release ratio defines the amount of P-released for 1 mg of acetate sequestered in the form of PHA. At the same time, the yield of low PP indicates the fraction of phosphorus that is stored as releasable polyphosphate. In this study, the P/Ac release ratio and yield of low PP were kept at the default values of 0.51 mgP/mgCOD and 0.94 mgP/mgP, respectively. Aside from these parameters, PHA yield on sequestration demonstrates the stored-PHA amount when 1 mg of acetate is consumed, and aerobic P/PHA uptake indicates the amount of Stored-P per unit of PHA consumed in the aerobic phase, are of importance. Therefore, these parameters were again kept at the default values. The yield of PHA on sequestration (mgP/mgP) and aerobic P/PHA uptake was equal to 0.889 and 0.93, respectively. In this research, the parameters associated with PAOs that were mentioned were not modified in the calibration process. However, the adjusted kinetic and stoichiometric parameters that have proven as sensitive values are available in Table 8.2.

Table 8.2 adjusted kinetic and stoichiometric parameters

	Parameter	***Unit***	***Default value***	***Calibrated value***
		kinetics		
OHO	Max. spec. growth rate	[1/d]	3.2	2.5
	Substrate half sat.	[mgCOD/L]	5	10
	Anoxic growth factor	[-]	0.5	0.45
	Aerobic decay rate	[1/d]	0.62	0.9
PAO	Max. spec. growth rate	[1/d]	0.95	1.5
	Max. spec. growth rate, P-limited	[1/d]	0.42	0.6
	Aerobic/anoxic decay rate	[1/d]	0.1	0.15
	Anaerobic decay rate	[1/d]	0.04	0.06
Switches	Aerobic/anoxic DO half sat.	[mgO_2/L]	0.05	0.25
		Stoichiometric		
Common	Particulate substrate COD:VSS ratio	[mgCOD/mgVSS]	1.6	1.41
	Particulate inert COD:VSS ratio	[mgCOD/mgVSS]	1.6	1.41
OHO	Yield (aerobic)	[-]	0.66	0.74

In this study, the kinetics and stoichiometric parameters of PAOs and OHOs for growth and decay were mainly focused on, in sensitivity analysis. As seen from Table 8.2, the estimated maximum specific growth rate and the anoxic growth factor of heterotrophs were lower than the default values. In comparison, the model's maximum specific growth rate of the PAOs was higher than the default values. The $\mu_{max-heterotrophic}$ and $\mu_{max-PAO}$ were estimated to be 2.5 d^{-1} and 1.5 d^{-1}, respectively. By varying the maximum growth rate of OHOs and PAOs by ±50% of the default value, the total phosphorus concentration changed by ±50%. In contrast, a smaller variation of changes of ±30% in aerobic decay rate and ±20% in the anaerobic decay rate of PAOs indicated a 30% TP sensitivity in the effluent. This suggests the larger impact of PAO anaerobic decay rate on TP effluent concentration. Under constant influent characteristics, variation in simulation output was plotted against the variation of effluent TP concentration (Figure 8-3), proposing a substantial impact of maximum growth rate and aerobic decay of OHOs and PAOs and PAO anaerobic decay rate on TP simulation output. As depicted in the figure, phosphorus removal performance greatly decreased with increasing the OHO maximum specific growth rate while the performance shows that TP removal has a parabolic curve with a change in PAO anaerobic decay rate, where TP removal is greatly decreased at low and high PAO anaerobic decay rates.

Further, as seen in Table 8.2, the estimated aerobic decay rate of OHOs, maximum specific growth rate, and anaerobic decay rate of PAOs were higher than the model's default values. The optimum $\mu_{max-heterotrophic}$, aerobic OHO decay rate, $\mu_{max-PAO}$, aerobic PAO decay rate and anaerobic PAO decay rate were estimated to be 1.28 1/d, 0.99 1/d, 1.52 1/d, 0.07 1/d, and 0.06 1/d, respectively. The estimated anoxic and anaerobic decay rate and fermentation rate of OHOs were similar to the default values available in the model. Therefore, the selected parameters were manually calibrated based on the experimental results and literature review. The model was then

simulated with the calibrated parameters under dynamic-state conditions, where the simulation results were compared with experimental outcomes to study the model fitness.

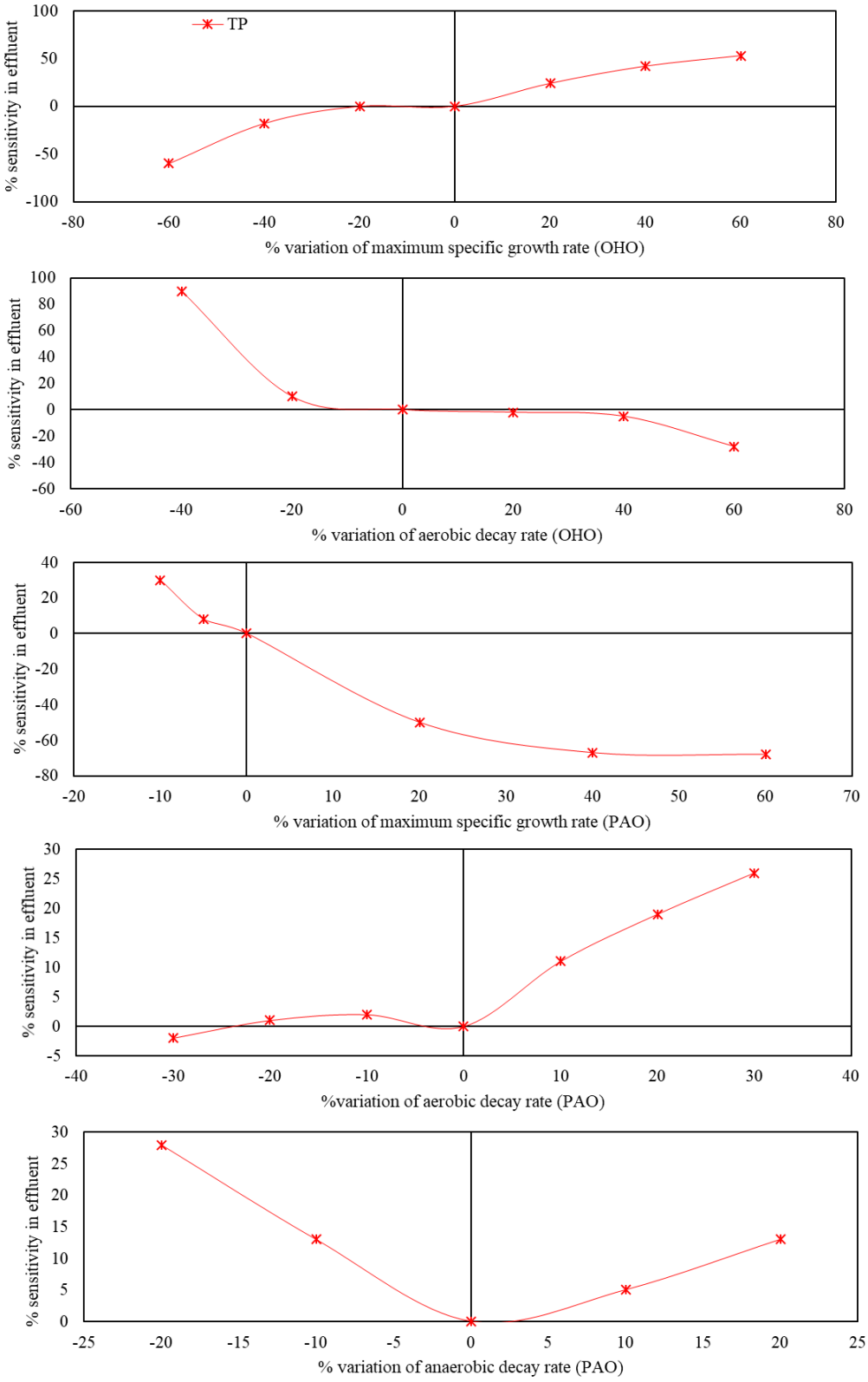

Figure 8-3 Sensitivity analysis of the impact of maximum growth rate and decay rate of PAOs and OHOs as effective parameters on the simulation TP concentration output

8.4.4 Model prediction and accuracy

The simulation model ran for 10 days as a pre-calibration phase for model development, reaching a rather stable nutrient removal rate. There were inconsistencies between the simulation model outcomes and experimental values in this phase. Running the simulation model with default values generated a rather noticeable distinction between the two sets of results. Effluent's phosphorus and nitrogen content were predicted higher than the measured values when default parameters were used. These discrepancies concerned almost all output variables, including nitrogen and phosphorus compounds removal, revealing the necessity of model calibration. By adjusting the most sensitive parameters based on the sensitivity analysis, calibrated model was reached with significant effect. The calibrated model was dynamically validated using the experimental data. By applying kinetic and stoichiometric parameter calibration, the model met the experimental system requirements by agreeing on output variables for the model and measured values. when reaching a fully calibrated BioWin® model, next step was to compare model predicted values and measured data. From days 20 to 60, the dynamic simulation indicated a robust TP, PO_4-P, and oxidized nitrogen effluent concentration prediction. A comparison of model predictions using BioWin® and experimental results for all four DO concentrations and aerobic HRT is available in Figure 8-4. The model showed a satisfactory representation of the measured data in all experiments. The model predicted effluent PO_4-P and TP in 0.8-120 and 0.8-200 of 0.44 mg/L, 0.84 mg/L and 0.19 mg/L, 0.7 mg/L respectively, which compared quite accurately with the experimental data of 0.52 mg/L, 1.02 mg/L and 0.25 mg/L and 0.95 mg/L respectively. The effluent P-content increased by decreasing the DO concentration to 0.4 mg/L in different aerobic HRTs, yet the model predictions and experimental data were closely comparable. The model anticipated an effluent PO_4-P and TP of 2.22, 2.59 mg/L, and 5.42 and 5.75 mg/L in 0.4-120 and 0.4-200, respectively. These observations efficiently matched the experimental effluent results of 2.28, 3.31 mg/L, and

5.14 and 5.66 mg/L of PO_4-P and TP in 0.4-120 and 0.4-200, respectively. The average percentage error (APE) calculation, [381] showed less than 10% divergence between predicted and observed data in TP, PO_4-P, NO_4-N, NO_3-N, and TKN. Statistical analysis procedures were used to determine and further justify the statistical significance of the values. Mean values were calculated for TP, PO_4-P, and nitrate and nitrite effluent concentrations. Phosphorus and nitrogen contents showed a rather subtle difference between mean measured value and mean simulated value. For effluent-TP concentration, the *p*-value is greater than 0.05, the outcome fails to reject the null hypothesis, demonstrating a not statistically significant difference between the population means. Furthermore, effluent PO_4-P and nitrate/nitrite have shown a good match between data from the model and the experiment due to a *p*-value higher than the significance level. In addition, a correlation coefficient (R-value) of higher than 80% indicates the accuracy and reliability of the simulation. The correlation coefficient between simulated and measured effluent oxidized nitrogen, phosphate, and total phosphorus were 0.8, 0.85, and 0.86. Considering the effluent quality, phosphorus content had a higher correlation. The higher accuracy could be attributed to a balanced electron acceptor/donor availability, provided for PAO activity and growth.

Moreover, the simulation model predicted a 35% abundance of PAOs, which is gradually higher than the measured value of 29.6%, yet it precisely predicts the microbial composition and structure of the system. A 98% acetate uptake, 31.4 mgCOD/L of PHA storage, 10.1 mgN/L of TN, and 0.7 mgP/L of TP in effluent demonstrate the high prediction capability of the model. However, a high APE in solids and COD results showed the difference between simulated and experimented outcomes (Table 8.3). In all scenarios, the model under-predicted the COD and solids, while other measurements were relative to each other. The model efficiently predicted the nutrient level in effluent while due to insufficient deliberation on soluble microbial products (SMP), under-

prediction of SCOD and TCOD occurred. Also, these parameters are difficult to calibrate as the influent WW characteristics of each feeding source affect the model.

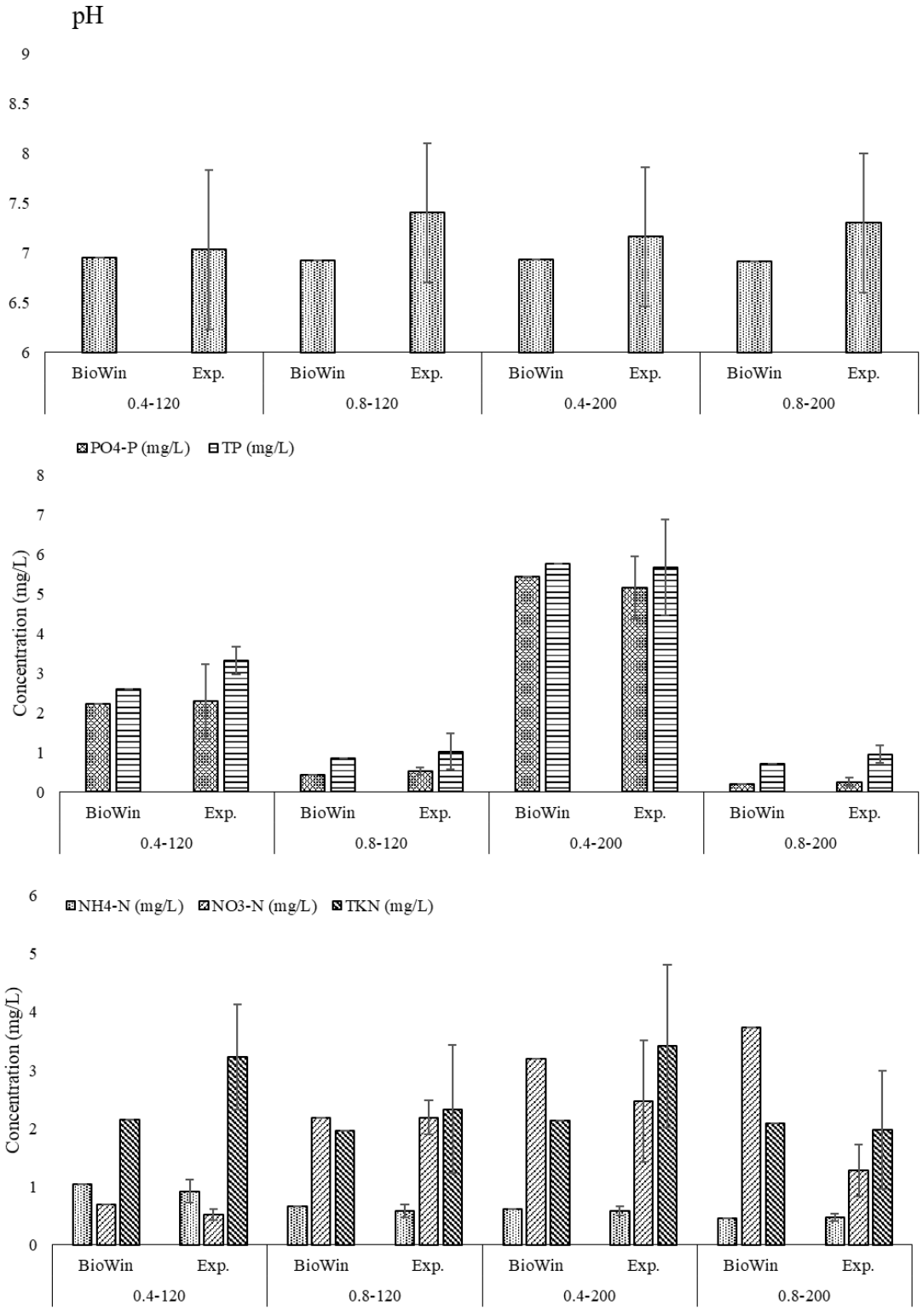

Figure 8-4 SBRs pH, phosphorus and nitrogen concentration of model vs. average measured data profile

Table 8.3 Experimental and under-predicted model effluent quality values of COD and solids at calibrated BioWin® model

	0.4-120		0.8-120		0.4-200		0.8-200	
Parameters	**BioWin®**	**Exp.**	**BioWin®**	**Exp.**	**BioWin®**	**Exp.**	**BioWin®**	**Exp.**
COD (mg/L)	36.58	40.3±5.4	18.78	23.6±3.3	32.69	70±10.5	17.72	25.1±10.1
SCOD (mg/L)	29.69	33.5±5.5	15.46	14.5±4.1	25.57	41.2±9.6	11.36	10±4.5
TSS (mg/L)	6.77	23.5±3.3	6.73	16.6±2.6	6.61	23.7±2.9	6.97	20.1±2.9
VSS (mg/L)	4.87	16.6±2.4	4.72	10.8±1.9	4.83	17.9±1.45	4.49	11.4±2.01

The simulation data and measured results were evaluated using Nash–Sutcliffe efficiency (NSE) and correlation coefficient R^2 methods as indicated by [378]. An NSE value between 0 and 1 and an R^2 value close to 1 was generally considered the acceptable level of performance, indicating a good model prediction for observed values. The NSE and R^2 values related to the observed and simulation results comparison of 0.97 and 0.89, respectively, indicate a satisfactory level of effectiveness in the simulated model. With a value of 0.97 between 0 and 1 for NSE, an acceptable level of performance is viewed. Moreover, an R^2 of 0.89, close to 1, further justifies the model prediction for phosphorus removal. The dynamic SBR-mode model by BioWin® examines different aspects of process performance, nutrient removal efficiencies, effluent characteristics, and process yields. Therefore, based on the results, the simulated optimal DO concentration and aerobic HRT, as well as parameters of phosphorus compound removal, were similar to those obtained from the experimental setup. In which it demonstrates a high predictive ability of

mathematical modeling of the P-removal process. (A list of measured data is available in **Appendices, H, Table 10.9**)

8.5 Conclusion

Of the four SBRs experimented with, the configuration with 0.8 mg/L of DO concentration and 200 minutes aerobic HRT was the system with the highest TP and TN removal. This configuration had the highest PHB storage and P-release rate with a 0.65 P-rel/VFA uptake ratio indicating adequate EBPR activity. The SBR0.8-200 was applied in the model platform, and the simulation indicated an efficient phosphorus removal under proposed conditions. In this study, model calibration was carried out by directly determining the carbonaceous and nutrient fractions of synthetic wastewater and stoichiometric and kinetic parameters of biomass, combined with calculation and prediction of process performance through BioWin®. Comparison between calibrated BioWin® model and experimental data from lab-scale SBR-EBPR showed a rather good prediction potential for the simulated model. The simulation accuracy for effluent TP and phosphate was both higher than 80%. Therefore, model simulation in BioWin®, along with efficient analysis of sensitive parameters, calibration, and validation, will monitor process performance and effluent quality.

9 Conclusion and future directions

Enhanced Biological P-removal is highly effective in the environment and costs of chemical treatment; however, most WWTPs still incorporate chemical precipitation with biological methods. Notwithstanding, EBPR as a biological system with alternating anaerobic, aerobic, and anoxic phases is highly dependent on microbiological and operational sectors. Understanding the microbial identification fundamentals specifically for PAOs and GAOs and optimization of operation, design, and configuration offers potential high P-removal, P-recovery, and sustainability, as reviewed in chapter 2. In addition, a greater understanding of the microbiology and metabolic modeling of EBPR as an engineered ecosystem helps develop EBPR technology (chapter 3). Despite extensive research with valuable improvement and full-scale developments in these areas for removing and recovering phosphorus, there are still numerous unresolved issues to reconsider and untangle. This thesis concentrated on building on past research by investigating the EBPR performance, nutrient removal efficiency, and microbial potential through aeration approaches (Chapters 4 to 8). Recent findings regarding the effect of aeration concentration, aeration pattern, and duration were incorporated into the experimental scheme. The results could be exploited for further research and engineering insights.

9.1 Contribution, limitations and future directions

This Ph.D. study aimed to investigate the possibility of applying an efficient aeration system to the EBPR process in lab-scale SBR-mode by characterizing the different aspect of aeration according to oxygen concentration, aerobic retention time, and aeration pattern to assess the condition the Bio-P-activity and nutrient removal from synthetic wastewater. In this series of experiments, a DO concentration of 2 mg/L was selected as the baseline. This DO level is commonly interpreted as the sufficient oxygen concentration for phosphorus removal and

simultaneous nitrification and denitrification. Lower and higher DO levels in comparison to baseline were investigated in different experimental stages to look into the effect of excessive and limited oxygen availability on EBPR performance. Considering the baseline for DO concentration, other aeration strategies were applied, including a change in aerobic HRT and the pattern of aeration.

Initially, oxygen concentration and aerobic retention time were studied in various scenarios. The main results from assessing dissolved oxygen concentration in a range of 0.8 to 4 mg/L show that DO level as an operational factor highly influences nutrient removal performance both at an operational and microbial level, where high P-removal is achieved at lower DO levels. This study showed that 0.8 mg/L DO concentration could achieve successful biological P-removal with higher than 90% removal efficiency with an efficient aerobic HRT due to a shift in bacterial population towards PAOs. However, several studies have reported low-level DO conditions leading to potential nitrification and denitrification separation, lack of COD availability, and secondary phosphorus release.

Therefore, investigation on gradual and instant DO decrease in SBR-mode reactor was identified as an applicable strategy for changing high-aeration level systems to low-aeration level processes with a reduction in energy requirement. Under the gradual DO-decrease mode, the highest maximum volumetric acetate uptake ratio and maximum volumetric PO_4 uptake rate were achieved since a higher biomass growth rate could take full advantage of the carbon source available for nutrient removal. Higher PAO abundance driven by gradual oxygen concentration decrease illustrated the higher degree of phosphorus removal. In contrast to a balanced EBPR in a gradual DO-decrease scenario with minimum disruption to process performance, abrupt low-DO concentration in an instant DO decrease resulted in a jump of effluent-P concentration to higher

than 8 mg/L. The activity of the EBPR process in the instant-DO decrease strategy was disturbed when the system experienced sudden operational change, which caused a drastic drop in PAO abundance.

Moreover, an investigation on high aerobic HRT indicated an upset of EBPR activity due to a decline in P-removing organism's population and out-competition by ordinary heterotrophs and GAOs. It is suggested that efficient short cyclic periods in SBR mode reactors tend to resist process failure and maintain biomass activity. By reducing the HRT, the F/M ratio increases, elevating the biological treatment capacity.

In addition, the aeration pattern was evaluated to consider the effect of change in arrangement on EBPR performance. By applying intermittent aeration with different interval periods, the results indicated higher process performance. The system exhibited approximately 30% higher nutrient removal by increasing the intermittent-aeration duration from 25 to 50 minutes. The primary factors affecting the P-removal performance in 25-minute intervals were insufficient reaction time, variation in DO concentration, and carbon source. In this case, due to invaded anaerobic conditions and the availability of electron acceptors, processes requiring oxygen, including carbonaceous oxidation, nitrification, and P-uptake, lead to inefficient anaerobic PAO performance. In addition, due to high DO levels, the remaining NO_2-N and NO_3-N from the preceding cycle will not be removed by means of denitrification, resulting in an accumulation of nitrite/nitrate in the system. Therefore, the highest nutrient removal is achieved when nitrification, denitrification, and P-removal, as the three major processes are all active in the reaction (aerobic/anoxic) period. Yet, their process rates and advantage over one another in the aerated/non-aerated phases specify the process performance efficiency.

Subsequently, running a simulation model in BioWin® for water quality prediction and operational effects under various treatment cases, with thorough sensitivity analysis, calibration, and validation steps, justified the importance of a balanced EBPR system, with sufficient DO concentration, aerobic HRT, and aeration pattern, which indicated the ability of BioWin® in predicting nitrification and denitrification performance and effluent phosphate and TP profile. The advantage of BioWin® in predicting the effluent values and its adaptability even with the change in effluent quality limitations and regulations was demonstrated in this study. As a robust asset for selecting optimal treatment processes, reducing cost and energy consumption, and for analysis and prediction, BioWin® is a powerful tool used by various water authorities, municipalities, and manufacturers to provide beneficial insights on applied processes WWTPs.

Overall, the results and knowledge obtained in these studies provided valuable information to improve real engineering practices. The findings are significant because they provide crucial insights into the effect of aeration as an operational factor on EBPR regarding anaerobic/anoxic/aerobic metabolism and the response of process performance to changes in operation. The findings also contribute to a better understanding of PAO population dynamics observed in various aerated cases concerning the abundance, stoichiometric and kinetic values.

Yet, this research has few limitations that can be addressed in future studies for a broader overview of the effect of aeration on EBPR.

- Due to limited time in research and resources, treatment of real wastewater was not assessed, and only synthetic wastewater mimicking real conditions was prepared and utilized. In this study, only acetate was used as the VFA source; however, most wastewater will probably have a composition that contains a mixture of short-chain fatty acids. As such, future investigations should combine aeration strategies with feeding alternatives.

- Considering practical applications, a detailed investigation on the effect of micro-aerated systems with a change in the DO baseline, in combination with natural wastewater feeding system under specific operational conditions, could help clarify if lower DO levels may correlate with high P-removal efficiency and goof EBPR performance.
- Although this study demonstrated the microbial population change in various strategies, a deeper understanding of the metabolic pathways of active microorganisms is still lacking. Further research is required to look into the effect of aeration on functional diversity and differences between microorganisms.
- A lab-based SBR system with a maximum volume of 6 liters was in use, though a pilot scale would have efficiently imitated the effect of operational changes on real WWTPs.
- In addition, lower aeration levels (<0.2 mg/L) were not considered with the capacity of the instruments in use. Although, reaching a stable EBPR in micro-aerated systems with minimum aeration levels favors enhancing energy efficiency and cost.

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10 Appendices

A. Population detection and PAOs identified

Table 10.1 summary of research on population detection and PAOs identified

Reactor Configuration	Feed Of Reactors	Community Analysis Methods	Populations Detected	PAO Identified	PH	Temp ˚C	COD/P Mg/Mg	Eff-P Mg/L	SRT	Reference
Sbr University Of Wisconsin—Madison (UWMS)	Acetate-based feed	qPCR-16s	β-proteobacteria	Candidatus Accumulibacter (51 ±3%)	_	_	14	_	4	(He et al. 2007)
		FISH	β-proteobacteria	Candidatus Accumulibacter (55±10%)						
SBR University Of California—Berkeley (UCB)	Acetate-based feed	qPCR-16s	β-proteobacteria	Candidatus Accumulibacter (67 ±7%)	_	_	14	_	4	(He et al. 2007)
		FISH	β-proteobacteria	Candidatus Accumulibacter (80±5%)						
EBPR Activated Sludge (BP Pilot Plant) (A/O Configuration)	wastewater	FISH	β,α,У proteobacteria actinobacteria	Rhodocyclus-related bacteria (6-18%)	_	_	0-21.2	0.3-7.9	4	(Lee et al. 2002)
EBPR Activated Sludge (BNP Pilot Plant) (A/O Configuration)	wastewater	FISH	β,α,У proteobacteria actinobacteria	Rhodocyclus-related bacteria (4-28%)	_	_	0-21.2	0.1-5.6	21	(Lee et al. 2002)

SBR (UCT Configuration)	Acetate-based feed	qPCR, ARISA	β-proteobacteria	Candidatus Accumulibacter (20%)	7-7.5	_	14	_	4	(He et al. 2010)
Bardenpho Configuration (Skagen WWTP) Pilot Plant	domestic WW	FISH	β-proteobacteria	Rhodocyclus-related PAO (9%)	_	_	60	<1	_	(Kong et al. 2007)
SBR A/O Configuration	synthetic WW acetate based	FISH, 16s rRNA-targeted	β-proteobacteria	accumulibacter Rhodocyclus group (81%)	7.35± 0.5	20	43	<0.5	7	(Hesselmann et al. 1999)
Pilot Plant A2/O	domestic WW	FISH	β-proteobacteria	Accumulibacter-related organisms (11%)	7.3	19	12.5-20	0.1	10.9	(He et al. 2008)
Pilot Plant UCT/AO	domestic WW	FISH	β-proteobacteria	Accumulibacter-related organisms (20%)	7.7	13	14-22.4	0.4	9	(He et al. 2008)
SBR A/O Configuration	domestic WW+ VFA mixture	FISH	β-proteobacteria	Candidatus Accumulibacter (53%)	7 to 9	23	35.7	_	10	(Levantesi et al. 2002)
SBR A/O Configuration	synthetic WW acetate based	FISH	actinobacteria, β-proteobacteria	_	6 to8	_	20	0.1-2.4	20	(Okunuki et al. 2004)
SBR A/O Configuration	synthetic WW acetate based	FISH	β-proteobacteria	Candidatus Accumulibacter (55.1%)	8	20	49.7	_	10	(Kim et al. 2013)
SBR A2/O Configuration	synthetic WW acetate based	FISH	β-proteobacteria	Candidatus Accumulibacter (29.2%)	8	20	49.7	_	10	(Kim et al. 2013)
UCT EBPR Configuration	municipal WW	FISH	β,α,У proteobacteria	Rhodocyclus-related PAO (20%)	_	_	_	<1	_	(Zilles et al. 2002)
Aerated Anoxic EBPR	municipal WW	FISH	β,α,У proteobacteria	Rhodocyclus-related PAO (6%)	_	_	_	<1	_	(Zilles et al. 2002)

SBR A/O Configuration	synthetic WW	FISH	β-proteobacteria	Candidatus Accumulibacter (50%)	7± 0.1	25	9	<0.5	9	(Pijuan et al. 2006)
SBR A/O Configuration	Acetate/propionate feed	FISH	β-proteobacteria	Candidatus Accumulibacter (3-64%)	7± 0.2	20-24	15	<0.5 -50	8	(Oehmen et al. 2006)

B. GAO anaerobic metabolism

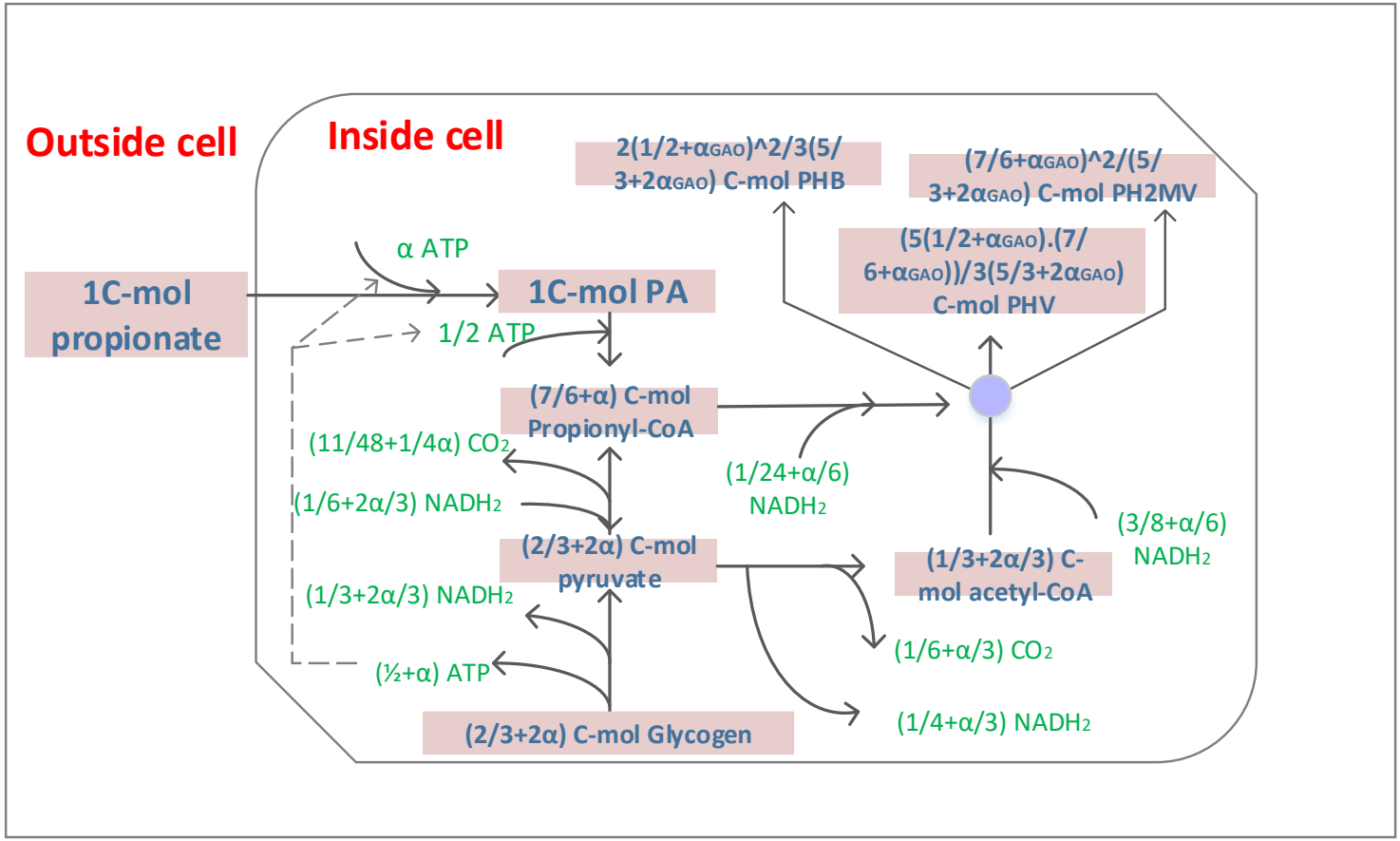

Figure 10-1 Anaerobic metabolism of GAOs in propionate-fed systems including the energy and reducing power production rates

C. PAO aerobic metabolic model in acetate and propionate-fed system

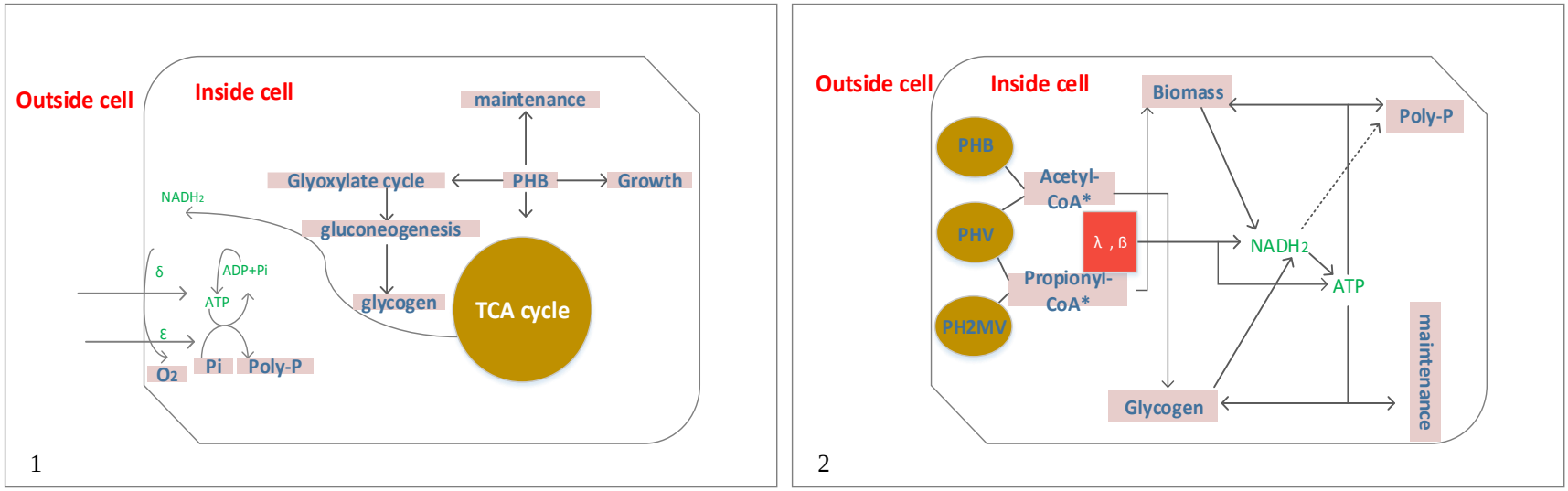

Figure 10-2 aerobic metabolic model for PAO in acetate-fed system 2. aerobic metabolic model for PAO with propionate uptake as substrate

D. Anaerobic and aerobic metabolic models

Table 10.2 acetate-fed metabolic reactions for PAOs and GAOs

acetate	PAO	***Anaerobic***	R1(4) :CH2O+(1/2+ α1) ATP+1/4 NADH→CH1.5 O0.5 (PHB)+1/2 H2O R1: Acetate+(1+2α)ATP →Acetyl-CoA R2:HPO3+ H2O→α2 ATP+ H3PO4 R2-a: Glycogen→2pyruvate+3ATP+2NADH2 R2-b: Poly-Pn+ADP→Poly-Pn-1+ATP R3-a:CH2O + (1/2+ α1) ATP + H2O→2NADH+CO2 R3-b:CH10/6O5.6+1/6 H2O→2/3 CH1.5O+1/3 CO2+1/2 NADH+1/2 ATP R3-a: Glycogen→2pyruvate+3ATP+2NADH2 R3-b: Pyruvate→Acetyl-CoA+CO2+ NADH2 R3-c: 2 acetyl-CoA→Propionyl-CoA+ CO2+NADH2 R4(1) :CH2O+(1/2+ α1) ATP+1/4 NADH→CH1.5 O0.5 (PHB)+1/2 H2O R4-a: 2 Acetyl-CoA+NADH2→3-hydroxybutyryl-CoA R4-b: Acetyl-CoA+propionyl-CoA+ NADH2→3-hydroxyvaleryl-CoA R5-a: CH2O+ (0.5+α1)/α2 HPO3+(1/3+(0.5+α1)/α2) H2O→0.89CH1.5O0.5+0.11CO2+(0.5+α1)/α2 H3PO4 R5-b: CH2O+1/2CH10/6O5.6+ (0.25+α1)/α2HPO3→1.33CH1.5O0.5+0.17CO2+(0.25+α1)/α2H3PO4+5/12-(0.25+α1)/α2 H2O R5: acetate+1/(6+3Fglx)glycogen→4/(6+3Fglx)PHB+Fglx/(6+3Fglx)PHV+1/3CO2+[1+2αPao=3/(6+3Fglx)]Pi
		aerobic	Ra:CH1.5 O0.5+1.5H2O→2.25NADH2+0.5ATP+CO2 Ra-1: H3PO4 (in)+ATP→HPO3+H2O Ra-2: (C4H6O2) (1/4) +1.5H2O →2.25NADH2+0.5ATP+CO2 Ra-1: NADH2+0.5O2→H2O+δATP Ra-2: NADH2+0.4HNO3→ δN ATP+0.2N2+1.2H2O Ra-1: NADH2+1/2O2→δ° ATP+H2O Ra-2: NADH2+2/5 HNO3→1/5 N2+δnATP+6/5 H2O Rc:1.27CH1.5O0.5+0.2NH3+0.015H3PO4+(K+m(atp/μ)) ATP+0.385H2O→CH2.09O0.54N0.2P0.015+0.615NADH2+0.27CO2 Rc: 1.27(C4H6O2) (1/4) +0.2NH3+0.015HPO3+H2O+(1.6+m (ATP/μ)) ATP→CH2.09 O0.54N0.2 P0.015+0.615NADH2+0.27CO2 Rd-1: H3PO4out+1/Ɛ NADH2+1/2ƐO2→H3PO4in+1/Ɛ H2O Rd-2: H3PO4in →α3ATP+HPO3+H2O

		Rd-1: ε0H3PO4(out)+NADH2+1/2 O2→ε0H3PO4(in) +H2O Rd-2: ε0H3PO4(out)+NADH2+2/5HNO3→εnH3PO4(in)+1/5N2+6/5H2O Re:4/3 CH1.5 O0.5+5/6 ATP+5/6 H2O→ CH10/6O5.6+1/3CO2+aNADH2 Re: 4/3(C4H6O2) (1/4) +1.5H2O→(C6H10O5) (1/6) +1/3 CO2+NADH2
GAO	***anaerobic***	R1*: acetate+(1+2αGAO) ATP→ Acetyl-CoA R2*: glycogen→2pyruvate+3ATP+2NADH2 R3*-a: Pyruvate→NADH2+CO2+Acetyl-CoA R3*-b: pyruvate+2NADH2→propionyl-CoA+ATP R3*-c: Acetyl-CoA+0.5NADH2→Acetyl-CoA* R3*-d: propionyl-CoA+0.5NADH2→propionyl-CoA* R4*: Acetate+(21/24+3/2 αGAO) Glycogen→ ((35/24+1/2 αGAO))/((19/12+αGAO)) PHB+(2.5(35/24+1/2 αGAO) (3/24+1/2 αGAO))/((19/12+αGAO)) PHV+(1.5(3/23+1/2 αGAO))/ ((19/12+ 1/2 αGAO)) PH2MV+(11/48+1/4 αGAO) CO2
	aerobic	Ra*: PHA→ʎCH1.5 O0.5* (Acetyl-CoA*) +βH5/3O (1/3) * (propionyl-CoA*) Rb*: NADH2+0.5O2→H2O+δATP Rc*-1: CH1.5 O0.5* (Acetyl-CoA*) +1.5 H2O→CO2+0.5ATP+2.25NADH2 Rc*-2: CH (5/3) O (1/3) * (propionyl-CoA*) +5/3 H2O+CO2+2/3 ATP+2.5 NADH2 Rc*-3: 4/3 CH1.5O0.5* (Acetyl-CoA*) +4/6 ATP+5/6 H2O→CH (10/6) O (5/6) +1/3 CO2+NADH2 Rc*-4: CH (5/3) O (1/3) * (propionyl-CoA*) +0.5H2O+1/3 ATP→CH (10/6) O (5/6) +0.5NADH2 Rd*: 1.06CH (5/3) O (1/3) * (propionyl-CoA*) +0.19NH3+K2 ATP+0.267H2O→CH1.84O0.5N0.19+0.515NADH2+0.06CO2 Re*: -ATP=0

Table 10.3 propionate-fed metabolic reactions for PAOs and GAOs

propionate	**PAO**	***anaerobic***	R1: propionate+(1+3αPAO) ATP→propionyl-CoA + H2O R2: PO3-1 (Poly-P) + H2O→ATP+ H2PO4-1 R3: glycogen+ H2O→2CO2+2ATP+4NADH2+2acetyl-CoA R4-a: Acetyl-CoA+0.5NADH2→Acetyl-CoA* R4-b: propionyl-CoA+ 0.5NADH2→propionyl-CoA* R4-c: CH2O (2/3) (propionate)+1/3 CH (10/6) O (5/6) (glycogen)+(2/9+ αPAO) HPO3 (Poly-P) +(αPAO-1/18) H2O→ (2/9+ αPAO) H3PO4+ 1/18 CH1.5O0.5 (PHB)+5/12CH1.6O0.4(PHV)+3/4CH5/8O1/3(PH2MV) +1/9CO2 R4-d: CH2O (2/3) (propionate)+1/3 CH (10/6) O (5/6) (glycogen)+(2/9+ αPAO) HPO3 (Poly-P) +(αPAO-1/18) H2O →9/5CH1.6 O0.4 (PHV)+(2/9+ αPAO) H3PO4+2/3 CH5/3 O1/3 (PH2MV) +1/9 CO2
		aerobic	Ra: PHA+(1.5λ+5/3 β) H2O→(λ+β) CO2+(0.5λ+2/3 β) ATP+(2.25λ+2.5β) NADH2 Rb: NADH2+0.5O2→H2O+δATP Rc: PHA+(0.21/1.27 λ+0.21/1.06 β) NH3+(1.7/1.27 λ+1.38/1.06 β) ATP+(0.69/1.27 λ+0.55/1.06β) H2O→ (1/1.27 λ+1/1.06 β) CH2.17 O0.84N0.21 P0.015+(0.89/1.27 λ+0.68/1.06 β) NADH2+(0.27/1.27 λ+0.06/1.06 β) CO2 Rd: H3PO4+1/εNADH2+1/2εO2+ATP→HPO3+(1+1/ε) H2O Re: PHA+(λ/2+β/3) ATP+(5/8 λ+0.5β) H2O→ (3/4 λ+β) CH (10/6) O (5/6) +λ/4 CO2+(3/4 λ+0.5β) NADH2
	GAO	***anaerobic***	R1*: acetate+(1+2αGAO) ATP→ Acetyl-CoA R2*: glycogen→2pyruvate+3ATP+2NADH2 R3*-a: Pyruvate→NADH2+CO2+Acetyl-CoA R3*-b: Pyruvate+2NADH2→2H2O+propionyl-CoA R3*-c: Acetyl-CoA+0.5NADH2→Acetyl-CoA* R3*-d: Propionyl-CoA+0.5NADH2→Propionyl-CoA* R3*-t: propionate+(1/2+ αGAO) glycogen→ (1+αGAO) H2O+(1/2+ αGAO) CO2+(1/2+ αGAO) Acetyl-CoA*+(7/6+ αGAO) Propionyl-CoA* R4*-a: CH2O (2/3) +(2/3+2αGAO) CH (10/6) O (5/6) →(1/3+αGAO/3) H2O+(5/6+5/(3αGAO)) CH1.6O0.4+2/3 CH (5/3) O (1/3) +(1/6+αGAO/3) CO2 R4*-b: CH2O (2/3) +(1+3αGAO) CH (10/6) O (5/6) → (2/9+αGAO/2) CO2+(4/9+ αGAO) Acetyl-CoA*+(4/3+(3αGAO)/2) Propionyl-CoA*
		aerobic	Ra*: PHA→ʎCH1.5 O0.5* (Acetyl-CoA*) +βH5/3O (1/3) * (propionyl-CoA*) Rb*: NADH2+0.5O2→H2O+δATP Rc*-1: CH1.5 O0.5* (Acetyl-CoA*) +1.5 H2O→CO2+0.5ATP+2.25NADH2 Rc*-2: CH (5/3) O (1/3) * (propionyl-CoA*) +5/3 H2O+CO2+2/3 ATP+2.5 NADH2

Rc*-3: 4/3 $CH_{1.5}O_{0.5}$* (Acetyl-CoA*) +4/6 ATP+5/6 H_2O→CH (10/6) O (5/6) +1/3 CO_2+$NADH_2$

Rc*-4: CH (5/3) O (1/3) * (propionyl-CoA*) +0.5H_2O+1/3 ATP→CH (10/6) O (5/6) +0.5$NADH_2$

Rd*: 1.06CH (5/3) O (1/3) * (propionyl-CoA*) +0.19NH_3+K2 ATP+0.267H_2O→$CH_{1.84}O_{0.5}N_{0.19}$+0.515$NADH_2$+0.06CO_2

Re*: -ATP=0

Table 10.4 glucose-fed metabolic reactions for PAOs

glucose	**PAO**	***anaerobic***	R1-a: 1/6 ATP+CH2O→CH(13/6)O(PO3)(1/6)+1/6 ADP R1-b: CH(13/6) O(PO3)(1/6)+1/6 ATP+1/6 H2O→CH(5/3) O(5/6)+1/6ADP+1/3H3PO4 R1-c: CH(5/3)O(5/6)+1/6H3PO4→CH(13/6) O(PO3)(1/6) R2-a: HPO3+H2O→H3PO4 R2-b (3): CH(13/6) O(PO3)(1/6)+1/3 ADP+1/(6H3PO4)+1/3NAD→CH(4/3)+1/3 ATP+1/3 NADH2+1/6 H2O R3 (2): CH(13/6) O(PO3)(1/6)+1/3 ADP+1/(6H3PO4)+1/3NAD→CH(4/3)+1/3 ATP+1/3 NADH2+1/6 H2O R4: 1.2CH(4/3)+2/5NADH2+1/5ATP→CH(8/5) O(2/5)+1/5 HO+2/5 NAD+1/5 CO2+1/5 ADP+1/5 H3PO4
		aerobic	Ra: CH(8/5)O(2/5)+12/5 NAD+6/5 H2O+2/5 H3PO4in+2/5 ADP→12/5 NADH2+CO2+2/5 ATP Rb: NADH2+0.5O2+1.85ADP+1/85H3PO4in→NAD+2.85H2O+1.85ATP Rc: 1.27CH(8/5)O(2/5)+0.2NH3+(2.012+μatp/μ) H2O+0.8055NAD+(1.5+μatp/μ)ATP→CH2.09 O0.54 N0.2 P0.015 +0.27CO2+0.8055NADH2+(1.5+μatp/μ)ADP+(1.485+μatp/μ) H3PO4in Rd-1:H3 PO4out+1/7 NADH2+1/14 O2→H3PO4in+1/7 H2O+1/7 NAD Rd-2:H3PO4in+ATP→2HPO3+H2O+ADP Re: 5/3CH(8/5)O(2/5)+2NAD+7/3H2O+5/6 ATP→CH(8/5)O(5/6) +2NADH2+2/3CO2+5/6 H3PO4in+5/6 ADP

E. Oxygen concentration and aerobic retention time study

Table 10.5 measured data for chapter 3- SBR mode reactors with different DO levels and 120 minute aeration duration

	time (min)	acetic acid	propionic acid	iso-butyric acid	butyric acid	iso-valeric acid	valeric acid	total VFA (mmol/L)	PHB (cmmol/gVSS)	Glycogen (cmmol/gVSS)
SBR 4mg/L-120 min	15	4.87	0	0	0.336	0	0.58	5.786	0.5	4.16
	60	0.356	0.407	0.538	0.397	0.489	0.34	2.57	2.16	4.1
	90	0.2	0	0	0.35	0.22	0.24	0.85	2.36	3.11
	180	0.187	0	0	0.339	0	0.26	0.7	1.01	3.8
	195	0.19	0	0	0	0	0	0.19	0.51	4.1
SBR 2mg/L-120 min	15	4.87	0	0	0.336	0	0.58	5.786	0.51	3.21
	60	0.22	0.38	0.53	0.13	0.15	0.17	1.58	3.05	3.12
	90	0.21	0	0	0	0	0	0.21	2.54	2.75
	180	0.26	0	0	0	0	0	0.26	1.54	3.13
	195	0	0	0	0	0	0	0	1.01	3.19
SBR 0.8mg/L-120 min	15	4.87	0	0	0.336	0	0.58	5.786	0.74	3.13
	60	0.22	0.2	0.43	0.1	0.05	0.18	1.18	3.34	3.01
	90	0.1	0	0	0	0	0	0.1	2.69	2.63
	180	0.05	0	0	0	0	0	0.05	1.95	3
	195	0	0	0	0	0	0	0	1.26	3.14

Table 10.6 measured data from chapter 3- glycogen concentration at different DO levels and 200 minute aeration duration

Glycogen Concentration mmol/gVSS						
	4 mg/L DO		2 mg/L DO		0.8 mg/L DO	
time (min)	4 mg/L DO day 10	4 mg/L DO day 28	2 mg/L DO day 10	2 mg/L DO day 28	0.8 mg/L DO day 10	0.8 mg/L DO day 28
15	3.710	5.300	3.194	4.015	3.117	3.229
60	3.125	4.965	3.095	3.965	3.095	3.138
90	3.338	4.600	2.886	3.757	3.018	2.925
115	3.338	4.524	2.645	3.571	2.643	2.550
150	3.542	4.439	3.033	3.902	3.005	2.977
195	3.565	4.905	3.018	3.879	3.005	3.005
210	3.586	5.000	3.122	3.989	3.093	3.093
240	3.565	4.905	3.221	3.971	3.067	3.205
260	3.460	4.905	3.207	3.990	3.078	3.191
285	3.543	5.000	3.223	4.014	3.175	3.183
305	3.553	5.100	3.223	4.139	3.159	3.207
345	3.553	5.130	3.207	4.139	3.144	3.193
360	3.604	5.100	3.223	4.139	3.138	3.223

F. Aeration pattern study

Table 10.7 measurement data chapter 4- phosphate, VFA and carbon concentrations in continuous and intermittent systems

	time (min)	Phosphate (mg/L)	Glycogen (Cmmol/gVSS)	PHB (Cmmol/gVSS)	acetic acid	propionic acid	iso-butyric acid	butyric acid	iso-valeric acid	valeric acid	total VFA (mg/L)
EBPRCONT	15	15	4.014634	0.5	4.87	0	0	0.336	0	0.58	5.786
	60	50.1	3.965	2.44	0.239	0.387	0.531	0.367	0.488	0.306	2.318
	90	69.1	3.757143	2.6	0.182	0	0	0.337	0	0.59	1.109
	105	75.15	3.571429	2.11	0.15	0	0	0.336	0	0	0.486
	150	33.65	3.902439	1.81	0.145	0	0	0.31	0	0	0.455
	195	30	3.878505	1.75	0.12	0	0	0.3	0	0	0.42
	210	30	3.988631	1.59	0.1	0	0	0.1	0	0	0.2
	240	28.4	3.971292	1.43	0.11	0	0	0	0	0	0.11
	260	28.1	3.990385	1.3	0	0	0	0	0	0	0
	285	27	4.014423	0.94	0	0	0	0	0	0	0
	305	27	4.138614	0.62	0	0	0	0	0	0	0
	345	10.24	4.138614	0.52	0	0	0	0	0	0	0
	360	4.59	4.138614	0.53	0	0	0	0	0	0	0
EBPRINT-50	0	2.5	3.19	0.51	4.87			0.336		0.58	5.786
	15	20.5	3.10	0.51	0.187			0.341		0	0.528
	60	60.4	2.89	3.21	0.15			0		0	0.15
	90	75.4	2.65	3.35	0			0		0	0
	115	78.9	2.76	3.41	0			0		0	0
	125	61.1	3.01	2.85	0			0		0	0
	130	40.3	3.00	2.75	0			0		0	0
	165	25.4	3.03	2.8	0			0		0	0
	185	26.3	3.02	2.79	0			0		0	0
	195	26.5	3.05	2.76	0			0		0	0
	215	15.6	3.11	1.95	0			0		0	0

	225	8.4	3.12	1.5	0			0		0	0
	245	4.9	3.14	1.02	0			0		0	0
	265	5	3.13	1	0			0		0	0
	275	5.1	3.15	0.64	0			0		0	0
	290	5	3.20	0.6	0			0		0	0
	315	4.43	3.19	0.53	0			0		0	0
	345	3.26	3.18	0.52	0			0		0	0
	360	2.83	3.23	0.5	0			0		0	0
	0	10.1	4.014634	0.51	4.87	0	0	0.336	0	0.58	5.786
	15	31.7	3.965	1.91	0.22	0.38	0.526	0.358	0.482	0.291	2.257
	60	45.4	3.757143	2.1	0.182	0	0	0.337	0	0.6	1.119
	90	46.4	3.357143	2.24	0.181	0	0	0.337	0	0.59	1.108
	115	46.9	3.619048	2.37	0.26	0	0	0.24	0	0	0.5
	125	45.1	3.642857	2.15	0.19	0	0	0	0	0	0.19
	130	46.54	3.666667	2.1	0.15	0	0	0	0	0	0.15
	145	44.53	3.661905	2.14	0.1	0	0	0	0	0	0.1
	150	43.1	3.660904	2.1	0	0	0	0	0	0	0
	155	42.59	3.657143	1.42	0	0	0	0	0	0	0
	170	34.4	3.638095	1.41	0	0	0	0	0	0	0
	180	28.4	3.671429	1.15	0	0	0	0	0	0	0
	185	29.5	3.684987	1.09	0	0	0	0	0	0	0
	195	30.9	3.690476	0.8	0	0	0	0	0	0	0
	205	31	3.761905	0.74	0	0	0	0	0	0	0
	215	29.94	3.809524	0.7	0	0	0	0	0	0	0
	230	21.85	3.857143	0.69	0	0	0	0	0	0	0
	235	20.45	3.859872	0.67	0	0	0	0	0	0	0
	240	19.67	3.859913	0.66	0	0	0	0	0	0	0
	245	18.46	3.866667	0.64	0	0	0	0	0	0	0
	255	17.43	3.904762	0.635	0	0	0	0	0	0	0
EBPRINT-25											

	265	10.64	3.909524	0.6	0	0	0	0	0	0	0
	275	10.14	3.893941	0.6	0	0	0	0	0	0	0
	280	9.46	3.880952	0.59	0	0	0	0	0	0	0
	295	9.46	3.952381	0.53	0	0	0	0	0	0	0
	305	9.46	4	0.53	0	0	0	0	0	0	0
	345	9.46	3.995238	0.52	0	0	0	0	0	0	0
	360	9.46	4.004762	0.52	0	0	0	0	0	0	0

G. Study of gradual and instant aeration change

Table 10.8 measurements of chapter 5- phosphorus concentrations and DO levels in gradual and instant DO decrease scenarios

		SBRgra				SBRins			
	time (days)	DO (mg/L)	P-release (mg/L)	Peff (mg/L)	P removal	DO (mg/L)	P-release (mg/L)	Peff (mg/L)	P removal
stage 1	1	2.1	45.6	4.25	82%	1.9	50.4	5.1	78%
	2	2.3	50.1	5.7	74%	1.98	52.3	6	73%
	3	2	52.3	6	72%	1.92	57	4.98	77%
	4	2	54.8	5.84	74%	2.1	52.1	5.1	77%
	5	1.98	52.1	5.3	78%	2.05	55.8	4.12	83%
	6	1.95	56.8	5.24	75%	2.14	49.6	4.53	78%
	7	2.3	54.8	5	77%	2.3	45.2	4.62	78%
	8	2.14	50	4.7	79%	2.1	54	4.75	79%
	9	2.15	49.7	3.98	82%	2.4	57	4.61	79%
	10	2	46	3.89	82%	2.06	51.4	4.75	78%
	11	1.97	48.9	4.65	80%	2.1	50.6	5.02	78%
	12	2.35	56.4	4.5	81%	2.23	48.8	4.8	79%
	13	2.4	53.2	4.23	82%	2.42	50.6	5.06	79%
	14	2.15	52.9	4.7	78%	2.32	47.4	4.7	78%
	15	2	50.4	5	76%	2.01	51.3	5.6	73%
stage 2	16	0.88	61.1	3	87%	0.5	50.8	9	62%
	17	0.89	58.2	3.1	86%	0.49	54.8	10.15	55%
	18	0.9	62.8	3.2	87%	0.42	52.1	10.2	59%
	19	1.1	65.2	2.54	89%	0.51	55.2	10.1	57%
	20	1.14	69.3	2.5	89%	0.44	46.79	10.11	55%
	21	1.2	65.2	3	86%	0.45	55.1	10.1	53%
	22	1.13	66.2	3.2	86%	0.42	56.1	10.2	55%
	23	1.13	64.3	3.05	86%	0.43	54.3	10.66	50%
	24	1	63	3.8	84%	0.4	53	12.2	48%
	25	0.8	64.7	2.25	90%	0.4	49.6	9.82	57%

	26	0.86	61	2.2	90%	0.4	51	9.2	59%
	27	0.87	64.2	2.48	89%	0.43	49.8	8.4	62%
	28	0.89	63.8	2.02	91%	0.48	44.3	9.49	59%
	29	0.84	69.5	2.12	90%	0.384	49.3	9.9	55%
	30	0.81	70.1	2.14	90%	0.38	50	10	55%
	31	0.8	74.1	2.1	91%	0.4	44.1	6.98	71%
	32	0.81	74	2.54	87%	0.41	44	9.5	53%
	33	0.81	70.4	2.8	87%	0.41	40.1	8.45	61%
	34	0.89	69.3	2.5	89%	0.39	39.2	8.2	64%
	35	0.9	65.1	2.104	90%	0.39	46.5	9	55%
	36	0.86	64.9	2.15	90%	0.36	44.9	10.5	52%
	37	0.84	60.9	2.3	89%	0.44	45.2	9.3	56%
	38	0.81	61.9	1.98	91%	0.31	51.02	7.9	65%
	39	0.82	69.5	1.8	92%	0.41	49.2	8.8	59%
	40	0.81	64.9	1.49	93%	0.41	44.2	7.89	65%
	41	0.8	62.8	1.48	93%	0.4	42.9	6.9	68%
	42	0.93	70.1	1.9	91%	0.43	40.1	7.5	64%
	43	0.92	74.2	2	90%	0.42	34.7	8	62%
	44	0.9	68.9	1.98	91%	0.4	38.2	8.1	64%
	45	0.91	74	1.5	94%	0.41	40	7.9	66%
stage 3	46	0.6	55.4	2.1	91%	0.4	31	8.1	66%
	47	0.54	53.1	3.3	85%	0.43	31.1	8	64%
	48	0.5	52	3.5	86%	0.41	30	7.89	68%
	49	0.5	51.8	3.56	85%	0.39	32	9.1	61%
	50	0.45	53.9	2.5	89%	0.38	41.5	7.6	66%
	51	0.42	55.6	3	86%	0.387	45.5	8.15	62%
	52	0.4	56.7	3.1	86%	0.4	40	8.8	61%
	53	0.43	60.4	3	86%	0.42	41	10.2	52%
	54	0.45	61.3	2.6	89%	0.45	39.8	9.5	60%
	55	0.44	64.8	3.4	85%	0.46	39	9.65	58%
	56	0.41	60.1	3.5	85%	0.432	40.2	10	56%

	57	0.39	59.7	3.1	86%	0.42	40.36	10.25	54%
	58	0.39	60.2	3.2	86%	0.4	41.6	11.3	51%
	59	0.4	59.4	3.5	84%	0.38	44	10.4	52%
	60	0.42	61.3	3	87%	0.46	44.56	10.2	54%
	61	0.41	60.7	3.9	84%	0.412	43.6	11	54%
	62	0.45	60.3	3.1	85%	0.4	43.8	10.1	50%
	63	0.46	65.8	2.45	89%	0.42	43.6	9.6	55%
	64	0.5	64.1	2.6	89%	0.47	40.2	9	61%
	65	0.42	59.4	3.25	84%	0.41	40.1	8.9	56%
	66	0.41	63.4	3	86%	0.4	43.2	8.9	60%
	67	0.43	66	3.4	84%	0.39	39.1	9.2	56%
	68	0.41	62.4	3.1	86%	0.43	40.6	9.6	57%
	69	0.46	60.5	3	86%	0.44	44	8.45	61%
	70	0.4	61.8	3.5	85%	0.41	41	6.98	69%
	71	0.39	60.8	3	86%	0.4	40	8.9	59%
	72	0.38	66	2.5	88%	0.4	41.2	7.98	62%
	73	0.4	64.1	3.1	85%	0.46	40.6	8.63	59%
	74	0.4	62	3.3	85%	0.42	42.1	8.45	62%
	75	0.41	63.4	2.8	88%	0.43	43	9.23	60%

H. Dynamic simulation model development

Table 10.9 measurements of chapter 6- nitrogen and phosphorus concentrations of SBR0.8-200

	SBR0.8-200								
days	initial TP (mg/L)	initial PO4-P (mg/L)	initial Nitrate+nitrite (mg/L)	exp. TP (mg/L)	model TP (mg/L)	exp. PO4-P (mg/L)	model PO4-P (mg/L)	exp. Nitrate+nitrate (mg/L)	model Nitrate+nitrite (mg/L)
1	6.64	5.12	15.48	-	-	-	-	-	-
2	7.25	5.66	14.69	-	-	-	-	-	-
3	6.49	5.1	16.8	-	-	-	-	-	-
4	6.05	5.5	14.62	-	-	-	-	-	-
5	4.13	3.2	14.263	-	-	-	-	-	-
6	4.156	3.33	13.2	-	-	-	-	-	-
7	3.78	3.02	11.4	-	-	-	-	-	-
8	2.65	1.56	10.62	-	-	-	-	-	-
9	1.98	1.02	10.02	-	-	-	-	-	-
10	1.24	0.89	9.4	-	-	-	-	-	-
11	-	-	-	0.95	2.1	0.3	0.56	11.1	13.4
12	-	-	-	0.99	2.2	0.36	0.589	10.6	12.4
13	-	-	-	1.05	2.35	0.5	0.59	9.5	10.9
14	-	-	-	0.9	2.32	0.34	0.59	10.2	13.35
15	-	-	-	0.88	2.1	0.31	0.64	10.35	12.5
16	-	-	-	0.89	2.1	0.35	0.55	10.4	12.3
17	-	-	-	0.87	1.98	0.3	0.53	11	12.4
18	-	-	-	0.75	1.25	0.32	0.54	9.56	10.4
19	-	-	-	0.76	1.65	0.26	0.61	9.4	10.9
20	-	-	-	0.778	1.57	0.32	0.62	9	11
21	-	-	-	0.89	0.884	0.33	0.27	8.55	8.45
22	-	-	-	0.8	0.81	0.3	0.29	8.65	8.5
23	-	-	-	0.9	0.88	0.25	0.21	8.69	8.56
24	-	-	-	0.89	0.75	0.26	0.24	8.8	8.68
25	-	-	-	0.876	0.88	0.29	0.27	8.94	8.89

26	-	-	-	0.84	0.83	0.3	0.31	8.45	8.4
27	-	-	-	0.74	0.8	0.21	0.2	8.1	8.23
28	-	-	-	0.71	0.73	0.16	0.19	9.2	8.45
29	-	-	-	0.76	0.752	0.22	0.21	8.45	8.56
30	-	-	-	0.77	0.74	0.22	0.22	8.69	8.98
31	-	-	-	0.76	0.73	0.23	0.2	8.7	8.4
32	-	-	-	0.79	0.736	0.24	0.24	8.89	8.45
33	-	-	-	0.88	0.88	0.22	0.22	8.98	8.46
34	-	-	-	0.83	0.83	0.2	0.21	7.69	7.65
35	-	-	-	0.99	0.98	0.2	0.21	8.12	7.98
36	-	-	-	0.9	0.88	0.25	0.24	8.9	8.7
37	-	-	-	0.8	0.78	0.23	0.23	9.1	9.2
38	-	-	-	0.87	0.88	0.25	0.26	9	9
39	-	-	-	0.765	0.77	0.24	0.25	9.12	9.2
40	-	-	-	0.81	0.82	0.26	0.27	9.15	9.2
41	-	-	-	0.836	0.84	0.26	0.28	9	9.1
42	-	-	-	0.742	0.74	0.2	0.2	8.46	8.45
43	-	-	-	0.79	0.8	0.21	0.2	8.69	8.6
44	-	-	-	0.77	0.77	0.2	0.22	8	8.3
45	-	-	-	0.79	0.8	0.21	0.22	8.75	8.1
46	-	-	-	0.84	0.841	0.23	0.24	8.4	8.24
47	-	-	-	0.86	0.88	0.23	0.23	8.23	8.2
48	-	-	-	0.88	0.87	0.24	0.24	8.35	8.23
49	-	-	-	0.82	0.812	0.21	0.21	8.58	8.5
50	-	-	-	0.86	0.88	0.25	0.219	8.45	8.29