The Effect of Slowly Biodegradable Carbon on the Morphology, Integrity and Performance of Aerobic Granular Sludge

By

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Submitted to the graduate degree program in Civil & Environmental and Architectural Engineering and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

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The Effect of Slowly Biodegradable Carbon on the Morphology, Integrity and Performance of Aerobic Granular Sludge

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Abstract

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Abstract

In the last two decades, a new biofilm process, aerobic granular sludge technology, has been applied by researchers for organic and nutrient removal from municipal wastewater. Most studies have been performed with high strength wastewater and reported granules with irregular shape and incomplete organic and nutrients removal. Slowly biodegradable organic compounds, including the particulate and colloidal fraction, constitute an essential fraction of municipal wastewater. The objective of this study was to investigate the influence of slowly biodegradable organic matters, including particulate compounds, on the morphology, integrity and performance of aerobic granular sludge. Three identical lab-scale aerobic granular reactors (AGRs) were operated for two experimental phases in a sequencing batch reactor (SBR) regime.

The objective of the first experimental phase was to develop stable, compact and regular aerobic granules that achieve biological nutrient removal (BNR) of both nitrogen and phosphorus. For both experimental phases, the hydraulic loading rate was 1.32 kg COD/m^3/day. For the 1st phase, the three reactors were fed soluble organic matter (sodium

acetate) as the sole carbon source with an anaerobic-aerobic SBR cycle. To achieve complete BNR, a post anoxic cycle was included in the SBR cycle. During the second experimental phase, the influence of particulate COD was studied with particulate potato starch being added with and without pretreatment. In the first reactor, particulate starch was included in a 1:1 soluble COD: particulate COD ratio. For the other AGRs, the potato starch was pretreated either with heat hydrolysis or biological fermentation, an anaerobic process. Both pretreatments were applied to increase the bioavailable specific substrate for diffusion into the granular structure and degradation. For both experimental phases, results are presented for granule morphology, integrity and reactor treatment performance.

The findings of this study demonstrated that implementing a post anoxic phase remarkably increased both the nitrogen and phosphorous removal performance of the aerobic granules. 96% COD, 94% N and 86% P removal was accomplished after applying the post anoxic period in the SBR cycle. Comparable to previous studies, the free nitrous acid (FNA) inhibited the selection of PAOs when nitrite accumulated in the AGRs. The microscopic investigations and the EPS extraction showed that the presence of particulate starch in the feeding solution influenced the morphology and the structure of the granules. The granule surface had more filamentous growth and a more porous structure. AGR fed with a 1:1 particulate:soluble COD feed also demonstrated reduced nitrogen and phosphorous removal (78 and 81%, respectively). Moreover, only 75% COD removal was accomplished. Microscopy suggests that particulate removal was achieved by surface adsorption, followed by hydrolysis and degradation by microorganisms.

Both pretreatments were capable of solubilizing most of the particulate starch. The heat hydrolysis broke down the starch polymers to produce mostly soluble starch, slowly

biodegradable compound. This pretreatment also lead to filamentous growth, indicating that both the size and nature of the organic matter affected the aerobic granular sludge. On the other hand, most of the fermentation pretreatment products were VFAs. The anaerobic fermentation pretreatment enhanced and maintained morphology, integrity and performance of the aerobic granular sludge. Therefore, aerobic granular sludge can be successfully applied for BNR from municipal wastewater. However, if high particulate COD fractions are present, a fermentation pretreatment may be necessary.

To My Husband and My Glimmer of Light, Yazen

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SYMBOLS

SBR	- sequencing batch reactor
AGR	- aerobic granular reactor
CER	- cation exchange ratio
cm	- centimeter
COD	- chemical oxygen demand
rbCOD	- readily biodegradable chemical oxygen demand
sbCOD	- slowly biodegradable chemical oxygen demand
sCOD	- soluble chemical oxygen demand
pCOD	-particulate chemical oxygen demand
ESS	- effluent suspended solids (mg L^{-1})
EPS	- extracellular polymeric substances
HRT	- hydraulic residence time
ANOVA	- analysis of significance level
L	- liter
m	- meter
min	- minute
mL	- milliliter
MLSS	- mixed liquor suspended solids (g L^{-1})
VSS	- volatile suspended solids
PS/PN	- polysaccharide to protein ratio
rpm	- rotations per minute

s	- second
SRT	- sludge residence time (days)
SVI	- sludge volume index (mL g^{-1})
DNS	- dinitrosalicylic acid
FNA	- free nitrous acid
RS	- reducing sugars
BNR	biological nutrient removal
PAOs	phosphate accumulating organisms
GAOs	glycogen accumulating organisms
DPAOs	denitrifying phosphate accumulating organisms
DGAOs	denitrifying glycogen accumulating organisms
FNA	free nitrous acid

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CHAPTER 1: INTRODUCTION

1-1 Introduction

The need for applying more cost-effective and sustainable wastewater treatment technologies has motivated researchers to find new wastewater treatment applications. Biofilm sludge technology has been widely utilized to treat municipal and industrial wastewater, and biofilm systems can be more efficient than suspended activated sludge (Miqueleto et al., 2010; Schnaitman, 1970; Turakhia et al., 1989). The biofilm formation involves initial attachment of the suspended microorganisms to a surface such as carrier materials, followed by colonization and cell adhesion (Evans et al., 1994). Several microbial populations aggregate in a single entity as a response to chemical and physical stresses in microbial environments, resulting in immobilization and colonization of bacteria cells.

Various biofilm systems have been applied for wastewater treatment such as tricking filters (TFs), moving bed biofilm reactors (MBBR) and fixed-film activated sludge (IFAS). TFs remove ammonia and organic compounds in the wastewater by microbial communities (aerobic, anaerobic, and facultative bacteria; fungi; algae; and protozoa) attached to a medium as a biofilm or slime layer. Rotating biological contactors and packed bed reactors are common TFs systems (Boller et al., 1986; Grady Jr et al., 2011). MBBR technology utilizes polyethylene biofilm carriers operating in an aerated wastewater treatment basin. The biocarrier provides suitable surface area to enhance the growth of heterotrophic and autotrophic bacteria within its cells (Andreottola et al., 2000). IFAS process integrates the conventional biofilm characteristics and the completely mixed conditions of activated sludge process by including floating plastic carriers onto which biofilm can establish (Rosso et al., 2011). In the last two decades, a new biofilm process, aerobic granular sludge-technology, has been applied in lab- and full-scale pilots as

biological treatment for domestic and industrial wastewater to remove high strength organic and nutrients compounds (S. S. Adav, Lee, Show, et al., 2008).

1-2 Characteristics of granular sludge

Figure 1-1 depicts the difference in morphology between flocculent sludge and aerobic granular sludge for organic and nutrient removal application. Compared to activated sludge flocs, aerobic granules are regular, smooth, nearly round in shape, dense, fast settling particles that are able to withstand a high loading rate. The sludge volume index (SVI) is usually used to indicate the compactness of the settled sludge. Research has reported SVIs for aerobic granules in the range of 18-75 ml/g biomass, compared to higher than 75 ml/g for activated sludge (J. J. Beun et al., 2001; de Kreuk, Pronk, et al., 2005; Kim et al., 2004; Y. Liu et al., 2002; BS McSwain et al., 2005). Additionally, the settling velocity of aerobic granules has been reported to be in the rage of 20-70 m/hr, while the reported velocity for activated flocs is in the range of 7-10 m/hr (Lee et al., 2010).

In BNR treatment, anaerobic, anoxic and aerobic conditions should be applied to select for polyphosphate- accumulating organisms (PAOs), glycogen-accumulating organisms (GAOs), nitrifiers and dentrifiers. In conventional activated sludge system, these conditions were accomplished by construction of anaerobic, anoxic and aerobic treatment train. On the other hand, the conditions necessary for BNR are achieved in a single aerobic granule. A single granule contains different redox zones, including aerobic, anoxic and anaerobic, depending on the oxygen gradient through the granule. Therefore, different bacterial species associated with different redox gradients accumulate in the granule, which is critical for biological nutrient removal applications that rely on the selection of aerobic and anaerobic organisms. The ability of aerobic granules to include a variety of microbial communities is necessary for successful

removal of organic and nutrient compounds within a single reactor. Aerobic granular sludge has been studied with readily biodegradable wastewater for nutrient removal, and this technology is an effective application for simultaneous nitrification-denitrification and phosphorous removal (J. J. Beun et al., 2001; de Kreuk, Heijnen, et al., 2005; Dulekgurgen et al., 2003). Aerobic granular sludge should experience two different stages (an anaerobic/anoxic and an aerobic stage) to perform simultaneous nitrification-denitrification and phosphorous removal.

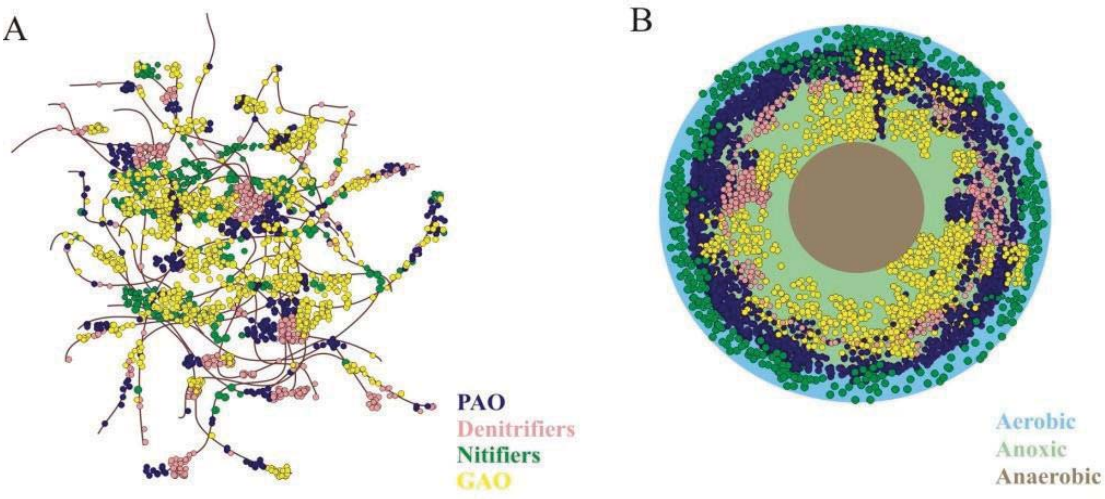

***Figure 1-1: Structural differences of A) flocculent activated sludge and B) aerobic granular sludge with nitrifiers (green) in the aerobic zone followed by PAOs (Blue) GAOs (yellow) and denitrifies (pink) in the up following anoxic zone. Phosphorous accumulating organisms (PAOs), glycogen accumulating organisms (GAOs), ammonium and nitrite oxidation organisms (Nitrifiers) and nitrate and nitrite reducing organisms (Denitrifiers), adapted from* (Winkler, 2012).**

Figure 1-2 shows the conversion processes taking place inside an aerobic granular reactor. In the anaerobic period, (PAOs), denitrifiers and (GAOs) store soluble organic compounds as intracellular polymers (Polyhydroxybutyrate, PHB); meanwhile, PAOs release phosphate to the bulk liquid (Dulekgurgen et al., 2003). In the aerated period, ammonia is

oxidized to nitrite, then nitrate in the outer zone of the granules; meanwhile, PAOs oxidize their intracellular storage polymers and take phosphate up from the bulk liquid. In the anoxic zone, denitrifiers convert nitrate and nitrite to nitrogen gas through a series of reactions.

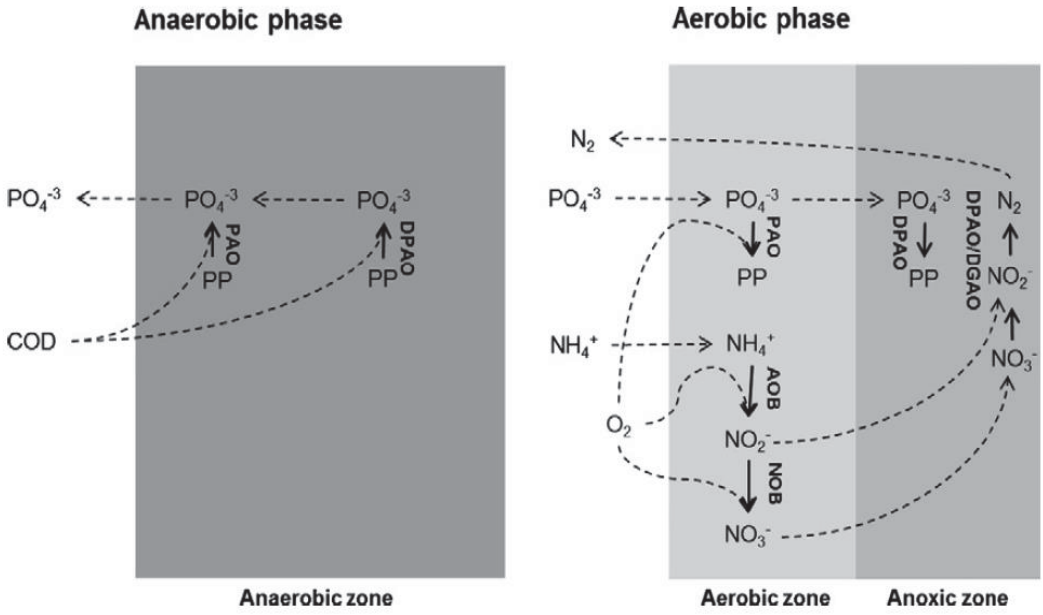

Figure 1-2: Schematic illustration of the conversion processes during the anaerobic and aerobic phases in the aerobic granular sludge structure. AOB: ammonium-oxidizing bacteria; NOB: nitrite-oxidizing bacteria; PAO: polyphosphate-accumulating organisms; DPAO: **denitrifying polyphosphate-accumulating organisms; GAO: glycogen-accumulating organisms; DGAO: denitrifying glycogen-accumulating organisms; COD: chemical oxygen demand; PP: polyphosphate adopted from *(Bassin et al., 2012a)*.**

1-3 Stability and formation of aerobic granulation

In contrast to conventional attached systems (i.e., IFAS, MBBR), aerobic granules aggregate without carrier material. Different microbial communities are grouped in a dense granule and bound to each other by a tight matrix of Extracellular Polymeric Substance (EPS). A granule is characterized by self-immobilization of microbial species as hypothesized in the literature (Show et al., 2012). EPS are metabolic products accumulating in the granule matrix. They consist of proteins, polysaccharides, humic acids, and lipids excreted by microbial communities

(Wingender et al., 1999). This secretion enhances the initiation of the aerobic granulation process (S. S. Adav, Lee, & Tay, 2008). Furthermore, it was hypothesized that EPS are responsible for bridging bacterial cells and other particulates into an integrated granule (Y.-Q. Liu et al., 2004). Studies have demonstrated most of the EPS are proteins, which enhance the stability of the granules (Dulekgurgen et al., 2003). The formation and integrity of the granule matrix depends on the reactor design and operation conditions, including shear force and settling time, loading rate, and the composition and nature of the substrate.

1-4 Parameters controlling aerobic granule formation and stability

1-4-1 Reactor configuration design

Typically wastewater treatment is performed with a completely continuous mixed reactor configuration. This configuration is associated with some drawbacks. One possible disadvantage is the overgrowth of filamentous bacteria, which leads to bulking and foaming formation (Huangfu, 2012). Moreover, this configuration needs a large footprint (Davies et al., 1998) and may not sustain a high loading rate (Steven C. Chiesa et al., 1985). In contrast, the sequencing batch reactor (SBR) typically selects against filamentous organisms (Steven C. Chiesa et al., 1985), requires less land (de Bruin et al., 2004), and can sustain high loading shocks (Chang et al., 1996). To date, aerobic granular technology has been successfully operated with SBRs. This configuration is modeled as a plug flow reactor or an infinite number of completely mixed reactors in series. Instead of substrate being removed across space, one reactor is subjected to a timed batch cycle, and substrate is removed as a function of time (Wilderer et al., 2001). Five distinguished phases--Feeding, React, Settle, Draw and Idle--are associated with SBR configuration. This sequencing in phases results in biomass with good settling velocity and high

substrate removal (Irvine et al., 1997). The period and strategy of each phase depends on the wastewater treatment application.

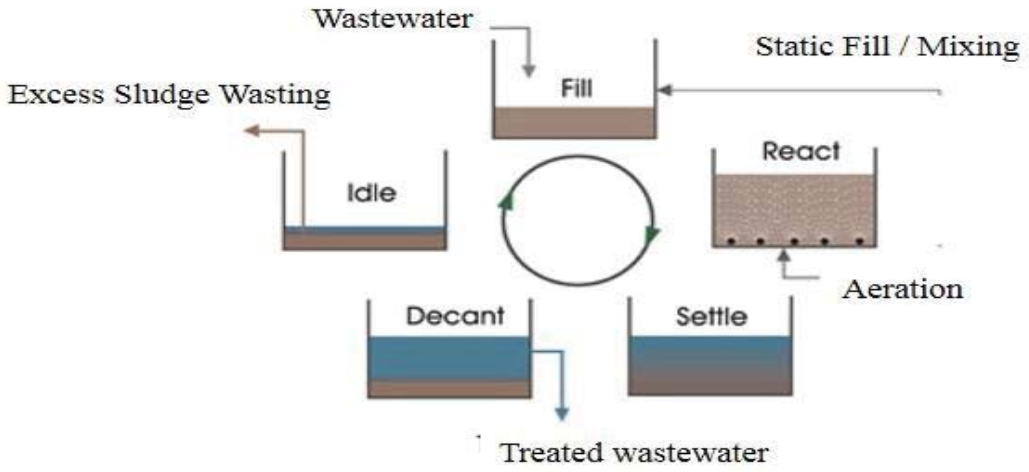

Figure 1-3: Schematic presentation of SBR operation applied for organic and nutrient removal application.

For organic and nutrient wastewater treatment, figure 1-3 represents the possible time sequence and SBR operation phases. The Feeding phase could be either static, mixed, or aerated Fill. If the feeding is performed with mixing, biological reactions will occur during this phase, either anaerobically (no aeration) or aerobically (with aeration). Studies have demonstrated that cultivating compact and dense granules can be achieved with a very short, or pulse feeding strategy (S. S. Adav, Lee, Show, et al., 2008). A short, static Fill creates a high substrate concentration in the reactor, which acts as an aerobic selector. This results in dense and compact granules with high COD and ammonia removal (J. Beun et al., 1999) B McSwain et al. (2004) investigated the role of different feeding strategies on aerobic granule formation. 100% static fill, 66% aerated fill and 33% aerated fill strategies were applied in a SBR regime. This study stated

100% static fill (dump fill) accumulates the substrates at the beginning of the aeration phase, which enhanced the formation of compact and stable granules. To cultivate aerobic granular sludge capable of performing simultaneous COD, nitrogen and phosphorous, other studies applied pulse feeding in which the feed solution is supplied to the biomass from the bottom of the reactor. This selects for the anaerobic selector, improving BNR process (Bassin et al., 2012b; de Kreuk, Heijnen, et al., 2005). The air and mixing requirements for the React phase are provided by sparging air through fine bubble diffusers. During the Settle phase, granules are selected with a short settle time, which washes out any slow-settling particles. This will be discussed in more detail in the next section.. While in the Draw phase, effluent wastewater is pumped from the extraction point. The Idle phase is employed for wasting purposes.

1-4-2 Operation conditions

1-4-2-1 Hydrodynamic shear force

In biofilm systems, the initiation of colonization and further aggregation of microbial cells occurs as a response to physical or chemical stresses (O'Toole et al., 1998). Hydrodynamic shear stress is considered one of the essential physical conditions controlling biofilm formation and diffusion of substrates into these immobilized microbial cells (Y. Liu et al., 2002; Vieira et al., 1993). For aerobic granule formation, relativity high hydrodynamic shear force is a key factor in forming strong and dense granules. Literature has studied the impact of shear stress in terms of superficial upflow air velocity (u_s) on formation and stability of granules. This velocity is defined as the air supply rate divided by the surface area of the reactor over which the air supplied. It has been reported that at least 0.87 cm/s superficial upflow air velocity is required to sustain stable granulation (Q. Wang et al., 2004). More compact and denser granules have been formed in reactors operated with superficial upflow air velocities equal to or higher than 1.2 cm/s

(J. J. Beun et al., 2001; de Kreuk, Heijnen, et al., 2005; Y. Liu et al., 2002; Tay et al., 2001). Furthermore, a study conducted by Adav et al (2007) supported these results. This study demonstrated the influence of applying different air flowrates (0.59, 1.18, 1.17 cm/s) on the stability and formation of granules, as well as EPS production. Compact flocs were formed at the lowest air flowrate. with no granule formation At the higher flowrates of 1.18 and 1.77 cm/s, the formation of granules accelerated, reaching 1 to 3.5 mm in diameter. The extracted EPS for the granules contained 309–537 mg proteins g^{-1} biomass and 61–109 mg carbohydrates g^{-1} biomass. In contrast, EPS extracted from flocs formed at the low air intensity contained 50.2–76.7 mg proteins g^{-1} biomass and 50.2–77.3 mg carbohydrates g^{-1} biomass. The extraction of the EPS data revealed that the formation of compact granules is associated with high extracellular protein content (S. Adav et al., 2007). Therefore, the shear force influences not only the shape of the granules but also its EPS composition.

1-4-2-2 Settling time and settling velocity

Settling time is another important parameter that influences the formation of aerobic granules. The effect of minimum particle settling velocity (v_s) has been well-defined in the literature. Although some studies have reported aerobic granule formation in SBRs operated with settling periods as long as 30 min (Moy et al., 2002), aerobic granules have largely been cultivated by applying shorter settling times equal to or less than 10 min (J. J. Beun et al., 2001; de Kreuk, Heijnen, et al., 2005). Short settling times retain only fast settling particles with settling velocities higher than 12 m/hr, causing washout of slowly settling flocs (de Kreuk, Pronk, et al., 2005).

In terms of granule integrity, the settling period has been shown to influence EPS composition. McSwain et al (2005) investigated the impact of settling time on granule formation.

Two SBRs were operated with 3- and 15-m/hr settling times. Results demonstrated flocs were predominant in the SBR with a 3-m/hr settle period with 120 ± 12 ml/g biomass SVI. The SBR with a 15-m/hr settle period enriched strong and dense granular sludge characterized by 50 ± 2 ml/g biomass SVI. Also, this study showed that the extracellular protein content for the formed granules was two times the content extracted from the flocculent sludge. This supports the hypothesis that a high extracellular protein content contributes to robust granule integrity (BS McSwain et al., 2005).

1-4-2-3 Organic loading rate

Studies have demonstrated the influence of organic loading rate on the morphology and stability of aerobic granules. A low organic loading rate enhances the growth of filamentous bacteria on the surface of the granules and in the reactor (Y. Liu et al., 2006). This yields granules with poor settling characteristics, which is demonstrated with a high SVI value and irregular shape. Recently, Lee et al (2010) investigated the fate and transformation of aerobic granules and filaments under different organic loading rates. Two 1-L SBRs were fed with 0.5 and 2 ($kg/m^3.d$) loading rates, respectively. Irregular aerobic granules (2–3 mm) with fungi-like black filamentous growth were collected from an SBR and used in this study as the reactor inoculum. The reactors were fed daily with glucose and aerated by fine bubble diffusers. Large granules with diameters larger than 20 mm were reported in both reactors after more than 100 d of cultivation. However, different granule morphologies were observed. For the reactor with a higher organic loading rate, the black filamentous bacteria were no longer observed on the granules, which were regular and round in shape. At the lower loading rate, filaments were dominant (A.-j. Li et al., 2010). These results are supported by the kinetic selection theory for filament selection in completely-mixed activated sludge systems. According to the theory, low

substrate concentrations favor the growth of filamentous over floc-forming bacteria as shown in figure 1-4 (J. Chudoba et al., 1973; Jan Chudoba, 1985).

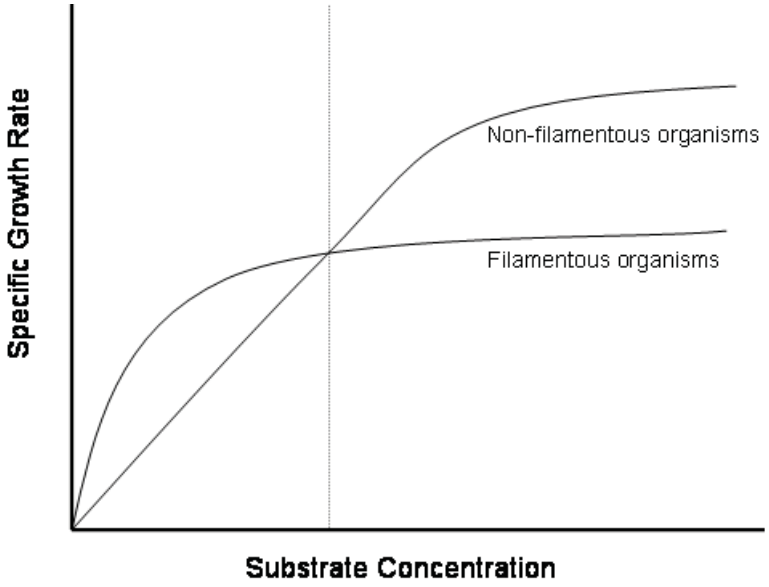

Figure 1-4: Microbial growth curves as proposed by the kinetic selection theory by J. Chudoba et al., 1973.

In a previous study, Liu et al (2007a) investigated the effect of the SBR cycle length on the morphology of aerobic granules and biomass yield. Activated sludge was cultivated in three SBRs with 1.5, 4 and 8 hr operation cycles with organic loading rates 8, 3 and 1.5 kg/(m^3 d) respectively. This experiment showed specific biomass growth rates of aerobic granules declined from 0.266 to 0.031 d^{-1}, while the corresponding biomass growth yield (Y_{obs}) declined from 0.316 to 0.063 g VSS g^{-1} COD. Moreover, the 1.5 hr cycle time yielded the biggest granules while the 4 hr cycle time yielded the most compact ones compared with those cultivated at other cycle times (Y.-Q. Liu et al., 2007). It is not surprising that the SBR with a higher loading rate yielded more biomass. In the Liu et al. (2007) study, the changes in loading rate and SBR cycle time would have greatly affected kinetic selection within the reactor as well. Implementing a long cycle creates a portion of time in which the substrate concentration approaches zero. This is

often referred to as a feast-famine regime for SBRs, and the famine period has been shown to select against starvation-sensitive filamentous bacteria (Steven C Chiesa et al., 1985). Therefore, for aerobic granules grown in SBRs, both the intensity of the substrate concentration and the duration of famine conditions are expected to influence species selection and granule formation (Y. Liu et al., 2006).

1-4-3 Composition and nature of wastewater constituents

Stable, robust and nearly round aerobic granules have been widely cultivated with various readily biodegradable substrates (rbCOD) such as, glucose, acetate, phenol and sucrose (S. S. Adav, Lee, & Tay, 2008; BS McSwain et al., 2005; Mosquera-Corral et al., 2005; Zheng et al., 2005). In fact, in addition to the rbCOD fraction, real wastewater consists of slowly biodegradable organic matter (sbCOD) including particulate and colloidal matter (Huang et al., 2010). Some studies have reported aerobic granule cultivation with real wastewater containing particulate COD such as abattoir wastewater (Cassidy et al., 2005), mixed of 40% domestic and 60% industrial (Y.-Q. Liu et al., 2010), diary wastewater (N. Schwarzenbeck et al., 2005), and malting wastewater (N Schwarzenbeck et al., 2004). S.-G. Wang et al. (2007) successfully formed aerobic granules with brewery wastewater to remove organic and nitrogen compounds in an 8.6 L SBR. The wastewater contained 1300-2300 mg/L COD and 30-37 mg/L total nitrogen, 77-87% of which was soluble COD. Ammonia chloride was added to the real wastewater to adjust the COD:ammonia ratio to 100:10. In this study, 88% COD removal and 89% total nitrogen removal was achieved, respectively. Yilmaz et al. (2008) investigated the performance of grown granules with organic and nutrient loading rate as 2.7 g COD L^{-1} day^{-1}, 0.43 g N L^{-1} day^{-1}, and 0.06 g P L^{-1} day^{-1} . The influent organic matter was (600–783) mg COD/L, including (265–384) mg COD/L soluble organic compounds. High effluent suspended solids

were observed, which limited the overall removal efficiency to 68%, 86%, and 74% for total COD, TN, and TP, respectively.

The variation of aerobic granular characteristics fed with different types of degradable compounds leads to further research inquiries. What would occur if wastewater contains a higher percentage of particulate COD fraction? How would this fraction affect the morphology, integrity and performance of aerobic granular? What are the mechanisms responsible for the hydrolysis and degradation of slowly biodegradable compounds within aerobic granular sludge?

1-5 Hydrolysis of slowly biodegradable organic matters

Within a full-scale wastewater treatment plant, large particulate COD is often removed with a primary clarification unit. However, primary clarifiers do not remove particle sizes ranging from 10^3 amu (atomic mass unit) to 100 μm (Morgenroth et al., 2002). Hydrolysis of these slowly biodegradable organic compounds is considered as the rate-limiting step in the removal of organic matter and nutrients from municipal and industrial wastewater (Drewnowski et al., 2011). The main concern related to the presence of polymers is the conversion processes to more readily biodegradable compounds, which are particularly necessary for phosphorus removal. The conversion processes are controlled by enzymatic hydrolysis occurring in the bulk liquid and on the organism's surface. Batstone et al. (2002) stated that hydrolysis of polymers can be represented by two conceptual mechanisms (Batstone et al., 2002):

- Enzymatic secretion by organisms to the bulk liquid where they are adsorbed onto a polymer or with a soluble substrate.
- Attachment of a polymer to an organism, which produces enzymes in its vicinity and received benefit from soluble products generated by the enzymatic reaction.

According to the enzyme-adsorption based kinetic model, availability of adsorption sites and concentration of hydrolytic enzymes are key factors for the increase of the rate of hydrolysis (Vavilin et al., 2008). Therefore, for biofilms and specifically granular sludge, it should be questioned how hydrolysis of slowly biodegradable compounds would take place and diffuse inside the biofilm. Furthermore, if diffusion occurs, how would this influence the stability and integrity of the biofilm matrix?

1-6 Effect of slowly biodegradable compounds on biofilm technology including aerobic granular

It has been hypothesized that the macromolecules characterized by low diffusion rate in the biofilm matrix could influence the biofilm formation if hydrolytic activity takes place inside the biofilm. Consequently, low density and a rough biofilm surface could enable transport and conversion of large polymeric molecules. Mosquera-Corral et al. (2003) studied the structure of biofilms converting two different polymers (soluble starch and soy-proteins) and their associated monomers (glucose and an amino acid, aspartate) in biofilm airlift suspension (BAS) reactors. Results showed more than 95% of the total hydrolytic activity occurred inside the biofilm entity. Also, biofilms formed with polymers were characterized by rough surfaces and low biomass density (Mosquera-Corral et al., 2003).

To date, limited research has been conducted on the stability and performance of aerobic granular sludge with a large proportion of slowly biodegradable influent substrate. Schwarzenbeck et al. (2004) treated malting wastewater with 1230 mg/L particulate COD and reported around 50 % of particulate COD was removed. However, the formed granules were irregular in shape and dominated with fluffy structure on the surface as presented in Figure 1-5-

A. Moreover, the authors attributed the removal of the particulate COD to the presence of protozoa growing on the granule surface or on the reactor wall (N Schwarzenbeck et al., 2004). Typically, particulate matters must be hydrolyzed extracellularly before soluble substrate can be transported into the cell and degraded. For aerobic granules, this could be challenging since the particulate fraction cannot penetrate or diffuse into the granule.

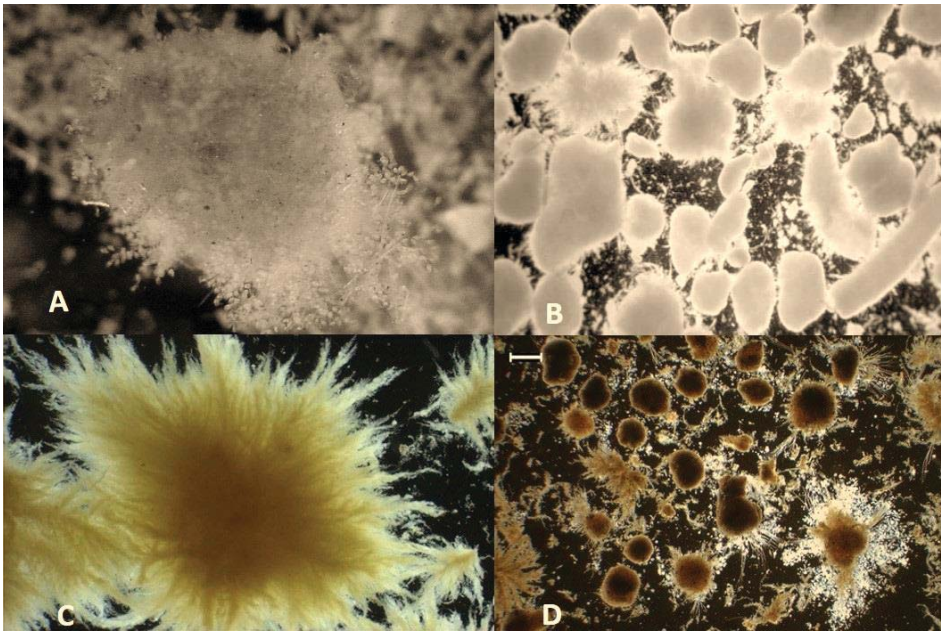

Figure 1-5: Aerobic granules fed with complex wastewater, malting wastewater (A) (N Schwarzenbeck et al., 2004), dairy wastewater (B) (N. Schwarzenbeck et al., 2005), starch (C) and real municipal wastewater in the Netherlands (D) (de Kreuk et al., 2010).

De Kreuk et al. (2010) examined the performance and morphology of aerobic granular sludge treating wastewater with a particulate and colloidal fraction of suspended and soluble starch. Adsorption of the starch was observed onto the granule surface. Moreover, although aerobic granules were maintained in the reactors, the granule matrix was associated with excessive filamentous surface (Figure 1-5-C). In terms of treatment performance, the low carbon diffusion inside the granules led to a noticeable decrease in the denitrification performance. Also, only 50% total COD removal was obtained in this study (de Kreuk et al., 2010). The irregular

granule morphology could be attributed to the extended substrate availability during the aeration phase, increasing the substrate gradient in the granule, and minimizing the famine period that is known to select against filaments (Steven C. Chiesa et al., 1985). Lastly, disintegration of the granule may be caused by loose Extracellular Polymer substances secretion from the granule network. Up to now, research addressing the effects of particulate compounds on EPS composition and distribution has not been done. Therefore, in order to obtain a practical approach towards control of aerobic granular structure treating real wastewater, research should discern the influence of sbCOD and particulate COD on the performance and integrity of granular sludge.

1-7 Scope of this study

The purpose of this study is to investigate the effect of particulate COD fraction contained in municipal wastewater on aerobic granule treatment performance and granule integrity in terms of EPS and granule shape and size. Based on low COD removal efficiencies reported by N Schwarzenbeck et al. (2004) and de Kreuk et al. (2010) when particulate COD was present, this study also examines the feasibility of implementing primary fermentation to convert the particulate organic fraction to a readily biodegradable organic fraction that can be easily removed by aerobic granular sludge, and which may meet the specific needs of phosphorus accumulating organisms for volatile fatty acids.

The total organic loading rate used in this study was 1.3 $kg/m^3/day$. In the first stage of the study, three SBRs were operated to cultivate aerobic granular sludge fed with acetate as the only carbon source. In the second stage, particulate starch was included in a 1:1 soluble COD: particulate COD ratio. Reactor (R1) was fed with the synthetic wastewater without pretreatment. For the other AGRs, the potato starch was pretreated before being fed to the aerobic granular

reactors. For reactor (R2), the starch was heat hydrolyzed before being fed the reactor. In the third reactor (R3), the synthetic wastewater, including the 0.5 particulate fraction, was pretreated with a primary fermenter before being fed to the aerobic granular reactor. Many full-scale wastewater treatment plants currently utilize fermentation reactors to produce volatile fatty acids for biological phosphorus removal, so this pretreatment step simulates a feasible unit that could be implemented to increase aerobic granule performance, should the particulate COD fraction be found to be detrimental.

CHAPTER 2: METHODS AND PROCEDURES

2-1 Experiment set up

2-1-1 Reactor set up and operation

Aerobic granular sludge experiments were carried out in three identical bubble columns as sequencing batch reactors (SBRs). The reactor operating conditions during the experimental phases are presented in Table 2-1. During the first 22 days of the experiment, aerobic granular reactors were operated with SBR cycle phases: Anoxic/Anaerobic Fill, Aeration, Settle and Decant periods. Nitrogen gas was sparged during the first five minutes in the Fill period to reduce the residual oxygen from the Aeration phase of the previous cycle. A post Anoxic phase was implemented on the 23rd day of the experiment to complete denitrification prior to the next SBR cycle (thereby reducing the NO_3 concentration as well), and the operating cycle was expanded to 6 hrs instead of 4 hrs. On day 30, the total cycle period was returned to 4-hr cycle period.

Table 2-1: Configuration of the SBR cycle through experimental phases.

	1st phase of the experiment			2nd phase of the experiment
Phase	day 1–22	day 23–30	day 31–44	day 45-97
Anoxic/Anaerobic Fill (min)	60	60	60	60
Aeration (min)	176	266	146	146
Post Anoxic (min)	_	30	30	30
Settling (min)	2	2	2	2
Decant (min)	2	2	2	2
Total cycle duration (min)	240	360	240	240

The SBRs were designed to handle 3-L working volume as detailed in Figure 2-1. A photo of the

three aerobic granular reactors (AGRs) installation is presented in Figure 2-2. The internal diameter was 6.4 cm and the height was 96 cm. The cycle was controlled with a programmable timer (ChronTrol, San Diego, CA). The treated wastewater was extracted at a height around 50 cm, which approximately yields 50% exchange ratio. Pumping the wastewater to and from the reactors was performed with peristaltic pumps (Cole-Primer model 7524, IL). Mixing during Aeration and Anoxic phases was accomplished by the supplying of air and nitrogen gas, respectively, from a fine porous stone at the bottom of each reactor. The dissolved oxygen (DO) level was not controlled and it was near saturation (around 7.8 mg O_2/L). Air was supplied by a pressurized service connection, and the rate was controlled by a flow meter in terms of liters per minutes. The air flowrate was set to 2.5 L/min to provide the sufficient superficial upflow gas velocity (1.3 cm/s) to maintain compact granules as mentioned in the literature (S. Adav et al., 2007; B. S. McSwain, 2005). During the anoxic phase, 0.5 L/min nitrogen gas flowrate was supplied to the reactors from solenoids controlled by a flow meter. The total settling time (Settle and Decant) was 4 min to allow for the settling of the high settling velocity flocs and granules. The pH was controlled to 7.5 by a pH electrode and controller by dosing carbon dioxide gas via fine porous stone. The temperature was controlled by a recirculating water bath (Isotemp model 3016P, Champaign, IL). The temperature was set to 25^0 C during the first two weeks of the experiment to maximize the nitrification kinetics. For the rest of the experiment, temperature was set to room temperature (20^0 C).

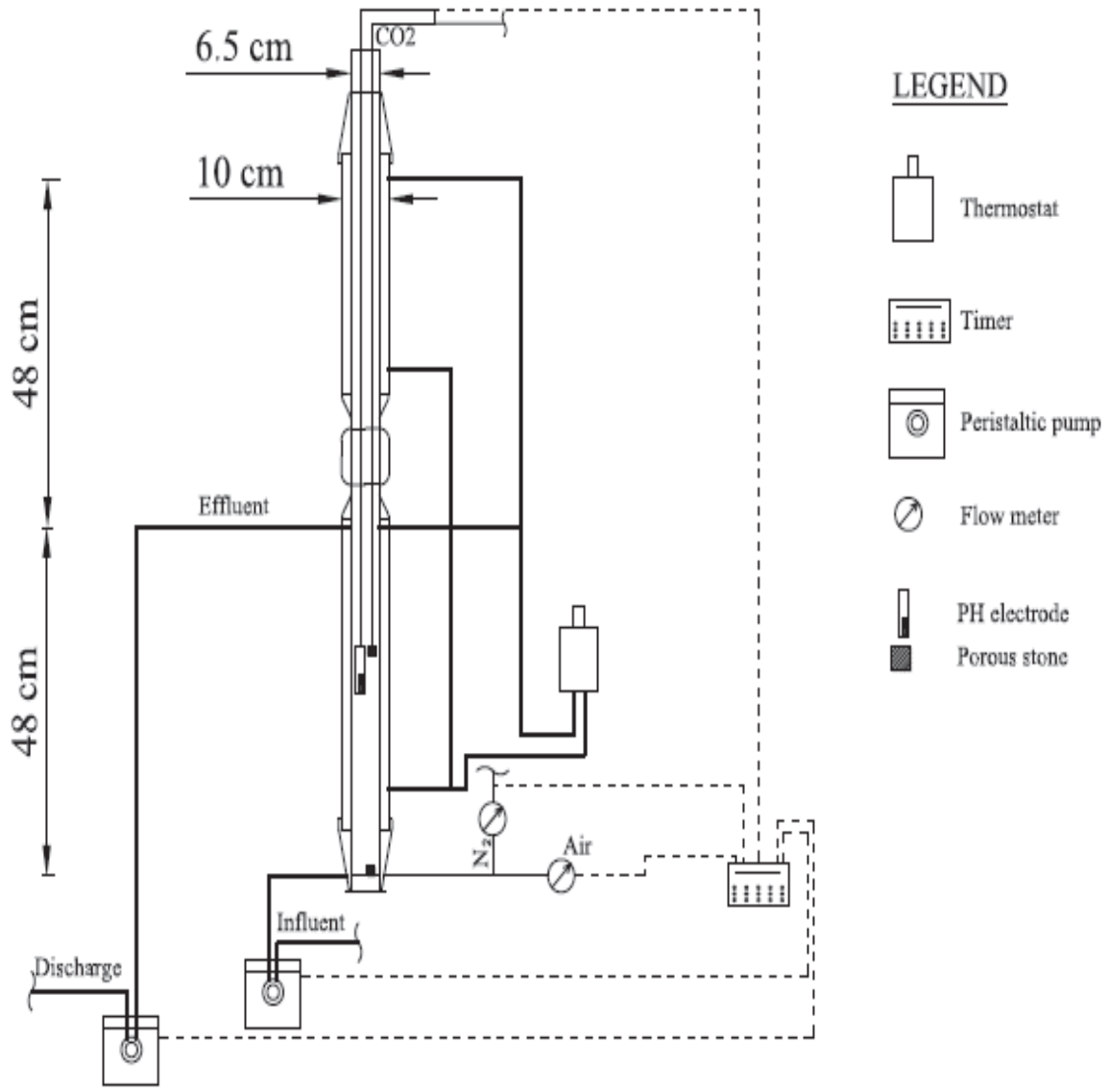

Figure 2-1: Schematic representation of the 3-L aerobic granular sequencing batch reactor (SBR) set-up.

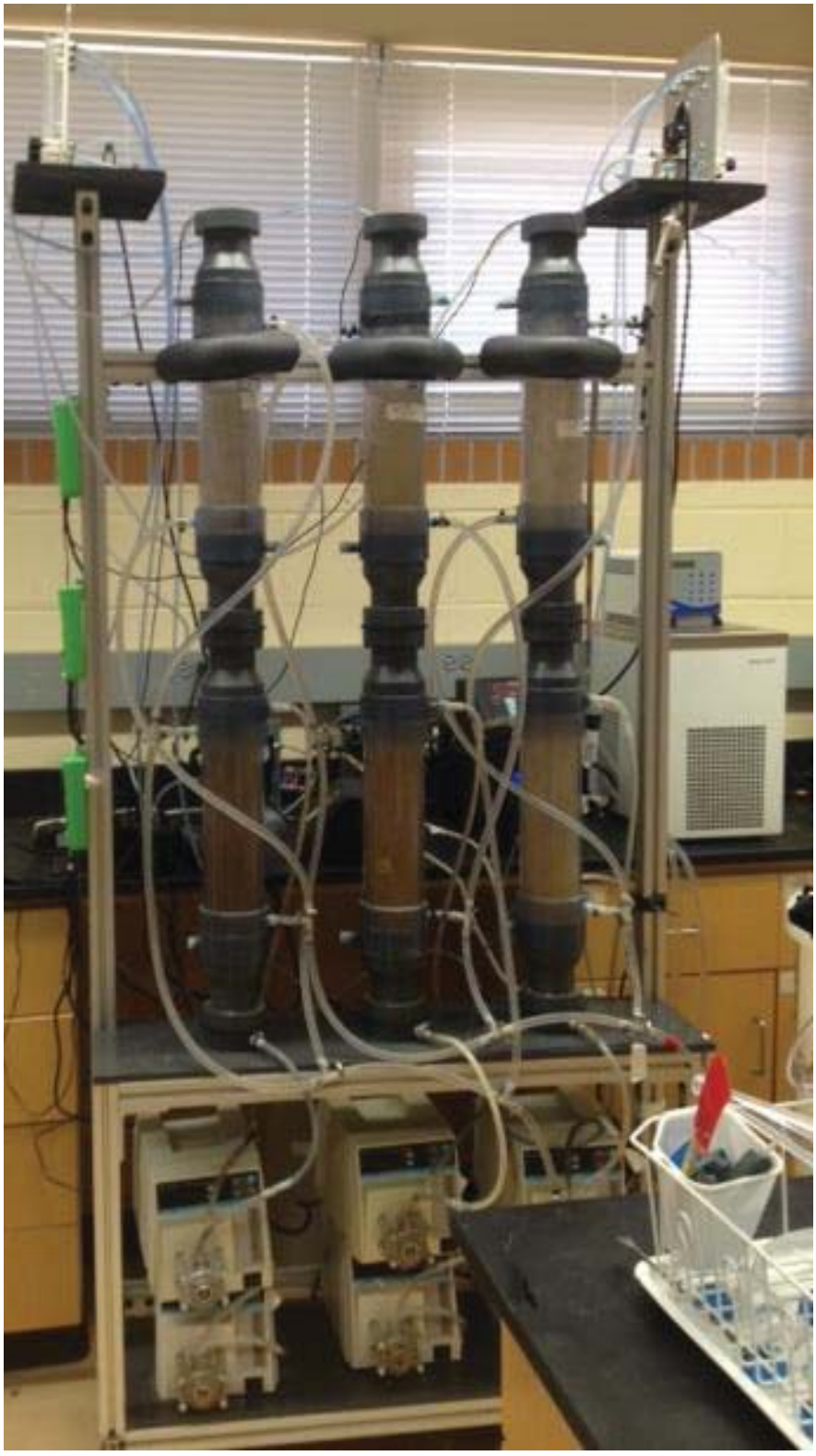

Figure 2-1: The aerobic granular reactors installation in the environmental laboratory at the University of Kansas.

2-1-2 Fermenter operation and design

During the second experimental phase, fermentation of the synthetic wastewater containing particulate COD was conducted in a cone-polyethylene tank operated in an SBR regime (Figure 2-3). The fermenter was operated with a 12 hr-cycle including 0.5 hr Feeding, 9.75 hrs Anaerobic Mixing, 1 hr Settling, 0.5 hr Decant and 0.25 hr Idle. The cycle was controlled with a programmable timer (ChronTrol, San Diego, CA). Peristaltic pumps (Cole-Primer model 77521, IL) were used to pump the wastewater to and from the fermenter during the Feeding and Decant periods. Mixing was achieved in the fermenter with one submersible pump (1/12 hp, Marineland, Ridgefield, CT). To enhance hydrolysis, enzymatic production and fermentation, the fermenter was run under a mesophilic temperature range (35- 50^0 C) (Darilek, 1996). Therefore, temperature was controlled to 40^0 C in the fermenter by a recirculating water bath passed through (Isotemp, Fisher Scientific, Champaign, IL). The working volume for the fermenter was 10 L and the hydraulic retention time (HRT) was 24 hrs. Each cycle, 5 Liters of fermented supernatant was pumped to a storage container to feed Reactor 3.

The solid retention time (SRT) was controlled to 2 days, as recommended in the literature (Bagchi, 1994) to prevent the growth of methanogens that could consume the volatile fatty acids produced. To control the SRT, mixed liquor suspended solids were wasted daily from the fermenter. The wastage process was done during the Idle period after the Decent period. The volume of the wasted MLSS was calculated based on the MLSS in the fermenter, working volume of the Fermenter and the effluent suspended solids (ESS).

Figure 2-2: Fermenter installation in the environmental laboratory at the University of Kansas.

2-1-3 Wastewater composition

A synthetic wastewater was prepared to represent municipal wastewater. For both experimental phases, the wastewater recipe was the same except the carbon source. The synthetic wastewater was usually prepared daily to prevent any degradation in storage prior to reactor feeding. During the SBR cycle, 1.5 and 5 L were pumped into the reactors and fermenter, respectively. The composition of the synthetic wastewater during the first experimental phase is presented in Table 2-2. Trace solution was prepared according to Smolders at al. (Smolders et al., 1995), and this is presented in Appendix A, Table A-1. Addition of particulate COD for the second experimental phase is described in the next section.

Table 2-2: Synthetic wastewater characteristics[1]

Feed compounds	Concentration mg/L
Carbon source	440[2]
$MgSO_4.7H_2O$	83
KCl	33
NH_4Cl	117[3]
K_2HPO_4	20[4]
KH_2PO_4	8[4]

[1] 2 mL of trace solution was added for each liter.
[2] as COD (mg O_2/L).
[3] Equals 30 mg N/L.
[4] Equals 5.4 mg P/L.

2-2 Experiment phases

The purpose of this study was to investigate the feasibility of aerobic granular sludge for organic and nutrient removal from municipal wastewater with a particulate COD fraction. Table 2-3 shows the organic matter particle size distribution in typical municipal wastewater and in this study's wastewater. To assess the particle size in the synthetic wastewater, membrane filtration test was used with different sizes (100, 73, 30, 20 and 10 micron). Membranes were sorted from the biggest size to the smallest size and 500 mL of the prepared wastewater was filtered through the sorted membranes. The filtrate was passed through a glass filter (GFF) to measure the soluble fraction. Then, membranes and filter was dried for 3 hours at 105 C. Membranes were cooled and weighted to measure the percentage of the remained carbon compounds amount on each membrane to the total used compound amount.

Table 2- 3: Particle size distribution (%) of organic matter in municipal wastewater the synthetic wastewater used in this study.

	Size (μm)				
	< 0.08 (Soluble)	(0.08-1) (Colloidal)	1-100 (Supra colloidal)	> 100 Settable	Reference
Raw wastewater	30[1]	-	60	10	(Huang et al., 2010)
Primary effluent	41	7	28	15	(Balmat, 1957)
1st experimental phase	100	-	-	-	This study
2nd experimental phase	50	-	47	3	This study

[1] This percentage represents organic matter particle size smaller than 1 μm.

2-2-1 Phase 1

Acetate was used as the sole carbon source in the first experimental phase. This experiment was conducted to evaluate the aerobic granule performance and stability when fed with readily biodegradable organic compounds (rbCOD). Also, a post anoxic period was implemented to enhance the denitrification process, without adding an additional carbon source. The three SBRs were seeded with unstable aerobic granules formed in a previous experiment. The mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were 1164±84 and 1085±60 mg/L, respectively. The operational conditions and the cycle configuration are mentioned in section 2-1-1. This phase lasted for 42 days before starting the 2nd phase of the experiment.

2-2-2 Phase 2

This phase was conducted to examine the fate of the slowly biodegradable organic portion (colloidal and particulate fraction) in municipal wastewater treatment, as well as its effect on the granule integrity and performance. Potato starch purchased from Thermo Fisher Scientific (part number, 9005-84-9) was used as a particulate carbon source, and this was added to sodium acetate in a 1:1 weight chemical oxygen demand (COD) ratio. The equivalent amount of starch

and sodium acetate used to satisfy this COD ratio was calculated based on the theoretical COD equivalent for starch and sodium acetate (1.27 mg O_2/mg starch ad 0.78 mg O_2/mg sodium acetate, respectively). The aerobic granular SBRs from the 1st phase were run with different pretreatments as described in Figure 2-4. Reactor 1 was fed with 1:1 particulate COD to soluble COD ratio. For Reactor 2, the particulate starch was heat-hydrolyzed before mixing with the synthetic wastewater to increase the available specific substrate surface area, thereby accelerating the degradation process to produce degradable starch and reducing sugars that are easier to diffuse inside the granules. Heat-aided hydrolysis was performed by heating the starch in boiling water for 5 min then autoclaving for 1 hr at 120^0 C. Reactor 3 was fed with the fermenter supernatant, described below.

The fermenter was fed with the same synthetic wastewater for reactor 1 (a 1:1 COD ratio of acetate: potato starch). The fermenter was seeded with 1 L of biomass from the anaerobic digester operated with 98^0 C and 4 L of activated sludge from the aeration basin from the Lawrence Municipal Wastewater Treatment Plant, Lawrence, KS. Two weeks before the startup of the 2nd phase, the fermenter was commissioned to allow the bacteria to acclimate to the new synthetic wastewater. 5 L of the fermenter supernatant was pumped every 12 hr to a plastic container, 1.5 L of which was pumped to Reactor 3 every 4 hrs. The fermenter SRT was maintained at 2 d; these procedures are detailed in section 2-1-2.

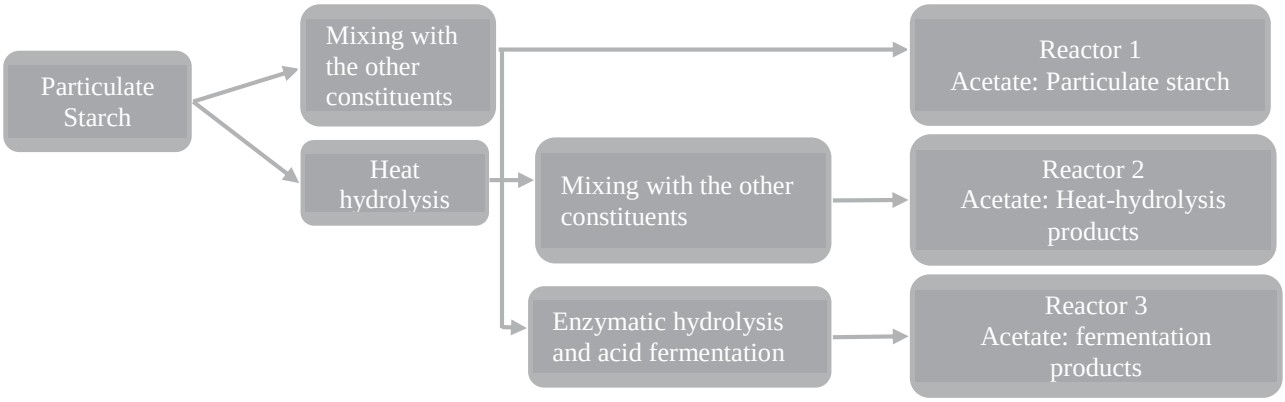

Figure 2-4: Schematic diagram of the 2nd phase of the study. The "other constituents" are outline in Table 2-2.

2-2-3 Batch experiments of anaerobic hydrolysis and fermentation process potential

A series of batch experiments were conducted to assess the efficiency of anaerobic hydrolysis and fermentation of particulate starch by mixed cultures. These batch fermentations were performed in 200 mL capped serum bottles for 10 hours. 200 mL mixed liquor (988 mg VSS/L) was taken from the fermenter and left for 1 hour to settle the biomass. After that, the supernatant was dumped and the biomass was washed with deionized water. The washed biomass was inoculated in the serum bottles with 200 mL synthetic wastewater with particulate starch as the sole carbon source (equivalent to 223 mg COD/L) and the other nutrient constituents in the synthetic wastewater presented in Table 2-2 (minus the acetate). The serum bottles s were sealed with rubber stopper and aluminum seals and incubated at 40^0 C in a water bath for 10 hr. The bottles were shaken every 15-30 min to provide sufficient contact between the biomass and the particulate starch. Samples were collected in 1-2 hr intervals, filtered through 0.45 μm, and analyzed for soluble COD and VFAs production.

2-3 Analytical methods

2-3-1 Sludge and effluent suspended solids characteristics

During the 1st phase, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), and the sludge volume index (SVI) were measured twice a week according to the *Standard Method for the Examination of Water and Wastewater* (APHA et al., 2005). During the 2nd phase, effluent suspended solids and volatile suspended solids (ESS and EVSS) were measured twice a week. For the fermenter, daily measurements of MLSS, MLVSS, ESS and EVSS were done to estimate the sludge volume needed to be wasted to maintain 2-day SRT.

2-3-2 Extracellular polymeric substances analysis

Extracellular polymeric substances were extracted and characterized to investigate the EPS content variations corresponding to readily and slowly biodegradable substrate concentrations. Since the main substances in the EPS are proteins and carbohydrates, these were the two EPS fractions quantified.

2-3-2-1 Extracellular polymeric substances extraction

EPS were extracted with the cation exchange resin (CER) method according to (Frølund et al., 1996) with slight modifications. Cation exchange resin (Dowex 50x8, Na^+Form) purchased from Sigma (part number, 44445) was used to extract the EPS with a 1 MLVSS: 70 CER weight ratio. For phase 1, mixed liquor samples were collected from each reactor before the end of the cycle. The samples were centrifuged at 4°C and 10,000 rpm for 15 min to separate the biomass pellets from the bulk liquid. CER with the mentioned ratio was washed with a phosphate buffer (pH 7.5) and filtered. The biomass pellets were re-suspended in 40 ml phosphate buffer and gently homogenized with a pestle for 2 min. The homogenized biomass was added to the washed CER and the volume was set to the original volume of the samples with phosphate buffer. The resulted

mixture along with the blank CER sample was stirred at 800 rpm for 4 hours in the dark at 4°C. After 4 hr of extraction, the samples were centrifuged at 4°C and 10,000 rpm for 15 min, and the supernatant was stored at -20°C for the protein and carbohydrate content analyses of the tightly-bound EPS (TB-EPS).

For phase 2, the same extraction method was followed with slight modifications in the pretreatment steps according to (Liang et al., 2010; Yu et al., 2008) to distinguish between the tightly (TB) and loosely (LB) bound EPS (Figure 2-5). The EPS extraction was performed in triplicate in three consecutive days. First, the MLSS samples were centrifuged at 4^0 C and 2000 rpm for 15 min. The supernatant was collected to separate the reactor wash or bulk liquid. After the homogenization step mentioned above, the resulted mixture was centrifuged at 40 C and 8000 rpm for 15 min. the supernatant liquor was collected and stored at -20^0 C to measure the LB-EPS composition. Then, the pellets were re-suspended with buffer solution to their original volumes and extracted with the CER flowing the same steps as in the 1st phase.

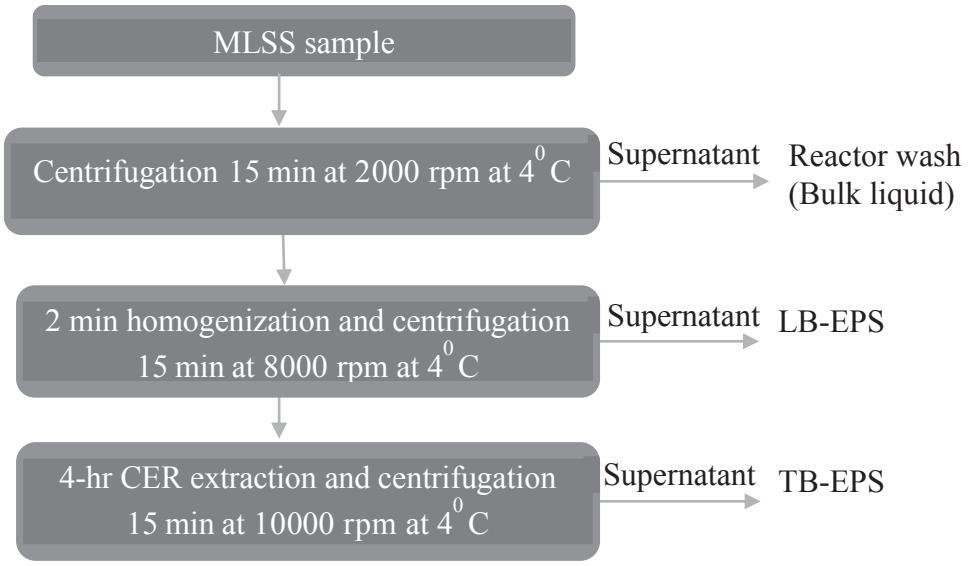

Figure 2-5: Procedure steps of the EPS extraction during the 2nd phase of the experiment.

2-3-2-2 Protein and carbohydrate content methods

Protein content was measured according to the Lowry method (Lowry et al., 1951). The extracted EPS, and CER blank samples were analyzed in triplicate to obtain the protein content. The Lowry solution was freshly prepared on the day of the measurement, and the recipe for preparing this solution is outlined in Appendix A, Table A-2. Folein solution was freshly prepared by adding 5 mL of 2N Folein and Ciocalteu's Phenol Reagent to 6 mL of Milli-Q water. 0.7 ml of Lowry solution was added to 0.5 ml of each sample. The mixture was vortexed and incubated in the dark for 20 min in room temperature. Then, 0.1 ml of the Folein solution was added to each sample and vortexed. A further sample incubation period was carried out for 30 min. The absorbance of the samples was read at 750 nm with Spectrophotometer UV-VIS. The absorbance readings were compared with Bovine Serum Albumin standards measured on the same day, and a standard curve was used to determine the concentration of unknowns.

Extracellular polymeric carbohydrate content was measured following the Anthrone method (Gerhardt et al., 1994). Anthrone solution (Table A-2) was freshly prepared at the day of the measurement. The extracted EPS, supernatant and CER blank samples were subjected to this method in triplicate to obtain the carbohydrate content. Carbohydrates were dehydrated by adding 2 ml of 75% H_2SO_4 to 1 ml of each sample, then the mixture was vortexed and 4 ml of the enthrone solution was added. The samples was heated to 100^0 C for 15 min. then the digested samples were cooled to room temperature and the absorbance was read at 570 nm with Spectrophotometer UV-VIS. Glucose standards were prepared and treated as the. The carbohydrate concentration was obtained from the standard curve.

2-3-3 Microscopic investigations and image analyses of aerobic granule

To characterize granule macro scale properties and observe the difference in granule

morphology during the experimental phases, image analyses were conducted. Samples were collected from the reactors and images were captured with a stereomaster microscope (Fisher Scientific). The images were analyzed with Image Pro Plus software (version 7.0, Media Cybernetics). Different parameters were measured with this software such as mean particle diameter and aspect ratio which is defined as the ratio between the minimum diameter and maximum diameter.

Additionally, to visualize the difference in structure between the granules fed with different carbon source, scanning electron microscopic (SEM) analysis were carried out. Granules were washed with phosphate buffer solution and fixed with 2.5% glutaraldehyde for 1h. The fixed granules were re-suspended in the buffer solution and washed with Milli-Q water. The washed granules were dehydrated via successive passages through 30%, 50%, 75%, 85%, 90%, 95%, and 100% ethanol, and subjected to critical drying for scanning electron microscopic (SEM) analysis. Moreover, to assess the starch hydrolysis within the granule, granules were collected at the end of a cycle. Iodine solution was added to the granules under fluorescence upright microscope (Zeiss).

2-3-4 Standard reactor measurements

To evaluate the biochemical conversion performance of the aerobic granules, weekly grab samples for the influent and effluent wastewater were collected. Additionally, cycle sampling was carried out twice, also one time during the 1^{st} and 2^{nd} phase, respectively, to assess the conversion processes occurring during a single SBR cycle. Grab samples were collected every 15-30 min during a single cycle. The samples collected weekly and during the SBR cycle were filtered through 0.45 μm filter (expect for TCOD measurement) and analyzed.

Total and soluble COD were measured by the closed reflux method according to the *Standard*

Methods for the Examination of Water and Wastewater (APHA et al., 2005). Ammonium concentration were measured by the phenate method according to the *Standard Methods for the Examination of Water and Wastewater* (APHA et al., 2005). Ortho phosphate, nitrate and nitrite were measured with Ionic Chromatography IC 2000 equipped with IonPac AS18 Analytical Column and IonPac AG18 Guard Column. Samples IC operation conditions to measure the mentioned anions are detailed in Appendix B, Table B-1. Occasionally, Ortho phosphate, nitrate and nitrite were measured according to the *Standard Methods for the Examination of Water and Wastewater* (APHA et al., 2005).

2-3-5 Determination of hydrolysis and acid fermentation products methods

To evaluate the pretreatments of the polymer starch, the hydrolyzed products should be quantified. The hydrolysis of starch involves multiple steps. First, large molecular size starch is broken down to smaller molecules including amylose and amylopectin (including particulate starch and soluble starch). Then, these molecules are further converted to reducing sugars such as maltose and glucose. The reducing sugars can be fermented by acid forming bacteria to volatile fatty acids.

2-3-5-1 Reducing sugars measurement by Dinitrosalicylic Acid method (DNS)

Reducing sugars (RS) measurement was carried out according to Dinitrosalicylic Acid method (Miller, 1959). Composition of dinitrosalicylic acid (DNS) and Rochelle salt reagents are presented in Appendix A, Table A-2. 3 mL of the DNS reagent was added to 3 mL of each sample. The mixtures along with glucose standards were heated at 90^0C for 5 min, then, 1 mL of 20% Rochelle salt was added to stabilize the red-brown color. After cooling the mixtures and standards to room temperature, the absorbance of the samples was read at 578 nm and compared with the absorbance of the standards to calculate reducing sugars concentration.

2-3-5-2 Soluble starch measurement by starch-iodine complex formation (SIC) method

Soluble starch was measured according to the starch-iodine complex formation (SIC) method (Stauffer, 1989). Starch standards were prepared by adding 500 mg starch to boiling water. Then, 50 to 400 mg/L starch standards series were prepared from the 500 mg/L starch standard. 2 mL of iodine solution (outlined in Appendix A, Table A-2) was added to 2 mL of each sample and standard. The volume of the mixture was replenished to 8 mL with Milli-Q water. After 20 min of color development at 20^0 C, the absorbance of the color was obtained at 570 nm ad converted to starch concentration by comparing with the starch standard curve.

2-3-5-3 Volatile fatty acids determination method

Since most of the acetogenesis fermentation process products are acetic and propionic acid, acetate and propionate were measured with Ionic Chromatography IC 2000 equipped with IonPac AS18 Analytical Column and IonPac AG18 Guard Column. Samples IC operation conditions to measure the mentioned anions are detailed in Appendix B, Table B-2.

2-3-6 Statistical analyses

Standard deviation was calculated to represent the error level between measurements. ANOVA analysis was performed to determine whether the characteristics and performance of the aerobic granule significantly differ between the three reactors. The significance level was based on 95% confidence interval ($\alpha = 0.05$). The differences were declared significant at $p<0.05$.

CHAPTER 3: RESULTS

3-1 First Experimental Phase: Development of stable aerobic granules for biological nutrient removal

This experiment was conducted to develop aerobic granules that can perform complete biological nutrient removal of nitrogen and phosphorus. The reactors were seeded with granules that were formed in a previous trial. In this experimental phase, all reactors were operated identically, and the reactors were fed with a sole carbon source of soluble acetate (440 mg O_2/L). To improve total nitrogen removal in the reactors, a post-anoxic period was implemented on Day 23 of the experiment.

3-1-1 Characterization of aerobic granular sludge:

3-1-1-1 Microscopic investigations and particle size distribution

A stereomicroscope was used to visualize granules and to analyze the diameter and shape of the granule using image analysis. During this experimental phase, compact and round granules developed in all the reactors as shown in Figure 3-1. The images were analyzed to estimate the bulk properties of granule shape, such as mean diameter and aspect ratio (the ratio of the minimum to maximum diameter of a particle). Table 3-1 summarizes the particle size distribution and shape for the granules present in each reactor on Day 40 of the experiment, which was after steady-state of sludge properties and nutrient removal was established.

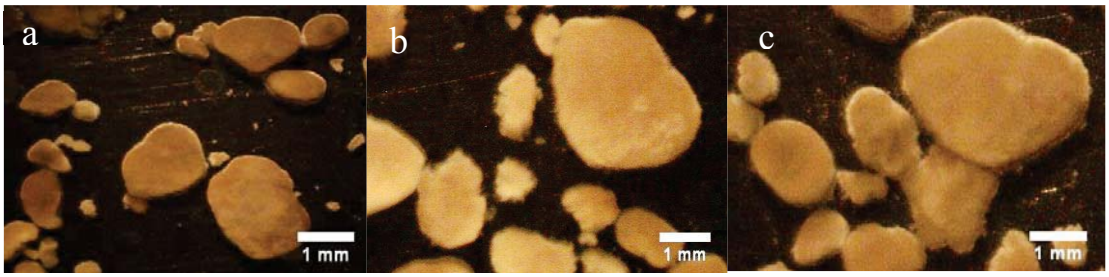

Figure 3- 1: developed aerobic granules on day 40 of the 1st phase of the study. Granules in Reactor 1 (a), granules in Reactor 2 (b) and granules in Reactor 3 (c)

The size analysis demonstrated that the percentage of flocs (defined as particles < 0.3 mm in diameter) of the total particles counted was 13-15% in all reactors on Day 40. Approximately 85% of the biomass particles had a larger diameter, with 32-39% having diameters greater than 2 mm. The mean diameter and aspect ratio didn't vary significantly ($p>0.05$) between the three reactors The mean diameter was 1.52 ± 1.11, 1.54 ± 1.00 and 1.60 ± 0.9mm corresponding to a 0.67 ± 0.20, 0.68 ± 0.18 and 0.69 ± 0.16aspect ratio in Reactor 1, 2 and 3, respectively.

Table 3-1: Size distribution of all particles counted on Day 40 of the 1st experimental phase, with all reactors operated identically and fed with soluble acetate as a carbon source

	Diameter (mm)	Reactor 1	Reactor 2	Reactor 3
		% Particles in range		
Flocs	< 0.3	15	15	13
Granules	0.3-1.0	31	21	21
Granules	1.0 -2.0	16	33	28
Granules	>2.0	39	32	38
Mean diameter (mm)		1.52 ± 1.11	1.54 ± 1.00	1.60 ± 0.97
Mean aspect ratio (unitless)		0.67 ± 0.20	0.68 ± 0.18	0.69 ± 0.16
Total particles counted		1007	783	792

3-1-1-2 EPS characterization and distribution

Tightly-bound EPS were extracted on day 38 from all reactors, and the protein and carbohydrate content was characterized. The protein content was greater than the polysaccharide content in EPS from granules formed in all the reactors. The analysis showed that the EPS content didn't significantly vary in the granule matrix developed in all the SBRs ($p>0.05$). The protein content was measured to be 230 +/- 21, 230 +/- 11 and 240 +/- 15 mg/g VSS for the EPS extracts from Reactor 1, 2 and 3, respectively. The carbohydrate content was 50 +/- 6, 53 +/- 6

and 51 +/- 3 mg/g VSS for the EPS extracts from Reactor 1, 2 and 3, respectively. These results yielded a 4.4 to 4.7 protein to carbohydrate ratio.

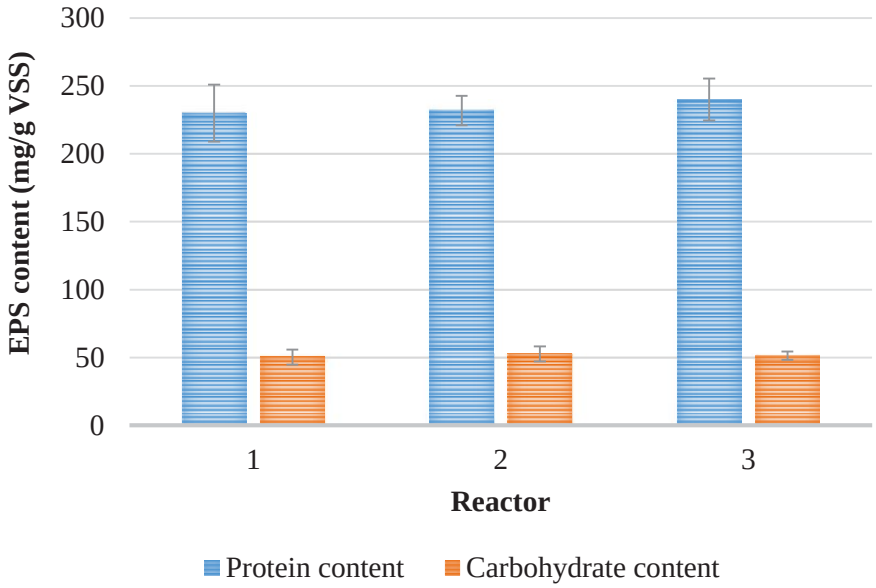

Figure 3- 2: Protein and carbohydrate content of the EPS in the granule matrix. Error bars represent the standard deviations of triplicate protein or carbohydrate measurements.

3-1-1-3 Suspended solids measurements

The suspended solids properties (MLSS, VSS and SVI) were statistically similar ($p>0.05$)in all the reactors. The mixed liquor suspended solids (MLSS) concentration fluctuated between 1000 and 1500 mg/L during the first three weeks. In the fourth week, the MLSS concentration increased in all the reactors up to 2230 mg/L. After that, the MLSS reached steady-state and did not change significantly the last two weeks of the experimental phase, averaging 2140, 2160 and 2180 mg/L in Reactor 1, 2 and 3, respectively, as displayed in Figure 3-3. The SVI_5 measurements, which represent the SVI after 5 min of settling, are presented in Figure 3-4 for the three SBRs. After one month of reactor operation, the SVI decreased from 80 to 90 mL/mg MLSS to 23 to 24 mL/mg MLSS in Reactor 1, 2 and 3, respectively. During steady-state operation the last two weeks of Phase 1, no significant difference ($p>0.05$) in the SVI was measured between reactors.

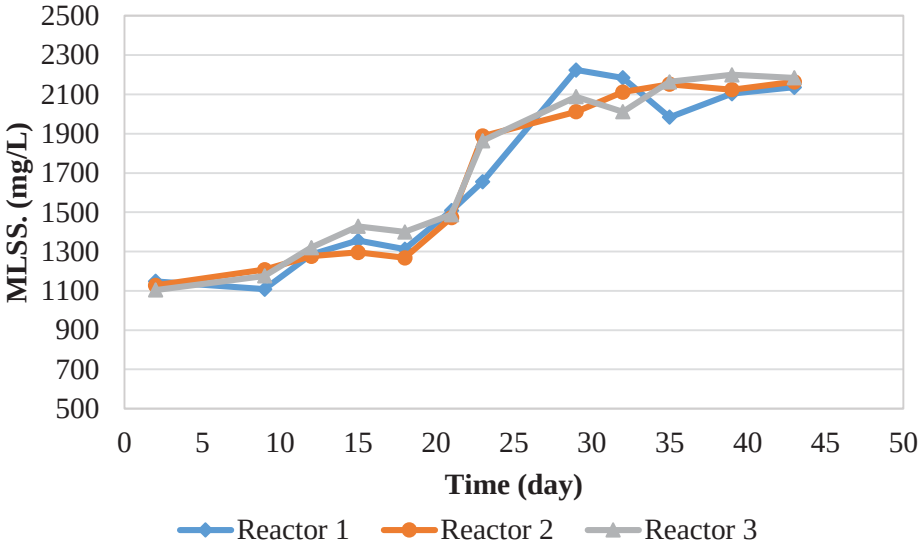

Figure 3- 3: Mixed liquor suspended solid concentrations in the SBRs during the 1st experimental phase, in which all reactors were operated identically and fed with acetate as a sole carbon source.

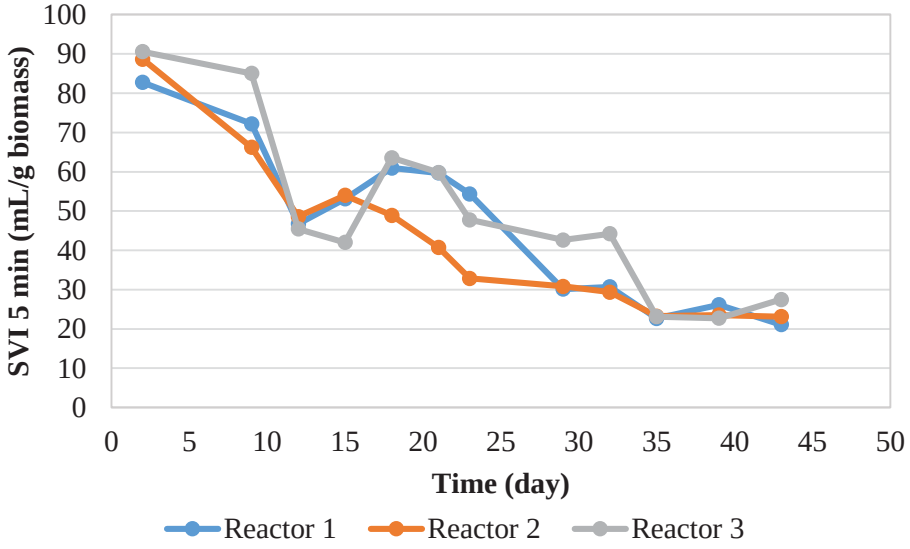

Figure 3- 4: Sludge volume index (SVI 5 min) measurements in the SBRs during the 1st experimental phase, in which all reactors were operated identically and fed with acetate as a sole carbon source.

3-1-1 Aerobic granular reactor treatment efficiency

Each reactor was seeded with aerobic granules formed in a preliminary trial, and the experimental phase began with the transfer of biomass to new reactors (represented as Day 0). The COD, N, NH_3 and P removal for the transferred biomass was 88%, 29%, 38% and 50%, respectively. To evaluate the performance of the aerobic granules, weekly samples were collected at the end of an SBR cycle and analyzed for soluble COD (sCOD), ammonia, nitrite, nitrate and orthophosphate. The COD removal efficiency increased from 87 to 91% removal for all reactors during initial operation to > 95% removal for all the reactors by the end of the experimental phase. The results for all reactors are displayed in Figure 3-5.

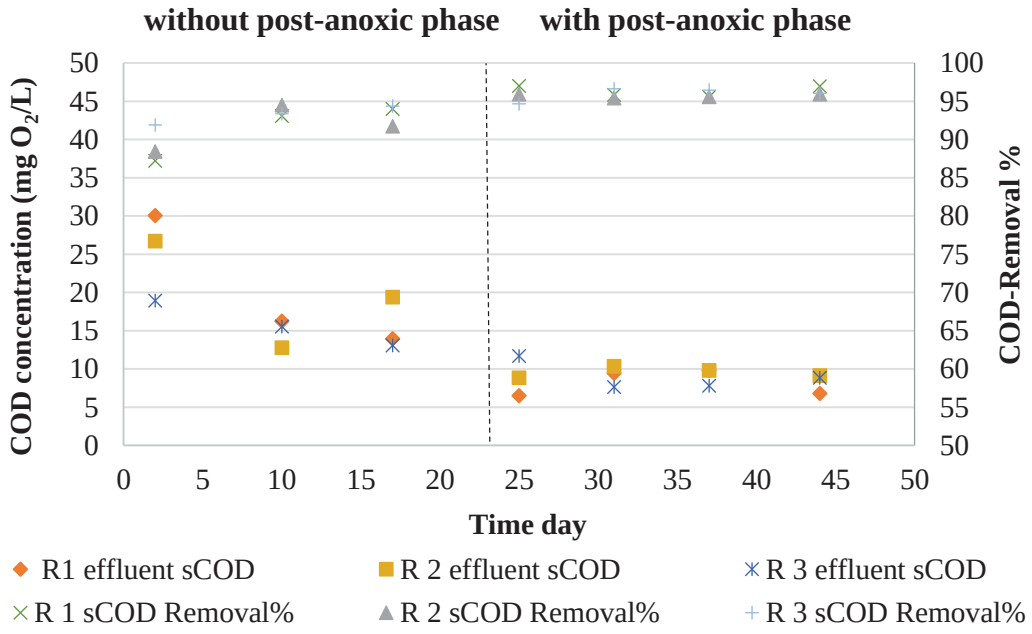

Figure 3- 5: COD concentrations (primary y-axis) and the total COD removal percentage (secondary y-axis) for the SBRs during the 1st experimental phase.

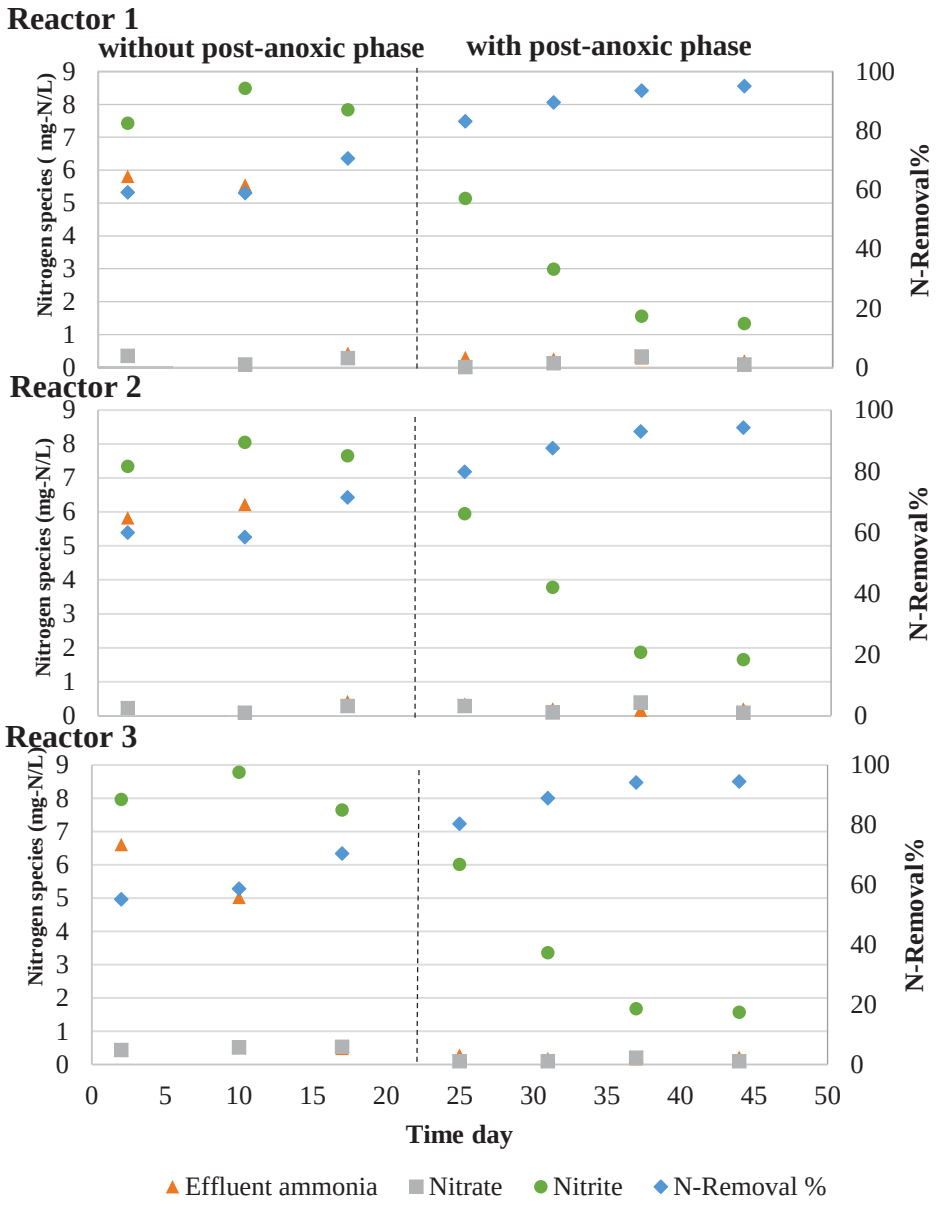

Figure 3- 6: Effluent nitrogen species concentrations (primary y-axis) and the total nitrogen removal percentage (secondary y-axis) for the SBRs during the 1st experimental phase.

As shown in Figure 3-6, complete nitrification was achieved (>96% ammonia removal) after two weeks of reactor operation, which corresponded to an increase in MLSS concentration in the reactors. Meanwhile, the total nitrogen removal was still fairly low (50-70%) due to

persistent nitrite concentrations produced by the nitrification process. Before the post-anoxic phase was implemented on Day 23, nitrite concentrations averaged 7.61 mg-N/L. However, it decreased to 1.7 mg-N/L in the effluent by Day 37 of operation. In all reactors, nitrate was determined less than the detection limit of the ion chromatograph (0.5 mg-N/L) over the experimental phase. By the end of the first experimental phase, the total nitrogen removal efficiency increased to 92 to 95% for all reactors.

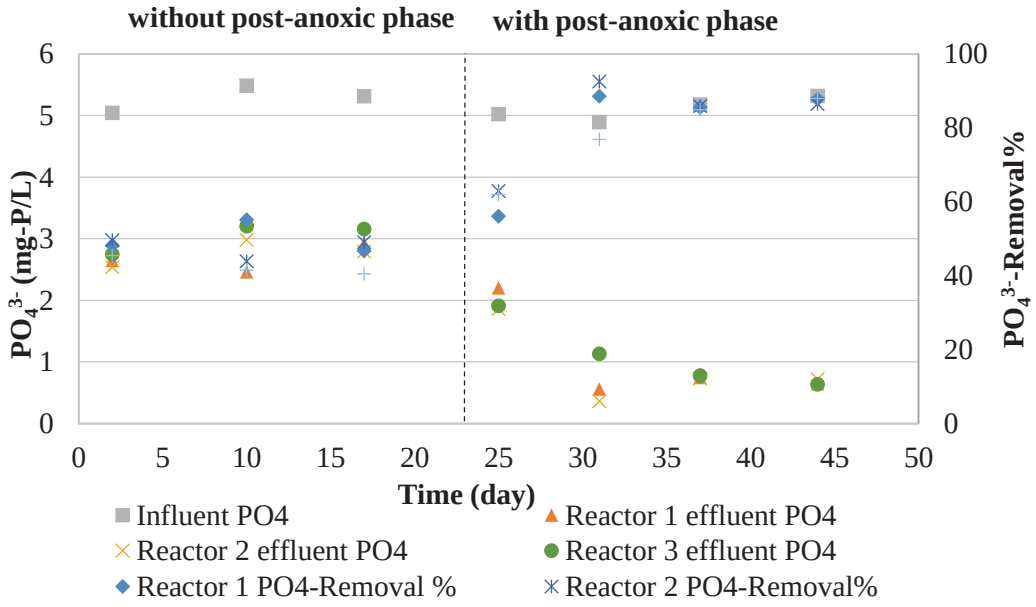

Figure 3- 7: Orthophosphate concentrations (primary y-axis) and the total phosphate removal percentage (secondary y-axis) for the SBRs during the 1st experimental phase.

Total phosphate removal varied between 40 to 55% during the first three weeks of SBR operation, as shown in Figure 3-7. Interestingly, improvement in the phosphate removal was observed after implementing the post-anoxic period. The orthophosphate concentration decreased from 2.78 to 3.16 mg-P/L for all reactors on Day 17 to 0.72 to 0.75 mg-P/L on Day 37 of operation. In order for phosphorus accumulating organisms (PAOs) to be active during the anaerobic phase, the reactor needs to have negligible external electron acceptors available (i.e.,

oxygen, nitrate, and nitrite). Because nitrite remained in the reactor at the end of an SBR cycle, and 50% of this volume was returned to the beginning of the next SBR cycle, the residual nitrite inhibited phosphorus removal. When complete nitrification was realized with the addition of the post-anoxic SBR phase, total phosphate removal also improved.

The graphs presented in 3-5 through 3-7 illustrate the total COD, TN, and orthophosphate removal during an SBR cycle over 44 days of operation. Chemical data was also collected and analyzed at smaller time intervals during an SBR cycle, and this data better illustrates the impact of the anaerobic / aerobic / and post-anoxic periods on the biochemical conversions occurring within the granule matrix. In Figure 3-8, cycle analyses are presented before and after the post-anoxic period were applied. Overall, the three SBRs achieved statistically similar treatment performance ($p>0.05$) for COD, nitrification, denitrification, and phosphorus removal.

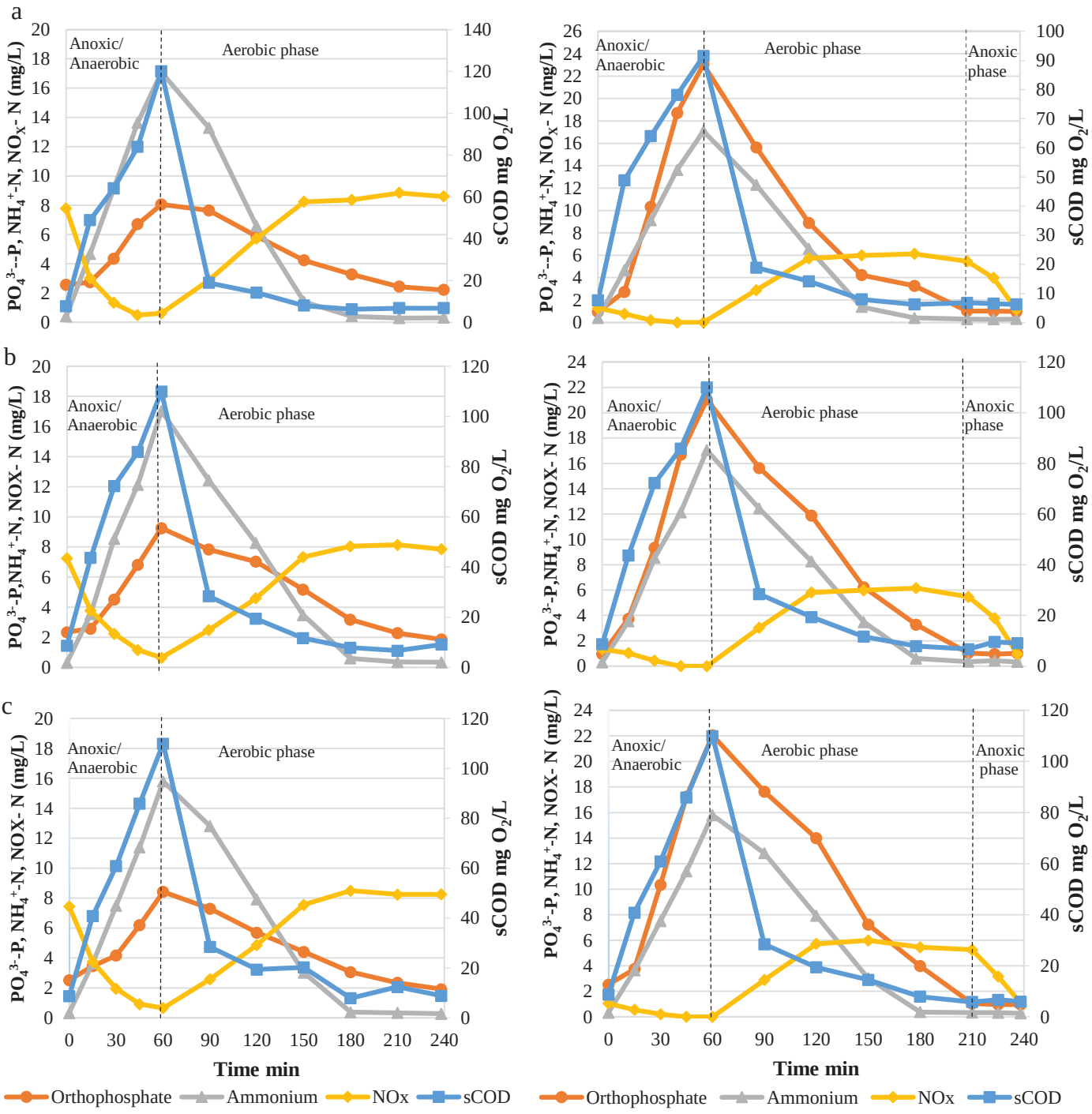

Figure 3- 5: Biochemical conversion processes occurring during a single SBR cycle on Day 22 (left side) and Day 44 (right side) of the 1st phase. Reactor 1 (a), Reactor 2 (b) and Reactor 3 (c).

Figure 3-8 also shows that including a post-anoxic phase mainly influenced the denitrification process and the phosphate removal. The effluent nitrite level was measured around 8 mg-N/L in the cycles without the post-anoxic period. When the post-anoxic period was instituted on Day 47, effluent nitrite decreased to around 6 mg-N/L at the end of the aeration period and then dropped to less than 2 mg-N/L by the end of the SBR cycle. All reactors responded identically to the application of a post-anoxic phase. The post-anoxic phase also improved the phosphate removal. On Day 22, phosphate release during the anaerobic phase began after the nitrite that was produced and recycled from the previous cycle was entirely removed (likely from denitrification occurring in the anaerobic phase). As a result, phosphate concentrations reached approximately 8.5 mg-P/L by the end of the anaerobic period. In the aerobic period, the released phosphate was taken up by the PAOs, leaving approximately 2 mg-P/L by the end of the aeration period, which was the concentration in the effluent. Once the post-anoxic phase was added and nitrite was more completely removed to nearly 1.7 mg-N/L by the end of the SBR cycle, faster and more total phosphate was released in the next SBR cycle's anaerobic period. The phosphate at the end of the anaerobic period was approximately 22 mg-P/L. This measured concentration was reduced to less than 1 mg-P/L by the end on the SBR cycle. Once external electron acceptors (nitrite) were removed from the anaerobic phase, PAOs became more active and efficient during the SBR cycle.

Based on the sludge properties and treatment performance during the last two weeks of the experimental phase, the reactors were defined as being in steady-state operation. The average values for performance are presented in Table 3-2. For this experimental phase, no significant difference ($p>0.05$) was observed between any reactor for any measured parameter. The

similarity between reactors before the second experimental phase is critical for the interpretation of those results.

Table 3- 2: Steady-state characteristics of the aerobic granular sludge reactor for the first experimental phase, in which all reactors were fed with readily biodegradable acetate[1].

	Reactor 1	Reactor 2	Reactor 3
Biomass concentration in reactor (mg VSS/L)	1740 ± 83	1710 ± 150	1880 ± 90
Biomass concentration in reactor (mg MLSS/L)	2130 ± 92	2110 ± 60	2130 ± 79
SVI 5min (mL/mg)	26 ± 4	26 ± 4	28 ± 5
N- Removal efficiency (%)	94 ± 1	94 ± 1	94 ± 1
P- Removal efficiency (%)	87 ± 2	86 ± 1	86 ± 2
sCOD- Removal efficiency (%)	96 ± 1	96 ± 1	96 ± 1

[1] Reported with standard deviation of the steady-state data.

3-2 Second Experimental Phase: Investigation of the effect of slowly biodegradable carbon fractions on the performance of aerobic granular sludge for biological nutrient removal

The key purpose of this study was to understand the effect of the slowly biodegradable COD (sbCOD) fraction, which is typically present in municipal wastewater to some extent, on the performance of aerobic granular sludge technology. At full-scale, when municipal wastewater is deficient in the volatile fatty acids needed to encourage biological phosphorus removal, treatment plants may utilize a fermentation reactor to perform hydrolysis of particulate COD and acidogenesis, which is the anaerobic, biological production of volatile fatty acids. In this study, two different pretreatments of a synthetic wastewater containing soluble acetate and particulate starch were performed: a heat hydrolysis and fermentation. The characteristics and efficiency of the aerobic granular sludge reactor in treating these wastewater streams were compared with an

SBR fed with equal parts soluble acetate and particulate starch.

3-2-1 Characterization of the hydrolysis products and influent wastewater to the SBRs

The fermenter was inoculated two weeks before the start of the 2nd experimental phase of the study, in order to select the organisms capable to hydrolyze the particulate starch. As displayed in Figure 3-9, it took one month to increase the soluble COD fraction from 220 to 376 mg O_2/L. Subsequently, the measured sCOD was in the range 365-376 mg O_2/L for the duration of the experiment. After one month of fermenter operation, an increase in acetate concentration and detection of propionate took place. The acetate concentration increased by 30+/-2 mg O_2/L, and 4.5+/-0.5 mg O_2/L propionate was produced after one month of operation. Afterward, the acetate and propionate level in the effluent was in the range 290-300 and 18-20 mg O_2/L, respectively.

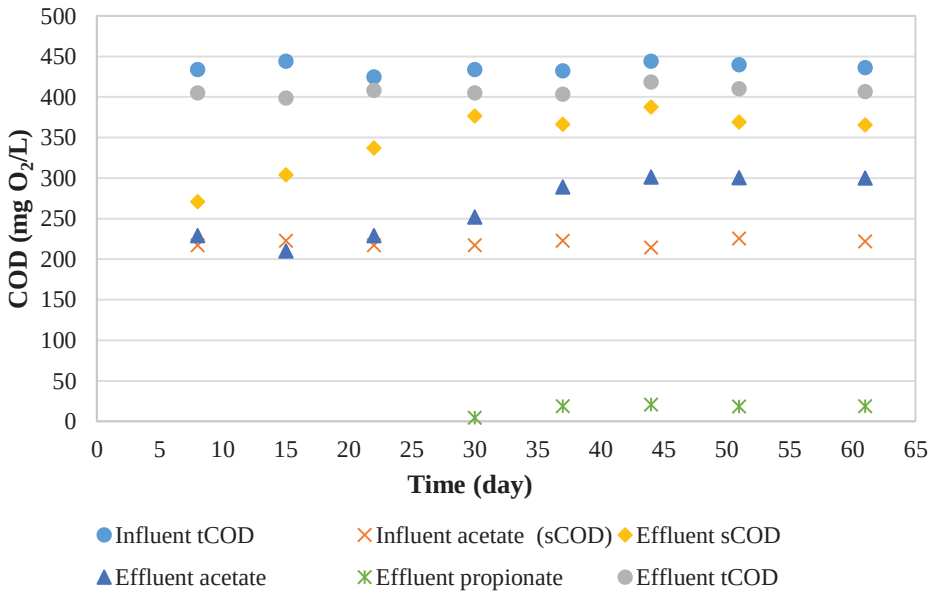

Figure 3- 9: Characterization of the influent and effluent carbon concentrations of the fermenter during the fermenter operation.

A series of batch experiments were performed in duplicate to determine the hydrolysis and sCOD production potential of the fermenter as a function of time, and results from these are presented in Figure 3-10. Each batch was fed influent particulate COD of 220 mg O_2/L, and 83% of the particulate starch was hydrolyzed during the 10 hr, forming soluble COD. 73% of the hydrolyzed products were fermented to volatile fatty acids, acetic and propionic acids. The remaining fraction of sCOD products was unidentified in these batch experiments.

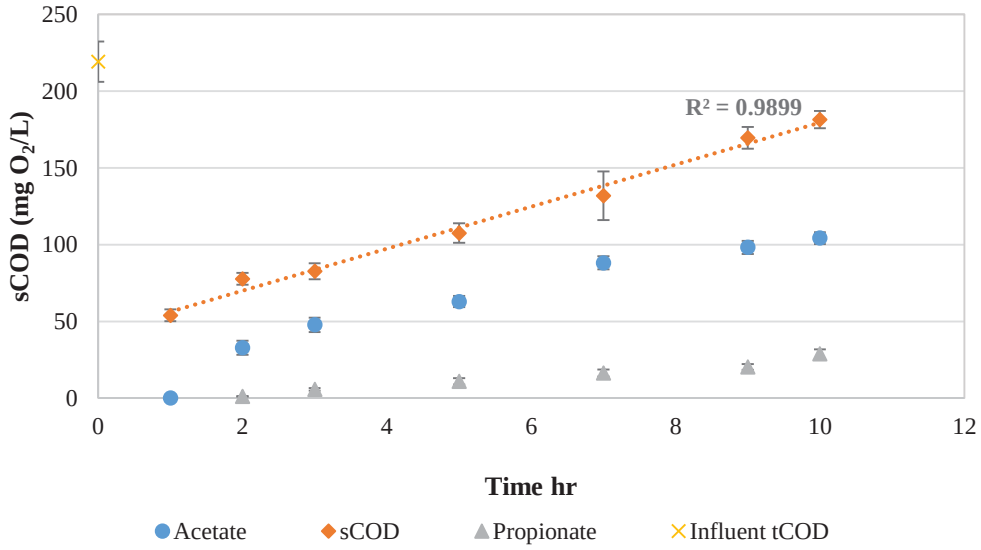

Figure 3- 10: Anaerobic hydrolysis and fermentation production potential of starch polymers at 40^{0}C, standard variation is the error bar in the y-axis.

The heat hydrolysis process showed break down of the starch polymers and hydrolyzed 78-80% of the particulate starch (PS) to sCOD. To evaluate the biodegradability of the hydrolyzed products from both pretreatments, the pretreated wastewater was analyzed for expected hydrolyzed compounds of soluble starch (SS), reducing sugars, and VFAs. Soluble starch (SS) was measured to estimate the hydrolysable slowly biodegradable compounds (hsbCOD). Reducing sugars (RS) were determined along with the VFAs to represent the readily biodegradable COD (rbCOD) portion in the pretreated wastewater.

Figure 3-11 displays the composition of the pretreated wastewaters, which were then fed to Reactors 2 (heat hydrolysis pretreatment mixed with 50% acetate) and 3 (fermentation pretreatment mixed with 50% acetate). Reactor 1 was fed without pretreatment (440 ± 6 mg total COD/L). Consequently, 50% of the total COD was particulate, slowly biodegradable (psbCOD) and the remaining was acetate (rbCOD). For the heat hydrolysis, 66-68% of the particulate starch (PS) fraction was solubilized to mainly soluble starch (hsbCOD), which became 33-34% of the total COD entering Reactor 2. Also, 16-18% of the particulate starch became reducing sugars (RS) during the heat hydrolysis. This increased the rbCOD fraction from 50 to 59 % of the total COD (440 ± 7 mg total COD/L). 9% total COD loss of the total COD was measured in the fermenter, leaving 400 ± 11 mg total COD/L feeding Reactor 3. The anaerobic hydrolysis pretreatment yielded 80-82% solubility of the PS before feeding Reactor 3. The VFAs fraction percentage increased from 50-51% to 77-79%, and RS became 11-12% of the total COD. This production of VFAs and RS increased the rbCOD percentage up to 90% of the total organic compounds.

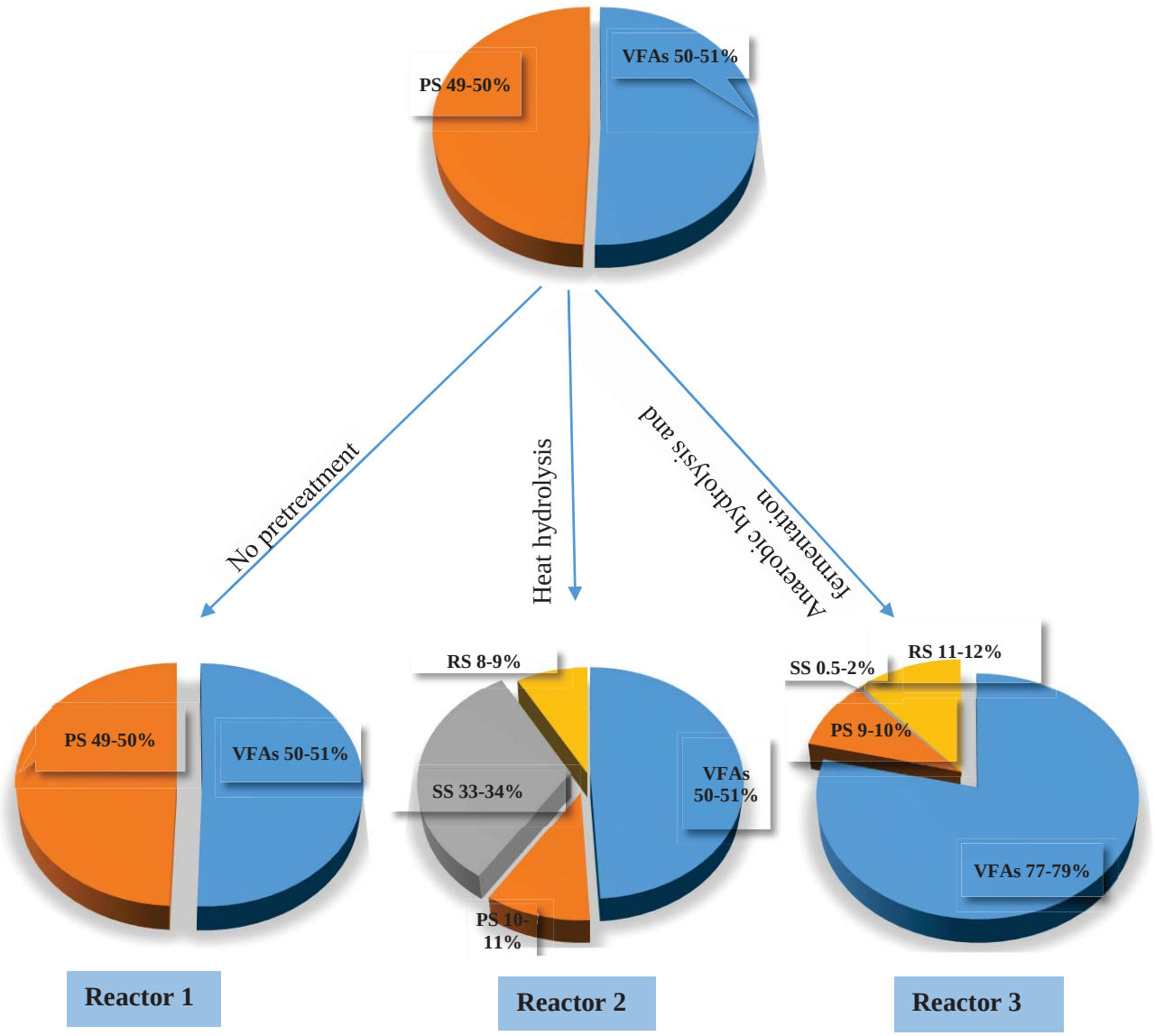

Figure 3- 11: The chemical oxygen demand of organic carbon fractions (particulate starch (PS), soluble starch (SS), volatile fatty acids (VFAs), and reducing sugars (RS)) in the synthetic wastewater before and after the pretreatment processes. The final wastewater compositions were fed to Reactors 1, 2, and 3, respectively.

3-2-2 Reactor Performance

Reactor 1 fed was fed with non-pretreated wastewater containing 50% particulate starch.

Reactor 2 and 3 received the pretreated wastewater having the organic matter composition

detailed in the previous section.

3-2-2-1 Aerobic granular sludge properties:

3-2-2-1-1 Microscopic investigations and particle size distribution:

The stereomicroscope images presented in Figure 3-12 and 3-13 capture the macroscale structure of granules. Figure 3-12 displays the differences in the morphology and particle size of the granules in each reactor on Day 14 of this experimental phase. The predominant granules fed with non-pretreated wastewater in Reactor 1 were smaller in particle size than the granules in the other reactors, and their surface was filamentous. The granules in Reactor 2 had a fluffy surface and the largest average diameter. Compared with the other reactors, the granules fed with the fermentor-pretreated wastewater in Reactor 3 were more stable in size and more regular in shape.

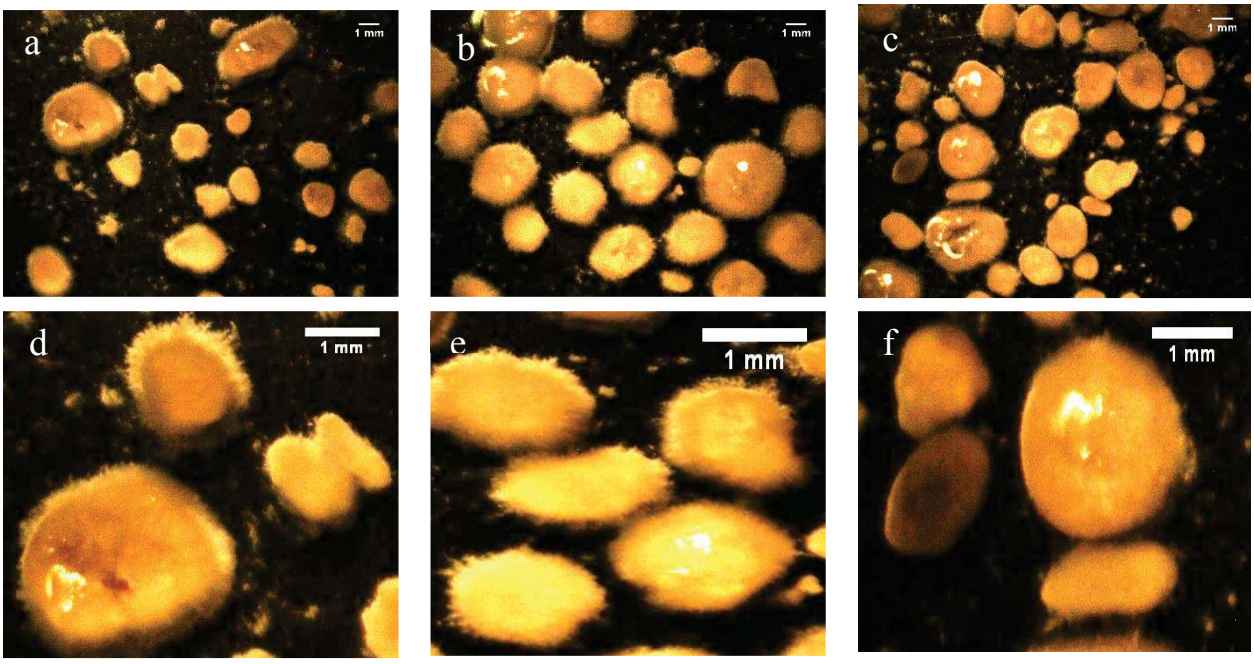

Figure 3-12: Stereomicroscope images captured on Day 14 during the 2nd experimental phase. Granules present in Reactor 1 (a,d), Reactor 2 (b,e) and Reactor 3 (c,f).

During the rest of the 2nd experimental phase, the morphology and structure of the granules further diverged from the smooth granules observed in the first experimental phase. On

Day 40 of this phase, not all the granules in Reactor 2 and 3 had the fluffy surface. However, the rest of the granules developed to be more filamentous and less dense. In contrast, granules fed with the fermenter-pretreated wastewater retained their regular and stable morphology as shown in Figure 3-13. Scanning electron microscopic images were obtained to further observe how the COD fractions affected the surface structure of the granules (Figure 3-13). The granules in Reactor 1 had a more porous structure than the granules in the other reactors. In contrast, a dense and compact structure was observed for the granules in Reactor 3.

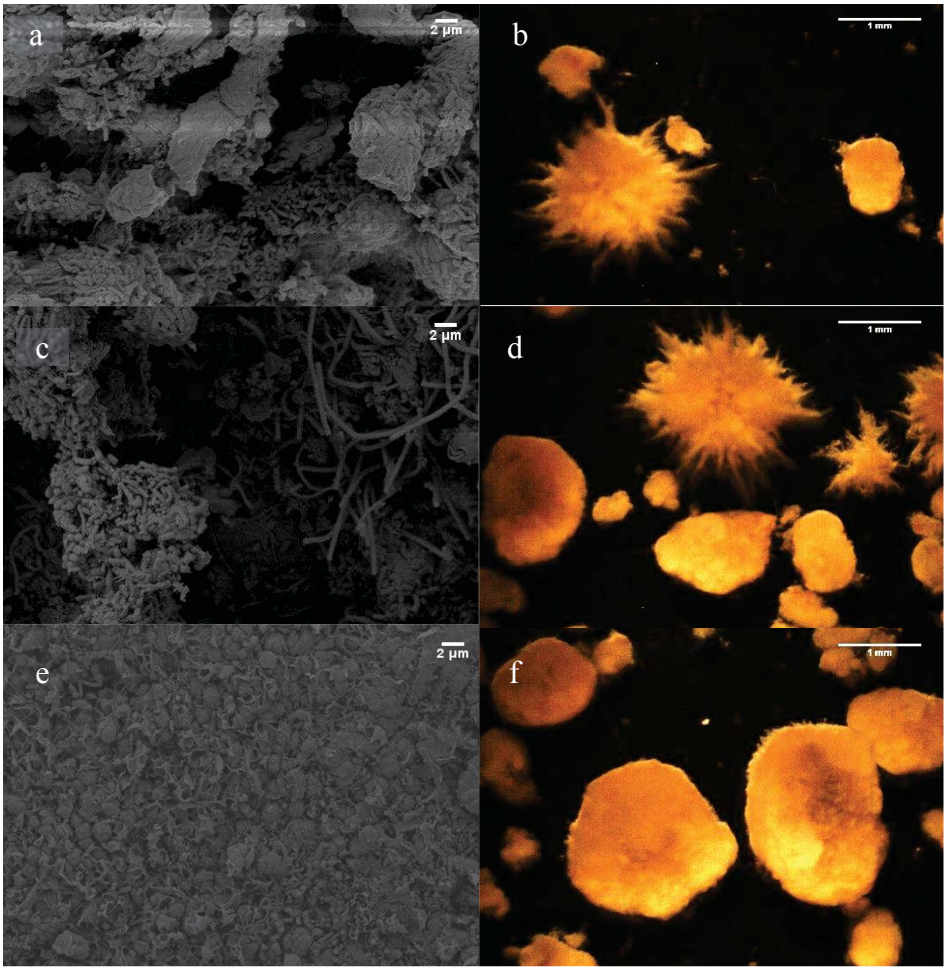

Figure 3- 63: SEM (a,c,e) and stereomicroscopic (b,d,f) observations of the morphology and structure of the granules on Day 40 of the 2nd experimental phase. Reactor 1 (a,b), Reactor 2(c,d) and Reactor 3 (e,f). Scale bar is 2 µm for SEM images and 1 mm for stereomicroscope images.

To estimate the particle size distribution, image analysis was performed for stereomicroscope images of granules taken on Day 44 of this experimental phase, when reactor operation was stable. Average values are summarized in Table 3-3. The analysis confirmed the microscopic observation. The mean diameter and aspect ratio significantly ($p<0.05$) deferred between all reactors. The highest percentage of flocs (defined as particles with a diameter < 0.3 mm) was measured in Reactor 1, whereas the lowest percentage was found in Reactor 3. Additionally, the mean diameter was determined to be 0.82 ± 0.88, 0.92 ± 0.94 and 1.43 ± 0.96 in Reactor 1, 2 and 3, respectively. The corresponding aspect ratio was measured to be 0.60 ± 0.17, 0.60 ± 0.18 and 0.66 ± 0.16. Since the aspect ratio represents the roundness and regularity of the granules, these results demonstrated that the largest and smoothest granules were developed in Reactor 3, which fed with the fermented pretreated wastewater. Compared with the granules fed with solely acetate during the 1st phase, smaller and rougher granules were developed when half of the acetate was altered with PS and the heat hydrolyzed starch.

Table 3- 3: Size distribution of all particles counted on Day 44 of the 2nd experimental phase.

		% Particles in range		
	Diameter (mm)	Reactor 1	Reactor 2	Reactor 3
Flocs	< 0.3	36	26	15
Granules	0.3-1.0	39	12	25
Granules	1.0 -2.0	14	20	31
Granules	>2.0	11	13	29
Mean diameter		0.82 ± 0.88	0.92 ± 0.94	1.43 ± 0.96
Mean aspect ratio		0.60 ± 0.17	0.60 ± 0.18	0.66 ± 0.16
Total particles counted		1028	657	672

3-2-2-1-2 Characterization and distribution of EPS:

The EPS analyses showed that the protein content of the EPS extracted from the granules in each reactor was significantly ($p<0.05$) different from reactor to reactor. The tightly bound EPS (TB-EPS), loosely bound EPS (LB-EPS) and total EPS composition contents were measured and presented in Figure 3-14 and 3-15. Figure 3-14 displays the protein content for the different granules along with the concentration of the measured readily biodegradable COD (rbCOD). The order of the total protein content of the EPS in the granule matrix was Reactor 3> Reactor 2 > Reactor 1. This order was corresponding to rbCOD feeding concentration in order of Reactor 3> Reactor 2 > Reactor 1. Additionally, Figure 3-14 shows that the loosely bound protein content was the highest for the granules fed with non-pretreated wastewater in Reactor 1 among the other reactors.

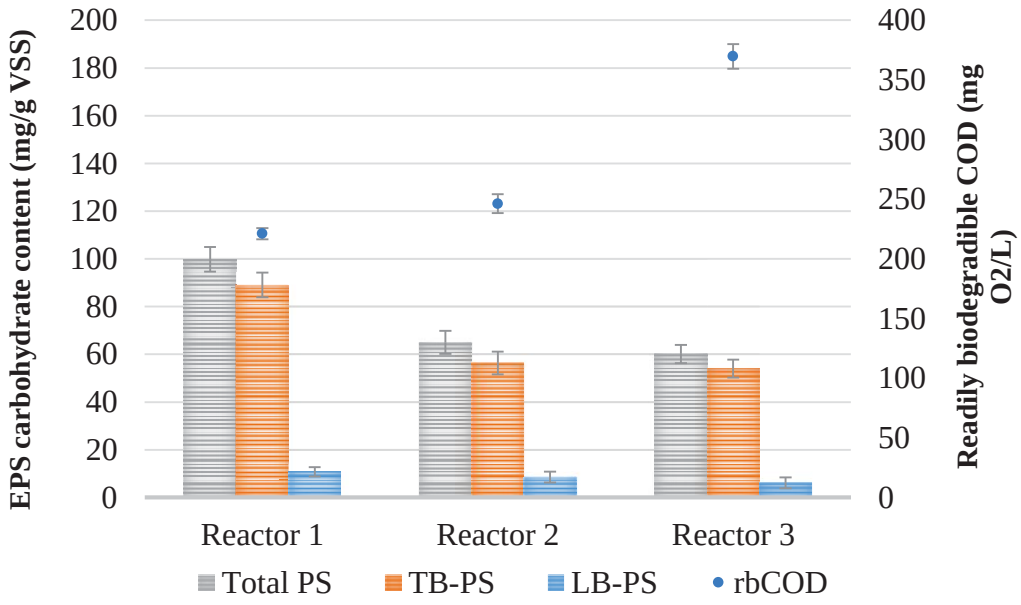

Figure 3- 14: The extracellular polymeric substance protein content extracted from the aerobic granular sludge fed with different fractions of readily biodegradable organic matter in the influent wastewater. The error bars represent the standard deviation of triplicate extractions for loosely-bound (LB-PN), tightly-bound (TB-PN), and total EPS protein (Total PN).

The EPS carbohydrate content data had a different trend than the protein content data, as displayed in Figure 3-15. The TB-PS and LB-PS concentrations were significantly higher ($p<0.05$) for the EPS extracted from granules in Reactor 1 than the other reactors. No significant difference ($p>0.05$) in the TB-PS and LB-PS concentrations was measured between the EPS extracts in Reactor 2 and Reactor 3. The protein to polysaccharide ratio in the extracts was 1.6, 3.7 and 4.8 for Reactor 1, 2 and 3, respectively.

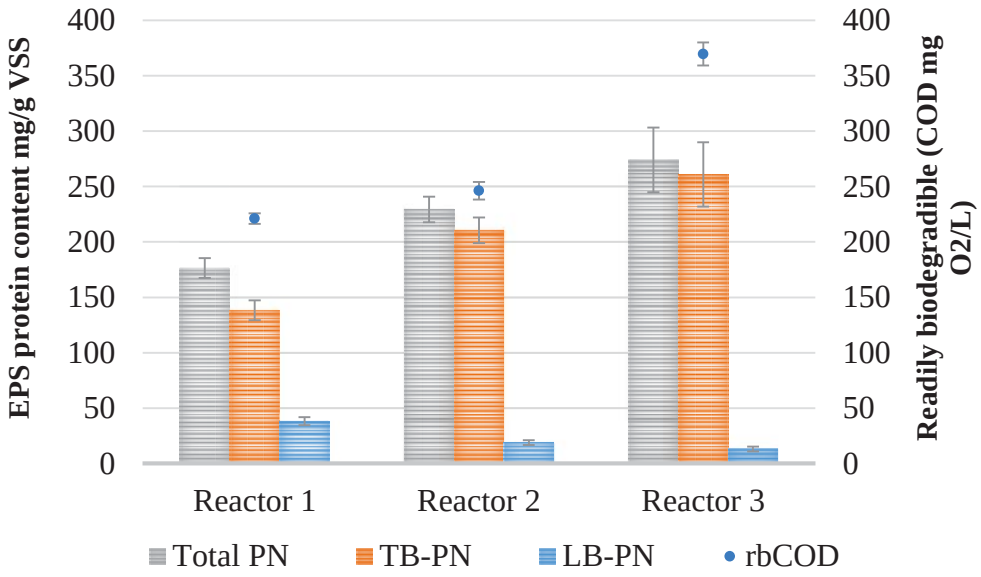

Figure 3- 75: The extracellular polymeric substance carbohydrate content extracted from the aerobic granular sludge fed with different fractions of readily biodegradable organic matter in the influent wastewater. The error bars represent the standard deviation of triplicate extractions for loosely-bound (LB-PN), tightly-bound (TB-PN), and total EPS protein (Total PN).

3-2-2-1-3 Suspended solid measurements

All the SBRs in this experimental phase were inoculated with the same amount and type of granules. However, during the first two weeks of this phase, significant change ($p<0.05$) was observed in all the reactors as shown in Figure 3-16. The MLSS of Reactor 1 decreased rapidly, whereas it immediately increased in Reactors 2 and 3. For Reactor 1 the MLSS decreased from

1900 to around 1000 mg/L while the MLSS for Reactors 2 and 3 increased from 2040 and 2200 mg/ L to 2790 and 4050 mg/L, respectively.

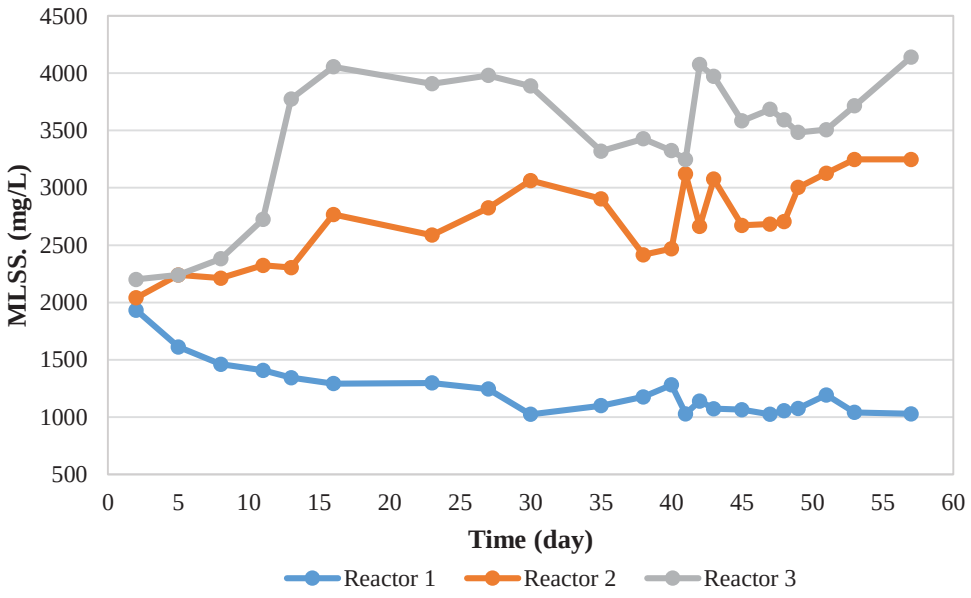

Figure 3- 16: Mixed liquor suspended solids (MLSS) concentrations in the reactors during the 2nd experimental phase of the study.

Similar to the MLSS trend, the effluent suspended solid (ESS) concentrations of the SBRs changed quickly after the second experimental phase began. Reactor 1 showed excessive effluent suspended solid concentrations during the first two weeks as exhibited in Figure 3-17. Some crushed granules were observed in the effluent wastewater of this reactor. For Reactor 2, the effluent suspended solids also increased during the first two weeks of being fed heat-hydrolysed PS, then stabilized at an elevated level, compared to the first experimental phase. No significant difference ($P>0.05$) in the ESS for Reactor 3 was measured during the 2nd phase of this study.

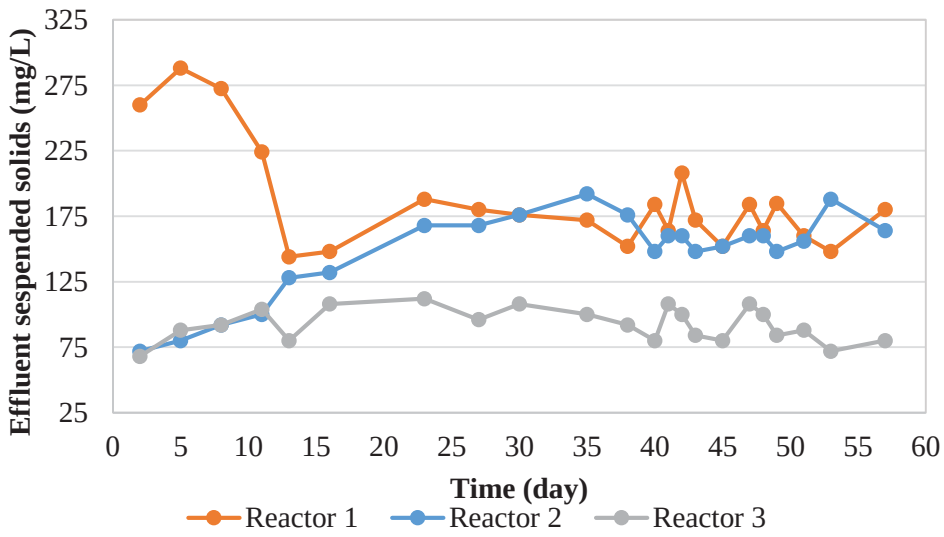

Figure 3- 17: Effluent suspended solids for the AGRs during the 2nd phase.

After the first two weeks of the 2nd experimental phase, the change in MLSS and other sludge characteristics (VSS and SVI) was not significant ($p>0.05$). Based on this evaluation and the treatment performance detailed in the next section, the reactors were determined as being in steady-state, and average sludge characteristics for this period are presented in Table 3-4. The MLSS and VSS were the highest in Reactor 3 and the lowest in Reactor 1. In contrast, the SVI and the effluent suspended solids were the highest for Reactor 1 and the lowest for Reactor 3.

Table 3- 4: Steady-state sludge and effluent suspended solids characteristics, averaged from Days 16 to 56 during the second experimental phase[1]

	Reactor 1	Reactor 2	Reactor 3
Volatile Suspended Solids (mg VSS/L)	970 ± 100	2860 ± 260	3220 ± 220
Mixed Liquor Suspended Solids (mg MLSS/L)	1130 ± 100	2470 ± 200	3700 ± 290
Effluent suspended solids (TSS mg/L)	170 ± 17	160 ± 15	94 ± 13
SVI_5 (mL/g)	35 ± 11	26 ± 5	18 ± 3

[1] Reported with standard deviation.

3-2-2-1 Aerobic sludge treatment performance

Weekly sampling of the reactors treatment performance demonstrated that the variation in the sCOD fraction of the influent wastewater affected the total nitrogen and COD removal. Figure 3-18 displays the effluent ammonia, nitrite, and nitrate concentrations as well as the total nitrogen removal in each reactor during the 2nd experimental phase. Almost complete nitrification was achieved in all the aerobic granular reactors. The ammonia removal for Reactor 1 slightly changed from the first two weeks (99%) to steady-state operation (around 96%). However, compared to the other reactors, Reactor 1 showed the greatest decrease in the denitrification rate, characterized by the nitrite concentration increasing in the effluent after the first week of this phase. By steady-state, the effluent nitrite concentration in Reactor 1 was approximately 5 mg-N/L, which decreased the overall nitrogen removal from 96% in the first experimental phase to 79% in the second. No significant difference ($p>0.05$) in the nitrogen removal between the experimental phases was observed in Reactors 2 or 3.

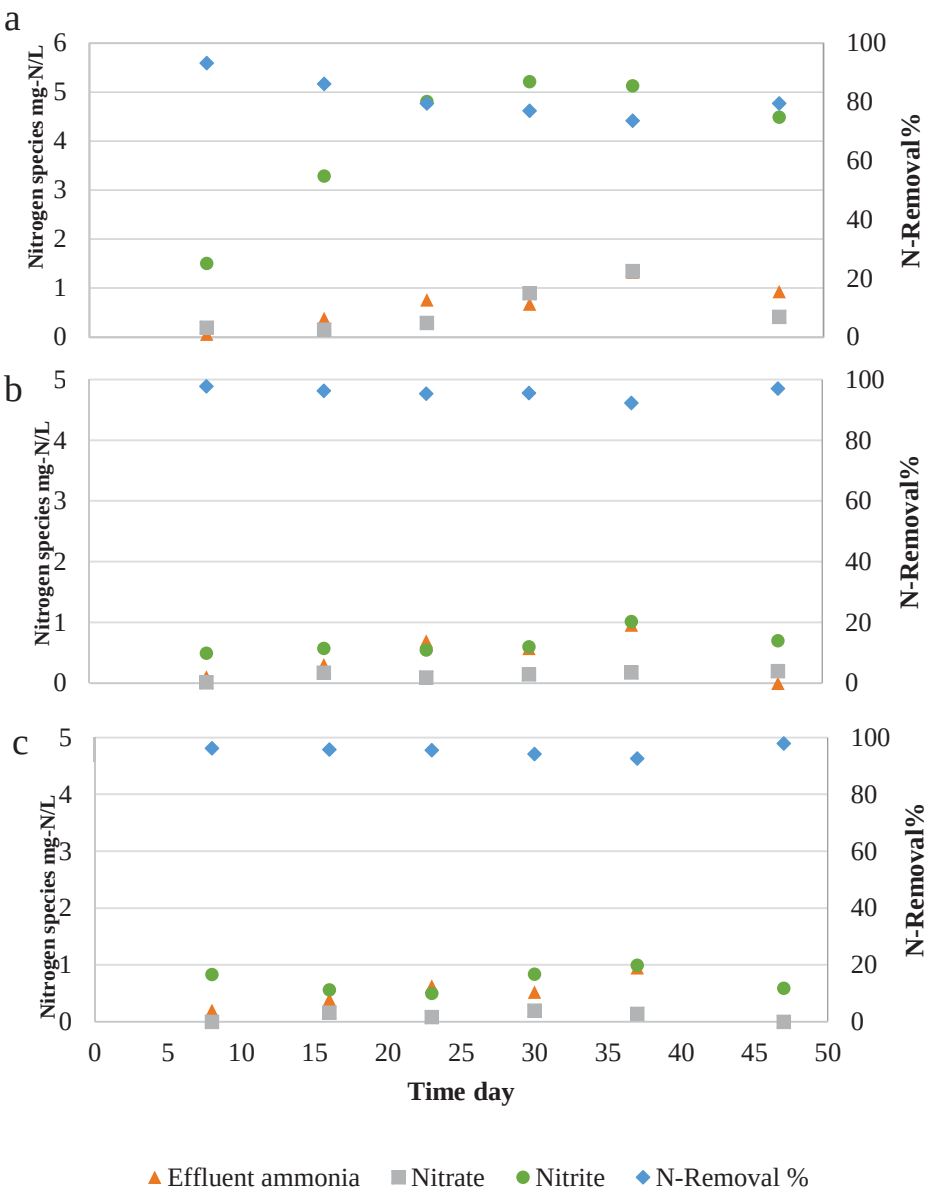

Figure 3- 18: Effluent nitrogen species concentrations along with the total nitrogen removal % during the 2nd experimental phase of this study. Reactor 1 (a), Reactor 2 (b) and Reactor 3 (c).

From the weekly sampling data, the initiation of the second experimental phase caused a slight variation in the phosphorous removal, as shown in Figure 3-19. For Reactor 1, the

phosphorous removal dropped from 87 to 81 % during the first week. Approximately the same removal efficiency was measured for the following three weeks, before stabilizing to around 75% in the last two weeks. For Reactors 2 and 3, the phosphorous removal efficiency increased during this phase from 86 and 86% to 87 and 91%, respectively.

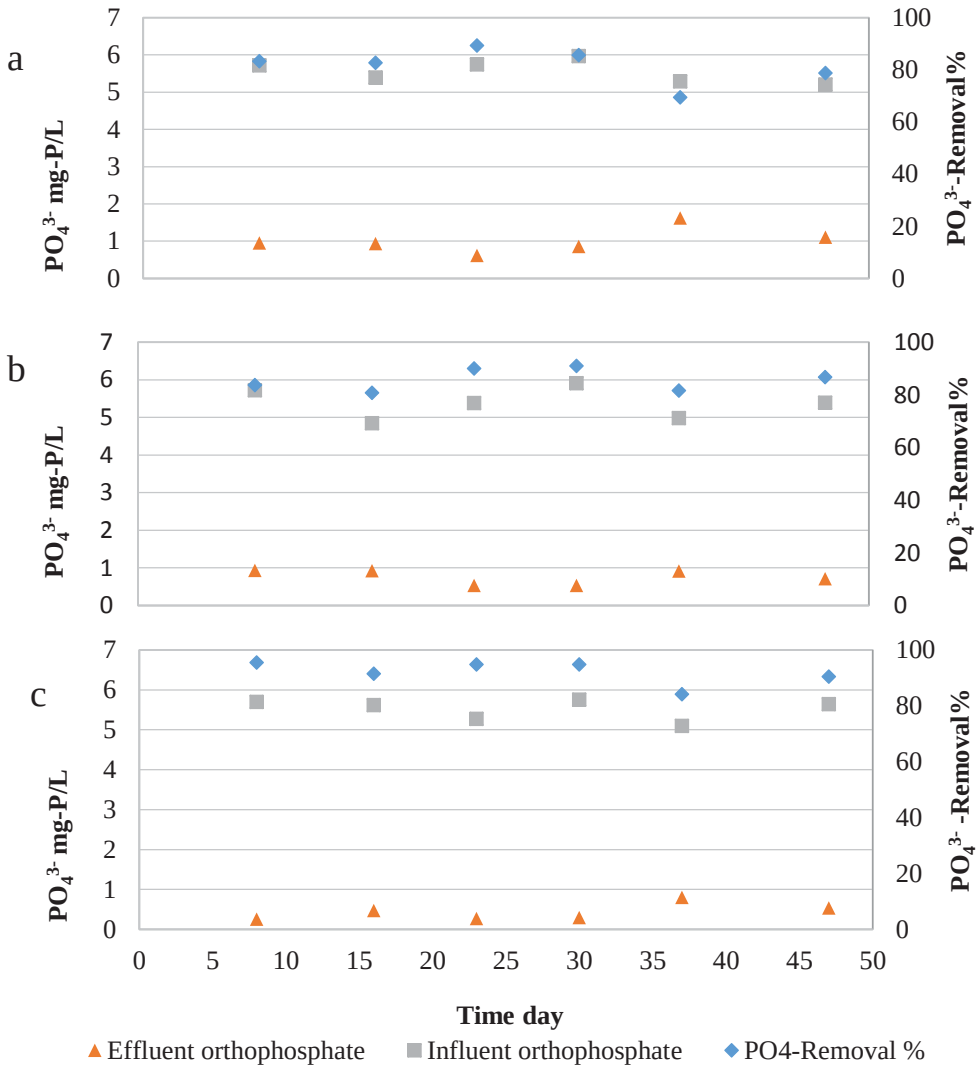

Figure 3-19: Effluent and influent orthophosphate concentrations in the treated wastewater along with the removal percentage during the 2nd phase of this study. Reactor 1 (a), Reactor 2 (b) and Reactor 3 (c).

The soluble COD fractions were completely consumed in all the reactors (>96). For Reactor

1, the total COD removal at the end of the first week was very low, 19%, then it increased to approximately 45% by steady-state operation. The total COD removal efficiency in Reactors 2 and 3 was fairly consistent throughout the experimental phase and varied between (63-69%) and (68-79%), respectively (Figure 3-20).

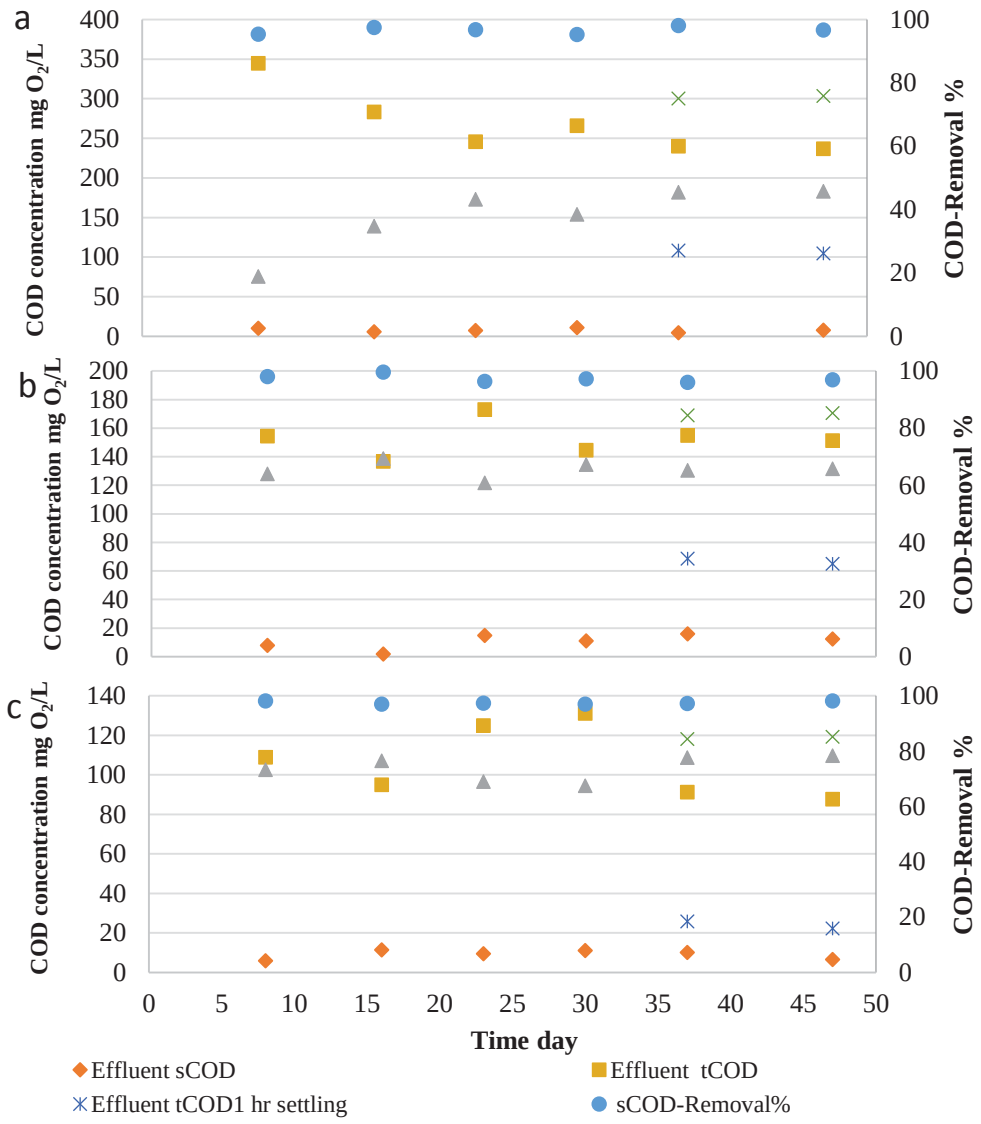

Figure 3- 20: Effluent and influent COD concentrations in the treated wastewater along with the removal percentage during the 2nd experimental phase of this study. Reactor 1 (a), Reactor 2 (b) and Reactor 3 (c).

Since the measured effluent COD concentration includes the washed out biomass, methods were adapted to determine the removal of the COD in the influent wastewater, without measuring ESS. The effluent wastewater from a cycle (1.5 L) was left to settle for one hour to separate the biomass from the bulk liquid. After that, the supernatant was collected and the total COD concentration was determined as described before. The results showed that Reactor 3 fed with the fermenter-pretreated wastewater yielded the best performance with 94% total COD removal, whereas 75 and 85% removal were achieved for Reactor 1 and 2. Since all reactors removed 97% of the sCOD, the differences in total COD removal was attributed to differences in the sbCOD (both particulate and hydrolyzed) fractions in the wastewater. The average treatment performance for each reactor during steady-state operation is presented in Table 3-5.

Table 3- 5: Summary of steady-state aerobic granular sludge treatment performance for the 2nd experimental phase[1].

	Reactor 1	Reactor 2	Reactor 3
Total N Removal %	78 ± 3	95 ± 2	95 ± 2
Phosphate Removal %	81 ± 9	86 ± 4	91 ± 5
Soluble COD Removal %	97 ± 1	97 ± 1	97 ± 1
Total COD Removal % (including effluent suspended solids)	42 ± 5	66 ± 2	74 ± 5
Total COD Removal % (after 1 hr settleable biomass was removed)	75 ± 1	85 ± 1	94 ± 1

[1] reported with standard deviation errors.

Beyond considering steady-state effluent characteristics, biochemical analyses were investigated at smaller time intervals during a single SBR cycle on Day 59 during the steady-state operation, as presented in Figure 3-21. The data demonstrated that the biochemical

conversion processes were slower in Reactor 1, which was fed the unpretreated wastewater with 50% particulate starch. The ammonia was totally consumed after 210 min in Reactor 1, while it was totally oxidized after just 180 and 150 min in Reactors 2 and 3, respectively. Interestingly, the denitrification process was the least efficient in Reactor 1. At the end of the aeration, the sum of the oxidized nitrogen compounds (nitrite and nitrate) was 8, 2 and 3 mg-N/L in Reactor 1, 2 and 3, respectively. These concentrations were further reduced during the post-anoxic period to 5, 0.9 and 0.6 in Reactor 1, 2 and 3, respectively.

The cycle analysis also showed different kinetics for the orthophosphate release and uptake by PAOs during the anaerobic and aerobic phases. The orthophosphate released at the end of the anaerobic period was 8.2 mg-P/L in Reactor 1, whereas 16.1 and 18.5 mg-P/L in Reactor 2 and 3, respectively. This orthophosphate release was followed by uptake of the released orthophosphate in the reactors during the aerobic phase, with different rates in each reactor. It took all the aeration time to reduce the concentration of the orthophosphate to 1.1 mg-P/L, where it acquired 180 and 150 to achieve 0.7 and 0.5 mg-P/L in Reactor 2 and 3, respectively.

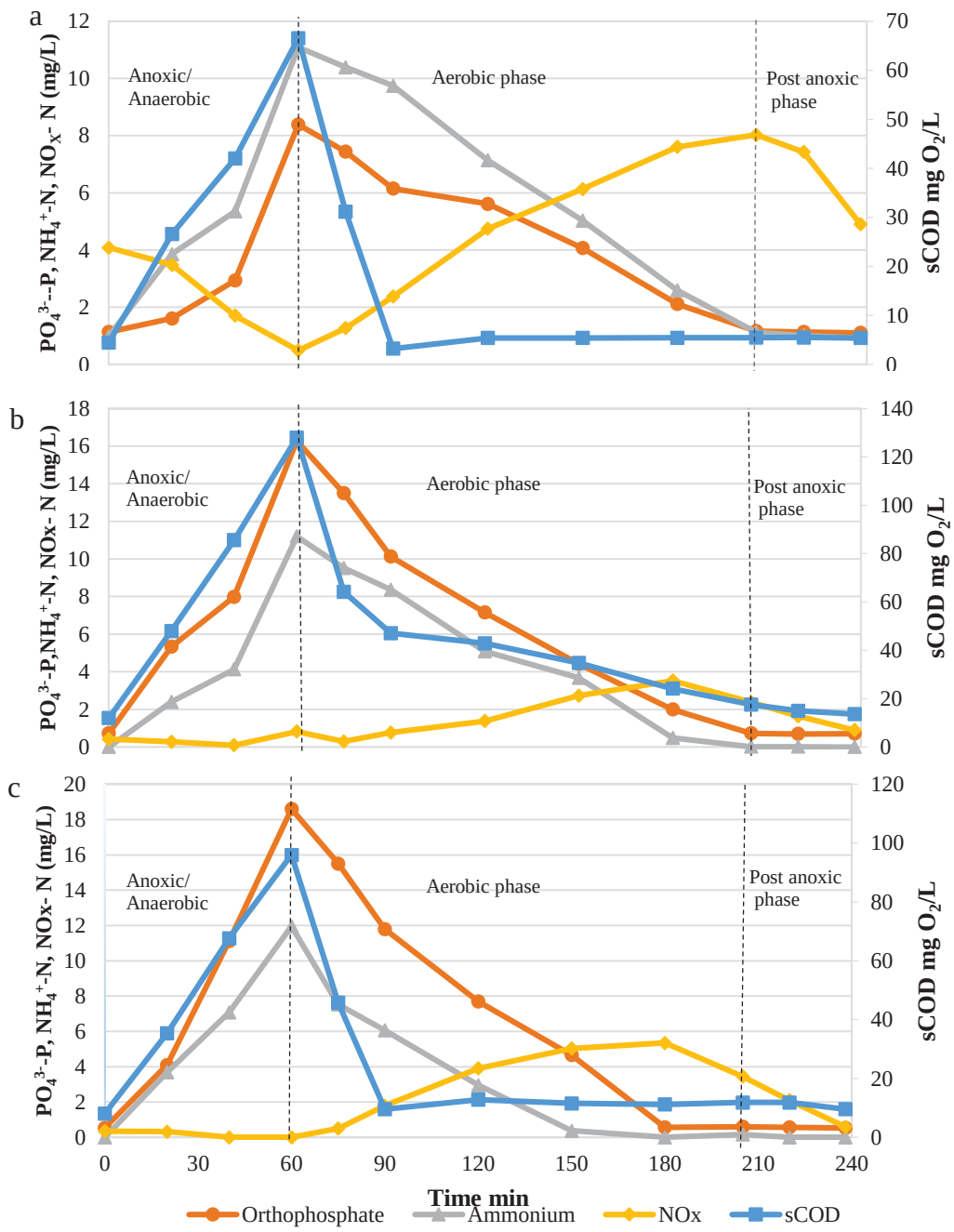

Figure 3-21: Biochemical conversion processes occurring during a single SBR cycle on Day 47 of the 2nd phase. Reactor 1 (a), Reactor 2 (b) and Reactor 3 (c).

Figure 3-21 depicts the soluble organic matter consumption during a typical SBR cycle during the 2nd experimental phase. By comparison, the soluble COD removal in Reactor 2 during the aeration phase was different than that observed in the other reactors. In Reactors 1 and 3, the

residual sCOD after 60 min of the cycle was completely degraded and consumed (>96% removal) after 30 min of the start of the aeration period. In contrast in Reactor 2, not all the sCOD was degraded after 30 min, but the sCOD then degraded slower for the rest of the aerobic period. However by the end of the cycle, the overall sCOD removal in Reactor 2 was comparable to the effluent removal percentages obtained in the other reactors.

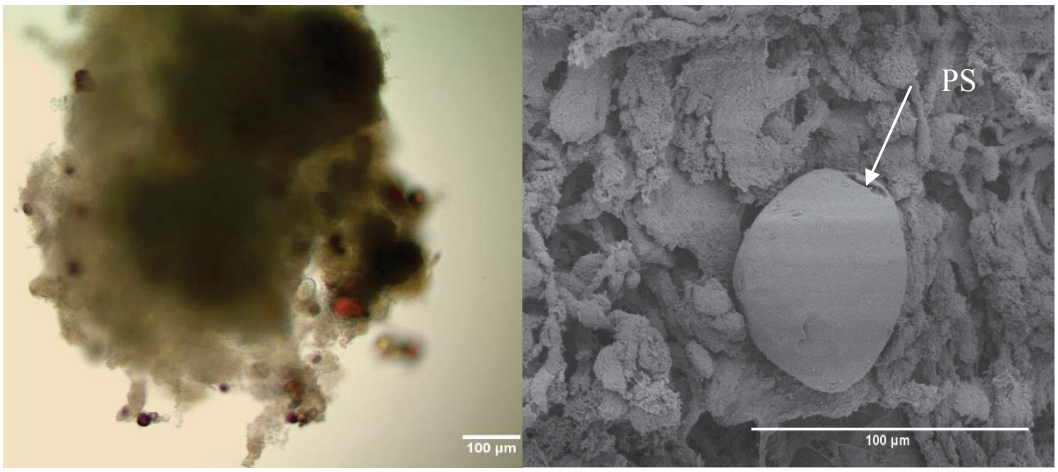

Figure 3- 22: Bright field microscopy and SEM observations of the starch adsorption onto a granule surface. Purple dots in the left image represents the starch particles stained with iodine.

It was difficult to estimate the total COD during an operating cycle because of the suspended biomass in the COD measurement. Therefore, bright field and SEM images of granules after the end of the operating cycle were taken to help understand the mechanism responsible for the removal of the psbCOD portion of the particulate starch. Figure 3-22 shows that starch polymers were embedded into both the surface and matrix of the granule. These observations demonstrate that the 4 hr operating cycle were insufficient to hydrolyze and degrade the particulate starch.

CHAPTER 4: DISCUSSION

4-1 Phase 1: Development of stable aerobic granules

4-1-1 Aerobic granules characteristics

The overall structure of sludge particles controls the sludge settleability and compactness. During this experiment, regular, stable and compact granules were developed. The majority of the sludge particles were larger than 0.3 mm with aspect ratio higher than 0.65. Particles larger than 0.3 mm are categorized as typically granules (de Kreuk, Pronk, et al., 2005). Thus, most of aggregates in the SBRs were granular. The aspect ratio is equal to the minimum diameter of each particle divided by the maximum diameter and reflects the granules' roundness. An aspect ratio of 1 would reflect a perfect sphere, and the nearest value to 1 means the most regular the granule has. Therefore, spherical granules were developed in this experiment.

Besides the size and regularity of the sludge particles, the extracellular substances (EPS) content secreted by the cells play a major role in the granule formation and development. The EPS work as a network to promote cells aggregation and biofilm formation through chemical bonding and physical interactions (Nielsen et al., 1997; Zhu et al., 2012). The high EPS content in the granule matrix increases the density and compactness of the granules. The extraction data showed that the developed granules in the AGRs had high EPS content. Most of the extracted EPS content was extracellular protein, which was more concentrated than the carbohydrate content in the EPS matrix. It is difficult to perform a direct comparison of EPS composition with the literature because a range of extraction techniques have been employed, and the reactor operation influences the results. However, in this study the protein content was the dominant component of the EPS, which was similar to several reported aerobic granule studies (BS

McSwain et al., 2005; Su et al., 2005; Z.-W. Wang et al., 2006; Zhu et al., 2012). It is believed that the high EPS protein concentration in the granule could strengthen the stability and maintain the integrity of the aerobic granular sludge (S. S. Adav, Lee, & Tay, 2008; BS McSwain et al., 2005). As a result, granules with low SVI and high settleability properties were formed in the SBRs.

4-1-2 Aerobic granules treatment performance

4-1-2-1 The treatment of the organic matter and ammonia

After two weeks of operation, the granules in the SBRs were able to perform almost complete COD and ammonia removal (> 95% and > 96%, respectively). This means the selection of nitrifiers was accomplished. However, the nitrite concentration in the effluent was the predominant product compared with nitrate. The nitrification process proceeds first by oxidizing ammonium to nitrite, followed by nitrite oxidization to nitrate. The ammonium oxidizing bacteria (AOB) must have been either more abundant or more active than the nitrite oxidizing bacteria (NOB). Nitrite accumulation in aerobic granular reactors has been reported previously (X.-Y. Shi et al., 2010; Y.-J. Shi et al., 2011).

4-1-2-2 Improvement of simultaneous nitrification denitrification

The total nitrogen removal was limited due to the incomplete nitrification and accumulation of nitrite. Typically in a granule structure, simultaneous nitrification and denitrification (SND) occurs because complementary populations are active in the aerobic and anoxic zones. After the oxidation of ammonia by nitrifiers in the aerobic zone, the nitrate is denitrified to nitrogen gas in the anoxic layer. Mainly, this process depends on the penetration depth of oxygen inside the granule matrix. A greater diffusion depth of oxygen results in a smaller anoxic layer, which hinders the selection of denitrifiers, or inhibits the denitrification

process. In this study, the DO level was not controlled and it was near saturation (around 7.8 mg O_2/L). The cycle samples collected on Day 22 of the 1st phase showed that when all ammonium was consumed, the NOx concentration remained constant indicating no further denitrification. This could be attributed to excessive penetration of oxygen into the granules, which would have prevented denitrifiers from being actice.

Previous studies investigated different strategies to lessen the effect of oxygen penetration on the total nitrogen removal. In some studies the bulk fluid oxygen concentration during the aeration period was decreased to improve the denitrification process, and the required shear force was accomplished by mixing nitrogen gas along with the decreased oxygen concentration (J. J. Beun et al., 2001; de Kreuk, Heijnen, et al., 2005). In this study, a post-anoxic phase was implemented to increase the denitrification efficiency. Consequently, 94% nitrogen removal was accomplished at the end of this phase. Applying anoxic conditions after the aeration period not only resulted in depletion in the nitrite concentration during the anoxic phase but also enhanced the denitrification activity during the aerobic phase as shown in Figure 3-8. These results are likely due to an increase in the anoxic layer depth and enrichment of denitrifiers in the granule matrix. No external carbon source was needed to achieve the denitrification process. Two guilds of organisms that may have contributed to this are the denitrifying phosphate accumulating organisms (DPAOs) and denitrifying glycogen accumulating organisms (DGAOs). These species utilize their stored carbon, such as polyhydroxybutyrate (PHB) for PAOs, in the denitrification process (Bassin et al., 2012a; de Kreuk, Pronk, et al., 2005). Thus, enrichment of these species, and other denitrifiers, is advantageous for nutrients removal from wastewater.

4-1-2-3 Improvement of phosphorous removal

Phosphorous removal was positively influenced by applying the post-anoxic period. Without the post anoxic phase, the phosphate removal ranged from 40 to 55%. Afterwards, the removal was increased to 86-87% in all the reactors at the steady-state, which corresponded to 0.70-0.73 mg-P/L phosphate concentration in the reactors. The improvement in the removal performance is probably due to the decrease in the nitrite concentration during the operation process. Nitrite mainly inhibits the PAOs in the nitrous acid form (FNA) (Zhou et al., 2011). Saito et al. (2004) investigated the effect of nitrite on the phosphate uptake rate in aerobic and anoxic conditions. This study found the phosphate uptake rate significantly decreased as the nitrite concentration increased. At pH 7.4 ± 1 and 1.2×10^{-3} mg-N/L FNA (calculated based on the acid equilibrium constant), the drop in the aerobic phosphate uptake was almost 100%, compared with no FNA. In the Saito et al. study (2004), nitrite inhibition of the phosphate uptake rate during the aeration period caused severe limits on the metabolism and competitiveness of PAOs. This influence could be accomplished by decreasing the phosphate release and PHA storage under the start of the subsequent cycle. Lower PHA storage results in less phosphate uptake under subsequent aerobic conditions.

Recently, Pijuan et al. (2010) discovered that FNA intensely prevents aerobic P-uptake, glycogen production and growth of the *Accumulibacter* PAO. 50% inhibition was determined at 0.52×10^{-3}, 0.48×10^{-3} and 0.36×10^{-3} mg-N/L FNA for the above three processes, respectively. For EPBR, GAOs exist in the system and they are less sensitive to nitrite as compared to PAO. The competition for organic carbon could be much more severe for PAOs, resulting in less PAOs in the system (Zeng et al., 2003). These previous findings confirmed the cycle analysis (Figure 3-8) where the nitrite concentration reached higher than 8 mg-N/L,

corresponding to 0.6×10^{-3} mg-N/L FNA at pH 7.5. It is apparent that the high nitrite concentration affected both the phosphate release and uptake during an SBR cycle, causing low phosphorus removal performance. On the other hand, after implementing the post-anoxic phase, the PAOs were more active with higher rates of phosphate release and uptake, since the nitrite concentration dropped to 6 mg-N/L at the end of the aeration and to less than 2 mg-N/L. At pH 7.5, these nitrite concentrations are corresponding to 0.47×10^{-3} and less than 0.16×10^{-3} mg-N/L FNA, respectively. This yielded more acetate accumulation and P release during the anoxic/ anaerobic period, resulting in active PAOs.

4-2 Phase 2: The effect of organic biodegradability nature on the aerobic granular sludge

4-2-1 Evaluation of hydrolysis processes

79% of the particulate starch (PS) was most often solubilized by heat hydrolysis. A previous study compared the effect of different heating conditions on the hydrolysis of different types of starch (McQuade et al., 1998). It was concluded that the solubility of starch depends on the type of the starch, heating time and heating temperature. In the current study, 80% solubility of the potato starch was achieved with heating at 120^0 C for 1 hr, which is consistent with the findings by McQuade et al. ((McQuade et al., 1998). However, approximately 82% of the soluble product was SS, which is also slowly biodegradable (Kayabali, 1997).

For the other pretreatment, anaerobic hydrolysis and fermentation enhanced the biodegradability of the organic matter in the wastewater. This experiment showed that higher rbCOD production potential was obtained by anaerobic treatment than by heat treatment. This is particularly due to the conversion of hydrolysis products to further monobasic molecules. Hydrolysis proceeds by microorganisms in contact with the complex, particulate organic matter producing extracellular enzymes. These enzymes break down the macromolecules' bonds,

resulting in soluble products. However, some loss (9%) of the total COD was observed in the fermented wastewater. This loss could be stored by the microorganisms as intracellular energy source for cell growth (Y. Wang et al., 2013).

The batch experiments showed that the VFAs were produced as soon as the particulate starch was hydrolyzed. Previous studies indicated that the fermentation process of the soluble products is simultaneously taking place with enzymatic hydrolysis (Bagchi, 1994; GonCalves et al., 1994). The results showed the VFAs concentration in the wastewater increased by 27-28 % of the total COD. This is advantageous for the treatment of real municipal wastewater, since the fraction of VFAs in municipal wastewater can be insufficient for biological phosphorus removal.

4-2-2 Effect of the different organic compounds on the aerobic granule characteristics

4-2-2-1 Effect of the different organic composition on the morphology of the aerobic granules

The microscopic investigation revealed that the carbon composition influenced aerobic granule characteristics. Feeding the granules with the non-pretreated and the heat hydrolyzed-pretreated wastewater caused granules to develop filamentous and finger-type structures on the granule surface and caused a decrease in the aspect ratio and the particle size distribution. The growth of filaments is probably due to a lower substrate gradient inside the granules caused by the hydrolysis of the starch. The bright field and scanning electron microscope images indicated that some of the PS was attached to the surface of the granule. Studies have pointed out that after the attachment of the starch macromolecules on the granule surface, hydrolysis to smaller compounds occurs, followed by local degradation by the microorganisms on the surface (de Kreuk et al., 2010; Mosquera-Corral et al., 2003). This leads to a small soluble substrate gradient inside the granule. Extensive research in the 1970s showed that filamentous organisms have a

competitive advantage when low substrate concentrations are present for extended times, and this is often referred to as the kinetic selection theory (J. Chudoba et al., 1973). In Reactor 1, the rbCOD concentration at and within the granule surface was small, and soluble COD was continually produced with hydrolysis, which created the extended substrate condition that is known to favor filamentous growth.

Reactor 2 was fed with mainly solubilized starch after the heat hydrolyzed-pretreatment. In contrast to Reactor 1, the SS could attach to the granule surface as well as penetrate into the granule. Because the SS was still slowly biodegradable, the extended presence of a low substrate concentration still selected for filamentous organisms. B McSwain et al. (2004) demonstrated that a clear feast-famine regime was necessary within the SBR cycle in order to select for compact and spherical aerobic granules. McSwain et al's previous work only tested rbCOD wastewater feeds. This study shows that sbCOD in the wastewater influent can act as an extended source of substrate, which reduces the starvation period represented as "famine" in the SBR cycle. The cycle sampling showed that the degradation of the soluble COD, including the SS in Reactor 2, was not complete after 30 min like the other reactors. It took all the aeration period to degrade the SS. Consequently, a fluffy surface and irregular shape were observed for the granules. Correspondingly, due to the applied high shear force, these filaments could slough away from the granule surface and be wasted from the reactors. This led to an overall smaller particle size present in these reactors and higher effluent suspended solids.

4-2-2-2 Effect of the carbon source on the structure and integrity of the aerobic granules

The total EPS protein and carbohydrate content varied considerably with the influent carbon source in the wastewater. The fermentor-pretreated wastewater led to the highest extracted protein content followed by feeding with heat hydrolyzed and non-pretreated

wastewater, respectively. However, the extracted carbohydrate content did not follow the same trend as the protein content. The granules fed with 50% particulate starch had the highest extracellular polysaccharide content, while no significant carbohydrate difference was measured between granules from Reactors 2 or 3, which were fed with pretreated, soluble COD wastewaters. The increase in the extracted polysaccharides in the granules fed with the non-pretreated wastewater could be attributed to the incorporation of the particulate starch in the granule matrix. The bright field and scanning microscope images of the granules collected at the end of the operation cycle (Figure 3-20) showed that a portion of the particulate starch was adsorbed and incorporated in the granule matrix. Nielsen et al., (1997) stated that the EPS include bacterially produced polymers, lysis products, hydrolysis products, as well as polymers embedded in the biofilm matrix from the surrounding environment. Therefore, the higher EPS polysaccharide content in the granules grown in Reactor 1 was likely due to the sorbed particulate matter.

The EPS production by cells was the maximum in Reactor 3, fed with the highest readily biodegradable COD level, followed by Reactor 2 and 1, respectively. The difference of the EPS protein concentrations may be attributed to the increased feeding of readily biodegradable COD level in Reactor 3. Figure 3-11 indicated the highest measured proteins corresponded with the highest influent rbCOD concentration. The easy degradation and uptake of readily biodegradable organic matter resulted in higher biomass growth as well. Previous studies stated that the EPS production rate is proportional to the rate of substrate utilization (Laspidou et al., 2002; Okabe et al., 1998). Turakhia et al. (1989) indicated that a higher substrate degradation rate yields higher cell growth rate, resulting in an elevation in the EPS production, which could support this study's findings for Reactors 2 and 3. On the other hand, the granules fed with 50% PS had significantly

lower EPS protein content than the other reactors. The measured protein content in these granules was even lower than the measured value before starting the 2nd experimental phase. The biomass concentration in this Reactor was very low compared with the biomass in the other reactors, and it decreased after changing the carbon source in the feeding solution.

The increase in the extracellular protein content with increased rbCOD may be explained by a higher growth rate, but cell lysis and cell degradation may also contribute to the EPS measurements. Nielsen et al. (1997) indicated that the easy degradation and uptake of readily biodegradable organic substrates probably results in a high level of exoenzymes in the EPS matrix. It is possible that these exoenzymes transport to the core of the granule, mainly consisting of the dead cells, and degrade the dead cell to proteins / amino acids and polysaccharides. Consequently, these degraded compounds are incorporate into the whole granule network, increasing the EPS in the network. This significantly alters the EPS protein content, since the proteins constitute the largest portion of all the cell compounds, around 70% of the total cell constituents (Schnaitman, 1970). This is probably the mechanism responsible for the high level of EPS protein content in the granules grown in Reactors 2 and 3. Overall, different mechanisms are responsible for the EPS production in the aerobic granular sludge, and it is difficult to distinguish which mechanisms are dominant at any given time.

4-2-2-3 Effect of the different organic composition on the structure and integrity of the aerobic granules

The SEM images indicated that granules fed with fermented wastewater had the most compact structure, and granules fed with 50% starch polymers had the least compact and most porous structure. Similar observations were made by Okabe et al. (1998) on biofilm structures grown on domestic wastewater with a particulate matter fraction. The results showed the biofilm

consisted of intertwined filaments functioning as a network in the biofilm, resulting in a very porous structure. The EPS extraction analyses could be used in conjunction with the microscopic investigation to describe this porous structure.

The protein to polysaccharide ratios in the extracts were 1.6, 3.7 and 4.8 for Reactor 1, 2 and 3, respectively. In the literature, a high protein to polysaccharide ratio is expected to play an important role in the stability and integrity of granules (S. S. Adav, Lee, Show, et al., 2008). It is hypothesized that the high protein content includes positively charged amino groups that are able to neutralize the negative charge of the other EPS compounds. Thus, higher Protein/ Polysaccharide may be advantageous for reducing the granule surface charge and enhancing the sludge properties (Y.-Q. Liu et al., 2004; Z. Wang et al., 2006). As a result, low protein content in the EPS network could lead to granule disintegration. This could cause the noticeable decrease in the MLSS during the first two weeks of the 2nd experimental phase, corresponding to high effluent suspended solids.

The LB-EPS results indicated that granules in Reactor 1 had the highest amount of the LB-PN and LB-PS and the lowest TB-PN. EPS can function as the maintenance energy source for microbial communities, when external carbon or nutrients become limiting (J. Wang et al., 2008). Therefore, microorganisms can utilize the EPS to maintain their growth, resulting in a decrease in the EPS and more loosely bounds EPS. These loosely-bond EPS could erode from the EPS framework in the granules and transport to the bulk liquid, leaving cavities and pores in the granule matrix. This would deteriorate the cell to cell adhesion and decrease the density of the granule, resulting in less stability and settleabilty. X. Li et al. (2007) correlated the amount of LB-EPS in activated sludge with the settleabilty and dewatering of the sludge. In that study, it was claimed that the increase in the LB-EPS in the activated sludge floc led to an increase in the

SVI of the sludge, resulting in poor bioflocculation and greater cell erosion. Like the Li et al. (2007) study, an increased SVI was associated with an increase in the LB-EPS concentration in the current study.

4-2-2-4 Treatment performance under different carbon source

The data showed that the aerobic granular sludge in all the reactors treated almost all the soluble COD (>96). However, the total organic removal varied for the reactors with the different influent wastewaters. The aerobic granules fed the 50% PS were not able to degrade all the particulate starch in a 4-hr SBR cycle. Figure 3-20 shows that the starch polymers were sorbed and embedded into the granule structure at the end of an operation cycle. Previous studies attributed the removal of suspended solids and sbCOD to the presence of protozoa on the surface of the aerobic granules and the SBRs wall (de Kreuk et al., 2010; N Schwarzenbeck et al., 2004; N. Schwarzenbeck et al., 2005). Excessive wall growth was observed in Reactor 1 and 2. The tCOD removal for Reactors 1, 2 and 3 was 42 ± 5, 66 ± 2, 74 ± 5, respectively, at steady-state. Not only the starch polymers contributed to the low COD removal, but also oughing of filaments from the granule surface and LB-EPS, as discussed in the previous sections. Therefore, settleable particles would need to be removed from the effluent of a granular SBR, prior to discharge at full-scale. This was tested with a 1 hr settling of the effluent wastewater, which increased the removal for Reactors 1, 2 and 3 to 75±1, 85±1, and 94±1, respectively. This indicates considerable amount of the washed out biomass or the sloughed filaments and LB-EPS contributed in the effluent COD quality.

Ammonia removal was unaffected by the presence of particulate and sbCOD in the wastewater. The effluent ammonia was less than 1 mg-N/L for all the reactors over the course of the 2nd experimental phase. However, 50% PS presence in the influent of Reactor 1 influenced

the total nitrogen, particularly the nitrite oxidation and denitrification processes. The nitrite gradually increased to around 5 mg-N/L in the effluent during the first three weeks, then continued the same during the rest of the 2nd phase. Compared to the other reactors, the cycle analysis demonstrated that the denitrification rate during the aerobic period was the slowest. The accumulation of nitrite in the reactor was probably due to the porous structure of the developed granules in the reactor. For the other reactors, the granules were observed with a more compact structure, and the total nitrogen was unchanged. As mentioned in section 4-1-2-2 in discussion, the oxygen diffusion controls the denitrification process. In a biofilm system, the biofilm structure influences the mass transport of the oxygen from the bulk liquid (Coma et al., 2012). The oxygen transport rate not only depends on the size of the granule but also on the permeability of the granule. This means the more porous the structure, the higher the oxygen transport rate inside the granule. Consequently, the anoxic zone volume would decrease, resulting in reduced denitrification. This led to a drop in the total nitrogen removal from 94±1 before dosing the PS to 78±3 after dosing the PS in Reactor 1.

As stated in section 4-1-2-3, the phosphorus removal was negatively affected by the elevated nitrite and FNA concentration in Reactor 1. Figure 3-19 showed that the P-uptake and release during the operation cycle was the slowest in Reactor 1 compared with the other reactors. The P-removal was 81 ± 9%, whereas it was 87 ± 2% before dosing the PS.

Overall, it seems that the 50% PS mainly increased the effluent suspended solids quality, and decreased the tCOD removal and denitrification process. The heat hydrolysis pretreatment maintained the nutrient removal performance of the aerobic granules. However, it also caused high effluent suspended solids. The fermentor pretreatment maintained both the organic and nutrient removal performance.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5-1 Conclusions

This experiment was conducted to evaluate the stability and performance of aerobic granular sludge treating municipal wastewater containing 50% slowly biodegradable matter. Also, this study investigates the feasibility of heat and fermentor pretreatments to enhance the performance of biological nutrient removal by aerobic granules.

Stable, regular and compact aerobic granules were developed in the 1st phase of this study after applying the post-anoxic phase. The developed sludge showed high settleabilty performance (26±4, 26±4 and 28±5 mL/mg SV1 in the three reactors). This study demonstrated the role of implementing post-anoxic conditions on enhancing the nutrient removal of the aerobic granules. The post-anoxic phase improved denitrification during the operation cycle. Additionally, it reduced the inhibition of the FNA on the PAOs. Consequently, denitrifiers and PAOs were enriched in the SBRs. The aerobic granules were capable of removing the organic carbon and nutrients from the synthetic municipal wastewater. Complete COD and nitrogen removal were achieved (96 ± 1% and 94 ± 1%, respectively), and 86-87% of phosphorus was removed during steady-state operation of this phase.

In the 2nd phase of this study, the presence of 50% particulate matter in municipal wastewater negatively affected the morphology, structure and performance of the aerobic granular sludge. The substrate gradient caused by the hydrolysis of a portion of the particulate starch on the surface of the granules resulted in a filamentous growth. This growth led to high effluent suspended solids and high effluent COD. In addition, the low readily biodegradable COD feed led to a decrease in the EPS production in the granule framework, resulting in less

dense granules and a more porous structure. This porous structure increased the oxygen diffusion inside the granule, leading to nitrite accumulation in the effluent. As a result, the total nitrogen and phosphorus removal also decreased.

Both pretreatments of the particulate wastewater were effective in solubilizing the particulate starch by decreasing its fraction from 50% to around 10%. Moreover, both pretreatments maintained the structure of the aerobic granular sludge and performance of nutrient removal in the SBRs. However, the granules fed with the heat hydrolyzed-pretreated wastewater developed a filamentous surface due to the fact that the produced soluble starch was a slowly biodegradable compound. The sloughing of this filamentous surface increased the effluent suspended solids and effluent COD concentration. Also, the heat hydrolysis pretreatment is an unrealistic method for full-scale application. On the other hand, pretreating the wastewater through a fermenter before pumping to the SBR maintained and enhanced the aerobic granules' characteristics, morphology, integrity and performance. Overall, anaerobic hydrolysis and fermentation pretreatment yielded the best strategy to enhance the aerobic granular treatment to treat municipal wastewater with 50% particulate matter. Several full-scale wastewater treatment plants already utilize fermentation reactors in order to increase the VFA concentration of influent wastewater for biological phosphorus removal, so using a fermentor to increase wastewater biodegradability should be feasible at full-scale.

5-2 Future directions

The particulate matter in this study was a starch polymer. The assessment of the hydrolysis products and the particulate fraction was done based on knowledge of the expected hydrolysis products. In real wastewater, different suspended solids contribute to the particulate COD fraction, and only a portion of particulate COD or sbCOD would pass through a primary clarifier, if present. For future studies, it is important to understand the typical composition of municipal wastewater regarding particulate COD, hsbCOD, and rbCOD fractions before and after any fermentation pretreatment. It would be very useful to specifically characterize the hydrolysis products of the particulate fraction. Also, this study investigated the effect of a 50% particulate matter wastewater fraction. For future work, different percentages and absolute concentration of the sbCOD should be tested with aerobic granular reactors, to determine the limits for treatment with aerobic granules.

REFRENCES

Adav, Sunil S., Lee, Duu-Jong, Show, Kuan-Yeow, & Tay, Joo-Hwa. (2008). Aerobic granular sludge: Recent advances. *Biotechnology Advances, 26*(5), 411-423. doi: http://dx.doi.org/10.1016/j.biotechadv.2008.05.002

Adav, Sunil S., Lee, Duu-Jong, & Tay, Joo-Hwa. (2008). Extracellular polymeric substances and structural stability of aerobic granule. *Water Research, 42*(6–7), 1644-1650. doi: http://dx.doi.org/10.1016/j.watres.2007.10.013

Adav, SunilS, Lee, Duu-Jong, & Lai, J. Y. (2007). Effects of aeration intensity on formation of phenol-fed aerobic granules and extracellular polymeric substances. *Applied Microbiology and Biotechnology, 77*(1), 175-182. doi: 10.1007/s00253-007-1125-3

Andreottola, G, Foladori, P, & Ragazzi, M. (2000). Upgrading of a small wastewater treatment plant in a cold climate region using a moving bed biofilm reactor (MBBR) system. *Water science and technology, 41*(1), 177-185.

APHA, AWWA, & WEF. (2005). *Standard methods for the examination of water and wastewater* (21 ed.). American Public Heath Association, Washington, DC

Bagchi, Amalendu. (1994). Design, construction, and monitoring of landfills.

Balmat, JL. (1957). Biochemical oxidation of various particulate fractions of sewage. *Sewage and Industrial Wastes*, 757-761.

Bassin, J. P., Kleerebezem, R., Dezotti, M., & van Loosdrecht, M. C. M. (2012a). Measuring biomass specific ammonium, nitrite and phosphate uptake rates in aerobic granular sludge. *Chemosphere, 89*(10), 1161-1168. doi: http://dx.doi.org/10.1016/j.chemosphere.2012.07.050

Bassin, J. P., Kleerebezem, R., Dezotti, M., & van Loosdrecht, M. C. M. (2012b). Simultaneous nitrogen and phosphate removal in aerobic granular sludge reactors operated at different temperatures. *Water Research, 46*(12), 3805-3816. doi: http://dx.doi.org/10.1016/j.watres.2012.04.015

Batstone, Damien J, Keller, J, Angelidaki, Irini, Kalyuzhnyi, SV, Pavlostathis, SG, Rozzi, A, Sanders, WTM, Siegrist, H, & Vavilin, VA. (2002). The IWA Anaerobic Digestion Model No 1(ADM 1). *Water Science & Technology, 45*(10), 65-73.

Beun, J. J., Heijnen, J. J., & van Loosdrecht, M. C. M. (2001). N-Removal in a granular sludge sequencing batch airlift reactor. *Biotechnology and Bioengineering, 75*(1), 82-92. doi: 10.1002/bit.1167

Beun, JJ, Hendriks, A, Van Loosdrecht, MCM, Morgenroth, E, Wilderer, PA, & Heijnen, JJ. (1999). Aerobic granulation in a sequencing batch reactor. *Water Research, 33*(10), 2283-2290.

Boller, M, & Gujer, W. (1986). Nitrification in tertiary trickling filters followed by deep-bed filters. *Water research, 20*(11), 1363-1373.

Cassidy, D. P., & Belia, E. (2005). Nitrogen and phosphorus removal from an abattoir wastewater in a SBR with aerobic granular sludge. *Water Research, 39*(19), 4817-4823. doi: http://dx.doi.org/10.1016/j.watres.2005.09.025

Chang, Chao H, & Hao, Oliver J. (1996). Sequencing batch reactor system for nutrient removal: ORP and pH profiles. *Journal of chemical technology and biotechnology, 67*(1), 27-38.
Chiesa, Steven C, Irvine, Robert L, & Manning, John F. (1985). Feast/famine growth environments and activated sludge population selection. *Biotechnology and bioengineering, 27*(5), 562-568.
Chiesa, Steven C., & Irvine, Robert L. (1985). Growth and control of filamentous microbes in activated sludge: an integrated hypothesis. *Water Research, 19*(4), 471-479. doi: http://dx.doi.org/10.1016/0043-1354(85)90039-9
Chudoba, J., Grau, P., & Ottová, V. (1973). Control of activated-sludge filamentous bulking–II. Selection of microorganisms by means of a selector. *Water Research, 7*(10), 1389-1406. doi: http://dx.doi.org/10.1016/0043-1354(73)90113-9
Chudoba, Jan. (1985). Control of activated sludge filamentous bulking—VI. Formulation of basic principles. *Water Research, 19*(8), 1017-1022.
Coma, M., Verawaty, M., Pijuan, M., Yuan, Z., & Bond, P. L. (2012). Enhancing aerobic granulation for biological nutrient removal from domestic wastewater. *Bioresource Technology, 103*(1), 101-108. doi: http://dx.doi.org/10.1016/j.biortech.2011.10.014
Darilek, Glenn T. Corapcioglu M. YavuzYeung Albert T. (1996). Sealing Leaks in Geomembrane Liners Using Electrophoresis. *Journal of Environmental Engineering, 122*(6), 540.
Davies, WJ, Le, MS, & Heath, CR. (1998). Intensified activated sludge process with submerged membrane microfiltration. *Water Science and Technology, 38*(4), 421-428.
de Bruin, L, de Kreuk, M, van der Roest, H, Uijterlinde, C, & van Loosdrecht, M. (2004). Aerobic granular sludge technology: an alternative to activated sludge? *Water Science & Technology, 49*(11-12), 11-12.
de Kreuk, M. K., Heijnen, J. J., & van Loosdrecht, M. C. M. (2005). Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. *Biotechnology and Bioengineering, 90*(6), 761-769. doi: 10.1002/bit.20470
de Kreuk, M. K., Kishida, N., Tsuneda, S., & van Loosdrecht, M. C. M. (2010). Behavior of polymeric substrates in an aerobic granular sludge system. *Water Research, 44*(20), 5929-5938. doi: 10.1016/j.watres.2010.07.033
de Kreuk, M. K., Pronk, M., & van Loosdrecht, M. C. M. (2005). Formation of aerobic granules and conversion processes in an aerobic granular sludge reactor at moderate and low temperatures. *Water Research, 39*(18), 4476-4484. doi: http://dx.doi.org/10.1016/j.watres.2005.08.031
Drewnowski, J., & Makinia, J. (2011). The role of colloidal and particulate organic compounds in denitrification and EBPR occurring in a full-scale activated sludge system. *Water Science & Technology, 63*(2), 318-324. doi: 10.2166/wst.2011.056
Dulekgurgen, Ebru, Ovez, Suleyman, Artan, Nazik, & Orhon, Derin. (2003). Enhanced biological phosphate removal by granular sludge in a sequencing batch reactor. *Biotechnology letters, 25*(9), 687-693.
Evans, Elwyn, Brown, Michael RW, & Gilbert, Peter. (1994). Iron chelator, exopolysaccharide and protease production in Staphylococcus epidermidis: a comparative study of the effects of specific growth rate in biofilm and planktonic culture. *Microbiology, 140*(1), 153-157.

Frølund, Bo, Palmgren, Rikke, Keiding, Kristian, & Nielsen, Per Halkjær. (1996). Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Research, 30*(8), 1749-1758. doi: http://dx.doi.org/10.1016/0043-1354(95)00323-1

Gerhardt, P, Murray, RGE, Wood, WA, & Krieg, NR. (1994). Methods for General and Molecular Bacteriology, ASM, Washington DC: ISBN 1-55581-048-9, p 518.

GonCalves, R, Charlier, A, & Sammut, François. (1994). Primary fermentation of soluble and particulate organic matter for wastewater treatment. *Water Science and Technology, 30*(6), 53-62.

Grady Jr, CP Leslie, Daigger, Glen T, Love, Nancy G, Filipe, Carlos DM, & Leslie Grady, CP. (2011). *Biological wastewater treatment*: IWA Publishing.

Huang, Man-hong, Li, Yong-mei, & Gu, Guo-wei. (2010). Chemical composition of organic matters in domestic wastewater. *Desalination, 262*(1–3), 36-42. doi: http://dx.doi.org/10.1016/j.desal.2010.05.037

Huangfu, Yanchong. (2012). Filamentous foaming problem control in activated sludge.

Irvine, Robert L., Wilderer, Peter A., & Flemming, Hams-Curt. (1997). Controlled unsteady state processes and technologies - An overview. *Water Science and Technology, 35*(1), 1-10. doi: http://dx.doi.org/10.1016/S0273-1223(96)00872-4

Kayabali, Kamil. (1997). Engineering aspects of a novel landfill liner material: bentonite-amended natural zeolite. *Engineering Geology, 46*(2), 105-114.

Kim, S, Choi, H, & Kim, I. (2004). Enhanced aerobic floc-like granulation and nitrogen removal in a sequencing batch reactor by selection of settling velocity. *Water Science & Technology, 50*(6), 157-162.

Laspidou, Chrysi S., & Rittmann, Bruce E. (2002). A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. *Water Research, 36*(11), 2711-2720. doi: http://dx.doi.org/10.1016/S0043-1354(01)00413-4

Lee, Duu-Jong, Chen, Yu-You, Show, Kuan-Yeow, Whiteley, Chris G., & Tay, Joo-Hwa. (2010). Advances in aerobic granule formation and granule stability in the course of storage and reactor operation. *Biotechnology Advances, 28*(6), 919-934. doi: http://dx.doi.org/10.1016/j.biotechadv.2010.08.007

Li, An-jie, Zhang, Tong, & Li, Xiao-yan. (2010). Fate of aerobic bacterial granules with fungal contamination under different organic loading conditions. *Chemosphere, 78*(5), 500-509. doi: http://dx.doi.org/10.1016/j.chemosphere.2009.11.040

Li, XY, & Yang, SF. (2007). Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. *Water Research, 41*(5), 1022-1030.

Liang, Zhiwei, Li, Wenhong, Yang, Shangyuan, & Du, Ping. (2010). Extraction and structural characteristics of extracellular polymeric substances (EPS), pellets in autotrophic nitrifying biofilm and activated sludge. *Chemosphere, 81*(5), 626-632.

Liu, Yong-Qiang, Liu, Yu, & Tay, Joo-Hwa. (2004). The effects of extracellular polymeric substances on the formation and stability of biogranules. *Applied Microbiology and Biotechnology, 65*(2), 143-148.

Liu, Yong-Qiang, Moy, Benjamin, Kong, Yun-Hua, & Tay, Joo-Hwa. (2010). Formation, physical characteristics and microbial community structure of aerobic granules in a pilot-scale sequencing batch reactor for real wastewater treatment. *Enzyme and Microbial Technology, 46*(6), 520-525. doi: http://dx.doi.org/10.1016/j.enzmictec.2010.02.001

Liu, Yong-Qiang, & Tay, Joo-Hwa. (2007). Influence of cycle time on kinetic behaviors of steady-state aerobic granules in sequencing batch reactors. *Enzyme and Microbial Technology, 41*(4), 516-522. doi: http://dx.doi.org/10.1016/j.enzmictec.2007.04.005

Liu, Yu, & Liu, Qi-Shan. (2006). Causes and control of filamentous growth in aerobic granular sludge sequencing batch reactors. *Biotechnology Advances, 24*(1), 115-127. doi: http://dx.doi.org/10.1016/j.biotechadv.2005.08.001

Liu, Yu, & Tay, Joo-Hwa. (2002). The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. *Water Research, 36*(7), 1653-1665. doi: http://dx.doi.org/10.1016/S0043-1354(01)00379-7

Lowry, Oliver H, Rosebrough, Nira J, Farr, A Lewis, & Randall, Rose J. (1951). Protein measurement with the Folin phenol reagent. *J biol chem, 193*(1), 265-275.

McQuade, SJ, & Needham, AD. (1998). Geomembrane liner defects 衍 auses, frequency and avoidance.

McSwain, B, Irvine, R, & Wilderer, P. (2004). The effect of intermittent feeding on aerobic granule structure. *Water Science & Technology, 49*(11-12), 19-25.

McSwain, Belinda Sue. (2005). BSPH, 2005. *Molecular investigation of aerobic granular sludge formation. The University of Notre Damein Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy. Indiana*.

McSwain, BS, Irvine, RL, Hausner, M, & Wilderer, PA. (2005). Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. *Applied and Environmental Microbiology, 71*(2), 1051-1057.

Miller, Gail Lorenz. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry, 31*(3), 426-428.

Miqueleto, A. P., Dolosic, C. C., Pozzi, E., Foresti, E., & Zaiat, M. (2010). Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing batch biofilm reactors for wastewater treatment. *Bioresource Technology, 101*(4), 1324-1330. doi: http://dx.doi.org/10.1016/j.biortech.2009.09.026

Morgenroth, E, Kommedal, Roald, & Harremos, P. (2002). Processes and modeling of hydrolysis of particulate organic matter in aerobic wastewater treatment-a review. *Water Science & Technology, 45*(6), 25-40.

Mosquera-Corral, A., de Kreuk, M. K., Heijnen, J. J., & van Loosdrecht, M. C. M. (2005). Effects of oxygen concentration on N-removal in an aerobic granular sludge reactor. *Water Research, 39*(12), 2676-2686. doi: http://dx.doi.org/10.1016/j.watres.2005.04.065

Mosquera-Corral, A., Montràs, A., Heijnen, J. J., & van Loosdrecht, M. C. M. (2003). Degradation of polymers in a biofilm airlift suspension reactor. *Water Research, 37*(3), 485-492. doi: http://dx.doi.org/10.1016/S0043-1354(02)00309-3

Moy, BY-P, Tay, J-H, Toh, S-K, Liu, Y, & Tay, ST-L. (2002). High organic loading influences the physical characteristics of aerobic sludge granules. *Letters in Applied Microbiology, 34*(6), 407-412.

Nielsen, Per Halkjær, Jahn, Andreas, & Palmgren, Rikke. (1997). Conceptual model for production and composition of exopolymers in biofilms. *Water Science and Technology, 36*(1), 11-19.

O'Toole, George A, & Kolter, Roberto. (1998). Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular microbiology, 28*(3), 449-461.

Okabe, S., Kuroda, H., & Watanabe, Y. (1998). Significance of biofilm structure on transport of inert particulates into biofilms. *Water Science and Technology, 38*(8–9), 163-170. doi: http://dx.doi.org/10.1016/S0273-1223(98)00690-8

Pijuan, Maite, Ye, Liu, & Yuan, Zhiguo. (2010). Free nitrous acid inhibition on the aerobic metabolism of poly-phosphate accumulating organisms. *Water Research, 44*(20), 6063-6072. doi: http://dx.doi.org/10.1016/j.watres.2010.07.075

Rosso, Diego, Lothman, Sarah E., Jeung, Matthew K., Pitt, Paul, Gellner, W. James, Stone, Alan L., & Howard, Don. (2011). Oxygen transfer and uptake, nutrient removal, and energy footprint of parallel full-scale IFAS and activated sludge processes. *Water Research, 45*(18), 5987-5996. doi: http://dx.doi.org/10.1016/j.watres.2011.08.060

Saito, T., Brdjanovic, D., & van Loosdrecht, M. C. M. (2004). Effect of nitrite on phosphate uptake by phosphate accumulating organisms. *Water Research, 38*(17), 3760-3768. doi: http://dx.doi.org/10.1016/j.watres.2004.05.023

Schnaitman, Carl A. (1970). Protein composition of the cell wall and cytoplasmic membrane of Escherichia coli. *Journal of bacteriology, 104*(2), 890-901.

Schwarzenbeck, N, Erley, R, & Wilderer, P. (2004). Aerobic granular sludge in an SBR-system treating wastewater rich in particulate matter. *Water Science & Technology, 49*(11-12), 41-46.

Schwarzenbeck, N., Borges, J. M., & Wilderer, P. A. (2005). Treatment of dairy effluents in an aerobic granular sludge sequencing batch reactor. *Applied Microbiology and Biotechnology, 66*(6), 711-718. doi: 10.1007/s00253-004-1748-6

Shi, Xian-Yang, Sheng, Guo-Ping, Li, Xiao-Yan, & Yu, Han-Qing. (2010). Operation of a sequencing batch reactor for cultivating autotrophic nitrifying granules. *Bioresource Technology, 101*(9), 2960-2964. doi: http://dx.doi.org/10.1016/j.biortech.2009.11.099

Shi, Yi-Jing, Wang, Xin-Hua, Yu, Hai-Bo, Xie, Hui-Jun, Teng, Shao-Xiang, Sun, Xue-Fei, Tian, Bing-Hui, & Wang, Shu-Guang. (2011). Aerobic granulation for nitrogen removal via nitrite in a sequencing batch reactor and the emission of nitrous oxide. *Bioresource Technology, 102*(3), 2536-2541. doi: http://dx.doi.org/10.1016/j.biortech.2010.11.081

Show, Kuan-Yeow, Lee, Duu-Jong, & Tay, Joo-Hwa. (2012). Aerobic Granulation: Advances and Challenges. *Applied Biochemistry and Biotechnology, 167*(6), 1622-1640. doi: 10.1007/s12010-012-9609-8

Smolders, GJF, Klop, JM, Van Loosdrecht, MCM, & Heijnen, JJ. (1995). A metabolic model of the biological phosphorus removal process: I. Effect of the sludge retention time. *Biotechnology and bioengineering, 48*(3), 222-233.

Stauffer, Clyde E. (1989). *Enzyme assays for food scientists*. New York.

Su, Kui-Zu, & Yu, Han-Qing. (2005). Formation and characterization of aerobic granules in a sequencing batch reactor treating soybean-processing wastewater. *Environmental Science & Technology, 39*(8), 2818-2827.

Tay, J-H, Liu, Q-S, & Liu, Y. (2001). The role of cellular polysaccharides in the formation and stability of aerobic granules. *Letters in Applied Microbiology, 33*(3), 222-226.

Turakhia, MH, & Characklis, WG. (1989). Activity of Pseudomonas aeruginosa in biofilms: effect of calcium. *Biotechnology and bioengineering, 33*(4), 406-414.

Vavilin, V. A., Fernandez, B., Palatsi, J., & Flotats, X. (2008). Hydrolysis kinetics in anaerobic degradation of particulate organic material: An overview. *Waste Management, 28*(6), 939-951. doi: http://dx.doi.org/10.1016/j.wasman.2007.03.028

Vieira, Maria João, Melo, Luis F, & Pinheiro, Maria Manuela. (1993). Biofilm formation: hydrodynamic effects on internal diffusion and structure. *Biofouling*, *7*(1), 67-80.
Wang, Jingfeng, Wang, Xuan, Zhao, Zuguo, & Li, Junwen. (2008). Organics and nitrogen removal and sludge stability in aerobic granular sludge membrane bioreactor. *Applied Microbiology and Biotechnology*, *79*(4), 679-685. doi: 10.1007/s00253-008-1466-6
Wang, Qiang, Du, Guocheng, & Chen, Jian. (2004). Aerobic granular sludge cultivated under the selective pressure as a driving force. *Process Biochemistry*, *39*(5), 557-563. doi: http://dx.doi.org/10.1016/S0032-9592(03)00128-6
Wang, Shu-Guang, Liu, Xian-Wei, Gong, Wen-Xin, Gao, Bao-Yu, Zhang, Dong-Hua, & Yu, Han-Qing. (2007). Aerobic granulation with brewery wastewater in a sequencing batch reactor. *Bioresource Technology*, *98*(11), 2142-2147. doi: http://dx.doi.org/10.1016/j.biortech.2006.08.018
Wang, Yayi, Guo, Gang, Wang, Hong, Stephenson, Tom, Guo, Jianhua, & Ye, Liu. (2013). Long-term impact of anaerobic reaction time on the performance and granular characteristics of granular denitrifying biological phosphorus removal systems. *Water Research*(0). doi: http://dx.doi.org/10.1016/j.watres.2013.06.013
Wang, Zhi-Wu, Liu, Yu, & Tay, Joo-Hwa. (2006). The role of SBR mixed liquor volume exchange ratio in aerobic granulation. *Chemosphere*, *62*(5), 767-771. doi: http://dx.doi.org/10.1016/j.chemosphere.2005.04.081
Wang, Zhiping, Liu, Lili, Yao, Jie, & Cai, Weimin. (2006). Effects of extracellular polymeric substances on aerobic granulation in sequencing batch reactors. *Chemosphere*, *63*(10), 1728-1735. doi: http://dx.doi.org/10.1016/j.chemosphere.2005.09.018
Wilderer, Peter A, Irvine, Robert L, & Goronszy, Mervyn C. (2001). *Sequencing batch reactor technology* (Vol. 10): IWA publishing.
Wingender, Jost, Neu, Thomas R, & Flemming, Hans-Curt. (1999). *What are bacterial extracellular polymeric substances?* : Springer.
Winkler, Mari-Karoliina Henriikka. (2012). *Magic granules*: [Sl: sn].
Yilmaz, Gulsum, Lemaire, Romain, Keller, Jurg, & Yuan, Zhiguo. (2008). Simultaneous nitrification, denitrification, and phosphorus removal from nutrient-rich industrial wastewater using granular sludge. *Biotechnology and Bioengineering*, *100*(3), 529-541. doi: 10.1002/bit.21774
Yu, Guang-Hui, He, Pin-Jing, Shao, Li-Ming, & He, Pei-Pei. (2008). Stratification structure of sludge flocs with implications to dewaterability. *Environmental science & technology*, *42*(21), 7944-7949.
Zeng, Raymond J., van Loosdrecht, Mark C. M., Yuan, Zhiguo, & Keller, Jürg. (2003). Metabolic model for glycogen-accumulating organisms in anaerobic/aerobic activated sludge systems. *Biotechnology and Bioengineering*, *81*(1), 92-105. doi: 10.1002/bit.10455
Zheng, Yu-Ming, Yu, Han-Qing, & Sheng, Guo-Ping. (2005). Physical and chemical characteristics of granular activated sludge from a sequencing batch airlift reactor. *Process Biochemistry*, *40*(2), 645-650. doi: http://dx.doi.org/10.1016/j.procbio.2004.01.056
Zhou, Yan, Oehmen, Adrian, Lim, Melvin, Vadivelu, Vel, & Ng, Wun Jern. (2011). The role of nitrite and free nitrous acid (FNA) in wastewater treatment plants. *Water Research*, *45*(15), 4672-4682. doi: http://dx.doi.org/10.1016/j.watres.2011.06.025

Zhu, Liang, Lv, Mei-le, Dai, Xin, Yu, Yan-wen, Qi, Han-ying, & Xu, Xiang-yang. (2012). Role and significance of extracellular polymeric substances on the property of aerobic granule. *Bioresource Technology, 107*(0), 46-54. doi: http://dx.doi.org/10.1016/j.biortech.2011.12.008

APPENDIXES

Appendix A: Chemical solutions recipes used in this study

Table A- 1: Trace elements used in the synthetic wastewater recipe.

Compound	Concentration. g/L
FeCl3. 6H2O	1.5
H3BO3	0.15
CuSO4.5H2O	0.03
KI	0.03
MnCl2.4H2O	0.12
Na2MoO4.2H2O	0.06
ZnSO4.7H2O	0.12
CoCl2.6H2O	0.15

Table A- 2: Recipe of reagents used in the analytical analyses in this study.

Reagent	Chemical compound	Concentration g/L
Lowry solution	NaOH	5.72
	Na_2CO_3	28.62
	$CuSO_4.5(H_2O)$	0.14
	$Na_2Tartrate.2(H_2O)$	0.29
Anthrone solution	$C_{14}H_{10}O$	2
	75% H_2SO_4	985
	Ethanol	15
1% dinitrosalicylic acid (DNS) solution	$C_7H_4N_2O_7$	10
	Na_2SO_4	0.5
	NaOH	10
Rochelle salt solution	$KNaC_4H_4O_6 \cdot 4H_2O$	200
Iodine solution	I_2	0.176
	KI	40.1

Appendix B: Ionic Chromatography operation conditions:

Table B- 1: Ionic chromatography operation conditions used for the determination of nitrite, nitrate and phosphate.

Columns	IonPac AS18 Analytical, 4 × 250 mm (Dionex P/N 060549) IonPac AG18 Guard, 4 × 50 mm (Dionex P/N 060551)
Eluent source	ICS-2000
Eluent concentration	23 mM KOH
Flow rate	1 mL/min
Temperature	30°C
Injection volume	5 mL
Sample run time	10 min
Detection	Suppressed conductivity, ASRS ULTRA, 4 mm (Dionex P/N 053947)
Suppresser current	57 mA

Table B- 2: Ionic chromatography operation conditions used for the determination of acetate and propionate.

Columns	IonPac AS18 Analytical, 4 × 250 mm (Dionex P/N 060549) IonPac AG18 Guard, 4 × 50 mm (Dionex P/N 060551)
Eluent source	ICS-2000
Eluent concentration	5 mM KOH
Flow rate	1 mL/min
Temperature	30°C
Injection volume	5 mL
Sample run time	6 min
Detection	Suppressed conductivity, ASRS ULTRA, 4 mm (Dionex P/N 053947)
Suppresser current	13 mA