

OPERATING pH AND FEED COMPOSITION AS FACTORS AFFECTING
STABILITY OF AEROBIC GRANULAR SLUDGE

By

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Abstract

Aerobic granular sludge is considered to be a paradigm shift in biological wastewater treatment because of its unique characteristics including: (1) rapid settling under a variety of conditions; (2) simultaneous biological nutrient removal without the need of different tanks; and (3) high chemical oxygen demand (COD) removal efficiency in conditions of variable-strength wastewaters. To understand the optimum operating conditions for aerobic granules cultivation, different studies have focused on investigating the factors affecting this process. However, after the successful cultivation of aerobic granules, one of the main challenges for the large-scale application of this technology is the stability of aerobic granules during the long term operation and under variable operating conditions. In the current study, variations in operating pH and growth medium were explored as the parameters affecting the stability and bioactivity of aerobic granules.

In the first phase of the experiment, the impact of pH variation on aerobic granular sludge stability and conversion processes was investigated. After cultivating granules in three identical sequencing batch reactors (SBRs), a 9-day alkaline (pH = 9) and acidic (pH = 6) pH shocks were imposed on mature granules (cultured at pH = 7.8-8) with simultaneous COD, nitrogen and phosphorus removal. During the shock, changes in COD and nutrient removal and the stability of granules were investigated. Alkaline pH shock had no significant impact on COD removal, while, nitrogen and phosphorus removal decreased from 88% and 95% to 66% and 50%, respectively, with no further recovery. On the other hand, both nitrifiers and polyphosphate accumulating organisms (PAOs) showed less sensitivity to the low pH; moreover, nutrient removal efficiencies recovered to their initial values after three days of operation at pH 6. Operating the reactor at pH = 9 induced granules breakage and resulted in an increased biomass

concentration in the effluent (up to 500 mg L^{-1} after 5 days) and in a significant decrease of bio-particles size. Granules disintegration coincided with a decline in PN/PS ratio. Changes in the composition of EPS matrix and in chemical structure of the gel-forming biopolymers were presumed as the main reasons for granules instability under high pH.

In the second phase of the study, the impact of growth medium (carbon source) variation on physical-chemical characteristics of aerobic granular sludge as well as their performance was investigated. Mature granules were cultivated in two identical SBRs using acetate-based synthetic wastewater. After mature granules were achieved in both reactors with simultaneous COD, ammonium and phosphate removal capability, the feed of one of the reactors, R2, was changed to municipal wastewater and the other reactor, R1, (served as control) was kept using the same synthetic wastewater. The results showed that biological phosphate removal was completely inhibited in R2 due to the lack of volatile fatty acids (VFA) in the municipal wastewater; however, the biomass maintained high ammonium and COD removal efficiencies. The disintegration of the granules in R2 occurred during the first two weeks after changing the feed. After the acclimation of bacteria to the new growth medium, re-granulation of the biomass in R2 was then observed within 30 days. The granules breakage in R2 did not exert significant impact on settling property of biomass, average sludge volume index (SVI) stayed close to 47 mL g^{-1} . The chemical composition of the extracellular polymeric substances matrix and granules disintegration found to be closely linked to the type of wastewater. It was observed that changing the growth medium from acetate-based synthetic wastewater to municipal wastewater did not have significant impacts on aerobic granular sludge characteristics; especially it did not affect its settling properties. However, sufficient amount of readily biodegradable carbon source

should be provided to maintain simultaneous biological nitrogen and phosphorus removal in the system.

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List of Abbreviations

AGS	Aerobic granular sludge
Anammox	Anaerobic ammonium oxidation
AOB	Ammonium oxidizing bacteria
bCOD	Biodegradable chemical oxygen demand
BNR	Biological nutrient removal
CAFB	Continuous flow airlift fluidized bed
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
DGAO	Denitrifying glycogen accumulating organisms
DPAO	Denitrifying poly-phosphate accumulating organisms
DO	Dissolved oxygen
EPS	Extracellular polymeric substances
ETSS	Effluent total suspended solid
FA	Free ammonia
GAO	Glycogen accumulating organisms
HRT	Hydraulic retention time
H/D	Height to diameter
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
NOB	Nitrite oxidizing bacteria
OLR	Organic loading rate
ORP	Oxidation-reduction potential
PAO	Poly-phosphate accumulating organisms
PHB	Poly-hydroxybutyrate
PN	Protein

PS	Polysaccharide
SBR	Sequencing batch reactor
SBAR	Sequencing batch airlift reactor
sCOD	Soluble chemical oxygen demand
SG	Specific gravity
SND	Simultaneous nitrification-denitrification
SRT	Solids retention time
SVI	Sludge volume index
TCOD	Total chemical oxygen demand
TN	Total nitrogen
TP	Total phosphorus
TSS	Total suspended solids
VER	Volume exchange ratio
VFA	Volatile fatty acids
WAS	Waste activated sludge
WWTP	Wastewater treatment plant
ZSV	Zone settling velocity
K_s	Half saturation constant
μ_{\max}	Maximum specific growth rate
Y	Biomass yield
S	Concentration of growth-limiting substrate in solution
k_d	Endogenous decay coefficient

Chapter 1

Introduction and Research Objective

1.1. Background

The activated sludge process is the most widespread biotechnology for wastewater treatment. However, filamentous bulking and poor settleability, low volumetric load conversion rates, and production of large amount of waste activated sludge (WAS) affect activated sludge process when applied for biological nutrient removal (BNR). Poorly settling biomass leads to additional wastewater treatment costs from chemical dosing and process modification, as well as higher nutrient loads in the treated effluent from increased suspended solids (Jenkins et al., 2004; Monti et al. 2007).

The settling properties of biomass can be significantly improved by converting flocculent activated sludge into a denser and more compact microbial structure such as a biofilm or the recently developed aerobic granular sludge. By eliminating secondary clarifiers and providing simultaneous nitrogen and phosphorus removal in an aerated sequencing batch reactor (SBR)

configuration, granular sludge can decrease the required plant footprint and energy consumption (Inocêncio et al., 2013). The compact and dense microbial aggregates of granular sludge allow a greater biomass concentration and longer solids retention time (SRT) in the reactor. Higher biomass concentration significantly increases the volumetric load conversion rates of the system (Di Iaconi et al., 2007; Dangcong et al. 1999) and a longer SRT results in decreased WAS production (Di Iaconi et al., 2007). Granular sludge was first described in 1980 for anaerobic systems (Lettinga et al., 1980) while aerobic granulation was first reported in the 1990's (Mishima and Nakamura, 1991; Morgenroth et al. 1997). Bio-granules contain different microbial species in a compact spherical shape (Adav et al., 2008a) and make it possible to have various biological pathways in one microbial consortium. As a result aerobic granular sludge has been extensively studied in the last two decades with the goal of removing carbon, nitrogen, and phosphorus simultaneously under various temperatures and influent loading conditions (Inocêncio et al. 2013).

The application of aerobic granular sludge progressed from lab to pilot and finally full-scale treatment plant. Large-scale applications of granular sludge, e.g. Nereda[®] technology, are now implemented in Europe and South Africa.

1.2. Full-Scale Application

After the successful performance of pilot- and full-scale Nereda[®] aerobic granular sludge technology in the Netherlands, Portugal, and South Africa, the advantages of aerobic granular sludge process such as lower energy consumption and footprint have been proven. The specific characteristics of aerobic granular sludge have also allowed for the combination of fill and draw steps in full-scale SBR processes (Giesen and Thompson, 2013)

Giesen and Thompson, (2013) stated that currently there are about 40 international aerobic granular sludge plants at different stages of application (i.e., under construction or under design). The first full-scale application of Nereda[®] started in 2005 by retrofitting a storage tank to treat 250 m³ d⁻¹ from a cheese production factory in Ede, Netherlands (Giesen et al., 2013a). In 2009 the on-site treatment facility was relocated and upgraded to a Nereda treatment plant with a capacity of 500 m³ d⁻¹. The process was seeded with conventional activated sludge and achieved granulation within three months. The biomass concentration is currently maintained at 10-20 g L⁻¹ with a SVI of 20-30 ml g⁻¹. The first green-field application started in 2006 for the treatment of 250 m³ d⁻¹ food industry wastewater.

At the Frielas wastewater treatment plant (WWTP) in Portugal one of the six conventional activated sludge bioreactors has been converted to Nereda[®] technology. Almost 80% granulation has been achieved in the system with a biomass concentration of 6 to 8 g L⁻¹ (Giesen et al., 2013b). Monitoring the air consumption per mass of COD removal in both the Nereda[®] and existing activated sludge systems indicated a 30% energy savings in aeration (Inocêncio et al. 2013). The demonstration plant located in Gansbaai, South Africa has been able to achieve effective treatment of organics and nutrient, Table 1.1.

Table 1.1. Performance of the demonstration installation in Gansbaai in 2011 (Giesen and Thompson, 2013).

Parameter	Influent (mg L ⁻¹)	Effluent (mg L ⁻¹)
Total COD (TCOD)	1265	40
NH ₄ -N	75	<1
Total nitrogen (TN)	-	<10
Total phosphorus (TP)	19	3
Total suspended solids (TSS)	450	<5

After the successful application of Nereda[®] at the Gansbaai WWTP, the Epe full-scale plant was designed and built between 2010 and 2011 in Epe, Netherlands (Giesen et al., 2013b). Influent

passes through screens, grit removal, three aerobic granular sludge SBRs, and gravity sand filters at the Epe WWTP. Table 1.2 shows the performance of Epe plant in 2012. The Nereda[®] system was seeded with conventional suspended growth activated sludge and the start-up period lasted four months. According to Giesen et al., (2013b) the previous energy consumption of 3500 kWh d⁻¹ was reduced to 2000 - 2500 kWh d⁻¹ with the introduction of Nereda[®] technology.

Table 1.2. Performance of Epe WWTP during process verification March-May 2012 (Giesen et al., 2013b).

Parameter	Influent (mg L ⁻¹)	Effluent (mg L ⁻¹)
COD	879	27
BOD	333	<2
NH ₄ -N	54	0.1
TN	-	<4
TP	9.3	0.3
TSS	341	<5

Overall the Nereda[®] technology has the potential for cost-effective treatment of wastewater. The only drawback could be associated with the implementation of this technology is the possibility of granules instability during the long term operation and under variable operating conditions.

1.3. Sustainability and Cost-Effectiveness Associated With Stability of Aerobic Granules

Previous studies indicated that operational conditions can affect the surface properties of the bio-flocs (Ye et al., 2011). Variable operating condition can disturb the EPS matrix and subsequently affect granules stability. Variations in operating pH, temperature, salinity and wastewater composition can frequently happen in wastewater treatment plants. For example, discharge of wastewater from textile or potato processing industry with high pH or other industrial wastewaters with low pH such as winery or fruit canning industry can alter the pH of the influent to WWTP. The instability of aerobic granules under variable conditions results in granules breakage, VSS breakthrough in the effluent and might affect the nutrient removal efficiency.

Higher biomass concentration in the effluent and higher nutrient concentration violate the effluent limits and requires tertiary treatment which is associated with higher costs. Moreover, to achieve re-granulation with high nutrient removal efficiencies can last for two to three months.

On the other hand, in sludge treatment facilities the biomass dewaterability is considered as the bottleneck of the process. Flocs' size can be mentioned as one of the most important factors affecting sludge dewatering (Karr and Keinath, 1978). The reduced particle size, as a result of granules disintegration, can decrease the sludge dewaterability and consequently increase the demand for chemical addition.

Instability of aerobic granules is one of the main factors affecting the sustainability of this technology; however, identifying the factors influencing the stability and bioactivity of aerobic granules can significantly enhance its sustainable application.

1.4. Problem Statement and Research Objectives

Aerobic granular sludge is considered as a great shift in biological wastewater treatment due to the unique granules attributes such as compact and dense structure, high settling velocity and multilayer composition which can provide a compact and very cost-effective full-scale application of biological wastewater treatment. However, full-scale application of this technology could face some difficulties when granules lose their compactness and stability and change to flocculent biomass. The instability of aerobic granules is considered as a threat to the performance of the systems and achieving the required effluent quality. Thus, predicting and being aware of the causes and parameters influencing and disturbing the stability and bioactivity of aerobic granular sludge is crucial prior to the full-scale application of this technology. So far, literature has mainly focused on a wide range of parameters affecting aerobic granules formation; however, some of these parameters such as OLR, growth medium, pH, temperature

and aeration rate may also influence the stability of the cultivated granules. The impacts of some parameters that might have seasonal variations (such as temperature and OLR) or might vary by changing the type of wastewater (such as carbon source or pH) on granules stability and bioactivity have not been fully addressed yet. According to the fact that extracellular polymeric substances (EPS) matrix has a decisive role in aerobic granule formation and its stability, any parameter altering EPS matrix production, composition or chemical structure can threaten the granules stability as well. The mentioned conditions bring up the need of a research focusing on the relationship between operating conditions such as operating pH, temperature, salinity and growth medium on the stability of aerobic granules. The objective of this study was to investigate the stability of aerobic granular sludge under variable operating conditions including variable pH and growth medium. The flow diagram of the research plan is shown in Figure 1.1.

The specified objectives of this study are as follows:

1. Assessing the impact of alkaline and acidic pH shocks on aerobic granules stability, EPS matrix compositions and in general physical- chemical characteristics of granules.
2. Assessing the impact of alkaline and acidic pH shocks on biological nutrient removal efficiencies and main biological conversions within granules.
3. Assessing the impact of substrate composition and carbon source on aerobic granules stability and their physical-chemical characteristics.
4. Assessing the impact of substrate composition and carbon source on biological nutrient removal efficiencies of aerobic granules and main biological conversions within them.

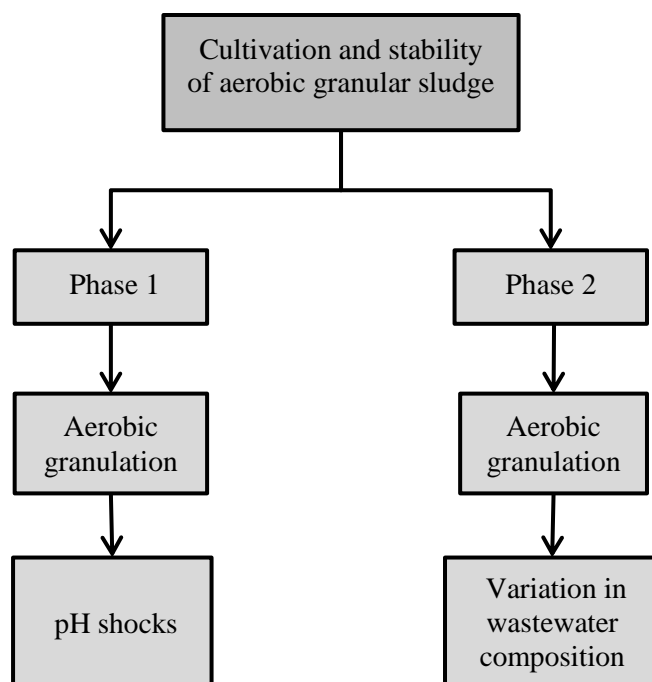


Figure 1.1. Flow diagram of the research plan

Chapter 2

Literature Review

Stricter regulations to protect the water bodies requires further treatment of wastewater to a more stringent effluent limits which forces most of the conventional biological treatment plants to retrofit their systems to BNR plants. However, increasing the population requires enlarging the infrastructure of WWTPs to meet the future loads. The mentioned difficulties on the way of biological wastewater treatment could be mitigated by using a technology such as aerobic granular sludge being able to perform complete nutrient removal in a more compact system while requiring less energy and chemicals (Giesen et al., 2013a). Aerobic granular sludge as an emerging technology has the potential to provide simultaneous nutrient removal and treatment of higher organic loading rates in a single tank (Bassin et al., 2012). These advantages of aerobic granules are thanks to their unique characteristics such as compact and dense structure and different oxidation-reduction potential (ORP) levels as the result of oxygen diffusion limitations

within the inner layers. Moreover, taking the advantage of the fast settling velocity of aerobic granules, the required infrastructure could significantly be reduced by the omission of secondary clarifiers (de Kreuk et al., 2005a). Combination of the mentioned characteristics of aerobic granular sludge provides the opportunity to design and build a compact BNR plant benefiting from this technology.

So far, characteristic of aerobic granules, mechanisms of granulation and factors influencing the process have been extensively studied by researchers (de Kreuk et al., 2005a; Liu and Tay, 2002; Tay et al., 2004; Li et al., 2009; Liu and Tay, 2007; Sun et al., 2006; Liu et al., 2007; Zhu et al., 2013; Nor-Anuar et al., 2012; Bassin et al., 2012; Coma et al., 2012). Aerobic granulation, characteristics and stability of the formed granules significantly depends on the operating conditions.

2.1. Characteristics of Aerobic Granular Sludge

2.1.1. Physical Characteristics

2.1.1.1. Shape and Size

Aerobic granules are typically spherical or ellipsoidal with a smooth outer surface (Chen et al. 2008). The average size of granules in aerobic processes is the result of two opposing phenomena: (1) biomass growth; and (2) cell detachment due to hydrodynamic shear forces (Liu and Tay, 2002). It has been shown that the average diameter of granules varies from 0.2 to 5.0 mm and is greater than the average diameter of activated sludge flocs (Dangcong et al. 1999; Tay et al., 2001a; Tay et al., 2001b; Tay et al., 2001c; Zhu and Wilderer, 2003; Toh et al., 2003).

The average diameter of granular sludge does not correlate well with VSS concentration, Figure 2.1. Toh et al., (2003) suggested that larger diameters could be due to higher inorganic content in the granules rather than an overall increase in organic matter contents (i.e., biomass

and EPS). Toh et al., (2003) also stated that an increase in granule size beyond a certain threshold (i.e., 4.0 mm in diameter) could have negative impacts on the strength and stability of granular biomass. Their study suggested that average granule diameters between 1.0 and 3.0 mm are optimum for cost-effective and stable performance of granular sludge processes.

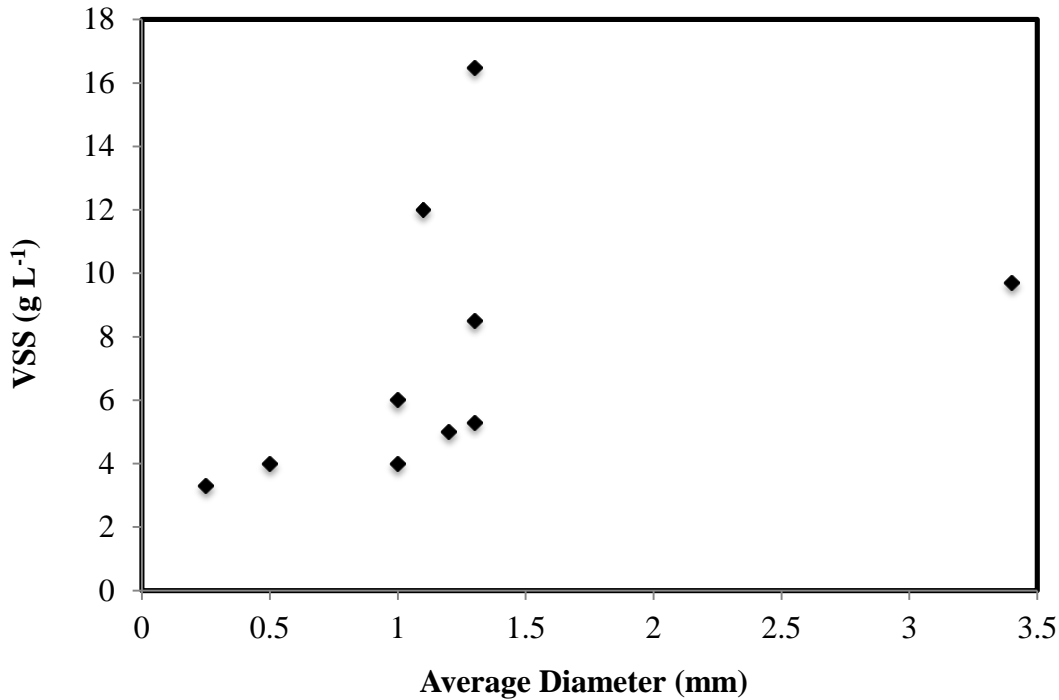


Figure 2.1. Volatile suspended solids (VSS) concentration in various sizes of granules (Data from: Dangcong et al. 1999; Jang et al., 2003; de Kreuk et al., 2005; Linlin et al., 2005; Zheng et al., 2005; Bao et al., 2009; Wan and Sperandio, 2009).

2.1.1.2. Settleability

The settleability of activated sludge is directly related to the biomass concentration and solid-liquid separation efficiency. The settling velocity of aerobic granular sludge can vary from 25 to 70 m h⁻¹ and is significantly higher than activated sludge flocs (i.e., 7 to 10 m h⁻¹) (Qin et al., 2004a). Higher settling velocities allow for greater biomass concentrations that consequently enhance removal capacity by increasing the active biomass and maintaining slow-growing bacteria (Schwarzenbeck et al., 2005).

The sludge volume index (SVI) of granular sludge is usually below 80 mL g^{-1} and values as low as 20 mL g^{-1} have been reported (Su et al., 2012). Low SVI values are due to the high density and compact structure of granules. The specific gravity for granules ranges from 1.004 to 1.100, while flocculent activated sludge shows values from 1.002 to 1.006 g cm^{-3} (Li et al., 2009; Shi et al., 2009).

2.1.2. Chemical Characteristics

2.1.2.1. Hydrophobicity and Surface Charge

Microbial adhesion is the result of interactions between attractive and repulsive forces from different approaching surfaces. Most bacterial and natural surfaces are negatively charged between pH values of 5 and 7, and electric repulsion is one of the main factors inhibiting bio-granulation (Poortinga et al., 2002).

Bio-granulation is usually initiated by an increase in cell hydrophobicity brought on by stressful environmental conditions such as hydrodynamic shear forces, feast/famine periods, and pH variations. Microorganisms can improve their resistance to stressful conditions by increasing surface hydrophobicity resulting in aggregation (Liu and Tay, 2002). Cell surface hydrophobicity is usually associated with the presence of specific bacterial fibrils and cell wall proteins (Dworkin, 1999; McNab et al., 1999; Singleton et al., 2001). Polysaccharides (e.g., alginate) can be hydrophobic or include hydrophobic regions based on their molecular structure (Lin et al., 2010).

According to van Oss, (1997) hydrophobic interactions are usually the strongest among all non-covalent biological interactions. An increase in hydrophobicity of cell surfaces causes a decrease in the Gibbs free energy of the system and promotes adhesion between bacterial cells in aqueous solutions (Liu and Tay, 2002). The decrease in Gibb's free energy is a result of displaced ordered

water molecules from the surfaces of interacting molecules to the bulk solution (Wilschut and Hoekstra, 1984).

An inverse correlation between cell surface hydrophobicity and surface negative charge has been demonstrated. Higher surface charges result in stronger polar interactions between EPS and water molecules (Liao et al., 2001). Therefore, higher cell hydrophobicity corresponds with reduced cell surface charge and leads to weakened repulsive forces between microbial cells. The surface hydrophobicity and charge of granules is affected by the production, composition, and physical characteristics of EPS (Liao et al., 2001).

2.1.2.2. Extracellular Polymeric Substances

Geesey, (1982) defined EPS as extracellular polymeric substances of microbiological origin that participate in the formation of microbial aggregates. EPS has been found in both anaerobic and aerobic systems, and is an important factor for bio-granulation and long-term granule stability. The basic role of EPS is the formation of an extracellular matrix that fixes individual cells.

EPS are either bound to the cell surface (i.e., cell-bound EPS) or excreted in the growth medium (i.e., soluble free EPS). The ability to secrete EPS is widespread among microorganisms because it decreases floc-water loss, promotes adherence to surfaces, facilitates microbial aggregation, and protects against stressful conditions. The final placement of EPS is the result of: (1) active transport from the metabolic origin to the cell surface; (2) adsorption from the surrounding medium; or (3) release from cell lysis (Wingender et al., 1999).

Under standard cultivation conditions the production of EPS is normally low. An increase in production occurs when bacterial cells are exposed to external stresses that can be divided into two main groups (Liu et al., 2004): (1) environmental changes which can alter the microbial community, increasing or decreasing the amount of EPS-producing bacteria; and (2)

environmental changes which can modify the metabolic pathway of EPS production of the unchanged microbial community. To date, the major factors identified as enhancing the production of EPS are: (1) providing alternative feast/famine periods by using SBRs; and (2) imposing selective pressures such as high shear stress and short settling times (Lin et al., 2008).

The amount and composition of EPS produced is strictly related to the microbial species involved, their growth phase and physiology, the applied operating conditions, and the feed composition (Nielsen et al., 1997; Batstone and Keller, 2001; Tay et al., 2001a; Sponza, 2002). Previous studies indicated that a matrix of EPS surrounds the bacterial cell walls (Forster, 1991; de Beer et al., 1996; Veiga et al., 1997) in aerobic granules. Since cell surface hydrophobicity is related to specific fibrillar structures and cell wall proteins (Dworkin, 1999; McNab et al., 1999; Singleton et al., 2001) a correlation between cell surface hydrophobicity and the EPS content of granules is conceivable.

Granular sludge was found to be less negatively charged than activated sludge (Morgan et al., 1990) and it has been proposed that EPS can enhance microbial adhesion by decreasing the negative charge of cell surfaces through cross-links with divalent cations (e.g., Ca^{2+}) (Liu et al., 2004; Lin et al., 2008).

The predominant macromolecules in EPS extracted from aerobic granular sludge are: (1) polysaccharides; (2) proteins; (3) lipids; and (4) nucleic, humic, and uronic acids (Adav and Lee, 2008). In addition, non-polymeric substituents such as acetyl, succinyl, pyruvyl, and inorganic groups can be found in the EPS composition. Polysaccharides are the only components synthesized extracellularly, while the others are synthesized inside the cytoplasm and excreted outside the cell wall (Durmaz and Sanin, 2001; Mahmoud et al., 2003). Proteins can be associated with lipids (i.e., lipoproteins) or covalently bound to carbohydrates (i.e.,

glycoproteins) within the EPS (Bitton, 2005; Czaczyk and Myszka, 2007). Since EPS is heterogeneous, hydrophobic and hydrophilic groups can be simultaneously present. The overall hydrophobicity of the EPS matrix is the result of the average between its hydrophobic and hydrophilic components (e.g., the protein to polysaccharide ratio affects the hydrophobicity of EPS matrix) (Daffonchio et al., 1995).

There are two different points of view regarding the main components of granular EPS matrix. Some studies presented proteins as the predominant constituent (Quarmby and Forster, 1995; Nielsen et al., 1997; Dignac et al., 1998; Mcswain et al., 2005; Zhang et al., 2007). It is stated that proteins are more involved than polysaccharides in electrostatic bonds with divalent cations since proteins have a high content of negatively charged amino acids and appear to represent a higher fraction of EPS. Proteins are usually hydrophobic while polysaccharides are usually hydrophilic (Dignac et al., 1998). According to Zhang et al., (2007) and Liu et al., (2003b) higher surface hydrophobicity was associated with higher protein (PN) to polysaccharide (PS) ratios and lower surface negative charge. On the other hand, some of the recent research on granular biomass introduced some specific polysaccharides (such as alginate) as the gel-forming biopolymers and responsible for the stability of granular EPS matrix (Lin et al., 2008; Seviour et al., 2012). In the mentioned studies two polysaccharides, alginate and a novel polysaccharide called granulan, are presented as the main components of the EPS matrix that stay as a strong gel under acidic and neutral operating pH.

2.1.2.3. Inorganic Content

High concentrations of calcium precipitates (e.g., calcium carbonates) have been widely reported within the cores of granular sludge. Calcium precipitates are especially observable in granules fed with acetate (Wang et al., 2007; Ren et al., 2008) and their production is usually observed in

granules larger than 1.0 mm in diameter (Wang et al., 2007). According to Ren et al., (2008) and Qin et al., (2004) granules containing calcium precipitates have a stronger structure and larger size compared to those without calcium accumulation. However, a negative impact on bioactivity based on the specific oxygen uptake rate was observed with calcium precipitation.

Qin et al., (2004) and Liu and Tay, (2004) analyzed the metal composition of aerobic granules and found iron, magnesium, and aluminum to be much lower than calcium. Concentrations of the less prominent metals remained constant during bio-granulation whereas calcium concentration showed an increasing trend. Elevated calcium concentrations could promote the formation of granules by decreasing the required settling time.

Angela et al., (2011) found high concentration of calcium and phosphate in the inorganic core of aerobic granules with biological phosphorus removal capability. Further analyses confirmed the crystalline form of calcium-phosphate precipitation in the form of hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3(\text{OH})$). In the mentioned study, it was shown that phosphorus precipitation in the form of hydroxyapatite inside the granules does not have any contrast with biological phosphorus removal in these systems.

2.1.3. Biological Characteristics

2.1.3.1. Microbial Composition

The biological composition of granules has been studied extensively and found to fluctuate with many parameters (Tay et al., 2002; Meyer et al., 2003; Tsuneda et al., 2003; Yi et al., 2003). Carbon sources (Tay et al., 2001a) and operating conditions such as feast/famine periods (McSwain et al., 2004) and dissolved oxygen (DO) concentration (Xavier et al., 2007) can affect the bacterial composition. It has also been shown that composition is strongly related to the nature of the seed sludge and the size of the granule (Fang et al., 1994; Tay et al., 2002; Liu and

Tay, 2004; Weber et al., 2007). In addition, the composition can be affected by shear force intensity, organic loading rates, and SRT (Dangcong et al. 1999; Gao et al., 2011).

All granules are characterized by a concentric multi-layered structure containing channels and pores for the transport of oxygen and substrates (Paul and Liu, 2012). Aerobic, anoxic, and anaerobic zones characterized by different redox potentials can be defined along the direction of mass transfer within the granule. The different redox potentials allow for simultaneous COD, nitrogen, and phosphorus removal by encouraging the growth of aerobic, facultative, and obligate anaerobic bacteria (Wang et al., 2009). To date several different microbial activities have been observed in aerobic granular sludge processes, including (Lin et al., 2003; Yang et al., 2003; Bassin et al., 2012; Winkler et al., 2012):

- Carbon removal;
- Nitrification (i.e., ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB));
- Denitrification (e.g., denitrifying glycogen accumulating organisms (DGAO), denitrifying polyphosphate accumulating organisms (DPAO) or ordinary facultative heterotrophs, and anaerobic ammonium oxidizers (Anammox);
- Phosphorus accumulation (e.g., polyphosphate accumulating organisms PAO or DPAO); and
- Glycogen accumulation (e.g., glycogen accumulating organisms GAO or DGAO).

The general structure of aerobic granules is composed of two main parts: (1) an outer aerobic layer where autotrophic and some of the heterotrophic bacteria (e.g., PAO) are present; and (2) an inner anoxic/anaerobic layer where denitrifying bacteria (e.g., DPAO, DGAO, and

anammox), dead cells, and precipitates prevail in the absence of oxygen, Figure 2.2 (Beun et al., 2001; de Kreuk et al., 2005; Ni et al., 2008; Bassin et al., 2012).

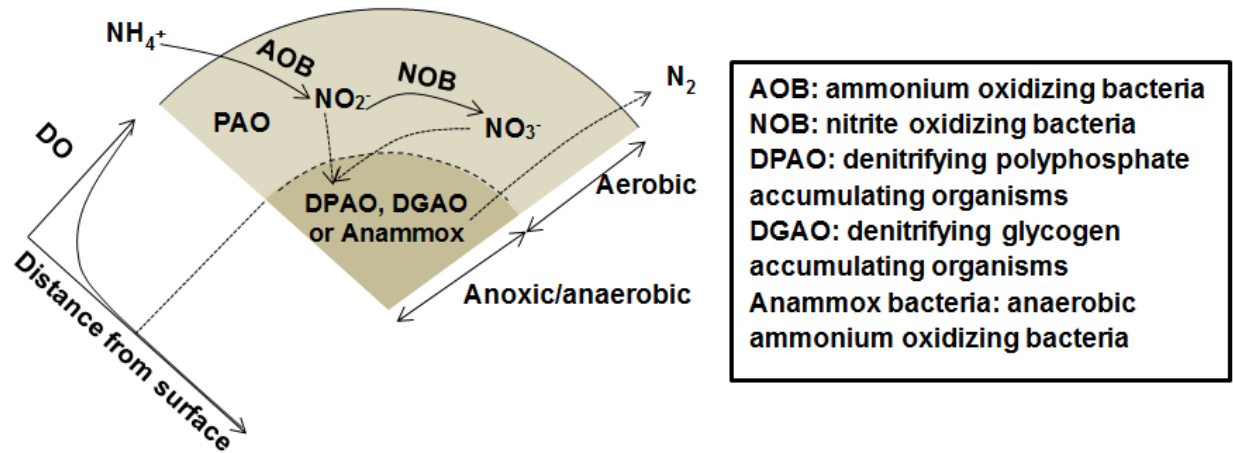


Figure 2.2. Aerobic granule structure and possible microbial distribution with simultaneous COD, nitrogen, and phosphorus removal (Developed after de Kreuk et al., 2005; Bassin et al., 2012; Winkler et al., 2012).

Processes of nitrification and aerobic phosphorus uptake occur during the aeration phase of granular sludge treatment. Denitrification and anoxic phosphorus removal can be achieved simultaneously during the aerobic phase in the anoxic zone of the granule by DPAOs (de Kreuk et al., 2005; Bassin et al., 2012). According to Winkler et al., (2012), limited oxygen concentrations (e.g. $1.5 \text{ mg O}_2 \text{ L}^{-1}$) can support complete autotrophic nitrogen removal through partial nitrification-anammox processes in the anoxic layer of the granules.

The distribution of microbial communities within aerobic granules could result from a difference in growth rate due to competition for space and substrates, and oxygen diffusion limitation across the depth of the granule (Lemaire et al., 2008b). Similarly, Tay et al., (2002) and Ivanov et al., (2005) stated that the depth of the anaerobic layer depends on microbial activity and oxygen mass transfer. The presence of nitrifiers has been detected in the aerobic layer at a depth of 70–100 μm from the surface of the granule, while heterotrophs (e.g., PAOs and denitrifiers)

can exist in both the outer aerobic layer and the center (Ni et al., 2008). Lemaire et al., (2008b) stated that in granules larger than 500 μm in diameter, PAOs are dominant in the outer layers of the granule (200 μm) while GAOs exist in the central layer. Ivanov et al., (2005) found a layer of obligate anaerobic bacteria at a depth of 850-1000 μm while dead microbial cells were found at depths greater than 1000 μm .

2.1.3.2. Filamentous Growth

The presence of filamentous bacteria has been commonly detected in aerobic granular sludge processes (Linlin et al., 2005; Schwarzenbeck et al., 2005; Liu and Liu, 2006; Tay et al., 2004). Filamentous microorganisms typically include aerobic, heterotrophic bacteria, and can also include some elongated fungal species. When the presence of filamentous bacteria is low they do not cause operational problems and may even stabilize the granule structure by acting as a rigid backbone (Liu and Liu, 2006). However, once filamentous bacteria become dominant in the reactor they can increase granule surface area without a corresponding increase in mass. This leads to a decrease in settleability and consequently biomass washout (Liu and Liu, 2006).

Many factors such as substrate concentration DO deficiency, SRT, and feed composition can trigger filamentous growth. Filamentous bacteria are K-strategists, characterized by a lower half-saturation constant (K_s) and lower maximum specific growth rate (μ_{max}) than granule-forming bacteria (i.e., r-strategists) (Chudoba et al., 1973a). Therefore, filamentous bacteria can easily outcompete the bacteria favored for bio-granulation under substrate and nutrient limiting conditions. A high COD/N ratio or nitrogen deficiency is considered to be one of the main factors triggering bulking (Bitton, 2005). It has also been shown that a deficiency in nutrient or trace elements may cause filamentous overgrowth (Sponza, 2002; Wood and Tchobanoglous,

1975). These observations are confirmed by the kinetic selection theory for filamentous growth (Chudoba et al., 1973b).

Low ammonia diffusion coefficients ($1.01 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$) that cause nitrogen deficiency inside the granules may subsequently trigger filamentous overgrowth (Liu and Liu, 2006). DO concentrations lower than 1.1 mg L^{-1} could also promote filamentous growth (Martins et al., 2003) and a minimum concentration of 2.0 mg L^{-1} is suggested to control filamentous overgrowth (Chudoba, 1985). DO penetration into the granule's core is related both to its concentration in the bulk solution and to the oxygen consumption rate inside the granule. Due to the compact structure and large size of granular biomass, the bulk DO concentration in granular sludge SBRs is not a good representation of the DO concentration within the aerobic granules (Liu and Liu, 2006).

A long SRT has also been reported as a favorable parameter for filamentous growth in flocculent biomass, although some species of filamentous bacteria are able to grow in a wide range of sludge ages (Jenkins, 1992). According to Liu and Liu, (2006) the optimum suggested SRT for controlling filamentous bacteria overgrowth in aerobic granular sludge is 10 days. The authors stated that aerobic granules developed at a SRT of 10 days showed a stable structure characterized by good density, smooth outer layers, and a low percentage of filamentous organisms.

Tay et al., (2001a) studied the impact of feed composition on granulation and showed that granules fed with glucose-based substrate contained more filamentous bacteria than those fed with acetate-based substrate. It is in fact generally accepted that carbohydrates like glucose, maltose, and lactose (Chudoba, 1985) and readily biodegradable organic substrates can promote filamentous growth.

2.2. Biogranulation

2.2.1. Mechanisms of Aerobic Granulation

Bio-granulation can be defined as the aggregation of cells to form a stable, contiguous, and multicellular consortium (Calleja, 1984). Aerobic granulation can be obtained using conventional activated sludge without external carriers (e.g. media or sand) (Tay et al., 2001a). Cell aggregation is influenced by various conditions including hydrophobicity (van Loosdrecht et al., 1987), the production of EPS, and bacterial morphology. Surface hydrophobicity is one of the main factors in immobilization and aggregation during the first steps of bio-granulation (Marshall and Cruickshank, 1973; Liu et al., 2003a). The process of bio-granulation has been examined in lab- and full-scale studies with conventional sequencing batch reactors (SBR) (Liu and Tay, 2007) or modified SBRs such as sequencing batch airlift reactors (SBAR) (de Kreuk et al., 2005; Bao et al., 2009). Aerobic granulation has also been reported in some modified continuous systems that impose the required selection pressures by reducing the hydraulic retention time (Morales et al., 2012).

The different proposed mechanisms for aerobic granulation are summarized as follows:

- Beun et al., (1999) stated that fungi become dominant in the first stage of the aerobic granulation process. Fungi are able to form pellets with diameters of 5 to 6 mm. The formed pellets can settle faster than the bacterial flocs that consequently wash out of the reactor. The dominant fungi pellets then act as a matrix upon which bacteria can grow. Overgrowth of fungi dominated pellets causes disintegration of the fungi due oxygen transfer limitations. The established bacterial colonies are thus able to be retained in the system and form granules.
- Liu and Tay (2002)suggested a four-phase model for aerobic granulation:

- First phase: cell movement initiates bacterium-to-bacterium contact or bacterial attachment onto an external carrier which acts as a nuclei;
 - Second phase: aggregation and stabilization results from attractive forces such as van der Waals, ionic pairing, and cell surface dehydration;
 - Third phase: granulation occurs and a highly organized microbial structure is established; and
 - Fourth phase: the granules mature in terms of stability and compactness, maintained by hydrodynamic shear forces.
- Weber et al., (2007) presented a three-phase process:
 - First phase: ciliated protozoa settle on activated sludge flocs and form fluffy-branched colonies;
 - Second phase: ciliate stalks condense and act as a backbone for bacterial growth and bio-granulation; and
 - Third phase: after the overgrowth of bacteria on ciliate stalks, most ciliates die and leave behind compact and smooth granules.
 - Barr et al., (2010) suggested two different mechanisms for granulation based on their observations from three different SBRs operating for biological phosphorus removal. They noticed two different types of granules in the system: (1) compact white; and (2) loose yellow. According to the characteristics of these two types of granules the proposed mechanisms are as follows:

- The outgrowth of microcolonies from one type of bacteria formed the compact and dense white granules; and
- The aggregation of various small microcolonies composed of multi-cultural bacteria formed the loose and rough yellow granules.
- Verawaty et al., (2012) stated that a mixture of flocculent sludge and crushed granules as inoculum can reduce the initial start-up period for granulation. The attachment of flocs onto the surface of the crushed granules prevents excess biomass washout and maintains nutrient removal performance. Some aggregations of flocculent biomass form granules, but the majority of granules result from interactions between the flocculent and crushed granular biomass.
- Ahn et al., (2009) showed that in a SBR operated to achieve enhanced biological phosphorus removal (EBPR), PAO microcolonies that withstood high shear forces became the inoculating flocs in granulation. They stated that an increase in the size of flocs and bio-granulation was a subsequent stage of accumulibacter-enriched flocs co-aggregation.

2.2.2. Factors Affecting Aerobic Granulation

2.2.2.1. Shear Forces

The applied shear force, usually by aeration or mechanical mixing, is an important selective pressure for bio-granulation. Shear forces influence the hydrodynamic pattern of liquid flow which can be correlated with microbial aggregation (Liu and Tay, 2002). High aeration rates force microorganisms to attach in round and compact shapes by imposing circular flow patterns. The use of mechanical mixers and aeration at the same time causes the irregular and multi-directional movement of biomass, leading to irregular aggregations of microorganisms which are

neither compact nor round (Liu and Tay, 2002).

2.2.2.2. Aeration

Aeration provides the majority of shear stress in aerobic granular sludge processes and plays an important role in the characteristics of granules. Shear forces from aeration are usually imposed through an up-flow delivery system and the strength of the shear force depends on the superficial air velocity. Up-flow aeration leads to circular flow patterns in the liquid that form round and regularly shaped granules.

Low air velocities (i.e., $\leq 0.008 \text{ m s}^{-1}$) cannot form granules while medium velocities form large, filament-dominated granules that lead to system failure. Only high air velocities (i.e., $> 0.025 \text{ m s}^{-1}$) favour the formation of round and dense granules characterized by higher settling velocities and higher biomass concentrations (Gao et al., 2011). High shear forces also increase EPS production and cell hydrophobicity, two important factors affecting granulation (Liu and Tay, 2002).

Hydrodynamic turbulence brought on by high air velocities can increase substrate mass transfer in granular sludge. However, the dense and compact characteristics of granules formed under high air velocities negatively affect substrate transfer through the biomass (Tanyolac and Beyenal, 1998). The lack of substrate transfer between the different layers within the granule could be one of the main factors inducing granule disintegration (Wäsche et al., 2000) and the impact of shear force on mass transfer must be considered.

Tay et al., (2001b) showed that among four identically sized SBRs, compact and dense granules were cultivated under superficial gas velocities higher than 0.012 m s^{-1} while no granulation was observed in the reactor with a velocity of 0.003 m s^{-1} . Liu et al., (2006) reported that granules can be operated at reduced aeration rates (i.e., from 0.0166 m s^{-1} to 0.0055 m s^{-1}) after their

formation. Different aeration rates during the aerobic phase do not affect the settling properties of the granules and just affect granule appearance. Under consistently high aeration rates (i.e., 0.0166 m s^{-1}) granules appear round and compact, while under low aeration rates granules change to elongated-shape aggregations.

2.2.2.3. Mechanical Mixing

Mechanical mixing is not common for aerobic granular sludge processes because of the fragile texture of the granules. However, compact granules have been cultivated when mechanical mixing was used to apply additional shear force.

Shin et al., (1992) investigated the cultivation of granules under mixing speeds of 3 and 6 rpm. They reported that COD removal efficiency was higher with the application of 6 rpm, while the overgrowth of large filamentous bacteria caused bulking in the system with 3 rpm. The induced shear stress from 6 rpm prevented the bulking phenomena. Mosquera-Corral et al., (2011) used a 6-blade Rushton turbine impeller with a speed of 100 rpm to cultivate granular sludge. They obtained compact and dense granules with a final average diameter of 1.75 mm. Zhang et al., (2011) applied mechanical mixing during the anoxic and anaerobic phase to cultivate granules using low strength wastewater. They achieved compact and dense granules after 50 days.

2.2.2.4. Settling Time

Short settling time is considered one of the most important selective pressures for granulation at early stages. Reducing the settling time washes out slowly settling flocs and facilitates the retention of biomass with higher settling velocities and denser (Cassidy and Belia, 2005). Removing flocculent bacteria decreases the number of competing microorganisms and promotes bio-granulation (Adav et al., 2009). Short settling times can also induce EPS production as a microbial response to stressful conditions (Xiong and Liu, 2012).

McSwain et al., (2004) reported that between two SBRs with 2 and 10 min settling times, only the SBR with 2 min settling time achieved bio-granulation. Qin et al., (2004) found that SBRs with a settling time of less than 15 min were able to cultivate granules. According to the data from previous studies, settling velocities correlate well with granule size, Figure 2.3. Decreasing settling time could select for denser and larger granules that have higher settling velocities.

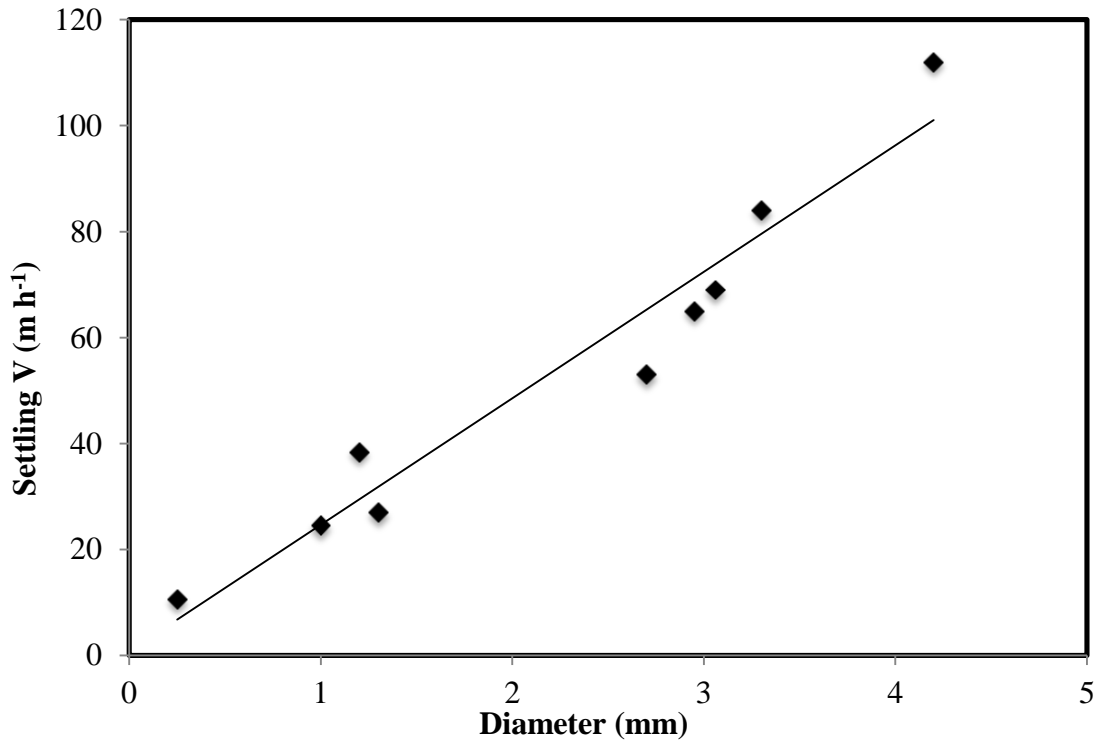


Figure 2.3. Correlation between settling velocity (V) and granule size in different studies using lab-scale SBRs. (Data from: Moy et al., 2002; Jang et al., 2003; Linlin et al., 2005; Zheng et al., 2005; Wan and Sperandio, 2009).

2.2.2.5. Volume Exchange Ratio

The volume exchange ratio (VER) can be defined as the ratio of volume discharged to the total working volume of the reactor. High VERs (i.e., >60%) impose a selective pressure by selecting bio-particles with higher settling velocities. Both an increased VER and decreased settling time result in keeping biomass with higher settling velocities and tendencies for EPS production in the system (Wang et al., 2006b; Qin et al., 2004b). In systems with identical reactor configuration

and operation, higher VERs can impose selective pressure on the system for bio-granulation.

Most aerobic granular sludge processes use VERs of 50% (Lochmatter and Holliger, 2014; Bao et al., 2009; Jungles et al., 2011). Wang et al., (2006b) studied the impact of VER on aerobic granulation in four identical SBRs with the same operating conditions (e.g., settling time, organic loading rate, and aeration intensity) and varying VERs from 20-80%. They observed that under higher VERs (i.e., 80 and 60%) granulation occurred faster and the granules formed were bigger and rounder with lower SVI. Under lower VERs (i.e., 20 and 40%), complete granulation did not occur and a mixture of granules and suspended particles developed in the reactors.

Liu et al., (2005) introduced the unified selection pressure theory for optimizing the formation and specific characteristics of aerobic granular sludge. According to this theory, the three main selective pressures of settling time, VER, and discharge time can be unified into one parameter called the minimum settling velocity of bio-particles. The calculation for minimum settling velocity considers: (1) the designed settling time; and (2) the settling time that is provided for the biomass during the discharge time.

2.2.2.6. Cycle Parameters

Shorter cycle times are thought to be more advantageous for bio-granulation. Long cycle times can lead to an increase in the starvation phase and cause granular disintegration due to the consumption of EPS (Li et al., 2006). Although cycle time is not considered as a determining factor in granule formation (Liu et al., 2005a), a reasonable cycle time between 3 and 6 h (Beun et al., 1999; de Kreuk et al., 2005; Zhu and Wilderer, 2003) is required to allow biomass to grow and accumulate without disintegration or biomass washout. Short cycle times can also negatively affect the granulation process due to:

- The loss of starvation phases required for long-term stability by selecting accumulating bacteria (van Loosdrecht et al., 1997; de Kreuk and van Loosdrecht, 2004);
- Excessive bacterial washout and a decrease in SRT due to solids discharge through effluent withdrawal (Tay et al., 2002); and
- An increase in the organic loading rate that could encourage the overgrowth of heterotrophic bacteria and result in large, unstable granules (Zheng et al., 2006).

Liu and Tay, (2007) conducted a study to investigate the impact of cycle time on granulation. Three SBRs with cycle times ranging from 1.5 to 8.0 h were run. Granules cultivated at 1.5 h cycle time were the largest. This could be due to imposing higher organic loads on the reactors and the subsequent increase in the heterotrophic growth rate. Granules cultivated with a 4 h cycle time were the most compact. The large and loose granules cultivated at 1.5 h cycle time were unstable due to low substrate and oxygen transfer and an increasing dead zone inside the granules. Liu et al., (2007) obtained unstable granules at a cycle time of 1 h without starvation. They observed that increasing the cycle time to 1.5 h led to the formation of granules with higher stability.

2.2.2.7. Feast/Famine Periods

Alternating feast and famine periods significantly influences bio-granulation. The periodic nature of cycling between high and low substrate concentrations selects for accumulating bacteria (e.g., PAO and GAO) in the reactor. Accumulating bacteria decrease hydrophobicity by secreting EPS and therefore promote bio-granulation (Li et al., 2006; Lin et al., 2008).

SBRs have been used in most of the studies conducted on aerobic granular sludge formation because of their ability to impose alternating feast/famine periods. (Jang et al., 2003;

Arrojo et al., 2004; Cassidy and Belia, 2005; Di Iaconi et al., 2007). However, bio-granulation has also been studied in modified continuous flow systems. Zhou et al., (2013) cultivated aerobic granules in a continuous flow airlift fluidized bed reactor (CAFB) by imposing high shear forces operating without a distinct feast-famine period. They acquired more than 55% granules after 12 days of operation. Morales et al., (2012) cultivated granules without imposing feast-famine periods in a continuously stirred tank reactor (CSTR). The CSTR had the ability to select for fast settling bio-particles and granulation was achieved with an HRT of 1 h and an organic loading rate (OLR) between 4.8-12 g COD L⁻¹ d⁻¹.

It was believed that short feeding times and therefore feast periods facilitated fast granulation by imposing a higher starvation time (Beun et al., 1999). Further research showed that longer anaerobic feeding periods (i.e., 1 h) are preferable since they select for accumulating microorganisms like PAO and GAO that promote robust and stable granules (de Kreuk et al., 2005). Accumulating bacteria convert readily biodegradable substrates to slowly biodegradable storage polymers as glycogen or polyhydroxybutyrate (PHB) under anaerobic conditions (Villaseñor et al., 2000; de Kreuk et al., 2005). Long anaerobic feeding periods stimulate the conversion of all easily biodegradable carbon sources into internally stored glycogen or PHB, decreasing heterotrophic growth rates during famine aerobic periods. The enrichment of PAO or GAO increases EPS production and induces the formation of compact granules (de Kreuk and van Loosdrecht, 2004).

2.2.2.8. Reactor Configuration

Reactor configuration, particularly the height to diameter ratio (H/D), can affect granulation by imposing a selective pressure. High H/D ratios (i.e., > 5) increase the distance the biomass has to travel to be kept inside the reactor during the settling time. Therefore, sludge with higher settling

velocities is retained (Liu and Tay, 2004). Moreover, higher H/D ratios provide longer circular flow patterns during aeration that are necessary for the formation of compact and dense granules (Tay et al., 2006).

Contrary to previous studies, Kong et al., (2009) reported that the H/D ratio of the reactor is not by itself a decisive factor in granule formation and that H/D ratios could be variable in full-scale applications. Four reactors with H/D ratios from 4 to 24 with a volume exchange ratio of 50% were run to study the impact of this parameter. An equal percentage of granules with the same microbial structure and size were achieved simultaneously in all four reactors. The results indicate that as long as other selective pressures such as the reduced settling time and high shear stress are provided the configuration of the reactor does not have significant impacts on bio-granulation.

2.2.2.9. Seed Sludge

Properties of the seed sludge such as settleability, surface charge, and hydrophobicity can affect bio-granulation. Hydrophobic bacteria promote bio-granulation as they are more likely to aggregate together than hydrophilic bacteria (Wilén et al., 2008). Therefore, seed from biological phosphorus removal systems or systems with accumulating bacteria would promote the start-up of bio-granulation.

In most granulation studies the systems are inoculated with conventional suspended growth activated sludge. A recent study by Verawaty et al., (2012) showed that a mixture of flocculent sludge and crushed granules enhanced bio-granulation by reducing the start-up time and maintaining nutrient removal during the first phases of granulation. Excessive biomass washout during the first steps of bio-granulation was prevented by attachment of flocculent sludge to the

crushed granules surface. Such a start-up mode provides an opportunity for the slower growing bacteria responsible for nutrient removal (e.g., PAO and nitrifiers) to be retained in the system.

2.2.2.10. Solids Retention Time

During the start-up period of aerobic granular sludge processes a variable SRT is present due to high biomass washout from decreased settling times (Qin et al., 2004). The SRT of the system can be fixed after full bio-granulation is achieved and effluent biomass concentrations are low. SRT can affect the activity of autotrophic and heterotrophic bacteria and consequently the selection of microorganisms within the granules and their ability for nutrient removal and long-term stability. SRT also affects the overgrowth of filamentous bacteria, directly influencing the stability of formed granules (Liu and Liu, 2006).

Li et al., (2008) studied the effect of SRT on granulation. They stated that reactors operating at different SRTs, from 3 to 40 days, with a settling time of 30 min (i.e., low selective pressure) have not developed stable or compact granules. Therefore, SRT was discounted as a key influencing factor for granulation in favor of other parameters, such as settling time and shear force. However, long SRT is reported as an influencing factor on the EPS production (Kaddouri et al., 2013). Kaddouri et al., (2013) observed that by increasing the SRT from 10 to 90 days the concentration of bound EPS is increasing; while, it stayed constant at lower SRTs (10-30 days). Changes in bound EPS production during long SRTs might affect the granulation process. In granulated systems SRTs up to 71 days (de Kreuk et al., 2005) have been reported.

2.2.2.11. Organic Loading Rates and Carbon Sources

High organic loading rates could increase the diameter of granules by encouraging the growth of heterotrophic bacteria, Figure 2.4. According to Kim et al., (2008) the optimum OLR for aerobic granulation in SBRs is about $2.5 \text{ kg COD m}^{-3} \text{ d}^{-1}$; however, it should be taken into account that

the optimum OLR depends on the type of carbon source. Other studies have demonstrated that various OLRs can be used to cultivate aerobic granules (Zhang et al., 2013; Coma et al., 2012; Othman et al., 2013). Imposing high organic loading rates (i.e., above $6 \text{ kg COD m}^{-3} \text{ d}^{-1}$) under an aerobic feeding mechanism may select for filamentous bacteria and consequently reduce long-term stability (Xavier et al., 2007; Zheng et al., 2006).

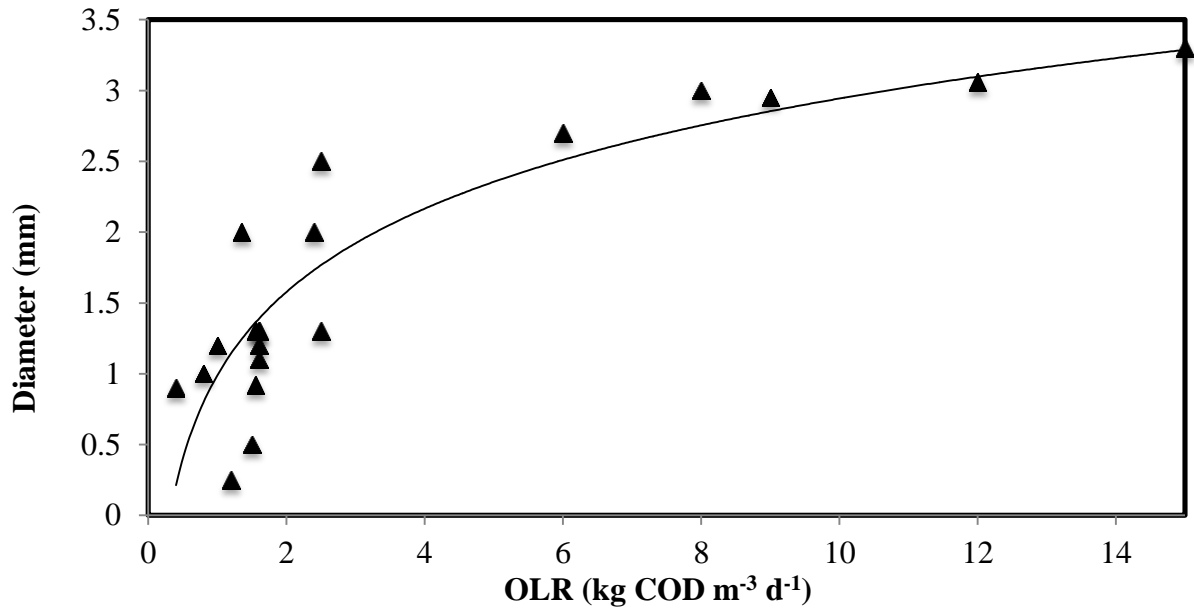


Figure 2.4. Correlation between granules diameter and organic loading rate in different studies (Data combined from: Dangcong et al. 1999; Moy et al., 2002; Jang et al., 2003; de Kreuk et al., 2005; Linlin et al., 2005).

Aerobic granules have been cultivated under OLRs ranging from 0.547 to $13.0 \text{ kg COD m}^{-3} \text{ d}^{-1}$ (Coma et al., 2012; Tay et al., 2004). COD removal efficiencies of 70 to 90% above OLRs of $9 \text{ kg COD m}^{-3} \text{ d}^{-1}$ indicate that aerobic granular processes are capable of withstanding concentrated organic loading rates (Othman et al., 2013, Val del Río et al., 2013). In most studies high organic strength wastewater has been used, however the use of municipal and other low strength wastewaters have been reported (Ni et al., 2009; Coma et al., 2012; Isanta et al., 2012). Tay et al., (2004) could not obtain granules under $2 \text{ kg COD m}^{-3} \text{ d}^{-1}$ and the granules obtained at

8 kg COD m⁻³ d⁻¹ were loose and unstable, disintegrating a few days after formation. Stable and dense granules were cultivated after 14 days with an OLR of 4 kg COD m⁻³ d⁻¹.

Different type substrates and carbon sources such as easily biodegradable (e.g. acetate and ethanol), toxic wastewaters (e.g. phenol and pentachlorophenol), municipal and industrial wastewater (e.g. dairy and pharmaceutical wastewater) have been used to cultivate aerobic granules (Tay et al., 2001a; Beun et al., 1999; Jiang et al., 2002; Lan et al., 2005; Coma et al., 2012; Arrojo et al., 2004; Inizan et al., 2005). Besides organic loading rate, various carbon sources can influence the strength and microbial composition of granules. Various types of carbon sources can affect the bacteria cells growth and their bioactivity. Changes in the bacteria metabolism can have subsequent impacts on EPS production and composition, which are crucial parameters in granule formation and stability. Cerning et al. (1994) observed changes in the EPS production yield by changing the carbon source. They also stated that the biopolymer produced with glucose as the carbon source was different from the one produced in a lactose-based media. Moreover, some studies showed that granules cultivated using glucose are more resistant under high OLRs than those cultivated using acetate. At low OLRs glucose-fed granules appear to be loose and filamentous dominated while acetate fed granules are more compact and dense, with a smooth outer layer and better settling velocity (Moy et al., 2002, Gao et al., 2011).

2.2.2.12. Metal Ions

The presence of metal ions such as magnesium and calcium in the feed will accelerate bio-granulation. Divalent calcium can form a bond between two negatively charged cell surfaces and enhance the aggregation of microorganisms. Moreover, calcium precipitates can act as a core for granules and further accelerate bio-granulation. Adding 100 mg L⁻¹ calcium and 10 mg L⁻¹ magnesium can reduce the time for bio-granulation by half (Jiang et al., 2003; Li et al., 2009).

Bruus et al., (1992) studied the effect of calcium, extraction on flocs stability and observed deterioration as a result of calcium extraction. The authors stated that calcium has a bridging role in the EPS matrix and acts as the backbone of sludge flocs. The same bridging role of divalent cations could affect the EPS matrix of granules and their long-term stability.

On the other hand, increasing calcium concentrations in the feed can change the metabolism of PAOs. Barat et al., (2008) reported that high influent calcium concentration and the precipitation of calcium phosphate affects biological phosphorus removal by decreasing the phosphorus release to acetate uptake ratio. These changes promote the growth of GAO in place of PAO and decrease the efficiency of biological phosphorus removal. Therefore biological phosphorus removal should be considered before divalent cations are dosed in the feed to promote bio-granulation.

2.2.2.13.pH

Low pH conditions promote faster bio-granulation, but granules formed under low pH conditions are fungi-dominated with an unstable structure (Yang et al., 2008). The overgrowth of fungi is one cause of system failure (McSwain et al., 2004; Yang et al., 2008). Yang et al., (2008) reported that granules formed under pH 4 are much bigger (i.e., 7 mm) and looser than those grown under pH 8 (i.e., 4.8 mm). In agreement with Yang et al., (2008), Lemaire et al., (2008a) reported that after 1 hour in pH-controlled vessels most of the large granules started to become loose while the majority of the smaller granules disintegrated at pH values of 6.5.

On the other hand, Wan et al., (2014) presented pH as the decisive parameter in the domination of filamentous microorganisms in aerobic granules rather than the carbon source. They observed that at neutral pH (7) the glucose-fed granules were filamentous-dominated while acetate-fed ones were majority floc-forming. By decreasing the pH to 4.5 the acetate-fed granules also

showed filamentous overgrowth. Moreover, they stated that by increasing the pH to 8 the floc-forming bacteria dominated granules with glucose as the carbon source. Lochmatter and Holliger, (2014) stated that the optimum pH for reducing the start-up period while maintaining nutrient removal capability is neutral pH. Moreover, it should be taken into account that the proportion of the total non-ionized ammonia (free ammonia, FA) is a function of the pH and temperature. In this regard, Yang et al., (2004) investigated the role of free ammonia in the development of aerobic granules, pointing out how high pH values could inhibit the activity of nitrifying bacteria due to the pH-enhanced production of free ammonia. The authors concluded that as the free ammonia concentration increased, a significant decrease in cell hydrophobicity and EPS production occurred, preventing the development of aerobic granules.

2.2.2.14. Dissolved Oxygen

Aeration induces the shear force necessary for successful bio-granulation and also provides the DO gradient inside the granule. Bio-granulation with low DO (i.e., saturation under 40%) is characterized by oversized granules with low densities. These properties lead to deterioration and failure of the system. de Kreuk et al. (2005) found that controlling the DO concentration at very low oxygen saturation levels (i.e., 20%) after the start-up period did not affect the properties of the granules and allowed the highest COD, nitrogen, and phosphate removal efficiencies.

Granules cultivated at higher DO concentration (i.e., 2-5 mg L⁻¹) are mostly compact and dense, with high settling velocities (Wilén and Balmér, 1998; Liu et al., 2009). DO values at least higher than 2 mg L⁻¹ during the aerobic phase were favorable for granule formation both in lab- and pilot-scale studies (Arrojo et al., 2004; Wang et al., 2007; Yilmaz et al., 2008; Bao et al., 2009; Liu et al., 2010; Zhang et al., 2011; Jungles et al., 2011; Liu et al., 2011; Isanta et al., 2012; Val del Río et al., 2012; Zhang et al., 2013).

2.2.2.15. Temperature

According to de Kreuk et al., (2005b) the morphology of aerobic granular biomass is affected by the type of microorganisms present in the granules and their growth rate. A temperature change could affect the bioactivity of the microorganisms and consequently the stability of the aerobic granules.

Most aerobic bench-scale granulation studies were conducted at room temperature (i.e., 20-25°C). de Kreuk et al., (2005b) studied the impact of low temperature on aerobic granulation and reported that granules formed slower at low temperatures (i.e., 8°C) compared to higher temperatures (25°C). Lochmatter and Holliger, (2014) observed that temperatures of 20°C decreased the start-up period and better maintained phosphorus removal efficiency than temperature of 15°C. Song et al., (2009) found that granules formed at 30°C are characterized by higher compactness, settleability, and bioactivity compared to the ones cultivated at 25°C and 35°C.

Up to now aerobic granulation at low temperature has not been successfully achieved (Ebrahimi et al., 2010). Granules obtained at low temperature are unstable and readily change to flocculated sludge during settling periods. Flocculation leads to high biomass washout during effluent discharge. de Kreuk et al., (2005b) reported that granules working at low temperatures could be stable by starting up the granulation phase at higher temperature and then decreasing the temperature after bio-granulation is accomplished.

2.3. Stability of Aerobic Granular Sludge

After understanding the mechanism of aerobic granulation and factors affecting this process, in order to have a robust and reliable industrial application of this technology knowledge about the granules stability is crucial. The instability and breakage of the granules affects their settling

velocity and induces the biomass washout (Nor-Anuar et al., 2012). Moreover, variations in granules diameter, decreasing by granules breakage, might affect the biological conversions such as simultaneous nitrification/ denitrofcation and phosphorus removal due to the changes in the volume of anoxic and anaerobic layers inside the granules (Nor-Anuar et al., 2012).

2.3.1. Parameters Influencing the Stability of Aerobic Granules

So far, some operating parameters such as pH, temperature, salinity, solid retention time, organic loading rate, carbon source, COD/N ratio, toxic compounds such as free ammonia (FA) are known as the influencing factors on granules stability (Seviour et al., 2009a; Zhu et al., 2013; Adav et al., 2010; Nor-Anuar et al., 2012; Zheng et al., 2013) .

Seviour et al., (2009a) stated that environmental pH, temperature and ionic strength can induce aerobic granules instability. Dissolution of aerobic granules under pH values greater than 10 and temperature above 50 °C has been reported (Seviour et al., 2009a). According to the previous studies high salinity, compared to other parameters such as operating pH, does not have significant impacts on aerobic granules stability (Pronk et al., 2014 and Seviour et al., 2009a). Pronk et al., (2014) stated that high salinity (20 mg L⁻¹) resulted in an increase in supernatant turbidity which was hypothesized to be due to the weaker granular gel structure.

In a study by Zhu et al., (2013) on the impact of sludge discharge on aerobic granules stability, it has been proven that long SRT of granular sludge induces the instability of the granules and an appropriate biomass discharge is required to have stable granular biomass.

High organic loading rate is also proposed to be one of the factors enhancing granules breakage. Adav et al., (2010) stated that the disintegration of granules under high organic loading rate is due to the decrease in protein production by bacteria species. They reported that the separated strains in their study lost their auto-aggregation under high OLR.

In addition to the organic matters concentration, carbon source might affect the granules stability. According to Ye et al. (2011), EPS production by bacteria is affected by the influent carbon source which can have subsequent impacts on granules stability.

Luo et al., (2014) stated that influent COD/N ratio and its variation causes granules disintegration. A low COD/N ratio of 1 and 2 resulted in a reduction in extracellular polysaccharide production, microbial community shift and subsequently to granules disintegration.

Moreover, toxic compounds such as free ammonia (FA) are reported as influencing factors on EPS excretion (Yang et al., 2004; Zheng et al. 2013). Zheng et al. (2013) stated that high concentration of FA have deteriorating impacts on granules stability. A threshold FA concentration of $17.76 \text{ mg N L}^{-1}$ was suggested. The disintegration of granules was hypothesized to be due to inhibitions of polysaccharide production. On the other hand, it was noted that FA provides a more favorable condition for GAOs rather than PAOs and PAOs were eliminated under high FA.

Chapter 3

Material and Methods

In this chapter the overall description on reactors configuration, wastewater composition and analytical methods used to monitor different parameters are presented. The more detailed explanations about experimental conditions and analytical methods used in each phase of the experiment are given in the related chapters.

3.1. Experimental Set Up

So far, sequencing Batch Reactors (SBRs) are the most common type of reactors used to cultivate aerobic granular sludge (Cassidy and Belia, 2005; Di Iaconi et al., 2007; Jang et al., 2003; Arrojo et al., 2004). The SBRs used in the current study were operated following the order presented in Figure 3.1.

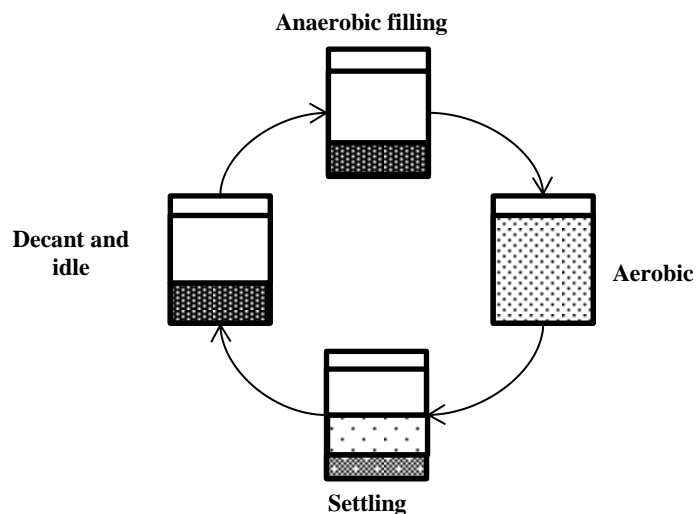


Figure 3.1. SBR phases

In the current study column-type SBRs with a total volume of 6.7 L and working volume of 5.7 L were used to cultivate aerobic granular sludge. Figure 3.2 shows the schematic of the used SBRs. The reactors were 12 cm in diameter and 60 cm in height, having a height to diameter ratio (H/D) of 5. The SBRs used in the current study were operated following the order presented in Figure 3.1. The detailed description of the SBRs cycles and operating conditions in each phase are presented in the related chapters.

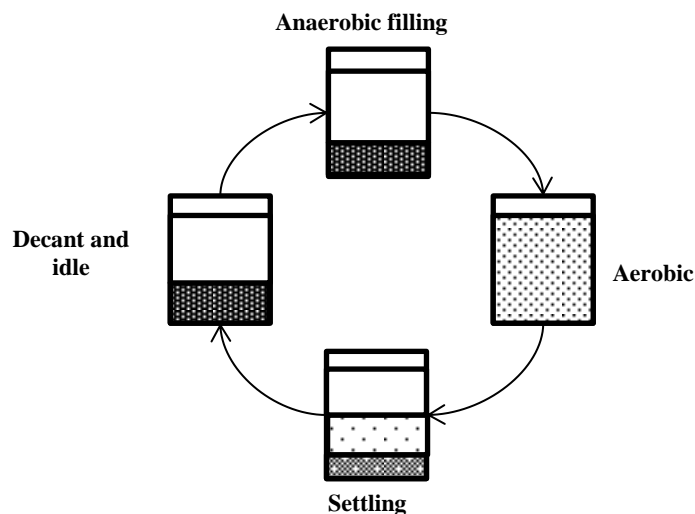


Figure 3.2. Different phases in one cycle in SBRs

Aeration was introduced using fine bubble air diffusers from bottom of the reactor to provide circular hydrodynamic flow pattern. Air flow meters were used to control the air flow rate at 6 and 5 L min⁻¹ during the first and second phase of the experiment, respectively. Superficial gas velocity was calculated to normalize the air flow rate considering the surface area of the SBRs, refer to Appendix A for more information.

The decant port was located at the middle of the reactor (25 cm above the bottom of the reactors) providing a volume exchange ratio (VER) of 50%. The peristaltic pumps were used to discharge the effluent.

During the most of the experimental period the feeding was static (no mixing) through the settled biomass and from the bottom of the reactors.

All experiments were conducted at room temperature, 20-22°C. DO concentration was not controlled, providing 100% oxygen saturation in the reactors (9.09 mg L⁻¹ at 20°C). The pH set point was varying for different phases of the experiment which will be discussed in detailed in the individual chapters.

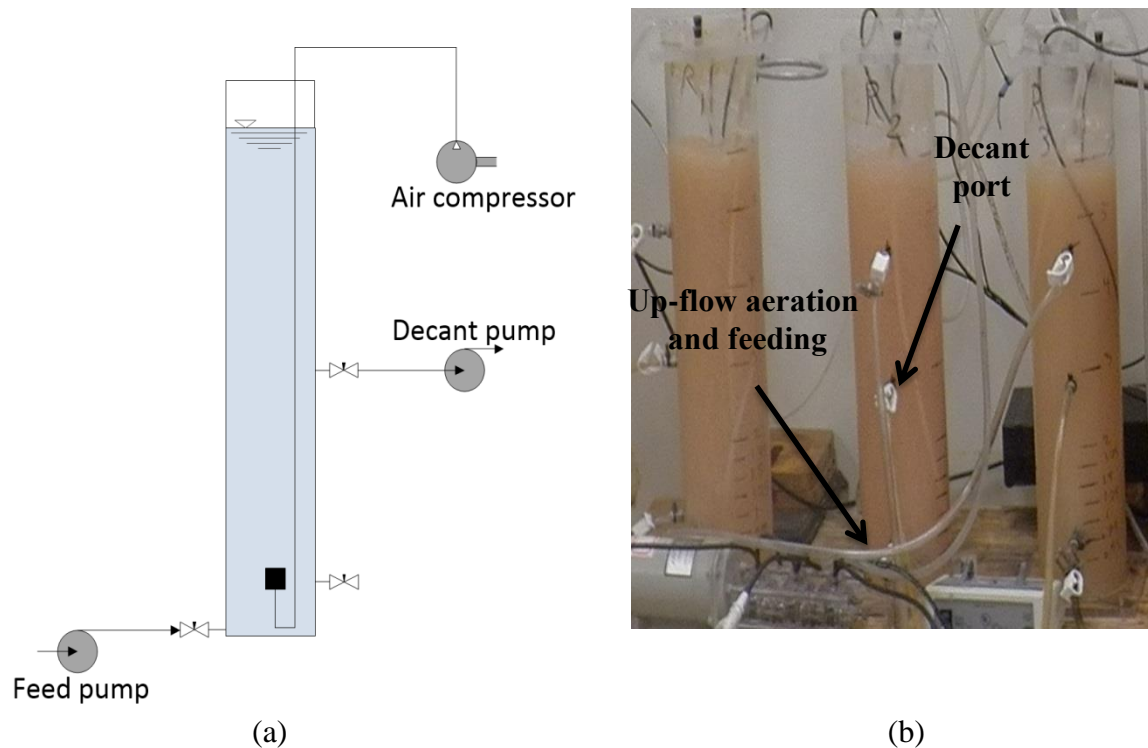


Figure 3.3. a) Schematic of the designed SBRs, b) picture of the SBRs

3.2. Characteristics of Wastewater and Seed Sludge

3.2.1. Feed Composition

Synthetic wastewater, with acetate as only carbon source was used to cultivate aerobic granules. Ammonium and phosphorus concentration were fixed at about 54 mg L^{-1} and 9 mg L^{-1} , respectively. Primary effluent from South End Water Pollution Control Centre was used as the influent in the last experiment; refer to sections 4.2 and 5.2 for the detailed description of the synthetic and municipal wastewaters.

3.2.2. Seed Sludge

Conventional suspended growth activated sludge from a full- scale BNR plant, West End Water Pollution Control Centre, Winnipeg, MB, was used as seed sludge for both phases of the study. Primary characteristics of the seed sludge such as mixed liquor suspended solid (MLSS), mixed

liquor volatile suspended solids (MLVSS), sludge volume index (SVI) were measured prior to the experiment to be able to monitor the changes during the study, the results presented in the related chapters. The reactors were seeded with two liters of settled biomass to have an initial MLSS concentration of more than 2000 mg L⁻¹; details are presented in sections 4.2 and 5.2.

3.3. Analytical Methods

3.3.1. Liquid Phase Analysis

Chemical Oxygen Demand was measured using the Hach digestion vials (High Rate vials measuring COD concentration in the range of 20-1500 mg COD L⁻¹).

Soluble Phosphate (PO₄³⁻-P), ammonium (NH₄⁺-N), nitrite (NO₂⁻-N) and nitrate (NO₃⁻-N) were measured using an automatic flow injection analyser (Quick Chem 8500, Lachat instruments).

Dissolved oxygen (DO) was measured in the reactors using portable DO meter (Thermo Scientific Orion star series).

pH was controlled using pH controller (Eutech instruments pH 200 series). 1M HCL and 1M NaOH were used to control the pH during the regular cycles and pH shocks.

3.3.2. Solid Phase Analysis

MLSS, MLVSS, Specific gravity (SG), zone settling velocity (ZSV) and Sludge Volume Index (SVI30) were measured according to standard methods (APHA et al., 2012) sections 2540D, 2540E, 2710F, 2710E and 2710D, respectively.

Size distribution of the flocs and granules were measured using Malvern laser light scattering instrument, Mastersizer 2000 series (Malvern Instruments, Worcestershire, UK), able to measure particle sizes ranging 0.02-2000µm.

The morphology and structure of the biomass was monitored using Nikon Microscope Eclipse E400 equipped with Olympus DP70 camera.

3.3.2.1. EPS Extraction

EPS was extracted according to Adav and Lee, (2008). For EPS measurement 10 mL of biomass was taken at the beginning of the aerobic phase. The EPS extraction method can be summarized in the following steps:

1. Adding 0.06 mL formaldehyde and incubating at 4°C for 1 hr.
2. Adding 4 mL 1N NaOH and incubating at 4°C for 3 hr.
3. Centrifuging at 4500 rpm for 25 min at 4°C.
4. Filtering by 0.2 µm filter.

Then extracted sample is ready for measuring different components.

3.3.2.2. Polysaccharide Measurement

Polysaccharide concentration in the extracted sample was measured following phenol-sulfuric acid colorimetric method (Dubois et al., 1956) and using Glucose as standard solution, refer to appendix A for detailed explanation on standard curve and polysaccharide measurement. The detailed procedure was as follows:

1. Adding 0.05 mL of 80% phenol to 2 mL of the sample containing polysaccharide.
2. Adding 5 mL of concentrated sulfuric acid (95%) rapidly against the liquid surface. Waiting for 10 min and shaking the tubes carefully
3. Incubating in a water bath at 25-30°C for 10-20 min before reading. The color of the solution should be stable before measurement.

4. Reading absorbance of the sample using spectrophotometer, Novaspec Plus, Biochrom, at wave length of 490 nm after calibrating with blank solution, using distilled water instead of polysaccharide solution.

3.3.2.3. Protein Measurement

Protein content of the EPS solution was measured using The Modified Lowry Assay Kit with bovine serum albumin (BSA) as standard solution; refer to appendix A for detailed explanation on standard curve and protein measurement. The following procedure shows the sample preparation for protein measurements:

1. Adding 1 mL modified Lowry reagent to 0.2 mL sample containing protein.
2. Mixing well and incubating for 10 min at room temperature.
3. Adding 0.1 mL, 1N phenol reagent.
4. Mixing well and incubating at room temperature for 30 min
5. Reading absorbance of the sample using spectrophotometer, Novaspec Plus, Biochrom, at wave length of 750 nm after calibrating with blank solution, using distilled water instead of protein solution.

Chapter 4

The Impacts of Acidic and Alkaline pH Shocks on Stability and Performance of Mature Aerobic Granules with Simultaneous COD, Nitrogen and Phosphorus Removal

4.1. Introduction

Aerobic granular sludge (AGS) technology is a potentially cost-effective and energy efficient alternative to suspended growth activated sludge processes (Giesen and Thompson, 2013). The specific characteristics of aerobic granules such as high settling velocity and compact structure reduces the required space for a treatment plant, allows higher biomass concentration in the reactors and subsequently provides the ability of operating with higher volumetric load conversion rates than conventional suspended biomass systems. The multilayer structure of aerobic granules provides different oxidation-reduction potential levels inside the biofilm and promotes the simultaneous removal of COD, nitrogen and phosphorus (de Kreuk et al., 2005; Liu and Tay, 2004). As a result, compared to other activated sludge systems, granular biomass requires up to 75% smaller footprint, allows up to 50% reduction in energy consumption and up

to 25% savings in investment and operational costs as shown during the full-scale applications (Inocêncio et al., 2013). Full-scale application of AGS, however, requires relatively long start-up period and could be affected by the uncertain granules stability in the presence of variable operating conditions.

Several studies are carried out on the mechanisms of aerobic granulation and on the variables affecting AGS formation (Adav et al., 2008a; Gao et al., 2011; Liu and Tay, 2002; Su and Yu, 2005). On the contrary, only a few works have focused on the stability of mature aerobic granules under variable influent carbon source, pH, temperature and salinity (Seviour et al., 2009a; Pronk et al., 2014).

Several studies reported a strong correlation between stability of aerobic granules and characteristics of EPS matrix (Wang et al., 2006; Zhang et al., 2007; Liu et al., 2004). As fully described in chapter 2, EPS are biopolymers secreted by microorganisms and enhance the granules formation and stability by facilitating bacterial cells adhesion. There are two different standpoints regarding the essential biopolymers in granule formation and stability. Zhang et al. (2007) introduced extracellular protein as the biopolymer responsible for granules formation and their stability. On the other hand, some recent studies indicated that specific types of gel-forming polysaccharides (e.g. alginate) are the key biopolymers in granules formation and their stability (Lin et al., 2008; Seviour et al., 2012).

Different operating conditions such as variable salinity, temperature and pH can alter the composition and structure of EPS by affecting the diversity and bioactivity of microorganisms. The changes in EPS matrix can subsequently disturb the stability of the granules (Seviour et al., 2009a). Pronk et al., (2014) reported that operation under salt concentration of $20 \text{ g Cl}^- \text{ L}^{-1}$ resulted in a decrease in granules size and an increase in effluent turbidity, possibly due to the

weaker structure of the EPS matrix under high saline conditions. Some of the previous studies indicated the disintegration and instability of bioflocs, anaerobic and aerobic granules under high pH (Gao et al., 2010; Sandberg and Ahring, 1992; Seviour et al. 2009a). Sandberg and Ahring, (1992) observed the anaerobic granular sludge disintegration and system failure at pH of 8.3. Seviour et al. (2009a) studied the impact of various environmental pH on rheological properties of aerobic granules after 24 h preservation of the granules at the desired pH. They reported the dissolution of aerobic granules sampled from a lab-scale SBR treating abattoir wastewater at pH>10, while the granules were stable when pH varied from 2 to 10. Moreover, Seviour et al. (2009b) observed a reversible process from an integrated network (gel) into a solution (sol), called sol-gel transition, at pH 9-12 in granular EPS extracted from aerobic granules treating abattoir wastewater. The test was conducted on the soluble EPS extracted from aerobic granules which were sampled from a lab-scale reactor. They stated that the EPS matrix exists as a strong gel at the operating pH range of less than 9, however, the extracted EPS was in sol state at pH above 12.

No research studies were found to focus on both stability and bioactivity of mature aerobic granules under rapid pH changes to either acidic or alkaline regions which might happen in full-scale wastewater treatment plants.

4.1.1. Objectives

The main goal of this study, after cultivating mature, dense and compact granules with simultaneous COD, nitrogen and phosphorus removal, was to investigate the stability and biological conversion processes of aerobic granular sludge under rapid acidic and alkaline pH changes. The performance of aerobic granules during the pH study was evaluated in terms of COD, nitrogen and phosphorus removal over a period of 9 days. The stability of granules in

terms of their physical and chemical characteristics was also monitored and compared in reactors operating under different pH regions, acidic (pH6), usual operational (pH7.8) and alkaline (pH9).

4.2. Materials and Methods

4.2.1. Experimental Set Up

The experiment was conducted in three identical column-type sequencing batch reactors (SBRs), R1, R2 and R3, characterized by 60 cm in height, 12 cm in diameter and a working volume of 5.7 L. Aeration was provided through fine bubble diffusers from the bottom of the reactor that caused a circular hydrodynamic flow trajectory. The airflow rate was controlled at 6 L min^{-1} , providing superficial gas velocity of $\approx 0.9 \text{ cm s}^{-1}$ during the aerobic phase; refer to Appendix A for detailed calculation. In order to provide higher shear stress, mechanical mixers (high-speed, low-torque mixer head, Cole-Parmer) with three-blade propellers (316 stainless steel three-blade propeller, Cole-Parmer) were used at the beginning of the experiment.

The SBRs were operated in cycles of 4 h, consisting of 60 min anaerobic filling, 168 min aerobic, 4 min settling, 5 min effluent decant and 3 min idle time. The sludge retention time was not controlled in the system and was variable, depending on the concentration of biomass in the effluent.

The decant port was located at the middle of the reactor (25 cm from the bottom) providing a volume exchange ratio of 50% and a hydraulic retention time (HRT) of 8 h. The test was conducted at room temperature (20-22 °C). DO concentration was not controlled in the reactor, resulting in almost 100% oxygen saturation in the reactors (9.09 mg L^{-1} at 20°C) during the aerobic phase. During the start-up period, pH was controlled between 7.8 - 8 using 1M HCl and 1M NaOH.

Synthetic wastewater, with acetate as the sole carbon source was used in this study. The influent COD concentration was maintained at $850 \text{ mg COD L}^{-1}$, imposing an OLR of $2.55 \text{ kg COD m}^{-3} \text{ d}^{-1}$. The influent $\text{NH}_4^+\text{-N}$ and $\text{PO}_4^{3-}\text{-P}$ concentrations were fixed at 54 mg L^{-1} and 9 mg L^{-1} , respectively. The composition of the synthetic wastewater was as follows (all in mg L^{-1}): sodium acetate (NaAc): 1000; NH_4Cl : 200; K_2HPO_4 : 50; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$: 15; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 12.5; $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$: 10 and 1 mL L^{-1} micronutrient with the following composition (all in g L^{-1}): $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$: 1.5; H_3BO_3 : 0.15; $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$: 0.03; KI: 0.03; $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$: 0.12; $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$: 0.06; $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$: 0.12; $\text{CoCl}_2\cdot 2\text{H}_2\text{O}$: 0.15; EDTA: 10. A COD:N:P ratio of about 100:6:1 provided the required concentration of N and P in the feed to encourage biological nutrient removal, while trace elements allowed avoiding micronutrient deficiency for microbial growth.

The reactors were inoculated with suspended growth activated sludge from West End Water Pollution Control Center (Winnipeg, MB), a BNR plant. Two liters of settled biomass with SVI of 108 mL g^{-1} were added to the reactors resulting in a final MLSS of 2500 mg L^{-1} and MLVSS of 2076 mg L^{-1} .

4.2.2. Tests on Effect of pH Changes

After the start-up period and achieving compact and mature granules with high efficiencies in COD, nitrogen and phosphorus removal, nine-day acidic or alkaline pH shocks were imposed on the SBRs by keeping the other operating conditions the same. In order to mitigate the differences between the three SBRs and have an identical biomass concentration and activity in the reactors prior to starting the pH study, biomass from all three SBRs were mixed and divided into three equal portions and returned to the reactors. The operating pH was changed from 7.8-8 to 6 and 9 in R1 and R3, respectively, and was controlled at the new pH for a period of nine days which

was longer than the SRT of the SBRs (in the range of 5 to 7). R2 was kept as the control reactor and operated under normal pH condition (7.8-8).

4.2.3. Analytical Methods

Mixed liquor suspended solid (MLSS/MLVSS), effluent total suspended solid (ETSS) and sludge volume index (SVI_{30}) were measured according to standard methods (APHA et al., 2012). Size distribution of the flocs was measured using Malvern laser light scattering instrument, Mastersizer 2000 series (Malvern Instruments, Worcestershire, UK). COD was measured using Hach digestion vials (High Rate vials 20-1500 mg COD L⁻¹). Soluble phosphate (PO_4^{3-} -P), ammonium (NH_4^+ -N), nitrite (NO_2^- -N) and nitrate (NO_3^- -N) were measured using an automatic flow injection analyser (Quick Chem 8500, Lachat instruments). MLSS/MLVSS, effluent total suspended solid (ETSS), SVI , COD, PO_4^{3-} -P, NO_2^- -N and NO_3^- -N were monitored every week, while the size distribution of the biomass was measured every two weeks. The samples for size, SVI and MLSS/MLVSS measurements were collected from the mixed liquor at the beginning of the aerobic phase (three samples for each parameter). For determining the average diameter of the granules, only particles with diameter of greater than 200 μm (Coma et al. 2012). The samples for liquid phase analyses and ETSS were collected from the supernatant at the end of the settling time. For EPS measurement 10 mL of biomass was taken from the mixed liquor after 30 min from the beginning of the aerobic phase. EPS were extracted following the method reported by Adav and Lee, (2008) using formaldehyde and sodium hydroxide addition and centrifugation. Polysaccharide concentration in the extracted EPS solution was measured following the phenol-sulfuric acid colorimetric method (Dubois et al., 1956), using glucose as standard. Protein content of the EPS solution was measured using the modified Lowry Assay Kit with bovine serum albumin (BSA) as the standard solution.

4.3. Result and Discussion

4.3.1. Aerobic Granulation

Selective pressure was imposed on biomass by gradual decrease in the settling time, from 20 min to 4 min within 4 weeks. Decrease in the settling time resulted in biomass washout, short SRT and subsequently loss of nutrient removal. The first granules formed within three weeks from the start of decreasing the settling time (at settling time of about 7 min), Figure 4.1b. One week after the granules formation, signs of filamentous bacteria overgrowth on the surface of granules were observed. Filamentous bacteria dominated granules structure and gradually changed the appearance of granules from round to star-shape in all three reactors, Figure 4.1c. As presented in Figure 4.2b, between days 20 to 45, the irregular aggregates showed fluffy structure with low settling velocity and high SVI.

The conversion of granules to fluffy aggregates and keeping a relatively short settling time (4 min) in the systems resulted in excess biomass washout and decrease in solid concentration (MLSS) inside the SBRs, Figure 4.2a. In order to suppress the filamentous overgrowth, induce the disintegration of the star-shape aggregations and promote the formation of compact granules some modifications have been applied on the operating conditions of the SBRs. The modifications can be summarized as follows:

1. Omission of mechanical mixers: mechanical mixers could cause the formation of the star-shape pellets by providing irregular flow patterns when combined with aeration. Omitting the mixers and using aeration as the only shear force can provide circular flow pattern to encourage formation of regular-aggregation (Liu and Tay, 2002).

2. Addition of 20 min to the idle phase: 20 min anaerobic phase was added before the feeding, in order to suppress the filamentous overgrowth (Bao et al., 2009). The anaerobic phase addition can affect the system in two ways:
 - I. Reducing the DO concentration to a very low value during the feeding time in order to help the storing bacteria such as PAOs and GAOs to dominate the system and suppress the filamentous bacteria by reducing the COD concentration entering aerobic phase.
 - II. Providing anaerobic famine period enhances deflocculation (Wilén et al., 2000). Deflocculating the irregular aggregates in combination with providing appropriate operating condition (such as such as circular hydrodynamic flow pattern) increases the probability of granules formation.
3. Modification of feeding: feeding was changed from top of the reactor to the bottom in order to provide an up-flow feeding through the settled sludge blanket. Providing fresh food with higher concentration of biodegradable COD (bCOD) can improve the robustness of the formed granules. The granules and flocs containing mainly storing bacteria are heavier; so, they will settle faster than fluffy aggregates and they mostly present in the bottom of the reactors. Up-flow feeding can help granulation by selecting for storing bacteria; therefore, granules can overcome filament-dominated aggregates.

After 20 days from imposing the modifications to the systems, the star-shape pellets started to disappear and smooth and round granules formed in all three reactors, Figure 4.1d. The results were consistent with the observations by Bao et al., (2009). They also stated that after the addition of 30 min anaerobic idle phase before feeding period, they were able to recover filament- dominated aggregates to round and smooth granules.

During the breakage of the aggregates, the supernatant turbidity increased and the disintegrated particles washed out from the SBRs.

After the disintegration of the irregular aggregates, small and smooth granules appeared in the SBRs resulting in an increased solids concentration.

After 160 days from the start-up of the reactors, mature and compact granules with simultaneous COD, nitrogen and phosphorus removal dominated the systems (80% by volume, calculated based on size distribution of the biomass), Figure 4.1e. Monitoring MLSS, SVI and SRT in all three SBRs, indicated an increase in these three parameters, Figure 4.2a, b and c, respectively.

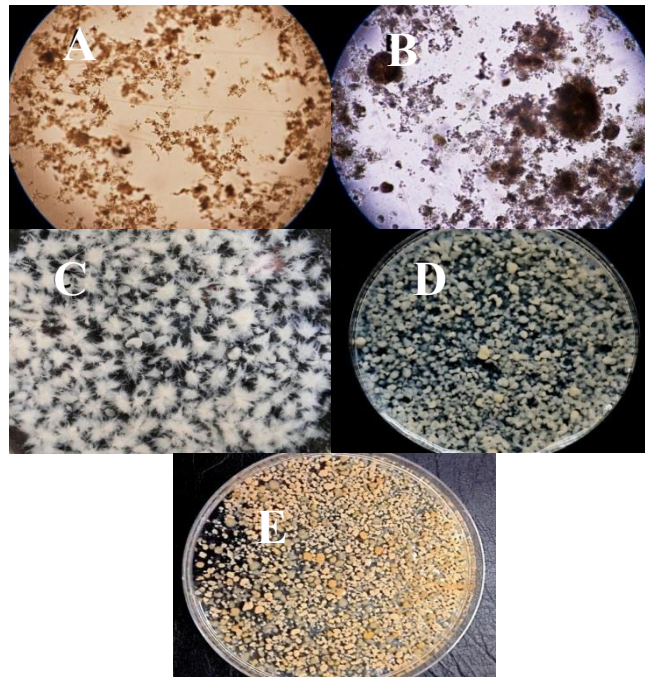


Figure 4.1. Sludge morphology at different stages of granulation: microscopic images (40X) of (A) seed sludge and (B) at day 15, digital camera images of (C) filamentous dominated granules (D) granules on day 94 and (E) on day 160.

Mature and compact granules had an average diameter of 1.3 mm, only considering particles with diameter of greater than 200 μm . The 20 min which was added to the anaerobic idle phase in order to disintegrate the star-shape pellets was removed by achieving fully granulated systems.

Granules maturation coincided with a decrease in SVI; the biomass SVI decreased from 150 mL g⁻¹ (seed sludge) to 20 mL g⁻¹ (matured granules). During the first stage of granulation, SRT was fluctuating (depending on the biomass washout in the effluent with no additional sludge waste) which is conventional during aerobic granulation (de Kreuk et al., 2005; Winkler et al., 2011; Beun et al., 2002). During the start-up period SRT was fluctuating between 0.4-3 days, after achieving the granulated systems it increased up to 18 days, Figure 4.2c. Gradual increase in SRT provided the appropriate conditions to keep microorganisms performing biological nutrient removal in the systems.

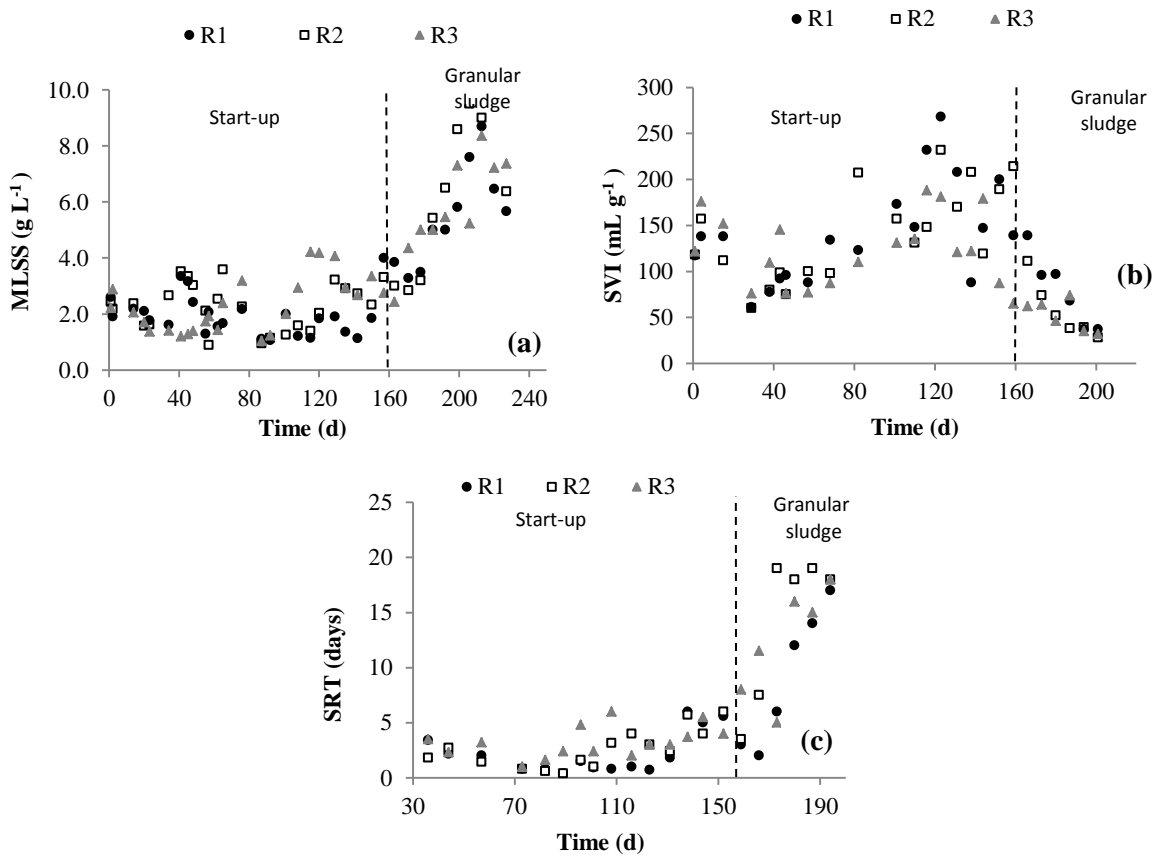


Figure 4.2. The variation of (a) MLSS, (b) SVI and (c) SRT, during the start-up (1) and steady-state conditions (2).

COD removal stayed at more than 95% in all three reactors during both start up and steady periods. Nitrogen removal was fluctuating during the experimental period. The concentration of the ammonium in the effluent was varying between 8-20 mg L⁻¹ and no nitrate or nitrite accumulation was observed in the reactors, Figure 4.3b and c, during the first 150 days. After dominance of compact granules in the systems, the concentration of ammonium in the effluent decreased to almost zero, Figure 4.3a.

Having mature granules dominating the systems, accumulation of nitrite indicates the occurrence of partial nitrification. The most important reason for nitrite accumulation rather than nitrate, while having COD/N ratio close to 15 and high concentration of oxygen (≈ 9 mg L⁻¹), can be either compact structure of granules preventing oxygen penetration in to the inner layers or having low SRT (< 3days) in the systems (during the first 150 days). SRT is reported to have a significant impact on AOBs and NOBs suppression when oxygen is not a limiting (Pollice et al. 2002).

By transferring the systems from flocculent dominated biomass to mature granules and increasing the SRT up to 18 days, the NOBs activity was observed in the SBRs, Figure 4.3b. The NOBs activity has been induced by increasing the SRT up to 18 days. Aerobic granules attained an average removal efficiency of 88%, 95% and 95% in nitrogen, phosphorus and COD removal, respectively. The granules maintained high efficiencies in nutrient removal during the steady state.

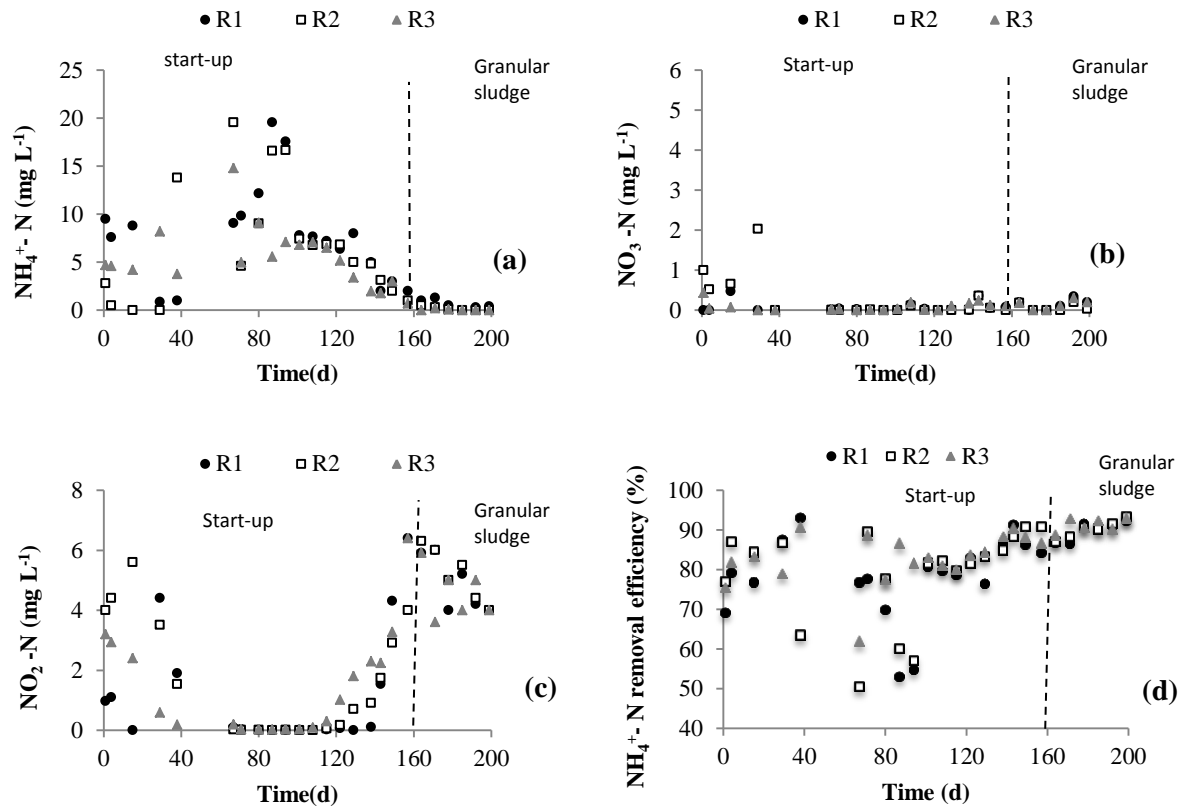


Figure 4.3. Changes in ammonium (a), $\text{NO}_3\text{-N}$ (b); $\text{NO}_2\text{-N}$ (c) concentrations and ammonium removal efficiency (d) during the start-up and steady-state conditions.

A simple nitrogen mass balance, shown in Appendix A, having 850 mg L⁻¹ of bCOD and 54 mg L⁻¹ of total nitrogen in the feed and very low total nitrogen concentration in the effluent, close to 5 mg L⁻¹, indicates the presence of simultaneous nitrification-denitrification (SND) in the systems. Most of the studies have reported that SND happens in the systems with low oxygen concentrations (Beun et al., 2001; Mosquera-Corral et al., 2005). In this study SND occurred at oxygen concentration of close to 9 mg L⁻¹ (saturation at 20°C). Occurrence of SND in the mentioned operating conditions can be explained by the size (1.3mm), compactness and dense structure of the granules. Compactness of the granules prevents oxygen penetration and provides the required anoxic phase for denitrification in the inner layers of the granules. Different oxygen penetration depths can select for different species within the layers of aerobic granules by

providing various ORPs levels. Simultaneous nitrification and denitrification using aerobic granular sludge at high DO concentration is also reported by Di Bella and Torregrossa (2013). They achieved more than 90% nitrogen removal through SND process at high DO concentration ($7-8 \text{ mg L}^{-1}$) using large granules having an average diameter greater than 1.5mm.

According to the changes in COD and nitrogen concentrations in one cycle, Figure 4.4, during the feast aerobic period, presence of high concentration of COD, nitrification hardly occurs. In this period only 2.7 mg L^{-1} of ammonium was consumed due to cells assimilation. That can be explained by DO consumption by some heterotrophs presenting in outer layers which is limiting the penetration of oxygen in to the inner layers. However, during famine aerobic period, when COD is depleted, almost 28 mg L^{-1} of ammonium was consumed while only $5 \text{ mg L}^{-1} \text{ NO}_x\text{-N}$ was produced. The observations indicate the presence of simultaneous nitrification- denitrification. During the famine aerobic phase the stored PHB can be used as electron donor in the process of denitrification (using NO_x as electron acceptor) by DPAO or DGAO (Third et al., 2003). In this study, a maximum of 94% total nitrogen removal was achieved through SND process over nitrite.

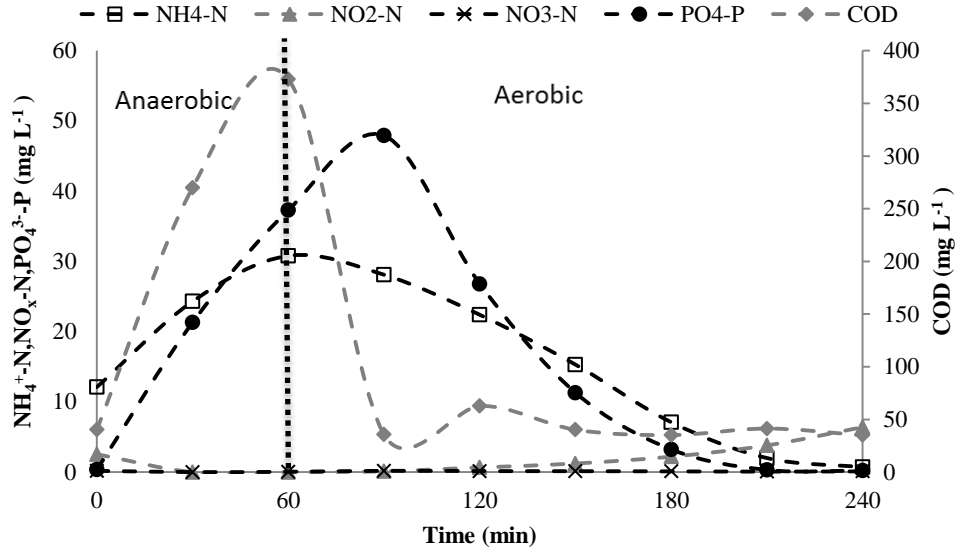


Figure 4.4. COD, ammonium, phosphate and NO_x-N (NO₂-N and NO₃-N) profiles during one cycle at steady-state conditions.

During the start-up period (first 150 days) no biological phosphorus removal was observed. The percentage of P removal was fluctuating between 40 to 65%, mainly due to the cells assimilation. After granules maturation and PAOs enrichment by applying alternative anaerobic/aerobic periods, phosphorus release was observed during the anaerobic phase and in the first 30 min of the aerobic phase, Figure 4.4. However, the release of phosphorus during the first 30 min after the start of the aerobic phase indicated the presence of PAOs in the inner layers of granules, where oxygen cannot penetrate easily.

After almost 160 days, phosphorus concentration in the effluent decreased to as low as 0.01 mg L⁻¹ and up to 99.88% of PO₄-P removal was achieved, Figure 4.5b.

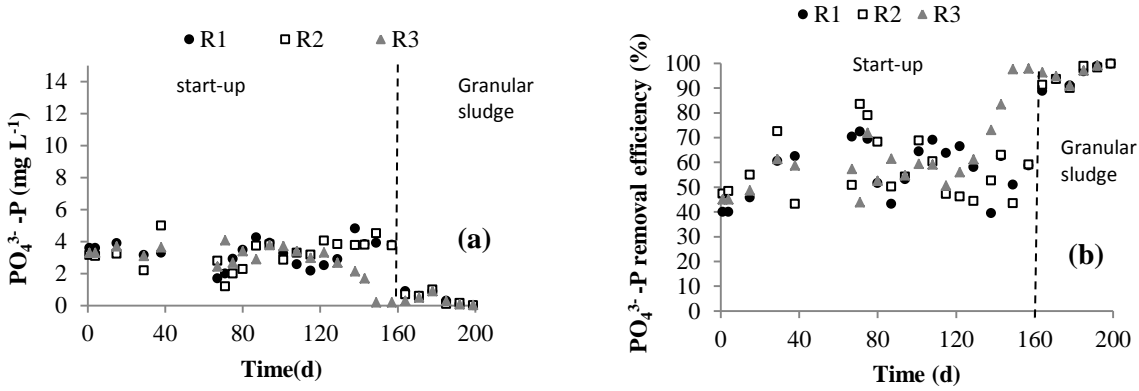


Figure 4.5. Phosphorus concentration (a) and removal efficiency (b) during the startup and steady-state periods.

The specific characteristics (MLSS, SVI and average diameter) and nutrient removal efficiencies of mature granules cultivated in R1, R2 and R3 are shown in Table 4.1.

Table 4.1. The mature granular biomass characteristics and nutrient removal efficiencies in R1, R2 and R3 (12 samples)

Parameters	Units	R1	R2	R3
MLSS	(g L ⁻¹)	5.3±1.6	6.0±2.6	5.4±1.7
SVI	(mL g ⁻¹)	39.0±13.8	34.5±15.2	39.5±11.4
Average diameter [*]	(μm)	1326±90	1365±90	1318±96
TN removal	(%)	88.8±3.4	88.7±3.8	89.0±4.1
PO ₄ ³⁻ -P removal	(%)	95.4±3.7	95.7±3.4	96.5±2.6
COD removal	(%)	95.0±0.7	95.0±1.0	95.0±1.2

^{*} Particles with a diameter greater than 200 μm

4.3.2. The Effect of pH Variation on Physical-Chemical Characteristics of Aerobic Granules

The physical-chemical characteristics of granules were evaluated in terms of ETSS, size distribution and EPS composition (PN/PS ratio). The acidic pH shock had minor impacts on granules stability and morphology, Figure 4.6. As shown in Figure 4.7a and b, the size distribution in R1 and R2 before and after the pH test is almost the same; moreover, Figure 4.8c

indicates that the ETSS did not exhibit any remarkable changes during the acidic shock, showing the stability of the system under pH as low as 6.

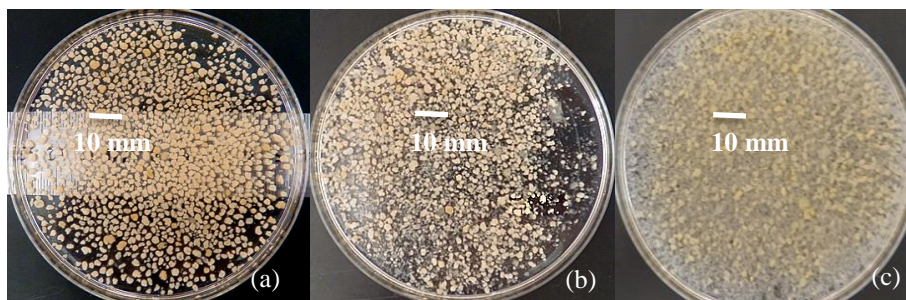


Figure 4.6. Granules morphology before (a) and nine days after shock in R1 (b) and R3 (c)

According to previous studies, EPS composition or PN/PS ratio can be used as an indication of granule maturation and their stability (Adav and Lee, 2008; Wang et al., 2006; Zhang et al., 2007). Figure 4.8a presents the concentration of the main biopolymers (PN and PS) and their ratio (PN/PS) in the extracted EPS from the biomass after eight days of operation under the new pH values (pH 6 and 9) which are compared with the control reactor (pH 7.8). Lower concentration of polysaccharide and higher concentration of protein in R1 (pH 6) compared to R2 (control) and R3 (pH 9) resulted in higher PN/PS ratio in R1 than in R2 and R3. By decreasing the pH to 6, PN/PS ratio decreased from 2 to 1.8 but after two days of operation under the new condition it recovered to 2.4. The slight changes in this ratio during the first days of the pH test could be due to the changes in the metabolism of microbial community (Ahn et al. 2009). Recovery of PN/PS ratio to 2.4 indicates the acclimation of the bacteria to the new condition which can also be benefited by the pH buffer capacity inside the granules (Ahn et al., 2009; Lemaire et al., 2008a).

Despite the parameters discussed above regarding the negligible impacts of acidic pH shock on granular biomass, SVI_{30} had an increase from 31 to 76 mL g⁻¹, Figure 4.8b. The increase in SVI_{30}

can be due to the reduction in biomass density as a result of changes in EPS composition. However, granular sludge with SVI_{30} of 76 mL g^{-1} is still considered as fast settling biomass.

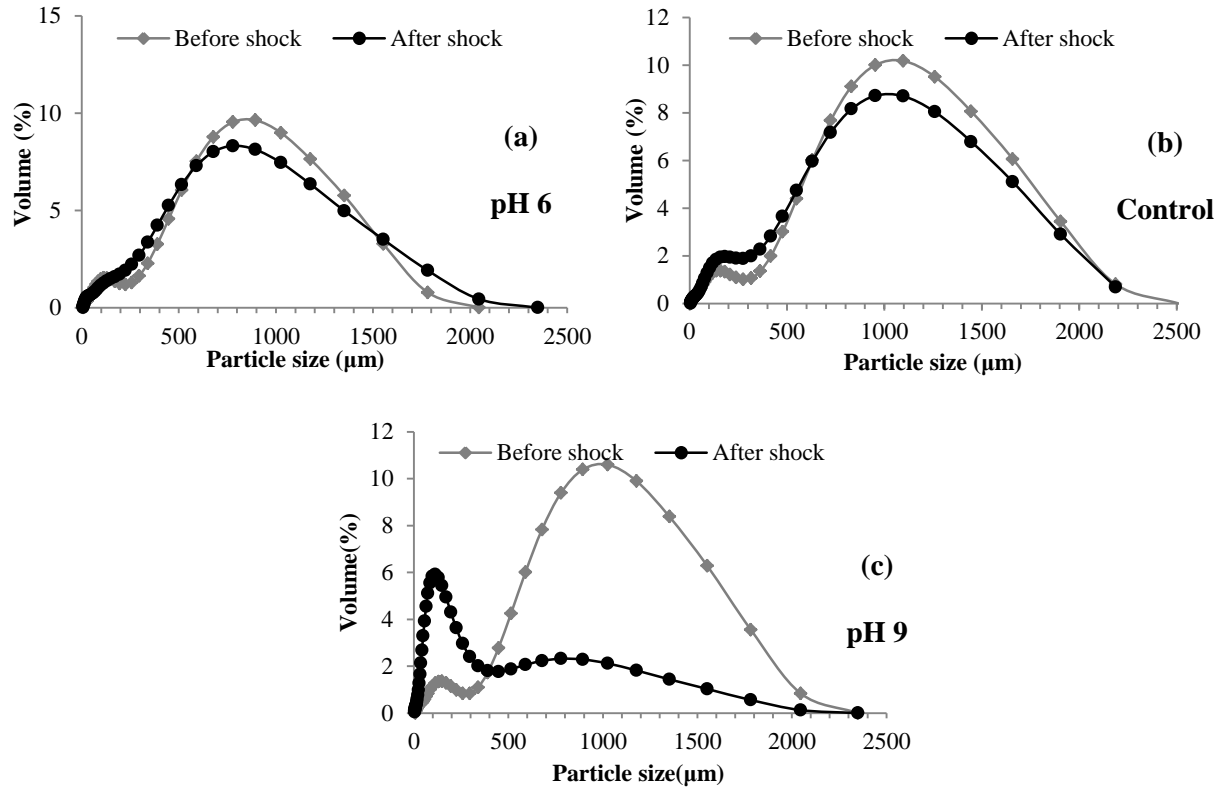


Figure 4.7. Size distribution before and after nine days of operation under pH shock: R1 (a), R2 (b) and R3 (c).

As shown in Figure 4.6, unlike the very slight impact of low pH on granules morphology, high pH had major effects on granules structure and morphology. Particles size distribution in R3 (pH 9) showed significant changes after nine days of pH test, Figure 4.7c. The peak in size distribution graph moved toward smaller size ranges, from 1000 μm to less than 100 μm , indicating granules breakage.

As presented in Figure 4.8c, after increasing the pH to 9, ETSS increased up to 500 mg L^{-1} indicating granules disintegration and biomass washout; however, ETSS decreased to 210 mg L^{-1} after 7 days of operation under pH 9. Compared to the other parameters influenced by alkaline pH, SVI_{30} was not significantly affected by high pH, Figure 4.8b. Even though the alkaline pH

led to the disintegration of granular biomass, the residue of granules mixed with bio-flocs could maintain a relatively low SVI_{30} of 70 mL g^{-1} to the end of the experimental period. Negligible difference in SVI_{30} between R1 and R3 does not indicate identical settling velocities of the biomass. High biomass concentration in the effluent from R3 during the first days of the pH test indicates biomass washout due to decrease in settling velocity and insufficient settling time for the mixture of crushed granules and bio-flocs.

The observations show that the disintegration of the granular sludge results in lower settling velocity compared to mature granules, however, the mixture of bio-flocs and crushed granules with SVI_{30} of 70 mL g^{-1} is still characterized by a higher settling velocity compared to the conventional flocculent biomass with a typical SVI_{30} of more than 120 mL g^{-1} (Toh et al., 2003).

The disintegration of granules coincided with changes in EPS concentration under alkaline pH. As presented in Figure 4.8a, both protein and polysaccharide concentrations decreased in the extracted EPS after eight days of operation under alkaline pH and PN/PS ratio in R3 is the lowest among the three SBRs. By changing the operating pH to 9 in R3, PN/PS ratio decreased from 1.7 to almost 1.0 with no further recovery during the nine days of pH study. Similar observations were reported by Gao et al. (2010) during their study on submerged anaerobic membrane bioreactor. They observed that under alkaline pH (pH 9.1) the PN/PS ratio decreased in sludge flocs and the bio-particles size distribution moved toward smaller size ranges.

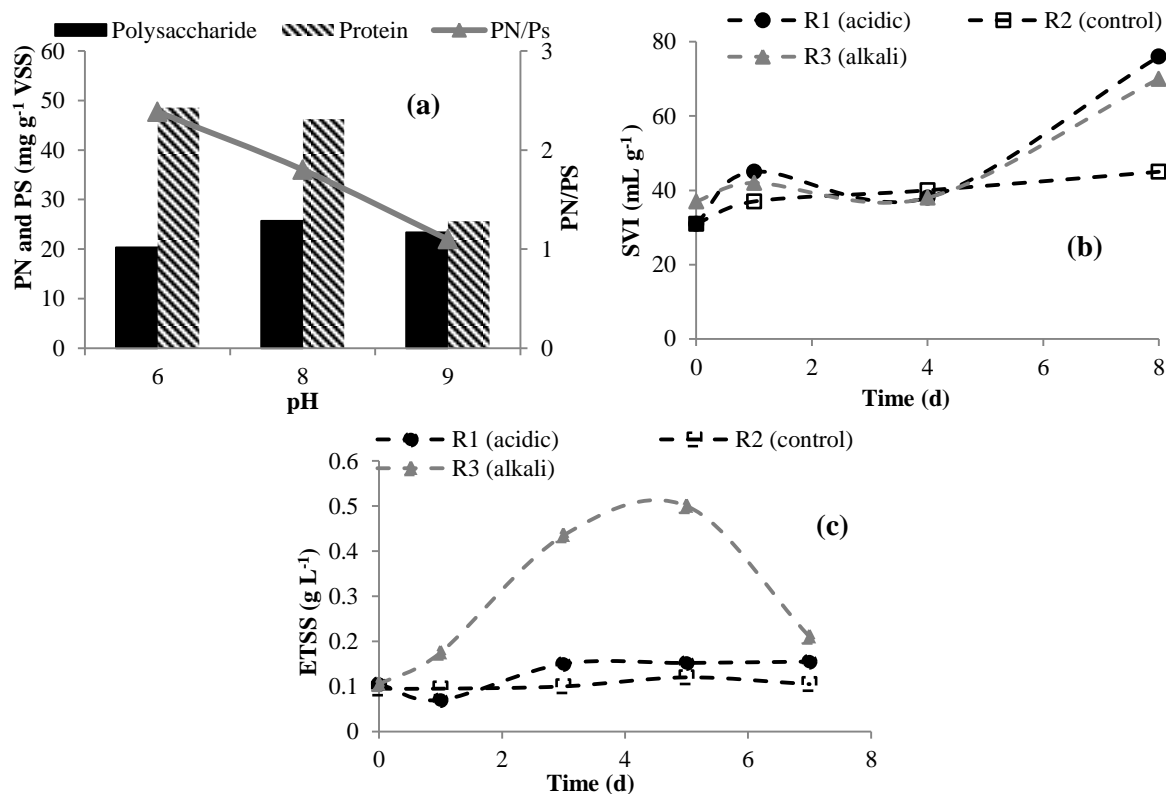


Figure 4.8. Protein, polysaccharide concentration and PN/PS ratio after eight days of operation under pH shocks (pH 6 and 9) (a), SVI30 (b) and the effluent TSS (c) (point zero represents the relevant parameter before the shock).

Besides influencing the EPS composition and PN/PS ratio, high pH can affect the EPS matrix by altering the functional groups in the chemical structure of biopolymers and increase their solubility. Depending on the biopolymers chemical structure and their acidity (pKa), under conditions with $\text{pH} > \text{pKa}$ (Vaccari et al., 2006) environmental pH can induce their dissolution by ionizing the associated functional groups (e.g. hydroxyl group that presents in all polysaccharides). Alkaline pH has been used to extract EPS from aerobic granular sludge (Seviour et al., 2010; Lin et al., 2010; and Adav and Lee, 2008) indicating the solubility of EPS and instability of granular polymeric matrix at high pH. Moreover, granular EPS matrix is considered as a strong hydrogel at neutral and acidic pH regions, while dissolution of this

hydrogel has been reported at higher pH values (greater than 9) (Seviour et al., 2009a and 2009b).

Granules are made of diverse microbial consortia, so various compositions of EPS could be produced depending on the specific species selected in the structure of the granules. Even though, proteins are known as the most important biopolymers in granules formation (Liao et al., 2001; Zhang et al., 2007; Adav and Lee, 2008), some recent studies introduced specific polysaccharides as responsible biopolymers in granular hydrogel formation and stability (Seviour et al., 2010; Lin et al., 2008; Adav et al., 2008b). Alginate-like exopolysaccharide (ALE) and a novel polysaccharide named as Granulan are the two gel-forming polysaccharides so far investigated in the granular EPS matrix (Lin et al. 2008; Seviour et al. 2011). Alginate is a linear polymer and composed of two residues of uronic acids: (1→4) linked β -D- mannuronic acid (M) and α -L-guluronic acid (G) which may be arranged in homopolymeric blocks as polymannuronic acid (MM) and polyguluronic acid (GG) or heteropolymeric block (MG). The distribution and proportion of these blocks affects the physical and chemical characteristics of the formed alginate (Lin et al., 2010). ALE is one of the prevalent components of polysacchrides in granular EPS and the gel forming capacity of alginates mostly depends on the GG blocks and their crosslink with divalent cations (e.g. Ca^{2+}) (Lin et al., 2010; Rastello De Boisseson et al., 2004). The cross-linkage of GG block with divalent cations makes them insoluble in water and produces a hydrophobic structure while the MM and MG blocks stay hydrophilic. Moreover, the ratio of GG to MM increases by forming granular sludge indicating the compact and dense structure of aerobic granules is related to the GG blocks (Lin et al., 2008).

Seviour et al. (2010 and 2011) presented Granulan as a complex and branched biopolymer. Seviour et al. (2012) stated that these two polysacchrides are both polar and soluble under alkaline conditions. The functional groups of the polymers (hydroxyl and carboxyl groups) could be ionized (protonated and deprotonated) under specific environmental pH depending on their pKa. According to Seviour et al. (2012), Granulan has a pKa of 9. Ionization of hydroxyl group under $\text{pH} > \text{pKa}$ (Vaccari et al., 2006) could explain the start of the sol-gel transition and instability of granular EPS at pH values ≥ 9 . Under pH 9, the presence of higher pH (greater than 9) inside the granules due to the biological conversions explains Granulan dissolution.

Moreover, according to Seviour et al. (2012) ALE has a pKa of 4.5 and it contains carboxylic acids residues in its structure which become deprotonated under high pH ($\text{pH} > \text{pKa}$) resulting in alginate solubility. In order to show the magnitude of the impacts of acidic and alkaline pH shocks on the physical characteristics of the granular biomass, the average value and standard deviation (SD) of SVI_{30} , biomass diameter and ETSS for 7 samples during the steady state and pH study are compared in Table 4.2.

Table 4.2. The average and standard deviation comparison during the steady-state and shock conditions in R1 and R3 (7 samples)

Parameters (SD)	Units	R1 (acidic shock)		R3 (alkaline shock)	
		Steady-state	Shock	Steady-state	Shock
SVI_{30}	(mL g^{-1})	50 ± 5.8	47 ± 19.8	46 ± 3.7	46 ± 15.6
Ave. diameter	(μm)	1305 ± 55	1351 ± 79	1313 ± 42	1240 ± 234
ETSS	(mg L^{-1})	262 ± 7.4	126 ± 37.6	262 ± 7.1	304 ± 172.3

4.3.3. Granular Sludge Bioactivity During the pH Test

Granules showed different reactions to the acidic and alkaline pH in terms of both bioactivity and physical-chemical characteristics. Figure 4.9 shows the variations in the COD (a), phosphorus (b) and nitrogen (c) removal efficiencies during the pH test.

As presented in Figure 4.9a, COD removal decreased from 95% to 86% and 91% in R3 and R1, respectively, and it recovered to its initial value after three days of operation under the new pH conditions.

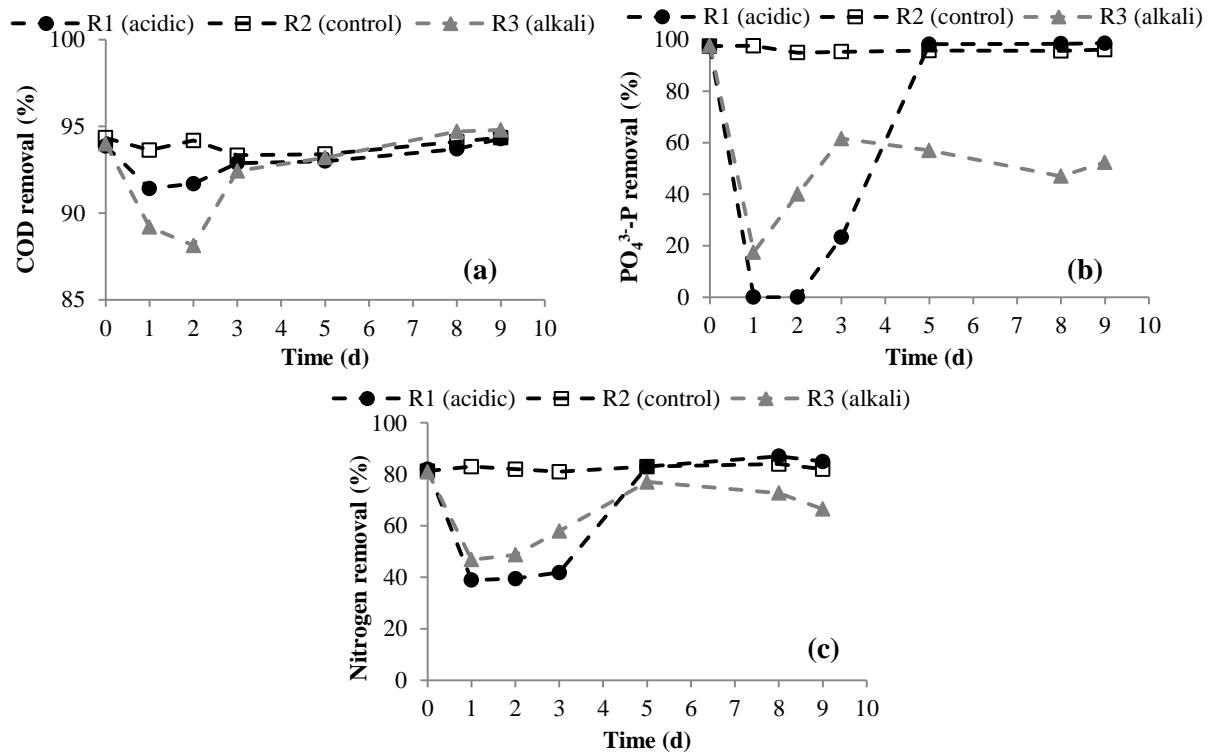


Figure 4.9. COD (a) phosphorus (b) and nitrogen (c) removal efficiencies during the pH test (point zero represents the relevant parameter before the shock).

Phosphorus removal efficiency decreased to almost zero in R1 during the first two days, Figure 4.9b. After three days phosphorus removal increased and recovered to more than 99% within five days. On the contrary, in R3 phosphorus removal decreased to 17% in the first day of operation under alkaline pH and it could not recover to more than 50%, even after nine days of operation under pH 9, corresponding to a P removal due to the cells assimilation only.

Altering the pH resulted in an increase in ammonium concentration in the effluent and a subsequent reduction in nitrogen removal to less than 50% both in R1 and R3, Figure 4.9c. Even though nitrification rate typically decreases at pH 6 (Princic et al. 1998), in this study, after three

days of operation under low pH nitrogen removal started to increase and recovered to 85% after five days. By contrast, under alkaline condition in R3, nitrogen removal started to increase after one day but it could not recover to more than 66%, even after nine days of operation under pH 9. There are two possible hypotheses explaining loss of biological nutrient removal at high pH: (1) washout of PAOs and nitrifiers from R3 due to granules disintegration during the first five days of pH study; and (2) inhibition of these microorganisms under high pH.

During the nine days of pH study, PAOs and nitrifiers showed satisfying performances under acidic pH in aerobic granular sludge which is opposite to what was reported in studies of conventional activated sludge systems (Jeon et al., 2001; Weissbrodt et al. 2013; Princic et al. 1998); however, it has been demonstrated that nitrifiers' response to inhibition and environmental conditions may depend on the specific microbial ecosystem aggregate characteristics (Munz et al., 2010). The different behavior of these microorganisms could be due to the pH buffer capacity within the granules due to the diffusion limitations (Lemaire et al., 2008a). With the EPS matrix disturbed and subsequent dissolution of the granules under alkaline condition, microorganisms were exposed to the operating pH of the bulk solution which may alter their bioactivity. Due to the slight impact of pH 6 on EPS matrix and granules structure, the pH buffer capacity was maintained inside the granules and microorganisms were able to recover their bioactivity in spite of the low pH conditions in the bulk liquid. On the other hand, the higher pH in the inner layers of the granules as a result of denitrification process (de Kreuk et al., 2005 and Angela et al., 2011) compensated the low pH during the acidic shock, while raising the pH under the alkaline shock.

The current study indicated the instability of aerobic granular sludge under short term increase of pH from 7.8 to 9. It should be pointed out that the granules disintegration might have been

induced by the presence of higher pH inside the granules which is the result of biological conversions and the type of carbon sources consumed in these conversions. According to previous studies (de Kreuk et al., 2005 and Angela et al., 2011) as a result of denitrification process, pH inside aerobic granules was higher compared to that of the bulk solution. In the current study, presence of denitrification in the system during the first cycles after the start of the pH study could increase the pH inside the granules to more than 9 (the bulk pH) which can solubilize the granular EPS matrix (e.g. Granulan) and induce granules breakage. Moreover, the pH increase as a result of denitrification while using acetate as electron donor can be higher compared to other carbon sources (Zhou et al., 2010; Tam et al., 1992). Thus using acetate as the carbon source might have induced the granules disintegration under pH 9 by providing higher pH in the inner layers of the granules. On the other hand, the composition and stability of EPS matrix can vary depending on the growth medium and carbon source (Lashkarizadeh et al., 2015; Ye et al., 2011). The growth medium can affect the EPS matrix by affecting the microbial species and their metabolism (Staudt, 2009). According to Lin et al. (2008 and 2010), the extracted alginate-like polysaccharide concentration using alkali treatment from acetate-fed aerobic granular sludge was much higher than that of extracted from the municipal wastewater-fed one: $310 \pm 16 \text{ mg g}^{-1}$ and $160 \pm 4 \text{ mg g}^{-1}$, respectively. These observations indicate the difference in the composition of granular EPS matrix and subsequently granules stability cultivated using different carbon sources. A summary of the observation from this study on the impacts of acidic and alkaline pH shocks is presented in Table 4.3.

Table 4.3. The summarization of the impacts of 9-day acidic and alkaline pH study on aerobic granules

Parameters	pH 6	pH 9
Size of the biomass	No major changes	Decreased
ETSS	No major changes	Increased (during the first 5 days)
SVI ₃₀	Increased	Increased
EPS composition (PN/PS ratio)	Increased	Decreased
Stability of AGS	stable	Unstable
COD removal	Recovered after 3 days [*]	Recovered after 3 days
Nitrogen removal	Recovered after 5 days	No recovery after 9 days ^{**}
Phosphorus removal	Recovered after 5 days	No recovery after 9 days
[*] Recovery is defined as achieving values equal to or higher relative to the initial values (after the specified period)		
^{**} Did not recover to the initial values even after 9 days		

4.4. Conclusion

The results from the current study indicated that alkaline condition (pH 9) results in deterioration of granules structure, physical characteristics, stability and biological activity. The granules disintegrated under pH 9 and the system changed to mainly (more than 70%, by volume) flocculent biomass resulting in a significant decrease in biomass particle diameter. Acidic pH shock did not induce granules breakage and the size distribution stayed the same even after nine days of operation under pH values as low as 6.

The phosphorus removal efficiency of the aerobic granules decreased from 98% to 50% under pH 9; PAOs activity was clearly interrupted under alkaline pH and no recovery was observed during the nine days of the study. Nitrogen removal also decreased under alkaline condition and it could not recover to more than 66% within 9 days. The acidic (pH 6.0) shock had no long-lasting impacts on granules bioactivity, both phosphorus and nitrogen removals recovered to 99% and 85%, respectively, within five days of operation under acidic pH after an initial

significant decrease (from 98% to 0% and from 80% to 39%, respectively). The results indicate the instability of aerobic granules under high pH during a short (nine-day) shock. However, it has to be taken in to account that the granules used in this study were cultivated using acetate-based synthetic wastewater which could have affected the microbial species selection, the EPS matrix and, thus, the granules stability.

Chapter 5

The Impacts of Influent Carbon Source on Stability and Biological Nutrient Removal Performance of Aerobic Granular Sludge ¹

5.1. Introduction

The unique characteristics of aerobic granular sludge such as high settling velocity, compact structure, simultaneous nutrient removal capability and ability to sustain high biomass concentration in the reactor provide the opportunity to design a compact wastewater treatment plant, making it an attractive alternative to the conventional activated sludge systems (de Bruin et al., 2004 and de Kreuk et al., 2005). Stable structure of aerobic granules is strongly correlated with their settling property and the biological conversions within different layers. Granules breakage and diameter reduction can affect the simultaneous nitrification/denitrification due to the changes in the anoxic zone inside the granules (Nor-Anuar et al., 2012). The stability of

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aerobic granular sludge under variable operating conditions is one of the most important factors that must be considered in industrial application of this technology (Wan et al., 2014). The strength and stability of the aerobic granules was proven to be a function of the gel-forming characteristics of EPS matrix (Seviour et al., 2009b). Parameters altering the gel structure of the granules such as high salinity can have subsequent negative impacts on their stability and bioactivity (Pronk et al. 2013).

Variations in the amount and chemical composition of the produced EPS can be the result of changes in environmental conditions such as nitrogen and phosphorus limitation, carbon substrate, ionic strength, pH and temperature (Nielsen et al. 1997; Staudt, 2009). Changes in these environmental conditions can affect the yield of EPS production and their composition while both are considered as threats to granules stability. Staud (2009) and Ye et al. (2011) observed that the EPS production by bacteria was influenced by changing the type of carbon source. The authors also stated that the growth medium with different carbon sources affects the chemical composition and backbone structure of the produced EPS.

The concentration and type of carbon source and carbon to nitrogen ratio (COD/N) in the growth medium can affect the bacterial culture and the yield of EPS production and its composition (Miqueleto et al., 2010; Ye et al. 2011) . Miqueleto et al., (2010) stated that in nutrient-limited conditions (e.g. nitrogen) the growth rate of microorganisms decreases by increasing the C/N ratio in the medium and by suppressing the microbial growth the excess carbon is used for EPS production. Moreover, EPS production is shown to be proportional to the substrate utilization rate (Laspidou and Rittmann, 2002); therefore, the biodegradability and concentration of carbon substrate should be considered as effective factors in EPS production yield (Cerning et al. 1994).

5.1.1. Objectives

This study aimed to investigate the stability and nutrient removal efficiency of aerobic granules after changing the feed composition (carbon source) from readily biodegradable acetate-based synthetic wastewater to more complex municipal wastewater. The effect of variation in the influent carbon source on aerobic granules was investigated by changing the feed of one of the reactors (R1 and R2) containing mature granules with simultaneous COD, ammonium and phosphorus removal capability. The feed was changed from acetate-based synthetic wastewater to municipal wastewater in R2, while R1 was kept as control reactor using acetate-based synthetic wastewater. COD, nitrogen and phosphorus removal efficiencies of aerobic granules were monitored and compared in R1 and R2 over 120 days. Changes in physical and chemical characteristics such as diameter, SVI and EPS composition of the aerobic granules operating in the new growth medium were also evaluated.

5.2. Materials and Methods

5.2.1. Experimental Set Up

Two identical column-type SBRs, R1 and R2 were setup. The reactors were 60 cm in height, 12 cm in diameter and had a working volume of 5.7 L. The SBRs were inoculated with suspended growth activated sludge from West End Water Pollution Control Center, a BNR plant, located in Winnipeg, MB. Two liters of settled biomass with SVI of 105 mL g⁻¹ was added to the reactors resulting in a final MLSS of 2100 mg L⁻¹ and MLVSS of 1680 mg L⁻¹. Both SBRs were operated with 4 h per cycle. Each cycle includes 60 min static filling period (through the sludge bed), 168 min aerobic period, 4 min settling period, 5 min effluent decant period and 3 min idle period. Up-flow aeration was used by pumping air through fine bubble diffusers at the bottom of the reactor. This created a circular hydrodynamic flow pattern. During the aerobic phase the airflow

rate was controlled at 5 L min^{-1} , providing superficial gas velocity of $\approx 0.7 \text{ cm s}^{-1}$ as well as 100% oxygen saturation in the reactors (9.09 mg L^{-1} at 20°C). The decant port was located in the middle of the reactors (25 cm from the bottom) providing volume exchange ratio of 50% and HRT of 8 h. The sludge retention time of both reactors was not controlled and was variable between 7 to 20 days depending on the concentration of biomass in the effluent. The experiment was conducted at room temperature, $20\text{-}22^\circ\text{C}$. The pH was controlled between 7.8-8 during the experiment using 1M HCl and 1M NaOH. During the granules cultivation period, synthetic wastewater with acetate as the sole carbon source was used. The influent COD concentration was maintained at $550 \text{ mg COD L}^{-1}$, imposing an OLR of $1.65 \text{ kg COD m}^{-3} \text{ d}^{-1}$. The influent $\text{NH}_4^+\text{-N}$ and $\text{PO}_4^{3-}\text{-P}$ concentrations were maintained at 54 mg L^{-1} and 9 mg L^{-1} , respectively. The synthetic wastewater composition was as following (in mg L^{-1}): Sodium acetate (NaAc): 1000; NH_4Cl : 200; K_2HPO_4 : 50; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$: 15; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$: 12.5; $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$: 10 and 1 mL of micronutrient solution. The composition of micronutrient was as following (in g L^{-1}): $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$: 1.5; H_3BO_3 : 0.15; $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$: 0.03; KI: 0.03; $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$: 0.12; $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$: 0.06; $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$: 0.12; $\text{CoCl}_2\cdot 2\text{H}_2\text{O}$: 0.15; EDTA: 10.

It took about 70 days in both reactors to cultivate mature granules with simultaneous COD, phosphorus and ammonium removal. After achieving complete granulated systems with 98%, 97% and 99.7% in COD, phosphorus and ammonium removal, respectively, the feed of R2 was changed from acetate-based synthetic wastewater to municipal wastewater by gradual dilution of the synthetic wastewater with the municipal sewage, while keeping all other operating conditions the same in R2. The percentage of the municipal wastewater increased from 30% to 100% within seven days. Municipal sewage consisted of primary effluent from a primary clarifier used for co-thickening waste activated sludge from a high-purity oxygen activated sludge reactor in the

South End Water Pollution Control Center in Winnipeg, MB. The average wastewater characteristics with standard deviations for 16 samples are shown in Table 5.1. After the feed of R2 was switched to municipal wastewater, both reactors were monitored for 120 days. R1 served as the control reactor using the acetate-based synthetic feed.

Table 5.1. Municipal wastewater compositions (16 samples)

Component	Municipal (mg L ⁻¹)
TCOD	480±30
SCOD	240±70
NH ₄ ⁺ -N	37±8
PO ₄ ³⁻ -P	5.1±1.2
TN	40±6
TP	6.4±2.3
TSS	185±20

5.2.2. Analytical Methods

Mixed liquor suspended solid (MLSS/MLVSS), effluent total suspended solid (ETSS), sludge volume index (SVI₃₀), zone settling velocity (ZSV) and specific gravity (SG) were measured according to the standard methods (APHA et al., 2012). Size distribution of the biomass was measured using Malvern laser light scattering instrument, Mastersizer 2000 series (Malvern Instruments, Worcestershire, UK), with the capacity to measure particle sizes ranging from 0.02 to 2000µm.

COD was measured using Hach digestion vials (High Rate vials 20-1500 mg COD L⁻¹). Phosphorus (PO₄³⁻-P), ammonium (NH₄⁺-N), nitrite (NO₂⁻-N) and nitrate (NO₃⁻-N) were measured using an automatic flow injection analyser (Quick Chem 8500, Lachat instruments). EPS was extracted following the method reported by Adav and Lee (2008) using formaldehyde and sodium hydroxide addition and centrifugation. Polysaccharide concentration in the extracted EPS solution was measured following phenol-sulfuric acid colorimetric method (Dubois et

al.1956), using glucose as standard. Protein content of the EPS solution was measured using the modified Lowry Assay Kit with bovine serum albumin (BSA) as standard solution.

5.3. Result and Discussion

5.3.1. Granules Formation

It took about 70 days in both reactors to cultivate mature granules with simultaneous COD, phosphorus and ammonium removal. All the lessons learned from the first experiment were implemented in this experiment. No filamentous dominated pellet was observed in this study indicating the effectiveness of the changes made to achieve smooth granules in the previous experiment. After achieving complete granulated systems with 98%, 96% and 99.7% in COD, phosphorus and ammonium removal, respectively, the feed of R2 was changed from acetate-based synthetic wastewater to municipal wastewater by gradual dilution of the synthetic wastewater with the municipal one, while keeping all other operating conditions the same in R2. The percentage of the municipal wastewater increased from 30% to 100% within seven days.

5.3.2. Nutrient Removal After Changing the Feed in R2

5.3.2.1. COD Removal

After changing the feed, COD removal of R2 decreased from 98% to 86% Figure 5.1a. The average concentration of COD in the effluent was about 34 mg L^{-1} . This was mostly due to the presence of non-biodegradable COD in the municipal wastewater. Figure 5.2 presents a typical trend COD, nitrogen and phosphorus concentration during one cycle (60 days after changing the feed). It was observed that in R2 with $344 \text{ mg SCOD L}^{-1}$ in the influent (providing an initial SCOD concentration of 189 mg L^{-1} in the reactor), the COD concentration at the end of anaerobic and aerobic phase was almost 107 mg L^{-1} and 30 mg L^{-1} , respectively. However, in R1 with $550 \text{ mg SCOD L}^{-1}$ in the influent (an initial SCOD concentration of 280 mg L^{-1} in the

reactor), the SCOD concentration was about 139 mg L^{-1} and 10 mg L^{-1} at the end of anaerobic and aerobic phase, respectively.

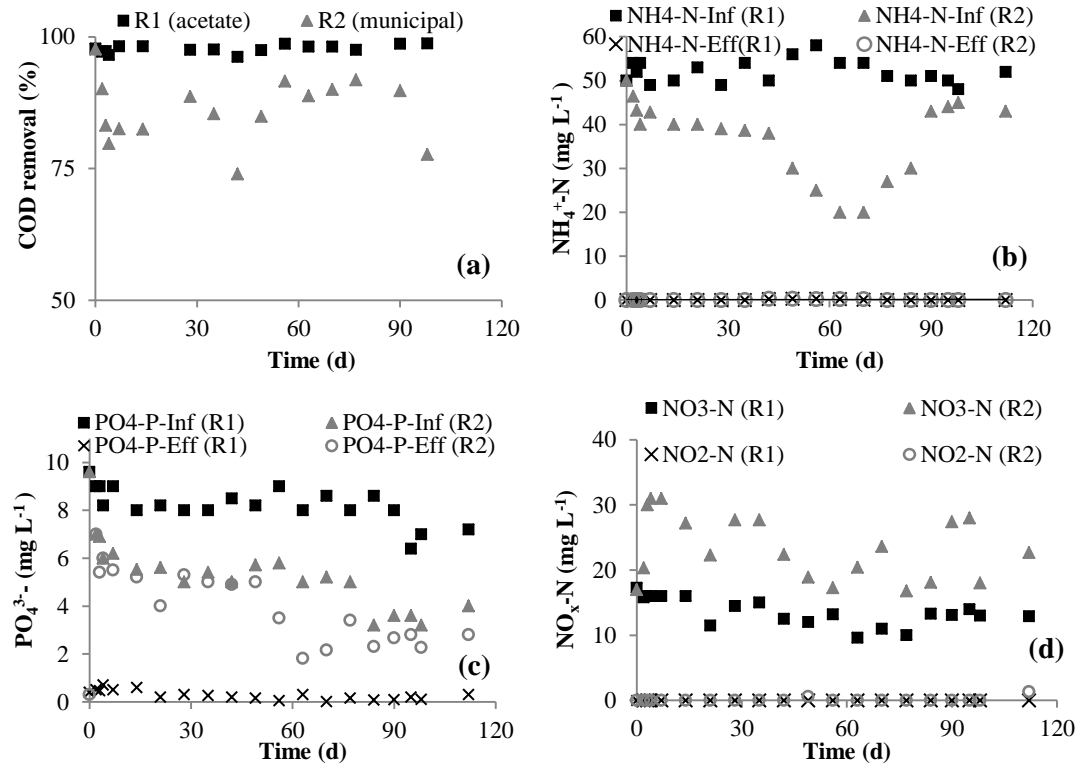


Figure 5.1. Variations in COD removal (a) ammonium (b) phosphorus (c) and $\text{NO}_x\text{-N}$ ($\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$) (d) concentrations in R1 and R2 (Inf= influent, Eff= effluent).

5.3.2.2. Nitrogen Removal

Changing the feed composition did not affect nitrification in R2 as it is shown in Figure 5.1b. Ammonium removal stayed at more than 99.7% during the experimental period. However, the nitrate concentration increased in R2 effluent. As presented in Figure 5.1c, the nitrate concentration of R2 was higher than R1. This difference could be due to the higher concentration of biodegradable COD in R1 feed which resulted in a higher biomass growth rate. The soluble COD (SCOD) concentration in R1 was 550 mg L^{-1} in the form of acetate and ammonium concentration was 54 mg L^{-1} , whereas in R2 the SCOD concentration in municipal wastewater was only $256 \pm 70 \text{ mg L}^{-1}$, with considerable fraction of non-biodegradable SCOD and

ammonium concentration at $37 \pm 8 \text{ mg L}^{-1}$. The higher biomass growth rate in R1 resulted in higher ammonium consumption for cells biosynthesis and subsequently higher biomass washout in the effluent, Figure 5.3c. The larger amount of biomass synthesis decreased the available ammonium for nitrification in R1; therefore, lesser amount of nitrate was produced.

5.3.2.3. Phosphorus Removal

Changing the substrate of R2 had negative impacts on biological phosphorus removal. Complete loss of PAOs activity was observed in R2. As shown in Figure 5.1d, phosphorus removal in R2 decreased from 97% to almost zero immediately after changing the feed. The municipal sewage composition indicates an average ratio of 40:6:1 in COD:N:P, while the preferred ratio for biosynthesis of the new cells should be close to 100:5:1 (Wang et al., 2006 and Krishnan et al., 2008). Moreover, a COD:P ratio greater than 40:1 (in this study was close to 40:1) was suggested for successful biological phosphorus removal (Mulkerrins et al., 2004). In a study conducted by Tasli et al., (1999) using suspended growth activated sludge, simultaneous nitrogen and phosphorus removal using a moderate strength domestic wastewater with C/P ratio between 33 to 39 was not achievable in a lab-scale SBR. However, in the same study by strengthening the feed with a spike of acetate (300 mg L^{-1}) and providing a C/P ratio of 66 resulted in high efficiencies in nitrogen and phosphorus removal. Thus higher nitrogen and lower COD concentration in the current experiment could cause disturbance in bacteria activity and biological nutrient removal. In order to encourage biological phosphorus removal without altering the composition of the feed, on day 20, 30 min anaerobic phase was added after feeding and before the aeration phase. The reason behind the addition of anaerobic phase was to ferment and enhance the hydrolysis of the non-readily biodegradable organic compounds in the municipal wastewater to provide more available volatile fatty acids (VFA) for PAOs. However,

no improvement in phosphorus removal was observed. There were two possible explanations for the loss of biological phosphorus removal: 1) Lack of available carbon for PAOs. 2) Most of the PAOs that existed initially in R2 were almost washed out.

The cycle mass balance of SCOD revealed that the removed SCOD during anaerobic phase was 82 mg L^{-1} in R2, Figure 5.2. Denitrification, nitrate (about 10.5 mg L^{-1}) left in the system from the previous cycle was fully removed during the first 60 minutes, consumed close to 73 mg L^{-1} SCOD (based on an average SCOD to nitrate ratio of 7; Surampalli and Tyagi, 2004). This resulted in only 9 mg L^{-1} of SCOD available to be consumed by PAOs during the anaerobic period. On the other hand, in R1 the total removed SCOD during anaerobic phase was 141 mg L^{-1} . With 42 mg L^{-1} of COD consumption for denitrification (6 mg L^{-1} nitrate), there was still about 99 mg L^{-1} COD available for PAOs. This SCOD (in the form of acetate) was readily biodegradable and the amount was sufficient for PAOs to perform an excellent biological phosphorus removal.

The cycle profile showed high concentration of anaerobic phosphorus release (about $86 \text{ mg PO}_4^{3-} - \text{P L}^{-1}$) in R1, Figure 5.2b. The release of phosphorus continued for 20 min after the start of aeration which was consistent with the observations from phase 1 of the experiment. It was presumed that PAOs were located in the inner layers of the granules and it took some time for oxygen to penetrate to the deeper layers. Nevertheless, R1 maintained excellent phosphorus removal rate during the experimental period. The average removal rate was approximately 97%. On the other hand, Figure 5.2b shows that in R2, very small amount of phosphorus ($4.7 \text{ mg PO}_4^{3-} - \text{P L}^{-1}$) was released during the anaerobic period and the aerobic phosphorus uptake was also very low (close to the same amount that was released).

An improvement in biological phosphorus removal (up to 64%) in R2 was observed between days 56 to 77, Figure 5.1c. During this period the ammonium concentration in municipal wastewater decreased from 39 to 20 mg L⁻¹, Figure 5.1b, due to the wet weather, while COD did not decrease and stayed at about 300 mg L⁻¹. The decreased ammonium concentration in the influent resulted in a reduced nitrate concentration in the effluent. Therefore, at the beginning of the next cycle, where denitrification happened, less carbon was consumed for denitrification. This provided more carbon available for PAOs for phosphorus removal. When the ammonium concentration in the influent increased back to the average concentration after day 77, the phosphorus removal efficiency again decreased to close to zero. This observation proved the competition between denitrifiers and PAOs for carbon source in R2 and PAOs were out-competed due to the low VFA availability in municipal wastewater. It also indicated the presence of PAOs in granular biomass and they were able to re-start functioning if there were sufficient amount of VFA in the system.

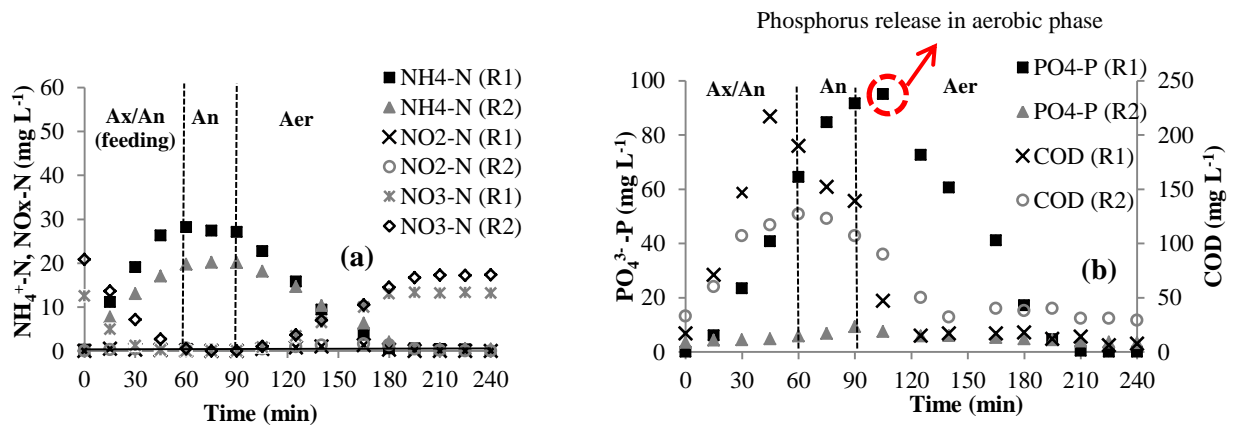


Figure 5.2. COD, Phosphorus and Nitrogen profile during one cycle on day 60 (NO_x-N : NO_2^--N and NO_3^--N , Ax: anoxic; An: anaerobic; and Aer: aerobic).

5.3.3. Changes in Physical-Chemical Characteristics of Granules After Changing the Feed

The granules in R2 disintegrated during the first month after changing the feed to municipal wastewater. Characterizing granules as particles with a diameter greater than 200 μm (Coma et al. 2012), about 50% of the granules were disintegrated and changed to bio-flocs by changing the influent. The granules disintegration resulted in a decrease in average diameter of biomass from 1mm to 0.57 mm in 30 days, Figure 5.3a; however, after one month acclimation, re-granulation started and the system became a complete granulated SBR within two month (after changing the feed) as shown in Figure 5.4. The granules size was measured at more than 1.2 mm on day 58, Figure 5.3a. It was noticed that the breakage of granules did not have negative impacts on the settling properties of the biomass. The mixture of the granular and flocculent biomass maintained high settling velocities. The SVI of the biomass in R2 stayed comparable with that of the biomass in R1, Figure 5.3b.

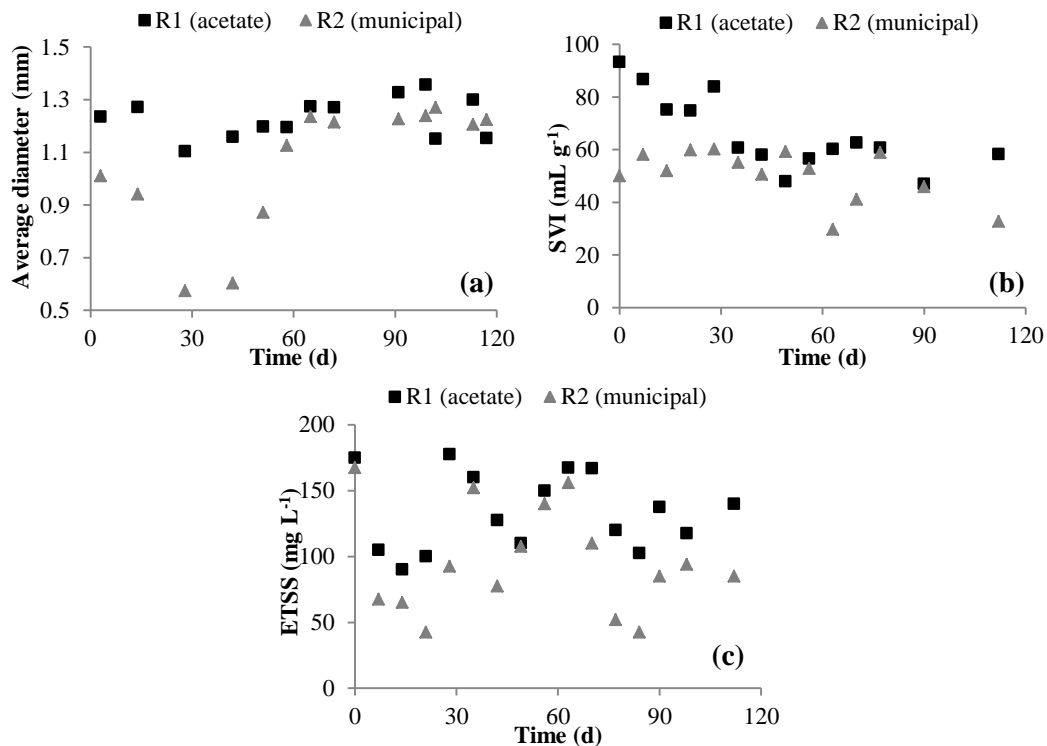


Figure 5.3. Changes in average diameter (a), SVI (b) and ETSS (c) (point zero represent the parameters before changing the feed composition in R2).

The characteristics of the granules in R1 and R2 were measured and the results are shown in Table 5.2. The results indicate that the granules in R2 possess comparable characteristics with those of the granules grown in R1. The granules in both reactors had almost identical ZSV, SG, and average diameter.

Table 5.2. Characteristics of granules in R1 and R2 after 120 days (14 samples)

Parameter	Unit	R1 (Acetate feed)	R2 (Municipal wastewater)
SVI (120 days)	(mL g ⁻¹)	60±12	47±10
ZSV	(cm min ⁻¹)	41.28±0.24	40.92±0.26
SG	-	1.020±0.004	1.012±0.002
Average diameter	(µm)	1230±70	1220±27

As shown in Figure 5.3c, neither excess biomass washout nor increase in ETSS was observed as the result of the granules disintegration. Lower ETSS was observed in R2 than in R1 indicating higher settling velocity of the biomass in R2. This suggested the absence of excess biomass

washout and maintaining high settling velocity by the biomass after partial granules disintegration.

In the study conducted by Ye et al. (2011) on conventional activated sludge, changing the substrate composition from complex municipal wastewater to easily biodegradable acetate-based resulted in an increase in SVI and ETSS during the first days. They also found that the flocs size decreased initially and it started to increase by the acclimation of the bacteria to the new growth medium which is in agreement with the results from the current study on granular biomass.

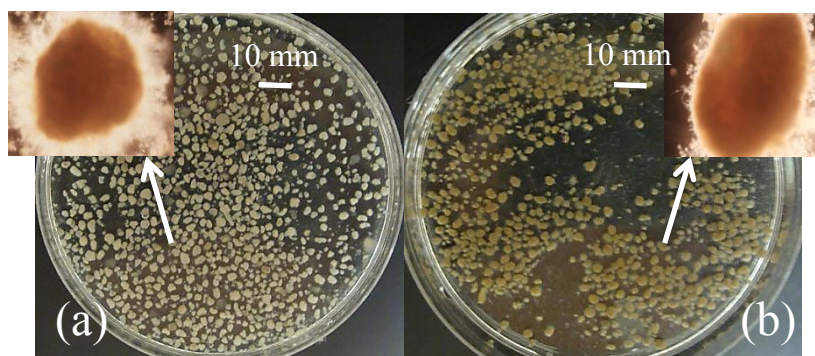


Figure 5.4. Granules at day 70 (granules magnification= 40X): (a) R1; (b) R2.

Different carbon source changed the EPS composition in R2. Comparing to R1, after changing the feed, R2 had a lower polysaccharide and higher protein concentrations throughout the experimental period. This resulted in an increased PN/PS ratio from 1.7 to almost 7.5 in R2 within 70 days after changing the feed composition, Figure 5.5c. These observations are in agreement with the results from the previous studies (Ye et al. 2011) on the impact of carbon source on EPS composition in conventional activated sludge. In their study they also observed that by using readily biodegradable carbon source (acetate) the amount of protein in the EPS composition decreased. Higher concentrations of protein in the EPS were observed when using complex municipal wastewater. Cerning et al. (1994) showed that various carbon sources have different impacts on microbial growth and consequently on the EPS production yield and its

composition. Zhang et al. (2007) presented extracellular protein as the responsible biopolymer for granules stability. However, Seviour et al., (2010), Lin et al., (2008) and Adav et al., (2008b) introduced specific type of polysaccharides as the main gel-forming and backbone components of EPS participating in granules stability. Changing the microbial diversity as a result of changing the growth medium can affect the production and composition of gel-forming components of EPS and weaken the granules structure. However, in this study after the acclimation of the bacteria to the new environmental condition and reproduction of the responsible biopolymers for granules stability, compact granules were able to dominate the system. R2 was able to change to a fully granulated SBR within two months. The specific characteristics of granules grown in R1 and R2 also showed no major differences (Table 5.2).

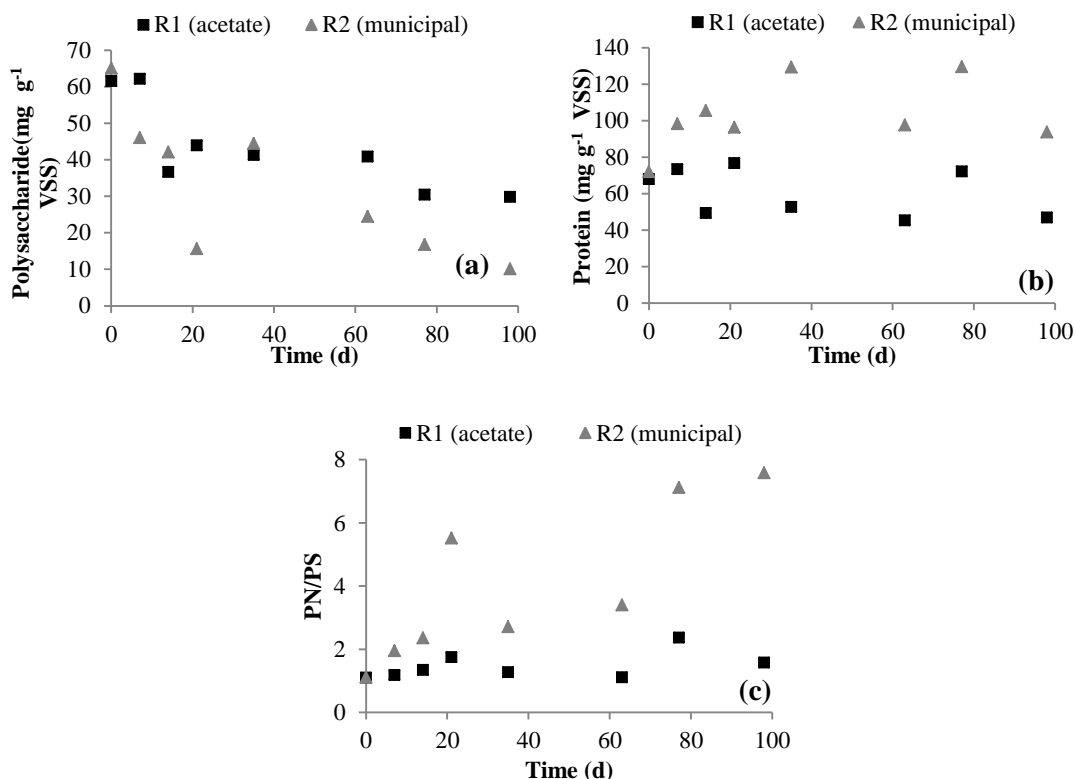


Figure 5.5. Polysaccharide (a) protein concentrations (b) and PN/PS ratio (c) in R1 and R2.

5.4. Conclusion

The results from this study indicated that changing the growth medium resulted in temporary granules disintegration in R2 and decreased the average diameter of the biomass (from 1mm to 0.57 mm). The disintegration of the granules did not affect the settling property of the sludge in R2. After the change, R2 maintained high efficiencies of ammonium and COD removal. However, biological phosphorus removal and denitrification were lost in R2, due to lack of VFA in the municipal wastewater. Low SVI ($\approx 47 \text{ mL g}^{-1}$) was maintained throughout the experimental period in R2 compared to SVI 60 mL g^{-1} in R1. After the acclimation of the bacteria to the new carbon source, re-granulation started and a complete mature granular system was obtained in R2 within two months after the change of feed. Therefore, changing the carbon source from readily biodegradable acetate to a more complex ones present in municipal wastewater does not have significant impact on aerobic granular sludge characteristics, particularly it does not affect its settling properties. However, after changing the feed composition, in order to maintain simultaneous biological phosphorus and nitrogen removal in the system sufficient readily biodegradable carbon source is required.

Chapter 6

Conclusions

The disturbance of the EPS matrix was presumed to be one of the main factors affecting the stability of aerobic granules. pH and carbon source were investigated as two of the potential parameters affecting the composition of EPS matrix by altering the diversity of microorganisms, their bioactivity and chemical structure of the EPS matrix.

In the first phase of the experiment, having filamentous pellets dominating the systems, it took almost 160 days to achieve mature granules with simultaneous COD and nutrient removal. The filament-dominated pellets changed to smooth and round granules after applying a number of operational changes including: (1) changing the feeding type from top of the reactor to up-flow feeding from the bottom; (2) increasing the idle time before the feeding period; and (3) omitting mechanical mixers. After achieving stable and matured granular systems with high nutrient removal efficiency, the impact of pH variation on granules stability and their nutrients removal efficiency were investigated.

The results indicated that high pH (greater than 9) had negative impacts on both granules stability and their nutrient removal efficiencies. Nitrogen and phosphorus removal efficiencies dropped significantly with no further recovery, while COD removal recovered to its initial values.

The experimental result indicated that low pH (as low as 6) had very minor impacts on granules structure and their nutrient removal capability. According to the changes in EPS composition (PN/PS ratio) and the concentration of the extracted EPS, the disintegration of granules under high pH was presumed to be due to the disturbance of the EPS matrix and subsequent weakening of granules structure.

The aforementioned results indicated the importance of pH variation which mostly happens in the treatment plants receiving industrial wastewater. Equalization tanks combined with neutralization of the wastewaters characterized by high pH (>9) could be considered as a solution to prevent or eliminate the failure of the granular system.

In the second phase of the experiment after implementing the lessons learned from the first experiment, no filament-dominated aggregates were observed in the reactors. Compact granules with simultaneous COD, ammonium and phosphorus removal were cultivated in two identical SBRs within 70 days, using acetate-based synthetic wastewater. After the dominance of mature granules in the systems, the influent of one of the reactors was changed from readily biodegradable acetate-based synthetic wastewater to municipal wastewater. Changing the growth medium resulted in partial disintegration of granules during the first two weeks; however, they recovered after two month of operation in the new growth medium (one month after disintegration). After changing the feed composition, granules maintained their high efficiencies in COD and ammonium removal while they lost their phosphorus removal with no further

recovery. The loss of phosphorus removal was presumed to be due to lack of available bCOD in the used municipal wastewater and PAOs were outcompeted by denitrifiers in using available bCOD. Comparing the granules grown on municipal and acetate-based wastewater, for two months, did not show any obvious difference in terms of their physical characteristics.

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Appendix A

A.1. EPS Measurement

A.1.1. Protein Measurement

After extracting EPS from granules following the method proposed by Adav and Lee, (2008), protein was measured using Modified Lowry Assay Kit with bovine serum albumin (BSA) as the standard solution. Different concentration of BSA was prepared using a stock BSA serum. The different prepared concentrations are shown in Table A.1.

Table A.1. The concentration of BSA solutions used to prepare the standard curve

Vial ID	Final concentration of BSA
A	1500 mg L ⁻¹
B	1000 mg L ⁻¹
C	750 mg L ⁻¹
D	500 mg L ⁻¹
E	250 mg L ⁻¹
F	125 mg L ⁻¹
G	0 mg L ⁻¹ = Blank

The absorbance of the prepared solutions with the known concentration of BSA was measured at wavelength of 750 nm using Novaspec Plus visible spectrophotometer, Biochrom, covering wavelength between 330 to 800nm. Distilled water was used instead of BSA contained solution

in order to prepare the blank solution. The achieved calibration curve for protein (absorbance versus concentration) is shown in Figure A.1.

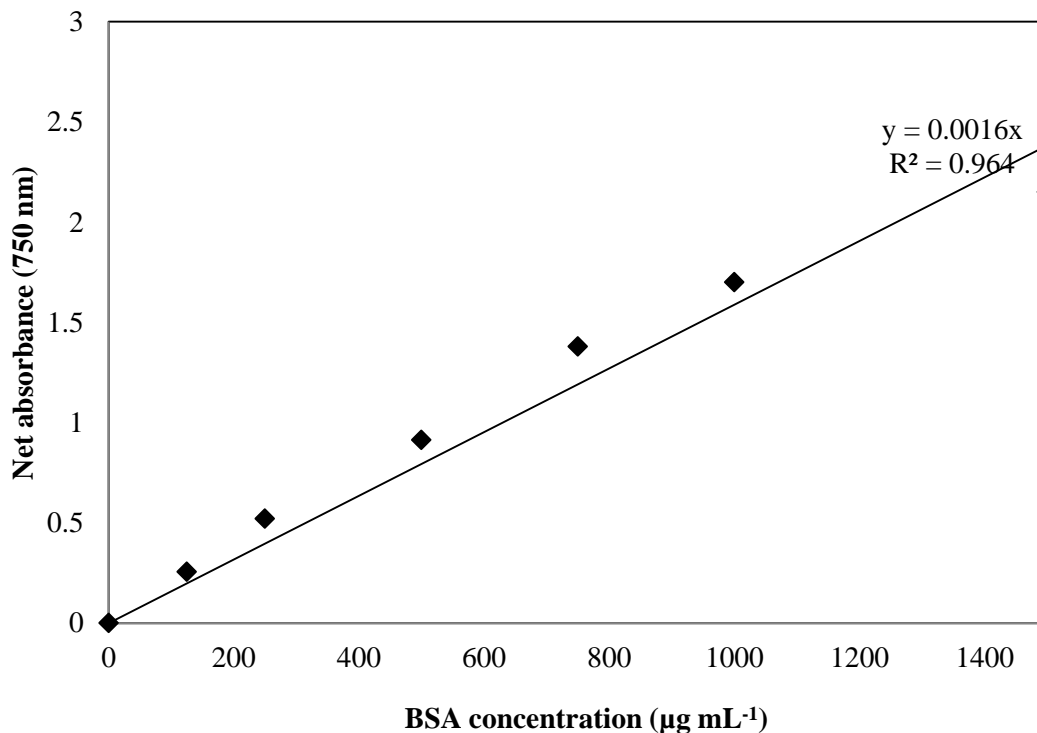


Figure A.1. Standard curve for protein measurement using BSA as standard

The absorbance of the unknown solutions was measured at 750 nm using spectrophotometer. The measured absorbance was used to calculate the unknown protein concentration using the prepared standard curve.

A.1.2. Polysaccharide Measurement

After extracting EPS, polysaccharide was measured using phenol-sulfuric acid colorimetric method and glucose was used as the standard solution. Different concentration of glucose was prepared using a stock solution of glucose. The prepared concentrations are shown in the Table A.2 below.

Table A.2. The concentration of glucose solutions used to prepare the standard curve

Vial ID	Final concentration
A	150 mg L ⁻¹
B	100 mg L ⁻¹
C	80 mg L ⁻¹
D	40 mg L ⁻¹
E	20 mg L ⁻¹
F	10 mg L ⁻¹
G	0 mg L ⁻¹ = Blank

The absorbance of the glucose solutions with the known concentrations was measured at wavelength of 490 nm using Novaspec Plus visible spectrophotometer, Biochrom, covering wavelength of 330-800nm. Distilled water was used instead of glucose contained solution in order to prepare the blank solution. The achieved calibration curve for polysaccharide is shown in Figure A.2.

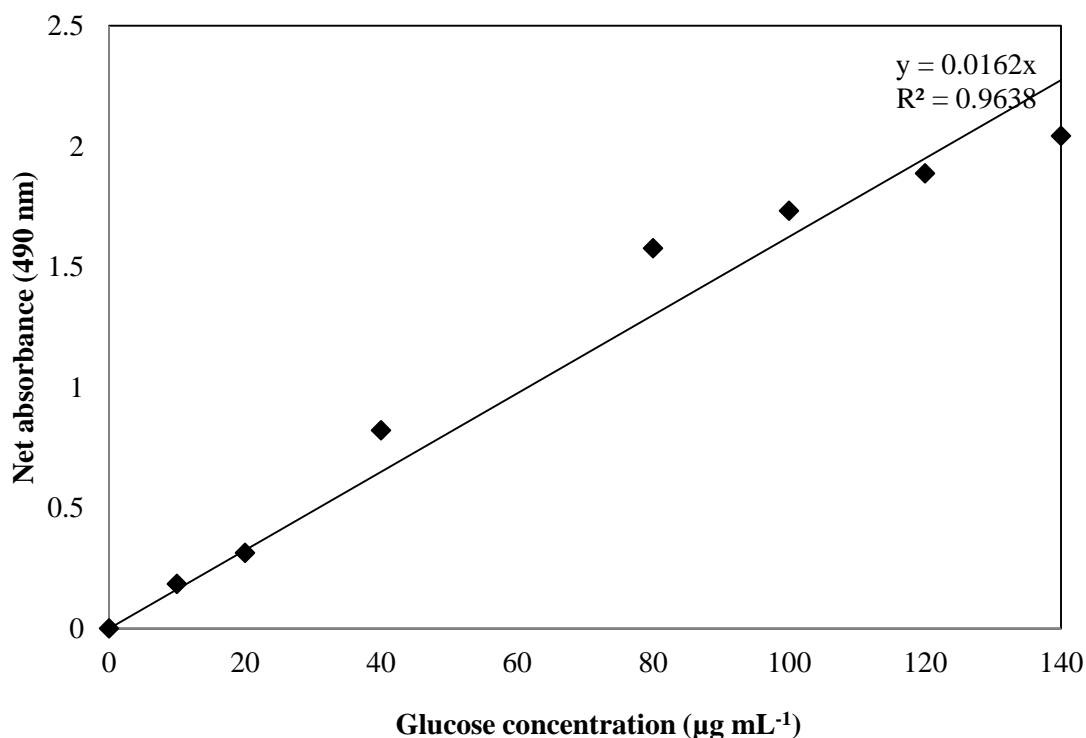


Figure A.2. Standard curve for polysaccharide measurement using glucose as standard

The absorbance of the unknown solution was measured at 490 nm after dilution with distilled water in a volumetric ratio of 1:1 (V: V). The measured absorbance was used to calculate the concentration of the unknown polysaccharide solution using the prepared standard curve with glucose.

A.2. Calculating Superficial Gas Velocity

The superficial gas velocity in the reactors was calculated by dividing the applied air flow rate by the surface area of the reactors.

$$\text{Superficial gas velocity (cm/s)} = \frac{\text{air flow rate (cm}^3/\text{s)}}{\text{surface area of the reactor (cm}^2\text{)}} \quad (\text{A-1})$$

A.3. Simultaneous Nitrification-Denitrification

In the first phase of the experiment, the bCOD concentration in the feed was close to 850 mg L⁻¹ and TN was 54 mg L⁻¹. The heterotrophic and autotrophic biomass growth were calculated according to Metcalf & Eddy et al., (2003) using Equations A-2 (Heterotrophic biomass growth) and A-3 (Autotrophic biomass growth).

$$\frac{QY(S_0 - S)}{1 + (k_d)SRT} \quad (\text{A-2})$$

$$\frac{QY_n(NO_x)}{1 + (k_{dn})SRT} \quad (\text{A-3})$$

Where, Y and Y_n are synthesis yield coefficient for heterotrophs and autotrophs, respectively. S₀ and S are the amount of bCOD in the feed and effluent, respectively. k_d and k_{dn} are endogenous decay coefficients for heterotrophs and autotrophs, respectively. The calculations are based on SRT of 8 days. Flowrate to the reactors (Q) per cycle was set at 2.85 L cycle⁻¹ with an influent bCOD (S₀) of 850 mg L⁻¹ and effluent bCOD (S) of 35 mg L⁻¹. The amount of NO_x-N was

assumed as 80% of the influent ammonium (54 mg L^{-1}). The kinetic coefficients are presented in Table A.3.

Table A.3. Kinetic coefficient for heterotrophs and autotrophs at 20°C (Metcalf & Eddy et al., 2003)

Parameters (at 20°C)	Value
$Y \text{ (gVSS g}^{-1}\text{bCOD)}$	0.40
$k_d \text{ (g VSS g}^{-1}\text{VSS d}^{-1}\text{)}$	0.12
$Y_n \text{ (g VSS g}^{-1}\text{NH}_4\text{-N)}$	0.12
$k_{dn} \text{ (g VSS g}^{-1}\text{VSS d}^{-1}\text{)}$	0.08

Using the formulas and assumptions presented above showed close to 483 mg VSS was produced in each cycle (9 mg cycle^{-1} of autotrophs and $474 \text{ mg cycle}^{-1}$ of heterotrophs). Considering the chemical formula of $\text{C}_5\text{H}_7\text{O}_2\text{N}$ as a representation of cells tissue, close to 12% of the biomass is composed of nitrogen. Therefore, for production of 483 mg biomass, almost 58 mg of nitrogen is required per cycle. The influent nitrogen is 154 mg per cycle. Considering 58 mg consumed for cells assimilation, the concentration of nitrogen in the reactor (with a working volume of 5.7 L) should be close to 17 mg L^{-1} while it was measured at 5 mg L^{-1} . The nitrogen mass balance shown above indicates the occurrence of simultaneous nitrification-denitrification in the system, in which almost 68 mg of nitrogen (12 mg L^{-1}) is removed.