THE EFFECTS OF SEED SLUDGE TYPE AND ANOXIC/AEROBIC PERIOD SEQUENCE ON AEROBIC GRANULATION AND COD, N TREATMENT PERFORMANCE

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submitted by **YUSUF ÇAĞATAY ERŞAN** in partial fulfillment of the requirements for the degree of **Master of Science in Environmental Engineering Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen ______________________
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Faika Dilek Sanin ______________________
Head of Department, **Environmental Engineering**

Assist. Prof. Dr. Tuba Hande Ergüder ______________________
Supervisor, **Environmental Engineering Dept., METU**

Examining Committee Members:

Prof. Dr. Celal Ferdi Gökçay ______________________
Environmental Engineering Dept., METU

Assist. Prof. Dr. Tuba Hande Ergüder ______________________
Environmental Engineering Dept., METU

Prof. Dr. Filiz Bengü Dilek ______________________
Environmental Engineering Dept., METU

Prof. Dr. Faika Dilek Sanin ______________________
Environmental Engineering Dept., METU

Assist. Prof. Dr. Can Özen ______________________
Biotechnology Dept., METU

Date: January 16, 2013

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all the material and results that are not original to this work.

Name, Last name: Yusuf Çağatay Erşan

Signature:

ABSTRACT

THE EFFECTS OF SEED SLUDGE TYPE AND ANOXIC/AEROBIC PERIOD SEQUENCE ON AEROBIC GRANULATION AND COD, N TREATMENT PERFORMANCE

Erşan, Yusuf Çağatay
M.S., Department of Environmental Engineering
Supervisor: Assist. Prof. Dr. Tuba H. Ergüder

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The aim of this master thesis study was improvement of the required operational conditions for aerobic granulation in sequencing batch reactors (SBRs).

In the first part of the study, membrane bioreactor sludge (MBS) and conventional activated sludge (CAS), were used to investigate the effect of suspended seed sludge type on granulation in SBRs. The MBS granules were found to be advantageous in terms of size, resistance to toxic effects, stability and recovery compared to CAS granules. During non-inhibitory conditions, sCOD removal efficiencies were 70±13% and 67±11% for MBS and CAS, and total nitrogen (TN) removal efficiencies were 38±8% and 26±8%, respectively.

In the second part of the study, the effects of period sequence (anoxic-aerobic and aerobic-anoxic) on aerobic granulation from MBS, and sCOD, N removal efficiencies were investigated. Granules developed in anoxic-aerobic period sequence were more stable and larger (1.8-3.5 mm) than granules developed in aerobic-anoxic sequence. Under steady conditions, almost 95% sCOD, 90% Total Ammonia Nitrogen (TAN) and around 39-47 % of TN removal was achieved. Almost 100% denitrification in anoxic period was achieved in anoxic-aerobic period sequence and it was observed around 40% in aerobic-anoxic period sequence.

The effects of influent sulfate (from 35.1 mg/L to 70.2 mg/L) on treatment efficiencies of aerobic granules were also investigated. The influent SO_4^{2-} concentrations of 52.6 mg/L to 70.2 mg/L promoted sulfate reduction. The produced sulfide (0.24 mg/L to 0.62 mg/L) inhibited the ammonia-oxidizing bacteria (AOB) performance by 10 to 50%.

Keywords: Extracellular Polymeric Substances (EPS), simultaneous nitrification-denitrification, inhibition, recovery, high loading rate.

ÖZ

AŞI ÇAMUR TİPİ VE ANOKSİK/AEROBİK PERİYOT SIRALAMASININ AEROBİK GRANÜLASYON VE KOİ, N ARITIM PERFORMANSINA ETKİSİ

Erşan, Yusuf Çağatay
Yüksek Lisans Tezi, Çevre Mühendisliği Bölümü
Tez Danışmanı: Yrd. Doç. Dr. Tuba H. Ergüder

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Bu yüksek lisans tezinin amacı ardışık kesikli reaktörlerde (AKR) aerobik granülasyon için gerekli işletim koşullarının iyileştirilmesidir.

Çalışmanın birinci bölümünde, membran biyoreaktör çamuru (MBÇ) ve konvansiyonel aktif çamur (AÇ), askıda aşı çamuru tipinin AKR'lerde granülasyona etkisini araştırmak amacıyla kullanılmıştır. MBÇ granüllerinin büyüklük, toksik etkilere dayanıklılık, stabilite ve iyileşme açısından AÇ granüllerinden daha avantajlı olduğu ortaya çıkarılmıştır. İnhibisyon olmayan koşullarda, çKOİ giderim verimleri MBÇ ve AÇ için sırasıyla %70±13 ve %67±11 ve toplam azot (TN) giderim verimleri de sırasıyla %38±8 ve %26±8 olmuştur.

Çalışmanın ikinci bölümünde, periyot sıralamasının (anoksik-aerobik ve aerobik-anoksik) MBÇ'den aerobik granülasyona ve çKOİ, N giderim verimlerine etkisi incelenmiştir. Anoksik-aerobik periyotta üretilen granüller aerobik-anoksik periyotta üretilenlerden daha stabil ve büyük (1,8-3,5 mm) olmuştur. Kararlı koşullarda, her iki reaktörde de yaklaşık %95 çKOİ, %90 TAN ve %39-47 TN giderimi elde edilmiştir. Anoksik-aerobik periyot sıralamasında anoksik periyotta yaklaşık %100 denitrifikasyon elde edilmiştir ve bu aerobik-anoksik işletimde 40%olarak gözlenmiştir.

Giriş suyundaki sülfatın (35,1 mg/L'den 70,1 mg/L ye) granüllerin arıtım verimleri üzerindeki etkileri incelenmiştir. Giriş SO_4^{2-} derişimleri 52,6 mg/L ile 70,2 mg/L arasındayken reaktör içerisinde sülfat indirgenmesi artmıştır. Üretilen sülfür (0,24 mg/L'den 0,62 mg/L'ye) amonyak oksitleyen bakterilerin (AOB) performansını %10'dan 50'ye kadar inhibe etmiştir.

Anahtar kelimeler: Hücre dışı polimer madde (EPS), simultane nitrifikasyon-denitrifikasyon, inhibisyon, iyileşme, yüksek yükleme hızı.

To My Beloved Family and Eternal Love…

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ABBREVIATIONS

AOB	Ammonia-oxidizing Bacteria
CAS	Conventional activated sludge
CMFR	Completely mixed flow reactor
CASP	Conventional activated sludge process
COD	Chemical oxygen demand
C/N	Carbon to nitrogen ratio
DO	Dissolved oxygen
DGGE	Denaturing gradient gel electrophoresis
EPS	Extracellular polymeric substance
FA	Free-ammonia (NH_3-N)
H/D	Height to diameter ratio
HRT	Hydraulic retention time
IA	Image analyzer
LB	Loosely bound
LR	Loading rate
MBR	Membrane bioreactor
MBS	Membrane bioreactor sludge
METU	Middle East Technical University
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
NOB	Nitrite-oxidizing bacteria
OLR	Organic loading rate
OUR	Oxygen uptake rate
PN	Protein
PS	Polysaccharide
RE	Removal efficiency
SAV	Superficial air velocity
SBR	Sequencing batch reactor
sCOD	Soluble chemical oxygen demand
SEM	Scanning electron microscope
SNDN	Simultaneous nitrification-denitrification
SOUR	Specific oxygen uptake rate
SRB	Sulfate reducing bacteria
SRT	Solid retention time
SVI	Sludge volume index
TAN	Total ammonia nitrogen (NH_4-N + NH_3-N)
TB	Tightly bound
TN	Total nitrogen
TON	Total oxidized nitrogen
USB	Upflow sludge blanket
UASB	Upflow anaerobic sludge blanket

CHAPTER 1

INTRODUCTION

Granular sludge technology had started its historical progress in 1980's via anaerobic systems. Basic concept behind the granular sludge formation (development of granules from suspended culture) is microbial aggregation that cells come together and form quite stable, concentrate microbial community under stressful operational conditions (Calleja, 1984). Granular sludge can be categorized as aerobic and anaerobic granules. Granular sludge contains various types of species and numbers of microorganisms per gram biomass in a compact form. The compactness and high amount of microorganisms make granular sludge advantageous over suspended sludge in terms of their density, structural properties, settling velocities and resistance to toxicity (Liu and Tay, 2004). Furthermore, since they are easily separable from water media and they contain large amount of microorganisms in smaller volumes, 75% saving from construction site and in turn 20% saving from total cost is possible (Beun et al., 1999)

Biogranulation was first studiead anaerobically and anaerobic granules were first reported by Lettinga et al. (1980). Anaerobic granules were investigated and, according to our knowledge, the applications in upflow anaerobic sludge blanket (UASB) reactors were found as best in terms of cultivation of granules. UASB reactors packed with anaerobic granules became very useful in industrial wastewater treatment since high amount of biomass could be operated in compact form, which enabled the treatment of high amount of wastewaters. In addition to that, anaerobic granules are more resistant to toxic metals, shock loads and influent fluctuations which are among the properties of industrial wastewaters, than the suspended sludge (Liu and Tay, 2004). As it was expected, the feasibility and efficiency of anaerobic granular sludge's applications and its modifications had been proven via treating various types of wastewater by industrial-scale systems (Lettinga et al, 1980; Fang and Chui 1993; Schmidt and Ahring, 1996). Yet, anaerobic granules have major drawbacks; start-up period takes long time, nutrient removal (N and P) is not possible and low-strength wastewater treatment might not be that efficient (Liu and Tay, 2004). These drawbacks let scientists to search aerobic granulation. Mishima and Nakamura (1991) reported the first aerobic granules having 2 – 8 mm diameter size in aerobic upflow sludge blanket (USB) reactors. Within the scope of available literature information, almost all aerobic granules were cultivated later on in laboratory-scale sequencing batch reactors (SBRs) (Morgenroth et al., 1997; Beun et al., 1999; Peng et al., 1999; Etterer and Wilderer, 2001; Tay et al., 2001a; Liu and Tay, 2002; Su and Yu, 2005; Adav et al., 2008). Since one of the advantages of aerobic granules to anaerobic granules is nutrient removal (Liu and Tay, 2004), aerobic granules capable of treating nutrients (N, P) were developed. Furthermore, aerobic granules even treating high-strength wastewaters containing toxic compounds as well as nutrient removal were cultivated (Tay et al., 2001a; Adav et al., 2007b; Adav and Lee, 2008; Wang et al., 2009).

Pilot-scale installations for aerobic granular sludge development started a decade ago. These successful installations revealed that wastewater treatment with aerobic granular sludge is a promising technology providing efficient organic and nutrient removal (Ni et al., 2009; Gao et al., 2011; Giesen et al., 2012). However, to our knowledge, there are only 2 municipal plants (1 pilot, 1 full-scale) and 3 industrial pilot plants started to operate since 2005 (Giesen et al., 2012). Main reason of this few number of installation is explained with the low stability of aerobic granules due to high growth rates of heterotrophic microorganisms (Adav et al., 2008). Therefore, in order to avoid weaknesses and improve sustainability of aerobic granular systems, studies continue to investigate the problems encountered with stability.

The strategies to improve the stability of the aerobic granules developed in SBRs are well reviewed by Adav et al. (2008), Lee et al. (2010) and Nor-Anuar et al. (2012). One of the main parameter improving stability is stated as the flocculating capability of strains in seed (Lee et al., 2010). Extracellular Polymeric Substance (EPS) production and predominant EPS content are claimed to be other important parameters (Adav et al., 2008). Liu and Tay (2008) stated that reasonable starvation time also promotes long-term stability. With the studies focusing on nutrient removal with aerobic granules, an additional parameter, non-aerated periods, came into consideration (Jang et al., 2003; Qin and Liu, 2006; Wang et al., 2007; Yilmaz et al., 2007; Wan et al., 2009). The studies investigating the effect of these oxygen-lacking periods on granulation proved that the presence of anoxic and/or anaerobic periods in an SBR cycle is beneficial to enhance granular density, compactness and microbial diversity (Wan et al., 2009; Pijuan et al., 2009; Su et al., 2012).

Among the parameters cited above, two important parameters that can affect the development of aerobic granular sludge from suspended sludge and their nutrient (N, P) removal efficiency are seed sludge type and non-aerated periods. Seed sludge is important in terms of its morphological properties and EPS content to cultivate granules. Non-aerated periods are important in terms of SBR's operational conditions, formation of stress conditions for granulation and treatment performances. Yet, to our knowledge, there are unanswered questions in literature. One of the questions is "how does seed sludge type affect granule development, granular stability and granular treatment performances?" and the other is "how does non-aerated period sequence affect granule development, granular stability and treatment performance". Therefore, the main objectives of this thesis study are to investigate;

- The effects of seed sludge type on
    - development of aerobic granular sludge from suspended sludge,
    - treatment performances of cultivated granules,
- The effects of anoxic/aerobic period sequence on
    - development of aerobic granular sludge from suspended sludge,
    - COD, N treatment performances of cultivated granules.

Additionally, with the inspiration drawn from the results of the studies above

- The effects of influent sulfate and soluble sulfide concentrations on treatment performances of cultivated granules and granular structure,

were also investigated.

The results are expected to fill the gaps in the aerobic granulation field about the effects of seed sludge type and non-aerated periods on granulation, thus contribute to the stable granular sludge cultivation and enhance treatment performances.

CHAPTER 2

LITERATURE REVIEW

In this chapter, theoretical background information on aerobic granulation technology is given. In particular, the factors affecting aerobic granulation, the applications with aerobic granular sludge and other relevant literature information related to the thesis study are provided.

2.1. Aerobic Granulation Mechanism

Granular sludge differs from flocculent sludge with its round shape and clear outline (Gao et al, 2011). The color of the aerobic granules was mostly defined as yellow (Zheng et al., 2006). Size is an important parameter for discrimination of flocculent sludge and granule. Flocs with sizes larger than 200 µm are defined as granules (Beun et al. 1999; Gao et al. 2011).

Tay et al. (2001a) investigated aerobic granule formation procedure microscopically. Their aim was to obtain visual evidence for aerobic granulation progress. Two SBRs were operated after seeding with conventional activated sludge (CAS) having filaments. Granulation progress was observed via optical microscopy, image analyzer (IA) and scanning electron microscope (SEM) (
Figure 2.1). At the end of the first week, sludge became denser and aggregates were formed. In the second week, granules with apparent round outer shape developed in both reactors. At the end of the third week, mature granules, which had more regular outer shape and homogenous morphology, were obtained. Granulation process was described to follow the path shown in
Figure 2.1;

- development of seed sludge to dense aggregates,
- formation of granules via continuous aggregation,
- development of granular sludge to mature granules.

It was stated that cell structure became tighter and the rod-like species, which did not exist in seed sludge, appeared; proving the basic theory behind granulation (Tay et al. 2001a).

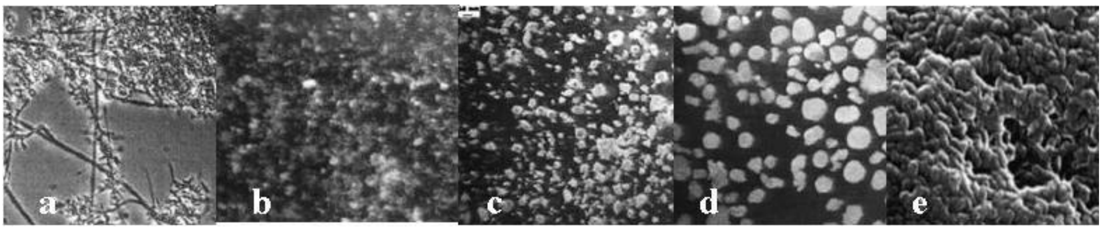

Figure 2.1 Granulation mechanism
a- seed sludge (optical microscopy), b- 1st week of operation (image analysis),
c- 2nd week (image analysis), d- 3rd week (image analysis), e- mature granules (SEM) (Tat et al., 2001a)

Bacteria produce extracellular polymeric substance (EPS) which play crucial role in development of aggregates (Tay et al., 2001b). EPS forms a three dimensional structure via physically or electrostatically bridging with each other and enable cells to attach and aggregate (Ross, 1984). These aggregates contain high amount of biomass and spherical outer shape. According to Quarmby and Forster (1995), EPS deficiency causes weakness in aerobic granules. It is indicated that as stress conditions on microorganisms increase, EPS release and production also increase (Liu et al., 2004a). However, excess EPS production was claimed to decline mass transfer efficiency of substrates by clogging the pores of aggregates (Mu et al., 2006; Zheng and Yu, 2007; Sheng et al., 2010b). Detailed information about the effects of EPS on granulation is given in Section 2.2.9.

In addition to EPS, there are other factors that might affect the granulation process such as superficial air velocity, reactor configuration, exchange ratio, starvation. Detailed information is given in the following section (Section 2.2)

2.2. Factors Affecting Aerobic Granulation

It is known that development of a technology highly depends on research and minimization of unknowns in the phenomena. At the beginning of 1990's, researchers started to investigate factors affecting granulation and granule properties. Granulation was thought to be affected by a number of operational parameters such as substrate composition, organic loading rate (OLR), settling time, reactor design, volumetric exchange ratio, hydraulic retention time (HRT), feeding strategy, superficial air velocity (SAV), starvation, oxygen deficiency and seed sludge type. These factors are discussed in the following sections.

2.2.1. Substrate Type

2.2.1.1. Synthetic wastewater – Carbon Source Type

Progress in granulation is mainly depended on aggregation of microorganisms, therefore carbon source is found to be an insignificant parameter in granulation process. Carbon source was stated to be affecting the dominant species composition in the granules and granule properties such as stability (Tay et al., 2001a; Sun et al., 2006). Studies (Liu and Tay, 2004; Zheng et al., 2005; Adav et al., 2008a) revealed that there is no specific carbon source for granule cultivation (development of granules from suspended culture). Various carbon sources were used to develop aerobic granular sludge such as glucose, acetate, phenol, starch, ethanol, molasses, sucrose (Liu and Tay, 2004; Zheng et al., 2005; Adav et al., 2008a).

In the study of Tay et al. (2001a), two different carbon sources, acetate and glucose, were used to cultivate aerobic granules in two identical SBRs. Conventional activated sludge (CAS) having filaments was used as seed sludge. It was stated based on image analysis results that at the first week filamentous sludge in acetate-fed reactor vanished, but it still existed in glucose-fed reactor. In both reactors, developed sludge was more compact and denser compared to the seed sludge at the end of the first week. It was stated that filamentous sludge still existed at the second week in glucose-fed reactor; but aggregates had filamentous-free view in acetate-fed reactor. In the third week, main difference between the mature granules cultivated with acetate and glucose was clarified in terms of compactness and outer shape. Acetate-fed sludge became rigid granules having smooth outer shape, whereas glucose-fed sludge became loose granules having fluffy outer shape. Since there is no granulation in CAS process, it is indicated that main factor encouraging aggregation is SBR operation which includes periodic operation but not the carbon source type. As the SBR operation proceeds, the microbial structure significantly approaches to granular form in terms of compactness and regular shape. The former is defined as the higher microorganism content in smaller volume (Liu and Tay, 2004).

In another study (Sun et al., 2006), 4 different substrate types, acetate, glucose, peptone and fecula were investigated. Aerobic granules were cultivated from all of the substrates. Granules cultivated from peptone were found as the most stable ones because of their higher settling velocities. Main reason behind cultivating stable granules with peptone was correlated with the study of van Loosdrecht et al. (1995) investigating the relation between the stability and the bacterial growth rate. It was stated that substrate gradient had a crucial role in stability of aerobic granules. As substrate gradient increased inside the granule, heterogeneity occurred which would lead to the enrichment of floc-like bacteria thus decreased granular stability. Yet, when the substrate gradient was small, slow-growing bacteria would become dominant and granule's stability increased. Therefore, using peptone which is hardly biodegradable substrate compared to acetate and glucose, selected the slow-growing microorganisms and increased stability. Liu et al. (2004b) also suggested that to improve granular stability selection of slow-growing nitrifying bacteria would be better.

2.2.1.2. Real Wastewater

There are studies in which real wastewater such as textile, soybean, dairy, brewery sanitary, palm oil mill wastewater, refineries and municipal wastewater are used for the development of granules (de Kreuk and van Loosdrecht, 2006; Schwarzenbeck et al., 2005; Su and Yu, 2005; Adav et al., 2008a; Ni et al., 2009; Gobi et al., 2011). In almost all of the studies, granules were cultivated from suspended sludge in SBRs of similar operation. This indicates that substrate or wastewater type has no or little effect on granulation process.

De Kreuk and van Loosdrecht (2006) experimented domestic sewage to investigate if it would be proper to achieve aerobic granulation in SBR. After 20 days of operation 1.1 mm sized aerobic granules were achieved from domestic sewage.

Arrojo et al. (2004) cultivated granular sludge by using dairy wastewater. Granules having average sizes of 1.05 mm were achieved after three weeks operation. At Day 23, granules with sizes of 1.2 – 3mm were mostly abounded. At day 219, granules with sizes of 2.4-4 mm were abounded. Not only cultivation of granules but also C and N removals of granules were investigated. Granules capable of 80% N and 70% C removal were cultivated.

Su and Yu (2005) developed aerobic granules from soybean-processing wastewater. At week 2, size of the cultivated granules was 0.3 mm and at Day 46 it became 0.9 mm. Mean size of the matured granules was 1.22±0.85 mm. Treatment performances of granules were investigated in terms of COD removal and reported as 98 – 99 %.

Wang et al. (2007a) reported that aerobic granules of 2- 7 mm were obtained after 9 weeks of SBR operation with brewery wastewater. The treatment performances of granules were considerably high; 88% COD, 89% NH_4-N removal efficiencies were reported.

Muda et al. (2010) developed granular sludge with textile wastewater as the sole carbon source. Granule size increase from 0.2 mm to 2.3 mm was reported. COD removal efficiency of 94 - 95% and even color removal efficiency of 62% were reported.

2.2.2. Organic Loading Rate (OLR)

Organic loading rate (OLR) is a decisive operational parameter affecting the aerobic granulation process and in turn, the characteristics and the treatment performances of granules (Liu and Tay, 2004). It mostly affects the cell hydrophobicity and EPS production which are thought essential for cell aggregation and stability (Tay et al., 2004a,b). Therefore, in order to have more compact, stable and stronger granules as well as higher treatment efficiencies, OLR should also be taken into consideration.

It was stated that low OLR such as 1 kg COD/m^3.d is not appropriate for granule formation. On the other hand, at high OLR such as 9 kg COD/m^3.d, highly compact granules can be cultivated, yet this highly compact structure prevents oxygen diffusion to the deeper parts and causes disintegration of granules due to oxygen deficiency in the core of the granules (Moy et al., 2002; Liu et al. 2003a; Tay et al., 2004a,b). Moy et al. (2002) investigated the effect of high OLR on aerobic granule properties. They developed acetate-fed granules and glucose-fed granules in two identical SBRs. After granular performance reached steady-state, OLR was increased from 6 kg COD/m^3.d to 15 kg COD/m^3.d gradually and differences in granule properties were observed. It was claimed that acetate-fed granules disintegrated at 9 kg COD/m^3.d since they had compact structure which limited the substrate penetration in contrast to glucose-fed granules which had looser structure that delayed the substrate diffusion limitation and sustained the maximum OLR tested (15 kg COD/m^3.d).

Although it cleared some points, Moy et al.'s study (2002) could not explain the role of OLR on granulation process. That is why, Tay et al. (2004a) performed a detailed study on the effect of OLR on aerobic granulation. They had operated 4 identical SBRs with OLRs of 1, 2, 4 and 8 kg COD/m^3.d. It was reported that aerobic granules could not be obtained at low OLRs (1 and 2 kg COD/m^3.d). In the reactor operated at higher OLR of 4 kg COD/m^3.d, stable aerobic development was reported. In the reactor operated at the highest OLR (8 kg COD/m^3.d), granules were developed but they were not stable and thus easily washed out. Muda et al. (2011) revealed that there was a significant relation between OLR and HRT. They reported that increasing HRT caused decrease in OLR. The decrease in OLR caused deterioration of granular properties, i.e. biomass content (MLVSS) of granules decreased, the average granule size decreased, settling ability of the granules worsened, biologic activity and oxygen uptake rate (OUR) of granules decreased. On the contrary, at constant HRT, they increased OLR and observed that biomass concentration, mean granule size, settling ability and OUR of granules improved. The authors concluded that granulation can be favored and treatment performance of granular sludge can be enhanced by choosing an optimal OLR. After influence of OLR on aerobic granulation process was clearly understood, Tay et al. (2004b) investigated the effects of OLRs of 1, 2, 4 and 8 kg COD/m^3.d on granular characteristics. Experimental set-up same with the study of Tay et al. (2004a) was conducted and specific gravities, strength, cell hydrophobicities and EPS production of granules were compared. Granular sludge could not be developed at OLRs of 1 and 2 kg/m^3.d. Granules cultivated at an OLR of 4 kg COD/m^3.d were found to have higher specific gravity of 1.064, higher strength with an integrated coefficient of 99.5%, 75% higher cell surface hydrophobicity and higher polysaccharide/protein (PS/PN) ratio compared to looser and more amorphous flocs developed at 8 kg COD /m^3.d.

2.2.3. Reactor Configuration

Reactor configuration is thought to be an effective parameter for granulation. Reactor type is important in terms of the relation between microbial aggregation and liquid flow pattern, in other words, hydraulic behavior in the reactor and its role on microbial aggregation (Liu and Tay, 2004). Almost all aerobic granule cultivation studies performed in SBRs. The stress-related parameters such as settling time in SBRs, height/diameter ratio, and hydraulic shear force let microorganisms produce more EPS and hence ease the aggregation and granule cultivation (Liu and Tay, 2004). Since SBR operation is suitable for control of stress-related parameters on microorganisms, granule cultivation is easier in SBR (Liu et al., 2004a; Liu and Tay, 2004). In other reactor configurations such as completely mixed flow reactor (CMFR) and membrane bioreactor (MBR), no granulation has been reported. However, there are applications such as seeding MBR directly with granular sludge for treatment purposes (Wang et al, 2008a; Li et al., 2007a). It was stated that MBR is an appropriate reactor configuration for preserving granular sludge properties and activity only if operational conditions are modified to preserve rod-like species, thus granular structure (Li et al., 2007a).

Column type reactor differs from CMFR in terms of interaction between flocs and flow. It was stated that column type reactor is suitable for hydraulic attrition which makes flocs and granules in round shape and increases their compactness, since relatively homogenous circular flow and local vortex along reactor column is originated by upflow air or liquid flow, the latter for anaerobic granulation (Liu and Tay 2004). In CMFR, on the other hand, microbial colonies scatter with randomly distributed flow. Hydraulic shear force is distributed along any direction. Thus, only irregular shaped flocs are formed and aggregates could not progress to granule in CMFRs (Liu and Tay, 2002).

Height to diameter ratio (H/D) of the reactor is stated as an important parameter in SBR configuration, since it influences the hydraulic properties and the selective stress conditions such as settling time particular to SBR operation (Beun et al., 1999). Furthermore, H/D in SBR affects minimal settling velocity which is defined as the minimum settling velocity of aggregates required not to be washed out from the reactor. It is stated that minimal settling velocity should be 10 m/h to cultivate granules and it is believed that cultivation of aggregates with high settling velocity can be promoted by using reactors having higher H/D ratio (Heijnen and van Loosdrecht, 1998). It was reported that higher H/D such as 20 – 30 enhanced the effectiveness of selective pressure and increased the settling velocity of aggregates, hence granulation in SBR (Heijnen and van Loosdrecht, 1998; Beun et al., 1999). Furthermore higher H/D is thought to provide a longer circular flow trajectory and more uniform hydraulic shear force which enhance granulation (Liu and Tay, 2002). In contrast to ideas about H/D, Kong et al. (2009) reported that granulation was successfully observed in all four reactors with 4 different H/D ratios from 4 to 24. It was established that species forming the granules developed at different H/D ratios might have been different but granular properties showed almost same results. They implied that settling time was a crucial parameter, but minimal settling velocity and H/D ratio of the reactor had no influence on granulation process and granule properties (Kong et al., 2009).

2.2.4. Hydraulic Selection Pressure

Hydraulic selection pressure is another parameter that affects granulation. Hydraulic selection on bacterial community is mainly provided by settling period, hydraulic retention time and exchange ratio of the SBRs (Liu and Tay, 2004; Gao et al., 2011). They are all related with each other.

2.2.4.1. Settling Period (Settling Time)

In SBR operation settling period is the last period before effluent withdrawal and the main step is the segregation of effluent from sludge. Literature information reveals that settling time takes role in progress of granulation (Liu and Tay, 2004). Short settling period eliminates species with poor settling ability and encourages the growth of easily settleable microorganisms and this phenomenon is defined as hydraulic selection (Liu and Tay, 2004). In addition, stronger and larger granules with higher settleability can be obtained since decreasing settling time helps the wash-out of non-flocculating microorganisms from the system (Adav et al., 2009a).

Qin et al. (2004b) investigated the effect of settling time on granulation. They concluded that weak selection pressure which means long settling period (more than 15 min), does not favor aerobic granulation. It was stated that short settling time of 5 min increased EPS production and cell hydrophobicity in addition to microbial activity. These hydraulic selection-related changes were believed to reinforce aerobic granulation (Qin et al., 2004a). In another study, Qin et al. (2004b) operated 4 different SBRs with different settling times such as 20 min, 15 min, 10 min and 5 min and investigated the sludge properties. It was reported that reactor with the shortest settling time (5 min) became granular sludge-dominated unlike the others. Authors reported that granular sludge percentage decreased when settling time increased. Following this outcome, settling times of the other SBRs also shortened down to 1 min and conversion from suspended sludge to granular sludge was observed in the reactors. It was stated that settling time also played crucial role in accumulation of calcium in aerobic granules which is likely to influence the granule properties (Qin et al., 2004b). In their study Adav et al. (2009a) operated 3 identical SBRs with different settling times. In addition to the similar results as those of Qin et al.'s study (2004b), the microbial community change was reported via denaturing gradient gel electrophoresis (DGGE) method. It was reported that microbial community changed for the different settling times studied. Decrease in settling time selects highly settleable microbial communities such as *Z.resiniphila, C.gleum* and *Rhodocyclales* from the seed sludge. Mostly non-flocculating strains such as *Pseudomonas* and *Flavobacterium* were washed out with short settling time which favored granulation (Adav et al., 2009a).

2.2.4.2. Volumetric Exchange Ratio

Another important parameter directly related with the hydraulic selection and in turn the aerobic granulation is exchange ratio of the SBR. Volumetric exchange ratio is defined as the ratio of the discharge port from the water surface level (L), to the water height of the reactor (H) (Figure 2.2). In other words, the exchange ratio is the volume of the discharged water divided to the liquid volume of the SBR (Liu and Tay, 2004).

In literature (Tay et al. 2001a, Wang et al. 2007; Yilmaz et al. 2007; Wan et al. 2009; Adav et al. 2009b; Zhang et al., 2012; Su et al. 2012) mostly 35-50% exchange ratio was suggested for development of granular sludge.

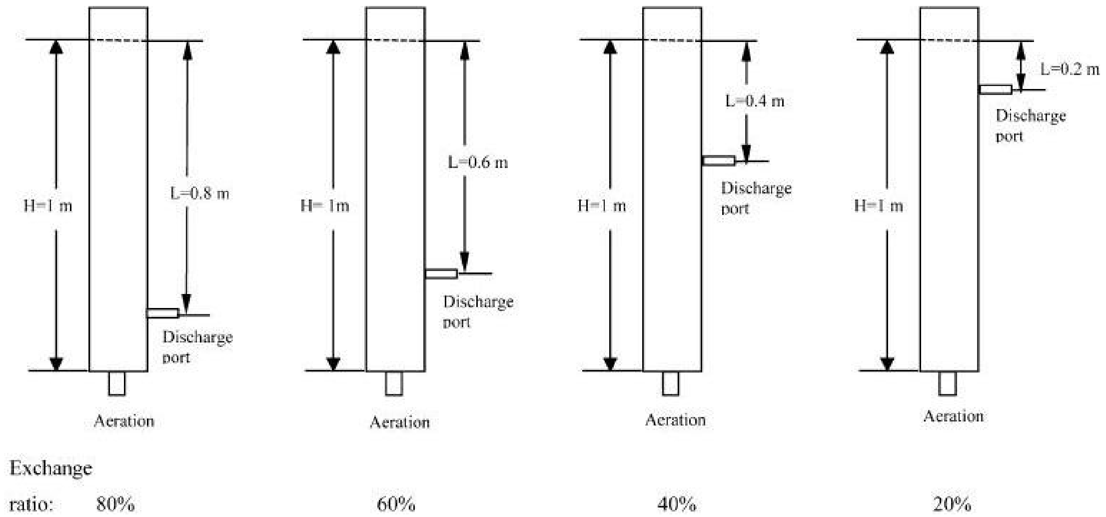

Figure 2.2 Different Exchange Ratios (L/H) of SBR (Liu et al., 2005c)

Effect of volumetric exchange ratio at a constant settling time was investigated by Wang et al. (2006b). They investigated exchange ratios of 20% to 80% with 20% increments. Granular sludge was reported to dominate in reactors operated with 60% and 80% exchange ratios. Investigated volumetric exchange ratios less than 60% were found inappropriate for full granulation, since reactor contents were mixture of suspended and granular sludge. Time required for granulation was also investigated. It was reported that granules were seen in 20 days in the reactor operated with 20% volumetric exchange ratio while granules were cultivated in 2 days in the reactor operated with 80% volumetric exchange ratio. It was concluded that increase of exchange ratio of reactors decreased the time of granule cultivation. It was also stated that increase in exchange ratio increased the granule percentage (granular portion of total sludge) in the reactors. Additionally, it was reported that as the exchange ratio increased, calcium accumulation in granules increased. Calcium ions were reported to be used in EPS production, thus it could be said that higher exchange ratio enhanced EPS production and fastened granulation.

Liu et al. (2005c) found consistent results with Wang et al. (2006b) and stated that higher exchange ratio required higher settling velocity, thus bioparticles were selected according to their settling velocity, shape, compactness and density. According to Stoke's law, aggregates which are heavy (dense) and with round shape settle faster than aggregates which are irregularly shaped, light and tiny. Therefore spherical bioparticles were selected and fluffy, light aggregates were discharged from the system.

De Clippeleir et al. (2009) investigated the volumetric exchange ratio in SBR operation for N-removing granule cultivation. It was stated that the volumetric exchange ratio should be determined by considering the growth rate of the enriching microorganism; the lower the growth rate the lower the exchange ratio to avoid the wash-out of microorganisms. Authors investigated cultivation of granules containing anaerobic ammonia oxidizing bacteria and aerobic ammonia oxidizing bacteria at the same time. Therefore, they found out that low volumetric exchange ratio of 25% was essential to facilitate a fast start-up for nitrogen removal. It was reported that operation at 25% exchange ratio shortened the time required for cultivation of N-removing microorganisms (both aerobic and anaerobic) and in turn N removal. However, 25% exchange ratio delayed the stable granular sludge formation and granular sludge dominated the system after 135 days of operation. It can be said that although it has some benefits it takes long time to develop granular sludge at 25% exchange ratio operation.

2.2.4.3. Hydraulic Retention Time (HRT)

Hydraulic selection is important in granulation process since it suppresses suspended growth and eliminates non-flocculating microorganisms as it was previously mentioned. Frequency of hydraulic selection is related with the hydraulic retention time (HRT) of the SBR. Therefore, HRT has indirect effect on hydraulic selection, thus granulation by avoiding floaty and dispersed sludge with frequent washout mechanism. The washout mechanism suppresses the suspended sludge accumulation in the reactors. At the end of each SBR cycle granular sludge is kept in the reactor and rest is withdrawn from the system based on the determined exchange ratio and the period of settling time (Beun et al, 1999; Liu and Tay, 2004).

In their study, Liu and Tay (2004) suggested that, HRT should be set carefully and it should be neither too long to enable suspended growth nor too short to prevent microbial growth. Liu and Tay (2008) suggested HRT of 8 h to develop stable granules. Muda et al. (2011) claimed that increasing HRT was an indirect way of decreasing OLR, thus granular properties such as settleability, stability, OUR decreased alike in the case of OLR decrease. It was also stated that at constant OLR, stable granules can be developed at HRTs of 6 h to 24 h. Beun et al. (1999) stated that, critical point for HRT can be determined by using $1/\mu_{max}$. In order to suppress suspended biomass growth, HRT values lower than $1/\mu_{max}$ should be used. Muda et al. (2011) investigated the properties and the performances of aerobic granules in treating textile wastewater at varying HRTs. It was stated that biomass concentration, mean granule size, settling ability, and OUR decreased with the increasing HRT from 6 h to 24 h. It was concluded that, increase in HRT (decreasing OLR) caused reduction in biomass content. Settling properties of the granules became poorer while HRT increased from 6 h to 24 h. Poor settling properties caused wash out of sludge and decreased mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) contents of the reactor which determine (indirectly proportional) the solid retention time (SRT). Therefore, HRT increase indirectly caused increase in SRT. Since SRT and $\mu_{overall}$ are also inversely proportional, increase of HRT indirectly decreased the overall microbial activity of the reactor (Muda et al., 2011).

HRT is related with the number of cycles in a day in SBR operation. Additionally, number of cycles in a day is related with the cycle time (i.e. number of cycles in a day equals the ratio of 24h to cycle time) in SBR operation (Tchobanoglous et al. 2004). Therefore, HRT is related with the cycle time of SBR operation and proportionally increases with cycle time. Liu and Tay (2008) claimed that granules were cultivated earlier in reactor operated with 1.5 h cycle time compared to granules cultivated with 8 h cycle time. It was concluded that the time required for granulation (cultivation time) increased with the increasing cycle time. Decreasing the cycle time (i.e. increased number of cycles per day), on the other hand increased the frequency of hydraulic selection. Frequency of hydraulic selection is related with HRT, thus an increase in frequency of hydraulic selection means decrease in HRT and shortened the period for granulation. However, granules developed with a shorter HRT (HRT of 3h) were found to be not stable in long-term operation, because decreasing HRT also decreased the starvation time which negatively affected the stability (Liu and Tay, 2008). Therefore, HRT selected should be long enough to provide starvation for the development of more stable granules.

Aerobic granules are also used for nutrient removal which requires granules containing nitrifying organisms. HRT plays an important role in cultivation of nitrifying granules, because of its aforementioned relations with SRT and cycle time. Tay et al. (2002a) investigated the cycle time and operated an SBR with different cycle times. It was concluded that nitrifying granules can be obtained at cycle times of 6 h to 12 h. It was discussed that longer cycle times decreased the hydraulic selection pressure which prevented healthy granule formation and shorter cycle times affected the SRT which caused wash-out of nitrifying microorganisms.

2.2.5. Solid Retention Time

Li et al. (2008) stated that SRT is not a controlling parameter in granulation process. Authors investigated the effect of SRT on granulation under negligible hydraulic selection pressure. They operated the reactors with 30 min of settling period and 8 h of HRT to avoid hydraulic selection. SRTs in the range of 3 days to 40 days were investigated and no granulation was reported. It was proved that without hydraulic selection pressure, SRT is not an indispensable parameter on granulation.

SRT is controlled by mostly settling time and settleability of aggregates in SBR. During cultivation of aerobic granules from suspended sludge, hydraulic selection mechanism (controlled via predetermined HRT, settling period, volumetric exchange ratio, cycle time) provides discharge of considerable amount of non-flocculating suspended sludge (Liu et al. 2005c; Liu and Liu, 2006). Therefore, it can be said that combination of settling time and HRT directly affects the SRT (Liu and Tay, 2004). As the granular sludge cultivates, settleability increases, thus SRT starts to increase and stabilizes at 25 days. However, since settleability of granules is one of the parameter affecting the resultant SRT, in most of the studies SRT has not been strictly controlled (Liu and Liu, 2006).

SRT determines the dominant microorganism in the cultivated granules, thus their capabilities and stability (Lin, 2003; Liu and Liu 2006; Li et al. 2008). SRT and specific microbial growth rate are inversely proportional with each other. As SRT increases, microorganisms having low specific growth rate are favored. It was stated that filamentous and non-flocculating microorganisms have lower maximum specific growth rate compared to floc-forming microorganisms (Chudoba, 1985). Therefore, unless SRT is controlled, filamentous microorganisms such as *M. parvicella* growth, and fluffy outer surface on granular sludge, which causes poor settleability, would occur (Lin, 2003). Lin (2003) compared the granules operated with 10 days SRT and 70 days SRT. It was concluded that granules operated with 10 days SRT were compact and stable while granules operated with 70 days were fluffy and filamentous dominated with poor settleability.

2.2.6. Superficial Air Velocity

Superficial air velocity (SAV) is a delicate parameter in granulation process. Studies showed that hydrodynamic shear force or aeration intensity or in other words SAV is one of the main parameter affecting the granulation process (Tay et al. 2001a). Hydrodynamic shear force physically contributes in granular stability. High shear force easily scraped and avoided filamentous species from the surface of granules by abrasion (Adav et al. 2007a). It can also be said that hydrodynamic shear force is mostly effective on granulation process via facilitating collision, rather than improving the granular stability. (Tay et al., 2001b; Tay et al., 2003; Adav et al., 2007a). Van Loosdrecht (1995), on the other hand, stated that hydrodynamic shear force is very important for stability of granules, since it balances biomass growth and detachment.

Tay et al. (2001a) stated that aerobic granules could not be obtained with low hydrodynamic shear force when SAV is less than 1.2 cm/s. As it was mentioned previously, EPS played crucial role in aggregation of microorganisms. Adav et al. (2007a) uttered that with the increase of aeration intensity, EPS production of cells increases. It was also stated by Tay et al. (2003) that granular strength and density are proportional to the SAV, hence hydrodynamic shear force. Main reason is that EPS, production of which increases with increasing SAV, has primary role on cohesion and adhesion of cells, thus improves the strength and density of the granules (Liu and Tay, 2004; Beun et al., 1999). Di Iaconi et al. (2006), on the other hand, investigated the effect of hydrodynamic shear force on granule properties in sequencing batch bio-filter reactor (SBBR). It was stated that high hydrodynamic shear force (15-20 dyne/cm^2) caused quasi-linear increase in biomass density, but EPS content and composition were not affected. It was reported that starvation affected biomass hydrophobicity and protein content of granules more than hydrodynamic shear force.

SAV also takes place in shaving of granules. Air velocity induced hydrodynamic shear force shaves surface of the granules and clean them from the filamentous species. Granular sludge having filamentous outer surface could not settle as fast as granular sludge free from filamentous outer surface developed at superficial air velocity of 4.1 cm/s (Beun et al. 1999). Increasing SAV enhanced the surface smoothness and increased the granular sphericity (Beun et al. 1999).

Adav et al. (2007a) investigated aeration intensity and reported that low aeration intensity (1 L/min; 0.6 cm/s) inhibited the granule formation. It was also claimed that low aeration intensity such as 0.6 cm/s stimulated the filamentous growth and blocked mass transfer into flocs. Achievement of more rigid granules with increasing aeration intensity from 1 L/min to 3 L/min (0.6 cm/s to 1.8 cm/s) was reported. There were only flocs at 1 L/min (0.6 cm/s) aeration intensity; while 3.3 mm granules were developed at 3 L/min (1.8 cm/s).

2.2.7. Starvation

Starvation is one of the parameters affecting internal granular stability (Li et al. 2006). SBR is the only reactor type appropriate for aerobic granulation, since it has periodic operation different from other reactors (Liu and Tay, 2008). SBR is not a continuously-fed reactor, durations of SBR periods can be longer than that is required for degradation of substrate. Therefore, starvation can be observed which is called as intermittent cyclic starvation (feast/famine regime) (Li et al. 2006; Liu et al. 2007a; Liu and Tay, 2008). It was stated that starvation increased bacterial hydrophobicity, thus facilitated aggregation (Bossier and Verstrate, 1996). On the other hand, around 4 h of starvation for one of the substances such as C, K, P, N deteriorates granule properties (Wang et al., 2006a).

Li et al. (2006) investigated the effect of starvation on granulation and granule properties. It was stated in the study that starvation phase in SBR operation initiated the granulation from flocs. In contrast to Li et al.'s study (2006), Liu et al. (2007a) investigated whether starvation is a prerequisite or not for cultivation of aerobic granules. They operated 2 SBRs with 1 h cycle time with no starvation portion. After one week operation they obtained aerobic granules of sizes larger than 400 μm, however instability was observed. In the same study, they seeded cultivated granules to another SBR having cycle time of 1.5 h with starvation portion and compared the stability of granules operated with starvation and without starvation. It was concluded that starvation had positive effects on granular stability. In order to investigate these positive effects and any existing negative effects of starvation duration, Liu and Tay (2008) operated 3 identical SBRs having cycle time of 1.5 h, 4 h, and 8 h. Since same feed was used, starvation length increased with the increasing cycle time. It was reported that granules cultivated in the SBR having cycle time of 1.5 h were unstable, whereas granules cultivated with cycle time of 4 h and 8 h and thus had higher starvation period were stable and found appropriate for long-term operation. It was concluded that starvation is not the main factor behind granulation, but have positive effects on granular properties such as improved stability.

Wang et al. (2006a) set 5 identical batch reactors with cultivated granules and fed them with C-lacking, N-lacking, P-lacking and K-lacking wastewaters. They compared the results and concluded that short-term starvation of these compounds caused negative effects on granule properties such as decreased EPS content, microbial activity, settleability and stability of granules. This conclusion, on the other hand, does not conflict with the previously mentioned results pointing that starvation increases stability. Wang et al. (2006a) investigated the 4 h starvation of different substances in batch reactors, while aforementioned studies investigated the effect of intermittent cyclic starvation (feast/famine regime) in SBRs, the feeding and hydrodynamic properties of which were totally different than that in batch reactors. (Li et al., 2006; Liu et al., 2007a; Liu and Tay, 2008).

2.2.8. Effect of divalent metal ions

Divalent metal ions, especially Ca^{2+} and Mg^{2+}, have positive effects on granulation process and granule properties (Yu et al., 2001; Jiang et al., 2003; Ren et al., 2008; Li et al., 2009; Liu et al., 2010; Gao et al., 2011). It was discussed that bacterial aggregation can be enhanced by divalent atoms, since they are able to neutralize the negative charges on the surface of microorganisms (Morgan et al., 1990; Gao et al, 2011). Divalent atoms can also form precipitates which behave like nuclei and promote microbial aggregation. In addition, they are able to enhance granulation acting as a cation bridge via forming ionic bonds on the surface of flocs (Yu et al. 2001; Ren et al. 2008).

2.2.8.1. Ca^{2+} ion

It was mentioned in the studies on the effects of hydraulic selection (Tay et al. 2001a; Liu et al., 2004a,c; Liu et al. 2005a), especially those about settling time (Qin et al., 2004a,b; Liu et al., 2004a; Adav et al., 2009) and exchange ratio (Wang et al. 2006b) that calcium ion accumulation in granules increased proportionally with the increase of hydraulic selection pressure. Calcium ions are reported to be used in EPS production, thus it can be said that with the increase of hydraulic selection pressure, EPS production increases and calcium content also increases in granules (Wang et al. 2006b). Calcium ions act like a bridge between EPS and strengthen the granular structure as well as fasten granulation (Yu et al., 2001; Ren et al., 2008; Gao et al., 2011).

Jiang et al. (2003) investigated the effects of Ca^{2+} ions on granulation process. They operated two SBRs; one as a control with no Ca^{2+} feed and the other one augmented with 100 mg/L of Ca^{2+} as test reactor. It was verified that augmentation with 100 mg/L of Ca^{2+} decreased the cultivation time of granules from 32 days to 16 days. Additionally, in the Ca^{2+}-enriched reactor, granular properties such as polysaccharide content, density, compactness, strength and settleability of granules improved compared to granules in control reactor. Ren et al. (2008) reported similar results about the effect of calcium ion on granular properties and stated that more rigid structure and higher strength can be achieved with Ca^{2+}-accumulated granules. However, Ca^{2+} might have negative effects on bioactivity of granules. It was remarked that specific oxygen uptake rate (SOUR) of Ca^{2+}-accumulated granules were lower than that of non-accumulated ones. While Ca^{2+} content of granules was less than 90 mg/g SS, SOUR of granules was 26 mg O_2/g VSS.h, unlike the SOUR of 9.5 mg O_2/g VSS.h when Ca^{2+} content of granules was 150 mg/g SS. It was reported that influent concentration of more than 20 mg/L Ca^{2+} decreased SOUR of granules.

2.2.8.2. Mg^{2+} ion

Another divalent atom having positive effects on granulation is Mg^{2+} (Li et al. 2009; Gao et al., 2011). In 2009, Li and his group investigated the effect of Mg^{2+} on cultivation of granules. Experimental set-up involved two SBRs; one as a control fed with no Mg^{2+} and the other augmented with 10 mg/L of Mg^{2+} as test reactor. Decrease in granulation time from 32 days to 18 days was reported when Mg^{2+} was used in the feed. Furthermore, it was stated that mean granule size of Mg^{+2}-augmented reactor was 2.9 mm while mean granular size of control reactor was 1.8 mm. Similar to Ca^{2+}-fed granules, better granular properties such as high polysaccharide content, density, compactness, strength and settleability were reported for Mg^{2+}-fed granules compared to granules in control reactor. It was also indicated that no morphological difference was observed between granules.

In order to better understand whether Ca^{2+} augmentation or Mg^{2+} augmentation have more influence on granulation, Liu et al. (2010) compared the Ca^{2+}- and Mg^{2+}- enhanced aerobic granulation in SBR. They operated two SBRs, one of which was augmented with 40 mg/L Ca^{2+} and the other with 40 mg/L Mg^{2+}. It was found that granules were developed in Ca^{2+}-fed reactor earlier than Mg^{2+}-fed reactor. Physical characteristics such as settling velocity, density, and compactness of Ca^{2+}-fed mature granules were much better than the ones augmented with Mg^{2+}. On the other hand, EPS production yield and microbial diversity of Mg^{2+}-fed granules were reported as higher than Ca^{2+}-fed granules. It was also expressed that substrate degradation of Mg^{2+}-fed granules was faster than Ca^{2+}-augmented granules. As a result of the study, it was concluded that physical properties of granules were sensitive to Ca^{2+} and biological properties were mostly affected by Mg^{2+} during granulation process.

It can be commented about the effect of divalent atoms, especially Mg^{2+} and Ca^{2+}, on granulation that they are not vital for granulation; however, they decrease the cultivation time as well as improve the granular stability. Faster start-up period and more efficient granular systems can be achieved by Ca^{2+} and Mg^{2+} augmentation.

2.2.9. Extracellular Polymeric Substances

Extracellular polymeric substance (EPS) is secreted by cells and behaves like glue as represented in Figure 2.3 (Liu et al., 2004a). Since the theory behind granulation is aggregation of cells and formation of matrix structure, EPS is thought to influence granulation and granular properties such as stability, compactness, size and microbial activity. It is indicated that EPS takes part in sticking of cells to each other, formation of matrix structure and improvement of long-term stability of granules (Schmidt and Ahring, 1994; Tay et al., 2001c; Qin et al., 2004a).

EPS (polysaccharides, proteins, lipids, nucleic acids) is known to exist in microbial flocs, biofilms and granules. Tay et al. (2001c) found in their study that granular sludge had much higher EPS content than that of flocs and biofilms. EPS can also be described as a protective barrier as well as a supportive media needed for stabilization of membrane structure, in a microbiological perspective (Liu et al., 2004a). EPS secretion is reported to increase with increasing stressful conditions (Qin et al., 2004a; Liu et al., 2004a). It can be seen from the reviewed studies that parameters which play crucial role in granulation also affect the EPS production (Liu and Tay, 2004; Adav et al., 2009a; Gao et al., 2011). As noted before, short settling time, high superficial air velocity (hydrodynamic shear force), high organic loading rate and starvation increases the EPS production (Liu et al., 2004a). Yu et al. (2001b) claimed that produced EPS surrounded the aggregates and provided higher surface area to interact with organic and inorganic materials. In the study of Tsuneda et al. (2003), it was asserted that negative surface charge density of native cell surface declined with the EPS production of the cell. It was also stated that electrostatic interaction and cell adhesion, which are the phenomena behind granulation, was promoted with the increase of EPS amount. Therefore, it can be said that EPS amount is likely to be very important in granulation process.

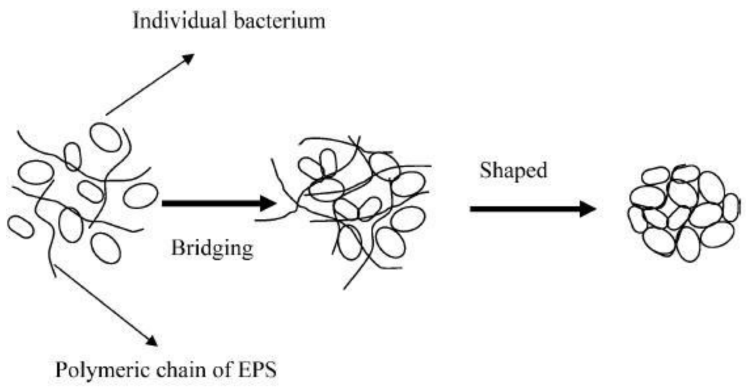

Figure 2.3 Role of EPS in granulation (Liu et al., 2004a)

Wang et al. (2006c) reported that EPS production followed an exponential trend and EPS itself became carbon and nitrogen source during starvation period. This phenomenon was claimed to control the granular enlargement by setting internal and external bacterial growth.

The only study indicating the negative effects of EPS content of seed sludge was conducted by Yu et al. (2009). Authors indicated that EPS in seed sludge delayed granulation and EPS would promote granulation only if it was released by aggregated microorganisms. They investigated the properties of seed sludge in terms of EPS content. They used two identical SBRs and seeded them with CAS flocs and EPS-free pellets. It was found that granulation occurred earlier in the reactor seeded with EPS-free pellets than that seeded with CAS flocs. It was claimed that tightly bound (TB) and loosely bound (LB) EPS content of the seed sludge affected the microbial aggregation negatively and delayed the granule formation. In other words, granulation might be delayed due to the fact that EPS bounded to flocs prevented cell to cell collision. However, there was no other adverse effect of EPS content of seed sludge on granular properties reported.

As it is mentioned above, EPS is composed of polysaccharides, proteins, lipids, nucleic acids and humic acids. The composition of EPS was reported to be effective on granular compactness and stability (Liu et al. 2004a, Qin et al. 2004a). However, there are conflicts about the effective polymer composition for aerobic granulation as well as appropriate EPS amount of granules as summarized in Table 2.1, since there is no standardized common method for EPS extraction. Main conflict is about the ratio of polysaccharides to protein (PS/PN) (Liu et al., 2004a). Tay et al. (2001c) stated that polysaccharides (PS) rather than proteins (PN) increase sharply in granulation process. It is claimed that the more the PS/PN ratio is the more the specific gravity and the mechanical strength of the granules will be (Tay et al., 2001b; Tay et al., 2002). Tay et al. (2001b) revealed that PS/PN ratio increased from 5.7 to 13 with the development of aerobic granules. In contrast to that, Zhu et al. (2012) claimed that protein was the sensitive EPS component and contributed granular stability and specific gravity. Ratios for protein/polysaccharide (PN/PS) were found in the range of 5 and 12. They concluded that settleability, stability, surface charge, hydrophobicity of granular sludge increased proportionally with the increase of PN/PS ratio. Moreover, Zhang et al. (2007) reported an increase in PN/PS ratio from 2.3 to 4.9 when aerobic granules were cultivated.

Table 2.1 EPS content of aerobic granules in different studies

Protein (mg/g MLVSS)	**Polysaccharide (mg/g MLVSS)**	**References**
20 – 80	50 – 80	Jiang et al. (2004)
20-30	105-115	Fang et al. (2002)
120 – 150	25 – 35	Zhang et al. (2007)
273 – 325	10.6 – 20.3	Juang et al. (2010)
40 – 70	15 – 35	Gao et al. (2011)
400 – 450	150 – 175	Adav and Lee (2011)
25 – 32	2.5 - 5	Zhu et al. (2012)

In order to better understand the concept, Adav et al. (2008b) had a closer look at this conflict and investigated each of the components separately. Phenol-fed granular sludge was used. Granular sludge was protein abundant and PN/PS ratio of the granule was reported as about 3.9. They hydrolyzed each EPS component (α- and β-polysaccharides, proteins, lipids) individually by using selective enzymes from granular sludge and assessed the variances. Therefore, they obtained detailed information about the contribution of each component in granular stability. Minimal alteration was observed after removal of proteins from granule's EPS. Hydrolysis of lipids and α–polysaccharides also led to minimal changes. However, disintegration of granules was reported following hydrolysis of β–polysaccharides. It was concluded that β-polysaccharides are forming the skeleton of granules. In other words, the β-polysaccharides are the main component deciding stability, and both protein and polysaccharide content of sludge can increase during granulation.

Which one of the EPS components will be dominant during granulation is determined by the nutrient content of the feed (Wu et al., 2012). Wu et al. (2012) investigated the effect of COD/N ratio on PS/PN ratio of aerobic granules. It was found that substrate COD/N ratio of 0/200 led to PS/PN ratio of 0.5 while substrate COD/N ratio 800/200 led to PS/PN ratio of 4.1 in aerobic granulation. It was also investigated by Durmaz (2001) whether C/N ratio of the wastewater affects the PS and PN production of CAS flocs. Dominant type of EPS released by flocs was investigated by feeding identical reactors with wastewaters having C/N ratio of 5, 17.5 and 40. While microorganisms exposed to wastewater having C/N ratio of 5, PN/PS ratio was 4. In contrast to that microorganisms exposed to wastewater having C/N ratio of 40 released EPS with the PS/PN ratio of 4. It was proved that, with the increase of C/N, PS content of EPS increased and PN content decreased. It can be said that dominant proportion of EPS released by sludge flocs depends on the wastewater type. Since CAS is used in aerobic granule cultivation, it is possible to see variations in the dominant EPS component of aerobic granules, which is mainly due to the wastewater type, in particular C/N ratio.

2.2.10. Seed Sludge

As it is mentioned before, main theory behind granulation is the agglomeration of flocs and floc-like microorganisms (Tay et al., 2001a). In anaerobic granulation seed sludge type is known as a decisive parameter in formation and stability of granules (Liu and Tay, 2004). In aerobic granulation concept, it is thought to be effecting the settleability, microbial activity, hydrophobicity and other macroscopic characteristics of granular sludge (Liu and Tay, 2004). In studies on aerobic granulation CAS is usually used as suspended seed sludge (Wilen et al., 2007; Yu et al., 2009; Sheng et al., 2010a).

Wilen et al. (2007) investigated the microbial community structure in seed sludge and concluded that hydrophobic bacteria such as floc-forming *Alphaproteobacteria Actinobacteria* and floc-affiliated *Betaproteobacteria* content in seed was very effective on granulation. The higher the number of floc-formers, the earlier the granular sludge with higher settleability can be cultivated.

Sheng et al. (2010a) conducted an experimental set-up to investigate the effect of floc properties of the seed sludge on granulation. Small-loose flocs and large-dense flocs were separated from conventional activated sludge and used in seeding of two identical SBRs. Granular sludge development was reported in both reactors. It was claimed that obtaining granules from small-loose flocs was completely related with the selective biomass discharge. The authors asserted that selective discharge of slow-settling biomass, which is competitive in substrate utilization, provides more substrate to granule-forming microorganisms. It was also confirmed that there was no microbial difference between the granules obtained from large-dense flocs and small-loose flocs. This similarity proved the idea that granule forming microorganisms existed in both large-dense flocs and small-loose flocs and should be selected from the community for effective granulation.

Xu et al. (2011) investigated two different sludge forms. Two identical reactors were seeded by activated sludge flocs and sludge pellets (achieved from sludge flocs). It was found that sludge pellets responded better than sludge flocs and granulation occurred earlier with sludge pellets. It was reported that granules developed from sludge pellets also had better COD removal, larger diameter, higher hydrophobicity and Mg^{2+} content than those achieved from normal sludge flocs. It is suggested that for pilot-scale or full-scale applications, sludge pellets would be a better inoculant than sludge flocs.

In a very recent study, Verawaty et al. (2012) investigated the effect of seed sludge on time required for granule cultivation. They seeded an SBR with a mixture of floccular sludge and crashed granules. It was stated that crashed granular sludge acted like a packing material and flocs directly attached to them, hence the use of crashed granules significantly decreased the time required to reach steady-state. It was concluded that seeding with crashed granules is an effective strategy to decrease start-up period and achieve better biological nutrient removal by improving biomass holding capacity.

The effect of seed sludge on aerobic granulation is a recent subject. Common traits in all of these studies are mainly the investigation of the physical properties of the seed sludge and in all studies CAS was used as seed sludge. There is still a gap in literature about the possibility of aerobic granule cultivation by using seed sludge different than conventional activated sludge.

2.2.11. pH, Temperature and Dissolved Oxygen

Studies about the effects of pH on anaerobic granule formation and treatment performances exist in many studies (de Beer et al. 1992; Bitton 1999). However, there is not much information about the effects of pH on aerobic granulation in literature. Most of the aerobic granulation studies were conducted under standart conditions with variable pH values (Liu et al., 2003a; Qin and Liu, 2006). Liu and Tay (2004) stated that pH and temperature are not as decisive as they are in anaerobic granulation. Findings in the study of Bao et al. (2009) support the idea that temperature is not a decisive parameter in aerobic granulation. In their study, they operated an SBR at 10^{o}C and achieved aerobic granules having mean size of 3.4 mm and treatment efficiencies of 90.6 – 95.4% COD, 72.8 – 82.1 % NH_4-N and 95.8 – 97.9% PO_4-P. It was stated that low temperature affected the nitrification negatively and intermittent nitrite accumulation was observed during operation.

Temperature and pH are important in determining the optimum conditions for enrichment of desired specie. For example, Yang et al. (2008) cultivated fungi-dominated fluffy aerobic granules at pH 3 and bacteria-dominated compact aerobic granules at pH 8.1. Therefore, it can also be said that pH and temperature become important when granules are aimed for a specific utilization such as nutrient removal, nickel biosorption (Xu et al., 2006). In nutrient removal, especially in nitrification process, pH and temperature have significant influence on enrichment of nitrifiers and their activity (Anthonisen et al., 1976; Kim et al., 2008). It was concluded that pH and temperature control were not important for granule cultivation from suspended sludge but affect the microbial activity and treatment performance of granules like other conventional systems.

There are studies where aerobic granules were cultivated at DO levels of 0.7-1 mg/L as well as DO levels higher than 2 mg/L (Peng et al., 1999; Tay et al. 2001a). It was claimed for the effect of pH and temperature that DO was not a decisive parameter in granulation process (Liu and Tay, 2004).

2.2.12. Carbon / Nitrogen Ratio

Aerobic granular sludge technology is used to achieve both organic and nutrient removal. In order to increase the efficiency of granules, influent C/N ratio and its effects on granulation and granules were investigated in several studies (Wu et al., 2012; Li et al., 2011; Yang et al., 2004a). Liu et al. (2003) conducted an experimental study on 4 SBRs with 4 different C/N ratios. It was stated that C/N ratio of 100/5 to 100/30 was appropriate for aerobic granulation. It was also reported that decreasing C/N ratio increased the nitrifying community content of the granules while decreased the granule size.

Cydzik-Kwiatkowska and Wojnowska-Baryla (2011) used C/N ratio of 1 and 2 to cultivate nitrifying granules. Influent NH_4-N concentration was 162±12 mg/L with COD/N ratio of 2 and 292±12 mg/L with COD/N ratio of 1. Granules achieving more than 90% COD and NH_4-N removal efficiencies were developed. Dominant oxidized form of nitrogen was observed as nitrite rather than nitrate. Similar to Liu et al.'s study (2003a) amount of nitrifying microorganisms increased with decreasing COD/N ratio.

Li et al. (2011) investigated the formation and characteristics of granules by stepwise increasing of N/C. Similar results with those of Liu et al. (2003) were found. Granules having spherical shape and clear outer surface were reported. Li et al. (2011) stated that the selection of ammonia-oxidizing bacteria (AOB) in aerobic granules enhanced with the stepwise increase of N/C and decrease of settling time. It was asserted that 14.9±0.5% of the granule was AOB, whereas 0.89±0.1% of the granule was nitrite oxidizing bacteria (NOB). Partial nitrification (nitrite accumulation) was observed and it was discussed that main reason of partial nitrification was the percentage difference between AOB and NOB in granules. Ammonia oxidation capacity of more than 95% was reported. In a very recent study, Wu et al. (2012) operated 4 identical SBRs having 0 to 4 COD/N ratios. In reactors operated with COD/N ratio of 1 and 2, achievement of granules with clear outer shape were reported. On the other hand, in reactors having COD/N ratio of 4 and 0, no granules were developed and the reason was attributed to the either excess or no EPS production, respectively.

For the C/N ratio range of 1-20, granules can be developed but microbial characteristics totally vary. Stability of granules can be increased by using low C/N (3-5) ratio, since it is appropriate for the selection of nitrifying microorganisms (slow growing microorganisms) in aerobic granules which increases stability (Liu et al., 2004b; Yang et al., 2004a). Therefore, low C/N ratio is good for nitrification; on the other hand, nitrite accumulation and smaller granule size were reported at low C/N ratio. To conclude, although C/N ratio is not a decisive parameter in granulation process, operator should take the C/N ratio into consideration to achieve best treatment performance from the granules for any type of wastewater.

2.2.13. Non-aerated periods in SBR cycle

Studies in which nutrient removal with aerobic granules was aimed, an additional operational parameter, non-aerated periods (lacking of oxygen) in SBR cycle, came into consideration (Jang et al., 2003; Qin and Liu, 2006; Wang et al., 2007a; Yilmaz et al., 2007; Wan et al., 2009). Effects of non-aerated periods on granulation were investigated and studies proved that the presence of anoxic and/or anaerobic periods in an SBR cycle were also beneficial in terms of granular density, compactness and microbial diversity (Wan et al., 2009; Pijuan et al., 2009; Su et al., 2012). Influence of SBR operation with pre-anoxic sequence on granulation was further investigated by Wan et al. (2009). They conducted an experimental set-up with two identical SBRs one of which was operated fully aerobic and the other with anoxic-aerobic (pre-anoxic) sequence. According to the experimental results, it was asserted that aerobic granulation was affected positively with pre-anoxic operation. It was claimed that the growth and accumulation of denitrifying heterotrophic organisms in the core of the granules increased with the decrease in autotrophic-heterotrophic competition. This enhancement was reported to increase the density of granules, since there was an alternative electron source such as NO_2 and NO_3 which compensated the oxygen limitation during granulation process. Therefore, it was suggested that, in order to cultivate denser and more versatile granules, addition of anoxic period in an SBR cycle would be advantageous for granulation process. De Kreuk and van Loosdrecht (2006) stated that most of the organic matter was used during anaerobic/anoxic period and starvation time in the following aerobic period increased and in turn enhanced the microbial auto-aggregation. Zhang et al. (2011) stated that long-term stable granules could be achieved by SBR operation with the sequence of anaerobic-aerobic-anoxic periods. Adav et al. (2009b) stated that DO deficiency and substrate deficiency in the core of the granules which are reasons for filamentous growth, could be avoided by the addition of a non-aerated period. The addition of non-aerated periods provided the enrichment and placement of denitrifiers in the core of the granules. Denitrifiers in the core would use NO_x as electron source under DO deficient conditions to consume carbon and hence suppressed filamentous growth.

There are researches in the literature that randomly integrate the non-aerated periods in SBR cycle to investigate the effects of these periods on granule cultivation and treatment performance of granules (Jang et al., 2003; Qin and Liu, 2006; Wang et al., 2007a; Yilmaz et al., 2007; Adav et al., 2009b; Wan et al., 2009; Su et al., 2012). However, to our knowledge, there has been no detailed research that investigated and compared the period sequence effect (aerobic-anoxic sequence to anoxic-aerobic sequence) on granulation process and treatment efficiency of the granules, yet.

2.3. Applications with Aerobic Granular Sludge

Aerobic granulation is a recent phenomenon and still needs time to be used in real-scale applications. Utilization of aerobic granules in wastewater treatment was mostly investigated in laboratory-scale tests. Treatment capability of aerobic granules on various types of wastewater including toxic compounds, metals, nutrients, dyestuffs is still being investigated (Gao et al., 2011).

2.3.1. Laboratory Scale Tests

2.3.1.1. Organic and Nutrient Removal Performances

Studies (Arrojo et al., 2004; Schwarzenbeck et al., 2005; Su and Yu, 2005; de Kreuk and van Loosdrecht, 2006; Adav et al. 2008a) reported that granular sludge can be cultivated from different type of wastewaters such as wastewaters of brewery, soybean-processing, dairy product industries and domestic wastewater. In addition to studies on cultivation of granules, treatment efficiency of granules was also investigated in several studies (Jiang et al., 2004; De Kreuk et al., 2005; Zhu et al., 2012) and it was found that aerobic granules can treat organic-containing wastewaters with high efficiency.

As explained earlier, aerobic granules are also capable of treating N and P containing wastewater since each granule contains aerobic heterotrophs, nitrifiers and denitrifiers at the same time as it is depicted in Figure 2.4 (Liu and Tay, 2004; Yilmaz et al., 2007). It is revealed that possible DO diffusion range in flocs is 200-400 µm (Lens et al., 1995a). Therefore, the size of each zone in aerobic granule depends on DO concentration and granule size (Gao et al., 2011). Mostly, the outmost, the inner and the innermost shell is composed of aerobic heterotrophs, nitrifiers and denitrifiers/anaerobic autotrophs, respectively.

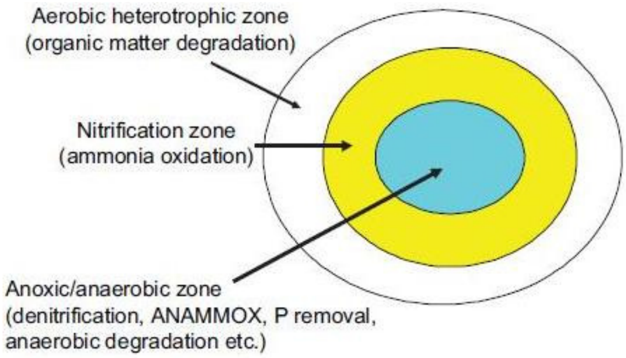

Figure 2.4 Conceptual aerobic granule structure (Gao et al., 2011)

Main theory behind nutrient removal in aerobic granular systems is almost same with the biological nutrient removal (BNR) systems (Yilmaz et al., 2007). However, in granular systems, anaerobic, anoxic and aerobic portions (zones) exist in granular structure (Figure 2.4). Thus placement of microorganisms in granules provides nutrient removal in some extent, independent from non-aerated periods. In BNR systems, on the other hand, anaerobic or anoxic tanks/phases are used for nutrient (N,P) removal (Gao et al., 2011).

It is revealed in Table 2.2 that aerobic granules have considerable potential for organic and nutrient (N,P) removal for both low-strength and high-strength wastewaters (Jang et al., 2003; Qin and Liu, 2006; Wang et al., 2007a,b; Yilmaz et al., 2007; Wan et al., 2009). Wan et al. (2009) compared full aerobic operation with anoxic-aerobic operation and stated that nitrification rate of granules was still higher in the reactor operated with anoxic-aerobic operation. Anoxic-aerobic operation improved density of cultivated bioflocs, thus MLSS concentration and SRT increased. This SRT increase was also claimed to improve nitrification stability. Qin and Liu (2006) also stated that anoxic periods enabled the enrichment of denitrifiers by the availability of external carbon source.

Adav et al. (2009b) also stated that, with 2 h oxic and 2 h anoxic operation, nitrifiers were located at the surface and heterotrophs were located in the core of granules. This gradual placement enhanced the simultaneous nitrification-denitrification capacity and more than 90% nitrogen removal was achieved at a nitrogen loading rate (NLR) of 6.4 kg NH_4-N/m^3.d. Yet, 99% NH_4-N oxidation and NO_3-N accumulation observed, when the conditions were completely aerobic for the same NLR.

In an alternating aerobic-anoxic operation, Qin and Liu (2006) reported 95% COD removal and 100% NH_4-N oxidation at an NLR of 0.15-0.45 kg/m^3.d. However, an external carbon addition was required for NO_3-N removal in subsequent anoxic period. Yilmaz et al. (2007) operated an SBR for 13 months with period sequence of anaerobic-aerobic-anoxic. They cultivated aerobic granules capable of 85% sCOD, 86% total nitrogen and 74% total phosphorus removal efficiencies at the loading rates of 2.7 kg COD /m^3.d, 0.43 kg N/m^3.d and 0.06 kg P/m^3.d.

Table 2.2 Literature studies concerning organic and nutrient removal

Wastewater Type	COD Inf. (mg/L)	TN Inf. (mg/L)	TP Inf. (mg/L)	COD RE (%)	TAN RE (%)	TN RE (%)	TP RE (%)	References
Synthetic	-	35	20	100	100	94	94	De Kreuk et al., 2005
Abattoir	7685	1057	217	98.6	-	97.4	98.2	Cassidy and Belia, 2005
Synthetic	640	40	20	95	-	71	99.6	Dulekgurgen et al., 2003
Dairy	2000-4000	50-210	20-30	90	-	80	67	Schwarzenbeck et al., 2005
Brewery	1300-2300	30-37	3.2-4.3	88.7	88.9	-	-	Wang et al., 2007a
Phenol-containing	333-833 phenol 250-650 TOC	-	-	95 TOC 100 Phenol	-	-	-	Jiang et al., 2004
Landfill leachate & municipal	400-24400	56-160	2-14	80	87	-	-	Di Iaconi et al., 2007
Domestic	1000	50	-	>80	>90	-	-	Liu et al., 2007b
Synthetic	300	40	-	95	97	-	-	Jang et al., 2003
Synthetic	500	37.5-112.5	-	95	100	-	-	Qin and Liu, 2006
Abattoir	600-783	225-277	35-42	85	99.7	86	74	Yilmaz et al., 2007
Synthetic	750-1000	37.5-50	-	81	100	-	-	Wan et al., 2009
Synthetic	1280	100-1700	-	94-96	93.6	-	-	Adav et al., 2009b
Synthetic	600	60	10	-	100	100	100	Kishida et al., 2006
Synthetic	427	30-35	5-6	99.1	97.8		98.6	Gao et al., 2012
Synthetic	1600	-	-	90	-	-	-	Zhu et al., 2012
Synthetic	0-800	0-200	-	80	72	-	-	Wu et al., 2012
Abattoir	862-1137	200-254	31-40	95	80	-	-	Verawaty et al., 2012
Inf: Influent RE: Removal Efficiency								

2.3.1.2. Removal Performances for Toxic Substances

Most of the industrial wastewaters contain toxic compounds for conventional biological treatment systems. Phenols, chlorinated phenolic compounds, dyestuffs, heavy metals have severe effects on environment and they are also toxic for conventional biological treatment (Sun et al., 2008a; Carucci et al, 2009). Nevertheless, studies demonstrated that biological treatment of these substances is possible by using aerobic granules. Results of Tay et al.'s study (2004c) indicated that specific phenol degradation rate of aerobic granules was higher than 1 g phenol/g VSS for wastewater containing 500 mg/L phenol. Moussavi et al. (2010) also asserted that as high as 1 g/L phenol containing saline wastewater could be treated with 99% phenol removal efficiency. Furthermore, acclimation of aerobic granules to phenolic toxicity was reported by Ho et al. (2010). Authors stated that 5 g/L phenol containing wastewater could be efficiently treated by aerobic granules without performance reduction. Carucci et al. (2009) demonstrated that 4-chlorophenol could be treated from wastewater after acclimation of aerobic granules. Wang et al. (2007b) reported 94% 2-4-dichlorophenol treatment and 95% COD removal by using aerobic granules. Liu et al. (2008) compared the effectiveness of anaerobic granules and aerobic granules on 4-t-octylphenol removal from wastewaters. Results indicated that Bacillus and γ-Proteobacteria were the dominating species in aerobic granules which provided better performance than anaerobic granular sludge.

In addition to removal of toxic organic compounds, heavy metals and color removal with aerobic granules is also possible (Muda et al. 2010; Gao et al. 2011). According to the study of Muda et al. (2010), 62% color removal from textile wastewater was achieved. Sun et al. (2008a) also investigated the biosorption of malachite green by aerobic granules. It was reported that 1 g of aerobic granule can adsorb 56.8 mg malachite green dye at 30^{o}C.

Heavy metal removal capacities of aerobic granules were investigated in different studies. Liu et al. (2002) stated that aerobic granules were capable to adsorb 566 mg/g Cd^{2+} and 270 mg/g Zn^{2+}. In addition to removal of heavy metals such as Zn^{2+}, Cd^{2+}, Co^{2+} by adsorption, removal of Cd^{2+}, Cu^{2+}, Ni^{2+} and Cr^{3+} can be achieved by aerobic granular sludge via ion exchange mechanism. Moreover, these metals can attach to the EPS secreted by aerobic granules and further removed by chemical precipitation mechanism (Xu et al., 2006; Xu and Liu, 2008; Sun et al., 2008b).

2.3.2. Pilot-Scale and Full-Scale Applications

Developing technologies and non-conventional systems have a drawback of being risky for the investors, since maintenance parameters and sustainability of recently developed systems are shadowy. Wastewater treatment by aerobic granular sludge can also be rated among this class. There is quite few information in the literature about pilot-scale and full-scale applications of aerobic granular sludge technology. There were only lab-scale applications until 2003 (Giesen et al. 2012). Initiation of a pilot plant was reported in Epe, Netherland, in 2003. It was reported that the volume of reactor was 1.5 m^3 (Ni et al., 2009). Another pilot reactor having a volume of 3.1 m^3 was reported in Italy. Additionally, construction of pilot plants with volumes of 2.7 m^3 and 1 m^3 was reported in Hungary and China, respectively (Ni et al., 2009). These pilot plants were integrated to the wastewater treatment plants which were already serving (Ni et al., 2009; Gao et al., 2011).

According Giesen et al.'s report (2012), a group in Netherlands, namely, National Nereda Onderzoeks Programma (NNOP) focused on scaling up of the aerobic granule technology to pilot-scale and full-scale. The wastewater treatment technology by using aerobic granular sludge named as "Nereda technology". According to the report, in 2005, mini full-scale Nereda with a treatment capacity of 50 – 250 m^3/day was constructed (Giesen et al. 2012). The construction of an industrial pilot plant in food industry was reported in 2006. NNOP retrofitted this pilot plant and constructed a new plant in 2009 which was capable of handling 1 – 8 g COD /L with 95% removal efficiency. Municipal wastewater with a flow rate of 4000 m^3/d and high strength septic influent was started to be treated using Nereda in South Africa in 2008. This application was important since it provided irrigational reuse of the wastewater after treatment. NNOP has successfully applied first municipal full-scale Nereda to sewage treatment at Epe, Netherlands. The capacity was reported as 1500 m^3/h. It was reported that influent wastewater was containing industrial wastewater such as slaughterhouses. Effluent quality was reported as N< 5 mg/L and P < 0.3 mg/L. After experimental observation was completed, the plant was opened on May 8, 2012 by Dutch Prince Willem-Alexander. It was the first literally available full-scale municipal wastewater treatment plant. Giesen et al. (2012) asserted that 3 municipal treatment plants for Municipality Stellenbosch in South Africa, District Water Board Ijssel and Rjin are still under construction.

Although aerobic granulation studies started in 1990's, pilot-scale installations started their development decade ago. However, these successful installations reveal that wastewater treatment with aerobic granular sludge is a promising technology providing efficient organic removal and nutrient removal. In near future, conventional biological treatment systems might be replaced by aerobic granular systems. Furthermore, being resistant to toxic compounds and ability to handle high strength wastewaters as well as dyestuffs and metal ions containing wastewaters, make aerobic granular sludge possible to be a good alternative for treatment of various types of industrial wastewater. Yet, studies continue to investigate the problems encountered, in order to avoid weaknesses and improve sustainability of aerobic granular systems.

2.4. Storage and Reactivation Performances of Aerobic Granules

Response of aerobic granules to long-term idle periods should be investigated in order to assure the full-scale applicability of aerobic granular sludge technology (Lee et al., 2010). There are several studies focused on storage of aerobic granules and their physicochemical properties (Zhu and Wilderer, 2003; Liu, et al., 2005b; Zeng, et al., 2007). Wang et al. (2008b) conducted an experiment not only to investigate the long-term storage of aerobic granules, but also to investigate the reactivation of them after long-term storage. Mature granules of 7.5 g/L VSS were stored at 4^{o}C without DO stripping. However, at 15th day of the operation, the DO concentration was below detection limit. According to experimental results, no significant break up of granules was reported and it was stated that granules preserved their spherical shape with pleats and cavities on them which inhibited the settling properties. Color change from brownish yellow to dark black was reported. After this long-term storage of 6 months, they investigated the reactivation of granules and claimed that 11-16 days were enough to achieve full microbial activation of heterotrophs and nitrifiers.

Aerobic granules can also be stored after drying (Lee et al. 2010). It was stated that cultivated aerobic granules with a mean size of 5.4 mm were dried at 25^{o}C for 24 h and granule size decreased to 2.6 mm. Dried granules were stored for 21 days at room temperature (24-29 ^{o}C). After reactivation, granule sizes increased to 5.4 mm. COD removal efficiencies of these reactivated granules were almost the same as those of granules before drying (Lee et al., 2010). In another study, Gao et al. (2012) compared different storage strategies on the basis of reactivation performance of aerobic granules. Six bottles of granule samples taken from the main reactor were stored at -25^{o}C, +4^{o}C and ambient room temperature (20 – 26^{o}C) with substrate compositions of either distilled water or 400 mg/L glucose solution. After 8 months of storage reactivation experiments were conducted. It was claimed that storage substrate had no influence for long-term storage of aerobic granules. On the other hand, temperature was reported as a crucial parameter for storage. It was stated that granules stored at 4^{o}C showed the best performance in recovery. They were also reported as preserving their spherical shape unlike the others stored at -25^{o}C and room temperature. Settling properties of the granules stored at -25^{o}C were asserted to be better than others, which was attributed to the better preserved internal microstructure at -25^{o}C. Granules stored at room temperature were reported to display the worst performance in both physical characteristics and biological recovery.

Yuan et al. (2012) approached the recovery concept from another perspective. They investigated different operational conditions for optimization of recovery. In their study, 3 different strategies; different organic loading rates (from 0.4 to 2.4 kg COD/m^3.d), ammonia concentrations (from 15 to 80 mg/L) and shear forces (from 0.65 to 2.6 cm/s superficial air velocity) were investigated. Firstly, aerobic granules were developed and stored for 12 months under ambient temperature (20 – 26^{o}C) conditions. Then, reactivation experiments were conducted in 11 SBRs having different operational conditions. It was claimed that the best recovery performance can be achieved by operating the SBR with 0.8 kg COD/m^3.d of OLR, 15-20 mg/L of influent ammonia concentration and at 2.6 cm/s of superficial air velocity. Recovery under these conditions was achieved in 7 days with the COD removal of 97.44% and SOUR of 40.63 mg O_2/g SS.h.

It can be concluded that aerobic granules are appropriate for long-term storage and their reactivation period is quite fast. Therefore, it can be said that aerobic granular sludge technology has a potential for very fast start-up by seeding reactors with stored granules. These results also reveal that wastewater treatment by using aerobic granules is not limited with the continuously produced wastewater sources to achieve considerable performance. Storage and reactivation capacity of aerobic granules enable the utilization of this technology also for the seasonally-working industries.

CHAPTER 3

MATERIALS AND METHODS

In this thesis study, three reactor sets were conducted. In this chapter, the seed sludge, the experimental sets, reactor operations and the analytical methods used in each set are described.

3.1. Seed Sludge

One of the sludge type used in experimental studies was conventional activated sludge (CAS). It was taken from return activated sludge line of secondary clarifier of the Greater Municipality of Ankara Domestic Wastewater Treatment Plant. Membrane bioreactor sludge (MBS), which was also used as seed sludge in experiments, was obtained from membrane unit of METU-Vacuum Rotation Biomembrane Plant. Properties of seed sludge in each sequencing batch reactor experiments are given in Table 3.1.

Before being used as seed in the reactors, both sludge types were concentrated or diluted (if necessary) to achieve 5000 mg/L initial MLVSS concentration in Set-1 and Set-2. Set-3 was conducted by using aerobic granular system which was continuation of Set-2.

Table 3.1 The properties of seed sludge used in each set

Sludge Properties	Membrane Bioreactor Sludge (MBS) (R1)	Conventional Activated Sludge (CAS) (R2)
Set – 1		
MLSS (mg/L)	8560±226	4640±289
MLVSS (mg/L)	5740±113	3860±158
SVI_{30} (mL/g)	103	134
Set – 2		
MLSS (mg/L)	2730±320	NA
MLVSS (mg/L)	1980±114	NA
SVI_{30} (mL/g)	256	NA
Set – 3		
	Granules cultivated from Membrane Bioreactor Sludge	
MLSS (mg/L)	5750±212	
MLVSS (mg/L)	5300±113	
SVI_{30} (mL/g)	34	

3.2. Wastewater Composition

Synthetic wastewater was used in all reactor operations. It was composed of all the necessary micro- and macro-nutrients for an optimum aerobic microbial growth. The composition of wastewater is slight modification of wastewater composition used in aerobic, anoxic or micro-aerobic granulation and nutrient removal studies in literature (Smolders et al., 1994; Erguder and Demirer 2005a,b; Fang et al., 2009; Shi et al., 2010). Acetic acid (HAc) was used to supply carbon source for aerobic heterotrophs and denitrifiers, on the other hand, bicarbonate (HCO_3^-) was used as carbon source for nitrifiers. The synthetic wastewater composition is given in Table 3.2. Under the light of results obtained in each set, HAc, NH_4Cl, $NaHCO_3$ were modified for the next reactor set. Table 3.3 represents the values of modified parameters in each set.

Table 3.2 The synthetic wastewater compositions in each set

General Wastewater Composition		Micro-nutrient Solution*	
HAc (mL/L)	Varied in sets	**$FeCl_2.4H_2O$ (g/L)**	1.5
NH_4Cl (mg/L)	Varied in sets	**H_3BO_3 (g/L)**	0.15
$NaNO_3$ (mg/L)	243	**$CuCl_2.2H_2O$ (g/L)**	0.02
$NaHCO_3$ (mg/L)	Varied in sets	**KI (g/L)**	0.18
$MgSO_4.7H_2O$ (mg/L)	180	**$MnSO_4.H_2O$ (g/L)**	0.1
$CaCl_2.2H_2O$ (mg/L)	160	**$(NH_4)_6Mo_7O_{24}.4H_2O$ (g/L)**	0.044
$Na_3PO_4.12H_2O$ (mg/L)	244	**$ZnCl_2$ (g/L)**	0.057
Yeast extract (mg/L)	2	**$CoCl_2.6H_2O$(g/L)**	0.15
Micro-nutrient solution*(mL/L)	0.6	**EDTA (g/L)**	10

Table 3.3 Modified ingredients in each set

SET	HAc (mL/L)	COD (mg/L)	NH_4Cl (mg/L)	Total Ammonium Nitrogen (TAN*) (mg/L)	$NaHCO_3$ (mg/L)
Set-1	1.78	2000	1530	400	3000
Set-2	1.34	1500	765	200	1500
Set-3	1.34	1500	765	200	1500
*TAN: NH_4-N + NH_3-N					

3.3. Experimental Procedure

The three sets conducted in this study and the aim of each set was given as follows;

- Set-1: the effects of seed sludge type on granulation and treatment efficiency of granules,
- Set-2: the effects of anoxic and aerobic period sequence on granulation and treatment efficiency of granules,
- Set-3: the effects of influent sulfate concentration and soluble sulfide on treatment performance of granules.

3.3.1. Reactor Configuration

Plexiglas cylindrical sequencing batch reactors (SBRs) each with a height of 60 cm, inlet diameter of 8 cm, effective volume of 2.45 L and exchange ratio of 50% were used in experiments. The exchange ratio is the ratio of discharged effluent volume to the working volume (Liu and Tay, 2004). Two identical reactors were used in Set-1 and Set-2 and one reactor was used in Set-3.

3.3.2. Sequencing Batch Reactor (SBR) Operational Conditions

In this thesis study, same SBR operational conditions were applied. In each set, SBRs were operated for 4 cycles in a day. Each cycle was 6 h and composed of feeding, anoxic, aerobic, settling and withdrawal periods. Durations of the periods were controlled by automatic time controllers. Hydraulic retention time (HRT) of the reactors was 12.3 h. One of the main purposes in Set-1 and Set-2 was granule cultivation. Since SRT has no influence on granulation process (Li et al., 2008), SRT was not controlled during the operational period of Set-1 and Set-2. Beun et al. (2000) stated that, there was an increase in SRT from 2 days to 30 days during granulation and then stabilized at 17 days when granules achieved. Therefore, in Set-3 SRT was not controlled either, because full granulation had already been achieved and no severe changes were observed on sludge properties during the application of Set-3.

Air was supplied by using aeration pumps with capacity of 360 L air/h which also provided hydraulic shear force (2 cm/s SAV). Steel mixers (48 rpm) were used to mix reactors' contents. In order to prevent possible mechanical destruction on cultivated granules, mixers were removed from the reactors after 75% granulation was achieved (Nor Anuar et al., 2012). Reactors were mixed during aerobic periods only by aeration, thereafter. Therefore, aeration was not only applied for air supply but also for well-mixing of the reactor content.

3.3.2.1. Set-1: The Effects of Seed Sludge Type on Granulation and Treatment Efficiency

The purpose of Set-1 was to compare 2 sludge types, namely, MBS and CAS in terms of granule cultivation and COD, TN removal performances of granules during and after cultivation.

Two SBRs (R1 and R2) with exchange ratio of 50% were operated for 37 days. Both reactors were operated under similar operational conditions. The only difference was the seed sludge type. R1 was seeded with MBS while R2 was seeded with CAS. Depending on the results of a preliminary study (Appendix A), an acclimation period was applied. The purpose of the acclimation period was to minimize detrimental effects of new operation type (from MBR to SBR and CASP to SBR) and new wastewater composition on seeded microorganisms. After 2-days of acclimation period, reactors were operated for 37 days. At the beginning of the operational period of 37 days, MLVSS concentrations of R1 and R2 were 7670±325 and 4570±183 mg/L, respectively.

During acclimation period, one cycle (6 h) consisted of 5 min of feeding, 120 min of anoxic period, 198 min of aerobic period, 35 min of settling period and 2 min of withdrawal period. After 2-days of acclimation, anoxic, aerobic and settling periods were changed in time as given in Table 3.4 to promote granulation and improve treatment efficiency. Reactors were operated at an upflow air velocity of 2 cm/s (during aerobic period) and a HRT of 12.3 h. Theoretical organic and total nitrogen loading rates of the reactors were 3.92 g COD/L.d and 0.86 g N/L.d, respectively during the entire operation of Set-1.

Table 3.4 Cycle details during operational period of 37 days in Set-1

Periods (min)	**Operation days**						
	1-5	**6-7**	**8-13**	**14-20**	**21-24**	**25-26**	**27-37**
Feeding period	5	5	5	5	5	5	5
Anoxic period	120	120	120	90	90	90	90
Aerobic period	208	213	218	248	253	255	258
Settling period	25	20	15	15	10	8	5
Withdrawal period	2	2	2	2	2	2	2

3.3.2.2. Set-2: The Effects of Anoxic and Aerobic Periods' Sequence on Granulation and Treatment Efficiency

The purpose of Set-2 was to determine the effects of anoxic/aerobic period sequence on granule cultivation from suspended sludge and COD, TAN removal performances of granules during and after cultivation.

MBS was used as suspended sludge seed. The seed sludge was initially acclimated to the SBR operation and synthetic wastewater. To this purpose, one SBR was seeded with MLSS and MLVSS concentrations of 7300±212 and 5000±71 mg/L, respectively. Operational conditions of SBR during 14 days of acclimation period are represented in Table 3.5.

Table 3.5 Cycle details during acclimation period of 14 days in Set-2

Operational Conditions	**Acclimation period (days)**					
	1	**2-4**	**5**	**6-9**	**10**	**11-14**
Number of cycles per day	1	1	2	2	2	4
Organic loading rate (g COD/L.d)	0.19	0.39	0.78	1.18	1.95	2.94
Total N loading rate (g N/L.d)	0.024	0.048	0.094	0.14	0.24	0.47

After acclimation period, acclimated seed sludge was equally divided into 2 SBRs, namely, R1 and R2. R1 and R2 were operated under similar operational conditions for 63 days (Table 3.6). The only difference was the anoxic/aerobic period sequence. R1 was operated with anoxic-aerobic period sequence while R2 was operated with aerobic-anoxic sequence. Initial MLVSS concentrations of R1 and R2 at the beginning of the operational period were 1180±118 and 1230±102 mg/L, respectively.

Both reactors were operated at 6 h-cycles composed of; 10 min of feeding, 45 min of anoxic, 288-302 min of aerobic, gradually decreasing settling (from 15 to 1 min) and 2 min of withdrawal periods (Table 3.6).

Table 3.6 Cycle details during operational period of 63 days in Set-2

Operational Conditions	**Operation (days)**							
	1-9	**10-13**	**14**	**15-16**	**17-25**	**26-39**	**40-48**	**49-63**
Number of cycles /d	4	4	4	4	4	4	4	4
HRT (h)	12.3	12.3	12.3	12.3	12.3	12.3	12.3	12.3
Organic LR* (g COD/L.d)	1.47	2.94	2.94	2.94	2.94	2.94	2.94	2.94
Total N LR* (g N/L.d)	0.24	0.47	0.47	0.47	0.43	0.43	0.43	0.43
Cycle duration (h)	6	6	6	6	6	6	6	6
Periods and durations in one (1) cycle								
Feeding (min)	10	10	10	10	10	10	10	10
Anoxic (min)	45	45	45	45	45	45	45	45
Aerobic (min)	288	298	299	301	301	298	301	302
Settling (min)	15	5	4	2	2	5	2	1
Withdrawal (min)	2	2	2	2	2	2	2	2
*LR: Loading Rate								

In order to achieve almost the same F/M ratio as that of the final conditions of the acclimation period (1.2 g/g.d for COD and 0.19 g/g.d for N), feed solution was diluted by half and influent COD:TAN concentrations of synthetic wastewater were set as 750:100 (mg/L : mg/L) for both reactors for the first 9 days of operation. Corresponding loading rates were 1.47 g COD/L.d and 0.24 g N/L.d (leading to F/M ratio of 1 g/g.d for COD and 0.23 g/g.d for N). After both reactors reached MLVSS concentrations of more than 2000 mg/L (Day 10), influent COD:TAN concentrations were set as 1500:200 (mg/L : mg/L). Therefore, theoretical organic and nitrogen loading rates of the reactors were 2.94 g COD/L.d and 0.47 g N/L.d, respectively (Table 3.6). There was 40 mg/L of influent NO_3-N to enrich heterotrophic denitrifiers during anoxic period. After 75% granular sludge was achieved in the reactors (Day 15), influent NO_3-N concentration and in turn total N loading rate was decreased to 20 mg/L and 0.43 g N/L.d, respectively (Day 17).

Both reactors were operated at upflow air velocity of 1-2 cm/s (during aerobic period) and a hydraulic retention time (HRT) of 12.3 h. The reactor contents were mixed with 48 rpm-mixers. After full granulation was achieved in both reactors, mixers were removed to prevent mechanical destruction of granules (Day 25) (Nor-Anuar et al., 2012). After removal of the mixers, in order to protect the hydraulic selection provided by settling time in R2 (aerobic-anoxic sequence), mixing of the reactor content was provided by 1 min aeration before starting of the settling period.

3.3.2.3. Set-3: The Effects of Influent Sulfate and Soluble Sulfide Concentration on Treatment Performance of Granules and Granular Structure

The results of Set-2 revealed that sulfate and total soluble sulfide might have adversely influenced the treatment performance. Therefore, Set-3 was conducted in order to investigate the effects of influent sulfate on sulfide production and this sulfide production-related change on treatment performance of the granules.

Set-3 was conducted by using the SBR (R1) used in Set-2 and operated for 28 days. The seed sludge was the granular sludge (with 95% granule percentage) developed in R1 in Set-2.

One cycle was composed of 5 min of feeding, 45 min of anoxic period, 306 min of aerobic period, 2 min of settling period and 2 min of withdrawal period. The initial MLVSS and MLSS concentrations in the reactor were 5300±113 mg/L and 5750±212 mg/L, respectively.

Synthetic wastewater composition was same as that of Set-2 except the influent SO_4^{2-} concentrations (Table 3.3). Loading rates were 2.94 g COD/L.d (influent COD of 1500 mg/L) and 0.43 g N/L.d (influent TAN of 200 mg/L) similar to Set-2 (Table 3.6). Reactor was exposed to 35.1 mg/L, 46.8 mg/L, 52.6 mg/L, 58.5 mg/L and 70.2 mg/L SO_4^{2-} by changing $MgSO_4.7H_2O$ concentration in wastewater solution. The variations on treatment efficiencies, granule size and properties were investigated. Table 3.7 reveals the applied $MgSO_4.7H_2O$ concentrations in the wastewater solution with their corresponding influent SO_4^{2-} concentrations.

Table 3.7 Influent sulfate concentrations during 28 days operation in Set-3

Modified Parameters in Synthetic Wastewater	**Days**						
	1	**2-6**	**7-11**	**12-15**	**16-20**	**21-23**	**24-28**
$MgSO_4.7H_2O$ (mg/L)	90	120	135	90	150	90	180
SO_4^{2-} (mg/L)	35.1	46.8	52.6	35.1	58.5	35.1	70.2

3.4. Analytical Methods

3.4.1. Reactor Performance

In order to investigate the performances of the SBRs operated in this work, pH, dissolved oxygen (DO) were measured daily and soluble chemical oxygen demand (sCOD), total ammonia nitrogen (TAN), nitrate (NO_3-N), nitrite (NO_2-N), sulfate (SO_4^{2-}) and total soluble sulfide (S^{2-}+HS+H_2S) were analyzed on alternate days.

In order to do the mass balance and determine nitrification, denitrification and sCOD removal efficiencies on cycle basis for each reactor, alternate day sampling was done. Sampling was done for 4 points in one cycle as follows; wastewater composition (influent), reactor content after feeding period (initial) reactor content after the first reaction period (anoxic or aerobic) and discharged effluent. Experiments were performed after filtering samples from 0.45 µm cellulose acetate filter.

Accuracy of the devices and kits used for measured compounds were determined by calibration curves and given in Appendix B.

_pH:_ Daily pH measurements were performed with a pH meter (Eutech Instruments, Cyberscan pH 510) and a pH probe (Hanna Instruments HI 1230 pH probe).

Dissolved Oxygen (DO): Daily DO measurements were performed with a DO meter (Extech Instruments Model 407510 Heavy Duty DO Meter) during anoxic and aerobic period of reactors.

Soluble Chemical Oxygen Demand (sCOD): sCOD of filtered samples were measured according to an EPA approved reactor digestion method (for sCOD range of 0-150 mg/L and 0-1500 mg/L) (Hach Water Analysis Handbook, 2012). For sCOD measurements, Aqualytic AL 38 heater and PC Multidirect Spectrophotometer (Program 130-131) were used..

Total Ammonia Nitrogen (NH_4-N + NH_3-N): TAN concentrations of filtered samples were analyzed by following standard methods (4500-NH_3 Steps B and C) (APHA, AWWA, WEF, 2005). Gerhardt Vapodest was used for distillation of ammonium nitrogen and 0.02 N H_2SO_4 was used as titrant.

Nitrate-Nitrogen (NO_3-N): Nitrate-nitrogen (NO_3-N) concentrations of filtered samples were analyzed by EPA approved NitraVer® cadmium reduction method (for a range of 0.3 -30 mg/L NO_3-N) (Hach Water Analysis Handbook, 2012). Spectrophotometric measurement was done via Hach DR2800 Spectrophotometer (Hach program no 355).

Nitrite-Nitrogen (NO_2-N): Nitrite-nitrogen (NO_2-N) concentrations of filtered samples were analyzed by EPA approved NitriVer® ferrous sulfate method (for a range of 2 – 250 mg/L NO_2) (Hach Water Analysis Handbook, 2012). Spectrophotometric measurement was done via Hach DR2800 Spectrophotometer (Hach program no 373).

Sulfate (SO_4^{2-}): SO_4^{2-} concentrations of filtered samples were analyzed by EPA approved SulfaVer® turbidity method (for a range of 2 – 70 mg/L SO_4^{2-}) (Hach Water Analysis Handbook, 2012). Spectrophotometric measurement was done via Hach DR2800 Spectrophotometer (Hach program no 680).

Total Soluble Sulfide (S^{2-}+HS^-+H_2S): Sulfide concentrations of filtered samples were analyzed by EPA approved methylene blue method (for a range of 5-800 µg/L S^{2-}) (Hach Water Analysis Handbook, 2012). Spectrophotometric measurement was done via Hach DR2800 Spectrophotometer (Hach program no 690).

3.4.2. Granule Cultivation and Granule Properties

In order to determine granulation process, the sludge content of the SBRs was weekly sampled for suspended solids (SS), volatile suspended solids (VSS), extracellular polymeric substances (EPS), average granule sizes and sludge volume index (SVI) analysis. The analytical methods followed for each parameter's analysis are given below.

Suspended solids (SS) and volatile suspended solids (VSS): SS and VSS were measured by following standard methods (2540 D, E) (APHA, AWWA and WEF 2005).

Extraction of Extracellular Polymeric Substance (EPS): Extraction method for Extracellular Polymeric Substances (EPS) was determined after a preliminary study comparing the appropriate extraction methods used in literature (Appendix C). As a result of this preliminary study, the ultrasound+formamide+NaOH+Centrifuge method described by Adav and Lee (2008) was selected and used to extract EPS from sludge samples.

Protein content of EPS: Protein content was measured by using colorimetric method described in the study of Lowry et al. (1951). Extracts were diluted 5 to 10 times with PBS solution in order to have results in calibrated absorbance range (0-250 µg/2ml). Calibration curves are given in Appendix B.

Carbohydrate content of EPS: Carbohydrate portion of the EPS was analyzed by colorimetric method defined in the study of Dubois et al. (1956). Extracts were diluted 10 to 20 times in order have results in calibrated absorbance range of (0-80µg/2ml). Calibration curves are given in Appendix B.

Average Granule Size: Granule samples were taken to falcons during completely mixed, aerobic period of reactors. Random samples were taken from falcons three times and sizes of 8 granules from each random sample (24 granules in total) were measured via ocular micrometer and light microscope (Leitz Wetzlar Microscope, 6.3 x 4 magnification). Standard deviations and mean sizes were calculated from the data obtained. Granule pictures were taken by 3.2 Megapixel camera (Sony Ericsson W890i).

Sludge Volume Index (SVI): Sludge volume index for 30 minutes and 5 minutes were measured following the Standard Methods (2710 D) (APHA, AWWA, WEF, 2005).

Granule Percentage: In order to calculate the granular sludge portion of the reactor sludge (granule-flocculent-suspended sludge mixture), dynamic SVI_x proposed by Schwarzenbeck et al. (2005) was used. SVI_5 was used to define granular sludge and SVI_{30} was used for all granular, suspended and flocculant sludge. Granule percentage was calculated by using SVI_5 and SVI_{30} as it is mentioned in the study of Liu et al. (2010b) (Eqn 3.1) when mean size of granules were larger than 0.2 mm.

$$Granule\% = \frac{SVI_{30}}{SVI_5} \times 100 \qquad \text{Eqn 3.1}$$

Settling Velocity of Granules: In order to determine settling properties, settling velocities of granules were measured according to the method described by Etterer and Wilderer (2001). Granules were dropped to free-fall in a cylindrical tube (5 cm diameter, 1 m height) with effective height of 90 cm (filled with water) and last 50 cm of the fall were recorded to define terminal settling velocity of granules. In each test at least 15 granules were used and average values were calculated and given in figures, tables.

CHAPTER 4

RESULTS AND DISCUSSION

There were three sets conducted namely, Set-1 (the effects of seed sludge type), Set-2 (the effects of anoxic/aerobic period sequence) and Set-3 (the effects of influent sulfate and soluble sulfide). In each set, reactors were operated until inspected parameters and their effects could be discriminated significantly.

4.1. Set-1: The Effects of Seed Sludge Type on Granulation and Treatment Efficiency

Seed sludge properties are thought to be effective on settleability, microbial activity, hydrophobicity and other macroscopic characteristics of granular sludge (Liu and Tay, 2004). In studies on aerobic granulation, conventional activated sludge (CAS) is usually used as suspended seed sludge (Wilen et al., 2008; Yu et al. 2009; Sheng et al. 2010a).

MBS differs from CAS in terms of sludge morphology, EPS content and bacterial composition. MBS has mean floc size of 100 – 240 µm and CAS has mean floc size of 70 – 160 µm (Le Clech et al., 2006; Mass'e et al. 2006). Furthermore, EPS amount of MBS flocs is also higher than that in CAS flocs, thus they are good at holding bounded EPS (Le Clech et al., 2006; Mass'e et al., 2006). It is also stated that MBS contains denser flocs and fewer amounts of long filamentous bacteria than CAS sludge (Cicek et al., 1999; Mass'e et al., 2006). Therefore, Set-1 was conducted to investigate the effects of seed sludge type, namely, CAS and MBS, on granulation and treatment efficiency of granules.

Reactors operated in Set-1 were named based on their seed sludge type. The reactor seeded with MBS was named as R1 and the reactor seeded with CAS was named as R2. Due to some operational problems, 3 phases occurred instinctively during the operation. Therefore, for better representation of operational conditions and results, operational period of 37 days was described in 3 phases. Phase-1 corresponds to the Days 1-10, Phase-2 (overloading conditions) to the Days 11-27 and Phase-3 (recovery) to the Days 28-37.

Observations and results obtained for both reactors were similar for operational parameters such as pH and DO. On the other hand, results obtained from granulation and treatment performances were more significant for discrimination of seed sludge types. Therefore, results were discussed in the order of operational parameters, granulation performance and treatment performance.

4.1.1. Operational Parameters

4.1.1.1. pH

During the 37 days of operation daily measured pH values of R1 and R2 for synthetic wastewater (feed solution) and reactor content in anoxic periods and aerobic periods are represented in Figure 4.1 and Figure 4.2, respectively.

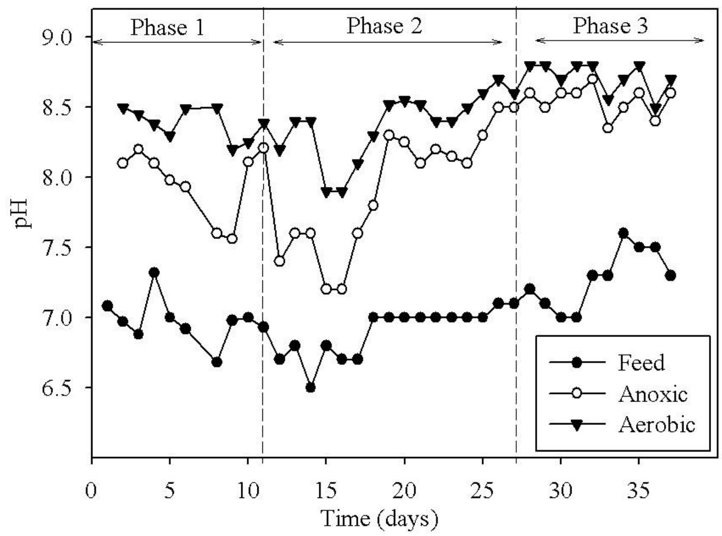

Figure 4.1 pH variations of R1 during operation (MBS) in Set-1
(-○- anoxic period -▼- aerobic period)

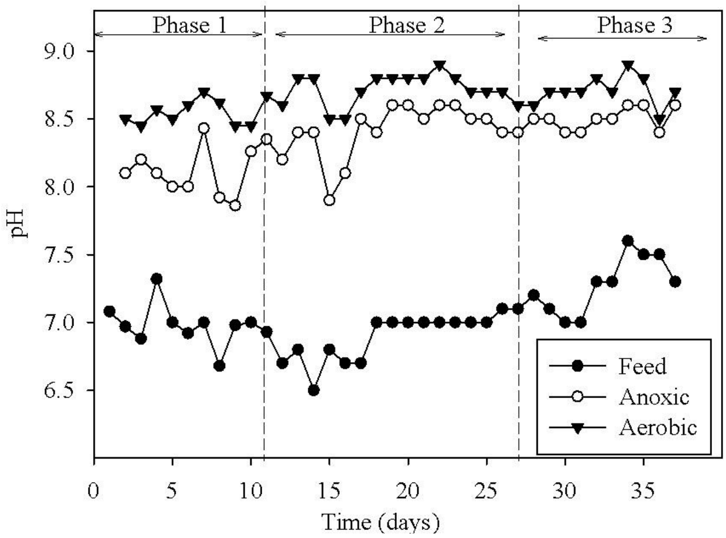

Figure 4.2 pH variations of R2 during operation (CAS) in Set-1
(-○- anoxic period -▼- aerobic period)

As can be seen from Figure 4.1 and Figure 4.2, pH values in both reactors were higher than that of feed solution. Average pH value of feed solution was 7±0.2. Yet, during 37 days of operation, average pH values in anoxic periods were 8.2±0.4 and 8.3±0.2 for R1 and R2, respectively. During non-aerated periods, if NO_x exists, heterotrophs use NO_x as electron acceptor and denitrification occurs (Tchobanoglous et al., 2004). One of the products of denitrification reaction is alkalinity (Eqn 4.1). Therefore, in anoxic period, increase in pH was an expected situation.

$$5CH_3COOH + 8NO_3^- \rightarrow 4N_2 + 6H_2O + 8OH^- \qquad \text{Eqn 4.1}$$

The other possible mechanism for pH increase in non-aerated periods is sulfate reduction. Sulfate reducing bacteria (SRB) can reduce SO_4^{2-} by using an electron acceptor such as acetate, hydrogen etc. (Eqn 4.2). The products can be S^{2-}, HS^- or H_2S according to the pH of the environment (Sawyer et al., 2003). As can be seen from Eqn 4.2, consumption of H^+ ion and production of bicarbonate can be a reason for pH increase when SRB is enriched in the sludge.

$$CH_3COO^- + SO_4^{2-} + H^+ \xrightarrow{SRB} CO_2 + HCO_3^- + HS^- + H_2O \qquad \text{Eqn 4.2}$$

In the periods following the anoxic periods, which are aerobic periods of each cycle, 0.3-0.4 unit of pH increase was observed in both reactors. In 37 days of operation, average pH values in aerobic periods were 8.5±0.3 and 8.7±0.1 for R1 and R2, respectively. Total oxidized nitrogen (TON) removal during aerobic period, which is called as simultaneous nitrification-denitrification (SNDN), can be possible with aerobic granules (de Kreuk et al., 2005;2006; Yilmaz et al., 2007; Gao et al., 2011). Therefore, one of the reasons behind the 0.3-0.4 unit pH increase in aerobic periods might be SNDN-related alkalinity production.

4.1.1.2. Dissolved Oxygen (DO)

As can be seen from Figure 4.3 and Figure 4.4, during the operation period of 37 days, in anoxic periods, appropriate anoxic conditions were provided with the DO concentrations of 0.3±0.1 mg/L and 0.3±0.2 mg/L in R1 and R2, respectively. In aerated periods, appropriate aerobic conditions were provided with DO concentrations of 8.5±0.4 mg/L and 8.5±0.3 mg/L in R1 and R2, respectively (Figure 4.3 and Figure 4.4).

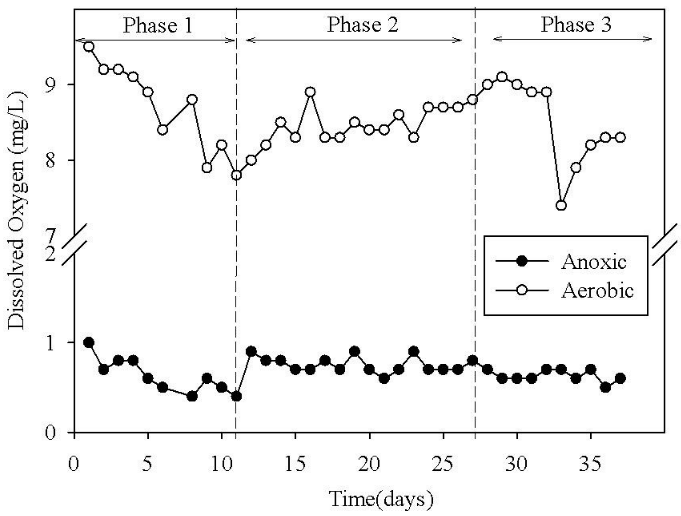

Figure 4.3 DO concentrations in R1 during operation in Set-1
(-●- anoxic period -○- aerobic period)

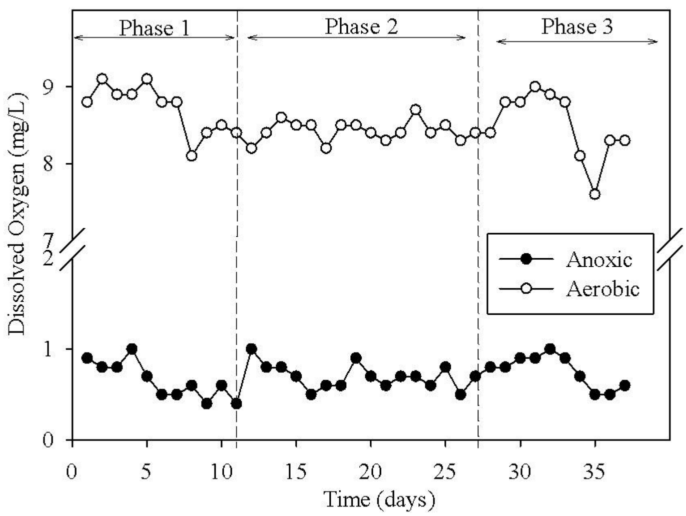

Figure 4.4 DO concentrations in R2 during operation in Set-1
(-●- anoxic period -○- aerobic period)

4.1.2. Granulation Performance

The effects of seed sludge type on granule cultivation from suspended sludge were investigated. Granulation process was observed by periodically taking samples from the reactors. Evolutions of two seed sludge types were depicted in Figure 4.5 and Figure 4.6. Granular sludge was developed in both reactors. However, full granulation could not be achieved in both reactors, till the end of the operation. In 37 days of operation, the sludge in both reactors was granule-floc mixture dominated.

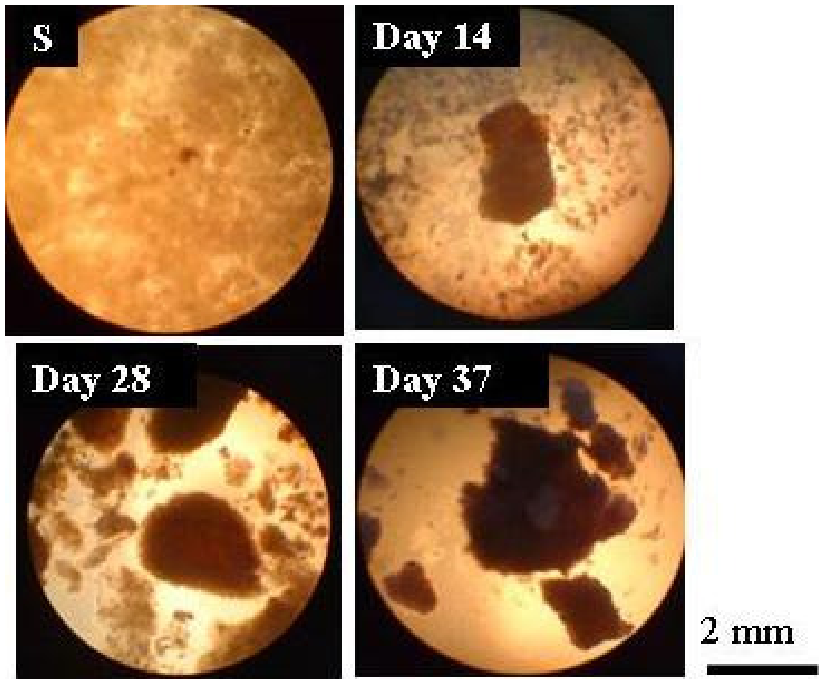

Figure 4.5 Microscopic view of sludge in R1 in Set-1
(S: Seed Sludge)

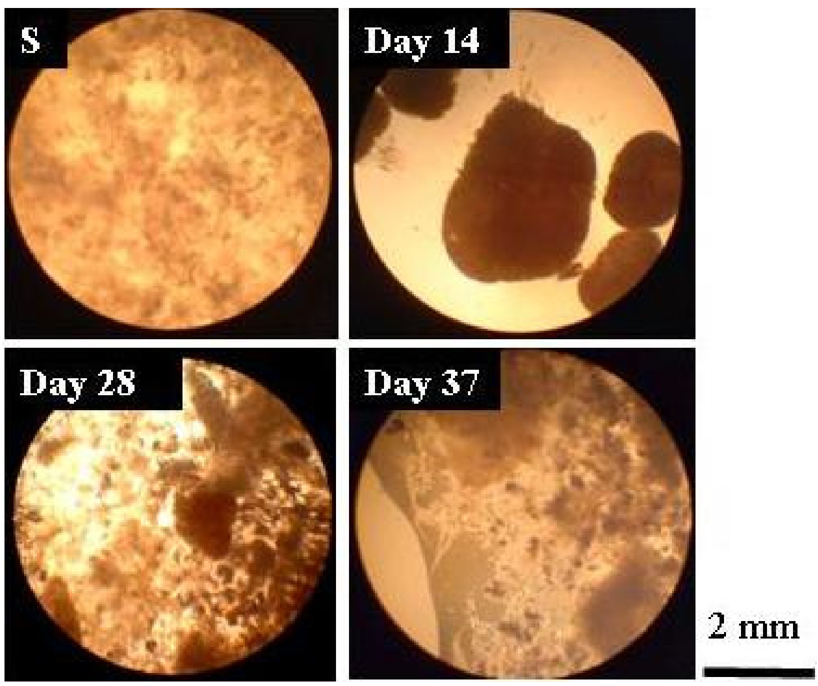

Figure 4.6 Microscopic view of sludge in R2 in Set-1
(S: Seed Sludge)

As can be seen from Figure 4.5 and Figure 4.6, seed sludge of both reactors was totally suspended and floccular, and they had no granular sludge at the beginning. The colors of seeds were reddish-brown and dark-brown for R1 and R2, respectively. In both reactors, granular sludge was developed and granules with a compact structure first appeared on Day 9. The color of active aerobic granules is known to be yellow-brown (Zhang et al. 2005). In this study, the color of the seed sludge of both reactors also turned from dark reddish-brown to light yellow-brown during granulation (Figure 4.7, Figure 4.8). Yet, the color of the sludge in R2 was lighter than that in R1. The color of the sludge is generally an indicator of the predominant microorganisms or the chemical composition of the content in reactors (Gao et al., 2011). Luxmy et al. (2000) stated that bacterial community in MBS is significantly different than CAS. Therefore, the color difference observed in reactors might be due to the predominance of the different bacterial communities.

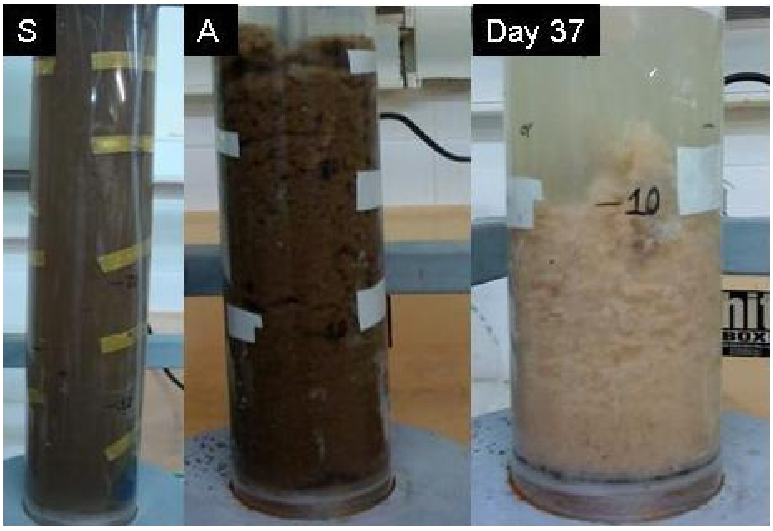

Figure 4.7 Change of sludge properties and color in R1 in Set-1
(S: Seed Sludge, A: Acclimated Sludge)

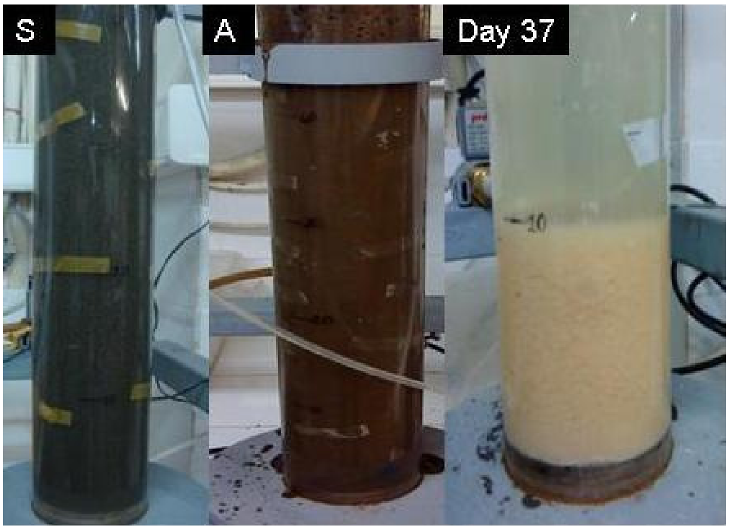

Figure 4.8 Change of sludge properties and color in R2 in Set-1
(S: Seed Sludge, A: Acclimated Sludge)

Settling velocities of the granules developed in R1 and R2 were 36-39 m/h and 19-20 m/h, respectively (Table 4.1). Granular sludge can have settling velocities varying from 18 m/h to more than 91 m/h (Jang et al., 2003; Qin and Liu, 2006; Wang et al., 2006; Adav et al., 2009b; Gao et al., 2011). Thus, although settling velocities of R1 granules were higher than that of R2 granules, they were even not in the mid-range of literature values. Microscopic analysis of sludge were also conducted on Day 14 and granules having average size of 1.58±0.83 mm with dense non-filamentous structure and irregular shape were observed in R1 (Figure 4.5, Table 4.1). On the other hand, granules cultivated in R2 had average size of 2.19±0.82 mm, filamentous structure and spherical shape (Figure 4.6, Table 4.1). Janczukowicz et al. (2001) reported that filamentous microorganisms are the indicators of sludge bulking which enrich under low F/M conditions, low or high DO concentrations or due to inappropriate reactor configuration and have adverse effects on settling properties. Therefore, non-filamentous structure of granules in R1 was an indicator of successful adaptation of MBS to the stressful conditions applied for granulation. Yet in R2, granular sludge having filamentous structure revealed that adaptation of CAS sludge to the stressful conditions was still not successfully completed. As Cicek et al. (1999) stated that filamentous microorganisms were found in greater amounts in CAS compared to that in MBS (and mostly placed inside the flocs). Therefore, the delay in adaptation of sludge and the filamentous structure of granules in R2 were not surprising. The granules with filamentous structure as observed in R2 might also result in deterioration of settling properties and stability of the granules (Janczukowicz et al., 2001; Gao et al., 2011). Hence, settleability of granules in R1 was better than that in R2.

During Phase-2 (Days 11-27), deterioration of the granular properties (stability, settleability) was observed for both reactors. Disintegrated granules were washed out from the system and caused decrease in MLVSS in both reactors (Table 4.1). Granular sludge percentage (the ratio of SVI_{30} to SVI_5) slightly increased since suspended sludge and disintegrated granular sludge were washed out from the reactors (Table 4.1). In other words, both disintegrated granular and existing suspended content of the sludge inside the reactors declined. The average particle sizes of the granules of R1 and R2 were also measured on Day 28 (at the end of Phase-2) and it was observed that granules of R2 decreased in size (1.08±0.54 mm), became loose and were dominated with filamentous structure (Figure 4.6, Table 4.1). Granules in R1 were also disintegrated and washed out; however, the granules which were not disintegrated continued to greaten in size and average size increased to 2.48±1.28 mm at the end of Phase-2 (Figure 4.5, Table 4.1). These average granule sizes indicated that there were still large granules which were not disintegrated in R1. On the other hand, the reason behind severe disintegration of granules in R2 might be due to operational conditions in Phase-2. Yang et al. (2004b) indicated that free-ammonia (FA) concentration higher than 23.5 mg/L prevents granule formation. Due to cyclic nature of SBR operation, treatment efficiency of each cycle affects the initial concentration of the next cycle. In other words, the decrease in the removal efficiency of one cycle means the increased effluent concentrations, which results in an increase in the initial wastewater concentrations of the following cycle based on the volumetric exchange ratio.

Table 4.1 Granular sludge properties of each reactor in Set-1

Parameter	**R1**			**R2**		
	Phase-1*	**Phase-2***	**Phase-3***	**Phase-1**	**Phase-2**	**Phase-3**
Settling velocity (m/h)	-	36	39.6	-	19.1	20.2
Average size (mm)	1.58±0.83	2.48±1.28	0.97±0.47	2.19±0.82	1.08±0.54	0.56±0.23
MLVSS (g/L) (beginning-end of phase)	4.6 -2.4	2.4-1.5	1.5-3	4.5-2.4	2.4-1.4	1.4-1.7
MLVSS/MLSS	0.71	0.84	0.75	0.90	0.77	0.52
Granule (%)	34	37	32	33	37	22
*Phase-1: Days 1-10, Phase-2: Days 11-27, Phase-3: Days 28-37						

Parallel to this case, during Phase-2, low treatment efficiency related TAN accumulation was observed in both reactors. TAN accumulation resulted in average free-ammonia concentrations of 38±8 mg/L and 46±6 mg/L in R1 and R2, respectively. Therefore, disintegration of granules and decrease in MLVSS concentrations (Table 4.1) were observed for both reactors, being severe in R2. Size increase of the granules in R1 represented that although free-ammonia concentration (38±8 mg/L) caused disintegration of granules, it did not severely prevent agglomeration of MBS. Therefore, it can be said that MBS could form granules under free-ammonia concentrations (38±8 mg/L) which mostly prevented agglomeration of CAS.

During Phase-3, R1 granules, despite the decrease in size, remained in their intact structure, while R2 granules decreased in size and had a fluffy structure. The average particle sizes of the granules sampled from R1 and R2 at the end of the experiment (Day 37) were measured as 0.97± 0.47 mm and 0.56±0.23 mm, respectively (Table 4.1). It was stated that optimum granule size for effective nutrient removal is 1.2-1.4 mm (de Kreuk et al., 2006). Therefore, granules of R1 were close to the size range for efficient nutrient removal, but granules of R2 were smaller in size for effective nutrient removal. Disintegration/degradation of the granules in R2 was also confirmed by the decreasing trends of MLVSS/MLSS ratio values and the granular sludge percentages of the total biomass content through Phases 1 to 3 (Table 4.1). MLVSS/MLSS ratio was more or less constant in R1, while it decreased from 0.9 to 0.52 in R2. MLVSS/MLSS ratio gives an approximate idea about the biologically active biomass content of the sludge (Woodside and Kocurek, 1997). Therefore, continuous decrease in MLVSS/MLSS ratio indicated that the amount of active microorganisms was decreasing in R2. Therefore, it can be said that granule-floc mixture of R1 was more resistant to the studied loading rates and potential toxicity (occurred during Phase-2) than those of R2.

Granulation and disintegration patterns were also followed by extracellular polymeric substances (EPS) of the granules developed (Figure 4.9). Protein (PN)-EPS and polysaccharide (PS)-EPS contents of seed sludge of both reactors were around 100 mg/g VSS and 75 mg/g VSS, respectively. In Phase-1, PN-EPS increased to 180 and 160 mg/g VSS for R1 and R2 sludge, respectively. PS -EPS were almost stable during granulation in Phase-1. PN/PS ratio was reported to increase sharply during granulation (Zhang et al. 2007; Jiang and Liu, 2010). Therefore, the increase in PN amount was attributed to the granule formation in Phase-1. In Phase-2, both protein and polysaccharide contents of EPS significantly increased from 180 to 300 mg/g VSS and from 160 to 250 mg/g VSS in R1 and R2, respectively. It was revealed that increase in OLR and stressful conditions caused increase in EPS concentration (Liu et al., 2004a; Zhu et al., 2012). As stated before, free-ammonia toxicity (stressful conditions) and high initial concentrations were observed in reactors during Phase-2. These stressful conditions might be the possible reason of the sharp EPS increase in both reactors. PN and PS contents of granules in R1 almost stabilized in Phase-3. However, PN increase in R2 continued significantly up to concentration of 500 mg/g VSS, while PS increased to a value of 186 mg/g VSS. It was revealed that high level of EPS clogged the pores of granular sludge and decreased the substrate gradient which would lead to the disintegration of granules (Mu et al. 2006). Durmaz and Sanin (2001) also stated that, PN content of EPS increased if cell lysis occurred. Therefore, continuous increase of EPS content observed during Phase-2 and Phase-3 might have negatively affected the granules and taken role in disintegration of R2 granules.

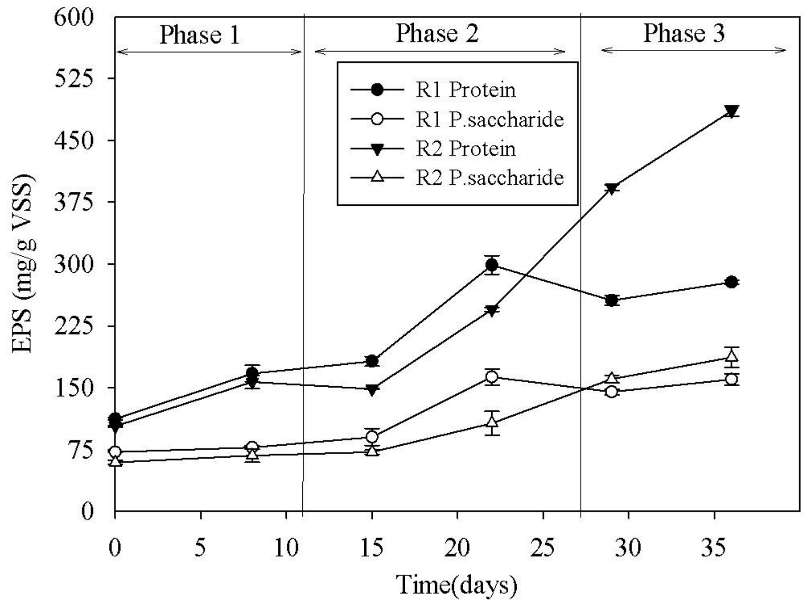

Figure 4.9 Variations in the EPS contents of the granules developed during operation in Set-1

In literature, there is no typical range of PN/PS ratio representing the stable granular form (Liu et al., 2004a). PN/PS ratios of 0.1 to 12 were reported in literature (Tay et al., 2001b; Juang et al., 2012). Zhu et al. (2012) stated that if PN was the predominant compound of the EPS content as it is in this study, decrease in PN/PS ratio would cause loss of granular compactness and disintegration of granular sludge. In this study, it was observed that during Phase-1, in which the granular sludge developed in both reactors, PN/PS ranges of the sludge in both reactors were between 1.8 and 2.0. During Phase-2 where deterioration of granules was observed in both reactors, PN/PS ratio was still 1.8-2.0 for R1 and it was calculated as 2.0-2.3 for R2. These observations indicated that, although PN/PS ratio was more or less same with the values that granulation occurred (Phase-1) in both reactors, granule disintegration could occur. During Phase-3; PN/PS ratio was still around 1.8 in R1, while it increased from 2.3 to 2.6 in R2 where granule sizes decreased in both reactors. Therefore, it can be said that the change in PN/PS ratio was not an indicator of granule disintegration in this study. However, the changes in EPS contents (either PN or PS) relative to seed sludge might reveal the granulation process. In addition, the changes in EPS contents (i.e. excess EPS production) relative to stable conditions (EPS content during well operating conditions) might be an indicator of the disintegration.

4.1.3. Treatment Performances

4.1.3.1. Nitrogen Removal

Nitrogen removal performances of reactors were investigated in terms of TAN removal (TAN oxidation), Total Oxidized Nitrogen (TON) removal and Total Nitrogen (TN) removal (N loss). The removal efficiencies for TAN and TON during anoxic and/or aerobic periods of cycles and for TN during whole cycle were calculated by following equations (Eqn 4.3, Eqn 4.4,
Eqn 4.5, Eqn 4.6, Eqn 4.7,
Eqn 4.8). The subscripts used in the equations namely, initial and effluent, refer to the concentration of the samples taken after feeding period and during withdrawal period of a cycle, respectively.

$$TAN\ RE\,\% = \frac{(TAN_{initial} - TAN_{effluent})}{TAN_{initial}} \times 100 \qquad \text{Eqn 4.3}$$

$$Nitrification\ \% = \frac{[(TAN_{initial} + NO_{2initial}) - (TAN_{effluent} + NO_{2effluent})]}{(TAN_{initial} + NO_{2initial})} \times 100 \qquad \text{Eqn 4.4}$$

$$TN_{anoxic}\ RE\,\% = \frac{(NO_{3initial} + NO_{2initial}) - (NO_{3effluent} + NO_{2effluent})}{(NO_{3initial} + NO_{2initial})} \times 100 \qquad \text{Eqn 4.5}$$

$$TN_{anoxic}\ RE\,\% = Denitrification\,\% \qquad \text{Eqn 4.6}$$

$$TN_{aerobic}\ RE\,\% = SNDN\,\% \qquad \text{Eqn 4.7}$$

$$TN\,RE\,\% = \frac{(TAN_{initial} + NO_{3initial} + NO_{2initial}) - (TAN_{effluent} + NO_{3effluent} + NO_{2effluent})}{(TAN_{initial} + NO_{3initial} + NO_{2initial})} \times 100 \qquad \text{Eqn 4.8}$$

Where,
RE: Removal Efficiency
TAN: Total Ammonia Nitrogen
TN: Total Nitrogen,
SNDN: Simultaneous Nitrification-Denitrification

Analyses revealed that the average initial TAN concentrations of R1 during Phases-1, 2 and 3 were 263±73 mg/L, 343±25 mg/L and 250±44 mg/L, respectively, and of R2 were 240±73 mg/L, 327±21 mg/L, 248±45 mg/L for Phases-1, 2 and 3, respectively. Figure 4.10 and Figure 4.11 show the profiles of TAN loading rate, TAN oxidation efficiencies and TAN effluent concentrations of anoxic and aerobic periods of monitored cycles. As can be seen from Figure 4.10 and Figure 4.11, during the 37 days of reactor operation, TAN removal efficiencies of reactors were low (5 - 45%) compared to literature studies (more than 96-99%) (Qin and Liu, 2006; Yilmaz et al., 2007; Adav et al., 2009b). However, R1 achieved slightly better TAN treatment performance than R2.

As seen in Figure 4.10 and Figure 4.11, TAN loading rates were 0.8 g N/L.d, for both reactors during the experiments. Considering the initial and effluent TAN concentrations of the anoxic periods of the monitored cycles, it was understood that there was no or negligible TAN oxidation during anoxic periods in both reactors (Figure 4.10b, Figure 4.11b). During Phase-1, TAN oxidation efficiencies of R1 and R2 were fluctuating and in the range of 25-40% and 10-30% under aerobic conditions (aerobic periods of the cycles), respectively (Figure 4.10b, Figure 4.11b). Despite fluctuations in Phase-1, TAN removal efficiencies displayed an increasing trend for both reactors. The TAN removal efficiencies on Day 11 were 3 times higher than that in Day 1 for R1 and R2, respectively, which proved the enrichment of the nitrifying bacteria. Average TAN oxidation efficiencies of R1 and R2 were 32±11% and 20±8% in Phase-1, respectively (Table 4.2).

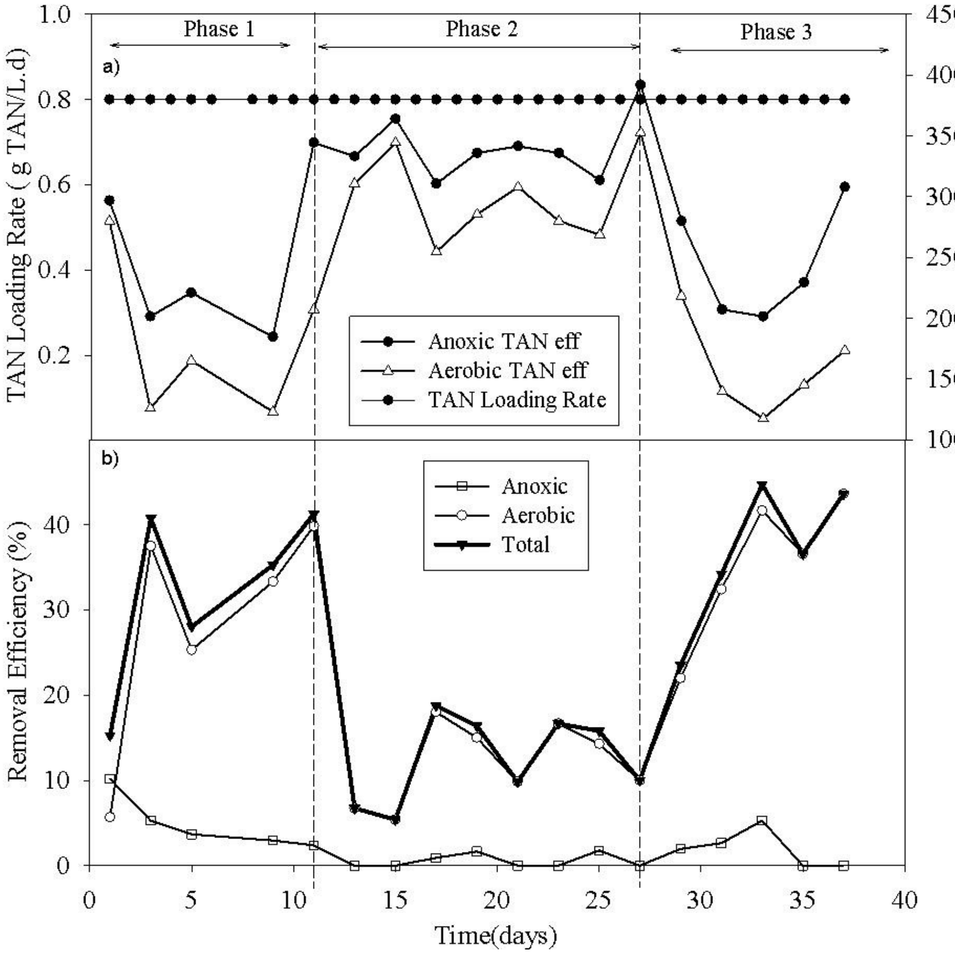

Figure 4.10 TAN overview of R1 during operation in Set-1
a) Effluent concentrations and Loading Rate (LR), b) Removal Efficiencies

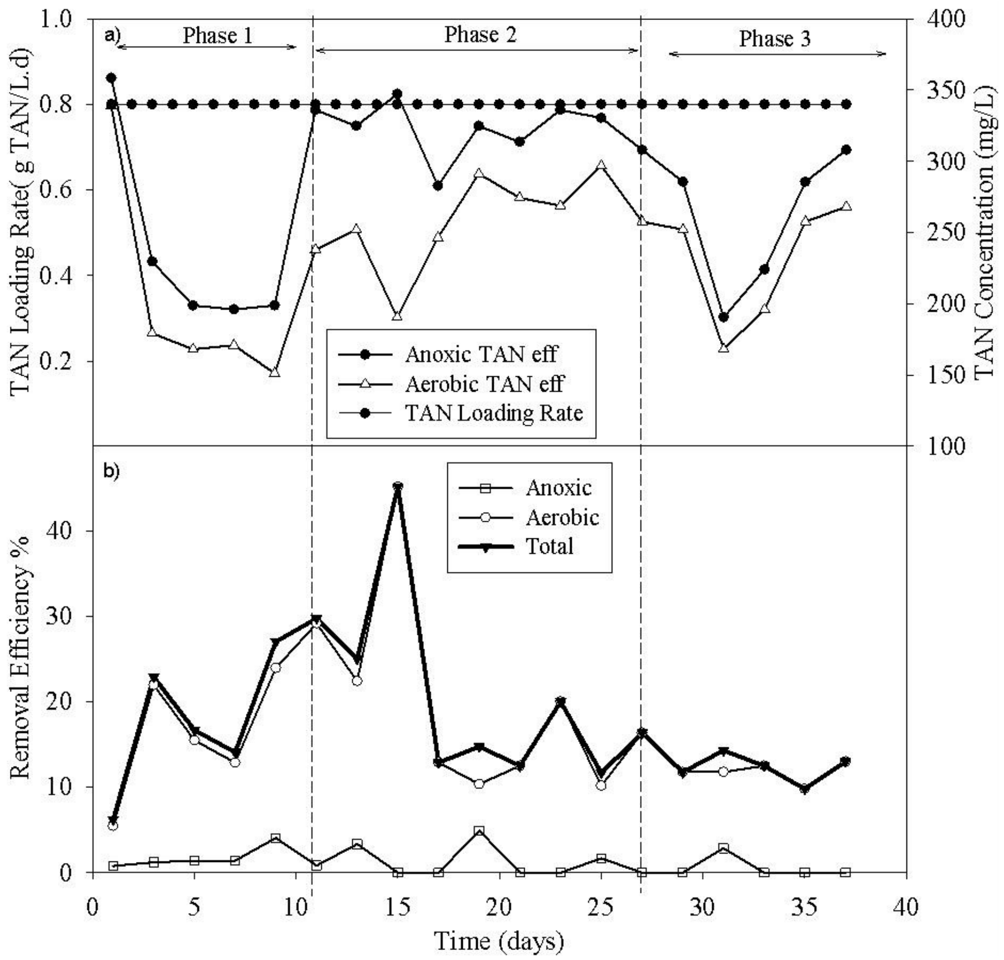

Figure 4.11 TAN overview of R2 during operation in Set-1
a) Effluent concentrations and Loading Rate (LR), b) Removal Efficiencies

Table 4.2 Average cyclic removal efficiencies of each operational period in Set-1

Parameter	R1			R2		
	Phase-1	Phase-2	Phase-3	Phase-1	Phase-2	Phase-3
TAN RE* (%)	32±11	13±5	37±8	20±8	16±11	12±2
TN RE (%)	34±10	14±3	38±8	26±8	19±11	20±3
Denitrification (%)	77±30	87±17	70±8	82±20	88±8	74±10
N loss in aerobic period (mg/L)	9±1	-	18±11	10±3	-	8±1
FA concentration (mg NH_3-N/L)**	16±4	38±8	24±8	23±6	46±6	24±7
COD RE (%)	70±13	39±12	70±16	67±11	54±15	36±4

*RE: Removal Efficiency, **FA: free-ammonia
Phase-1: Days 1-10, Phase-2: Days 11-27, Phase-3: Days 28-37

In the following 16 days in Phase-2, TAN removal efficiency in R1 decreased, fluctuated around 10-20% and average TAN removal efficiency was calculated as 13±5% (Figure 4.10, Table 4.2). TAN oxidation in R2, on the other hand, reached its peak value (45%) at the beginning of Phase-2 only for one day (Figure 4.11). Following that day (Day 15), TAN oxidation efficiency of R2 also drastically decreased to 10-15%. The reason of low TAN removal efficiency might be the pH values measured in aerobic periods which were at the upper limit of the optimum pH range (pH 7.5-8.6) defined in literature for nitrification (Yoo et al. 1998) (Figure 4.1, Figure 4.2). In addition the pH effect, free-ammonia (NH_3-N) toxicity was a potential TAN removal inhibitory parameter. Low TAN removal efficiencies resulted in TAN accumulation in the following cycle. High initial TAN concentration (293±62 mg/L for R1, 285±59 mg/L for R2) led to high free-ammonia concentration at pH conditions observed during aerobic periods of both reactors in this study. Anthonisen et al. (1976) reported that 10-150 mg/L free-ammonia (8.2-123 mg/L NH_3-N) concentration inhibited the activity of nitrifiers. The effects of free-ammonia on nitrifying granular sludge were investigated by Yang et al. (2004b). Authors stated that 2.5 – 39.6 mg/L NH_3-N inhibited microbial activity (respirometric activity) of nitrifiers 2.5 times. The free-ammonia concentrations that microorganisms exposed to during aerobic periods of each cycle were calculated according to Anthonisen et al. (1976) by using monitored pH, temperature and measured TAN concentrations (Eqn 4.9 and Eqn 4.10). The average values are given for each Phase in Table 4.2.

$$K_b / K_w = e^{(6344/273+^{o}C)} \qquad \text{Eqn 4.9}$$

$$NH_3 - N(mg/L) = \frac{TAN(mg\ N/L) \times 10^{pH}}{K_b / K_w + 10^{pH}} \qquad \text{Eqn 4.10}$$

Where,

K_b= Base dissociation constant
K_w= Self-ionization constant of water

During Phase-2, free-ammonia concentrations in reactors were around aforementioned toxic levels (Table 4.2). Peak values observed were 47 mg/L NH_3-N and 55 mg/L NH_3-N for R1 and R2, respectively. The removed TAN concentrations were found to be almost equal to the calculated free-ammonia concentrations (Figure 4.10, Table 4.2). Thus, it was concluded that in Phase-2 TAN removal during aerobic periods was due to free-ammonia stripping rather than TAN oxidation. Ammonia oxidizers might have been inhibited in both reactors.

Due to TAN accumulation and mentioned free-ammonia inhibition, the reactors' contents were washed with water on Day 27. Thereafter, free-ammonia concentration of both reactors was measured 16 mg/L NH_3-N at the beginning of Phase-3, (Day 28). During Phase-3 TAN oxidation efficiency of R1 gradually recovered and increased to 40-45% (Figure 4.10b). Yet, the nitrification efficiency of R2 remained same as in Phase-2 and the accumulation of TAN was observed again (Figure 4.11b). Therefore, it was concluded that self-recovery of CAS floc-granule mixture was not possible under the TAN loading rate (0.8 g TAN/L.d) experimented in this study. It was likely that 12±2% TAN removal efficiency in R2 was related to the free-ammonia stripping rather than oxidation reaction. On the other hand, under similar conditions, MBS floc-granule mixture showed better performance in terms of self-recovery.

During 37 days of operation, denitrification was achieved during anoxic periods of the cycles for both reactors (Table 4.2). Denitrification efficiencies fluctuated between 60% and 90%. Since steady-state conditions could not be achieved during the operation, noted fluctuations were observed in anoxic period. On the other hand, TON removal during aerobic period is also possible with aerobic granules since in granular systems, anaerobic, anoxic and aerobic portions (zones) exist in granular structure due to limited DO diffusion (de Kreuk et al., 2005;2006; Yilmaz et al., 2007; Gao et al., 2011). In order to determine TON removal performances of reactors, N losses in aerobic periods were calculated in terms of mg/L according to N mass balance. However since free-ammonia stripping may interfere with N loss during aerobic periods, actual assessment of TON removal or SNDN performance was not possible.

Overall, since steady conditions could not be achieved in both reactors, nitrogen removal performances of the granules cultivated from different seed sludge types were partially discriminated in this study. However, the response of the seed sludge and the developed granules to the varying operational conditions could be compared. Nitrification performances of MBS granules were found to be slightly higher than that of CAS granules during granule cultivation. Moreover, ammonia oxidation performance of MBS granules rapidly recovered to some extent when the inhibitory conditions were minimized, while no recovery was observed for CAS granules in terms of ammonia oxidation performance. Manser et al. (2005) stated that there are no differences between MBS and CAS systems in terms of nitrifier population and treatment performances. Authors also revealed that nitrifiers were located in the MBS flocs much deeper than those located in CAS flocs. Therefore, it might be concluded that MBS flocs and thereby developed granules had more appropriate structure for conservation of ammonia oxidizers and nitrifiers from toxic environment which led to better recovery performance.

4.1.3.2. Organic Removal

Organic removal performances of reactors were investigated in terms of sCOD removal. Average influent sCOD values of R1 were 1252±92 mg/L, 1411±311 mg/L, 1154±73 mg/L and of R2 were 1243±97 mg/L, 1226±145 mg/L, 1187±161 mg/L sCOD in Phase-1, 2 and 3, respectively. As represented in Figure 4.12 and Figure 4.13, during the 37 days of reactor operation, sCOD removal efficiencies of reactors were low (45-80 %) compared to literature studies (85-97 %) (Qin and Liu, 2006; Yilmaz et al. 2007; Adav et al. 2009b). However, R1 performed slightly better COD treatment than R2.

Figure 4.12 and Figure 4.13 show the profiles of organic loading rate (OLR), sCOD removal efficiencies and effluent concentrations of anoxic and aerobic periods of monitored cycles.

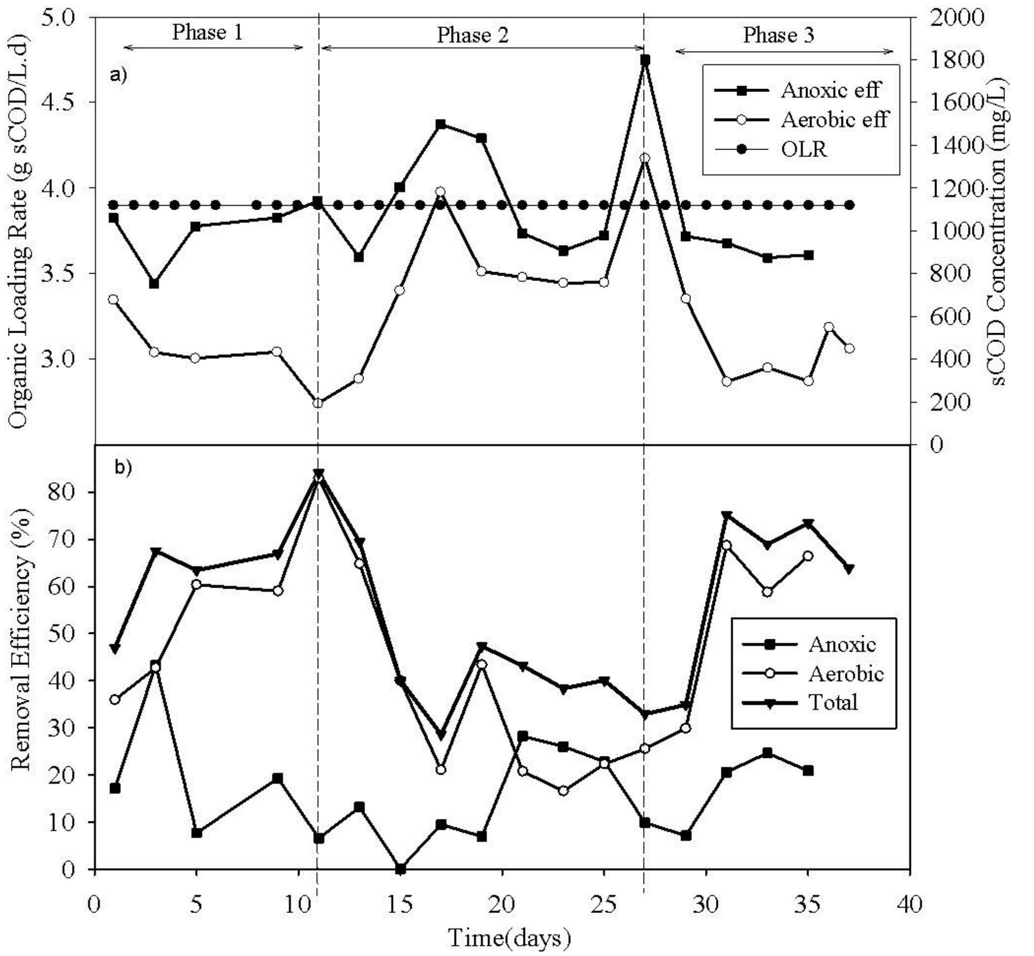

Figure 4.12 sCOD overview of R1 during operation in Set-1
a) Effluent concentrations and loading rate (LR), b) Removal Efficiencies

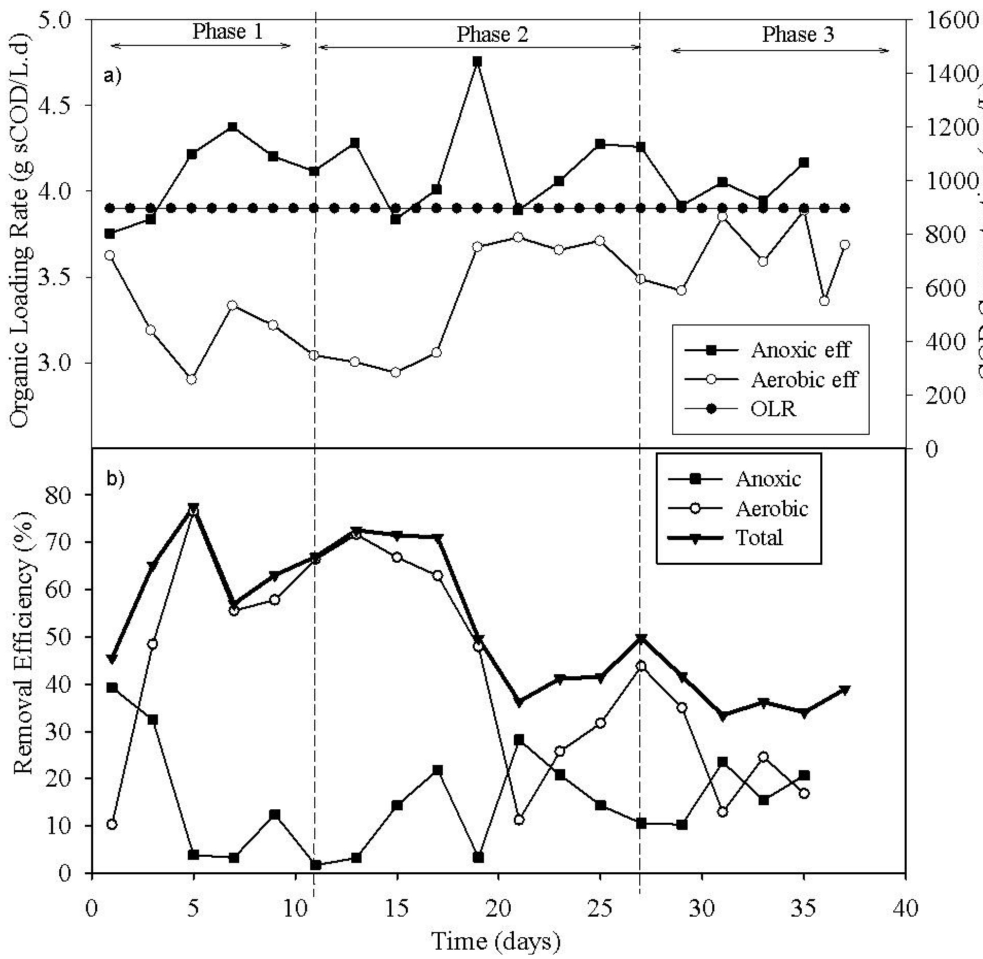

Figure 4.13 sCOD overview of R2 during operation in Set-1
a) Effluent concentrations and loading rate (LR), b) Removal Efficiencies

Both reactors, seeded with different sludge types, displayed similar sCOD treatment performances during Phase-1. Total sCOD removal efficiencies of R1 and R2 gradually increased from 47 to 85% and from 46 to 77%, respectively, following the adaptation of both MBS and CAS to the reactor operation, during Phase-1. However, during Phase-2, the removal efficiencies of both reactors decreased to 30-35% (Figure 4.12 and Figure 4.13). This decrease was attributed to the free-ammonia inhibition. Yang et al. (2004b) stated that 2.5-39.6 mg/L NH_3-N free-ammonia can inhibit specific oxygen uptake rate (SOUR) of heterotrophs 5 times. Compared to R1, there were 4 days of delay in inhibition of organic removal performance of R2, which was attributed to the granule size and shell structure of the granules. At the end of Phase-1, granules developed in R2 had greater sizes than those of R1 (Table 4.1). Gao et al. (2011) states that granules have shell structure and microorganisms are systematically located. The outmost, the inner and the innermost shells are composed of aerobic heterotrophs, nitrifiers and denitrifiers/anaerobic autotrophs, respectively (Gao et al., 2011). Therefore, if the granular properties (compactness, biomass content) are similar, granules of greater sizes can resist to high loading rates more than smaller-sized granules (Moy et al. 2002). During Phase-1, MLVSS concentrations were very similar for both R1 and R2 (Table 4.1). Furthermore, R2 granules were greater in size than R1 granules as previously mentioned. Hence, they might have contained more aerobic heterotrophs than that of R1 granules which resulted in 4 days (16 cycles) of delay in organic removal inhibition in R2 (Figure 4.12 and Figure 4.13). During Phase-3, COD removal efficiency of R1 gradually increased, yet, the low COD removal performance of R2 remained still (Figure 4.12 and Figure 4.13). The average COD removal efficiencies of R1 and R2 during Phase-3 were calculated as 70±16% and 36±4%, respectively (Table 4.2). As it was discussed in "Nitrogen Removal" section (Section 4.1.3.1), there was no recovery of TAN oxidation in R2 and TAN accumulation and high pH related free-ammonia inhibition still remained. No sign of recovery of organic removal efficiency in R2 was, therefore, attributed to the existing free-ammonia inhibition on aerobic heterotrophs (Yang et al. 2004) during Phase-3. Therefore, the organic removal efficiency did not recover in R2.

Considering the experimental results of Set-1, it can be said that there were enough implications to compare seed sludge types in terms of effectiveness on development of aerobic granules from suspended culture. Granules cultivated from membrane bioreactor sludge were found to be advantageous in terms of greater size, higher resistance to toxic effects, higher stability and shorter recovery periods. As Manser et al. (2005) stated nitrifiers might have located much deeper in the MBS flocs than those located in CAS flocs. Therefore, under toxic conditions (35-40 mg/L NH_3-N), nitrifiers were conserved in granules cultivated from MBS seed. Conservation of nitrifiers helped the rapid recovery of TAN oxidation in R1. Additionally, better TAN removal and in turn lower free-ammonia concentrations positively affected recovery of organic removal of in R1. Liu et al. (2004b) suggested that in order to increase granular stability granules should be cultivated by selecting slow-growing nitrifying bacteria. Thus, for having better TAN oxidation efficiencies, the granules cultivated with MBS seed might have higher amount of nitrifiers and thus had more stable structure with a regular smooth shape than the ones cultivated with CAS seed. In addition to that, MBS granules having non-filamentous structure had better settleability than CAS granules having filamentous structure. Lastly, granules of MBS seed had higher biomass retention and higher ability to withstand high loading rates (0.8 g TAN/L.d and 3.92 g COD/L.d) which might decrease the start-up duration.

4.2. Set-2: The Effects of Anoxic/Aerobic Period Sequence on Granulation and Treatment Efficiency of Granules

There are studies in literature that randomly integrate the anoxic or anaerobic periods in SBR cycle to investigate the effects of these periods on granule cultivation and treatment performance of granules (Jang et al., 2003; Qin and Liu, 2006; Wang et al., 2007; Yilmaz et al., 2007; Adav et al., 2009b; Wan et al., 2009; Su et al., 2012). However, to our knowledge, there is no detailed investigation about how the sequence difference (anoxic-aerobic and aerobic-anoxic) affects the granulation process and treatment efficiency of the granules. Therefore, Set-2 was conducted to investigate the effects of the anoxic/aerobic period sequence on granule cultivation in SBR and treatment performances of cultivated granules. In other words, the effects of pre-anoxic SBR operation and post-anoxic SBR operation on granulation and treatment performance were investigated. Reactors in Set-2 were named based on their periodic sequences. The reactor having anoxic-aerobic period sequence was named as R1 and the reactor having aerobic-anoxic period sequence was named as R2.
Both reactors, seeded with same MBS, were operated at the same SBR operational conditions (Table 3.6) except for the difference in the order of anoxic and aerobic periods. The results are discussed in terms of treatment performances and granulation performances of the reactors, respectively.

4.2.1. Treatment Performances

Seeds of the two reactors were provided by equally dividing the acclimated suspended sludge to R1 and R2 (1180±42 mg/L MLVSS for R1 and 1230±14 mg/L MLVSS for R2). Therefore, to hold the F/M ratio same as that of the acclimation reactor (0.66 g/g.d for sCOD and 0.13 g/g.d for TAN), the loading rates were initially set to 1.47 g sCOD/L.d and 0.2 g TAN/L.d for both reactors, in the first 9 days. Analyses revealed that the average initial sCOD and TAN concentrations were 446±25 and 65±8 mg/L for R1, 428±32 and 78±15 mg/L for R2, respectively, during the first 9 days. After 9 days of operation, MLVSS concentrations in reactors were almost doubled (1990±155 mg/L in R1 and 1880±70 mg/L in R2) which enabled the increase in loading rates to 2.94 g sCOD/L.d and 0.4 g TAN/L.d on Day 10 (Modification I). Average initial sCOD and TAN concentrations were measured as 803±66 and 131±17 mg/L for R1 and 808±65 and 139±18 for R2, respectively, in the rest of operation (Days 10-63).

Daily measured pH values of R1 and R2 for synthetic wastewater (feed solution) and reactor content in anoxic periods and aerobic periods are represented in Figure 4.14 and Figure 4.15, respectively.

In the first 17 days of operation pH increased from 6.9-7.4 to 8-8.3 in R1 during anoxic periods (Figure 4.14). Following anoxic periods, pH increase from 8-8.3 to 8.7-8.8 in the first 15 minutes of aerobic periods was observed in R1. In R2, premier period after feeding period was aerobic period and pH increase in the first 15 minutes from 6.9-7.4 to 7.7-8.5 was also observed during the first 17 days. Following aerobic periods, pH increase from 7.7-8.5 to 8.6-8.8 was observed in anoxic periods of R2 (Figure 4.15).

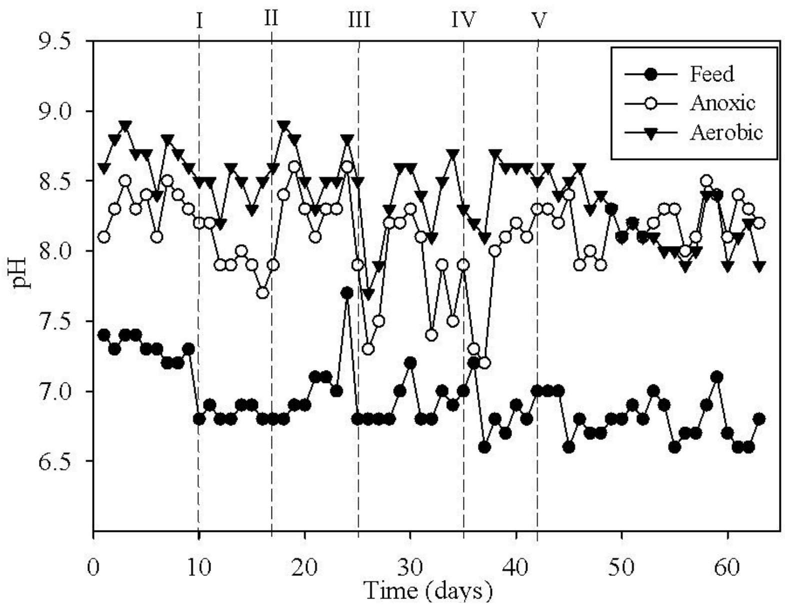

Figure 4.14 pH variations in R1 (anoxic-aerobic) during operation in Set-2
(-○- anoxic period -▼- aerobic period)

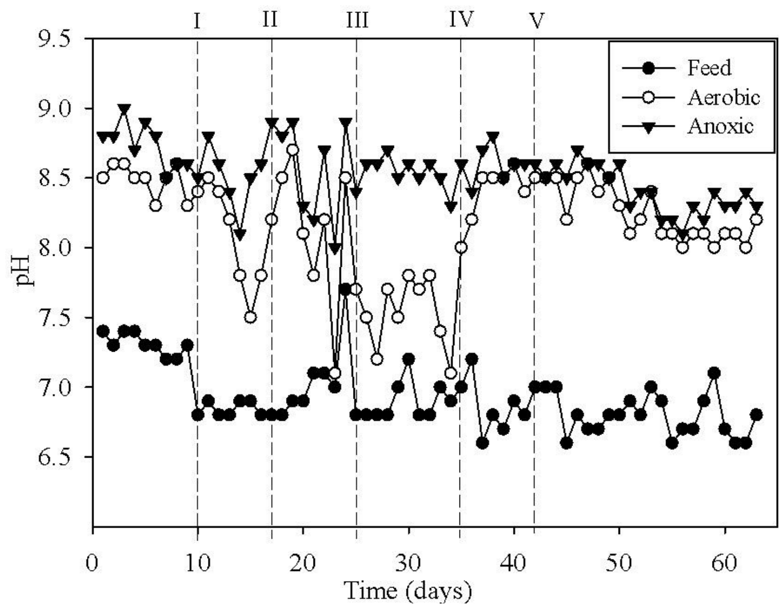

Figure 4.15 pH variations in R2 (aerobic-anoxic) during operation in Set-2
(-○- aerobic period -▼- anoxic period)

Figure 4.16 and Figure 4.17 show the profiles of TAN loading rate, TAN oxidation efficiencies and TAN effluent concentrations of anoxic and aerobic periods of monitored cycles in both reactors during the entire operation. As represented in Figure 4.16 and Figure 4.17, TAN oxidation efficiencies of both reactors had an increasing trend from 10-20% to 55-63% in the first 17 days.

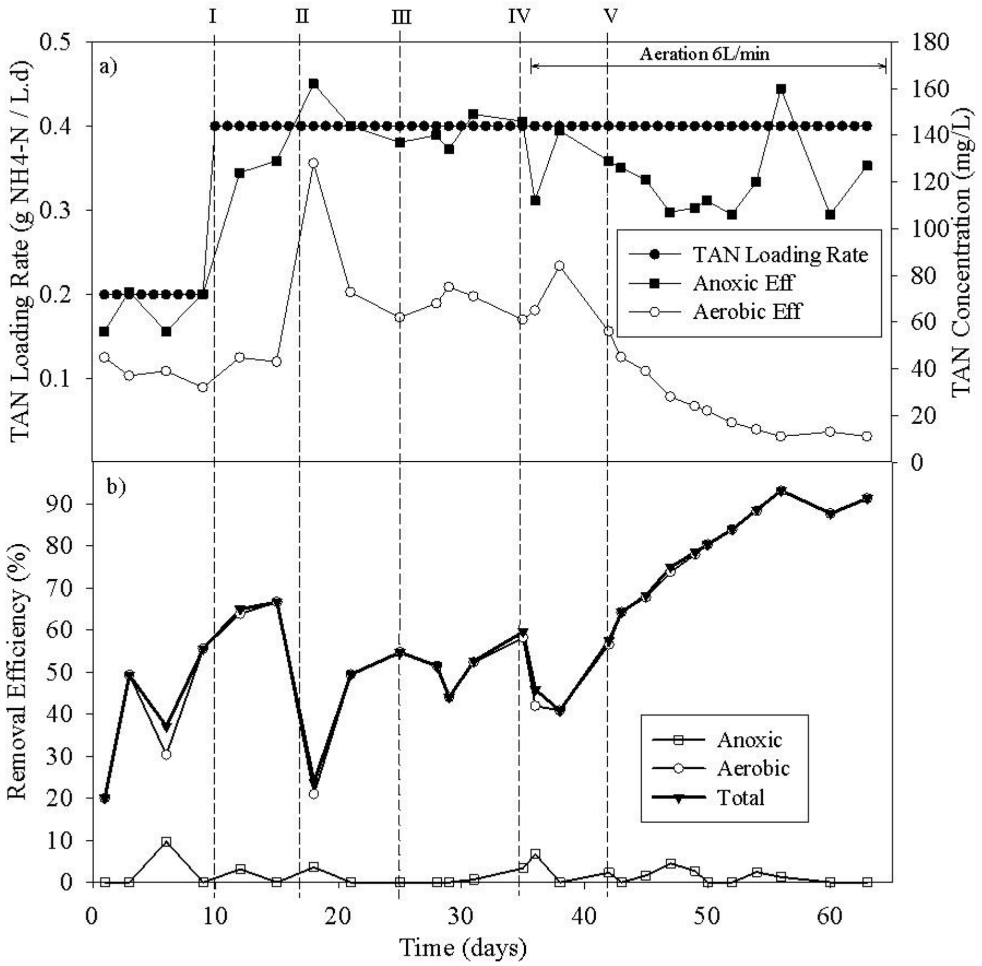

Figure 4.16 TAN overview of R1 during operation in Set-2
a) Effluent concentrations and loading rate (LR), b) Removal efficiencies

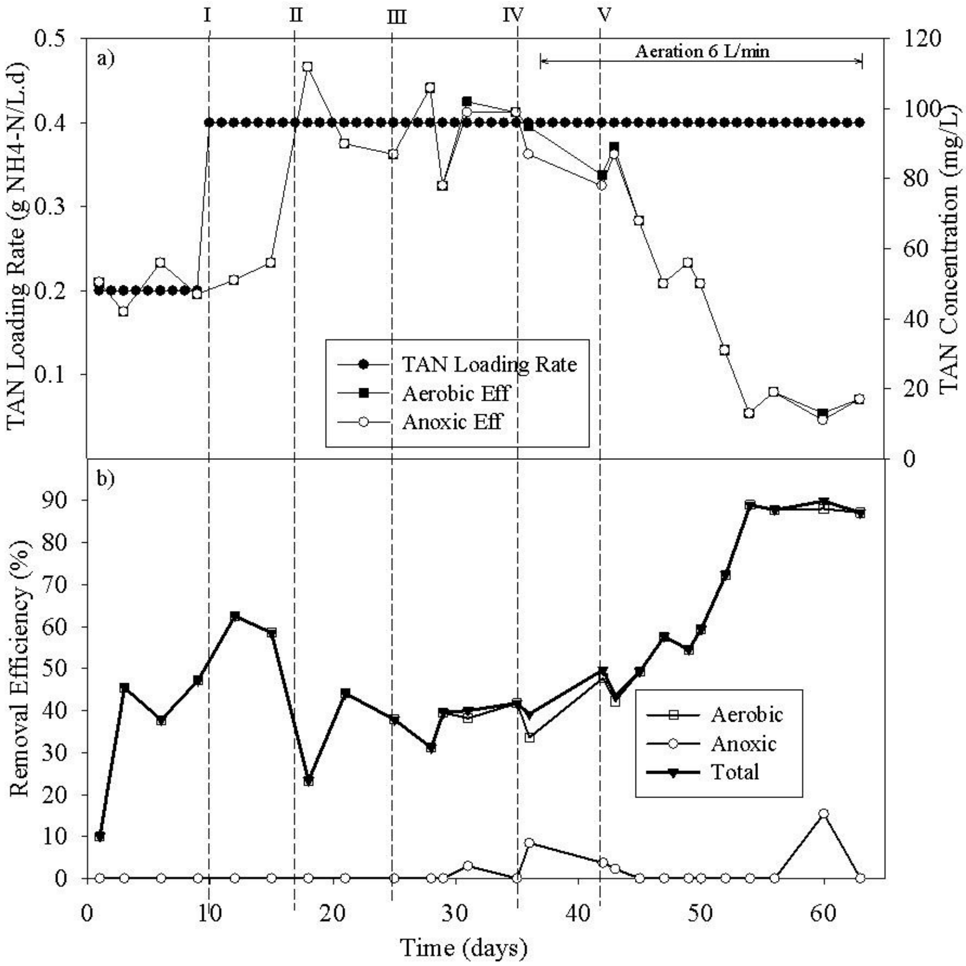

Figure 4.17 TAN overview of R2 during operation in Set-2
a) Effluent concentrations and loading rate (LR), b) Removal efficiencies

Figure 4.18 and Figure 4.19 show the profiles of organic loading rate, sCOD removal efficiencies and sCOD effluent concentrations of anoxic and aerobic periods of monitored cycles in both reactors during the operation. As shown in Figure 4.18 and Figure 4.19, sCOD removal efficiencies were around 70-80% during the first 17 days of operation for both reactors.

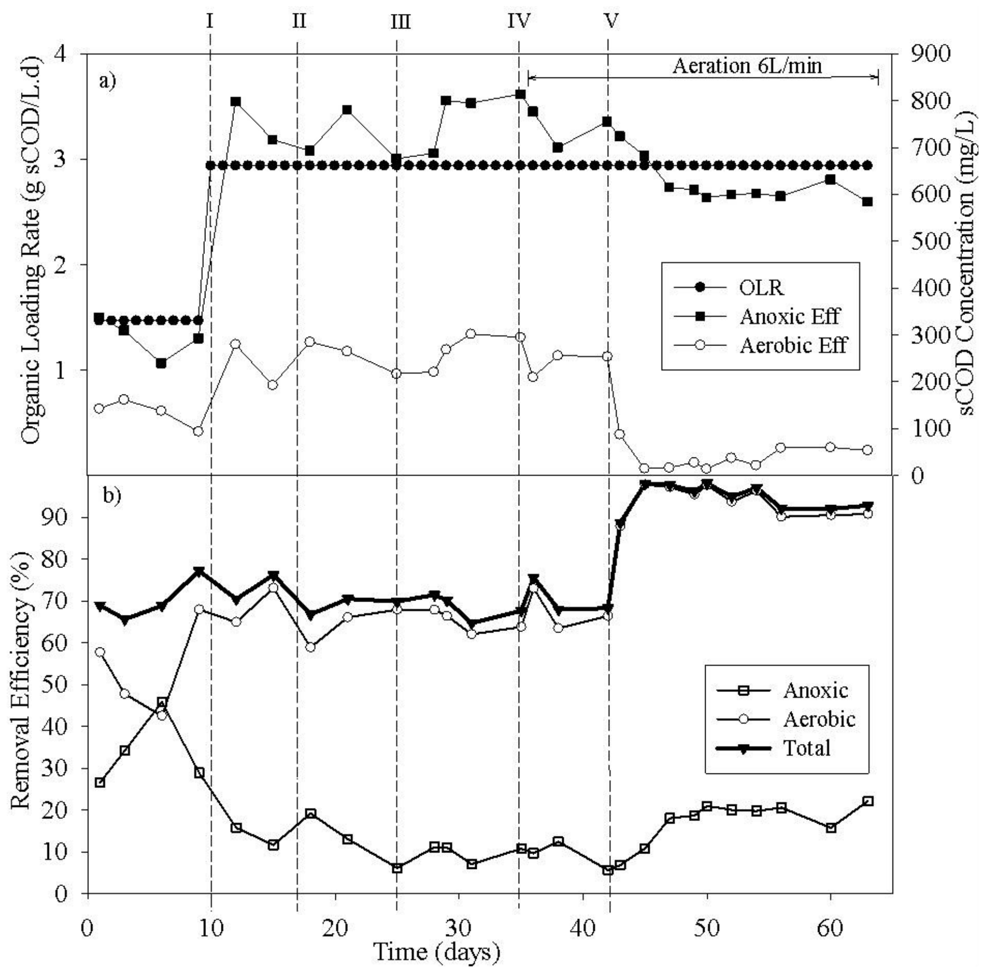

Figure 4.18 sCOD overview of R1 during operation in Set-2
a) Effluent concentrations and loading rate (LR), b) Removal efficiencies

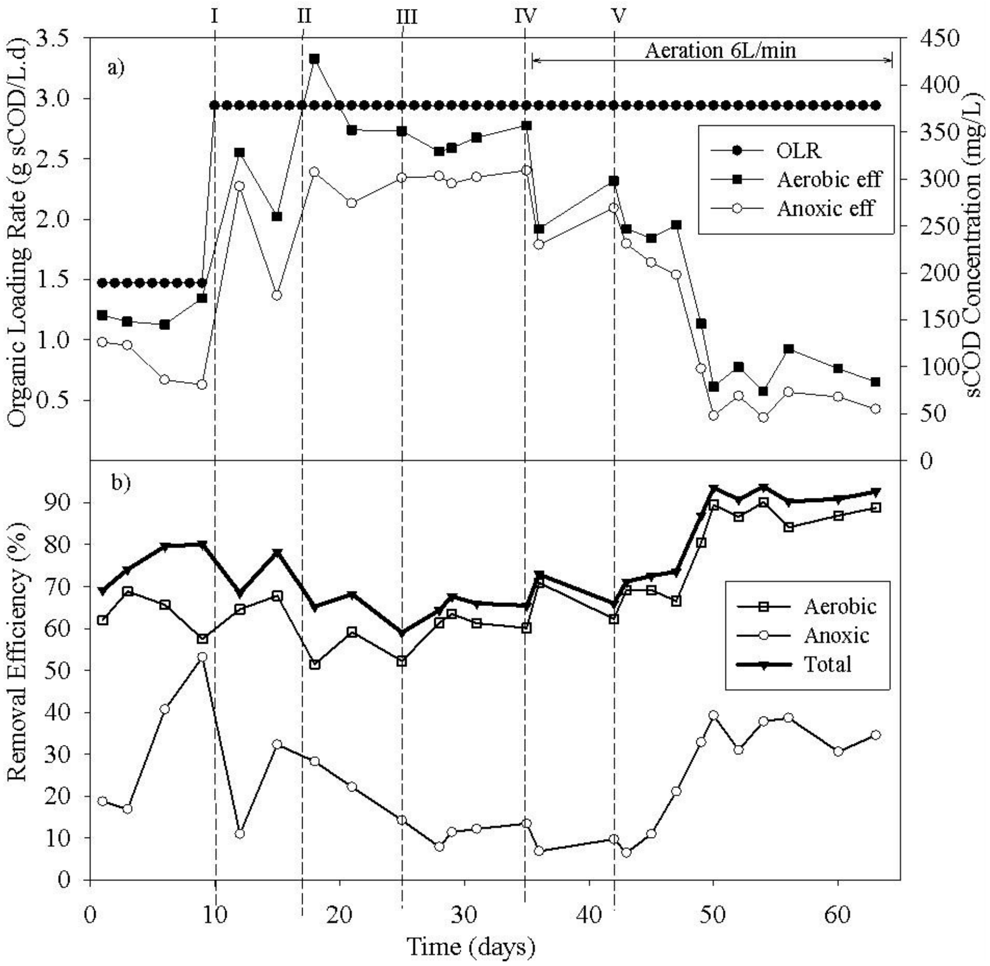

Figure 4.19 sCOD overview of R2 during operation in Set-2
a) Effluent concentrations and loading rate (LR), b) Removal efficiencies

As stated, during the first 17 days of operation, TAN and sCOD removal were lower than expected and trend of pH values were very similar to Set-1. The initial results and treatment performances (TAN oxidation, sCOD removal, denitrification performances and pH conditions) of the reactors were summarized on average values at the end of 17 days of operation and represented in Table 4.3. As seen in Table 4.3, average TAN oxidation efficiencies were similar for both reactors and observed as 54±12% and 50±10%, respectively. Organic removal performances were also similar and averaged out 69±4% and 63±5% for R1 and R2, respectively. Average pH values in aerobic periods were 8.6±0.2 and 8.3±0.3 for R1 and R2, respectively, which might be critical at high TAN loading rate (0.4 g TAN/L.d) due to potential inhibitory free-ammonia production at high pH values. Studies in literature reported that TAN removal efficiencies of 98-100% and organic removal efficiencies of 85-95% can be achieved with aerobic granules (Jang et al., 2003; Qin and Liu, 2006; Yilmaz et al., 2007; Adav et al., 2009b; Gao et al., 2012). From this general overview, it was figured out that possible pH-related problems might occur in the reactors in coming days and treatment performances might become worse. Therefore, it was necessary to prevent potential further inhibition and to improve the treatment performances for better comparison of the period sequence effect. To this purpose, the underlying reasons for low efficiency observed in both reactors were investigated in detail. The observations, underlying reasons of the problematic situation and possible solutions were investigated one by one. The observations were summarized as follows;

- High pH in reactors (pH 8.6-8.7)
- pH increase in the first 15 minutes of aerobic periods
- Low TAN Removal Efficiency (<60%)
    - o Low TAN removal-related TAN accumulation (~160 mg/L initial TAN concentration at the beginning of cycles)

    - Day by day increasing free-ammonia concentrations (up to 12-17.5 mg/L NH_3-N)
- Low COD Removal (<70%)

Table 4.3 First overview of reactor performances in Set-2 (Days 1-17)

Parameters	Average Values			
	R1 (Periods)		R2 (Periods)	
	Anoxic	Aerobic	Aerobic	Anoxic
pH	8.2±0.2	8.6±0.2	8.3±0.3	8.7±0.2
TAN RE* (%)	-	54±12	50±10	-
Denitrification (%)	94±1	-	-	86±5
sCOD RE (%)	19±9	69±4	63±5	24±11
*RE: Removal Efficiency				

4.2.2. Possible Problems and Related Modifications

The first underlying reason for the limitation of removal efficiencies was hypothesized as the "high pH and TAN accumulation" related free-ammonia toxicity likewise in Set-1. In Set-2, however, the TAN loading rate was 0.4 g TAN/L.d (half of the value applied in Set-1), so the TAN accumulation and free-ammonia inhibition was not as drastic as in Set-1. As it was mentioned before, in the first 15 min of aerobic periods in both reactors, pH increase up to 8.4-8.6 was observed. It was thought that accumulated initial TAN concentrations (130-150 mg/L NH_4-N) at pH 8.5 were already causing free-ammonia inhibition (12.3 mg/L and 17 mg/L) immediately at the beginning of the aerobic periods. Therefore, if the high pH conditions in the cycles were avoided, free-ammonia toxicity would also have been prevented. To this purpose, modifications (II, IV, V) were done.

4.2.2.1. Reduction of NO_3-N influent (Modification II, Days 17-35)

Possible reasons for pH increase (in the first 15 min of aerobic periods) were analyzed. The first potential mechanism for pH increase in R1 was denitrification performed during anoxic period as discussed in Section 4.1.1.1. Denitrification performances during anoxic periods in the first 17 days indicated that R1 (anoxic-aerobic) was able to denitrify 94±1% of the initial NO_3-N (40 mg/L). Average denitrification performance of R2 (aerobic-anoxic) was 86±5% (Table 4.3). There was no problem with denitrification performances of both reactors which meant denitrifiers were enriched in both SBRs successfully. One of the products of denitrification process is OH^-, which increases pH (Eqn 4.1). However, the pH increase observed in anoxic period was not as rapid as that was observed in aerobic period in R1. During anoxic periods, reactor contents were mixed with 48 rpm steel mixers. However, 48 rpm did not provide efficient mixing during anoxic periods since the sludge had 60-70% granular portion. Therefore it was thought that rapid pH increase observed at the beginning of aerobic periods might have been due to well-mixing and homogenous distribution of the alkalinity (produced in anoxic periods) by aeration applied as soon as the anoxic periods ended and aerobic periods started. In other words, alkalinity produced in anoxic periods might have further resulted in sudden pH increase in aerobic periods with the effective mixing by aeration in R1. In R2, similar to R1, during the first 15 minutes of the aerobic periods pH increase was observed. However, since the first period of R2 was aerobic period, there were two possible reasons. The first reason was similar to aforementioned situation in R1 which was the contribution of alkalinity produced in the previous cycles' last period (anoxic period) to the aerobic periods. The 50% volumetric exchange ratio (mixing of feed solution with the residual of previous cycle during feeding) played the main role in the mentioned pH contribution of anoxic periods of previous cycle to the following cycles' aerobic periods. The second and more possible reason was the simultaneous denitrification of influent NO_3-N under low DO concentrations (during feeding period) and further alkalinity production. Therefore, it was concluded that pH increase in both reactors was mostly related with influent NO_3-N concentration.

In order to decrease the level of pH resultant of denitrification (R1) and simultaneous denitrification (R2), 40 mg/L NO_3-N influent concentration of reactors was decreased to 20 mg/L NO_3-N on Day 17 (Modification II). As seen in Figure 4.14, pH variation in R1 (anoxic-aerobic) was not affected from the change in influent NO_3-N, pH values of 8.3-8.6 were still observed (Days 17-25). However, in R2, it was observed that pH values fluctuated during aerobic periods (Days 17-25). Unlike the fluctuations and decrease of pH in aerobic periods of R2, pH values during anoxic periods were mostly above 8.6 which was a critical value in terms of free-ammonia production in the system (Anthonisen et al. 1976). The peak free-ammonia concentrations were calculated as 15 mg/L NH_3-N and 16 mg/L NH_3-N for R1 and R2, respectively. Therefore, as seen in Figure 4.16 and Figure 4.17, despite the Modification II (Days 17-25), pH increase during aerobic periods could not controlled and TAN oxidation performances of both reactors were still low 40-50%. Yet, denitrification performances of the reactors were not significantly affected and recorded as 94±7% and 83±6% in R1 and R2, respectively for Days 17-25.

Heterotrophic denitrifiers use sCOD as carbon source (Eqn 4.1), therefore, influent NO_3-N concentration has an influence on anoxic organic removal efficiency. The stochiometric sCOD requirement for denitrification of 40 mg/L NO_3-N was 114.4 mg/L sCOD which corresponds to 15% of the influent sCOD. As represented in Figure 4.18, anoxic organic removal efficiency of R1 decreased from 19% to 8% with Modification II. However, there was no significant change in overall sCOD removal efficiencies of reactors after Modification II. The sCOD removal efficiencies of both reactors for Days 17-25 were around 65-70%, similar to the first 17 days of operation (Figure 4.18 and Figure 4.19).

In order to decrease ongoing high pH values at the beginning of aerobic periods and further NH_3-N inhibition in the reactors, pH adjustment was done between Days 25-35. At the beginning of aerobic periods rapid pH increase was compensated by adding 0.02N H_2SO_4 to the reactors and adjusting the pH to 7.5 (Days 25-35). In R2, although pH was increased somewhat, it was usually hold below 8 during aerobic periods (Figure 4.15). The possible free-ammonia inhibition starting at the beginning of aerobic period was avoided in some cycles in R2, by adjusting the pH at 7.5. However, as seen in Figure 4.14, fluctuations and pH increase up to 8.6 was still valid during aerobic and anoxic periods in R1 and R2, respectively. Neither the TAN oxidation nor the organic removal performances of R1 increased (Figure 4.16 and Figure 4.18). Similar to R1, no change in TAN oxidation and organic removal performances of R2 was observed either (Figure 4.17 and Figure 4.19). Furthermore, pH increase was still valid in some extent.

Modification II indicated that pH increase during aerobic periods could not be decreased by decreasing influent NO_3-N concentration and by manually controlling the pH increase during aerobic periods. It was concluded with Modification II that there might have been other reasons for pH increase and other factors for inhibition of TAN oxidation during aerobic periods.

By the time it was being tried to solve pH-realated problems and increase treatment efficiency of both reactors, the sludge content of both reactors reached almost full granulation (granule percentage of 80-90%) on Day 25. Nor-Anuar et al. (2012) stated that mixers might have adverse effects on granular structure and mechanical destruction of granules might occur. Therefore, on Day 25 mixers were removed from the reactors (Modification III) to avoid possible mechanical deterioration of granules which might adversely affect the treatment performance. The removal of mixers would avoid any possible inhibition related to destruction of granules.

4.2.2.2. Increasing Air Supply (Modification IV, Days 35-42)

The attempt to decrease the level of pH observed during aerobic periods, by decreasing the influent NO_3-N concentrations of previous anoxic periods and in turn alkalinity production, did not result in recovery of TAN removal efficiencies. The pH increase observed during aerobic periods might be related with SNDN potential to occur during aerobic periods. Yoo et al. (1999) stated that, at DO level of 2.5 mg/L effective SNDN can be observed in SBR even with suspended sludge. Denitrification can simultaneously occur even during aerobic periods at the inner part of the granules (de Kreuk et al. 2005; 2006), since DO diffusion into the core of the flocs, especially the granules, is limited (Tay et al., 2002a; 2002b). DO might diffuse up to 200-400 μm in flocs (Lens et al., 1995a). Since reactors in Set-2 were full of granular sludge at the end of 25 days of operation, it was possible to have SNDN during aerobic periods which would promote pH increase. Granule size of 1.2-1.4 mm is claimed to be the optimum size for nutrient removal via SNDN, which indicates the importance of aerobic and anoxic layer volumes in a granule (de Kreuk et al., 2006). In the first 35 days, average granule sizes were 2.9±0.2 mm and 1.9±0.3 mm for R1 and R2, respectively. Granules of those sizes were appropriate to prevent DO diffusion to inner parts of the granules and to form anoxic zones, thus SNDN related pH increase might have occurred. However, for the mentioned granule sizes of almost 2-3 mm, DO diffusion might have been also limited for nitrifiers (located at the inner shell between the aerobic heterotrophs at the surface and the denitrifiers in the core), if the bulk DO concentrations and mixing conditions were not sufficient. Therefore, in order to clarify if the nitrification performances of reactors were limited due to the limitations in DO diffusion, aeration or mixing problems, air supplies of reactors were doubled to 6 L/min (2 cm/s superficial air velocity) on Day 35 (Modification IV). The increase in aeration intensity would provide effective mixing and avoid possible inhibition of ammonia oxidizers due to oxygen transfer limitation. Thus, it would be possible under proper DO conditions to increase ammonia oxidation which would also increase H^+ production and compensate the pH increase observed at the beginning of aerobic periods. At lower pH values the dominant TAN form is NH_4-N rather than the inhibitory NH_3-N. As seen in Figure 4.20 and Figure 4.21, DO concentrations of the reactors were increased up to 7 mg/L during aerobic periods with Modification IV.

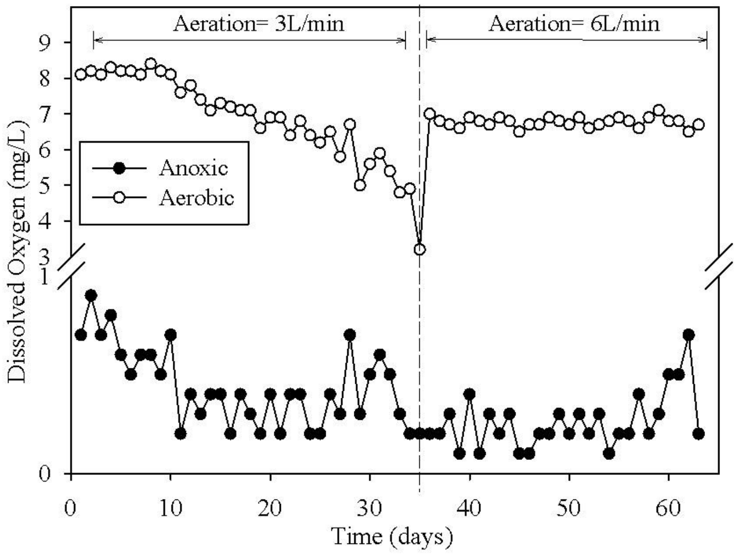

Figure 4.20 DO values in R1 during operation in Set-2
(-●- anoxic period -○- aerobic period)

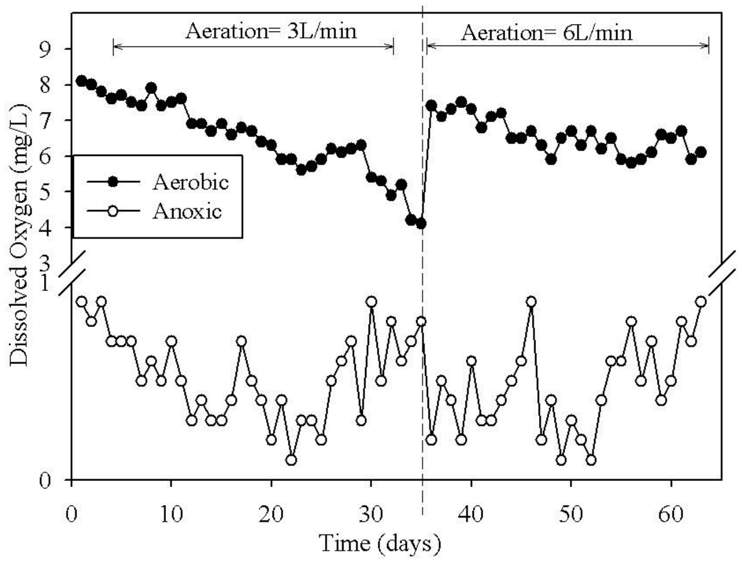

Figure 4.21 DO values in R2 during operation in Set-2
(-●- aerobic period -○-anoxic period)

After Modification IV, reactors were monitored for one week from Day 35 to Day 42 (28 cycles, 9 HRTs). Yet, there was no significant change observed in pH trend of the reactors (Figure 4.14 and Figure 4.15). pH values were around 8.5-8.7 in aerobic periods. TAN oxidation performances of reactors did not change either (Figure 4.16 and Figure 4.17). As represented in Figure 4.16 and Figure 4.17, TAN oxidation performances of both reactors were still 40-50%, at DO levels of 7 mg/L. Low TAN removal efficiencies, in turn high initial TAN concentrations and pH increase were still leading to inhibitory free-ammonia concentrations in both reactors. During the week after Modification IV (Days 35-41), free-ammonia concentrations during aerobic periods were 23.5 mg/L and 17.3 mg/L in R1 and R2, respectively. The inhibitory free-ammonia concentrations also limited the aerobic heterotrophs' activity; the maximum sCOD removal efficiencies of 75% and 72% were recorded for R1 and R2, respectively, between Days 35-41 (Figure 4.18 and Figure 4.19).

Denitrification performances of 95±5% and 83±9% measured for R1 and R2, respectively, during Days (1-35) did not significantly change. Performances during Days 35-41 (after Modification IV), were 92±5% and 78±7% for R1 and R2, respectively.

It was concluded with Modification IV that DO concentrations in the reactors were not the limiting factor for obtained low TAN oxidation performances.

It was seen in the first 42 days that R1 (anoxic-aerobic sequence) was able to denitrify 93±7% of the initial NO_3-N (during anoxic periods). On the other hand, denitrification (during anoxic periods) was 80±14% in R2 (aerobic-anoxic sequence). The average total N losses in the first 42 days were 63±15 mg/L and 50±17 mg/L during aerobic periods in R1 and R2, respectively. The N loss during aerobic periods attributed to the SNDN process performed by granular sludge developed. However, free-ammonia concentrations calculated were 16-28 mg/L NH_3-N (Days 10–42). Therefore, observed N loss might have been also due to ammonia stripping; thus N loss could not be directly correlated with the SNDN performance.

4.2.2.3. Decreasing Influent Sulfate Concentration (Modification V)

The other hypothesis of the pH increase observed was possible sulfate reduction (Eqn 4.2) promoted in the core of the granules during anoxic period as discussed in Section 4.1.1.1. Soluble sulfide, which is produced by sulfate reduction, might be one of the inhibition sources of ammonia oxidation (Æsøy et al. 1998, Sears et al. 2004). Sears et al. (2004) stated that 0.25 mg/L of soluble sulfide can completely inhibit ammonia oxidation. Therefore, on Day 42, influent SO_4^{2-} concentration (in the form of $MgSO_4.7H_2O$ used as Mg^{2+} source for granulation) was decreased by half, from 70.2 mg/L to 35.2 mg/L SO_4 (Modification V).

As seen in Figure 4.14 and Figure 4.15, pH values became more stable and fluctuations decreased. Furthermore, after Modification V, the average pH values of both R1 and R2 decreased and become stable at 8.2±0.2 during aerobic periods. On the other hand, the level of pH increase during anoxic periods of R2 decreased and the average pH value was measured as 8.4±0.1 between Days 42-63.
As illustrated in Figure 4.16 and Figure 4.17, after Modification V, TAN oxidation performances of both reactors gradually increased from 50% up to 90%. Effluent TAN concentrations decreased down to around 11-13 mg/L in both R1 and R2. The increased TAN oxidation efficiency means higher amount of H^+ production and further pH decrease (Eqn 4.11). The decrease in pH during aerobic periods in R1, therefore, was resultant of effective TAN oxidation

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H_2O + 2H^+ \quad \text{Eqn 4.11}$$

When the sCOD removal efficiencies were considered, it was observed with Modification V that sCOD removal efficiency increased significantly in both reactors. Soluble sulfide and sulfate do not have significant toxic effects on aerobic heterotrophs (Lens et al., 1995a,b). Therefore, the main reason limiting the performance of aerobic heterotrophs until Day 42 was high initial TAN and high pH values related free-ammonia inhibition. After Modification V, the prevention of free-ammonia production provided a sharp increase in TAN oxidation and minimized the effluent TAN concentrations. Due to the cyclic nature of SBR initial TAN concentrations also decreased to desired values (121±16 for R1 and 128±16 for R2). The prevention of free-ammonia production provided increase in organic removal efficiencies up to 95%. Steady-state conditions for sCOD removal were achieved on Day 49 in both reactors (Figure 4.18 and Figure 4.19).

Soluble sulfide production by sulfate reduction (Eqn 4.2, Section 4.1.1.1) led to both increase in pH and inhibition of ammonia oxidizers. In other words, soluble sulfide production was likely to be the mechanism responsible from high pH values and the sulfide toxicity on TAN oxidation. As stated before, untreated TAN concentrations caused TAN accumulation which further triggered free-ammonia production at high pH conditions. Therefore, produced free-ammonia was likely to be a second inhibitory parameter for TAN oxidation. It was concluded with Modification V that sulfate reduction, following sulfide production and soluble sulfide inhibition was also leading to the free-ammonia inhibition by limiting the TAN oxidation performance at levels of 50% (TAN accumulation) and increasing the pH over 8.5.

The increasing trend in sCOD and TAN removal efficiencies reached steady conditions for sCOD on Day 49 and for TAN on Day 54. In other words, after Day 54, both reactors reached steady-state conditions in terms of sCOD and TAN removal. Steady-state removal efficiencies were investigated for Days 54-63 and (for 40 cycles; 12 HRTs) performances summarized in Table 4.4. At steady-state, average free-ammonia concentrations were 5±1.6 mg/L and 6.2±1 mg/L NH_3-N for R1 and R2, respectively.

Table 4.4 Reactor performances after Day 54 (Steady conditions) in Set-2

Parameter*	**sCOD**		**TAN**		**TON****	
	R1	**R2**	**R1**	**R2**	**R1**	**R2**
Influent (mg/L)	1500	1500	200	200	20	20
Initial (mg/L)	754±9	757±16	121±19	124±15	42±8	40±16
Effluent (mg/L)	39±19	60±12	12±2	15±3	65±8	71±9
RE. (%)	95±2	92±1	90±2.5	89±1	-	-
Denitrification in anoxic period (%)	-	-	-	-	95±7	37±15
TN RE. (%)	-	-	-	-	46±9	38±11
SNDN in aerobic period (%)	-	-	-	-	46±10	30±4

* Influent: Feed solution, Initial: sample taken at the beginning of feeding period, Effluent: sample taken at the end of the cycle,
RE: Removal Efficiency, TN: Total Nitrogen, TAN: Total Ammonia Nitrogen, SNDN: Simultaneous nitrification-denitrification
**TON≈NO_3-N (NO_2-N was negligible)

TAN oxidation efficiency: As represented in Table 4.4, under steady conditions, average TAN oxidation performances of R1 and R2 were 90±2.5% and 89±1%, respectively. Effluent TAN concentrations were observed as 12±2 and 15±3 mg/L, respectively. Accordingly, the effluent NO_3-N (TON) concentrations of aerobic periods also increased in both reactors. The average effluent TON (97-99% of TON was NO_3-N) concentrations after Day 54 were measured as 65±8 mg/L and 71±9 mg/L for R1 and R2, respectively (Table 4.4). In both reactors, effluent nitrite concentrations were negligible (4±2 mg/L and 0.1±0.1 mg/L NO_2-N for R1 and R2).

Organic Removal Efficiency: Table 4.4 illustrates organic removal performances of reactors in terms of sCOD removal. At steady conditions, average sCOD removal efficiencies of reactors were calculated as 95±2% and 92±1% for R1 and R2, respectively. In consequence, average effluent sCOD concentrations were 39±19 mg/L and 60±12 mg/L for R1 and R2, respectively.

Denitrification and SNDN efficiency: After Day 42, with the decrease in the influent sulfate concentration and further recovery of pH to lower values (pH 8.1±0.2 for R1 and 8.2±0.1 for R2) in both reactors (Figure 4.14 and Figure 4.15), potential free-ammonia concentration was minimized (5-7 mg/L) during aerobic periods (Anthonisen et al., 1976). Therefore, neglecting the ammonia stripping effect, the N loss in aerobic period can be clearly correlated with the SNDN performance of the reactors. Results of N balance revealed that 44±12 mg/L and 25±8 mg/L TON was lost during aerobic period in R1 and R2, respectively. The corresponding SNDN efficiencies were 46±10 % and 30±4% in R1 and R2, respectively (Table 4.4).

Under steady conditions, denitrification performances during anoxic periods were also investigated. The average denitrification efficiencies (in anoxic periods) were 95±7% in R1 (with a stable trend) and 37±15% in R2 (with a stable trend) (Table 4.4). In order to determine the reason of different denitrification performances of R1 and R2, one cycle (Day 50 – cycle 197) was investigated in detail with respect to time.

Observation of one cycle (Day 50): Figure 4.22 and Figure 4.23 represent the change of sCOD, TAN, NO_2-N and NO_3-N concentrations in cycle 197 (Day 50). In R1, 30% of the initial sCOD was removed in anoxic period (45 min) and 75% of the remaining sCOD was removed from the system in the first 2 h of the aerobic period. In R2, almost 50% of the initial sCOD was consumed in the first 75 min of aerobic period. sCOD consumption of reactors followed the first-order kinetics with 99% confidence interval. The aerobic sCOD consumption rates of R1 and R2 were calculated as 0.72 h^{-1} and 0.48 h^{-1}, respectively, indicating the 1.5 times higher degradation rates in the former during aerobic period.

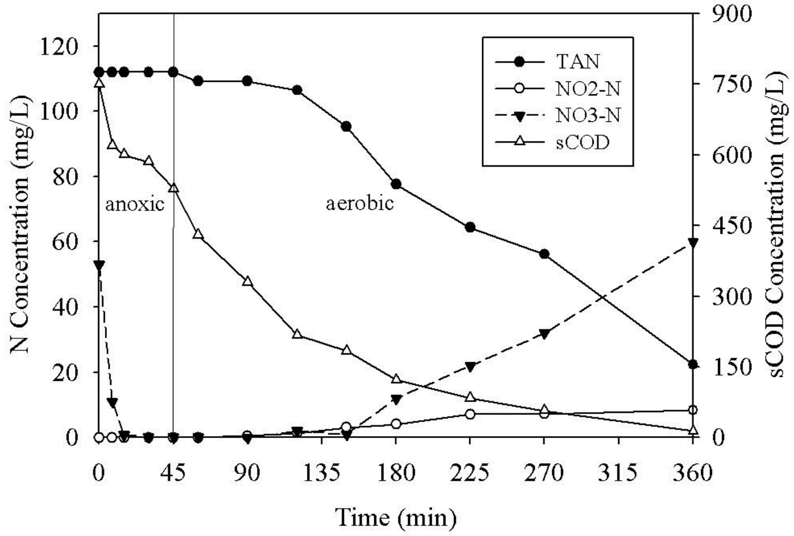

Figure 4.22 Cycle performance of R1 (Day 50) in Set-2

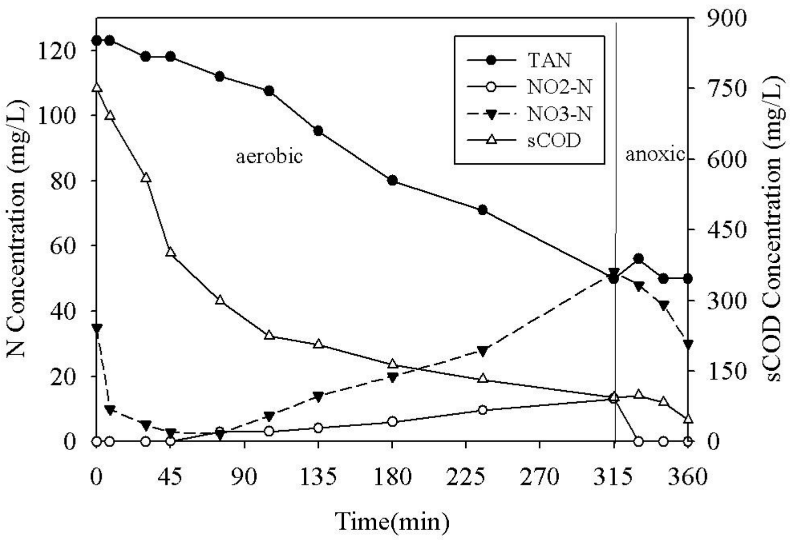

Figure 4.23 Cycle performance of R2 (Day 50) in Set-2

TAN removal was not observed during the anoxic periods of both reactors. Significant TAN concentration decrease was observed during aerobic period (due to nitrification) in both reactors only after ~70% of the initial sCOD was depleted. It was observed during aerobic period of R1 that 68.4 mg/L TON (NO_3-N being 88% of TON) accumulated while 89.6 mg/L TAN consumed in R1 (Figure 4.22). In R2, 60.2 mg/L TON (NO_3-N being %80 of TON) accumulated after the consumption of 73 mg/L TAN (Figure 4.23). Since the pH value was in the range of 7.7-8.1, 98% of the initial TAN was in ammonium ion form and N loss due to NH_3-N was negligible. This observation supported the idea that SNDN occurred in both reactors during aerobic phase. The N loss due to SNDN was measured as 25% in R1 and 18 % in R2.

As can be seen in Figure 4.22, there was enough carbon source (752 mg/L COD) at the beginning of the cycle in R1 (anoxic-aerobic sequence) for heterotrophic denitrification to occur. However, for having the sequence of aerobic-anoxic periods, the sCOD concentration remained at the beginning of the anoxic period in R2 was only 94 mg/L (Figure 4.23). For the reduction of 65.2 mg/L of TON (measured at the end of aerobic period) of R2 via denitrification during anoxic period, 186 mg/L sCOD was required stochiometrically. Therefore, 94 mg/L sCOD was not enough for 100% denitrification of the TON concentration of 65.2 mg/L (at the beginning of anoxic period in R2). Eventually, denitrification efficiencies during anoxic periods of R1 and R2 were measured as 99% and 54%, respectively which were verifying the stochiometric calculations. Therefore, the poor denitrification performance of R2 was attributed to the limited electron donor (COD) concentration resultant of its aerobic-anoxic periodic sequence. In order to verify this conclusion, batch test-1 was conducted.

Batch Test-1: Anoxic period of R2 was simulated with two batch reactors, one of which was fed with external carbon (500 mg/L sCOD) before anoxic period and the other one without any external carbon feed. It was found that initial 80 mg/L NO_3-N concentration was denitrified to 15 mg/L (81% denitrification) in 45 min when (unlimited) external carbon source (500 mg/L sCOD) was added. However, without external carbon source (sCOD at the end of aerobic period was 108 mg/L), effluent concentration was measured as 55 mg/L NO_3-N for the same initial concentration of 80 mg/L NO_3-N after 45 min of anoxic period. Denitrified NO_3-N amount (25 mg/L NO_3-N, 31% denitrification) without carbon source was very similar to the consumption in the cycle analysis (30 mg/L NO_3-N) and steady-state conditions (37±15 %) (Figure 4.23, Table 4.4). In study of Qin and Liu (2006), similar conclusion was obtained. SBR having granular sludge was operated with aerobic-anoxic period sequence and 45-55% TON (12-27 mg/L TON) consumption was observed after 2 h of anoxic period due to the carbon source limitation (Qin and Liu, 2006).

It was also observed in Figure 4.22 and Figure 4.23 that almost 90% and 50% of the initial NO_3-N was consumed during the feeding period (first 10 min of the cycle, just before the anoxic or aerobic periods started) in R1 and R2, respectively. In order to clarify that NO_3-N consumption during feeding period was whether due to denitrification or abiotic chemical consumption, batch test-2 was conducted.

Batch Test-2: Feeding period of R1 was simulated in two batch reactors, one of which was biologically active and the other was inactive. It was observed in biologically active reactor that 58 mg/L of initial NO_3-N concentration decreased to 10 mg/L NO_3-N due to denitrification. On the other hand, in abiotic reactor 38 mg/L initial concentration did not change at the end of 10 min of feeding. This result also revealed that, once granular sludge was developed, the anoxic period might be shortened or even removed considering the available carbon source amount and denitrification rate, for nitrate removal occurred even during the feeding period.

4.2.3. Granulation Performances

The evolution of flocs and granules in R1 (anoxic-aerobic) and R2 (aerobic-anoxic) during the operational period of 63 days are weekly shown in Figure 4.24 and Figure 4.25, respectively. Acclimated suspended seed sludge was composed of flocs, had loose/irregular structure and no filamentous growth (Figure 4.24-A, Figure 4.25-A). First granules with small sizes, round shape and dense structure were observed on Day 5 in R1 and on Day 8 in R2 (Figure 4.24 and Figure 4.25).

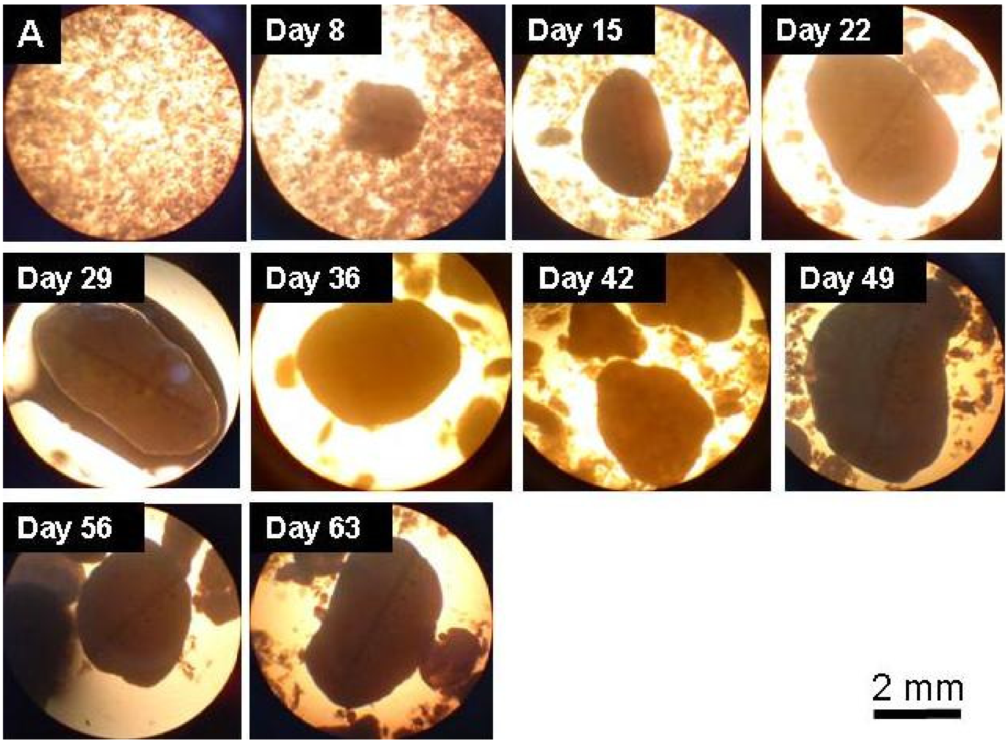

Figure 4.24 Evolution of flocs and granules during the operational period in R1 (anoxic-aerobic) in Set-2 (A: images taken at the end of the acclimation period)

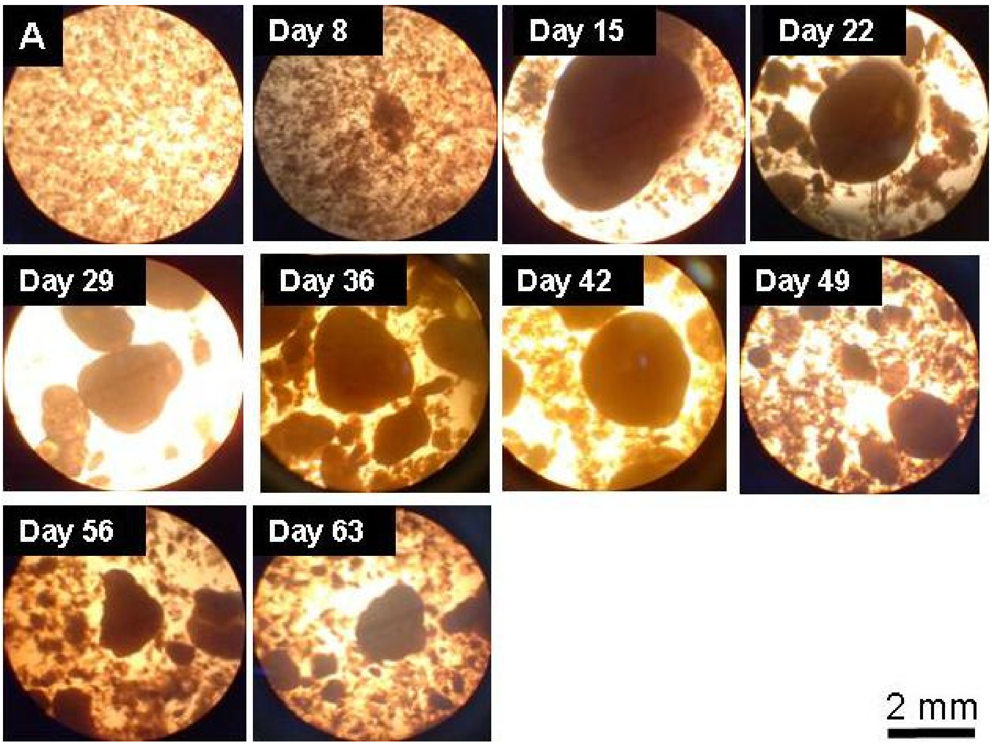

Figure 4.25 Evolution of flocs and granules during the operational period in R2 (aerobic-anoxic) in Set-2 (A: images taken at the end of the acclimation period)

Average diameters of the granules (developed in the first week) in R1 and R2 were 1.8±0.6 mm and 0.4±0.05 mm, respectively (Figure 4.26). Following the decrease in the settling period from 15 min to 5 min, a sharp increase in the average sizes was observed for both reactors. Yet, the average granule sizes were always higher in R1. The average sizes were measured as 3±0.85 mm and 2.1±0.9 mm for R1 and R2 on Day 15, respectively (Figure 4.26). As can be seen from Figure 4.26 and Figure 4.27, granule percentages of 93% and 84% were achieved with average granule sizes of 3.5±0.9 mm and 2.7±0.7 mm in R1 and R2, respectively, on Day 25. The granule cultivation rate was also higher in R1 than that of R2 (Figure 4.27). Despite the decrease in the average granule sizes following Day 25 (2.4±1 mm and 2±1.2 mm in R1 and R2, respectively, by Day 42), granular sludge percentage did not significantly change and were more or less constant in both reactors till Day 42. However, both the average granules' sizes and the percent of the granular sludge content of both reactors started to decrease after Day 42 (Figure 4.26 and Figure 4.27). This was attributed to the decrease in the influent magnesium concentration. As it was described in Modification V (Section 4.2.2.3), the influent sulfate concentration (in the form of $MgSO_4.7H_2O$) was decreased from 180 mg/L to 90 mg/L on Day 42, which also led to the decrease in the magnesium concentration. It was reported that divalent atoms such as Ca^{2+} and Mg^{2+} have positive effects on granulation and enhances granular structure (Gao et al., 2011). After Day 49, average granule sizes in R1 started to increase. After a week, on Day 56, granule size increase was also observed in R2 (Figure 4.26); therefore, it was claimed that the decrease in the Mg^{2+} concentration might have decreased the granulation rate of flocculant sludge but did not harm the existing granules. The granular sludge content of R1 started to slowly increase by Day 49 and reached a steady level at 88% by Day 56, when sCOD and TAN removal efficiencies had also reached steady conditions (Figure 4.27, Table 4.4). Recovery/steadiness of the granule percentage and the increase in the granule sizes observed in R1 revealed that the new Mg^{2+} level applied was not catastrophic for granules, and granulation might continue steadily (Figure 4.26 and Figure 4.27). On the other hand, the percent of granular sludge of R2 continued to decrease and reached 57% by the end of the study. In addition, the average granule sizes of R2 displayed a decreasing trend with fluctuations, which was not on the wane but in the way of disintegration (Figure 4.26 and Figure 4.27). At the end of operation (Day 63) granules in R2 mainly disintegrated, became loose and had an average size of 1.5±0.6 mm, thus granular sludge percentage decreased down to 55% (Figure 4.25, Figure 4.26 and Figure 4.27). On the other hand, granules in R1 were still compact/dense and had an average size of 2.9±0.7 mm (Figure 4.24 and Figure 4.26). There might be a reason for granule disintegration in R2. Denitrifying heterotrophs in the core of the granules were lack of carbon since the operation was post-anoxic and carbon-limiting conditions occurred for denitrification during anoxic periods in R2. This starvation during anoxic period in the core of the granules, in which heterotrophic denitrifiers are expected to be dominant, might have caused corruption of the core and disintegration of the granules (Adav et al., 2009b) (Figure 4.26).

The settling velocities of the granules in R1 and R2 measured at the end of the study were 72 m/h and 36 m/h, respectively, which were comparable to the literature data given as 18 m/h to more than 91 m/h (Jang et al., 2003; Qin and Liu, 2006; Wang et al., 2006; Adav et al., 2009b; Gao et al., 2011). Higher settling velocities obtained for R1 granules were expected because of their higher particle sizes and compact structure and hence, potentially higher densities than those of R2 granules.

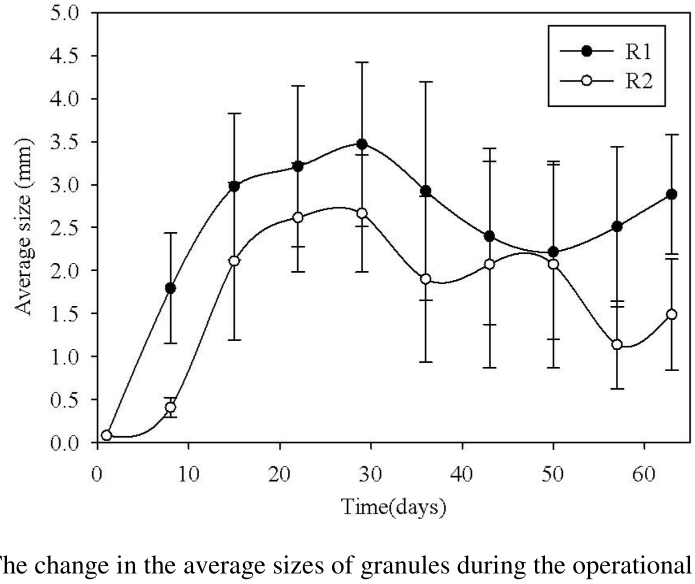

Figure 4.26 The change in the average sizes of granules during the operational period in Set-2

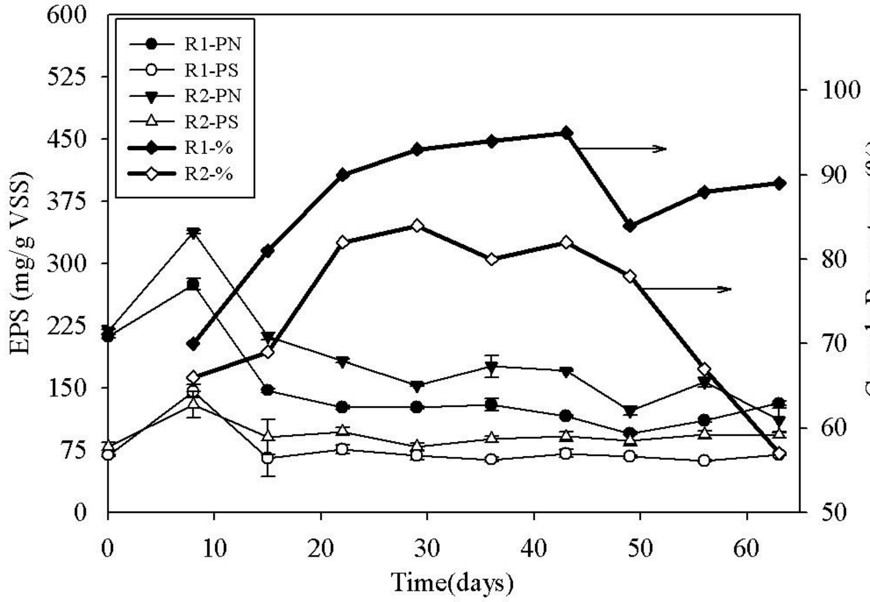

Figure 4.27 Variations of EPS and granule percentage in Set-2

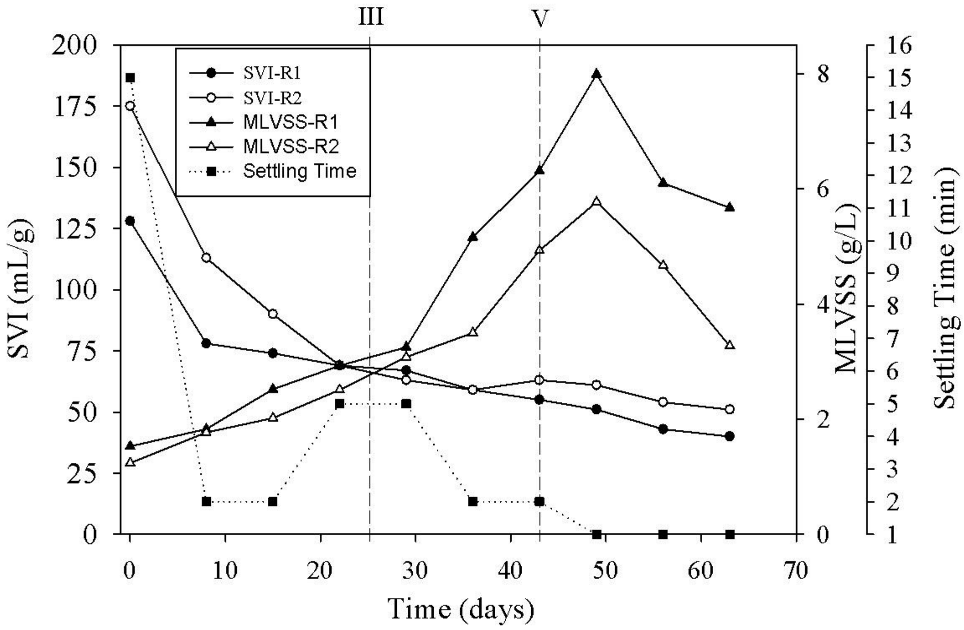

Figure 4.28 Variations of SVI_{30}, MLVSS concentrations inside the reactors and settling time during granulation in Set-2

Figure 4.28 represents the changes in sludge properties in both reactors during the entire 63 days of operation. The SVI_{30} values decreased in the first 9 days, meanwhile the first granules appeared in both reactors. It was reported that SVI_{30} values decreased with the granulation and SVI_{30} values stay mostly below 80 mL/g for aerobic granules (Gao et al. 2011). In this study, SVI_{30} values of 40 and 51 mL/g were observed for R1 and R2, respectively (Figure 4.28). Mature granules having SVI_{30} values as low as 10-20 mL/g were also reported in literature (Qin and Liu, 2006; Su et al., 2012). Therefore, it can be said that in both reactors granular sludge with good settleability, being slightly better in R1, were cultivated which supports the aforementioned settling velocity difference.

An increasing trend for MLVSS concentrations were observed during the first 25 days of operation (Figure 4.28). Nor-Anuar et al. (2012) reported that rotators have adverse effects on granular stability due to extensive shear stress such as causing disintegration. Since, homogenous mixing of reactors was not possible with 48 rpm after 80% granulation and physical destruction can occur in mechanically-mixed reactors, on Day 25 mixers were plugged out from the system (Modification III). Following Modification III, (between Days 25-49) when granulation percentage was around at 92-94% and ~80-84% for R1 and R2, respectively, MLVSS concentrations inside the reactors sharply increased and average granule sizes decreased (Figure 4.26 and Figure 4.28). Yilmaz et al. (2007) reported that density of granules increased with increasing MLVSS at stable granule size. There were increase in MLVSS and decrease in average granule size while there was no granule disintegration (more or less same granular percentage) between Days 28-49. Therefore, it can be said that between Day 28 and Day 49 density of granules increased in both reactors (Figure 4.26, Figure 4.27 and Figure 4.28). In other words, while volume of granules decreased with decreasing size, their MLVSS concentrations reached up to 8 g/L and 6 g/L in R1 and R2, which indicated that granules contained more microorganisms in smaller volumes (compactness increased). In granular systems MLVSS concentrations much higher than that of suspended sludge processes were reported in literature (Yilmaz et al., 2007; Ni et al., 2009; Adav et al. 2009b). Therefore, MLVSS increase up to 6-8 g/L during granulation was consistent with the relevant granulation studies. In order to maintain the biomass concentration at 6 g/L inside the reactors, settling period was decreased to 1 min after Day 50. Following the decrease in settling period, MLVSS concentrations decreased in both reactors and granule percentage of R1 increased (Figure 4.27 and Figure 4.28). As it is known, settling time significantly effect granulation (Liu and Tay, 2004; Gao et al., 2011). Therefore, increase in granule percentage in R1 might be due to the decrease in settling time (Figure 4.27 and Figure 4.28). On the other hand, MLVSS concentration continued its decrease in R2 at 1 min settling time until the end of operation. It is reported that, settling time provides selection of the microorganisms according to their settling velocities (Zhang et al., 2011; Gao et al., 2011). As represented in Figure 4.25, Figure 4.27 and Figure 4.28, granule percentage in R2 started to decrease which indicated the suspended form of sludge domination in the reactor and its further washout (MLVSS decrease) at settling time of as low as 1 min (Days 50-63).

EPS release is important for the agglomeration of microorganisms (Morgan et al., 1990; Liu et al., 2004a; McSwain et al., 2005; Wang et al., 2006c; Ren et al., 2010-179; Sheng et al., 2010b). Stressful conditions such as hydraulic selection, starvation and non-aerated periods increase EPS release, especially the PN release, during granulation process (Nichols et al., 2004; Qin et al., 2004; Zhang et al. 2007; Yu et al., 2009). The effect of anoxic-aerobic periods' sequence was, therefore, also compared with respect to the changes in the EPS content of the granules (Figure 4.27). EPS results also indicated that anoxic-aerobic operation (R1) resulted in more stable granules as well as granulation process.

During the first 10 days, where granulation started in both reactors, EPS amount in both reactors increased from 200 to approximately 300 mg/g VSS PN and from 70 to approximately 140 mg/g VSS PS. PN content and PN/PS ratio were reported to increase sharply during granulation (Zhang et al., 2007; Jiang and Liu, 2010). PN/PS ratio in aerobic granules typically varies between 0.1–5 (Liu and Tay, 2002; Beun et al., 2002). The sharp increase in EPS in the first 10 days and PN/PS increase (from 1.94 to 3.1 in R1 and from 1.94 to 2.8 in R2) was, therefore, related with the granulation process. With the development of over 80% granular biomass in the reactors (after Day 22), PN/PS ratio was more or less stable around 1.9 till Day 42 for both reactors (Figure 4.27). After Day 42, despite the slight decrease in PN/PS ratio (1.4) in R1, PN/PS ratio further increased to 1.9 and remained still till Day 63. In R2, on the other hand, PN content of granules started to fluctuate and decreased from 170±1 to 110±14 mg/g VSS between Days 42-63, which indicated the disrupted stability (Figure 4.27). Although some researchers indicated that granular skeleton was composed of β-polysaccharide, some others asserted that PN played the major role in core stability and sharp PN increases observed during granulation and hence long-term stability of the granules depended on PN content (Di Iaconi et al., 2006; Zhang et al., 2007; Adav et al. 2008b). In R2 between Days 42-63 PN/PS ratio decreased from 1.9 to 1.2 with the decrease of PN content. Decreasing PN/PS ratio caused disintegration of the granules since hydrophobicity decreased (Zhu et al. 2012). Similarly, between Day 42 and Day 63, granule disintegration was observed in R2. Therefore it can be said that, the observations in this study were consistent with the idea that PN was the major component of core stability.

As mentioned earlier in observation of one cycle at steady-state (Section 4.2.2.3), the aerobic-anoxic period sequence of R2 led to carbon-lacking conditions during anoxic period, because most of the carbon was already consumed by aerobic heterotrophs during aerobic period. It is reported that the decrease in F/M ratio decreased the EPS content, especially the protein content, of the aggregates (Sponza, 2003; Dvorak et al., 2011; Li et al., 2011). Moreover, Wang et al. (2005) indicated that biodegradable portion of EPS mostly existed in the core of granules. It is also claimed that PN is the main EPS component in the core of granules (Di Iaconi et al., 2006; Zhang et al., 2007). Therefore, C-lacking conditions might have resulted starvation of denitrifiers and further possible biodegradation of PN, decrease in PN content and PN/PS ratio which resulted in disintegration of R2 granules.

Despite the lower PN/PS ratio values, the EPS content of the granules in R2 was always higher than that of R1, for both PS and PN contents. Wang et al. (2010) stated that flocculant sludge has higher EPS content than granular sludge. As represented in Figure 4.27, granule percentage in R1 was always higher than that in R2 which means flocculant sludge content was higher in R2. Therefore, the higher EPS obtained in R2 was explained by its higher flocculant sludge percentage.

4.3. Set-3: The Effects of Influent Sulfate and Soluble Sulfide Concentrations on Treatment Performance of Granules and Granular Structure

At the beginning of the operation, reactor had already been operated at an influent SO_4^{2-} concentration of 35.1 mg/L for 67 days (Appendix D). The SRT was tried to be adjusted by periodic sludge wastage from the system and it was varying between 22-26 days during the 67 days of operation. The average removal efficiencies were observed as 94±3% for sCOD, 90±4% for TAN and 43±10% for TN at steady conditions (Figure D.1). In order to investigate the effects of influent sulfate concentration on treatment efficiency of granules, an SBR (anoxic-aerobic sequence) was fed with varied influent SO_4^{2-} concentrations of 35.1, 46.8, 52.6, 58.5 and 70.2 mg/L SO_4^{2-} (i.e. from 90 to 180 mg/L $MgSO_4.7H_2O$). The range of influent SO_4^{2-} concentrations applied was determined according to the results of Set-2. In Set-2, when influent SO_4^{2-} concentration was decreased from 70.2 mg/L SO_4^{2-} to 35.1 mg/L SO_4^{2-}, significant changes in the COD and TAN removal performances had been observed. Therefore, concentrations of 35.1 mg/L SO_4^{2-} and 70.2 mg/L SO_4^{2-} were decided to be the minimum and maximum concentrations of Set-3, respectively. Each different SO_4^{2-} dose was applied for 5 days.

As stated previously, granular sludge has steady removal performance at 35.1 mg/L SO_4^{2-}. That is why, in Set-3, after application of each different SO_4^{2-} dose for 5 days if any deterioration was observed, the SO_4^{2-} does was decreased back to 35.1 mg/L SO_4^{2-} for recovery purposes. As soon as the steady conditions were achieved, next dose of higher concentration was applied.

4.3.1. Treatment Performances

The corresponding treatment efficiencies for the investigated influent SO_4^{2-} concentrations are given in Figure 4.29. As seen in Figure 4.29, for the influent SO_4^{2-} concentrations of 35.1 and 46.8 mg/L (90 and 120 mg/L $MgSO_4.7H_2O$), sCOD and TAN oxidation efficiencies were not negatively affected and both were measured as 97% (Days 0-6, Phase I-II). When the influent SO_4^{2-} concentration was increased to 52.6 mg/L (135 mg/L $MgSO_4.7H_2O$, Phase III), sulfate reduction and sulfide generation (0.26 mg/L S^{2-}) was observed during anoxic period of the anoxic-aerobic sequence (Table 4.5, Figure 4.29a,b). TAN and sCOD oxidation efficiencies decreased by 20% and 7%, respectively (Figure 4.29c) and the pH of reactor content increased during aerobic period (Table 4.5). Following the inhibition, reactor was operated again with 35.1 mg/L of influent SO_4^{2-} concentration (Days 11 – 15, Phase IV) to decrease the possible stress on microorganisms and provide recovery of the treatment performances. After sCOD and TAN removal efficiencies increased back to almost 90%, the influent SO_4^{2-} concentration was increased to the next dose of 58.5 mg/L (150 mg/L $MgSO_4.7H_2O$) (Phase V). Following that, sulfide generation in anoxic period doubled (0.54 mg/L S^{2-}) and TAN oxidation decreased down to 47-53%, which was similar to that observed in Set-2; (R1) (Section 4.2.1) in the first 42 days of unsteady operation (Table 4.5, Figure 4.29). sCOD removal efficiency did not significantly decrease with the influent SO_4^{2-} dose of 58.5 mg/L, however, still slight decrease of 10% was recorded. After repetition of the recovery session with 35.1 mg/L of influent SO_4^{2-} concentration between Day 20 and Day 23 (Phase VI), reactor was exposed to the final dose of 70.2 mg/L SO_4^{2-} concentration (180 mg/L $MgSO_4.7H_2O$) (Phase VII). TAN oxidation decreased to 44±2%, while COD removal efficiency also decreased from 91.5±2.3 to 80±0.5%.

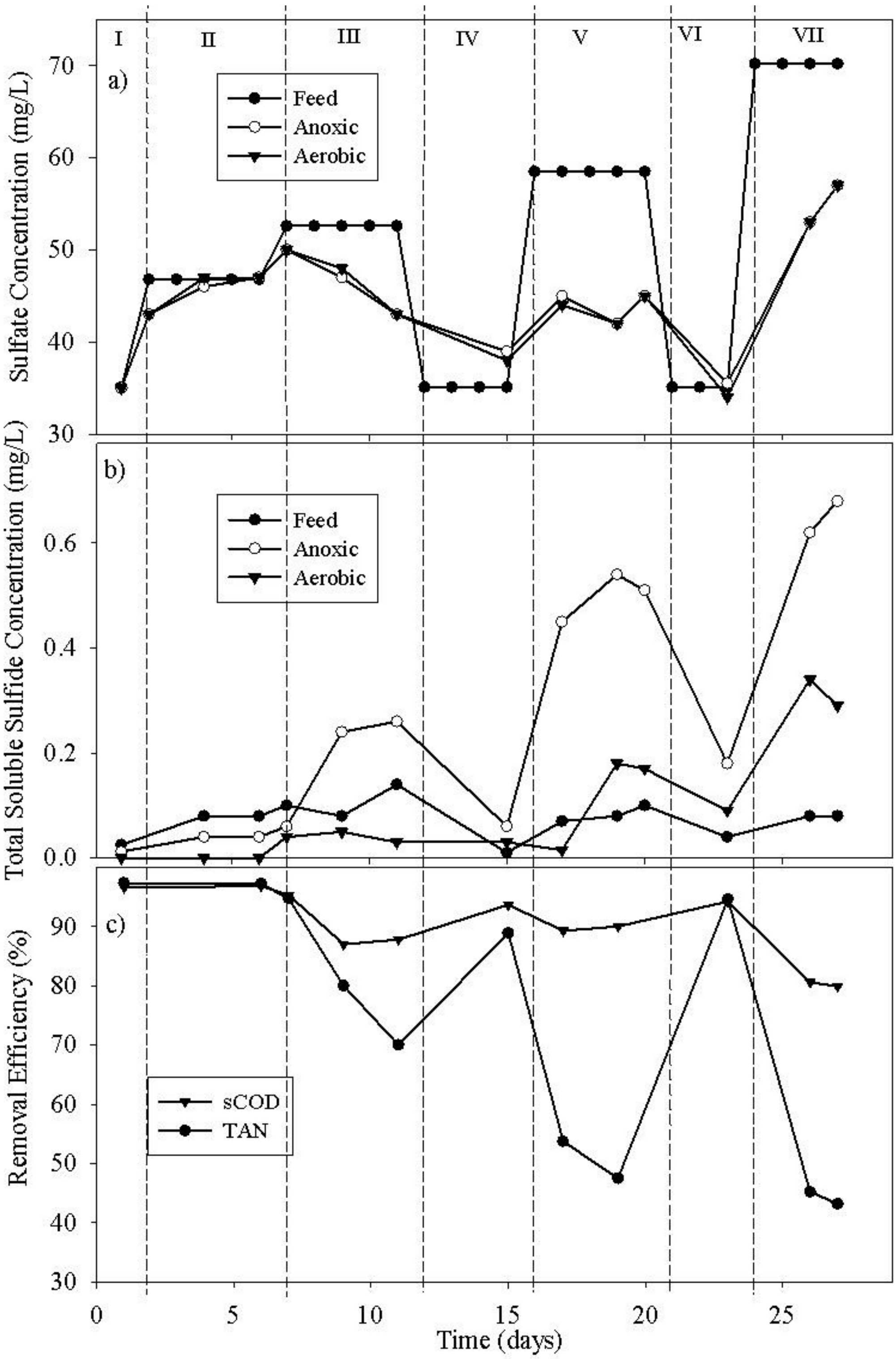

Figure 4.29 Changes during operation (Phases I-VII)
a)SO_4 influent dose, b)Total soluble sulfide concentration, c)Removal efficiency at $MgSO_4.7H_2O$ concentrations of I- 90 mg/L, II- 120 mg/L, III-135 mg/L, IV-90 mg/L, V- 150 mg/L, VI-90 mg/L, VII- 180 mg/L

Table 4.5 The pH values and corresponding free-ammonia concentrations with varying influent SO_4 concentrations

Influent SO_4 (mg/L)	Total soluble sulfide produced (mg/L)	pH at the end of anoxic period	pH of aerobic period (after 15 min- the end of period)	NH_3-N (mg/L)
35.1	< 0.1	8.5	8.5 to 7.7	2.06
46.8	< 0.1	8.3	8.7 to 8.1	4.8
52.6	0.26	8.4	9.1 to 8.6	15.2
58.5	0.54	8.5	9.1 to 8.8	27.8
70.2	0.62	8.4	8.9 to 8.6	18.9

Figure 4.29 and Table 4.5 reveals that the increasing influent sulfate concentrations led to increased amount of sulfate consumption, and further increased amount of sulfide production. It was also observed that pH increased with the increasing influent SO_4^{2-} dose, especially for the doses promoting sulfide production (from 52.6 to 70.2 mg/L SO_4^{2-}) as represented in Table 4.5. Sulfate reduction, sulfide production and further related pH increase were attributed to the presence of sulfate reducing bacteria in the inner cores of granules. Reduced sulfur compounds are known as toxic for aerobic oxidation of TAN and 0.25 mg/L of soluble sulfide can completely inhibit ammonia-oxidation (Æsøy et al. 1998; Sears et al., 2004). In this study, the produced total soluble sulfide concentrations (0.24-0.62 mg/L S^{2-}) potential to be inhibitory for TAN removal were close or greater than the literature data. It should also be noted that increase in pH with the increasing SO_4^{2-} dose led to the increase in free-ammonia concentration. Free-ammonia is stated as an inhibitory compound for nitrification (Anthonisen et al. 1976; Yoo et al., 1998; Yang et al. 2004b). Yang et al. (2004b) stated that 2.5 – 39.6 mg/L NH_3-N concentration inhibits the specific oxygen uptake rate of nitrifiers and aerobic heterotrophs by 2.5 and 5 times, respectively. It was also stated that 10 – 150 mg/L NH_3-N is toxic for nitrifiers (Anthonisen et al., 1976). Therefore, in this study, not only sulfide concentrations of 0.24-0.68 mg/L S^{2-}, but also free-ammonia concentrations of 15.2-27.8 mg/L NH_3-N might be inhibitory for TAN oxidation (Table 4.5). The same result was also valid for sCOD removal performances. sCOD removal efficiency was slightly inhibited (7-12%) by free-ammonia (15.2-27.8 mg/L NH_3-N) and potentially total soluble sulfide (0.26-0.62 mg/L S^{2-}). Sulfide related COD removal inhibition was not mentioned so far in the literature. Moreover, it was stated that sulfide and existence of SRB in aerobic systems have negligible effects on COD removal performance (Lens et al. 1995a,b). Therefore, it can be said that COD removal efficiency was individually inhibited by free-ammonia concentration inside the reactors during operation.

The inhibitory sulfide and free-ammonia concentrations (0.62 mg/L S^{2-} and 27.8 mg/L NH_3-N, respectively) were almost twice of the levels given in literature for severe toxicity (0.25 mg/L S^{2-} and 10 mg/L NH_3-N). However, 80% sCOD removal and 50% TAN oxidation could still be achieved at the highest dose. The level of inhibition observed during operation was more severe for ammonia oxidizers than that of aerobic heterotrophs. That might be due to exposure of ammonia oxidizers to both sulfide and free-ammonia, while aerobic heterotrophs were only affected by free-ammonia.

The inhibitory sulfide and free-ammonia concentrations (0.25 mg/L S^{2-} and 10 mg/L NH_3-N) given in literature are for conventional activated sludge systems. Since granular sludge is denoted to be more resistant to toxic effects than suspended sludge, inhibition observed on treatment efficiencies in this study was less than the values determined in literature. For example, Sears et al. (2004) stated total inhibition of ammonia oxidizers when exposed to 0.25 mg/L to 0.5 mg/L soluble sulfide while in this study 50% of inhibition was detected at doses of 0.24-0.62 mg/L soluble sulfide. Rapid recovery was also observed for the inhibitory doses studied (Figure 4.29), which signifies the advantages of aerobic granular sludge to suspended sludge. These results support the idea that granular sludge and granules cultivated in this study are much more resistant than suspended sludge to toxic effects and high loading rates.

There was no inhibition detected for denitrifiers. Denitrification performances during anoxic conditions were 94±3%, which was not affected by the increasing sulfate and further sulfide doses and also free-ammonia concentrations. It was hard to make distinctive conclusions on the performances of denitrification during aerobic periods (SNDN), because with the increasing sulfate doses, both pH and free-ammonia concentrations increased which might resulted in ammonia stripping and interfered with the SNDN related N loss.

As seen in Figure 4.29a, effluent SO_4^{2-} concentrations of anoxic and aerobic periods were more or less same, that indicated there was no SO_4^{2-} consumption or production during aerobic periods. However, there was no consistency between the consumed SO_4^{2}-S and the produced soluble sulfide in anoxic periods (Figure 4.29a,b). The produced soluble sulfide concentrations were always 80-90% less than SO_4^{2-}-S concentrations consumed. It was likely that the sulfide produced might have been used in other potential reactions during anoxic periods. There are possible mechanisms for consumption of the sulfide produced during anoxic periods (Eqn 4.12, Eqn 4.13, Eqn 4.14, Eqn 4.15, Eqn 4.16). Possibility of these reactions to occur during the anoxic periods of Set-3 was stochiometrically investigated.

$$1.25S^{2-} + 2NO_3^- + 2H^+ \rightarrow 1.25SO_4^{2-} + N_2 + H_2O$$

ΔG= -972.8 kJ/reaction
Eqn 4.12

$$5S^{2-} + 2NO_3^- + 6H_2O \rightarrow 5S^0 + N_2 + 12OH^-$$

ΔG= -1168.4 kJ/reaction
Eqn 4.13

$$HS^- + 0.4NO_3^- + 1.4H^+ \rightarrow S^0 + 0.2N_2 + 1.2H_2O$$

ΔG= -251.9 kJ/reaction
Eqn 4.14

$$2HS^- + O_2 \rightarrow S^0 + 2OH^-$$

ΔG= -169.35 kJ/mole
Eqn 4.15

$$2HS^- + 2O_2 \rightarrow H_2O + S_2O_3^{2-}$$

ΔG= -387.35 kJ/mole
Eqn 4.16

Eqn 4.12 and Eqn 4.13 are lithotrophic nitrate removal mechanisms where reduced sulfur (S^{2-}) is used as energy source. The heterotrophic denitrification (Eqn 4.17), on the other hand, can be a high rate process and preferable one under anoxic conditions (Reyes-Avila, 2004).

$$1.25CH_3COOH + 2NO_3^- \rightarrow N_2 + 1.5H_2O + 2OH^-$$

ΔG= -1054.8 kJ/reaction
Eqn 4.17

The initial NO_3-N concentrations were 30-50 mg/L during operation. Corresponding stochiometric sCOD values required for denitrification of 30-50 mg/L NO_3-N were calculated and compared with the observed sCOD consumption in anoxic periods. It was found that sCOD consumed during anoxic periods was slightly higher than that was required for the NO_3-N consumed in anoxic period. This comparison indicated that heterotrophic denitrification (Eqn 4.17) during anoxic period was more possible than litotrophic nitrate reduction (Eqn 4.12, Eqn 4.13). Additionally, Eqn 4.12 represents SO_4^{2-} production under anoxic conditions, while sulfide is used for NO_3-N reduction. However, as seen in Figure 4.29a,b, just the opposite was realized, i.e. SO_4^{2-} was consumed while sulfide was produced during anoxic periods. Moreover, pH values varied in the range of 7-9.1 during anoxic periods in which S^{2-} can not be the dominant sulfide form (Figure 4.30) for Eqn 4.12 to occur. For the experienced pH values, HS^- was the dominant sulfide form. Similarly, Eqn 4.13 can not occur for the studied pH values, where the dominant type of sulfide was HS^-. According to Figure 4.30, total soluble sulfide produced should have been composed of 50% H_2S+50% HS^- to 99% HS^- for the pH ranges experienced (7-9.1) during anoxic periods in this study. Since there was no mixing during anoxic periods, H_2S stripping was not possible. Therefore, with the increase in pH values, all soluble sulfide should be in the form of HS^-.

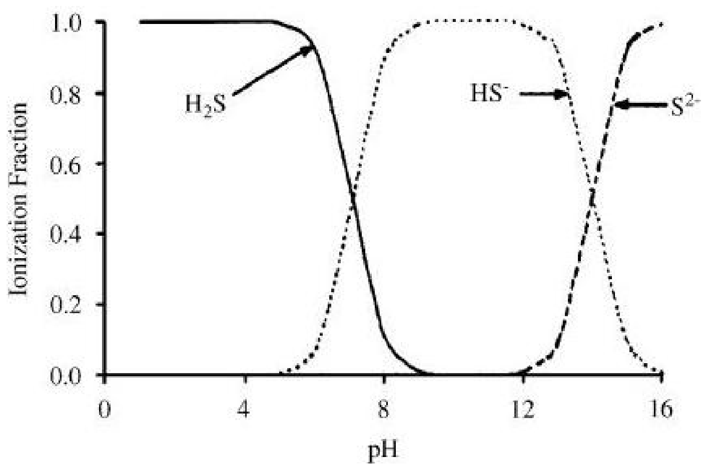

Figure 4.30 The effect of pH on fractions of sulfide species (S^{2-}, HS^-, H_2S) and related distribution in aqueous solutions at 25°C (Sawyer et al., 2003)

HS^- might also be oxidized via reduction of NO_3 to N_2 under anoxic conditions (Wong and Liu, 2011). However, compared to heterotrophic denitrification (Eqn 4.17, ΔG= -1054.8 kJ/reaction), Gibbs free-energy of nitrate reduction with HS^- (Eqn 4.14, ΔG=-251.9 kJ/reaction) was considerably higher which agreed with the idea that heterotrophic denitrification was the dominant mechanism for NO_3-N reduction (Eqn 4.17) instead of NO_3-N reduction with HS^- (Eqn 4.14) during anoxic periods.

The aforementioned information still does not answer the unbalance between SO_4-S consumed and sulfide-S produced; that is missing sulfide during anoxic periods. There were 2 possible (one biological, Eqn 4.15, one chemical Eqn 4.16) mechanisms for oxidation of HS^- under oxygen-limited conditions (DO<0.1 mg/L) (Janssen et al., 1995). Janssen et al. (1995) also stated that under oxygen-limited conditions (DO<0.1 mg/L) chemical oxidation of HS^- by the mechanism of Eqn 4.16 should be considered, especially in highly-loaded bioreactors. Atomic S balance performed for anoxic periods revealed that both chemical and biological consumption of HS^-, (Eqn 4.15 and Eqn 4.16) were stochiometrically possible. However, detailed analysis was not conducted.

4.3.2. Granule Properties

The change in the granule properties with respect to each $MgSO_4.7H_2O$ was also investigated. Possible effects of Mg^{2+} on granules were clarified. As represented in Figure 4.31, granular compactness, outer surface properties and shape did not change during the operational period.

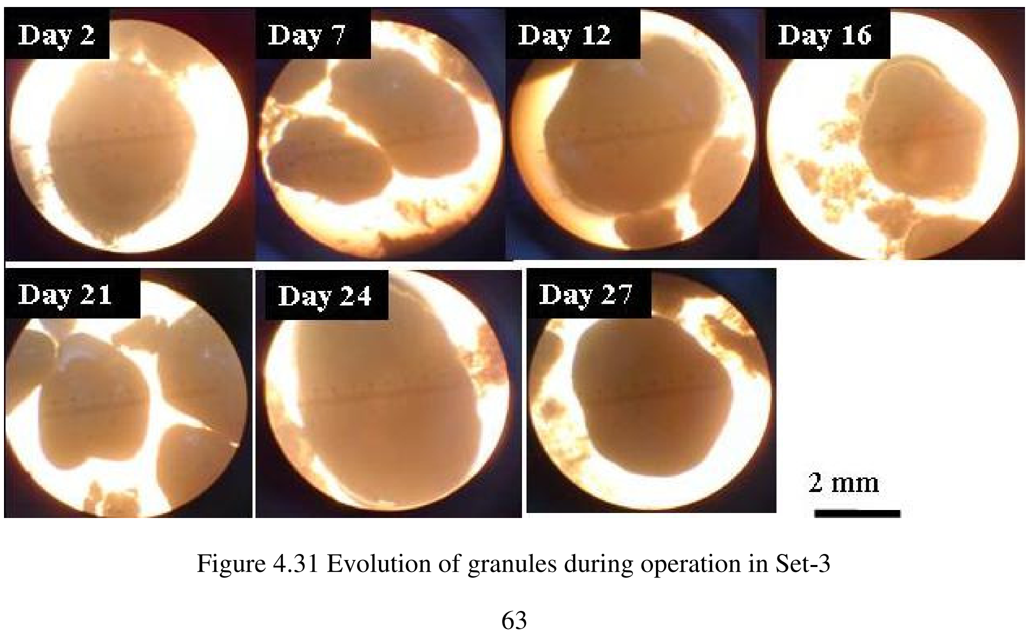

Figure 4.31 Evolution of granules during operation in Set-3

Average granule sizes were also investigated. As can be seen in Figure 4.32, average granule sizes were more or less same for all $MgSO_4.7H_2O$ concentrations studied. The granules' average sizes were 3.2±0.8 mm on Day 1 and 3.6±1 mm at the end of experiments. The granular sludge percentage in the reactor slightly decreased (from 94% to 90%) during the operation (Figure 4.32). However, SVI_{30} values of the sludge of the reactor remained almost same (34-37 mL/g) which indicated that settleability of granules was not deteriorated (Figure 4.33).

Granular properties such as MLVSS and MLSS concentrations were measured and represented in Figure 4.33. It can be seen that there was no drastic change during Set-3. Therefore, it can be said that varying influent SO_4^{2-} concentrations (from 35.1 to 70.2 mg/L) did not affect the granular sludge properties. In addition, the produced sulfide concentrations, the further increase in pH and free-ammonia doses experienced under the studied conditions did not lead to any significant change in granular cultures in terms of settleability, MLSS-MLVSS contents, granule percentage and granular size.

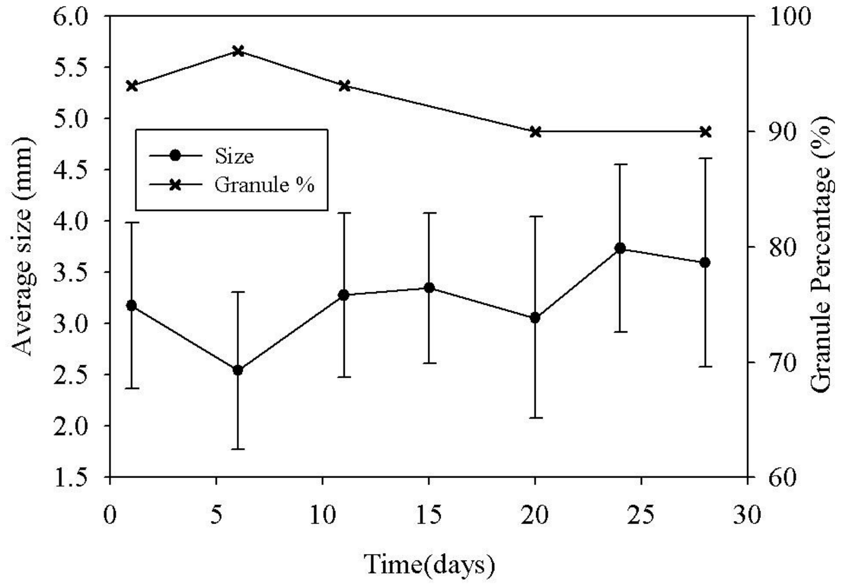

Figure 4.32 Granule percentage and average sizes in Set-3

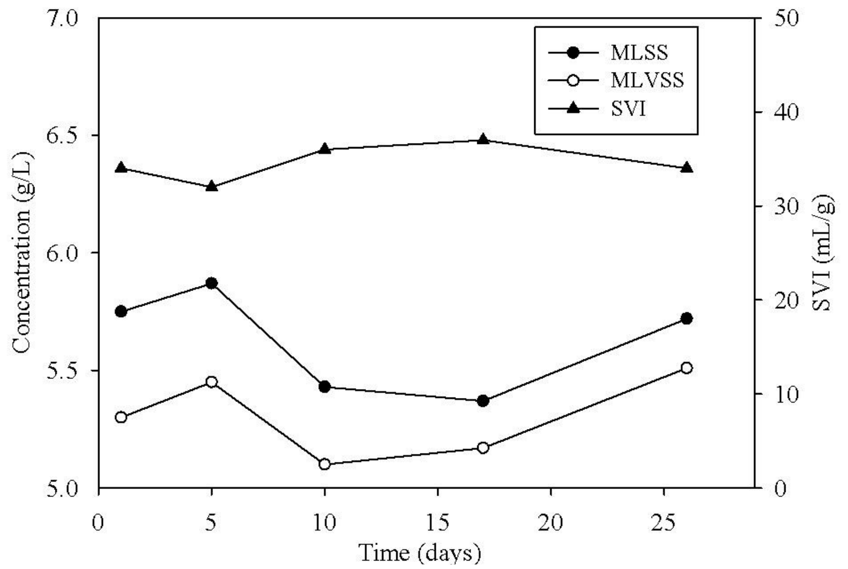

Figure 4.33 Variations of SVI_{30} and MLSS-MLVSS concentrations at a constant settling time (2 min) during operation in Set-3

CHAPTER 5

CONCLUSION

In this thesis study, the effects of seed sludge type and anoxic/aerobic period sequence on granulation and sCOD, N treatment of granules were investigated. The results and outcomes were summarized as follows;

- Aerobic granules of 0.56±0.23 mm to 2.48±1.28 mm can be successfully developed with both MBS and CAS.

- The granules cultivated from MBS seed performed better than CAS seed in terms of
  - having stable structure with smooth shape (non-filamentous structure in MBS, filamentous structure in CAS),
  - having higher settling velocity (36-39 m/h MBS, 19-20 m/h CAS),
  - having higher biomass retention (2.8 g/L MBS granules, 1.5 g/L CAS granules),
  - conserving granular structure better at high loading rates (0.86 g N/L and 3.92 g COD/L)
  - being more tolerant to toxicity (38-46 mg/L NH_3-N)

- In addition, granules of MBS are capable of providing better protection for nitrifiers and acclimating to the new conditions faster, thus they have better recovery performance than those of CAS. Therefore;
  - MBS granules performed better TAN oxidation efficiency (37±8%) and COD removal efficiency (70±16%) than CAS granules (TAN oxidation 12±2%, COD removal 36±4%) after recovery period.

- Free-ammonia concentrations of 38-46 mg/L totally inhibited TAN oxidation and decreased COD oxidation by 55-60%.

- Aerobic granules can be successfully developed in both anoxic-aerobic (sizes of 1.8±0.6 mm-3.5±0.9 mm) and aerobic-anoxic (sizes of 0.4±0.05 mm-2.7±0.7 mm) period sequence.

- The main influence of period sequence was observed on granule properties and stability. Granule percentage achieved in anoxic-aerobic sequence (>90%) was higher than that in aerobic-anoxic sequence (<85%). Additionally, the anoxic-aerobic sequence was found to be more advantageous than aerobic-anoxic sequence in terms of cultivating granules with;
  - higher granular sludge cultivation rate (5 days in anoxic-aerobic, 9 days in aerobic-anoxic sequence)
  - more stable structure (stable denitrifying core in anoxic-aerobic sequence, core disintegration due to C-lacking in aerobic-anoxic sequence)
  - higher settling velocities (72 m/h in anoxic-aerobic sequence, 36 m/h in aerobic-anoxic sequence)
  - higher biomass content (8 g/L in anoxic-aerobic sequence, 5.77 g/L in aerobic-anoxic sequence)
  - lower SVI (40 mL/g in anoxic-aerobic sequence, 51 mL/g in aerobic-anoxic sequence)

- Due to lack of carbon source, the granules developed at aerobic-anoxic sequence disintegrated. The anoxic-aerobic period sequence is, therefore, found to be better for long-term operation. If total nitrogen removal is aimed, adequate carbon source is needed to protect the stability of the granules developed at aerobic-anoxic sequence.

- Periodic sequence had no significant effect on COD removal and TAN removal efficiencies of granules. Yet, the sequence of aerobic-anoxic periods led to lower denitrification performance due to limited-carbon source and 40% lower SNDN performance compared to that of anoxic-aerobic sequence. Under steady-state conditions, granules cultivated with the operational sequence of anoxic-aerobic and aerobic-anoxic periods achieved 95±2% and 92±1% COD removal, 90±2.5% and 89±1% TAN removal, 46±9% and 38±11% TN removal, 46±10% and 30±4% SNDN, respectively, at an OLR of 2.94 g COD/L.d and NLR of 0.43 g N/L.d.

- Influent SO_4 concentration of more than 52.6 mg/L increased biological activity of sulfate reducing bacteria (SRB) potentially located in the core of the granules and sulfate reduction. The mentioned increase in biological activity of SRBs increased pH and related free-ammonia production at high TAN loading rate conditions (0.4 g TAN/L.d).

- Total soluble sulfide concentrations of 0.24-0.62 mg/L and free-ammonia concentrations of 15-27 mg/L were found to inhibit TAN oxidation by 30-50%. Since inhibition of ammonia oxidizing bacteria (AOB) caused TAN accumulation and dominant free-ammonia production at high pH values, sCOD oxidation was also indirectly inhibited, by 25-30%. But the recovery is possible with the developed aerobic granules under the studied conditions.

- It was clarified that influent SO_4^{2-} concentrations of 35.1 to 70.2 mg/L did not significantly affect granular properties such as, settleability, granular stability, granule size and granule percentage.

To summarize, under high influent TAN concentrations and loading rates, aerobic granules can oxidize 95% of the influent sCOD, TAN and remove 50% of the influent TN, if the well operating conditions (sulfate limited and non-inhibitory conditions) are provided. In order to increase TN removal efficiency, removal of anoxic period from the operation only after the 90% or more granular sludge achieved and operation of the granular system with lower DO concentrations (DO< 3 mg/L) are recommended.

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APPENDIX A

DEVELOPMENT OF INITIAL START-UP SBR CONDITIONS FOR GRANULE CULTIVATION FROM CONVENTIONAL ACTIVATED SLUDGE (CAS) AND MEMBRANE BIOREACTOR SLUDGE (MBS)

Purpose
The aim of this preliminary study was to determine initial SBR conditions for granule cultivation from both Conventional Activated Sludge (CAS) and Membrane Bioreactor Sludge (MBS) for further investigation of the effects of seed sludge type on granule cultivation and nitrogen and COD removal performances.

Reactor Seeding and Operation
One of the sludge type used in experimental studies was conventional activated sludge (CAS). It was taken from return activated sludge line of secondary clarifier of the Greater Municipality of Ankara Domestic Wastewater Treatment Plant. Membrane bioreactor sludge (MBS), which was also used as seed sludge in experiments, was obtained from membrane unit of METU-Vacuum Rotation Biomembrane Plant. Properties of seed sludge in each sequencing batch reactor experiments are given in Table A.1.

Before being used as seed in the reactors, sludge were concentrated or diluted (if necessary) to achieve 2000 mg/L initial MLVSS concentration.

Table A.1 The properties of seed sludge

Preliminary Set-up		
	CAS	**MBS**
MLSS (mg/L)	3786 ± 46	2150 ± 79
MLVSS (mg/L)	3000 ± 34	1553 ± 38
SVI_{30} (mL/g)	195	135

During the preliminary study, investigated operational conditions were as follows;

- Requirement of acclimation,
- MLVSS concentration of seed for start-up,
- Settling period range,
- Exchange ratio
- Anoxic period duration

In order to investigate the necessity of acclimation, two SBRs were seeded with either CAS or MBS (MLVSS concentration of 2040 mg/L) and operated without acclimation period with a settling time of 25 minute. However, considerable amount of sludge reactor content were washed out. MLVSS decrease during hydraulic selection was an expected situation (Erguder and Demirer, 2008), but MLVSS concentrations inside the reactors were as low as ~800-1000 mg/L which was lower than the optimum values determined (2800-3000 mg/L) for SBR operation (Tchobanoglous et al., 2003). It was therefore concluded that seed sludge either CAS or MBS should be acclimated to the SBR operation and synthetic wastewater composition before start-up.

A new reactor set (2 SBRs) was conducted. One of the SBRs was seeded with CAS, while the other with MBS. Reactors were operated 5 days for acclimation. After the acclimation period, reactors were operated for 33 days. Synthetic wastewater composition fed during the 33 days of operation is given in Table A.2. Influent sCOD and Total Ammonia Nitrogen (TAN) concentrations were 630 mg/L sCOD (2.14 g COD/L.d) and 200 mg/L NH_4-N (0.68 g TAN/L.d), respectively. Operational conditions during the 33 days of operation are given in Table A.2.

Table A.2 Cycle details during operational period of 33 days

Operational Conditions	**Preliminary Study**		
	Day 1	**Days 2-8**	**Days 9-33**
Number of cycles per day	4	4	4
HRT (h)	7.5	7.5	7.5
Organic LR* (g COD/L.d)	2.14	2.14	2.14
Total N LR (g N/L.d)	0.76	0.76	0.76
Cycle duration (h)	6	6	6
Volumetric Exchange Ratio (%)	85	85	85
Periods and durations in one (1) cycle			
Feeding (min)	8	8	8
Anoxic (min)	160	160	90
Aerobic (min)	168	170	250
Settling (min)	20	18	8
Withdrawal (min)	4	4	4
*LR: Loading rate			

Results and Discussion

Floccular sludge was dominant in the system during the first 9 days. Qin et al. (2004a,b) stated that decreasing settling time promotes granulation and granule dominated sludge can be achieved when settling period is less than 15 min. After Day 9, settling period decreased to 8 min to increase selection pressure and to promote granulation of floccular sludge. Figure A. 1 represents the sludge content of the reactors at the end of 8 days operation. Following the decrease in settling period, first granules appeared in both reactors on Day 10, which was consistent with the results in Qin et al.'s (2004a,b) studies. However, MLVSS concentrations inside the reactors were 1000 mg/L and 729 mg/L for MBS and CAS sludge, respectively. These MLVSS concentrations were fewer than that of determined MLVSS concentrations (2800-3000 mg/L) for SBR systems (Tchobanoglous et al. 2003). Liu and Tay (2004) stated that exchange ratio is one of the parameters for hydraulic selection which selects microorganisms according to their settling velocities. However, the studied exchange ratio (85%) might have caused excess washout and rapid MLVSS decrease inside the reactors when combined with the decrease in settling time. In order to better control the MLVSS concentration and hydraulic selection inside the reactors during granulation, lower exchange ratio might be useful. Appearance of granules also indicated that, settling period of 15-18 min at the beginning of the operation was long enough to prevent washout and short enough to trigger granulation, but it should be gradually shortened to less than 8 min to enhance granulation in the system. Meanwhile, MLVSS concentrations should be controlled to prevent drastic changes such as increasing or decreasing F/M ratio (overloading or starvation), SRT.

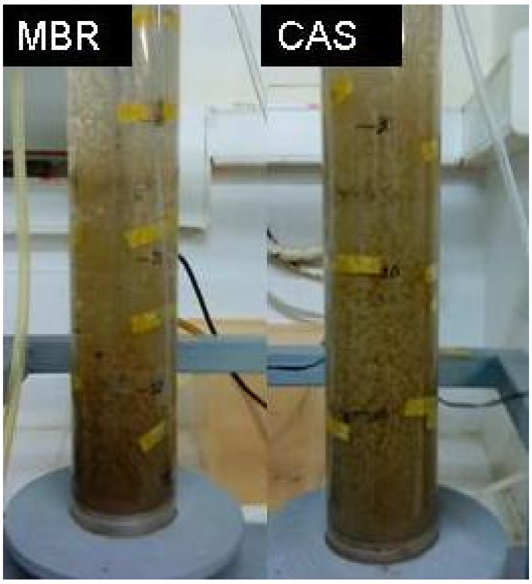

Figure A. 1 Reactor view during settling at the end of Day 9

Microscopic analyses were conducted to investigate sludge properties for the evolution of seed sludge from suspended to granular form during operation for both reactors (Figure A. 2, Figure A.3). In both reactors, granular sludge was observed and the sizes of granules developed from both MBS and CAS seeds were given in Table A.3.

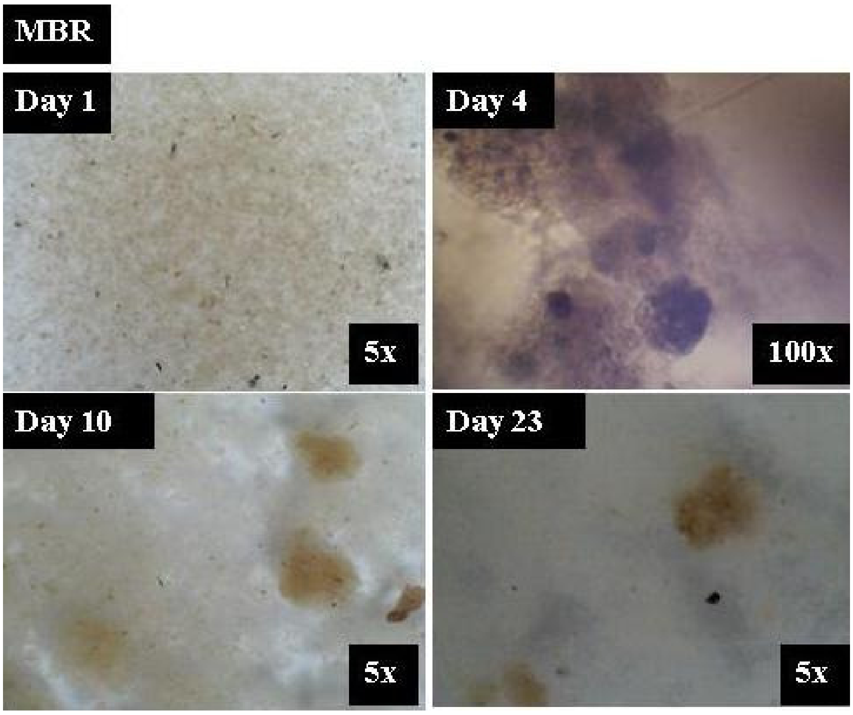

Figure A. 2 Evolution of MBS seed sludge

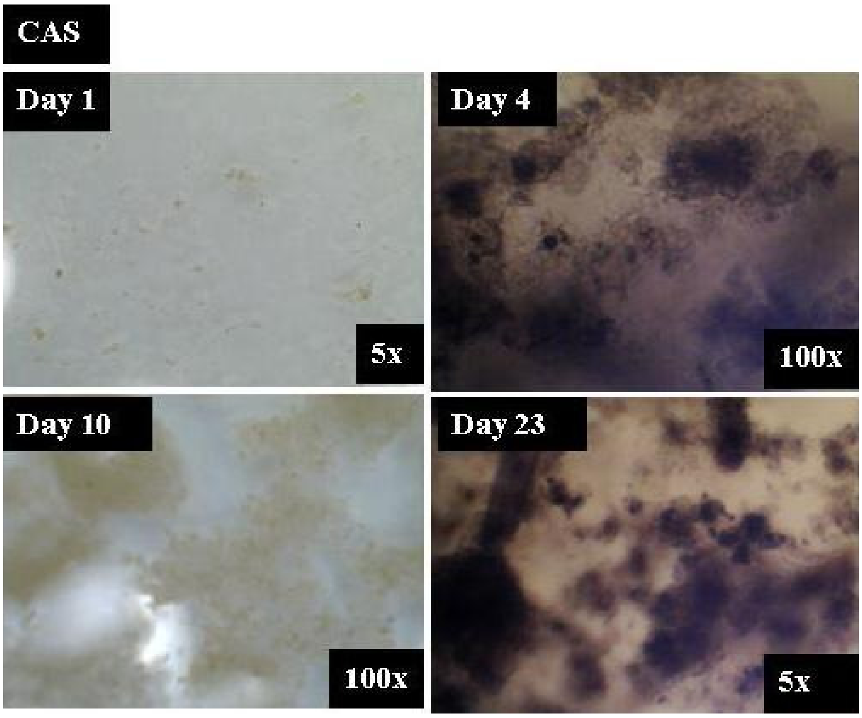

Figure A.3 Evolution of CAS seed sludge

Table A.3 Average granules' sizes

Operation days	Granule Size (mm)	
	MBS	CAS
Day 10	2.56±0.80	1.45±0.65
Day 23	2.08±0.63	
Day 33	2.05±0.73	1.82±0.60

Few amount of granules were visually observed in both reactors. Hydrodynamic shear force, created by aeration, is stated to have positive effects on granulation (Tay et al., 2001b; Tay et al., 2002a). In order to increase duration of hydrodynamic shear force and to promote granulation, aerobic period duration should be increased. As seen in Table A.2, after start-up reactors were operated with 160 min of anoxic period and 168 min of aerobic period. Increase in the duration of aerobic period means decrease in the anoxic period duration for a constant cycle time. Shortening the anoxic period should not affect the denitrification performance. In other words, anoxic period duration should be long enough for denitrification of 40 mg/L of influent NO_3-N. Therefore, to determine effective anoxic period duration, initial and effluent NO_3-N concentrations were measured by sampling from the reactors at the beginning and at the end of anoxic periods, respectively. It was found that when anoxic period lasted for 160 min, total denitrification of NO_3-N was achieved. After Day 9, in order to increase hydrodynamic shear force aerobic period duration was lengthen to 240 min and anoxic period was shorten to 90 min. Following this modification, denitrification performance for influent 40 mg/L NO_3-N remained same and effluent NO_3-N concentrations were negligible. Since granules appeared in the system and total denitrification was achieved during anoxic period, initial 90 min of anoxic period was found appropriate.

Conclusion
At the end of 33 days of operation, sludge inside the reactors was still composed of granules and flocs, and full granular biomass could not be achieved. However, effect of operational parameters was observed in detail. To summarize, following should be considered in further studies.

- Acclimation period is required for better start-up and for acclimation of microorganisms to SBR operational conditions and wastewater composition.

- Initial MLVSS concentration to seed reactors should be increased up to 5000 mg/L to compensate MLVSS wash-out during granulation.

- To prevent excess sludge wash-out before the granulation (promoted by decrease in settling time), exchange ratio of the reactors should be decreased from 85% to 50%.

- Providing selection pressure with gradual decrease of settling time (from 15 to 2 min) is more advantageous option to better control of the effects of hydraulic selection on parameters (MLVSS, F/M, SRT).

- Initial settling time of 15-18 min is enough to prevent excess sludge washout for initial SVI_{30} values of 150-200 mL/g.

- Anoxic period of 90 min is sufficient enough for denitrification of 40 mg/L of influent NO_3-N concentration.

APPENDIX B

CALIBRATION AND ABSORBANCE CURVES

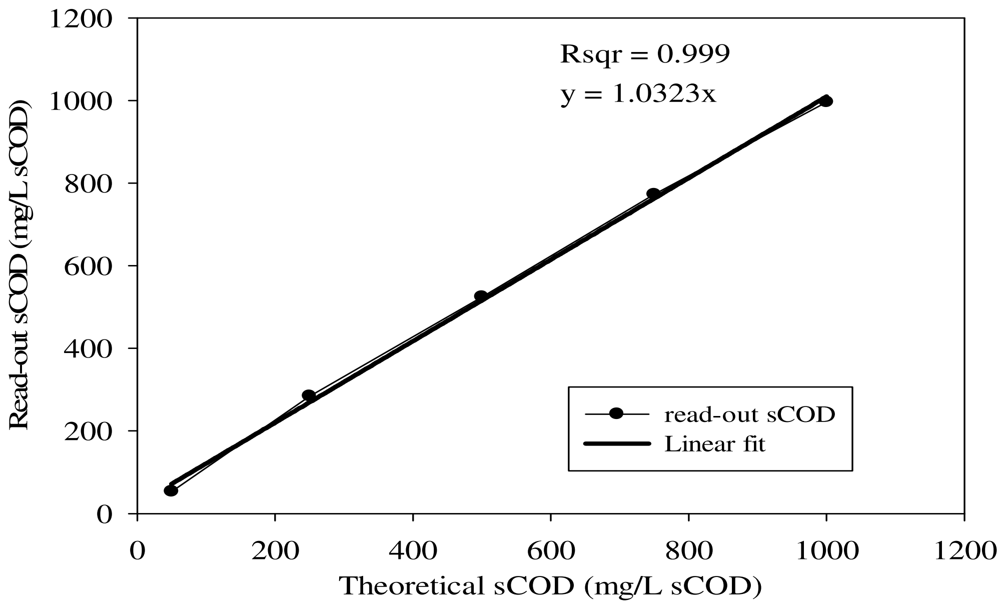

Figure B. 1 Calibration curve for sCOD range of 0-1500 mg/L

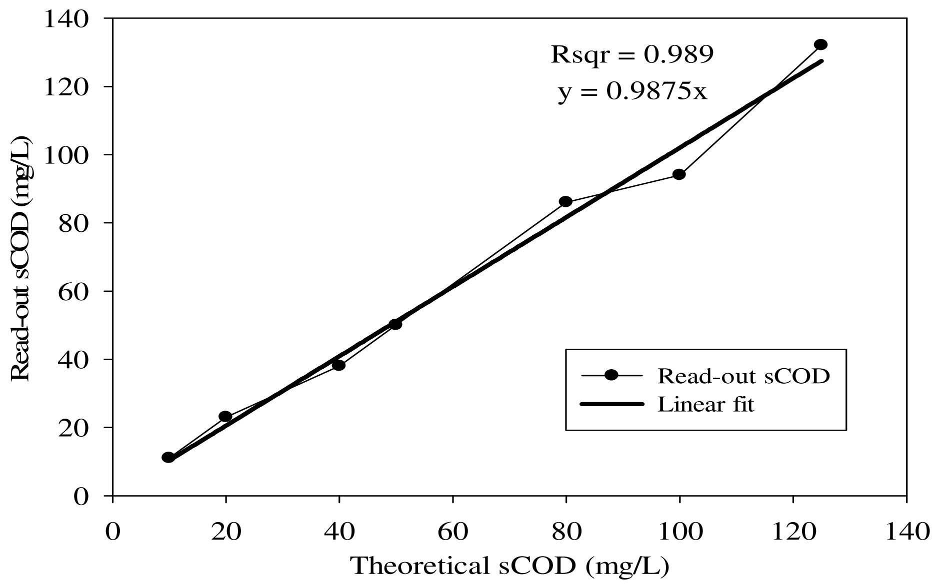

Figure B. 2 Calibration curve for sCOD range of 0-150 mg/L

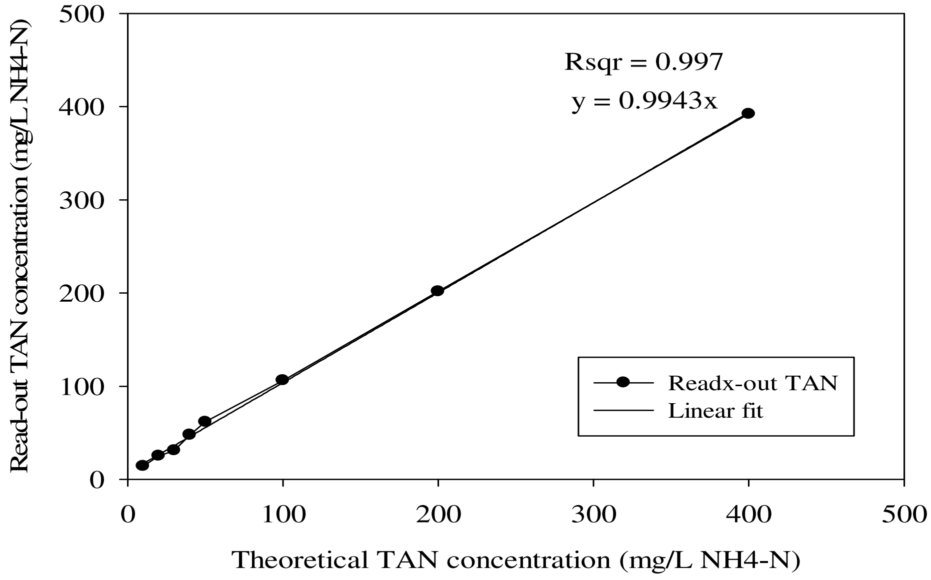

Figure B. 3 Calibration curve for TAN range of 10-400 mg/L

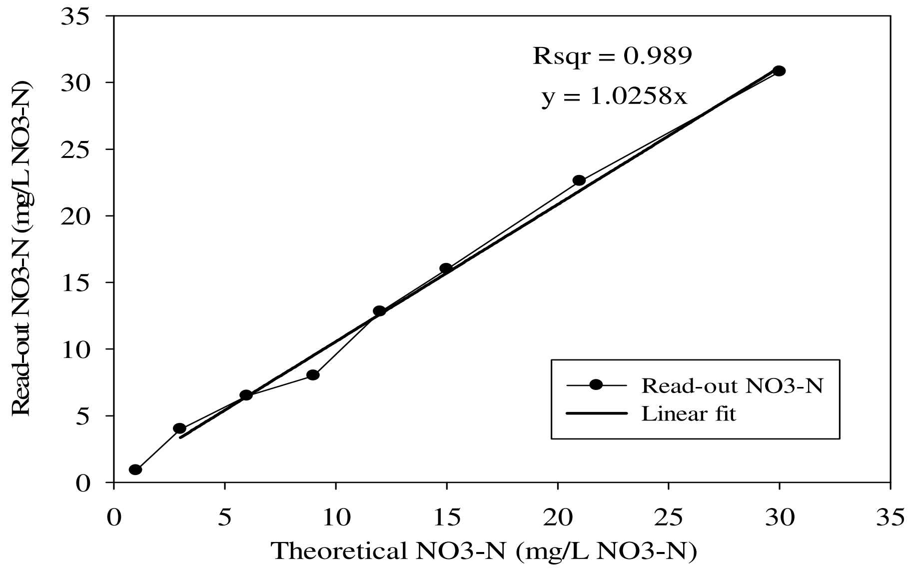

Figure B. 4 Calibration curve for NO_3-N range of 0-30 mg/L

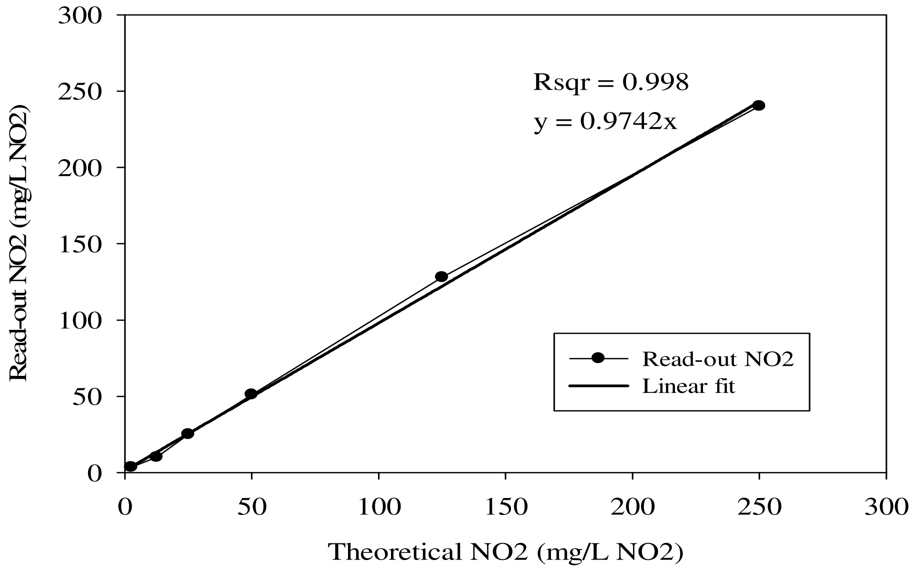

Figure B. 5 Calibration curve for NO_2 range of 0-250 mg/L

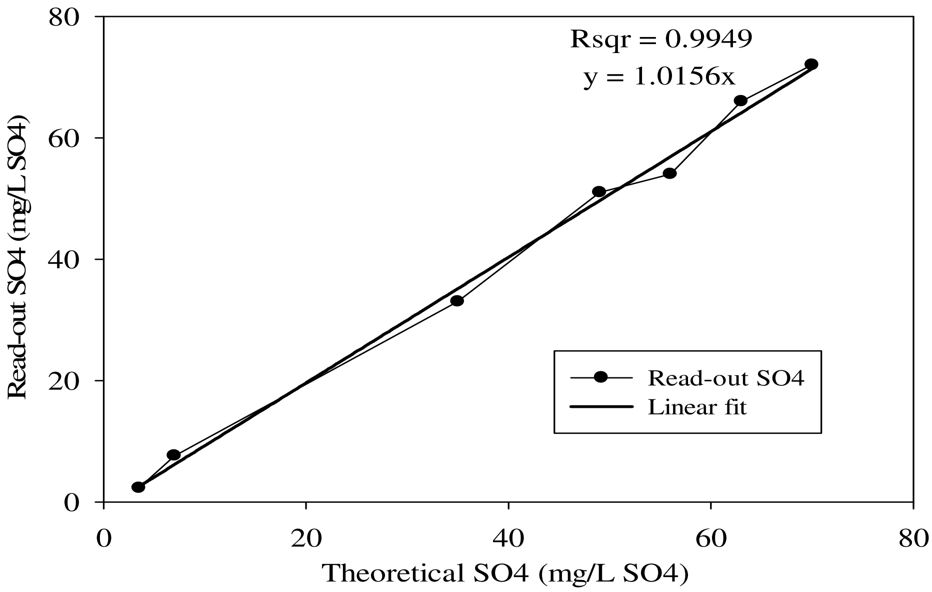

Figure B. 6 Calibration curve for SO_4^2 range of 2-70 mg/L $^-$

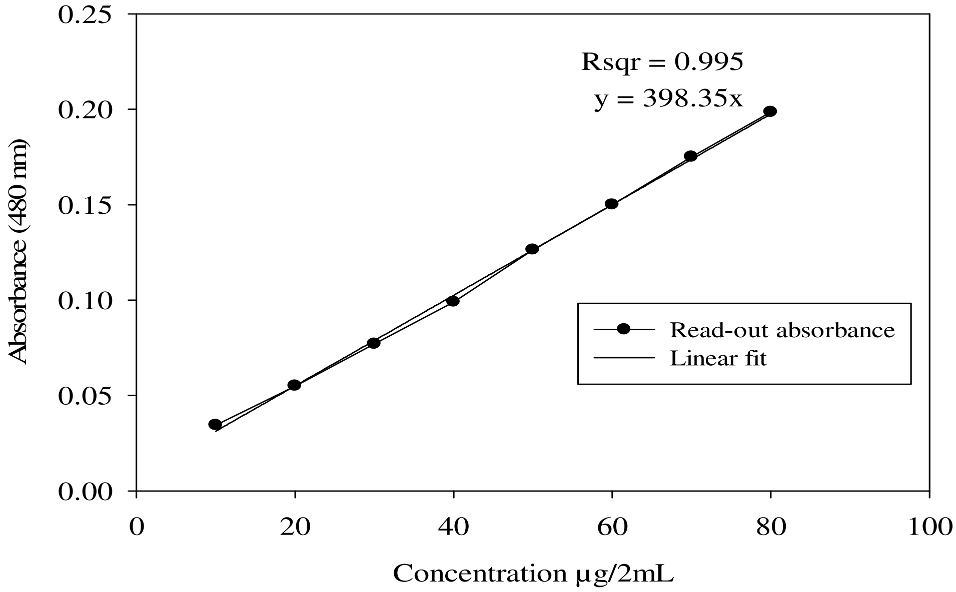

Figure B. 7 Absorbance curve for polysaccharide range of 0-80 µg/2mL

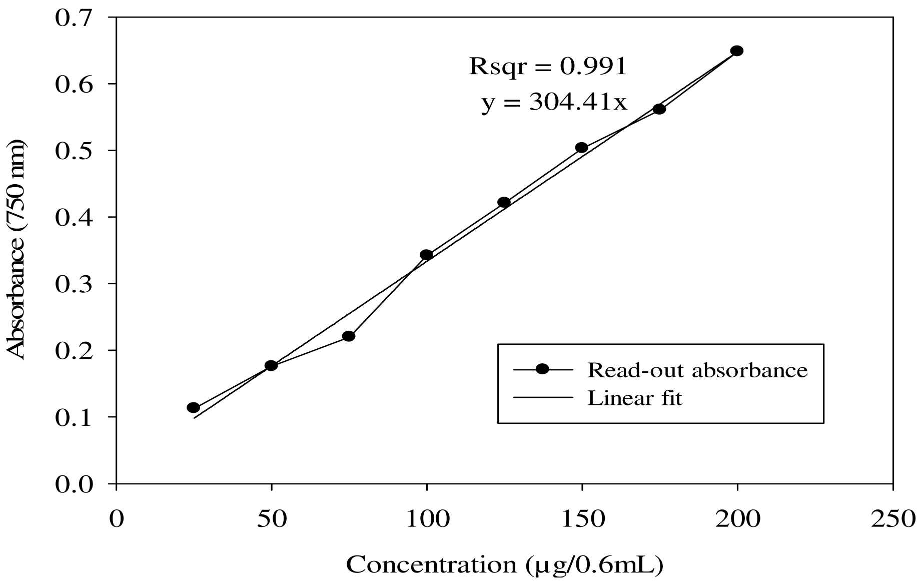

Figure B. 8 Absorbance curve for protein range of 0-200 µg/0.6mL

APPENDIX C

DETERMINATION OF THE EXTRACELLULAR POLYMERIC SUBSTANCE (EPS) EXTRACTION METHOD FOR AEROBIC GRANULES

One of the tools used in the detection of granulation process in this thesis was the determination of the variations in EPS content of the suspended sludge and aerobic granular sludge during the entire operation. Extraction methods can differ from each other according to their EPS extraction efficiency. Different EPS extraction techniques from suspended sludge and aerobic granules were reported (Adav et al., 2008; Durmaz, 2001; Wang et al. 2007c; Juang et al, 2010). Optimum EPS extraction method can be defined as the method that is extracting the EPS efficiently without causing cell lysis (Frolound et al. 1996). EPS extraction efficiency is the total amount of EPS extracted from the total organic matter (Nielsen and Jahn, 1999). The more the EPS is extracted without cell lysis, the better the EPS extraction efficiency will be. In the light of this information extraction methods were compared to select an effective EPS extraction method for granular sludge. Some of the EPS extraction methods are as follows;

- Cation Exchange Resin (CER) (Durmaz, 2001; Liu and Fang, 2002; Wang et al., 2007c)
- Ultrasound + Formamide + NaOH + Centrifuge (Adav and Lee, 2008-139)
- Ultrasound + Centrifuge (Juang et al., 2010; Dignac et al., 1998)
- Formaldehyde + NaOH + Centrifuge (Liu and Fang, 2002; Adav and Lee, 2008)
- Heating + Centrifuge (Zhang et al., 1999)
- EDTA + Centrifuge (Liu and Fang, 2002)
- Centrifuge + Ethanol (Ratto et al., 2006)

Among the aforementioned methods, three of them namely, CER extraction method, Ultrasound + Formamide + NaOH + Centrifuge extraction method (UFNC) and Ultrasound + Centrifuge extraction method (UC), were selected for comparison. The CER extraction method was chosen as an option since it was proposed as the best physical extraction technique for suspended sludge and sludge flocs (D'Abzac et al., 2010). The UFNC extraction method was chosen as an option since it was stated as the best EPS extraction method for granules (Adav and Lee, 2008; D'Abzac et al., 2010,). The UC extraction method was the third option, since it was claimed to be capable of extracting loosely-bound and tightly-bound EPS content from granules physically (Juang et al., 2009). Therefore, 1 chemical and 2 physical extraction methods were compared.

One of the aims in this thesis work was to investigate cultivation of aerobic granules from suspended sludge. Therefore, extraction method should be applicable for both granular sludge and suspended sludge flocs. In order to achieve information about the applicability of methods, each option was applied to both granular sludge and suspended sludge. Anaerobic granular sludge was taken from UASB-Wastewater Treatment Plant of Ankara Anadolu Efes Brewery Factory and membrane bioreactor sludge (MBS) was taken from METU-Vacuum Rotation Biomembrane Plant.

In each method, samples used for comparison were as follows; control, suspended MBS, anaerobic granular sludge, standard solution. Standard solution composition and MLVSS concentrations of sludge samples used in each experiment are given in Table C.1.

Table C.1 Standard Solution and MLVSS Concentration of Sludge Samples

Method	Standard Solution (mg/L)		MLVSS Concentration (mg/L)	
	PN[1]	PS[2]	Granular Sludge	MBS
CER	41.66	20	3500±212	2500±85
UFNC	83.33	40	4250±71	5320±42
UC	83.33	40	4250±71	3500±226
*: Protein **: Polysaccharide				

In CER extraction method, CER (Sigma Aldrich Dowex® Marathon® C Na^+ form strongly acidic) was used as a cation exchange resin. In order to prevent any interference from CER, 150 g of CER was initially stirred in 300 mL phosphate buffer saline (PBS) solution at 120 rpm for at least 1 h and further dried in a room temperature (20-24^oC)(Durmaz, 2001). PBS solution was composed of 4 g/L NaCl, 0.1 g/L KCl, 0.06 g/L KH_2PO_4, 0.455 g/L Na_2HPO_4. Experiment was performed with 250 mL suspended sludge sample and 250 mL anaerobic granular sludge sample.

Extraction method was applied to six different samples namely;

- CER control,
- MBS control,
- MBS + CER,
- Standard Solution,
- anaerobic granular sludge control,
- anaerobic granular sludge + CER,

Sludge samples of 250 mL were washed 3 times with PBS solution by centrifuging at 3500 rpm and resuspended with PBS solution. Solids analyses (SS-VSS) were done with 50 mL portion of 250 mL sample. MLVSS concentrations of anaerobic granular sludge and MBS were measured as 35 g/L and 7.5g/L, respectively. For EPS extraction from samples in this method, CER dose of 100 g CER/g VSS was used (Durmaz, 2001). Therefore, in order to decrease necessary amount of CER, anaerobic granular sludge and MBS samples were diluted 10 and 3 times, respectively to the values given in Table C.1 before extraction. Samples of 200 mL were taken from new diluted samples (Table C.1) of both sludge types. Each sample sludge was separated from PBS solution by centrifuging at 3500 rpm, then corresponding 70 g and 50 g CER (100 g CER g^{-1} VSS) were added to the anaerobic granular sludge precipitate and MBS precipitate, respectively. PBS solution was further added to the jars to achieve final volume of 200 mL. These samples were named as *MBS + CER* and *anaerobic granular sludge + CER*. As sludge control, diluted sludge samples (200 mL) free of CER were also prepared. These control samples were named as *anaerobic granule control* and *MBS control*. As *CER control*, 200 mL sample free of sludge and containing 70 g CER was prepared. In order to follow recovery performance of method, 200 mL of standard polymer solution containing 70 g CER (Table C.1) was also prepared. This jar was named as *standard solution*. Polymers were extracted by using a standard jar test apparatus operated at a stirring speed of 120 rpm for 5 h. At the end of extraction, CER was separated from the samples by filtration, and filtrate was taken for EPS (protein and polysaccharide) analysis.

In the UFNC extraction method, 50 mL of samples were used. Extraction method was applied to 3 different samples namely;

- MBS,
- anaerobic granular sludge,
- standard solution

Sludge samples of 50 mL were washed 3 times with PBS solution (without changing the sample volume) by centrifuging at 3500 rpm and resuspended with PBS solution. Possible soluble interferences were avoided by this washing. Solids analyses (SS-VSS) were done with 40 mL portion of 50 mL washed samples. MLVSS concentrations of anaerobic granules and MBS were 42.5 g/L and 10.5 g/L, respectively. Anaerobic granular sludge and MBS were diluted 10 times and 2 times, respectively, to the values given in Table C.1 before extraction. Sludge samples of 10 mL were taken from the diluted stock and ultrasound at 120W was applied to these 10 mL samples for 5 minutes in an ice bath. Then, 0.06 mL formamide was added to each sample and waited for 1 hour at 4^{o}C. After that, 4 ml of 1N NaOH was added to the sample tubes and waited again for 3 hours at 4^{o}C. Then samples were centrifuged at 4^{o}C and 10000 rpm for 20 minutes. Supernatant was taken and filtered through 0.45µm filter for EPS content analysis (Adav et al., 2008-6).

In the UC extraction method, again 50 mL of samples were used. This method was repeated for 3 samples namely;

- MBS,
- anaerobic granular sludge,
- standard solution

Samples were washed 3 times with PBS solution (without changing the sample volume) by using 3500 rpm for 10 min and resuspended with PBS solution. Solid analyses (SS-VSS) were done with 40 mL portion of 50 mL washed samples. MLVSS concentrations of anaerobic granules and MBS were 42.5 g/L and 10.5 g/L, respectively. Anaerobic granular sludge and MBS were diluted 10 times and 3 times respectively, to the values given in Table C.1 before extraction. Samples of 10 mL were taken and exposed to ultrasound at 120 W for 10 minutes. After that, they were centrifuged at 4^{o}C and 10000 rpm for 20 minutes. Juang et al. (2010-9) stated that tightly bounded polysaccharides and protein content were extracted into the supernatant following these processes. Supernatant was, therefore, filtered through 0.45µm filter, for EPS content analysis.

After each extraction method, protein and polysaccharide amounts of extracts were measured by using methods described by Lowry et al. (1951) and Dubois et al. (1956), respectively. For protein measurements, in order to measure concentrations in the range of the calibration curves (25-200 µg/0.6ml) (Appendix B), extracts were diluted 5 times with PBS solution. For polysaccharide measurements, based on the methods, dilutions were either 10 times (CER method and Ultrasound+Centrifuge method) or 20 times (Ultrasound + formamide + NaOH + Centrifuge) to be in the range of polysaccharide calibration curves given as 10-80 µg/2mL (Appendix B).

Results

Standard polymer solutions were exposed to the procedure of each extraction method. Recovery performances were measured and given for eah method in Table C.2. Extracted EPS components of MBS and anaerobic granular sludge were measured after each extraction method and given in Table C.3.

According to recovery performances, all three methods were in the range given as appropriate (70-125% recovery performance) to be used for EPS extraction (APHA, AWWA, WEF, 2005). Despite being in the range; the recovery performances varied significantly for PS. UC method was not as efficient as the others in recovery of PS.

Table C.2 Standard Solution

Methods	**Concentrations of the standard solutions (µg/2mL)**		**Concentrations of the extracted PN and PS (µg/2mL)**		**Recovery (%)**	
	PN*	**PS****	**PN***	**PS****	**PN***	**PS****
CER	41	22.5	36	18.9	88	84
UFNC	86.4	40.2	76.9	37	89	92
UC	78.4	44.8	74	31.3	94	70
*: Protein **: Polysaccharide						

Table C.3 EPS components of sludge extracted by three different methods

Methods	MBS EPS (mg/g VSS)		Granular Sludge EPS (mg/g VSS)	
	PN*	**PS****	**PN***	**PS****
CER method	79.55 ± 11.5	38.5 ± 4	150 ± 22	88 ± 5
UFNC	84.5 ± 9.1	53 ± 9.8	265 ± 14	113 ± 18
UC	6.3 ± 7.4	15.8 ± 1.3	14 ± 2.3	26 ± 6

*: Protein
**: Polysaccharide

The extracted EPS concentrations from the suspended sludge and granular sludge significantly varied with the applied extraction methods. Table C.3 summarizes the quantities of EPS extracted from suspended sludge and anaerobic granular sludge. Extracts of UC method were poor in terms of protein and polysaccharide for both MBS and granular sludge compared to the other methods. It was seen that UC method can extract around 7-15% of the protein content and 30-50% of the polysaccharide content from MBS compared to other methods. In addition, the UC method's performance was also poor for granular sludge. Around 5-10% of the protein content and 20-30% of the polysaccharide content could be extracted compared to other methods. This revealed that UC extraction method was not an efficient EPS extraction method for both suspended sludge and granular sludge.

Application of methods to suspended MBS revealed that quantities of extracted protein amount by CER method and UFNC method were almost same. Quantities of polysaccharide concentrations were also close to each other with slight difference. It was concluded that in respect of EPS extraction for MBS, both CER method and UFNC method can be applicable.

Application of methods to anaerobic granular sludge revealed that CER method was not as efficient as UFNC method for extracting EPS, especially protein, from granular sludge. It was seen that extraction of polysaccharide from granular sludge by using CER method was comparable to that using UFNC method. More specifically, 88-113 mg/g VSS were extracted from anaerobic granular sample by using the mentioned two methods. However, extracted protein contents were totally different. Quantity of proteins extracted by UFNC method was 1.7 times higher than that by CER method. In literature it is stated that high amount of protein exists in the composition of EPS, if intracellular polymers are excreted or cell lysis occurs, thus cell lysis can be detected by protein content of EPS (Brown and Lester, 1980; Durmaz and Sanin, 2001). However, it was stated that large amount of proteins and little nucleic acids usually exist in EPS matrix, thus proteins itself can not be an accurate indicator of cell lysis but nucleic acids can be if they exist in large amount (Frolound et al. 1996). In a very detailed study about comparison of varying EPS extraction methods, Adav and Lee (2008) conducted DNA extraction measurement and also 2-keto-3-deoxyoctonate (KDO) which is part of the cell membrane of bacteria in order to determine cell lysis. It was concluded that, DNA and KDO concentrations were in negligible amount and there were no interference with intracellular material when UFNC method was applied for EPS extraction. Therefore, it can be concluded in this comparative study that UFNC method seemed to have more efficient protein extraction performance for granular sludge than CER method. It was also revealed in literature studies (D'Abzac et al. 2010; Adav and Lee, 2008) that, UFNC method is more applicable EPS extraction method for granular sludge than CER extraction method.

In this thesis work, it was aimed to observe EPS variations during granule cultivation from suspended sludge and during steady state granular operation. Therefore, UFNC method was used in the entire work.

APPENDIX D

SBR OPERATION WITH AEROBIC GRANULES IN ANOXIC-AEROBIC PERIOD SEQUENCE UNDER STEADY CONDITIONS FOR 67 DAYS

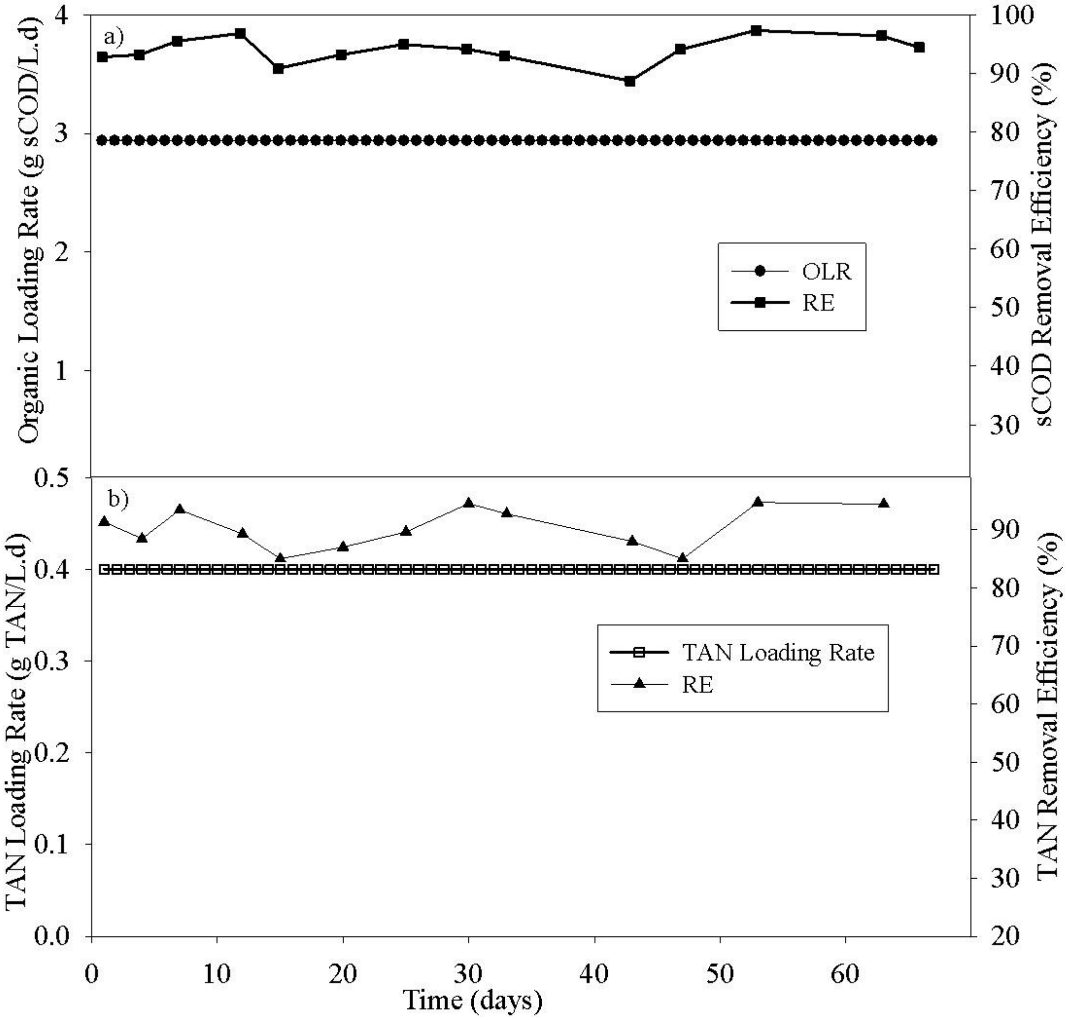

Figure D.1 Removal Performances for 67 days of operation under steady conditions
a) sCOD Loading Rate (LR) and Removal Efficiency b) TAN Loading Rate (LR) and Removal Efficiency