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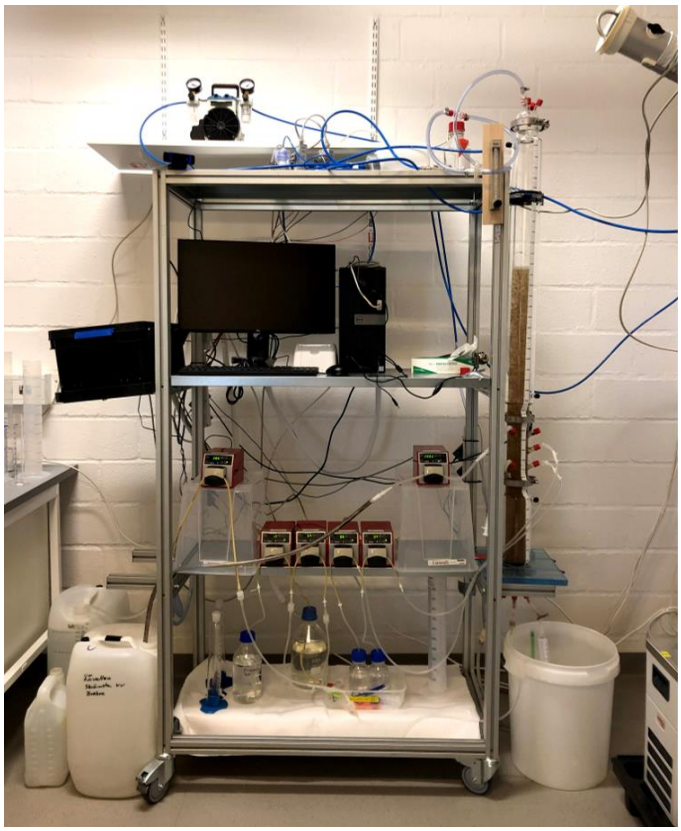

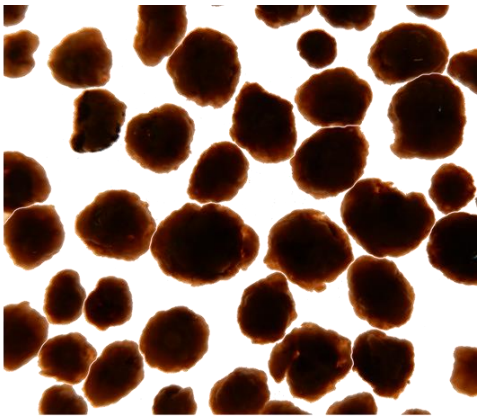

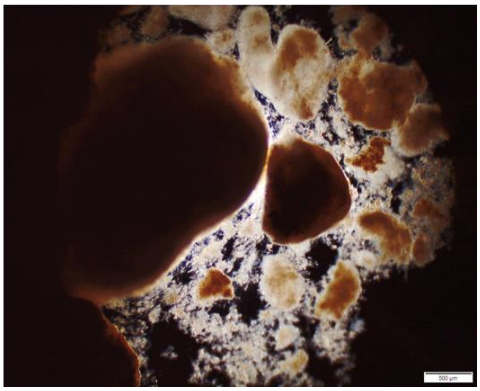

Start-up of aerobic granular sludge (AGS) reactors inoculated with granular sludge from a full-scale plant

Comparison of organics and nutrients removal from synthetic wastewater and granule development in lab-scale AGS sequencing batch reactors

Master's thesis in Nordic Master in Environmental Engineering

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DIVISION OF WATER ENVIRONMENT TECHNOLOGY

CHALMERS UNIVERSITY OF TECHNOLOGY
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MASTER'S THESIS ACEX30

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Examensarbete ACEX30
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Cover: AGS lab-scale reactor set up with microscopic image of 2x magnification and digital image from granules observation in R2.

Department of Architecture and Civil Engineering
Göteborg, Sweden, 2021

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ABSTRACT

Aerobic granular sludge (AGS) has shown to be a promising sustainable technology for wastewater treatment due to compact treatment, energy-efficient and its ability to treat different contaminants such as organic matter, nutrients, and xenobiotic compounds, including pharmaceuticals. The present study aimed to evaluate the AGS development and process performances during the start-up period by using AGS as the inoculum. Three lab-scale sequencing batch reactors (R0, R1, R2) were used and fed by complex synthetic wastewater: two reactors (R0 and R1) were dosed with pharmaceutically active compounds (PhACs). The results showed that the start-up period which lasted for 44 days was limited by nitrogen removal and low biomass concentration in all reactors. Despite the disturbance of operational conditions during the operation, granulation was observed and good settling properties with SVI_{15}/SVI_5 ratio > 0.9, SVI_5 of 29−79 mL/g, and average diameter of 2.2−3.67 mm was maintained. Dense and compact granules were predominant in R0 and R1 while R2 had fluffy and loose structured granules. An average of 86−89% TOC removal, 57−100% TP removal, and 51−62% TN removal was observed. It can be concluded that AGS seeding sludge indeed contributed to a rapid AGS start-up with no acclimation period of TOC and TP removal and increasing granule sizes which are beneficial for conversion processes. Further research such as a cycle study and optimization of operational conditions are recommended for better understanding of the conversion processes and optimal AGS performance.

Key words: aerobic granular sludge, seeding, reactor start-up, nutrients removal, pharmaceutical wastewater, sequencing batch reactors

Contents

Preface

This master thesis was a pre-study of two projects about AGS at division of Water Environment Technology, Chalmers University of Technology, Sweden, performed by two PhD candidates, Cecilia Burzio and Jennifer Ekholm, focusing on pharmaceuticals removal and operation at low temperature. The project was started in January 2021 but the lab work presented in this thesis were limited to the period from beginning of March until the end of April 2021 while the project is still on-going.

Here, I would like to thank my supervisors, Britt-Marie Wilén, for the opportunities and her guidance in this project, and also Stein Wold Østerhus, for his insights and supports. My special thanks also to Cecilia and Jennifer for supervising me during the lab works, sharing their experiences, discussions and their full supports during this project. I would also send my best gratitude to Amir who have always been a huge help for me to work in the lab, people at WET, and friends in the WET lab. Thank you for your warm welcome and kindness so that I had such good time in Sweden. Last, thank you for my family in Indonesia, friends and colleagues who always send their prayer and support me during my studies.

Göteborg, Sweden, 2021
Fenny Clara Ardiati

Abbreviations

AGS – aerobic granular sludge
Anammox – anaerobic ammonium oxidation
AND – alternating nitrification denitrification
AOB – ammonium oxidizing bacteria
AS – activated sludge
Bio-P – biological phosphorus removal
COD – chemical oxygen demand
Comammox – complete ammonia oxidizer
CO_2 – carbon dioxide
DO – dissolved oxygen
DOHO – denitrifying ordinary heterotrophic organisms
DGAO – denitrifying glycogen accumulating organisms
DPAO – denitrifying polyphosphate accumulating organisms
EPBR – enhanced biological phosphorus removal
EPS – extracellular polymeric substances
GAO – glycogen accumulating organisms
HCl – hydrochloric acid
H_2O – water
IC – ion chromatography
MFC – mass flow controller
MLSS – mixed liquor suspended solids
MLVSS – mixed liquor volatile suspended solids
NaOH – sodium hydroxide
N – nitrogen
N_2 – nitrogen gas
NH_4^+ – ammonium ion
NH_4-N – ammonium nitrogen
NOB – nitrite oxidizing bacteria
NO_2^- – nitrite ion
NO_2-N – nitrite nitrogen
NO_3^- – nitrate ion
NO_3-N – nitrate nitrogen
OHO – ordinary heterotrophic organisms
OLR – organic loading rate
O_2 – oxygen gas
PAO – polyphosphate accumulating organisms
PHA – polyhydroxyalkanoates
PhACs – pharmaceutically active compounds
PN – protein
PO_4^{3-} – orthophosphate
PO_4-P – phosphorus from phosphate
PS – polysaccharide
SBR – sequencing batch reactors
SND – simultaneous nitrification and denitrification
SRT – solids retention time
SVI – sludge volume index
TN – total nitrogen
TOC – total organic carbon

TP – total phosphorus
TSS – total suspended solids
VFA – volatile fatty acid
VSS – volatile suspended solids
WWTP − wastewater treatment plant

1 Introduction

Aerobic granular sludge (AGS) is a promising sustainable alternative technology for wastewater treatment. However, technical challenges are still posed such as long start-up period needed for stable operation to occur. In this study, AGS was used as the seeding sludge for start-up operation as the strategies to improve the start-up period. This master thesis is a pre-study of PhD projects about AGS at division of Water Environment Technology, Chalmers University of Technology, Sweden by Cecilia Burzio and Jennifer Ekholm, focusing on removal of pharmaceuticals and operation at low temperature.

1.1 Background

Wastewater generation is an unavoidable issue as long as it is produced by mostly anthropogenic activities such as industry, agriculture and households. In fact, it is predicted that 80% of the global wastewater is potentially released to water environment without a proper treatment in 2030 (WWAP, 2017). It seems that there could be lack of public interest concerning the wastewater treatment, yet the discharge of untreated wastewater has been known to negatively impact human's health, environment and economic activities (Hernández-Sancho et al., 2015). Therefore, the development and application of wastewater treatment have been urged to keep and improve the better quality of water sources. Today, the urgency is also supported in a global level through the 2030 Agenda for Sustainable Development with target 6.3 which aims at higher percentage of safely treated wastewater and keep good ambient quality of water bodies (UNGA, 2016).

As in other countries, Sweden also faces many challenges with wastewater treatment. The rapid urbanization in Sweden has been observed (Statistic Sweden, 2019) and it is reported that more centralized connections will be built, replacing the smaller treatment plants (Swedish EPA, 2020). Thus, these situations are not only potentially putting higher pressures on the wastewater load to wastewater treatment plants (WWTP) but also decreasing the space available for extensions. In terms of pollutants, Sweden has been focusing on treating organic matter, nitrogen and phosphorus since the eutrophication in water bodies occurred in the 1960s (Swedish EPA, 2020). Therefore, the stricter demand on their removal has been regulated in the Urban Wastewater Treatment Directive (1991) and a minimum of secondary treatment such as biological treatment method should be applied in the WWTP. Last, the challenges posed by climate change such as: 1) untreated overflows due to extreme weather; 2) greenhouse gas emissions; and 3) wastewater processes affected by extreme temperature are still occurring (Zouboulis and Tolkou, 2015; Abdulla and Farahat, 2020).

Besides the organic matter and nutrients, various unwanted substances can be also released into wastewater, such as micro-pollutants. In Sweden, during 2018, the Swedish government started the initiation to support the development of wastewater treatment for pharmaceutical residue removal (Swedish EPA, 2020). Pharmaceuticals may discharge into the water bodies through several pathways from the industrial wastewater, medication of human or animal, domestic sludge, or even landfill leachate (O'Flynn et al., 2021). According to the screening study by Fick and Lindberg (2014) on behalf of Swedish EPA, 101 pharmaceuticals were found in the surface water, WWTPs (influent, effluent and sludge), drinking water and biota from several locations

in Sweden. The assessment of pharmaceuticals in the WWTPs was also conducted by Wallberg et al., (2016) which showed at least three pharmaceuticals (ethinyl estradiol, estradiol, and diclofenac) exceeded the allowed maximum concentrations and three pharmaceuticals (levenorgestrel, ibuprofen, metoprolol) were higher than the effect level values in the surveyed WWTPs in Sweden. Today, as the world is facing a Covid-19 pandemic, the load of pharmaceuticals in the environment would be also expected to increase (Bandala et al., 2021; O'Flynn et al., 2021). As a result, this condition was predicted to be a high risk, jeopardizing the aquatic biota as well as humans through the drinking water sources. On the other hand, the current practices in WWTPs are commonly not designed for pharmaceuticals residue removals (Sundin et al., 2017). Relevant study by Hörsing et al., (2014) reported only 25% of pharmaceuticals were removed in WWTPs. Therefore, the urgency to develop and apply for the advanced treatment is needed, not only for pharmaceuticals removal but also to reduce the antibiotic-resistant genes and dispersion of other micro-pollutants in water bodies (Sundin et al., 2017).

As part of coping with the wastewater challenges, the attention to develop an efficient and environmentally friendly wastewater treatment has been increasing in the recent years. In terms of organics and nutrients removal, biological treatment has been widely used in practice and known to be an economically attractive and eco-friendly (Crini and Lichtfouse, 2019). Among various biological treatment processes, aerobic granular sludge (AGS) has shown to be one of the promising technologies since the late 1990s (Mishima and Nakamura, 1991; Bengtsson et al., 2018). Many researches have been conducted and reported various benefits with AGS application compared to the conventional activated sludge treatment. The compact and large granules of the AGS lead to a rapid settling velocity and thus enable a short settling time and high sludge concentration for its operation. This condition makes a compact treatment to save space for the WWTP as well as smaller carbon footprint (de Bruin et al., 2004). Moreover, as the common application of the AGS in the sequential batch reactor (SBR), it can reduce the pumping requirements as it is commonly needed within the activated sludge process. Thus, the AGS system is also believed to be energy-efficient (Pronk et al., 2015a) and has less investment and operational cost (Gonzalez-Martinez et al., 2017).

Studies have been performed to investigate the pollutant removal and the AGS shows great ability to treat different contaminants such as organic matter, nutrients, heavy metals, toxic organic compounds, including pharmaceuticals (Gao et al., 2011a; Zhao et al., 2015; Amorim et al., 2016; de Sousa Rollemberg et al., 2018). Based on its proven high performance, the full-scale of AGS has been developed in some countries, including Sweden. As a part of the full-scale study, the first AGS plant in the Nordic countries has been built and studied within the AGNES II project (Aerobic Granular sludge – Nutrient removal and recovery Efficiency in Sweden) since March 2018. The chosen location is the Österröd Wastewater Treatment Plant which is located in the municipality of Strömstad (Sweden Water Research, 2017). The technology belongs to Nereda® and the studies involved various stakeholders from the universities, research institutions and wastewater treatment plant. Among a total of 70 plants in the world, excellent performance is reported from the Nereda plant in Poland which achieved 5 mg/L of total nitrogen, 0.8 mg/L of total phosphorus and 4.4 of BOD5 and 39 mg/L COD in the effluent (Nereda, n.d).

Despite the great potential of the AGS system, challenges occur in the practical application and one of the major issue is the start-up period (Zhang et al., 2019). Strategies to accelerate the start-up of AGS systems are required to facilitate its application. Studies have been focused on the effects of different environmental and operational conditions on AGS granulation processes, including settling time, organic loading rate, shear forces and exchange ratio (Liu and Tay, 2015; Nancharaiah and Kiran Kumar Reddy, 2018; Szabó et al., 2016; Tay et al., 2001a; Wan et al., 2015). In addition, the type of seed sludge has been found to be important for starting-up AGS processes.

The most common inoculum for AGS is activated sludge (AS) with relatively long period of granulation, more than 25-35 days in a lab-scale AGS (de Kreuk and van Loosdrecht, 2006; Deng et al., 2016). Attempts of using other inoculums have been done such as crushed AGS (Pijuan et al., 2011), mixture of flocculent sludge and crushed granules (Verawaty et al., 2012), mixed AS and AGS (Long et al., 2014), and well-acclimated AGS from lab-scale SBR (Liu et al., 2005a; Wang et al., 2019). Most results showed that inoculation with granular biomass can decrease the start-up period for AGS and thus it is recommended to seed with granular biomass or mixed with flocculated sludge (Bengtsson et al., 2018). Few studies have investigated the AGS start-up by using granular sludge from full-scale AGS plants. Therefore, this project aims to study the AGS performance and granules development during the start-up period of AGS in a lab-scale sequencing batch reactor (SBR). The seeding sludge was obtained from the full-scale AGS reactors at the Österröd Wastewater Treatment Plant, Strömstad, Sweden. The project also evaluated the AGS system in treating the pharmaceuticals and complex synthetic wastewater. The results are expected to be beneficial for the information of AGS processes for further practical application and further studies.

1.2 Aims

The primary aim of the project is to increase the understanding of the AGS performance in a lab-scale sequencing batch reactor (SBR) during the start-up period. Three lab-scale SBRs of 3.2 litres were used in which all reactors were fed by complex synthetic wastewater and two reactors were additionally dosed by pharmaceutically active compounds (PhACs). The pollutant removal of organics and nutrients as well as the granules development were evaluated in the study. Based on these goals, research questions to answer are as follows:

1. How is the comparison of granule development in three AGS reactors when seeded with granules from a full-scale plant? Are the aerobic granules successfully maintained in the lab-scale SBR?

2. What are the observed conversion processes of the pollutants (organic and nutrients) that occur in the AGS system?

3. What is the removal efficiency of total organic carbon (TOC), total nitrogen (TN) and total phosphorus (TP) in the three AGS reactors? Does the performance meet the criteria from local regulations?

4. Is a successful start-up of the AGS system achieved in the study?

5. What are the suggestions or recommendations for future research?

1.3 Scope and limitations

The project was performed as the pre-study of two projects at Chalmers University of Technology, performed by PhD candidates Cecilia Burzio and Jennifer Ekholm. These two studies include the investigation of pharmaceutically active compounds (PhACs) removal and low temperature effects on AGS system performance. However, this project is only focused on the start-up period of the lab-scale AGS reactors. Due to some circumstances, there are several limitations of this study as follows:

- Due to a dynamic condition during experiments with limited amount of time, the study is limited to 44 days of operation while the project is still ongoing.
- Most of the operational parameters and conditions for the reactor were decided beforehand.
- The removal of PhACs is not included in the evaluation of the AGS performance.
- The synthetic wastewater was used as the influent instead of real wastewater.
- The microbial community analysis will not be carried out but samples were collected for further investigation.

2 Literature review

2.1 Wastewater treatment

Wastewater can be defined as a mixture of liquid or water which contains waste generated by activities (Tchobanoglous et al., 2003). The term wastewater is not limited to sewage which often implies the used water from domestic, industrial, and institutions sources but also the polluted urban and agricultural runoff and groundwater (UN Water, 2015). Wastewater can carry diverse constituents such as suspended solids, nutrients, organics, pathogens and toxic metals which can cause problems to the environment and human. For this reason, treatments are needed before it discharges to the water bodies to reduce the level of pollutants based on the use-specific standard requirements (Tchobanoglous et al., 2003; UN Water, 2015).

Based on the treatment level, wastewater treatment can be divided into: 1) preliminary; 2) primary; 3) secondary; and 4) tertiary (Crites and Tchobanoglous, 1998). The goals of preliminary treatment are to remove the large objects, grit and grease to avoid problems in the treatment processes and operations. In the primary treatment, the settleable particles in wastewater are removed by physical operations such as sedimentation. In the secondary treatment, biological and chemical processes are often used in a combination to remove the soluble and particulate organic matter, in the form of suspended and colloids, as well as nutrients such as nitrogen and phosphorus. Last, the tertiary treatment usually refers to filtration and disinfection process to remove the residual suspended solids after the secondary treatment (Tchobanoglous et al., 2003).

In Sweden, the discharge from wastewater treatment plants (WWTPs) is regulated by several acts such as the EU urban wastewater treatment directive (91/271/EEC) and Swedish EPA's regulations (NFS 2016:6). In this directive, it is stated that at least secondary treatment should be used in the WWTP and thus the biological treatment method is often applied. As regards, the requirements are often set for organic matter and nutrients. In the NFS 2016:6 regulations, it is stated the effluent limit for total nitrogen (TN) and chemical oxygen demand (COD) or total organic carbon (TOC) that can be achieved in two ways. Either as minimum reduction as an annual mean value or maximum concentration. These limits were considered to be met in this project and further discussed in Section 3.4.

2.2 Biological wastewater treatment

Biological wastewater treatment can be used to: 1) transform dissolved and particulate biodegradable substances into acceptable end products; 2) capture and incorporate suspended and non-settleable colloidal solids into a biofilm; 3) transform or remove nutrients (nitrogen and phosphorus); and 4) remove specific trace organic contaminants (Tchobanoglous et al., 2003). The main principle of biological treatment is to utilize microorganisms and provide favourable conditions for them to grow and perform these processes.

Based on the treatment processes, there are two main categories of biological wastewater treatment: suspended-growth and attached-growth processes. Activated sludge is an example of suspended-growth processes because the microorganisms are maintained in suspension within the wastewater while in the attached-growth process,

the microorganisms are attached to carriers made from various materials such as plastic, gravel, and rock and kept within the wastewater (Tchobanoglous et al., 2003). AGS is a relatively new type of process used in biological wastewater treatment. It has been extensively developed since the discovery in the 1990s (Mishima and Nakamura, 1991; Morgenroth et al., 1997).

2.3 Aerobic granular sludge

In the first International Water Association (IWA) workshop on AGS (2004), the researchers stated the definition of AGS as: "the aggregates of microbial origin which do not coagulate under reduced hydrodynamic shear and which settle significantly faster than activated sludge flocs" (De Kreuk et al., 2007a). In this chapter, characteristics and morphology of aerobic granules, granulation process, application of AGS and the conversion processes by AGS system are discussed.

2.3.1 Characteristics and morphology of aerobic granules

As the comparison of AGS and activated sludge is often discussed, the different characteristics between them can help to distinguish these types of aggregate, see Table 2.1. The most well-known properties of AGS are large aggregate size and compact structure. This leads to higher settling velocity which enables short settling times which give a very compact process (Bengtsson et al., 2018).

Table 2.1 Typical characteristics of activated sludge and aerobic granular sludge (Tchobanoglous et al., 2003; De Kreuk et al., 2007a; Coma et al., 2012; Nancharaiah and G. Kiran Kumar Reddy, 2018).

Parameter	Activated sludge	Aerobic granular sludge
Shape and average size	Irregular, less than (< 0.2 mm)	Well-defined spherical shape, more than (> 0.2 mm)
Specific gravity	0.997−1.01	1.010−1.017
Settling velocity	Low, less than (< 10 m/h)	High, more than (> 10 m/h)
Sludge volume index (SVI)	$SVI_{10} \neq SVI_{30}$	$SVI_{10} \approx SVI_{30}$
SVI_{30}	Around 100 mg/L	Around 30-60 mg/L
Layer's structure of granules	Minimum possibility for anaerobic zones	High possibility of aerobic, anaerobic, and anoxic zones
Coagulation under reduced hydrodynamic shear	Tend to coagulate when settle	Do not coagulate and settle as separate units
Extracellular polymeric substances (EPS)	Lower content	Higher content

In general, the granules are visible due to the larger sizes compare than flocs. Figure 2.1 shows the example of activated sludge and aerobic granules from the microscopy analysis. The colour of granules could be varied as it has been reported, aerobic and denitrifying granules are commonly yellow, methanogenic granules are black whereas

active annamox granules are carmine (Pol et al., 2004; Chen et al., 2014). Meanwhile, the shape can be varied depends on the operational conditions and hydrodynamic shear forces (Xiao et al., 2008). For instance, in a high organic loading rate (OLR) condition, the granules were more irregular in shape and less smooth on the surface while in the low OLR, the granules were observed to be smaller and tightly packed (Li et al., 2008). Zhou et al. (2016) also found that granules tend to be smaller, smoother and compact at higher shear force.

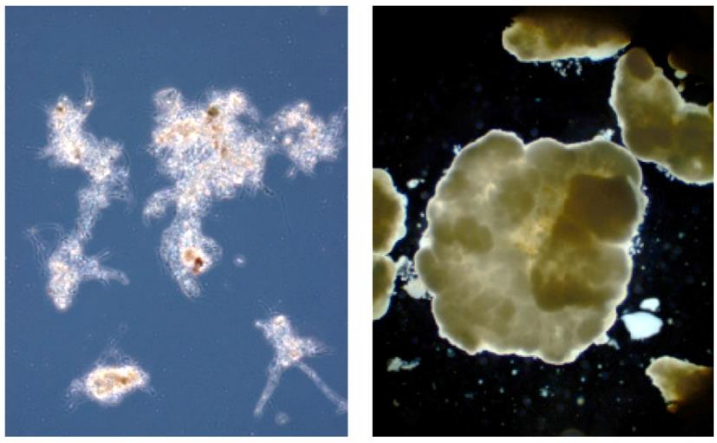

Figure 2.1 Microscopy image of activated sludge flocs (left) and aerobic granules (right) (Bengtsson et al., 2017).

Granular structure and diameter are the most interesting parameters of AGS since they are linked to mass transfer and the oxygen diffusion gradients within the granules (Liébana, 2019). In terms of structure, granule is often described as a multilayer sphere consists of aerobic, anoxic and anaerobic layer, see Figure 2.2. Aerobic shows the presence of free oxygen (O_2), anoxic means the presence of bound oxygen such as nitrates (NO_3) and nitrites (NO_2) while anaerobic occurs when the oxygen is absent (Frankel, 2020). Thus, these layers are based on decreasing oxygen and substrate gradients from the outer layer to the inner layer. This condition results in the differences of microbial community in each layer, allowing simultaneous nitrification-denitrification and biological phosphorus removal within granules (Coma et al., 2012; Nancharaiah et al., 2016).

2.3.2 Granulation processes

The granule formation is one of the key factors to achieve successful wastewater treatment using the AGS. Granulation can be described as a particle aggregation (Verawaty et al., 2012) or microcolony outgrowth (Barr et al., 2010). In general, there are four stages of granulation mechanism explained by (Liu and Tay, 2002): i) cell-to-cell contact; ii) initial attachment of microorganisms to form aggregates; iii) enhanced attachment by EPS production to form the mature aggregates; and iv) shaping up of granules by hydrodynamic shear force for development of AGS. The first and second stage are usually pre-condition for initiating the granulation process since the AGS development is often done by inoculation of sludge in the form of flocs (Adav et al., 2008; Nancharaiah and Kiran Kumar Reddy, 2018). On the other hand, the third and fourth stage are continuously observed during the AGS process as it is a part of dynamic

growth system in which granules can attach and detach at all times (Nancharaiah and Kiran Kumar Reddy, 2018).

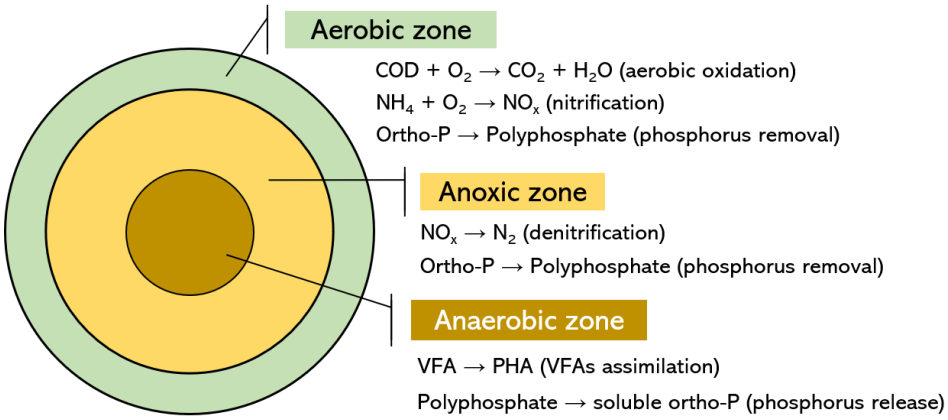

Figure 2.2 *Structural layers of aerobic granules which allow different biological conversion processes inside the granule (Figure by author, adapted from Liébana (2019)). The conversion process is often called simultaneous carbon, nitrogen and phosphorus removal.*

Figure 2.3 shows the granules formation which is caused by selected forces in the reactor. In general, the selection forces for granulation is mostly found to be (1) high hydrodynamic shear forces, (2) feast-famine regimes, and (3) washing-out of non-granulated biomass (Lee et al., 2010; Show et al., 2012; Wilén et al., 2018). Shear forces are generally caused by aeration, inducing EPS production, cell surface hydrophobicity and thus trigger interactions between cells and lead to granulation (Nancharaiah and Kiran Kumar Reddy, 2018; Tay et al., 2001a). Feast-famine regimes allow the alternating condition of high (”feast”) and low (”famine”) available organic matter exposure to the granules (Bengtsson et al., 2018). It promotes the internal storage of organic matter and thus decreasing bacterial growth, promoting slow-growing microorganisms to develop within the granules and form compact granules (De Kreuk et al., 2007a). Moreover, this condition also increases bacterial cell surface hydrophobicity that allow a faster microbial aggregation (Gao et al., 2011a).

Washing-out the non-granulated biomass allows the domination of larger particles within the reactor, promoting the sludge granulation. Szabó et al. (2017) found that most genera were washing out proportionally to their relative abundance on the flocs which means that they will be proportionally washed out until the granule’s formation. Overall, these forces can be defined as the trigger factors in which that they cannot stand alone and are not a solid requirement for granulation as long as other favourable conditions are established (Bengtsson et al., 2018; de Kreuk and Van Loosdrecht, 2004; Wilén et al., 2018). Furthermore, the selection pressure that is given to the reactor’s operation allows the granule formation which may consist of other forces or factors such as dissolved oxygen, solids retention time, cycle time, settling time and exchange ratio (Liu et al., 2005b). This topic is discussed in Section 2.3.3.2.

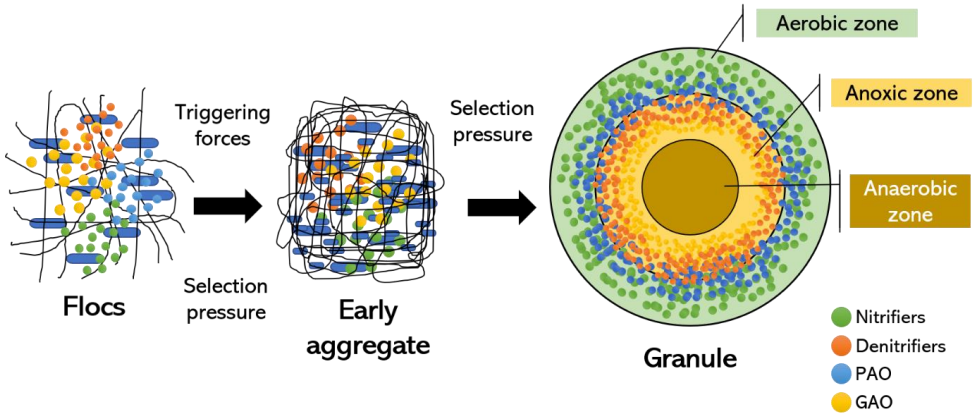

Figure 2.3 *Illustration of the gradual process of granulation from the flocs as the most common seeding sludge for AGS to compact granules. The trigger and selection forces are involved for granules formation (Figure by author, adapted from Nancharaiah and Kiran Kumar Reddy (2018)).*

2.3.3 Application of AGS

Most studies on AGS have reported the successful granulation in sequencing batch reactors (SBRs) since the first lab-scale study was conducted in the middle of 1990s (Mishima and Nakamura, 1991; Morgenroth et al., 1997). Thus, many researches have been doing investigations into the optimization of SBR operations to obtain effective granulation in the AGS process. In this chapter, the concept of SBR, operational parameters affecting granulation, and studies on pharmaceuticals wastewater using the AGS system are discussed.

2.3.3.1 AGS in the sequencing batch reactor (SBR)

The AGS process is often performed in a single tank called the sequencing batch reactor (SBR) which is operated in batch-mode with a continuous cycle consisting of: (1) influent filling; (2) react/mixing; (3) settle; (4) decant/draw and (5) idle (Orhon et al., 2009; Vigneswaran et al., 2009). The SBR is able to serve as biological treatment as well as the clarifier/solid separation in one reactor so it can save space compared to activated sludge process (Shah and Rodriguez-Couto, 2019). For the AGS process, SBR is beneficial as the biomass can be exposed to relatively high concentration of organic matter and nutrients during and after filling of influent from the bottom of the reactor. This condition will cope with diffusion limitation of substrate to the core of granules and also support the granulation process (Bengtsson et al., 2018; McSwain et al., 2004a). Figure 2.4 shows the illustration of SBR operation.

During the filling phase, the wastewater influent is filled to the reactor. Next, in the react phase, aeration is applied to supply the biomass with sufficient dissolved oxygen for the conversion processes and create mixing at the same time. After that, in the settling phase, the solids/biomass will settle and thus the SBR acts as the clarifier. Then, the effluent is discharged from the reactor during the decant/draw phase and in the AGS process, it is common to have a variation from 20-80% of exchange ratio (Wang et al., 2006), so there is a residual treated wastewater in the tank which will be mixed with the new influent during the next filling phase. At the end, the idle phase is the stable condition for the AGS and it is the final step before the new cycle begins (Shah and

Rodriguez-Couto, 2019). In this study, the idle phase is often used to do maintenance such as sensors calibration.

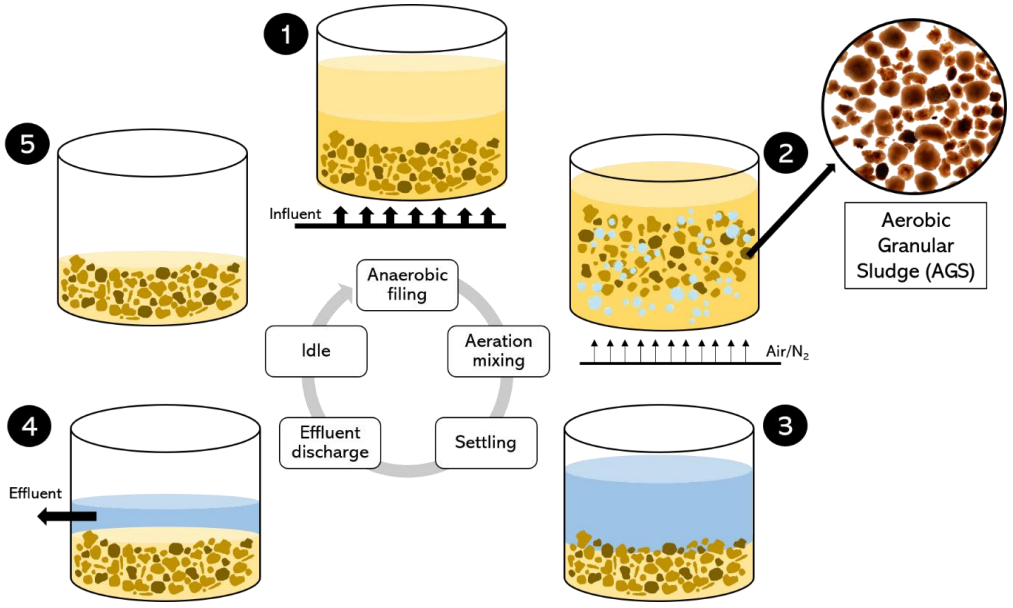

Figure 2.4 *Illustration of AGS process in sequencing batch reactor (SBR) operation. The SBR operates in a continuous cycle which consists of five phases: (1) anaerobic filling; (2) react/aeration mixing; (3) settling; (4) effluent discharge/ draw and (5) idle. During aeration phase, the reactor can be injected with air to increase dissolved oxygen (DO) concentration in the reactor or nitrogen gas (N_2) when the DO is too high (Figure illustrated by author, adapted from Bengtsson et al. (2018)).*

2.3.3.2 Operational parameters affecting granulation and AGS performance

In order to achieve granulation and stable performances from the AGS, several parameters can be controlled during the operation of the AGS in the SBR. Controlling these operational conditions can also be known as the selection pressures that are put in the reactors to achieve granulation. In this section, these parameters are discussed, limited to parameters which were observed to change during the start-up period of AGS.

pH and dissolved oxygen (DO) concentration

pH has an important role in the wastewater treatment process which can be expressed as the acidity (pH less than 7 is acidic) and alkalinity (pH more than 7 is alkaline) of water or the neutral solution in which the pH 7 (Madigan et al., 2015). The pH values in the wastewater can be influenced by the conversion processes which occur during the operation which can affect the microbial community and stability of the granules (Lashkarizadeh et al., 2016; Madigan et al., 2015). On the other hand, the dissolved oxygen (DO) is also crucial to control the conversion process such as nitrification, denitrification and phosphorus removal. In terms of AGS, the DO concentration is controlled in a range of 0.7 to 7 mg/L (Winkler et al., 2018).

Settling time

One of the strategies to obtain granulation is to select and keep the rapid settling aggregates within the AGS system. Thus, it is important to operate the settling time

along the process to wash-out the freely suspended microorganisms and flocs and let the retained granules grow (Heijnen and Van Loosdrecht, 1998; McSwain et al., 2004b). Applying a short settling time has been known as a common strategy for granulation (McSwain et al., 2004b). However, a harsh wash-out of the desired microbial community in the system may occur and thus, a stepwise decrease in settling time has been introduced (Szabó et al., 2016). Various studies have found different settling time to achieve granulation within a range of 2 minutes to 30 minutes from a lab-scale, pilot-scale and full-scale of AGS (Gao et al., 2011b; Liu et al., 2016; Ni et al., 2009; Pronk et al., 2015a)

Solids retention time (SRT)
Solids or sludge retention time (SRT) can be defined as the average time the biomass or sludge remain in the system and thus it is often called sludge age (Tchobanoglous et al., 2003). It has been known to be an important operational parameter in the activated sludge process, affecting the bioflocculation. In the activated sludge process, the range of SRT was reported to be around 2 to 8 days (Rittmann et al., 1987) while in the AGS process it was in a range of 1 to 40 days with a fluctuation along the operation (Pan, 2003). The SRT may also trigger the microbial selection in the system (Liébana, 2019). For instance, the slow-growing bacteria such as ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) can be washed out in the low SRT which can impact the nitrogen removal efficiency (Szabó et al., 2016).

2.3.3.3 AGS system in remediation of pharmaceuticals

In Sweden, Nykvarnsverket in Linköping was the first WWTP to install advanced treatment to remove pharmaceuticals in 2017 by applying ozonation (Tekniska verken, n.d.; Swedish EPA, 2020). Other technologies have been known to be able to treat pharmaceuticals: physical, oxidative, biological, adsorptive or the combination of them (Baresel et al., 2017). Among these techniques, using biological treatment has been widely reported for its benefits in the relatively low-cost treatment, sustainability, and high removal efficiency (Crini and Lichtfouse, 2019). Today, AGS has been shown as one of the more promising alternatives for removal of pharmaceuticals (Xia et al., 2015; Zhao et al., 2015; Amorim et al., 2016).

A few studies report on pharmaceutical removal in AGS. Two studies performed experiments with pharmaceutical removal after reaching a stable AGS system in a system fed by the synthetic wastewater (Zhao et al., 2015; Amorim et al., 2016) while one study successfully compared the directly fed AGS system with a hypersaline pharmaceutical wastewater (R_p) and hypersaline synthetic wastewater during the start-up (R_s) (Jiang et al., 2021). Results showed that granulation could not be achieved after 90-days of operation when R_p was used in the start-up period (Jiang et al., 2021). In terms of the sludge or granule's morphology, a high portion of smaller diameter granules was observed after PhACs was dosed and the AGS was irregular and loose-structured (Amorim et al., 2016). The MLSS was declining in the beginning of dosing of PhACs to the system. The studies which investigated the EPS concentration in the system similarly reported an increase of protein (PN) and polysaccharide (PS) was observed when adding the PhACs that was assumed to act as protection from the toxicity. In terms of AGS organic matter and nutrients removal performance, PhACs probably affected the microorganisms responsible for phosphorus removal, nitrification and denitrification as the decrease in removal of COD, P, and N was observed. Similar

studies also reported the inhibitory effect of pharmaceuticals on COD, P, and N-removal (Amorim et al., 2014; Moreira et al., 2015).

2.3.4 Conversion processes

The AGS system has been known to be able to allow different microorganisms with multiple functions distributed within the granules (Liébana, 2019; Winkler et al., 2013). Therefore, different biological conversion processes occur in the granules, instead of taking place in different treatment stages or tanks such in the activated sludge process (Bengtsson et al., 2018). In this section, organic matters and nutrients removal mechanisms in the AGS are discussed.

2.3.4.1 Organic matter removal

The removal of organic matter in the wastewater can be a result of degradation processes of particulate organic matter and utilization by microorganisms that occur through several processes in the AGS reactor or SBR cycle. During the anaerobic or anoxic phase, organic matter is required as the electron donors in the denitrification process. In addition, at anaerobic conditions, it is also removed through fermentation and assimilation of volatile fatty acids (VFA) by polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) (de Sousa Rollemberg et al., 2018). The PAOs and GAOs can store VFA as PHA (polyhydroxyalkanoates) for their growth under aerobic or anoxic conditions but only PAOs can also further store polyphosphate (Bengtsson et al., 2018).

On the other hand, during aerobic phase, organic matter can be converted to simple end products through aerobic oxidation which is often dominated by heterotrophic microorganisms with additional biomass production or synthesis of new cells (Tchobanoglous et al., 2003; de Sousa Rollemberg et al., 2018; Davis, 2020). In aerobic granules, the hydrolysis of particulate organic matter and further its consumption are known to happen on the granule's surface, promoting filamentous outgrowth and more irregular and porous surfaces of granules (De Kreuk et al., 2010; Wagner et al., 2015). Table 2.2 shows the summary of organic matter removal mechanisms in the AGS system (de Sousa Rollemberg et al., 2018).

Table 2.2 Mechanisms of organic removals by aerobic granules.

Phase	Process	Microbial groups
Anaerobic, anoxic	Denitrification	DOHO, DPAO, DGAO
Anaerobic	VFA fermentation and assimilation	PAO, GAO
Aerobic	Aerobic oxidation	OHO
Aerobic, anoxic	Simultaneous nitrification and denitrification	AOB, NOB, DOHO

2.3.4.2 Nitrogen removal

The removal of nitrogen in wastewater occurs through nitrification and denitrification processes which requires aerobic and anaerobic/anoxic conditions, respectively. In the AGS reactor, nitrogen removal can be performed not only in the separate process, but

also within the granules which is called simultaneous nitrification and denitrification (SND) process (Pochana and Keller, 1999; Coma et al., 2012; Nancharaiah and Kiran Kumar Reddy, 2018). In addition, nitrogen as ammonia (NH_3) can also be removed by assimilation and through ammonium (NH_4^+) adsorption into aerobic granules that may bind to extracellular polymeric substances (EPS) (Bassin et al., 2011).

Nitrification
Nitrification is a process in which the ammonium (NH_4^+) is oxidized to nitrite (NO_2^-) and further to nitrate (NO_3^-) with the oxidation reaction as follows (Davis, 2020):

$$2\ NH_4^+ + 3\ O_2 \xrightarrow{AOB} 2\ NO_2^- + 4\ H^+ + 2\ H_2O \quad (2.1)$$

$$2\ NO_2^- + O_2 \xrightarrow{NOB} 2\ NO_3^- \quad (2.2)$$

Besides the aerobic autotrophic bacteria such as AOB and NOB, nitrification can be also performed by COMAMMOX (complete ammonia oxidizer) and Anammox (anaerobic ammonium oxidation) in aerobic and anoxic conditions, respectively. COMMAMOX can do a complete nitrification while Anammox perform both nitrification and denitrification processes (de Sousa Rollemberg et al., 2018).

Denitrification
Denitrification can be defined as a process in which nitrate is reduced to nitrogen gases, primarily N_2, under anoxic conditions (Davis, 2020). In the biological process of denitrification, the electron donor is the organic matter which can be in different forms in the wastewater such as: (1) readily biodegradable organic matter in the influent; (2) readily biodegradable organic matter produced during endogenous decay; and (3) external source such as methanol or acetate (Tchobanoglous et al., 2003). Thus, in the AGS, high portion of internally stored PHA is often required as the carbon source for denitrification, especially in the SND process (De Kreuk et al., 2007b; Third et al., 2003; Xavier et al., 2007). The reaction stoichiometry for denitrification using the general representation of wastewater influent can be described as follow (Davis, 2020):

$$C_{10}H_9O_3N + 10\ NO_3^- \xrightarrow{denitrifiers} 5\ N_2 + 10\ CO_2 + 3\ H_2O + 3\ NH_3 + 10\ OH^- \quad (2.3)$$

2.3.4.3 Biological phosphorus removal

The removal of phosphorus through biological processes is often mentioned as biological phosphorus removal (BPR or Bio-P) or enhanced biological phosphorus removal (EBPR). The process requires anaerobic and aerobic/anoxic condition and is performed by PAOs. During the anaerobic phase, the readily biodegradable organic matter such as VFA is taken up by PAOs and converted to PHA. At the same time, there will be a release of soluble orthophosphate (PO_4^{3-}) to the solution. Then, during aerobic/anoxic phase, the stored PHA provides the energy for PAOs to grow and form polyphosphate bonds in the cell storage, removing the soluble orthophosphate from the solution and thus PO_4 is stored in PAOs. Therefore, the phosphorus in wastewater can be removed as the biomass/sludge is removed from the process (Davis, 2020; Madigan et al., 2015; Tchobanoglous et al., 2003). This model was proposed based on studies on BPR biochemical pathways (Comeau et al., 1986; Wentzel et al., 1986). Another model was developed by involving glycogen degradation during anaerobic phase which acts

as the electron donor for PHA production and glycogen synthesis in aerobic phase (Mino et al., 1998). Figure 2.5 shows how the concentration of compounds changes during EPBR process.

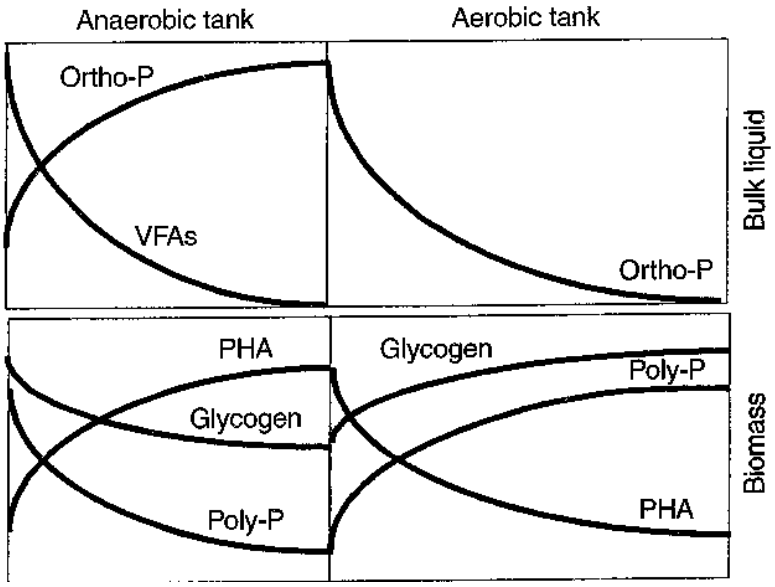

Figure 2.5 *Illustration of EPBR process during anaerobic and aerobic condition. The graph represents the changes in concentration of soluble orthophosphate and VFAs in bulk liquid (wastewater) as well as PHA, glycogen and polyphosphate biomass (PAOs) (Van Loosdrecht et al., 1997).*

3 Materials and methods

3.1.1 Österröd wastewater treatment plant

In this project, the granular sludge was obtained from a full-scale AGS reactors at the Österröd WWTP, Strömstad, Sweden. The WWTP was rebuilt in 2017-2018 and is designed for a maximum of 30,000 population equivalent. The AGS is a part of upgrading the biological treatment of wastewater which was constructed in March 2017 and start operating in May 2018 (Alfredsson, 2020). It uses a sequencing batch reactors (SBR) process which consist of three steps in a cycle: (1) simultaneous fill/draw; (2) aeration; and (3) fast settling. Figure 3.1 shows the wastewater treatment processes at the Österröd WWTP. The AGS line consists of several steps as follows (Mark de Blois and Jonatan Flodin, 2018):

- Buffer tank for flow equalization with a volume of 320 m^3. The wastewater is pumped to the AGS reactors with three pumps of 200 m^3/h each.
- AGS reactors: two AGS reactors for biological purification and sedimentation with a volume of 750 m^3 each.
- Buffer out: a buffer tank for flow equalization with a volume of 250 m^3. The wastewater is pumped to a flocculation tank with two pumps of 200 m^3/h each.
- Sludge buffer: a tank for leveling excess sludge of 50 m^3 with two sludge pumps with a capacity of 20 m^3/h each in which only one pump used in a normal operation.

The outcoming wastewater from AGS reactors is mixed with the water from the parallel activated sludge process and enters the flocculation tank and final sedimentation before it is discharged. The AGS is designed to treat approximately 60% of the flow whereas the rest (40%) is treated in the activated sludge process.

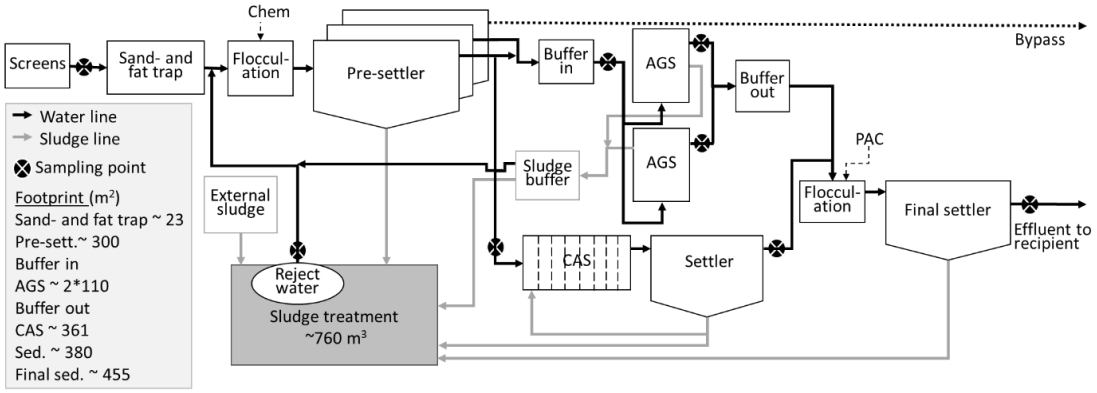

Figure 3.1 Wastewater treatment processes at Österröd Wastewater Treatment Plant, Strömstad, Sweden (modified by Jennifer Ekholm from Mark de Blois and Jonatan Flodin, 2018).

3.2 Reactor configuration and operational conditions

The thesis study was limited to 44 operational days while the project is still ongoing. The experiments were performed by using three parallel lab-scale sequencing batch reactors (SBRs) with a volume of 3.2 litres of each reactor, see Figure 3.2. All reactors (R0, R1, R2) were fed by complex synthetic wastewater: and two reactors (R0 and R1) were additionally dosed by pharmaceutically active compounds (PhACs). As a start-up, the reactors were filled with 3.2 litres of synthetic wastewater and inoculated with aerobic granular sludge from a full-scale AGS plant at Österröd WWTP. The cultivated seeding aerobic granular sludge had concentrations of 8.25 g MLSS/L, 7.1 g VSS/L and sludge volume index (SVI) after 5, 10, and 30 minutes of settling of 52, 48, and 48 mL/g, respectively. The influent was pumped to the bottom of the reactor and composed of 300 mg COD/L (110 mg TOC/L), 50 mg TN/L and 6 mg TP/L with addition of micronutrients (30−50 mg/L) and pharmaceutical compounds (PhACs) (10 µg/L) for R0 and R1.

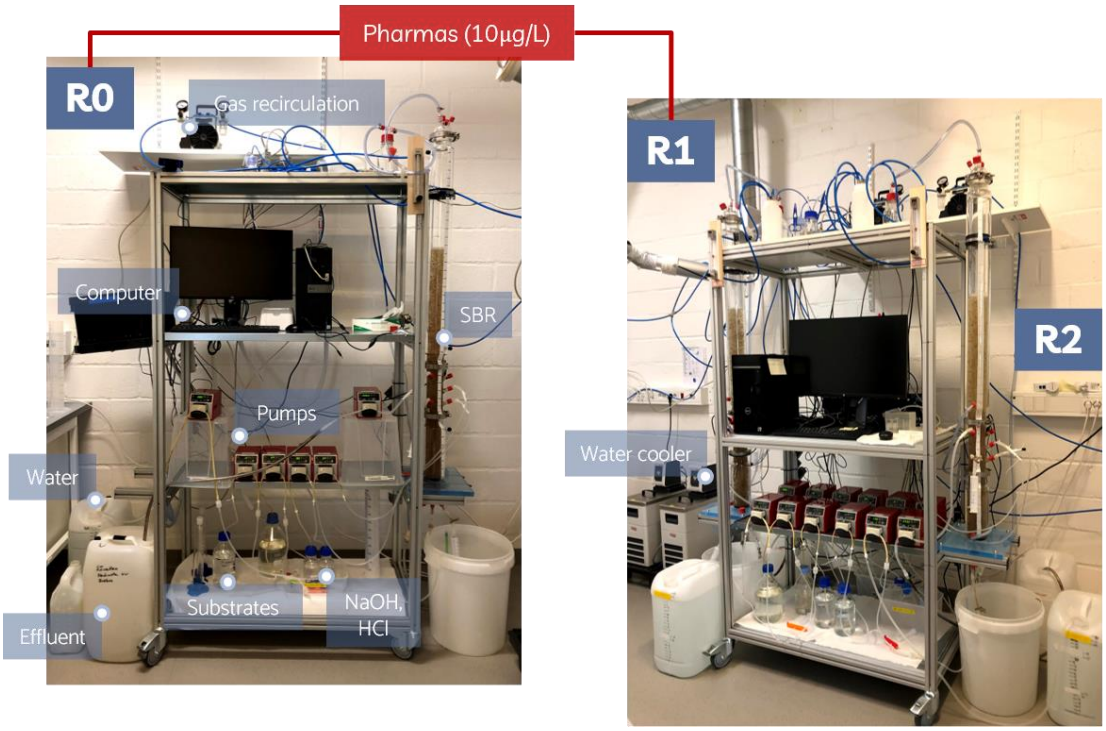

Figure 3.2 Three AGS reactors set up in WET laboratory, called R0, R1 and R2.

Figure 3.3 shows a schematic illustration of the SBR configuration for the three reactors. It is a column-type reactor which is operated in room temperature controlled at 20 ± 1°C. The reactor was connected to a water cooler to control the temperature of the system for the further temperature study. During the experiment, the level of pH and dissolved oxygen (DO) in the reactors were maintained and monitored by using sensors and the data was obtained through data acquisition hardware in the computer connected to the sensors. At least every three days of operation, the DO sensors were calibrated by exposing the sensors in air in which the DO value should be approximately 100%. For controlling the pH, 1 M of hydrochloric acid (HCl) and sodium hydroxide (NaOH) solutions were prepared and automatically dosed to the reactor by using two pumps.

The aeration sparger was introduced from the bottom of the reactor. The DO level was adjusted by injecting nitrogen gas (N_2) when the DO was higher than the set-point and air when the DO was lower by a mass flow controller (MFC). The gas from the reactor flowed into a gas mixing vessel and it was recirculated to the reactor. A gas wash bottle was put before the filter to act as a foaming trap that may be produced from the reactor and the gas filter was installed to avoid problems with liquid and biomass before entering the gas pump. A rotameter was placed before the gas reached the inlet of the reactor to measure the volumetric flow rate of the gas which was kept at a constant value of 3 L/s.

The wastewater influent was prepared in separate bottles or bucket which consisted of water (H_2O) bucket, carbon and nitrogen (C+N) bottle and phosphorus and micronutrients (P+micronutrients) bottle. For R0 and R1, the PhACs was dosed in the C+N solutions. The purpose of separating the C+N and P solutions is to avoid the massive growth of microorganisms in the substrate bottle. The water and substrate solutions were then pumped and mixed before entering the bottom of the reactor. The wastewater effluent was pumped out from the reactor at around 1.6 L height of the reactor which corresponding to a 50% exchange ratio. There are two sampling ports which were placed at the middle and the bottom of the reactor for taking the samples as well as acting as the biomass withdrawal or the excess sludge removal point. The design of the SBR and operational conditions is summarized in Table 3.1.

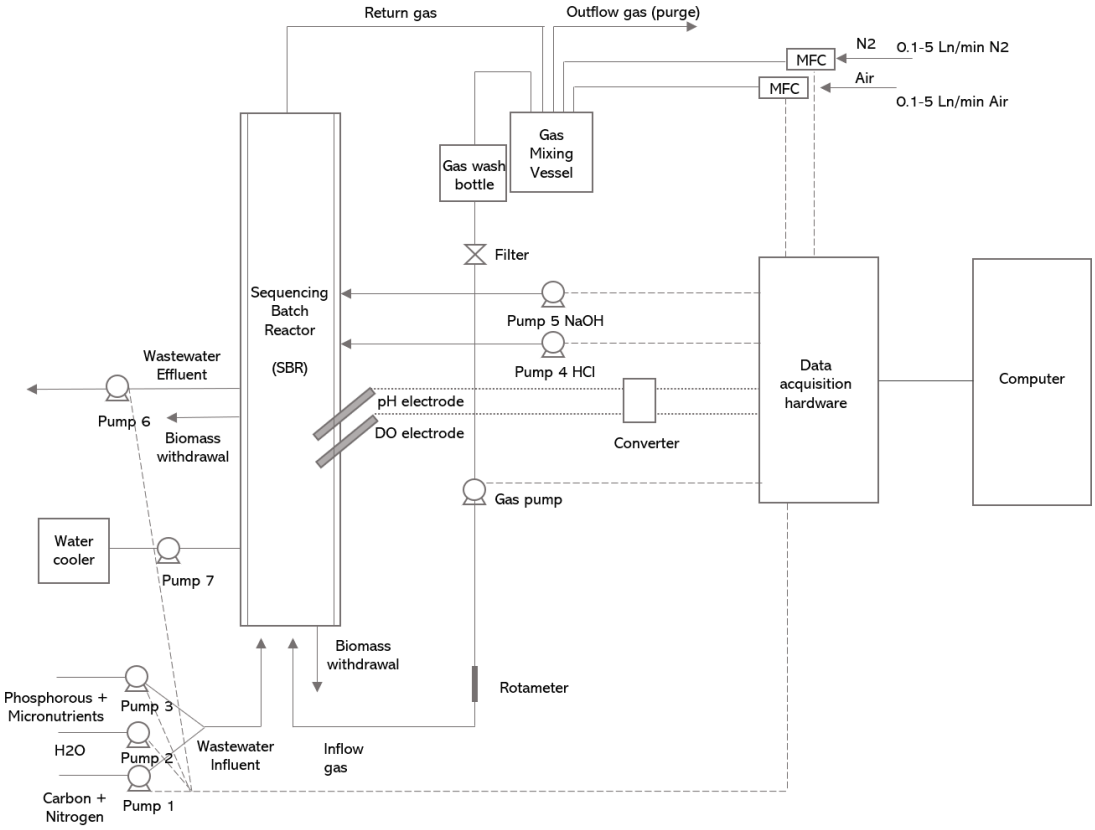

Figure 3.3 SBR configuration (Figure by Burzio, C. and Ekholm, J.)

Table 3.1 The SBR design and operational conditions.

Parameter	Value
Volume	3.2 L
pH	7.5 ± 0.3
Dissolved oxygen (DO)	20 ± 10 %
Temperature	21°C
Gas/air flow	3 L/s
Exchange ratio	50%
Cycle time	6 hours

3.2.1 Cycle setting

The SBR operates in a continuous cycle which consists of five phases: (1) anaerobic filling/feed; (2) aerobic react/aeration; (3) settling/rest; (4) effluent discharge/ withdrawal and (5) idle. The total time of a cycle is set to be 6 hours and different time was set up for each phase, see Figure 3.4. The feeding or filling of wastewater influent to the reactor was performed in a static anaerobic condition, meaning there is no aeration and no mechanical mixing of the influent. In this phase, the uptake of VFAs and release of soluble orthophosphate (PO_4^{3-}) by PAOs in the bio-phosphorus removal mechanism is expected to occur. Next, in the aeration phase, the wastewater was aerated with a mixture of air and nitrogen gas which was automatically adjusted by the system based on the set DO value. The period of this phase was the longest compared to other phases to give a longer time for organics removal and nitrification to happen (Shah and Rodriguez-Couto, 2019). In this phase, the aeration also acted to increase hydrodynamic shear force in the reactor contributing to the granulation and the stability of the granules (Nancharaiah and Kiran Kumar Reddy, 2018; Tay et al., 2001a).

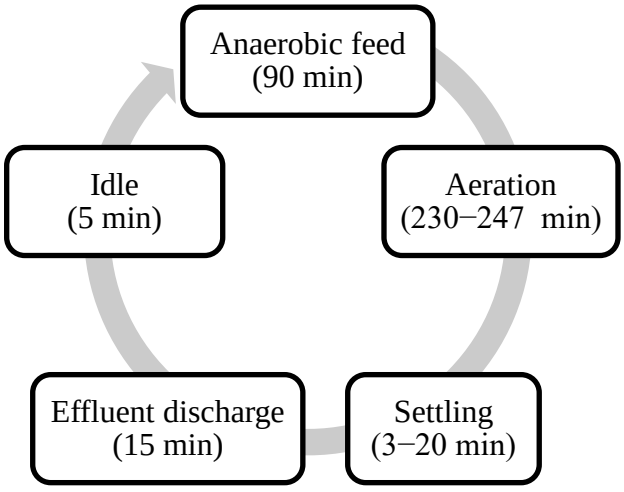

Figure 3.4 Time setting for each phase in one cycle of SBR operation. As the settling time was decreasing from 20 minutes to 3 minutes, the aeration time was also adjusted from 230 minutes to 247 minutes to keep the total time of one cycle to be 6 hours or 360 minutes.

The settling phase allowed the sludge or the biomass to settle and, in this study, the settling time was gradually decreased during the SBR operation. This strategy was based on the study by Szabó et al. (2016) that introduced a stepwise decrease of settling time, resulting in a balanced microbial population during the biomass wash-out, to favour of slow growing microorganisms such as nitrifiers, and thus a fast AGS start-up period. In the beginning, the settling time was decided to start with 20 minutes since mature granules were used as the seeding sludge for the start-up. Compared to activated sludge process, the AGS process is operated with shorter settling time that aims to select for the fast-settling flocs and granules (Beun et al., 1999; McSwain et al., 2004b). Therefore, along the operation of the reactors, the settling time was reduced up to 3 minutes, see Table 3.2. This is also a common strategy based on findings in the successful cultivation of a domination of granules with a settling time less than 5 minutes (Liu et al., 2005; Qin et al., 2004). The performance of the AGS and the concentration of the biomass in the reactor, called mixed liquor suspended solids (MLSS), were taken into account in the decision to gradually reduce the settling time.

Table 3.2 The changes in settling and aeration time for three AGS reactors during the operational days.

Operation day	Settling time (min)			Aeration time (min)		
	R0	R1	R2	R0	R1	R2
0	20			230		
13	15			235		
16	12			238		
19	9	12	9	241	238	241
22	7	12	7	243	238	243
23	6	12	6	244	238	244
30	5	7	5	245	243	245
34	4	7	4	246	243	246
35	3	5	3	247	245	247
43	5	5	3	245	245	247

3.2.2 Synthetic wastewater composition

Synthetic wastewater was used as the wastewater influent that was fed to the reactors. The recipe was adjusted based on the study of Layer et al. (2019) which investigated different synthetic and real-municipal wastewater as the influent in the start-up of AGS reactors. The research found that the synthetic wastewater with a higher portion of non-diffusible organic substrate, called complex synthetic wastewater, simulating real wastewater better compared to the 100%-VFA synthetic wastewater that is often applied in lab-scale studies. The micronutrients composition was adapted from Tay and Yan (1996). The target concentration of the synthetic wastewater in this experiment was 300 mg COD/L or 110 mg TOC/L, 50 mg N/L and 6 mg P/L. Table 3.3 and Table

3.4 show the detailed composition of carbon and nitrogen (C+N) solution and phosphorus (P) and micronutrients solution, respectively. The stock solution of mixed PhACs consisted of eleven compounds with concentration of 10 μg/L: atenolol, carbamazepine, ciprofloxacin, diclofenac, ketoprofen, mefenamic acid, metoprolol, sertraline, sulfamethoxazole, and venlafaxine.

Table 3.3 The carbon (C) and nitrogen (N) substrate recipe for the complex synthetic wastewater.

Substrate	Concentration (g/L)
Sodium acetate ($NaC_2H_3O_2$)	0.10
Sodium propionate ($NaC_3H_5O_2$)	0.06
Glucose ($C_6H_{12}O_6$)	0.07
Peptone	0.06
Ammonium chloride ($(NH_4)Cl$)	0.16
Calcium chloride hydrate ($CaCl_2.2H_2O$)	0.02
Magnesium sulphate ($MgSO_4$)	0.02
Potassium chloride (KCl)	0.03

Table 3.4 The phosphorus (P) and micronutrients substrate recipe for the complex synthetic wastewater.

Substrate	Concentration (g/L)
P substrate	
Potassium dihydrogen phosphate (KH_2PO_4)	0.01
Dipotassium hydrogen phosphate (K_2HPO_4)	0.02
Sodium hydrogen carbonate ($NaHCO_3$)	0.2
Micronutrients	
Boric acid (H_3BO_3)	0.05
Zinc chloride ($ZnCl_2$)	0.05
Copper chloride ($CuCl_2$)	0.03
Manganese sulphate monohydrate ($MnSO_4.H_2O$)	0.05
Ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	0.05
Aluminium chloride ($AlCl_3$)	0.05
Cobaltous chloride hexahydrate ($CoCl_2.6H_2O$)	0.05
Nickel chloride ($NiCl_2$)	0.05

As it is mentioned in Section 3.2, the synthetic wastewater was prepared in separate C+N solution and P+micronutrients solution to avoid bacteria growth in the storage bottles. Other actions to prevent the microbial contamination were done by using the autoclaved substrate bottles and pipes as well as a routine changing of the wastewater influent pipes when a massive biofilm was observed within the pipes. However, during the experiments, the C+N substrate solutions were sometimes filtered with a 0.2 μm pore size filter paper when microbial growth was noticed in the substrate solution. Figure 3.5 shows the comparison of the clean C+N substrate solution and wastewater pipes and the contaminated solution and pipes.

(a) (b)

Figure 3.5 Substrate conditions: (a) C+N substrate solution in the bottle and the influent wastewater pipes were observed to be clean and (b) microbial growth was observed in the substrate bottle and pipes.

The substrate solutions were concentrated 20 times and 100 times for C+N substrate and P substrate, respectively, considering an efficient preparation for the experiments. The micronutrients solution was added at 1 mL per litre of wastewater while the PhACs solution was added at 1 mL per 2 litres of wastewater only to R0 and R1. The concentrated substrate solutions were then diluted by adding the water which was prepared in the 30 L bucket. At day 28 of operation, the antifoam agent (0.005%) was dosed in the water since the foaming problem arose in the reactors and it was stopped at the day 40 of operation.

3.2.3 Pumps set up

There were a total of 8 pumps, including the gas pump, that were used in one AGS reactor, see Figure 3.3. During the experiments, the wastewater influent pumps which consist of C+N pump, P+micronutrients pump and water pump were the most concerned because they will affect the influent concentration to the AGS system. Therefore, they were routinely calibrated, especially the C+N and P+micronutrients pumps, by comparing the measured flow and the target flow for each pump. The measured flow (Q) for calibration was done during anaerobic feeding phase by calculating how much solution was taken in a 50 mL measuring cylinder around 1 hour, see equation (3.1).

$$Q = \frac{V_{initial} - V_{final}}{60 \text{ minutes}} \left(\frac{mL}{min}\right) \quad (3.1)$$

On the other hand, the target flow for the substrate solution and water were calculated by using equation (3.2) and equation (3.3), respectively. The calculation was based on the fact that the influent flow (Q_{inf}) is the sum of the P+micronutrients flow (Q_{p+m}), C+N flow (Q_{C+N}) and water flow (Q_w), see Figure 3.6.

$$Q_{C+N} = Q_{p+m} = \frac{V_{influent}}{CF \times t_{feed}} \left(\frac{mL}{min}\right) \quad (3.2)$$

where,
$V_{influent}$ = influent volume (mL) = 1600 mL
CF = concentration factor; CF for Q_{C+N} = 20; CF for Q_{p+m} = 100
t_{feed} = anaerobic feeding time = 90 minutes

$$Q_w = Q_{inf} - Q_{C+N} - Q_{p+m} = \frac{V_{influent}}{t_{feed}} - Q_{C+N} - Q_{p+m} \left(\frac{mL}{min}\right) \quad (3.3)$$

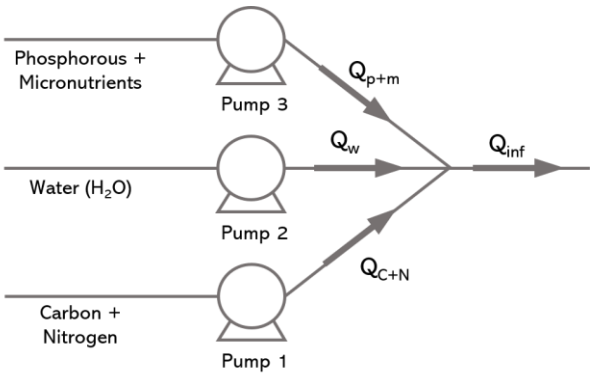

Figure 3.6 Illustration of pumps setting for the wastewater influent in the AGS system. The wastewater influent consists of P+micronutrients, water and C+N flows and thus three pumps were provided for one AGS reactor. The arrows show the direction of the flow.

3.3 Analytical methods

The analysis in this study is divided into the wastewater analysis which used the liquid samples and the biomass analysis which observed the sludge or biomass samples. The purpose of the wastewater analysis is to know the AGS performance of removing the organics, nitrogen and phosphorus in the wastewater. On the other hand, the biomass analysis aims to investigate the granule development in the AGS system. Table 3.5 shows the summary of parameters that were observed in the study and the time to take the samples. In general, the sampling was done twice a week except the sludge morphology and size analysis which were done only once a week. In order to avoid much disturbance to the system, the samples were analysed in duplicates and the average value was used.

For the wastewater analysis, the analysis was focused on the dissolved fractions of organics, nitrogen and phosphorus since all the samples were filtered. The influent and effluent samples were taken to calculate the removal efficiency of the organics, nitrogen and phosphorus removal in the treatment. The influent sample was obtained by collecting 500 to 1000 mL wastewater influent during the anaerobic feeding phase in

order to have a mixed and representative sample. The sample from the anaerobic feeding phase was also taken mainly for investigate the bio-P removal in the system. The sampling was done during 1 to 2 minutes after aeration phase started in order to have a homogenous sample with the assumption of biodegradation process in the aerobic phase could be negligible during this short period (Calgaro and Trieb, 2020). The solid analysis such as TSS and VSS in the reactor and in the effluent were done during the aerobic and decant/effluent discharge phase, respectively. Most of the liquid samples were taken by mixing the sample from the middle sampling port and the bottom sampling port, except for the anaerobic feeding sample. Table 3.6 provides information about the methods for each monitoring parameter which are discussed further in the next section.

Table 3.5 Monitoring parameters in the wastewater and biomass analysis.

Sampling phase	Monitoring parameter
Wastewater analysis	
Anaerobic feeding/ influent	TOC, TN, NH_4^+-N, NO_2^--N, NO_3^--N, PO_4^{3-}-P
Anaerobic feeding (end)/ 1−2 min aerobic	TOC, TN, NH_4^+-N, NO_2^--N, NO_3^--N, PO_4^{3-}P
Aerobic (end)	TSS, VSS (reactor)
Effluent discharge	TSS, VSS (effluent), NH_4^+-N, NO_2^--N, NO_3^--N, PO_4^{3-}P
Biomass analysis	
Aerobic	Sludge morphology, size analysis
Settling	SVI_5, SVI_{10} and SVI_{15}
	solids retention time (SRT)/ sludge age

Table 3.6 Summary of methods for wastewater and biomass analysis.

Parameter	Method
Liquid/wastewater sample	
NO_2^--N, NO_3^--N, PO_4^{3-}-P	Ion chromatography (ISO, 1995)
NH_4^+-N	Ion chromatography (ISO, 1998)
TOC, TN	TOC analyzer coupled with TN module
Biomass sample	
TSS	Standard method 2540 D (APHA, 1998)
VSS	Standard method 2540 E (APHA, 1998)
SVI	Standard method 2710 D (APHA, 1998)
Sludge morphology	Light microscopy
Size analysis	Image analysis

3.3.1 Wastewater analysis

3.3.1.1 Measurement of organic matter and total nitrogen

In this study, the organic matter as total organic carbon (TOC) and total nitrogen (TN) were measured by a TOC analyzer coupled with TN module (TOC-V, Shimadzu, Japan). The sample was filtered through 0.2 μm pore size filters and diluted 8 times for influent sample, 4 times for anaerobic feeding sample and no dilution for the effluent sample. The results from TOC analyzer that were used were the value of TOC as well as the total nitrogen (TN) in mg/L. The organic matter in wastewater is also often measured as chemical oxygen demand (COD). COD is defined as "a measure of the oxygen equivalent of the organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant" (APHA, 1995). However, the COD test is not preferred during the experiments due to the toxic waste generation. Thus, COD test was only conducted once for influent, effluent and VFAs samples to make an estimation of COD value from the TOC analysis. The COD test was done by using Hach test based on Hach Method 8000 (Hach, n.d.), see Appendix A.1 – organics analysis. Figure 3.7 presents the linear regression of COD and TOC values based on the measurements of influent, effluent and VFAs (acetate, propionate, glucose and peptone) samples.

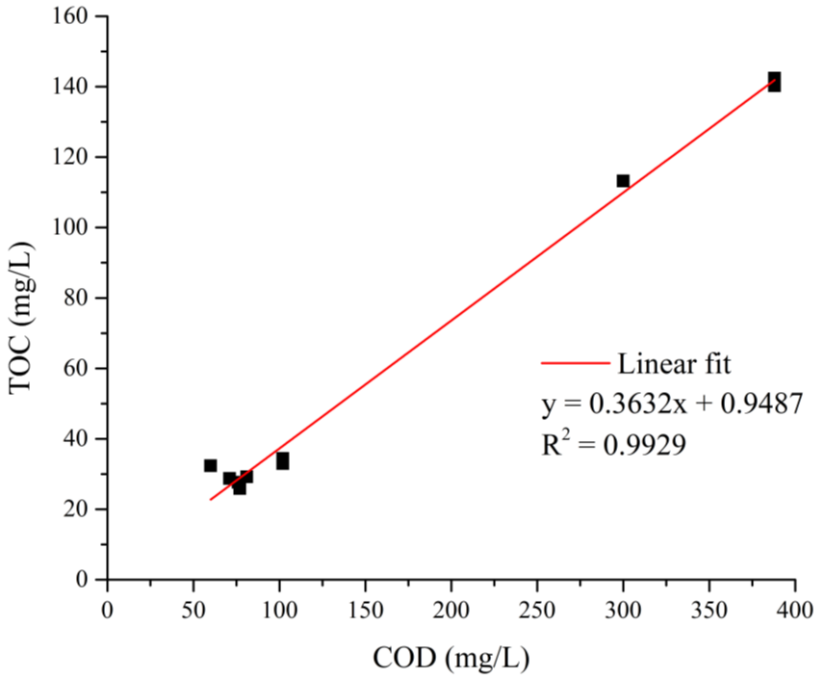

Figure 3.7 Linear regression of COD and TOC relationships based on influent, effluent and VFAs samples from the experiments.

3.3.1.2 Measurement of nutrients

The nitrogen species and phosphorus concentration in wastewater were measured as the ions, cations and anions, by using a Dionex ICS-900 ion chromatograph. The cation measured ammonium (NH_4^+) while the anions measured nitrite (NO_2^-), nitrate (NO_3^-) and orthophosphate (PO_4^{3-}). The method for dissolved cations was based on ISO 14911 (1998) while the dissolved anions was based on ISO 10304-2 (1995). The sample was filtered through 0.2 μm pore size filters and should be diluted until the conductivity

<200 µS/cm. Thus, the samples were diluted 4 times, 5 times and 2 times for influent, anaerobic feeding and effluent samples, respectively. The results from IC analysis were defined in concentration of millimolar (mM). Therefore, the concentration was converted to mg/L by multiplying the concentration in mM with the molar mass of nitrogen for NH_4^+, NO_2^-, and NO_3^- and phosphorus for PO_4^{3-}. From these concentration, total nitrogen (TN) and total phosphorus (TP) can be calculated. The calibration was conducted by using the standards solution in different concentrations. Detailed procedure is explained in the Appendix A.2 – nutrients analysis.

3.3.1.3 Removal efficiency

The removal efficiency was calculated for the removal of TOC, TN and TP to know the performance of the AGS system. Equation 3.4 shows the formula of removal efficiency. The results were be compared to the requirements set by the regulations, see Table 3.8. In the calculation, the target influent concentration is used which are 300 mg COD/L, 50 mg N/L and 6 mg P/L. For the organic concentration, the COD is converted to TOC by using the estimation from the comparison of TOC and COD values, see Figure 3.7. As a result, approximately 110 mg TOC/L was used as the target TOC influent concentration.

$$\text{Removal efficiency (\%)} = \frac{C_{influent} - C_{effluent}}{C_{influent}} \times 100\% \quad (3.4)$$

where,
$C_{influent}$ = target influent concentration (mg/L)
$C_{effluent}$ = effluent concentration (mg/L)

3.3.2 Biomass analysis

3.3.2.1 Total suspended solids (TSS) and volatile suspended solids (VSS)

The biomass concentration is one of the most important parameters of biological treatment which is commonly measured as the total suspended solids (TSS) and volatile suspended solids (VSS). In another term, it is also often referred to as mixed liquor suspended solids (MLSS) or mixed liquor volatile suspended solids (MLVSS), if one uses a mixture of influent wastewater and the microbial suspension in a bioreactor (Tchobanoglous et al., 2003). The VSS is classified as the solids which is volatilized at 550°C and usually are the organic compounds (Patnaik, 2017). In a biological treatment, VSS represents the amount of active biomass, cell debris, endogenous decay and non-biodegradable VSS in the influent wastewater (Tchobanoglous et al., 2003). On the other hand, the TSS is defined as the solids that are retained on a 2 µm pore size filter and dried to constant weight at 103−105°C (Patnaik, 2017). In a biological treatment, TSS includes the VSS and the inorganic solids (Tchobanoglous et al., 2003). The TSS and VSS analysis were done based on standard method 2540 D and E (APHA, 1998), respectively, see Appendix A.3 – solids analysis.

3.3.2.2 Sludge volume index (SVI)

The sludge volume index (SVI) is defined as “the volume in millilitres occupied by 1 gram of a suspension after 30 minutes settling” according to standard method 2710 D (APHA, 1998). It is commonly used as the monitoring parameter of the sludge settling

characteristics (Sezgin, 1982). In the AGS system, SVI is used to determine the degree of granule formation as its value differs compared to activated sludge flocs (De Kreuk et al., 2007a). Equation 3.5 shows the calculation of SVI (APHA, 1998).

$$SVI = \frac{\text{settled sludge volume (mL/L) x 1000}}{\text{suspended solids (mg/L)}} \quad (3.5)$$

During the experiment, the SVI was calculated based on the sludge bed volume in the reactor after 5, 10 and 15 minutes, known as SVI_5, SVI_{10} and SVI_{15}, respectively. This is possible since the reactor can act as the graduated cylinder (Calgaro and Trieb, 2020). The calculation of SVI in the study is calculated based on Equation 3.6.

$$SVI_x = \frac{\frac{SV_x}{V_R}}{TSS_R}\left(\frac{mL}{g}\right) \quad (3.6)$$

where,
SVI_x = SVI after x minutes (mL/g)
SV_x = sludge bed volume after x minutes (mL)
V_R = volume of the reactor (L)
TSS_R = TSS concentration in the reactor (g TSS/L)

3.3.2.3 Solids retention time (SRT)

As it is mentioned in Section 2.3.3.2, the SRT is monitored to know the sludge age or the time of the biomass is remaining in the system. Equation 3.7 is used to calculate the SRT (Layer et al., 2019). In this study, the SRT is calculated on a weekly basis or 7 days operation with a constant volume of reactor around 3.2 L.

$$SRT = \frac{V_R \text{ x } TSS_R}{Q_{ex} \text{ x } TSS_{ex} + Q_{eff} \text{ x } TSS_{eff}} (\text{days}) \quad (3.7)$$

where,
V_R = volume of the reactor (L)
TSS_R = TSS concentration in the reactor (g TSS/L)
Q_{ex} = flow rate of excess sludge (L/d)
TSS_{ex} = TSS concentration of the excess sludge (g TSS/L)
Q_{eff} = flow rate of effluent (L/d)
TSS_{eff} = TSS concentration in the effluent (g TSS/L)

The flow rate of the excess sludge is assumed to be based on the volume of samples which were taken out during one week while the flow rate of effluent is a constant value based on 50% of exchange ratio in the SBR in a day, see Equation 3.8 and Equation 3.9, respectively. The TSS concentration in the reactor is equal to the TSS concentration in the excess sludge since it is the same MLSS.

$$Q_{ex} = \frac{V_{ex}}{t} (\text{L/d}) \quad (3.8)$$

where,
V_{ex} = volume of samples taken out during t−days (L)
t = time interval of SRT calculation (days)

$$Q_{eff} = \frac{V_{eff}}{t} (L/d) \quad (3.9)$$

where,
V_{eff} = volume of the effluent discharge (L)
t = time (day)

3.3.2.4 Granule morphology and size analysis

The sludge and granule morphology was observed by using an Olympus BX60 light microscope and the particle size was performed in ImageJ software (Schneider et al., 2012). The samples of biomass from the reactor were taken as 100 mL from each reactor during the aeration phase. For the microscopy analysis, two to three drops of sample were placed on the glass slide and in some cases covered by a cover glass to be observed in 2 times (2x) and 10 times (10x) magnification. For the size analysis, the biomass sample was placed on a Petri dish and diluted with tap water in order to remove the fraction of smaller flocs before the pictures were taken. A camera was fixed above the Petri dish which was put in the fixed position on the top of an overhead projector (OHP), see Figure 3.8. At least 10 to 15 pictures should be taken from the light microscope for the sludge morphology and from the camera for the size analysis.

Five pictures of the granules from each reactor were edited in Adobe Photoshop CS6 in order to remove the picture's background before they were processed in the ImageJ software. ImageJ is an open-source software for image processing and analysis that can be downloaded from https://imagej.nih.gov/ij/download.html. In this study, the program was used to do the particle analysis to count the area of the granules which is further converted to granules diameter (ImageJ, 2020). The circularity was also measured in the program and the particle size distribution was calculated to see the granules development over time. The detailed procedure is explained in Appendix A.4 – size analysis.

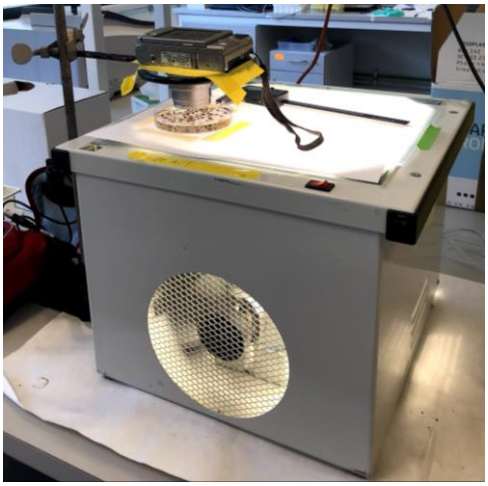

Figure 3.8 A set up for granule's size analysis. The overhead projector (OHP) was used to provide the light and the pictures of the granules on the Petri dish were taken by a camera placed in a fixed position.

3.4 Start-up definition

Successful start-up AGS systems have been observed to vary in time in several previous studies. However, most of them defined the start-up period based on the biomass or physical properties and the substrate removal. For instance, Layer et al., (2019) had the start-up AGS definition based on SVI_{30} < 90 mL/g, SVI_{30}/SVI_{10} > 0.8, size fraction with diameter > 0.25 mm for at least 50% of the TSS, granule morphology, and stable nutrient removal. Another research highlighted the fresh aerobic granules that were observed by the change of the colour to a brownish-yellow colour (Liu et al., 2005). The start-up period could also be achieved by specifically defined criteria such as the study by Lochmatter and Holliger (2014) which reported five criteria: 1) 95% COD-removal; 2) 80% P-removal; 3) 85% NH_4^+-removal; 4) 65% N-removal; and 5) biomass growth ($VSS > VSS_{start}$) as well as granulation (SVI_{30} < 50 mL/g). In this study, the success of start-up runs will be defined based on definitions from previous studies and the initial definition of granules according to the experts in the 2006 AGS workshop (De Kreuk et al., 2007a), see Table 3.7. As a reference for target TOC, TN and TP removals, Table 3.8 shows the summary of effluent limits that should be met in this project.

Table 3.7 Criteria for a successful start-up definition during experiments.

Parameter	Definition	Note
SVI_{15}/SVI_5	> 0.9	SVI_{15} is used instead of SVI_{30} since it was not measured
Granule size	> 0.2 mm	Volume based median size from size distribution
Granule morphology	brownish-yellow colour; no coagulation; stable, regular-shaped and fast-settling granular biomass	Granules do not coagulate and settle as separate units
Biomass growth	$VSS > VSS_{start}$	Indicator of sufficient biomass concentration
Substrate removal	Minimum of removal: TOC=75%; TN=70%; TP=80%	Stable performance

Table 3.8 Effluent discharge requirements for wastewater.

Parameter	Limit value	Reference
Chemical oxygen demand (COD)/ total organic carbon (TOC)	75% minimum reduction	Naturvårdsverket (2016)
Total nitrogen (TN)	70% minimum reduction	
Total phosphorus (TP)	80% minimum reduction	European Union (1991)

4 Results

4.1 Operational parameters of AGS reactors

4.1.1 Dissolved oxygen and pH

The dissolved oxygen (DO) and pH levels were monitored in one-minute intervals. Figure 4.1, Figure 4.2, and Figure 4.3 show the data of DO percentage and pH in the beginning, middle and end of this study, respectively. The values were selected from a random cycle, consisting of 6 hours, in one day. At the first day of AGS operation, the average DO and pH levels were in a range of the default values, see Table 3.1. The results for the average DO% in the aeration phase were 21.94 ± 8.22 %, 18.8 ± 8.31 %, and 20.87 ± 11.35 % in R0, R1 and R2, respectively. During this phase, although the average value of DO% were in a range of the operational settings, the upper range of DO% in R0 and R2 showed a little bit higher than 30%. On the other hand, in general, the pH values seem to be stable during the aeration phase with the average of 7.51 ± 0.17, 7.30 ± 0.12, and 7.40 ± 0.24 in R0, R1 and R2, respectively.

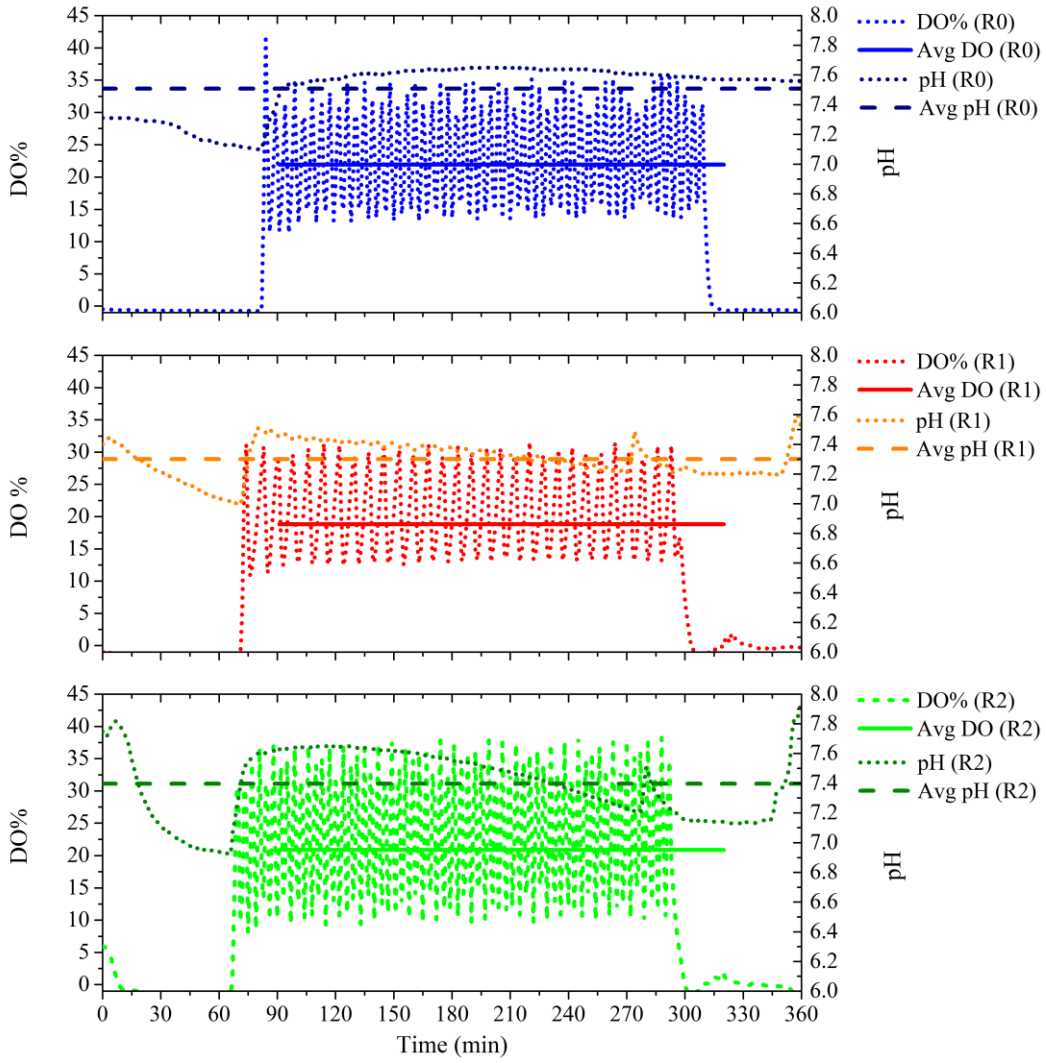

Figure 4.1 DO and pH level in R0, R1 and R2 during one cycle at day−1 of AGS operation. The average DO during the aeration phase are shown while the average pH in the graphs were determined from the whole cycle.

In the middle of the study at day−18, problems occurred in R0 and R1 while the condition of DO and pH in R2 was quite stable. The average DO% at day−18 were 4.99 ± 4.13 % in R0 and 22.95 ± 0.52 % in R2 while it was almost 0% in R1. In R0, the DO% values were very low and the reading was only about 1% when the DO sensor was exposed in the air. Prior to this condition, the DO sensor in R0 was also observed to be often very low when the calibrations were done in all reactors. Therefore, the DO sensor for R0 was able to be changed in day−26. The pH values were 7.36 ± 0.07 in R0 and 7.33 ± 0.09 in R2 within the whole day while R1 was experienced unstable pH condition at day−18. In this day, the aeration sparger was accidentally detached from R1, resulting in the anaerobic phase for around 33 hours. As there was no aeration in the reactor, the wastewater was also not mixed while the pH was adjusted automatically by the system based on the pH sensor. Since the pH sensor was located in the middle of the reactor, the system translated the high pH which was around 8 to 9 by continously injecting acid to the reactor without a mixing process. Therefore, the pH significantly dropped from 8 to the lowest of 1.98 when the aeration started again, see Figure 4.2. However, the pH values could be stabilized within the subsequent days.

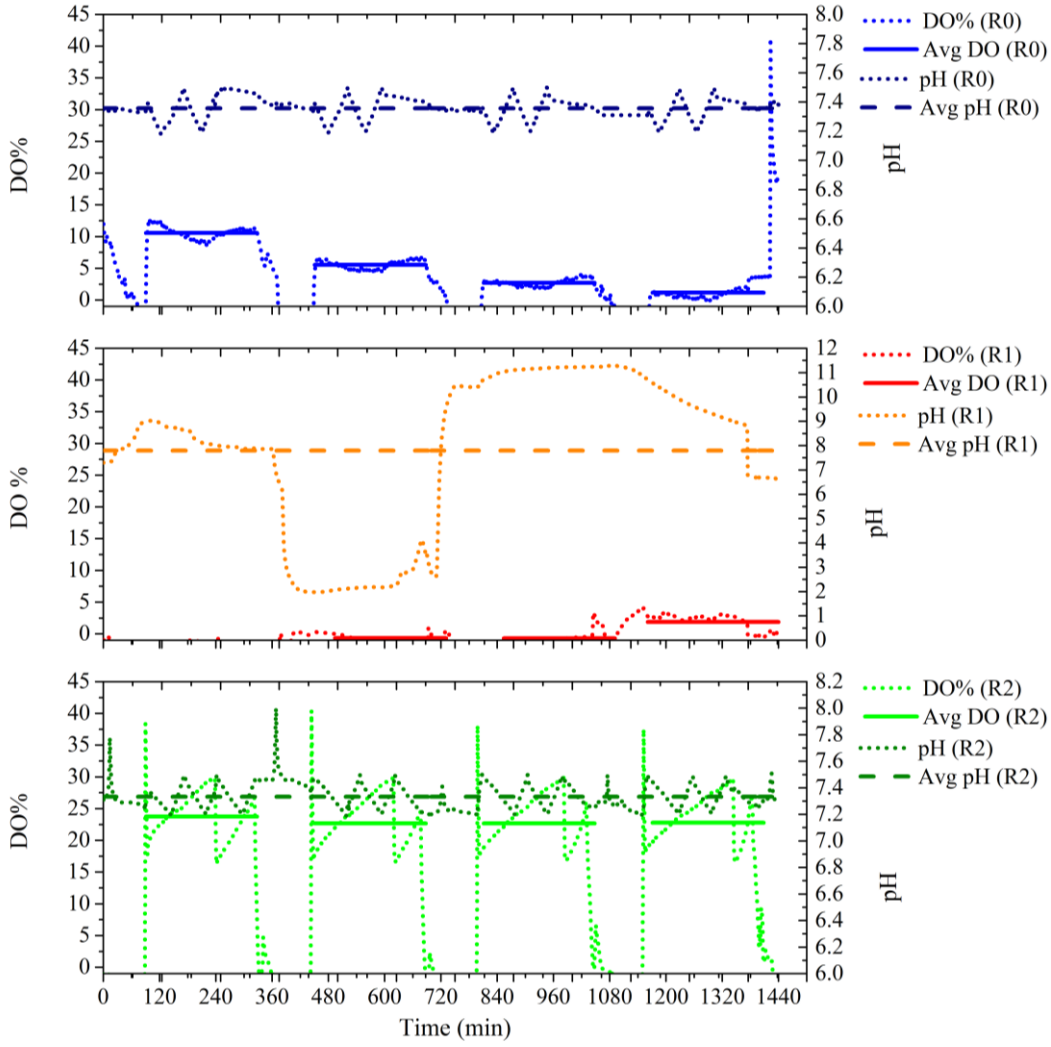

Figure 4.2 *DO and pH level in R0, R1 and R2 during four cycles at day−18 of AGS operation. The average DO during the aeration phase are shown while the average pH in the graphs were determined from the 4 cycles.*

At the end of this study, the average DO% in three reactors were observed to be in a range of the default values although it was a little bit higher in R1 and lower in R2. Problem with DO was also once happened in R0 at day−43 due to the detachment of aeration sparger for around 12 hours. However, the DO was still continuously controlled, resulting in the average DO% during the aeration phase to be 20.82 ± 6.68 %, 21.11 ± 7.06 % and 19.11 ± 8.3 % in R0, R1 and R2, accordingly. It is interesting to discover a different pattern of DO% changes at day−44 compared to day−1. There was a bigger gap in the fluctuation of the DO% values in the end of the study which could presumably show a better oxygen uptake by the microorganisms during the aeration phase. The DO% changes were also observed to be different between the reactors. In terms of pH, the values were able to be stable towards the end of the study although in day−44, the average pH in R2 was slightly lower. The average values for pH in this day were 7.58 ± 0.13, 7.34 ± 0.07, and 6.99 ± 0.12 in R0, R1 and R2, respectively.

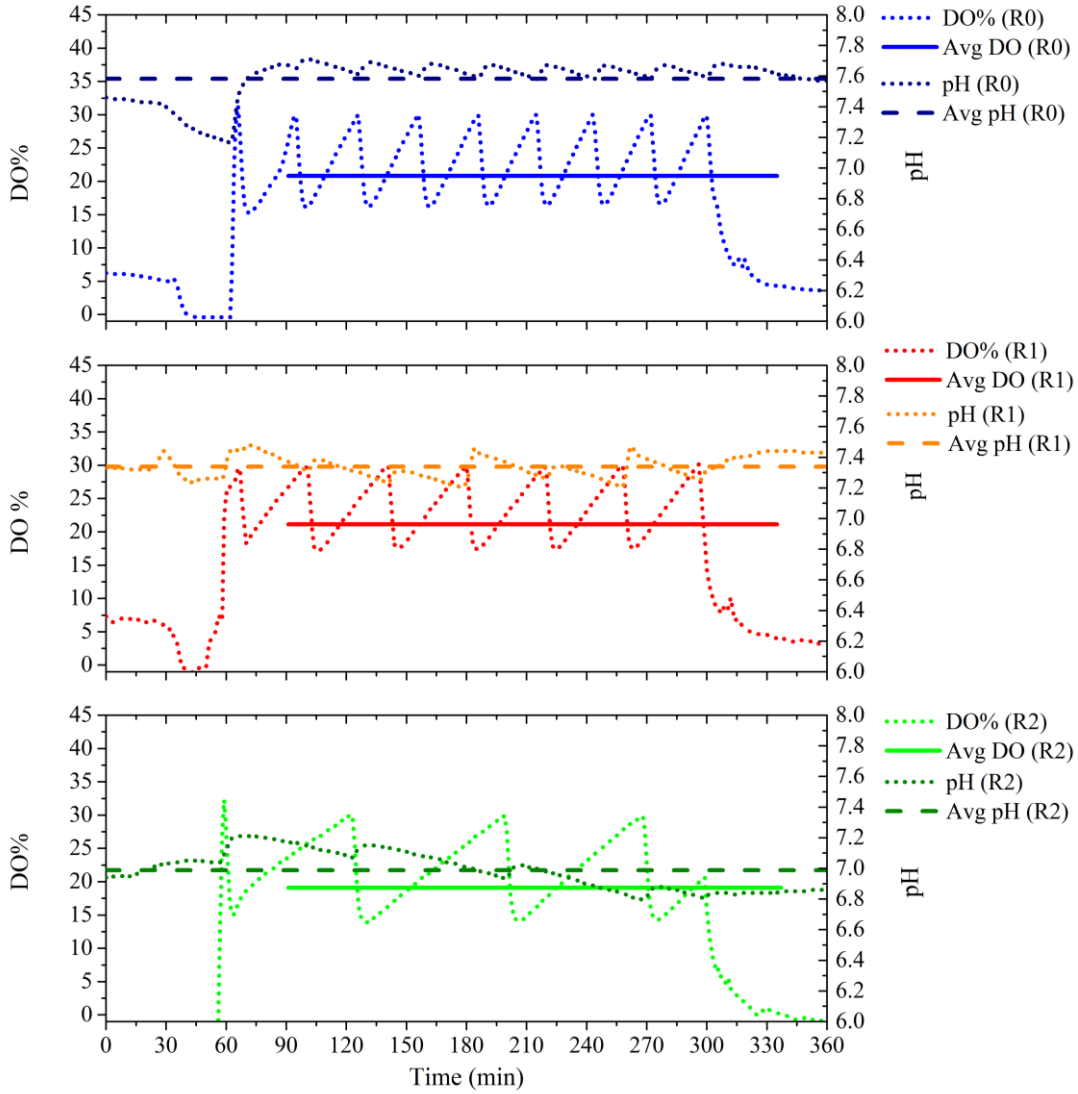

Figure 4.3 *DO and pH level in R0, R1 and R2 during four cycles at day−44 of AGS operation. The average DO during the aeration phase are shown while the average pH in the graphs were determined from the whole cycle.*

4.1.2 Solids retention time

The solids retention time (SRT) during the AGS operation was determined since the first five days and was further calculated every 7 days of observation. The results were shown in Figure 4.4 along with the changes of the settling time in three reactors. During this study, the settling time in all reactors could be reduced in a relatively short intervals based on the SRT ≥ 20 days and the AGS performance on the organics and nutrients removal. The average SRT in R0 during the whole operational days was 33 days, 28 days in R1 and 36 days in R2. In general, a decreasing trend of SRT was observed in all reactors in accordance to the reduction of settling time in each reactor. However, when the SRT was lower than 20 days which was spotted in R1 around day−33, the settling time was decided to be kept at 5 minutes compared to R0 and R2 which was lowered to 3 minutes. Therefore, the SRT in R1 was able to increase again. Towards the end of this study, the SRT in R2 also significantly decreased and later after this study, the settling time were set to be 5 minutes in all reactors to provide more time for the microorganisms to perform the conversion processes.

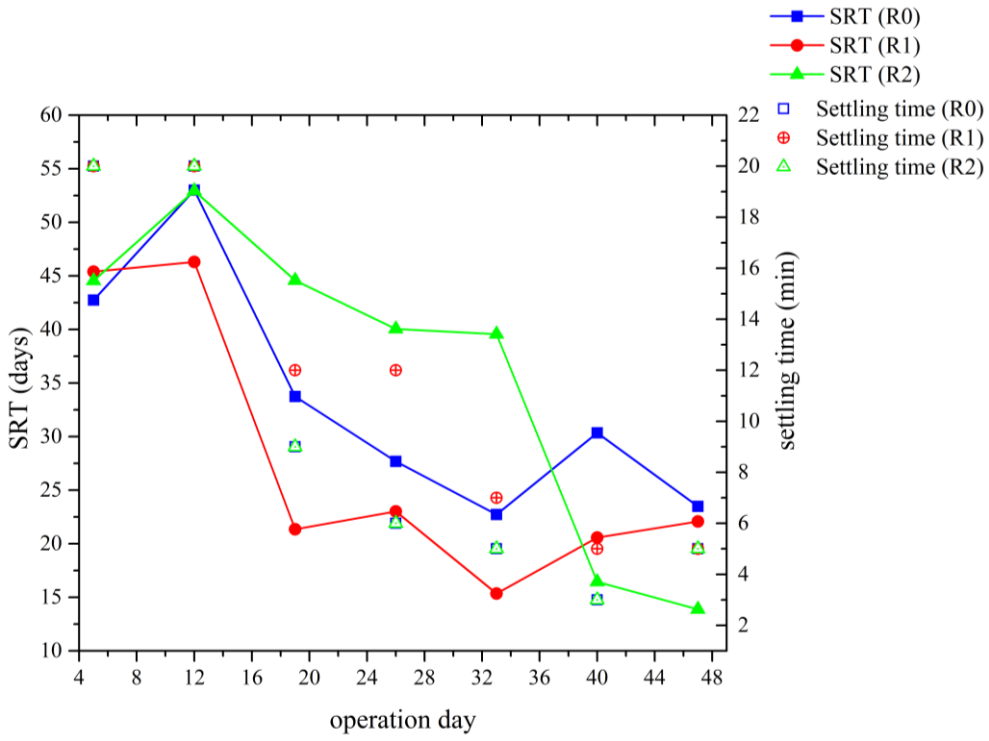

Figure 4.4 Calculated SRT and the changes in settling time during AGS operation in R0, R1 and R2.

4.2 Conversion processes

4.2.1 Influent

The results of TOC, TN and TP concentrations in the influent wastewater in a comparison with the target concentration are presented in Figure 4.5, Figure 4.6 and Figure 4.7, respectively. It was obviously noticed that all concentrations were fluctuating during the AGS operation. Therefore, the flow calibration in the substrates were routinely done in all reactors to adjust the pumps if the concentrations were higher

or lower than the target concentration. Towards the end of this study, the TOC, TN and TP influent concentration in all reactors were able to become more stable and closer to the target concentration of 110 mg TOC/L, 50 mg TN/L and 6 mg TP/L, respectively.

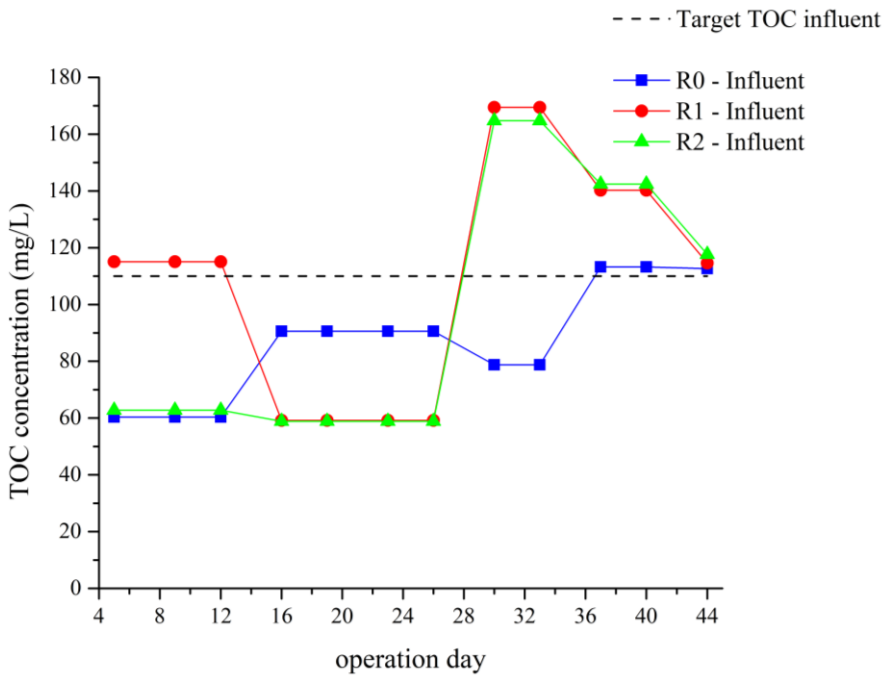

Figure 4.5 *TOC concentration in the influent wastewater in the three AGS reactors, compared to the target concentration of TOC.*

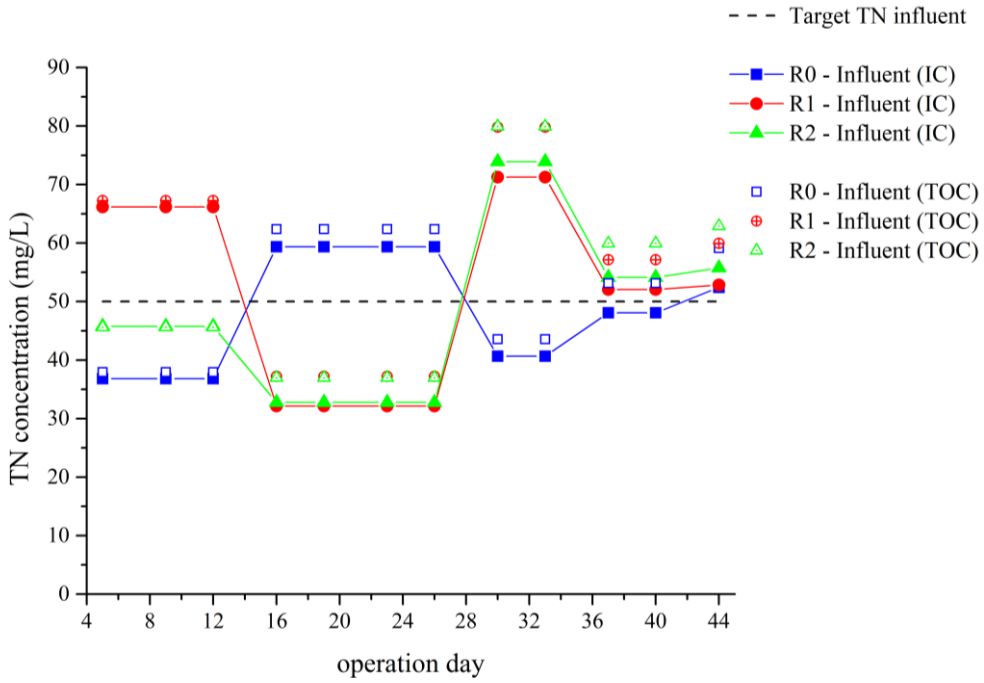

Figure 4.6 *TN concentration in the influent wastewater in the three AGS reactors which was measured by ion chromatograph (IC) and TOC analyzer. The values were compared to the target concentration of TN.*

The range of influent concentration during the study was 58 to 170 mg TOC/L, 32 to 80 mg TN/L and 4 to 15 mg TP/L. In terms of TOC concentration, a significant increase was observed between the measurement at day−26 and day−30 in R1 and R2 while the concentration also increased after day−33 in R0. As the problem of foaming in all reactors arose, the antifoam was starting to be added in the influent water at day−30. Therefore, it was assumed that the antifoam caused the higher TOC concentration in the influent. Then, the water samples from all reactors were taken and the results showed that the water dosed with the antifoam was given a TOC concentration of 38.2 mg TOC/L, 35.21 mg TOC/L and 32.75 mg TOC/L in R0, R1 and R2, respectively. Indeed, reduction of TOC concentration in the influent was observed after day−40 when the antifoam dosing was stopped. In terms of TN concentration, the results from the TOC analyzer were mostly higher than the measured concentration from IC.

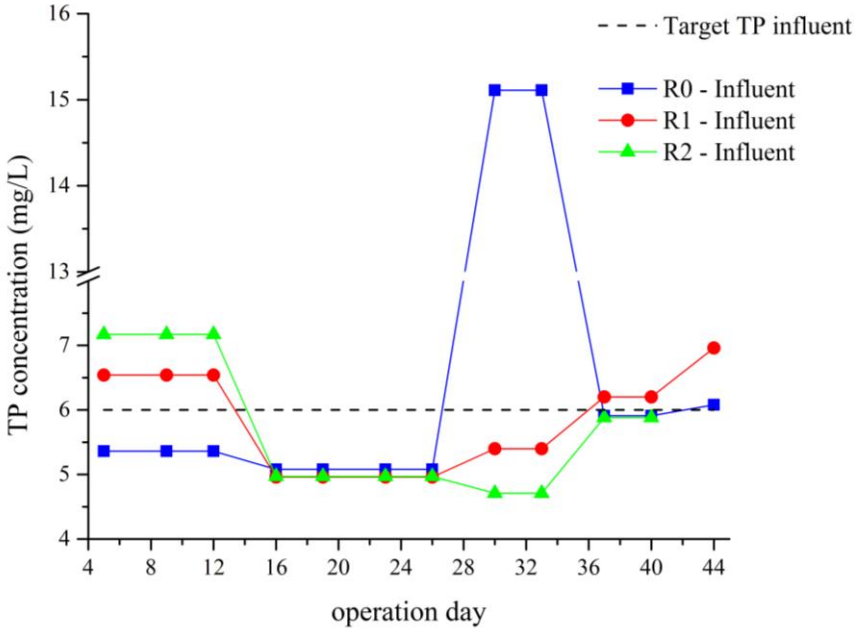

Figure 4.7 TP concentration in the influent wastewater in the three AGS reactors, compared to the target concentration of TP.

4.2.2 Conversion of organics

The results of TOC concentration and removal in the wastewater during the AGS operation are presented in Figure 4.8. The TOC concentration in all reactors during the anaerobic feeding phase were quite stable and mostly below 20 mg TOC/L in the beginning of operation and starting to increase after the measurement at day−26. The significant increase of TOC concentration in R1 was observed at day−19 from 9.32 to 69.16 mg TOC/L after the aeration problem occurred at day−18. After day−40, the TOC concentration in the anaerobic feeding phase was starting to reduce in all reactors. The effluent concentration of TOC during the first 26 days were in a range of 2.15 to 10.88 mg TOC/L, except the high TOC concentration in R1 at day−19. Between day−30 and day−40, the effluent TOC concentration increased in a range of 22.06 to 35.6 mg TOC/L but was able to decrease again at day−44 to 10.62 mg TOC/L, 4.09 mg TOC/L and 5.48 mg TOC/L in R0, R1 and R2, respectively.

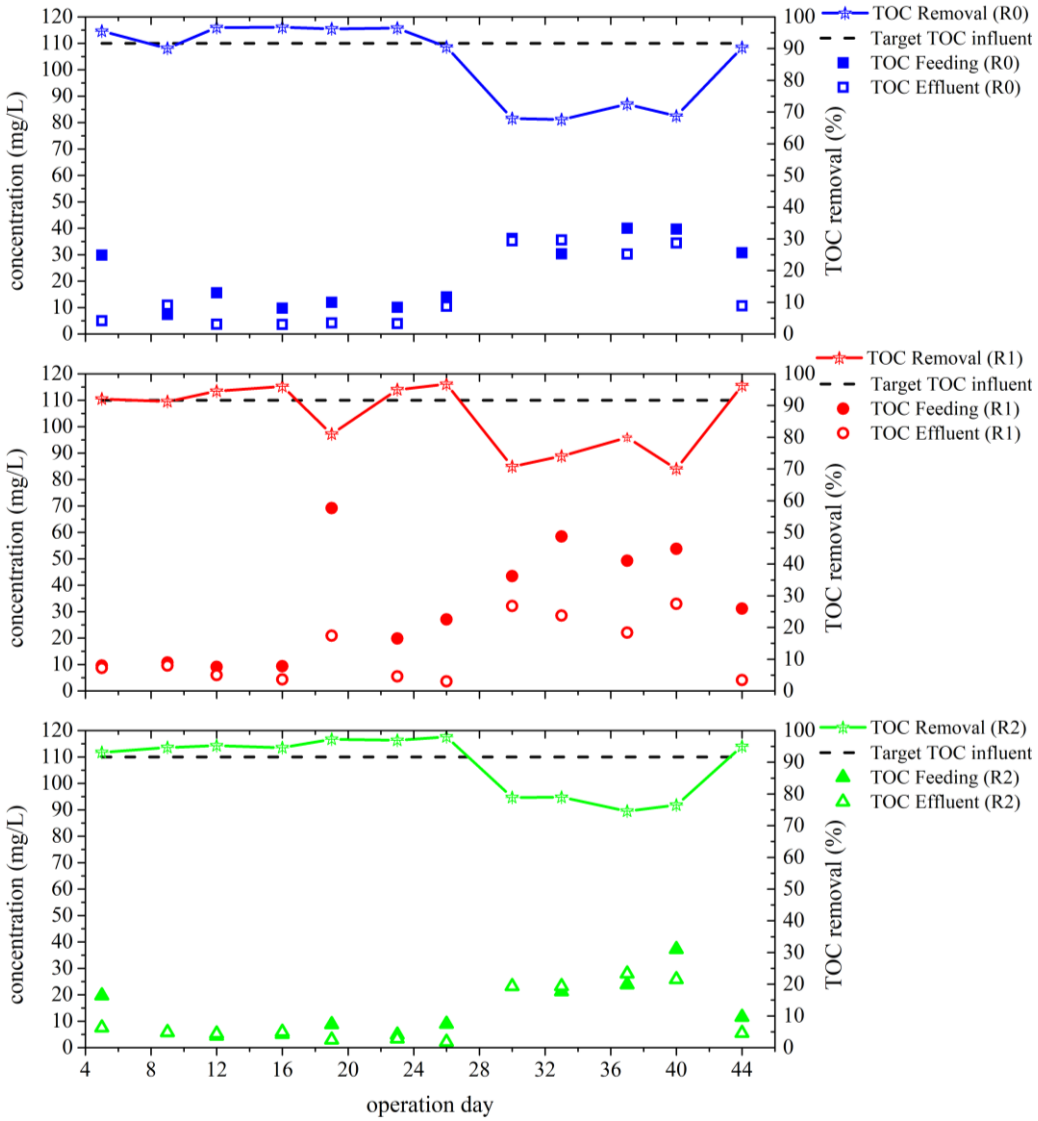

Figure 4.8 *The concentration of TOC in the wastewater at the anaerobic feeding phase and effluent in three AGS reactors, compared to the target TOC concentration in the influent. The secondary axis shows the percentage of TOC removal.*

In terms of the TOC removal, the AGS started with a high TOC removal percentage in the beginning of the operation in a range of 90.11 to 98.04%, except the 80.98% in R1 when the aeration problem occurred. Between the days when the antifoam dosing was introduced, the reduction of TOC removal was observed in all reactors in a range of 67.64 to 79.95%. However, the removal was able to increase to 90.35% in R0, 96.28% in R1, and 95.02% in R2 at the end of the study.

4.2.3 Conversion of nitrogen

Figure 4.9 presents the results of TN removal and nitrogen species concentration which consists of NH_4-N, NO_3-N and NO_2-N in the wastewater in the AGS operation, including the influent, anaerobic feeding and effluent. During the anaerobic feeding phase, the NH_4-N concentration was almost reduced by half in all reactors, except in R1 when the aeration problem started to occur. The average NH_4-N concentration in

the feeding phase during the AGS operation were 28 ± 5.5 mg NH_4-N/L, 30.8 ± 6.9 mg NH_4-N/L and 24.6 ± 4.5 mg NH_4-N/L in R0, R1 and R2, respectively. On the other hand, a relatively low NO_2-N concentration was also detected in the feeding phase with an average of 1.9 ± 0.6 mg NO_2-N/L in R0, 2.6 ± 0.6 mg NO_2-N/L in R1 and 2 ± 0.4 mg NO_2-N/L in R2. The NO_2-N conversion to NO_3-N was also started to occur slowly during the feeding phase, showing an average of 5.8 ± 2.1 mg NO_3-N/L, 2.9 ± 2.1 mg NO_3-N/L and 5.3 ± 1.1 mg NO_3-N/L in R0, R1 and R2, respectively.

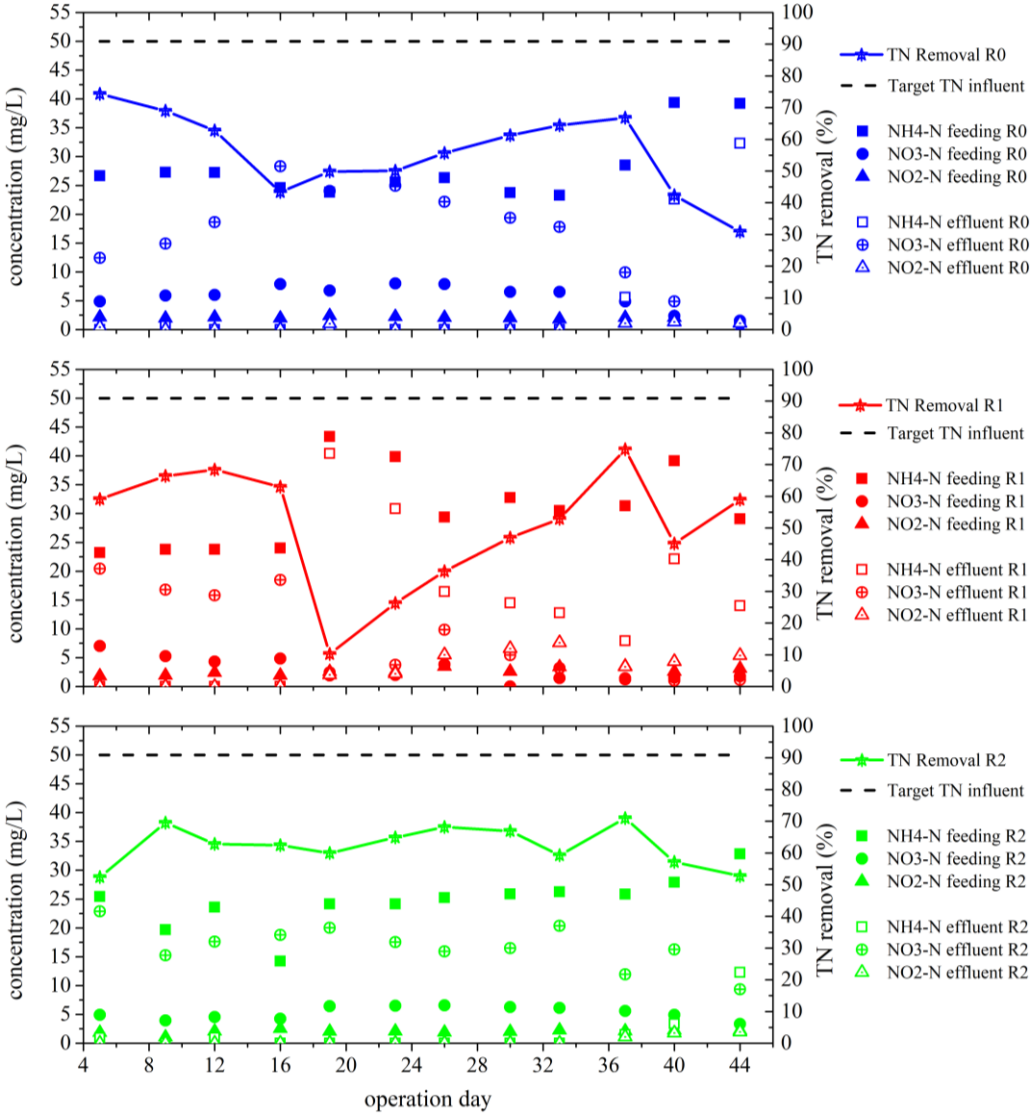

Figure 4.9 *The concentration of NH_4-N, NO_3-N, NO_2-N in the wastewater at the anaerobic feeding phase and effluent in three AGS reactors, compared to the target TN concentration in the influent. The concentrations were measured by IC. The secondary axis shows the percentage of TN removal.*

In the effluent concentration, there were mostly 0 mg NH_4-N/L in R0 and R2 until day−33 of AGS operation and the concentration started to increase, reaching 32.29 mg NH_4-N/L in R0 and 12.29 mg NH_4-N/L in R2. An exception in R1 in which the highest NH_4-N concentration was 40.42 mg NH_4-N/L in day−19 after the aeration problem

occurred but it was able to decrease to 14.01 mg NH_4-N/L in day−44. In terms of NO_2-N concentration, it was not detected in R0 and R2 up to day−33 of AGS operation and then it has been spotted since day−37 and increased to 1.1 and 1.97 mg NO_2-N/L in R0 and R2, respectively. In general, the NO_2-N concentration was maintained at a relatively low concentration and lower than the NO_3-N concentration, except in R1 when the aeration problem occurred. At the end of the study, the NO_2-N concentration in R1 was accumulated and reached 5.38 mg NO_2-N/L and only slightly converted to NO_3-N. Meanwhile, the NO_3-N concentration in the effluent started with an increasing trend but it decreased in all reactors since day−26, ending up with a low concentration of 1.18 mg NO_3-N/L, 1.15 mg NO_3-N/L, and 9.36 mg NO_3-N/L in R0, R1 and R2, respectively.

In terms of TN removal, R2 showed a relatively stable performance compared to R0 and R1 by a variation between 52 to 71% removal. The removal of TN in R0 was started with a decreasing trend but it was able to increase slowly to 66.77% at day−37 before it dropped to 30.87% at the end of the study. The nitrogen conversion was lost significantly in R1 when the aeration problem happened at day−19, falling to 10.23%. Then, the TN removal in R1 started to increase and reached the highest at 74.82% in day−37 and later it was fluctuating in the last two measurements. At the end of the study, the TN removal in all reactors were relatively low which ended up with 30.87%, 58.93% and 52.76% in R0, R1, and R2, respectively. The TN removal was also calculated based on the measurements by TOC analyzer, see Figure 4.10. The results showed that both TOC and IC measurements followed a similar trend in all reactors although overall, a higher removal was observed based on the IC analysis. However, at the end of the study, the TN removal based on the TOC analyzer was only slightly different than the IC analysis, reaching around 37.62% in R0, 60.52% in R1 and 50.93% in R2.

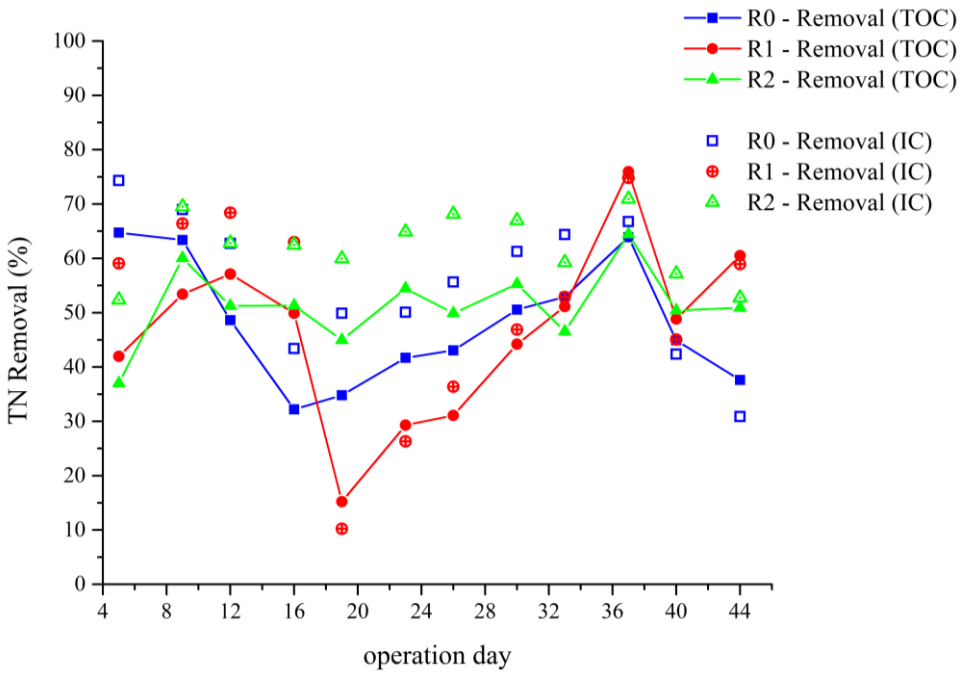

Figure 4.10 Comparison of TN removal (%) between the measurement by TOC analyzer and IC in three reactors.

4.2.4 Conversion of phosphorus

The results of TP concentration and removal during the AGS operation are presented in Figure 4.11. The release of soluble orthophosphate (PO_4^{3-}) to the solution by the PAOs were shown in the graphs by the TP concentration during the anaerobic feeding phase. At this phase, the released concentrations were varied in a range of 14 to 29.8 mg PO_4^{3-}/L in R0, 7.5 to 23.8 mg PO_4^{3-}/L in R1 and 13.6 to 24.5 mg PO_4^{3-}/L in R2. As the increasing trend of released TP concentration was observed in R0 and R2, MLSS samples were taken as much as 100 mL per day at day−36 in all reactors, except during the sampling days. As a result, the TP concentration in the feeding phase decreased in R0 from the highest at 29.84 to 13.98 mg PO_4^{3-}/L and 12.67 to 9.91 mg PO_4^{3-}/L in R1 at the end of the study. Meanwhile, it increased in R2 but it was still below 25 mg PO_4^{3-}/L at day−44.

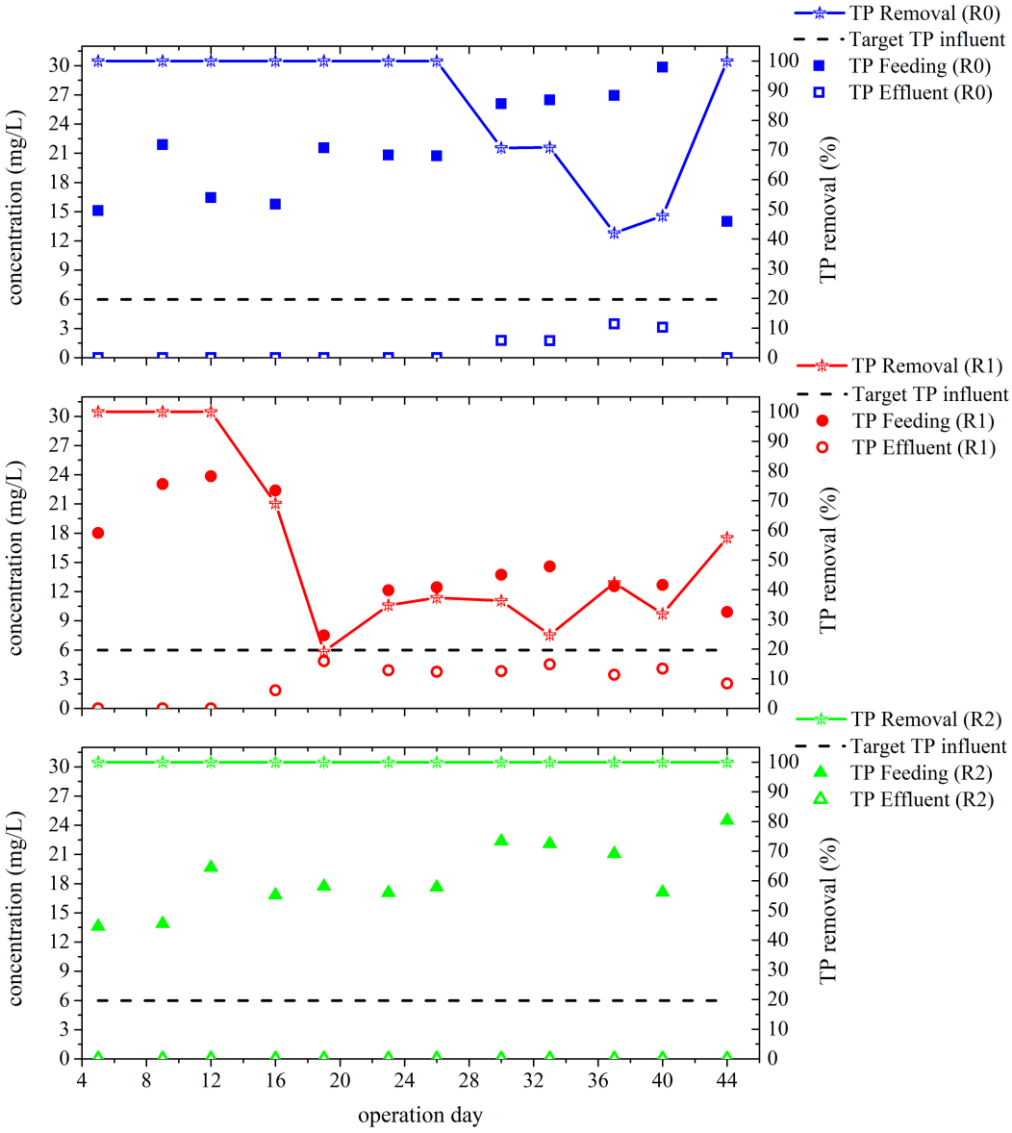

Figure 4.11 The concentration of TP in the wastewater at the anaerobic feeding phase and effluent in three AGS reactors, compared to the target TP concentration in the influent. The secondary axis shows the percentage of TP removal.

The effluent concentration of TP in all reactors were diverse which were also shown by the percentage of TP removal during the AGS operation. The R2 showed a stable performance of 100% Bio-P removal compared to other reactors. In R0, the Bio-P removal was decreasing between day−30 to day−40 from 70.73% to the lowest of 47.87%. However, it was able to recover in day−44 and reached 100% of TP removal. On the other hand, the TP removal was observed to drop from 100% to 69.08% at day−16 and it was falling off to 19.17% when the aeration problem occurred in R1. After that, it reached 57.52% of TP removal at the end of the study.

4.3 Granule development

4.3.1 TSS and VSS

Figure 4.12 shows the results of TSS or MLSS and VSS or MLVSS concentration in the reactor along with the settling time settings during the AGS operation. A decreasing trend was seen in the TSS and VSS concentration when the settling time was reduced. At day−1, all reactors started in a range of 6.32 to 7 g TSS/L and 5.67 to 6.22 g VSS/L and kept increasing within 9 days operation to the highest concentration of 11.98 to 15.9 g TSS/L and 10 to 13.23 g VSS/L. The settling time was gradually decreased from 20 minutes to the lowest at 5 minutes in R0 and R1 and 3 minutes in R2. In accordance with this condition, the TSS concentration in reactors were at a relatively low range: 3.28 to 6.7 g TSS/L in R0; 2.73 to 6.9 g TSS/L in R1; and 3.38 to 7.15 g TSS/L in R2. During the last study period, the VSS concentration was constantly slightly lower than the TSS concentration and also kept at a relatively low range: 3.35 to 5.85 g VSS/L in R0; 2.93 to 6 g VSS/L in R1; and 3.23 to 6.03 g VSS/L.

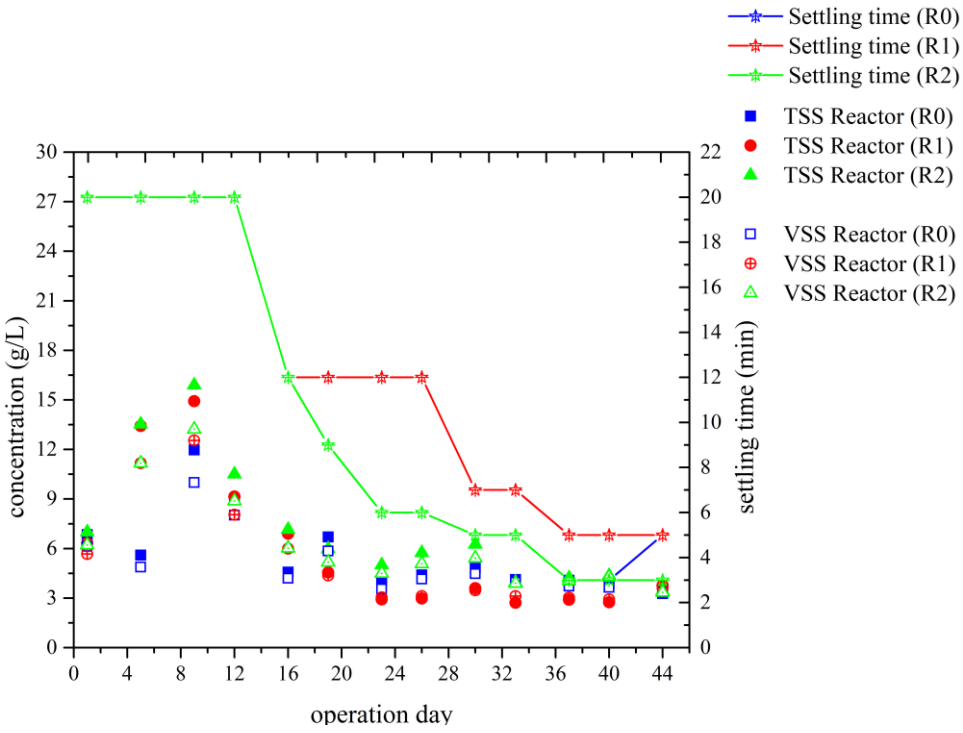

Figure 4.12 The TSS and VSS concentration in the reactors based on the analysis of the mixed liquor samples during aeration phase. The secondary axis shows the settling time during the AGS operation.

On the contrary, an increasing trend was observed of the TSS and VSS concentrations in the effluent and were in accordance with the reduced settling time during the AGS operation, see Figure 4.13. In this study, the blank test of TSS and VSS analysis were done by using the 0.2 µm pore size filter with a MQ water. The results of the weight loss from these filters were used to deduct the measured VSS concentration in the effluent. It was decided since the modified method in the study resulted in a very low concentration so that a small difference in the measured filter weight could give a higher VSS concentration. The average TSS concentrations in the first nine days of operation were very low: 0.021 ± 0.007 g TSS/L in R0; 0.025 ± 0.01 g TSS/L in R1; and 0.023 ± 0.005 g TSS/L in R2. After that, more biomass was flushed out in all reactors as the settling time was reduced. At the end of this study, the highest TSS concentration in the effluent was in R2 at 0.097 g TSS/L with the lowest settling time of 3 minutes. Meanwhile, the TSS concentration in R0 and R1 at day−44 were slightly different with 0.044 g TSS/L and 0.057 g TSS/L, respectively. In general, the VSS concentration in the effluent was maintained at a slightly lower concentration than the TSS.

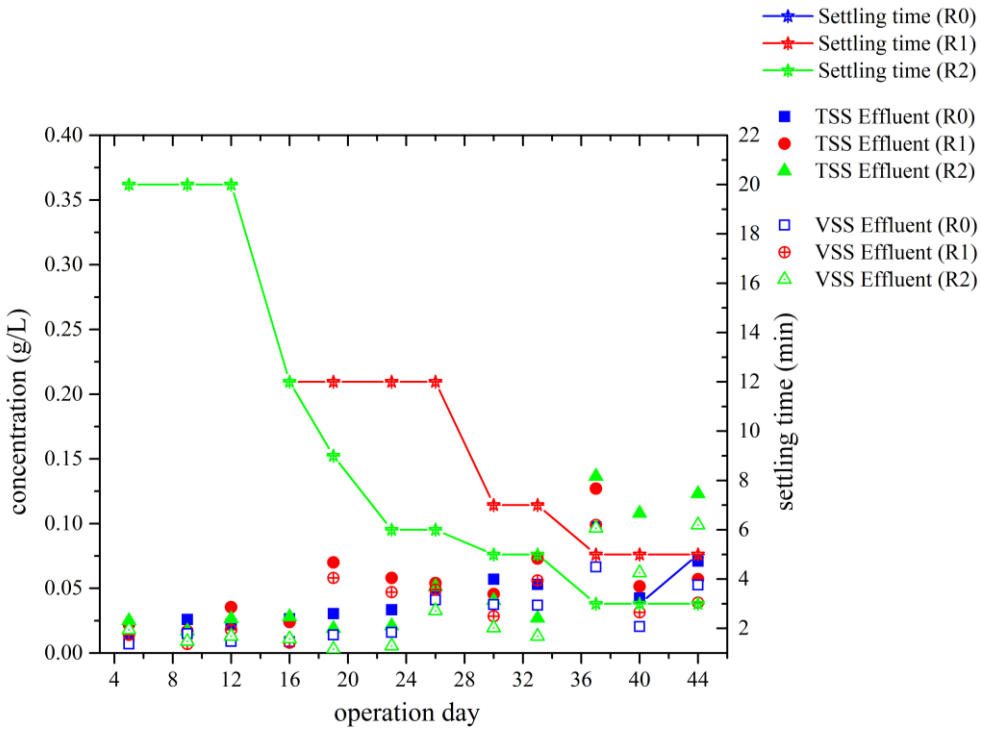

Figure 4.13 The TSS and VSS concentration in the effluent along with the settling time during the AGS operation.

4.3.2 Sludge volume index

The SVI in 5 minutes (SVI_5), 10 minutes (SVI_{10}) and 15 minutes (SVI_{15}) were monitored during the AGS operation, see Figure 4.14. In general, the SVI_5 in all reactors started to increase until the middle of the AGS operations to the highest values 129 mL/g, 258 mL/g and 113 mL/g in R0, R1 and R2, respectively. A significant increase of the SVI_5 in R1 occurred after the aeration problem happened at day−19. After that, the values were decreasing and reached a similar value as SVI_{15} in R0 and R1 meanwhile it was slightly higher in R2. As a result, an increasing trend was observed in the ratio of SVI_{15} to SVI_5 in all reactors. At the end of the study, the SVI_5 and SVI_{15}

were measured to be 39 mL/g in R0 and 27 mL/g in R1. On the other hand, the SVI_5 in R2 at day−44 was 76 mL/g and the SVI_{15} was 67 mL/g.

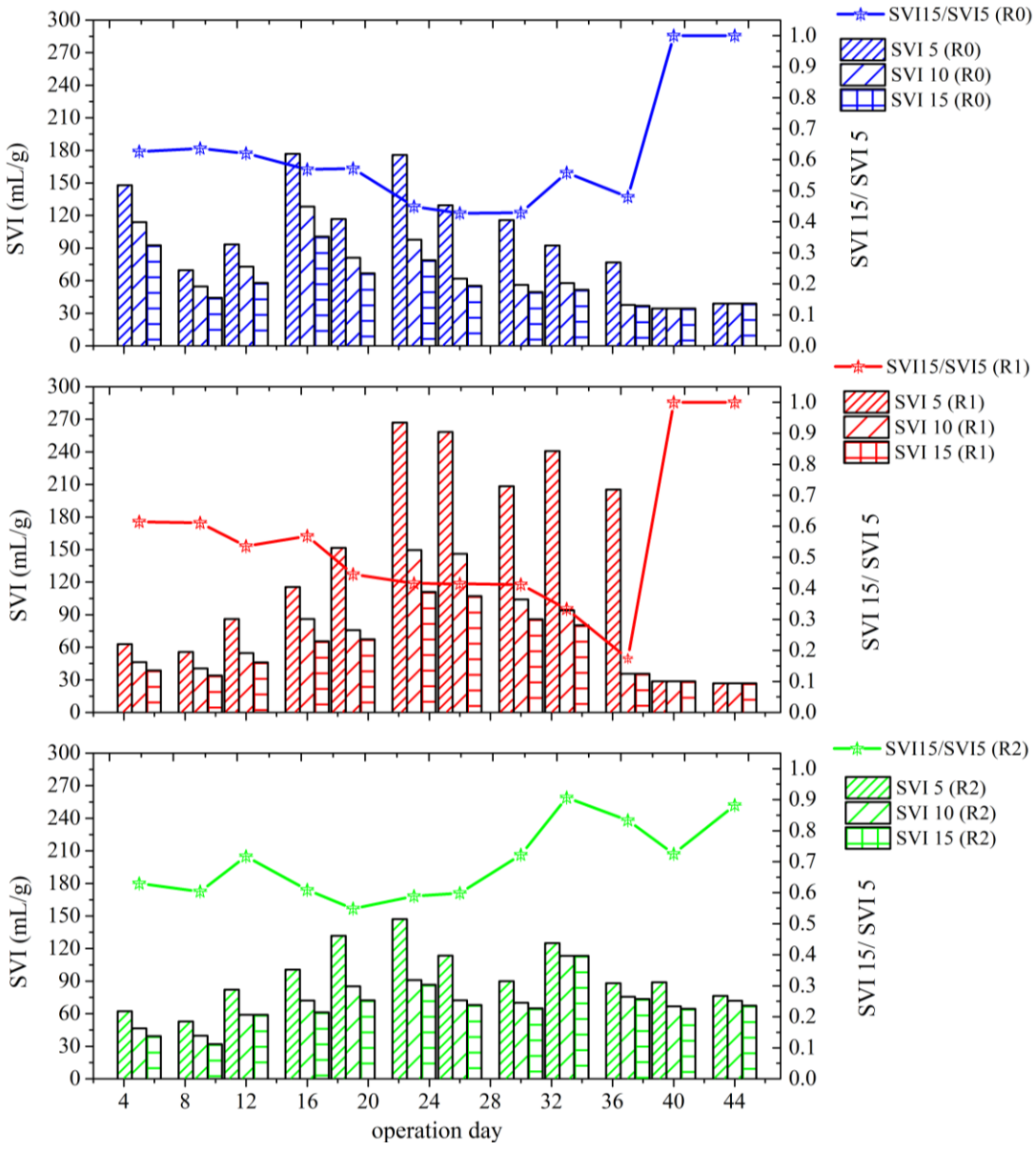

Figure 4.14 The SVI values consist of SVI_5, SVI_{10} and SVI_{15} in three reactors along with the ratio of SVI_{15} to SVI_5 in the secondary axis.

4.3.3 Granule morphology

Figure 4.15 shows the observation under the light microscope of the seeding sludge from the AGS plant at Österröd WWTP. A compact and dense structure of the granules was observed with an extensive amount of extended filaments surrounding the granules outer surface. Moreover, an excessive blooming of sessile ciliates (likely as *Vorticella* spp., *Carchesium* spp. and *Epistylis* spp.) (De Kreuk *et al.*, 2010) was spotted in the seeding sludge. In terms of the colour, the significant changes were seen in all reactors from the brown granules to light-yellow granules, see Figure 4.16. At the beginning of the study, the appearance of round-shape and large granules was dominating in R1 and

R2 while it was irregular and smaller in R0. After 44 days of the start-up operation, small to medium sized granules were similarly shown in R0 and R1 with a dense and compact structure. On the other hand, loose-structured granules were observed in R2 but they were mostly larger than the granules in R0 and R1.

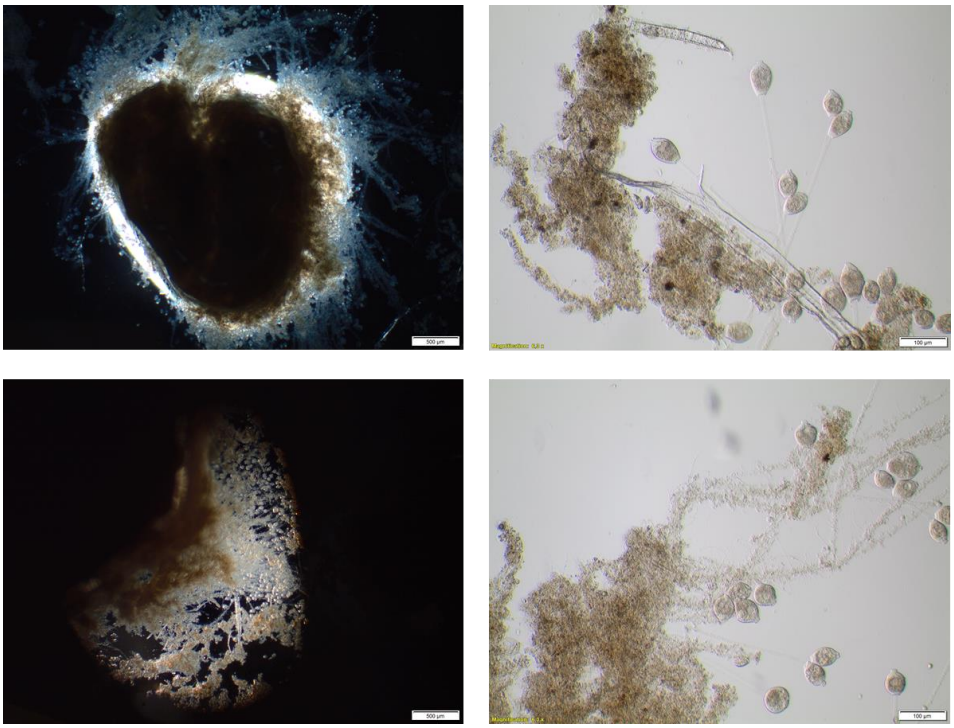

Figure 4.15 The observation of AGS morphology which was used as the seeding sludge under the light microscope with 2x magnification (left-hand side) and 10x magnification (right-hand side).

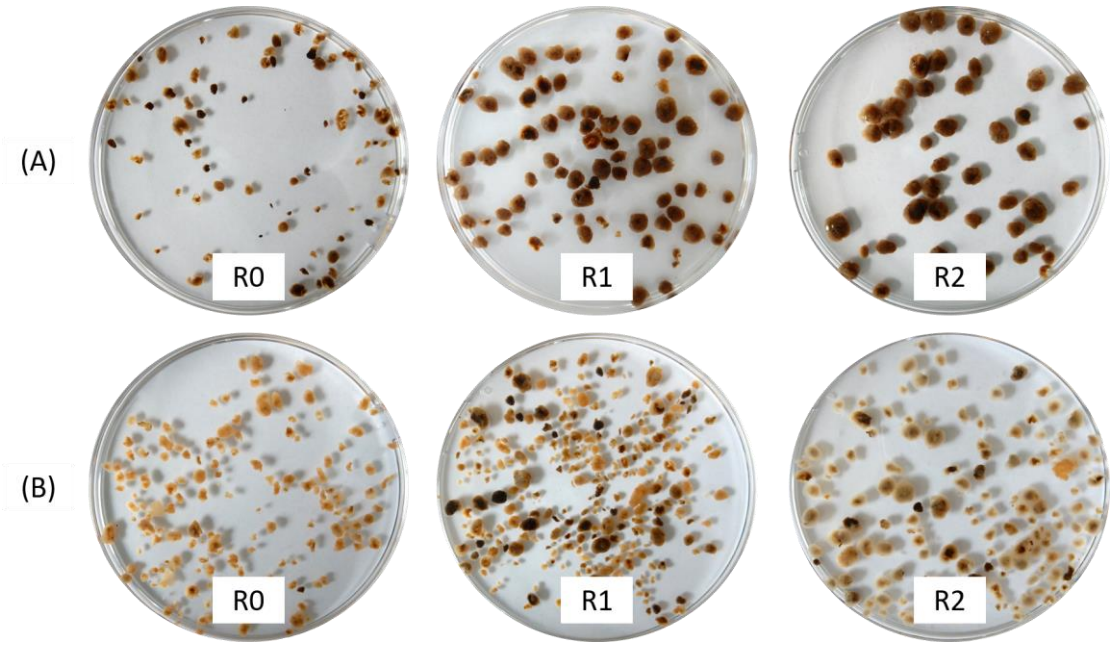

Figure 4.16 The observation of granules morphology: (A) at the beginning of the study (day−14) and (B) at the end of the study in three reactors (day-43).

The detail observation from the microscopy images in R0, R1 and R2 are presented in Figure 4.17, Figure 4.18, and Figure 4.19, respectively. The monitoring was done once a week during the AGS operation. In a contrast with the seeding sludge, dense granule with smooth surfaces was observed in all reactors at least until day−28 or day−35 of the start-up. An exception condition in R2 in which the granules started with the filamentous growth on the surface at day−7. At the end of the study, a more fibrillose or finger-type structure of granules was observed in R0. Meanwhile, filamentous fungi around granules were spotted in R1 at day−42. A relatively smooth granules surface with a small amount of extended filaments was observed in R2 at day−42. However, they changed into fluffy granules and small black granules could be spotted inside the granules. Moreover, more of floccular sludge was predominant in R2 since day−30.

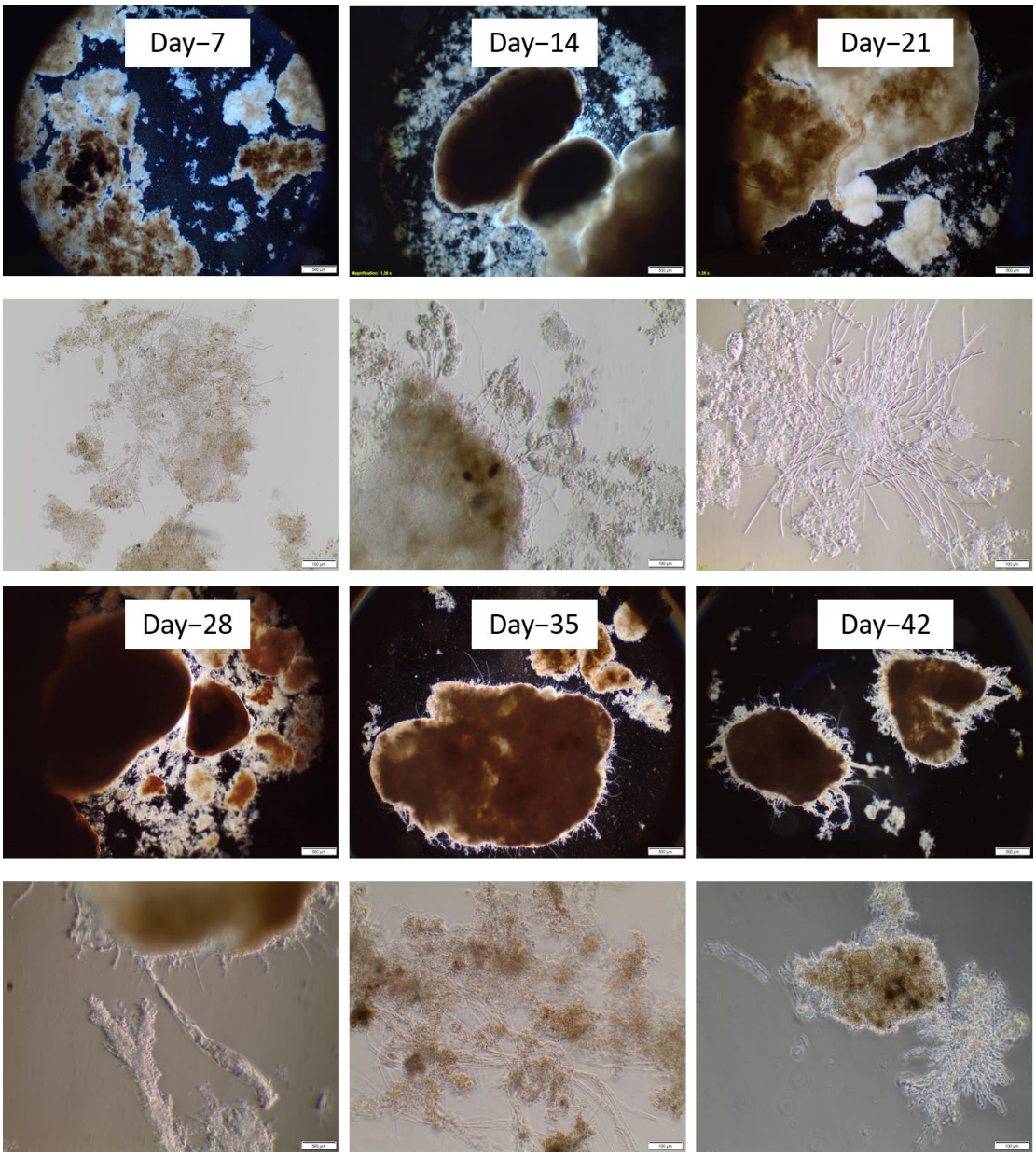

Figure 4.17 The observation of granules morphology in R0 by light microscope with 2x magnification (1st and 3rd row) and 10x magnification (2nd and 4th row).

Based on the 10x magnification of microscopy images, the variation of microbiota could be observed. For instance, the appearance of filamentous bacteria was observed in R0 at day−21, see Figure 4.17. The presence of protozoa was predominant in the all reactors since the beginning of the start-up such as the sessile ciliates (likely as *Vorticella* spp., *Carchesium* spp. and *Epistylis* spp.). The metazoan was also likely spotted such as the rotifers. *Zoogloea* was also observed under the microscope in all reactors. The filamentous fungi started to be spotted in R1 at day−42, see Figure 4.18. Further details of microscopic images were also taken in 20x magnification can be seen in Appendix A.5 – microscopic images of 20x magnification.

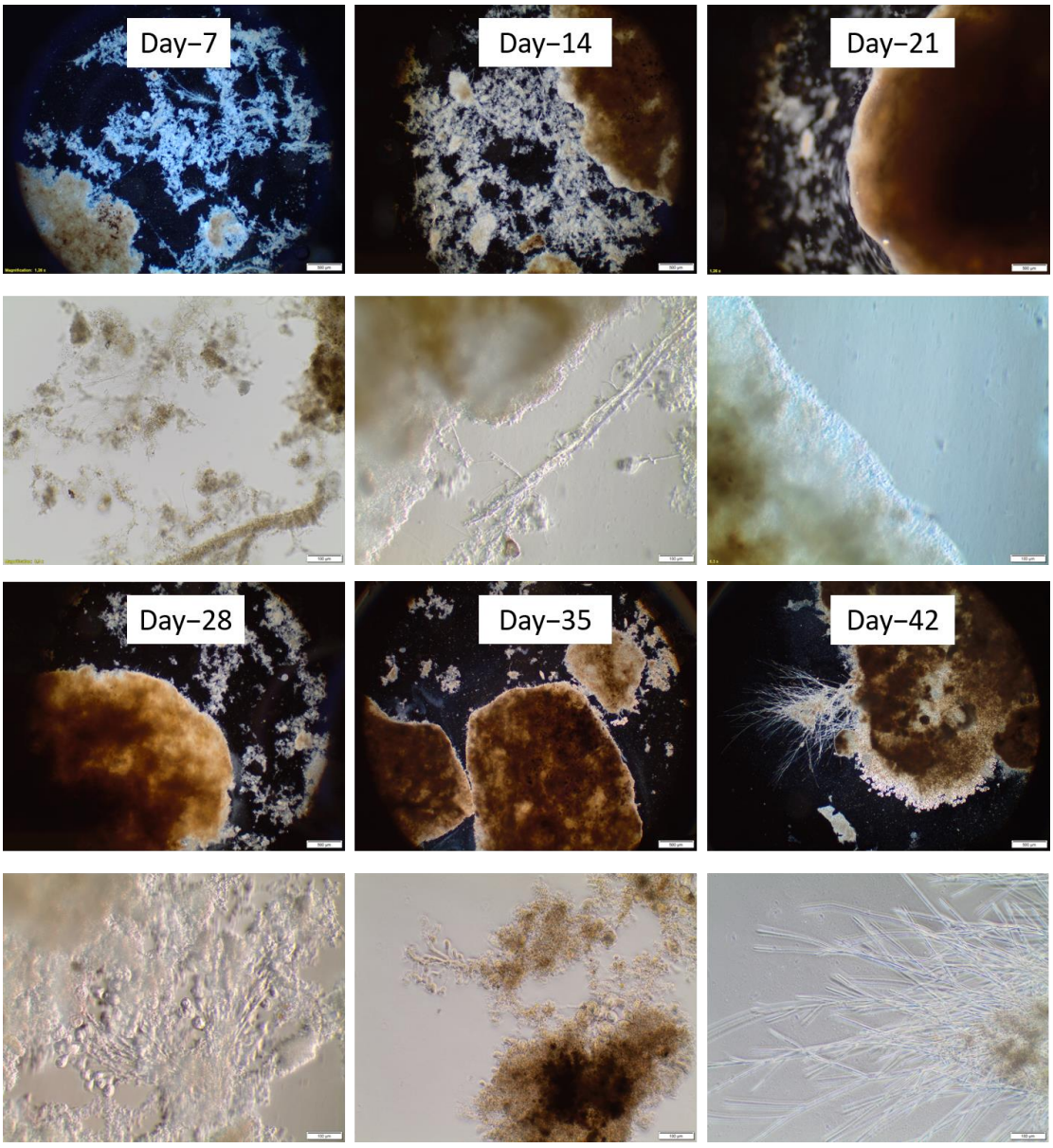

Figure 4.18 The observation of granules morphology in R1 by light microscope with 2x magnification (1st and 3rd row) and 10x magnification (2nd and 4th row).

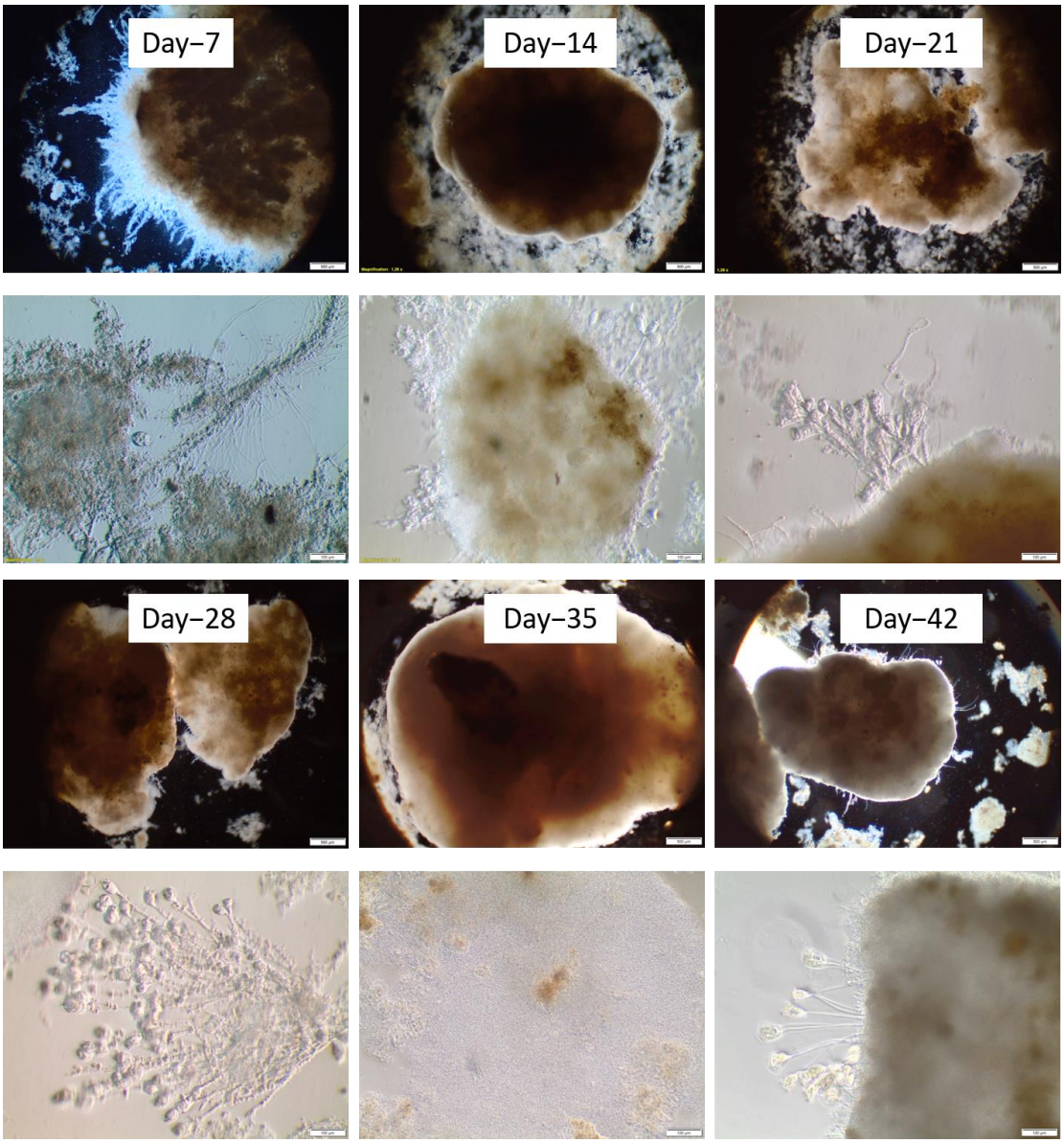

Figure 4.19 The observation of granules morphology in R2 by light microscope with 2x magnification (1st and 3rd row) and 10x magnification (2nd and 4th row).

4.3.4 Granule size

The diameter of the granules in the three reactors were measured based on the average of 150-300 granules which were detected by using ImageJ analysis from five pictures selected from 10 to 20 pictures. The seeding sludge had the average diameter of 2.3 ± 1.6 mm at the beginning of the study. After 14 days of operation, larger granules were observed in R1 and R2 with the largest diameter of 4 ± 1.4 mm and 4.7 ± 1.3 mm, respectively. In the same period, the granules size decreased in R0 to 1.7 ± 1.3 mm. Towards the end of the study, the granules size in all reactors tend to be similar although it was still higher in R2, see Figure 4.20. Overall, the averages of the granules diameter during the study period were 2.21 ± 0.32 mm in R0, 2.94 ± 0.56 mm in R1 and 3.67 ± 0.85 mm in R2. Table 4.1 presents the circularity and the maximum diameter of the granules during the AGS start-up operation. on an average, the shape of the newly

developed granules were maintained to be similar to the seeding sludge with a moderate circularity (Blott and Pye, 2008). The largest size of the granules was observed to be 6.85 mm in R0 and 7.97 mm in R1 and R2.

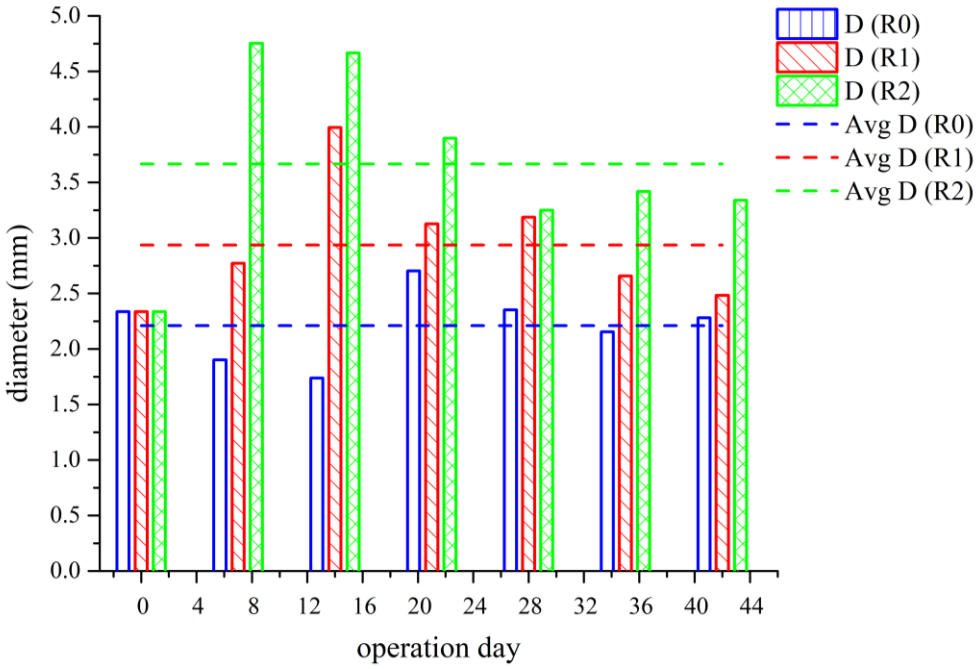

Figure 4.20 The development of granules sizes in three reactors by ImageJ analysis. The diameter (D) of the granules were measured and the average of these values during 44 days of the start-up are shown as Avg D.

Table 4.1 The measurements of circularity and maximum diameter of granules.

Operation day	Circularity			Maximum diameter (mm)		
	R0	R1	R2	R0	R1	R2
0	0.71	0.71	0.71	6.85	6.85	6.85
7	0.70	0.69	0.80	5.05	7.83	7.03
14	0.64	0.68	0.70	5.00	6.33	7.97
21	0.69	0.69	0.67	4.87	7.48	7.08
28	0.64	0.61	0.62	5.56	7.97	6.80
35	0.61	0.65	0.61	4.68	5.19	6.88
42	0.64	0.62	0.79	4.83	5.20	6.28
Average	0.66	0.66	0.70	5.26	6.69	6.98

The size distribution of the granules is presented in Figure 4.21 which was based on the cumulative distribution by using 0.1 mm intervals of the measured granules diameter. The results showed that the volume based median sizes of the granules were varied

between the three reactors with a range of 2.5 to 3.3 mm, 3 to 4.75 mm and 3.3 to 5.5 mm in R0, R1 and R2, respectively. A different trend was also observed among three reactors. At the end of the study, R0 still had around 0.79% of the smaller flocs less than 0.2 mm while the smallest size was observed to be 1 mm in R1 and 1.1 mm in R2. Around 30% of the smaller flocs less than 2 mm was shown in R0 at end of the study while smaller portion showed in R1 with around 10% and 4% in R2.

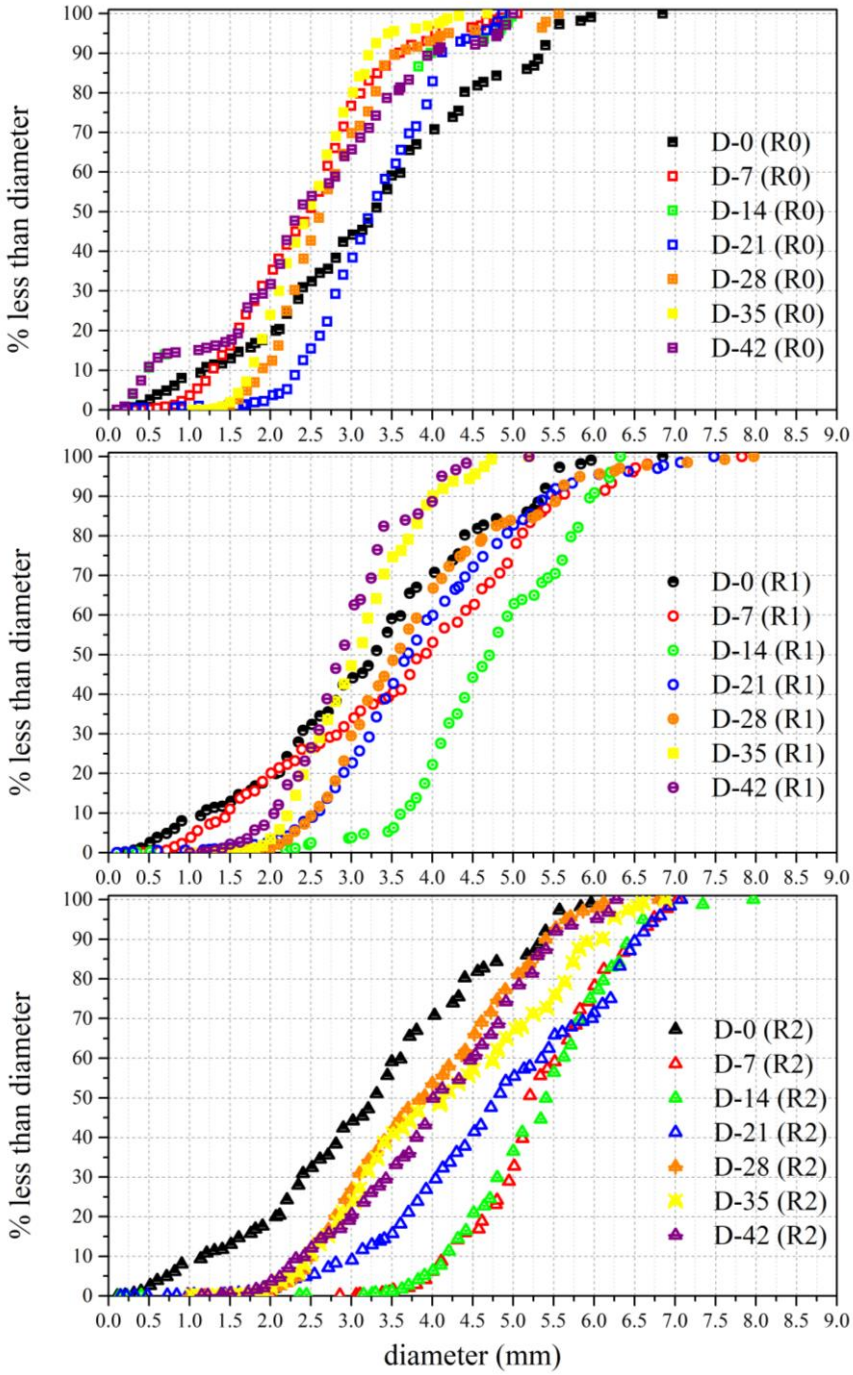

Figure 4.21 The cumulative size distribution by volume for granules in three reactors during the AGS start-up operation.

5 Discussions

5.1 AGS start-up operations

5.1.1 Dissolved oxygen

Based on the SBR design for the AGS operations, see Table 3.1, DO was set to 20 ± 10 % for all reactors in the aeration phase. The DO monitoring data from the randomly selected cycle showed that the average DO% in all reactors were in a range of SBR operational settings and close to 20% during the aeration phase, except in the middle of AGS operations in R0 and R1. However, some disturbances should be taken into consideration regarding the DO level during the operations. First, the DO level was not controlled in all reactors between day−2 and day−5 as the nitrogen gas was over at the end of the 1st day of operations. During this period, foaming was observed in R0 and thus the foam traps were installed in all reactors on day−6 to avoid problems with the gas pump.

Second, during the DO calibration until the 16th day of the AGS operations, the DO sensor in R0 showed a relatively low percentage in a range of 35% to 60% readings when it was exposed in the air. Therefore, the adjustment of the DO settings was frequently done in R0 to match the 100% readings. This problem seems also occurred in the previous project which explained a drift-up problem of the DO sensor when it was exposed to a DO level of less than 20% (Calgaro and Trieb, 2020). As a result, a higher DO level was presumably exposed in R0 compared to other reactors during this period. This problem was getting worst on day−18 when the DO level kept decreasing during the aeration phase in the whole day operations with an average of 4.99 ± 4.13 %. Based on the monitoring data, the air flow was observed to be continuously injected into the reactors since the system may read a low DO% in the reactor. This condition may probably explain the accumulation of a higher DO level in R0 since the beginning of the study, resulting in an oversaturated oxygen level in the reactor and creating lower DO% readings from the DO sensor. Therefore, the DO sensor in R0 was switched at day−18 and better DO% readings were observed towards the end of the study.

Third, a disturbance also occurred in R1 on day−18 of operation. During this day, there was no aeration in the reactor for approximately 33 hours due to the detachment of the aeration sparger at the bottom of the reactor. As a result, the anaerobic condition was continued and had a huge impact on the AGS performance. The investigation was done and it was found out that the gas filter clogged due to the foams produced from the reactor, see Figure 5.1. There were also some little granules inside the foam trap. This condition created high pressure within the reactor due to the closed air circulation system. As a result, the aeration sparger detached from the bottom of the reactor, creating an anaerobic condition for a relatively long time. The DO was not controlled in R1 for 9 days to prevent a similar problem due to the foaming that was still observed in the reactor. A similar condition also once happened on day−43 in R0 in which it stayed in anaerobic condition for around 12 hours due to the detachment of aeration sparger. However, the DO was still controlled later after the accident.

Overall, the DO levels may reflect reliable values in R2 meanwhile in R0, the DO% readings have been stable since the 19th day of operation. On the other hand, the DO level in R1 was adequate except in the day when the aeration problem occurred. It is also interesting to see the difference in the DO% between the 1st day and the 44th day

of the AGS start-up. Figure 4.1 shows that the DO% in all reactors reached close to the highest (30%) and the lowest (10%) limit of the DO settings. The reason behind this could be that the nitrogen gas was almost completely consumed on the first day of the operation. Meanwhile, it should be also considered that based on the monitoring data, the DO levels were mostly in a range between 15% to 30% in all reactors. Hence, the DO% average could be slightly higher than 20% during the aeration phase. Towards the end of the study, a bigger interval was observed between the lowest and the highest DO% in all reactors. This condition was similar in R0 and R1 while the biggest interval was shown in R2, see Figure 4.3. The results could show a faster oxygen consumption in R2 while it was slower in R0 and R1 which may relate to the AGS performance in each reactor.

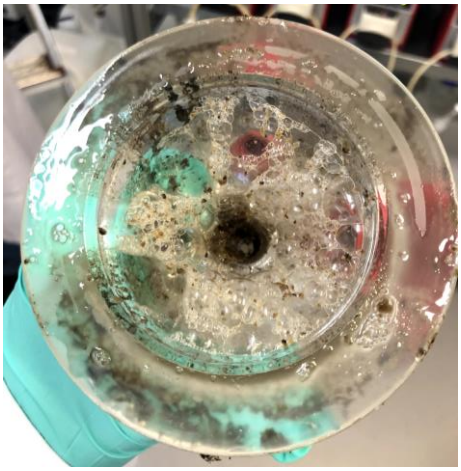

Figure 5.1 The foams at the top of the reactor R1 on the 18th day of AGS operation.

Studies found that the dissolved oxygen has an important role within the AGS process (De Kreuk et al., 2005; Sturm and Irvine, 2008; Yuan and Gao, 2010). As it is mentioned in Section 2.3.4, the oxygen provides aerobic condition which is required in the organics and nutrients removals through mechanisms such as aerobic oxidation, SND, nitrification, and internal storage of soluble orthophosphate. In this study, the importance of DO was reflected during the disturbance in R1 with the absence of aeration for 33 hours. In this period, the TN and TP removal significantly dropped about 53% and 50%, respectively. In a contrast, only a 15% reduction was observed in TOC removal. This condition could be similarly explained by Mosquera-Corral et al. (2005) which found that a short-term oxygen reduction did not influence the acetate uptake rate. On the other hand, many AGS studies focused on investigating the DO effect on nitrogen removal (De Kreuk et al., 2005; Lochmatter et al., 2013; Sturm and Irvine, 2008; Yuan and Gao, 2010). Related findings have been presented that a higher DO enhances nitrification while a lower DO favour the denitrification process (Beun et al., 2001; De Kreuk et al., 2005; Pronk et al., 2015a). Further discussions on nitrogen removal are discussed in Section 5.3.2.

Although the granulation process has been known to happen in a wide range of DO (Winkler et al., 2018), many AGS start-up studies performed in a relatively higher DO level or even an uncontrolled DO (100% saturation) (De Kreuk et al., 2005; Jiang et al., 2021; Lochmatter et al., 2013; Mosquera-Corral et al., 2005). For instance, a study by Lochmatter and Holliger (2014) reported that the aeration strategy with a constant high

DO (60% saturation) could improve nitrification and SVI values while by gradually decreasing DO from 50% to 30% during the aeration phase in a cycle, positively impacted the nutrients removal. As a result, it led to a rapid AGS start-up with a combination of other operational conditions such as settling time, adaptation of contaminant load, temperature and pH. In a contrast, a relatively lower DO (20% saturation) was used in this study and other research (De Kreuk et al., 2010). The overall results on the AGS performance showed a potentially good average efficiencies on TOC removal (86−89%), TN removal (51−62%) and TP removal (54−100%) even with some disturbances during the experiments and a stable operation has not been achieved. In a specific AGS process, the applied DO concentration also influences the oxygen diffusion limitation within the granules which has been further known to affect the granules morphology and stability (Wilén et al., 2018).

5.1.2 pH

pH is one of the crucial parameters in the AGS as part of the biological treatment process since it can affect microorganisms' activities and the treatment performance (Lashkarizadeh et al., 2016; Jiang et al., 2019). In this study, the pH was set to be 7.5 ± 0.3, and based on the monitoring data, the pH values were relatively stable in a range of operational settings. A minor fluctuation was observed at day−44 in R2 which showed a slightly lower pH around 6.99 ± 0.12 due to the absence of a base solution for a short while. However, a major disturbance arose in R1 on day−18 as is explained in Section 4.1.1. Due to the absence of mixing and the automatic adjustment of pH was continued in the reactor, leading to a pH shock in R1 which was exposed to a pH of 1.98 and the highest pH of 11.32. before it was stabilized to neutral pH. Based on the system, the alkaline shock (pH above 8) was continued around 11 hours whereas the acidic shock (pH below 6) was believed to be more than 6 hours since the pH sensor did not properly read the real values.

Few AGS studies reported the investigation of pH shock in AGS systems with relatively extreme pH values but mostly during a few days (Lashkarizadeh et al., 2016; Jiang et al., 2019; Zhang et al., 2021). For instance, Lashkarizadeh et al. (2016a) compared AGS performance between alkaline (pH=9) and acidic (pH=6) pH shocks during 6 days of operation. The results showed that the high pH had a huge impact on N and P-removal with a reduction to 66% and 50%, respectively, with no further recovery. The alkaline shock also created granules breakage while only a slight impact was observed at low pH. Although it was not intended in this study, the AGS could recover relatively fast to reach the highest TOC removal of 97% within 7 days and TN removal of 75% in 18 days. However, it did not show a stable Bio-P removal until the end of the study. On the other hand, granulation seems to occur in spite of the pH disturbance with the observation of dense and large granule size with an average diameter of 2.94 ± 0.56 mm. Although at the end of this study, fungi were observed under the light microscope in R1, see Figure 4.18. Fungi have been known to favour a low pH environment (Yang et al., 2008) and many of them can grow in a wide range of pH of 3.0−9.0 (Deacon, 2013). Thus, it may be assumed that the extreme acidic pH could have triggered the growth of fungi whereas it prevents the growth of most bacteria.

Overall, despite the major pH disturbance in R1, pH values in all reactors are supposed to be reliable. In a previous similar project by Calgaro and Trieb (2020), the pH was set slightly lower in the neutral pH of 7 ± 0.3. The AGS start-up was conducted in 29 days

and resulted in high TOC, TP, and TN removals with average efficiencies of 92%, 81%, and 86%, respectively. In this study, the pH was in a range of 7.5 ± 0.3. A study by Lochmatter and Holliger (2014) concluded a neutral pH (7−7.3) showed a faster AGS start-up and highly impacted better nitrification while the AGS runs with a higher pH (7.5−7.8) favoured the Bio-P removal. The findings similarly explained that a higher selection of PAOs was found in a slightly alkaline environment (pH > 7.3) (Weissbrodt et al., 2013) whereas a pH range of 7−8.5 had been commonly reported to perform stable nitrification values (Painter and Loveless, 1983; Antoniou et al., 1990). Therefore, in this study, it was expected that the AGS system could perform good organics and nutrients removal although a stable operation has not been achieved in the experimental period presented in this report.

5.1.3 Settling time

A gradually decreased settling time in the reactors was applied based on a study by Szabó et al. (2016) which was able to achieve rapid AGS start-up and avoid harsh wash-out of nitrifying bacteria. In this study, the settling time in all reactors was started from 20 minutes and similarly decreased to 15 and finally 12 minutes on day−16. The settling time was continuously reduced to 9, 7, 6, 5, 4, and the lowest to 3 minutes at day−35 in R0 and R2. Meanwhile, the settling time was constant at 12 minutes in R1 due to the major disturbance at day−18 until it was lower down to 7 and 5 minutes at day−35. This strategy has been known to aim for fast-settling granules and thus enhance the granulation in the reactors (McSwain et al., 2004b; Gao et al., 2011b; Szabó et al., 2016; Bengtsson et al., 2018). By using this strategy, more than 90% TOC removal, 98% NH_4-N removal, and 100% TP removal were achieved in R0 and R2 within the first five days and continued for 26, 33, and 26 days of operation, respectively. However, after the reduction from 4 to 3 minutes, NH_4-N concentration was observed in the effluent from R0 and R2 as well as the PO_4^{3-}P concentration in the effluent from R0. Despite the slower performance in R1, a decreased nutrients removal was also observed after 5 minutes of settling time. A similar condition was observed in terms of biomass profile that after 5 minutes settling time, the TSS concentration in the effluent kept increasing with low biomass concentration in the reactors. Therefore, it may assume that the strategy to reduce the last lower settling time was too fast, resulting in a wash-out of AOB, NOB, and PAOs.

Similar findings were reported by Lochmatter and Holliger (2014) which compared two strategies of stepwise decrease from 30 minutes to 5 and 3 minutes settling time with different time intervals within 20 and 29 days. The study concluded that 10 minutes settling time should be a precaution to avoid important biomass washout. However, the results were varied from other prior studies. For instance, Su et al. (2013) showed an optimal settling time based on keeping the biomass discharge ratio between 0.01 and 0.05 whereas, in this experiment, the optimal performance has not been achieved with a similar range of discharge ratio. However, one should consider different experimental settings that could make a variety of results. Indeed, the settling time control in the AGS operation should be adapted while monitoring the AGS performance (Szabó et al., 2016).

5.1.4 Solids retention time

SRT values were controlled by discharging excess sludge and the biomass concentration in the reactors (TSS_R) and effluent (TSS_{eff}), see Equation 3.7. Thus, it is reasonable that SRT values are also linked to the stepwise decrease of the settling time. The results showed that by gradually reducing the settling time, TSS_R decreased while the TSS_{eff} increased which resulted in the lower SRTs, see Figure 4.4. As is discussed in Section 2.3.3.2, a wide range of SRT in the AGS operation has been commonly reported. In the laboratory studies by using synthetic wastewater, relatively low SRTs were observed such as Beun et al. (1999) showed rapid AGS formation with SRTs varied between 0.6 to 5.8 days, SRT below 6 days, and 3 days in the first 10 days of operation (Szabó et al., 2016) and a wider range of 5 to 18 days in long term operations (Val del Río et al., 2012). Meanwhile, Bengtsson et al. (2018) reported a higher SRT between 20 and 50 days to treat municipal wastewater to maintain the nitrification process. Investigation with a different microbial population in the AGS was also done by Winkler et al. (2012) which showed SRT of 11 ± 3 days for nitrifiers and 13 ± 4 days for PAOs and GAOs. In the experimental run, the average of SRT was 33, 28, and 36 days and the lowest SRT was observed to be 23, 15, and 14 days in R0, R1, and R2, respectively.

During the AGS operation, the SRT was decided to be ≥ 20 days since the removal efficiencies decreased in the lower SRTs. Similar findings had been demonstrated that the nitrogen and biological phosphorus removals linked to the SRT with a variation of optimum SRT for these processes (Lee et al., 2007; Li et al., 2016; Bengtsson et al., 2018; Castellanos et al., 2021). Moreover, it is interesting that within the AGS system, a granule allows different ecological niches such that nitrifiers in the aerobic zone (outer layer), denitrifies, PAOs, and GAOs in the anoxic zone (inner layer) (Nancharaiah and Kiran Kumar Reddy, 2018). Therefore, the average SRT values may present non-homogenous biomass distribution, especially during the AGS start-up when the microbial population is easily changed (Li et al., 2008; Bengtsson et al., 2018).

Bassin et al. (2012) and Winkler et al. (2011) investigated different sludge removal strategies to wash out more GAOs in the upper part of the reactor and maintain PAOs in the bottom fraction of granules. In this study, the excess sludge was taken from the middle port of the reactor during the aerobic phase so that homogenous sludge is expected to be a wash-out. However, some excess sludge was also discharged during the withdrawal phase, especially when the settling time was significantly reduced. Hence, possibilities to wash out important biomass can still occur and the effect of different sludge discharge operations through the SRT controlled is remained unclear (Zhu et al., 2013). Therefore, it can be concluded that there will always be a variation of optimum SRT but it is still an important parameter that is needed to be adjusted along with monitoring of AGS performances, especially with the attention of slow-growing bacteria such as nitrifiers (Liu and Liu, 2006; Cydzik-Kwiatkowska and Wojnowska-Baryła, 2011; Castellanos et al., 2021).

5.1.5 Influent wastewater

As it is shown in Section 4.2.1, the substrate concentration in the influent has fluctuated although the flow calibration was routinely done. Therefore, it is important to frequently check the flows from the substrate and water pumps to quickly adjust the influent concentration. During the AGS operation, attention was put to the highest TOC

concentration that was observed in all reactors after the antifoam dosing. A significant increase of 2.8 times higher TOC concentration was observed in R1 and R2 at day−30 of operation. Although the TOC influent concentration was not as high as in the two other reactors, R0 has also experienced a decreased TOC removal around 22.5% reduction whereas 26% and 19% reduction were observed in R1 and R2, respectively. The sudden high organic load in the AGS reactors seemed to impact the conversion processes. It was reflected that residual VFAs were observed at the end of the anaerobic feeding phase (Tormachen, 2021). This condition was undesirable since it may favour the out competition of slow-growing organisms in favour of heterotrophic microorganisms (Liu et al., 2004b; Wan et al., 2009; Lochmatter and Holliger, 2014). Moreover, the COD availability during the aeration phase has been reported to affect the granulation by promoting the presence of filamentous bacteria and biofilm on the reactor wall (McSwain et al., 2004a; Lochmatter and Holliger, 2014) which was also observed in this study, see Figure 5.2. Therefore, actions were taken to avoid further problems such as cleaning the reactors and stopped the antifoam dosing once the foam was relatively less observed in the reactors.

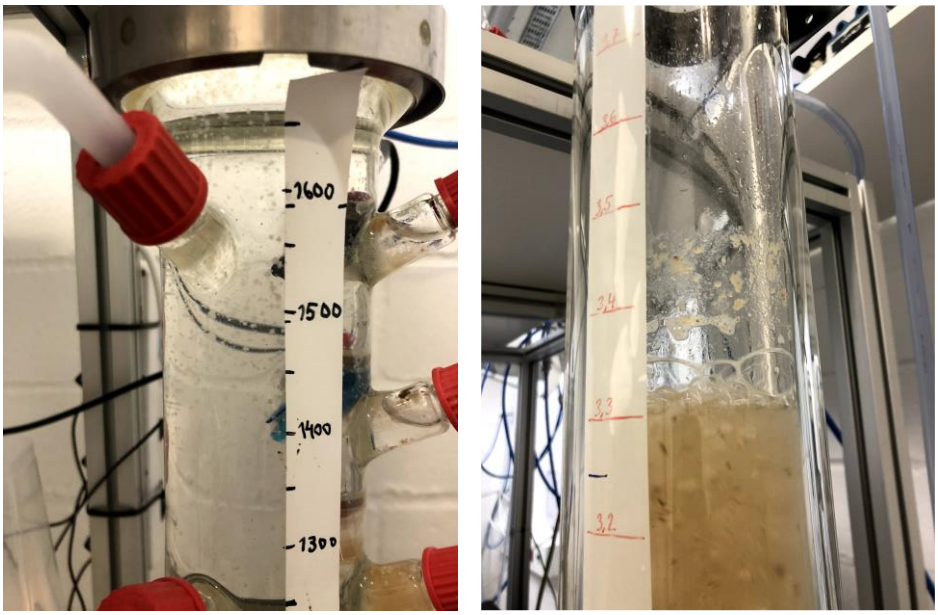

Figure 5.2 Example of biofilm wall growth which was observed in R0 (left) and R2 (right). The appearance of fluffy granules is clearly shown in R2.

Another disturbance was noticed in TP influent concentration in which twice as high as the target concentration was measured only in R0 at day−30, see Figure 4.7. However, the probable reason was the uncertainty of the influent sampling since a smaller volume was taken that particular day which might result in the non-homogenous concentration. Moreover, the flow was properly calibrated and only a very low VFAs was observed at the end of the anaerobic feeding phase, something that has also been observed in a previous study (Tormachen, 2021).

5.2 Granule development in AGS start-up

5.2.1 Granule morphology

Based on microscopic observation, the outgrowth of filaments in the seeding sludge disappeared, except in the first seven days of operation in R2. Although filamentous bacteria were observed at 10x magnification along with the AGS operation, fibrillose granules started to be present only in R0 whereas a few extended filaments surrounded granules in R1 and R2 at the end of the study. The seeding sludge was compact and dense and the structure was maintained in R0 and R1. On the other hand, loose-structured granules with a dark brown core were observed in R2. Figure 5.3 shows the illustration of the hypothesis on comparison of AGS development in three reactors.

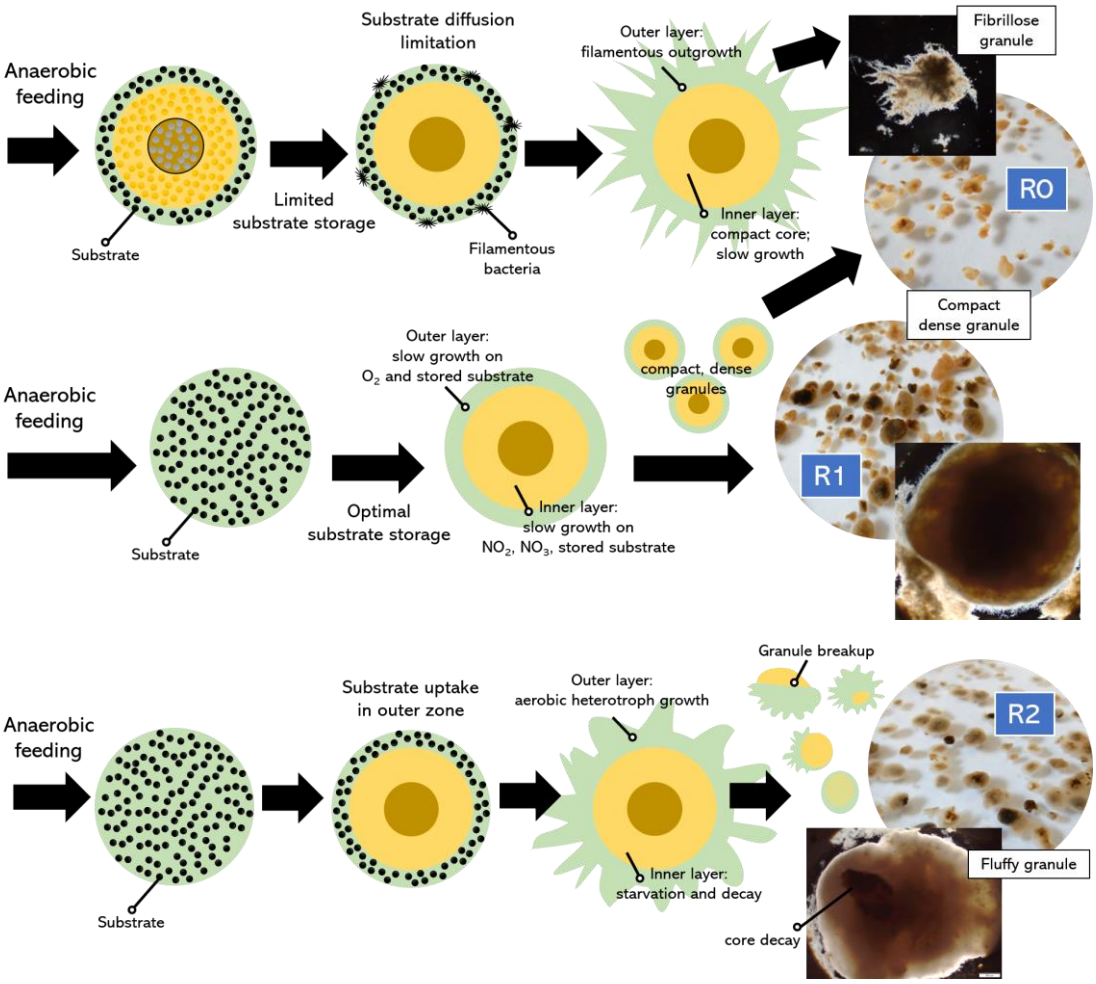

Figure 5.3 Hypothesis of AGS development during the operation in R0, R1, and R2 (Figure by author, adapted from Pronk et al. (2015b)).

As is discussed in Section 4.3.3, dense and compact granules with relatively smooth surfaces were observed in all reactors in the early phase of operation. Studies have been demonstrated that the formation of these granules was possible when multiple operational factors were maintained, providing an ideal condition for granulation. The main mechanism was commonly reported through optimal substrate storage which leads to different zones formation within a granule. Figure 2.2 presents the ideal structural layers of aerobic granules consist of aerobic, anoxic, and anaerobic zone.

During this condition, the substrate or VFAs in wastewater should be taken up by PAOs or GAOs type of bacteria and converted to PHA and stored in the cells (Tchobanoglous et al., 2003; Madigan et al., 2015; Davis, 2020). During anaerobic plug flow feeding with a relatively high substrate concentration in this phase, the substrate uptake throughout the granule is possible, leading to optimal substrate storage in the granule (Pronk et al., 2015b). On the other hand, in the aerobic phase, the intercellular stored substrate will be utilized for the slow-growth in the outer layer and inner layer of the granule (de Kreuk and Van Loosdrecht, 2004; Pronk et al., 2015b). Here, the storage polymers can be oxidized based on the available electron acceptor such as oxygen (O_2), nitrite (NO_2), or nitrate (NO_3). Hence, this condition can lead to a stable and compact granules formation, see Figure 5.3. However, other factors indeed supported the denser granules such as high shear stress (Bengtsson et al., 2018; Beun et al., 2002; Liu and Tay, 2002; Zhou et al., 2016), feast-famine regimes (Bengtsson et al., 2018; De Kreuk et al., 2007a; Gao et al., 2011b), settling time, SRT and biomass wash-out control (Weissbrodt et al., 2012; Zhu et al., 2013; Szabó et al., 2017).

Towards the end of the study, the larger granules with dense structure seem were only maintained in R0 and R1 whereas fluffy and loose structured larger granules were observed in R2. Many studies investigated filamentous overgrowth in the AGS system which can cause instability of granules and fluffy structure (Franca et al., 2018; Liu and Liu, 2006; Tay et al., 2001b; C. Wan et al., 2014). However, a relatively lower level of filamentous growth was seen in R2 compared to other reactors, see Figure 5.4. Instead, the appearance of protozoans such as sessile ciliates (likely as *Vorticella* spp., *Carchesium* spp., and *Epistylis* spp.) was observed even in 2x magnification, see Figure 4.19. According to Kocaturk and Erguder (2016), higher COD/TAN ratios (7.5−30) favoured the growth of heterotrophs and caused white, fluffy, and large granules. This finding leads to the assumptions of heterotrophs overgrowth in R2 which started to detect after the TOC concentration significantly increased in the influent at day−30, giving COD/TN around 7.6−7.8. Moreover, a sticky and starch texture were also observed during the solid analysis. It is hypothesized that this might be due to high EPS (extracellular polymeric substance), a sticky substance, that was secreted by microorganisms during the growth and lysis which should support the cells attachment (Liu and Tay, 2002; Lin *et al.*, 2015; Deng et al., 2016). The theory is supported by the fact that granules breakups were noticed mostly in R2, leading to biomass lysis or disintegration of granules. A similar phenomenon has been reported due to aerobic heterotrophs which create substrate starvation in the inner layer of granule, cause decay of granule's core and thus lead to granule breakups (Pronk et al., 2015b), see Figure 5.3.

Although dense structured granules were mostly observed in the reactors, the gradual growth of filamentous organisms was seen in smaller granules within all reactors at the end of the study. In Figure 5.4, one can observe different levels of filament growth: 1) filamentous overgrowth in R0 which causes fibrillose granules; 2) moderate level of filamentous growth in R1; 2) low level of filamentous growth in R2. Liu and Liu (2006) conducted a comprehensive review of the causes and control of filamentous growth in AGS reactors. Multiple factors and simultaneous conditions were identified to favour filamentous growth in AGS such as DO, substrate concentration, and SRT. Studies demonstrated that the DO deficiency could cause filamentous growth (Palm et al., 1980; Gaval and Pernelle, 2003; Martins et al., 2003), especially in relatively low DO concentration (< 1.1 mg/L) (Martins et al., 2003). Chudoba (1985) recommended a

minimum DO concentration of 2 mg/L to avoid filamentous overgrowth in AGS reactors. In the experimental runs, DO deficiency was most likely to occur in R0 and R1 due to aeration problem for a relatively long time (hours). However, it should be noted that the DO levels in reactors may not describe the actual DO gradients within the granule and it is indeed, a dynamic process that occurred along the operation (Liu and Liu, 2006).

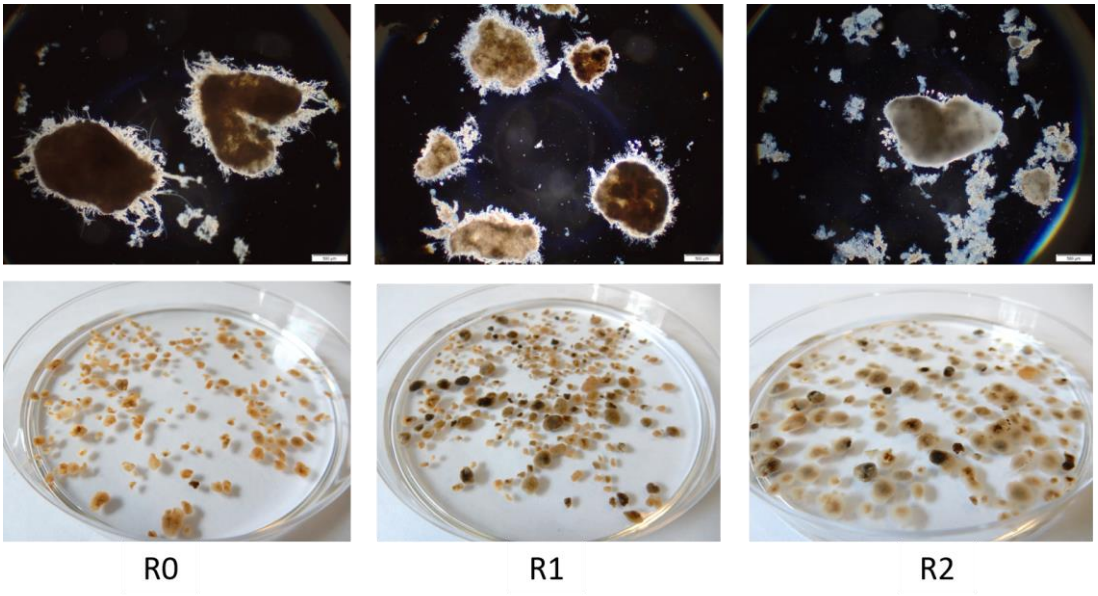

Figure 5.4 Comparison of microscopic images by 2x magnification in R0, R1, and R2 (upper) and photo images (lower) at day−42 of operation.

Substrate concentration was also highlighted with the observation of filamentous growth. A typical constant substrate concentration will be controlled in the AGS operation whereas the biomass concentration will tend to increase along with the operation. Under this condition, filamentous growth was observed (Liu and Liu, 2006). It was pertinent to the findings of filamentous growth that was favoured under low concentration of substrates or when substrate diffusion limitation occurred (Eckenfelder, 2000), see Figure 5.5. Moreover, filamentous growth was also observed under low levels of nutrients due to its high surface-to-volume ratio (Chudoba et al., 1973). Due to nutrients deficiency, higher production of extracellular polysaccharides (ECP) has been reported (Aquino and Stuckey, 2003; Liu et al., 2004a) and jelly-like and viscous granules were formed (Liu and Liu, 2006). Last, the long SRT was hypothesized to favour filamentous growth due to the low specific growth rate (Chudoba, 1985; Liu and Liu, 2006). According to these theories, indeed, a combination of these factors could happen and thus the progressive filamentous growth was observed in all reactors with varied abundance. It should be also noted that filamentous organisms could support the granule's structure for bacteria to grow and thus lead to granules formation in the early phase of AGS operation (Liu and Liu, 2006; Nancharaiah and Kiran Kumar Reddy, 2018). As long as the overgrowth did not happen, it will not impact further operational problems (Liu and Liu, 2006) such as sludge bulking or foaming which has been commonly reported (Chudoba, 1985; Guo and Zhang, 2012; He et al., 2019) and also observed in reactors.

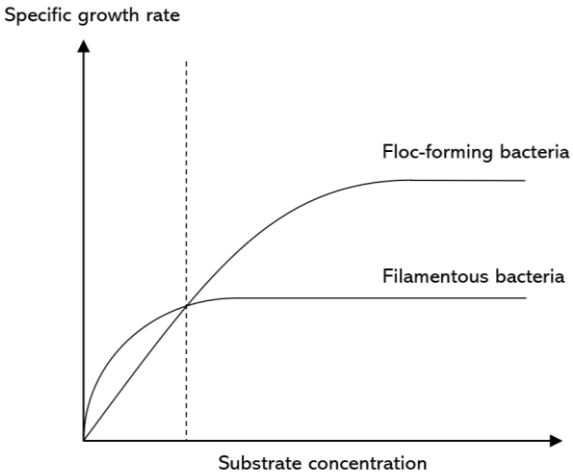

Figure 5.5 Comparison of the specific growth rate of floc-forming bacteria and filamentous bacteria along with the substrate concentration (Chudoba, 1985; Liu and Liu, 2006).

Other than filamentous growth, fungi were detected microscopically at day−42 in R1 which tend to be unwanted in the AGS system. A study by Wan et al. (2014) found that fungus was a predominant filamentous microorganism in acidic pH and thus, the alkaline condition was recommended in the AGS system. This finding seems to show a similar condition of acidic pH in R1 for a relatively long time. Filamentous fungi overgrowth has been demonstrated to be detrimental for the AGS system since it resulted in poor settling of granules, biomass disintegration, and poor nutrients removal (Sharaf et al., 2019). Hence, all reactors were completely cleaned after the early observation of fungi. Another action could also be done such as adding the anti-fungal agent for 24 hours (De Kreuk et al., 2010). Figure 5.5 shows the microscopic images of fungi in R0 and the comparison with similar findings in a study by Wan et al. (2014).

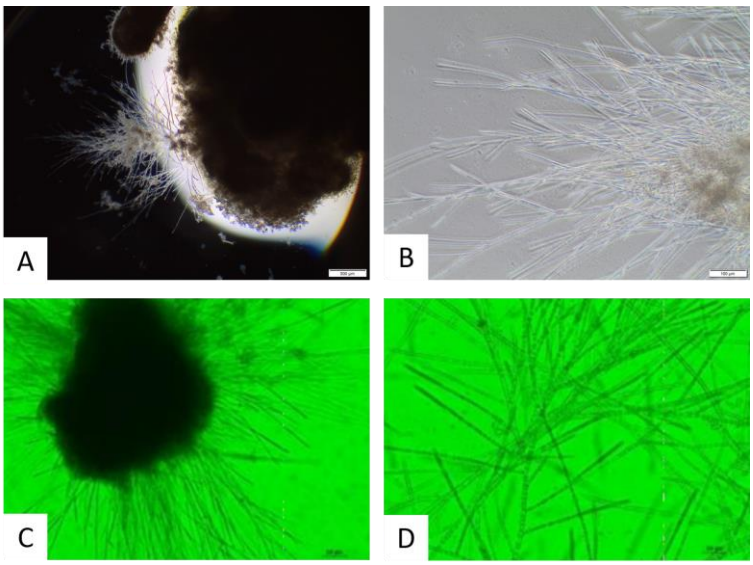

Figure 5.6 Observation of fungi in R0 at day−42 of operation: A) 2x magnification; B) 10x magnification. Optic images of filamentous fungal granules (Wan *et al.*, 2014)*: C) micro-aggregate; D) inner core.*

5.2.2 Granule size

The size of granules has been known to be examined and defined in the granulation process (De Kreuk et al., 2007a). Previous studies have been reported the importance of granule size concerning AGS stability which could be in terms of the activity or process performance, the size distribution of granules, and granules morphology (Zheng et al., 2006; Long et al., 2015; Long et al., 2019; Franca et al., 2018). However, due to dynamic changes in operational conditions, granules sizes can be varied during the AGS processes (Long et al., 2019). It is reflected in this study which a similar trend was observed in R1 and R2 that the size of the granules significantly increased after the first seven days of operation and seems to progressively disintegrate. Meanwhile, the size of the granules in R0 began to increase later at day−21 and gradually decrease towards the end of the study. During the 44 days of operation, similar and even larger average granules sizes were observed in all reactors compared to the seeding sludge with an average diameter of 2.3 ± 1.6 mm.

A wide range of average granule sizes has been reported with varied average granules diameter. For instance, start-up studies with a lab-scale AGS using the real wastewater had an average diameter in a range of 0.2 to 4 mm with different operational conditions and AGS configurations (Nancharaiah and Kiran Kumar Reddy, 2018). These studies investigated AGS cultivation by using activated sludge as the inoculums for the start-up while AGS was used in this study. However, scholars have also been reported different granules size by using different seeding sludge. Long et al. (2014) tried a mixture of mature AGS with AS and observed a rapid increase of granule size during the first-18 days of operation and was further maintained with an average size of 1.58 mm and no obvious disintegration occurred. Another study used a combination of crushed granules and floccular sludge and obtained the 50^{th} percentile of particles around 0.8 mm after 90 days with the highest percentage of 30% crushed granules (Pijuan et al., 2011). Compared to these findings, a larger granule size was observed and maintained in the experimental runs with consideration of the larger diameter of inoculum was used. Moreover, based on the former granule's definition by De Kreuk et al. (2007a), a minimum size of 0.2 mm granules is still maintained in this study.

Based on particle size analysis, only particles larger than 0.1 mm were measured in this study. The seeding sludge at day−0 had a smaller fraction around 0.15% of measured particles less than 0.2 mm. The flocs fraction gradually disappeared in R1 and R2 towards the end of the study while it increased to 0.8% in R0 at day−42, see Figure 4.21. It may occur due to a little number of particles compared to other observations. Although more accurate sizes could be obtained and the fact that the mixed liquor sample was diluted to avoid multiple scattering, the results were still worth considering since granules (>0.2 mm) were the main focus in this study. Although the average granule sizes decreased, according to particle size distribution results, it seems that granulation occurred with the appearance of newly developed granules and an increased fraction of big granules, except in R1 due to process failure. It is reflected by the 50^{th} percentile of particle size which was progressively increased and a higher fraction of granules larger than 0.2 mm. Obvious disintegration of granules was seen in R2 which also showed more granules breakage as well as in R1. It was hypothesized that domination of granules breakup in R2, process disturbances in R1 such as lower DO levels, and too fast lower settling time in all reactors might lead to smaller particles (Li et al., 2008; Long et al., 2019; Zhou et al., 2016).

5.2.3 Biomass analysis and settling properties

All reactors started with SVI_5 of 52 mL/g but the values gradually increased within 26 days and reached a relatively high SVI in a range of 113 to 258 mL/g. It might reflect that the settling properties of AGS gradually decreased in the early phase of operation, showing the SVI values close to properties of activated sludge (100−150 mL/g) (De Kreuk et al., 2005) instead of AGS (35−70 mL/g) (Pronk et al., 2015a; Sharaf et al., 2019). Based on SVI_5 values, granulation time was estimated to be 40 days in R0 and R1 which were demonstrated by SVI_5 of 35 mL/g and 29 mL/g, respectively. Meanwhile, granulation was about to occur after around 30 days in R2 but it seems to be disturbed and unstable settling properties were still observed until the end of the study. Figure 5.7 shows the observation of fluffy and porous granules in R2 after day−30 which probably caused low granules density and thus relatively high SVI values. As it is discussed in Section 5.2.1, it was assumed that high TOC concentration might be the main reason since other operational conditions were relatively stable in R2 compared to R0 and R1.

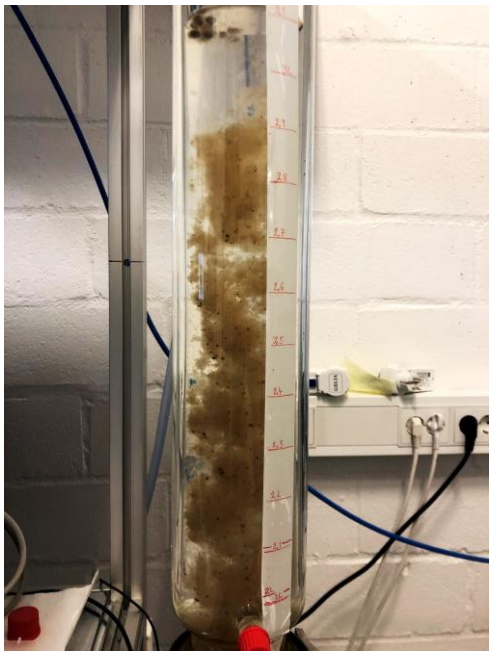

Figure 5.7 Granules in R2 at the beginning of settling phase at day−30.

In terms of solids concentrations, the VSS/TSS ratio in all reactors was above 95% which could mean that TSS concentrations may represent VSS concentration and thus also biomass concentration. All reactors started with a range of 6.3 to 7 g TSS/L and it gradually decreased within 44 days to 3.3 to 3.7 g TSS/L. However, at some point, it could increase when the same settling time was set in all reactors, meaning that biomass growth was observed. Regardless of this, the biomass concentrations in all reactors were still relatively low compared to other AGS studies. For instance, 8.5 g MLSS/L was obtained on day−22 of AGS start-up by using stored AGS as the inoculum and synthetic wastewater with a COD concentration of 1000 mg /L (Wang et al., 2019). Another study by De Kreuk et al. (2010) showed a biomass concentration of 6.4 g VSS/L by using AGS as the seeding sludge and soluble starch as the substrate. In a contrast, lower TSS concentration was obtained when activated sludge was used as the inoculum. A previous similar study by Calgaro and Trieb (2020) of AGS fed by

synthetic pharmaceutical wastewater reached a stable TSS concentration of 3.5 g TSS/L within 29 days and in a range of 3 to 4 g MLSS/L in other research (De Kreuk et al., 2010; Wang et al., 2019). A common consequence of higher TSS concentration in the effluent was observed during the operation. At the end of the study, TSS in the effluent remained higher than 43 mg/L, whereas it has been reported in a range of 5 to 14 mg/L in other lab-scale studies (Bengtsson et al., 2018) and 20 mg/L in the AGS plant (Pronk et al., 2015a). A similar finding was reported in a previous study and that the most likely reason was the increase of biomass wash-out from the reactors as a result of reducing the settling time. Moreover, it was also reflected from the increased fraction of small granules and decreased SRT (De Kreuk et al., 2010) which was also observed in this study.

5.3 AGS start-up performance

5.3.1 Conversion of organics

Based on TOC analysis, all reactors started with high TOC removal but a significant drop was observed after day−30, yet they were able to recover within 2 weeks, reaching even higher TOC removal than the early phase of operation with 90% in R0 and above 95% in R1 and R2. As discussed in Section 5.1.5, the most likely reason for the decreased removal efficiency was due to a sudden increase of TOC concentration in the influent from the antifoam agent. A similar finding was reported by (Long et al., 2015) which investigated different organic loading rates (OLR) in the AGS system. It was found that with the increase of OLR, the COD effluent concentration rose and the COD removal declined which was also observed in this study. However, during 10 days of antifoam dosing, TOC removal gradually increased and this might be assumed that heterotrophs also took a part in removing carbons (Bengtsson et al., 2018). This assumption was reflected since carbon leakage into the aeration phase was observed and denitrification seemed did not improve. In the case of R1, the falling of carbon removals at day−19 was expected due to the huge loss of biomass caused by a process failure. Another possibility is some kind of surface interaction between granules and antifoam agent that causes aggregate disintegration.

As it is shown in Table 2.2, organic removals by aerobic granules could occur through different mechanisms. It is seen through the experimental runs that several probable mechanisms occurred: 1) TOC/COD anaerobic uptake by PAOs and GAOs; 2) aerobic oxidation of organics by OHO; and 3) simultaneous nitrification and denitrification by AOB, NOB, and DOHO. The first assumption was reflected by low VFAs concentration (acetate, propionate, glucose) at the end of the anaerobic phase (Tormachen, 2021). The latter hypothesis was likely to happen since high TOC removals were maintained in all reactors. In terms of aerobic oxidation by OHO, it was also pertinent with the observation of filamentous growth in all reactors and porous granules in R2 as it was also reported in other studies due to hydrolysis of organic matter on granule's surface (De Kreuk et al., 2010; Wagner et al., 2015).

5.3.2 Conversion of nitrogen

The average TN removal performance from all reactors within 44 days of operation was measured to be 51%, 56%, and 62% in R0, R1, and R2, respectively. These values seem to be relatively low compared to other AGS studies. Higher N-removal in a range of

70−90% have been reported by using synthetic wastewater during AGS start-up (Pijuan et al., 2011; Long et al., 2014; Lashkarizadeh et al., 2016; Szabó et al., 2016) and around 70−80 % by using a low concentration of pharmaceuticals (Calgaro and Trieb, 2020; Jiang et al., 2021). An increasing trend was observed in all reactors along with the operation but the performance was falling since day−37, reaching a lower range of 38 to 61% among all reactors at the end of the study.

In this study, ammonium removal had already been observed in all reactors during the first five days. Based on the calculation, it was shown that around 40−50% of NH_4-N removal occurred in the anaerobic phase at least during the first 37 days. Similar findings were reported in the previous study (Calgaro and Trieb, 2020) and it was hypothesized that ammonium removal was performed by anaerobic ammonium oxidation (anammox). It was supported by the fact that no observation of NO_2-N in the effluent, meaning that it would not be present in the next anaerobic feeding phase. Moreover, the pH gradually decreased during the anaerobic feeding phase and most likely due to H^+ release from nitritation, see Equation 2.1. Compared to this result, there was also zero concentration of NO_2-N within 37 days in the effluent and pH tended to decrease during the anaerobic phase. Moreover, there was gas release, probably N_2, which was sometimes observed during the feeding phase in the reactors. By the fact that the DO level was sometimes above 0% during the anaerobic feeding phase in all reactors, hence enabling partial nitrification and hence making it possible for anammox bacteria to thrive, something that is known to occur in the AGS process (de Sousa Rollemberg et al., 2018).

Despite good NH_4-N removal, the TN removal efficiencies seemed to be relatively low although an increasing trend was observed within 37 days. It is most likely due to inhibition of denitrification during this period which was reflected by the accumulation of NO_3-N in the effluent, in a range of 12−28 mg NO_3-N/L in all reactors during the first 33 days. Based on this fact, the NO_3-N might enter the next anaerobic feeding phase, giving anoxic condition instead of anaerobic during the feeding phase. One could consider that DPAOs and DGAOs might also be present, showing that a lower concentration of NO_3-N was observed after the feeding phase (de Sousa Rollemberg et al., 2018). However, a higher NO3-N concentration was observed in the effluent, meaning poor denitrification. In the AGS system, denitrification could occur through simultaneous nitrification and denitrification (SND) processes (Coma et al., 2012; Pochana and Keller, 1999) due to different redox conditions within a granule (Nancharaiah et al., 2016), see Figure 2.2. However, SND seems to be challenging since multiple factors may affect the process. Previous studies have demonstrated that smaller granule diameters led to decrease N-removal efficiency, especially in a long-term operation (De Kreuk et al., 2005; Pochana and Keller, 1999). The authors highlighted the probable cause was the decrease of the anoxic zone within the granule. This theory may happen in the experimental runs since the size of the granules decreased towards the end of the study. However, to what degrees this condition impacts the denitrification process could not be predicted in this study.

Another consideration might be the dissolved oxygen concentration which has been commonly found to be one of the parameter that controls the N-removal efficiency (De Kreuk et al., 2010; Nancharaiah and Kiran Kumar Reddy, 2018). A study by (Mosquera-Corral et al., 2005) observed the highest N-removal (94%) using a steady-state AGS operation with 20% of oxygen saturation. Although a 20% DO level was set

during the study, the oxygen concentration fluctuated, and even DO was not controlled during some periods due to foaming problems or absence of N_2 gas in the early phase. Thus, it is assumed that higher DO levels could cause inhibition of denitrification which was also observed in other studies (Amorim et al. 2014; Beun et al., 2001; Mosquera-Corral et al. 2005). Based on this fact, some aeration strategies could be done such as alternating nitrification and denitrification (AND) instead of SND strategies (Purba et al., 2020). A study by Lochmatter et al. (2013) focused on different aeration strategies for both SND and AND. The results showed that AND is more efficient than SND by favouring denitrifiers during the low DO period which can be done by two strategies: 1) alternating high and low DO phase (gradually decreased of 50%, 40%, 30% DO with 5% DO interruption) and 2) intermittent aeration (6−10 minutes aeration pulses with 7−12 minutes no aeration and mixing). The later strategy, also the so-called post-anoxic phase, was found to be the most effective to improve N-removal and seems more realistic for full-scale application. Therefore, this strategy can be considered for further operation in this study.

The last consideration in the denitrification process was the availability of organics which is required as the electron donor, see Equation 2.3. During the study, TOC concentration fluctuated but it was observed to be lower than the target concentration of 110 mg TOC/L before the antifoam dosing in all reactors. In the last 2 weeks when the TOC concentration was higher in the influent, better denitrification was observed. This condition might reflect that sufficient organic matter should be assured in the reactors to promote denitrification which was also demonstrated in the previous study (Lochmatter et al., 2013). As is discussed in Section 2.3.4.2, different forms of organic matter may be presented such as organic matter or VFAs, external sources, and internally stored PHA in granules. However, it should be also noted that this factor is not solely responsible for the inhibition of denitrification.

Towards the end of the operation, the TN removal was decreasing in all reactors within the last 7 days. During this period, relatively high NH_4-N concentration was measured in the effluent around 32, 14, and 12 mg NH_4-N/L in R0, R1, and R2, respectively. This condition was unwanted since the high level of ammonium has been known to inhibit the growth of nitrifiers (Vadivelu et al., 2006). Since relatively long aeration was applied, around 4 hours, it was reasonable that nitrification was supposed to occur. Therefore, it was assumed that the significant dropped of N-removal was caused by harsh biomass wash-out since more biomass was removed through daily excess sludge removal by taking out around 100 mL MLSS per day in each reactor, starting from day−36. Many studies have been reported similar observations about biomass wash-out that leads to lower SRT, carbon leakage in the aeration phase and thus promoting filaments and zoogloeal overgrowth rather than growth of slow-growing microorganisms such as nitrifiers (Wilén et al., 2018). Moreover, due to the fast alternation of microbial community during AGS start-up (Li et al., 2008), one could not assure that a homogenous biomass size distribution was discharged from the reactors. Thus, increasing the settling time after this observation was a wise decision to have longer SRT and keep the slow growers in the reactors.

5.3.3 Conversion of phosphorus

In general, biological phosphorus removal was achieved in this study. Stable TP performance was observed in R2 with 100% removal efficiencies within 5 days and it

was maintained until 44 days even with the lowest settling time. The result was expected since R2 received the most stable operational conditions such as pH, DO, and controlled substrate concentration in the influent along with the operation. On the other hand, R0 and R1 started with 100% TP removal but a significant drop was noticed on day−30 and day−19, respectively. EBPR was able to recover within 2 weeks in R0 back to 100% TP removal while it took more than 25 days in R1 to achieve 57% TP removal and thus full recovery of EBPR has not been achieved until the end of the study.

By reflecting the IC analysis results, PO_4-P release was shown along with the operation in all reactors, and VFAs were mostly taken up during the anaerobic phase (Tormachen, 2021). This observation may reflect that VFAs uptake was performed by PAOs and soluble orthophosphate released to the solution. The release of PO_4-P during the anaerobic feeding phase was measured in a range of 13−22 mg PO_4-P/L in all reactors. However, higher concentration was indeed observed in R0 in a range of 26−30 mg PO_4-P/L between day−30 and day−40. During this period, PO_4-P concentration started to be detected in the effluent, and first, it was assumed that the PAOs might be saturated with phosphorus that they could not take up all the soluble orthophosphate from the solution. Therefore, it was decided to remove some biomass by taking MLSS samples every day from day−36, which acted as daily excess sludge removal. However, it turned out that this strategy affected other processes, mainly nitrogen removal, as was discussed in Section 5.3.2.

PO_4-P concentration in the influent was three times higher than the target concentration which was only observed in R0 on day−30, see Figure 4.7. The reason was not known and the influent samples from R1 and R2 showed relatively low PO_4-P concentration, close to target concentration. As soon as the influent concentration was lowered down and the settling time increased to 5 minutes, the TP removal was improved. This finding leads to an assumption of possible carbon limitation within the reactors when too high PO_4-P concentration was observed in the reactor. A similar result has been demonstrated based on a study of different COD:N:P ratios in the AGS system and it showed that TP removal increased at the lowest P concentration (Hamza et al., 2019).

One may keep in mind that the DO concentration in R0 and R1 during the anaerobic feeding phase was observed in a range of 0 to 7% saturation, meaning that it was not completely anaerobic meaning that the PAOs might compete for the carbon source with such microorganisms as denitrifiers or heterotrophs (Peng et al., 2010; Lochmatter and Holliger, 2014). However, since denitrification activity has not completely observed during this period, the possibility was unlikely to occur. As the biomass concentration in the reactors were low, it was also possible that the slow growers such as PAOs were washed out during the sludge removal. Thus, there were not enough of them in the reactors to perform EPBR. Therefore, the settling time was set back to 5 minutes to let the slow-growing organisms grow.

In R1, the most likely reason behind the low TP removal efficiencies was the significant disturbance on day−19, resulting in the pH shock where the lowest pH value was 1.98 and 11 as the highest. At this condition, the TP removal was falling from 100% to 19% and was only back to 57% within 25 days. A similar finding was reported by Lashkarizadeh et al. (2016) which demonstrated a reduction of phosphorus removal efficiency from 98% to 50% at alkaline pH shock (pH=9) with no further recovery. It was hypothesized that PAOs were inhibited during the high pH.

6 Conclusion

The aerobic granules were successfully maintained in all AGS reactors during the 44 days of operation by using granules from a full-scale plant as the seeding sludge. The settling properties were similar in all reactors with an SVI_{15}/SVI_5 ratio of more than 0.9 and SVI_5 showed a typical value of AGS by reaching 35, 29, and 79 mL/g in R0, R1, and R2, respectively. Dense and compact structured granules were maintained and predominant in R0 and R1 while fluffy and loose structured granules were seen in R2 at the end of the study. Multiple stresses were assumed to affect granules morphology and caused filamentous overgrowth in the smaller granules in R0 and R1 while the growth of heterotrophs was hypothesized for causing disintegration of granules in R2. In addition, filamentous fungi started to be observed in R1 towards the end of the study, and thus, it should be monitored to avoid further problems. In terms of size, the flocs fraction in the seeding sludge gradually disappeared and similar and larger average granules sizes were observed in all reactors compared to the seeding sludge with an average diameter of 2.3 mm.

In terms of process performances, all reactors could meet the criteria for a minimum of 75% TOC removal by average TOC removal in a range of 86−89%. Meanwhile, the minimum 80% TP removal was reached in R0 and R2 with average removal efficiencies between 86 to 100% TP removal whereas 57% TP removal was measured in R1. TN removal was still relatively low on average of 51 to 62% in all reactors and it was also lower than the target TN removal of 70% minimum reduction. The disturbance of operational conditions was presumably taking a significant role in the inhibition of AGS performance such as dissolved oxygen concentration, pH, fluctuating substrate influent concentration, higher organic loading due to antifoam dosing at day−30, harsh biomass wash-out due too early decreased settling time to 3 minutes and more excess sludge removal since day−36. Process failure has been noticed in R1 since day−19 due to the absence of aeration and acidic and alkaline pH shock ($1.98 \leq pH \leq 11$).

Despite the disruptions, conversion processes of organics and nutrients were still able to be observed during the operation. Organic removals might perform through mechanisms such as COD anaerobic uptake by PAOs and GAOs which was reflected by low VFAs at the end of the anaerobic phase. Moreover, aerobic oxidation by OHOs during the aerobic phase and carbon uptake by denitrifiers during the anoxic phase within the SND process also possibly occurred. Observation of filamentous and heterotrophs growth might support aerobic oxidation. In terms of the fate of nitrogen, it was hypothesized that NH_4-N removal (40−50%) during the anaerobic phase was performed by anaerobic ammonium oxidation (anammox). The presence of DPAOs and DGAOs might also be considered since an accumulation of NO_3-N and 0−7% DO saturation was observed during the anaerobic phase. Nitrification seems to work along with the operation but denitrification was most likely inhibited, causing low TN removal. Assumptions were made on a smaller anoxic zone layer in granules, high uncontrolled DO level in the early phase, and fluctuated TOC concentration as the required electron donor.

Biological phosphorus removal was demonstrated during the operation based on VFAs uptake and PO_4-P release during the anaerobic phase and PO_4-P was removed in the effluent since it was taken up by PAOs and GAOs. The situation of pH shock in the middle of an operation in R1 impacted EPBR and thus it was assumed that PAOs were

inhibited by this condition. Meanwhile, a sudden dropped of TP removal in R0 on day−30 was predicted due to too high release of PO_4-P concentration, carbon limitation caused by three times higher PO_4-P concentration in the influent as well as limited PO_4-P uptake by PAOs.

In the end, it can be concluded that the AGS start-up period has not finished within 44 days of operation. The main reason is the instable nitrogen removal and low biomass growth in all reactors. However, granulation was presumably achieved in all reactors as seen by the good settling properties and maintained granule size and compact morphology. Given by similar performances observed in all reactors, it showed that low concentration of PhACs in wastewater did not affect the granules formation and process performances on treating organics and nutrients. The results show that the AGS operations was on a good track to reach almost complete start-up period.

The AGS seeding sludge indeed helped the rapid start-up with no acclimation period of TP and TOC removal as well as increasing granule sizes which are beneficial for the conversion processes. Solutions focusing on more stable operational conditions such as DO, pH, substrates concentration, and settling time strategies could be considered to achieve a faster start-up period. To confirm the possible mechanisms in the conversion of organics and nutrients, a cycle study could be done in future research. This can be beneficial to optimize the AGS operation as a strong relationship has been observed between the operational conditions and AGS performances.

7 References

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8 Appendix

Appendix A.1 – organics analysis

Total organic carbon (TOC) – by using TOC-TN analyzer

Procedure:

1. Check synthetic air gas valve (G2) is open.
2. Check the acid bottles to the left (phosphoric and hydrochloc acid) and the water bottle for rinsing the syringe (should be up to 2000 mL for needle rinse). Also check the water levels inside the instrument; the one in the front to the left should be filled with MQ water to the maximum level and the bottle in the rear should be filled to the overflow by filling MQ water in the small needle to the right.
3. Turn on the instrument.
4. Turn on the ventilation arm.
5. Turn on the TOC-control V program and choose H/W Settings.
6. Go to ASI and check vial volume.
7. Close and select Sample Table Editor.
8. Click new.
9. Connect to the computer to the machine.
10. Check that air pressure is at 150 (front of instrument).
11. Go to the sample editor and click insert multiple samples. Choose method to TOC+TN.
12. Make the sample table; give the number of samples and name. Click save and place MQ in the beginning and at the end.
13. Wait until the green sign is on and check the waste bucket (should not be full).
14. The map of samples will turn up and check so that the samples should be where they are.
15. Start the run when the green lamp is on (”start”). A menu will turn up where you click in ”shut down” after running the instrument.
16. Check that the needles are taking the samples.

Chemical oxygen demand (COD) – based on Hach Method 8000 (0-150 mg/L)

Procedure:

1. Preheat the thermostat to 148°C (298.4°F).
2. Invert the Cuvette test a few times to bring the sediment into suspension.
3. Carefully pipet the sample into the Cuvette test: 2.0 mL for reagent blank (MQ water) and homogenized sample.
4. Close the cuvettes, throughly clean the outside.
5. Invert the cuvette test.
6. Heat the cuvette test in the preheated thermostat at 148°C (298.4°F) for 2 hours.
7. Remove the hot cuvettes. Allow to cool to approximately 60°C (140°F) and invert a few times.

8. Allow to cool to room temperature.
9. Clean the outside of the cuvettes and evaluate.
10. Insert the blank into the cell holder, select the test and push READ.
11. Remove the blank.
12. Insert the sample cuvette into the cell holder and push READ.

Appendix A.2 – nutrients analysis

Anions (NO_2^-, NO_3^-, PO_4^{3-}) and cations (NH_4^+) – based on Ion Chromatograph (IC)

Procedure:

Sample preparation

1. Filter the samples through 0.2 μm pore size filters
2. Dilute the sample with miliQ water that the conductivity should be < 200 μS/cm. The concentration of the ion of interest should ideally be between 0.01 mM and 1.5 mM.
3. Prepare the sample vials and put the sample into the vial. Approximately 6 mL of sample is needed for each analysis.

Ion chromatograph preparation

1. Check eluent and regenerant solutions. The instrument consumes 1 mL/min of eluent and one sample takes 20 min to run.
2. If it is too little eluent is left, new solution should be prepared:
   Anion eluent: dilute 20 mL of carbonate buffer to a total of 2 L with MQ water.
   Anion regenerant: add 20 mL of sulfuric acid solution (2.5 M H_2SO_4). Fill up the bottle completely with MQ water (no air space in bottle).
   Cation eluent: dilute 400 mL of methanesulfonic acid to a total of 2 L with MQ water.
   Cation regenerant: add 137.5 mL of TBAOH solution. Fill up the bottle completely with MQ water (no air space in bottle).

Ion chromatograph running

1. Turn on the autosampler, anion IC and cation IC.
2. Log into the computer and open software Chromelon.
3. Start the IC pumps, both anion and cation IC. The pumps should run for at least half an hour before starting the analysis. This is for the baseline to become stable.
4. Make the sample sheet: write name of the samples, position of the vials in the autosampler, and choose the correct method.
5. Put the vials in the autosampler.
6. Start the run.

Table 8.1 Standard solutions for IC analysis.

Chemicals	Molecular weight (mg/mmol)	Mass (mg)	Mass (g)
KNO_3	101.11	101.11	0.10
$NaNO_2$	68.99	68.99	0.07
KH_2PO_4	136.09	136.09	0.14
NH_4Cl	53.49	53.49	0.05

Appendix A.3 – solids analysis

Total suspended solids (TSS) and volatile suspended solids (VSS) − method 2540 D and E (APHA, 1998)

Procedure for TSS:

1. Select a sample volume (max. of 200 mL) that will yield no more than 200 mg of total suspended solids.
2. Place the filter on the base and clamp on funnel and apply vacuum. Wet the filter with a small volume of MQ water to seal the filter against the base.
3. Shake the sample vigorously and quantitatively transfer the sample to the filter using a large orifice, volumetric pipet. Removal all traces of water by continuing to apply vacuum after sample has passed through.
4. Rinse the pipet and funnel onto the filter with small volume of MQ water. Remove all traces of water by continuing to apply vacuum after water has passed through.
5. Carefully remove the filter from the base. Dry at least one hour at 103−105°C. Cool in a desicator and weigh.
6. Retain the sample in the dish for subsequent ignition at 550°C.

Calculation of TSS:

Total suspended solids (mg/L) = (A-B) x 1000/C

where:
A = weight of filter and dish + residue in mg
B = weight of filter and dish in mg
C = volume of sample filtered in mL

Procedure for VSS:

1. After determining the final weight in the total suspended solids analysis, place the filter and dish in the muffle furnace and ignite at 550°C ± 50°C for 15 minutes.
2. Allow to partially air cool, desiccate and weigh.

Calculation of VSS:

Volatile suspended solids (mg/L) = (A-B) x 1000/C

where:
A = weight of residue + filter and crucible in mg from TSS test
B = weight of residue + filter and crucible in mg after ignition
C = volume of sample filtered in mL

Appendix A.4 – size analysis

Size analysis – ImageJ software

Procedure:

1. 100 mL of mixed liquor samples are taken and pour into the Petri dish.
2. Samples are diluted with tap water to get rid of the flocs.
3. Picture of caliper, showing 5000 μm, is taken for the measurement settings in ImageJ.
4. A camera fixed above the Petri dish which was put in the fixed position on the top of an overhead projector (OHP). At least 10 to 15 pictures should be taken from the light microscope for the sludge morphology and from the camera for the size analysis.

Image processing:

1. Open ImageJ software.
2. Open the picture of caliper and do measurement by click ”measure” for around 10 times and use the average value of the length in pixels.
3. Go to analyze and choose set scale. Enter the average value of the length in pixels and the known distance of 5000 μm. Click global to keep the same settings.
4. Go to set measurements and choose: area, shape descriptors, limit to threshold, display label, min & max gray value, mean gray value.
5. Open sample image. Go to image, type and set it to be 8bit.
6. Go to image, adjust and go to threshold and adjust the level so that the black colour fulfill all the granules surface.
7. Go to analyze, analyze particles and set the size (unit^2) to be 8000-infinity, circularity for 0-1.00, show outlines display results and exclude on edges.
8. Use the measurements results and convert the area to diameter by using formula:

$$\text{Radius} = \sqrt{\frac{\text{Area}}{3.14}} \times 2$$

Appendix A.5 – microscopic images of 20x magnification

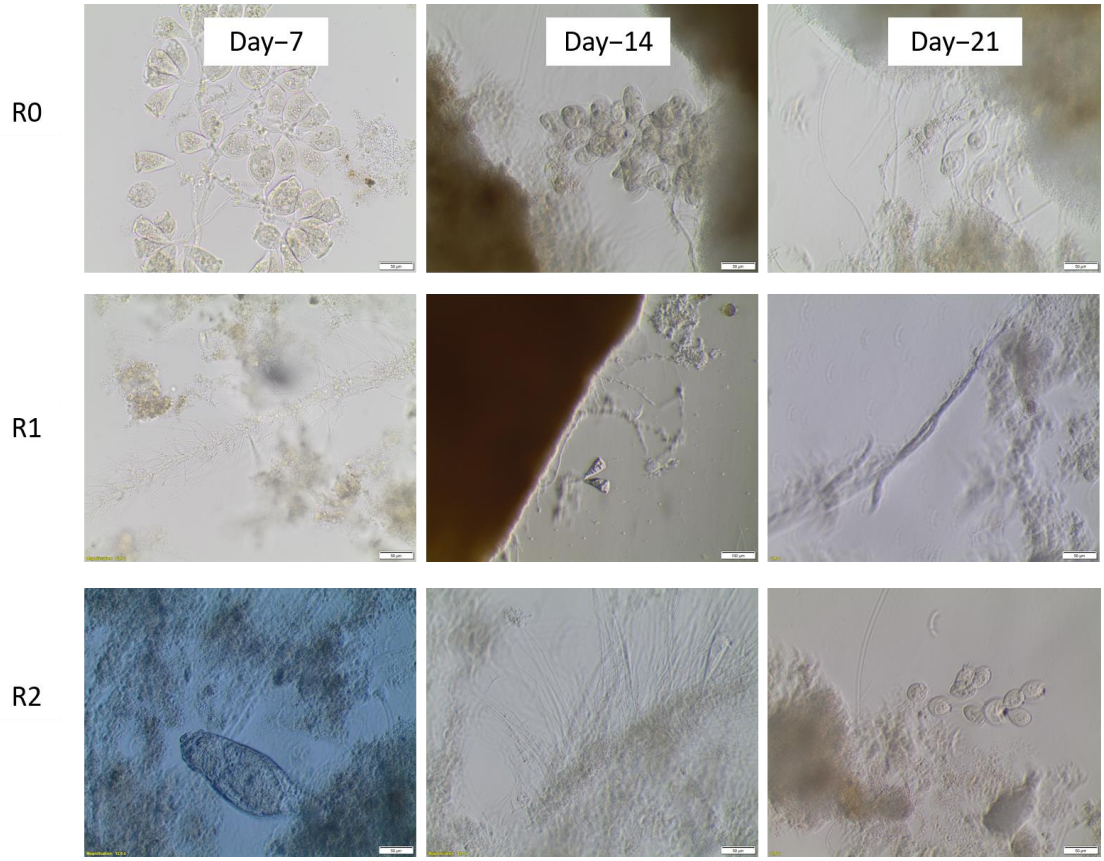

Figure 8.1 Microscopy image of 20x magnification from all reactors on day−7, day−14 and day−21.

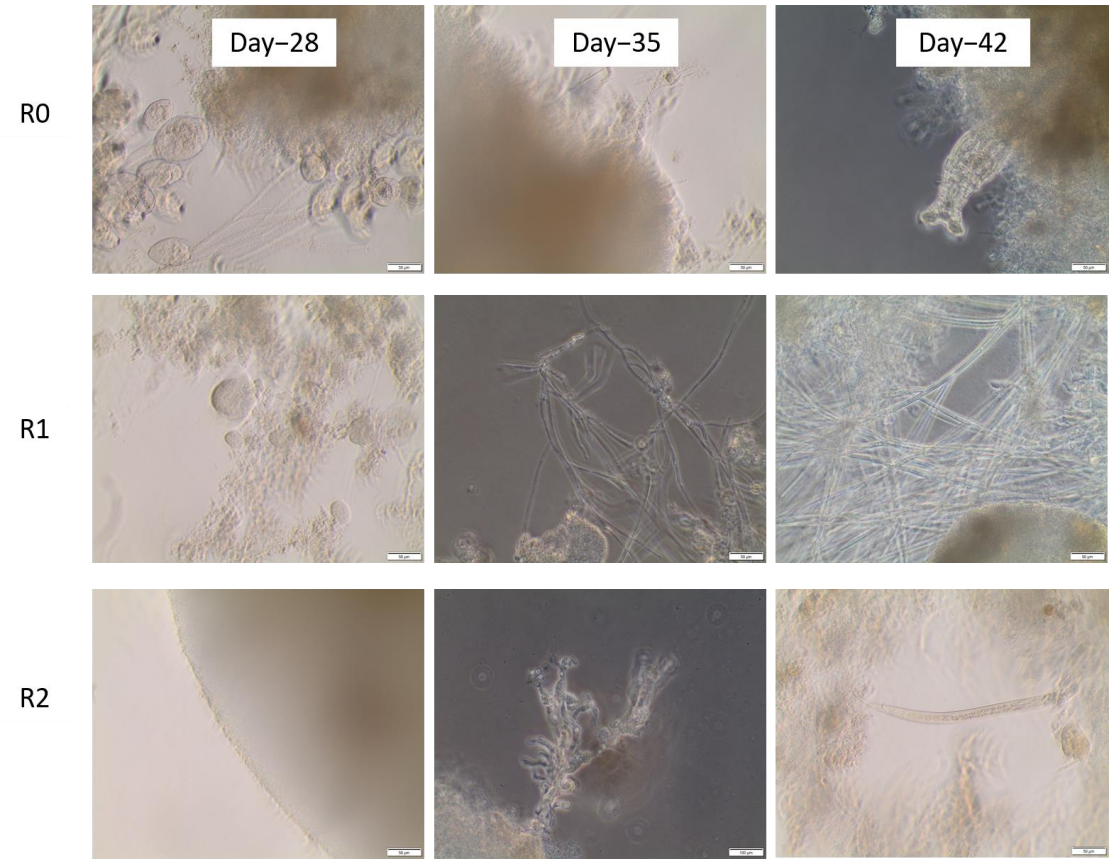

Figure 8.2 Microscopy image of 20x magnification from all reactors on day−28, day−35 and day−42.

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