Cascade anaerobic digestion system treating waste activated sludge under short sludge retention time: process performance and role of recirculation

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By

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ABSTRACT

Hydrolysis, where the complex insoluble organic matter is hydrolyzed by hydrolytic enzymes, is regarded as rate-limiting step for anaerobic digestion of waste activated sludge (WAS). The cascade system, which consisted of small-reactors in series, was developed in this study to enhance the hydrolysis rate. Degradation efficiency of sludge was strongly related to the sludge retention time (SRT) and recirculation ratio (RR). Therefore, this study investigated the effect of SRT and RR on process performance and hydrolysis enzymatic activity of WAS treatment in the cascade system and conventional single continuous stirred tank reactor (CSTR) system. The cascade system's chemical oxygen demand (COD), volatile solid (VS), protein and carbohydrates reduction, and methane production could be maintained despite the decrease of SRT, which was associated with an increase in organic loading rate (OLR) (15 d = 3.33 $gCOD\ L^{-1}d^{-1}$, 12 d = 4.16 $gCOD\ L^{-1}d^{-1}$). The performance of reference CSTR system deteriorated at shorter SRT. Hydrolysis, acidogenesis and methanogenesis analysis indicated that shorter SRT accelerated the hydrolysis rate of cascade system from $1.29 \pm 0.23\ gCOD\ L^{-1}d^{-1}$ (SRT=15 d) to $1.59 \pm 0.19\ gCOD\ L^{-1}d^{-1}$ (SRT=12 d), leading to the increase of overall degradation of WAS. The enhancement of hydrolysis rate was supported by the increased protease and cellulase activity detected at shorter SRT. Recirculating the effluent from reactor3 (R3) to reactor 1 (R1) was also essential to maintain the performance. The results not only showed deteriorating performance of cascade system regarding lower COD, VS, protein, carbohydrates reduction and methane production, but also presented lower hydrolysis enzymatic activity at 2% RR compared to 10% RR. 10% recirculation of solid phase, which contained biomass and sludge-attached enzymes, was beneficial to recover the performance of cascade system but the performance was not as good as that with 10 % recirculation of both solid and liquid phase of R3 effluent. The cations analysis revealed that the required quantities of Cobalt (Co), Nickel (Ni) and Zinc (Zn) was highest in R1 while the concentration of soluble Co, Ni and Zn was lowest in R1. The concentration of Co, Zn and Ni was increased along AD of cascade system, providing the opportunities of offering increment of trace elements to the growth of microbial community and enzymes formation in R1. In conclusion, cascade system had better performance at shorter SRT compared to single CSTR. The recirculation played an important role to maintain the superior performance of cascade system under high OLR.

Key words: cascade system; hydrolysis; sludge retention time; recirculation ratio; hydrolysis enzymatic activity; trace elements; waste activated sludge

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NOMENCLATURE

WAS	Wasted activated sludge
SRT	Solid retention time
OLR	Organic loading rate
AD	Anaerobic digestion
RR	Recirculation ratio
VFA	Volatile fatty acid
CSTR	Continuously stirred tank reactor
PFR	Plug-flow reactor
HRT	Hydraulic retention time
Cobalt	Co
Zinc	Zn
Nickel	Ni
Magnesium	Mg
Calcium	Ca
Iron	Fe
PAO	Phosphate-accumulating organisms
GAO	Glycose-accumulating organisms
PHA	Polyhydroxyalkanoates
Total COD	tCOD
Soluble COD	sCOD
BMP	Biochemical methane potential
Volatile solid	VS
Total solid	TS

1. INTRODUCTION

Activated sludge has been widely used in municipal wastewater treatment and large amount of residual waste activated sludge (WAS) are generated. The handling and disposal of WAS is a problem of growing importance, which takes up 60% of the total operational cost (Wei et al., 2003). Effective management of WAS, including removing degradable organic matter, reducing its volume and minimizing subsequent odors and pathogen vectors, is crucial for the operation of the wastewater treatment plant. Anaerobic digestion (AD) is widely accepted and highly recommended for WAS treatment due to its lower energy requirement and comparably moderate performance (Claire Bougrier et al., 2006). Hydrolysis, acidogenesis, acetogenesis and methanogenesis are the biological steps involved in AD. Compared to primary sludge, WAS is only half digestible as primary sludge (Ji et al., 2010; L.-J. Wu et al., 2015). Hydrolysis is generally identified as an overall rate-limiting step among the four steps for the WAS digestion and the first-order kinetics may be the most appropriate kinetics to explain the hydrolysis of WAS which has dominant particulate organic matter.

A slow hydrolysis rate exists in conventional mesophilic single CSTR for WAS treatment, leading to a low overall degradation efficiency of organic matter. In order to enhance the hydrolysis rate of WAS, the applications of different pretreatment technologies, such as thermal hydrolysis mechanical-, biological-, chemical- and alkali- pretreatment, can increase the speed of degradation as well as improve the biodegradability of WAS (Carrère et al., 2010). Apart from pretreatment, thermophilic digesters can also increase solubility of organic matter and chemical-biological reaction rates. However, they are not economically feasible with high-cost commercial enzymatic preparation and sustainable energy input (Huang et al., 2019; Parmar et al., 2001). There is little attention paid on modifying the configurations of bioreactors to enhance hydrolysis rate of WAS anaerobic digestion. Single CSTR is the most common form of AD but it is more suitable for homogeneous and easily biodegradable substrate. A long HRT (20-28days) is always required in AD of WAS (Bolzonella et al., 2005). A serial CSTRs configuration is proposed to improve the biogas production. For easily degradable substrates, such as fruits and vegetables, serial CSTRs function as two-phase system to achieve high efficiency of degradation and to maintain the stability of methanogens (Smith et al., 1989; Wang et al., 1999). For the AD of the WAS, in which hydrolysis is the rate limiting step, it is suggested that a higher methane production can be achieved by adopting serial CSTRs (Athanasoulia et al., 2012). According to first-order kinetics, a smaller volume of CSTR can increase the concentration of non-hydrolyzed organic matter thus increased hydrolysis rate is expected. In that case, cascade system is proposed in this study and it is simulated with CSTR in series.

SRT and recirculation play important roles in determining the efficiency of WAS reduction. Partial sludge stabilization can occur at long SRT while it also results in low reduction efficiency due to the low concentration of non-hydrolyzed organic matter. Higher degradation efficiency was already achieved at SRT 15 days compared to SRT 22 days in cascade system (Nair, 2019). Therefore, a further reduction of SRT from 15 days to 12 days is expected to exhibit accelerated enzymatic activity in cascade system. During the operation of staged AD system for easily-

hydrolyzed solid waste treatment, generally a high recirculation ratio (50-200%) is conducted to maintain the stability of the system by avoiding VFA concentration to reach toxicity level to methanogens. A much smaller recirculation ratio (10%) is already adopted in the novel cascade system for WAS treatment and lower recirculation ratio (RR) may provide higher substrate concentration thus higher hydrolysis rate. No detailed information related to the role of recirculation in cascade system for WAS reduction has been provided. Thus, a lower RR (2%) is conducted in this study to investigate the importance of the recirculation ration to AD of WAS.

A cascade system is proposed to achieve a higher degradation efficiency compared to single CSTR system. Aiming at investigating the effect of SRT and recirculation on the efficiency of WAS reduction, several SRT and recirculation ratio are adopted in this study. Lowering SRT from 15 days to 12 days is expected to increase the efficiency of organic matter degradation and improve biogas production with higher concentration of non-hydrolyzed organic matter. The role of recirculation for WAS anaerobic digestion is investigated in this study by lowering the recirculation ratio from 10% to 2%.

1.1 Thesis outline

The thesis is divided into the following sections:

1. **Introduction**

2. **Literature review**

This section provides information about the biological process in anaerobic digestion of WAS with emphasis on hydrolysis. The hydrolysis is the rate limiting step of AD of WAS and it follows the first order kinetics. Except pre-treatment, changing the configurations of anaerobic digestion has been developed to enhance hydrolysis of WAS. A detailed overview of single CSTR and serial CSTRs for different substrates are presented. The effects of operational parameters on AD of WAS are listed in this section with emphasis on sludge retention time and recirculation ratio. Based on the literature review, the research gap in this area of study was listed. Two main research questions regarding to the research gaps are then formulated in this section.

3. **Materials and methodology**

The details of the set-up of cascade system as well as reference CSTR are introduced here. Methodology of operations are characterized as feeding characterization, operational conditions and sampling. Furthermore, the analytical methods conducted in this study are described precisely with regard to online monitoring, physicochemical analysis and enzymatic activity analysis. Finally, the equations related to the assessment calculations of hydrolysis, acidogenesis and methanogenesis rate as well as cations requirement are presented in this section.

4. **Results and discussion**

The results of this study will firstly show the overall performance of cascade system and reference CSTR system under different SRT and RR. Based on COD balance, the hydrolysis, acidogenesis, methanogenesis rates and degree are calculated to evaluate the change of biological process under different operational conditions. As direct indicator of hydrolysis, the results of hydrolysis enzymatic activity are also shown to further evaluate the contributions of SRT and RR. Last but not least, the data of cations analysis is presented to further investigate the role of RR in terms of nutrients increment.

5. **Conclusion and recommendation**

2. LITERATURE REVIEW

2.1 Waste activated sludge

Over the last few decades, activated sludge has been widely used in municipal wastewater treatment as the microorganisms present in it helps to transform the dissolved organic pollutants into biomass and carbon dioxide. The main byproduct of municipal wastewater treatment is the disposed activated sludge. The expansion of wastewater treatment plants and the increasingly stringent effluent regulations contribute to higher production of wasted activated sludge (WAS) from wastewater treatment plants (Generation, 1999). The handling and disposal of WAS are quite expensive and the traditional WAS treatment accounts for up to 60% of the total operational cost of a wastewater treatment plant (Wei et al., 2003). The objectives of sludge treatment are to remove degradable organic matter as well as prevent subsequent odors and pathogen vectors (Koorse, 1993).

2.1.1 Anaerobic digestion

In the context of decreasing fossil fuel reserve, anaerobic digestion (AD) is commonly suggested to be a sustainable technology for WAS treatment due to the fact that it not only allows a reduction of sludge quantity of about 40-50%, but also generates energy by methane production (Claire Bougrier et al., 2006). Lower cost, lower energy footprint, less energy requirement, and comparably moderate performance enable AD to become a favored treatment of WAS compared to aerobic digestion. Instead of oxygen, AD utilizes SO_4^{2-}, and organic matter as electron acceptor. Without the presence of oxygen, AD utilizes anaerobic microorganisms to convert organic matter to biogas, which includes 55-75 vol% methane and 25-40 vol% carbon dioxide (Simate et al., 2011). AD goes through four interdependent, complex sequential and parallel biological steps, which are hydrolysis, acidogenesis (fermentation), acetogenesis and methanogenesis, shown in Figure 1 (Henze et al., 2008).

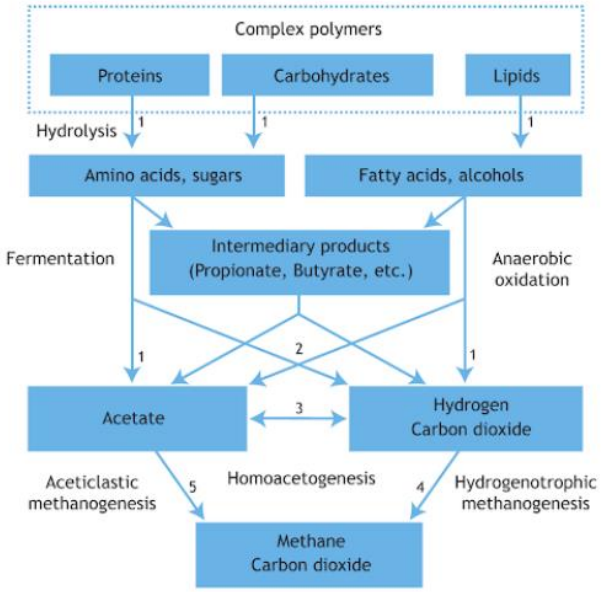


Figure 1 Review Scheme for the Anaerobic Digestion. Numbers suggest the microorganisms related: 1. Hydrolysis and fermentation microorganisms, 2. Acetogenic microorganisms, 3. Homo-acetogenic microorganisms, 4. Hydrogenotrophic methanogens, 5. Aceticlastic methanogens (Henze et al., 2008)

Hydrolysis is a process breaking down the proteins, carbohydrates, lipids and other minor components into a soluble and smaller size organic compounds like amino acids and sugars by multiple extracellular enzymes from microorganisms (Ayol, 2005). During acidogenesis, the dissolved compounds produced are converted into volatile fatty acid (VFA), alcohols, lactic acid, CO_2, H_2, NH_3 and H_2S as well as cell materials by fermentative bacteria. Then acetogenic bacteria digest the products from acidogenesis to acetate, CO_2, H_2 and cell materials. During methanogenesis, acetolactic methanogens convert acetate to methane (CH_4) and hydrogenotrophic methanogens utilize CO_2 and H_2 as substrate to produce CH_4(Henze et al., 2008).

Unlike primary sludge, WAS is characterized with relatively low degradability at long sludge ages (Gossett et al., 1982). Primary sludge is generated through a mechanical wastewater treatment process, mainly consisting of kitchen and toilet garbage, such as feces, vegetables, fruits, textiles and paper(Ji et al., 2010). The main components of WAS are biomass and non-hydrolysable particulate materials because of biological metabolism (Ji et al., 2010). And it is reported that only 30-40% organic matter content of WAS can be degraded with 10-20 days SRT under mesophilic conditions (Takashima, 2008). Due to the fact that most organic matter of WAS are located inside the cells and there is a semi-rigid cell envelop protecting the cells from osmotic lysis, hydrolysis is generally identified as an overall rate-limiting step among the four steps for the WAS digestion (Eastman et al., 1981c; Kim et al., 2011; Y Li et al., 1992). Hydrolysis process relies on effective and efficient reaction of various enzymes. There are some specific enzymes synthesized and secreted during hydrolysis, including proteases for proteins (Whiteley et al., 2003), amylase and cellulase for carbohydrates(Van Der Maarel et al., 2002), and lipases for lipids (Whiteley et al., 2003).

2.1.2 First-order kinetics

The hydrolysis of particulate organic matter has traditionally been modeled by the first-order kinetics which reflects cumulative effects of all the microscopic processes occurring in the anaerobic digestion (Eastman et al., 1981a). Not all the particulate organic matter in the substrate can be degraded with equal facility. Particulates hydrolysis is a surface related phenomenon, which is affected by the specific surface area of the particle (the particle size). Large particles with a low surface-to volume ratio has higher hydrolysis rate than small particles Different types of organic matter, such as proteins, polysaccharides, lipids are hydrolyzed at different rates as well. In this case, the overall hydrolysis function represents the sum of the individual processes taking place in the anaerobic digestion. First-order kinetics can only be applied when the rate-limiting factor is the surface of the particulate substrate (Vavilin et al., 2008). Leiyu et al. (2009) demonstrated that the hydrolysis of WAS particulate COD obeyed the first-order kinetics. Mahmoud et al. (2004) also mentioned that the hydrolysis of the main biopolymers and overall particulate COD of the primary sludge digested in CSTRs were well described by first-order kinetics. Therefore, for WAS, a kind of complex and heterogeneous substrate, with dominant particulate organic matter, first-order kinetics may be most appropriate to describe its degradation function (Eastman et al., 1981b).
Several factors, such as pH, temperature, enzyme types and hydrolytic substance, has effects on the hydrolysis process of WAS. From the past investigation, it is proposed that the hydrolysis rate is linearly related to the amount of biodegradable substrate in the anaerobic digestors at constant pH and temperature, shown in Equation 1 (W. T. M. Sanders, 2001)

$$\frac{dX_{degr.}}{dt} = -k_h X_{degr.} \quad \text{(Equation 1)}$$

In which:

$X_{degr.} = biodegradable\ substrate\ (kg\ COD\ m^{-3})$

$k_h = first\ order\ hydrolysis\ constant\ (day^{-1})$

$t = time\ (day)$

2.2 Efforts to enhance hydrolysis

In order to enhance hydrolysis, several efforts have been made in terms of pre-treatment to increase enzymatic rate by accelerating the solubilization and reducing the particle size of WAS, including thermal hydrolysis mechanical (like ultrasound, high pressure or lysis), biological (mainly thermal phased anaerobic), chemical (largely ozonation) and alkali pre-treatments (Carrère et al., 2010). In addition, past research demonstrated that adding certain enzymes or specific bacteria which can secrete certain enzymes prior to anaerobic digestion can enhance the enzymatic activity of hydrolysis (Yu et al., 2013). Even though these methods can increase speed of degradation and improve biodegradability of WAS in different extents due to increased accessible surface area, they are not economically feasible because of high-cost commercial enzymatic preparation and substantial energy input (Huang et al., 2019; Parmar et al., 2001). Only limited mechanical, thermal and thermo-chemical methods were effectively applied in full scale due to the disadvantages related to pretreatments, such as increasing operational cost, byproducts-poisoning of the AD system, secondary pollution due to the chemicals etc. (Jain et al., 2015). However, little attention has been given to the investigating AD process of WAS to improve hydrolysis rate and the overall energy recovery. It is observed that a slow hydrolysis rate of WAS exists in the conventional one stage anaerobic digester (Eastman et al., 1981c; Kim et al., 2011; Y Li et al., 1992). Therefore, it is crucial to develop the original bioreactor designs to improve the hydrolysis efficiency, thus increasing overall degradation efficiency and biogas production.

2.3 Different types of bioreactors

Apart from adopting pretreatment before the WAS digestion, a variety of new bioreactor designs have been developed in recent years to facilitate a higher rate of reaction for the treatment of organic wastes (Bouallagui et al., 2003; Mumme et al., 2010). Different types of bioreactors used in AD are discussed below.

2.3.1 Single CSTR

Continuously stirred tank reactor (CSTR) is the most common form of large-scale anaerobic digesters (Manyi-Loh et al., 2013). In a single CSTR, feed is introduced to reactor and the biomass is suspended in the main liquid through intermittent or continuous mixing, contributing to good substrate-sludge contact with slight mass transfer resistance (Mao et al., 2015). One of the biggest technical drawbacks of CSTR is the occurrence of short-circuiting, which indicates that the remaining organic matter have a shorter residence time than the nominal residence time (Y. Liu et al., 2019). It is more suitable to treat homogeneous and easily biodegradable substrate. Generally, a long hydraulic retention time (HRT), which is between 20-28 days, is set to reach the required degradation of WAS and avoid washing out the slow growing methanogens for WAS treatment (Boe et al., 2006).

2.3.2 Serial CSTRs

In recent years, several bioreactors in series are introduced to overcome restrictions of single CSTRs and improve the performance of biogas production. Generally, serial CSTRs can provide superior performance with longer biomass retention time and less fresh feed loss due to the elimination of the 'short-circuiting' phenomenon. For the substrate with different characteristics, the configurations of serial CSTR have different effects on the biological process in the anaerobic digesters. Based on different substrates, the functions of serial CSTRs are discussed below:

(1) For easily biodegradable substrates

For the AD of easily biodegradable solid wastes, which contain high fraction of easily degradable substrate, serial CSTRs functioned as two-phase anaerobic digesters, which are aimed at improving AD by having separate reactors for the different phases to provide flexibility to optimize each of these reactors. The high efficiency of hydrolysis and acidogenesis in easily biodegradable substrates may cause accumulation of VFA and induce a sharp drop of pH, which is toxic to the methanogens. The growth of hydrolytic bacteria, acidogenic bacteria and methanogenic archaea adapts to different environment. Typically, given the optimum pH for hydrolysis and acidogenesis are 5.5-6.5 while for methanogenesis is 6.5-8.2, hydrolysis/acetogenesis and methanogenesis are separated in two different reactors to maintain the high efficiency of organic matter degradation as well as supply instability of methanogens (Lee et al., 2009). However, the operation of two-phase anaerobic digesters are complicated and separation processes are costly. In addition, the disruption of the syntrophic relationship between syntrophic bacteria and methanogens may cause more product inhibition in acidogenic reactor (Smith et al., 1989). Moreover, accumulation of high hydrogen partial pressure can promote the accumulation of higher molecule VFA than acetate and the degradation of acetogenesis would be inhibited by high concentration of acetate (Smith et al., 1989; Wang et al., 1999). Nevertheless, it is reported that the degradation efficiency of lignocellulosic-rich substrates in a two-phase anaerobic digesters was much lower than that of substrates with high sugar content (Lindner et al., 2016). Therefore, two-phased anaerobic bioreactors are more suitable for easily biodegradable solid waste, like fruits and vegetables.

(2) For hard biodegradable substrates

For the AD of the substrates in which hydrolysis is yield limiting step for methanogenesis and the conversion of VFA is the rate limiting step for achieving stable process, such as manure, corn stover, mixture of primary sludge and WAS, single CSTR and two-phase system are not efficient enough to achieve high methane production. The introduction of serial CSTR configuration on methanogenic phase reactors has attracted considerable attention. The application of serial CSTRs in different substrates can be summarized in Table 1. The researches of Kaparaju et al. (2009) and Boe et al. (2009) revealed that two methanogenic reactors for manure treatment produced more biogas and contained less VFA as well as residual methane potential loss in the effluent compared to single CSTR. Two thermophilic CSTRs connected serially with equal volumes distribution were installed to treat meadow grass and cattle manure, leading to 24% more methane than a single CSTR (Feng et al., 2017). The increase of methane production of manure AD can be ascribed to similar syntrophic relationship between acetogens and methanogens in both methanogenic

reactors. The longer HRT can be achieved by bigger volume distribution of the 1st reactor due to the hydrolysis of the particulate organic matter (Kaparaju et al., 2009). The 1st reactor is regarded as a main mixed-culture methanogenic reactor, and the 2nd reactor as a recovery stage or effluent (Boe et al., 2009). Furthermore, the accumulation of intermediate process inhibitors in the 1st reactor can be partly removed to the 2nd reactor. In addition, due to the fact that corn has complex three-dimensional structures and the hydrolysis is rate-limiting step during AD, a combination of the two-stage and serial CSTRs was developed to achieve high biogas efficiency AD of corn stover, in which 33.2%-50.5% higher methane yield was observed than that of single CSTR and two-phased system (Y. Liu et al., 2019). The main reason of higher AD efficiency is because that there are three main peaks of gas production during the AD of lignocellulosic materials: (I) hydrolysis and acidogenesis stage, (II) first methanogenesis stage and (III) second methanogenesis stage (Y. Liu et al., 2019). A similar research done by YuQian Li et al. (2017) demonstrated the conversion rate of VS, cellulose and hemicellulose were enhanced in serial CSTRs with 20-20 days and 30-10 days HRT distribution with higher overall AD efficiency. The increase of methane production in the serial CSTRs can also be attributed to the increased biomass retention, contributing to less loss of relatively fresh feed due to 'short-circuiting' in single CSTR (Angelidaki et al., 2005). Nevertheless, it must be noticed that a higher volume is adopted in the 1st reactor since the higher hydrolysis can be achieved while the VFA concentration is too high for the methanogens during the AD of manure or food. The volume allocated to the main reactor (first reactor in serial digestion) must be sufficient to maintain a stable process with a reasonably low VFA level, as a healthy first step is a precondition for a successful serial digestion (Boe et al., 2005).

Table 1 The applications of serial CSTRs

Substrate	Number of CSTR	System configurations	Effects compared to single CSTR	Literature source
Manure	2	2 mesophilic CSTRs connected in series with 70/30%, 50/50% volume distribution	13-17.8% more CH_4 production	(Kaparaju et al., 2009)
Manure	2	2 mesophilic CSTRs connected in series with 80/20%, 90/10% volume distribution	11% higher biogas yield	(Boe et al., 2009)
Corn stover	3	3 CSTRs are divided as 1 acidogenic stage and 2 methanogenic stage	33.2–50.5% higher CH_4 yield	(Y. Liu et al., 2019)
Corn stover	4	2 mesophilic CSTRs connected in series with 20/20 days, 30/10 days HRT distribution	8.3-12.2% and 13.8-14.6% higher CH_4 production	(YuQian Li et al., 2017)
Grass and manure	2	2 thermophilic CSTRs are connected serially with equal working volumes	24% more CH_4 production	(Feng et al., 2017)
Waste activated sludge	2	2 CSTRs connected in series with 40 L and 60 L volume distribution	9.5% higher biogas production at 12.3 d SRT	(Athanasoulia et al., 2012)

(3) For waste activated sludge

WAS is a heterogeneous substrate with dominant particulate organic matter. The hydrolysis of WAS is rate limiting step and it follows first-order kinetics. Conventional single CSTR is not attractive for degradation of concentrated WAS at low SRT and high OLR. There is a need for developing an innovative configuration to AD of WAS. It is reported that the volumetric gas production rate of 2 methanogenic reactors connected in series with 40 L and 60 L working volume was 0.21-0.32 m^3 biogas/m^3 reactor/d while that of single CSTR was 0.12-0.27 m^3 biogas/m^3 reactor/d, shown in Table 1 (Athanasoulia et al., 2012). According to first-order kinetics, the smaller volume of CSTR, the higher concentration of non-hydrolysis organic matter and higher hydrolysis rate can be achieved. However, there is limited information about the applications of the different configurations of serial CSTRs in WAS treatment. Moreover, the applications mentioned above were all operated without recirculation (Table 1). To date, limited attention is paid on the effect of recirculation on serial CSTRs for AD of hard degradable substrates, especially for WAS. Taking the anaerobic digestion characteristics of WAS and the features of different configurations of serial CSTRs into consideration, a novel cascade system is proposed. The cascade system is a plug-flow mode and it is simulated with CSTR in series. In the cascade system, three smaller CSTRs are connected after each other followed by a connection to a bigger CSTR at the end. The third smaller CSTR has additionally a return flow to the first CSTR in cascade system. Compared to conventional single CSTR, cascade system provides better mass transfer rate with higher agitation. Also, cascade system allows to achieve higher non-hydrolyzed substrate concentration with smaller-volume CSTRs to enhance overall hydrolysis rate.

Based on the previous research from Nair (2019), the novel cascade system showed a superior performance at 22 days SRT compared to the conventional CSTR system. Various indicators have been conducted to show enhancement of cascade system in comparison to the conventional CSTR system. For example, the efficiency of the volatile solids (VS) fraction reduction of sludge can be used to evaluate the stability of digested sludge. Under 22 days SRT, VS removal efficiency was $35 \pm 6\%$ in cascade system while that was $33 \pm 5\%$ in single CSTR, illustrating higher stability of cascade system (Regulations, 2003). More importantly, under 22 days SRT, the total COD reduction efficiency and the methane production in the novel cascade system were higher than in conventional CSTR system, which were $39 \pm 3\%$ and 6.4 ± 0.5 L/d (cascade system) and $30 \pm 4\%$ and 5.8 ± 0.4 L/d (single CSTR) respectively, revealing higher organic matter degradable was achieved in cascade system. Moreover, it was observed that the highest hydrolysis enzymatic activity of cascade system was located in first three reactor, with only 2.2 L of each reactor compared to the final reactor with 15.4 L working volume. The hydrolysis enzymatic activity of single CSTR was somewhere between the last two reactors in the cascade system (Guo, 2019). Thus, the smaller sub-reactors of cascade system was able to provide higher enzymatic activity with higher non-hydrolyzed concentration of substrate, contributing to higher efficiency of the overall WAS degradation (Nair, 2019).

2.4 Factors influencing performance of cascade system

Within the anaerobic environment, various operating parameters, i.e. pH and alkalinity, temperature, retention times, recirculation, and sufficient nutrients, affect the rates of the different steps of the AD of WAS.

2.4.1 Temperature

Temperature has direct effect on the physical-chemical properties of all the components as well as thermodynamic and kinetic of biological process in digesters (Boe et al., 2006). The practical operating temperature of WAS digestion for mesophilic condition is 35-38℃ and for thermophilic condition is 52-56℃. There are several advantages in thermophilic digestions of WAS, such as increased solubility of organic compounds, increase chemical-biological reaction rates, improved physical chemical properties of soluble substrates and high death rates of pathogenic bacteria (van Lier, 1995). It is reported that the COD removal efficiency increased from 35% to 45% when the mesophilic conditions are changed to thermophilic conditions for WAS treatment (Bolzonella et al., 2012). Nonetheless, decrease pKa of ammonia is observed in thermophilic digesters. Higher free-ammonia fraction may inhibit the growth of microorganisms. Also, increased pKa of VFA thermophilic digestors contributes to more undissociated fraction, especially at low pH (4-5), which makes the thermophilic process more susceptible to inhibition (van Lier, 1995). Other disadvantages have also been identified, such as larger investment, higher net energy input, decreased stability and low-quality effluent (Mao et al., 2015). Compared to thermophilic AD, even though lower methane yield and poor biodegradability are observed in mesophilic AD, it exists higher richness in bacteria and better process stability (Mao et al., 2015).

2.4.2 pH and alkalinity

pH has an effect on enzymatic activity in microorganisms and different groups of microorganisms have different optimum pH range (Lay et al., 1997). The optimal pH range for methanogenic archaea is 6.5-8.0 while a wider pH range (4-8.5) is suitable for growth of fermentative bacteria (Lee et al., 2009). However, different types of fermentation products are generated under different pH condition. The study from Horiuchi et al. (2002) demonstrates that at pH 8.0, the main products are acetic and propionic acid, while at pH 5-7, the main products are acetic and butyric acid. The system pH is mainly influenced by alkalinity. Alkalinity in system results from the presence of the hydroxides, carbonates and bicarbonates of elements such as calcium, magnesium or ammonia. The ratio of VFA and alkalinity is one of the important criteria for judging digester stability. It is reported that if VFA:alkalinity ratio is in between 0.4 and 0.8, some instability will occur in the system and if it is larger than 0.8, the system is significantly instable (Switzenbaum et al., 1990; Zickefoose et al., 1976). Therefore, a VFA:alkalinity ratio between 0.1 and 0.35 or the alkalinity between 1000 and 5000 mg$CaCO_3$/L is recommended in a healthy digester (Switzenbaum et al., 1990).

2.4.3 Solid retention time

SRT is associated with microbial growth rates and it plays an important role in determining the efficiency of WAS reduction. For the CSTR in series of WAS treatment, the hydraulic retention time (HRT) is equal to SRT. Conventional CSTR system of WAS treatment requires long retention time, between 20-40 days, in order to prevent washout of methanogens due to the slow-growth rate of methanogens and achieve the optimal degradation efficiency of WAS (Bolzonella et al., 2005). The hydrolysis rate is related to the particle size of the particles or the number of adsorption sites at the particle surface. Long SRT results in relatively low non-hydrolyzed substrate concentration in CSTR, leading to lower hydrolysis rate. The study from Bolzonella et al. (2005) also observed lower biogas production with higher SRT (from 8 to 35 days) adopted, reporting that the specific gas production decreased from 0.18 to 0.07 m^3/kg VS_{fed} when SRT increased from 8 to 35 days. Following first-order kinetics, the research of O'Rourke (1968) illustrated that with the increase of SRT, the volumetric rate of hydrolysis is dropping. Therefore, higher enzymatic activity during hydrolysis process can be acquired by adopting lower SRT, which gives access to maximize the substrate concentration (de Gooijer et al., 1996). The research of Guo (2019) showed that the hydrolysis enzymes activities (protease and cellulase) with SRT of 15 days were higher than that with 22 days, indicating a shorter SRT can contribute to a higher hydrolysis rate. In particular, the novel cascade system and the CSTR with shorter SRT released more ammonia and phosphate, corresponding to more protein and carbohydrate degradation (Nair, 2019). Moreover, shorter SRT while still maintaining digestion performance is welcomed in industry because it provides the opportunity to reduce reactor size for a given load (Maspolim et al., 2015). During 15 days running, the part research from Nair (2019) revealed that the cascade system as well as the conventional CSTR system were stable, indicating that lower SRT can be set to further accelerate the hydrolysis efficiency of both systems.

2.4.4 Recirculation

Recirculation ratio (ratio of recycle flow rate to feeding flow rate) plays a significant role on the operation of the staged AD system (Romli et al., 1994). It has multiple benefits, such as the reduction of VFA concentration to avoid it reaching toxicity level for methanogens, improving suitable buffer capacity by alkalinity increment and supplying external microbes by sludge exchange (Aslanzadeh et al., 2013). Moreover, it is also demonstrated that high recirculation ratio (RR) can accelerate hydrolysis and the total extracellular enzyme activities have been improved due to the rapidly refreshment of the niche around the hydrolytic enzyme, increment of enzyme concomitantly and the introduction of an established microbial population from methanogenic reactor (Confer et al., 1998; Zhang et al., 2007).

However, compared to other studies (Kafle et al., 2011; Romli et al., 1994; Xu et al., 2011) where RR of 50-200% RR was applied, a much smaller RR (10%) was adopted in the novel cascade system. Most of researches which adopted high RR in WAS treatment focused on thermophilic anaerobic digesters or the two-phased anaerobic digesters after pretreatment (Kafle et al., 2011; Wu et al., 2016; L. Wu et al., 2015; Xu et al., 2011). The reasons of using high RR is because the AD of WAS in thermophilic digesters or after pretreatment accumulated high VFA and result in low pH (<6.5), causing toxicity to methanogens, thus reducing the methane production of the whole AD. It is reported that the VFA concentration is over 2000 mg/L which can be inhibitive to methanogens and the high VFA concentration can be ascribed to the high efficiency of hydrolysis and acidogenesis (Zhang et al., 2007; Zuo et al., 2015). Hydrolysis is the rate-limiting step of the overall WAS anaerobic digestion, especially in mesophilic condition. Thus the VFA in AD of WAS is not accumulated as fast as easily-hydrolyzed solid waste. According to the result from Nair (2019), the highest total VFA concentration existed in R1 of cascade system, which was $568 \pm 137\ mg/L$. In that case, the incentive of reducing toxicity of VFA to maintain stability of the cascade system is not as strong as other research with AD for easily-hydrolyzed solid waste. Also, lower recirculation ratio would reduce the operational cost.

In the previous study, recirculation was applied in two-phased thermophilic systems to provide alkalinity in order to avoid the collapse of the systems due to VFA accumulation. However, up to now, no previous study has examined the roles of recirculation in a serial digestion system for AD of WAS, especially in mesophilic condition. Such information is especially important where several smaller reactors in series are applied, especially under short SRT. Reducing the RR in cascade system from 10% to 2% may influence the stability of the system as well as the hydrolysis enzymatic activity, thus has impacts on overall degradation efficiency. However, whether it is feasible to reach such low RR in cascade system is not clear yet. Therefore, it is crucial to investigate the importance of recirculation on the cascade system for WAS anaerobic digestion.

2.5 Research gaps & research questions

2.5.1 Research gaps

According to the literature review, the following research gap can be listed below:

(1) **The applications of serial CSTRs in anaerobic digestion of waste activated sludge have not been well investigated and whether the serial CSTRs achieve higher degradation efficiency and higher methane production in anaerobic digestion of waste activated sludge at short SRT compared to conventional single CSTR is not clear yet.**

(2) **The option of decreasing SRT to enhance enzymatic activity thus increase overall degradation efficiency requires further investigation till it reaches boundary condition.**

(3) **Although the importance of recirculation has studied well recently for the anaerobic digestion of easily degradable solid waste, the role of recirculation on the anaerobic digestion of waste activated sludge is not clear yet.**

2.5.2 Research questions

Based on the research gaps, two main research questions and sub questions can be formulated:

(1) **To what extent will the performance of cascade system and CSTR change when the SRT drops from 15 days to 12 days under 10% RR?**

a) What are the differences for cascade system and conventional CSTR between SRT 15 days and 12 days with respect to general indicators, such as COD, N, P, methane production, total solid, volatile solid, biogas composition, total proteins, total polysaccharides, alkalinity and VFA?

b) What are the changes of the protease and cellulase activity in free enzymes and attached enzymes under the shorten SRT? Will the hydrolysis rate evaluated by COD balance and the measured enzymatic activity show the same trend with the change of SRT?

(2) **To what extent will the performance of cascade system change when recirculation ratio drops from 10% to 2%?**

a) Set the SRT to the minimum in which the system still function based on the previous experiment. With the change of recirculation set up, what are the changes of COD, N, P, methane production, total solid, volatile solid, biogas composition, total proteins, total polysaccharides, alkalinity and VFA for cascade system and conventional CSTR?

b) What are the changes of the protease and cellulase activity in free enzymes and attached enzymes under the different RR? Will the hydrolysis rate evaluated by COD balance and the measured enzymatic activity show the same trend with the change of recirculation?

3. MATERIALS & METHODOLOGY

3.1 Reactor set-up

The schematic diagram of the set-up of Cascade system as well as reference single CSTR is shown in Figure 2. The Cascade system consisted of four CSTR, which were connected in series. The operational volume of first three CSTRs (R1-R3) were 2.2 L, whereas the fourth reactor (R4) was 15.4 L. The exit of previous reactor located at the top of CSTR and it leads to the bottom side of next reactor. The conventional single CSTR was regarded as reference bioreactor and the operational volume was as the same as the total volume of Cascade system, 22 L. The recirculation was introduced in cascade system from R3 to R1. Reactors were fed semi-continuously using 520 Du peristaltic pumps (Watson Marlow). Ritter® wet tip biogas meters were connected to CSTRs to measure the biogas production. A pH probe and temperature probes were installed at the top of CSTRs. The feed bucket located in a fridge to keep 4 ℃. The temperature of all the bioreactors in this set-up were maintain within mesophilic range (35 ± 1℃) by a water jacket recirculation system (PMT TC16, Tamson, Holland). During the operation of the reactor, 40 g/L antifoaming solution (Silicone anti-foaming emulsion) was added to R1. The tank which stored antifoaming solution was continuously mixed by magnetic stir (L23, LABINCO) to keep homogeneity of the antifoaming solution and it was operated in room temperature.

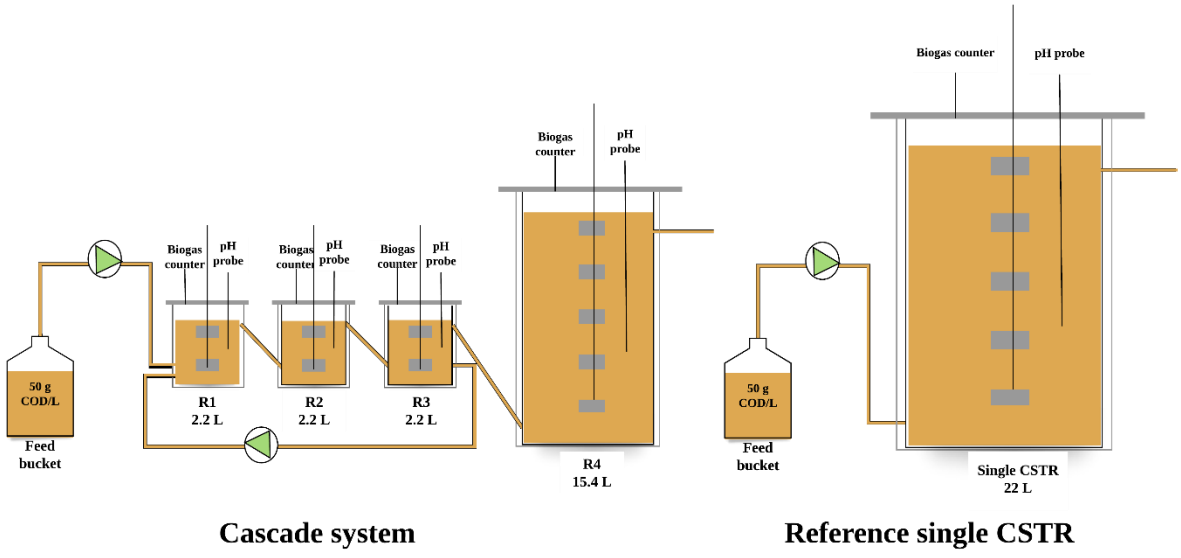


Figure 2 Scheme of the set-up of cascade system (left) and reference CSTR (right)

3.2 Methodology

3.2.1 Feeding characterization

The influent fed into the system was the concentrated WAS, obtained from sewage treatment plant Harnaschpolder, Den Hoorn, Netherlands. The WAS was originally performed in the enhanced biological phosphorous removal process. The raw sludge from sewage treatment plant was then diluted and the influent sludge concentration was maintained at about 50 gCOD/L. During the whole operation, the influent sludge was stored in fridge at 4 ℃ to guarantee the stable characteristics of the substrate.

3.2.2 Operational conditions

In order to investigate the effects of SRT and recirculation ratio to the digestion efficiency of cascade system, the whole operation period was divided into 2 phases: Phase I, Phase II with different SRTs and different RR:

- Phase I (0-96 days): Sequential feeding (2.5-hour feeding, 0.5-hour reaction) at two different operational conditions. First, SRT was set at 15 days by keeping the feeding flow rate at 1.47 L/day (0-43 days). Second, SRT was reduced to 12 days by increasing the feeding flow rate to 1.83 L/day (44-96 days). In this phase, 10% recirculation was introduced from R3 to R1.

- Phase II (97-165 days): Based on the 12 days SRT as well as 10% recirculation ratio set from last phase, recirculation ratio was firstly reduced to 2% (97-133 days) to investigate the role of recirculation ratio. Finally, in order to investigate the functions of biomass and sludge-attached enzymes, the soluble and pellets of effluent of R3 was separated. 10% of effluent of R3 was centrifuged under 13500 rpm for 15 min. The pellet was collected and the demi-water was combined to the volume of 10% of flow. Then the mixed liquid was added to R1. This period can be called as recovery period. (133-165 days).

3.2.3 Sampling and analysis

Samples were taken at regular basis from the Cascade system and reference CSTR to evaluate systems' performance. The operational parameters as well as their frequency were summarized in Table 2:

Table 2 Biological performance parameters

Parameters	Unit	Sample and frequency	
		Sludge	Biogas
pH	-	3/w	
Temperature	℃	c	
Biogas volume	L		7/w
Biogas CH_4 content	%		1/w
Total COD	g COD/L	3/w	
Soluble COD	g COD/L	3/w	
NH_4^+	mg N/L	1/w	
PO_3^-	mg P/L	1/w	
Total solid		1/w	
Volatile solid		1/w	
VFA	mg VFA/L	1/w	
Alkalinity		once stable	
Anions		once stable	
Cations		once stable	
Protein		once stable	
Polysaccharides		once stable	

Reference: c-continuous, x/w-x times a week, once stable-once the systems were stable

3.3 Analytical methods

3.3.1 Online monitored parameters

The following parameters were continuously monitored and registered by the LabVIEW 2016:

- **pH**
- **temperature**
- **biogas production**
- **Redox**

3.3.2 Physicochemical analysis

The sludge samples from each reactor and influent as well as biogas samples were taken and analyzed regularly or once the reactors were stable to evaluate the performance of the systems. The analytical methods applied to determine the physicochemical parameters, which can be summarized in Table 3:

Table 3 The analytical methods of physiochemical analysis for cascade system and reference CSTR

Parameters	Analytical methods
Total COD	Hach Lange test kits 014
Soluble COD	HACH Lange test kits 314
pH	pH meter (Multi 9620 IDS, WTW)
NH_4^+	HACH Lange test kits 303
PO_3^-	HACH Lange test kits 350
VS/TS	Standard method gravimetric analysis (APHA)
VFA	Gas chromatography (GC) (7890A, Agilent Technologies, USA) with a flame ionization detector (FID) (Agilent 7890A, USA)
Biogas volume	Ritter® wet tip biogas meters
Biogas Composition	Gas chromatography (GC) (7890A, Agilent Technologies, USA)
Anions	Inductively coupled plasma mass spectrometry (ICP-MS) (Plasma Quant) measurement after centrifugation (ST 16R, Thermo Scientific) and filtration with glass fiber filter as well as acidified by 1% HNO_3
Cations	Inductively coupled plasma mass spectrometry (ICP-MS) (Plasma Quant) measurement after centrifugation (ST 16R, Thermo Scientific) and filtration with glass fiber filter as well as acidified by 1% HNO_3
Alkalinity	Digital titration apparatus with 0.1 M hydrochloric acid
Protein	Modified Lowry method with bovine serum albumin (Sigma, USA) (Frolund et al., 1995)
Polysaccharides	Standard method: phenol-sulfate examination method using glucose (Sigma, USA) (Dubois et al., 1956)

3.3.3 Enzymatic activity analysis

In terms of two conceptual models represented for hydrolysis disintegration of complex organic matter, especially WAS, the hydrolytic enzymes can be characterized as free enzymes and sludge-attached enzymes. The free enzymes are defined as the enzymes secreted by the organisms to the bulk liquid, from which the enzymes can be absorbed onto particle or reacts with soluble substrates (Batstone et al., 2002). For the sludge-attached enzymes, it is demonstrated that enzymes are produced by the organisms which attach to the particles and benefit from soluble products in liquid (Batstone et al., 2002).

The activities of total protease and cellulase (including free and attached enzymes) are individually analysed by Pierce fluorescent protease assay kit (Thermo Fisher, USA) and MarkerGene™ fluorescent cellulase assay kit (MarkerGene, USA), using a 96-well microplate spectrophotometer (Synergy HTX, BioTek, USA) at 35 °C.

Firstly, 1 mL sludge samples were centrifuged at 3000 rpm for 30 seconds to separate block things from cell bulk. The supernatant was then centrifuged at 14000 rpm for 1 min to separate free (supernatant) and sludge-attached (pellets) enzymes. The pellets were washed and resuspended in 1 mL PBS, followed by 3000 rpm centrifuging for 30 seconds to separate cell-attached enzymes and cell. The liquid with free and sludge-attached was transferred to a container plate.

For the protease measurement, PBS buffer and 0.5 mM substrate reagent were added to the container plate. Finally, the fluorescence results were measured in a microtiter plate reader.

For the cellulase measurement, PBS buffer and 100μL of substrate working reagent were add to the plate, followed by 5-60 min incubation at room temperature. Finally, the fluorescence results were measured in fluorescein excitation/emission filter set

3.4 Assessment calculation

3.4.1 COD balance-based calculation of hydrolysis, acidogenesis and methanogenesis

The equations related to COD conversion ratios of hydrolysis, acidogenesis, methanogenesis were calculated from a COD balance in the anaerobic digestion process (L.-J. Wu et al., 2015). Soluble COD, total COD, VS, COD_{VFA} and COD_{CH4} were used to define the total COD conversion ratios of hydrolysis, acidogenesis, methanogenesis, which were shown below:

(1) Hydrolysis equations

$$\boldsymbol{Hydrolysis\ degree\ (COD\%)} = \frac{\left(\frac{massSCOD + massCOD_{CH_4}}{d}\right)_{eff.} - \left(\frac{massSCOD}{d}\right)_{inf.}}{\left(\frac{massTCOD - massSCOD}{d}\right)_{inf.}} \times 100\% \quad \text{(Equation 2)}$$

$$\boldsymbol{Volumetric\ hydrolysis\ rate\ (gCOD/L/d)} = \frac{\left(\frac{massSCOD + massCOD_{CH_4}}{d}\right)_{eff.} - \left(\frac{massSCOD}{d}\right)_{inf.}}{Volume\ of\ reactor} \quad \text{(Equation 3)}$$

$$\boldsymbol{Specific\ hydrolysis\ rate\ (gCOD/gVS/d)} = \frac{\left(\frac{massSCOD + massCOD_{CH_4}}{d}\right)_{eff.} - \left(\frac{massSCOD}{d}\right)_{inf.}}{Mass\ of\ VS\ within\ reactor} \quad \text{(Equation 4)}$$

(2) Acidogenesis equations

$$\boldsymbol{Acidogenesis\ degree\ (COD\%)} = \frac{\left(\frac{massCOD_{VFA} + massCOD_{CH_4}}{d}\right)_{eff.} - \left(\frac{massCOD_{VFA}}{d}\right)_{inf.}}{\left(\frac{massTCOD - massCOD_{VFA}}{d}\right)_{inf.}} \times 100\% \quad \text{(Equation 5)}$$

$$\boldsymbol{Volumetric\ acidogenesis\ rate\ (gCOD/L/d)} = \frac{\left(\frac{massCOD_{VFA} + massCOD_{CH_4}}{d}\right)_{eff.} - \left(\frac{massCOD_{VFA}}{d}\right)_{inf.}}{Volume\ of\ reactor}$$

(Equation 6)

$$\boldsymbol{Specific\ acidogenesis\ rate\ (gCOD/gVS/d)} = \frac{\left(\frac{massCOD_{VFA} + massCOD_{CH_4}}{d}\right)_{eff.} - \left(\frac{massCOD_{VFA}}{d}\right)_{inf.}}{Mass\ of\ VS\ within\ reactor}$$

(Equation 7)

(3) Methanogenesis equations

$$\boldsymbol{Methanogenesis\ degree(COD\%)} = \frac{\frac{massCOD_{CH_4}}{d}}{massTCOD_{inf.}} \times 100\% \quad \text{(Equation 8)}$$

$$\boldsymbol{Volimertic\ methanogenesis\ rate\ (gCOD/L/d)} = \frac{\frac{massCOD_{CH_4}}{d}}{Volume\ of\ reactor} \quad \text{(Equation 9)}$$

$$\boldsymbol{Specific\ methanogenesis\ rate\ (gCOD/gVS/d)} = \frac{\frac{massCOD_{CH_4}}{d}}{Mass\ of\ VS\ within\ reactor} \quad \text{(Equation 10)}$$

Where $massSCOD$ represented the soluble COD weight (g/L); $massTCOD$ represented the total COD weight (g/L) $massCOD_{CH_4}$ was the CH_4 weight calculated as COD (g/L); $massCOD_{VFA}$ was the VFA weight calculated as COD (g/L); inf. was influent and eff. Was effluent. Especially, VFA concentration were converted to COD concentration by the conversion factors respective to each type of VFA: 1.07 for acetic acid, 1.51 for propionic acid, 1.82 for butyric (C4) acid and iso-butyric acid (IC4), 2.04 for valeric (C5) and isovaleric acid (IC5), 2.21 for caproic and iso-caproic acid (IC6) (Cokgor et al., 2006; Yuan et al., 2011).

3.4.2 The calculations for required amount of nutrients

(1) Macronutrients

The required quantities of macronutrients depend on substrate loading rate and biomass yield due to the fact that macronutrients are mainly used for anabolism of microorganisms (Hendriks et al., 2018). According to Hendriks et al. (2018), the required amount of macronutrients can be calculated as:

$$C_{E-macronutrinets} = COD_{bio-influent} \times Yield \times E_{biomass} \quad \text{(Equation 11)}$$

Under the operational conditions of cascade system (SRT=12 days, 35℃), the substrate loading rate was be set as the differences of total COD between influent of each reactor and effluent. The biomass yield for the combination of hydrolytic, acidogenic, acetogenic and methanogenic microorganisms under mesophilic conditions are in the range of 0.18-0.22 gVSS/g CODbio (de Kok et al., 2013). In order to avoid macronutrients limitations, the maximum yield (0.22 gVSS/g CODbio) was used to calculate the macronutrients requirement. $E_{biomass}$ represented the elemental composition biomass regarding to macronutrients. Scherer et al. (1983) reported the range of Ca composition in biomass was 0.078-3.8 mg/gVSS and the range of Mg composition in biomass was 0.077-0.45 mg/gVSS.

(2) Micronutrients

The required quantities of micronutrients rely on the biodegradable COD concentration of WAS to be treated and not on the biomass yield because micronutrients are mainly used for the production and functioning of enzymes and co-factors. According to Hendriks et al. (2018), the required amount of micronutrients can be calculated as:

$$C_{E-micronutrient} = COD_{bio-influent} \times E \quad \text{(Equation 12)}$$

Similar as the calculation of macronutrients, the biodegradable COD concentration was be set as the differences of total COD between influent of each reactor and effluent under the operational conditions of cascade system. The range of Cobalt and Zinc requirement for mesophilic system was provided by Scherer et al. (1983), which was 0.9-5.4 $\mu g/g\ COD_{bio-inlfuent}$ and 0.7-18.9 $\mu g/g\ COD_{bio-inlfuent}$ respectively.

4. RESULTS AND DISCUSSION

In this chapter, the results from monitoring cascade system in comparison to reference single CSTR system under different SRT (15 days and 12 days) and different recirculation ratio (10% and 2%) are demonstrated. A total of five different sections are defined as operational performance, COD conversion ratios, hydrolysis enzymatic activity, cations analysis and overview discussion.

The reactors were operated for 165 days. During the entire operational period of this study, the operational performance of both systems under different operational conditions were illustrated by the indicators, including total COD removal, methane production, solid reduction, ammonia and phosphate release, pH, alkalinity, soluble COD and VFA concentration. In order to verify the biological process during the AD of both systems under different conditions, COD conversion ratios of hydrolysis, acidogenesis and methanogenesis as well as the corresponding conversion rates and specific conversion rates were also calculated based on COD balance. Since hydrolysis is the rate limiting step in AD of WAS, apart from hydrolysis ratio calculated from COD balance, the hydrolysis enzymatic activity was also presented to evaluate the trend of enzymatic activity with shorter SRT and lower RR. Finally, the concentration of cations in different reactors under 2% RR was presented to further analyze the role of recirculation from R3 to R1 on cascade system.

4.1 Operational performance

4.1.1 Total COD and Methane production

Total COD removal and biogas production are the two most important indicators of anaerobic digestion efficiency. The time profile of total COD (tCOD) and daily methane production in phase I and phase II of cascade system and reference CSTR system are shown in Figure 3 and Figure 4. The feeding concentration for the reactor during the whole period of study was maintained at about 50 gCOD/L. The fluctuations came from the dilution of raw sludge from WWTP. It was observed that along the AD of cascade system, the tCOD concentration was gradually decreased from R1 to R3, followed with a sharp decrease in R4. With bigger volume of R4 (15.4 L), higher SRT was achieved in R4 compared to R1 to R3 (2.2 L), contributing to higher tCOD removal efficiency. The biogas generated in AD of WAS mainly consisted of CH_4 and CO_2. Since the ratio of CH_4 and CO_2 is normally stable under specific conditions (Appendix table 1) and dissolution of CO_2 is strongly dependent on pH, methane is a better indicator for overall performance of process (Hansson et al., 2002; J. Liu, 2003). In accordance with the COD reduction, CH_4 production of R4 (5.23 LCH_4/day) was higher than that from R1 to R3 in cascade system.

In phase I, both cascade system and reference CSTR were operated at 15 days SRT (Day 1-43) and 12 days SRT (Day 44-96). The lower SRT was achieved by increasing flow rate from 1.47L/d to 1.83 L/d, resulting in increasing OLR from 3.33 $gCOD\ L^{-1}d^{-1}$ to 4.16 $gCOD\ L^{-1}d^{-1}$. It was observed that tCOD in each reactor firstly gradually increased then decreased, finally maintained at a stable value when SRT was decreased. Before the system was stable, the biomass requires time to adapt to the higher OLR. For cascade system, there was no significant difference of effluent tCOD between 15 d SRT and 12 d SRT. However, it was observed that effluent tCOD in reference CSTR increased at lower SRT. The results presented that cascade system was capable of obtaining similar tCOD removal efficiency at lower SRT, which were $42 \pm 2\ \%$ (SRT=15 D) and $40 \pm 2\ \%$ (SRT=12 D) respectively. The average tCOD removal efficiency of reference CSTR system decreased from $35 \pm 3\%$ to $26 \pm 2\%$. Similar removal efficiency of WAS was reported by Maspolim et al. (2015), demonstrating that COD reduction in a 2-phase system decreased from 42.3% to 40.7% while that in single CSTR decreased from 35.5% to 31.6% when SRT decreased from 30 to 12 days. The results of CH_4 production were in corresponding to the reduction of tCOD. With the decrease of SRT, the daily CH_4 production of cascade system increased from $11.0 \pm 1.2\ LCH_4/day$ to $12.9 \pm 0.7\ LCH_4/day$ while that in reference CSTR system increased from $9.4 \pm 1.2\ LCH_4/day$ to $10.1 \pm 0.9\ LCH_4/day$. The significant difference of tCOD removal efficiency and the daily CH_4 production between cascade system and CSTR revealed a higher biological conversion capacity could not be achieved by decreasing SRT in conventional CSTR system while cascade system could obtain higher capacity at shorter SRT.

10% recirculation ratio introduced from R3 to R1 was decreased to 2% in phase II (Day 97-133). According to Figure 3, the tCOD concentration was first increased in R1, R2 and R3 then increased in R4 at 2% RR. Correspondingly, decrease of CH_4 production first happened to reactor 1-3 then to R4. As the applied RR decreased, tCOD concentration of R1-R4 was increased to different extent,

resulting in a sharp reduction (from 40 ± 2 % to 33 ± 4 %) of average tCOD removal efficiency in cascade system. The result was correlated with the significant decrease of CH_4 production in cascade system from 12.9 ± 0.7 to 11.1 ± 0.4 LCH_4/day (Figure 4). With more available substrates in R3 effluent, it should be noted that the tCOD removal efficiency in R4 was increased, which was correlated to small increase of methane production in R4. The final phase was the recovery phase, in which the 10% of solid phase of R3 effluent was recycled to R1. Even though the system required longer time to get stable, it can already be noticed that by recycling 10% solid phase of R3 effluent, the tCOD removal efficiency increased to 37 ± 4 % and CH_4 production increased to to 12.0 ± 0.6 LCH_4/day.

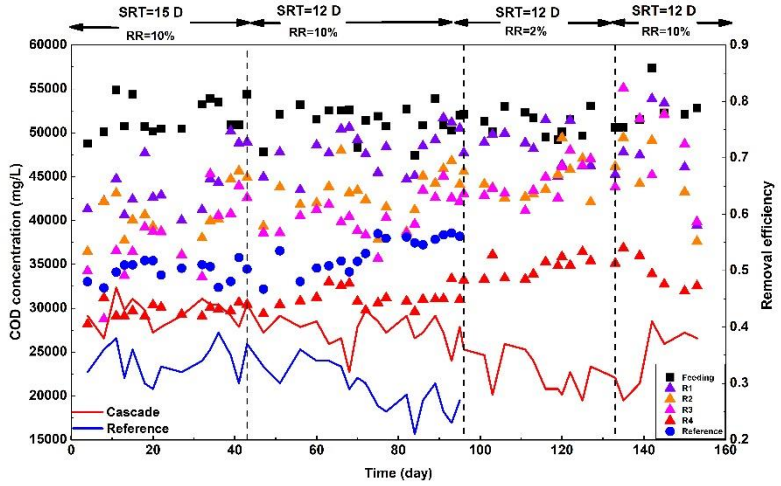


Figure 3 Time profile of total COD concentration in cascade system and reference CSTR

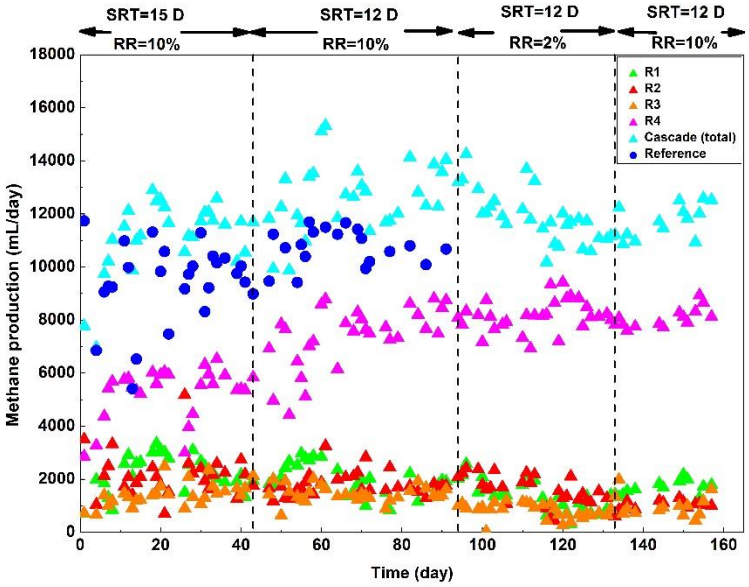


Figure 4 Time profile of daily methane production in cascade system and reference CSTR

4.1.2 Total solids and Volatile solids

The solid reduction is associated with SRT and it can be used to express the degree of stabilization of AD (Athanasoulia et al., 2012). Figure 5 and Figure 6 showed the total solid (TS) and volatile solid (VS) content of cascade system and reference CSTR in different phases. During the AD of WAS, TS and VS were degraded to a certain extent and converted to biogas, which was correlated to the trend of COD concentration along the AD of cascade system.

The TS of feeding WAS maintained at about 40 g/L. The TS reduction efficiency was $35 \pm 5\%$ (SRT=15 D) and $32 \pm 2\%$ (SRT=12 D) in cascade system and the values were $25 \pm 7\%$ (SRT=15 D) and $24 \pm 2\%$ (SRT=12 D) in reference CSTR system. Higher removal efficiency was obtained for VS, which was $41 \pm 5\%$ (SRT=15 D) and $37 \pm 2\%$ (SRT=12 D) in cascade system and was $32 \pm 7\%$ (SRT=15 D) and $30 \pm 1\%$ (SRT=12 D) in reference CSTR system. The maximum VS reduction of mesophilic AD of WAS at 15 days SRT in the research of Gossett et al. (1982) was about 31%, which was similar to the result of reference CSTR system in this study. Nevertheless, some higher VS reduction results were observed from other literatures. For example, 61% VS reduction can be achieved in a mesophilic acidogenic reactor at 10 days SRT or a thermophilic AD at 2 HRT with mixture of WAS (60%) and primary sludge (40%) (Huyard et al., 2000). Song et al. (2004) also attained 43.5% at 20 days SRT under mesophilic condition for sewage sludge treatment. A range of 27-70% was presented by Speece (1988). Various VS reduction values were published due to different operating temperature, SRT, feeding sludge characteristics and type of sludge digested, such as primary sludge, WAS, trickling filter and the mixture of these sludge (De la Rubia et al., 2006). The researchers in 1960's and 1970's reported that conventional AD of WAS can exhibit VS reduction only up to 40% (Garrison et al., 1978; Ghosh, 1991; Malina Jr, 1961). Apart of that, the VS reduction also differed from the types of WAS and the loading rates in digesters. The WAS from carrousel systems or enhanced biological phosphorus removal (EBPR) systems were less biodegradable than those from high loaded systems. The WAS in this study was originated from an EBPR system, Harnaschpolder, and the biochemical methane potential (BMP) of this WAS was 232 mL-CH_4/g-VS (Guo, 2019). The methane yield of both systems under different SRT and RR was shown in Table 4, revealing that the biodegradability of the WAS in cascade system already reach 93% of BMP under 15 d SRT and 10% RR. The TS and VS reduction was relatively similar at 15 days SRT and 12 days SRT for both cascade systems. Appels et al. (2008) also claimed that the changes in increasing VS destruction were relatively small when detention time exceeds 12-13 days at 35 ℃. Besides, at two different SRT, the level of solid reduction in cascade system was consistently 10% higher than that in reference CSTR, which exhibited the superiority of utilizing cascade system to degrade solid fraction of WAS.

The overall TS, VS reduction and methane yield encountered remarkable drop when RR was decreased from 10% to 2%. In cascade system, the mean TS reduction declined from 32% to 21% and the mean VS reduction decreased from 37% to 26%. Significant increasing values of the effluent TS and VS were observed in R1-R3. The methane yield was decreased from 207 ± 15 $mL\,CH_4/g\,VS$ to 185 ± 9 $mL\,CH_4/g\,VS$ when RR was decreased from 10% to 2%. However, the TS, VS reduction and methane yield increased when 10% solid phase of R3 effluent was recycled to R1, indicating the reduction of TS and VS removal efficiency was due to the decrease of RR.

Table 4 Methane yield for both systems under different SRT and RR

Methane yield	SRT=15 days RR=10%	SRT=12 days RR=10%	SRT=12 days RR=2%	SRT=12 days RR=10%
unit	$mL\,CH_4/g\,VS$	$mL\,CH_4/g\,VS$	$mL\,CH_4/g\,VS$	$mL\,CH_4/g\,VS$
Cascade system	217 ± 24	207 ± 15	185 ± 9	201 ± 9
Reference CSTR	185 ± 23	164 ± 14	-	-

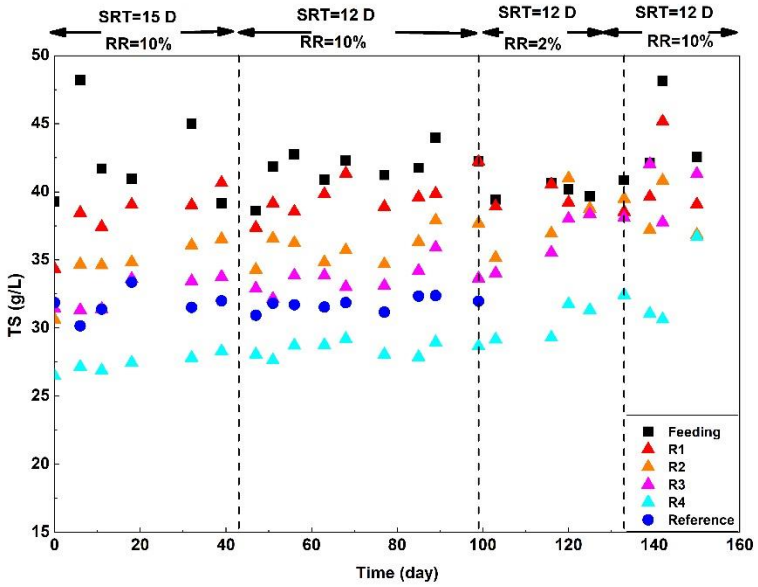


Figure 5 Time profile of total solid in cascade system and reference CSTR

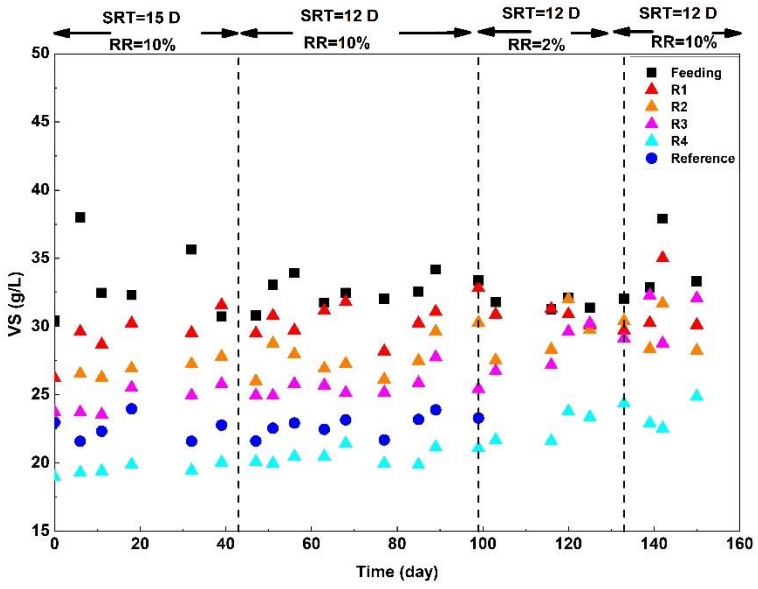


Figure 6 Time profile of volatile solid in cascade system and reference CSTR

4.1.3 VFA and soluble COD

During the hydrolysis of WAS, organic matter is solubilized to small transportable components. The products from the disintegration of carbohydrates, proteins and fats are monosaccharides, amino acids and long fatty acids, respectively. Apart from that, inert particulates and inert soluble materials can also be generated after hydrolysis. On the other hand, soluble COD (sCOD) is then rapidly metabolized into biogas. sCOD can be used to indicate the hydrolysis capacity or the digestion capability of WAS due to the fact that hydrolysis is the rate limiting step in AD of WAS. Since VFA was the major fraction of sCOD, the time profile of VFA followed the same trend as sCOD, shown in Figure 7 and Figure 8. During AD, hydrolysis was followed by acetogenesis and acidogenesis, which were relatively fast compared to hydrolysis. VFAs are the most common intermediates among fermentation products and their concentration is generally regarded as the best indicators for process stability (Boe et al., 2006). The accumulation of VFA reflects the kinetics uncoupling between acid producers and consumers and high concentration of VFA can be inhibitive to the activity of methanogens (Switzenbaum et al., 1990). VFA becomes more toxic at lower pH with its undissociated fraction, which can freely cross the cell membrane and disrupts homeostasis (Switzenbaum et al., 1990). Therefore, the buffering capacity as well as the pH has impact on VFA inhibition. Also, it was stated by Viéitez et al. (1999) that if the VFA concentration exceeded 13000 mg/L, the AD would stop. In this study, the total concentrations of VFA in each reactor was relatively low, with no more than 600 mg/L total VFA in R1. The value of pH (Appendix figure 2) was at the range of 6.6-7.2 of cascade system and reference CSTR during the whole operation, which was located at the optimal pH range of AD (Moosbrugger et al., 1993). In this study, the highest VFA concentration of cascade system obtained in R1, followed by R2 and R3. The VFA in R4 and reference CSTR was negligible. With highest total VFA and lowest alkalinity, R1 was more likely to be influenced by VFA. The ratio of total VFA (TVFA) and alkalinity, which was TVFAs/alkalinity, was 0.37 (SRT=15 D, RR=10%), 0.40 (SRT=12 D, RR=10%) and 0.42 (SRT=12 D, RR=2%). It is believed that if the TVFAs/alkalinity ratio is lower than 0.8, the AD process is stable (Callaghan et al., 2002). Apart from total VFA, individual VFA can give more important information about the biological pathways and the digesters performance (Feng et al., 2017). It is suggested that if propionic acid to acetic acid ratio exceeding 1.4 implies impending digester failure (Hill et al., 1987). At 15 days SRT, the dominant VFA of reactors were acetate (316 mg/L), occupying over than 50% of total VFA (582 mg/L), followed by 172 mg/L propionic (approximately 30%). These results indicated that the dominant fermentation product was acetate and the reactors during the whole experiment were operated at stable conditions. Under stable conditions, the fermentation pathway to acetate and hydrogen contributed the main carbon flow to CH_4 formation.

After adopting shorter SRT, a small increase was observed in sCOD as well as VFA in R1 and R2. A similar phenomenon was examined by Athanasoulia et al. (2012), demonstrating that VFA concentration in the single-stage process increased from 0.20 ± 0.04 g/L (SRT=16.4 days) to 0.29 ± 0.11 g/L (SRT=12.3 days). With lower SRT, higher hydrolysis rate can be achieved thus higher concentration of soluble COD and more VFA intermediate can be attained.

The results in terms of the reduced RR presented lower sCOD release as well as relatively low total VFA generation. The mean total VFA in R1 was 562 ± 183 mg/L at 10% RR while it dropped to

384 ± 104 mg/L at 2% RR. The declined total VFA and sCOD generation were also correlated to the lower TCOD removal efficiency. After recycling only solid phase of 10% R3 effluent to R1, increase trend was shown in sCOD release and VFA generation in R1, implying hydrolysis was recovered by established microbial community and sludge-attached enzymes recirculation.

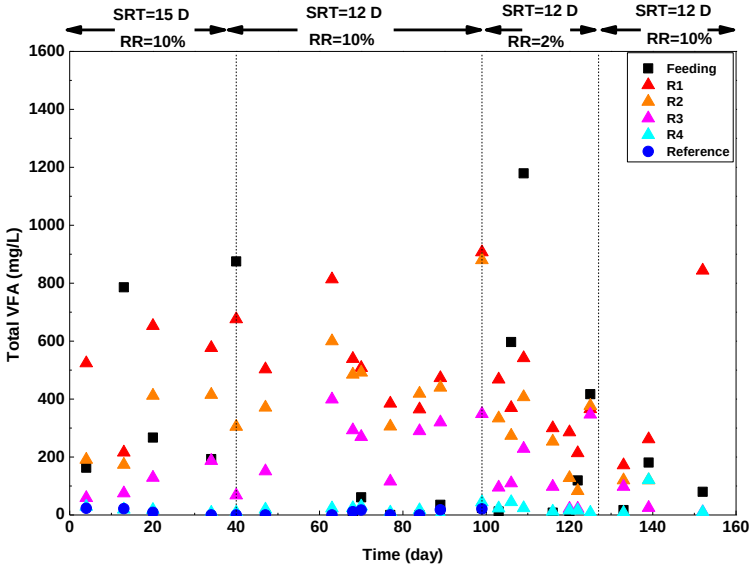


Figure 7 Time profile of total VFA in cascade system and reference CSTR

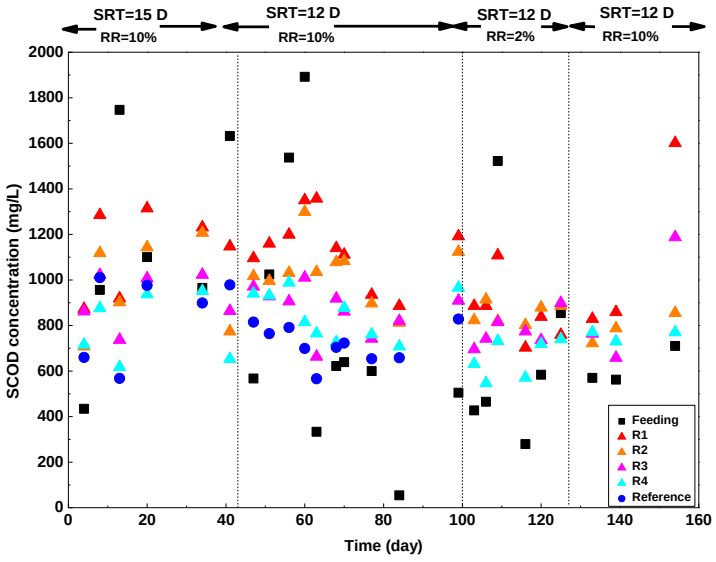


Figure 8 Time profile of soluble COD in cascade system and reference CSTR

4.1.4 Ammonia and phosphate

Ammonia comes from AD of nitrogenous matter from WAS, mostly in the form of protein (Kayhanian, 1999). Ammonia is released during hydrolysis and it is regarded as a significant factor reflecting the degradation efficiency of the systems and affecting the process stability (Boe et al., 2006; Kayhanian, 1999). Figure 9 demonstrates that the ammonia concentration increased along cascade system during the whole operation. The toxicity of ammonia is related to pH and temperature. It is reported that ammonia concentration between 1500 and 3000 mg/L are inhibitory at pH higher than 7.4 whereas if it exceeds 3000 mg/L, the ammonia ion itself can become quite toxic regardless of the pH (Boe et al., 2006; McCarty, 1964). In this study, the ammonia concentration in both systems was maintained in the range of 900-1200 mg/L, suggesting that the ammonia concentration was appropriate to not cause inhibition. L. J. Wu et al. (2015) also reported similar result ($1280 \pm 170\ mg/L$) in a mesophilic WAS digester at 10 days SRT with similar feeding concentration. At 15 days SRT, the effluent ammonia concentration of reference CSTR (1065 ± 25 mg/L) was slightly lower than cascade system (1116 ± 22 mg/L), suggesting that higher hydrolysis rate existed in cascade system. After lowering SRT, the ammonia concentration in reference CSTR decreased to 957 ± 33, mg/L which was correlated to relatively lower tCOD reduction and lower solid reduction. Reducing RR also had negative effects on ammonia release, which means that relatively lower ammonia concentration was observed at 2% 882 ± 27 mg/L) compared to 10% (1020 ± 35mg/L), indicating lower RR hampered hydrolysis rate of protein. It was also demonstrated the concentration of ammonia gradually increased back to 925 ± 20 mg/L after recycling solid phase of R3 effluent to R1, indicating the reduction of ammonia release was attributed to the decrease of RR.

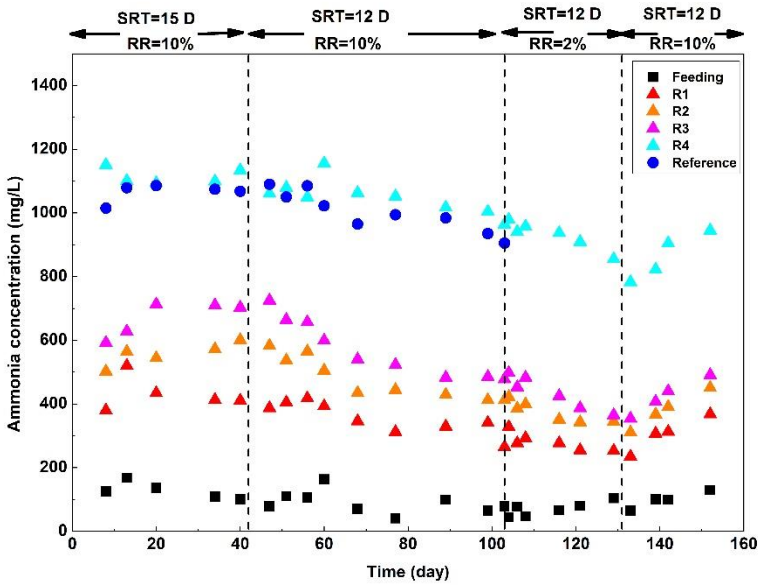


Figure 9 Time profile of ammonia concentration in cascade system and reference CSTR

P in WAS is typically present in three different forms: organically bound, inorganic compounds and free orthophosphate. Figure 10 showed a sharp increase of phosphate release in R1. On one hand, during hydrolysis and acidification, organically bound P can be released to orthophosphate by the decomposition of extracellular polymers substances and lysis of microorganism cells (Ji et al., 2010). On the other hand, orthophosphate could be released due to the existence of PAO (Coats et al., 2011). When WAS entered the reactors, it was detected that the Phosphorus-Accumulating Organisms (PAO) and Glycogen-Accumulating Organisms (GAO) were still active in both systems (Guo, 2019). In the anaerobic condition, PAO blend in VFAs into storage products, polyhydroxyalkanoates (PHA), within the cells with phosphate by using the energy obtained from hydrolysis of polyphosphate (Coats et al., 2011; Satoh et al., 1992). Especially, the WAS was originated from EBPR process, containing relatively high concentration of poly-phosphate, contributing to relatively lower VFA concentration in reactors, especially in R1 and R2 of cascade system. It was also observed that the orthophosphate concentration in R4 was decreased compared to R3. With the increase of pH in R4, the orthophosphate can bind with cations in the reactors, such as Mg^{2+}, Ca^{2+}, Al^{3+} and Fe^{2+}, to form P-precipitants (Latif et al., 2015). Typical P-precipitants are inorganic salts in reactors, like hydroxyapatite ($Ca_5(OH)(PO_4)_3, HAP$) and struvite ($MgNH_4PO_4 \cdot 6H_2O, MAP$) (Bolzonella et al., 2012). Comparing the differences of phosphate and cations concentration between R3 and R4, a molar ratio in the liquid phase being of 0.65 for Ca/P and 0.63 for Mg/P on average can be calculated under 12 days SRT. The values were compatible with the moderate precipitation of phosphorous salts inside the reactor. The past research also revealed the similar phenomenon, stating that the saturation index (SI) of hydroxyapatite was greater than 0 and the calcium concentration was decreased along cascade system (Nair, 2019).

After lowering SRT from 15 days to 12 days, phosphate concentration in R1 increased from 793 ± 40 mg/L to 822 ± 43 mg/L, which might have effects on the ammonia concentration and VFA. With formation of struvite, ammonia was bound with Mg^{2+} and orthophosphate, leading to a lower ammonia concentration observed in 12 days SRT. There was a significant difference of orthophosphate concentration at 2% RR and 10% RR. Regardless of the influence of dilution due to recirculation, it was observed that the overall orthophosphate concentration in cascade system dropped significantly when RR was decreased. On one hand, the lower hydrolysis rate leaded to less phosphate release. On the other hand, lower VFA concentration was already observed at 2% RR (Figure 7), indicating that less VFA was able to be utilized by PAO to release orthophosphate. The recovery phase with 10% recirculation of solid phase from R3 effluent induced higher ammonia and phosphate release, indicating that the reduce of ammonia and phosphate release was mainly due to 2% RR.

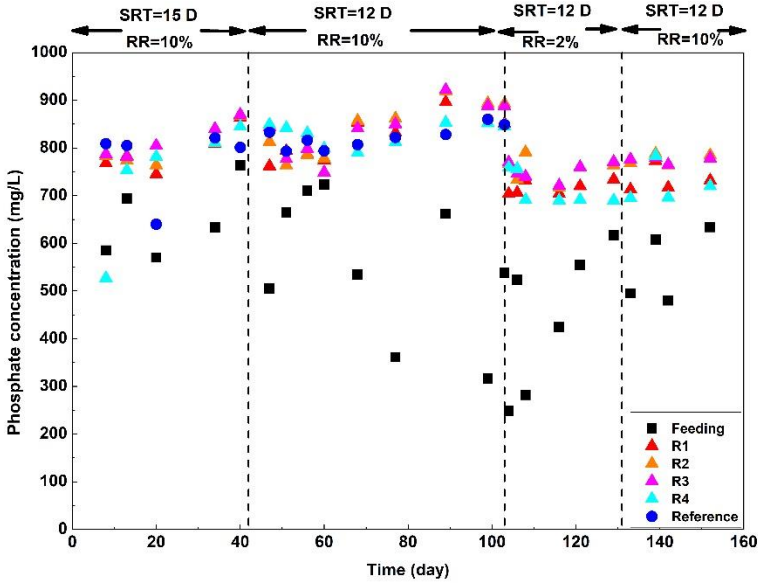


Figure 10 Time profile of phosphate concentration in cascade system and reference CSTR

4.2 Hydrolysis, acidogenesis and methanogenesis analysis

In order to evaluate the comprehensive effects of shorter SRT and lower RR on the biological process in both systems, the calculated results of the COD conversion degrees and conversion rates of hydrolysis, acidogenesis and methanogenesis are shown in Table 5. In cascade system, higher volumetric hydrolysis rate and specific hydrolysis rate were observed in R1, R2 and R3 in comparison to R4. With only 6.6 L out of 22 L working volumes, the hydrolysis degree of R1-R3 accounted for almost half of total cascade hydrolysis degree, which were $28.0 \pm 5.1\%$ out of $56.9 \pm 9.4\ \%$ at 15 days SRT and $28.4 \pm 2.9\ \%$ out of $69.4 \pm 8.1\ \%$ at 12 days SRT. At 15 days SRT, the hydrolysis degree, volumetric hydrolysis rate of cascade system were $56.9 \pm 9.4\ \%$, 1.29 ± 0.23 gCOD/L/day respectively, which were higher than the values of conventional mesophilic digester, 37%, 0.61 gCOD/L/day from L. J. Wu et al. (2015). By comparing cascade system and reference CSTR, it was clear that higher hydrolysis rate was obtained in cascade system, which indicated that smaller volume of reactors in series, cascade system, was capable of achieving higher hydrolysis rate compared to conventional CSTR system. The acidogenesis and methanogenesis rate followed the same trend as hydrolysis rate, higher values examined in R1, R2, R3 compared to R4 and reference CSTR. No big difference was observed among the trend of hydrolysis, acidogenesis, methanogens rates was due to the fact that hydrolysis was the rate limiting step in AD of WAS.

It was observed that for cascade system, all the conversion rates of hydrolysis, acidogenesis and methanogenesis at 12 days SRT were higher than those at 15 days SRT and the improvement percentages of conversion degree were 12.5%, 8.9%, 11.9% respectively. As presented before, higher soluble COD and VFA were observed, which was correlated to higher hydrolysis rate at lower SRT. Higher concentration of non-hydrolyzed substrates was added to the reactor, leading to higher hydrolysis rate, thus providing more available substrates for acidogenesis and methanogenesis and finally enhancing the overall degradation efficiency.

When RR was reduced from 10% to 2%, the results of all COD conversion ratios exhibited a sharp drop. It could be noticed that the hydrolysis, acidogenesis and methanogenesis rates with 2% RR at 12 days SRT were even lower than those with 10% at 15 days SRT. Lower COD conversion at lower RR was also correlated to lower COD reduction rate and rather low ammonia and VFA release in all reactors. The rates in hydrolysis, acidogenesis and methanogenesis were close to each other, indicating that the hydrolysis was inhibited and the overall degradation efficiency was decreased when lower RR ratio was adopted in cascade system.

Combined with the results of COD conversion degree and COD conversion rates, it is indicated that smaller reactor could attain enhancement on hydrolysis rate of WAS, leading to improvement of overall degradation efficiency. Each steps of AD, including hydrolysis, acidogenesis and methanogenesis could be further enhanced by adopting shorter SRT whereas lower RR can be inhibitive to the biological process of WAS degradation.

Table 5 Conversion degree and rate of hydrolysis, acidogenesis and methanogenesis in Cascade system and reference CSTR system

Biological process unit		Conversion degree			Volumetric reaction rate			Specific reaction rate		
		Hydrolysis	Acidogenesis	Methanogenesis	Hydrolysis	Acidogenesis	Methanogenesis	Hydrolysis	Acidogenesis	Methanogenesis
		%			gCOD/L-reactor/d			gCOD/gVS/d		
SRT=15 D RR=10%	R1	10.3 ± 4.0	9.6 ± 3.8	10.1 ± 3.8	2.33 ± 0.9	2.23 ± 0.58	2.34 ± 0.86	0.07 ± 0.03	0.07 ± 0.03	0.07 ± 0.03
	R2	12.7 ± 4.8	13.7 ± 4.4	12.7 ± 4.7	2.45 ± 0.93	2.35 ± 0.56	2.52 ± 0.93	0.08 ± 0.03	0.10 ± 0.03	0.09 ± 0.03
	R3	8.5 ± 2.3	8.7 ± 1.8	8.4 ± 2.3	1.53 ± 0.46	1.62 ± 0.36	1.56 ± 0.48	0.06 ± 0.02	0.07 ± 0.01	0.06 ± 0.02
	R4	40.1 ± 9.0	41.9 ± 6.7	39.4 ± 8.6	0.93 ± 0.18	1.01 ± 0.07	0.94 ± 0.18	0.04 ± 0.01	0.05 ± 0.00	0.04 ± 0.01
	Reference	45.5 ± 9.4	45.8 ± 8.7	45.1 ± 8.7	1.05 ± 0.22	1.05 ± 0.20	1.04 ± 0.21	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
SRT=12 D RR=10%	R1	10.3 ± 3.1	10.8 ± 3.0	9.4 ± 3.3	2.36 ± 0.73	2.51 ± 0.72	2.19 ± 0.79	0.07 ± 0.02	0.08 ± 0.03	0.07 ± 0.02
	R2	11.3 + 2.3	10.5 ± 1.9	11.2 ± 2.3	2.41 ± 0.51	2.26 ± 0.43	2.45 ± 0.51	0.08 ± 0.02	0.09 ± 0.02	0.08 ± 0.02
	R3	9.0 ± 1.8	9.1 ± 2.1	9.1 ± 1.8	1.73 ± 0.32	1.74 ± 0.38	1.79 ± 0.33	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
	R4	51.4 ± 7.7	50.5 ± 8.5	50.4 ± 7.6	1.34 ± 0.21	1.31 ± 0.22	1.34 ± 0.21	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
	Reference	53.7 ± 12.4	55.3 ± 6.0	51.6 ± 9.1	1.22 ± 0.29	1.27 ± 0.13	1.20 ± 0.20	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
SRT=12 D RR=2%	R1	6.4 ± 2.0	6.0 ± 2.0	5.9 ± 1.7	1.46 ± 0.46	1.39 ± 0.47	1.38 ± 0.39	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
	R2	8.4 ± 2.5	8.1 ± 2.5	8.0 ± 1.4	1.82 ± 0.53	1.79 ± 0.55	1.78 ± 0.53	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02
	R3	4.1 ± 2.0	4.0 ± 1.9	4.9 ± 2.8	0.82 ± 0.36	0.82 ± 0.36	1.02 ± 0.56	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.02
	R4	36.9 ± 9.0	36.4 ± 9.1	36.2 ± 8.5	1.05 ± 0.24	1.05 ± 0.25	1.04 ± 0.24	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01

4.3 The hydrolysis enzymatic activity

4.3.1 Protein and carbohydrates

Degradation of protein and carbohydrates is vital for AD performance of WAS. It has been reported that the proteins and carbohydrate are the predominant organic matter of WAS, which account for around 90% of the volatile suspended solids of WAS. Mostly, WAS consists of 30%-40% protein and 20% carbohydrates (Chen et al., 2007). Figure 11 and Figure 12 displayed the protein and carbohydrates concentration in both systems under different SRT and RR. The research of Tanaka et al. (1997) reported 39% of protein and 52% of carbohydrate removal efficiency of WAS. Similar values were obtained in the research of C Bougrier et al. (2007), which was 35% protein removal efficiency and 50% carbohydrates removal efficiency respectively. Higher than past research, the final removal efficiency of protein and carbohydrate at 15 days SRT in cascade system were 47% and 71% respectively whereas those in reference system were 50% and 60% respectively. The rest of the protein and carbohydrates might be refractory organic matter. It was clear that the final removal efficiency of carbohydrates was higher than protein, which was consistent with the research of C Bougrier et al. (2007) and Tanaka et al. (1997). And it can be noticed that the degradation of carbohydrate was prior to protein. The sharp reduction of carbohydrate existed at the beginning of cascade system whereas the protein had more degradation at later reactors. The delay of protein degradation can be ascribed to the rapid hydrolysis of carbohydrates, leading to release of glucose, which can cause repression of protease formation (Yang et al., 2015). After sharp reduction of carbohydrates, the restriction effect became less to protease formation thus the removal efficiency of protein became higher at later AD of WAS.

Relatively lower carbohydrates removal efficiency was observed at lower SRT, which was consistent with the results of total COD concentration and TS/VS concentration. Similar removal efficiency of protein was obtained in cascade system at both SRT. At shorter SRT, the relatively higher effluent COD concentration might be attributed to carbohydrates with its reduced removal efficiency. When RR was reduced to 2%, removal efficiency of protein and carbohydrates was substantially affected in each reactor, which was correlated to the lower hydrolysis rate examined at lower RR.

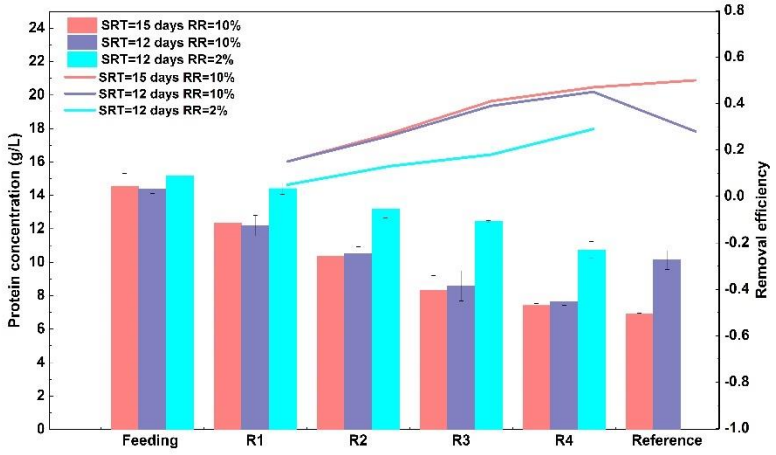


Figure 11 Protein concentration of cascade system and reference CSTR

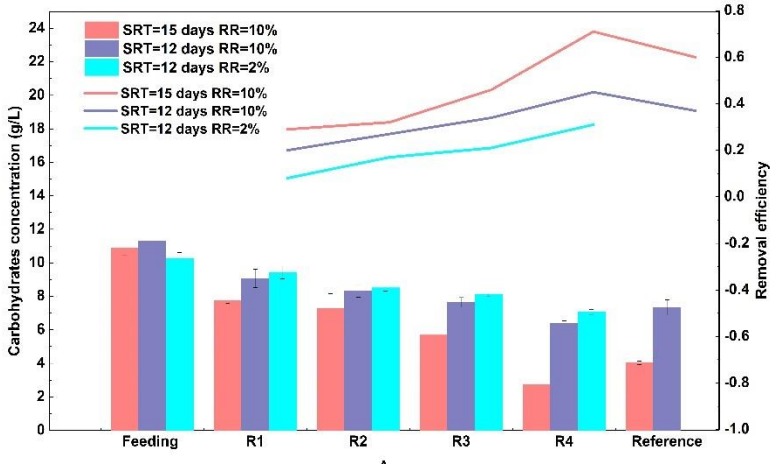


Figure 12 Carbohydrates concentration of cascade system and reference CSTR

4.3.2 Free and sludge-attached enzymatic activity

Conventional process parameters related to process performance such as VS, TS and COD reduction as well as methane production determine the end products of metabolism, which cannot directly measure the microbial activity related to hydrolysis (Yamaguchi et al., 1991). As discussed before, hydrolysis is the overall rate-limiting step and during hydrolysis, specific enzymes are synthesized and secreted to catalyzes specific reactions. Determination of enzymatic activity is one of the direct ways to evaluate the hydrolysis efficiency (Parawira et al., 2005). Based on whether the enzymes are in the vicinity of the particles or released to the bulk liquid, hydrolytic enzymes can be divided to free or sludge-attached enzyme (Batstone et al., 2002). It is critical to investigate whether the hydrolysis occurs in bulk solution or attached particles in order to understand the macromolecule metabolism in AD of WAS. The activity of both free and sludge-attached enzyme was measured for protease and cellulase in this study, shown in Figure 13, Figure 14, Figure 15 and Figure 16. It was detected that the enzymatic activity of sludge-attached protease and cellulase was higher than that of free protease and cellulase due to the fact that dominant organic matter in WAS were protein and carbohydrates. The sludge-attached enzymes were more dominant in digesting protein and carbohydrates compared to free enzymes, which was proved by Confer et al. (1998), illustrating that the sludge-attached hydrolysis and release of protein and polysaccharides can be repeated until the hydrolytic fragments are small enough to be assimilated by cells. Same phenomenon was reported by W. Sanders et al. (2000) and Vavilin et al. (1996). Therefore, the dominant mechanisms of hydrolysis of WAS were related to sludge-attached enzymes. It was furthermore seen that enzymatic activity was increased after it entered the reactors due to the increase of temperature (from 4 ℃ to 35 ℃) and optimum pH in reactor. Under 15 days and 12 days SRT, the hydrolysis activity for both protease and cellulase reached highest level in R1, then it was decreased along the AD in cascade system. Compared to reference CSTR, higher enzymatic activity was observed in cascade system. The smaller reactor volume was adopted in R1, R2, R3, leading to higher non-hydrolyzed substrate concentration compared to R4 and reference CSTR. Higher hydrolysis rate can be achieved in the first three smaller reactors in cascade system due to first order kinetics, which was on the other hand, proved by the observation of higher hydrolysis enzymatic activity.

The sludge retention time in R1 was 1.5 days at 15 days SRT and that was 1.2 days at 12 days SRT. Maspolim et al. (2015) reported that hydrolytic and acidogenic bacteria have magnitudes faster growth rate than methanogenic bacteria. With the growth rate of 5.1 d^{-1}, hydrolytic and acidogenic bacteria can grow in the R1 easily. However, since the growth rate of acetolactic was 0.6 d^{-1} and that of hydrogenotrophic methanogens 2.85 d^{-1}, the retention time in R1 was lower than the required time of acetolactic methanogens but sufficient for the growth of hydrogenotrophic methanogens (Koster et al., 1988; Stams et al., 2003). The microbial community analysis of (Guo, 2019) also detected that *Methanosprillum* as dominant hydrogenotrophic group were dominant in R1-R3 wile higher relative abundance of *Methanosaeta* was shifted in R4.

In accordance with the results of superior performance of cascade with lower SRT, higher enzymatic activity was detected with shorter SRT for both free and sludge-bounded protease and cellulase. According to first order kinetics, higher non-biodegradable substrates concentration

promoted higher hydrolysis rate. Under shorter SRT, higher OLR loaded to reactors was likely promoting the growth of hydrolytic and acidogenic microorganisms, contributing to higher hydrolysis enzyme activity. The investigation of microbial analysis from Guo (2019) also revealed that the relative abundance of syntrophic bacteria and fermenters, such as *Sedimentibacter*, *Enterococcus*, *Thermovirga*, *Smithella*, *Syntrophomonas,* was increased when SRT was decreased from 15 days to 12 days. The trend of organic matter reduction as well as COD conversion rate regarding to hydrolysis was consistent with the trend of hydrolysis enzymatic activity in both systems. Hence, both protease and cellulase at shorter SRT preferentially and rapidly degrades protein and carbohydrates respectively.

After lowering RR from 10% to 2%, it was observed that both protease and cellulase activity was decreased sharply. In particular, the sludge-attached protease dropped from 13006 to 6648 U/mL in R1, from 11792 to 8037 U/mL in R2, from 10028 to 8068 U/mL in R3 respectively. Similarly, Zhang et al. (2007) also reported that increasing RR can enhance hydrolysis rate due to the improved enzymatic activity. Moreover, it is observed that the sludge-attached enzymatic activity showed increased trend from R1 to R4 in cascade system under 2% RR, which was opposite trend at 10% RR. The opposite trend revealed that the hydrolysis rate was greatly deteriorated in R1, R2 and R3 of cascade system at 2% RR. The phenomenon of enzymatic activity was consistent with the observation of the removal efficiency of organic matter. Figure 11 and Figure 12 demonstrated the lower hydrolysis ability in terms of protein and carbohydrates at lower RR. The results were therefore illustrated that lower RR had negative impacts on hydrolysis activity as result of lower hydrolysis enzymatic activity.

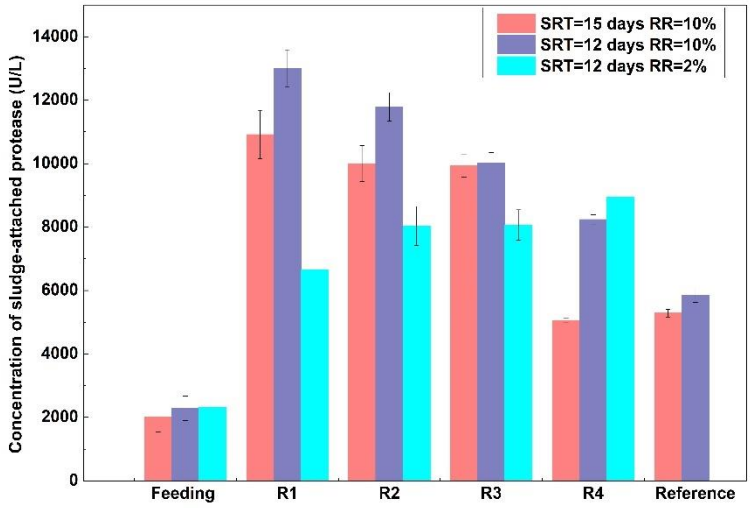


Figure 13 Sludge-attached- Protease concentration in cascade system and reference CSTR

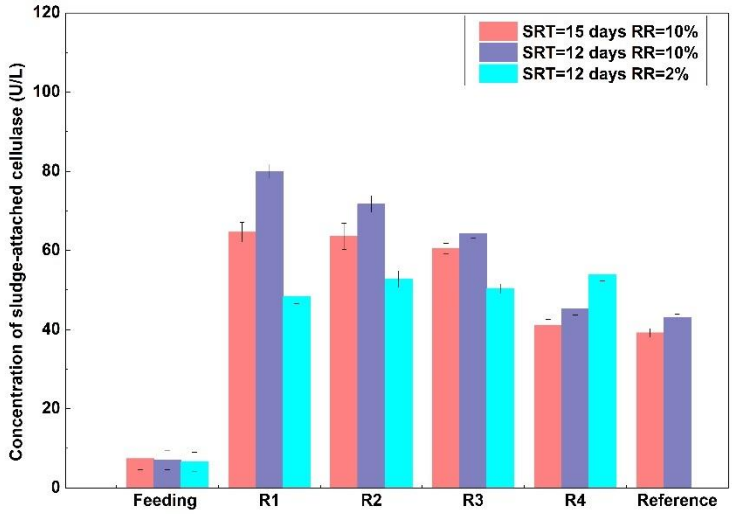


Figure 14 Sludge-attached- Cellulase concentration in cascade system and reference CSTR

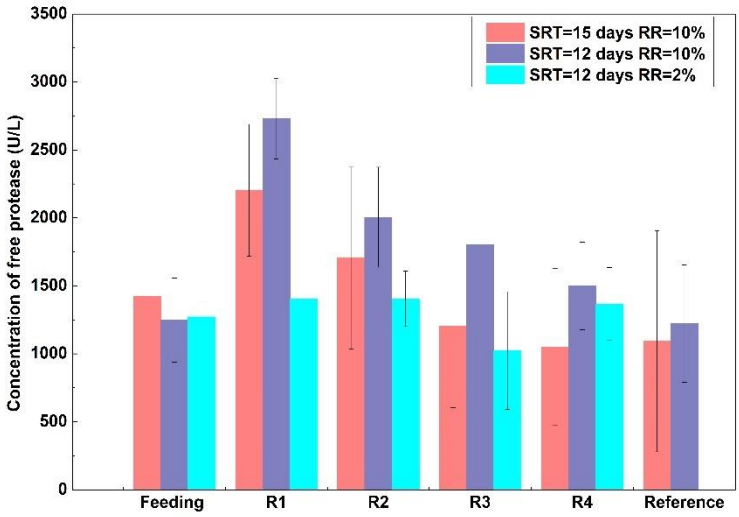


Figure 15 Free-Protease concentration in cascade system and reference CSTR

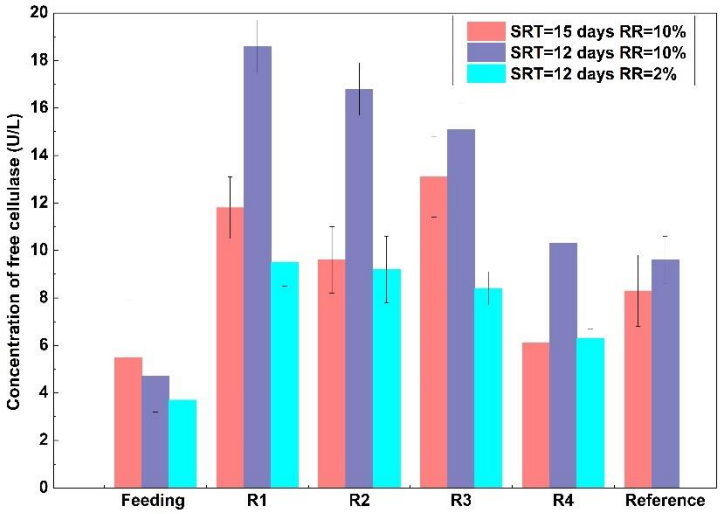


Figure 16 Free- Cellulase concentration in cascade system and reference CSTR

4.4 Cations analysis

When RR was decreased from 10% to 2%, the hydrolysis rate as well as enzymatic activity of cascade system was dropped sharply, contributing to deteriorated overall degradation efficiency. During the recovery phase, only 10% solid phase of R3 effluent was recycled from R3 to R1. The organic matters removal efficiency as well as methane production was increased compared to 2% RR due to the increment of biomass and sludge-attached enzymes. However, it should be noted that the removal efficiency during the recovery phase was not increased back to the same level as the phase with 10% recirculation of both solid and liquid phase from R3 effluent. It is indicated that the liquid fraction also played an important role to enhance the hydrolysis of WAS anaerobic digestion. Hence, the cations analysis of soluble fraction was conducted to investigate the cations recirculation on cascade system for WAS treatment.

The disintegration of WAS can release metal ions due to their multiple functions in activated sludge formation (Park et al., 2007). On the other hand, the soluble metal ions can be absorbed and utilized by anaerobic microorganisms. It is reported that not only operational parameters, but also growth media regarding to metal ions can influence biological process during AD (Angelidaki et al., 2009). Growth media consists of macronutrients (C, N, P, K, Na, S, Ca and Mg) and micronutrients (Hendriks et al., 2018). With regards to micronutrients, trace elements iron (Fe), cobalt (Co), nickel (Ni), zinc (Zn) and vitamins are generally used for enzyme or co-factor production (Hendriks et al., 2018). Past research suggest that a shortage of trace metals can cause restrictions to biological process in AD and the addition of trace metals can stimulate higher methane production (Ezebuiro et al., 2017; Speece, 1988). Regarding to hydrolysis, it is demonstrated that Ca is important for the folding of cellulase whereas the presence of Zn, Co, Mg and Ca is essential to protease formation (Brahmachari, 2016; Jisha et al., 2013; LUCHINAT, 1994). The supplement of Co is beneficial to acidogenesis due to the fact that vitamin B_{12} is Co-containing vitamin and the formation of propionate requires vitamin B_{12} (Dryden et al., 1962). Important trace elements in hydrogenotrophic methanogenesis are Fe, Ni, Co, selenium, molybdenum and tungsten while those in acetolactic methanogenesis are Fe, Ni, Co and Zn (Banks et al., 2012; Boonyakitsombut et al., 2002).

The required macronutrients depend on biomass concentration and the substrate concentration. The required quantities of macronutrients in this study were calculated by Equation 11, shown in Table 7. The range of Mg and Ca required quantities in cascade system were decreased along the AD of cascade system due to the lower biodegradable COD concentration at later reactors. According to the results retrieved from Table 6, the concentration of soluble Mg and Ca was decreased along the AD of cascade system, implying that even though Mg and Ca was released with the degradation of WAS, more Mg and Ca was absorbed as macronutrients to stimulate the growth of anaerobic microorganisms. The sharp drop of them between R3 and R4 may be caused by the precipitation of Mg and Ca at pH 7.2, which was consistent with the drop of orthophosphate concentration from R3 to R4. Furthermore, it was observed that the results of the concentration of soluble $Mg^{2=}$ and Ca^{2+} (Table 6) were much higher than the required quantities Table 7, indicating that the concentration of Mg and Ca was sufficient to promote the growth of anaerobic organisms.

According to Table 6, it is observed that along the AD of cascade system, the concentration of Co, Ni and Zn was increased, implying that degradation of WAS in AD system released micronutrients gradually. Based on Equation 12, the required quantities of trace elements regarding Fe, Co, Zn and Ni were calculated, shown in Table 7. The concentration of soluble Fe and Zn in cascade system was higher than the minimum required concentration while the concentration of Zn under 2% RR was located at rather low value. By comparing Table 6 and Table 7, it was clear that the lowest concentration of Co, Zn and Ni existed in R1 while the required quantities of these elements in R1 were highest. The recirculation from R3 of trace metals in terms of Co, Ni and Zn can be used to supplement the uptake of micronutrients to R1 in order to avoid the insufficient micronutrients. Higher RR was likely to stimulate the formation of hydrolysis enzymes and the growth of hydrolytic, acidogenesis and methanogenic microorganisms by supplying higher concentration of trace elements in R1. 2% RR reduced the increment of micronutrients in R1, probably leading to lower hydrolysis enzymatic formation and less populations of hydrolytic, acidogenic and methanogenic bacteria.

All the detected metal ions concentration at 10% RR was higher than that at 2% RR, especially for Ca and Mg, which was reasonable because the degradation efficiency of WAS was higher at 10%RR, leading to more cations release. The higher RR also recirculated more trace elements, such as Co, Zn and Ni, from R3 to R1, which on the other hand, further enhanced the hydrolysis enzymes formation as well as microorganism growth. The increment of hydrolysis enzymatic activity was consistent with increase of trace metals concentration at higher RR. It is therefore increasing RR from R3 to R1 could stimulate the hydrolysis rate, thus improving overall degradation efficiency in cascade system.

Table 6 Soluble metal ions concentration in cascade system under 2% RR and 10% RR

Cations	SRT=15 D RR=10%						SRT=15 D RR=2%					
	Mg	Ca	Fe	Co	Zn	Ni	Mg	Ca	Fe	Co	Zn	Ni
	mg/L	mg/L	mg/L	ug/L	ug/L	ug/L	mg/L	mg/L	mg/L	ug/L	ug/L	ug/L
Feeding	69.4	54.3	3.1	3.4	61.6	14.5	84.3	59.5	2.7	2.6	26.4	6.4
R1	98.6	60.3	4.7	4.5	131.5	23.2	62.1	44.9	2.0	3.3	75.4	12.1
R2	51.2	49.6	4.0	5.8	158.3	26.4	31.9	41.7	1.9	3.9	89.6	13.6
R3	32.3	43.3	3.6	6.4	170.6	28.3	15.7	32.9	1.7	5.3	131.8	14.4
R4	6.7	15.7	1.9	13.4	77.0	31.7	1.4	12.3	1.4	13.8	75.9	29.8

Table 7 Required nutrients regarding the macronutrients and micronutrients in cascade system at 12 d SRT

Cations	Mg	Ca	Fe	Co	Zn	Ni
	mg/L	mg/L	mg/L	$\mu g/L$	$\mu g/L$	$\mu g/L$
R1	0.34-2.01	0.35-17.01	0.4-1.7	73.23-508.55	14.24-384.46	18.3-109.8
R2	0.29-1.71	0.30-14.42	0.4-1.4	62.11-413.30	12.08-326.06	15.5-93.2
R3	0.21-1.21	0.21-10.18	0.3-1.0	43.82-304.31	8.52-230.06	11.0-65.7
R4	0.15-0.89	0.15-7.54	0.2-0.8	32.47-225.49	6.31-170.47	8.1-48.7

4.5 Overall discussion

By combining the performance indicators with the hydrolysis enzymatic activity and cations analysis, the overall effects with regards to SRT and RR could be evaluated. The reasons why the SRT and RR had great impacts on cascade system and reference CSTR were discussed below.

4.5.1 The effect of lower sludge retention time

This study compared applications of cascade system and reference single CSTR in treatment of WAS at different SRT. The same working conditions in terms of substrate concentration, temperature, recirculation ratio were applied to both configurations at 15 days SRT and 12 days SRT. The effect of decreasing SRT which inevitably increased OLR was evaluated. The competitive advantage of cascade system at shorter SRT of 12 days was shown to maintain the sludge digestion and biogas production compared to reference CSTR.

When SRT decreased from 15 days to 12 days, stable COD, TS, VS, ammonia concentration in effluent and daily methane production could be maintained in cascade system. Conversely, the effluent COD, TS, VS concentration in reference CSTR was increased, resulting in lower daily methane production. As predominant organic matter in WAS, protein and carbohydrates removal efficiency remained similar in cascade system at 15 days SRT and 12 days SRT while that dropped in reference CSTR at 12 days SRT. Besides, a small increase of soluble COD and total VFA concentration was observed in R1, R2 and R3 of cascade system whereas the mean residual VFA of both systems were found to be close to 0 mg/L at both SRT. COD conversion rate regarding hydrolysis, acidogenesis and methanogenesis calculated based on COD balance revealed that higher hydrolysis rate was achieved at shorter SRT in cascade system, contributing to higher overall degradation efficiency. These results were also in accordance with the higher hydrolysis enzymatic activity detected in terms of protease and cellulase in cascade system at shorter SRT. Based on experimental performance of both systems at different SRT, it was clear that the cascade system could tolerate shorter SRT and higher OLR than reference CSTR with its improvement of hydrolysis rates as result of enhancement of hydrolysis enzymatic activity.

The improved performance in the cascade system at shorter SRT could be due to chemically and biologically induced reasons. Considering the predominant organic matter in WAS is particulate organic matter and the dominant hydrolysis enzymes were sludge-attached enzymes, first order kinetics was generally applied to represent the overall hydrolysis functions. According to first order kinetics, higher OLR at shorter SRT provided cascade system with higher non-hydrolyzed substrates, contributing to higher hydrolysis rate and furthermore higher overall degradation efficiency of WAS. In addition, the populations of hydrolytic/acidogenic bacteria as well as methanogenic archaea were likely increased at shorter SRT as they were provided with more available substrates at higher OLR with shorter SRT. The pH was stable (6.6-7.2) and it was located in the range where bacteria can grow without inhibition regardless of the change of SRT (J. Li et al., 2012). According to the microbial community evaluation from Guo (2019), specific fermenters and syntrophic bacteria existed in cascade system at both SRT, including *Sedimentibacter*, *Enterococcus*, *Thermovirga*, *Smithella*, *Syntrophomonas* and etc, which appeared to contribute to

hydrolysis, acidogenic and acetogenic activity during AD of WAS. Nonetheless, the relative abundance of these syntrophic bacteria and fermenters was increased at shorter SRT. It was likely that the presence of these specific bacteria with higher relative abundance in the neutral pH and higher available substrates stimulate the cultivation of the syntrophic fermenters, which may lead to the enhancement of hydrolysis and acidogenesis, contributing to higher organic loading tolerance of cascade system at 12 days SRT. Moreover, the activity of methanogens was also evaluated by Guo (2019). For methanogens, clear shift in relative abundance of hydrogenotrophic methanogens to acetolactic methanogens was observed along cascade system, from which *Methanosprillum* as dominant hydrogenotrophic group were dominant in R1-R3 and *Methanosaeta* as dominant acetolactic group were dominant in R4. Not only the syntrophic relationship between butyrate, propionate oxidation bacteria and hydrogenotrophic methanogens but also the faster growth rate of hydrogenotrophic methanogens compared to acetolactic methanogens contributed to the higher abundance of *Methanosprillum* in R1-R3. The high population of *Methanosaeta* in R4 implied methane pathway went mainly through this way because they can only use acetate. Nevertheless, the effluent VFA maintained at low level because it was directly consumed to produce biogas, indicating that methanogenesis was not restricted in the cascade system despite higher OLR.

The effect of decreasing the operational SRT could be drastic and it was proved in this study that with lower SRT like 12 days, the WAS digestion performance was decreased dramatically in conventional single CSTR system. However, shortening the SRT of WAS digestion is advantageous in sizing the reactor volume. The superior performance of cascade system which had CSTRs in serial gave an opportunity to further shorten SRT from 15 days to 12 days because it was proved in this study that it could maintain the organic removal efficiency at 12 days SRT. The tolerance of cascade system at shorter SRT could be ascribed to the higher hydrolysis rate due to first order kinetics and higher hydrolytic and acidogenic activity in cascade system.

4.5.2 The effect of lower recirculation ratio

In this study, the recirculation ratio, introduced from R3 to R1, applied to the cascade system ranged from 10% to 2%. The cascade system was running stably at 12 days SRT and 10% RR. The decrease of RR inhibited the performance of cascade system in terms of the removal efficiency of total COD, TS, VS, protein and carbohydrates as well as methane production. The COD conversion rates of hydrolysis, acidogenesis and methanogenesis decreased dramatically at 2% RR. Correspondingly, less total VFA and relatively lower soluble COD was detected in the cascade system, implying 2% contributed to lower hydrolysis rate, which was consistent with the lower hydrolysis enzymatic activity. During the recovery phase, only 10% solid fraction of R3 effluent was recycled from R3 to R1, the organic matter degradation efficiency and methane production of cascade system increased compared to 2%. However, it was not increased back to the level at the previous phase when 10% of total R3 effluent was recycled. Therefore, the appropriate recirculation ratio from R3 to R1 could help the maintenance of hydrolytic capacity and overall degradation efficiency as result of the changed environment. Also, the effects of recirculation could be significant in terms of biomass and sludge-attached enzymes increment as well as cations recirculation.

The hydrolysis was inhibited at 2% RR, resulting from decreased total enzymatic activity compared to that at 10% RR. The hydrolysis of WAS was mainly attributed to sludge-attached enzyme, followed by free enzyme. Zhang et al. (2007) reported that higher RR was not only beneficial to allow enzymes attach to the surface of organic matter, but also help rapidly refresh the niche around the hydrolysis enzymes. That was likely to provide higher opportunities for the enzymes to touch the surface of organic matter, leading to higher sludge-attached enzymes activity. It was suggested that lower RR was not favorable to the hydrolysis of organic matter, especially proteins and carbohydrates, due to the shortage of sludge-attached enzymatic activity as well as slow synthesis and refreshment of free hydrolytic enzymatic activity.

The R3 effluent also contained a more established microbial population. Especially, the growth rate of syntrophic fermenters and methanogens is relatively slow, the functional microorganisms in R1, R2 might have been washed out especially at lower SRT. During the recovery phase, 10% of biomass was recycled from R3 to R1 and the overall degradation efficiency was increased, indicating that the recycling of biomass indeed was able to enhance the performance of cascade system. It was implying that the recirculation from R3 to R1 could provide additional microbial populations and avoid the rapid washing out of functional microorganisms in terms of hydrolytic, acidogenesis, syntrophic bacteria and methanogens, which can accelerate WAS degradation. Past researches of Zuo et al. (2015) and Zhang et al. (2007) also demonstrated that that increasing RR could afford the high OLR system with increment of established microbial populations, thus stimulating more release of hydrolytic enzymes and improving overall performance of system.

It was also reported that that increasing recirculation was beneficial to minimize local shortage of nutrients (Zhang et al., 2007). During the recovery phase, the performance of cascade system was not recovered back to the previous phase with complete 10% recirculation of R3 effluent. In order to evaluate the effect of recirculation of soluble fractions on hydrolysis and acidogenesis, further

investigation was conducted with the analysis of soluble cations concentration. The cations analysis showed that the concentration of Mg, Ca and Fe was decreased along the AD of cascade system, which indicated that they can be utilized as macronutrients for hydrolytic microorganisms since it was reported they were essential for the cells aggregation and protease formation. Compared to the required quantities, high concentration of soluble Mg, Ca and Fe existed in all reactors of cascade system, implying that the concentration of Mg and Ca was sufficient to provide the growth media for microorganism growth. According to Banks et al. (2012), Co, Zn and Ni play important roles in the growth of microorganisms and the formation of hydrolytic enzymes. It was also observed that R1 had highest required quantities of trace elements, such as Co, Ni and Zn while the soluble concentration of them in R1 was lowest. The increasing trend of soluble Co, Ni and Zn concentration along the AD of cascade system provided the opportunity to supplement Co, Zn and Ni from R3 to R1 by adopting recirculation. 10% recirculation from R3 to R1 was able to offer more increment of Co, Zn and Ni compared to 2% RR to enhance hydrolysis rate of cascade system, thus contributing to higher overall degradation efficiency, which was proved by the more cations release at 10% RR. Therefore, it was likely that 10% RR ensured the micro-environment of the growth of microorganisms and the formation of hydrolytic enzymes by appropriate trace elements increment, contributing to higher enzymatic activity thus high hydrolysis rate and overall degradation efficiency.

Apart from the reasons mentioned above, there were also a range of advantages provided with higher recirculation ratio reported from other researchers, such as redistricting the inoculum and diluting potential toxins by alkalinity increment, thus the pH can be adjusted to the optimal range (Namsree et al., 2012; Romli et al., 1994; Zhang et al., 2007; Zuo et al., 2015). However, other researches focused on the thermophilic anaerobic digestion or the WAS digesters after pretreatment, where the hydrolysis was not restricted and the accumulation of VFA caused great inhibition to methanogenesis of AD. While in this study, the digesters operated in mesophilic conditions and without pretreatment, thus the degradation of WAS did not accumulate VFA to the toxic level. Apart from that, the alkalinity was sufficient enough to buffer the system and the pH was maintained at the optimal range for the stability of systems. Therefore, the role of RR in cascade system for AD of WAS was different from other researches. Instead of avoiding toxicity from VFA accumulation, the role of recirculation in cascade system could be mainly boiled down to the refreshment of hydrolytic enzymes, replacement of the established microbial community and the increment of trace elements in terms of Co, Zn and Ni.

5. CONCLUSION & RECOMMENDATION

5.1 Conclusion

Based on this study, the following conclusions can be made:

1. The cascade system was capable of maintaining COD, VS, protein, carbohydrates reduction, and methane production when operated at 15 and 12 days SRT; while the reference CSTR system performance deteriorated as the SRT was reduced from 15 days to 12 days.

2. According to first order kinetics, non-hydrolyzed substrate concentration was achieved at shorter SRT to enhance the hydrolysis rate. Hydrolysis, acidogenesis and methanogenesis analysis in cascade system and reference CSTR system revealed that the tolerance of cascade system at shorter SRT was ascribed to the higher hydrolysis rate, resulting from the associated increase of the total hydrolysis enzymatic activity in terms of protease and cellulase.

3. 10% RR introduced from R3 to R1 showed better performance regarding to higher removal efficiency of COD, VS, protein, carbohydrates and methane production in cascade system compared to that at 2% RR, which was strongly supported by higher level of hydrolytic enzymatic activity at 10% RR. Especially, it was verified that lower rate of hydrolysis, acidogenesis and methanogenesis was associated with deteriorated performance of cascade system at 2% RR.

4. 10% RR not only favored the increment of established microbial community and sludge-attached enzymes, but also provided R1 with more increment of trace elements regarding to cobalt, zinc and nickel.

Consequently, the cascade system showed superior performance in anaerobic digestion of waste activated sludge than single CSTR despite the lower sludge retention time. 10 % recirculation ratio was essential to maintain the performance of the cascade system by providing established microbial community, hydrolytic enzymes and trace elements increment.

5.2 Recommendation

To further investigate the operational conditions of cascade system, the following recommendations could be made:

1. In further research of measurement, it is recommended to quantitatively characterize bacterial and archaeal community structures at different SRT and RR, which could give an insight on how the relative abundance and quantities of hydrolysis, acidogenic, acetogenic bacteria and methanogenic archaeal change with the change of operational conditions. This measurement can not only provide a better understanding of the fundamental roles of microbial community in the AD of WAS but also help further investigating the pathway transitions under different operational conditions, which could be regarded as direct indicators to evaluate and control of process operation.

2. The effects of PAO, GAO on anaerobic digesters of WAS could also be evaluated as well. During the AD, the VFA was formed after hydrolysis and acidogenesis process. However, the net production of VFA was low in R1 was due to the presence of PAO, GAO and poly-phosphate, which would utilize VFA for orthophosphate formation. Therefore, the role of PAO, GAO is recommended to be investigated to distinguish the enhancement of hydrolysis rate under different operational conditions and the effects of denitrification.

3. In order to obtain a complete picture of the nutrients shortage of cascade system under different RR, another experiment regarding to the effects of trace elements can be conducted. After the recovery phase, 10% of both the solid fraction and the liquid fraction of R3 effluent should be recycled back to R1. After the system was stabilized, the performance of cascade system as well as the hydrolysis enzymatic activity should be evaluated to check whether the performance recovers to the phase with 10% RR previously.

4. In this study, only soluble phase of metal ions was analyzed. However, the presence of metal ions is determined by the liquid phase and solid phase of both influent and effluent streams of each reactor of cascade system. The soluble Ni, Co and Zn concentration was relatively low but only whether they are sufficient to provide trace element for microorganism growth and enzymatic activity formation was not clear due to the shortage information of solid phase of these metal ions. Therefore, it is recommended to measure the metal ions concentration in cell-attached EPS as well as that in the cells. With combination of solid phase and liquid phase, the role of metal ions, especially trace elements can be investigated.

6. REFERENCES

Angelidaki, I., et al. (2009). Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays. *Water Science and Technology, 59*(5), 927-934.

Angelidaki, I., et al. (2005). Effect of operating conditions and reactor configuration on efficiency of full-scale biogas plants. *Water Science and Technology, 52*(1-2), 189-194.

Appels, L., et al. (2008). Principles and potential of the anaerobic digestion of waste-activated sludge. *Progress in energy and combustion science, 34*(6), 755-781.

Aslanzadeh, S., et al. (2013). The effect of effluent recirculation in a semi-continuous two-stage anaerobic digestion system. *6*(6), 2966-2981.

Athanasoulia, E., et al. (2012). Optimization of biogas production from waste activated sludge through serial digestion. *Renewable energy, 47*, 147-151.

Ayol, A. (2005). Enzymatic treatment effects on dewaterability of anaerobically digested biosolids-I: performance evaluations. *Process Biochemistry, 40*(7), 2427-2434.

Banks, C. J., et al. (2012). Trace element requirements for stable food waste digestion at elevated ammonia concentrations. *Bioresource Technology, 104*, 127-135.

Batstone, D. J., et al. (2002). The IWA anaerobic digestion model no 1 (ADM1). *Water Science and Technology, 45*(10), 65-73.

Boe, K., et al. (2006). Online monitoring and control of the biogas process.

Boe, K., et al. (2009). Serial CSTR digester configuration for improving biogas production from manure. *Water research, 43*(1), 166-172.

Boe, K., et al. (2005). *Optimisation of serial-CSTR biogas reactors using modeling by ADM1.* Paper presented at the International Workshop on the IWA Anaerobic Digestion Model No. 1.

Bolzonella, D., et al. (2012). High rate mesophilic, thermophilic, and temperature phased anaerobic digestion of waste activated sludge: a pilot scale study. *Waste management, 32*(6), 1196-1201.

Bolzonella, D., et al. (2005). Mesophilic anaerobic digestion of waste activated sludge: influence of the solid retention time in the wastewater treatment process. *Process Biochemistry, 40*(3-4), 1453-1460.

Boonyakitsombut, S., et al. (2002). Degradation of propionate and its precursors: The role of nutrient supplementation. *KSCE Journal of Civil Engineering, 6*(4), 379-387.

Bouallagui, H., et al. (2003). Mesophilic biogas production from fruit and vegetable waste in a tubular digester. *Bioresource Technology, 86*(1), 85-89.

Bougrier, C., et al. (2006). Effect of ultrasonic, thermal and ozone pre-treatments on waste activated sludge solubilisation and anaerobic biodegradability. *Chemical Engineering Processing: Process Intensification, 45*(8), 711-718.

Bougrier, C., et al. (2007). Impacts of thermal pre-treatments on the semi-continuous anaerobic digestion of waste activated sludge. *Biochemical engineering journal, 34*(1), 20-27.

Brahmachari, G. (2016). *Biotechnology of microbial enzymes: production, biocatalysis and Industrial applications*: Academic Press.

Callaghan, F., et al. (2002). Continuous co-digestion of cattle slurry with fruit and vegetable wastes and chicken manure. *Biomass and Bioenergy, 22*(1), 71-77.

Carrère, H., et al. (2010). Pretreatment methods to improve sludge anaerobic degradability: a review. *Journal of hazardous materials, 183*(1-3), 1-15.

Chen, Y., et al. (2007). Hydrolysis and acidification of waste activated sludge at different pHs. *41*(3), 683-689.

Coats, E. R., et al. (2011). Effect of anaerobic HRT on biological phosphorus removal and the enrichment of phosphorus accumulating organisms. *Water environment research, 83*(5), 461-469.

Cokgor, E. U., et al. (2006). Respirometric assessment of primary sludge fermentation products. *Journal of Environmental Engineering, 132*(1), 68-74.

Confer, D. R., et al. (1998). Location of protein and polysaccharide hydrolytic activity in suspended and biofilm wastewater cultures. *Water research, 32*(1), 31-38.

de Gooijer, C. D., et al. (1996). Bioreactors in series: an overview of design procedures and practical applications. *Enzyme Microbial Technology, 18*(3), 202-219.

de Kok, S., et al. (2013). Impact of dissolved hydrogen partial pressure on mixed culture fermentations. *Applied microbiology and biotechnology, 97*(6), 2617-2625.

De la Rubia, M., et al. (2006). Effect of solids retention time (SRT) on pilot scale anaerobic thermophilic sludge digestion. *Process Biochemistry, 41*(1), 79-86.

Dryden, L., et al. (1962). Production of vitamin B12 and vitamin B12 analogues by pure cultures of ruminal bacteria. *Nature, 195*(4837), 201.

Dubois, M., et al. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry, 28*(3), 350-356. Retrieved from <Go to ISI>://WOS:A1956WC83000017. doi:DOI 10.1021/ac60111a017

Eastman, J. A., et al. (1981a). Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. *Journal (Water Pollution Control Federation)*, 352-366.

Eastman, J. A., et al. (1981b). Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. *Water Pollution Control Federation*, 352-366.

Eastman, J. A., et al. (1981c). Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. *Water Pollut*, 352-366.

Ezebuiro, N. C., et al. (2017). Characterisation of anaerobic digestion substrates regarding trace elements and determination of the influence of trace elements on the hydrolysis and

acidification phases during the methanisation of a maize silage-based feedstock. *Journal of environmental chemical engineering, 5*(1), 341-351.

Feng, L., et al. (2017). Anaerobic co-digestion of cattle manure and meadow grass: Effect of serial configurations of continuous stirred tank reactors (CSTRs). *Biosystems engineering, 160*, 1-11.

Frolund, B., et al. (1995). Enzymatic-Activity in the Activated-Sludge Floc Matrix. *Applied Microbiology and Biotechnology, 43*(4), 755-761. Retrieved from <Go to ISI>://WOS:A1995RT23200028.

Garrison, W. E., et al. (1978). Pilot-plant studies of waste activated sludge processing. *Journal (Water Pollution Control Federation)*, 2374-2387.

Generation, B. (1999). Use, and Disposal in the United States. *US Environmental Protection Agency, Washington, DC*.

Ghosh, S. (1991). Pilot-scale demonstration of two-phase anaerobic digestion of activated sludge. *Water Science and Technology, 23*(7-9), 1179-1188.

Gossett, J. M., et al. (1982). Anaerobic digestion of waste activated sludge. *Journal of the Environmental Engineering Division, 108*(6), 1101-1120.

Guo, H. (2019). Cascading digesters to increase hydrolysis and overall reduction in anaerobic waste activated sludge digestion *Unpublish data*.

Hansson, M., et al. (2002). Early warning of disturbances in a laboratory-scale MSW biogas process. *Water Science and Technology, 45*(10), 255-260. Retrieved from <Go to ISI>://WOS:000177331900041.

Hendriks, A., et al. (2018). Growth media in anaerobic fermentative processes: the underestimated potential of thermophilic fermentation and anaerobic digestion. *Biotechnology advances, 36*(1), 1-13.

Henze, M., et al. (2008). *Biological wastewater treatment*: IWA publishing.

Hill, D., et al. (1987). Using volatile fatty acid relationships to predict anaerobic digester failure. *Transactions of the ASAE, 30*(2), 502-0508.

Horiuchi, J.-I., et al. (2002). Selective production of organic acids in anaerobic acid reactor by pH control. *Bioresource Technology, 82*(3), 209-213.

Huang, W., et al. (2019). Modification of corn stover for improving biodegradability and anaerobic digestion performance by Ceriporiopsis subvermispora. *Bioresource Technology, 283*, 76-85.

Huyard, A., et al. (2000). The two phase anaerobic digestion process: sludge stabilization and pathogens reduction. *Water Science and Technology, 42*(9), 41-47.

Jain, S., et al. (2015). A comprehensive review on operating parameters and different pretreatment methodologies for anaerobic digestion of municipal solid waste. *Renewable and Sustainable Energy Reviews, 52*, 142-154.

Ji, Z., et al. (2010). Effects of waste activated sludge and surfactant addition on primary sludge hydrolysis and short-chain fatty acids accumulation. *Bioresource Technology, 101*(10), 3457-3462.

Jisha, V. N., et al. (2013). Versatility of microbial proteases. *Advances in enzyme research, 1*(03), 39.

Kafle, G. K., et al. (2011). Sludge exchange process on two serial CSTRs anaerobic digestions: process failure and recovery. *Bioresource Technology, 102*(13), 6815-6822.

Kaparaju, P., et al. (2009). Optimisation of biogas production from manure through serial digestion: Lab-scale and pilot-scale studies. *Bioresource Technology, 100*(2), 701-709.

Kayhanian, M. (1999). Ammonia inhibition in high-solids biogasification: an overview and practical solutions. *Environmental technology, 20*(4), 355-365.

Kim, H.-W., et al. (2011). A comparison study on the high-rate co-digestion of sewage sludge and food waste using a temperature-phased anaerobic sequencing batch reactor system. *Bioresource Technology, 102*(15), 7272-7279.

Koorse, S. J. (1993). The role of residuals disposal laws in treatment plant design. *Journal-American Water Works Association, 85*(10), 57-62.

Koster, I. W., et al. (1988). Ammonia inhibition of the maximum growth rate (μ m) of hydrogenotrophic methanogens at various pH-levels and temperatures. *Applied microbiology and biotechnology, 28*(4-5), 500-505.

Latif, M. A., et al. (2015). Low pH anaerobic digestion of waste activated sludge for enhanced phosphorous release. *Water research, 81*, 288-293.

Lay, J., et al. (1997). Analysis of environmental factors affecting methane production from high-solids organic waste. *Water Science and Technology, 36*(6-7), 493-500.

Lee, D. H., et al. (2009). Methane production potential of leachate generated from Korean food waste recycling facilities: a lab-scale study. *Waste management, 29*(2), 876-882.

Leiyu, F., et al. (2009). Kinetic analysis of waste activated sludge hydrolysis and short-chain fatty acids production at pH 10. *Journal of Environmental Sciences, 21*(5), 589-594.

Li, J., et al. (2012). Syntrophic propionate degradation in anaerobic digestion: a review. *International Journal of Agriculture and Biology, 14*(5).

Li, Y., et al. (2017). Serial completely stirred tank reactors for improving biogas production and substance degradation during anaerobic digestion of corn stover. *Bioresource Technology, 235*, 380-388.

Li, Y., et al. (1992). Upgrading of anaerobic digestion of waste activated sludge by thermal pretreatment. *Water Science Technology, 26*(3-4), 857-866.

Lindner, J., et al. (2016). Is the continuous two-stage anaerobic digestion process well suited for all substrates? *Bioresource Technology, 200*, 470-476.

Liu, J. (2003). *Instrumentation, control and automation in anaerobic digestion*: Siv Holmqvist, Department of Biotechnology, Lund University.

Liu, Y., et al. (2019). Anaerobic digestion performance and microbial community structure of corn stover in three-stage continuously stirred tank reactors. *Bioresource Technology, 287*, 121339.

LUCHINAT, C. (1994). The Reaction Pathways of Zinc Enzymes and Related Biological Catalysts. *IVANO BERTINI*, 37.

Mahmoud, N., et al. (2004). Anaerobic stabilisation and conversion of biopolymers in primary sludge—effect of temperature and sludge retention time. *Water research, 38*(4), 983-991.

Malina Jr, J. (1961). *The effect of temperature on high-rate digestion of activated sludge.* Paper presented at the Proc. 16th. Ind. Waste Conf., Purdue Univ., Waste Lafayette, Ind.

Manyi-Loh, C., et al. (2013). Microbial anaerobic digestion (bio-digesters) as an approach to the decontamination of animal wastes in pollution control and the generation of renewable energy. *International journal of environmental research and public health, 10*(9), 4390-4417.

Mao, C., et al. (2015). Review on research achievements of biogas from anaerobic digestion. *Renewable and Sustainable Energy Reviews, 45*, 540-555.

Maspolim, Y., et al. (2015). Comparison of single-stage and two-phase anaerobic sludge digestion systems–Performance and microbial community dynamics. *Chemosphere, 140*, 54-62.

McCarty, P. L. (1964). Anaerobic waste treatment fundamentals, Part III, Toxic materials and their control. *Public works, 95*, 91-94.

Moosbrugger, R., et al. (1993). A 5 pH point titration method for determining the carbonate and SCFA weak acid/bases in anaerobic systems. *Water Science and Technology, 28*(2), 237-245.

Mumme, J., et al. (2010). Novel upflow anaerobic solid-state (UASS) reactor. *Bioresource Technology, 101*(2), 592-599.

Nair, R. (2019). Cascade anaerobic digestion system to enhance waste activated sludge degradation.

Namsree, P., et al. (2012). Anaerobic digestion of pineapple pulp and peel in a plug-flow reactor. *Journal of Environmental Management, 110*, 40-47.

O'Rourke, J. T. (1968). *Kinetics of anaerobic treatment at reduced temperatures*.

Parawira, W., et al. (2005). Profile of hydrolases and biogas production during two-stage mesophilic anaerobic digestion of solid potato waste. *Process Biochemistry, 40*(9), 2945-2952.

Park, C., et al. (2007). Characterization of activated sludge exocellular polymers using several cation-associated extraction methods. *Water research, 41*(8), 1679-1688.

Parmar, N., et al. (2001). Enzyme treatment to reduce solids and improve settling of sewage sludge. *Journal of industrial microbiology biotechnology advances, 26*(6), 383-386.

Regulations, E. (2003). Technology: Control of pathogens and vector attraction in sewage sludge. *USEPA, Office of Research Development*.

Romli, M., et al. (1994). Effect of recycle on a two-phase high-rate anaerobic wastewater treatment system. *Water research, 28*(2), 475-482.

Sanders, W., et al. (2000). Anaerobic hydrolysis kinetics of particulate substrates. *Water Science and Technology, 41*(3), 17-24.

Sanders, W. T. M. (2001). *Anaerobic hydrolysis during digestion of complex substrates*.

Satoh, H., et al. (1992). Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal processes. *Water Science and Technology, 26*(5-6), 933-942.

Scherer, P., et al. (1983). Composition of the major elements and trace elements of 10 methanogenic bacteria determined by inductively coupled plasma emission spectrometry. *Biological trace element research, 5*(3), 149-163.

Simate, G. S., et al. (2011). The treatment of brewery wastewater for reuse: State of the art. *Desalination, 273*(2-3), 235-247.

Smith, D. P., et al. (1989). Reduced product formation following perturbation of ethanol-and propionate-fed methanogenic CSTRs. *Biotechnology and bioengineering, 34*(7), 885-895.

Song, Y.-C., et al. (2004). Mesophilic and thermophilic temperature co-phase anaerobic digestion compared with single-stage mesophilic-and thermophilic digestion of sewage sludge. *Water research, 38*(7), 1653-1662.

Speece, R. (1988). A survey of municipal anaerobic sludge digesters and diagnostic activity assays. *Water research, 22*(3), 365-372.

Stams, A., et al. (2003). Metabolic interactions between methanogenic consortia and anaerobic respiring bacteria. In *Biomethanation I* (pp. 31-56): Springer.

Switzenbaum, M. S., et al. (1990). Monitoring of the anaerobic methane fermentation process. *Enzyme and Microbial Technology, 12*(10), 722-730.

Takashima, M. (2008). Examination on process configurations incorporating thermal treatment for anaerobic digestion of sewage sludge. *Journal of Environmental Engineering, 134*(7), 543-549.

Tanaka, S., et al. (1997). Effects of thermochemical pretreatment on the anaerobic digestion of waste activated sludge. *Water Science and Technology, 35*(8), 209-215.

Van Der Maarel, M. J., et al. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of biotechnology, 94*(2), 137-155.

van Lier, J. B. (1995). *Thermophilic anaerobic wastewater treatment: temperature aspects and process stability*: Van Lier.

Vavilin, V., et al. (2008). Hydrolysis kinetics in anaerobic degradation of particulate organic material: an overview. *Waste management, 28*(6), 939-951.

Vavilin, V., et al. (1996). Modeling of volatile fatty acids degradation kinetics and evaluation of microorganism activity. *Bioresource Technology, 57*(1), 69-80.

Viéitez, E., et al. (1999). Biogasification of solid wastes by two-phase anaerobic fermentation. *Biomass and Bioenergy, 16*(5), 299-309.

Wang, Q., et al. (1999). Degradation of volatile fatty acids in highly efficient anaerobic digestion. *Biomass and Bioenergy, 16*(6), 407-416.

Wei, Y., et al. (2003). Comparison performances of membrane bioreactor and conventional activated sludge processes on sludge reduction induced by Oligochaete. *Environmental science & technology, 37*(14), 3171-3180.

Whiteley, C., et al. (2003). Co-digestion of primary sewage sludge and industrial wastewater under anaerobic sulphate reducing conditions: enzymatic profiles in a recycling sludge bed reactor. *Water Science Technology, 48*(4), 129-138.

Wu, L.-J., et al. (2016). Comparison of hyper-thermophilic–mesophilic two-stage with single-stage mesophilic anaerobic digestion of waste activated sludge: process performance and microbial community analysis. *Chemical Engineering Journal, 290*, 290-301.

Wu, L.-J., et al. (2015). Upgrading of anaerobic digestion of waste activated sludge by temperature-phased process with recycle. *Energy, 87*, 381-389.

Wu, L., et al. (2015). Upgrading of anaerobic digestion of waste activated sludge by a hyper-thermophilic–mesophilic temperature-phased process with a recycle system. *RSC Advances, 5*(84), 68531-68541.

Wu, L. J., et al. (2015). Upgrading of anaerobic digestion of waste activated sludge by a hyper-thermophilic-mesophilic temperature-phased process with a recycle system. *RSC Advances, 5*(84), 68531-68541. Retrieved from <Go to ISI>://WOS:000359568400037. doi:10.1039/c5ra08811a

Xu, S. Y., et al. (2011). Optimization of food waste hydrolysis in leach bed coupled with methanogenic reactor: effect of pH and bulking agent. *Bioresource Technology, 102*(4), 3702-3708.

Yamaguchi, M., et al. (1991). Enzyme activity for monitoring the stability in a thermophilic anaerobic digestion of wastewater containing methanol. *Journal of fermentation and bioengineering, 71*(4), 264-269.

Yang, G., et al. (2015). Degradation properties of protein and carbohydrate during sludge anaerobic digestion. *Bioresource Technology, 192*, 126-130. doi:10.1016/j.biortech.2015.05.076

Yu, S., et al. (2013). Effect of endogenous hydrolytic enzymes pretreatment on the anaerobic digestion of sludge. *Bioresource Technology, 146*, 758-761.

Yuan, Q., et al. (2011). VFA generation from waste activated sludge: effect of temperature and mixing. *Chemosphere, 82*(4), 603-607.

Zhang, B., et al. (2007). Extracellular enzyme activities during regulated hydrolysis of high-solid organic wastes. *Water research, 41*(19), 4468-4478.

Zickefoose, C., et al. (1976). *Operation manual anaerobic sludge digestion*: US Environmental Protection Agency, Office of Water Program Operations.

Zuo, Z., et al. (2015). Performance enhancement of leaf vegetable waste in two-stage anaerobic systems under high organic loading rate: Role of recirculation and hydraulic retention time. *Applied energy, 147*, 279-286.

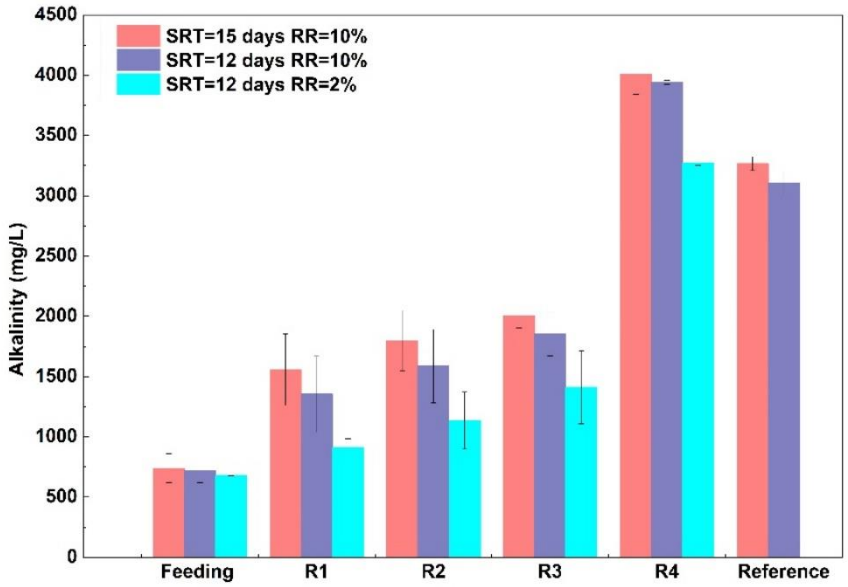
7. APPENDICES

Appendix A Methane composition

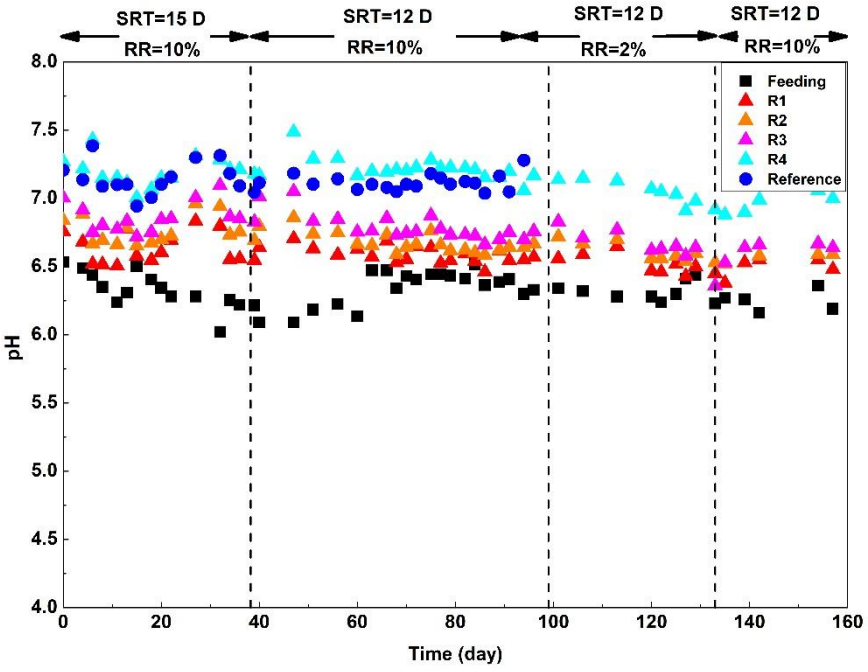
Appendix table 1 The percentage of methane in biogas

Phase	Day	Percentage of methane				
		R1	R2	R3	R4	R5
SRT=15 days RR=10%	6	0.54	0.58	0.55	0.58	0.56
	14	0.54	0.72	0.57	0.57	0.58
	21	0.53	0.70	0.57	0.59	0.54
SRT=12 days RR=10%	42	0.53	0.53	0.56	0.59	0.56
	50	0.54	0.54	0.58	0.59	0.56
	56	0.54	0.56	0.59	0.59	0.56
	63	0.54	0.55	0.57	0.57	0.56
	68	0.53	0.55	0.57	0.60	0.56
	77	0.52	0.54	0.60	0.59	0.57
	84	0.53	0.54	0.59	0.58	0.56
	89	0.53	0.54	0.57	0.58	0.55
	99	0.52	0.53	0.59	0.58	0.56
SRT=12 days RR=2%	103	0.53	0.63	0.57	0.59	0.57
	110	0.54	0.54	0.59	0.58	0.56
	116	0.54	0.55	0.58	0.59	0.56
	125	0.55	0.54	0.57	0.58	0.54
	103	0.54	0.55	0.59	0.60	0.58
	106	0.55	0.55	0.58	0.61	0.58
	110	0.50	0.53	0.58	0.55	0.53
	113	0.50	0.51	0.56	0.55	0.54
	118	0.52	0.54	0.53	0.53	0.53
	126	0.51	0.53	0.54	0.53	0.52
	133	0.54	0.53	0.55	0.53	0.51
SRT=12 days RR=10%	139	0.51	0.56	0.56	0.52	0.53
	154	0.51	0.57	0.64	0.55	0.52
	162	0.52	0.52	0.56	0.52	0.53

Appendix B Alkalinity and pH

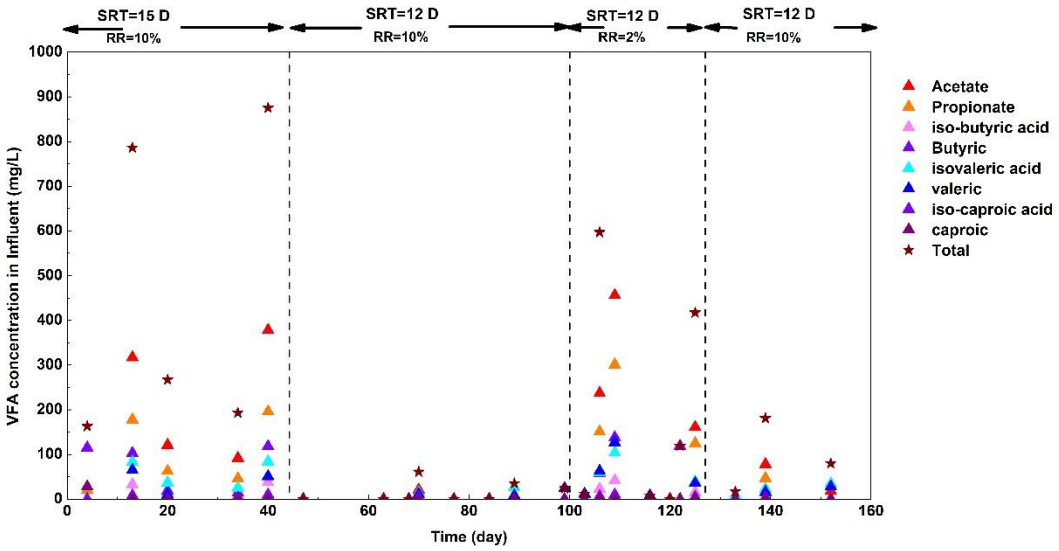


Appendix figure 1 Alkalinity of cascade system and reference CSTR

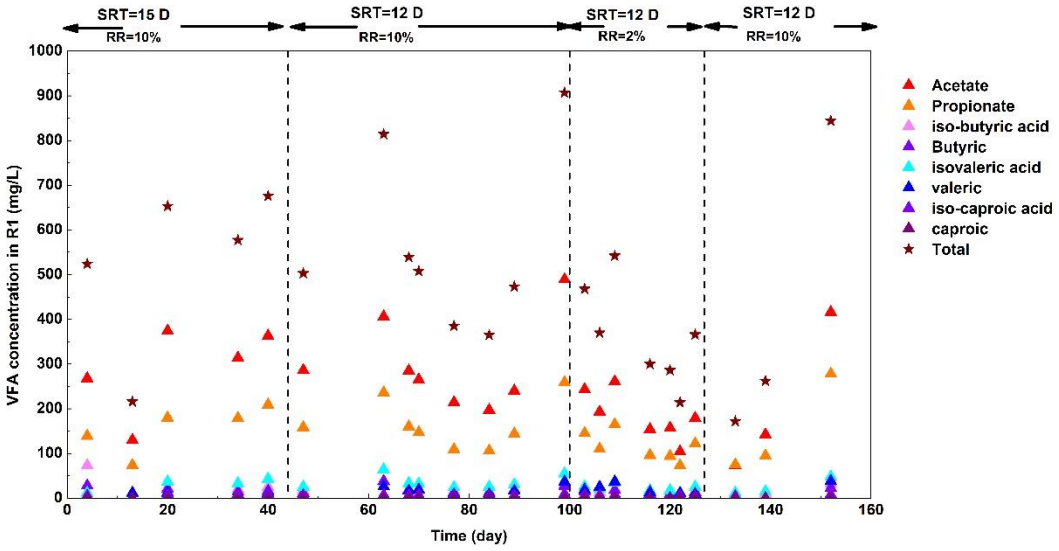


Appendix figure 2 pH of cascade system and reference CSTR

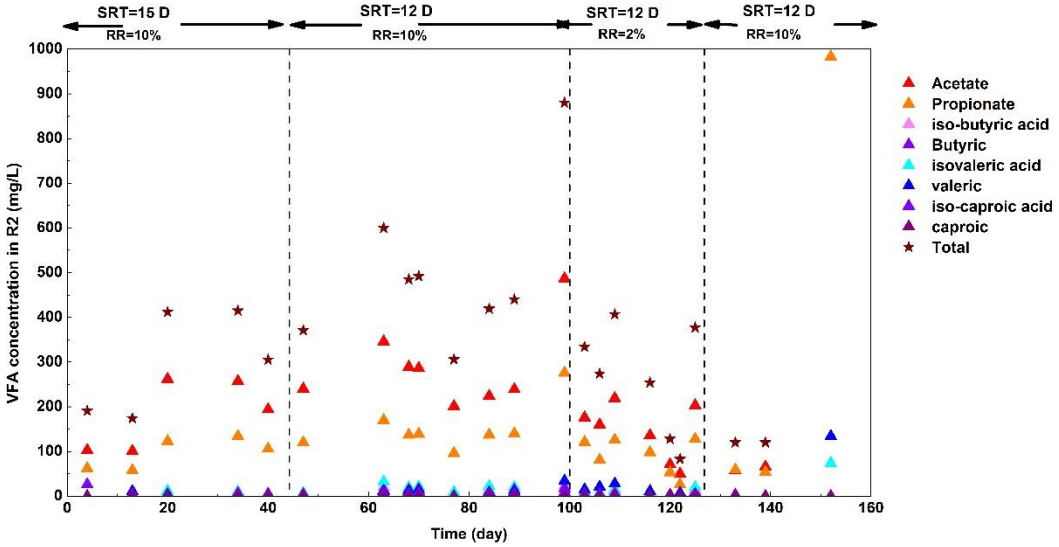
Appendix C VFA concentration in each reactor



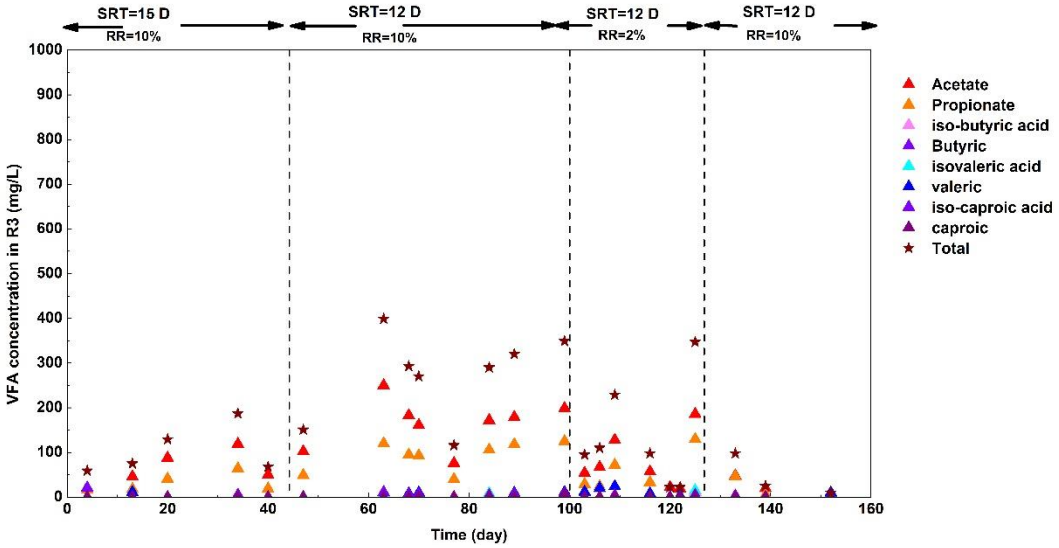
Appendix figure 3 VFA concentration of influent



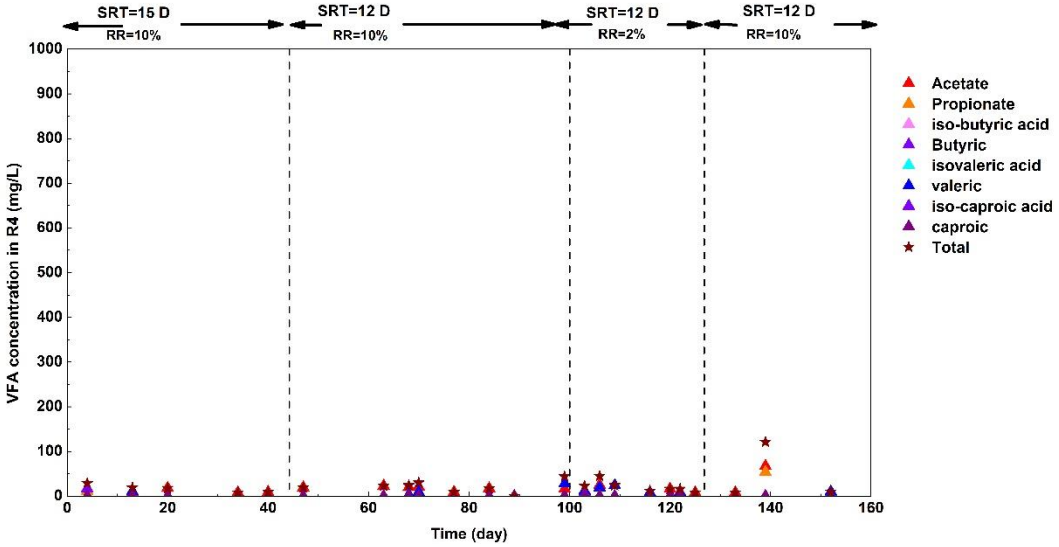
Appendix figure 4 VFA concentration in R1 of cascade system



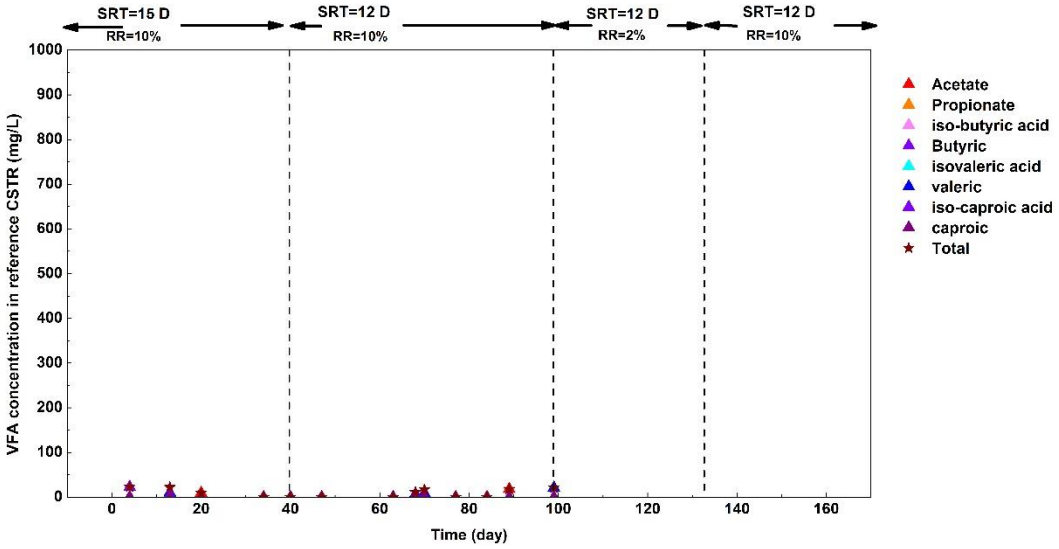
Appendix figure 5 VFA concentration in R2 of cascade system



Appendix figure 6 VFA concentration in R3 of cascade system



Appendix figure 7 VFA concentration in R4 of cascade system



Appendix figure 8 VFA concentration in reference CSTR

Appendix D Cations concentration in cascade system

Appendix table 2 Metal ions concentration in cascade system under 10% RR

Cations	SRT=12 D RR=10%										
	K	Na	Si	Al	Mn	Li	Ti	Cu	As	Se	Mo
	mg/L	mg/L	mg/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L
Feeding	247.8	84.0	10.8	324.7	90.5	9.1	6.9	43.9	12.0	2.5	1.7
R1	415.6	89.3	16.8	635.4	104.3	14.2	15.7	79.3	24.2	2.2	2.9
R2	438.5	89.4	18.9	750.6	49.6	16.4	18.7	91.3	27.4	2.8	3.8
R3	479.1	90.2	22.4	834.3	38.9	22.5	20.8	94.0	28.1	3.0	4.1
R4	492.4	85.6	26.7	378.6	18.6	27.3	22.1	42.1	33.8	3.2	5.3

Appendix table 3 Metal ions concentration in cascade system under 2% RR

Cations	SRT=12 D RR=2%										
	K	Na	Si	Al	Mn	Li	Ti	Cu	As	Se	Mo
	mg/L	mg/L	mg/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L
Feeding	219.0	77.3	9.2	15.9	257.6	8.4	1.9	9.8	8.4	2.6	0.8
R1	374.6	79.1	14.8	168.7	30.6	13.5	7.3	37.4	18.9	2.3	1.7
R2	406.3	81.3	13.0	337.0	19.2	18.4	8.0	66.4	23.2	2.9	3.1
R3	438.6	82.2	21.6	325.7	10.6	24.6	10.8	45.8	26.5	2.6	2.9
R4	518.8	92.6	28.3	190.5	3.7	30.4	17.9	25.1	32.4	2.4	6.3