Anaerobic treatment of source-diverted blackwater-maximizing biomethane recovery

by

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ABSTRACT

New sanitation is developed based on wastewater source-diversion and on-site treatment to maximize resource recovery from domestic wastewater. Blackwater stream collected from toilets is rich in organics and nutrients, and the rest greywater stream contains major water content. The primary objective of this thesis was to obtain high biomethane recovery from blackwater using anaerobic digestion (AD) technology. Bioreactor operational performances accompanied the microbial community developments in various treatment conditions were demonstrated. The limitations in blackwater AD processes were systematically revealed and resolved to help establish a high-rate blackwater treatment process.

The research chain started from characterizing blackwater collected from different types of toilet flushing systems to gain insights in the limiting factors in biomethane generation. Vacuum toilet blackwater (1 L water/flush) generated 29% lower biomethane production potential (BMP) than conventional and dual flush toilet blackwater (9 L and 6 L water/flush) when treated at 35°C. The high free ammonia concentration of 393 mg/L in vacuum toilet blackwater was identified as the inhibition factor, and the methanogenesis process was found to be directly inhibited by free ammonia while the substrate hydrolysis and fermentation processes were not significantly affected.

Continuous upflow anaerobic sludge blanket (UASB) reactors were operated at 35°C to treat different types of blackwater from water-conserving (vacuum) and water-wasting (conventional) toilets. Sulfate inhibition was observed in conventional toilet blackwater UASB treatment, which resulted in a low methane production rate of 58.0 mL/L reactor /d. A high organic loading rate

(OLR) of 4.1 kg COD/m^3/d was obtained in vacuum toilet blackwater UASB operation through stepwise acclimatizing the system to increasing OLRs. Methane production rate of 0.68 m^3 CH_4/ m^3 reactor/d and chemical oxygen demand (COD) removal efficiency of 84% were obtained, representing the highest blackwater treatment efficiency up to date. When the OLR was further increased to 4.9 kg COD/m^3/d, a 40% reduction in solid substrate hydrolysis was observed due to sludge loss. Food waste and vacuum toilet blackwater co-digestion was then performed to resolve the substrate hydrolysis limitation. The maximum OLR of 10 kg COD/m^3/d and methane production rate of 2.42 m^3 CH_4/ m^3 reactor/d were obtained for blackwater and food waste co-digestion process using a UASB reactor, which was attributed to the enhanced bioreaction conditions with more favorable carbon/nitrogen (C/N) ratio and readily biodegradable substrates. The treatment performances for blackwater and food waste co-digestion in the current research represented the maximum bioenergy recovery efficacy from household biowaste up to date.

The 16S rRNA gene sequencing results revealed that different groups of bacteria and archaea were enriched with different blackwater sources (conventional toilet blackwater or vacuum toilet blackwater with and without food waste addition) and changed OLRs in UASB operations. The combined syntrophic acetate oxidation (SAO) and hydrogenotrophic methanogenic (HM) pathway was established in blackwater treatment processes. Hydrogenotrophic methanogens dominated the archaeal communities in all blackwater UASB operations. Methanogens from genus *Methanospirillum* and *Methanolinea* dominated in conventional toilet blackwater UASB reactor, and their abundances shifted with different feed sulfate concentrations. Methanogens from genus *Methanogenium* were predominant in vacuum toilet blackwater mono-digestion system using the UASB reactor, and the dominant communities shifted to genus *Methanoculleus*

and uncultured genus from family *Methanospirillaceae* in food waste co-digestion condition. SAO bacteria groups, e.g. from order Clostridiales were enriched in all blackwater treatment systems. The establishment of the SAO-HM methanogenic pathway in the vacuum toilet blackwater treatment system was associated with the environmental factors of high ammonium concentration and/or high OLRs.

PREFACE

This thesis is an original work conducted by Mengjiao Gao. All research involved in this thesis were designed by me and my supervisor Dr. Yang Liu at the University of Alberta. I conducted all experiments, collected and analyzed the data, wrote and revised the manuscript. Dr. Yang Liu contributed to the conceptualization, supervision, review and editing of the manuscript. Some colleagues also contributed to the manuscript and their contributions are listed as following:

Chapter 3:

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Dr. Lei Zhang contributed to the methodology, review and editing of the manuscript. Dr. Anna P. Florentino contributed to the analysis of raw Miseq data.

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Chapter 7:

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Dr. Lei Zhang contributed to the review and editing of the manuscript.

Chapter 8:

Dr. Bing Guo contributed to the analysis of raw Miseq data.

DEDICATION

To my dear parents: Zhanchun & Lijuan

To all my family and friends

To the souls of my grandfather and uncle

To the brave souls who sacrificed in the pandemic

To justice

To peace and love

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Fate is unpredictable. When I look back at my past four years' Ph.D. study, many thanks that I would like to say to people who have helped me, guided me and supported me. I would like to give thanks to Dr. Xiaochun Chen, my master's supervisor, who not only provided help and guidance to my study but also provided me the precious opportunity to meet Dr. Yang Liu, my Ph.D. supervisor, the role model for my whole life.

Words are not enough to express my gratitude to Dr. Yang Liu. Four years ago, I changed my major from Chemical Engineering to Environmental Engineering and started my Ph.D. study in the University of Alberta under Dr. Liu's supervision. At the beginning, I did not even know what COD was, let alone understand any wastewater treatment technology. Hard as it was, Dr. Liu put faith in me. We started to work on the promising topic on blackwater bioenergy recovery, and my journey began on exploring anaerobic digestion and the new sanitation system. Dear Dr. Liu, all the discussions with you, inspirations from you, your comments and edits for every of my write-up and presentations, all the encouragement, all the caring I've received and felt from you are treasures of my life. You have shown me the great characteristics that a scientist should have : being confident, self-disciplined, with critical thinking and being rigorous to research. I felt your caring when I was upset, and I felt your gladness when I made progress. Thank you for being my supervisor, thank you for your patience, your guidance, your encouragement and your generous help to me.

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At this time when I am preparing for my final Ph.D. defense, the world is experiencing a pandemic. Millions of people are suffering from this disaster and many people have lost their beloved ones. I would like to give my salute to the doctors, nurses, scientists, firefighters, soldiers, workers, medical and food suppliers, volunteers and people who are sacrificing themselves to protect others. I would like to thank people who are helping each other and supporting each other to live through this hard time. To people who lost their families and friends, I wish you don't lose hope. We will keep fighting until the day when we are back to normal life. I hope to shake hands with friends. I hope to see people's smiles. I hope that day will come soon.

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ABBREVIATIONS

AD: anaerobic digestion

AM: acetoclastic methanogenesis

ANAMMOX: anaerobic ammonia oxidation

ANOVA: analysis of variance

BW: blackwater

COD: chemical oxygen demand

CODt: total COD

CODss: suspended COD

CODcol: colloidal COD

CODs: soluble COD

CODsolids: total COD exclude soluble fraction

CSTR: continuous stirred tank reactor

C/N: carbon/nitrogen ratio

CT: conventional toilet

DI: deionized

FA: free ammonia

FW: food waste

GC: gas chromatography

HRT: hydraulic retention time

HM: hydrogenotrophic methanogenesis

IC: ionic chromatography

k_h: first order hydrolysis rate coefficient

OLR: organic loading rate

SRT: sludge retention time

SMA: specific methanogenic activity

SAO: syntrophic acetate oxidation

SeqBR: sequencing batch reactor.

TSS: total suspended solids

TOC: total organic carbon.

TDS: total dissolved solids

TS: total solids

VS: volatile solids

TN: total nitrogen

TAN: total ammonia nitrogen

TP: total phosphorous

UASB: upflow anaerobic sludge blanket

VFA: volatile fatty acids

VT: vacuum toilet

VSS: volatile suspended solids

CHAPTER 1. GENERAL INTRODUCTION AND RESEARCH OBJECTIVES

1.1 Background and Motivations

Wastewater treatment is critical in protecting human and aquatic environment from pollution. With the evolvement of technology and human habits, in the 19th and 20th century, the centralized sanitation system has been installed in cities for hygiene purposes. However, millions of people from less developed areas, e.g. Africa, are exposed to the risks from diarrhoeal and other infectious diseases spread from polluted water due to the lack of sanitation. Even for developed countries where sanitation systems are widely installed and maintained, pathogenic risks exist due to the improper sanitation pattern (Lens et al., 2015). Further, the current sanitation system consumes a large amount of energy in the transportation and biological treatment processes accompanied by excess sludge generation, which makes it vulnerable to the challenge of energy crisis rising from the global population increase and fast urbanization. Approximate 84% of the energy consumption in human society is through fossil fuel combustion, and the large demand has been stressing the petroleum and natural gas industry and stimulating CO_2 emission (Rittmann, 2015). Facing such issues, a new sanitation concept was brought up in recent decades, aiming at reducing the environmental risks and enhancing resource recovery from wastewater (Otterpohl and Oldenburg, 2007). Comparing to the conventional sanitation systems with centralized sewage collection and treatment, the new sanitation system enables separate collection and treatment of the heavily polluted blackwater (toilet wastewater) from the other less polluted wastewater stream-greywater at the household level. As blackwater contains most pathogens and micropollutants (Lienert et al., 2007), half the load of the organic materials, and most of the total nitrogen and phosphorus (De Graaff et al., 2011) in the total household wastewater, but only accounts for a small fraction of the total wastewater volume, a lower level of pathogenic risk, and bioenergy and nutrients (nitrogen & phosphorus) recovery can be achieved from treating blackwater. Water re-use can be simultaneously achieved through treating the greywater which contains large quantities of water.

In the new sanitation design, bioenergy recovery from the source-diverted blackwater serves as one of the core components ensuring the overall process sustainability. A simple and effective

approach to recover the bioenergy is through anaerobic digestion (AD) which can convert organic pollutants into biogas with the help of anaerobic microorganisms at low operational cost. In traditional sanitation system, anaerobic treatment has been utilized in treating conventional combined sewage, especially in the tropical area due to its strong dependence on temperature; intensive research have also been conducted on enhancing the treatment performances under low temperatures in the recent years (Zhang et al., 2018). As the anaerobic treatment process favors organic-rich substrates, it is economically more beneficial to directly apply anaerobic digestion in the decentralized sanitation system where blackwater serves as the bioenergy resource. However, due to the limited blackwater collection sites, only a few studies about blackwater anaerobic digestion have been reported in the recent twenty years, and with various blackwater collection sources and different operational systems and conditions applied, the treatment performances varied significantly in these reported studies. Relatively low organic loading rates (OLR) with low methane generation have been observed in most of these systems, but research questions about the optimal operational conditions for achieving high-rate blackwater treatment and the limiting factors for obtaining effective biomethane recovery remain unaddressed. Further, insightful information such as the functional microbial community involved in the blackwater anaerobic treatment processes have never been reported.

1.2 Research Objective

The overall objectives of this research were to maximize biomethane recovery from blackwater anaerobic treatment process and to illustrate insightful information on process limitations and optimization strategies as well as the accompanied microbial community development. The specific objectives include:

1) Evaluate characteristics of blackwater collected from different types of toilets and identify the critical limiting factors on biomethane production.
2) Investigate process limitations in continuous blackwater treatment processes using upflow anaerobic sludge blanket (UASB) reactors.
3) Mitigate blackwater treatment limitations towards enhancing organic loading rate with high biomethane production.

4) Reveal the microbial community development and identify the feasible methanogenic pathways in blackwater treatment system.

1.3 Research Approach

The research objectives have been approached in the following ways:

1) *Evaluation of source-diverted blackwater from different toilet flushing systems*

Variations in toilet flushing system can result in significant differences in blackwater volume and chemical properties. Different toilets that are currently widely utilized were selected for blackwater characterization, including conventional, dual flush and vacuum flush toilets. The biomethane potential (BMP) test was performed to evaluate the maximum methane yield. The inhibition factor that limited the biomethane yield of vacuum toilet blackwater was verified through toxicity assays.

2) *Blackwater UASB treatment*

The current research selected UASB reactors to demonstrate continuous blackwater treatment because UASB enables a long solids retention time (SRT) with a short hydraulic retention time (HRT), which potentially enables high OLRs. Optimization strategies against the free ammonia inhibition (identified from the BMP test) were applied for treating vacuum toilet blackwater, including pH control and microbial acclimatization. Impacts from influent sulfate on the biomethane yield of conventional toilet blackwater was evaluated through changing toilet flushing water from tap water to DI water in the UASB operation. The OLR was challenged in the UASB reactor treating vacuum toilet blackwater. Process limitations under the overloaded condition was illustrated by investigating hydrolysis and methanogenesis processes.

3) *Blackwater and food waste anaerobic co-digestion using a UASB reactor*

This section followed the previous vacuum toilet blackwater UASB treatment, which aims to help resolve the hydrolysis limitation in the blackwater mono-digestion system at the high organic loading condition. Food waste contains readily biodegradable organics that can potentially help enhance microbial activities, and with high carbon but low nitrogen contents of food waste, their addition can help balance the carbon to nitrogen (C/N) ratio of the feedstock.

The UASB reactor was operated to demonstrate its feasibility of treating blackwater and food waste co-substrates. The OLR was gradually challenged to demonstrate the threshold of the UASB treatment capacity for household biowaste.

4) *Reveal methanogenic pathways in blackwater anaerobic treatment processes*

An effective methanogenic pathway plays the key role in extracting bioenergy from organic pollutants in blackwater. The current research evaluated the development of the methanogenic pathway through approaches of specific methanogenic activity (SMA) tests, carbon isotopic signatures analysis and 16S rRNA gene sequencing. These approaches were applied to different treatment/operational conditions of i) different treated feedstocks, i.e. blackwater collected from conventional and vacuum toilets, and vacuum toilet blackwater with food waste; ii) different bioreactor operational mode, i.e. in batch reactors and continuous UASB reactors; iii) different bioreactor operational conditions, i.e. different organic loading rates; iv) conventional toilet blackwater with different types of toilet flushing water. The interactions between hydrolytic/fermentative bacteria and methanogenic archaea were interpreted. The dynamic shifts of microbial communities and the establishment of methanogenic pathway were correlated with blackwater treatment performances.

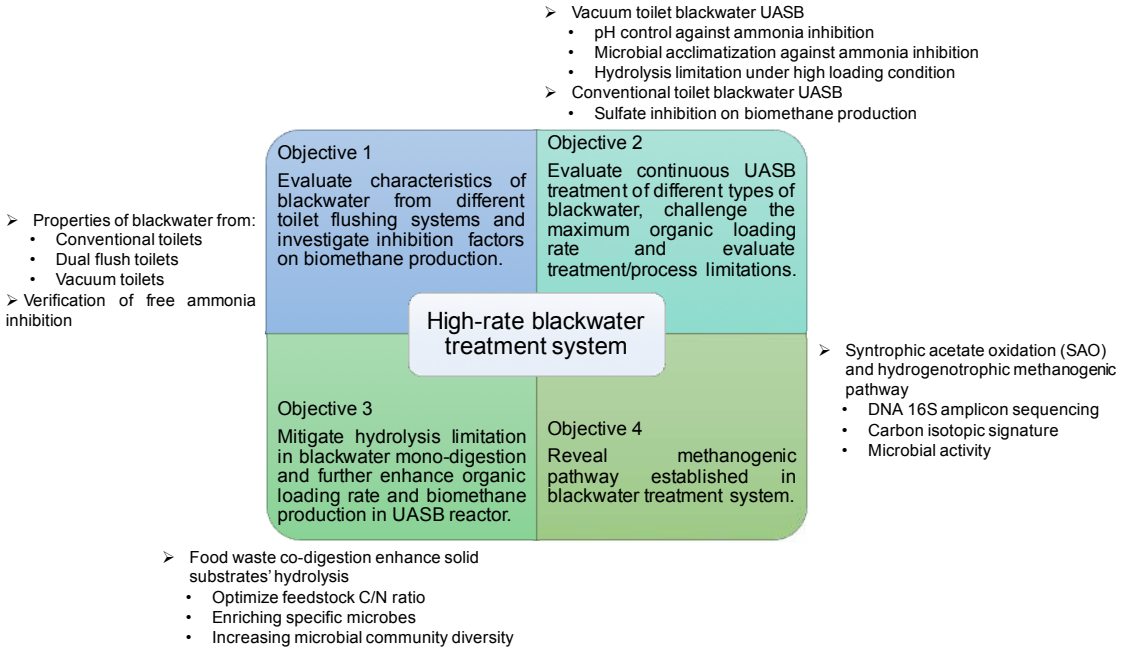

Fig. 1.1. Overview of thesis objectives and research approaches.

1.4 Thesis Organization

Chapter 1 contains a general introduction to the background, motivations, objectives and approaches of this research. The concept of the decentralized sanitation system is briefly described.

Chapter 2 provides a literature review and identified the research gaps and challenges in blackwater anaerobic treatment processes. Detailed descriptions of the decentralized wastewater treatment and reuse system and the anaerobic digestion technology are included. This chapter highlights the current research achievements and limitations in the blackwater anaerobic treatment field.

Chapter 3 investigates the impacts of toilet flushing systems on blackwater biomethane production potential. Blackwater collected from water-wasting and water-conserving types of toilets were compared. The inhibition effect on biomethane recovery potential from high free ammonia concentration was elucidated. Microbial community structures developed in various types of blackwater batch reactor operations were analyzed and linked with different biomethane yield. This chapter is directed to objectives 1 and 4. This chapter provided information on blackwater characteristics that guided the following studies. The biomethane potential values are utilized to evaluate the blackwater treatment performance in continuous operations in Chapters 4 and 5, and the identified free ammonia inhibition was mitigated in continuous vacuum toilet blackwater UASB treatment in Chapter 5.

Chapter 4 investigates the influence of sulfate content on the biomethane production process in a continuous UASB reactor treating conventional toilet blackwater. The sulfate inhibition effects on the treatment performances and microbial community shifts were illustrated. This chapter is directed to objectives 2 and 4.

Chapter 5 investigates the UASB treatment performances for vacuum toilet blackwater, focusing on demonstrating the threshold reactor treatment capacity (maximum OLR) and the microbial community dynamic shifts under increasing OLR conditions. The limiting factors under high

organic loading condition were evaluated, which guided the process optimization in Chapter 7. This chapter demonstrated objectives 2 and 4.

Chapter 6 investigates and compares the microbial community developments in continuous UASB treatment of conventional and vacuum toilet collected blackwater at different organic loading rates. The analysis of microbial communities' diversity, composition and functions were conducted. This chapter demonstrated objectives 1 and 4.

Chapter 7 investigates vacuum toilet blackwater and food waste co-digestion using the UASB reactor. The effects of anaerobic co-digestion of blackwater and food waste on the enhancement of blackwater hydrolysis, methanogenesis, and the UASB reactor maximum treatment capacity were demonstrated. This chapter is directed to objective 3. This chapter provides a mitigation strategy against the hydrolysis limitation in blackwater mono-digestion demonstrated in Chapter 5.

Chapter 8 compares the microbial community development in the high-rate blackwater mono-digestion system and the food waste and blackwater co-digestion system. The microbial community dynamic shifts under increasing organic loading rate conditions and the methanogenic pathways were elucidated. This chapter demonstrates objectives 3 and 4. This chapter revealed the fundamentals of process optimizations from the perspectives of microbial community development, and deeply interpreted the different treatment performances observed in the operations demonstrated in Chapters 5 and 7.

Chapter 9 gives the conclusion of the research findings and achievements from chapters 3-8. Recommendations for future work are presented in this chapter.

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CHAPTER 2. LITERATURE REVIEW

2.1 New sanitation towards sustainable development

"Sustainable development" was defined as "*development that meets the needs of the present generation without compromising the ability of future generations to meet their own needs*" (*Report of the World Commission on Environment and Development: Our Common Future [The Brundtland Report]*, 1987). A sustainable system can adapt to changing environment and be resilient in accommodating local circumstances. Due to the fast city expansion and industrialization, pollutions and resource scarcity have become the primary challenges to a sustainable future. To tackle these challenges, recovery and reuse of resources in terms of water, energy and nutrients have become the urgent necessity for the current society.

The conventional centralized sanitation systems have been developed to meet the needs of human hygiene, yet this design lacks sustainability due to the energy and water consumption and the large footprint requirement. In recent decades, a new sanitation concept of "decentralized sanitation and reuse" has been brought up to meet the sustainable goal. This new sanitation system exhibits benefits towards sustainability from perspectives of economics, energy, ecological, social-cultural and human health impact compared with the conventional sanitation system. The major benefits are discussed as following:

Economic cost

The conventional centralized sanitation pattern is costly due to the maintenance and operational expenses of the city-wide wastewater conveyance networks and the large-scale wastewater treatment plants (WWTP). While the decentralized sanitation system can reduce the cost with the short pipework for small-scale systems and the replacement of the conventional energy-intensive aeration process. The investment costs for the new sanitation pattern were estimated to be 640-2,170 US$/capita for the sewer disconnected cases, and 260-680 US$/capita for the sewer retained new sanitation scenarios, assuming that the new sanitation system can only replace the conventional scenario at similar costs to the current centralized system (2,600-2,800 US$/capita) (Maurer et al., 2005).

Resource recovery and reuse

In spite of the reliability in securing environmental and human health protections, the conventional centralized collection mixed different wastewater streams and diluted the pathogens and toxicants in a large water volume, resulting in high space and energy input along with emissions throughout the treatment processes. Resource recovery is difficult from sewage. The new sanitation system offers to maximize resource recovery from household wastewater through wastewater source diversion and on-site treatment approaches (Fig. 2.1). Bioenergy and nutrients can be recovered from treating the organic and nutrient-rich blackwater stream collected from toilets. The rest less polluted greywater from handwashing, shower, laundry can be treated for water re-use. This design enables closing the water, nutrients and energy cycle at the household level (Kujawa-Roeleveld et al., 2006). The recovered bioenergy can be utilized to heat up the treatment facilities and neighborhood communities. The treated water can potentially be utilized for toilet flushing, gardening and floor cleaning, and the recovered nutrients can be utilized as fertilizers for crops.

Ecological impact

The large-scale centralized wastewater collection and transportation networks are posing risks at a wider range in the spread of pathogens and micropollutants (hormones and pharmaceutical residues) when system failure occurs, while the decentralized system controls risks within a small scale. Further, the design of on-site treatment and reuse minimizes the effluent discharge into the waterbodies, which helps reduce cumulative impacts to aquatic environment (Fane and Fane, 2005).

In addition to the descriptions above, the impact on human health from pathogens can be lower (Fane et al., 2002) and social interactions can be enhanced with greater community involvement in the local treatment systems compared with the conventional centralized sanitation.

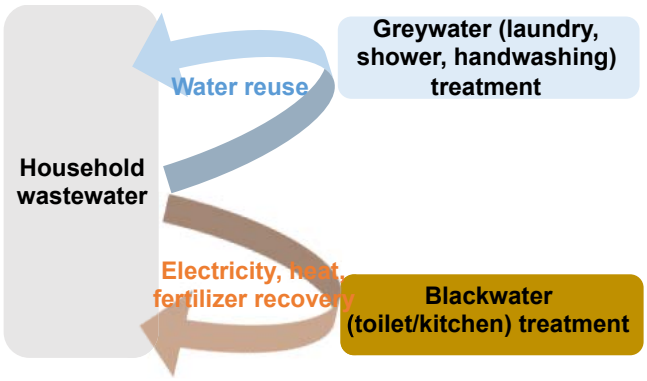

Fig. 2.1. Scheme of the decentralized sanitation and re-use system.

2.2 Blackwater

The current research work is mainly concerned with the treatment of source-diverted blackwater. The separate collection and treatment of blackwater at the local level provides benefits of i) minimize the spread of hazardous materials: pathogens, pharmaceutical residues, hormones from human excreta to the water cycles; ii) produce biogas to support local energy consumption, either for the treatment plants themselves or for residential utilization; iii) produce nutrient-rich effluent which can be applied as fertilizers after proper post-treatment for pathogenic removal; iv) reduce water consumption with the implementation of water-conserving toilet flushing systems.

2.2.1 Variance in blackwater sources

Blackwater characteristics can be affected by different factors. The human diet is one major aspect that could generally be affected by local economics, traditions, eating habits, and ages. For instance, it has been reported that fecal wet weights from low-income countries were twice those from high-income countries due to the higher fiber intake in the local population (Rose et al., 2015). The collection site/area from either work or at home also contributes to the variance in blackwater chemical properties. The different types of toilet flushing system is another key influencing factor to the blackwater characteristics in terms of both volume and chemical properties. Toilets in the current market can be separated based on their water consumption amounts. Water-conserving toilets such as vacuum toilets consume only 0.5–1.2 L water per flush and produce 5–7 L of blackwater per person per day, whereas water-wasting toilets such as conventional toilets use 5–9 L (up to 12 L) water per flush and produce 6–15 times more blackwater in volume (Elmitwalli et al., 2006; Moges et al., 2018; Zeeman et al., 2008). So far,

only a few studies have reported blackwater chemical properties, which have been summarized and listed in Table 2.1. Despite the different collection areas, the reported blackwater studies can be categorized based on the types of toilet flushing systems, either with vacuum toilets or conventional toilets.

Due to its lower dilution, blackwater collected from vacuum toilets includes relatively rich organics and nutrients contents with the chemical oxygen demand (COD) concentrations ranging from 5,500 (±1,300) mg/L to 15,500 (±3,300) mg/L. Conventional toilets, however, exhibited lower COD values ranging from 932 (±244) mg/L to 2,887 (±793) mg/L. Overall, the reported pH values were at the range of 7.4-9.0. The ammonium nitrogen (NH_{4+}-N) contents vary with the water amounts, which showed the high range of 499-1,400 mg/L for concentrated blackwater and the low range of 54-202 mg/L for the less concentrated type of blackwater (De Graaff et al., 2010; Gallagher and Sharvelle, 2010; Knerr et al., 2011; Zamalloa et al., 2013).

Table 2.1. Blackwater characteristics reported in literature.

	Conventional toilets					Vacuum toilets					
Literature	(Gallagher and Sharvelle, 2010)	(van Voorthuizen et al., 2008)	(Abdel-Shafy et al., 2009)	(Sharma et al., 2016)	(Knerr et al., 2011)	(Moges et al., 2018)	(Hertel et al., 2015)	(De Graaff et al., 2010)	(Wendland et al., 2007)	(Kujawa-Roeleveld et al., 2006)	(Zamalloa et al., 2013)
pH	8.9 (±0.4)		7.4-8.3	8.1 (±0.21)	9.0 (±0.1)	9 (±0.3)	7.58 (±0.17)	8.6-8.8	7.7		7.9 (±0.5)
CODt (mg/L)	932 (±244)	1,139	1,160 (±391)	1,712 (±225)	2,887 (±793)	5,500 (±1,300)	7,615 (±2,990)	7,700-9,800	8,700 (±3,980)	9,500-12,300	15,500 (±3,300)
CODss (mg/L)	-	391	-	-	-	-	-	4,900-5,100	-	7,000-9,600	-
CODcol (mg/L)	-	215	-	-	-	-	-	500-1,300	-	-	-
CODs (mg/L)	234 (±47)	530	-	927 (±256)	-	1,200 (±330)	-	2,300-3,400	2,400 (±650)	1,400-2,800	-
VFA (g COD/L)	-	-	-	0.2 (±0.08)	-	0.4 (±0.2)	-	1.2-1.5	-	0.5-1.9	0.4 (±0.3)
TN (mg/L)	77 (±11)	169	-	117.5 (±28)	-	-	1,455 (±220)	1,200-1,900	1,500 (±250)	-	-
NH_{4+}-N (mg/L)	54 (±13)	138	150 (±16)	88.7 (±19)	202 (±32)	900 (±180)	1,090 (±121)	850-1,400	1,100 (±140)	600-1,000	499 (±83)
TP (mg/L)	-	121	35.6	44.5 (±25)	34.4 (±6.0)	120 (±20)	-	150-220	202	90-140	324 (±232)
PO_{43-}-P (mg/L)	-	27	-	-	23.3 (±2.4)	60 (±20)	-	54-79	-	30-60	-
TDS (mg/L)	-	-	1,401 (±256)	-	-	-	-	-	-	-	-
TSS (mg/L)	435 (±147)	-	363 (±131)	1,053 (±571)	1,697 (±395)	3,000 (±900)	-	-	4,500 (±2,700)	-	8,300 (±2,500)
VSS (mg/L)	379 (±119)	-	-	683 (±278)	96.1 (±1.8)	-	-	-	-	-	-
TOC (mg/L)	-	-	-	1,028 (±227)	1,178 (±272)	-	2,428 (±878)	-	2,500 (±950)	-	-
SO_{42-}-S (mg/L)	-	-	10.9	14.7	-	-	-	-	43	-	-

2.2.2 Blackwater treatment options reported in literature

Different types of sustainable treatment options have been researched for blackwater treatment. For instance, composting, which can generate biofertilizers from blackwater, has been tested (Oarga Mulec et al., 2016). Despite its obvious advantages of the overall low treatment cost, effective pathogen removal, and economic end products' generation, its treatment efficiency and practical application are limited by the factors of long retention time, aeration control, proper selection of the composting bulk additives due to the low carbon to nitrogen (C/N) ratio of blackwater (Hashemi et al., 2019). A blackwater treatment process using membrane bioreactor (MBR) has been demonstrated at a semi-technical scale to obtain high effluent water quality (Knerr et al., 2011). In a single house demonstration case in Brazil, a maintenance-free evapotranspiration tank has been tested possible for blackwater management (Paulo et al., 2013). A hybrid process with granular activated carbon (GAC) filter and electrochemical treatment has been demonstrated for the on-site reuse and/or discharge of blackwater (Rogers et al., 2018). Microwave treatment has been tested as an effective method in rapidly reducing blackwater volume and pathogens, which makes it promising in emergency situations (Mawioo et al., 2016). The feasibility of applying "Microbial Fuel Cell (MFC)" on blackwater treatment for power generation has also been evaluated (Fangzhou et al., 2011; Vogl et al., 2016). Among the reported blackwater treatment technologies, anaerobic digestion is one of the most promising approaches for practical application. This technology enables on-site bioenergy recovery from blackwater with low maintenance requirements, which was superior cost-effectiveness.

2.3 Anaerobic digestion

After its discovery in the 10th century, anaerobic digestion technology has been widely utilized in wastewater treatment. Compared with the biological aerobic treatment processes, the premium advantages of anaerobic processes include the energy generation in the form of biogas, low sludge production, and small footprint. Due to the global energy deficiency and climate change in the past decades, increasing research interests have been put in anaerobic digestion towards deeper comprehension of the reactor design, reaction mechanisms, and process limitations.

2.3.1 Process description

The overall anaerobic digestion process is a biological treatment process carried out by a group of microorganisms that converts organics into biogas under anaerobic condition. Wastewater usually contains complex organic matters comprised of carbohydrates, proteins, and lipids. To be converted into biogas, the organics would go through the following four reaction steps:

A. Hydrolysis: acidogenic bacteria excrete enzymes for biopolymers solubilization, the products are monomers and dimers such as sugars, amino acids, and long-chain fatty acids;

B. Acidogenesis: soluble organics are converted to volatile fatty acids (VFA), alcohol, and carbon dioxide (CO_2);

C. Acetogenesis: alcohol and VFAs are converted to acetate and hydrogen (H_2) and CO_2;

D. Methanogenesis: acetate and H_2&CO_2 are converted to CH_4.

2.3.2 Reactor types

A proper selection of reactor configuration determines the success of the treatment process. Parameters of hydraulic retention time (HRT) and sludge retention time (SRT) are critical for configuration designs. High SRTs are preferable for process stability and low HRTs enable small reactor volumes. The so-far developed anaerobic bioreactors can be categorized as with or without biomass retention. The reactor type without biomass retention, such as continuous stirred tank reactor (CSTR), provides equal HRT and SRT and these reactors are generally utilized under low volumetric organic loading rates conditions with relatively long HRT/SRT. The type of reactors with sludge retention, such as upflow anaerobic sludge blanket (UASB), can offer an extensively longer SRT than HRT. The selection of reactor type is correlated with wastewater properties. In general, bioreactors with equal SRT and HRT tend to be applied in treating high solids types of waste/wastewater (Li et al., 2011), e.g. waste activated sludge, while bioreactors with SRT>HRT are usually applied for wastewater with low solids content.

Batch bioreactors

Batch reactors are easy to operate. These reactors are operated with a single time fed inoculum and feedstock and are emptied once the reaction completed. With better flexibility of controlling the operational parameters compared to the continuous mode bioreactors, the bench-scale batch

systems are widely applied to test the microbial responses towards the different types of feedstocks, changed environmental factors and toxicants/inhibitors (Hosseini, 2019).

Continuous stirred tank reactor (CSTR)

CSTR reactors are the very first generation of anaerobic bioreactors that applied in municipal sewage treatment. The system is completely mixed, and the constant feeding and discharging happen continuously. HRTs of 15-30 days are usually applied for CSTR reactors and the organic loading rates are normally within 4 kg COD/m^3/day (Metcalf and Eddy, 2003). Although such systems have advantages of simple control/operation and homogenous contact between microbes and organics, there are clear drawbacks such as the large reactor installation footprint due to the long HRT/SRT.

Upflow anaerobic sludge blanket (UASB)

The sludge retention is critical for obtaining a high-rate wastewater treatment process. With a deeper understanding of the importance of sludge retention, anaerobic bioreactor designs have been modified. A typical high-rate bioreactor type UASB, and its variant and modified reactor types including expanded granular sludge blanket (EGSB), internal circulation UASB (IC), anaerobic baffled reactor (ABR) have been utilized for high-rate wastewater treatment (Metcalf and Eddy, 2003).

Developed in the 1960s in the Netherlands, the upflow anaerobic sludge blanket (UASB) reactor was designed based on the concept of biomass retention (Lettinga et al., 1980). In a UASB reactor, wastewater is fed from the bottom of the reactor, evenly distributed and flow upwards through a sludge bed. Biomass retention can be accomplished by the sludge aggregates settling and the gas/liquid/sludge separation system in the reactors. The microbes in the sludge bed can entrap the suspended organics from the wastewater stream and convert the organics into biogas, which results in high biodegradation rates (Speece, 1983). UASB reactors have been widely applied to treat industrial wastewater, dairy wastewater, domestic sewage, and agricultural waste (Lettinga and Hulshoff Pol, 1991). The effects from temperature, OLR, and sludge granulation are of great importance in the operation of UASB reactors.

2.3.3 Limiting factors

It is important for each of the anaerobic reaction steps to work in a balanced state to maintain a stable anaerobic digestion process. Limitations in anaerobic digestion process correlate with both feedstock characteristics and treatment conditions. The predominant drawbacks of anaerobic digestion technology include i) the process slow start-up due to the slow growth rate of methanogens; ii) the strong dependence on temperature, e.g. hydrolysis of solid organics can be limited under cold climate; iii) the probable instability caused by methanogenesis inhibition, e.g. from toxicants such as high free ammonia (FA) and long-chain fatty acids (LCFA). Besides, the post-treatment processes are usually required to meet the effluent discharge limits, and the odorous problem might need to be encountered when treating sulfate-containing or protein-rich wastewater due to sulfide generation.

Temperature

Anaerobic digestion can be conducted under all three temperature ranges of psychrophilic (10-20 °C), mesophilic (20-40 °C) and thermophilic (50-60 °C). Low-temperature operation is generally not favorable. The microbial growth rate and substrate utilization rate are low under such conditions. The gas solubility is higher under lower temperatures, which may result in inhibitory effects, for instance, higher H_2S content can be toxic to microbes (Lettinga et al., 2001). It is even more difficult to treat complex wastewater steams under low-temperature conditions, and a long solids retention time (SRT) and hydraulic retention time (HRT) are usually required for hydrolyzing solid organics. For instance, a longer SRT of 75 days was required for sufficient hydrolysis and methanogenesis to happen when treating sewage at 15 °C compared with the SRT of 15 days under 25 °C (Miron et al., 2000; Zeeman and Lettinga, 1999). Compared with the psychrophilic condition, the thermophilic condition promotes microbial growth rate, solid organics hydrolysis rate and the substrate utilization rate, which typically results in smaller bioreactor footprints and shorter treatment time (Ruffino et al., 2015). Such advantages make this temperature condition superior to treat high-solid substrates, and the generally considered countereffect from high heating cost only plays a smaller role as compared to the benefits (Harzevili and Hiligsmann, 2017). Further, the high-temperature condition can help with pathogen removal. One disadvantage of the thermophilic condition is that the microbes are more sensitive towards the environmental parameters, process failures occurred when facing

VFAs accumulation/pH drop or the toxicants such as high free ammonia concentration (Chen et al., 2008). Compared with the above two conditions, bioreactors operated at mesophilic temperatures are widely applied because of their relatively high treatment efficiencies and stability.

pH

Among the several steps in the anaerobic digestion process, the final methanogenesis step for gas production favors a narrow pH range of 6.8-7.2, while the acid production phases favor more acidic pH ranging at 5.2-6.3 (Rajeshwari et al., 2000). To avoid the methanogenic inhibition, a pH level over 6.5 and alkalinity concentration over 1,000 mg $CaCO_3$/L are generally recommended (Tchobanoglous et al., 2003). pH adjustments are usually required for treating acidic wastewater streams, such as wastewater from fermentation industries with a pH range of 4.5-5.5 (Khanal et al., 2017). The impact of pH largely depends on the feedstock characteristics and the limiting steps. When the methanogenesis step could not catch up with the rate of substrates' acidification, the accumulated VFAs could probably result in a pH drop below 6, which can be harmful to methanogens. This type of effect can be observed when treating fast-acidified substrates such as food waste under high a loading state with insufficient alkalinity (Xu et al., 2018). In addition to the direct impact on microorganisms, pH level also governs the ionization state of some typical inhibitors in the anaerobic digestion process, where high pH value can contribute to the higher formation of free ammonia (FA) (Borja et al., 1996) and low pH value can lead to increasing fractions of unionized sulfide products i.e. H_2S (Khanal et al., 2017), and both of these unionized molecules have been considered as toxic agents to microorganisms, especially methanogens.

Organic loading rate

The organic loading rate represents the capability of an anaerobic bioreactor treating certain types of waste/wastewater, and high OLRs are preferable due to the low capital cost (low bioreactor volume). The maximum applicable OLRs are dependent on the reactor type, operational conditions, and substrates properties. For instance, high-rate bioreactors with sludge bed, e.g. expanded granular sludge blanket (EGSB) can tolerate high loadings up to 35 kg COD/m^3/day, while the low-rate types of reactors, e.g. anaerobic lagoon may not exceed 2 kg

COD/m^3/day (Metcalf and Eddy, 2003). Stable biogas generation and effluent quality can generally be obtained within the threshold OLRs. The overloaded condition can occur when the rates of methanogenesis and hydrolysis-fermentation processes could not match. VFA accumulation would happen when the fermentation rate is faster, which could result in a sour system and even process failure (Ferguson et al., 2016). When the rate of solid organics hydrolysis could not satisfy the methanogenesis rate, the methane yield would decrease; this typically happens when the active biomass gets lost under an overloaded state in the systems handling high solid fraction organic materials.

Toxicants/inhibitors

The toxic substances can be introduced from the waste/wastewater (e.g. heavy metals) streams or be produced during the anaerobic digestion processes (e.g. H_2S).

Ammonium is important to anaerobic digestion process as it can provide buffer capacity and be utilized by microbes as a nitrogen source. However, excess ammonium content has been commonly reported to inhibit methane production process, and the free ammonia (FA) has been considered as the major toxicants. The level of FA can be determined by total ammonium concentration (TAN), pH and temperature. Compared with the ionized ammonium, FA can diffuse into the cells and cause the proton imbalance. The FA inhibition effect generally acts on methanogens (Angelidaki and Ahring, 1993; Koster and Lettinga, 1984). Various FA inhibitory concentrations have been reported in different AD systems. The concentrations ranged from 55-80 mg FA/L (fed with acetate/propionate) to up to 1,600-2,600 mg FA/L (fed with swine manure) have been found generated inhibitory effects (Rajagopal et al., 2013; Yenigün and Demirel, 2013). The strategies of pH control, feedstock dilution, co-digestion, and microbial communities' acclimatization can be applied to mitigate ammonia inhibition.

Sulfate and sulfide (typically H_2S) inhibitions are commonly reported in AD systems. Excess sulfate contents are commonly found in industrial wastewater streams, such as from molasses fermentation, acid mine drainage, food processing industries, and domestic wastewater (Liamleam and Annachhatre, 2007). Their impacts on the biogas production processes are mainly from i) the competition of electron donors between sulfate reducing bacteria (SRB) and

other anaerobes (e.g. fermentative bacteria and methanogens), and ii) the toxic effects from H_2S generated during the reactions. SRB can utilize various types of electron donors including hydrogen, short-chain and long-chain fatty acids, alcohols, and other organics such as aromatic compounds (J.W.H. et al., 1994), which makes them competitive against multiple stages in the anaerobic digestion process, yet research efforts were mostly put into the methanogenesis stage. With the thermodynamic favorability and high H_2 affinity to SRB (Laanbroek et al., 1984), it is generally believed that H_2 utilizing SRB can outcompete hydrogenotrophic methanogens (O'Flaherty et al., 1999). Due to the variances in the chemical properties of substrates and the operational conditions, different inhibitory threshold levels and results were reported. For instance, the inhibitory threshold of H_2S varied in the reported studies with a level of approximately 50-400 mg/L unionized H_2S (Parkin et al., 1990). Factors such as the substrates' COD/sulfate ratio, the microbial community structures and the amount of produced unionized H_2S have been the main focus in sulfate competition studies. A low COD/sulfate ratio has been generally considered as generating inhibition effects on the methane yield, while contradictory results were reported that the COD/sulfate ratio did not affect methane yield or microbial diversity (Cetecioglu et al., 2019; Kiyuna et al., 2017).

Other inhibitors/toxicants including heavy metals, long-chain fatty acids and toxic organics have all been reported in anaerobic digestion processes (Chen et al., 2008).

2.4 Anaerobic treatment of source-diverted blackwater

2.4.1 Blackwater anaerobic treatment studies reported in literature

Only limited studies have been conducted in blackwater anaerobic treatment so far, and the reported research are mostly focused on demonstrating process feasibility. Various reactor types and operational conditions including temperatures and OLRs have been applied in these work. Table 2.2 lists the literature reported treatment performances regarding the COD removal efficiencies and methane yields.

The treatment performance in the reported work differed significantly upon the variances in the reactor type and the operational temperature. In terms of reactor type, accumulation system (AC) (not provided in Table 2.2) has been applied for vacuum toilet blackwater treatment at 20 °C and

achieved 80% of COD removal and 58% of methanisation (Kujawa-Roeleveld et al., 2006). Although the AC system could provide a relatively high COD removal efficiency and methanisation, a large reactor volume of 1,000 L and long HRT/SRT of 150 days were required for sufficient treatment in the above study. This "Load-empty" type of system can be used when considering the direct application of the digested effluent (with optional hygiene) for irrigation/fertilization (Kujawa-Roeleveld et al., 2006). CSTR treatment for vacuum toilet blackwater at 37 °C obtained a COD removal of 61% and methane production of 0.24 m^3 CH_4/kg COD added (Wendland et al., 2007). Performance of such systems with no sludge retention was found to be poorer than those reactors with sludge bed in the system. Overall, UASB and UASB-septic tank systems generated higher COD removal efficiencies and methane production compared to the other types of treatment systems. A high COD removal efficiency of 85% was achieved in a septic tank (Zamalloa et al., 2013). As for the applied temperature, the demonstrated research in Table 2.2 were performed in a temperature range of 15-37 °C. It is observed that for the same type of reactor using UASB-septic tank with the same blackwater source provided, the COD removal efficiency increased from 61% to 78% and the methane yield increased from 1.3 m^3 CH_4/ m^3 BW to 2.0 m^3 CH_4/ m^3 BW when the temperature was increased from 15 °C to 25 °C (Kujawa-Roeleveld et al., 2006).

Despite the variances of the treatment performances, it is noticed that the reported studies were operated under relatively low OLRs of 0.27-2.3 kg COD/m^3/day. For the conventional toilet blackwater, the highest OLR of ~2.3 kg COD/m^3/day was found in a UASB reactor operation, and it obtained an overall COD removal efficiency of 91% (UASB + membrane) and methane yield of 0.27 g CH_4-COD/g influent COD (van Voorthuizen et al., 2008). For a vacuum toilet blackwater (COD 5,500 mg/L) study, a similar high OLR of 2.3 kg COD/m^3/day was obtained in a sludge blanket anaerobic baffled reactor, and a 78% COD removal and 0.69-0.73 g CH_4-COD/g influent COD methane production were achieved (Moges et al., 2018). For a more concentrated type vacuum toilet blackwater (COD ~10,000 mg/L), the maximum OLR reached 1.4 kg COD/m^3/day, and a 78% COD removal and 0.57 g CH_4-COD/g feed COD of methane production were obtained (Zeeman et al., 2008). The blackwater properties, reactor types and the treatment conditions can all constrain the applied OLRs in the blackwater AD system. Considering blackwater contains a high solid organic COD fraction, the substrate hydrolysis may

be a limiting factor. Although the hydrolysis might be promoted with an elevated temperature, the high ammonium concentration from urine and high pH value can contribute to a high free ammonia concentration and result in ammonia inhibition. Regarding the probable limitations in blackwater AD processes, the optimal process design for the selection of bioreactor configuration, operational temperature, and OLRs requires further systematic investigations.

Table 2.2. Summary of blackwater anaerobic treatment (mono-digestion) in the literature.

	(Sharma et al., 2016)	(Gallagher and Sharvelle, 2010)	(Abdel-Shafy et al., 2009)	(van Voorthuizen et al., 2008)	(Moges et al., 2018)	(Zamalloa et al., 2013)	(De Graaff et al., 2010)	(Zeeman et al., 2008)	(Wendland et al., 2007)	(Kujawa-Roeleveld et al., 2006)
Reactor type	Septic tank + upflow anaerobic filter	UASB	UASB	UASB+ membrane	Sludge blanket anaerobic baffled reactor	Septic tank	UASB	UASB	CSTR	UASB-septic tank
Scale (L)	1200	95	250	5	16.4	20	50	50	10	200
Toilet type	Conventional	Conventional	Conventional	Conventional	Vacuum	Vacuum	Vacuum	Vacuum	Vacuum	Vacuum
Temperature (°C)	-	34	Ambient	37	25-28	33 (±2)	25	25	37	15 & 25
OLR (kg COD/m_3/d)	0.82	0.27	1.16	2.28	2.3 (±0.5)	0.5	1.0	1.4	0.5	0.33-0.42
HRT (days)	2.08	3.5	1	0.5	3	20-40	8.7	8.3	20	27-29
COD removal (%)	72.6	72	65.1	91	78	85	78 (±9)	78	61(±12)	61 & 78
Methane yield	-	0.2 L CH_4/L BW	-	0.27 g CH_4-COD/g feed COD	0.69-0.73 g CH_4-COD/g feed COD	0.01 L CH_4/L BW/d	1.8 m_3 CH_4/m_3 BW	0.57 g CH_4-COD/g feed COD	0.24 m_3 CH_4/kg COD added	1.3 & 2.0 m_3 CH_4/m_3 BW

2.4.2 New sanitation system case study

Pilot and full-scale demonstrations of the decentralized sanitation system are being developed worldwide, i.e. in Europe, the United States, Asia and Africa (Nansubuga et al., 2016).

Sneek, The Netherlands

The Netherlands has been pioneering the development of new sanitation systems. Starting from the year 2000 in Waterland, Groningen, several demonstration projects based on the concept of "separation at the source" have been developed in the Netherlands (Reinhard and Folmer, 2011). In 2006, a 32 house demonstration project was developed in Sneek, Friesland. Vacuum toilets utilizing 1 L flushing water per flush were installed, and the blackwater anaerobic treatment has been conducted in two UASB-septic tank systems (200 L each) and a UASB reactor (50 L). Project scale-up was realized in 2011 when the project including 250 houses in Waterschoon was

started. In this demonstration, around 12% of the gas demand of the district for house and tap water heating is covered by blackwater digestion (Waterschoon, 2020). A 500-houses project is under construction in Amsterdam.

Hamburg, Germany

The concept of "Hamburg Water Cycle" was developed in Hamburg, which aims at installing the source-separation sanitation system to combine the wastewater treatment and the energy recovery and supply at the residential level. After demonstrating the small-scale systems at Hamburg Karlshöhe & Lübeck Flintenbreite, the source-separation sanitation system has been stepwise scaled up in the Jenfelder Au district. The district involves 835 homes with more than 2,000 inhabitants on 0.35 km^2, which is the largest source separation sanitation system in Europe (Biederbeck, 2017). Vacuum pipe transportation (3.7 km network) is applied to deliver the collected blackwater to the treatment site where fermenters are located. The blackwater is mixed with co-substrates of organic waste and sewage sludge, and separated into the liquid phase and solid phase and treated through the UASB (liquid phase) and CSTR (solid phase) reactors, respectively (Zimmermann et al., 2018). Combined heat and power plant is applied, which produces ~ 450,000 KWh/year of electricity (equals to 225 household electricity supply in Hamburg) (Eaton, 2020).

China

China has a long history of applying anaerobic digestion technology to treat animal manure/human waste/agricultural waste, which was due to the appealing biogas production to mitigate energy shortage. As early as the 1880s, a fermenter test was performed in Guangdong. In the year 1936, a simple design anaerobic bioreactor- Chinese fixed dome was installed in Jiangsu, China (Marchaim, 1992). In the 1970s-1980s, with the development of "biogas for every household" campaign, household biogas plants have been widely installed in China. The simple designed "Chinese dome" digesters are widely applied, which are considered as CSTR systems. The mixing in such a system is provided by natural gas production instead of forced mechanical or circulation mixing (Zeeman and Kujawa-Roeleveld, 2013).

With the development of the concept "Ecological sanitation (Eco-San)" in the 1990s (Esrey et al., 1998), resource-oriented sanitation based on blackwater separate collection and treatment has become a hot topic. Further, the launching of the "toilet revolution" in China in 2015 helped accelerate the establishment of new sanitation systems in rural areas in order to modify the poor sanitary conditions, minimize water consumption as well as close the resource flow cycle (Cheng et al., 2018). The first demonstration project (under development) with vacuum source-separation and nutrients recovery from urine and feces was located in Changshu, Jiangsu province with a total of 8,959 households and 35,000 inhabitants (Fan et al., 2017). A 50-house demonstration project of resource-recovery sanitation was developed in the villages in Rugao, Jiangsu province in 2006. Vacuum toilet collection and transportation systems were installed, which only consumed 0.5 L water for blackwater collection (Zhang and Fan, 2019).

In addition to the above cases, the full-scale demonstration of source-recovery sanitation has also been encouraged and established in other countries. For instance, a 400 household sanitation system has been set-up and operated in Ghent, Belgium. The system consisted of the processes for bioenergy recovery from blackwater and kitchen waste through anaerobic digestion, heat recovery from greywater through the heat exchanger, water reuse, and nutrients recovery as fertilizer through struvite precipitation (De Gusseme et al., 2019). Other demonstrations have been developed in Norway, Indonesia, and India (Halalsheh and Wendland, 2008; Todt, 2015). The benefits of cost reduction, water conservation, and resource recovery and reuse compared to the traditional centralized sanitation system have always acted as the driving force in advancing in the establishment of sustainable sanitation systems.

2.5 Research gaps and challenges in blackwater anaerobic treatment

This section identifies the research gaps in the current blackwater anaerobic treatment field and outlines the potential challenges in maximizing biomethane recovery efficiency, which helps clearly address the motivations of the current work.

2.5.1 Research gaps

The following aspects have not been well studied and understood:

1) Different toilet flushing system contributes to variations in blackwater volume and chemical properties, blackwater collection source can influence the treatment performances.
2) Organic loading rates were low in the reported work.
3) No systematical investigation of the process/treatment limitations in blackwater anaerobic digestion system.
4) No microbial information has been provided in previous studies. The functional microbial community structures facing different feedstock types and operational conditions were unknown.

2.5.2 Potential challenges in blackwater anaerobic digestion systems

In the anaerobic treatment process, the initial substrate hydrolysis is considered as a potential rate-limiting step due to the complexity of blackwater. Effective substrate hydrolysis requires the active function of multiple types of enzymes, such as protease and cellulase, which strongly correlates with the operational temperature and the amount of active biomass. Another potential limiting step is the methanogenesis step due to the vulnerability and sensitivity of methanogens. Most of the inhibitors, toxicants or infeasible treatment conditions, for instance, blackwater shows high pH value and contains high ammonium content, can generate significant negative impacts on the methanogens, probably resulting in process failure. This research is mainly concerned with enhancing biomethane recovery from blackwater and understanding microbial community development in the system.

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CHAPTER 3. PERFORMANCE OF ANAEROBIC TREATMENT OF BLACKWATER COLLECTED FROM DIFFERENT TOILET FLUSHING SYSTEMS: CAN WE ACHIEVE BOTH ENERGY RECOVERY AND WATER CONSERVATION?[1]

3.1. Introduction

The anaerobic digestion process is an energy efficient waste treatment technology that converts organics to energy rich methane and only generates low amount of sludge to be managed (Rajagopal et al., 2013). To date, only limited research has been conducted to evaluate the application of anaerobic digestion processes for blackwater treatment. The few reported studies showed a wide range of chemical oxygen demand (COD) removal efficiencies (61-80%) and methanisation percentages (39-60%) (Abdel-Shafy et al., 2009; De Graaff et al., 2010). These differences can be largely attributed to the bioreactor systems utilized (i.e., continuously stirred tank reactor [CSTR], accumulation system [AC], and upflow anaerobic sludge blanket [UASB]) and the operating temperatures adopted (psychrophilic vs. mesophilic condition). For instance, when treating vacuum collected blackwater, longer hydraulic retention time was normally applied to AC or CSTR systems (HRT in the range of 20-150 days) as compared to UASB reactors (HRT as low as 8.7 days), which can be explained by the fact that UASB reactors enable long SRT at relatively short HRT due to an internal gas/sludge/liquid separation system (De Graaff et al., 2010). Further, treatment efficiency varies with temperature. For example, UASB-septic tank treating vacuum collected blackwater showed a high COD removal of 78% at 25 ˚C compared to a 61% COD removal at a lower temperature of 15 ˚C (Kujawa-Roeleveld et al., 2006).

It should be noted that the impacts of various blackwater characteristics (due to the different blackwater collection systems, such as conventional toilet, dual-flush toilet, vacuum toilet, kitchen refuse addition) on blackwater digestibility can also contribute to the large variance in the reported methane production from the literature. Blackwater from three toilet flushing systems are commonly mentioned in the literature, i.e., conventional toilet flush systems using 9

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L water per flush, dual flush systems using 3/6 L per flush, and vacuum flush toilets using 0.5-1.2 L water per flush (Abdel-Shafy et al., 2009; De Graaff et al., 2010; Gallagher and Sharvelle, 2011; Sharma et al., 2016; Wendland et al., 2007; Zeeman et al., 2008). Vacuum toilets are getting more attention in recent years due to their great water-conserving potential. Previous study reported that UASB treatment of 9 L flushed blackwater (initial COD concentration of 1,160 mg/L) achieved 68% COD removal with an average HRT of 24 hours (Abdel-Shafy et al., 2009). Anaerobic digestion with an on-site two-stage package system (consisting a modified septic tank and an upflow anaerobic filter) treating 5 L water per flush blackwater achieved up to 72.6% total COD removal with an HRT of 50 hours (Sharma et al., 2016). A COD removal of 75.2% was obtained from a UASB reactor treating dual flush toilet blackwater with an HRT of 4 days at 28 ˚C (Gallagher and Sharvelle, 2011). Longer HRTs were used for the treatment of vacuum toilet collected blackwater. For instance, anaerobic CSTR operated with an HRT of 20 days at the mesophilic condition achieved 61% COD removal (Wendland et al., 2007). UASB treatment with an HRT of 8.7 days led to 78% COD removal at 25 ˚C (De Graaff et al., 2010). COD removal of 72-86% was achieved at 25 ˚C with a pilot-scale UASB septic tank system with an HRT of 29 days (Zeeman et al., 2008).

The feasibility of anaerobic blackwater treatment has been evaluated in the above-mentioned literature, which the observed large variations in blackwater treatment efficiency underlines the importance of understanding the digestibility of blackwater collected from different toilet flush systems. Vacuum toilet collected blackwater has benefits of saving water and concentrating organic matter to maximize blackwater energy recovery efficiency. However, high concentrations of other pollutants such as metal ions, sulfide, and ammonia might also inhibit the anaerobic digestion processes thus leading to reduced treatability (Chen et al., 2008). In particular, concentrations of free ammonia (FA) are of concern.

The main objective of the present study is to evaluate and compare the anaerobic energy recovery potential from blackwater collected from water-wasting toilets (e.g., conventional or dual-flush toilets) and water-conserving toilets (e.g., vacuum toilets). To achieve this goal, biological methane potential (BMP) experiments and microbial analysis were performed, and the hydrolysis and methanogenesis processes were interpreted to evaluate if any potential inhibitor

exists in the blackwater. The current study provides critical information and fundamental knowledge in designing source diverted blackwater treatment systems for energy recovery, which is currently unavailable in the literature.

3.2. Method and materials

3.2.1 Blackwater preparation and characterization

Blackwater stock (feces and urine) was collected using toilet waste bags with no flush water added from healthy adults, seniors, and children on the University of Alberta campus for two consecutive weekdays. Blackwater stock was well mixed and stored at 4 ˚C before further experiments. Blackwater stock was diluted using tap water to simulate commonly used conventional (9 L water per flush), dual flush (6 L water per flush), and vacuum (1 L water per flush) toilet flushing systems, respectively. The total chemical oxygen demand (COD_t), suspended COD (COD_{ss}), colloidal COD (COD_{col}), soluble COD (COD_s), total solids (TS), volatile solids (VS), total ammonia nitrogen (TAN), total nitrogen (TN), total phosphorous (TP), and pH were analyzed for characterization of different flushed blackwater. COD, TS, and VS were measured according to the Standard Methods of American Public Health Association (APHA/AWWA/WEF, 2012). Inoculum was collected from a mesophilic anaerobic digester treating primary sludge in a local wastewater treatment plant. pH was measured using a B40PCID pH meter (VWR, SympHony).

3.2.2 Batch assays

Biochemical methane potential (BMP) tests

Batch experiments were firstly conducted to compare the methane production potential of blackwater collected from conventional toilets (9 L water/flush), dual flush toilets (6 L water/flush) and vacuum toilets (1 L water/flush). BMP tests were conducted in 157 mL serum bottles in duplicate at 35 °C in a shaker incubator (120 rpm) under dark conditions. The bottles were flushed with nitrogen gas after the addition of blackwater and inoculum and sealed with a butyl rubber stopper and an aluminum cap. Methane generation was monitored through measuring the bottle headspace pressure and the gas composition. The volatile fatty acid (VFA) concentrations were analyzed at the end of the tests. Evaluation of the reason for the cessation of methane production at the end of the BMP test was performed with dual flush toilet blackwater

(6 L water/flush) and vacuum toilet blackwater (1 L water/flush) samples.

FA inhibition

Additional BMP tests were conducted to investigate the potential impacts of FA on the blackwater anaerobic digestion process. Two trials were performed. In *Trial 1*, different amounts of NH_4Cl solutions (300 g/L) were added to conventional toilet blackwater (9 L water/flush), to achieve targeted final FA concentrations (i.e., 17, 58, 108, 145, 205, 360, 657 and 2397 mg/L), representing blackwater FA concentrations, conditions ranging from blackwater collected from conventional 9 L water per flush toilets (FA=17 mg/L), dual-flush toilets (FA ranging from 58-108 mg/L), vacuum toilets (FA ranging from 145-657 mg/L) to raw blackwater from dry toilets with no flush water (FA=2397 mg/L). In *Trial 2*, different volumes of flush water (0, 0.5, 1, 1.5, 1.87, 3.23, 6, and 9 L) were applied directly to raw blackwater to obtain targeted FA concentrations similar to the *Trial 1* study (FA ranging from 17-2397 mg/L). In *Trial 1*, NaOH solution (1 M) was used to adjust the pH to the targeted FA concentrations. The TAN concentration and pH were measured for each group before incubation at 35 °C. The experiment was set up in duplicate.

With such design, *Trial 1* evaluates if the elevated FA concentration in blackwater with less flush water could directly impact the blackwater anaerobic digestion process; and *Trial 2* examines whether FA is the main inhibition factor in the blackwater anaerobic digestion process.

3.2.3 FA inhibition mechanisms

FA inhibition mechanisms were determined through batch hydrolysis experiments with the demonstration of the inhibition effects on different steps in blackwater anaerobic digestion. All blackwater samples were prepared using 9 L flushed blackwater as mentioned in 2.2.2 (*Trial 1*) to evaluate FA changes. Considering the hydrolysis step is commonly a rate-limiting step for complex substrates due to slow solubilisation (Wang et al., 2016), the ratio of input inoculum and substrate volume was set to 1:1, to expedite the start-up period and shorten the lag phase. To elucidate the effects of FA on each step, hydrolysis, acidogenesis, and methanogenesis efficiencies were calculated and compared. Three groups of blackwater samples with FA concentrations of 27 mg/L (representing 9 L flushed blackwater), 114 FA mg/L (representing

3.23 L flushed blackwater), and 405 FA mg/L (representing 1 L flushed blackwater) were selected for hydrolysis test. Each group included four bottles with substrates (blackwater and inoculum) and four blanks (water and inoculum). Each set of four bottles (substrates or blanks) consisted of two bottles for gas phase (biogas) measurement and two for liquid phase (COD_s and VFAs) measurements. The methane production in the gas phase, COD_s, and VFAs concentrations in the liquid phase were measured every 24 hours.

3.2.4 Microbial community analysis

DNA extraction was conducted using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. Unflushed raw blackwater and anaerobic digester sludge (200 μL) were directly used for DNA extraction without previous centrifugation, this is because the stock blackwater sample and digester sludge contained little water content. Digested 9 L, 6 L, and 1 L flushed blackwater samples (2 mL) were centrifuged at 3,000 g for 10 min, discarding the supernatant, and using the pellet for DNA analysis. The extracted DNA was eluted with 100 μL Tris-HCl buffer, and its concentration and quality were checked through NanoDrop One (ThermoFisher, Waltham MA, USA). All DNA samples were stored at -20 °C until PCR was performed. PCR was performed followed by the sequencing experiment on the Illumina MiSeq platform. Bacterial 16S rRNA genes were amplified using the universal primer-pair 515F (GTGCCAGCMGCCGCGG) and 806R (GGACTACHVGGGTWTCTAAT). Similarly, archaeal 16S rRNA genes were amplified using universal primer set 517F (GCYTAAAGSRNCCGTAGC) and 909R (TTTCAGYCTTGCGRCCGTAC). Microbial community analysis was performed by RTL Genomics (Texas, USA). The sequencing platform generated 16S Illumina paired-end reads data of both forward and reverse amplicons, which were further assembled in contigs and processed running a workflow on QIIME pipeline version 1.9.1 (Caporaso et al., 2012). The reads were clustered against the reference SILVA database project (Quast et al., 2013), and any reads, which do not match the reference data are subsequently clustered by using a de novo approach.

3.2.5 Calculation methods and equations

Calculation methods and equations applied in this study, including methane production in BMP and hydrolysis tests, methanogenesis efficiency (%), hydrolysis efficiency (%), hydrolysis rate

coefficient (k_h), acidogenesis efficiency (%) and free ammonia concentration, are provided in section 3.5.

3.2.6 T-test and ANOVA analysis

Student t-test analysis were performed on BMP values and lag phase duration between the two trials in FA inhibition tests. One-way analysis of variance (ANOVA) was performed on hydrolysis and acidogenesis efficiencies for the three conditions in the hydrolysis test. T-test and ANOVA were performed using the Microsoft Excel® software. A p-value smaller than 0.05 represents statistically significant difference.

3.3. Results and Discussion

3.3.1 Blackwater characterization

The characteristics of conventional toilet (9 L water/flush), dual flush toilet (6 L water/flush) and vacuum toilet (1 L water/flush) blackwater samples are presented in Table 3.1. The VS/TS ratio of different water flushed blackwater samples was in the range of 0.78-0.83, in which the high fraction of organic matters implied high biodegradability of blackwater. The COD_t values of 9 L water flushed blackwater were similar to the reported values (2,887 [±793] mg/L) of blackwater from conventional flushing toilets (~9 L/flush) (Knerr et al., 2011). For all blackwater samples, COD_{ss} was 60-66 % of COD_t, COD_s was 30-34 % of COD_t, and COD_{col} was only 1.3-5.7% of COD_t. Due to the small fraction, COD_{col} was not further considered. The pH of all samples was in the range of 8.4 to 8.6, which was comparable to the reported blackwater pH range of 7.4-9.0 (Abdel-Shafy et al., 2009; Knerr et al., 2011). Overall, characteristics of blackwater samples gathered in the present study is comparable to those reported in the literature (De Graaff et al., 2010; Zeeman et al., 2008) .

It should be noted that the TAN of 1 L flushed blackwater was high (1,040 mg/L), which can be attributed to protein degradation and the existence of urea. The high pH and high TAN of 1 L flushed blackwater lead to high FA concentration which may cause instability in anaerobic digestion systems. Similar observation was reported in a previous continuous blackwater treatment study, which showed that the shock loading of blackwater with 485 mg FA /L led to a temporary methanogenesis process inhibition (De Graaff et al., 2010). In this study, the initial

free ammonia concentrations were 393, 60, and 26 mg FA /L for vacuum toilet (1 L water/flush), dual flush toilet (6 L water/flush) and conventional toilet (9 L water/flush) collected blackwater at 35 °C, respectively.

Table 3.1. Characterization of different water-volume flushed blackwater.

Index (mg/L)	1 L flushed BW	6 L flushed BW	9 L flushed BW
pH	8.6	8.5	8.4
COD_t	29,520	4,710	2,580
COD_{ss}	19,320	3,105	1,544
COD_s	8,880	1,545	888
COD_{col}	1,320	60	148
TAN	1,040	182	96.4
TN	1,700	410	190
TP	330	70.5	38
TS	17,140	3,570	2,390
VS	14,200	2,825	1,847

3.3.2 Anaerobic digestion in batch tests

Biochemical methane potential (BMP)

BMP of conventional toilet (9 L water/flush), dual flush toilet (6 L water/flush) and vacuum toilet (1 L water/flush) collected blackwater is displayed in Fig. 3.1. A 5-days lag phase was observed for 9 L and 6 L flushed samples, and an 8-days lag phase was observed for 1 L flushed samples. All experiments lasted for 46 days before reaching steady state conditions. BMP values of 9 L and 6 L flushed blackwater were similar (48%), which was clearly higher than those obtained for 1 L flushed blackwater (34%). The biochemical oxygen demand (BOD) over COD (BOD/COD) ratio of blackwater was reported to be 48-71% in the studies treating relatively low-strength blackwater with COD 1,000 -2,000 mg/L (Abdel-Shafy et al., 2009; Sharma et al., 2016; van Voorthuizen et al., 2008). The vacuum toilet blackwater with biodegradability of 46-60% was also reported in the literature (De Graaff et al., 2010; Kujawa-Roeleveld et al., 2006). These reported values are comparable with the BMP values of the water-wasting toilet blackwater in this study, with similar digestibility and inhibition potentials. At the end of the BMP tests, VFAs concentrations were 79 and 75 mg COD /L for 9 L and 6 L flushed samples, respectively; and

was 222 mg COD /L for the 1 L flushed sample. The low BMP value and the high VFAs concentration observed for 1 L flushed blackwater conditions indicate the presence of inhibition in these reactors. The evaluation of the cessation of the methane production in 6 L and 1 L flushed samples at the end of the experiments indicated that methanogens in 1 L flushed samples lost their ability to produce methane while the methanogens were not inhibited and functioned well in 6 L flushed blackwater (section 3.5).

Previous studies reported ammonia inhibition in both industrial and municipal waste treatment systems, which suppress methane production especially under high temperature and pH conditions (Braun et al., 1981; Rajagopal et al., 2013). The reported FA inhibition concentration varied from 45 mg/L (Kayhanian, 1999) to 2473 mg/L (Shanmugam and Horan, 2009) in different treatment system. The inhibition in 1 L flushed blackwater could be attributed to the relatively high FA concentration (393 mg/L) and therefore, further studies were performed to evaluate impacts from FA in blackwater.

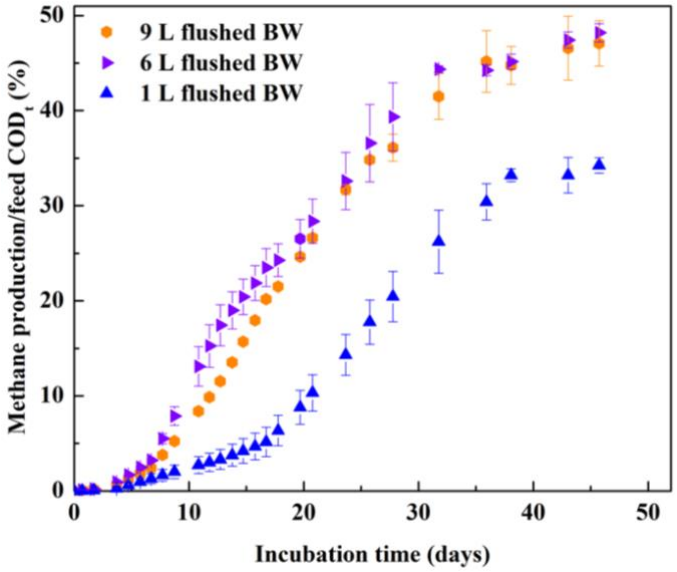


Fig. 3.1. Methane production from conventional toilet (9 L water/flush), dual flush toilet (6 L water/flush) and vacuum toilet (1 L water/flush) collected blackwater in the biochemical methane potential (BMP) test, represented as the percentage of initial feed CODt converted to methane (as COD).

FA inhibition

To examine the impacts of FA on blackwater BMP, *Trial 1* study was performed by directly adding additional ammonium source (NH_4Cl) into 9 L flushed blackwater to achieve targeted final concentrations of FA (17, 58, 108, 145, 205, 360, 657, and 2397 mg/L). FA concentrations in each trial and corresponding water flush amount are displayed in Table 3.2. The BMP results of the two trials are displayed in Fig. 3.2. As shown in Fig. 3.2A, the lag phase was approximately 5 days when FA concentration was below 108 mg/L, which increased to 8 days when FA concentration went up to 145 mg/L, while BMP values stayed at around 42%. Further, when the FA concentration reached 205 mg/L, BMP was significantly reduced from 42% (when FA was in the range of 17-145 mg/L) to 28%. No methane production was observed when FA concentration increased to 2397 mg/L indicating a completely inhibited process.

Further, *Trial 2* was conducted by directly diluting raw blackwater (with no water flush) with different volumes of dilution water to obtain targeted FA concentrations comparable to *Trial 1* study. As shown in Fig. 3.2B, similar to *Trial 1*, lag phase duration of 5 days and BMP of ~ 45% was detected for blackwater with FA concentrations ranging from 17-108 mg/L (9-3.23 L water flush), indicating that no significant inhibition existed within this range. Again, when the FA concentration was increased to 146 mg/L (1.87 L water flush), the lag phase length was increased to 7-8 days and BMP values were maintained at 45%. BMP decreased to 31% when the FA concentration went up to 218 mg/L (1.5 L water flush). No methane was detected in more than 90 days of incubation when the FA concentration was over 2336 mg/L.

Table 3.2. FA concentration (mg/L) obtained by the addition of NH_4Cl to 9 L water flushed sample or by dilution of raw blackwater in BMP test.

FA of Trial 1 NH_4Cl addition in 9 L blackwater	17	58	108	145	205	360	657	2,397
FA of Trial 2 Direct flushed blackwater	26 (9 L/flush)	60 (6 L/flush)	103 (3.23 L/flush)	146 (1.87 L/flush)	218 (1.5 L/flush)	393 (1 L/flush)	635 (0.5 L/flush)	2,336 (0 L/flush)

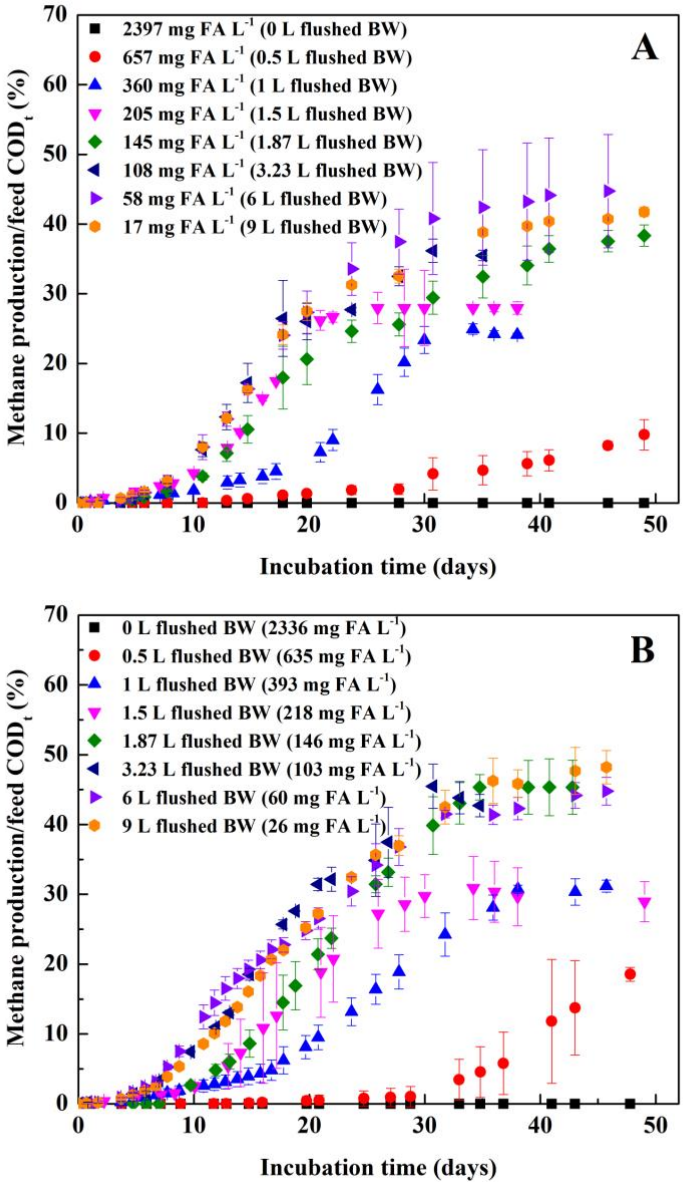


Fig. 3.2. Methane production of blackwater at various FA concentrations in *Trial 1* with different amount of NH_4Cl solution added to conventional toilet blackwater (9 L water/flush) (A), and *Trial 2* with different volumes of flush water applied to raw blackwater (B).

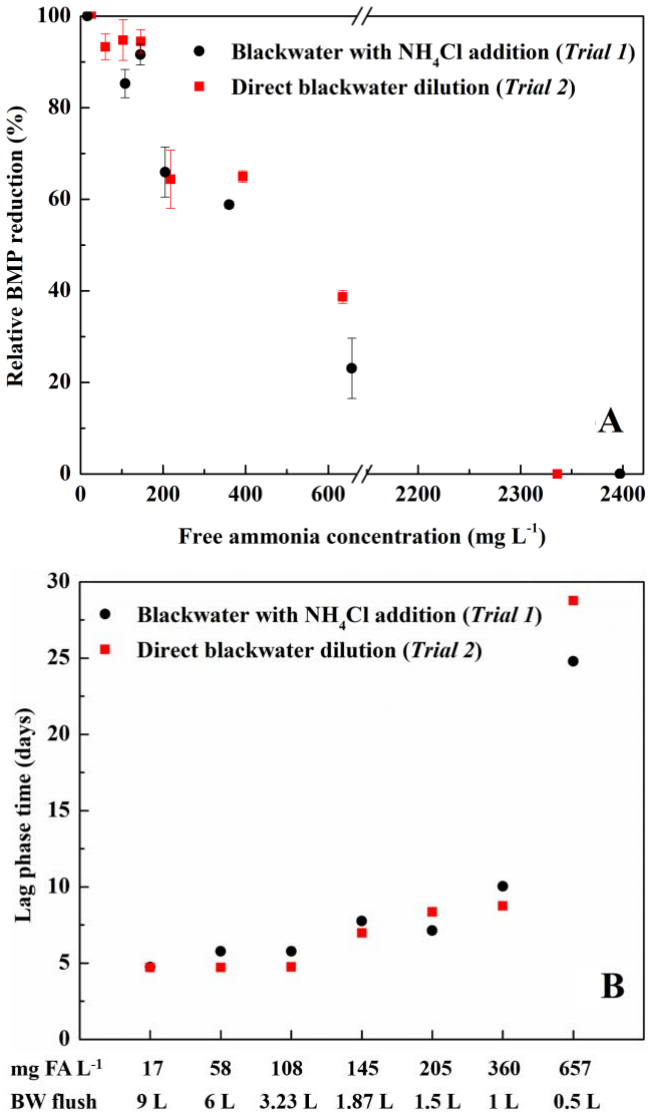


Fig. 3.3. Inhibition effects of blackwater from *Trial 1* and *Trial 2*: (A) the relative percentage of BMP achieved at different inhibition conditions compared with non-inhibition conditions (at the lowest free ammonia concentration in *Trial 1* and with the highest flushing water volume in *Trial 2*), the maximum BMP values achieved were shown as 100%; (B) the change of lag phase duration of methane production in the tests.

The BMP and lag phase duration of the two trials of FA inhibition analysis were statistically compared using t-tests. As shown in Fig. 3.3, the inhibition effects from *Trial 1* (when FA was added in increasing concentrations to 9 L flushed sample) and *Trial 2* (when raw blackwater received different water volumes to estimate FA concentrations based on dilution) were not

statistically different in terms of the BMP reduction ($p > 0.05$) and the lag phase extension ($p > 0.05$). The comparison of two trials illustrated that FA is the major inhibition factor of blackwater anaerobic digestion. Therefore, it can be concluded that the decreased BMP in 1 L flushed blackwater compared with 6 and 9 L flushed blackwater samples was a result of FA inhibition. Similar observations of ammonia inhibition have been reported widely previously for other waste sources. However, the FA inhibition concentration can vary significantly for different waste sources and treatment conditions. For instance, in the digested pig manure inoculated digester, a FA inhibition threshold was identified to be 15-130 mg/L (Astals et al., 2018).

3.3.3 Evaluation of the FA inhibition mechanisms

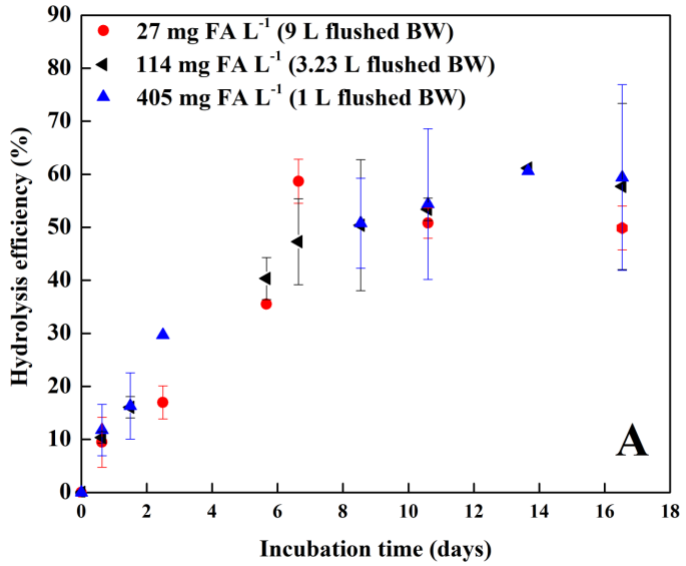
To further elucidate the impacts of FA on the different stages of the anaerobic digestion process (e.g., hydrolysis, acidogenesis and methanogenesis steps), inhibition mechanism studies were performed.

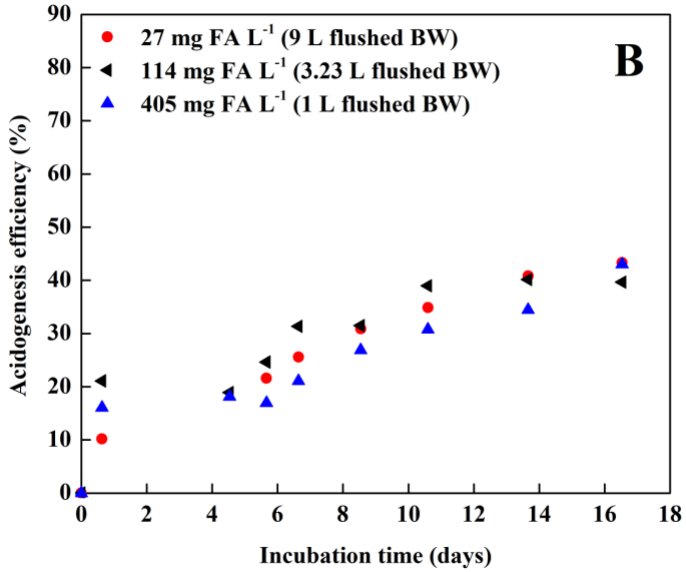
Fig. 3.4A shows the change of hydrolysis efficiency at various FA concentrations. The results indicated that blackwater with different amounts of FA demonstrated similar hydrolysis efficiency trends during 17 days of incubation ($p > 0.05$). The maximum hydrolysis efficiencies were in the range of 57-61% for blackwater with FA concentrations ranging from 27-405 mg/L. Unlike the reduction in BMP production observed for blackwater with high FA condition, no reduction in hydrolysis efficiency was observed with an increase of FA concentration. As shown in Table 3.3, the first order hydrolysis rate coefficient k_h had no significant variation among samples (all within the range of 0.198-0.209 d^{-1}), indicating that FA concentrations (in the range of 27-405 mg/L) had little impact on the blackwater hydrolysis rate. Moreover, no obvious lag phase was observed in the hydrolysis process for the analyzed conditions (Fig. 3.4A). It indicated that a lag phase time extension, with the increase of FA concentration in BMP tests from circa 5 days (< 108 mg/L) to greater than 8 days (> 145 mg/L), had no correlation to hydrolysis step inhibition. The large amount of inoculum addition at the beginning of the test led to sufficient microorganisms and enough hydrolysis enzymes excreted for hydrolysis of blackwater contents, demonstrating a capability to thrive in excessive FA concentrations.

In order to evaluate the acidogenesis efficiencies, the changes of three volatile fatty acids were monitored during the hydrolysis test, including acetate, propionate, and butyrate. As shown in Fig. 3.4B, the acidogenesis process started immediately after incubation, and the acidogenesis efficiency reached ~ 40-43% for all samples tested. ANOVA analysis indicated that no significant difference in the change of acidogenesis efficiency was observed in blackwater samples at various FA concentrations ($p > 0.05$). Therefore, the increasing FA (27-405 mg/L) showed no impact on acidogenesis in the blackwater digestion process in the present study. It is also important to notice that the acidogenesis products were mostly VFAs instead of CH_4 at FA 405 mg/L, with the VFAs accumulated to 590 mg COD /L after 17 days.

Same samples were then used to evaluate the potential FA impacts on the methanogenesis step. As shown in Fig. 3.4C, during the 17 days incubation, the methanogenesis efficiencies of the samples with 27 and 114 mg/L FA were similar, reaching 43% (± 3%) and 39% (± 4%), respectively. However, the methanogenesis efficiency value was only 8% (± 1%) for blackwater with 405 mg/L FA. The lag phase for methane production was less than 1 day when FA concentration was below 114 mg/L. As compared to the lag phase (5 days) observed in the BMP test, the observed short lag phase can be attributed to the application of large amounts of inoculum provided at the beginning of this inhibition mechanism study (also refer to the methodology section 3.2.3). On the contrary, an 8-day lag phase was observed for blackwater with initial FA concentration of 405 mg/L, despite the much larger amount of inoculum applied in this inhibition mechanism test.

With the combined analysis of different reaction steps in the blackwater digestion process, it can be concluded that the high FA concentration in blackwater mainly affected the methanogenesis step, which led to the decreased BMP at high FA concentrations. This is in agreement with previous studies where the methanogenesis step was considered as a rate-limiting step especially when the system was inhibited by excess FA (Wang et al., 2016). The results also indicated that compared with hydrolysis and fermentation bacteria, methanogens were more sensitive to the FA toxicity.


27 mg FA L-1 (9 L flushed BW)
114 mg FA L-1 (3.23 L flushed BW)
405 mg FA L-1 (1 L flushed BW)
Hydrolysis efficiency (%)
Incubation time (days)
A


27 mg FA L-1 (9 L flushed BW)
114 mg FA L-1 (3.23 L flushed BW)
405 mg FA L-1 (1 L flushed BW)
Acidogenesis efficiency (%)
Incubation time (days)
B

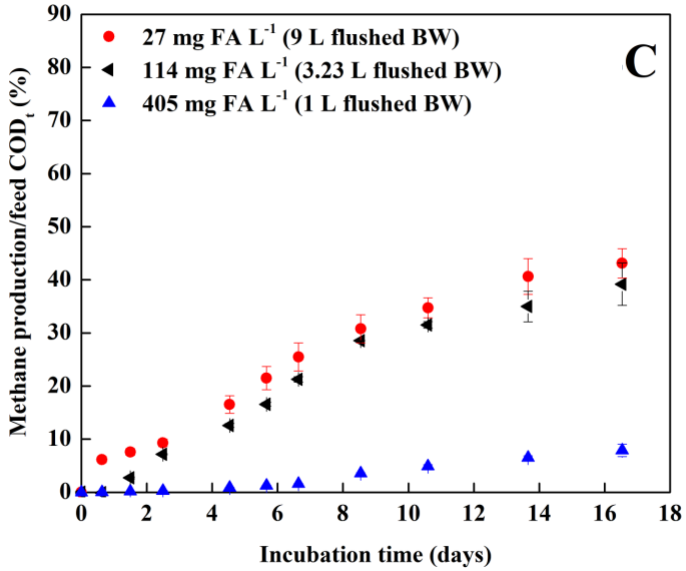

27 mg FA L-1 (9 L flushed BW)
114 mg FA L-1 (3.23 L flushed BW)
405 mg FA L-1 (1 L flushed BW)
Methane production/feed CODt (%)
Incubation time (days)
C

Fig. 3.4. Performance of different anaerobic treatment stages of conventional toilet blackwater (9 L water/flush) at various free ammonia concentrations (with different amount of NH_4Cl solution addition): (A) hydrolysis efficiency; (B) acidogenesis efficiency; (C) methanogenesis efficiency.

Table 3.3. Hydrolysis rate coefficients at various free ammonia concentrations.

Free ammonia concentration (mg/L)	Hydrolysis rate coefficients k_h (d^{-1})
27	0.198
114	0.204
405	0.209

3.3.4 Microbial population dynamics in blackwater anaerobic digestion processes

No study has been reported to date on the impacts of FA on the change of microbial communities in a blackwater anaerobic treatment system. In this study, the analysis of microbial population structure further elucidated the FA inhibition effects.

Microbial community analysis resulted in an average of 59,890 ± 6,655 reads and 8,322 OTUs for the bacterial community, while 23,688 ± 3,506 reads and 227 OTUs were obtained for the archaeal community. The relative abundance of bacterial taxonomic groups in the inoculum, raw blackwater, and digested blackwater samples (9 L and 1 L water flushed) are illustrated in Fig. 3.5. Overall, the bacterial community was mainly dominated by phylum Firmicutes, Bacteroidetes, and Proteobacteria for all samples. The inoculum did not show a distinct dominance of any of the bacterial group with the relative abundance of Firmicutes (10.1%), Bacteroidetes (17.2%), and Proteobacteria (21.7%) that were observed higher than others. Phylum Bacteroidetes (42.8%) and Firmicutes (53.9%) were predominant in raw blackwater sample. They also showed high relative abundance in 1 L (59.3% Bacteroidetes, 22.5% Firmicutes) and 9 L (48.3% Bacteroidetes, 21.3% Firmicutes) flushed samples, suggesting their tolerance to high ammonia levels. In phylum Bacteroidetes, the organisms from the order Bacteroidales presented the highest abundance in 9 L and 1 L flushed samples, with 48.3% and 59.3% of total bacterial reads, respectively. In phylum Firmicutes, members from the class Clostridia represented 20.4% and 21.6% in 9 L and 1 L flushed samples, wherein, the

Clostridiales order represented 20.4% and 15%, respectively. Phylum Proteobacteria and Synergistetes did not show high abundance in 1 L flushed samples (1% and 0.4%), but showed 13.8% and 2.3% relative abundance in 9 L condition. Overall, 9 L flushed samples showed higher microbial community diversity in comparison to 1 L flushed blackwater (Fig. 3.5).

Firmicutes and Bacteroidetes have been commonly found in anaerobic digesters (Kampmann et al., 2012), which include various genera capable of degrading wide range of carbohydrates and proteins (Cardinali-Rezende et al., 2011), and are mainly reported as acid-forming/fermentative bacteria in blackwater digestion. Members of Bacteroidetes were predominantly found in protein-rich systems and in human feces. They were mostly considered as acidogenic and involved in the proteolytic process (Liu et al., 2009). Previous research reported that Bacteroidetes showed high stability under the changes of pH and substrate types (Kampmann et al., 2012). Moreover, phylum Firmicutes has been reported as a predominant community at elevated ammonia levels (> TAN 3.0 g/L) (Li et al., 2015). These studies are consistent with the high abundance of Firmicutes and Bacteroidetes in both digested blackwater samples observed in the present study.

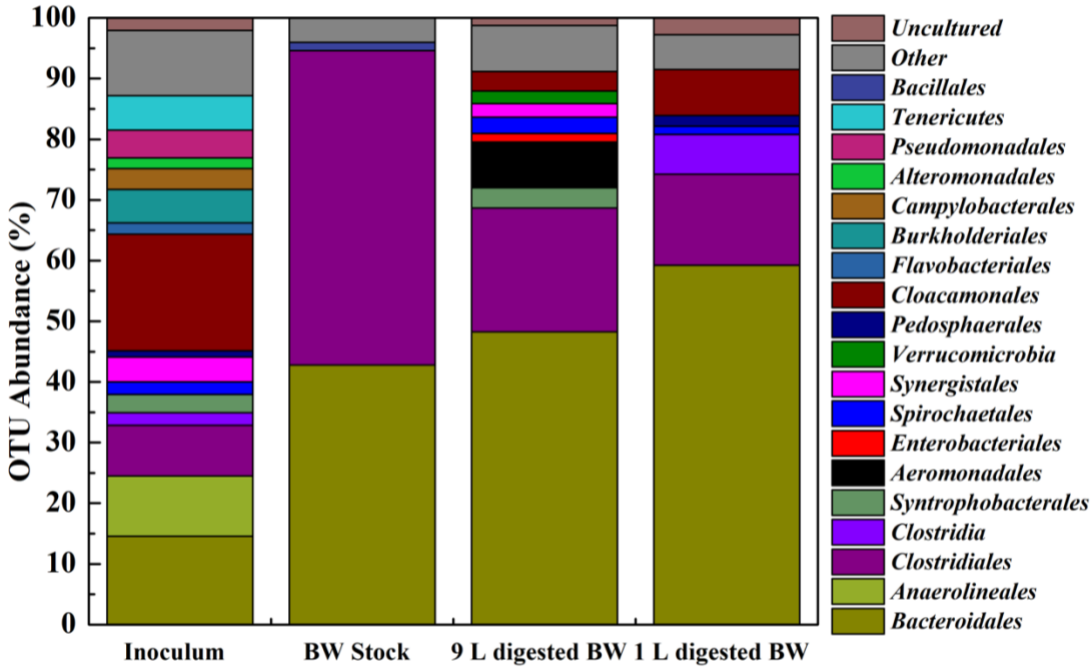


Fig. 3.5. Relative abundance of bacterial taxonomic groups of the inoculum, undigested raw blackwater (BW Stock), digested conventional toilet blackwater (9 L digested BW), and digested vacuum toilet blackwater (1 L digested BW) at the end of the BMP test.

Class Clostridia (in phylum Firmicutes) has been reported to be involved in hydrolysis, acidogenesis, and acetogenesis, in which members of the order Clostridiales can generate cellulosomes that degrade cellulose and provide substrates to help with the growth of acetogens and methanogens. They also contribute to the syntrophic acetate oxidation (SAO) process, which, as a separate path combined with hydrogenotrophic methanogenesis, can convert acetate to hydrogen when acetoclastic methanogens are inhibited due to ammonia inhibition (Schnürer and Nordberg, 2008). Genus *Clostridium* was found to generate hydrogen from complex substrates (Wang et al., 2017). Further, for phylum Proteobacteria and Synergistetes, it has been observed that they can be inhibited by excess FA in the manure digestion system (Li et al., 2015). It has also been reported that with TAN concentration below 3.0 g/L, phylum Synergistetes was dominant, while TAN over 3.0 g/L demonstrated a community shift to Firmicutes during mesophilic anaerobic digestion (Park et al., 2016). These previously reported studies correlated well with the results in this study that phylum Proteobacteria and Synergistetes showed higher fractions in 9 L flushed samples than in 1 L flushed samples, which was due to the high FA concentration in 1 L condition.

Varying types of methanogens respond differently to inhibition. Fig. 3.6 showed the relative abundance of archaeal taxonomic groups in the inoculum, 9 L, and 1 L water flushed digested blackwater samples. Overall, 99% of the archaeal reads could be assigned to the genus level, and all identified genera were involved in methanogenic processes. The dominant archaeal group in digested blackwater samples belong to the Euryarchaeota phylum suggesting the detected archaea were methanogens (Luo et al., 2009). *Methanobacterium, Methanoculleus, Methanolinea, Methanomicrobiales,* and *Methanosarcina* were identified in all the digested samples (Fig. 3.6).

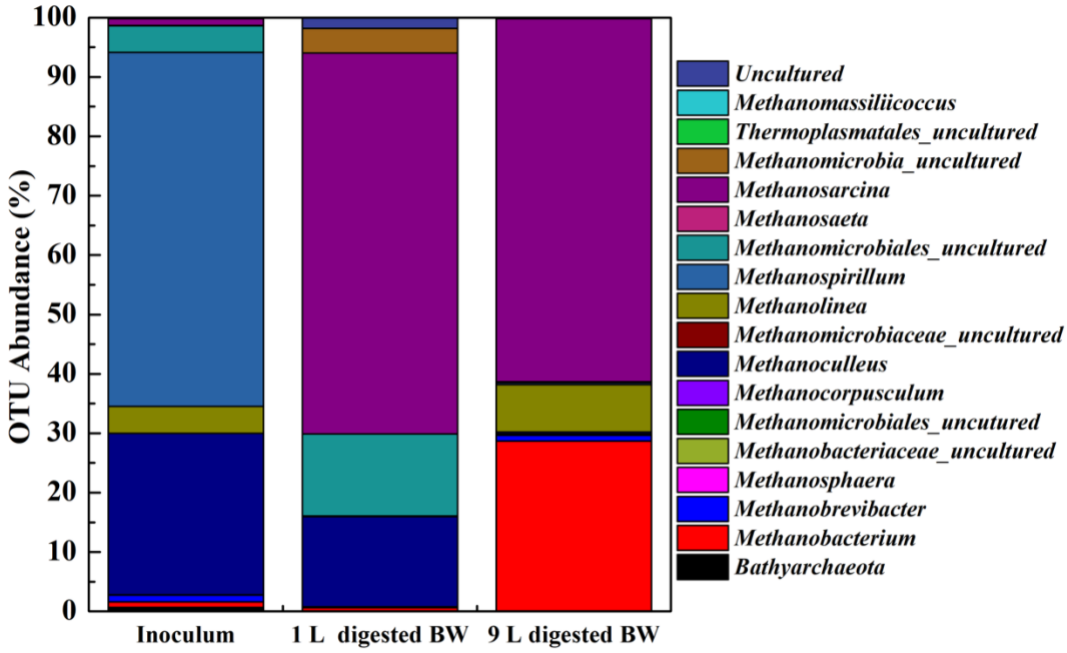


Fig. 3.6. Relative abundance of archaeal taxonomic groups of the inoculum, digested vacuum toilet blackwater (1 L digested BW) and digested conventional toilet blackwater (9 L digested BW) at the end of the BMP test.

Members of *Methanosarcina* are reported to be the most metabolically versatile group among the methanogens, able to use three pathways to generate methane including hydrogenotrophic, acetoclastic, or methylotrophic pathways (Lambie et al., 2015), while other than *Methanosarcina*, the rest detected methanogens in the present study would only use hydrogenotrophic pathway. The observation of these methanogens in digested samples indicated the systems could have multiple methanogenesis pathways for methane generation in blackwater digestion. *Methanosarcina* was the predominant genus members in 9 L and 1 L digested samples. This genus as mixotrophic methanogens has been reported with higher tolerance to ammonia toxicity compared to acetoclastic methanogens (Lü et al., 2013). With the morphology study, *Methanosarcina* is considered to be robust to ammonia inhibition due to its spherical cells, resulting in a lesser probability of FA diffusion into cells (Wiegant and Zeeman, 1986). But reported study showed that it could not actively transform acetate to methane under inhibition conditions when ammonia concentration increased above 4 g NH_4Cl /L with acetate accumulation (> 120 mM acetate) in a swine manure digestion system (Zhang et al., 2017). It

should be noted that although the abundance of genus *Methanosarcina* was similar in 1 L and 9 L flushed samples (64.1% in 1 L, 61.2% in 9 L), acetate accumulation and low methanogenesis efficiency were observed in 1 L condition. Greater diversity of methanogens was found in 9 L flushed samples at genus level, in which organisms from the genus *Methanosaeta* and *Methanomassiliicoccus* were exclusively detected. The genus *Methanosaeta,* as acetoclastic methanogens, was reported with low tolerance to ammonia compared to hydrogenotrophic methanogens (Angelidaki and Ahring, 1993). The specific growth rate of acetoclastic methanogens was halved at an ammonia concentration of 3.5 g N/L while the ammonia concentration was doubled for hydrogenotrophic methanogens to show the same reduction (Angelidaki and Ahring, 1993). *Methanobacterium* and *Methanolinea* showed higher abundance in 9 L flushed blackwater condition while *Methanoculleus* and *Methanomicrobiales* showed higher abundance in 1 L flushed samples. Strong correlation of archaeal community structure with ammonia concentration has also been reported in the literature, and it has been observed that members of *Methanoculleus* genus tend to exist at high ammonia level (Zhang et al., 2017; Ziganshin et al., 2013). Similarly, *Methanomicrobiales* methanogens were reported to be ammonia resistant, which correlates to our study that *Methanomicrobiales* was present only in the 1 L flushed blackwater (Angenent et al., 2002). As different methanogens have different tolerance to ammonia concentration, the higher diversity of archaeal structure in the 9 L flushed condition can be attributed to the more favorable microbial growth condition with low ammonia concentration compared to the 1 L flushed condition with high ammonia concentration.

It has also been reported that the methanogenic pathway could shift from acetoclastic methanogenesis to syntrophic acetate oxidization (SAO) combined with hydrogenotrophic methanogenesis against ammonia inhibition (Lü et al., 2013), which can be correlated to the abundance of bacterial taxonomic groups of *Clostridia* in our study. However, although the methanogenesis pathway tended to shift against ammonia inhibition and the ammonia tolerant methanogens were able to function, methanogenic efficiency was found to decrease under inhibition state at high FA concentration.

3.4. Conclusion

Our results indicated that the energy recovery potential of blackwater collected from different sources is different. Blackwater collected from water-conserving toilets generated lower BMP as compared to water-wasting toilets. Methane generation from vacuum toilet collected balckwater was low due to the FA inhibition on the methanogenesis step. Microbial analysis showed that the archaeal populations in the vacuum toilet collected blackwater reactor shifted to more ammonia tolerant methanogens. Our results demonstrated that when considering energy recovery from blackwater collected from water-conserving toilets, the wastewater properties, such as pH and ammonia concentration that may lead to process inhibition should be carefully considered.

3.5. Supplementary materials

3.5.1 Blackwater preparation and characterization methods

Blackwater stock (raw blackwater) was collected using toilet waste bags with no flush water added. Blackwater samples were obtained from 10 individuals, including healthy adults, seniors, and children (no medication intake prior to the blackwater collection) on the University of Alberta campus for two consecutive weekdays in December 2016. All blackwater samples were mixed, blended with a low speed blender, and stored at 4 ˚C before further experiments. To evaluate blackwater digestibility, anaerobic digester (AD) sludge from a local municipal wastewater treatment plant operating a steady biosolids anaerobic digestion system was used as the inoculum.

To determine the COD_{ss}, COD_{col}, and COD_{s}, samples were firstly filtered using 8 μm filter paper; the fraction retained on the filter paper was considered as COD_{ss}. Paper filtered samples were subsequently seeped into 0.45 μm membrane filters, and COD_{s} was the fraction that filtered through the membrane. COD_{col} was calculated as the samples that can pass through 8 μm filter but was retained on the 0.45 μm membrane (Zhang et al., 2013). TN was analyzed with the Hach TNT total nitrogen reagent set (DR-3900, Hach, CO, USA). TAN and TP were measured using Hach TNTplus vial tests (DR-3900, Hach, CO, USA).

3.5.2 Batch assays

Biochemical methane potential (BMP) tests

BMP tests were initially performed with 9 L, 6 L, and 1 L flushed blackwater in 157 mL serum bottles. Blank bottles with tap water and inoculum were used to evaluate biogas generated from the inoculum. Trace metals were considered to be sufficient in blackwater samples, and no additional trace elements were added. Biogas generation was monitored by measuring the headspace pressure using a GMH3151 manual pressure meter (Greisinger, Regenstauf, Germany). Biogas composition was measured using a 7890B gas chromatograph (Agilent Technologies, Santa Clara USA) equipped with a thermal conductivity detector (TCD) and two columns (Molsieve 5A 2.44 m 2 mm for CH_4 and Hayesep N 1.83 m 2 mm for N_2, O_2 and CO_2). The temperature of the oven, injector, and detector was 100, 150, and 200 °C, respectively. A volume of 2 mL headspace gas per sample was analyzed with the GC loop injection method. The headspace pressure was measured before and after each injection of the GC measurement. The incubation time for BMP tests lasted for 55 days to ensure completion of biogas production. Samples for VFA measurements were prepared by dilution and filtered through 0.2 μm nylon syringe filters. Concentrations of three main VFAs (acetate, propionate and butyrate) were determined with a DIONEX ICS-2100 Ionic chromatography (IC) (ThermoFisher, Waltham MA, USA) (De Graaff et al., 2010).

At the end of the BMP studies, the cause for the cessation of methane production was evaluated. Firstly, to investigate whether the lack of substrates led to the cease of methane production, sodium acetate solution, as additional carbon source, was added to blackwater samples with (1 L water flush) and without (6 L water flush) inhibition on day 55 to provide substrate of 1 g COD /L. The methane production was monitored after the addition of acetate. Further, to evaluate whether methanogens were inhibited, 100 μL fresh inoculum was added to the 1 L water flushed blackwater sample after the acetate was added and no additional methane production was observed; and 6 L water flushed blackwater sample after methane production stopped in the acetate addition step. The methane production was monitored after the addition of fresh inoculum with the same procedure as described above.

FA inhibition

Table S3.1. Total ammonia nitrogen (TAN) concentration (mg/L) and pH values in various types of blackwater samples in *Trial 1*.

TAN of *Trial 1* with NH_4Cl addition in 9 L blackwater (mg/L)	136	180	354	600	933	1,730	2,050	7,480
pH of *Trial 1* with NH_4Cl addition in 9 L blackwater	8.01	8.5	8.47	8.34	8.29	8.26	8.5	8.5

3.5.3 Calculation methods and equations

Methane production in BMP and hydrolysis tests was calculated using Equation (3S-1):

$$CH_{4t} = \frac{64 \cdot P_t \cdot C_t \cdot V_h}{R \cdot T} \qquad \text{(3S-1)}$$

Where:

CH_{4t}: Amount of methane production at time t (in mg COD);

P_t: Absolute headspace pressure at time t (in kpa);

V_h: Volume of headspace in serum bottles (in mL);

C_t: Methane composition in the headspace at time t (in %);

R: Gas law constant (in L kpa K^{-1} mo/L);

T: Absolute temperature (in K);

64: Conversion factor of 1 mol methane to 64 g COD.

Methanogenesis efficiency (%) also considered as BMP was calculated using Equation (3S-2):

$$Methanogenesis\ efficiency\ (\%) = \frac{CH_{4t}}{COD_{t0}} \qquad \text{(3S-2)}$$

Where:

COD_{t0}: Amount of total COD input (in mg COD).

Hydrolysis efficiency (%) was calculated using Equation (3S-3):

$$Hydrolysis\ efficiency\ (\%) = \frac{COD_{st} - COD_{s0} + COD_{CH_4}}{COD_{t0} - COD_{s0}} \text{ (3S-3)}$$

Where:

COD_{st}: Amount of hydrolysis products in liquid phase (COD_s) at time t (in mg COD);

COD_{s0}: Amount of soluble COD at time 0 (in mg COD);

COD_{CH4}: Amount of hydrolysis products in gas phase (CH_4) at time t (in mg COD);

The net amount of hydrolysis products of blackwater is calculated by subtracting the amount of hydrolysis products generated by AD sludge in the blank sample.

Hydrolysis rate coefficient (k_h) was modeled using first order kinetic Equation (3S-4):

$$Ln\left(1-\frac{\Delta COD_{s,t}}{\Delta COD_{s,end}}\right)=-k_h\cdot t \quad \text{(3S-4)}$$

$\Delta COD_{s,end}=COD_{CH4\ end}+COD_{s,end}-COD_{s,0}$ (in mg);

$\Delta COD_{s,t}= COD_{CH4t}+COD_{s,t}-COD_{s,0}$ (in mg);

Where:

$COD_{CH4,end}$: Amount of CH_4 at the end of the reaction;

$COD_{s,end}$: Amount of COD_s in the liquid phase at the end of the reaction;

t: Reaction time (in day);

k_h: First order hydrolysis rate coefficient (in d^{-1}).

Δ : Symbol of net increase amount.

Acidogenesis efficiency (%) was calculated using equation (5):

$$Acidogenesis\ effciency\ (\%)=\frac{COD_{VFAt}-COD_{VFA0}+COD_{CH_4}}{COD_{t0}-COD_{VFA0}} \quad \text{(3S-5)}$$

Where:

COD_{VFAt}: Amount of acidogenesis products (VFAs) at time t (in mg COD);

COD_{VFA0}: Amount of VFAs at time 0 (in mg COD);

The free ammonia concentration of blackwater was calculated using Equation (3S-6):

$$NH_3(FA)=1.214\times TAN\cdot\left(1+\frac{10^{-pH}}{10^{-(0.09018+\frac{2729.92}{T\,(K)})}}\right)^{-1} \text{ (3S-6)}$$

Where:

NH_3: Free ammonia (FA) (in mg/L);

TAN: Total ammonia nitrogen (in mg/L);

T (K): Kelvin temperature.

3.5.4 Results and Discussion of biochemical methane potential (BMP) test

The cessation of the methane production at the end of the experiments was assumed to be attributed to (i) the lack of substrate for methanogens and/or (ii) the reduction of methanogenic activities due to inhibition factors such as FA toxicity or the VFAs accumulation. To evaluate the mechanism of the methane production limitation, tests were performed with selected digested blackwater samples, including 6 L flushed blackwater and 1 L flushed blackwater groups after the methane production stopped.

After acetate addition, 6 L flushed blackwater samples immediately had methane production, and the methane generation lasted for 11 days (data not shown) until it reached its maximum capacity. The results showed that acetate was successfully converted into methane in 6 L flushed samples (CH_4/COD_t>95%). After the addition of fresh inoculum, no further methane was generated in these samples, which indicated methanogens were active and the cessation of methane production was due to the depletion of available substrates.

However, for 1 L flushed samples, no methane production was observed after acetate addition. Since acetate is the hydrolysis/fermentative product that could be directly utilized by methanogens, this result demonstrated that the methanogens were completely inhibited at this point which led to the cease of methane production. After the addition of fresh inoculum, methane generation was detected, which demonstrated that there was available substrate for methane production in 1 L flushed digested samples. It further verified that the methanogenesis step in low water flushed sample (1 L per flush) was inhibited. These results indicated that although a fraction of methanogens was able to work at the beginning of the BMP studies, they could not easily adapt to the inhibition condition and only maintain under a "inhibited steady state" (Chen et al., 2008), which refers to the process where methanogens are inhibited but can work steadily with a low methane yield. At the end of the experiment, methanogens completely lost their ability to produce methane in the 1 L flushed samples. For the less concentrated blackwater sample (6 L flushed blackwater), the methanogens were not inhibited and functioned well.

Table S3.2. Metal concentration in blackwater stock.

Na mg/L	K mg/L	Ca mg/L	Mg mg/L	Zn mg/L	Al mg/L	Cu mg/L	Mn mg/L	Pd mg/L	Fe mg/L	Cd mg/L	As mg/L	Cr mg/L
2361 (±808)	2161 (±3)	307 (±10)	66 (±3)	3.18 (±0.31)	1.85 (±0.39)	0.57 (±0.11)	0.33 (±0.02)	0.05 (±0.03)	<DL	<DL	ND	ND

ND=NONE DETECTED

DL=DETECTION LIMITS

3.6 References

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CHAPTER 4. BIOMETHANE RECOVERY FROM SOURCE-DIVERTED HOUSEHOLD BLACKWATER: IMPACTS FROM FEED SULFATE[2]

4.1. Introduction

The feasibility of anaerobic digestion with different system designs and operational parameters has been demonstrated for conventional toilet blackwater treatment around the world (Gallagher and Sharvelle, 2011; Sharma et al., 2016; van Voorthuizen et al., 2008). However, most of these reported studies focused on pursuing high effluent quality while low methanisation rates (ranging from 20-29%) were often observed (Bwapwa, 2012; Gao et al., 2019a; Luostarinen et al., 2007; van Voorthuizen et al., 2008), but has never been systematically studied. For instance, a methanisation rate of approximately 20% was obtained in a two-phased upflow anaerobic sludge blanket (UASB) septic tank reactor due to the limited substrate hydrolysis under low operational temperatures (10 and 20 °C) (Luostarinen et al., 2007). A 28% methanisation rate was demonstrated in a combined anaerobic baffled reactor (ABR) and membrane bioreactor (MBR) system and was attributed to the loss of biodegradable contents during blackwater collection (Bwapwa, 2012). Further, a methanisation rate of 27% was achieved in a combined UASB-membrane system, which was explained by the considerable substrate wash-out in the UASB treatment stage (van Voorthuizen et al., 2008).

Our previous batch experiments have demonstrated a high bio-methane potential (BMP) of 0.48 g CH_4-COD/g feed COD for conventional toilet collected blackwater (Gao et al., 2019b), while a low methanisation rate of less than 30% of feed COD (Gao et al., 2019a) was observed when treating it with UASB. In comparison, vacuum toilet collected blackwater (with less flushing water content) had a much higher methanisation rate (~44% of feed COD) when treated with the UASB (Gao et al., 2019c). However, it is unclear why the low methanisation was observed for conventional toilet collected blackwater. The current study aims to investigate the fundamental challenges associated with methane recovery from blackwater collected from conventional toilets. The major concerned factor was the sulfate content in blackwater which was mainly

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contributed from the toilet flushing water (60-70 mg SO_4^{2-}/L). Sulfate in wastewater stream can result in a reduced biomethane recovery potential and emission of hydrogen sulfide (H_2S). The emitted hydrogen sulfide causes sewer pipes corrosion, odorous problem and health concerns (Guisasola et al., 2008). The hydrogen sulfide also poses inhibition effects on methanogens in anaerobic bioreactors. This point is of significant importance to the overall sustainability and economics in decentralized sanitation system and has never been illustrated in other studies. The methanogenic pathways and microbial community structure development were investigated in this study to reveal the linkage between microorganisms and treatment performance. The current work could help to better understand the anaerobic treatment of conventional toilet blackwater and guide future operations and system designs.

4.2. Materials and methods

4.2.1 Feedstock collection and reactor operation

Raw blackwater (absent flush water) was collected from the University of Alberta campus (Edmonton, Canada). Raw blackwater was then stirred and mixed with toilet flushing water (i.e., tap water) to represent conventional blackwater, containing 9 L water per flush. The flushing water was changed to deionized (DI) water at the later stage (Phase II) of the experiment to demonstrate the impact of flush-water characteristics on blackwater methane recovery.

The anaerobic treatment of blackwater was conducted in a laboratory UASB reactor with a working volume of 4.2 L. The reactor was inoculated with 2.1 L of anaerobic digester sludge from a local anaerobic digester treating primary sludge (Edmonton, Canada) containing 13.1 (±0.6) g volatile suspended solids (VSS)/L. The blackwater-feed was pumped continuously into the UASB with a peristaltic pump (Longer pump BT 100-2J). The operating temperature was controlled at mesophilic temperature (35 °C) using a heating blanket. The bioreactor was optimized for blackwater treatment through stepwise reduction of HRT (over 240 days, data not shown). The optimized HRT for methane recovery from conventional blackwater was 2.2 days. For the current study, the total operation period lasted for 105 days, which was divided into three operation phases as shown in Table 4.1. The operation conditions were maintained with organic loading rates (OLR) of 0.45 kg COD/m^3/d and HRT of 2.2 days in all three phases; different flush-water (tap water or DI water) was used in certain phases.

4.2.2 Activity test

Specific methanogenic activity (SMA) tests were performed with UASB sludge at the end of each operation phase with hydrogen and carbon dioxide gases (H_2&CO_2) and sodium acetate substrates to investigate the specific activities from hydrogenotrophic methanogenesis and acetoclastic methanogenesis. Prior to each assay, UASB sludge was discharged from all side ports of the sludge bed, mixed, and characterized for pH, VSS, and COD concentrations. The test was then conducted in 166 mL serum bottles, with substrate concentration (H_2&CO_2 or acetate) of 1 g/L and pH adjusted to 7 using NaOH (5M) or HCl (5 M). H_2/CO_2 (80%/20%) gas and N_2 gas were applied to flush the headspace of the serum bottles for SMA (H_2&CO_2) and SMA (acetate) assays, respectively. The bottles were then sealed with rubber septums and aluminum caps. The tests were conducted in an incubator shaker (New Brunswick™ Innova® 44, Eppendorf, Canada) at 35 °C and 130 rpm in dark condition. Methane production was determined by measuring the headspace pressure of the serum bottles using a pressure meter (GHM 3151, Germany), and the methane fraction in the biogas was measured using a gas chromatography (GC) (7890B Agilent Technologies, USA) system equipped with a thermal conductivity detector (TCD). Each group of SMA tests was conducted in triplicates. The SMA values were calculated from dividing the initial linear slope of the methane production curve over the sludge VSS in each bottle. Blank control groups with only sludge were set-up simultaneously to subtract the methane production from sludge in the test group. The SMA (acetate and H_2 & CO_2) results were presented with average values of the triplicate bottles as g CH_4-COD/g sludge VSS/d with standard deviations.

4.2.3 DNA extraction and 16S rRNA gene sequencing

Triplicated sludge sampling from all ports of the UASB sludge bed were conducted, and the sampled sludge were mixed for each DNA extraction test at the end of each operation phase (Table 4.1) to investigate the microbial community structure variations. The genomic DNA of UASB sludge samples were extracted in duplicates. DNA was extracted from 1.5 mL mixed sludge samples from following the manufacturer's protocol using DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). The quality of the extracted DNA was checked in NanoDrop One (ThermoFisher, Waltham, MA), and the samples were stored at -20 °C before sending to the

RTL Genomics sequencing center (Texas, USA). Bacterial 16S rRNA genes were amplified using the universal primer-pair *357wF* (5'-*CCTACGGGNGGCWGCAG-3'*) and *785R* (5'-*GACTACHVGGGTATCTAATCC-3'*). Archaeal 16S rRNA genes were amplified using the universal primer set *517F* (5'-*GCYTAAAGSRNCCGTAGC-3'*) and *909R* (5'-*TTTCAGYCTTGCGRCCGTAC-3'*). Sequencing was performed on the Illumina MiSeq PE300 platform.

Paired-end reads data of forward and reverse sequences were analyzed using the QIIME2 (Bolyen et al., 2019) plugin "DADA2" (Callahan et al., 2016) to remove the low-quality sequences and chimeras. The phylogenetic tree was generated using the QIIME2 plugin "phylogeny", and taxonomy was assigned using the QIIME2 plugin "feature-classifier" (Bokulich et al., 2018) and Greengenes (version 13_8) 99% classifier (Campanaro et al., 2018).

4.2.4 Chemical analysis

The chemical properties of total COD (COD_t), soluble COD (COD_s), total suspended solids (TSS), and volatile suspended solids (VSS) were tested following standard methods (APHA/AWWA/WEF, 2012). The total ammonium-nitrogen (NH_4^+-N) (using 0.45 μm membrane filtered samples), sulfate (SO_4^{2-}) (using 0.45 μm membrane filtered samples) and total phosphorus (TP) concentrations were measured using Hach TNT vial tests (Hach, USA). The three volatile fatty acids (VFAs) acetate, propionate, and butyrate, and anions (F^-, Cl^-, NO_3^-, NO_2^-, Br^-) were tested using a DIONEX ICS-2100 Ionic chromatography (IC) system (Thermo Fisher Scientific, Waltham MA, USA) equipped with a conductivity detector. The total sulfides and dissolved sulfides (using 0.45 μm membrane filtered samples) were measured with the methylene blue method using Hach method 8131 (Hach, USA). The liquid pH was measured using a B40PCID pH meter (VWR, SympHony). The volume of produced biogas was measured from the gas bag at the top of the UASB, and the biogas composition was determined by gas chromatography. Chemical property measurements of the influent blackwater, UASB effluent, and the biogas production were carried out 3 – 5 times each week. UASB sludge characterizations including COD, TSS, VSS, and pH were performed 1 – 2 times in each phase.

4.2.5 Calculations

Sulfate and sulfides

In the anaerobic treatment system, a ratio of 1:0.67 for SO_4^{2-}:COD was used to calculate the COD consumed by sulfate reduction (Jeong et al., 2008). Sulfate (SO_4^{2-}) can be reduced to sulfides (*i.e.*, H_2S, HS^-, S^{2-}) in the liquid phase and H_2S in the gas phase. At a neutral pH under anaerobic digestion conditions, S^{2-} is negligible (Isa et al., 1986), and the free hydrogen sulfide (H_2S) in the liquid phase can be calculated by the first stage ionization equilibrium equation described below (Isa et al., 1986):

$$[H_2S]_s = f \times [TS]_s$$

$$f = \left(1 + \frac{K_1}{10^{-pH}}\right)^{-1}$$

Where:

$[H_2S]_s$ is the concentration of free H_2S in the liquid phase;

$[TS]_s$ is the concentration of total dissolved sulfides ($H_2S + HS^- + S^{2-}$) in the liquid phase;

f is the fraction of free H_2S of the total dissolved sulfides;

K_1 is the first stage equilibrium constant, and $K_1 = 1.49 \times 10^{-7}$ (35 °C) (Isa et al., 1986);

The equilibrium between gas phase H_2S and dissolved free H_2S concentration is controlled by Henry's Law (Isa et al., 1986).

Methane production rate and methanisation rate

The methane production rate was calculated from the amount of methane generated from per volume of the reactor daily and is expressed as mL CH_4/ L reactor/d. The methanisation rate was calculated from the amount of methane as COD (g CH_4-COD) generated from the feed blackwater COD and was presented as the percentage (%) of influent COD that converted to methane.

The average values of results shown for each operation phase (*e.g.*, COD removal efficiency, methane production rate, methanisation rate) were calculated from the steady-state results, where

the steady-state was indicated by relatively stabilized effluent quality and methane production from data of at least one week after the change of operation condition.

4.3. Results

4.3.1 Blackwater characteristics

Table 4.1. Operational conditions of UASB reactor and chemical properties of conventional toilet blackwater in all operation phases.

					UASB Influent							
	Flushing water	Operation duration	HRT	Organic loading rate	pH	COD_t	COD_s	SO_{42-}	NH_{4+}-N	TP	TSS	VSS
		day	day	$kg/m_3/d$		mg/L	mg/L	mg/L	mg/L	mg/L	g/L	g/L
Phase I	Tap	1-27	2.2 (±0.3)	0.45 (±0.05)	7.7 (±0.1)	1026 (±70)	263 (±13)	79.9 (±4.7)	113.6 (±2.8)	17.5 (±4.9)	0.43 (±0.06)	0.42 (±0.07)
Phase II	DI	28-61	2.2 (±0.0)	0.45 (±0.02)	7.9 (±0.1)	1021 (±49)	290 (±43)	24.0 (±3.1)	108.1 (±2.0)	21.6 (±6.5)	0.36 (±0.03)	0.35 (±0.03)
Phase III	Tap	62-105	2.2 (±0.1)	0.45 (±0.03)	7.8 (±0.1)	980 (±47)	256 (±20)	85.0 (±2.5)	107.5 (±3.2)	19.8 (±0.4)	0.32 (±0.1)	0.29 (±0.09)

Table 4.1 shows the chemical properties of blackwater used for different operation periods (Phases I – III). Blackwater containing tap-water collected in Phases I and III generated average total COD concentrations of 1026 (±70) and 980 (±47) mg/L, respectively. Comparable COD concentrations (1021 [± 49] mg/L) were obtained in the blackwater containing DI water used in Phase II. Only 26% of the total COD was contributed by soluble contents, which indicates a high fraction of solid organics was accounted for in the blackwater stream. These feed COD concentrations were comparable with the reported values of 932 – 1983 mg/L for blackwater collected from conventional toilets (Gallagher and Sharvelle, 2010; Knerr et al., 2011; Sharma et al., 2016; van Voorthuizen et al., 2008).

When tap water flushing was applied, sulfate concentrations of 79.9 (± 4.7) mg/L and 85.0 (± 2.5) (Phases I and III, respectively) were obtained, which was attributed to the sulfate contents in local tap water (61.1 [±1.2] mg/L) and human excreta (feces and urine). This result is comparable to the blackwater sulfate concentration reported previously (Rose et al., 2015). When DI water was used as toilet flushing water in Phase II, the feed sulfate was considerably less than in Phases I and III, measuring at 24.0 (± 3.1) mg/L.

4.3.2 UASB treatment performance of conventional toilets collected blackwater

The UASB treatment performance as indicated by COD removal, methane production, sulfate removal, and sulfides variations is exhibited in Fig. 4.1. In Phase I, with the system well adapted to the operational conditions (*i.e.*, OLR of 0.45 kg COD/m^3/d and HRT of 2.2 days), the influent COD was removed with a relatively stable effluent COD concentration of 167.1 (±34.3) mg/L and a COD removal efficiency of 82.4 (±1.6) % (Fig. 4.1A). The average methane production rate was 58.0 (±6.9) mL/L/d (Fig. 4.1B) and resulted in a methanisation rate of 31.9 (±2.7) %.

Sulfate reduction was observed with blackwater COD removal. Fig. 4.1C depicts that 81.7 (±4.0) % (Phase I) of the feed sulfate was removed and converted to sulfides under anaerobic conditions. The produced total sulfide and dissolved sulfide concentrations in the UASB effluent were 16.3 (±1.2) mg/L and 14.9 (±1.1) mg/L (Fig. 4.1D), respectively. Since the UASB effluent was at neutral pH ~7, most of the dissolved sulfide was in the form of free H_2S with an average concentration of 9.8 (±1.2) mg/L in Phase I. The concentration of free H_2S in the gas phase was calculated to be 4821.6 (±639.7) ppm (Fig. 4.1E). Similar levels of sulfides production have been observed previously in treating starch wastewater with a COD/sulfate ratio of 10 and similar feedstock characteristics (COD 1,000 mg/L) as the blackwater tested in the present study; the detected total and dissolved sulfide concentrations were 15.6 mg/L and 12.9 mg/L, respectively, and the free H_2S concentration was approximately 4.2 mg/L (Lu et al., 2016). Overall, 94.6 (±9.4) % of the removed sulfate content could be balanced out from the measured sulfides in the UASB effluent and the gas phase. Unaccounted sulfate portions may have been consumed by metal precipitation and cell synthesis (Khanal and Huang, 2005).

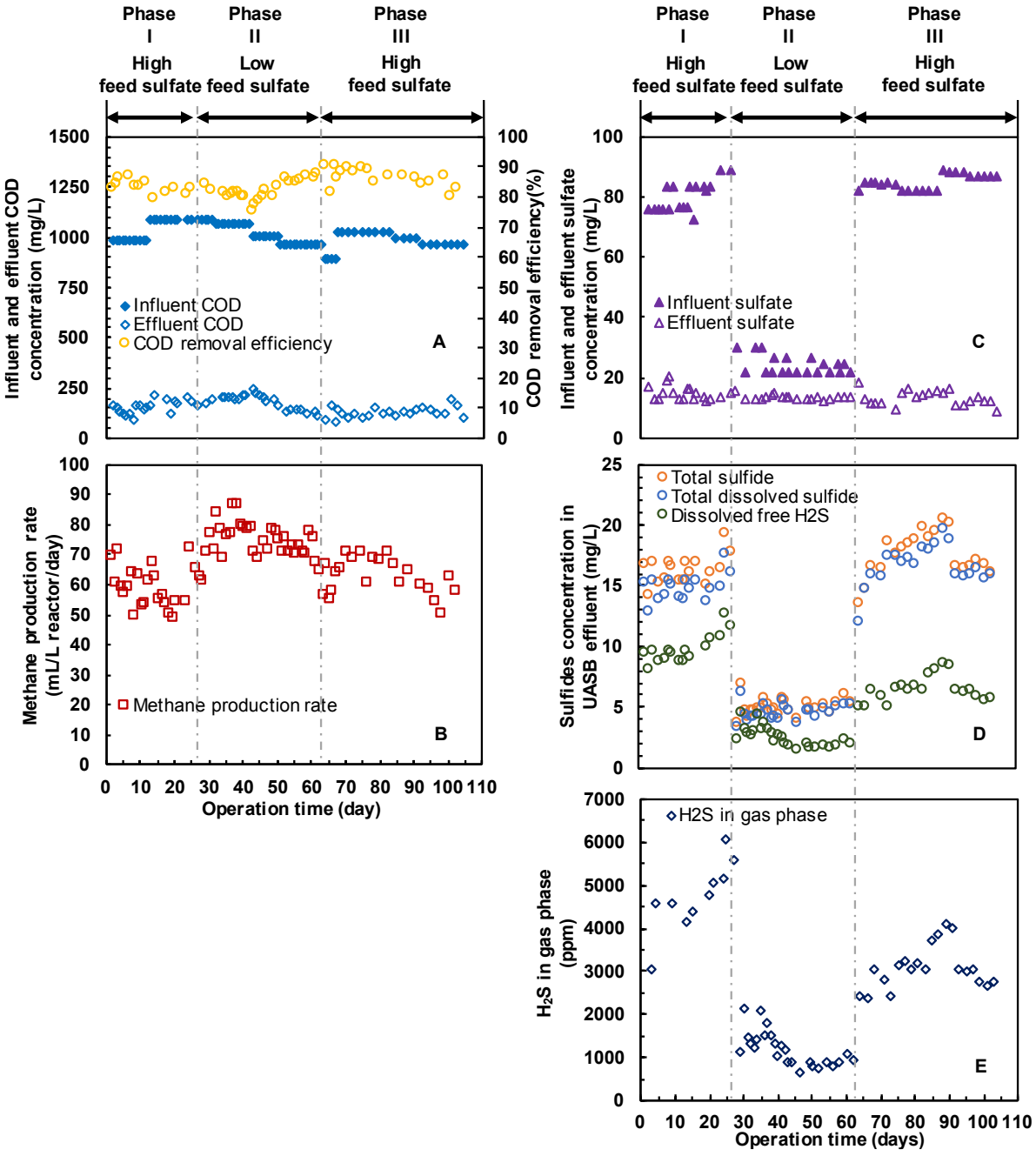

Fig. 4.1. UASB treatment performances of conventional toilet blackwater for operation Phase I (tap water flush, high feed sulfate), Phase II (DI water flush, low feed sulfate) and Phase III (tap water flush, high feed sulfate) under OLR 0.45 kg COD/m3/d: influent and effluent COD concentration (mg/L) and COD removal efficiency (%) (A); methane production rate (mL/L reactor/d) (B); influent and effluent sulfate concentration (mg/L) (C); total sulfide, total

dissolved sulfide, and dissolved free H_2S in the UASB effluent (mg/L) (D); H_2S in gas phase (ppm) (E) in time course.

4.3.3 Impact of toilet flushing water on UASB treatment performance

The impact of toilet flushing water was evaluated by feeding blackwater mixed with DI water (low feed sulfate) into the UASB under the same operational conditions as in Phase I.

The COD removal efficiency early in Phase II was 79.9 (±1.8) %, whereas a relatively low effluent COD concentration of 141.6 (±13.4) mg/L was achieved after 20 days of operation, which resulted in an increased COD removal to 85.4 (±1.1) %. The methane production and methanisation rate both gradually increased from 58.0 (±6.9) mL/L/d to 73.5 (±2.8) mL/L/d and from 31.9 (±2.7) % to 42.1 (±1.4) %, respectively, resulting in a 30% increase from Phase I. The effluent sulfate concentration in Phase II (13.5 [±0.5] mg/L) did not differ considerably from Phase I (14.5 [±3.0] mg/L), while a decreased sulfate removal efficiency to 42.8 (±6.9) % was observed. Consequently, the concentrations of total sulfide, dissolved sulfide, free H_2S in UASB effluent, and H_2S in gas phase decreased to 5.0 (±0.7) mg/L, 4.6 (±0.6) mg/L, 2.6 (±0.8) mg/L, and 1218.8 (±400.2) ppm, respectively.

In order to further verify the sulfate impact, the sulfate was supplemented to the blackwater feed by applying tap water for blackwater flushing, achieving a sulfate concentration of 85.0 (±2.5) mg/L in Phase III. The COD removal efficiency first slightly increased to 88.0 (±2.9) % and then gradually stabilized at 84.8 (±2.3) %. The methane production slowly decreased until it reached a relatively steady state of 62.0 (±6.3) mL/L/d, which was comparable to Phase I with 58.0 (±6.9) mL/L/d. Correspondingly, the methanisation rate in Phase III decreased to 34.4 (±2.9) %. The sulfate removal efficiency increased to 84.7 (±2.7) % with an effluent sulfate concentration of 13.0 (±2.1) mg/L. The total sulfide, dissolved sulfide, and free H_2S concentrations in UASB effluent were 17.9 (±1.4), 17.0 (±1.2), and 6.5 (±1.1) mg/L respectively, and the H_2S concentration in the gas phase was 3100.0 (±516.6) ppm. Overall, the variations of sulfate and sulfide components in the treatment process in Phase III was comparable to the values observed in Phase I.

Limited amount of VFAs (acetate, propionate, and butyrate) were detected in the UASB effluent throughout the whole operational period. When tap-water blackwater was treated, the total VFA concentrations of 31.6 (±3.4) mg COD/L were observed, which were composed of acetate 8.0 (±1.4) mg COD/L, propionate 21.0 (±2.4) mg COD/L, and butyrate 2.5 (±0.6) mg COD/L. In the DI-water blackwater fed condition, the total VFA concentration of 24.3 (±0.8) mg COD/L including acetate 6.3 (±0.6) mg COD/L, propionate 16.2 (±0.6) mg COD/L and butyrate 1.8 (±0.0) mg COD/L were detected. The low residual VFA contents correlated well with the high COD removal efficiencies (82.4-85.4%) obtained under both tap-water and DI-water blackwater fed conditions. The results are in agreement with previous reported studies, suggesting that although the electron transfer route shifted at lower COD/ sulfate ratios, the organics removal efficacies were not negatively impacted (Lu et al., 2016).

4.3.4 COD balance and specific methanogenic activity

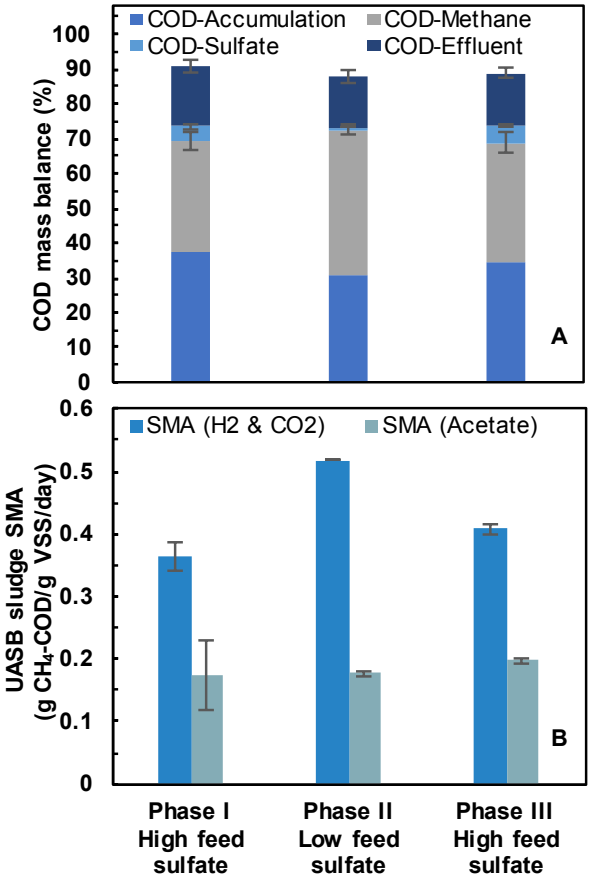

Fig. 4.2. COD balance represented by the COD distribution percentages into COD accumulated in sludge as COD-Accumulation, COD converted to methane as COD-Methane, COD consumed by sulfate reduction as COD-Sulfate and COD remained in the UASB effluent as COD-Effluent (A), UASB sludge specific methanogenic activity (SMA [H_2&CO_2], SMA [Acetate]) (B).

The COD mass balance was calculated for each operation phase at steady-state and presented as COD partitioned into accumulation in sludge, methane production, sulfate reduction, and effluent residue (Fig. 4.2A). Overall, a total of 88.0 – 91.1 % of the input COD could be balanced out in the effluent. Throughout the whole operation, the effluent COD only accounted for 14.6 – 17.6 % of the input COD, indicating relatively effective COD removal under all conditions. After lowering the sulfate content in the blackwater feed in Phase II, the proportion of the input COD that converted to methane showed a significant increase from 31.9 % (Phase I) to 42.1% (Phase II), while it dropped to 34.4 % (Phase III) after the sulfate was supplemented back into the feed. Correspondingly, the COD inflow that was consumed by sulfate reduction initially dropped from 4.2 % (Phase I) to 0.8 % (Phase II), then increased back to 4.9 % (Phase III). The COD accumulation in sludge decreased from 37.5 % to 30.4 % in Phase II and then increased to 34.4 % in Phase III.

Fig. 4.2B presents the UASB sludge properties of specific methanogenic activities (SMA) in each operation phase. Generally, the methanogenesis in anaerobic treatment systems was conducted through acetate and H_2&CO_2 utilization pathways. In the current study, the hydrogenotrophic methanogenic pathway was dominant throughout the whole blackwater treatment period. The values of SMA (H_2&CO_2) were always higher than SMA (acetate) throughout the blackwater treatment process. Moreover, the feed sulfate change impacted the hydrogenotrophic methanogenesis but not the acetoclastic methanogenesis activity. From Phase I to II, the SMA (H_2&CO_2) value significantly increased from 0.37 (±0.02) to 0.52 (±0.00) g CH_4-COD/g VSS/d after lowering the feed sulfate content; it then declined to 0.41 (±0.01) g CH_4-COD/g VSS/d when the sulfate was supplemented back in during Phase III. The SMA (acetate) values did not show considerable changes from Phase I to III, which ranged from 0.18 (±0.06) to 0.20 (±0.00) g CH_4-COD/g VSS/d.

4.3.5 Microbial community analysis

Microbial community diversity

The temporal development of the microbial community was analyzed using the beta-diversity based on Bray-Curtis distance to elucidate potential impacts from sulfate (Fig. S4.1). Both archaeal and bacterial communities shifted from inoculum in Phase I, indicating the significant impact from the substrate, *i.e.*, blackwater. The archaeal communities shifted drastically away from Phase I (tap water, high feed sulfate) along PCoA2 (20.6%) axis in Phase II (DI water, low feed sulfate), then shifted back towards Phase I with the supplementation of feed sulfate in Phase III (tap water, high feed sulfate). Nevertheless, the bacterial communities shifted along both PCoA1 (63.7%) and PCoA2 (27.3%) axes after feed sulfate was lowered in Phase II, then further shifted slightly away from all previous phases along PCoA2 (27.3%) axis in Phase III.

Archaeal community variation

A total of 17 archaeal taxa were detected at the genus level for inoculum and UASB samples for all studied operation conditions (Fig. 4.3). Methanogens from the genus *Methanolinea* (39.0%), two uncultured genera in the family (f_) *Methanospirillaceae* (38.1% and 13.7%) and the genus *Methanoculleus* (6.5%) dominated more than 97% of the abundance in the inoculum. The genus *Methanolinea* was then inherited from the inoculum and became the most dominant genera in Phase I with a relative abundance of 63.1 %. The genus *Methanospirillum* (21.0 %) became the second-most dominant group. In addition, the genus *Candidatus Methanoregula* and uncultured genera from the order Methanomicrobiales were enriched with relative abundances of 5.3% and 5.4%, respectively in Phase I.

Interestingly, the dominant methanogen group was the genus *Methanospirillum* (53.2%) after the feed sulfate concentration was lowered in Phase II, and the genus *Methanolinea* (38.1%) became the second-most dominant. The relative abundances of methanogens from the genus *Methanobacterium* and uncultured genera from the family *Methanoregulaceae* increased to 3.0% and 2.1%, respectively, compared to that in the high-sulfate condition in Phase I. In Phase III, with the reinstating of the high-sulfate blackwater feed, the genus *Methanolinea* rehabilitated to be the most abundant archaeal group with a relative abundance of 51.1%. Correspondingly, the

genus *Methanospirillum* decreased in relative abundance to a value of 38.0%. The genus *Methanobacterium* and uncultured genus from the family *Methanoregulaceae* showed slight increases in abundances to 3.7% and 4.7%, respectively.

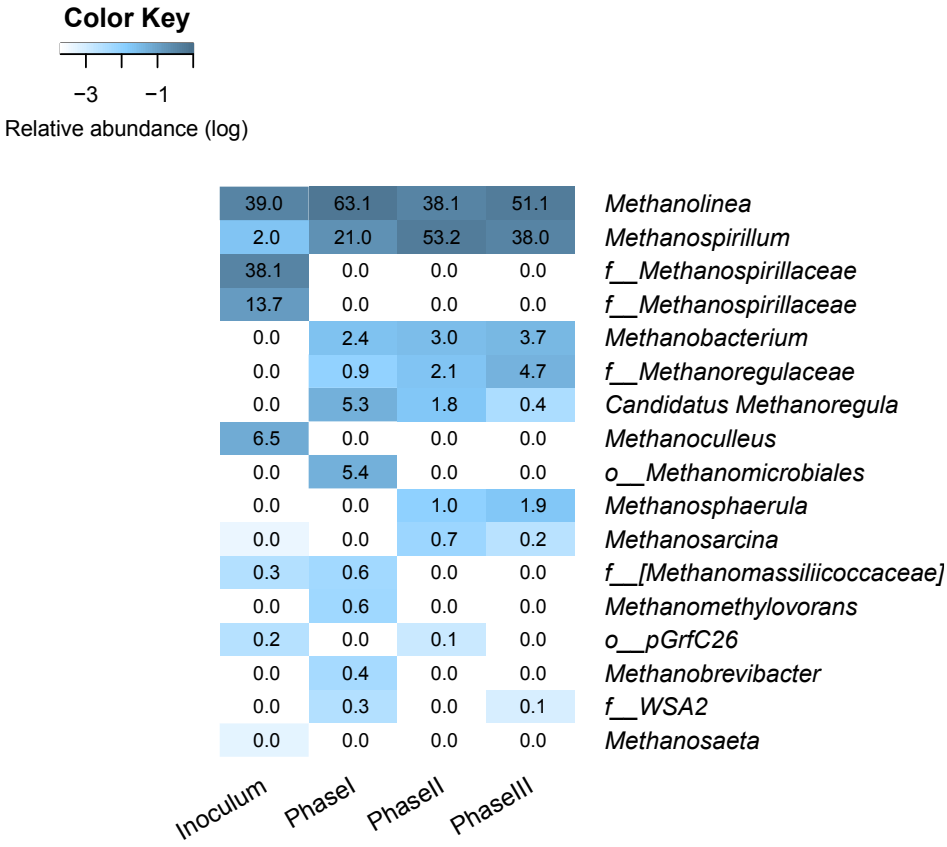

Fig. 4.3. Heatmap of archaeal communities at genus or higher level (family [f_]; order [o_]) in the inoculum and the operation Phase I (tap water flush, high feed sulfate), Phase II (DI water flush, low feed sulfate) and Phase III (tap water flush, high feed sulfate).

The genus *Methanolinea* and genus *Methanospirillum* have both been commonly found in anaerobic digesters treating waste such as biosolids and food-waste (Lee et al., 2018; Swiatczak et al., 2017). Both groups belong to the order *Methanomicrobiales*, and all members of this order have been reported as hydrogenotrophic methanogens that utilize H_2&CO_2 to produce CH_4 in bioreactors (Khan et al., 2018). This correlates well with the high SMA (H_2&CO_2) values demonstrated in this study, indicating the hydrogenotrophic methanogens dominated the system. The predominance of hydrogenotrophic methanogenesis pathway has been reported in both lab-scale and full-scale bioreactors under stable states, treating biosolids (Kim et al., 2013) and

blackwater collected from vacuum toilets (Gao et al., 2019c). The highest abundances of the genus *Methanolinea* in high-sulfate conditions (Phases I and III) were similar to the previously reported observation that *Methanolinea* dominated AD reactors at a sulfate concentration of 0.5 mM (Ma et al., 2017). The predominance of *Methanolinea* in the current study may indicate that it may be more resistant towards the high-sulfate conditions (~0.83 mM) than other methanogens. Species from genus *Methanospirillum* have been commonly reported with higher H_2 utilization rates (Ferry and Wolfe, 1977; Imachi et al., 2008), which is consistent with the higher SMA (H_2&CO_2) observed in Phase II (low sulfate condition) in the present study. Further, it has been reported that the genus *Methanospirillum* could function syntrophically to support SRB with the degradation of complex organics, such as aromatic and oil compounds (Ferry et al., 1974; Ferry and Wolfe, 1976; Morris et al., 2013). For example, an SRB strain (*Desulfatibacillum alkenivorans AK-01*) from the family *Desulfobacteraceae* was reported to be capable of degrading alkane with the presence of the genus *Methanospirillum* in the absence of sulfate (Morris et al., 2013). In the current study, the same family, *Desulfobacteraceae* (0.2%) (Table S4.3), was found in Phase II and may function syntrophically with methanogens *Methanospirillum* for a better organics' removal.

Bacterial community variation

The results for relative bacterial abundances at the family level (or higher levels, *i.e.*, order [o_], phylum [p_]) for inoculum and UASB operation (Phases I-III) are presented in Fig. 4.4. The inoculum was dominated by bacterial groups from the family *[Cloacamonaceae]* (23.2%), uncultured family from order Bacteroidales (14.8%), and the families *Rikenellaceae* (13.8%) *Anaerolinaceae* (12.0%). The detected bacteria have been commonly reported as fermenters for cellulose, nucleic acids, and fatty acids, *etc*. and degraders in digestion systems treating sewage sludge, agricultural waste, and food-waste, *etc*. (Graf, 2014; Lee et al., 2018; Swiatczak et al., 2017; Wojcieszak et al., 2017).

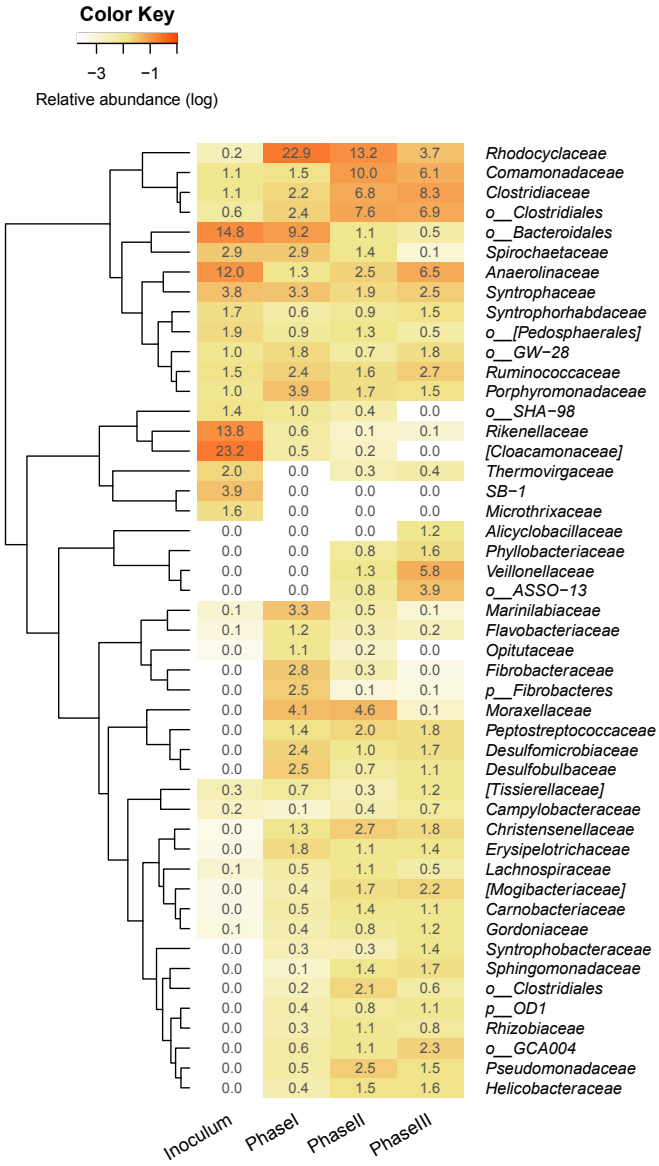

Fig. 4.4. Heatmap of bacterial communities at family or higher level (order [o_]; phylum [p_]) in the inoculum and the operation Phase I (tap water flush, high feed sulfate), Phase II (DI water flush, low feed sulfate) and Phase III (tap water flush, high feed sulfate).

The bacterial consortia shifted significantly when treating tap-water blackwater. In Phase I, the relative abundance of the bacterial family *Rhodocyclaceae* drastically increased to 22.9% and became the most abundant group. The family *Rhodocyclaceae*, which is a group of hydrogen (H_2) producing bacteria in sewage sludge digesters (Martínez et al., 2019), is a bacterium family that has been reported to play an important role in degrading organic matter, especially aromatic

substances (Xu et al., 2017; Zheng et al., 2016). The dominance of this bacterial group may indicate its strong syntrophic relationship with H_2 consuming communities in the blackwater treatment system. An uncultured family from the order Bacteroidales (9.2%) was inherited from the inoculum and was the second-most dominant in Phase I. The family *Moraxellaceae*, which had a relative abundance of 4.1%, has been reported in the human intestine and animal waste treatment systems, indicating its presence in blackwater (Lastovica et al., 2014; Pampillón-González et al., 2017). It should be noted that the family *Syntrophaceae* (3.3%) has been demonstrated as a syntrophic fatty acid oxidizer which can cooperate with the methanogens for carbon processing in digesters (Ziels et al., 2019).

In Phase II, the family *Rhodocyclaceae* (13.2%) remained the most abundant group. Three groups of bacteria from the family *Comamonadaceae* (10.0%), an uncultured family from the order *Clostridiales* (7.6%), and the family *Clostridiaceae* (6.8%) were enriched, while an uncultured family from the order *Bacteroidales* (1.1%), the family *Porphyromonadaceae* (1.7%), and the family *Syntrophaceae* (1.9%) decreased in abundance. These enriched bacterial groups have been commonly reported in animal manure AD treatment systems and functioned as key fermenters for hydrolysis and acetogenesis (Kushkevych et al., 2019; Pampillón-González et al., 2017; Sun et al., 2015). Species in the family *Clostridiaceae* have been reported as producing H_2 during fermentation (Wüst et al., 2011).

In the final and third phase, the most dominant bacterial group shifted to the family *Clostridiaceae* (8.3%), and an uncultured family from the order Clostridiales and the family *Comamonadaceae* remained dominant with relative abundances of 6.9% and 6.1%, respectively. The family *Rhodocyclaceae* (3.7%) decreased in abundance, while increases were observed for bacteria groups from the family *Anaerolinaceae* (6.5%), *Veillonellaceae* (5.8%), and an uncultured family from order *ASSO-13* (3.9%). These enriched bacteria have been widely reported as fermenters capable of degrading various types of organic wastes (Zamanzadeh et al., 2016).

No SRB was detected in the inoculum, while enrichment of SRB was observed after treating tap water-flushed blackwater with high feed sulfate. In Phase I, the enriched SRB comprised the

families *Desulfobulbaceae* (2.5%), *Desulfomicrobiaceae* (2.4%), *Desulfovibrionaceae* (0.3%), and *Desulfarculaceae* (0.1%), which had a total relative abundance of 5.4% of all enriched bacterium (Table S4.3). The abundances of both the families *Desulfobulbaceae* and *Desulfomicrobiaceae* significantly decreased to 0.7% and 1.0%, respectively, in Phase II when the feed sulfate concentration was lowered. A gradual recovery of SRB abundance with *Desulfobulbaceae* (1.1%) and *Desulfomicrobiaceae* (1.7%) was observed after sulfate was supplemented back in Phase III. Species from the overall dominant families *Desulfobulbaceae* and *Desulfomicrobiaceae* have been reported as mesophilic sulfate reducers that could utilize H_2 as electron donors (Boylan et al., 2019; Kuever and Galushko, 2014; Zhuang et al., 2019).

4.4. Discussion

To date, only limited studies have been reported on anaerobic treatment of conventional toilet blackwater (Abdel-Shafy et al., 2009; Bwapwa, 2012; Gallagher and Sharvelle, 2011; Gao et al., 2019a; Luostarinen et al., 2007; Sharma et al., 2016; van Voorthuizen et al., 2008). All studies reported low methanisation rates (20-29%), but no study systematically evaluated the potential inhibition factors. Although three studies reported the sulfate and sulfide values when treating this type of blackwater (Abdel-Shafy et al., 2009; Rose et al., 2015; Sharma et al., 2016), no investigation was performed to elucidate the impacts of flush water sulfate on conventional toilet blackwater biogas recovery.

Sulfate inhibition has been widely reported in anaerobic wastewater treatment processes, where various types of carbon sources and electron donors have been well studied. For instance, carbon sources like acetate, ethanol, lactate and benzoate *etc*. from industrial wastewater streams are commonly investigated (Bertolino et al., 2015; Hu et al., 2015; Li et al., 1996), and the complex substrates like sewage sludge have also been studied where the fermentative intermediate products such as VFAs, amino acids, aromatic compounds *etc*. can all be utilized by SRB (Hao et al., 2014; Nagpal et al., 2000). Bioreactor microbial communities have also been evaluated in these studies, where acetate utilizing sulfate reducers *Desulfobacter* and the H_2 utilizing sulfate reducers *Desulfovibrio* sp. were most commonly reported (Colleran et al., 1995). However, most of the reported work on sulfate inhibition focused on relatively high feed sulfate concentrations, i.e. from 0.5 g/L in starch wastewater (Lu et al., 2016) to as high as 40-50 g/L in oil refinery

wastewater (Colleran et al., 1995), which were higher than the values (~80 mg/L) often observed in municipal wastewater (as discussed in the current work). Only limited studies focused on low feed sulfate conditions like the current work, yet effects of low-level sulfate on methanogenesis kinetics have been reported. For instance, it has been observed in Lake Mendota sediments that methanogenesis was inhibited by the sulfate concentration as little as 0.2 mM (19.2 mg/L) (Winfrey and Zeikus, 1977). Similarly, sulfate concentrations of 60-105 μM (5.76-10.08 mg/L) have been found to result in the reduction in methanogen activities in lake sediments (Lovley and Klug, 1983). A recent reported study also demonstrated that different methanogenic consortia structure/composition reacted differently towards the level of sulfate and the carbon source (Chen et al., 2019). It should be noted that the H_2S concentration in the UASB effluent was low (2.6-9.8 mg/L) under all conditions in the current work. Previous studies have demonstrated that the inhibitory concentration of H_2S towards methanogenic activities varies depending on the wastewater characteristics and methanogenic consortia involved (Hilton and Oleszkiewicz, 1988; J.W.H. et al., 1994). For instance, a wide range of 2-615 mg/L H_2S in liquid phase has been reported to affect methanogens when treating sulfate-containing waste activated sludge (Jeong et al., 2009). The dynamic shift of the methanogenic community and the lower hydrogenotrophic methanogenic activities (SMA [H_2&CO_2]) observed under higher sulfate conditions in this study may indicate an effectual competition conducted by SRB over the electron donor H_2, which conversely suppressed the methanogens' substrate utilization rate.

In addition to the substrate types, the sulfate inhibitory effects have been reported to correlate with the COD:SO_4^{2-} ratio (Li et al., 1996). Lower COD:SO_4^{2-} ratios tend to intensify the competition for electron donors (Hulshoff Pol et al., 1998; Li et al., 2015; Lu et al., 2016; Siles et al., 2010), which could result in lower methane production. Our study clearly demonstrated the impact that sulfate in blackwater has on methane production, with a COD:SO_4^{2-} ratio of ~12, which is higher than commonly reported inhibition levels (1.5 – 10) (Li et al., 2015; Lu et al., 2016; Siles et al., 2010). For example, previous studies demonstrated that methanogenesis was inhibited when the COD:SO_4^{2-} ratio was lowered to 1.5 in pharmaceutical wastewater treatment due to the increased sulfide concentration (Li et al., 2015). Methane production was suppressed by 49% with the COD:SO_4^{2-} ratio decreased to equal or below 1.5 when treating antibiotic bio-waste (Qiang et al., 2018). The conversion of COD to methane decreased from 80.5 % to 75.9 %

with the COD:SO_4^{2-} ratio changing from 20 to 10 when treating wastewater containing acetate and ethanol (Hu et al., 2015). Similarly, the methane production rate decreased from 0.33 to 0.31 L CH_4/g COD when the COD:SO_4^{2-} ratio dropped from 20 to 10 in a sewage sludge treatment system (Jeong et al., 2009). Process failure for methane production has been reported with a COD:SO_4^{2-} ratio lower than 10 (Hulshoff Pol et al., 2001). However, it should be noted that operational conditions, substrate/ carbon source type, and metabolic pathways may also play important roles when considering the impacts of COD:SO_4^{2-} ratio on anaerobic treatment (Cetecioglu et al., 2019; Lu et al., 2016). For instance, a 60% decrease of methane production rate was observed with a relatively high COD:SO_4^{2-} ratio of 11.6 in the treatment of waste activated sludge (Jeong et al., 2008), while the rather low COD:SO_4^{2-} ratios (0.5, 1.5, 5) showed no impact on the methane production when treating acetate and propionate in a sulfate acclimated reactor (Cetecioglu et al., 2019). Moreover, in a hexadecane degradation process, the electron flow to SRB or methanogens was found determined by the initial sulfate concentration rather than the COD:SO_4^{2-} ratio (Ma et al., 2017).

It has been reported that the sulfate reduction process can outcompete methanogenesis due to its kinetic and thermodynamic favorability (Ueki et al., 1992). The current study has demonstrated a hydrogen consuming pathway with H_2 as electron donors. It has been commonly reported that hydrogenotrophic methanogens can be out competed by SRB (Chen et al., 2008; Oremland and Taylor, 1978) because SRB has a lower required hydrogen threshold concentration and a high hydrogen affinity (Laanbroek et al., 1984), whilst demonstrating thermodynamic favourability with methanogenesis in hydrogenotrophic pathways: $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$, ΔG^0= -135.6 kJ/mol and sulfate reduction: $SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O$, ΔG^0= -151.9 kJ/mol (the produced HS^- and H_2S was dependent on pH as shown in section 4.2.5) (McCartney and Oleszkiewicz, 1991). Previous studies detected that nearly all the H_2 oxidation was attributed to SRB instead of methanogens in reactors treating wastewater with sulfates (Chen et al., 2008), which could support the ascendancy of H_2 oxidizing SRB over hydrogenotrophic methanogens. Observations of inhibited methanogenesis have been reported in the literature; for instance, Ma et al. (2017) observed lower methanogenic activity with sulfate addition in a hydrogenotrophic methanogenic-dominated system. Jing et al. (2013) also reported a decreased SMA (H_2&CO_2) from 0.192 to 0.095 with sulfate addition in the treatment of synthetic sulfate-containing

wastewater. Similarly, Weijma et al. (2002) observed that sulfate reduction completely outcompeted methane production in a gas-lift reactor treating H_2&CO_2 and sulfate. These studies correlated well with our results that suppressed SMA (H_2&CO_2) values were obtained under high sulfate concentration conditions.

It is also important to note that the presence of SRB may result in significant impacts on the anaerobic environment. In marine sediments, it has been reported that in sulfate-rich condition, SRB and methanogens competed for substrates, while in sulfate-depleted conditions, they complemented each other for organic degradation (Plugge et al., 2011). This correlates well with the current blackwater treatment results that indicated that the absence of flushing water sulfate led to an enhanced COD biodegradation and enrichment of methanogens with higher substrate utilization rates. With the release from feed sulfate toxicity, organic matter biodegradation and methanogenesis were both facilitated with heightened biodegradation of organics (*i.e.*, higher COD removal efficiency, lower COD accumulation) and better biomethane recovery efficiency. Therefore, the present study demonstrated that the high sulfate concentration in blackwater generated toxic impacts in not only the electron donor competition between SRB and methanogens but also the inhibition of the methanogenesis process by altering methanogenic consortia and suppressing their activities.

4.5. Conclusions

This study showed that conventional toilet flushed blackwater can be effectively treated under anaerobic condition using an UASB reactor at 35 °C, a COD removal efficiency of >80% was achieved with an HRT 2.2 days and an OLR 0.45 kg COD/m^3/d. The tap water flushed blackwater with a sulfate concentration of 79.9 (±4.7) mg/L negatively impacted the methanogenesis, with a low methane production rate of 58.0 (±6.9) mL/L/d, and the removal of flush water sulfate resulted in a higher methane production rate of 73.5 (±2.8) mL/L/d. Hydrogenotrophic methanogens and hydrogen utilizing SRB were enriched in the blackwater treatment processes, whereas the change of feed sulfate concentration resulted in the variation in microbial communities. Advantageous methanogens with higher substrate utilization rates from the genus *Methanospirillum* dominated the archaeal population under low feed sulfate concentrations while *Methanolinea* was the most abundant archaeal genus under high feed

sulfate conditions. Subsequently, an increase in SMA (H_2&CO_2) from 0.37 (±0.02) g CH_4-COD/g VSS/d (high feed sulfate condition) to 0.52 (±0.00) g CH_4-COD/g VSS/d (low feed sulfate condition) was observed.

Overall, this work demonstrated the impact of sulfate concentration on blackwater biomethane recovery. Studies may be required to assess methane recovery efficiency and biogas quality in future practical applications.

4.6 Supplementary materials

4.6.1 Wastewater properties

Feces and urine accounted for the majority of major ions and metals in feed, thus the source of the flushing water (*i.e.*, tap vs. DI water) had little impact on such concentrations (See Tables S4.1 & S4.2). Similarly, nutrient components NH_{4+}-N ranged from 107.5 (±3.2) mg/L to 113.6 (±2.8) mg/L, and TP ranged from 17.5 (±4.9) mg/L to 21.6 (±6.5) mg/L (Table 4.1) were comparable in tap and DI-water blackwater, indicating their major origination was from human excreta. The TSS and VSS concentrations in all operational phases were mostly correlated with the natural properties of feces and urine collected with values ranged 0.32 (±0.1)- 0.43 (±0.06) g/L and 0.29 (±0.09)- 0.42 (±0.07) g/L, respectively (Table 4.1). pH values were similar within a range of 7.7 (±0.1) - 7.9 (±0.1) with different flushing water used.

Table S4.1. Anion concentrations of Tap water and DI water flushed blackwater.

	Fluoride (mg/L)	Chloride (mg/L)	Nitrite (mg/L)	Bromide (mg/L)	Nitrate (mg/L)
Tap water flushed BW	22.5 (±7.5)	195.8 (±64.1)	48.1 (±19.2)	5.5 (±0.9)	6.8 (±6.8)
DI water flushed BW	18.9 (±1.3)	149.7 (±2.9)	34.7 (±2.9)	3.4 (±0.1)	5.5 (±0.5)

Table S4.2. Metal concentrations of Tap water and DI water flushed blackwater.

	Na (mg/L)	K (mg/L)	Ca (mg/L)	Zn (mg/L)	Al (mg/L)	Cu (mg/L)	Mn (mg/L)
Tap water flushed BW	129.12 (±43.55)	111.49 (±0.05)	61.18 (±1.19)	0.17 (±0.2)	0.16 (±0.03)	0.03 (±0.01)	0.02 (±0.00)
DI water flushed BW	121.08 (±41.44)	110.82 (±0.15)	15.74 (±0.51)	0.16 (±0.2)	0.09 (±0.0.2)	0.03 (±0.01)	0.02 (±0.00)

4.6.2 Microbial community diversity

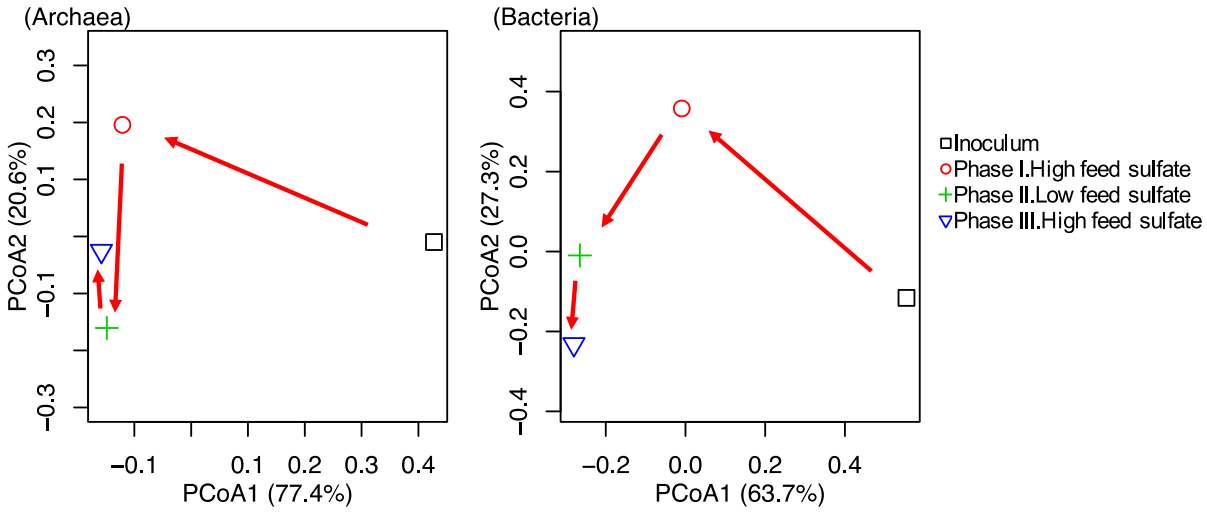

Fig. S4.1. Principal coordinate analysis (PCoA) of archaeal and bacterial communities of inoculum and reactor samples from operation Phase I (tap water flush, high feed sulfate), Phase II (DI water flush, low feed sulfate) and Phase III (tap water flush, high feed sulfate) computed using Bray-Curtis distance calculated using genus abundance data.

Table S4.3. Relative abundances (%) of sulfate reducing bacteria at family level.

Family	Inoculum	Phase I	Phase II	Phase III
Desulfobacteraceae	0.0	0.0	0.2	0.3
Desulfobulbaceae	0.0	2.5	0.7	1.1
Desulfomicrobiaceae	0.0	2.4	1.0	1.7
Desulfovibrionaceae	0.0	0.3	0.4	0.5
Desulfuromonadaceae	0.0	0.0	0.0	0.0
Desulfarculaceae	0.0	0.1	0.0	0.2

Table S4.4. Literature reported performances on conventional toilet blackwater anaerobic treatment.

Treatment system	UASB + membrane filter(van Voorthuizen et al., 2008)	UASB + septic tank (Luostarinen and Rintala, 2007)	UASB (Abdel-Shafy et al., 2009)	ABR (Bwapwa, 2012)	UASB (Gao et al., 2019a)
Blackwater source	5 L water flush toilet	Synthetic blackwater	Conventional flush toilet	Pit sludge	Conventional flush toilet
Temperature (°C)	37	20	Outdoor	Room	35
Reactor volume (L)	5	15	250	80	5
HRT (days)	0.5 day for UASB	5	1	3	1.4-5.5
OLR ($kgCOD/m^3/d$)	2.28	0.37-0.41	1.16	-	0.18-0.76
COD concentration (mg/L)	1,139	1,046 (±345)	1,160 (±391)	1,000-3,000	990 (±66)-1,076 (±5)
pH	7.2-7.4	5.8-6.6	7.4-8.3	8.9	7.4-7.9
BOD_5/COD	0.66	-	0.48	-	-
COD removal efficiency (%)	91% (64% from UASB)	91% (±4.6%)	68%	52-80%	72% (±6%)
Methanisation rate (%)	27%	20%*	-	28%	23-29%

*: calculated values.

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CHAPTER 5. ENHANCING BIOMETHANE RECOVERY FROM SOURCE-DIVERTED BLACKWATER THROUGH HYDROGENOTROPHIC METHANOGENESIS DOMINANT PATHWAY[3]

5.1. Introduction

Although only limited research has been performed to evaluate blackwater energy recovery due to the lack of available blackwater collection sites, the few reported studies have demonstrated the feasibility of applying anaerobic digestion processes for blackwater treatment and energy recovery. Various blackwater sources (e.g., high strength blackwater collected from vacuum toilets and low to medium strength blackwater collected from conventional or dual flush toilets), reactor types (e.g., upflow anaerobic sludge blanket [UASB], UASB-septic tank, continuous stirred tank reactor [CSTR], accumulation system [AC]), and various operating temperatures and operation conditions have been evaluated (Abdel-Shafy et al., 2009; Bwapwa, 2012; De Graaff et al., 2010; Elmitwalli et al., 2006; Gallagher and Sharvelle, 2011; Kujawa-Roeleveld et al., 2005; Kujawa-Roeleveld and Zeeman, 2006; Luostarinen et al., 2007; Luostarinen and Rintala, 2005; Moges et al., 2018; Sharma et al., 2016; Tervahauta et al., 2014; Wendland et al., 2007; Zeeman et al., 2008). However, the organic loading rates (OLR) achieved in these studies were low, and ranged from ~1.4 kg COD/m^3/d (for high strength vacuum toilets collected blackwater) (Zeeman et al., 2008) to ~2.3 kg COD/m^3/d (for low to medium strength blackwater) (van Voorthuizen et al., 2008), which resulted in long hydraulic retention time (HRT) or large reactor volume (large footprint) required for blackwater treatment. The relatively low OLRs achieved in comparison to other high organics waste streams (e.g., OLR up to 19.4 kg COD/m^3/d for dairy wastewater (Rico et al., 2015)) may be attributed to the limited hydrolysis for the high solid COD fraction (Wendland et al., 2007) and potential free ammonia inhibition in blackwater anaerobic treatment systems (De Graaff et al., 2010; Gao et al., 2019). Our recent studies have demonstrated that under the mesophilic conditions (35 °C), anaerobic digestion of vacuum collected blackwater was significantly inhibited by free ammonia (> 400 mg/L for 1L per flush vacuum collected blackwater) (Gao et al., 2019).

[3] A version of this chapter has been published: Gao, M., Zhang, L., Guo, B., Zhang, Y., Liu, Y., 2019c. Enhancing biomethane recovery from source-diverted blackwater through hydrogenotrophic methanogenesis dominant pathway. Chem. Eng. J. 378, 122258.

To maximize energy recovery from source-separated wastewater treatment and to enable more effective resource management from blackwater, optimization of the blackwater treatment processes through reducing the potential process inhibition is essential. Therefore, the current study challenged the maximum organic loading rate (OLR) for anaerobically treating vacuum toilet collected blackwater using an UASB reactor operated under mesophilic condition (35 °C) and acclimatized the microbial community by gradually increasing the OLR. UASB was selected based on its proven effectiveness in blackwater treatment (De Graaff et al., 2010), and its capability of treating high strength wastewater at high organic loading conditions (Rico et al., 2015). For the first time, microbial communities involved in the biodegradation processes and the microbial structure development through the various OLR conditions were evaluated. Correspondingly, the dominated methanogenic pathways throughout long-term (~304 days) operation were identified.

5.2. Methods and materials

5.2.1 Feedstock and reactor operation

Vacuum toilet collected blackwater (1 L flushing water per flush) used in this study was obtained from the University of Alberta campus (Edmonton, Canada) weekly over a two-day collection period, and stored at 4 °C until use. Raw blackwater was filled into a continuously stirred influent storage tank (storage time of 0.5 d) under room temperature condition (20 °C). Blackwater in the influent storage tank (UASB influent) was then pumped into a 5 L (working volume 4.7 L) upflow anaerobic sludge blanket (UASB) reactor (Fig. S5.1) with a peristaltic pump (Longer pump BT 100-2J).

The UASB reactor was operated at 35 °C with a heating blanket. The reactor inoculum at the beginning of the reactor operation was obtained from an anaerobic digester treating primary sludge at a local wastewater treatment plant. The produced biogas was collected with gas bags at the top of the reactor. The effluent was sampled from the top side ports of the reactor and was further characterized. The sludge bed height inside the UASB reactor was monitored throughout the reactor operation period to calculate the sludge volume inside the reactor. Reactor operational conditions are shown in Table 5.1. The OLRs were increased through increasing the influent

flow rate. Reactor operation lasted for 304 days, which is divided into seven Phases (from Phase I to VII), with varying OLR (0.28-4.87 kg COD/m^3/d) and HRT (2.08-36.42 days).

The steady state for each operation condition (OLR) was indicated by relatively stabilized COD removal efficiencies, methane productions and effluent properties obtained. The steady state was counted at least one week after each OLR change, and the steady state durations for each operation Phase lasted differently from 21 to 51 days, respectively.

Table 5.1. Operational parameters for UASB reactor treating vacuum toilet blackwater.

	Phase I	Phase II	Phase III	Phase IV	Phase V	Phase VI	Phase VII
Operation period (day)	Day 1-43	Day 44-88	Day 89-115	Day 116-171	Day 172-227	Day 228-269	Day 270-304
OLR (kg COD/m^3/d)	0.28	0.48	0.81	1.64	3.09	4.87	4.07
HRT (days)	36.42	20.24	11.84	6.28	3.34	2.08	2.56

5.2.2 Batch assays

Biological methane potential (BMP) test for blackwater

The BMP test in this study was performed to evaluate the maximum amount of methane that can be produced from the influent blackwater, and is expressed as the ratio of feed blackwater COD that can be converted to methane COD (g CH_4-COD/g influent COD_t). BMP tests followed previously reported procedures (Gao et al., 2019). All tests were conducted in triplicates.

Specific methanogenic activity (SMA) and stability test for UASB sludge

The batch assays were performed to determine specific methanogenic activity (SMA) and sludge stability of the anaerobic sludge accumulated in the UASB reactor sludge bed under all operation Phases. For each assay, UASB sludge was collected from all side ports, mixed and characterized for COD concentration, total suspended solids (TSS) and volatile suspended solids (VSS) concentrations before the batch experiments.

SMA represents the maximum methane production rate that the sludge can perform, and the results were expressed as g CH_4-COD/g sludge VSS/d in this study. For each group of SMA tests, batch serum bottles were filled with UASB mixed sludge and substrate (sodium acetate or H_2&CO_2). When acetate was applied, the initial substrate COD concentration was 1 g/L. The headspace of the serum bottles was flushed with nitrogen gas to achieve anaerobic conditions. When H_2&CO_2 was applied as substrate, a ratio of 80%/20% of H_2/CO_2 content was applied to flush the headspace of the test bottles. After flushed with gases, the bottles were then sealed with rubber septums and aluminum caps, incubated at 35 °C, and shaken at 130 rpm in the dark with an incubator shaker (New Brunswick™ Innova® 44, Eppendorf, Canada). Measurements of methane production were achieved through the measurement of headspace pressure using a pressure meter (GHM 3151, Germany) and the composition of biogas using gas chromatography (GC) (7890B Agilent Technologies, USA). SMA tests were performed in triplicates under steady state conditions of each operation Phase. The SMA values were calculated from dividing the initial linear slope of the methane production curve over the sludge VSS in each bottle. Blank controls with only sludge were set-up simultaneously to subtract the methane production from sludge in the test group. The SMA (acetate and H_2 & CO_2) results were presented with average values of the triplicates as g CH_4-COD/g sludge VSS/d with standard deviations in each operational Phase.

The sludge stability test was conducted using serum bottles with UASB mixed sludge at 35 °C. The set-up procedure for the sludge stability test was the same as that described in the BMP batch test. Methane production was monitored, and stability tests were stopped when no more methane production can be detected. Sludge stability tests evaluate the amount of biodegradable substrate present in the sludge and are expressed as g CH_4-COD/g sludge COD. Higher values indicate more biodegradable organics present in sludge, which represents less sludge stability.

5.2.3 DNA extraction and sequence analysis

Inoculum and the UASB sludge collected at the end of each operation Phase (Table 5.1) were used for genomic DNA extraction. 1.5 mL of each sludge sample was first centrifuged at 3,000 g for 10 min. The pellet was used for DNA extraction with DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. All DNA samples were extracted with

fresh sludge samples in duplicates. The quality of DNA samples was checked through NanoDrop One (ThermoFisher, Waltham, MA) and the DNA samples were stored at -20 °C until downstream analysis was performed. PCR was performed followed by the sequencing experiment on the Illumina MiSeq platform. 16S rRNA genes of the representative clones were sequenced to determine bacteria and archaea communities by RTL Genomics (Texas, USA). Bacterial 16S rRNA genes were amplified using the universal primer-pair *357wF* (*CCTACGGGNGGCWGCAG*) and *785R* (*GACTACHVGGGTATCTAATCC*). Archaeal 16S rRNA genes were amplified using universal primer set *517F* (*GCYTAAAGSRNCCGTAGC*) and *909R* (*TTTCAGYCTTGCGRCCGTAC*).

The raw sequences were processed using Qiime2 pipelines (Caporaso et al., 2010) DADA2 algorithm (Callahan et al., 2016), with the paired-end reads data of forward and reverse sequences achieved, and the low-quality sequences and chimeras removed. Taxonomy was assigned using 97% similarity in GreenGenes (version 13_8) reference database (McDonald et al., 2012; Werner et al., 2012).

5.2.4 Sampling and analysis

The wastewater characteristics including total COD (COD_t), suspended COD (COD_{ss}), colloidal COD (COD_{col}), soluble COD (COD_s), total suspended solids (TSS), volatile suspended solids (VSS), total dissolved solids (TDS), total ammonia nitrogen (TAN), total phosphorous (TP), reactive phosphorous (PO_{43}-P), volatile fatty acids (VFAs) and pH were routinely measured. All COD, TSS, VSS, and TDS measurements followed the procedure described in the standard methods from American Public Health Association (APHA/AWWA/WEF, 2012). TAN, TP, and PO_{43}-P were measured using Hach TNT vial tests (Hach, USA). Volatile fatty acids including acetate, propionate, and butyrate were measured using Ionic chromatography (IC) (DIONEX ICS-2100, ThermoFisher, USA). pH was measured with B40PCID pH meter (VWR, SympHony). The characterization of raw blackwater, UASB influent and UASB effluent was conducted three to five times a week depending on the reactor operation Phase and performance. Raw blackwater characterization was performed immediately after fresh blackwater preparation, and the UASB influent was characterized after 0.5-day storage in the blackwater influent tank (Section 5.6). The UASB influent characteristics shown were the results of average values

adopted for each operation Phase (Fig. 5.1A, error bars represent one standard deviation) and for the whole operation duration (Table 5.2), respectively. Characterization of UASB wasted sludge including COD, TSS, VSS, TAN, TP, and pH were conducted one to two times in each operation Phase with methods described above. The results of sludge TSS and VSS concentrations were presented as average values obtained (error bars represent one standard deviation) from all measurements counted within each operation Phase. Biogas production amount was measured daily through the gas bag collection. The concentration of methane and carbon dioxide were measured through GC (7890B Agilent Technologies, USA).

5.2.5 Calculations

All the calculations for average values and standard deviations of the COD mass balance, hydrolysis efficiencies, methane production rates and methanisation rates were obtained from the steady state data.

Solids retention time (SRT)

The theoretical SRT in different operation Phases were calculated based on the VSS concentration inside the UASB reactor and the amount of solids discharged and washed out (in VSS) from the reactor effluent. When the theoretical calculated SRT was longer than the operation duration, the operation duration was used as the operational SRT (Metcalf and Eddy, 2003).

COD mass balance

In each stable operation Phase, COD mass balance was determined, which assumes that the amount of influent COD was partitioned into: COD for methane production, COD accumulation in sludge bed (including discharged sludge and reactor sludge bed), and effluent COD. The COD balances were calculated from steady state COD loads and distributions (Wendland et al., 2007).

Methanogenic capacity

The methanogenic capacity of the UASB reactor was calculated based on the SMA of the sludge and the amount of VSS in the reactor. The reactor methanogenic capacity was calculated by

multiplying each SMA (H_2/CO_2) value obtained (2.2.2) by its corresponding VSS in UASB, and presented with average values (and standard deviations) adopted within each operation Phase. The methanogenic capacity of each operation Phase was compared with the detected methane production and the total feedstock input.

Solids COD hydrolysis efficiency

The solids COD hydrolysis efficiency refers to the percentage of hydrolyzed solids COD fraction of the influent blackwater, the influent solids COD refers to the sum of COD_{ss} and COD_{col}, which is defined as COD_{solids} in the present study. The hydrolysis efficiency of COD_{solids} can be determined through the following equation (De Graaff et al., 2010):

$$Hydrolysis\ efficiency\ of\ COD_{solids}(\%) = \frac{COD_{CH4} + COD_{s,effluent} - COD_{s,influent}}{COD_{t,influent} - COD_{s,influnet}}$$

Methane production

Methane production rate

Methane production rate shows the net amount of methane generated from the reactor daily, and represented as m^3 CH_4/ m^3 reactor/d.

Methanisation rate

The methanisation rate indicates the amount of methane (as COD) that converted from the influent blackwater COD, represented as g CH_4-COD/g influent COD_t.

5.2.6 Statistical analysis

One-way analysis of variance (ANOVA) was performed using Microsoft Excel® on comparisons of COD concentrations between raw blackwater and the UASB influent, and COD removal efficiencies between different operation Phases. A p-value less than 0.05 was considered statistically different.

5.3. Results

5.3.1 Feedstock characteristics

Table 5.2. Characteristics for UASB influent and UASB effluent.

	Unit	UASB influent	UASB effluent						
			Operation Phase I	Operation Phase II	Operation Phase III	Operation Phase IV	Operation Phase V	Operation Phase VI	Operation Phase VII
COD_t	mg/L	10,977 (±903)	1,140 (±147)	993 (±93)	1,175 (±106)	1,598 (±374)	1,694 (±500)	1,809 (±511)	1,424 (±299)
COD_{ss}	mg/L	7,019 (±897)	175 (±66)	154 (±61)	355 (±75)	458 (±232)	712 (±514)	632 (±295)	445 (±176)
COD_{col}	mg/L	490 (±103)	406 (±102)	267 (±45)	222 (±32)	320 (±144)	354 (±44)	346 (±57)	304 (±57)
COD_s	mg/L	3,482 (±204)	559 (±33)	571 (±43)	599 (±7)	820 (±137)	627 (±42)	686 (±38)	679 (±61)
TSS	g/L	4.93 (±0.73)	0.21 (±0.11)	0.10 (±0.04)	0.14 (±0.03)	0.19 (±0.07)	0.20 (±0.09)	0.50 (±0.16)	0.36 (±0.26)
VSS	g/L	4.68 (±0.73)	0.14 (±0.04)	0.09 (±0.04)	0.11 (±0.03)	0.16 (±0.06)	0.18 (±0.08)	0.39 (±0.19)	0.32 (±0.23)
pH		8.05 (±0.24)	7.87 (±0.19)						
Acetate	mg/L	884 (±24)	30 (±23)						
Propionate	mg/L	418 (±26)	32 (±11)						
Butyrate	mg/L	192 (±7)	4 (±3)						
NH_{4+}-N	mg/L	1,137.0 (±51.8)	1,218.0 (±72.7)						
FA	mg/L	154.6 (±86.4)	114.7 (±42.0)						
TP	mg/L	114 (±27)	94 (±35)						
PO_{43-}-P	mg/L	30 (±12)	41 (±22)						
TDS	g/L	2.83 (±0.48)	2.27 (±0.38)						

The average compositions of the UASB influent and the UASB effluent through the whole operational period are shown in Table 5.2. Raw vacuum toilet flushed blackwater has a pH of 8.62 (±0.06), COD_t of 9,985 (±714) mg/L and NH_{4+}-N concentration of 1,115.1 (±78.9) mg/L and the fraction of COD_{ss}, COD_{col} and COD_s accounted for 61%, 7%, and 33% of blackwater COD_t, respectively (Table S5.1).

Compared to the raw blackwater, the UASB influent (after 0.5 d storage in the influent tank) has similar COD concentrations ($p > 0.05$), higher VFA (~15% increase in acetate and propionate and ~18% increase in butyrate), and lower pH, which is attributed to the blackwater storage in the influent tank (blackwater feed storage time optimization is shown in section 5.6). As a result of

VFA increase and the associated pH decrease (pH dropped from 8.62 to 8.05), blackwater free ammonia concentration was reduced from the initial 431.3 (±44.5) mg/L to 154.6 (±86.4) mg/L, which helps to reduce free ammonia inhibition on blackwater digestion (Gao et al., 2019).

5.3.2 Performance of the UASB reactor

Fig. 5.1 shows the influent and effluent COD_t concentration and the methane production in various operation Phases with different HRT and OLR. One week after the reactor start-up, COD_t removal of ~80% and methanisation rate of 0.43 g CH_4-COD/g influent COD_t were achieved. After two weeks of operation, an average COD_t removal reached 87.0% (±0.8%), and the reactor operation was stable for the rest of the reactor start-up Phase. The change of HRT and OLR did not significantly impact COD_t removal in the first three operation Phases. In Phase II (OLR=0.48 kg COD/m^3/d) and III (OLR=0.81 kg COD/m^3/d), COD_t removal efficiency was 90.4% (±1.1%) and 87.3% (±4.1%), respectively. The COD_t removal efficiency started to decrease in Phase IV (OLR=1.64 kg COD/m^3/d), with an average of 83.5% (±3.8%) COD_t removal. Sludge wash-out was observed in this Phase, which led to an increase in effluent COD concentration from 1,175 (±106) mg/L (Phase III) to 1,598 (±374) mg/L (Phase IV) (Table 5.2). The COD_t removal efficiency was maintained at 85.0% (±3.9%) in Phase V (OLR=3.09 kg COD/m^3/d), which was then decreased to 81.4% (±4.4%) and 83.6% (±4.9%) respectively in the final two Phases with the high OLR (Phase VI 4.87 kg COD/m^3/d and Phase VII 4.07 kg COD/m^3/d, respectively) conditions. A substantial sludge wash-out was observed in Phase VI and the beginning of phase VII, which led to a poor effluent quality with an average COD concentration of 1,809 (±511) mg/L (Phase VI) (Table 5.2). The effluent quality recovered after the OLR decreased to 4.07 kg COD/m^3/d in Phase VII.

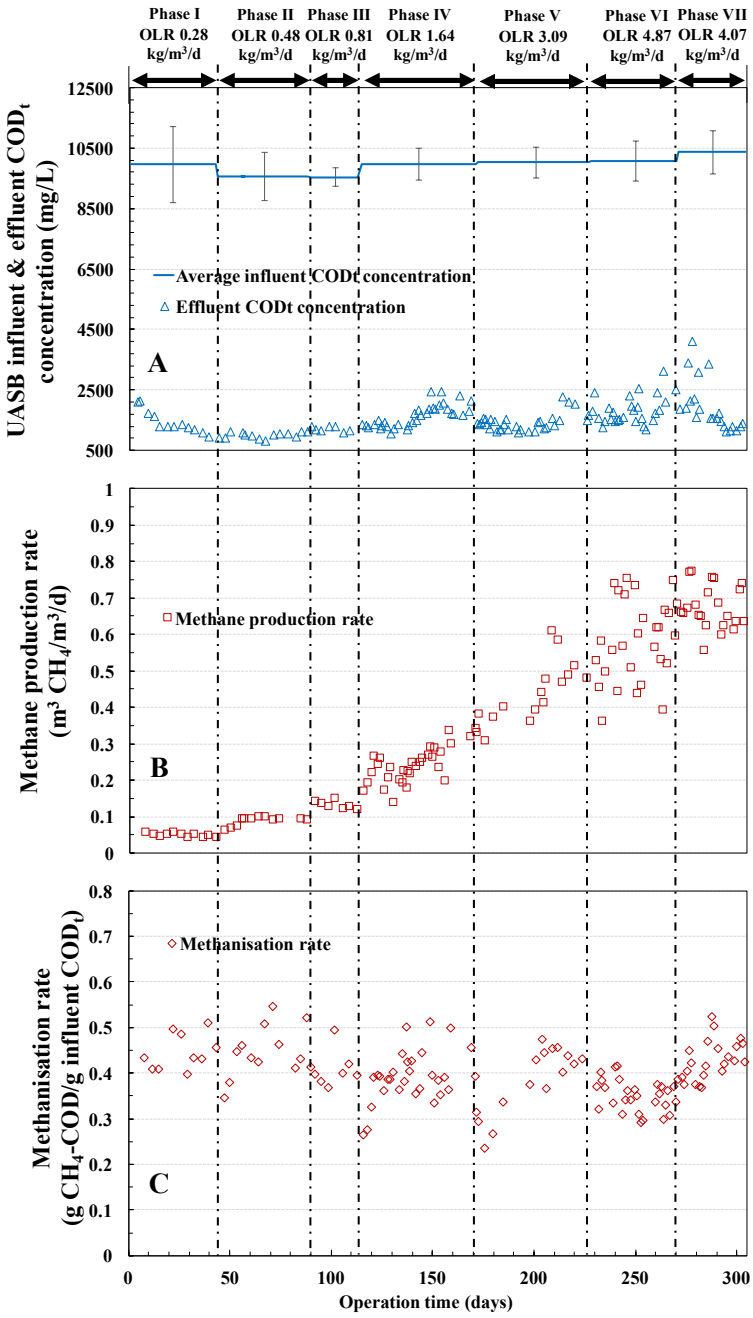

Fig. 5.1. Performance of UASB reactor treating vacuum toilet blackwater with step-wise increasing organic loading rates (OLR) and decreasing hydraulic retention time (HRT) at 35 °C. A. Influent and effluent COD_t concentrations (mg/L); B. Methane production rate (m_3 $CH_4/m_3/d$); C. Methanisation rate (g CH_4-COD/g influent COD_t). Error bars represent one standard deviation.

During the first five Phases (Phases I-V with OLR=0.28-3.09 kg COD/m^3/d), reactor operation was stable. As shown in Fig. 5.1, the methanisation rate ranged from 0.41 to 0.48 g CH_4-COD/g influent COD_t, which was comparable to the feedstock BMP values (0.48 g CH_4-COD/g influent COD_t, section 5.6), indicating effective methanogenesis with no free ammonia inhibition inside the UASB reactor. The methane production rate increased from 0.05 (±0.00) to 0.49 (±0.07) m^3 CH_4/ m^3 reactor/d (Phases I-V). The influent COD_s removal ranging from 76.7% (±3.9%) to 84.6% (±0.3%), COD_{col} removal ranging from 49.0% (±20.3%) to 73.0% (±4.6%), and COD_{ss} removal ranging from 84.4% (±5.4%) to 97.1% (±1.0%) were achieved (Table 5.2). Further, as shown in Fig. 5.2, COD_{solids} hydrolysis efficiency was stable, ranging from 29.5% (±5.2%) to 33.2% (±5.0%). However, when the influent OLR was further increased to 4.87 kg COD/m^3/d in Phase VI (HRT=2.08 days), significant sludge loss was observed, leading to a lowered theoretical SRT, which might also contribute to the the insufficient hydrolysis of COD_{solids}, suspended COD accumulation in the reactor, and subsequent reduction in the substrate methanisation rate, as demonstrated in Fig. 5.2. Similar observations have been reported previously (De Graaff et al., 2010; Wendland et al., 2007). As shown in Fig. 5.1 and Fig. 5.2, the methanisation rate dropped to 0.35 g CH_4-COD/g influent COD_t with methane production rate of 0.60 (±0.11) m^3 CH_4/ m^3 reactor/d, and the COD_{solids} hydrolysis efficiency was significantly reduced to 16.8% (±3.4%). The influent COD_{col} removal was found decreased to 17.1% (±3.9%); while the removal of average influent COD_s (77.7%±2.8%), and COD_{ss} (90.7%±4.5%) were not significantly affected compared with previous operational Phases ($p > 0.05$). In the last Phase, OLR was decreased to 4.07 kg COD/m^3/d and the HRT was increased to 2.6 days. With a reduced organic loading rate, it was observed that the COD_{solids} hydrolysis efficiency recovered back to 28.7% (±2.5%) (Fig. 5.2) and COD_{col} removal increased back to 63.4% (±7.5%) (Table 5.2). This observation correlates to the increase in the total COD reduction (84%), and the methanisation rate of 0.44 (±0.04) g CH_4-COD/g influent COD_t with methane production rate of 0.68 (±0.08) m^3 CH_4/ m^3 reactor/d (Fig. 5.1), indicating a successful system recovery.

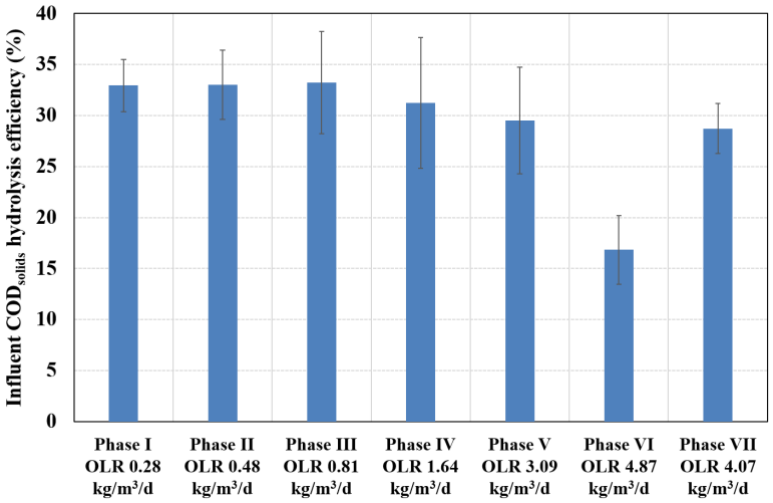

Fig. 5.2. The influent COD_{solids} hydrolysis efficiency of UASB reactor treating vacuum toilet blackwater at different organic loading rates (OLR). Error bars represent one standard deviation.

The three main volatile fatty acids (VFA) concentrations in the effluent of the UASB reactor through the whole operational period were monitored, and the results are shown in Fig. 5.3. Soon after the reactor start-up, total effluent VFA concentrations decreased to below 200 mg/L and were kept at a low level throughout the operational time. The effluent total VFA concentration of 66.1 (±33) mg COD/L observed in this study was within the range of commonly reported anaerobic digestion effluent VFA values (20-150 mg COD/L) for treating high strength blackwater (De Graaff et al., 2010; Tervahauta et al., 2014; Wendland et al., 2007). The observed low VFA concentration correlated well with the effective methane production observed in this study, suggesting that the produced VFA from the fermentation step can be utilized effectively by methanogens, and methanogenesis was not the limiting step in this treatment system (Murto et al., 2004). Increases of the VFA concentrations (mainly acetate) were occasionally observed, which were often accompanied by either a sudden increase in organic loading rates or sludge wash-out from the reactor. Nevertheless, our detected total VFA concentrations were always below VFA inhibition thresholds reported in blackwater treatments (e.g. 590-2,600 mg/L (De Graaff et al., 2010; Wendland et al., 2007)).

As shown in Table 5.2, along with the COD_t removal, relatively high TSS removal efficiencies of 95.1% (±3.2%)- 98% (±0.9%) were achieved in Phase I-V (OLR=0.28-3.09 kg COD/m_3/d).

The amount of effluent TSS increased under high OLR condition in Phase VI (OLR=4.87 kg COD/m3/d), which may be attributed to sludge wash out, leading to decreased TSS removal efficiency (86.1%). The TSS removal started to recover in Phase VII (90.7%) with a lower OLR 4.07 kg COD/m3/d applied. The overall high solids removal efficiency may be attributed to the UASB sludge bed, which not only provided microorganisms for biodegradation, but also enabled solids entrapment. The UASB effluent pH was maintained at 7.87 (±0.19), which was similar to the reported values in literature (De Graaff et al., 2010). Extra ammonium was released during the digestion process with a slight increase in effluent NH_4^+-N (~1,218 mg/L in effluent, as compared to ~1,137 mg/L in influent) observed, which can be explained by the hydrolysis of blackwater protein content. As a result, the free ammonia concentration was 115 mg/L, which was lower than the blackwater free ammonia inhibition level reported previously (Gao et al., 2019).

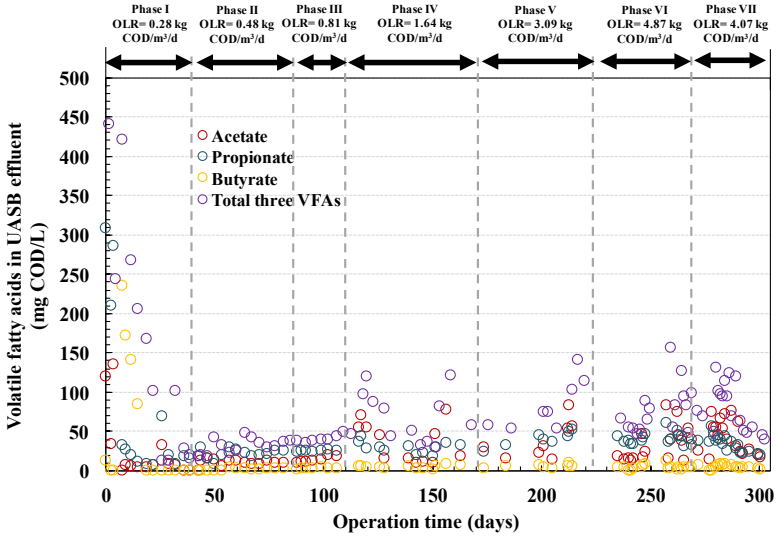

Fig. 5.3. Volatile fatty acids concentrations in UASB effluent at various reactor operation Phases. Acetate, propionate and butyrate concentrations in UASB effluent were presented as mg COD/L.

5.3.3 COD mass balance in UASB reactor

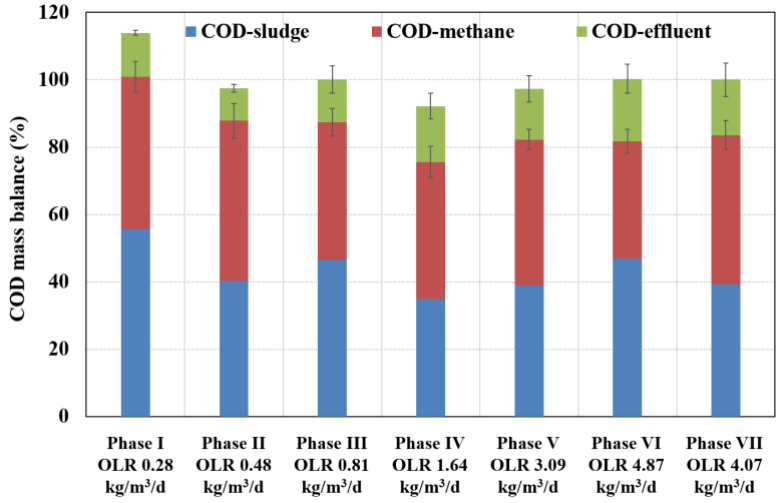

Fig. 5.4. COD mass balance of UASB reactor treating vacuum toilet blackwater at different organic loading rates (OLR), the partition of influent COD considered includes COD accumulation in sludge (sludge bed and discharged sludge), COD for methane production and effluent COD. Error bars represent one standard deviation.

The COD mass balance of the UASB reactor is displayed in Fig. 5.4. The partition of the influent COD_t includes (i) COD_t converted to methane, (ii) retained in sludge bed and discharged sludge, and (iii) COD in UASB effluent. As shown in Fig. 5.4, the sum of the detected COD counted for 92-114% of the total input COD, indicating satisfactory development of COD balance (Zhang et al., 2018). From Phases I-V (OLR=0.28-3.09 kg COD/m^3/d), with relatively high and stable COD_t removal (average 87%), the effluent COD only accounted for ~13% of influent COD_t, the methane production accounted for ~43% of influent COD_t, and the COD accumulation in sludge (wasted and accumulated) accounted for ~ 43%. Compared to the first five Phases, the amount of COD distributed in methane production decreased to 35%, along with the increased effluent COD of 19% and accumulated COD of 47% in Phase VI (OLR=4.87 kg COD/m^3/d), which, as discussed above, can be attributed to the reduced solids substrate hydrolysis efficiency in this Phase. In Phase VII, with a reduction in OLR to 4.07 kg COD/m^3/d, methane COD production increased to 44%, together with a decreased COD accumulation in sludge of 39%, and a reduced COD in reactor effluent (16%), suggesting a stable and satisfactory reactor operation.

5.3.4 Sludge bed development

The sludge TSS, VSS, VSS/TSS ratio, SMA and methanogenic capacity in various operation Phases are shown in Fig. 5.5. The sludge TSS and VSS concentrations (Fig. 5.5A) increased from the initial inoculum 20.7 (±0.9) g/L and 13.1 (±0.6) g/L, to 43.5 (±4.1) and 34 (±3.5) g/L in Phase V (OLR=3.09 kg COD/m^3/d), respectively. TSS and VSS further increased to 48.1 (±4.8) g/L and 37.5 (±3.1) g/L respectively, in Phase VI (OLR=4.87 kg COD/m^3/d), which may be explained by the COD_{ss} accumulation in the sludge bed. The VSS/TSS ratio was high at 0.73-0.82 through the whole operation period, indicating the high organic contents.

Further, as shown in Fig. 5.5B, SMA values obtained from acetate or H_2&CO_2 were significantly different throughout the reactor operation. Overall, SMA (H_2&CO_2) was greater than SMA (acetate), indicating the dominance of hydrogenotrophic methanogenic activities. SMA (H_2&CO_2) increased from 0.34 (±0.01) g CH_4-COD/g VSS/d in Phase I (OLR=0.28 kg COD/m^3/d) to 0.49 (±0.01) g CH_4-COD/g VSS/d in Phase V (OLR=3.09 kg COD/m^3/d). SMA (H_2/CO_2) reduced to 0.34 (±0.01) g CH_4-COD/g VSS/d under high loading conditions in Phase VI (OLR=4.87 kg COD/m^3/d) and 0.35 (±0.03) g CH_4-COD/g VSS/d in Phase VII (OLR=4.07 kg COD/m^3/d). The decreased SMA (H_2&CO_2) in phase VI can be attributed to the decreased substrate hydrolysis efficiency that led to higher amount of organics accumulated in sludge bed (with increased sludge VSS). In comparison, SMA (acetate) values were maintained in the range of 0.1-0.11 g CH_4-COD/g VSS/d in Phases I-IV (OLR=0.28-1.64 kg COD/m^3/d), which was then increased to 0.18 (±0.01) g CH_4-COD/g VSS/d in Phase V. Further SMA (acetate) increases in Phase VI (0.27 (±0.04) g CH_4-COD/g VSS/d) and VII (0.23 (±0.05) g CH_4-COD/g VSS/d) were observed, which may be attributed to the enhancement in syntrophic acetate oxidation (SAO) pathway with increased OLR that provided more readily available substrates as compared to the first four Phases.

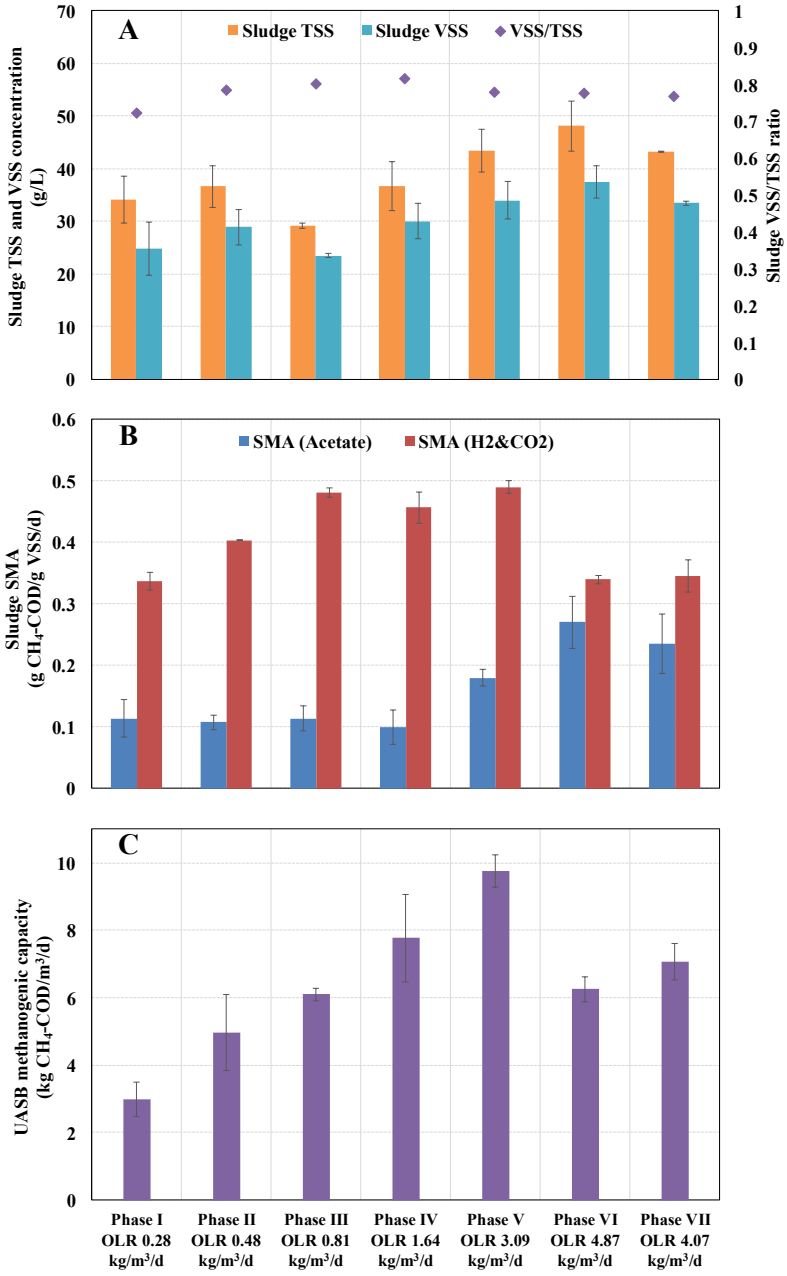

Fig. 5.5. Sludge properties for UASB reactor treating vacuum toilet blackwater at different organic loading rates (OLR). A. Sludge total suspended solids (TSS), volatile suspended solids (VSS) concentrations in g/L and VSS/TSS ratio in each operation phase; B. Sludge specific methanogenic activity (SMA) with acetate or H_2&CO_2 as substrate, represented as g CH_4-COD/g VSS/d; C. The methanogenic capacity of UASB reactor in each operation Phase, represented as kg CH_4-COD/m_3/d. Error bars represent one standard deviation.

Further, as shown in Fig. 5.5C, the methanogenic capacity varied from 2.98 (±0.5) kg CH_4-COD/m^3/d in Phase I to 7.07 (±0.5) kg CH_4-COD/m^3/d in Phase VII. The methanogenic capacity was the highest in Phase V (9.76 (±0.5) kg CH_4-COD/m^3/d) with an OLR of 3.09 kg COD/m^3/d. The relatively high methanogenic capacity observed in this Phase can be attributed to the relatively high SMA (H_2&CO_2) (0.49) achieved and the high VSS in the reactor that was capable to be maintained in the system when compared with higher OLR conditions (OLR=4.87 and 4.07 kg COD/m^3/d). As compared to Phase V, the decreased methanogenic capacity of 6.25 (±0.4) kg CH_4-COD/m^3/d in Phase VI was due to the reduced sludge SMA (H_2&CO_2) and the reduced sludge bed volume, which started to recover with the lower OLR applied in Phase VII. Throughout the operation, all the applied OLRs never reached the reactor methanogenic capacity. This was also verified by the fact of no VFA accumulation in the UASB effluent.

5.3.5 Microbial community analysis

Bacterial community structure

The results of the relative abundance of bacterial communities in inoculum, and UASB reactor Phase I, III, IV, and VII (OLR = 0.28, 0.81, 1.64 and 4.07 kg COD/m^3/d, respectively) are presented in Fig. 5.6. The given groups were at least with 1% relative abundance in the whole community structure. Overall, the bacterial domain was diverse under all investigated conditions and can be assigned to 12 phyla. The predominant phyla in the inoculum included Bacteroidetes (34.5%), Cloacimonetes (23.2%), Chloroflexi (12.5%) and Proteobacteria (9.5%), while the dominant phyla were shifted to Bacteroidetes (26.4-45.7%), Firmicutes (22.3-31.3%) and Proteobacteria (5.0-26.5%) in the backwater UASB. In particular, after a slight decrease in Phase I, the abundance of Bacteroidetes increased to 33.0% (Phase III), 45.7% (Phase IV) and 41.2% (Phase VII) with increasing OLR, and became the most dominant group in these Phases. It has been reported that phyla Bacteroidetes contain hydrolysis/fermentative bacteria that convert carbohydrates and proteins into acids (Kampmann et al., 2012), which have been found to dominate in anaerobic systems treating carbon rich substrates, e.g., food wastes (Lim et al., 2013). Further, phyla Firmicutes was also drastically enriched in the UASB reactor and was shown to be the second predominant phyla (22.3-31.3%) in the whole blackwater treatment period. Phyla Firmicutes has been reported to produce extracellular enzymes (e.g., proteases, lipases and cellulases) for the hydrolysis of protein, lipids and carbohydrates, and assist in acid

production in the fermentation process (Lim et al., 2013). Both Bacteroidetes and Firmicutes have been found in the human gut and rumen (Kampmann et al., 2012), which may explain their origin and enrichment in the UASB reactor. The relative abundance of phyla Cloacimonetes and Chloroflexi decreased to 3.0-6.4% and 2.6-6.0%, respectively, in the reactor. The abundance of phyla Proteobacteria was enriched in Phase I (26.5%), but then decreased to 5.0-8.7% at higher OLR conditions. Phyla Proteobacteria was reported with cellulolytic activity and is capable in degrading various types of carbohydrates and proteins (Cardinali-Rezende et al., 2011). Our current results demonstrate that phyla Proteobacteria was more preferable under low OLR conditions. A similar observation has been reported previously (Gao et al., 2019).

Community shifts at the genus level were also observed. The genus *Blvii28 wastewater-sludge group* (13.8%), *Candidatus Cloacimonas* (13.4%), uncultured genus from family *Anaerolineaceae* (9.7%) and uncultured genus from order Cloacimonadales (8.7%) contributed most in the inoculum. However, none of these genera were enriched in the blackwater reactor. *Blvii28 wastewater-sludge group* and *Candidatus Cloacimonas* were both reported as mesophilic acetogens in sewage sludge anaerobic digestion systems (Lee et al., 2018). The dominant genus included *Koukoulia* (21.7%), unknown genus from order Cloacimonadales (4.3%) and *Bacteroides* (4.2%) in Phase I; genus *Fibrobacter* (14.5%), uncultured genus from family *Clostridiales vadinBB60 group* (12.4%), and *Bacteroides* (6.1%) in Phase III; *Bacteroides* (21.2%), *Erysipelotrichaceae UCG-004* (7.8%) and *Fibrobacter* (6.5%) in Phase IV and *Bacteroides* (28.3%) in Phase VII. Overall, genus *Bacteroides* was found to be predominant under relatively high OLR conditions, demonstrating its robustness under such conditions. Previous studies showed that genus *Bacteroides* often inhabit the human intestine and function in carbohydrate transportation and protein metabolism (Karlsson et al., 2011). Further, *Fibrobacter* in Phase III (HRT 12 days, OLR 0.81 kg COD/m^3/d) has been reported to be essential for cellulose hydrolysis and are often found in herbivore gut (McDonald et al., 2009).

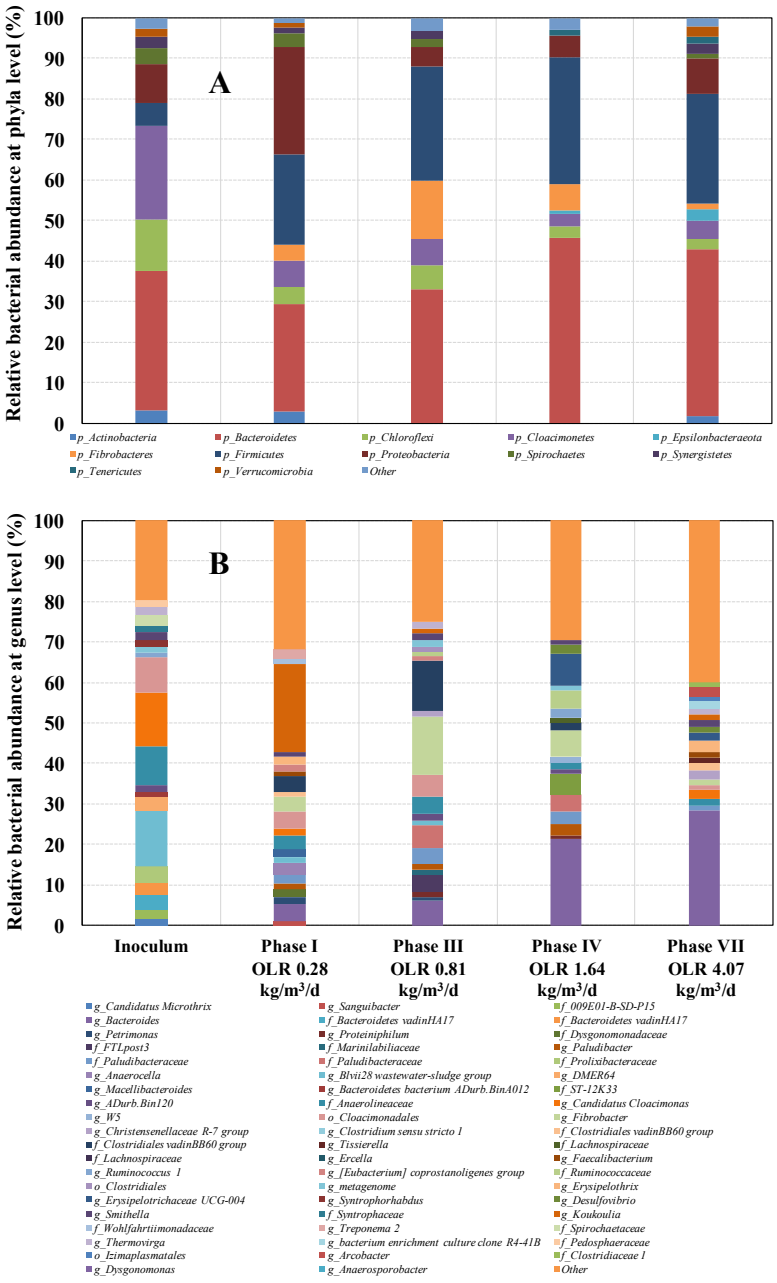

Fig. 5.6. Relative abundance of bacterial taxonomic groups of inoculum, and UASB reactor sludge in Phase I (OLR of 0.28 kg COD/m3/d, HRT of 36 days), Phase III (OLR of 0.81 kg COD/m3/d, HRT of 12 days), Phase IV (OLR of 1.64 kg COD/m3/d, HRT of 6.3 days), and Phase VII (OLR of 4.07 kg COD/m3/d, HRT of 2.6 days). A. Phyla level; B. Genus level.

Archaeal community structure

The results of the relative abundance of archaeal communities in inoculum, and UASB reactor in Phases I, III, IV, and VII (OLR=0.28, 0.81, 1.64 and 4.07 kg COD/m3/d, respectively) are presented in Fig. 5.7. The given groups contribute to at least 1% relative abundance in the community. Overall, more than 97% of the detected methanogens can be assigned to hydrogenotrophic methanogens in both inoculum and the UASB reactor. In the inoculum, the predominant methanogens were genus *Methanospirillum* (52.7%), *Methanolinea* (38.2%), and *Methanoculleus* (6.3%). In Phase I, the dominant methanogen groups were similar to those in the inoculum, including *Methanolinea* (49.6%), *Methanospirillum* (37.2%), and *Methanoculleus* (9.3%). The methanogenic species shifted significantly in Phases III, IV, and VII, with a significantly increased abundance of genus *Methanogenium* (52.0-61.2%), relatively lower abundance of genus *Methanolinea* (19.9-33.7%), and a significantly reduced abundance of *Methanospirillum* (4.5-11.8%) and *Methanoculleus* (3.6-7.4%), as compared to the Phase I community.

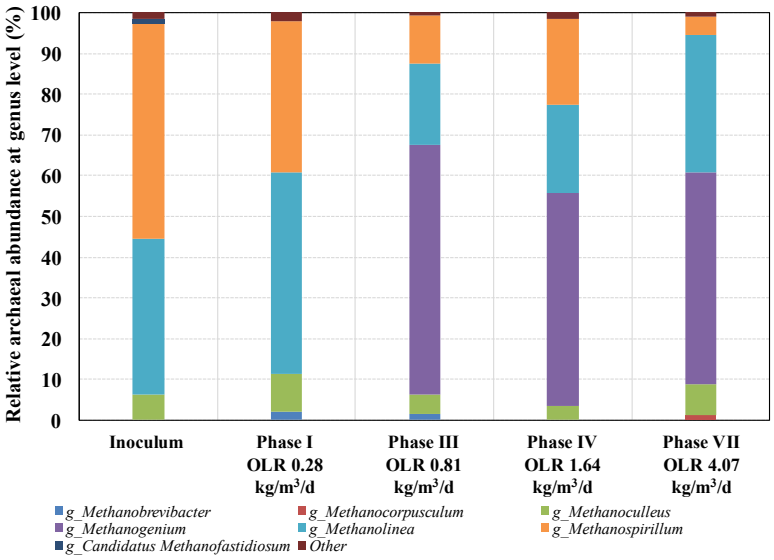

Fig. 5.7. Relative abundance of archaeal taxonomic groups of inoculum, and UASB reactor sludge in Phase I (OLR of 0.28 kg COD/m3/d, HRT of 36 days), Phase III (OLR of 0.81 kg COD/m3/d, HRT of 12 days), Phase IV (OLR of 1.64 kg COD/m3/d, HRT of 6.3 days), and Phase VII (OLR of 4.07 kg COD/m3/d, HRT of 2.6 days) at the genus level.

The diversity of methanogens was limited to four genus *Methanoculleus*, *Methanogenium*, *Methanolinea*, and *Methanospirillum*, which were all belong to the order *Methanomicrobiales*. Order *Methanomicrobiales*, as hydrogenotrophic methanogens, is commonly found in anaerobic digestion systems. The dominance of *Methanomicrobiales* in anaerobic reactors was correlated to the high ammonia concentration in previous studies (Werner et al., 2014). Genus *Methanolinea* was found in anaerobic reactors treating sewage sludge, and has been reported to metabolize H_2 or formate for growth and methane production (Imachi et al., 2008), and is able to couple with syntrophic oxidation (Wilkins et al., 2015). Genus *Methanospirillum* was found in an anaerobic system treating high-solids sewage sludge, and was reported to be strictly mesophilic hydrogenotrophic methanogen (Liu et al., 2016). *Methanoculleus* have been found to dominant in a liquid swine manure fed digester (Barret et al., 2013). As hydrogenotrophic methanogens, genus *Methanoculleus* has been reported tolerant to high ammonia concentrations (FA 1.1 g/L) in a thin stillage fed reactor (Moestedt et al., 2016). Further, genus *Methanogenium* was found in rural household digesters (Han et al., 2018) and in swine manure digestion systems (Qin et al., 2013). However, the studies of genera *Methanogenium* are still limited in anaerobic digestion systems.

Overall, with increases in the organic loading and methane production rate, bacterial and archaeal communities shifted. Under high OLR conditions (Phase VII, OLR=4.07 kg COD/m^3/d), microbial community in UASB was dominated with bacterial phylum *Bacteroidetes* (41.2%) and hydrogenotrophic methanogens *Methanogenium* (52.0%), which correlated to the high SMA observed in the present study and demonstrated their high robustness against high organic loading and ammonia stress. It should be noticed that although acetoclastic methanogenesis is commonly found in anaerobic digesters, the dominance of hydrogenotrophic methanogens has been reported previously for anaerobic digesters treating wastewater with high ammonia concentrations (Lee et al., 2018; Lü et al., 2013; Tian et al., 2018; Werner et al., 2014). Notably, methanogenic pathway through the syntrophic acetate oxidations (SAO) are generally found to be coupled in these processes, which may contribute to the enhanced methane production under this condition. Further studies on the microbial activities and methane production pathways under various operational conditions (e.g., RNA and protein analysis or

stable isotope studies) are needed to better elucidate the relationship of the microbial community structure and methane production.

5.4. Discussion

5.4.1 Superior treatment performance in comparison to the literature

As compared to the previous reported studies treating household blackwater through various types of system design and operational conditions, the present study demonstrated highest OLR of 4.1 kg COD/m^3/d and shortest HRT of 2.6 days, with satisfactory COD removal efficiency (~84%) and methane production (0.68 m^3 CH_4/ m^3 reactor/d). For instance, van Voorthuizen et al. (van Voorthuizen et al., 2008) used a combined UASB reactor (operating at 35 °C) and membrane treatment system to treat conventional toilet collected blackwater (COD = 1,139 mg/L) with an OLR of ~2.3 kg COD/m^3/d, and achieved an overall (UASB+membrane) COD_t removal efficiency of 91% (64% by UASB, 27% by membrane filtration) and methane production of 0.27 g CH_4-COD/g influent COD_t. The authors attributed their observed low COD removal efficiency (in UASB) and low methane production to the solids/sludge wash-out from the reactor. A study treating medium strength blackwater (COD = 5,500 mg/L) using a sludge blanket anaerobic baffled reactor (operating at 25-28 °C) with an OLR of 2.3 (±0.5) kg COD/m^3/d achieved 78% COD_t removal and 0.37-0.81 m^3 CH_4/ m^3 reactor/d (Moges et al., 2018). The high methane production reported could be attributed to the higher BMP (0.69-0.73 g CH_4-COD/g influent COD_t) of the feedstock used, as compared to the BMP (0.48 g CH_4-COD/g influent COD_t, section 5.6) of the blackwater used in the present study, and the reduction of potential ammonia inhibition with long blackwater pre-hydrolysis time (36-48 h). For the treatment of high strength vacuum toilet collected blackwater (COD = ~10,000 mg/L), relatively lower OLRs have been reported. For instance, a CSTR (operating at 35 °C) was operated with an OLR of 0.5 kg COD/m^3/d and achieved 61% COD removal (Wendland et al., 2007); a mesophilic UASB-septic tank system (operating at 25 °C) with an OLR of 0.42 kg COD/m^3/d achieved 78% COD removal (Kujawa-Roeleveld et al., 2006); and a pilot plant UASB-septic tank system (operating at 25 °C) with a lower OLR of 0.36 kg COD/m^3/d achieved 87% COD removal (De Graaff et al., 2010). A UASB reactor treating vacuum toilet collected blackwater with OLR = 1 kg COD/m^3/d (HRT = 8.7 d) operating at 25 °C achieved 78% COD_t removal (De Graaff et al., 2010). The high COD removal efficiency of 90% with a high methane production rate of 0.18 m^3 CH_4/ m^3

reactor/d were achieved in a UASB reactor at 25 °C with an OLR of 0.9 kg COD/m^3/d applied (Tervahauta et al., 2014). To date, the highest OLR used for treating high strength vacuum toilet collected blackwater was 1.4 kg COD/m^3/d (HRT=8.3 d) with a UASB reactor, which achieved 78% COD removal (Zeeman et al., 2008). All these reported operations treating high strength vacuum toilet blackwater generated lower methane production rates of 0.04-0.28 m^3 CH_4/ m^3 reactor/d, as compared with the current study (0.68 m^3 CH_4/ m^3 reactor/d).

The superior OLR achieved in the present study can be attributed to at least three reasons: (i) mitigation of blackwater free ammonia inhibition through adopting blackwater storage strategy in this work, (ii) well acclimatized microbial communities due to step-wisely increased OLR for acclimation, and (iii) the operation conditions applied (UASB reactor at 35 °C) for effective hydrolysis. Details of different reactor operation strategies are discussed below.

5.4.2 Feedwater composition

Blackwater characteristics vary largely depending on collection system types, and age groups, and food habits and choices of residents. Overall, the blackwater characteristics used in the present study are comparable with reported vacuum toilets collected blackwater (De Graaff et al., 2010). In particular, vacuum toilet collected raw blackwater contains high pH (8.6-8.8) and high ammonia nitrogen concentration (>1,000 mg NH_4-N/L). As a result, free ammonia inhibition can be significant when treating blackwater at 35 °C (Gao et al., 2019), which may help to explain the low OLR used for treating this type of blackwater. In the current study, the short-time (average 0.5 d) stored raw blackwater in the influent tank under 20 °C significantly reduced free ammonia concentration of the UASB influent (from 431 to 155 mg/L) through reducing the feedstock pH (8.05), demonstrating an effective simple free ammonia mitigation strategy.

5.4.3 Blackwater hydrolysis

Under high OLR (4.9 kg COD/m^3/d) and short HRT (2.1 days) conditions in Phase VI, clear limitation of substrate hydrolysis was observed, which led to decreased methane production. Limitation in hydrolysis is often observed under high OLR conditions for reactors treating complex wastes, when the insufficient SRT could not satisfy the hydrolysis of particulate COD (De Graaff et al., 2010; Zeeman and Sanders, 2001). As a result of hydrolysis limitation, COD

accumulation and methane production reduction have also been reported (Rajagopal et al., 2019; Rico et al., 2015), which can eventually cause system instability and process inhibition. For instance, a 33% increase in OLR led to a 20% reduction in particulate COD removal and 25% reduction in methane production for a CSTR reactor co-digesting blackwater and food waste (Wendland et al., 2007). In our study, 47% decrease in solids COD hydrolysis efficiency and 20% decrease in methanisation rate (compared to Phase I-V) have been observed. It was observed when OLR was reduced to 4.1 kg COD/m^3/d, system recovered with an increased hydrolysis efficiency (29%) and methanisation rate (0.44 g CH_4-COD/g influent COD_t). Future process optimization to further enhance blackwater treatment should consider options to enhance COD_{solids} hydrolysis, through pretreatment, implementing new reactor design that improves sludge retention, or optimizing operation conditions that facilitate faster hydrolysis (Cheng et al., 2019; Liu et al., 2019; Xu et al., 2019).

5.4.4 Methanogenesis pathway

As shown in the microbial analysis results, the hydrogenotrophic methanogens were predominant in the UASB reactor. This observation correlated well with our SMA results in that SMA (H_2&CO_2) was much greater than the SMA (acetate), indicating high H_2&CO_2 utilization capacities of the sludge. Similar community shifts towards the hydrogenotrophic pathway have been reported previously for feedstock with high ammonia contents (e.g., FA above 128-330 mg/L) (Schnürer and Nordberg, 2008), which was often accompanied with the syntrophic acetate oxidation (SAO) pathway (De Francisci et al., 2015). Our SMA (acetate) results demonstrated that the UASB sludge was capable of utilizing acetate as substrate; which may also be attributed to the possible syntrophic acetate oxidation activities from SAO bacteria that function syntrophically with the hydrogenotrophic methanogens (Karakashev et al., 2006). Unfortunately, information on the SAO community has not yet been fully developed. None of the five known species in the SAO communities, including *Thermacetogenium phaeum*, *Pseudothermotoga lettingae*, *Tepidanaerobacter acetatoxydans*, *Clostridium ultunense* and *Syntrophaceticus schinkii* (Westerholm et al., 2016) were detected in the current study. But it is worth noting that bacterial phyla *Chloroflexi* and *Synergistetes* in this study (with relative abundance of 2.6-6.0% and 0-2.5% in the detected operation Phases) have been reported to function through the SAO

pathway (Li et al., 2015). Further studies are needed to identify the diverse SAO communities and their interactions with hydrogenotrophic methanogens.

5.4.5 Environmental implications

With an average methane production of 0.68 (±0.08) m^3 CH_4/ m^3 reactor/d at an OLR of 4.07 kg COD/m^3/d achieved in this study, and the specific energy of methane being 40 MJ/ m^3 CH_4 (Zhang et al., 2018), the specific energy produced from our blackwater treatment study is 27.34 (±3.05) MJ/m^3 reactor/d as heat. In comparison with other high-strength (COD~10,000 mg/L) blackwater operations, the present work achieved higher biomethane recovery per m^3 reactor installed than the values from the reported literature, which generated heat energy varied from 1.6 to 11.2 MJ/m^3 reactor/d (De Graaff et al., 2010; Kujawa-Roeleveld et al., 2005; Tervahauta et al., 2014; Wendland et al., 2007; Zeeman et al., 2008). The overall recovered energy can be utilized for heating up the blackwater treatment system and nearby community, representing a sustainable approach for resource recovery and reuse from municipal wastewater. Overall, this work underlines the significance in reducing the process inhibition and the hydrogenotrophic methanogenesis pathway in treating high strength wastewater.

5.5. Conclusion

A continuous laboratory-scale UASB reactor treating vacuum toilet collected blackwater at 35 °C was demonstrated in this paper. The maximum reactor treatment capacity was challenged by step-wise OLR increase and HRT reduction. COD_t removal efficiency of 84% (±5%), and methane production of 0.68 (±0.08) m^3 CH_4/ m^3 reactor/d were successfully achieved with an OLR of 4.1 kg COD/m^3/d (HRT 2.6 days). Hydrogenotrophic methanogenic pathway was dominant in the blackwater digestion system, and the hydrogenotrophic methanogens *Methanogenium* were enriched in the UASB reactor.

5.6 Supplementary materials

5.6.1 Blackwater storage in influent tank

Preliminary tests have been conducted to evaluate the change in the blackwater chemical and biological properties, the results are shown in Table S5.1. The tests have been conducted on three groups of collected blackwater with each group in triplicated samples. Bio-methane potential

(BMP) tests were conducted following the description in the manuscript for the 0-day stored raw blackwater, 0.5-day stored blackwater and 1-day stored blackwater. Regarding the changes of influent characteristics due to blackwater storage, it is observed that 0.5-day storage has led to the change in VFA concentrations with ~15% increase in acetate and propionate and ~18% increase in butyrate (section 5.3.1), and further increase in blackwater storage time to 1 day led to an 21% increase in acetate concentration, 3% increase in propionate concentration and 24% increase in butyrate concentration, as compared with freshly collected raw blackwater. Under such condition, blackwater pH was lowered from initial 8.67 (±0.08) to 8.05 (±0.24) then 7.75 (±0.29), and the free ammonia (FA) concentration was dropped from initial 431.3 (±44.5) to 154.6 (±86.4) and then to 122.3 (±42) mg/L. Blackwater BMP increased from original 0.38 g CH_4-COD/g influent COD_t to 0.42 g CH_4-COD/g influent COD_t with 0.5-day storage. Further increase in storage time didn't significantly improve BMP. Thus, 0.5-day storage was applied in the blackwater treatment studies. During the whole UASB operation, the characteristics for raw blackwater and UASB influent have been monitored and compared (Table S5.2). The overall BMP of the UASB influent for the whole operation showed a value of 0.48 ± 0.05 g CH_4-COD/g influent COD_t.

5.6.2 Free ammonia

The free ammonia concentrations presented in the manuscript were calculated from the following equation:

$$NH_3(FA) = 1.214 \times TAN \cdot \left(1 + \frac{10^{-pH}}{10^{-\left(0.09018 + \frac{2729.92}{T\,(K)}\right)}}\right)^{-1}$$

NH_3: Free ammonia (FA) (in mg/L);

TAN: Total ammonia nitrogen (in mg/L);

T (K): Kelvin temperature.

5.6.3 Energy recovery

The calculations of energy recovery from blackwater anaerobic treatment systems were made based on the methane production rate (m^3 CH_4/ m^3 reactor/d) that one system achieved and the value of specific energy of methane as heat 40 MJ/m^3. Comparisons were made between the reported values and the current study. All presented values are listed in Table S5.3.

The total energy required to heat up the blackwater from 20°C to 35°C is 63 MJ/m^3 blackwater, while our produced methane can generate 67.76 MJ/ m^3 of heat which is sufficient for the heat consumption. The detailed calculation is shown below.

For the energy calculation: we consider heat up the inflow blackwater from 20 °C to 35 °C, we calculate the heat consumption as following:

Heating consumption per m^3 blackwater:
Δ Temperature × Specific heat capacity of water × blackwater volume
=15 °C ×4.2 ×10^3 kJ/m^3/°C ×1 m^3 =63 MJ/m^3 blackwater.

Our results showed a 44% of influent COD could be converted to methane, given the influent blackwater COD as 11 g/L, the methane heat value of 40 MJ/ m^3, we calculate the heat production as following:

Heat production per m^3 blackwater:
Heat value of methane × methane production
= 40 MJ/ m^3 × (11 kg/m^3 × 44% ×0.35 m^3 CH_4/kg COD)
= 67.76 MJ/ m^3

Energy balance
Energy balance = energy production- energy consumption =67.76 MJ/m^3- 63 MJ/m^3 blackwater=4.76 MJ/m^3 blackwater treated.

Table S5.1. Chemical and biological properties of the 0 day-stored raw blackwater, 0.5 day-stored and 1-day stored blackwater.

Influent blackwater storage time	pH	FA (mg/L)	Acetate (mg/L)			Propionate (mg/L)			Butyrate (mg/L)			Biological methane potential (g CH_4-COD/g influent COD_t)	
			MIN	AVERAGE	MAX	MIN	AVERAGE	MAX	MIN	AVERAGE	MAX	MIN	AVERAGE	MAX
0-day storage	8.67 (±0.08)	431.3 (±44.5)	1,004	1,279 (±238)	1,418	461	506 (±39)	530	175	260 (±73)	305	0.36 (±0.01)	0.38 (±0.00)	0.40 (±0.00)
0.5-day storage	8.05 (±0.24)	154.6 (±86.4)	1,421	1,466 (±39)	1,493	542	584 (±36)	605	300	308 (±12)	322	0.38 (±0.00)	0.42 (±0.00)	0.45 (±0.01)
1-day storage	7.75 (±0.29)	122.3 (±42.0)	1,392	1,552 (±139)	1,642	488	523 (±46)	576	275	322 (±42)	354	0.39 (±0.02)	0.41 (±0.03)	0.43 (±0.04)

Table S5.2. Characteristics for raw blackwater and UASB influent.

	Unit	Raw blackwater	UASB influent
COD_t	mg/L	9,985 (±714)	10,977 (±903)
COD_{ss}	mg/L	6,050 (±802)	7,019 (±897)
COD_{col}	mg/L	711 (±229)	490 (±103)
COD_s	mg/L	3,338 (±573)	3,482 (±204)
pH		8.62 (±0.06)	8.05 (±0.24)
Acetate	mg/L	771 (±381)	884 (±24)
Propionate	mg/L	362 (±169)	418 (±26)
Butyrate	mg/L	162 (±81)	192 (±7)
NH_{4+}-N	mg/L	1115.1 (±78.9)	1,137.0 (±51.8)
Free ammonia* (FA)	mg/L	431.3 (±44.5)	154.6 (±86.4)

*Calculated values.

Table S5.3. Vacuum toilet blackwater anaerobic treatment studies and comparisons.

	Temperature (°C)	Digester type	Size (L)	HRT (days)	OLR (kg COD/m^3/d)	Influent COD (g/L)	COD removal efficiency (%)	Methane production rate (m^3 CH_4/ m^3 reactor/d)	Energy recovery* (MJ/m^3 reactor/d)
Kujawa-Roeleveld et al.,(2005)	25	$UASB_d$ septic tank	200	29	0.42	12.3 (±7.8)	74	0.04	1.6
Wendland et al.,(2007)	37	CSTR	10	20	0.5	8.7 (±4.0)	61	0.12*	4.8
Zeeman et al.,(2008)	25	UASB	50	8.3	1.4	9.5-12.3	75*	0.28	11.2
De Graaff et al.,(2010)	25	UASB	50	8.7	1.0	7.7-9.8	78	0.21*	8.4
Tervahauta et al.,(2014)	25	UASB	50	9.3	0.9	11 (±4.1)	90	0.18	7.2
This study	35	UASB	5	2.6	4.1	11 (±0.9)	84	0.68	27.34

*Calculated values based on reported results.

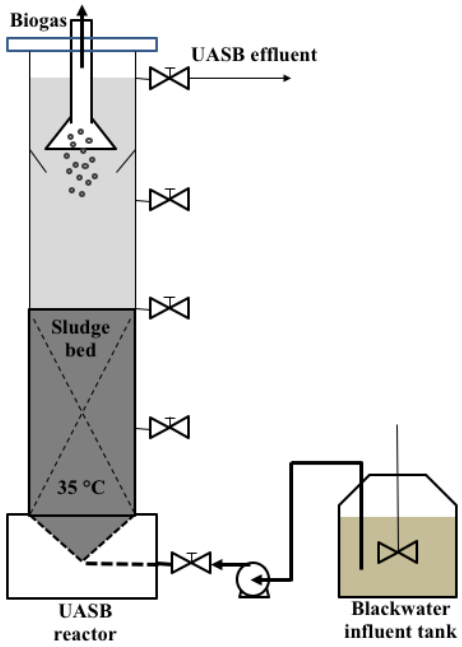

Fig. S5.1. Schematic of the UASB reactor (35 °C) and blackwater influent tank (room temperature) in this study.

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CHAPTER 6. MICROBIAL COMMUNITY DYNAMICS IN ANAEROBIC DIGESTERS TREATING CONVENTIONAL AND VACUUM TOILET FLUSHED BLACKWATER[4]

6.1 Introduction

Our previous results showed that distinct microbial communities were developed in batch reactors treating different types of blackwater (Gao et al., 2019). The microbial community development was associated with not only the organics composition and concentration, but also free ammonia concentration and pH. In particular, vacuum toilet collected blackwater contains a much greater free ammonia concentration as compared to blackwater collected from conventional toilets. High concentration of free ammonia has been reported as a stress factor in full-scale anaerobic digesters treating municipal sludge, manure, and other organic wastes (Sun et al., 2014; De Vrieze et al., 2015; Muller et al., 2016; Tian et al., 2018a). Free ammonia could inhibit microbial activities and reduce COD removal and methane production efficiencies. It has been recognized that hydrogenotrophic methanogens are dominant under high ammonia stress rather than acetoclastic methanogens (Werner et al., 2014; Tian et al., 2018a). Using acetate as the organic feed, it was demonstrated that microorganisms undergo a syntrophic acetate oxidation and hydrogenotrophic methanogenesis (SAO-HM) pathway under high ammonia conditions (Mosbaek et al., 2016; Westerholm et al., 2016; Tian et al., 2018b). For complex substrate feeding, *e.g.* blackwater, whether such a mechanism could occur remains unknown.

Further, microbial population development in continuous operating reactors differs from batch systems. One of the most widely applied anaerobic digestion system is the upflow anaerobic sludge blanket (UASB) reactor. The upflow operation enables the sludge maintenance inside the reactor and improves the contact between the wastewater stream and the microorganisms (Luostarinen and Rintala, 2007). Blackwater treatment using a continuous UASB reactor has been reported in a few recent studies at different operational conditions. A 91 % COD removal and a methane production of 0.27 gCH_4-COD/gCOD were achieved through the combination of UASB and membrane reactor at 37 °C, treating 5 L water flushed blackwater (van Voorthuizen

[4] A version of this chapter has been published: Gao, M., Guo, B., Zhang, L., Zhang, Y., Liu, Y., 2019a. Microbial community dynamics in anaerobic digesters treating conventional and vacuum toilet flushed blackwater. Water Res. 160, 249–258.

et al., 2008). An average of 78 % COD removal and methane production of 1.8 m_3 CH_4/m_3 of blackwater were reported in a UASB reactor treating vacuum toilet blackwater at 25 °C with an HRT of 8.7 days (De Graaff et al., 2010). A 90 % COD removal was achieved at low temperatures of 20 °C and 10 °C treating synthetic blackwater of 1 g/L total COD with a two-phased UASB-septic tank (Luostarinen and Rintala, 2007). However, no studies have reported the development of the microbial communities inside continuous operating blackwater treatment reactors at different operational conditions, or investigated the links between operational parameters, microbial communities, and reactor performances.

The current study aims to investigate the microbial community development in continuous operating UASB reactors treating conventional and vacuum toilet flushed blackwater with increments of organic loadings, and to compare the acclimatized microbial consortia between the two types of blackwater feedings. Conventional and vacuum toilet flushed blackwater generate different chemical properties due to the different amount of flushing water applied, and their treatment performances from the two UASB reactors were compared at similar organic loading rates. The microbial community structure, diversity, and composition at three development phases were investigated. The information can help elucidate the underlying microbial driving force, and guide future reactor design and operation.

6.2 Materials and methods

6.2.1 Sample collection and reactor operation

Fresh blackwater was collected from > 20 persons every week during the experimental period and stored at 4 °C until use. Dilution of blackwater was performed using tap water to mimic conventional toilets at 9 L water per flush and vacuum toilets at 1 L water per flush.

The influent was prepared daily and filled into storage bottles at room temperature, then fed into two 5 L upflow anaerobic sludge blanket (UASB) reactors (working volume 4.7 L) with peristaltic pumps (Longer pump BT 100-2J). The UASB reactors were operated at 35 °C, fed with 1 L water/flush or 9 L water/flush blackwater. Both UASB reactors were seeded with 2.35 L anaerobic flocculant sludge (volatile suspended solids [VSS] concentration of 13.1 (±0.6) g/L) from a full-scale mesophilic anaerobic digester treating primary sludge and waste activated sludge in a local wastewater treatment plant in Edmonton, Alberta, Canada.

The operational conditions of UASB reactors and treatment performances at three phases are shown in Table 6.1. Total operation durations for the UASB reactors treating conventional toilet blackwater and vacuum toilet blackwater were 120 and 115 days, respectively. The calculation of hydraulic retention time (HRT) was performed by dividing the reactor working volume (L) to the influent blackwater flowrate (L/d). The organic loading rates (OLR) were calculated by dividing the average influent blackwater chemical oxygen demand (COD) concentrations to the HRT of each operation phase for the two UASB reactors.

The HRT was stepwise reduced from phase 1 to phase 3, leading to increasing organic loading rates. For the conventional toilet blackwater reactor (CT), HRT decreased from 5.5 to 3 and 1.4 d, and the organic loading rate was increased from 0.18 to 0.36 and 0.76 kg COD/m^3/d. For the vacuum toilet blackwater reactor (VT), HRT were 36, 20, 12 days, and the organic loading rate was 0.28, 0.48 and 0.81 kg COD/m^3/d in phases 1, 2, and 3, respectively. For each operational phase, the two reactors' organic loading rates were maintained relatively similar ($p > 0.05$). HRTs (and corresponding OLRs) applied in the current study were selected based on literature values and tested in our reactors to ensure stable operation. For instance, the reported HRT for CT blackwater anaerobic treatment systems varied from 0.5-3.5 days (Abdel-Shafy et al., 2009; Gallagher and Sharvelle, 2010; van Voorthuizen et al., 2008); while much longer HRTs ranging from 8.3-30 days (Zeeman et al., 2008) were reported for VT blackwater treatment.

6.2.2 Chemical analysis

The influent and effluent characteristics of each phase were monitored to reveal chemical oxygen demand (COD) removal efficiency and methane production. Concentrations of influent and effluent COD were measured base on standard methods (APHA/AWWA/WEF, 2012), and ammonia nitrogen was measured using Hach TNT vial tests (Hach, USA). Free ammonia concentrations were calculated from total ammonia nitrogen (TAN) concentration, pH, and temperature using the following equation (Hansen et al., 1998):

$$NH_3(FA) = 1.214 \times TAN \cdot \left(1 + \frac{10^{-pH}}{10^{-\left(0.09018 + \frac{2729.92}{T(K)}\right)}}\right)^{-1} \qquad \text{(eq. 6.1)}$$

Three main volatile fatty acids (VFAs): acetate, propionate and butyrate were analyzed on a DIONEX ICS-2100 Ionic chromatography (IC) system (Thermo Fisher Scientific, Waltham MA,

USA) equipped with a conductivity detector and IonPac AS18 Analytical Column: 2 × 250 mm. Prior to sample analysis, 2 mL fresh collected UASB effluent was diluted with ultrapure water and filtered through 0.2 μm nylon membrane filter (Fisher Scientific, CA). Biogas composition was measured using GC 7890B (Agilent Technologies, Santa Clara USA) equipped with a thermal conductivity detector (TCD) and two columns (Molsieve 5A 2.44 m 2 mm for CH_4 and Hayesep N 1.83 m 2 mm for N_2, O_2 and CO_2). The temperature of the oven, injector, and detector was 100, 150, and 200 °C during detection, respectively. For each gas detection, 10 mL biogas was withdrawn with gas-tight syringe from the gas bag with 10 times rinse prior to ensure homogenous biogas contents. The amount of methane that generated from feed blackwater was defined as "methanisation" in this study, which was represented as gCH_4-COD/gfeedCOD. The chemical analysis including influent and effluent properties and the biogas production were all conducted 3-5 times a week during operation time.

Both the effluent quality and the biogas production were evaluated to demonstrate the steady state (stable operation of at least 20 consecutive days in each operation phase) before any change in operation conditions. Table 6.1 demonstrates the reactor performance under steady-state conditions.

Isotope fractions of biogas were measured at the end of operation at the Department of Earth and Atmospheric Sciences at the University of Alberta, with GC 5890 Series II (Hewlett Packard, USA). The apparent fractionation factor (α_c) was calculated by the following equation (Hao et al., 2017):

$$\alpha_c = \frac{(\delta^{13}CO_2 + 10^3)}{(\delta^{13}CH_4 + 10^3)} \qquad \text{(eq. 6.2)}$$

6.2.3 Statistical analysis

ANOVA was performed using Microsoft Excel® to compare the applied organic loading rates, and the obtained methanisation rates between the two UASB reactors. A p-value less than 0.05 was considered statistically different.

6.2.4 DNA extraction and 16S rRNA gene sequencing

Sludge samples for microbial community analysis were collected from the inoculum and mixed sludge from all sampling ports of the UASB reactors' sludge bed at steady state at the end of

each operation Phase. The collected mixed sludge samples were sampled for 1.5 mL and centrifuged at 3,000 g for 10 min. The supernatant was discarded, and the pellets were used for DNA extraction. Genomic DNA was extracted using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. All DNA samples were extracted with fresh sludge samples in duplicates. The extracted DNA concentration and quality were checked using NanoDrop One (ThermoFisher, Waltham, MA). Samples were stored at -20 °C until sent to sequencing center at RTL Genomics (Texas, USA). Bacterial 16S rRNA genes were amplified using the universal primer-pair 357wF (5'-CCT ACG GGN GGC WGC AG-3') and 785R (5'-GAC TAC HVG GGT ATC TAA TCC-3'). Archaeal 16S rRNA genes were amplified using universal primer set 517F (5'-GCY TAA AGS RNC CGT AGC-3') and 909R (5'-TTT CAG YCT TGC GRC CGT AC-3'). The amplicons were sequenced on Illumina MiSeq PE300 platform.

6.2.5 Bioinformatics analysis

The raw sequences were processed using the DADA2 algorithm (Callahan et al., 2016) in Qiime2 pipelines (Caporaso et al., 2010) to pair forward and reverse sequences, and remove low-quality sequences and chimeras. The total good-quality sequence reads were 92,080 for bacteria and 110,186 for archaea. Taxonomy was assigned using 97% similarity in GreenGenes (version 13_8) reference database (McDonald et al., 2012; Werner et al., 2012). The raw sequences were deposited in NCBI GenBank PRJNA521788.

Alpha and Beta diversity and principal coordinate analysis (PCoA) were analyzed using "vegan" package (Jari Oksanen et al., 2017) in R (RCoreTeam, 2017). Heatmap was produced using "gplot" package (Warnes et al., 2016). Metagenome, weighted average *rrn* operon copy number and functional gene annotation were calculated from 16S rRNA gene sequences with closed references in GreenGenes (version 13_8) using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013). The reference database used in PICRUSt was the Integrated Microbial Genomes (IMG) system (Markowitz et al., 2012) for metagenome and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2012) for functional gene annotation.

6.3 Results

6.3.1 Operation phases and performance

The treatment performances were shown in Table 6.1. The different toilet flushing systems provided different COD and TAN concentrations for the two types of collected blackwater, with the influent COD concentration at 900-1100 mg/L and TAN at 40-70 mg/L for the conventional toilet (CT) blackwater, and the influent COD concentration at 8500-11000 mg/L and TAN at 1000-1100 mg/L (supplemented with ammonia when the ammonia concentration of the collected vacuum toilet (VT) blackwater showed values lower than the 1,000-1,100 mg/L to maintain constant feedstock properties) for the vacuum toilet (VT) blackwater. The variations in chemical properties in both conventional toilet and vacuum toilet collected blackwater are due to the natural fluctuation of the blackwater production. The influent blackwater properties for the two types were similar to reported values in the literature (De Graaff et al., 2010; van Voorthuizen et al., 2008). The methanisation was significantly higher in the VT reactor ($p < 0.05$), but not significantly different between different phases in either reactor. The accumulated methane content in the biogas was stable through all phases, and was slightly higher in the VT reactor, 72 % in the CT reactor, and 75 % in the VT reactor. The CT reactor methanisation (0.23-0.29 gCH_4-COD/gfeedCOD) was comparable with reported results of 0.27 gCH_4-COD/gfeedCOD using the combined UASB and membrane reactor (van Voorthuizen et al., 2008).

The effluent qualities were relatively stable for both reactors in terms of effluent pH, COD, and TAN. The average COD removal efficiencies for the CT reactor was 72 % (± 6 %) for the three operational phases in this study, which was higher than the reported 64 % COD removal efficiency with similar operating temperature at 37 °C, but shorter HRT of 12 hrs (van Voorthuizen et al., 2008). The average COD removal efficiencies for VT reactor was 89% (± 2 %) for the three operational phases in this study, which was higher than the reported 78 % COD removal efficiency with a less favorable operating temperature at 25 °C and shorter HRT of 8.7 days (De Graaff et al., 2010). Each type of detected volatile fatty acids (VFAs) for both reactors were lower than 50 mg/L, indicating sufficient methanogenesis capacity inside the systems under the studied conditions. The TAN concentrations were increased in the effluent compared with the influent, due to the release of ammonia from the substrate hydrolysis process. Accordingly, the calculated free ammonia concentrations were higher in the VT reactor (98.9-123.5 mg/L) than in

the CT reactor (1.7-2.5 mg/L). The free ammonia levels were lower than the typically reported inhibition levels of >200 mg/L (De Graaff et al., 2010; Gao et al., 2019).

Table 6.1. Operational conditions and performances for UASB reactors treating conventional toilet (CT, 9 L/flush) and vacuum toilet (VT, 1 L/flush) blackwater.

		UASB Influent					UASB Effluent					Biogas	
	Operation duration (day)	COD	TAN	pH	HRT	Organic loading rate	pH	COD	VFA[a]	TAN	Free ammonia[b]	CH_4 production	CH_4 content
		mg/L	mg/L		day	kg/m^3/d		mg/L	mgCOD/L	mg/L	mg/L	gCH_4-COD/ g feedCOD	%
CT Phase 1	1-64	990 (±66)	45 (±7)	7.6 (±0.4)	5.5	0.18 (±0.02)	7.3 (±0.3)	270 (±60)	9 (±4)	94 (±42)	2.5 (±1.1)	0.23 (±0.04)	72
CT Phase 2	65-92	1,076 (±5)	49 (±5)	7.4 (±0.1)	3	0.36 ±0.00)	7.3 (±0.2)	209 (±56)	28 (±5)	66 (±6)	1.8 (±0.1)	0.29 (±0.03)	72
CT Phase 3	93-120	1,050 (±86)	64 (±9)	7.9 (±0.4)	1.4	0.76 (±0.07)	7.4 (±0.2)	281 (±50)	35 (±6)	52 (±8)	1.7 (±0.2)	0.26 (±0.05)	72
VT Phase 1	1-43	9,948 (±1,336)	1,073 (±27)	8.6 (±0.1)	36	0.28 (±0.05)	7.9 (±0.4)	1207 (±132)	41 (±41)	1,231 (±92)	123.5 (6.0)	0.45 (±0.05)	75
VT Phase 2	44-88	9,515 (±805)	1,095 (±19)	8.6 (±0.1)	20	0.48 (±0.07)	7.8 (±0.2)	991 (±91)	33 (±9)	1,220 (±78)	98.9 (±6.3)	0.48 (±0.05)	75
VT Phase 3	89-115	9,492 (±326)	1,121 (±56)	8.7 (±0.1)	12	0.81 (±0.07)	7.9 (±0.2)	1190 (±83)	42 (±5)	1,220 (±104)	123.0 (±10.4)	0.41 (±0.04)	75

[a] VFA: volatile fatty acids, the sum of measured acetate, propionate and butyrate.

[b] Calculated value using eq. 6.1.

In the VT reactor, the sludge VSS concentration increased from the initial concentration of 13.1 (±0.6) g/L, to 24.9 (±5.1), 28.9 (±3.4) and 23.5 (±0.4) g/L in Phases 1-3, respectively. The CT reactor also showed increases of sludge VSS concentrations to 15.6 (±1.4), 17.5 (±4.7) and 15.4 (±1.7) g/L in Phases 1-3, respectively. VSS concentrations were resulted from different microbial growth dynamics and developed microbial communities in the two reactors. The community development is a dynamic process, which can be affected by various factors. The

organic loading rates (OLRs) were kept similar between the two reactors, but the ammonia loading rates and concentrations were higher in the VT reactor. Therefore, the main factors for microbial community development were OLR increases through Phases 1-3, and ammonia concentration difference between the two reactors.

6.3.2 Microbial community diversity

The rank abundance distributions of archaeal and bacterial communities at the genus level and the total number of genera are presented in Fig. 6.1. In both CT and VT reactors, the archaeal communities (Fig. 6.1A) showed a steeper gradient than the inoculum, representing a less even distribution. The first ranked archaeal genus took up around 60 % of total community abundance, indicating mono-enrichment in the community. The total number of genera were similar, between 9 and 11 in each sample.

The distribution curves of the first 15 bacterial genera (Fig. 6.1B) of the three phases in the CT reactor were lower than the inoculum. On the contrary, the VT reactor showed much steeper slopes in phases 1 and 2, due to the higher relative abundance of the first ranked genus. In phase 3, the relative abundance of the first ranked genus dropped and the distribution curve became similar with the inoculum. The number of genera was 119 in the inoculum, 116 in CT phase 1, and increased to 146 and 172 in phases 2 and 3. A decrease was shown in the VT reactor to 81, 71, and 79 in the three phases respectively.

The evenness of the communities was measured using the Gini coefficient (Werner et al., 2014) (Fig. 6.1C). In archaea, the Gini coefficient fluctuated along different phases. In bacteria, the community changed to higher evenness (lower Gini coefficient) in the CT reactor than the inoculum while it remained similar in the VT reactor. Compared with the inoculum, the archaeal Shannon diversity (Fig. 6.1D) fluctuated in different phases. In the bacterial community, the Shannon index showed higher values in the CT reactor compared with the inoculum; the VT reactor showed similar levels with the inoculum.

The lower bacterial diversity in the VT reactor (fewer genera, evenness, and Shannon index) as compared to that in the CT reactor indicated a higher stress level, possibly attributed to the high

organic and ammonia concentrations in VT collected blackwater (Table 6.1). Nevertheless, lower bacterial diversity co-occurred with higher methane production in the VT reactor. This observation could be explained by the high substrate concentrations selecting microorganisms with higher growth rates, *i.e.* r-strategists (Klappenbach et al., 2000; Nemergut et al., 2016; Wu et al., 2017), resulting in higher substrate utilization and methane production. A previous study showed that higher organic loading rate selected r-strategists, which resulted an increased methane production with no inhibition on the microbial communities (Wu et al., 2017).

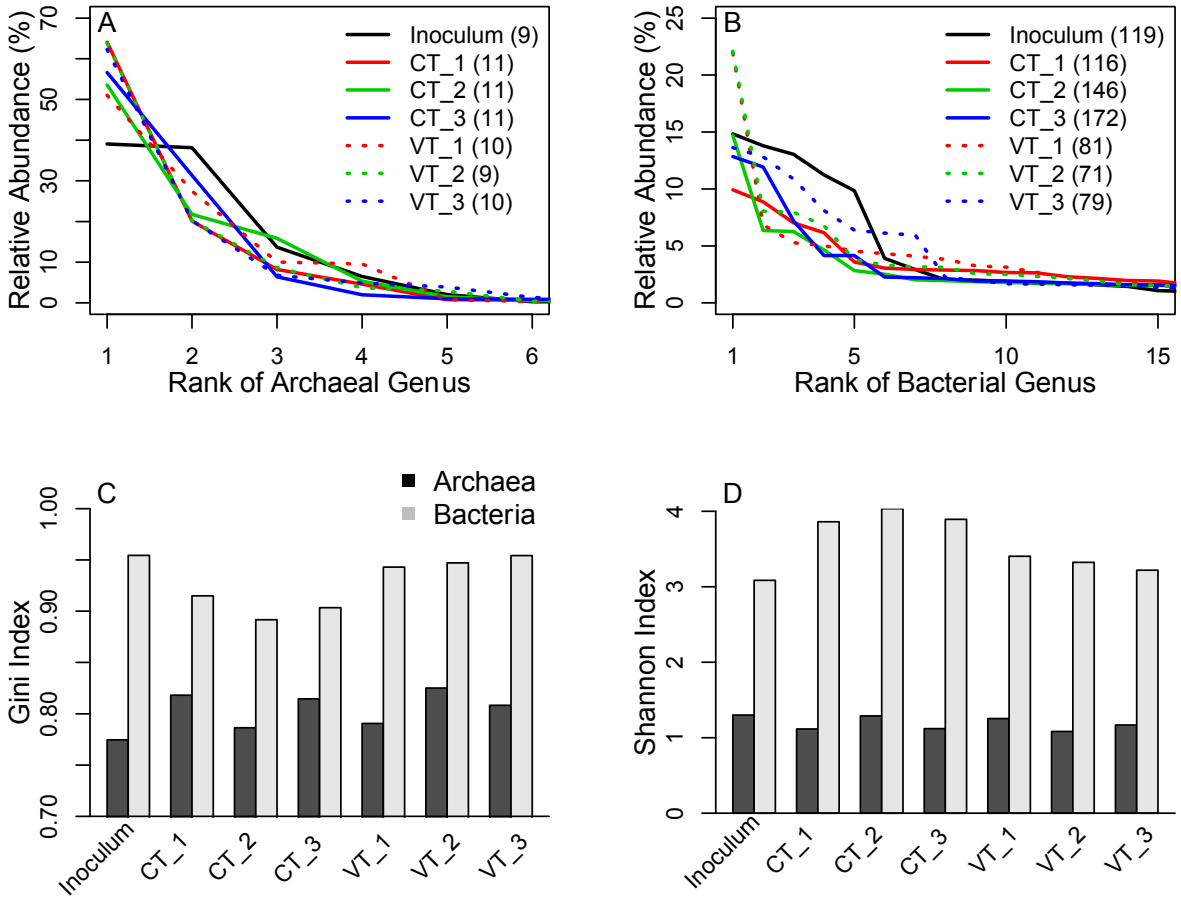

Fig. 6.1. Rank abundance distribution of archaea (A) and bacteria (B), total number of genera were indicated after the sample name; Gini index (C); and Shannon index (D). Samples were inoculum, conventional toilet blackwater (CT), and vacuum toilet blackwater (VT) reactors at phases 1, 2, and 3. Communities were analyzed at a genus level.

The beta-diversity of the archaeal and bacterial communities was presented on the principal coordinate analysis (PCoA) plot of Bray-Curtis distances among samples (Fig. 6.2). The archaeal communities in phase 1 of both reactors shifted towards the same direction (Fig. 6.2A). Then, the two reactors diverged to different directions, forming two clusters apart from each other along the PCoA1 axis, which explains 75.5 % of total sample variance. The bacterial communities of the inoculum, feed blackwater, CT, and VT reactors formed clusters distinct with each other (Fig. 6.2B). The community was stable through phase 1 to phase 3 in either reactor. The bacterial communities that developed in the two reactors were divergent, yet more similar than to the original community in the feeding blackwater, indicating that the operational conditions had greater impacts on the reactor communities than the communities in the feed water.

The archaeal community changed slowly from phases 1 to 3, whereas the bacterial community did not change from phase 1. Comparing the temporal changes, the archaeal community takes a longer time than the bacterial community to stabilize. This could be explained by the fact that archaea have higher doubling time, *i.e.* slower growth than bacteria; and the community turnover rate is also slower (Zinder, 1993). The concentrations of COD and TAN are putative leading factors that selected different communities. Werner et al. (2014) found that increases in ammonia concentration perturbed the bacterial community in anaerobic digesters, leading to more unevenness in the microbial community (higher Gini coefficient). Similarly, in our data, the VT reactor received a high ammonia loading, and showed greater unevenness than the CT reactor (Fig. 6.1C). Noticeably, the VT reactor had lower numbers of genera in both archaeal and bacterial communities than the CT reactor. The high concentrations of ammonia and other substrates probably inhibited more microbial genera, resulting in less diverse communities.

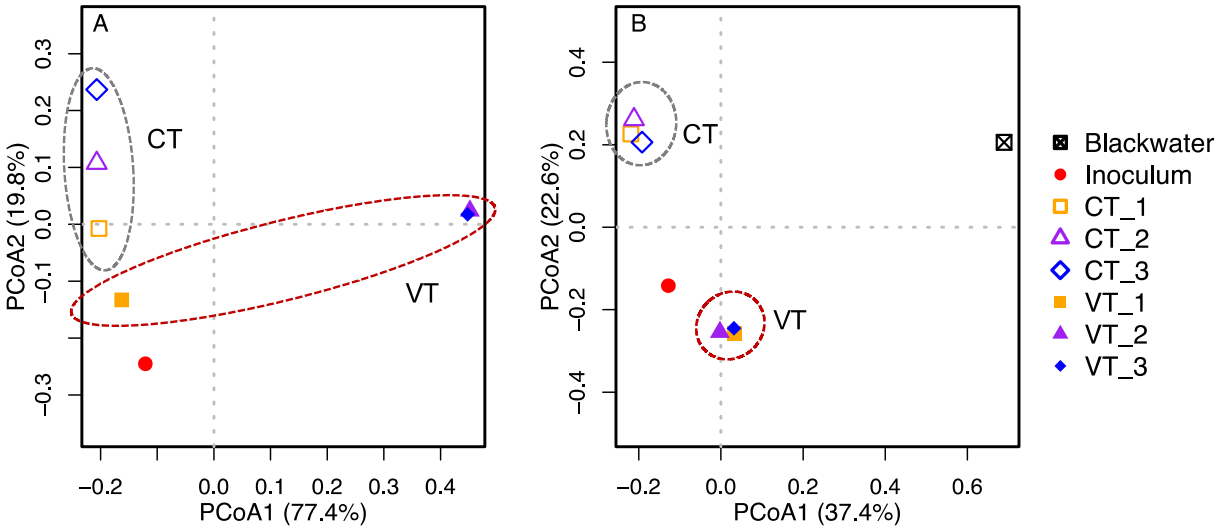

Fig. 6.2. Principal coordinate analysis (PCoA) of archaeal communities of inoculum and reactor samples (A), and bacterial communities of blackwater, inoculum, and reactor samples (B). PCoA was computed using Bray-Curtis distance calculated using genus abundance data.

6.3.3 Enriched archaeal consortia

At the genus level, 11 archaeal taxa were detected among all samples (Fig. 6.3). The inoculum was dominated by three methanogens (each >10 %): *Methanolinea* and two unidentified genera in the family (f_) *Methanospirillaceae*. Two other genera showed relative abundance >1 %, *Methanoculleus* and *Methanospirillum*. *Methanosarcina* and *Methanosaeta* were at very low abundance (0.03 % and 0.04 % respectively) in the inoculum. The CT reactor inherited the most abundant genus *Methanolinea* from the inoculum, which dominated through phase 1 to phase 3. *Methanospirillum* gradually increased from phase 1 to phase 3, becoming the second predominant genus. *Methanoculleus* and the two unidentified genera in f_ *Methanospirillaceae* deceased gradually. *Methanosarcina* increased in phases 1 and 2 (>1 %). *Methanosaeta* was not detected in phase 1 and appeared in phase 2 and 3 at low levels (<1 %). In the VT reactor, *Methanolinea* was the most abundant genus in phase 1 and second most abundant in phases 2 and 3. A remarkable increase was shown for *Methanogenium*, which was not detected in inoculum or the CT reactor, but became dominant in phases 2 and 3 in the VT reactor. The two unidentified genera in *Methanospirillaceae*, *Methanospirillum* and *Methanoculleus* decreased at

the end of phase 3 compared with the inoculum. *Methanosaeta* became non-detectable, while *Methanosarcina* slightly increased compared with the inoculum.

The predominant archaeal genera in the inoculum and reactors were different in composition (*Methanolinea,* the family *Methanospirillaceae*, *Methanoculleus*, *Methanospirillum* and *Methanogenium*), but they all belong to the order *Methanomicrobiales* which all perform hydrogenotrophic methanogenesis (Garcia et al., 2006). *Methanomicrobiales* is closely correlated to high TAN conditions in other studies, especially the genus *Methanoculleus* (Westerholm et al., 2012; Moestedt et al., 2016; Tian et al., 2018a). In full-scale reactors, *Methanomicrobiales* was less affected by the ammonia concentration compared to *Methanobacteriales*, *Methanosaetaceae* and *Methanosarcinaceae* (De Vrieze et al., 2015). *Methanomicrobiales* and *Methanobacteriales* were the most abundant archaeal orders in syntrophic acetate oxidization digesters (Werner et al., 2014). In our study, *Methanomicrobiales* took up 99 %, 97 %, and 99 % of archaeal communities of the inoculum, CT reactor, and VT reactor, respectively. *Methanoculleus* accounted for only 0.55-0.84 % and 2.89-9.56 % in CT and VT reactors, respectively.

Methanogenium was reported in a limited number of studies of anaerobic digesters. It was reported as the most abundant archaea in household digesters at low temperatures (11.1-15.7 ^{o}C) in high plateau environments (Han et al., 2018). It was grown at low abundance in mesophilic anaerobic digester treating swine manure (Zhu et al., 2011; Qin et al., 2013), as well as swine manure storage tanks (Barret et al., 2012; Barret et al., 2013). Pure culture of *Methanogenium* sp. showed slower growth rates with increasing concentration of ammonia at higher than 3 g NH_4^+-N/L (Hendriksen and Ahring, 1991). However, previous studies aiming for high TAN conditions rarely reported *Methanogenium* in the archaeal community. *Methanogenium* was found in marine methanogens (Romesser et al., 1979), which are tolerant to high salinity. In a high-salinity anaerobic digester treating secondary sludge, *Methanomicrobiales* dominated in the archaeal community and species closely related to *Methanogenium marinum* were abundant (Shin et al., 2010). Whether the salinity tolerance and ammonia tolerance could be compared may need further information.

Acetoclastic methanogenesis is more vulnerable to high ammonia concentration than hydrogenotrophic methanogenesis (Karakashev et al., 2006); thus acetoclastic methanogenesis may be inhibited in ammonia-rich reactors, such as reactors treating manure or slaughterhouse waste (Sun et al., 2014). In our blackwater-fed reactors, the acetoclastic methanogen *Methanosaeta* were in low abundance in the CT reactor, and decreased to undetectable levels in the VT reactor. *Methanosarcina* increased slightly in both reactors, possibly due to its versatile functionalities and resistance to harsh conditions (Sun et al., 2014; Mosbaek et al., 2016). Although they were most commonly reported methanogens in sewage sludge fed anaerobic digesters (De Vrieze et al., 2015), their abundance was lower than expected in our study. Noticeably, the sum of *Methanosaeta* and *Methanosarcina* only accounted for 0.07 % of inoculum archaeal community, which may have some impact on the community in our reactors.

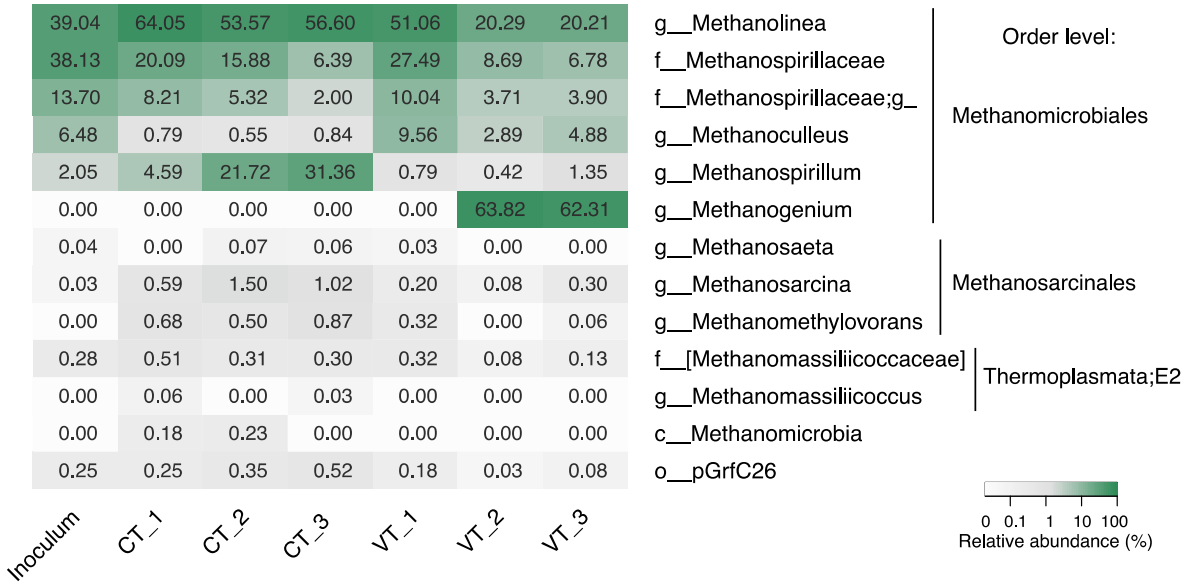

Fig. 6.3. Heatmap of archaeal genera, the taxonomic names were shown for genus level (g_), or higher level (family: f_ ; order: o_; class: c_) if not identified at genus level. Color key indicates relative abundance of genera in each sample.

6.3.4 Enriched bacterial consortia in CT and VT reactors

The most abundant families and fold-changes compared to the inoculum are shown in Fig. 6.4. Four groups of families were classified (groups A-D in Fig. 6.4) based on their fold-changes in relative abundances. In group A, bacterial families increased in both reactors. Families in group

B or C were enriched in the CT reactor only or VT reactor only, respectively. Group D represents families that remained at similar or decreased abundances in both reactors.

Enriched families in grouped A were low in the inoculum. The family *Porphyromonadaceae* increased to 15.9% in the CT reactor in phase 3, and 13.4% in the VT reactor in phase 3. *Fibrobacteraceae* increased remarkably in the VT reactor to 13.8% in phase 3. *Bacteroidaceae* grew to 1.8% and 7.0% and *Marinilabiaceae* increased to 1.8% and 1.6%, in phase 3 of CT and VT reactors respectively. *Lachnospiraceae* reached 2.8% in CT phase 3. One unidentified family in the order Clostridiales increased to 2.0% in the VT reactor in phase 3. Species in the families *Porphyromonadaceae*, *Lachnospiraceae*, and in the order Clostridiales were also observed in communities under high ammonium conditions (Muller et al., 2016).

Porphyromonadaceae abundance was significantly higher in high-ammonia reactors than in low-ammonia reactors (Muller et al., 2016) and a predominated anaerobic digester treating chicken wastes (Ziganshina et al., 2015). *Bacteroidaceae* was found at elevated abundance correlated with total ammonia concentration in full-scale reactors (De Vrieze et al., 2015). Clostridiales species were reported for their tolerance of high ammonia concentrations (De Vrieze et al., 2015; Mosbaek et al., 2016; Muller et al., 2016; Tian et al., 2018a). They were widely spread in the inoculum and reactors. Some of these bacteria were reported in anaerobic digesters treating animal wastes, *e.g.* swine manure, cattle manure, and chicken waste. *Marinilabiaceae* was highly abundant in ammonia-rich chicken-waste anaerobic digesters (Ziganshina et al., 2015). *Fibrobacteraceae* was the second most abundant family in cattle rumen fluid, *Bacteroidaceae* was the second most abundant family in cattle manure (Ozbayram et al., 2018b). *Fibrobacteraceae*, *Bacteroidaceae*, *Porphyromonadaceae* are important fiber-digesting bacteria capable of enhancing anaerobic digestion of lignocellulosic biomass (Yan et al., 2012; Ozbayram et al., 2018a; Ozbayram et al., 2018b). Their increased abundance in the blackwater-fed reactors could be associated with the presence of lignocellulosic matter in blackwater.

Group D showed families that reduced to non-detectable levels, such as *Microthrixaceae* in both reactors, *Comamonadaceae* and *Syntrophorhabdaceae* in the VT reactor. The well-known syntrophic families, *Syntrophorhabdaceae* and *Syntrophaceae*, decreased in both reactors. The

other families in group D decreased but still maintained their high abundance, such as *Anaerolinaceae*, *Cloacamonaceae*, *Spirochaetaceae*, *Thermovirgaceae*, and *Rikenellaceae*.

6.3.5 Enriched bacteria specific to the CT reactor

In the CT reactor, the enrichments (group B, Fig. 6.4) included sulfate-reducing bacterial families *Desulfobulbaceae*, *Desulfomicrobiaceae*, and *Desulfobacteraceae*, with total abundances of 7.3%, 5.2%, and 5.5% in phases 1-3, respectively. This observation is correlated with the relatively higher sulfate concentration in the CT collected blackwater. Since the blackwater sulfate source was supplemented from tap water (at a concentration of 61.9 ± 0.1 mg/L), CT collected blackwater contains a higher sulfate concentration per COD (90.5 ± 17.8 mg per gCOD) as compared to VT collected blackwater (28.3 ± 3.8 mg per gCOD). The COD/SO_4^{2-} ratios were 10.9-11.7 and 33.6-35.2 in the CT and VT feed respectively, which were higher than the reported inhibition threshold of 1.6 (Siles et al., 2010) and did not cause complete inhibition of methanogenesis. Negative effects of sulfate on methanogenesis have been reported previously, and mainly include (i) organic substrate competition between sulphate reducing bacteria and methanogens, and (ii) toxicity of sulphate reduction produced H_2S towards methanogens (J.W.H. et al., 1994; Chen et al., 2008; Siles et al., 2010; Dai et al., 2017). Hereby sulphate concentrations in CT collected blackwater should be monitored when designing anaerobic digestion systems for CT collected blackwater treatment.

Campylobacteraceae, *Rhodocyclaceae*, *Pseudanabaenaceae*, an unidentifed family in the class OPB56, *Geobacteraceae*, *Opitutaceae*, *Elusimicrobiaceae* and an unidentifed family in the class Bacteroidales were higher than 1% in the last phase, inferring their possible active roles in treating the CT collected blackwater.

6.3.6 Enriched bacteria specific to the VT reactor

In the VT reactor, *Fibrobacteraceae (*13.8 %*)*, an unidentified family in the order Clostridiales (13.6 %, Fig. 6.4 group C), together with *Porphyromonadaceae* (13.4 %), were the most abundant families. Specifically, less numbers of taxa (group C, Fig. 6.4) were enriched compared with the CT reactor (group B), in accordance with the lower community diversity (Fig. 6.1). *Ruminococcaceae*, *Tissierellaceae*, and *Clostridiaceae* showed higher than a 2-fold change

compared with the inoculum. *Xanthomonadaceae* and *Actinomycetales* were largely increased in phase 1, then decreased in phases 2 and 3.

Ruminococcaceae, *Tissierellaceae*, and Clostridiales have been reported in high-ammonia stressed anaerobic communities (Muller et al., 2016). Species in the family *Tissierellaceae* were related to salt tolerance, such as *Tissierella* and *Soehngeni* (Wang et al., 2017). *Sedimentibacter* in the family *Tissierellaceae* was enriched in low-ammonia SAO communities (Muller et al., 2016). SAO bacterial species include five known species, *Thermacetogenium phaeum*, *Pseudothermotoga lettingae*, *Tepidanaerobacter acetatoxydans*, *Clostridium ultunense*, and *Syntrophaceticus schinkii*, the latter two characterized as mesophilic SAOB (Westerholm et al., 2016). However, these species were not identified in our study. The enriched consortia in the VT reactor under high organic loading and ammonia stresses resemble previously reported SAO communities (Werner et al., 2014; Muller et al., 2016; Tian et al., 2018a), suggesting that a broader number of species may be associated with SAO function under the high ammonia stress (Westerholm et al., 2016).

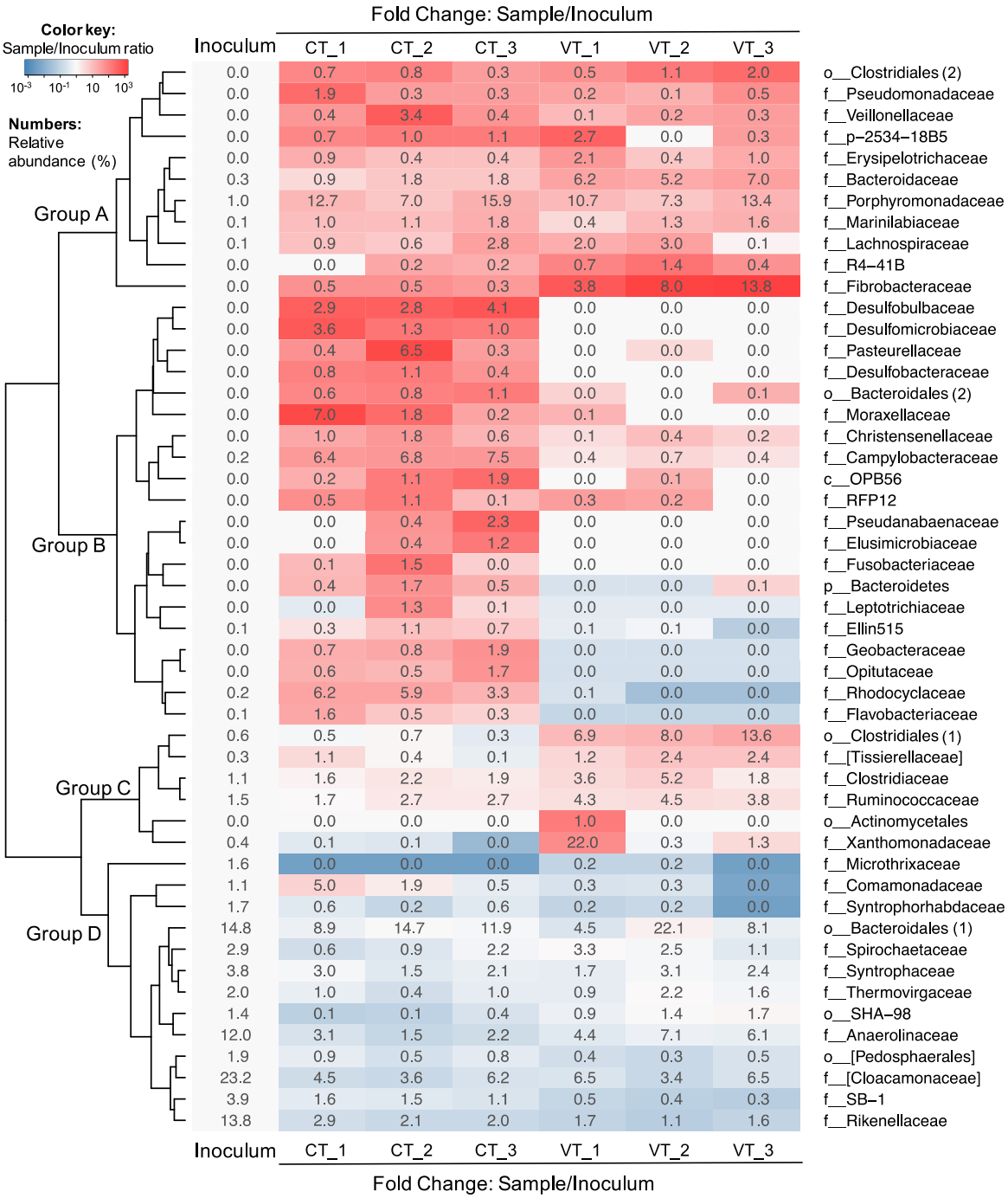

Fig. 6.4. Heatmap of fold changes of bacterial families with relative abundance >1 % in any sample, the taxonomic names were shown for family level (f_), or higher level (order: o_; class: c_) if not identified at family level. Numbers are relative abundances (%) of families in each sample. Color key indicates fold change of family relative abundances between reactor sample and inoculum, 0.01% was added to families in inoculum to avoid zero denominator. Hierarchical

clusters indicate similarities among families based on their fold changes using Euclidean distance method.

6.3.7 Functional shift

At the end of reactor operation, a carbon isotope test was performed for the methane and carbon dioxide gases. Our results showed that the biogas $\delta^{13}CH_4$ fraction was -46.71 ‰ and the $\delta^{13}CO_2$ fraction was -10.60 ‰ in the CT reactor, and -49.65 ‰ $\delta^{13}CH_4$ and -4.59 ‰ $\delta^{13}CO_2$ fraction in the VT reactor. The apparent fractionation factor (α_c) was 1.038 and 1.047 for the CT and VT reactors respectively. A higher α_c indicates that a methanogenesis pathway shifts from acetoclastic methanogenesis to hydrogenotrophic methanogenesis (Lü et al., 2013; Hao et al., 2017). Our results showed that the α_c value was higher in the VT reactor than that in the CT reactor, indicating that the archaeal communities were dominated by hydrogenotrophic methanogens. The ammonia concentration was higher in the VT reactor which may be related with the higher α_c.

The microbial community 16S rRNA gene data were analyzed to predict functional genes (Kanehisa et al., 2012; Markowitz et al., 2012) related with SAO-HM. The major methanogenesis pathways, hydrogenotrophic and acetoclastic methanogenesis, consisted of different functional genes (section 6.5). The total abundances are shown in Fig. 6.5A. Hydrogenotrophic methanogenesis genes slightly decreased in the CT reactor and increased in the VT reactor, whereas acetoclastic methanogenesis genes stayed at similar levels in all samples. The gene abundances were predicted from the 16S rRNA genes thus it reflects the taxonomic abundances in Fig. 6.3.

In the bacterial community, the prevalence of SAO indicator *fhs* gene (Muller et al., 2016) is shown in Fig. 6.5B. Compared to the inoculum, both 9L-BW and 1L-BW fed reactors had increased levels of the *fhs* gene. It increased gradually in the CT reactor. In the VT reactor, the *fhs* gene abundance showed a dramatic increase from phase 1 to phase 2, then marginally decreased in phase 3. The total relative abundances of OTUs contributing to *fhs* gene prevalence showed similar trends, accounting for 24 % of total bacterial OTU abundance in the inoculum, 34-40 % in the CT reactor and 30-38 % in the VT reactor. Detailed OTU relative contribution to

the *fhs* gene prevalence is shown in supplementary Fig. S6.2 and Fig. S6.3 at a family level. The main contributing families in the inoculum were syntrophic bacteria *Syntrophorhabdaceae* and *Syntrophaceae*, which decreased in both reactors. In the CT reactor, the highest contribution was from sulfate-reducing bacterial families *Desulfobulbaceae* and *Desulfomicrobiaceae*. *Porphyromonadaceae* and *Ruminococcaceae*, showed increased contribution in phase 3. In the VT reactor, the highest contribution was from an unidentified family in the order *Clostridiales*, which increased through phases 1 to 3.

Note that the CT and VT reactors did not show significant differences in their predicted functional profiles. Their reactor performances and enriched community compositions are different, but it could hardly be explained through the predicted prevalence of the *fhs* gene. The higher abundances of sulfate-reducing bacteria (Fig. 6.4) in the CT reactor may contribute to lower methane production yield (Liu et al., 2015) due to the competition of sulfate-reducing bacteria for carbon source, and/or the toxicity of hydrogen sulphide (produced from sulfate reduction reaction) to methanogens (J.W.H. et al., 1994; Siles et al., 2010; Dai et al., 2017). Ammonia concentration, together with other possible stress factors in the VT reactor, could contribute to the low microbial community diversity; which did not significantly impact the SAO-HM communities.

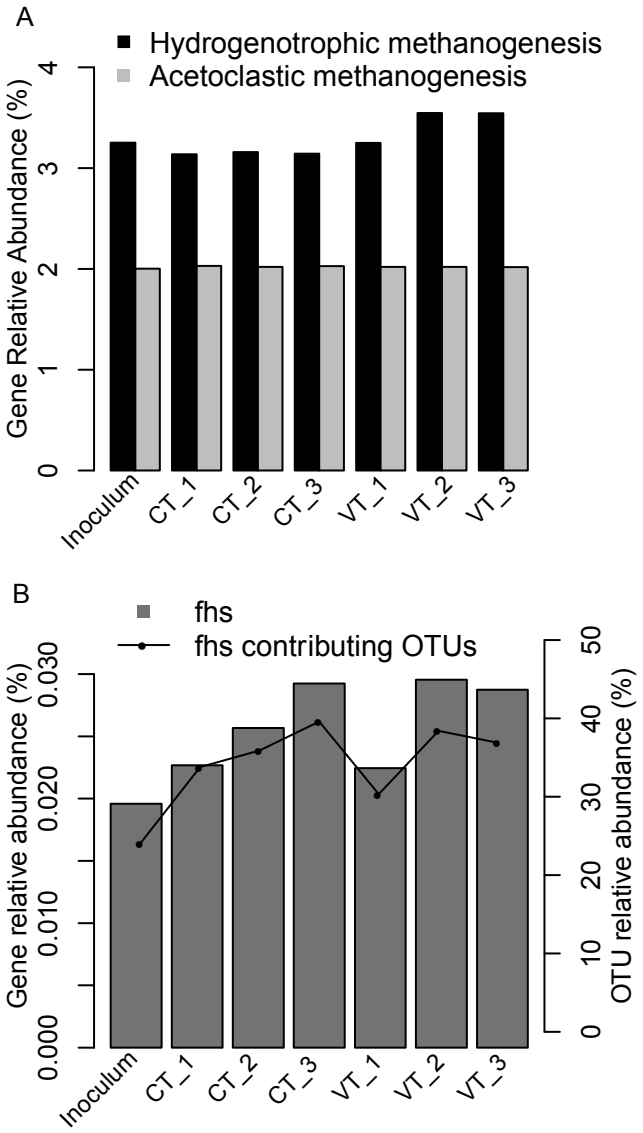

Fig. 6.5. Relative prevalence of hydrogenotrophic and acetoclastic methanogenesis genes in archaeal metagenome predicted using 16S rRNA gene sequence data using PICRUSt (A); Relative prevalence of gene *fhs* in bacterial metagenome and relative abundances of OTUs contributing to gene *fhs* prevalence (B). Genes were predicted using 16S rRNA gene sequence data using PICRUSt.

6.4 Conclusions

Water-saving vacuum toilets (VT) collected blackwater contains significantly higher concentrations of organics and ammonia compared to the conventional toilets (CT) collected

blackwater, which has led to different biogas production rates and microbial communities in anaerobic digestion reactors treating blackwater. The VT reactor showed a higher methane production rate, without any noticeable inhibition to the microbial community. The temporal dynamics of archaeal and bacterial communities indicated that the archaeal community had slowly adapted during different phases, resulting in a mono-dominance of a single genus; whereas the bacterial community was quickly adapted and remained stable through different phases. Lower alpha-diversity and higher methane production rates indicated selection of r-strategists in the VT reactor consortia due to the high substrate and ammonia concentrations in VT collected blackwater.

The enriched microorganisms were compared between the CT and VT reactors. *Methanolinea* was the most abundant archaeal genus in the CT reactor, and *Methanogenium* in the VT reactor. *Methanogenium* was reported to tolerate high ammonia concentrations but is infrequently reported as dominant in anaerobic digesters. Both reactors were dominated by hydrogenotrophic methanogens. The enriched bacteria were linked with high ammonia conditions, including *Porphyromonadaceae*, *Fibrobacteraceae*, *Ruminococcaceae*, *Bacteroidaceae*, *Clostridiales*, etc. The apparent fractionation factor (α_c) calculated from $\delta^{13}CO_2$ and $\delta^{13}CH_4$ fractions and predicted metagenomic functions indicated functional shifts to the syntrophic acetate oxidization and hydrogenotrophic methanogenesis pathway in the VT reactor. This study provided detailed information on microbial community dynamics and implied functions for concentrated and dilute types of blackwater treatment using anaerobic digestion.

6.5 Supplementary materials

6.5.1 Materials and Methods

Reference modules and genes in the KEGG database

1) Hydrogenotrophic methanogenesis

Pathway module: M00567, Methanogenesis, CO_2 => methane

Definition: (K00200 + K00201 + K00202 + K00203-K11261+(K00205, K11260, K00204)) K00672 K01499 (K00319, K13942) K00320 (K00577 + K00578 + K00579 + K00580 + K00581-K00582-K00583 + K00584) (K00399 + K00401 + K00402) (K22480 + K22481 +

K22482, K03388 + K03389 + K03390, K08264 + K08265, K03388 + K03389 + K03390 + K14127+(K14126 + K14128, K22516 + K00125))

2) Acetoclastic methanogenesis

Pathway module: M00357, Methanogenesis, acetate => methane

Definition: (K00925 (K00625, K13788), K01895) (K00193 + K00197 + K00194) (K00577 + K00578 + K00579 + K00580 + K00581-K00582-K00583 + K00584) (K00399 + K00401 + K00402) (K22480 + K22481 + K22482, K03388 + K03389 + K03390, K08264 + K08265, K03388 + K03389 + K03390 + K14127+(K14126 + K14128, K22516 + K00125))

3) *fhs* gene

KEGG orthology: K01938

Definition: formate--tetrahydrofolate ligase

6.5.2 Results

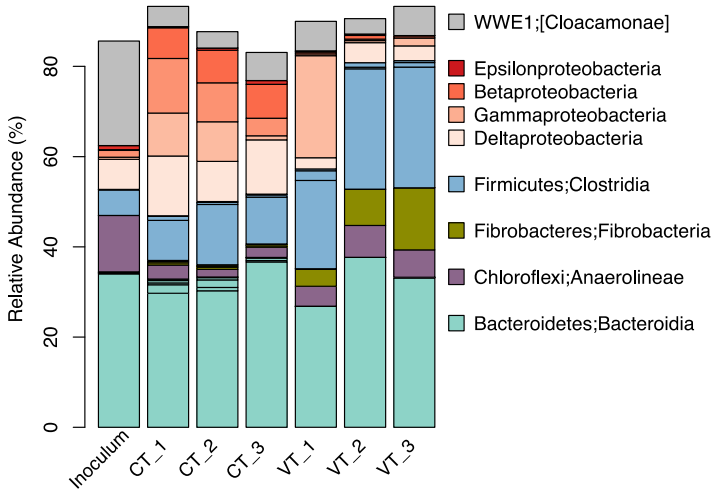

Fig. S6.1. Relative abundances of top 5 abundant classes in each sample. Samples were inoculum, conventional toilet blackwater (CT) and vacuum toilet blackwater (VT) reactors at phase 1, 2, and 3.

The predominant bacterial classes are shown in Fig. S6.1. *Bacteroidia* (phylum *Bacteroidetes*) was the most abundant class in the inoculum (34.0 %), and remained at high levels in reactors: 29.7 %, 30.2 %, 36.7 % in CT reactor phases 1-3 and 26.8 %, 37.7 %, 33.1 % in VT reactor phases 1-3, respectively. *Clostridia* (phylum *Firmicutes*) was the second most abundant class in the VT reactor (19.6-26.7 %), and in the CT reactor (8.9-13.4 %), with initial abundance of 5.7 % in the inoculum. *Fibrobacteria* (phylum *Fibrobacteres*) was not detected in inoculum and below 1 % in the CT reactor but increased gradually in the VT reactor (3.8 %, 8.0 % and 13.8 % in phases 1-3). In the phylum *Proteobacteria*, *Deltaproteobacteria* took up 6.7 % of inoculum and increased in the CT reactor, but decreased in the VT reactor. *Gammaproteobacteria*, *Betaproteobacteria* and *Epsilonproteobacteria* were all increased in the CT reactor. Only *Gammaproteobacteria* were significantly increased in VT reactor in phase 1. *Cloacamonae* (phylum WWE1) and *Anaerolineae* (phylum *Chloroflexi*) were the second and third highest classes in inoculum (23.2 % and 12.5 %) but all reduced to below 7 % in reactors.

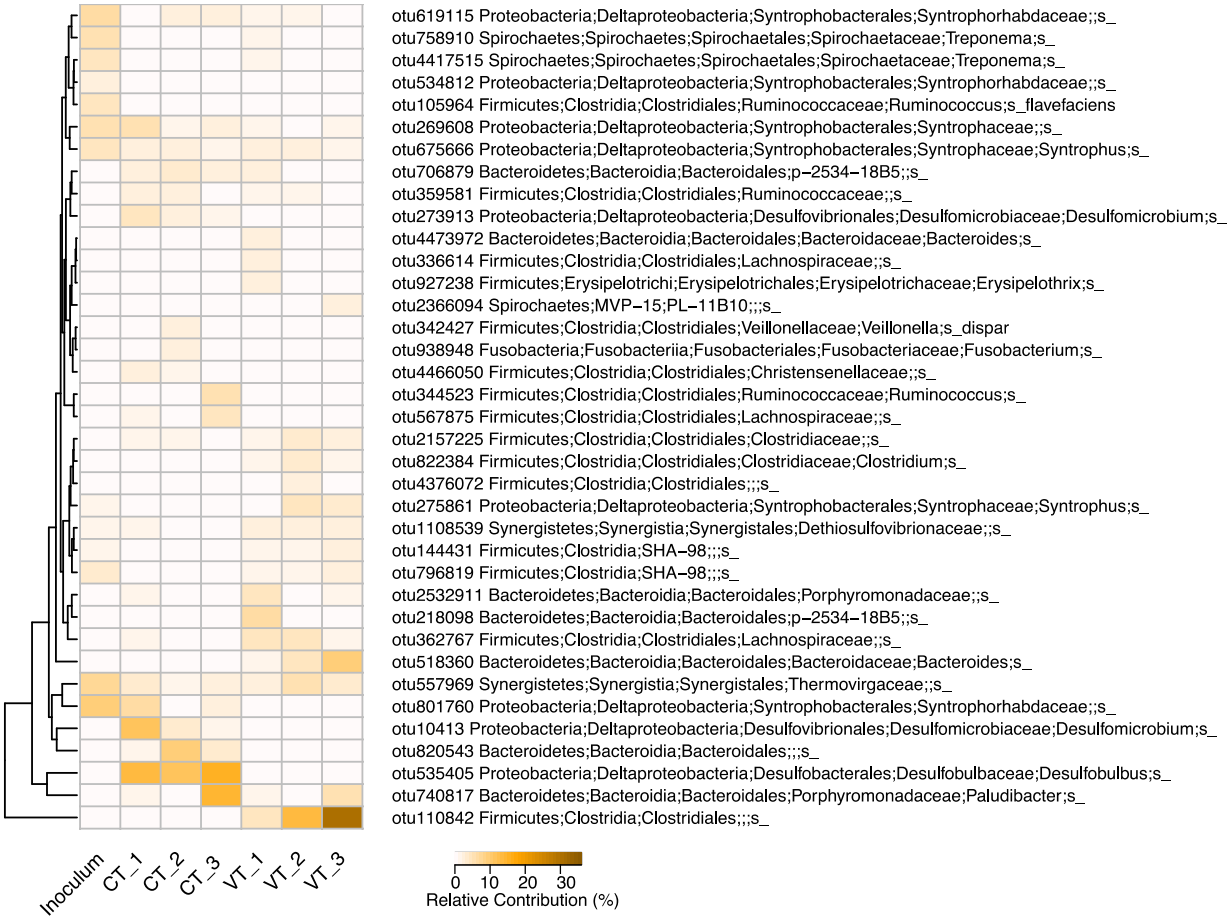

Fig. S6.2. Heatmap of top 10 OTU contributors on *fhs* gene abundance from each sample. Color key indicates relative contribution of OTUs.

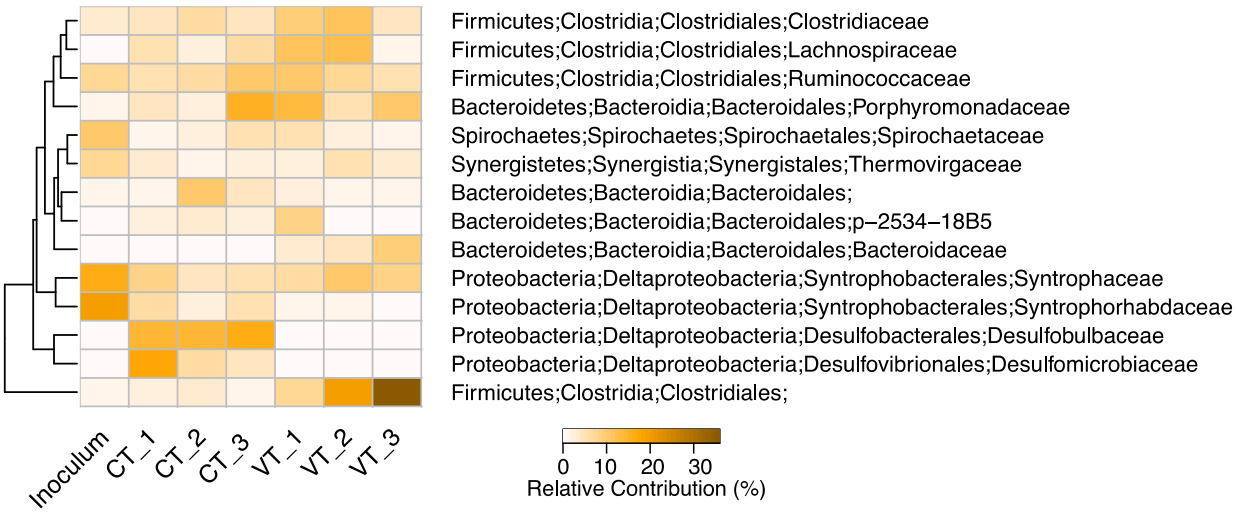

Fig. S6.3. Heatmap of top 5 family contributors on *fhs* gene abundance from each sample. Color key indicates relative contribution of families.

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CHAPTER 7. HIGH-LOADING FOOD WASTE AND BLACKWATER ANAEROBIC CO-DIGESTION: MAXIMIZING BIOENERGY RECOVERY[5]

7.1. Introduction

Limitations on biomethane recovery from source-diverted blackwater treatment have been identified in our previous studies. The high ammonia concentration in blackwater can inhibit the methanogenesis process. For instance, low methane yield (34%) was observed when treating blackwater with a high free ammonia (FA) concentration of 393 mg/L, as compared to a methane yield of 48% when treating blackwater with relatively lower FA concentrations of 26 and 60 mg/L (Gao et al., 2019a). Due to the high solid organic contents (53-85% of total organic matter (Kujawa-Roeleveld et al., 2005; van Voorthuizen et al., 2008)), substrate hydrolysis has been considered as a rate-limiting step when performing anaerobic blackwater treatment (Zeeman and Sanders, 2001). For example, a 8.2% reduction in the solids substrate hydrolysis efficiency can result in a 5 % decrease in methane production yield in a blackwater anaerobic digestion process when solids retention time (SRT) decreased from 254 days to 75 days (De Graaff et al., 2010). With such limiting factors, relatively low OLRs were observed in the established blackwater anaerobic treatment systems (Gao et al., 2019b; Luostarinen and Rintala, 2005; Zeeman et al., 2008). Our previous study has demonstrated the highest OLR of 4.1 kg COD/m^3/d for treating vacuum toilet collected blackwater reported to date using an upflow anaerobic sludge blanket (UASB) reactor at 35 °C (Gao et al., 2019b), and found that further increasing OLR resulted in hydrolysis limitation (44% reduction in solids COD hydrolysis efficiency).

Multiple types of optimization methods regarding enhancing solid substrate hydrolysis have been reported (Chen et al., 2008; Nguyen et al., 2017), yet, these strategies come with extra operational and capital costs, which are not ideal for industrial implementation. An alternative option is to perform anaerobic co-digestion with other organic wastes, such as food waste (Silvestre et al., 2014). With the readily biodegradable organics and high C/N ratio, food waste can help increase the organic load, lower pH, optimize substrate C/N ratio, and could ultimately facilitate microbial growth and enhance microbial activities in anaerobic treatment systems (Kim

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et al., 2011; Zhang et al., 2019). Extensive studies have been conducted on waste co-digestion over the past decade, especially for agricultural waste with high ammonium such as animal manure, and it has been well established that the better C/N ratios and buffer capacities of the co-substrates helped improved treatment efficiencies and enhanced the maximum OLRs (Kim et al., 2011; Zhang et al., 2013, 2019). However, only limited work has been conducted on blackwater and food waste co-digestion; most of these reported work were operated under low OLRs and no study has evaluated the feasibility of high-loading UASB treatment (Elmitwalli et al., 2006; Kujawa-Roeleveld et al., 2005; Lavagnolo et al., 2017; Rajagopal et al., 2013; Rounsefell et al., 2013; Wang et al., 2018; Zhang et al., 2019). A continuous stirred tank reactor (CSTR) system achieved 50-75% COD removal at various hydraulic retention time (HRT) of 10-20 days (OLRs 1-2 kg COD/m^3/d) for blackwater and food waste co-digestion (Wendland et al., 2007). A pilot scale UASB-septic tank (0.2 m^3) obtained 82% COD removal with an HRT ~29 days (Kujawa-Roeleveld et al., 2005). A sequencing batch reactor (SBR) treated feces and food waste and achieved 76.7% COD removal and obtained OLRs of 2-3 kg COD/m^3/d (Rajagopal et al., 2013). The highest OLR of 4.1 kg COD/m^3/d reported so far for blackwater and food waste co-digestion was demonstrated in an improved upflow solid reactor (IUSR), but the value did not exceed the highest OLR for treating only blackwater in our previous work. Organic loads of blackwater and food waste varied in these reported studies. An European demonstration study reported a feed BW and FW VSS load ratio of 1:1.6 with 5 L BW/capita/d and 200 g FW/capita/d production (Wendland et al., 2007). A study in Singapore reported 2 L brown water (only feces) and 150 g food waste per capita daily production that resulted in VS ratio of 1:5.5 for brown water : food waste [21], while a 1:1.1 BW : FW VS load ratio was reported in China with the 5 L BW/capita/d and 500 g FW/capita/d generation (Wang et al., 2018). In North America, the reported BW : FW VS collection ratio ranged between 1:2-1:3 in the decentralized household sanitation system (Zhang et al., 2019). These variations can be associated with local diet and living habitats, *etc.*, which contributed to variations in the reported treatment performance in addition to the different operational conditions and reactor types applied.

Our group's study has demonstrated a 53% increase in substrate hydrolysis efficiency from treating only blackwater to food waste and blackwater co-substrate in batch test, yet it has not been evaluated in continuous UASB operation (Zhang et al., 2019). In order to mitigate the

hydrolysis limitation in blackwater mono-digestion and to evaluate the feasibility of utilizing UASB for a high-loading blackwater and food waste co-digestion operation, food waste was added into the UASB reactor previously acclimatized to vacuum toilet blackwater treatment (with an OLR of 4.1 kg COD/m^3/d). The amount of food waste addition was stepwise increased to obtain increasing OLRs. The effectiveness of the co-digestion strategy was evaluated by the improvement of overall biomethane recovery and OLR achieved. The information provided by the present work should help to assess the environmental and economic feasibility of blackwater and food waste co-digestion strategy and potentially guide future decentralized waste/wastewater treatment system designs.

7.2. Materials and methods

7.2.1 Substrates

Vacuum toilet blackwater (utilizing 1 L flushing water per flush) and food waste were collected from University of Alberta campus (Edmonton, Alberta). Raw food waste collected from food courts and student lounges was mainly composed of grains (*e.g.*, rice, noodle, bread), vegetable residuals (*e.g.*, broccoli, carrot, onion), fruit residuals (*e.g.*, banana and orange peels, apple cores), and beverage residuals (*e.g.*, tea, coffee). Food waste were manually mixed and grinded with an electrical kitchen blender to reduce the particle size and further mixed with handheld mixer to obtain substrate homogeneity. Substrates were stored at 4 °C until use. In each operation phase, the blackwater and food waste co-substrates were prepared at the following VS mixing ratios (BW:FW): 1:0.3, 1:0.6, 1:1, and 1:1.5 for Phases I – IV, respectively (Table 7.1), where increasing amount of food waste was added on top of the fixed blackwater load (4.1 kg COD/m^3/d) to form the co-substrates. The VS concentrations of blackwater and food waste were measured prior to co-substrate preparation. In each co-substrate preparation, the food waste was added into blackwater, shaken for evenly mixing and then stored in a feedstock container equipped with a continuous mixing stirrer (120 rpm) (Fisher scientific, CA) to ensure homogenized feeding. The feedstock container was placed in a fridge (4 °C) next to the bioreactor to minimize the COD loss during the storage and feeding process.

7.2.2 Reactor set-up and operation

A 3.5-L (3.3-L working volume) UASB reactor was operated at 35 °C with a heating blanket. This study followed the previous blackwater mono-digestion treatment study, for which the initial inoculum was obtained from the blackwater acclimatized sludge (304 days' operation) with a volume of 1 L and volatile suspended solids (VSS) concentration of 27.9 g/L. The co-substrate was continuously pumped out from the feedstock container (4 °C) into the UASB using a peristaltic pump (Longer pump BT 100-2J). The biogas and effluent were collected at the top of the reactor. Daily methane generation was obtained by measuring the daily gas volume and the biogas contents using gas chromatography (GC) (7890B Agilent Technologies, USA). The height of sludge bed was constantly monitored to calculate the sludge volume change during the operation. Overall, the operation lasted for 130 days and was divided into four operational phases. For all four phases, the operational hydraulic retention time (HRT) was maintained at 2.6 days according to the previous optimized results in blackwater mono-digestion. The OLR was increased step-wise from 5.1 (± 0.2) kg COD/m^3/d (Phase I) to 11.6 (±0.6) kg COD/m^3/d (Phase IV) throughout the duration of operation by increasing the food waste load on top of the blackwater load (4.1 kg COD/m^3/d) (Section 2.1). The higher OLRs (while keeping the constant HRT) achieved can be also attributed to the stepwise substitution from blackwater to food waste as the major organic source in co-substrates, where the higher feedstock VS content resulted in higher energy density. Each phase was operated for at least 30 days to obtain stable biodegradation performance, which was determined by relatively constant COD removal efficiencies and biomethane generation rates (Kroeker et al., 1979; Yi et al., 2014) for at least two consecutive weeks in each operation phase.

7.2.3 Sludge properties

At the end of each operation phase, UASB sludge was collected from each port of the sludge bed and mixed. The sludge pH, COD, total suspended solids (TSS), and VSS were measured before performing batch tests to determine the stability and specific methanogenic activity (SMA) of the sludge.

Specific methanogenic activity (SMA)

Sludge SMA batch experiments were conducted to evaluate the maximum methane production rate that the sludge developed throughout the reactor's operation; SMA results were presented as g CH_4-COD/sludge VSS/d. In each operation phase, the test was performed with acetate or H_2 & CO_2 gas as precursor substrates to illustrate the methanogen production rates through acetoclastic or hydrogenotrophic pathways, respectively. Serum bottles with volumes of 166 mL per bottle were filled with the UASB mixed sludge and the substrate (sodium acetate or H_2 & CO_2 gas); the initial substrate concentration was designed to be 1 g/L. The pH of the mixed liquor was adjusted to neutral (~7) to provide fair comparison throughout the operation. When sodium acetate was applied as a substrate, the headspace of the bottles was flushed with N_2 gas to provide an anaerobic environment. When H_2 & CO_2 was applied as a substrate, the bottles were flushed with H_2 & CO_2 gas (80% & 20% by volume, respectively). Blank controls were set-up simultaneously (with no added substrates and flushed with N_2 gas) to evaluate CH_4 production from sludge in the test group. The bottles were sealed with rubber septums and aluminum caps after gas flushing and incubated at 35 °C in a shaking incubator (New Brunswick™ Innova® 44, Eppendorf, Canada) in dark conditions. Each group of batch test was conducted in triplicates. The gas production was obtained by measuring the headspace pressure using a pressure meter (GHM 3151, Germany) and the gas composition using GC. The SMA values were obtained from dividing the linear slope of methane production over the sludge VSS content in each bottle. The SMA (acetate and H_2 & CO_2) results were presented with average values of the triplicate measurements with standard deviations.

Sludge stability

The sludge stability test was performed to evaluate the amount of biodegradable COD presented in the sludge which could be converted to methane. Testing of each group of sludge stability was conducted at 35 °C using freshly collected sludge samples. The experimental setup followed a previous study (Gao et al., 2019b). The results of each sludge stability test are shown in g CH_4-COD/g sludge COD with an average value of the triplicate measurements and one standard deviation. Higher values indicate more biodegradable organics present in sludge, which represent less sludge stability.

7.2.4 Analytical methods

The UASB influent and effluent chemical properties were measured 3 – 5 times each week, including the pH, total COD (CODt), soluble COD (CODs), total solids (TS), VS, total nitrogen (TN), total ammonia nitrogen (TAN), reactive phosphorus (PO_4^{3-}-P), alkalinity (as $CaCO_3$), and three main volatile fatty acids (VFAs) including acetate, propionate, and butyrate. The sludge characteristics of pH, CODt, TSS, and VSS were measured twice in each operation phase as well as every time the sludge was discharged from the UASB. The COD, TS, VS, TSS, and VSS measurements were conducted following the standard methods described by American Public Health Association (APHA/AWWA/WEF, 2012). The TN, TAN, PO_4^{3-}-P, and alkalinity were measured using Hach TNT vial tests (Hach, USA). VFA measurements were conducted using ionic chromatography (IC) equipped with a conductivity detector (DIONEX ICS-2100, ThermoFisher, USA). The pH was measured using a B40PCID pH meter (VWR, SympHony). 20 L volume gas bags (Multilayer foil gas bag with Locking Combo Valve® with Septum, CHROMSPEC™, Canada) were connected to the UASB reactor for biogas collection. The biogas volume was manually measured with gas tight syringe (140 mL, equipped with stopcock). Biogas composition was measured using a 7890B gas chromatograph (Agilent Technologies, Santa Clara, USA) equipped with a thermal conductivity detector (TCD) and two columns (Molsieve 5A 2.44 m 2 mm for CH_4 and Hayesep N 1.83 m 2 mm for N_2, O_2 and CO_2). The temperature of the oven, injector, and detector was 100, 150, and 200 °C, respectively. Gas sample was withdrawn from the UASB gas bag using the gas tight sampling syringe with ten times flushing to ensure the gas homogeneity and was analyzed with the GC loop injection method. GC injections with a fixed standard gas (known composition of 40% CH_4, 25% CO_2, 1% O_2, 34 N_2%) (PRAXAIR, Canada) were conducted before and after biogas measurements to serve as a quality assurance and quality control (QA/QC) check.

7.2.5 Calculations

Methane production and methanisation rates

The methane production rate was calculated from the daily methane generation (biogas volume multiplied by the methane fraction in biogas) (L/d) from per volume (L) of reactor and presented as L/L/d. The methanisation rate represents the amount of feedstock COD that was converted

into methane COD through the treatment process and is shown as g CH_4-COD/g feed COD as a percentage (%).

Hydrolysis efficiency

The hydrolysis efficiency shown in this study indicates the hydrolyzed fraction of influent solid COD substrates. The solid COD content was calculated by subtracting the soluble COD from total COD in the influent; the hydrolysis efficiency was then calculated using the following equation:

$$Hydrolysis\ efficiency\ of\ COD_{solids}(\%) = \frac{COD_{CH4} + COD_{s,effluent} - COD_{s,influent}}{COD_{t,influent} - COD_{s,influnet}}$$

Solids retention time (SRT)

The theoretical SRT was calculated based on the amount of sludge wasted and washed out from the UASB (in VSS). Calculated SRT values were compared with the total operation time for each phase. When the theoretical SRT was longer than the practical reactor operation time, the real operation time was considered as the operational SRT for that phase (Metcalf and Eddy, 2003).

COD mass balance

For each operation phase, the amount of input COD was partitioned into methane production (COD-methane), COD discharge from wasted sludge (COD-discharge), COD accumulated in UASB sludge bed (COD-accumulation), and residual COD in UASB effluent (COD-effluent). The COD mass balance was calculated in the steady state of each operation phase.

Methanogenic capacity

The methanogenic capacity was calculated with the maximum sludge SMA and the amount of sludge VSS in the UASB at the steady state of each operation phase; this represents the highest potential methane production capability. For each operation phase, the methanogenic capacity was compared with the real methane production and the OLR to illustrate the process limitation.

VS removal efficiency

The VS removal efficiency was calculated weekly as the amount of VS removed from the influent VS after UASB treatment. Weekly average VS concentrations for UASB influent and effluent were utilized for the calculation.

7.3. Results and discussion

7.3.1 Feedstock properties

The chemical properties of the blackwater, food waste and food waste co-substrates in each co-digestion operation phase are presented in Table 7.1. The collected food waste contained TS and VS concentrations of 274.9 (± 28.7) and 241.6 (± 27.2) g VS/kg FW, respectively, which is within the general reported range of 135 – 280 g VS/kg FW (Gou et al., 2014; Rajagopal et al., 2013). The feedstock pH values decreased from 8.5 (VS ratio 1:0.3) to 7.8 (VS ratio 1:1.5) due to the increasing proportion of food waste (pH 4.9) in the co-substrates. Similar food waste pH values ranging between 4.4 – 5.3 have been reported in previous studies (Lavagnolo et al., 2017; Rajagopal et al., 2013). The co-substrate CODt concentration increased from 14.2 (± 0.6) to 30.5 (± 1.7) g/L throughout the operation; the CODs concentration increased from 5.1 (± 0.3) to 11.6 (± 0.8) g/L, respectively. Compared to blackwater having 32 % of the CODt as CODs, higher fractions of soluble organic contents were observed in the BW-FW co-substrate with between 36 – 38 % of the CODt as CODs. A comparable value of 37 % has been previously reported for BW-FW co-substrates (Rajagopal et al., 2013). Considering the low CODs fraction (21 %) in food waste, the higher CODs levels achieved in the co-substrate may be explained by the immediate hydrolysis of the solid organics after mixing blackwater and food waste (Wendland et al., 2007). Consequently, the short-chain VFA generation was also enhanced, where concentrations of acetate, propionate, and butyrate in the co-substrate drastically increased compared to blackwater alone (Table 7.1). Since food waste contains relatively low nitrogen levels (0.6 g TN/L & 0.1 g TAN/L), the TN concentrations of 2.5 – 3 g/L and the TAN concentrations of 1.1 g/L in co-substrates were primarily contributed by blackwater. The variations in TN contents were mainly due to the fluctuations in blackwater and food waste properties. As a result, the increased food waste proportion resulted in higher COD/TN ratios of the co-substrates. Compared to the blackwater COD/TN ratio of 4.1:1, the co-substrates showed increased values ranging from 5.6:1 to 11.0:1 in Phases I to IV, respectively, and were closer to the feasible COD/TN ratios of 20:1-40:1 demonstrated for effective anaerobic digestion (Fricke

et al., 2007; Speece, 2008). The higher COD/TN ratios achieved for the co-substrates tend to benefit the anaerobic treatment process with enhanced substrate digestibility. As demonstrated in our preliminary tests, bio-methane potential (BMP) values of 0.54 – 0.85 g CH_4-COD/g feed COD were obtained for BW-FW co-substrates compared to only 0.35 – 0.48 g CH_4-COD/g feed COD for blackwater alone, and no CH_4 production for food waste alone (due to high VFA accumulation) (Gao et al., 2019a; Zhang et al., 2019). The PO_{43}-P concentrations of co-substrates were in the range of 24.4 (±0.9) – 34.1 (±8.5) g/L, which is comparable with the values for blackwater, indicating the majority contribution from blackwater. The co-substrates alkalinity 1.63 (±0.01) – 1.87 (±0.08) g/L was contributed from the alkalinity of blackwater 1.99 (±0.13) g/L, indicating the high buffer capacity of blackwater. This observation was comparable with previous studies that feces and urine contained high alkalinity (Lavagnolo et al., 2017), but food waste treatment requires additional alkalinity for process stability (Chen et al., 2015).

Table 7.1. Operational conditions and feed properties.

	Unit	BW	FW	BW+FW (Phase I)	BW+FW (Phase II)	BW+FW (Phase III)	BW+FW (Phase IV)
BW:FW VS mixing ratio		-	-	1:0.3	1:0.6	1:1	1:1.5
Operation duration	day	-	-	1-30	31-60	61-98	99-130
Initial pH		8.7 (±0.1)	4.9	8.5 (±0.0)	8.4 (±0.0)	8.0 (±0.2)	7.8 (±0.3)
CODt	g/L	11.0 (±0.9)	413.1 (± 22.4)	14.2 (±0.6)	20.0 (±1.1)	25.4 (±1.2)	30.5 (±1.7)
CODs	g/L	3.5 (±0.2)	86.7 (± 4.7)	5.1 (±0.3)	7.5 (±0.4)	9.5 (±0.8)	11.6 (±0.8)
CODs /CODt	%	32	21	36	37.5	37.4	38
Acetate	g COD/L	0.45 (±0.18)	-	1.01 (±0.22)	1.20 (±0.12)	1.32 (±0.20)	1.25 (±0.17)
Propionate	g COD/L	0.32 (±0.08)	-	0.68 (±0.24)	0.61 (±0.12)	0.66 (±0.07)	0.61 (±0.08)
Butyrate	g COD/L	0.17 (±0.04)	-	0.22 (±0.04)	0.21 (±0.04)	0.32 (±0.05)	0.20 (±0.04)
TN	g/L	2.7	0.6	2.5	3.0	2.7	2.8

		(±0.2)	(± 0.1)	(±0.0)	(±0.3)	(±0.0)	(±0.0)
TAN	g/L	1.1 (±0.0)	0.1 (±0.0)	1.1 (±0.0)	1.1 (±0.0)	1.1 (±0.0)	1.1 (±0.0)
PO_{43}-P	mg/L	29.3 (±10.7)	-	27.6 (±5.3)	24.4 (±0.9)	34.1 (±8.5)	32.9 (±3.6)
Alkalinity	g $CaCO_3$/L	1.99 (±0.13)	-	1.87 (±0.08)	1.83 (±0.05)	1.72 (±0.06)	1.63 (±0.01)
COD/TN		4.1:1	688.5:1	5.6:1	6.6:1	9.4:1	11.0:1
TS	g/L	10.2 (±0.9)	274.9 (± 28.7)	13.4 (±1.4)	16.4 (±1.8)	20.6 (±0.9)	24.3 (±1.7)
VS	g/L	8.7 (±0.7)	241.6 (± 27.2)	11.7 (±1.2)	14.0 (±1.3)	17.5 (±0.6)	21.5 (±1.5)

7.3.2 Organics removal

The overall UASB treatment performance including COD removal efficiency (Fig. 7.1A), effluent VFA concentrations and VFA/alkalinity ratios (Fig. 7.1B), and methane production rate (Fig. 7.1C), are presented along with the UASB effluent properties in Table 7.2.

From Phase I-IV, the increased FW addition resulted in an increase in OLR from 5.1 (± 0.2) to 11.6 (± 0.6) kg COD/m_3/d (Fig. 7.1A, Table 7.1), which was 24 % - 183 % higher over the maximum feasible OLR (4.1 [± 0.4] kg COD/m_3/d) demonstrated in blackwater mono-digestion (Gao et al., 2019b). Throughout the operation, the residual COD in the effluent increased from 2.3 (± 0.4) g/L in Phase I to 5.3 (± 0.9) g/L in Phase IV, while comparable COD removal efficiencies were achieved in the range of 82.4 (± 2.8) – 83.6 (± 3.6) %. It should be noted that in order to maintain a good effluent quality, sludge was wasted from the UASB to avoid sludge wash-out. Correspondingly, the total VS removal efficiencies ranged from 80.8 (± 0.1) % to 85.8 (±0.9) % (Table 7.2). The relatively high COD removal efficiencies obtained in the food waste co-digestion reactor were similar to the value obtained in the previous study of blackwater mono-digestion (84 %) (Gao et al., 2019b). This result demonstrates that efficient organic reductions can be achieved under higher OLRs.

The level of pH, VFAs and alkalinity correlate with each other and are important for process stability in anaerobic digestion system. In the current study, the total VFA concentration in the effluent maintained at a relatively low level of 0.39 g COD/L in Phase I, where 62 % was from acetate residuals. When the OLR was increased to 7.0 (± 0.5) kg COD/m^3/d in Phase II, the effluent VFA concentration increased to 1.15 (± 0.4) g COD/L. When the OLR was further increased to 10.0 (± 0.5) kg COD/m^3/d in Phase III, the effluent VFA drastically increased at an early stage with concentrations reaching 2.69 (± 0.5) g COD/L, indicating a temporary VFA accumulation, while the effluent residual VFA contents dropped to 1.67 (± 0.4) g COD/L at the later steady state. A temporary VFA accumulation is generally observed in shock loading conditions when the system has not been well-acclimatized to the high OLR condition (Wu et al., 2018). In the final operation phase where the OLR reached 11.6 (± 0.6) kg COD/m^3/d, effluent VFA concentrations were 2.25 (±0.53) g COD/L. Similar high VFA values have been reported in a CSTR system with effluent VFA concentration up to 2.6 g COD/L when the OLR reached 2 kg COD/m^3/d, while steady operation was still achieved due to the system's buffering capacity (Wendland et al., 2007). VFA/alkalinity ratio has been suggested as an indicator for system stability, where values of 0.3-0.4 have been considered optimal and a level >0.8 may suggest system overloading (Wang et al., 2012). In the current work, the ratio of VFA/alkalinity increased from 0.11 (± 0.03) to 0.83 (± 0.07) with the increasing VFA concentrations and the decreased alkalinity (3.3 [± 0.2] g /L to 2.6 [± 0.3] g /L) from Phase I to IV. High VFA/alkalinity ratios of >0.8 were observed in Phase IV. Similar observations have been reported in the co-digestion of cattle slurry and fruit and vegetable wastes (FVW) that the VFA/alkalinity ratios increased to 0.4-0.8 after FVW addition was increased to over 30% of the feed and OLR over 4.52 kg VS/m^3/d (Callaghan et al., 2002). pH values in all operational phases changed in a limited range of 7.5 (± 0.1) – 7.8 (± 0.1), which may imply that pH was less sensitive compared to the VFA/alkalinity ratios in the current condition. The VFA accumulations, decreased alkalinity and the increased VFA/alkalinity ratios demonstrated the potential of system instability after high OLRs were applied in phases III and IV despite of the high organic removal and methane production achieved under such conditions.

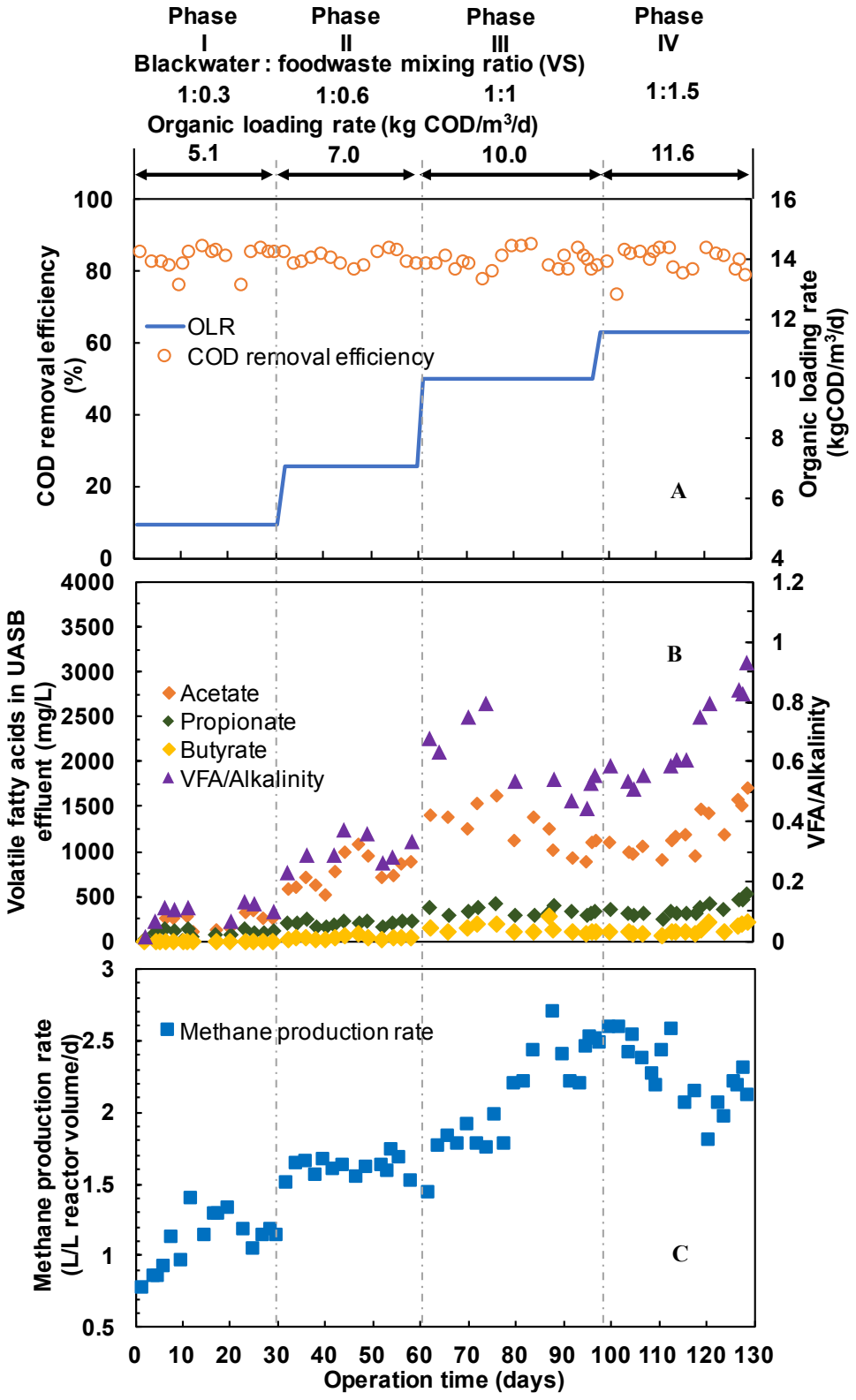

Fig. 7.1. Performance of blackwater and foodwaste co-digestion in Phase I-IV over time, OLR 5.1 (±0.2) kg COD/m3/d to 11.6 (±0.6) kg COD/m3/d, BW:FW mixing ratio (in VS) 1:0.3 to

1:1.5. COD removal efficiency (A); volatile fatty acids concentration in UASB effluent and VFA/Alkalinity ratios (B); methane production rate (L/L reactor/d) (C).

Table 7.2. Treatment performances and effluent properties.

	Unit	BW+FW (Phase I)	BW+FW (Phase II)	BW+FW (Phase III)	BW+FW (Phase IV)
CODt	g/L	2.3 (±0.4)	3.2 (±0.4)	4.2 (±0.8)	5.3 (±0.9)
CODs	g/L	1.1 (±0.1)	1.7 (±0.2)	2.1 (±0.6)	2.9 (±0.9)
TS	g/L	4.4 (±0.2)	5.5 (±0.3)	5.9 (±0.3)	6.2 (±0.3)
VS	g/L	2.3 (±0.1)	2.2 (±0.2)	2.9 (±0.3)	3.0 (±0.3)
VS removal efficiency	%	80.8 (±0.1)	84.2 (±1.9)	83.3 (±1.6)	85.8 (±0.9)
pH		7.8 (±0.1)	7.6 (±0.2)	7.6 (±0.2)	7.5 (±0.1)
TAN	g/L	1.2 (±0.0)	1.2 (±0.0)	1.2 (±0.0)	1.3 (±0.0)
PO_{43}-P	mg/L	28.3 (±6.9)	33.8 (±6.8)	21.2 (±6.4)	34.3 (±0.8)
Alkalinity	g $CaCO_3$/L	3.3 (±0.2)	3.3 (±0.0)	2.8 (±0.4)	2.6 (±0.3)

7.3.3 Biomethane production

Effective and stabilized biomethane production was achieved after 10 days of process start-up, which indicated the acclimatization of biomass to the BW-FW co-substrate. In operation Phase I, the methane production rate was 1.17 (± 0.12) L/L/d, which was 72 % higher than the highest value (0.68 [± 0.08] L/L/d) demonstrated in blackwater mono-digestion. A significantly enhanced methanisation rate of 58.8 % of the input COD was observed; this value is 34 % higher than treating blackwater alone (44.3 %). Similar to the current results, a 13 % higher methanisation rate was achieved in a BW-FW co-digestion system when compared to the treatment of blackwater alone (Wendland et al., 2007). From Phase I to III, the amount of

methane generated (1.17 [± 0.12] – 2.42 [± 0.15] L/L/d) was linearly correlated with the OLRs (5.1 [± 0.2] – 10.0 [± 0.5] kg COD/m^3/d, R^2 = 0.997, Fig. S7.1), with the methanisation rates in a range of 58.8 – 61.9 %. The high biomethane generation can be attributed to the enhanced substrate properties. Food waste addition provided more readily biodegradable organics and balanced the substrates' COD/TN ratio. As COD/TN ratio has been considered as one of the key parameters that regulates the biogas production (Vögeli et al., 2014), the co-substrate COD/TN ratios 5.6:1-9.4:1 (higher than 4.1:1 for blackwater) may have created better environmental conditions for microorganisms which facilitated effective methanogenesis process. However, when the OLR was further increased to 11.6 (± 0.6) kg COD/m^3/d in Phase IV, the methane production rate decreased and gradually stabilized at 2.07 (± 0.15) L/L/d, which was ~15% lower than the value observed in Phase III. Correspondingly, the methanisation rates decreased to 44.4 % in this phase. Since the methane production directly correlated with the OLR, the lower value demonstrated that the input COD could not be effectively converted into methane under such high OLR, indicating an overloaded condition. Similar observations have been reported that the methane production decreased under overloading conditions. For instance, in the co-digestion system treating food waste and grease-trap waste, a drastic reduction in the methane yield was observed when the shock loading of 10.4 (±0.9) kg COD/m^3/d was applied (Wu et al., 2018). Possible reasons that may contribute to the lower methane production include the wash-out of the active biomass under the high OLRs and short HRT, and the limited mass transfer due to the accumulation of solids in the sludge bed. Although a steady methane production can be achieved under the overloaded OLR condition in the current study, the system suffered from organics overloading where unstable sludge with accumulated organics would be produced. Overall, Phase III demonstrated the best performance regarding both COD reduction and methane production was obtained under OLR of 10.0 (± 0.5) kg COD/m^3/d.

7.3.4 Substrate hydrolysis

Blackwater contains high solids organics, thus the hydrolysis of these components has been identified as the limiting step during the anaerobic treatment process in blackwater mono-digestion (Gao et al., 2019b). Fig. 7.2A shows the solid substrates hydrolysis efficiencies of the co-substrates throughout the whole operation. For Phases I – III, the solid organics hydrolysis efficiencies were 48.6 – 53.4 %, respectively, which were 69.5 – 85.9 % higher than the values

obtained in the sole blackwater treatment process (28.7 %). The highest hydrolysis efficiency was also obtained under the conditions in Phase III, which corresponded to the highest methanisation rate achieved. The boosted hydrolysis efficiencies may have been influenced by the enhanced activities of the anaerobic microorganisms in the presence of readily biodegradable substrates (Kim et al., 2011) as discussed in section 3.3. In addition to the substrates properties, one important operational parameter that regulates the feedstock hydrolysis efficiency is the SRT, in which a sufficient amount of microbes are required for effective hydrolysis to occur (De Graaff et al., 2010). In the present study, when the overloaded condition occurred in Phase IV (OLR of 11.6 [±0.6] kg COD/m3/d), frequent sludge disposal was needed due to the sludge wash out, which resulted in a shortened SRT that is insufficient for efficient solid substrate hydrolysis. Thus, a low hydrolysis efficiency of 29.6 (± 7.5) % was observed under this operation condition.

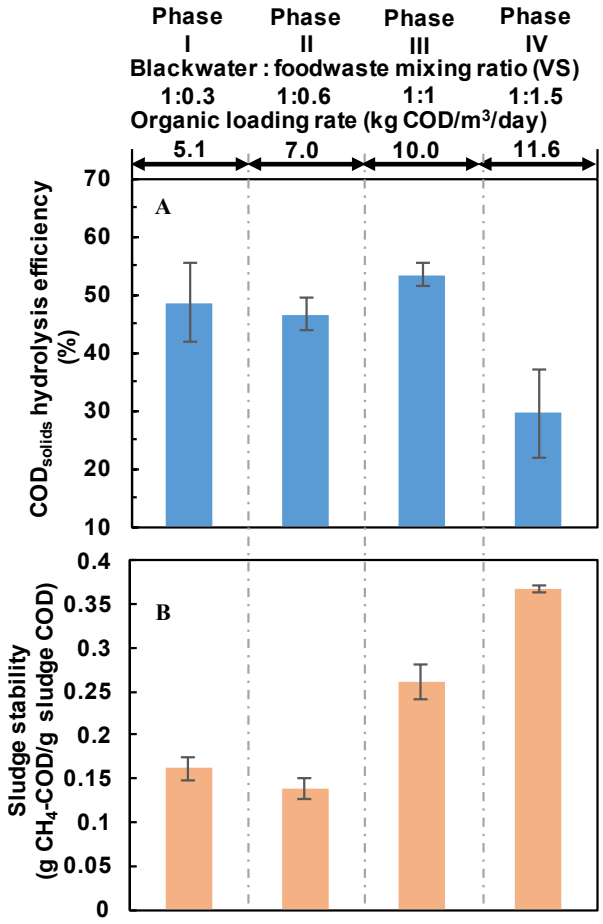

Fig. 7.2. Solid COD (COD_{solids}) substrate hydrolysis efficiency (%) (A); UASB sludge stability (g CH_4-COD/ g sludge COD) (B).

Sludge stability represents the fraction of undigested biodegradable substrates in sludge. In the present study, the enriched sludge exhibited relatively good stabilities with values of 0.16 (± 0.01) to 0.14 (± 0.01) g CH_4-COD/g sludge COD (Fig. 7.2B) in Phases I and II, respectively, indicating limited organic residuals in the sludge. This correlated well with the effective substrate hydrolysis and methane conversion observed under the operation conditions. However, increasing values were observed at higher OLRs in Phases III and IV. An especially high value of 0.37 (± 0.00) g CH_4-COD/g sludge COD was observed in the final operation phase with the high OLR of 11.6 (± 0.6) kg COD/m_3/d. Poor sludge stability has been reported when hydrolysis was the rate limiting step (Zhang et al., 2012). Due to the insufficient hydrolysis, solid organic matter accumulated in the sludge bed and replaced the active biomass (accompanied sludge wash-out), which can further result in reduced specific methanogenic activities of the biomass. In the current work, the high COD removal efficiency of 82.4 % and low substrate hydrolysis efficiency of 29.6 % in Phase IV implies that unhydrolyzed solid organics were entrapped in the UASB sludge bed under overloaded conditions, which resulted in poor sludge stability and lower methane generation compared with the previous phases. Sludge stabilization would be required before sludge final application or distribution (Zhang et al., 2012).

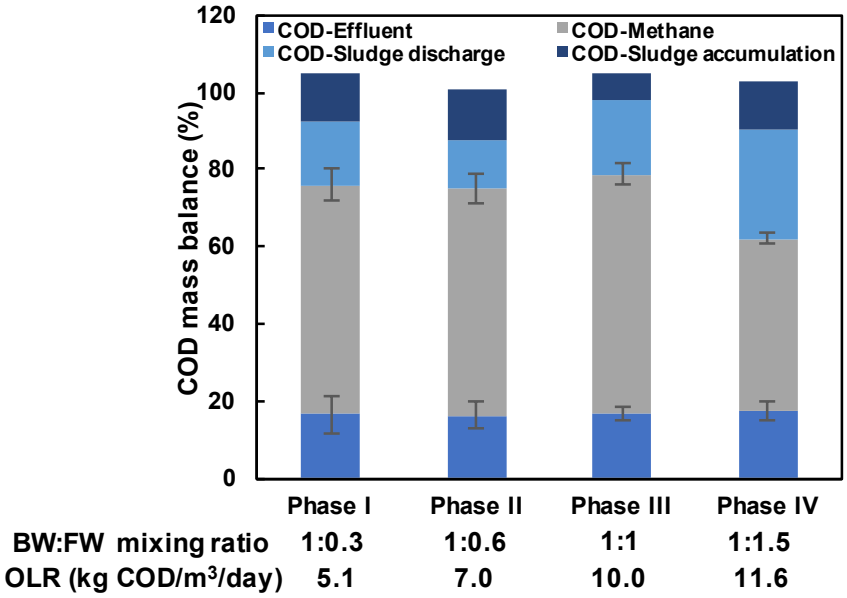

Fig. 7.3. COD balance represented by the output COD distribution into COD in effluent as COD-Effluent, COD converted to methane as COD-Methane, COD accumulated in discarded sludge as COD-Sludge discharge, COD accumulated in UASB sludge bed as COD-sludge accumulation.

7.3.5 COD mass balance

The COD balances for each operation phase are presented in Fig. 7.3. The COD output accounted for 100.9 – 105.1 % of the COD input, with the COD input considered to be 100 %. The limited differences (0.9 – 5.1 %) between influent and effluent CODs demonstrated the reliability of the measurements (Wu et al., 2018). The effluent COD residuals only accounted for 16.4 – 17.6 % of the COD input, indicating a relatively stable and effective organics removal through the UASB treatment. Most of the input COD (58.8 – 61.9 %) was converted into methane in Phases I – III, with the highest methane conversion rate of 61.9 % observed in Phase III with an OLR of 10.0 (± 0.5) kg COD/m^3/d. Compared to the blackwater treatment system with 44.3 % COD-methane conversion (Gao et al., 2019b), the BW-FW co-digestion clearly facilitated the biomethane recovery efficiency. A decreased COD-methane conversion rate of 44.4 % was observed in Phase IV when the system experienced overloading. More input COD was in sludge in Phase IV (40.7 % of the total organics input), which was significantly higher than Phases I – III (25.8 – 29.2 %). The discharged COD and accumulated COD inside UASB were 12.6 – 19.0 % and 6.9 – 13.2 % in Phases I – III, respectively. In Phase IV, 28.7 % of the input COD was washed out and discharged with sludge, likely relates to the poor retention of the feedstock as well as the biogas generation. The accumulated COD inside UASB accounted for 12.0 %. This correlated with results discussed in section 3.4 where sludge stability was poor under this condition.

7.3.6 Sludge specific methanogenic activity and methanogenic capacity

Sludge methanogenic activity is generally considered to be an important parameter to help evaluate reactor behavior (Silvestre et al., 2014) and to identify rate-limiting conditions (Kim et al., 2011). Precursor substrates of acetate and H_2 & CO_2 are typically used to identify the dominant methanogenesis pathway (acetoclastic or hydrogenotrophic methanogenesis). The results of SMA (acetate) and SMA (H_2 & CO_2) for each operation phase are presented in Fig. 7.4A. Throughout the entire operation of the UASB, relatively high SMA (H_2 & CO_2) values of 0.97 (± 0.01) – 1.20 (± 0.03) g CH_4-COD/g VSS/d were achieved, which were clearly higher than SMA (acetate) at 0.13 (± 0.01) – 0.18 (±0.01) g CH_4-COD/g VSS/d, indicating the ascendancy of the hydrogenotrophic methanogenesis pathway. A decreased SMA (H_2 & CO_2) value was observed in Phase IV due to the organics overload stress. Compared with the results

from blackwater mono-digestion, this co-digestion system generated over two-folds higher SMA (H_2 & CO_2), though comparable SMA (acetate) values. The higher hydrogenotrophic methanogenic activities achieved in the co-digestion process may indicate a grown population of hydrogenotrophic methanogens in the whole microbial community or a cohesive mass transfer between hydrogen-producing bacteria and the hydrogen-utilizing archaea, as the inoculated sludge was dominated by the hydrogenotrophic methanogens as illustrated in the previous study (Gao et al., 2019b). In such hydrogenotrophic methanogenesis dominated systems, the acetate degradation tended to be conducted through the combined syntrophic acetate oxidation (SAO) and hydrogenotrophic methanogenesis (HM) processes, where acetate was firstly converted into H_2 & CO_2 and then into methane (Karakashev et al., 2006; Li et al., 2015). Since the high SMA (H_2 & CO_2) enables effective conversion of H_2 & CO_2 into methane, the SAO pathway tended to be the limiting step for acetate degradation in the present study. This is supported by the observed residual acetate in the UASB effluent under high OLR conditions in the current study, where acetate may not be directly favored by methanogens. Similar observations have been previously reported in the treatment of food waste where residual propionate was frequently detected in the effluent when the dominant methanogenesis pathway was identified through utilizing acetate and butyrate (Han et al., 2005; Shin et al., 2001). In summary, the SMA (acetate) and SMA (H_2 & CO_2) results observed in the current system indicate efficient hydrogenotrophic methanogenesis and relatively inadequate SAO activities.

Although no SMA results have been reported in BW-FW co-digestion systems, the hydrogenotrophic methanogenesis dominant system has been previously reported in other food waste co-digestion systems. For instance, a high SMA (H_2 & CO_2) value of approximately 0.6 g CH_4-COD/g VS/d was observed in the co-digestion of manure and food waste in a mesophilic CSTR where the SMA (acetate) was only 0.06 g CH_4-COD/g VS/d (Neves et al., 2009). It should be noted that the current co-digestion process generated considerably high sludge methanogenic activities, which indicates a faster methane generation rate over blackwater mono-digestion system. A similar demonstration has also been made in the co-digestion of food waste and raw sludge (primary and secondary sludge); not only the methane yield was elevated but also the methane production rate was accelerated by the co-digestion strategy (Koch et al., 2015).

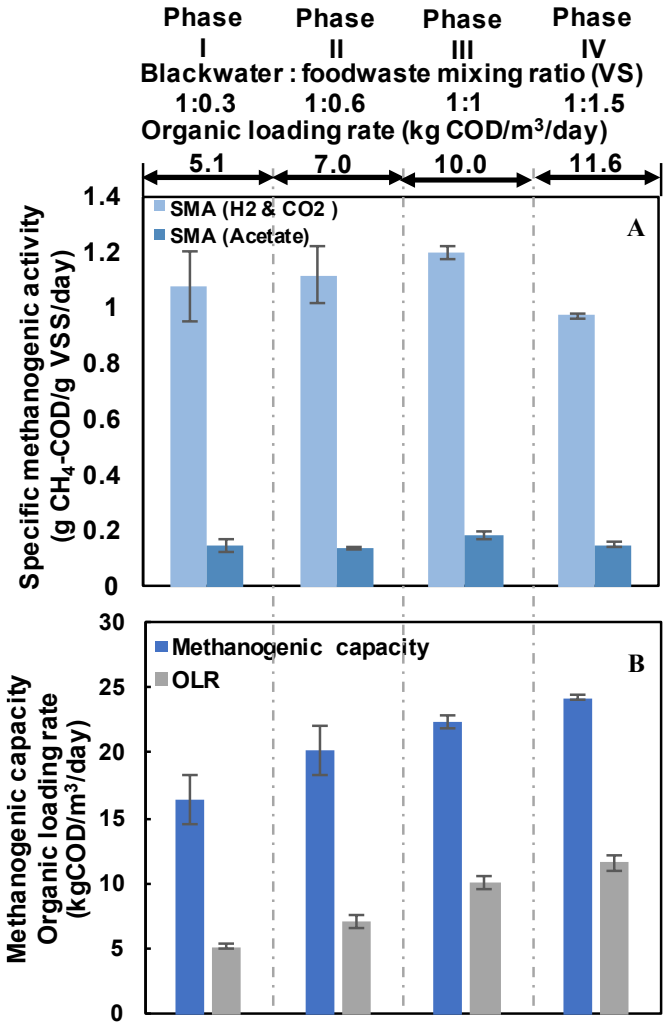

Fig. 7.4. UASB sludge specific methanogenic activity (SMA [H_2 & CO_2], SMA [Acetate]) (A); UASB methanogenic capacity and OLR (kg COD/m^3/d) (B).

The methanogenic capacity was calculated based on the sludge SMA (H_2 & CO_2) and the total amount of VSS in the UASB reactor at the end of each operation phase; this represents the maximum methane production capacity that the system can demonstrate under each operational condition. Methanogenic capacities of 16.4 (±1.9) – 24.3 (±0.3) kg CH_4-COD/m^3/d (Fig. 7.4B) were observed in Phases I-IV, which were 2.1 – 3.2 times of the OLRs (5.1 [±0.2] – 11.6 [±0.6] kg COD/m^3/d) in each operation phase. The increase in methanogenic capacities can be attributed to the increasing amount of VSS in the UASB reactor, indicating biomass growth. The highest methanogenic capacity observed in the final operation phase further verifies that the low

ultimate methane production was mainly limited by substrate hydrolysis. Yet, the high methanogenic capacities indicate a high biomethane recovery potential of the treatment system.

7.3.7 Evaluation on blackwater and food waste co-digestion

Energy conservation is the key in the decentralized wastewater treatment approach. An energy saving of ~40kWh/capita/year may be obtained through wastewater source-diversion and anaerobic treatment (Capodaglio and Olsson, 2020; Vaccari et al., 2018; Wendland et al., 2007; Zeeman et al., 2008). In this sanitation system, the anaerobic BW-FW co-digestion exhibited substantial advantages due to high biomethane generation.

Table 7.3. Performance comparison of the present study with the reported blackwater/brown water and food waste co-digestion studies.

		Kujawa-Roelevel d et al., 2005	Elmitwall i et al., 2006	Wendland et al., 2007	Rajagopal et al., 2013[a]	Rajagopal et al., 2013 [a]	Wang et al., 2018	Current study
Reactor type		UASB-septic tank	Accumul ation system	CSTR	Two-phase CSTR	SeqBR	IUSR	UASB
Temperature	°C	25	20	37	33	33	33	35
Feedstock COD	g/L	-	18.7	19.2	35.2	35.2	28.6	25.4
OLR	kg COD/m$_3$/d	-	0.1	1-2	1.5	2-3	4.1	10.0
Methane production rate	L/L/d	0.09*	-	0.27-0.41*	0.42-0.22*	0.39-0.49*	1.24	2.42
Energy recovery	MJ/m$_3$/d	3.6*	-	10.8-16.4*	16.8-8.8*	15.6-19.6*	49.6*	96.8*
COD removal efficiency	%	82	58	50-75	68.4	76.7	88	82.4

[a]: brown water and food waste co-digestion study.

*: calculated values.

Blackwater contains feces (undigested food and biomass [25 – 54% of dry mass in feces]) (Rose et al., 2015), urine, and often toilet papers. It exhibits relatively low C/N ratio and high solids organic matter, therefore, is not a desirable substrate for anaerobic digesters. Meanwhile, biomethane recovery from food waste was challenged by fast VFA accumulation and pH drop (Capson-Tojo et al., 2018). The food waste and blackwater co-digestion have triggered the advantageous synergistic effects, which facilitated the biomethane production. In addition to the readily biodegradable substrates supply (Lay et al., 1997) and a more balanced substrate C/N ratio (Kumar et al., 2010), enhanced microbial growth and activities (Kim et al., 2011; Mata-Alvarez et al., 2000) were observed in this study. Up to 85.9% increase in substrate hydrolysis efficiency through food waste co-digestion resulted in a higher biomethane generation of 2.42 L/L/d and higher OLR of 10.0 kg COD/m^3/d compared with blackwater mono-digestion with 0.68 L/L/d and 4.1 kg COD/m^3/d, respectively.

As described in Table 7.3, previous studies have evaluated the bioenergy recovery efficacy from blackwater and food waste co-digestion using various types of operation systems. The current study is the first work evaluating the feasibility of utilizing UASB reactor for blackwater and food waste co-digestion under high OLRs. Throughout the UASB operation, the solids concentrations of 7.5-18.9 g TSS/L (Table S7.1) for the co-substrates were within the suggested limits for applying UASB treatment (TSS<3%) (Abbasi et al., 2012), and it did not affect the smooth operation of the anerobic process. Compared to previous studies, the current work achieved relatively high COD removal, the highest methane production rate and OLR, which was 2.4 times of the highest OLR (4.1 kg COD/m^3/d) in the reported work. The higher OLR indicates less reactor volume requirement, thus lower capital cost. The methane generation of 2.42 L/L/d achieved in the current work represents a heat recovery potential of 96.8 MJ/m^3 reactor/d (2.35 kWh/kg COD input) (methane heat value 40 MJ/m^3, (Zhang et al., 2018)), which could assist the feedstock heat-up in wastewater treatment facilities and support neighborhood energy supply (Gao et al., 2019b). In addition, the optimal BW:FW VS ratio of 1:1 obtained in the present study resembles the practical collection ratios of 1:1.1-1:3 (BW:FW in VS) (Kujawa-Roeleveld et al., 2005; Rajagopal et al., 2013; Wang et al., 2018; Wendland et al., 2007), indicating its feasibility in practical application. It is important to notice that the large quantities of global food waste production (1.3 billion tons annually ("Key facts on food loss and waste

you should know!," 2019)) and the human excreta (5 L/capita/d blackwater using vacuum toilet (Wendland et al., 2007)) generation ensure a sustainable bioenergy resource.

Accordingly, the results obtained in the current work demonstrated a highly efficient and cost-effective method of treating source-diverted household wastewater, the high bioenergy recovery, waste treatment capacity, low construction and operational cost supported the feasibility of performing full-scale application with this strategy.

7.4. Conclusions

High-loading blackwater and food waste co-digestion was investigated using a UASB reactor with an HRT of 2.6 days at 35 °C. Substantial enhancement in biomethane recovery was achieved through stepwise increases of the food waste addition into blackwater. An OLR threshold was identified to be 10.0 (±0.5) kg COD/m^3/d when the co-substrate mixing ratio of 1:1 (BW:FW in VS) was applied in order to avoid process failure. Under this condition, the substrate methanisation rate and hydrolysis efficiency were 39.9 % and 85.9 % higher than the values observed from blackwater mono-digestion. A maximum methane production rate of 2.42 (± 0.15) L/L/d was achieved, which is the highest value in blackwater and food waste co-digestion reported to date. Insufficient sludge retention limited the biomethane recovery when OLR was further elevated to 11.6 (± 0.6) kg COD/m^3/d, leading to increased COD accumulation. Outstanding sludge hydrogenotrophic methanogenic activities of 0.97 (± 0.01) – 1.20 (± 0.03) g CH_4-COD/g VSS/d were achieved through blackwater and food waste co-digestion, which was up to 2.4 times higher than the blackwater mono-digestion process. The overall remarkable performance was likely due to better feedstock properties and subsequent boosted microbial activities.

7.5 Supplementary materials

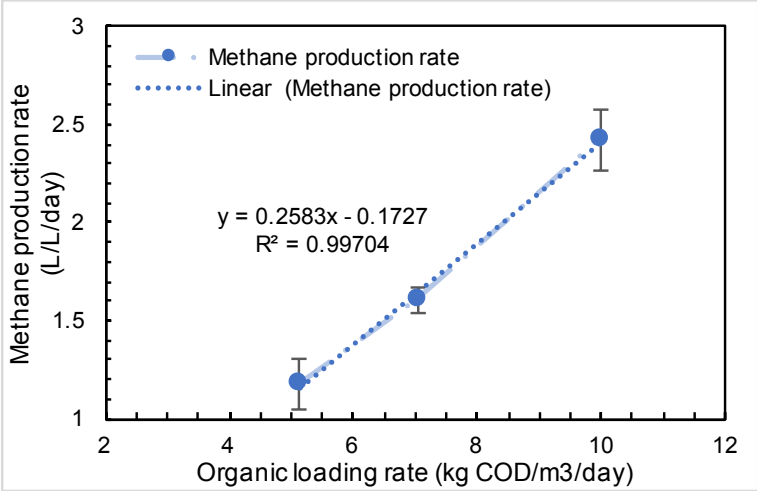

Fig. S7.1. Linear regression of organic loading rate and methane production rate for operational phases I, II and III.

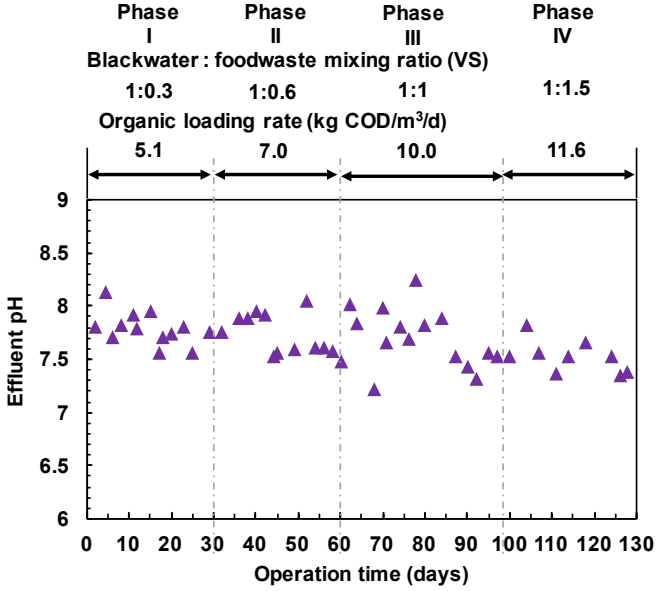

Fig. S7.2. UASB effluent pH in operational phases I, II, III, IV.

Table S7.1. Total suspended solids concentrations for co-substrates in operational phases I, II, III, IV.

	Unit	BW+FW (Phase I)	BW+FW (Phase II)	BW+FW (Phase III)	BW+FW (Phase IV)
Total suspended solids (TSS) concentration	g/L	7.5 (±0.3)	10.4 (±0.5)	15.0 (±0.7)	18.9 (±1.2)

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CHAPTER 8. ROLE OF SYNTROPHIC ACETATE OXIDATION AND HYDROGENOTROPHIC METHANOGENESIS IN CO-DIGESTION OF BLACKWATER WITH FOOD WASTE

8.1. Introduction

Compared with blackwater mono-digestion, anaerobic co-digestion (AcoD) with food waste has great potential to help mitigate the limitations and gain more energy benefits (Lim et al., 2013). The readily biodegradable organics and the high carbon but low nitrogen content of food waste can help enable better substrate hydrolysis and more balanced carbon to nitrogen (C/N) ratios of the co-substrate than treating only blackwater. To date, UASB reactors have never been tested for blackwater and food waste co-digestion, although it has been widely demonstrated that the UASB reactor design enables higher OLRs and biodegradation efficiencies as compared to configurations such as CSTR (Daud et al., 2018).

In the current study, we performed a continuous anaerobic co-digestion on blackwater and food waste using a UASB reactor under mesophilic condition (35°C). The objectives of this work are to (i) demonstrate high-rate blackwater and food waste co-digestion using the UASB reactor and (ii) illustrate the associated microbial community structure development. Increasing OLRs were achieved by stepwise increasing food waste proportions in the co-substrates until reaching the threshold. The dynamic shifts of the microbial community structures were correlated with the changed substrate properties and the applied OLRs and linked to the treatment performances. To the best of the author's knowledge, this is the first study that elucidates the microbial interactions in blackwater and food waste UASB treatment system. The crucial information provided could help guide the future design of the new sanitation system, especially for the household wastewater collection patterns.

8.2. Materials and method

8.2.1 Substrates collection and reactor operation

Vacuum toilet flushed blackwater (with 1 L flushing water per flush) and food waste (from cafeteria and student lounge) were collected from the University of Alberta campus (Edmonton, Canada). Both substrates were ground using an electric kitchen blender to achieve homogeneity,

then stored at 4°C prior to use. An upflow anaerobic sludge blanket (UASB) reactor with a working volume of 3.3 L was operated at 35°C (using a heating blanket) for the anaerobic treatment. The overall operational period was divided into five phases according to the different feeding substrates and organic loading rate (OLR) conditions adopted in each phase. The inoculum sludge (1 L, volatile suspended solids [VSS] 27.9 g/L) was obtained from a UASB reactor treating vacuum toilet blackwater (Gao et al., 2019b). In phase I, following the previous blackwater treatment operational condition, the UASB reactor was fed with only blackwater at an OLR of 4.1 kg COD/m^3/d. From phases II to V, the co-substrates of mixed blackwater and food waste were fed into the UASB reactor; the feeding substrates were prepared with increasing food waste volatile solids (VS) proportions in the co-substrates with the values of 23%, 38%, 50%, and 60% (Table 8.1). Throughout the whole operation, the hydraulic retention time (HRT) was fixed at 2.6 days with an upflow velocity of 5.2×10^{-3} m/h, and the increment in the organic loading rate for each operational phase was contributed from the increasing feedstock chemical oxygen demand (COD) concentrations. Phase I (blackwater mono-digestion) lasted for 21 days, and each of the other operational phases (Phases II-V, co-digestion) lasted for more than 30 days. Sludge was discharged from the reactor once per week for Phases I-IV and twice per week during Phase V. The sludge bed height was maintained at 17 cm (i.e., half the reactor height) throughout the operation with sludge discharge.

8.2.2 Chemical analysis

The influent feed samples and the UASB effluent samples were collected and measured three to five times each week for the detections of total COD (COD_t), soluble COD (COD_s), total nitrogen (TN), total ammonium nitrogen concentration, pH and volatile fatty acids (VFAs) including acetate, propionate, and butyrate. The total solids (TS) and volatile solids (VS) concentrations of the influent and UASB effluent were measured one to two times per week. The sludge properties including COD, total suspended solids (TSS), volatile suspended solids (VSS) and pH were measured at the end of each operational phase and at each time the sludge was wasted from the reactor. The measurements of COD, TS, VS, TSS, VSS were according to the standard methods and conducted in triplicate (APHA/AWWA/WEF, 2012). Hach TNT vial test methods (Hach, USA) were applied for the measurements of total nitrogen (TN) and total ammonium nitrogen concentrations. The methane production was obtained from the

measurements of the produced biogas amount in the gas bag, and the biogas composition using gas chromatography (GC) equipped with a thermal conductivity detector (TCD) (7890B Agilent Technologies, USA). Biogas composition was measured in duplicate. A fixed standard gas with a known composition of 40% CH_4, 25% CO_2, 1% O_2 and 34% N_2 (PRAXAIR, Canada) was used to calibrate GC before and after biogas measurements for quality control. The methane production rate was then calculated as the daily methane production volume per volume reactor and represented as m_3/m_3/d. The methanisation rate in this study represented the fraction of influent COD converted into methane and represented as g CH_4 /g feed COD in percentage (%) (the g CH_4 production was converted into g COD for calculation). A B40PCID pH meter (VWR, SympHony) was used to measure pH values. An ionic chromatography (IC) equipped with a conductivity detector (DIONEX ICS-2100, ThermoFisher, USA) was used for measuring VFA concentrations. A series of VFA standard mixtures in different concentrations were used for method calibration and check.

Isotopic detection on biogas was performed for each operational phase using GC 5890 Series II (Hewlett Packard, USA) at the Department of Earth and Atmospheric Sciences at the University of Alberta. The stable carbon isotopic signature $\boldsymbol{\delta}_{13}CH_4$ (‰) and $\boldsymbol{\delta}_{13}CO_2$ (‰) were obtained from the GC measurement and were utilized to calculate the apparent fractionation factor $\boldsymbol{\alpha}_c$ using the following equation (Hao et al., 2017):

$$\alpha_c = \frac{(\delta^{13}CO_2 + 10^3)}{(\delta^{13}CH_4 + 10^3)}$$

The solids COD hydrolysis efficiency represents the hydrolyzed fraction of influent solid organic contents, which was calculated using the following equation:

$$Hydrolysis\ efficiency\ of\ COD_{solids}(\%) = \frac{COD_{CH4} + COD_{s,effluent} - COD_{s,influent}}{COD_{t,influent} - COD_{s,influnet}}$$

8.2.3 Microbial community analysis

Triplicated sludge sampling from each port of the UASB sludge bed was performed for DNA extraction at the end of each operational phase. Fresh well-mixed sludge sample was centrifuged at 3,000 × g for 10 min, and after discarding the supernatant, the pellet was used for DNA

extraction following the manufacturer's protocol using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). The qualities of the extracted DNA samples were checked through NanoDrop One (ThermoFisher, Waltham, MA). The DNA samples were stored at -20°C before downstream analysis. The 16S rRNA gene sequencing was performed with the universal primer pair *357wF* (*5'-CCTACGGGNGGCWGCAG-3'*) and *785R* (*5'-GACTACHVGGGTATCTAATCC-3'*) for bacteria and *517F* (*5'-GCYTAAAGSRNCCGTAGC-3'*) and *909R* (*5'-TTTCAGYCTTGCGRCCGTAC-3'*) for archaea. The genes amplification and sequencing were performed in the sequencing center RTL Genomics (Texas, USA).

The raw sequences achieved from the sequencing center were processed using the Qiime2 pipelines (Caporaso et al., 2010) with DADA2 plugin (Callahan et al., 2016) to remove the low-quality sequences and chimeras. Taxonomy was assigned using the 99% similarity with the Greengenes (version 13_8) reference database (Werner et al., 2012).

8.3. Results and discussion

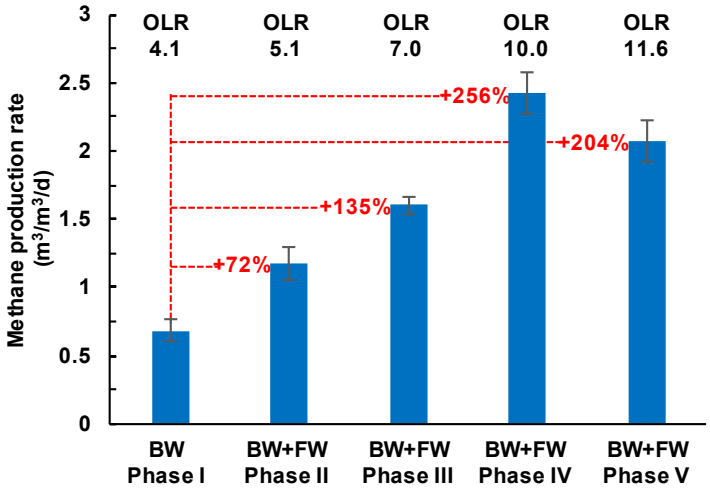

Fig. 8.1. Average methane production rates (m_3 CH_4/m_3 reactor/d), and comparisons for blackwater (BW) mono-digestion Phase I, and blackwater and food waste (FW) co-digestion Phases II, III, IV, and V. The organic loading rates (OLR) were shown for each operational phase separately in unit kg COD/m_3/d.

Table 8.1. Operational conditions, UASB treatment performances, and influent and effluent chemical properties.

		Unit	BW (Phase I)	BW+FW (Phase II)	BW+FW (Phase III)	BW+FW (Phase IV)	BW+FW (Phase V)
Operational conditions	Organic loading rate	kg COD/m_3/d	4.1 (±0.4)	5.1 (±0.2)	7.0 (±0.5)	10.0 (±0.5)	11.6 (±0.6)
	FW VS proportion in co-substrate	%	0	23	38	50	60
Treatment performances	COD removal efficiency	%	83.6 (±4.9)	83.6 (±3.6)	83.6 (±1.9)	83.1 (±2.5)	82.4 (±2.8)
	Solids COD hydrolysis efficiency	%	28.7 (±2.5)	48.6 (±6.8)	46.6 (±2.8)	53.4 (±2.1)	29.6 (±7.5)
	Methanisation rate	%	44.3 (±4.3)	59.5 (±4.0)	58.8 (±2.8)	61.9 (±1.3)	44.4 (±1.9)
	Methane production rate	m_3/m_3/d	0.68 (±0.08)	1.17 (±0.12)	1.60 (±0.06)	2.42 (±0.15)	2.07 (±0.15)
Influent chemical properties	Influent COD_t	g/L	11.0 (±0.9)	14.2 (±0.6)	20.0 (±1.1)	25.4 (±1.2)	30.5 (±1.7)
	Influent COD_s	g/L	3.5 (±0.2)	5.1 (±0.3)	7.5 (±0.4)	9.5 (±0.8)	11.6 (±0.8)
	Influent VFAs	g COD/L	0.95 (±0.26)	1.91 (±0.32)	2.02 (±0.15)	2.30 (±0.28)	2.06 (±0.17)
	Influent COD/TN		100:24	100:18	100:15	100:11	100:9
Effluent chemical properties	Effluent COD_t	g/L	1.4 (±0.3)	2.3 (±0.4)	3.2 (±0.4)	4.2 (±0.8)	5.3 (±0.9)
	Effluent COD_s	g/L	0.7 (±0.0)	1.1 (±0.1)	1.7 (±0.2)	2.1 (±0.6)	2.9 (±0.9)
	Effluent pH		8.0 (±0.1)	7.8 (±0.1)	7.6 (±0.2)	7.6 (±0.2)	7.5 (±0.1)
	Effluent FA	mg/L	146.1 (±46.1)	83.9 (±17.6)	56.2 (±6.4)	46.5 (±13.1)	53.2 (±14.7)
	Effluent VFAs	g COD /L	0.05 (±0.01)	0.39 (±0.13)	1.15 (±0.37)	1.67 (±0.41)	2.25 (±0.53)

Solids COD: influent COD excluded soluble fraction.

8.3.1 Treatment performances of blackwater mono-digestion and food waste and blackwater co-digestion

Table 8.1 showed the operational conditions, UASB influent and effluent properties, and treatment performances in blackwater mono-digestion (Phase I) and blackwater and food waste co-digestion (Phases II-V) processes. Fig. 8.1 showed the average methane production rates in each operational phase; the values obtained in the co-digestion Phases II-V were compared with the results from blackwater mono-digestion Phase I. The influent COD concentration was 11.0 (±0.9) g/L for blackwater only, and increasing values from 14.2 (±0.6) to 30.5 (±1.7) g/L were obtained for blackwater and food waste co-substrates with increasing food waste volatile solids (VS) proportions. The influent VFA concentrations in the co-substrates were more than doubled over the amount achieved from only blackwater, which was due to the readily biodegradable substrates in food waste that enables fast hydrolysis (Kim et al., 2011). Further, food waste additions resulted in higher COD/TN ratios of 100:18 -100:9 of the co-substrates compared to blackwater alone of 100:24. Effluent pH decreased from 8.0 in mono-digestion to 7.8-7.5 in the co-digestion period, which led to the reductions in the free ammonia (FA) concentrations from 146.1 mg/L to 83.9-46.5 mg/L. With these improved reaction environmental factors, significant enhancements in biomethane recovery were obtained, where 33-40% increases in the substrate methanisation rate and 72-256% increases in the methane production rates (Fig. 8.1) were achieved in co-digestion Phases II-IV over blackwater mono-digestion. The solids COD hydrolysis efficiencies ranging at 46.6-53.4% in co-digestion Phases II-IV were considerably higher than mono-digestion of 28.7%, which contributed to the elevated methanisation rates. The highest methanisation rate of 61.9% was obtained in Phase IV under the OLR of 10.0 kg COD/m^3/d, which resulted in a methane production rate of 2.42 m^3/m^3/d that was 256% higher than the value obtained from blackwater mono-digestion (0.68 m^3/m^3/d). When the OLR was further increased to 11.6 kg COD/m^3/d with food waste VS proportion of 60% in the co-substrate, a decreased substrate hydrolysis efficiency 29.6% and a reduced methane production rate 2.07 m^3/m^3/d was obtained, which indicated an overloaded condition. Increased effluent VFAs concentrations of 2.25 g/L were observed under such a condition. The reduced substrate hydrolysis and the increased effluent VFAs under the overloaded condition could be attributed to the insufficient sludge retention. The overloaded solid organics accumulated in sludge bed,

which may have caused significant loss of active biomass during sludge discharge and wash out. The low sludge retention time can then lead to insufficient solid organics hydrolysis and the loss of acids consuming microbes, eventually resulted in the decreased methane production in Phase V. The COD removal efficiencies of 82.4-83.6% obtained throughout the co-digestion period were comparable as the value of 83.6% achieved in treating blackwater alone. Overall, the threshold feasible OLR for blackwater and food waste co-digestion was demonstrated to be 10 kg COD/m3/d, which is 2.44 times over the highest OLR achieved from blackwater mono-digestion (4.1 kg COD/m3/d).

8.3.2. Microbial community development in blackwater mono- and blackwater and food waste co-digestion systems

The 16S rRNA gene sequencing results were analyzed to illustrate the microbial community developments in the blackwater mono-digestion, and blackwater and food waste co-digestion conditions.

Microbial community diversity

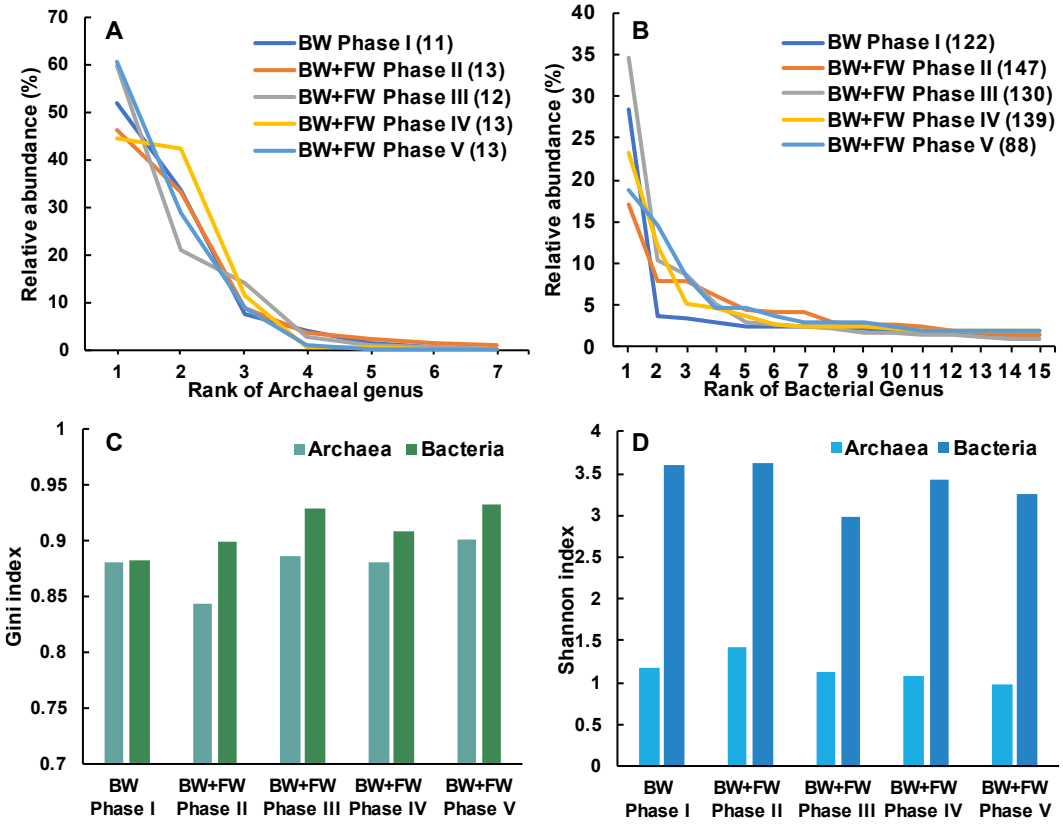

Fig. 8.2. Rank abundance distribution of archaea (A) and bacteria (B), the total number of genera were indicated after the sample name; Gini index (C) and Shannon index (D). Samples were for blackwater (BW) mono-digestion Phase I, and blackwater and food waste (FW) co-digestion Phases II, III, IV and V. Communities were analyzed at the genus level.

The total number of genus enriched in archaeal and bacterial communities and their rank abundance distributions were shown in Fig. 8.2. In Fig. 8.2A, the numbers of total enriched archaeal genera increased from 11 in blackwater mono-digestion to 12-13 in blackwater and food waste co-digestion systems; Phases III and V exhibited steeper gradients than the other phases, and the first ranked genus accounted for 60% of the total archaeal abundance in these two phases, demonstrating mono-enrichment under such conditions. In Fig. 8.2B, the total numbers of bacterial genera increased in co-digestion Phases II-IV (130-147) compared to the blackwater mono-digestion condition in Phase I (122), while a decrease was observed in the last operational phase (88) under organic overloaded condition. For the first 15 bacterial genera distributions, Phase I and III generated similar steep slopes, demonstrating the highest abundance of the first ranked bacterial genus.

The Gini coefficients were used to evaluate the evenness of the communities (Werner et al., 2014) and the results are shown in Fig. 8.2C. The values for archaea varied in a range of 0.88-0.90 in all phases except in Phase II where a lower value of 0.84 was obtained, indicating the highest evenness of the archaeal community in Phase II compared to the other operational conditions. The evenness of bacterial communities varied from phase to phase, and relatively lower evenness (higher Gini coefficients) were observed in Phases III and V in the co-digestion system. The Shannon index changed in similar trends for archaeal and bacterial communities, where the values increased from Phase I to Phase II and decreased in Phases III-V. The increased diversity of microbial communities in Phase II (with higher Shannon index and more genera richness) could be due to the improved environmental conditions, such as lower free ammonia concentration compared to mono-digestion condition (Xu et al., 2018), and the lower diversity in Phase V indicated a higher stress level, which may be due to the high OLR applied. Concurrently, lower solid substrate hydrolysis efficiency and methane production were observed under such an overloaded condition.

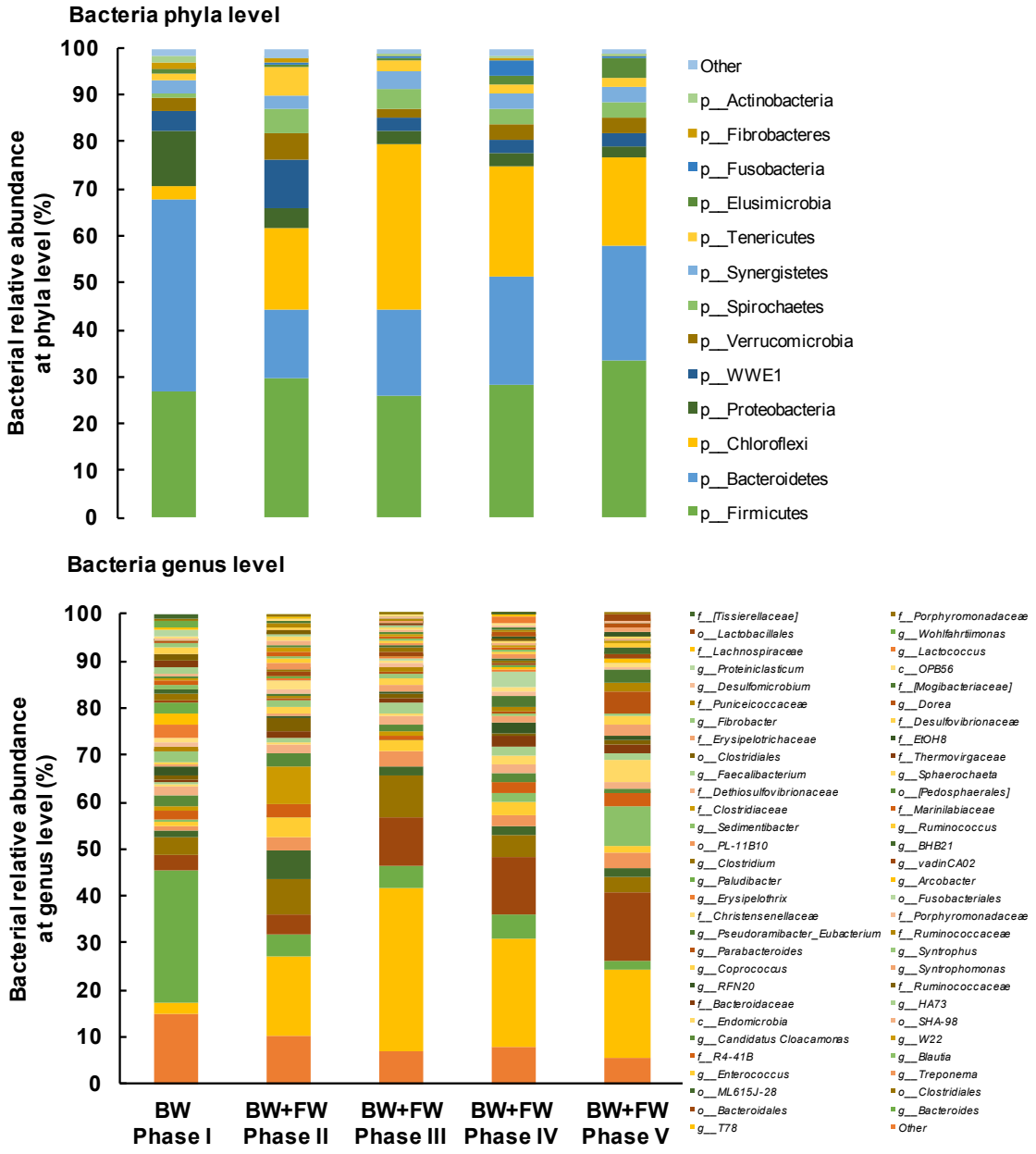

Fig. 8.3. Relative abundances and dynamics of bacterial taxonomic groups in the UASB samples collected at blackwater (BW) mono-digestion condition Phase I, at blackwater and food waste (FW) co-digestion conditions Phases II, III, IV, and V with food waste volatile solids (VS) proportions 23%, 38%, 50%, and 60% in co-substrates. The taxonomic classification of bacterial reads at phyla (p_) and genus (g_) levels are shown. Bacterial groups accounting for less than 1% of all classified sequences are summarized to the group "*Other*". Other levels included: class (c_), order (o_), family (f_).

Enriched bacterial consortia

The enriched bacterial communities with any of the abundance >1% were presented in Fig. 8.3, with the consortia assigned at phyla (p_) and genus (g_) levels. The blackwater mono-digestion system in Phase I was dominated by phyla Bacteroidetes (41.0%), Firmicutes (27.0%) and Proteobacteria (11.6%), which, as hydrolytic-fermentative bacteria (Guo et al., 2015), have been reported in human fecal microbiota (Harmsen et al., 2002), and in anaerobic bioreactors, e.g. treating biosolids. Genus *Bacteroides* (28.3%) from phyla Bacteroidetes was specifically enriched and dominated the bacterial community; they are well-known as mesophilic hydrolytic-fermentative bacteria and are capable of producing acids, ethanol and H_2 and CO_2 (Murray et al., 1984). Compared to blackwater mono-digestion, in the co-digestion condition in Phase II, decreases in phyla Bacteroidetes (14.8%) and Proteobacteria (4.2%) were observed while bacterium from phyla Chloroflexi (17.2%), WWE1 (10.5%), Tenericutes (6.3%) and Verrucomicrobia (5.6%) were enriched. Firmicutes (29.7%) exhibited comparable abundance as in blackwater mono-digestion condition and remained predominant. Genus *Bacteroides* showed a considerable reduction in Phase II (4.5%) which was consistent with the reported study that the abundances of *Bacteroides* negatively related to the methane yield in the co-digestion of food waste and pig manure (Jiang et al., 2019). It has been reported that a positive correlation existed between the increase in Firmicutes/ Bacteroidetes ratio and the methane yield (Chen et al., 2016), which is in accordance with the current study that with a 3 times higher Firmicutes/ Bacteroidetes ratio in Phase II, a 34% increase in the methanisation rate was obtained compared to Phase I. The enrichments of phyla WWE1 have been reported related to lignocellulosic substrates (Limam et al., 2014), which correlates well with the increased cellulosic content from food waste addition. Bacterial genus *T78* (17.1%) from phyla Chloroflexi was found specifically enriched in Phase II. This bacterial group has been detected in anaerobic digesters treating waste activated sludge under mesophilic conditions (Kirkegaard et al., 2017), and has been found functional of degrading carbohydrates and alcohols through syntrophic interactions (Praveckova et al., 2016). It has been suggested that family *Anaerolinaceae*, which genus *T78* were assigned to, can cooperate with hydrogenotrophic methanogens for hydrogen scavenging (Yamada and Sekiguchi, 2018). The enrichment and dominance of bacterial genus *T78* in the co-digestion system may have accompanied more cohesive and coordinately interactions between

fermentative and methanogenic microorganisms (Satoh et al., 2007), which indicated enhanced metabolic activities compared to blackwater mono-digestion system.

With the increasing food waste addition in Phases II-V, Firmicutes, Bacteroidetes, and Chloroflexi remained the most dominant bacterial phyla, while variations in their relative abundances were observed. Bacteria groups from phyla WWE1, Proteobacteria, Tenericutes, and Verrucomicrobia all showed reduced abundances with the increasing OLRs in Phases III-V compared to Phase II. Genus *T78* showed the highest abundance (34.7%) in Phase III (FW VS 38%), then gradually decreased to 23.2% and 18.7% in the last two phases. Since bacteria from genus *T78* function in the hydrolytic-acidogenic process (Wang et al., 2016), their reduction may possibly be affected by the high organic loading stress, where the amount of slow-growing SAO bacteria could be reduced by sludge loss. An uncultured genus from order Bacteroidales showed increasing abundances from 4.3% to 14.5% in co-digestion Phases II-V, while decreased abundances of an uncultured genus from order Clostridiales from 7.8% (Phase II) and 8.7% (Phase III) to 4.7% (Phase IV) and 3.6% (Phase V) were observed. As Bacteroidales was more active than Clostridiales in the biodegradation of cellulose (Xia et al., 2018), their enrichment could be due to the increasing food waste proportions. It was found that an uncultured bacterial genus from order Fusobacteriales (3.6%) was specifically enriched in Phase IV (FW VS 50%), and an uncultured bacterial genus from class Endomicrobia (4.7%), genus *Parabacteroides* (4.6%) and bacterial genus *Blautia* (8.5%) were specifically enriched in Phase V. *Blautia* from family *Lachnospiraceae* have commonly been isolated from human fecal samples, which can function as acetogens to produce various types of fermentative products such as short-chain fatty acids (SCFAs). It should be noted that a negative correlation has been reported between its abundance and acetate concentrations (Org et al., 2017). In the current study, the specific enrichment of genus *Blautia* was observed under the organic overloaded condition with relatively high acetate concentrations.

Enriched archaeal consortia

The archaeal community development throughout the operation is presented at order (o_) and genus (g_) levels in Fig. 8.4. Methanogens from order Methanomicrobiales accounted for over 97% abundances in Phases I and III-V, while the order Methanobacteriales (4.8%) and

Methanomicrobiales (93.5%) dominated in Phase II. Both of these groups were hydrogenotrophic archaeal consortia that produce methane through CO_2 reduction with electrons from H_2 (Wilkins et al., 2015); they have generally been detected in digesters treating sewage sludge and agriculture organics like silage (Demirel, 2014). Compared to acetoclastic methanogens, the hydrogenotrophic methanogens were generally reported with better adaptation to harsh environmental conditions, such as high ammonia stress, VFAs, sulfides, *etc*. and under high or over-load conditions (Westerholm and Schnürer, 2019), which was mainly attributed to their morphologic structures with smaller surfaces (Liu et al., 2016). In blackwater only condition (Phase I), the most abundant consortia were from genus *Methanogenium* (52.0%), followed by genus *Methanolinea* (33.7%), *Methanoculleus* (7.4%), and *Methanospirillum* (4.5%). *Methanogenium* has been observed in chicken feces (Miller and Wolin, 1986), anaerobic bioreactors treating industrial wastewaters (Zellner et al., 1990) and swine manure (Qin et al., 2013), and in blackwater digestion (Gao et al., 2019a). *Methanolinea* were commonly observed in anaerobic bioreactors treating organic wastes such as animal manure (Yıldırım et al., 2017), sewage sludge (Wu et al., 2013) and *etc*. After food waste was fed, both genus *Methanogenium* and *Methanolinea* showed decreased abundances. Especially, the relative abundances of genus *Methanogenium* gradually decreased with the increasing food waste addition, which could be correlated with the reduction of free ammonia concentration from 146.1 mg/L in blackwater mono-digestion Phase I to 53.2 mg/L in co-digestion Phase V, despite the OLR was more than two times higher. This indicated that the enrichment of genus *Methanogenium* might be positively correlated to free ammonia concentrations.

In the co-digestion period, the dominant methanogenic consortia shifted to genus *Methanoculleus*, uncultured genus from family *Methanospirillaceae* and genus *Methanolinea*. The abundances of genus *Methanolinea* fluctuated within 8.8-14.2% in Phases II-V. Genus *Methanoculleus* and uncultured genus from family *Methanospirillaceae* showed major dominances with relative abundances varied from phase to phase. In Phase II, the relative abundance of 46.1% was detected for *Methanoculleus* and 33.3% for *Methanospirillaceae*, while the abundance of *Methanoculleus* decreased to 21.1% and *Methanospirillaceae* increased to 59.6% in Phase III. Then in Phases IV and V under high OLRs, the abundances of *Methanoculleus* gradually increased to 44.4% and 60.4% and over competed

Methanospirillaceae whose abundances were 42.2% and 28.8%. Both *Methanoculleus* (Guo et al., 2014) and *Methanospirillaceae* (Han et al., 2017) have been previously reported in food waste mono-digestion and co-digestion systems. Methanogens from *Methanospirillaceae* have been identified with high H_2 utilization rates (Li et al., 2015), which resulted in faster methane productions in the co-digestion period. Compared with *Methanospirillaceae*, methanogens from *Methanoculleus* have a lower H_2 threshold, which was less advantageous in H_2 consumption (Shigematsu et al., 2006). Further, the enrichment of *Methanoculleus* has commonly been correlated with the stressful environmental conditions such as high ammonia concentrations and the increasing OLRs, *etc*. (De Francisci et al., 2015). These reported results helped explain the dominance of *Methanoculleus* under the high OLRs conditions in the current study. Contradictory results have been reported on the correlations between process stability and the abundance of *Methanoculleus*, where for a co-digestion system treating wheat straw and dairy cattle manure, *Methanoculleus* was correlated with enhanced process stability (Song and Zhang, 2015), while others reported their dominance during process deterioration (Li et al., 2015). In the current work, the community shift from *Methanogenium* in blackwater mono-digestion condition to *Methanoculleus* and *Methanospirillaceae*, in food waste co-digestion conditions may indicate a better metabolic environment, while the increasing abundances of *Methanoculleus* accompanied system instability in the final phases (Phase IV and V) may be attributed to the stress from high OLRs. It should be noted that a relatively high abundance of *Methanoculleus* has been reported in the co-digestion of food waste and brown water (only feces) (Lim et al., 2013). Overall, in both stable and deteriorative phases, methanogenic community structures showed no distinguished changes, while distinct differences existed in the relative abundances of each enriched methanogen genus; similar observation has been reported in the previous study (Xu et al., 2018).

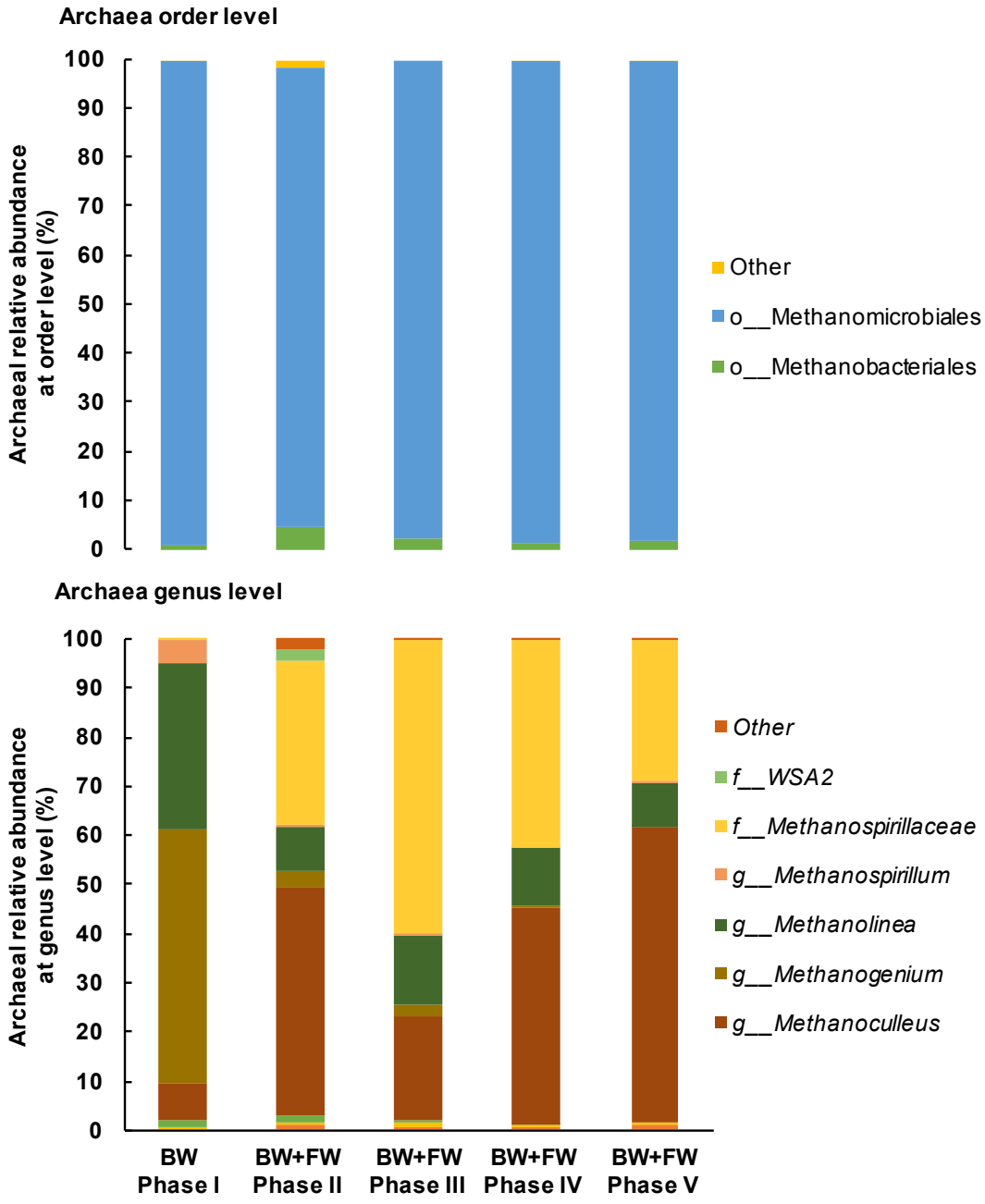

Fig. 8.4. Relative abundances and dynamics of archaeal taxonomic groups in the UASB samples collected at blackwater (BW) mono-digestion condition Phase I, at blackwater and food waste (FW) co-digestion conditions Phases II, III, IV, and V with food waste volatile solids (VS) proportions 23%, 38%, 50%, and 60% in co-substrates. The taxonomic classification of archaeal reads at order (o_) and genus (g_) levels are shown. Archaeal groups accounting for less than 1% of all classified sequences are summarized to the group "Other". Other levels included: family (f_).

8.3.3 Syntrophic pathways

The methanogenesis process was usually conducted through two major pathways, where acetoclastic methanogens utilize acetate through acetoclastic methanogenesis (AM) pathway and hydrogenotrophic methanogens utilize H_2 & CO_2 through hydrogenotrophic methanogenesis (HM) pathway for methane generation (Lim et al., 2013). *Methanosaeta* and *Methanosarcina* are two methanogen consortia capable of performing acetoclastic methanogenesis (Hattori, 2008). Instead of the acetoclastic pathway, acetate can be converted to methane through combined syntrophic acetate oxidation (SAO) and hydrogenotrophic methanogenesis (HM) without the presence of the above two acetoclastic methanogenic groups, which have been realized of significance in anaerobic bioreactors (Hao et al., 2011). Various types of environmental factors such as ammonia, temperature, VFAs, pH, *etc*. have been reported correlated with the establishment of the SAO-HM pathway (Lü et al., 2013). Especially, stressful reaction conditions with high ammonia concentration or high VFAs accumulation could contribute to the community shift towards this pathway (Karakashev et al., 2006). Due to the unfavorable thermodynamic properties of the SAO pathway (ΔG=+104.6 kJ/mol), this reaction can only proceed with the accompany of H_2 consuming methanogenesis (ΔG=-135.6 kJ/mol) to make the overall SAO-HM process feasible (ΔG=-31.0 kJ/mol) (Hattori, 2008).

The stable carbon isotopic signature $\boldsymbol{\delta}_{13}CH_4$ (‰) and $\boldsymbol{\delta}_{13}CO_2$ (‰) and the apparent fractionation factor $\boldsymbol{\alpha}_c$ are often utilized to illustrate the methanogenic pathways in anaerobic bioreactors (Hao et al., 2011). Fig. 8.5 shows the values obtained from each operational condition. Values of $\boldsymbol{\delta}_{13}CH_4$ (‰), $\boldsymbol{\delta}_{13}CO_2$ (‰) and $\boldsymbol{\alpha}_c$ obtained in each phase in the co-digestion period were higher than that from the mono-digestion condition. The $\boldsymbol{\delta}_{13}CH_4$ (‰) values were within a narrow range of -45.15 ‰ to -49.65‰ throughout the operation, while the $\boldsymbol{\delta}_{13}CO_2$ (‰) values generated an increasing trend from -4.59 ‰ to 5.68 ‰ from Phase I to III, then decreased to 0.97‰ and 0.48 ‰ at the high OLRs conditions. The apparent fractionation factor $\boldsymbol{\alpha}_c$ generated a similar trend with the changes in $\boldsymbol{\delta}_{13}CH_4$ (‰) and $\boldsymbol{\delta}_{13}CO_2$ (‰) with the values increased from 1.047 to 1.056 (Phases I to III) after the food waste addition then decreased to 1.048 (Phase IV) and 1.050 (Phase V) under higher OLRs. As suggested by the reported studies, compared to acetoclastic methanogenesis, the isotope fractionation in hydrogenotrophic pathways would generate elevated $\boldsymbol{\delta}_{13}CO_2$ (‰) (Lü et al., 2013). The higher $\boldsymbol{\delta}_{13}CO_2$ (‰) values obtained in the co-digestion period

may indicate a more active HM process compared to the mono-digestion condition. Further, the apparent fractionation factors have been utilized to differentiate the dominant methanogenic pathways, with values α_c <1.027 for AM and α_c >1.065 for HM (Conrad, 2005). The values obtained throughout the whole treatment (Phases I-V) in the present work were within a range of 1.047 to 1.056, which indicated a SAO-HM pathway.

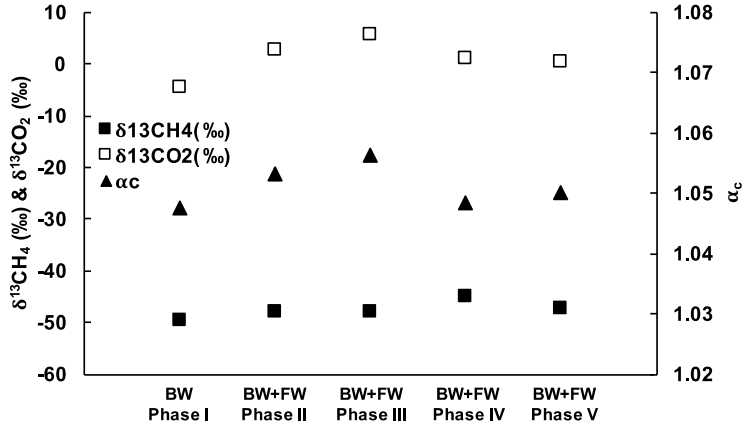

Fig. 8.5. Stable carbon isotopic signature $\delta_{13}CH_4$ (‰) and $\delta_{13}CO_2$ (‰) and the apparent fractionation factor α_c for biogas samples collected at different operational phases for blackwater (BW) mono-digestion Phase I, and at blackwater and food waste (FW) co-digestion Phases II, III, IV, and V.

It has been commonly reported that bacteria from phylum Firmicutes, Synergistetes and Chloriflexi could be correlated with the syntrophic acid oxidation (Ruiz-Sánchez et al., 2018). It has also been observed that another bacterial phylum Spirochaetes may be involved in the SAO-HM pathway (Lee et al., 2015). All of the above bacterial consortia were enriched in the current study, especially with Firmicutes 26.0-33.7% and Chloroflexi 2.6-35.0% dominated the system throughout the operation. Increased abundances of these bacterial consortia from Phase I to II, e.g. 2.6% to 17.2% for Chloroflexi and 1.1% to 5.0% for Spirochaetes, were observed with food waste addition, which could be due to the more available acetate produced through the enhanced substrate hydrolysis. The enriched bacterium from order Clostridiales, genus *Syntrophomonas, Syntrophus*, and *T78* have been reported correlated with syntrophic acetate/VFAs oxidation (Westerholm and Schnürer, 2019). Clostridiales (3.6-8.7%) and *T78* (17.1-34.7%) showed high abundances in the co-digestion period. As for methanogenic communities, both

Methanospirillaceae (Briones et al., 2009) and *Methanoculleus* (Zhao et al., 2017) have been reported to have cooperated with syntrophic bacteria in VFAs degradation. Interestingly, it was observed that the bacterial groups Clostridiales and *T78* shifted simultaneously with methanogenic group *Methanospirillaceae*, but opposite to *Methanoculleus* in the co-digestion period. This phenomenon may imply a synergistic correlation that existed between Clostridiales, *T78*, and *Methanospirillaceae*. As SAO are slow growers, the SRT impact on SAO-HM capacity can be significant; sufficient sludge retention is required to ensure effective acetate degradation (Sun et al., 2014). In the current work, the sludge loss under overloaded condition in Phases V reduced the SAO capacity, which resulted in the higher residual VFAs. The simultaneous reduction in abundances of the SAO bacteria Clostridiales and *T78*, and the HM methanogens *Methanospirillaceae* under high OLRs might contribute to the impairment of SAO-HM capability. Overall, the shifts of microbial groups involved in the SAO-HM process may correlate with the different environmental factors adopted, i.e. for blackwater, the main stress was from the high free ammonia concentration while in the co-digestion operation period, more stress was from the high OLRs.

8.4. Conclusions and implications

The current results demonstrated the apparent economic benefits of performing blackwater and food waste co-digestion using a UASB reactor. An OLR of 10 kg COD/m^3/d and methane production of 2.42 m^3/m^3/d were achieved in this study, which is higher than the bioenergy recovery efficiencies for blackwater and food waste co-digestion reported previously. Only 0.02 m^3/person of reactor volume is required using the current strategy for anaerobically treating source-diverted blackwater and food waste, indicating lower capital costs required as compared with other reported systems (0.05-1 m^3/person). It was also concluded that the combined SAO-HM methanogenic pathway played a key role in achieving effective biomethane production in blackwater and food waste treatment. The enhanced feeding substrate properties including VFAs, C/N ratios, and the environmental factors of free ammonia concentrations and pH values synergistically contributed to the enrichment of highly active microbial consortia with bacterial genus *T78*, and uncultured methanogenic genus from family *Methanospirillaceae* in the co-digestion system. Their simultaneously dynamic shifts during the co-digestion period indicated a syntrophic functional relationship. Methanogens from genus *Methanoculleus* showed

predominance under overloaded (11.6 kg COD/m^3/d in Phase V) condition in the co-digestion period, which illustrated its correlation with process deterioration. The dynamic shifts of *Methanoculleus* during the operation period also indicated that the early-stage community shift may be indicators of the process overloading.

It should be noted that the microbiota information as key performance indicators awaits more practical utilization. The knowledge provided by the current work can help with future operational design and process optimization in new sanitation systems for the treatment of source-diverted domestic wastewater.

8.5 Supplementary materials

The upflow velocity was calculated using the following equation:

$$v_{up} = \frac{H}{HRT}$$

H is the reactor height, in m;

HRT is the hydraulic retention time, in hour (h);

v_{up} is the upflow velocity, in m/h.

The upflow velocity in the current work was 5.2×10^{-3} m/h throughout the operation.

8.6 References

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CHAPTER 9. CONCLUSIONS AND RECOMMENDATIONS

9.1 Thesis Overview

The current applied centralized sanitation system is not compatible with the goal of sustainable development. Large amounts of water and energy are consumed for transporting and treating waste/pollutants; pathogens are conveyed and spread in the system while nutrients are lost after treatment. Reconceptualization out of the traditional centralized sanitation system design becomes a necessity facing global water scarcity, energy deficiency and poor sanitary conditions in some less developed/rural areas. The decentralized sanitation, also considered as new sanitation or sustainable sanitation, coping with the current challenges, is designed as a resource-recovery and reuse based wastewater treatment system. The profits come from recovering and reuse the water sources in greywater, and the nutrients and bioenergy in blackwater. In such a design, the energy circle can be closed at a small scale through utilizing the bioenergy recovered from blackwater anaerobic treatment. Promising as the process is, only a few studies have been carried out for blackwater anaerobic treatment. The process limitations, optimal treatment conditions, reaction mechanisms, and process optimization strategies, *etc*. have not been well comprehended and remain limitedly investigated.

Aiming at enhancing bioenergy recovery from domestic wastewater and closing the energy supply loop at the household level, this research systematically studied the blackwater anaerobic treatment processes from the perspectives of i) identifying the treatment/process limitations and ii) mitigating limitations and optimizing the treatment processes. As a start, blackwater characterization was conducted for different blackwater sources collected from various types of toilet flushing systems- water-wasting and water-conserving toilets, and the inhibitor identification studies were conducted using the bench-scale batch reactors. Chemical and biological properties of blackwater collected from conventional, dual flush and vacuum toilets were evaluated and the free ammonia inhibition was methodically studied. Then, continuous UASB treatments of conventional and vacuum toilet blackwater were conducted. High-rate treatments were pursued, and process limitations were evaluated. Sulfate inhibition in the methanogenesis process was identified in conventional toilet blackwater treatment processes. Solid fraction organic hydrolysis was realized to have limited the maximum treatment capacity

in the vacuum toilet blackwater treatment processes. Then the food waste and vacuum toilet blackwater co-digestion were performed to mitigate the hydrolysis limitation and to enhance the methane production. The involved biodegradation mechanisms were elucidated by the microbial communities' development and their dynamic shifts throughout the whole research.

9.2 Conclusions

The results obtained from the current research demonstrated that anaerobic digestion is an effective way of extracting bioenergy from source-diverted blackwater. With the mitigations against the process limitations, the high-rate blackwater treatment strategy with high organic removal efficiency and methane generation was demonstrated. The major conclusions are summarized as the following:

Blackwater characteristics, treatment limitations, and process optimizations

- The information on blackwater chemical and biological properties were obtained from studies described in Chapter 3. The organic and nutrients concentrations showed higher values in the vacuum toilet blackwater compared with the dual flush and conventional toilet blackwater. The high free ammonia (393 mg/L, 35 °C) concentration in vacuum toilet blackwater was found to inhibit the methanogenesis process, which resulted in a lower BMP value of 0.34 g CH_4-COD/g feed COD compared with the other water-wasting types of blackwater (BMP 0.48 g CH_4-COD/g feed COD) with less free ammonia contents (26 and 60 mg/L). The stepwise mechanism study revealed that the anaerobic digestion processes for substrates' hydrolysis and fermentation were not inhibited by the high free ammonia concentration. The free ammonia inhibitory level was identified as > 205 mg/L to impact the methane production in blackwater anaerobic treatment.
- The performances of continuous UASB treating conventional toilet blackwater at 35 °C were described in Chapters 4 and 6. Different OLRs and HRTs were tested in the study described in Chapter 6 with ranges of 0.18-0.76 kg COD/m^3/day and 1.4-5.5 days, respectively. The COD removal efficiencies were 72% and the methanisation rates were 23-29% of the feed COD in this demonstration. Compared with the BMP value obtained from the batch tests (Chapter 3), the continuous operation generated a lower methane yield. The treatment limitation was evaluated and verified in Chapter 4. Results showed that the sulfate contents of ~80 mg/L with the influent

COD/sulfate ratio of 12.2 in the conventional toilet blackwater inhibited the methanogenesis process and resulted in the lower methane production yield. The sulfate inhibition resulted in the microbial community shift and the inhibited methanogenesis activity. The toilet flushing water-tap water was found to have contributed to the major source of the blackwater sulfate. A 30% higher methanisation rate was observed when the major sulfate content was removed from blackwater (through replacing tap water with DI water flush).

- The performances of continuous UASB treating vacuum toilet blackwater at 35 °C were described in Chapters 5 and 6. Increasing OLRs from 0.28 to 4.87 kg COD/m^3/day were applied to elucidate the maximum feasible OLR. Results showed that the highest methane production rate of 0.68 m^3 CH_4/ m^3 reactor/day was achieved under the OLR of 4.07 kg COD/m^3/day condition, and the total COD removal efficiency could reach 84% with the methanisation rate reached 44% of the feed COD. The feedstock pH control and the stepwise increasing OLR strategies helped mitigated the free ammonia inhibition identified from the batch test, and they contributed to the development of the effective high-rate vacuum toilet blackwater treatment process. The methane generation was found limited by the insufficient solid substrate hydrolysis under the overloaded condition at OLR of 4.87 kg COD/m^3/day. Over 40% reduction in the solid organics' hydrolysis efficiency was observed.
- The performances of anaerobic co-digestion of vacuum toilet blackwater and food waste using a continuous UASB reactor at 35 °C were described in Chapters 7 and 8. The addition of food waste into blackwater successfully mitigated the limitation of substrate hydrolysis identified in vacuum toilet blackwater mono-digestion study and enhanced the biomethane recovery efficacy from domestic wastewater. The OLR was increased by stepwise increasing the food waste additions in the co-substrates with the blackwater: food waste VS mixing ratio changed from 1:0.3 to 1:1.5. The maximum feasible OLR was demonstrated to be 10 kg COD/m^3/day, which was 2.5 times of the maximum value obtained from blackwater mono-digestion system; the highest methane production rate of 2.42 m^3 CH_4/ m^3 reactor/day was 3.6 times of the value obtained for treating only blackwater; the COD removal efficiency remained at a similar level of 84% in the high-rate co-digestion system as compared with blackwater mono-digestion system. The optimal treatment performances achieved in the food waste and blackwater co-digestion system were attributed to the enhanced substrates properties including the more balanced C/N ratios, lower free ammonia concentrations, more readily hydrolyzed organics, and the enhanced

microorganisms with higher activities, which facilitated both substrate hydrolysis and methanogenesis.

Methanogenic pathway and microbial community development

Methods of the 16S rRNA gene sequencing analysis, carbon isotopic analysis, and specific methanogenic activity tests have been utilized to reveal the development of microbial community structures and the establishment of the methanogenic pathway in the blackwater treatment processes in this research. The combined syntrophic acetate oxidation (SAO) and hydrogenotrophic methanogenic (HM) pathway played a key role in obtaining effective biomethane production from blackwater treatment. Both the feedstock properties and the applied operational conditions affected the microbial community structure and microbial activities.

- The results described in Chapter 3 in the batch reactors showed that bacteria from Bacteroidales and the SAO bacterial group Clostridiales dominated in both vacuum and conventional toilet blackwater. Mixotrophic methanogens from *Methanosarcina* dominated the archaeal communities in both blackwater reactors. Hydrogenotrophic methanogens from *Methanoculleus* and *Methanomicrobiales* showed high abundances in vacuum toilet blackwater reactors while different groups of methanogens from *Methanobacterium* and *Methanolinea* were found abundant in conventional toilet blackwater condition. Such microbial structures indicated that a combined SAO and HM pathway might be established in both blackwater treatment groups for methane production. The microbial diversities in both bacterial and archaeal communities were observed less in vacuum toilet blackwater conditions, which can be attributed to the higher free ammonia concentration compared with the conventional toilet blackwater.
- From the results described in Chapters 4 and 6, the archaeal community was dominated by hydrogenotrophic methanogens from *Methanospirillum* and *Methanolinea* in the continuous treatment of conventional toilet blackwater in the UASB reactor. *Methanolinea* was dominant under all applied OLRs conditions. Different from the batch reactors, sulfate reducing bacteria (SRB) were enriched in the continuous operation system. The dominant SRB communities from *Desulfobulbaceae* and *Desulfomicrobiaceae* can consume H_2 for sulfate reduction, which resulted in competitions for electron donors between SRB and methanogens. The predominant methanogenic group shifted from *Methanolinea* to *Methanospirillum* when eliminating most

sulfate contents in the blackwater, and higher specific methanogenic activities of the biomass were achieved. The bacterial consortia from Clostridiales were enriched, which is capable of generating H_2 and functional for syntrophic acetate oxidation.

- Results from Chapters 5, 6 and 8 described the microbial community development in vacuum toilet blackwater UASB treatment system. Hydrogenotrophic methanogens dominated the system, with the methanogens from genus *Methanogenium* being predominant after the applied OLR exceeded 0.28 kg COD/m^3/day. It was noticed that the hydrolytic-fermentative bacteria group from genus *Bacteroides* generated increasing relative abundances with the increasing OLR applied and showed a considerably high value of 28.3% under the highest OLR condition of 4.07 kg COD/m^3/day. Bacteria from order Clostridiales also dominated the system throughout the operation. The functional genes prediction revealed that the prevalence of SAO functional genes in the vacuum toilet blackwater treatment system was contributed from the enrichment of an unidentified family in order Clostridiales. The isotopic carbon analysis for the produced biogas verified that the SAO-HM pathway was developed in the vacuum toilet blackwater treatment system.
- From the results of beta-diversity in both conventional and vacuum toilet blackwater continuous UASB treatment in Chapter 6, it was observed that the bacterial communities can quickly adapt to the feedstock-blackwater and remain relatively stable with the changing OLRs, while archaeal communities shifted slowly when adapting to different OLRs. Similar to results obtained from the batch test, the microbial community diversity was lower in the vacuum toilet blackwater UASB. The isotopic carbon fractionation factor α_c showed a higher value of 1.047 for the vacuum blackwater system than 1.038 obtained from the conventional blackwater system. These differences in the microbial community development between conventional and vacuum type blackwater were mostly attributed to the high ammonium concentration in the vacuum blackwater treatment system.
- The results from Chapter 8 described the microbial community development in the food waste and vacuum toilet blackwater co-digestion system in the UASB reactor. Multiple groups of bacteria consortia that are capable of performing syntrophic VFAs oxidation were enriched. The predominant bacterial group shifted from genus *Bacteroides* in blackwater mono-digestion system to genus *T78* in the co-digestion system. Hydrogenotrophic methanogens dominated the system. The predominant methanogens shifted from genus *Methanogenium* to genus

Methanoculleus and uncultured genus from family *Methanospirillaceae* from mono- to co-digestion treatment. *Methanospirillaceae* showed its highest relative abundance at OLR 7.0 kg COD/m3/day condition, while *Methanoculleus* generated the highest abundance under the organic overloaded condition at OLR 11.6 kg COD/m3/day. The simultaneous dynamic shifts of SAO bacterial groups Clostridiales and *T78* with methanogenic group *Methanospirillaceae* indicated their probable synergistic correlations. Their reduction under the overloaded condition indicated the impairment of the SAO-HM capacity. Isotopic fractionation factors (1.047 - 1.056) throughout the co-digestion operation illustrated the dominance of the SAO-HM pathway in the co-digestion system.

9.3 Outlook

Compared with the conventional centralized sanitation, the new sanitation system is more competitive towards the growing urbanization, especially is favorable for remote or developing areas that are lack of wastewater treatment infrastructures. The sustainability of the new sanitation system is contributed by the biomethane recovery from blackwater and kitchen waste, heat recovery from greywater, nutrients recovery and water reuse. The recovered biomethane, as demonstrated in the current work, can be directly utilized as a heating source or as electricity through the combined heat and power (CHP) system. The effluent, however, usually requires further treatment before being distributed or reused due to the presence of nutrients, pathogens, micropollutants and residual organic matters.

In addition to apply the liquid-phase anaerobic digestion effluent to crops, nutrients can be recovered in solid form in the post-treatment process after anaerobic digestion. Struvite precipitation has been considered as a popular process for recovering the nitrogen and phosphorus resources, and the recovered struvite can be utilized as a type of slow-release agricultural fertilizer. However, this process needs to be conducted in a separate process from the anaerobic bioreactor, and it requires additional chemical costs for magnesium, which increased the capital and operational costs. The profits are also restrained by the fact that struvite application is limited in the phosphorus industry due to the presence of magnesium and ammonia. Facing this situation, increasing research attention has been put on achieving the single-stage biogas and phosphorus recovery in anaerobic bioreactors, where the phosphorus can be

recovered in the form of calcium phosphate. In this process, biomass and calcium phosphate coprecipitated and formed granules in AD bioreactors (Tervahauta et al., 2014). While with limited studies being conducted, the favorable operational conditions remained unclear to enable a well-controlled and stabilized precipitation protocol for efficient phosphorous recovery. The risks and feasibility of the downstream utilization of such granules have not yet been investigated. Further studies are recommended to help establish a better phosphorous recovery process. The nitrogen concentration in AD effluent is high, even after struvite precipitation. The partial nitritation-anammox processes can be implemented as a post-treatment process for nitrogen removal (Pedrouso et al., 2020; Vlaeminck et al., 2009; Zhou et al., 2020).

Feces and urine contain pathogens and micropollutants (pharmaceuticals, hormones, bacteria containing antibiotic resistant genes), which are the major concerns for the recycle and reuse of the treated wastewater. It has been reported that the anaerobic digestion and the following nitrogen removal processes can partially degrade micropollutants, but the complete removal cannot be achieved (Gros et al., 2020). Chemo-physical processes such as electrochemical oxidation can be implemented for effective micropollutants' removal from the effluent (Radjenovic et al., 2011), and composting can be applied to the UASB sludge to remove micropollutants in the solid phase (Butkovskyi et al., 2016). Disinfection is indispensable before effluent utilization. The fates of the micropollutants in the end-products should be monitored for better risk assessment. Further investigations are required for establishing effective micropollutants' removal modules in the new sanitation system. The effluent residual COD can be removed through implementing an aerobic polishing system such as aerobic ponds. Otherwise, the effluent can be simply stored in a storage tank for a certain period time to meet the discharge standard.

The current work contributed to the knowledge of microbial community development and functional pathways in blackwater treatment at the DNA level, yet questions remain in transferring the information into engineering practice to guide the design and operations of the full-scale bioreactors. To promote applications such as bioaugmentation with functional microorganisms or enzymes to facilitate biogas production and pollutants removal, the RNA-level and proteomic analysis can be performed to further identify the core microorganisms. In

addition, the current work has realized that the syntrophic acetate oxidation (SAO) played a key role for biomethane generation in blackwater treatment process, but investigations on syntrophic oxidation remain inadequate: SAO species have not been well recognized or isolated so far, and the triggering parameters/conditions of the SAO pathway remain insufficiently addressed. Operational conditions such as temperature, VFA and ammonia concentrations have been correlated with SAO (Karakashev et al., 2006), but these factors may not necessarily be essential, taking into account that SAO has been observed in the conventional toilet flushed blackwater treatment system in the current study. Roles of inoculum, substrate type, reactor design, sludge retention time, operation duration could possibly contribute to SAO. Identifications of the influential factors can be promoted by manipulating operational factors and analyzing corresponding SAO genes expression, and more data collection and comparisons between other reported studies can facilitate this work. It should be noted that the role of SAO needs further elucidation as it appears to be robust against challenging conditions but is rate-limiting.

The anaerobic digestion model No.1 (ADM1) model has been developed for blackwater and kitchen waste treatment in a CSTR system (Feng et al., 2006), but it has never been tested for other bioreactor configurations (e.g. UASB in the current study). The ADM1 model in the UASB reactor can be developed based on the lab-scale data. Notably, the microbial interactions and the functional pathway are valuable information to be considered in the model. Their applications can better simulate the process and reveal the limitations in the blackwater (and kitchen waste) digestion processes, which could assist practical operations in full-scale demonstrations.

In the new sanitation system, vacuum transportation has played a key role in facilitating water conservation and high bioenergy recovery. Electricity is required to provide the air pressure under the low water conditions yet more benefits can be obtained from the less water resource consumption, the more concentrated blackwater flow (representing higher energy value) and the less effluent volume (for easier transportation and handling) in the vacuum system. Up to date, most of the established demonstrations utilized vacuum toilets with 1 L water per flush, while lower water demands such as 0.5 L/flush and even 0.25 L/flush could help further save freshwater and generate lower waste flow. Vacuum toilets with 0.5 L/flush are currently available in the market (e.g. Fann Roslagen Vacuum Toilet) but have not been well demonstrated

in blackwater treatment studies. It should be noted that with less water consumed, the ammonium concentration would rise, which may result in free ammonia inhibition in AD bioreactors. In this case, it is important to perform the kitchen waste co-digestion to mitigate the C/N ratio and pH. The pH values in the bioreactor should be monitored and well-controlled when necessary.

A major reason that has limited the worldwide popularization of the new sanitation system was the fact that the conventional sanitation systems have cost (continuously) large investments and remain reliable, and to replace them with new sanitation systems requires new investments. Further, public acceptance may be restrained by the concerns about micropollutants and pathogens in the end-products. It requires the cooperation of all stakeholders including the public, governments, water boards. to fulfill their duties to mitigate these restraints. Public education can help elucidate the advantages and risks of establishing new sanitation for the general public. End-product users, for instance, the agricultural actors that utilize the recovered nutrients and reclaimed water could be actively involved in illustrating the benefits and risks. In the transition of conventional to new sanitation, the government should play an essential role in policy and decision making and the integration of all stakeholders. New towns and communities, and remote areas that lack central sanitation systems can be encouraged to pioneer new sanitation demonstrations.

Overall, sustainable sanitation can be realized through closing energy, water and nutrients loops in the decentralized sanitation system. Despite the demonstrated feasibility and advantages, the global applications of the decentralized sanitation system remain low up to date. More investigations and modifications are needed to resolve constraints and further enhance the process's sustainability.

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