The Impact of Semi-continuous and Alternating Microbial Feeding Patterns on Methane Yield from UASB Reactors

by

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Abstract

The anaerobic treatment of wastewater is considered a promising technology for simultaneous organic matter removal and energy recovery. Compared to aerobic digestion, anaerobic digestion conserves energy and is cost-effective in handling high-strength wastewater and wastes. However, the high sensitivity of anaerobic systems to operational conditions such as temperature, pH, mixing conditions and feeding regimes has limited their application. In particular, feeding patterns have been shown to significantly impact the reactor operations, while a flexible feeding regime is commonly needed when treating the unequalised feedstock. To date, our understanding of the impact of bioreactor feeding strategies on anaerobic bioreactor performance is still limited.

The overall objective of this thesis is to evaluate the impact of various feeding strategies on the development of robust microbial communities in bioreactors and the anaerobic bioreactor treatment performance. Three feeding strategies, including continuous, semi-continuous (with starvation-feast cycle), and alternate feeding (with high loading-low loading cycle), were compared in this study to assess anaerobic digestion of synthetic wastewater and methane yields using three continuously operating laboratory-scale up-flow anaerobic sludge blanket (UASB) reactors. Observation of this study revealed that both long and short starvation-feast cycles provided by semi-continuous feeding did not sustain stable anaerobic digestion, whereas alternate feeding with high and low loading cycles promoted microbial community development. With dominant methanogens shifted from *Methanosaeta* in the continuous reactor to *Methanosarcina* in semi-continuous feeding reactors, significantly higher methanogenic activities were observed once reactors were recovered. Interestingly, the reactor undergoing the alternate feeding mode

successfully maintained a stable operation and thus the alternate feeding pattern is preferred over semi-continuous feeding when reactor feeding is not continuously and consistently available.

Preface

This thesis is an original work by Yiyang Yuan. A version of research findings in Chapters 3, 4, and 5 have been submitted as Yiyang Yuan, Lei Zhang, Yingdi Zhang and Yang Liu, "The impact of semi-continuous and alternating feeding patterns on methane yield from UASB reactors" to a peer-reviewed journal for publication. I was responsible for the experimental design, experimental operation, data collection and results analysis as well as the manuscript composition. Dr. Lei Zhang assisted with the experimental design and data collection. Dr. Yingdi Zhang contributed to the data collection and analysis. Professor Yang Liu was the supervisor of the project and has provided contributions to concept formation, results interpretation, and manuscript edits.

Acknowledgement

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Chapter 1 - Introduction

1.1 Background

Water is the most vital element for life on earth to survive and grow. About 71% of the Earth's surface is covered by water, of which only 3.5% is freshwater and the rest is salty water that is not suitable for human consumption. The freshwater on earth is stored over 68% in ice and glaciers, leaving only 2,120 km3 of freshwater available (USGS, 2019). To maintain sustainable water use, it is imperative that water is recycled and reused and thus wastewater treatment plays an increasingly important role in society today. On the other hand, wastewater also has a wealth of materials that, while damaging to people and the environment if dumped directly, may be beneficial if recycled and cleaned.

The depletion of non-renewable sources as a result of the growing population and needs, such as coal and natural gas, has drawn attention in recent days. Due to the high consumption of fossil fuels along with undesired climate change, and increased air and water pollution, renewable energy sources (RES) are urgently demanded to replace old fuels and benefit energy saving, food security, environmental conservation and sustainable development (Eswaran et al., 2021). Among all the types of renewable energy sources including biofuel, hydroelectricity, wind, geothermal, and solar, biofuel shows its advantage by turning wastes into reusable energy and thus being considered one of the most popular RES (Chu and Majumdar, 2012; Kumar and Samadder, 2017; Yikun et al., 2021). Biogas, especially methane, produced from anaerobic digestion is one kind of biofuel that is commonly utilized for heat and electricity generation (Weiland, 2010).

Anaerobic digestion is a promising method that may be used to treat wastewater while simultaneously recycling important resources. However, anaerobic digestion suffers from its extreme sensitivity when faced with variations in operating parameters such as temperature, pH, organic loading rate, feeding patterns, etc.) (Boiocchi et al., 2022; Gao et al., 2019b; Guo et al., 2022; Mariraj Mohan and Swathi, 2022; Panigrahi and Dubey, 2019; Sun et al., 2022a; Sun et al., 2022b; Zhang et al., 2020). It has been demonstrated that reactor functioning/performance and microbial community are impacted by reactor feeding patterns. Particularly, a semi-continuous feeding regime has drawn a lot of attention because a flexible feeding regime is desired for treating feedstock that is not equalized, as frequently observed in the diurnal/seasonal variations in sewage wastewater output and industrial wastewater production (e.g., effluent from food and beverage manufacturing) (Karadag et al., 2015; Ling and Lo, 2001; Sharma et al., 2008). Most of the previous studies conducted experiments on the continuously stirred tank reactor (CSTR). Results have shown that the semi-continuous feeding mode can promote chemical oxygen demand (COD) removal efficiency and functional stability (De Vrieze et al., 2013; Lv et al., 2014; Pagés-Díaz et al., 2015; Silva et al., 2021). However, only a few studies investigated the impact of semi-continuous feeding patterns on the modified up-flow anaerobic sludge blanket reactor (UASB). Contrary results were obtained when using internal circulation (IC) anaerobic reactors that are integrated by two UASBs in a vertical series, and a UASB integrated with a mixer by using the pump-fed method and syringe-fed method, respectively (Lu et al., 2019; Park et al., 2018). Therefore, investigating how feeding patterns affect the UASB reactor's performance and that of the reactor after recovery is worthwhile.

1.2 Research objectives and specific aims

The overall objective of this study is to elucidate the effects of feeding patterns (including semi-continuous feeding, alternating feeding and continuous feeding) on the microbiome development and treatment performance of UASB reactors. This study is crucial for UASB design, operation and optimization, as well as our understanding of enriching resilient microbial community dynamics in bioreactors. The specific aims of this study are to:

1. Access the feasibility and effects of applying a flexible feeding regime on stable anaerobic digestion.
2. Identify the species richness, evenness, and composition of microbial communities for reactors exposed to flexible feeding patterns.
3. Evaluate the correlation between reactor performance and microbiome development.

1.3 Thesis organization

A total of five chapters are included in this thesis dissertation. Chapter 1 introduced the background and objectives of this study. Chapter 2 documented the role of anaerobic digestion in wastewater treatment, operational factors that can affect anaerobic systems, application of flexible feeding regimes, types of anaerobic reactors and performance parameters. The experimental procedures and mathematic techniques employed in this study are described in Chapter 3. The findings and discussion related to the reactor performance and microbiological analysis were shown in Chapter 4. In addition, the overall results were summarised in chapter 5, along with recommendations for their implementation in the future. The results presented in this thesis dissertation will be published.

Chapter 2 - Literature Review

2.1 Overview of anaerobic digestion in wastewater treatment

With the rapid growth of the population and the development of energy-consuming technologies during the past century, water pollution and energy depletion are becoming major threats to human livelihood. According to the research conducted by Jones et al. (2021), global wastewater production is 358.0×10^9–361.4×10^9 m^3 yr^{-1} with 48% of produced wastewater discharged directly into the environment without undergoing any treatment. Discharged wastewater usually contains a high concentration of heavy metals, toxic compounds, and nutrients such as phosphorus/nitrogen with variable pH range and odour problems depending on the type of wastewater (von Sperling, 2007). An additional characteristic of wastewater is its massive production amount, which necessitates high-efficiency treatment processes to mitigate storage issues. The biological treatment process is typically classified as aerobic, anaerobic, or combined. Compared with anaerobic digestion, the aerobic process is noted by its better chemical oxygen demand (COD) and volatile suspended solid (VSS) removal efficiency (Chan et al., 2009). However, it also requires a large amount of input energy and a post-treatment for its excessive sludge production. On the other hand, anaerobic digestion has been developed to achieve comparable treatment efficiency with less energy consumption while generating biogas (renewable energy), potential valuable chemicals and nutrient-rich digestate that can be used for land application (Martin et al., 2011; von Sperling and Oliveira, 2009). The overall process of anaerobic digestion is shown in Fig.1. Anaerobic digestion is a biological process that converts organic carbon present in degradable compounds to reduced forms such as methane and carbon dioxide in the absence of oxygen. There are four steps involved in anaerobic digestion: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. During hydrolysis, particulate carbohydrate, protein, and lipid polymers were degraded enzymatically into monosaccharides, amino acids, and long-chain fatty acids (Vavilin et al., 2008). In acidogenesis and acetogenesis, simple organic compounds are broken down into intermediate products like propionate, butyrate, and valerate, and converted into acetate, H_2, and CO_2 which is used to produce biogas, mainly methane, during methanogenesis (Wang et al., 2018b). The degradation processes are each carried out by different consortiums of microorganisms and thus have various optimum operational and environmental conditions, which makes anaerobic digestion considered sensitive to the changes. As a result, certain substances may be over-accumulated in reactors and cause process failure subsequently.

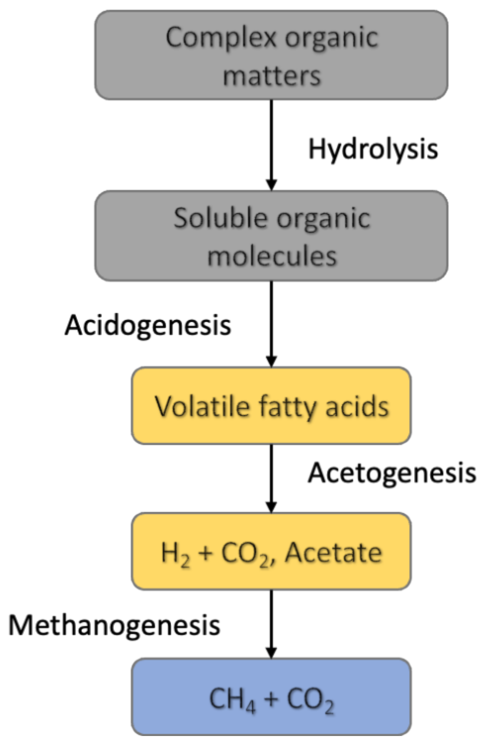


Fig. 1. Process of anaerobic digestion.

2.2 Operational factors that influence anaerobic digestion

To better control the anaerobic system, understanding the factors that can affect digestion is important. The entire process is driven by microorganisms. Operational conditions such as feedstock composition, reactor configuration, pH, temperature, feeding pattern, solid/hydraulic retention time, organic loading rate and mixing can all positively or negatively impact the composition and activity of the microbial community (Amani et al., 2010; Chow et al., 2020; Leitao et al., 2006). Thus, enhancing stability and efficiency may be achieved by manipulating operational conditions, especially factors that are easy to change without modifying the feedstock composition or adding chemicals.

2.2.1 Temperature

The operational temperature of anaerobic digestion is usually identified by three categories: psychrophilic (< 20 °C), mesophilic (20 – 43 °C) and thermophilic (50 – 60 °C) with mesophilic and thermophilic as two of the most commonly used temperature range (Nie et al., 2021). It is worth noting that the biogas production rate increased significantly when the temperature rise in the mesophilic temperature range without noticing any changes in microbial community structure, however, when the temperature is set above 35 °C, the microbial composition can be significantly affected. (Tian et al., 2018). The different temperature ranges may result in a completely different dominant metabolic pathway, for example, mesophilic reactors and thermophilic reactors (fed with food waste and wheat straw) were found to have the dominant genus *Methanosarcina* (hydrogenotrophic/acetoclastic methanogen) and *Methanothrix* (acetoclastic), respectively (Shi et al., 2018). However, due to the sensitivity of anaerobic microbes to the temperature, variations in temperature may cause severe changes in microbial communities and system instability or failure

(Ahn and Forster, 2002; Ciotola et al., 2013; Gao et al., 2011; Gimenez et al., 2014; Schmidt et al., 2019). Therefore, different microbes can be dominant at each temperature range and in each degradation process, which eventually can alter the dominant metabolic pathway to shift between three main pathways that are generally found in AD communities including hydrogenotrophic, acetoclastic and syntrophic acetate oxidation. Although thermophilic reactors can usually achieve higher biogas production, mesophilic reactors gain an advantage from their lower energy requirement and more stable system operation.

2.2.2 Mixing

Mixing, which is usually provided by mechanical mixing and recirculation, can be used to provide sufficient contact between microbes and substrates in order to build a homogeneous system. However, aggressive mixing may destroy the syntrophic relationship between microbes. It has been reported that reactors with a reduced rate of mixing show better digester performance and stability, especially during the start-up phase (Kim et al., 2002; McMahon et al., 2001; Stroot et al., 2001). Moreover, the optimum mixing rate in each kind of treatment is depending on the features of the system, such as the reactor type (Lettinga, 1995). For example, a Continuous stirred tank reactor (CSTR) is the most common reactor that is known to ensure the suspension of solids and good contact between microbes and substrates by providing good intensive mixing. One drawback of a high mixing rate is the destruction of flocs, however, the decreased amount of flocs may not necessarily impact the gas production in CSTR (Lindmark et al., 2014). On the other hand, to ensure good solid settleability in an up-flow anaerobic sludge blank reactor (UASB), mixing maybe not necessarily required. Even with a lower mixing intensity, the UASB reactor performance deteriorated when the effluent recirculation is provided caused by the disturbance made to the contact between bacteria and archaea groups as well as the hydrolysis efficiency (Sun et al., 2022a; Sun et al., 2022b; Zhang et al., 2020).

2.2.3 Retention time and organic loading rate

There are two main types of retention time: hydraulic retention time (HRT) and solid retention time (SRT). Hydraulic retention time is the average time taken by the water particle to move from the inlet to the outlet or the time length of the water particle staying in reactors. Solid retention time otherwise means the time of solid particles, usually the biomass remaining in the reactors (Mao et al., 2015; Panigrahi and Dubey, 2019). Short HRT may cause the washout of the biomass and poor effluent quality, while long HRT may affect the efficiency of the treatment. The SRT is closely related to factors such as the type of substrates, growth rate of microbes and types of reactor configuration (Elefsiniotis and Oldham, 1994; Li et al., 2011; Pilli et al., 2014). Because of the intrinsic characteristics of slow-growing microorganisms (especially, the methanogens) involved in anaerobic digestion, longer SRT is usually preferred to achieve high treatment efficiency and maintain good system stability (Wikandari and Taherzadeh, 2019). However, high SRT may result in insufficient utilization of digester components that is taken up by inactive microbes and thus reduce the efficiency of the system (Amani et al., 2010; Kanafin et al., 2021; Panigrahi and Dubey, 2019).

Organic loading rate (OLR) is defined as the amount of organic substrate fed per unit of time per unit volume of digester capacity and the maximum OLR is usually associated with parameters of reactor type/arrangement, wastewater characteristics, settleability and activity of biomass (Amani et al., 2010; Nkuna et al., 2022). There are two ways of adjusting the OLR of a reactor. One is to change the influent organic concentration and keep the same HRT or to change the HRT without modifying the influent feed. OLR that exceeds the threshold of digester capability can result in system instability together with reduced biogas production and viscous liquid medium, which is caused by the accumulation of volatile fatty acids (VFA)/acidification and imbalanced intermediates production and consumption (Guo et al., 2014; Jabeen et al., 2015; Lim et al., 2008; Wainaina et al., 2020). On the contrary, low OLR can cause an insufficient nutrient supply and the death or inactive state of microorganisms. Therefore, because of the high sensitivity of anaerobic digestion to the changes in operational conditions, the effects of loading variation should be carefully addressed. Generally, shock loading describes the instantaneous changes, while gradual or stepwise variation is defined as transient loading (Ketheesan and Stuckey, 2015). The shock can also further be characterized by hydraulic/organic shock loading depending on the type of factor utilized. To better control the anaerobic system, two extreme conditions as a result of sudden changes of OLR: overloading and starvation, should be carefully analyzed to understand the mechanism of causing system instability.

2.2.3.1 Overloading

Anaerobic reactors that operated with short-term or long-term pulsed overloading usually exhibited reduced biogas production and methane yield, as well as a drop in pH and alkalinity (Berninghaus and Radniecki, 2022; Braz et al., 2018; 2019; Razaviarani and Buchanan, 2014; Serrano et al., 2019). Interestingly, it was also found that pre-adapted digesters to OLR shock have shorter recovery times and higher tolerance to adverse conditions, which indicates the formation of a higher resilient and resistant system (Berninghaus and Radniecki, 2022; Meyer and Edwards, 2014). The rapid shift of the microbial community was observed within 3 hours after the loading shock (Braz et al., 2018). Specific bacterial groups were screened out from the stress such as the class *Actinobacteria*, the phylum *Bacteroidetes*, and the phylum *Firmicutes* (Braz et al., 2019; Regueiro et al., 2015). An increase in hydrogenotrophic methanogens and a decrease in acetoclastic methanogens is reported in several studies as a result of an increased hydrogen concentration due to VFA degradation (Braz et al., 2018; Lerm et al., 2012). In addition, *Methanosarcina*, which can utilize both hydrogenotrophic and acetoclastic pathways, is found to be more robust against perturbations because of its flexible function (De Vrieze et al., 2012). It was reported that a high loading rate has led to the shift of metabolic pathways for *Methanosarcina* and the promotion of syntrophic acetate oxidation (Hao et al., 2011; Qu et al., 2009). Furthermore, a more converged microbial community after loading stress was found by Wang et al. (2020) indicating a more even community was enriched and thus a higher methane yield after the recovery is expected.

2.2.3.2 Starvation

Starvation is another frequently occurring disturbance in full-scale AD operations, such as anaerobic reactors installed in the farms and reactors used for treating industrial wastewater that has seasonal variations (Chen et al., 2022; de Jonge et al., 2017; Hwang et al., 2010). Continuously operated systems that suddenly have a cessation of feeding usually experience starvation period. Both short-term and long-term starvation ranging from one day to more than 10 months were studied. The result shows that starvation of less than 1 day caused changes in endogenous respiration rate, ATP content and biomass level, however, long-term starvation can cause significant changes in microbial community for both the bacterial and archaeal populations and exhibit higher tolerance to a harsher environment, such as high VFA and ammonia concentration (de Jonge et al., 2017; Konopka et al., 2002). Magdalena et al. (2019) conducted research about the impact of two weeks of starvation on the anaerobic system and it was observed that the starvation period induced a shift of microbial community towards the syntrophic acetate-oxidizing bacteria coupled with hydrogenotrophic methanogens. Moreover, the repeated starvation period was also tested by providing pulsed nutrients. It was reported that the system that experienced pulsed nutrients every day has a higher growth rate and activities than systems that pulsed every 14 days (Carrero-Colon et al., 2006). Systems that were cultivated by repeated pulse supply were found to have a more even microbial community, higher substrate uptake rates and more efficient processes (Carrero-Colon et al., 2006; Jauregui-Jauregui et al., 2014; Ricao Canelhas et al., 2017).

2.2.4 Feeding patterns

Feeding patterns have been found to affect the biogas production, system stability and microbial community in the anaerobic digestion process (Guan et al., 2018; Li et al., 2021; Lu et al., 2019). Demand-driven flexible biogas production can be achieved by alternating feeding strategies to better integrate biogas plants into the energy supply systems (Feng et al., 2018; Mauky et al., 2015; Mauky et al., 2017). Also, instead of introducing a larger substrate amount at once, feeding the reactor with higher frequency may encourage higher digestion efficiency, which reflects the importance of gaining an understanding of different feeding strategies (Golkowska et al., 2012). There are four main types of feeding regimes that are widely discussed recently including continuous, batch, fed-batch and semi-continuous. Continuous feeding and batch feeding are the two most common feeding patterns. Continuous feeding ensures a constant flow rate at both input and output, while the batch feeding method allows substrates to react completely in a closed system before discharging the products and applying the pulse inputs again (Fig. 2 and Fig. 3). Nutrients can also be continuously supplied at predetermined intervals using semicontinuous and fed-batch modes, which are in between continuous and batch means.

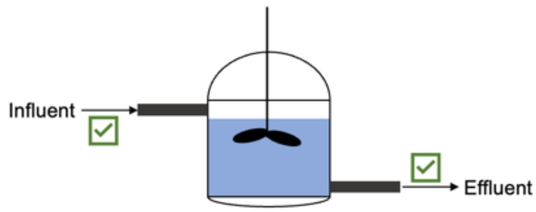


Fig. 2. Continuous feeding.

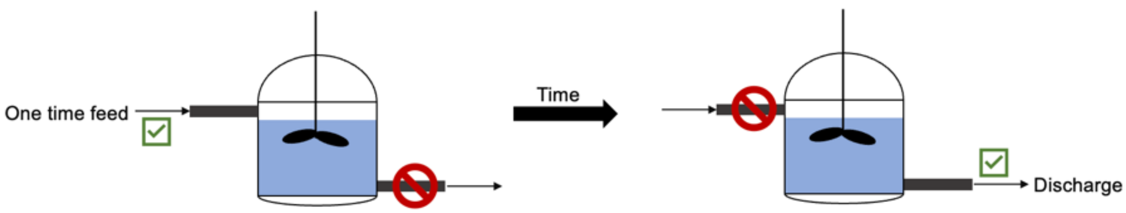


Fig. 3. Batch feeding.

Both continuous and batch feedings were proven to support very stable operations (Azis et al., 2018; Carbajo et al., 2010; Hua et al., 2020; Sabbah et al., 2004; Zytner et al., 2015). Generally, continuous reactors are employed for low-solid-content wastes (wet wastes) and batch reactors are selected to treat high-solid-content wastes (dry wastes) (Ghanimeh et al., 2020). Compared to the continuously fed reactors, the batch mode is easier to operate. However, only up to 50% of the inoculum can be recycled as new inoculum for the next round (Wang et al., 2021). A large quantity of required additional inoculum makes the batch operation not cost-effective.

As alternatives to the batch mode, the fed-batch feeding patterns were considered by dividing the total required amount of feed into several portions and adding them into the reactor periodically as shown in Fig. 4. Based on the research conducted by Wang et al. (2021) and Rodrigues et al. (2003), fed-batch reactors only need 45% of the inoculum for a batch reactor, while the cumulative methane yield is increased by 45%, probably due to reduced excessive volatile acids formation. Garcia-Pena et al. (2011) also reported stable performance and natural pH was maintained in a fed-batch reactor. In addition, it was also found that an increase in the population and activity of microbes and changes in microbial metabolic patterns may be induced by operating reactors with a fed-batch mode (Razaviarani and Buchanan, 2014; Wang et al., 2021).

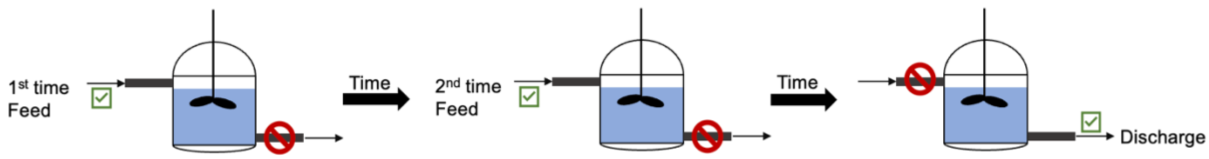


Fig. 4. Fed-batch feeding.

Similarly, a semi-continuous feeding mode can be defined as pulsed continuous feeding, where simultaneous input and output are maintained (Fig. 5). There are mainly two types of feeding methods, one is syringe-fed and the other is pump-fed. Both methods were commonly used in previous semi-continuous operations and achieved stable reactor operations that are fed with widely different feedstocks including food waste, manure, crop straw residues, grass, slaughterhouse waste, landfill leachate and industrial wastes (Borja-padilla and Banks, 1993; Camano Silvestrini et al., 2019; Feng et al., 2018; Li et al., 2014; Lim et al., 2008; Lu et al., 2019; Manser et al., 2015; Zhang et al., 2015). Compared to the continuous feeding mode, semi-continuous reactors showed superior performance for COD removal and biogas production and by utilizing pulse feeding, biogas production can be elevated in a short time (Feng et al., 2018; Lu et al., 2019). It is also worth noting that longer feeding cycles or less frequent feeding regimes under semi-continuous feeding mode have shown better digester performance and improved system stability when facing influent overloading (Camano Silvestrini et al., 2019; Mulat et al., 2016). Moreover, variations of operational parameters have been always shown to impact the microbial community. Both overloading and starvation stress that may exist in the application of semi-continuous feeding could provide selective forces to the microbiome. Results indicated that the microbial community after the change of feeding strategies promoted different dominant bacteria and archaea. For example, the hourly-fed reactor enriched the *Methanosaeta* and the daily-fed reactor promoted the growth of *Methanosarcina* with the latter being more tolerant to environmental shocks (Conklin et al., 2006). Based on the research done by Manser et al. (2015), semi-continuous feeding patterns induced alternation of the microbial community that contribute to higher specific methanogenic activities, especially with less frequent feeding events. Minimal microbial community diversity was formed with the cultivation of semi-continuous feeding, which leads to a higher degree of bacterial dynamics as well as higher resistance to high organic loading shock (De Vrieze et al., 2013; Lu et al., 2022). Nevertheless, not all of the studies recommended the semi-continuous feeding strategy. Previous studies have shown that semi-continuous reactors can only return 60% ~ 90% of methane production obtained from batch and fed-batch reactors. A study conducted by Park et al. (2018) reported better performance and stability of upflow anaerobic reactors (two stirrers were installed) with continuous feeding compared with a semi-continuous reactor fed with syringes once a day. Hence, there is still a lot of uncertainty regarding semi-continuous feeding's impact on reactor performance. In addition, alternate feeding (with high loading-low loading cycles) has not been considered in any study.

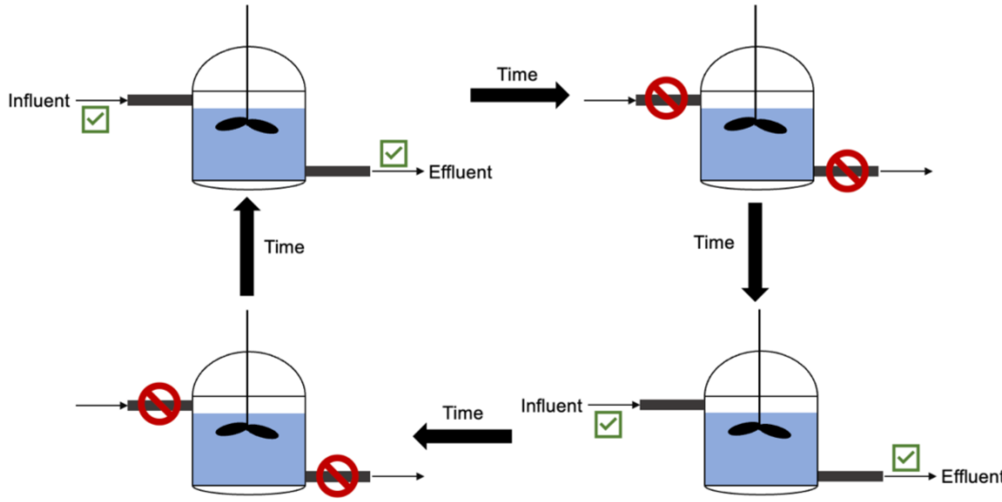


Fig. 5. Semi-continuous feeding.

2.3 Application of flexible feeding regime in wastewater treatment

Generally, wastewater is classified into two main types: Sewage wastewater and industrial wastewater (Hamiruddin et al., 2021). Each of them has distinct characteristics and thus treating them equally by using the same process design, such as a traditional continuous or batch system, without any modification may cause problems in practical application. Loading variation is a common phenomenon in some of the wastewater treatment systems/plants, mostly due to human activities. Wastewater flow rates can vary in a day, a week, one season or year to year, which leads to short-term, seasonal, multiyear and industrial variations (Metcalf & Eddy, 2014). Sewage wastewater originated from mainly three sources including domestic waste (ex. Bathrooms, washing, cooking), industrial wastewater that is directly discharged into the sewage system, as well as rain/stormwater (Seghezzo et al., 1998). Stormwater usually caused seasonal or regional variation; however, domestic wastewater may follow regular patterns during the day, which makes the flexible feeding regime applicable. Peak hours of domestic waste were generally found in the morning and early evening between 7 and 9 pm, which results in the daily variation of the flowrates in treatment plants, and the time and amplitude of the shock are dependent on the size of the community and the storage capacity of the system (Leitao et al., 2006; Metcalf & Eddy, 2014). Industrial wastewater usually contains heavy metals, toxic organics, high levels of COD, a wide range of pH, suspended solids and so on, depending on the type of industries, where the thermal power plants, steel plants and pulp and paper industries are considered the highest contributors to the industrial wastewater generation (Chan et al., 2009; Ranade and Bhandari, 2014). Karadag et al. (2015) conducted a study on the dairy industry. They mentioned that raw milk can be transformed into milk, yogurt, cheese, butter, ice cream and so on by various manufacturing processes, where wastewater is generated from the wash of equipment, containers, laboratory analysis and byproducts of processes. Depending on the type of processing and production plan, dairy effluents generated from an industry can have a wide range of organic content, the concentration of solids and nutrients contents, which means that the feedstock cannot be equalized. In addition, brewery wastewater production has been also reported to have a wide variation of both discharge volume and strength of pollutants because of the changing bear production and randomly collected wastewater (Ling and Lo, 2001). Because of the sensitivity of the anaerobic digestion system as mentioned before, varied feedstock/influent characteristics may cause process deterioration or instability in a continuous reactor that has reached steady state conditions. Therefore, compared to the constantly continuous reactor, flexible feeding regimes, such as a semi-continuous feeding pattern is valuable when the feedstock is not equalized or stays consistent.

2.4 Anaerobic bioreactor configurations

Studies have been conducted to investigate the impact of feeding regimes on different types of reactors. Contrary results may be found under different reactor configurations, feedstock characteristics and operational conditions.

2.4.1 Continuous stirred-tank reactor (CSTR)

The most commonly used reactor for analyzing different feeding regimes is the continuous stirred-tank reactor (CSTR) (De Vrieze et al., 2013; Egwu et al., 2022; Janke et al., 2016; Janke et al., 2019; Lv et al., 2014; Pagés-Díaz et al., 2015; Silva et al., 2021). The CSTR typically works with a mixer to maintain the solids in suspension and support a superior mass transfer efficiency. The digester is fed through an inlet and usually, an equal amount of mixed sludge/effluent was taken out simultaneously (Kariyama et al., 2018). Different temperatures, pH levels, solid concentrations, feeding methods (pump-fed or syringe-fed) and feeding intervals were adjusted in parallel among all the studies related to feeding regimes, which resulted in different outcomes. However, all of them were found to have an alternation of microbial community indicating the high sensitivity of anaerobic microbes. By changing the feeding frequency from daily to once every two days and once every three days, the CSTR fed with pig slurry had an increased methane yield (Silva et al., 2021). In the study performed by De Vrieze et al. (2013), CSTR that are fed once every two days by using synthetic domestic wastewater showed a comparable reactor performance, higher tolerance to the loading shock and higher functional stability compared with the other reactor that is fed every day. Similarly, Lv et al. (2014) demonstrated a similar gas production between once-per-day feeding and twice-per-day feeding reactors, suggesting a similar conversion efficiency was achieved. However, utilizing an equal feeding interval could be substantial in maintaining a stable operation. As indicated in the study performed by Egwu et al. (2022), unequal feeding intervals can lead to CSTR failure caused by nutritional and microbial imbalance.

2.4.2 Up-flow anaerobic sludge blanket reactor (UASB)

The up-flow anaerobic sludge blanket reactor (UASB) has been recognized as one of the most important anaerobic digesters for treating wastewater. During the operation of the UASB reactor, the influent was injected or pumped from the bottom of the reactor and passed through the sludge bed for digestion. As shown in Fig. 6, the three-phase separator located on the top of the reactor allows an easier collection of gas and effluent while preventing the escape of biomass. Compared to the CSTR reactor, UASB is designed for better solid settleability and solid retention time thus enhancing the formulation of highly active granules, which would eventually promote treatment efficiency and lower the footprint requirement (Schmidt and Ahring, 2000). It gains advantages over other technologies from its higher organic loading rates (OLRs), short hydraulic retention time (HRT) and low energy demand, which makes it suitable for treating medium to high-strength industrial wastewater as well as domestic wastewater (Daud et al., 2018; Latif et al., 2011; Odejobi et al., 2016). In addition, natural agitation provided by up-flow influent and biogas float can supply sufficient mixing and ensure good mass transfer efficiency between biomass and substrates. However, there are only a few studies investigating the impact of semi-continuous feeding patterns (ex. semi-continuous feeding patterns) on the UASB reactor. It was found that the traditional UASB fed intermittently (without specifying the frequency) showed a low COD reduction when treating the baker’s yeast (Manhokwe and Zvidzai, 2019). With the modification of traditional UASB reactors, the system performance was found positively or negatively related to the semi-continuous feeding mode. In the study conducted by de Mendonca et al. (2017), a semi-continuous

UASB implemented with an anaerobic filter exhibited higher biogas outputs compared with the previous report for treating cattle wastewater (de Mendonca et al., 2017). Furthermore, Lu et al. (2019) examined the performance of internal circulation (IC) anaerobic reactors that are integrated by two UASBs in a vertical series. They observed that a pump-fed semi-continuous IC reactor (fed one day every two days) outcompeted a continuous reactor for higher soluble COD removal and biogas production. However, according to Park et al. (2018), up-flow anaerobic reactors (added a stirrer) with continuous feeding performed better than semi-continuous reactors that are syringe-fed (once per day). As a result, research must be conducted to evaluate how feeding regimes affect the traditional UASB while keeping other factors constant in order to gain a better understanding of the mechanisms at play.

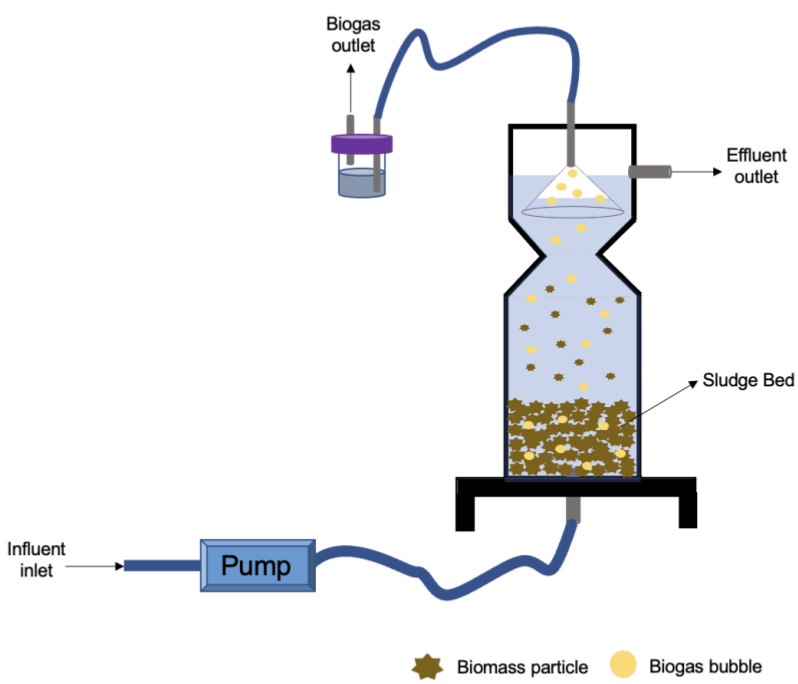


Fig. 6. Up-flow anaerobic sludge blanket (UASB) reactor.

2.5 Parameters of anaerobic digestion performance

2.5.1 Chemical oxygen demand (COD)

An important parameter in determining water quality is the chemical oxygen demand (COD), which represents the degree of organic contamination or pollution in a water sample (Dhanjai et al., 2018). Influent COD that was fed into the anaerobic system was composed of soluble COD and particulate COD and can also be characterized by biodegradable COD and non-biodegradable COD. The COD can then be converted to methane or be used by microbes for multiplication or move out with an anaerobic digestion effluent (Cheng et al., 2021). The influent total amount of COD should be equal to the output total COD from the three parts theoretically. The more methane is converted, and the less COD is left in the effluent, the more efficiently the system behaves.

2.5.2 Volatile fatty acids (VFA)

Volatile fatty acids (VFA) are the intermediate products produced during acidogenesis by acidogenic bacteria. Generally, VFA mainly consists of acetic, propionic and butyric acids. Among all the three types of VFA, butyric and acetic acids contribute to methane production and propionic acids remain unconverted (Khan et al., 2016). The propionate and butyrate accumulation are indicators of a stressful situation and excessive VFAs can increase the acidity of the digester and eventually cause the interruption of biogas production or process failure (Haririchi et al., 2022).

2.5.3 Methane production and methane yield

Methane yield can be defined as the amount of organic matter that is converted into methane. Theoretically, one gram of COD can be utilized by microbes to generate 0.35 L methane under standard temperature and pressure conditions (STP) (Angelidaki and Sanders, 2004). The value of methane yield should be constant during steady-state conditions (Michaud et al., 2002). The high methane production rate and methane yield reached by an anaerobic digester indicate stable and superior system performance

2.5.5 Specific methanogenic activity (SMA)

The specific sludge activity (SMA) is determined by the methane production rate and the sludge amount and it is a measure of the rate at which methanogens convert substrate to biogas (methane and carbon dioxide) (Hussain and Dubey, 2015; Subramanyam, 2013). As well as advising a suitable range of OLR that the system can handle at the start-up stage, SMA tested during the operation also positively correlated to a system's tolerance for OLR and process efficiency (Anderson and Kasapgil, 1995).

Chapter 3 - Methodology

3.1 Reactor start-up and operation

Three 1 L laboratory-scale UASB reactors $UASB_{cont}$, $UASB_{cont-semi-cont}$, and $UASB_{cont-semi-alt}$ were operated under mesophilic conditions (35 °C). The setup of UASB reactor was shown in Fig. 6. The temperature was maintained with a water bath and polystyrene foams. All three reactors were seeded with anaerobic digester sludge collected from a local wastewater treatment plant in Alberta, Canada. Reactors were filled to 40% with the inoculum sludge before reactor start-up. The contents of synthetic blackwater are provided in Table 1. Glucose and sodium acetate were the main substrates and the influent chemical oxygen demand (COD) was maintained at around 3.2 g COD/L. Detailed operation conditions of the three reactors are shown in Table 1. The experiment employed three different feeding patterns and was divided into three phases (Table 2). In phase I, all three reactors were operated continuously, and $UASB_{cont}$ maintained this operation condition for all three phases. In phase II, $UASB_{cont-semi-cont}$ and $UASB_{cont-semi-alt}$ reactors were operated using a semi-continuous feeding mode by implementing feeding (feast) and non-feeding (starvation) periods. In phase III, $UASB_{cont-semi-cont}$ was fed with continuous feeding, while $UASB_{cont-semi-alt}$ was fed with an alternating high/low feeding condition. The average organic loading rate (1 g COD/L/day) and hydraulic retention time (3.3 days) were maintained constant for all reactors during the 180 days' operation.

Table 1. The recipe of the synthetic blackwater for reactors.

Chemical	Characteristics	
Glucose Sodium Acetate	COD, mg/L	3,200
Na_2HPO_4	PO_4-P, mg/L	30
NH_4Cl	NH_4-N, mg/L	1000
KCl	K, mg/L	75
$CaCl_2$	Ca, mg/L	80
$MgCl_2 \cdot 6H_2O$	Mg, mg/L	30
$NaHCO_3$	Alkalinity mg $CaCO_3$/L	980
Urea, mg/L		0.736
Trace mineral stock, mL/L		1
Vitamin stock, mL/L		1

Table 2. Operational conditions of phases I, II and III for reactors.

	$UASB_{cont\text{-}semi\text{-}alt}$	$UASB_{cont\text{-}semi\text{-}cont}$	$UASB_{cont}$
Phase I Start-up	1 g COD/L/day	1 g COD/L/day	1 g COD/L/day
Phase II Semi-continuous feeding	Two-day repeated cycle: 2 g COD/L/day (24 hours) 0 g COD/L/day (24 hours)	One-day repeated cycle: 2 g COD/L/day (12 hours) 0 g COD/L/day (12 hours)	
Phase III Alternating high/low feeding vs. Continuous feeding	Alternating high/low feeding: 1.3 g COD/L/day (24 hours) 0.7 g COD/L/day (24 hours)	Continuous feeding: 1 g COD/L/day	

3.2 Analytical Methods

The characteristics of influent wastewater and reactor effluent were observed by measuring total COD (TCOD), soluble COD (SCOD), and phosphate phosphorus concentration according to the standard methods of the American Public Health Association (APHA) (APHA, 2012). Ammonia nitrogen in reactor influent and effluent was measured with the Nessler Ammonia Quantification Reagent Kit. Volatile fatty acids (VFAs), including acetate, propionate, and butyrate, were analyzed using an ionic chromatograph (Dionex ICS-2100) equipped with a conductivity detector (DIONEX ICS- 2100, ThermoFisher, USA). Influent/effluent pH was measured with a B40PCID pH meter (VWR, Radnor, USA). Sludge samples were collected from the reactors at the end of each phase and total suspended solids (TSS), volatile suspended solids (VSS), and specific methanogenic activity (SMA) were determined. TSS and VSS were measured using the standard methods of the American Public Health Association (APHA).

3.2.1 Specific methanogenic activity (SMA)

The specific methanogenic activity in the sludge was tested in 37 ml serum bottles containing 5 ml seed sludge, either sodium acetate or hydrogen (H_2), and carbon dioxide (CO_2). The substrate concentration was 1g COD/L (Zhang et al., 2020). Seed sludge was collected at the end of phases in each reactor. When sodium acetate was applied, the bottles were flushed with nitrogen gas to ensure an anaerobic environment. When H_2 (80%) and CO_2 (20%) were added as substrates, the bottles were instead flushed directly with H_2 and CO_2 to achieve anaerobic conditions. Blank tests (no substrates added) were included in each group of tests. The bottles were sealed with rubber stoppers and aluminum caps and incubated in a shaker (New Brunswick™ Innova® 44, Eppendorf, Canada) at 35 °C. The production of biogas was examined by measuring the headspace pressure with a hand-held pressure meter (GMH 3151, Germany) and gas samples were collected using syringes. The gas composition (nitrogen, oxygen, carbon dioxide, methane) was analyzed with a gas chromatograph (GC-7890B, Agilent Technologies, Santa Clara, USA). Each test was performed in triplicate and average values were calculated.

3.2.2 Microbial community analysis

Sludge samples (1 ml) were collected at the end of each phase. Genomic deoxyribonucleic acid (DAN) extraction was performed following the manufacturer's protocol using the PowerSoil Kit.

DNA samples stored at -20 °C were sent to Genome Quebec (Montreal, Canada) for sequencing. The DNA quality was tested with a NanoDrop One device (Thermo Fisher, Waltham, MA, USA). The polymerase chain reaction (PCR) was used to amplify DNA sequences using the primer pair 505F/806R. Forward and reverse raw sequences were paired with the Qiime2 and pipeline DADA2 algorithm (Callahan et al., 2016; Caporaso et al., 2010). Processed DNA sequences were compared with the Silva Database version 13_8 with 99% similarity (Quast et al., 2013). Microbial community analysis was performed by R software (RStudio 2022.02.3) using "pheatmap" packages for the heatmap, "corrplot" packages for correlation, "vegan" packages for Principal Coordinate Analysis (PCoA) of the Bray-Curtis distance, and the "psych" package for co-occurrence network analysis (Kolde, 2019; Oksanen et al., 2022; Revelle, 2022; Wei and Simko, 2021). Network analysis was further visualized by Gephi software (version 0.9.6).

3.2.3 Statistical analysis

A student's T-test and ANOVA analysis provided by Microsoft Excel were used to analyze the significance of the results. A p-value of less than 0.05 was considered to be significant.

3.3 Calculation

3.3.1 COD removal

$$\%\ COD\ removal = \frac{COD_{inf} - COD_{eff}}{COD_{inf}} * 100 \qquad (1)$$

Where:

COD_{inf} : concentration of COD in the influent (in g/L)

COD_{eff} : concentration of COD in the effluent (in g/L)

3.3.2 The COD equivalent of produced methane

$$COD_{methane} = Biogas\ production * CH_4\ composition/0.35 \quad (2)$$

Where:

$COD_{methane}$: COD equivalent of produced methane (in g/day)

Biogas production: the amount of biogas produced (in L/day)

CH_4 composition: composition of methane in biogas (%)

0.35: 1g COD is equivalent to 0.35L methane at standard temperature and pressure (STP)

3.3.3 Methane yield

$$\%\ Methane\ yield = \frac{COD_{methane}}{COD_{input}} * 100 \quad (3)$$

Where:

COD_{input}: the amount of COD input (in g/day)

3.3.4 Specific methanogenic activity (SMA)

The equations were adapted from studies conducted by Hussain and Dubey (2015) and Aquino et al. (2007).

$$Methane\ production = \frac{P_{headspace} * V_{headspace} * CH_4\ composition * 64}{R * T} \quad (4)$$

$$SMA\ (in\ mg\ CH_4 - COD/g\ VSS/day) = \frac{Methane\ production}{t * VSS_{sludge}} \quad (5)$$

Where:

Methane production: the amount of methane produced at time t (in mg COD)

$P_{pressure}$: absolute headspace pressure at time t (in kpa)

$V_{headspace}$: volume of headspace in serum bottles (ml)

CH_4 composition: methane composition in the headspace (%)

64: 1mol of methane is equivalent to 64g COD

R: Gas law constant (in ml kpa/K mol)

T: absolute temperature (K)

t: duration of the SMA test (day)

VSS_{sludge}: the amount of volatile suspended solid of the sludge used in the SMA test (g)

Chapter 4 - Results and Discussion

3.1. Anaerobic reactor performance

The methane yield and the COD removal efficiency of all three reactors are shown in Fig.1. In phase I, all three reactors were fed continuously and achieved similar performance, with an average methane yield of 60 ± 4.7 % and a COD removal efficiency of 84 ± 5.7% ($P > 0.05$). For the rest of the operation, $UASB_{cont}$ was operated with a continuous feeding strategy. Its performance did not vary significantly and the $UASB_{cont}$ reactor achieved overall an average methane yield of 60 ± 7.2 % and an average COD removal of 86 ± 9.7 %.

However, when $UASB_{cont\text{-}semi\text{-}alt}$ and $UASB_{cont\text{-}semi\text{-}cont}$ were operated in a repeated starvation/feast condition in phase II, the methane yield in $UASB_{cont\text{-}semi\text{-}alt}$ (2-day feeding cycle) decreased to 33 ± 5.4%, which corresponds to a drop in the COD removal efficiency to 60 ± 7.8%. Correspondingly, the methane yield of $UASB_{cont\text{-}semi\text{-}cont}$ (1-day feeding cycle) declined to 25 ± 1.9% and the COD removal efficiency decreased to 48 ± 4.1%. It was observed that semi-continuous operation led to the deteriorated reactor performance and methane yield, and a more frequent change in feeding pattern (as seen in $UASB_{cont\text{-}semi\text{-}cont}$) can lead to a less stable methane yield and a lower COD removal, which is consistent with previous studies conducted by Mulat et al. (2016).

In phase III, the continuous operation of $UASB_{cont\text{-}semi\text{-}cont}$ and the alternate feeding of $UASB_{cont\text{-}semi\text{-}alt}$ allowed these reactors to recover from the semi-continuous feeding phase that was imposed in phase II. A stable operation with fluctuated biogas production as shown in Fig. 7 and Fig. 8 was observed in $UASB_{cont\text{-}semi\text{-}alt}$ in phase III, with an alternate feeding strategy. The average methane yield and the average COD removal efficiency in $UASB_{cont\text{-}semi\text{-}alt}$ at the end of phase III were 74 ±

8.5% and 89 ± 2.8% (Fig. 7), respectively. Correspondingly, $UASB_{cont-semi-cont}$ reached a methane yield of 72 ± 2.4% and a COD removal efficiency of 87 ± 7.7%, in phase III, both values were comparable to the $UASB_{cont-semi-alt}$ performance (Fig.7). Interestingly, the performance of these two reactors in phase III is significantly better than that in the control reactor that was operated with continuous feeding without disturbance $UASB_{cont}$ ($P < 0.05$). At the same time, these results indicate that both continuous and alternating high/low feeding patterns achieved stable reactor operation and biogas production once starvation/feast stress was removed. However, a cyclic feast and starvation feeding pattern can harm the anaerobic digestion and eventually cause system instability.

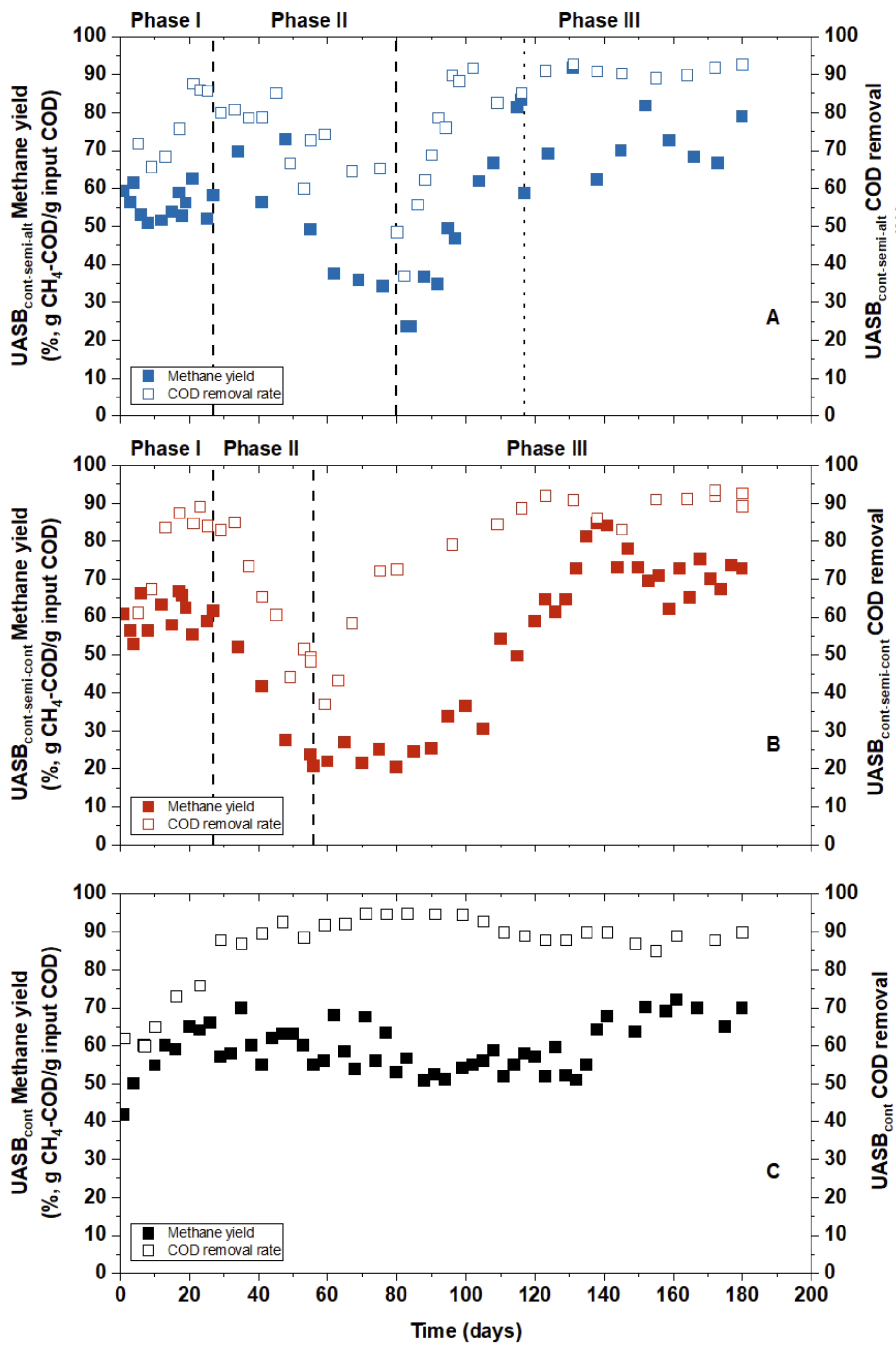


Fig. 7. Methane yield and COD removal over time from Phase I to Phase III for reactors: (A) $UASB_{cont-semi-alt}$; (B) $UASB_{cont-semi-cont}$; (C) $UASB_{cont}$.

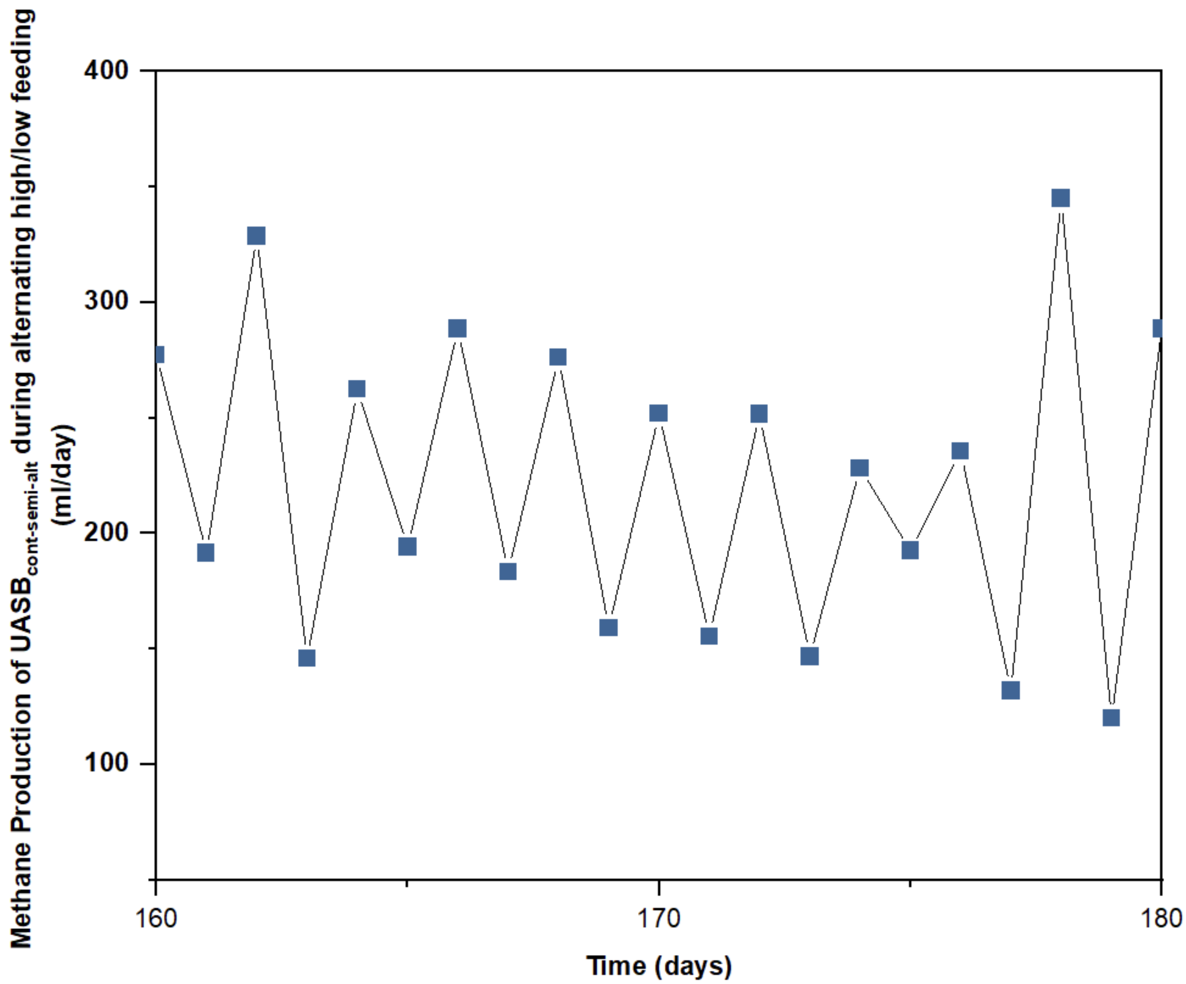


Fig. 8. Methane production of UASB$_{cont-semi-alt}$ in Phase III (alternating high/low feeding pattern).

3.2. Specific methanogenic activity (SMA) and sludge concentration

Fig. 9 and Fig. 10 illustrate the TSS/VSS and the SMA of the three reactors at the end of each phase. The SMAs of H_2/CO_2 and acetate at the end of phase I in all three reactors ranged from 212 mg CH_4-COD/g VSS d to 226 mg CH_4-COD/g VSS d and 127 mg CH_4-COD/g VSS d to 157 CH_4-COD/g VSS d, respectively. In phase II, the SMA of hydrogenotrophic methanogenesis and acetoclastic methanogenesis decreased to 133 ± 2.2 mg CH_4-COD/g VSS d and 154 ± 2.9 mg CH_4-

COD/g VSS d, respectively, in $UASB_{cont-semi-alt}$, and 45 ± 2.6 mg CH_4-COD/g VSS d and 0 mg CH_4-COD/g VSS d, respectively, in $UASB_{cont-semi-cont}$; indicating that the stress of the semi-continuous feeding (starvation/feast cycle) in phase II caused an adverse impact on the microbial activity in the sludge over time.

Interestingly, in phase III, $UASB_{cont-semi-alt}$ and $UASB_{cont-semi-cont}$ exhibited much higher hydrogenotrophic methanogen activities of 3,540 ± 382 mg CH_4-COD/g VSS d and 3,685 ± 54 mg CH_4-COD/g VSS d, respectively, compared with $UASB_{cont}$ (1478 ± 16 mg CH_4-COD/g VSS d) ($P<0.05$). It appears that semi-continuous feeding induces significantly higher hydrogenotrophic metabolism but has a less positive impact on acetoclastic metabolism. Rodriguez et al. (2009) reported that in an energy-limited anaerobic ecosystem, environmental conditions can be used to select microbes that provide the most energy for growth. The feasting/starvation feeding in phase II might have improved the selection of highly active microbes and eliminated relatively vulnerable microbes.

The VSS concentrations in $UASB_{cont-semi-alt}$ and $UASB_{cont-semi-cont}$ dropped from 14.5 ± 1.4 g/L to 5.05 ± 0.3 g/L and from 13.3 ± 1.1 g/L to 4.2 ± 0.1 g/L, respectively, from phase I to phase III. Having a higher capacity along with a low sludge concentration may indicate the high efficiency of the metabolism pathway performed by the newly formed microbial community after the starvation/feast stress imposed in phase II.

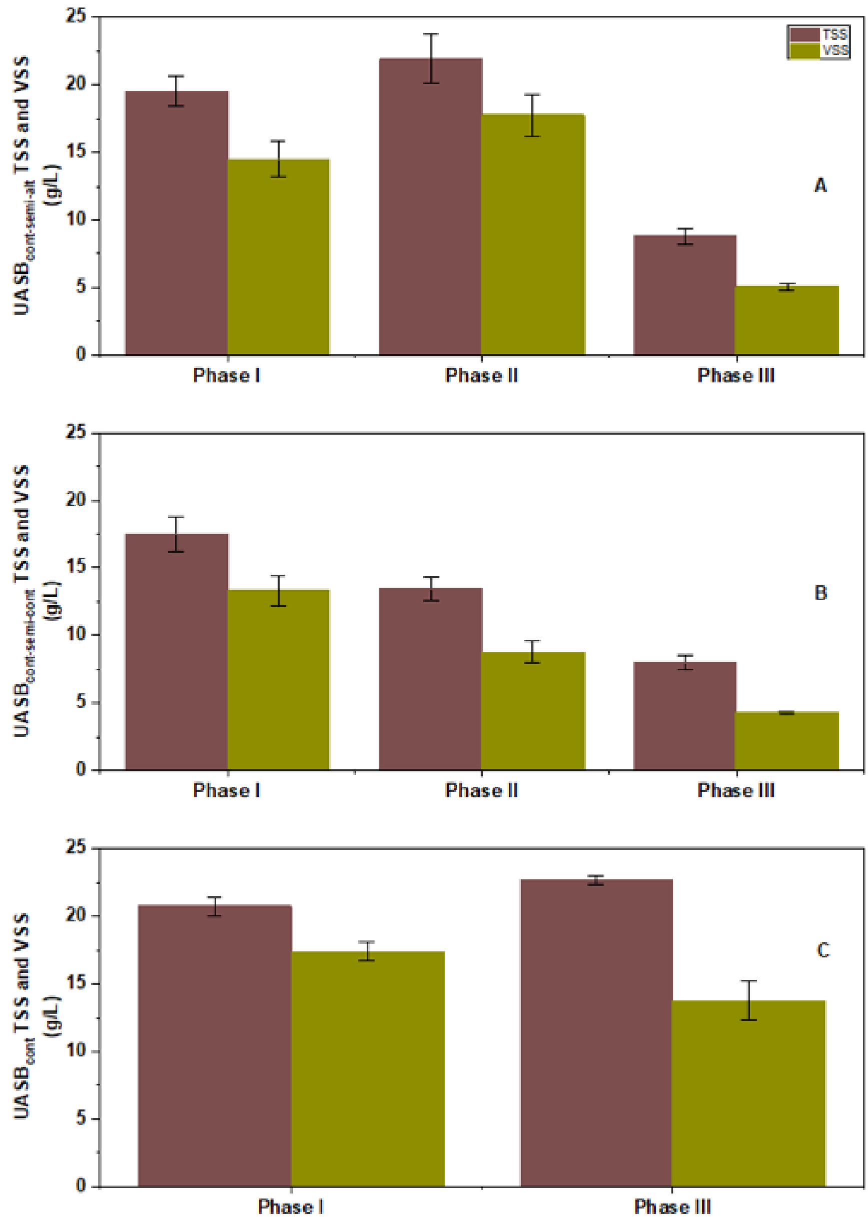


Fig. 9. Concentrations of TSS and VSS from Phase I to Phase III for reactors: (A) $UASB_{cont\text{-}semi\text{-}alt}$; (B) $UASB_{cont\text{-}semi\text{-}cont}$; (C) $UASB_{cont}$.

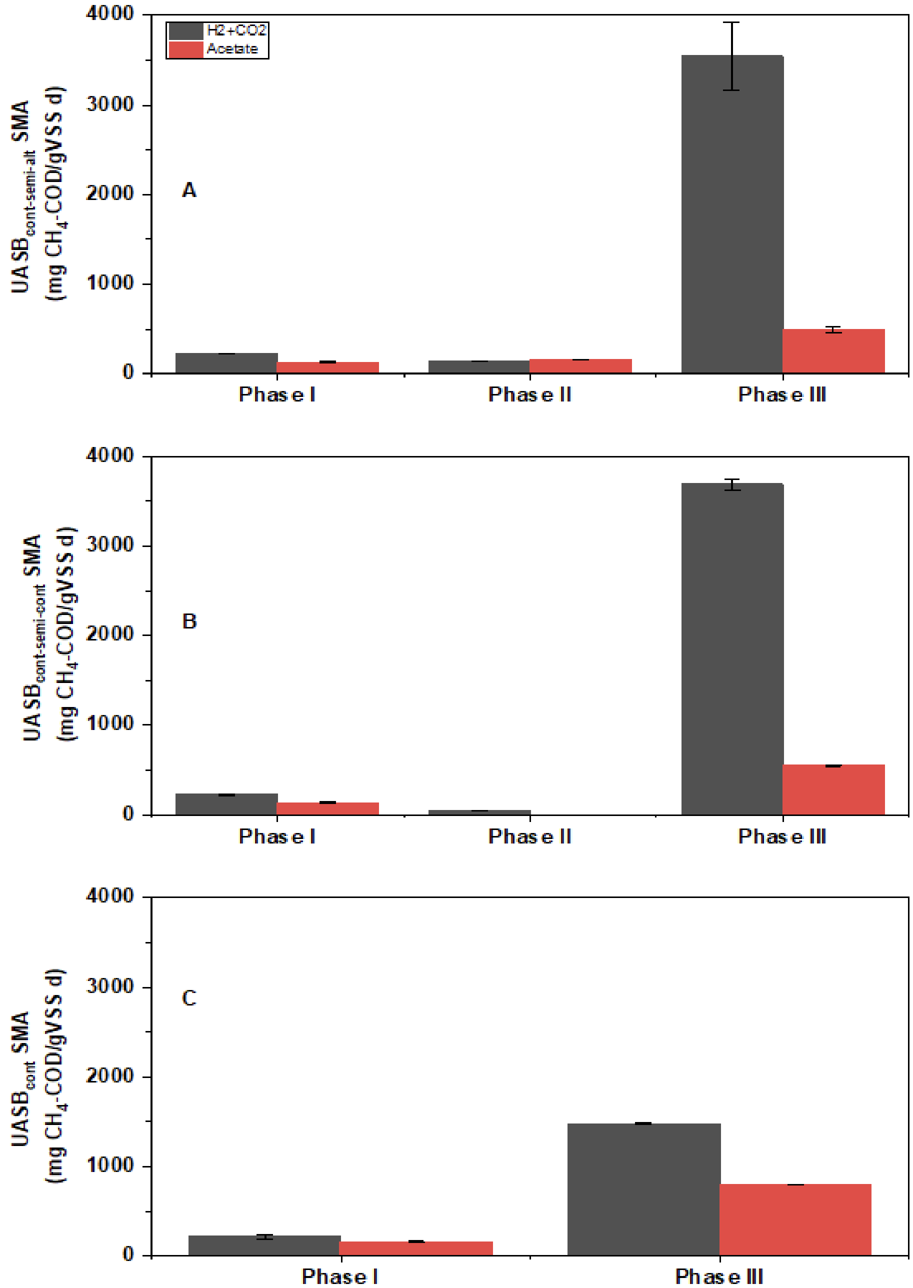


Fig. 10. Sludge SMA from Phase I to Phase III for reactors: (A) $UASB_{cont-semi-alt}$; (B) $UASB_{cont-semi-cont}$; (C) $UASB_{cont}$.

3.3. Microbial Community Analysis

3.3.1. Archaea

Archaeal communities (relative abundance > 1%) at the genus level are shown in Fig. 11. *Mehanosaeta* was found to predominate in phase I in all three reactors. In phase II, *Mehanosaeta* adapted with starvation/feast stress and maintained as the most and the second most dominant species in phase II in $UASB_{cont\text{-}semi\text{-}cont}$ and $UASB_{cont\text{-}semi\text{-}alt}$, respectively. *Methanobrevibacter* had a higher fraction in phase II (32.8%) in $UASB_{cont\text{-}semi\text{-}alt}$, however, its abundancy significantly decreased in phase III. This behaviour may suggest a stable environment is less favourable than a stressful environment to the growth of *Methanobrevibacter*. In phase III, the dominant archaea altered from *Mehanosaeta* to *Methanosarcina* in both $UASB_{cont\text{-}semi\text{-}alt}$ and $UASB_{cont\text{-}semi\text{-}cont}$. The same shift has been observed when a reactor is adapting to a new condition, such as an elevated organic loading rate (De Vrieze et al., 2012). The semi-continuous feeding mode in phase II introduced stress, inhibiting the metabolism of acetoclastic methanogens and suppressing the growth of *Methanosaeta*. Thus, the changes in feeding patterns from continuous to semi-continuous favoured the enrichment of hydrogenotrophic methanogens.

It should be noted that *Methanosarcina* can utilize both hydrogenotrophic and acetoclastic pathways. A high specific hydrogenotrophic methanogenic activity in phase III indicated that a hydrogenotrophic pathway was developed by *Methanosarcina* after the stress condition in phase II. On the other hand, *Methanosaeta* was the dominant archaea in $UASB_{cont}$ throughout the experiment. It has been reported that *Methanosaeta* was dominant at a low acetate concentration and *Methanosarcina* was dominant at a high acetate concentration (Conklin et al., 2006). However, without the semi-continuous feeding or a stress condition, the archaeal community may not shift from *Methanosaeta* to *Methanosarcina* even with a high acetate concentration in a long-term run, as shown by comparing UASB$_{cont}$ with UASB$_{cont-semi-alt}$ /UASB$_{cont-semi-cont}$.

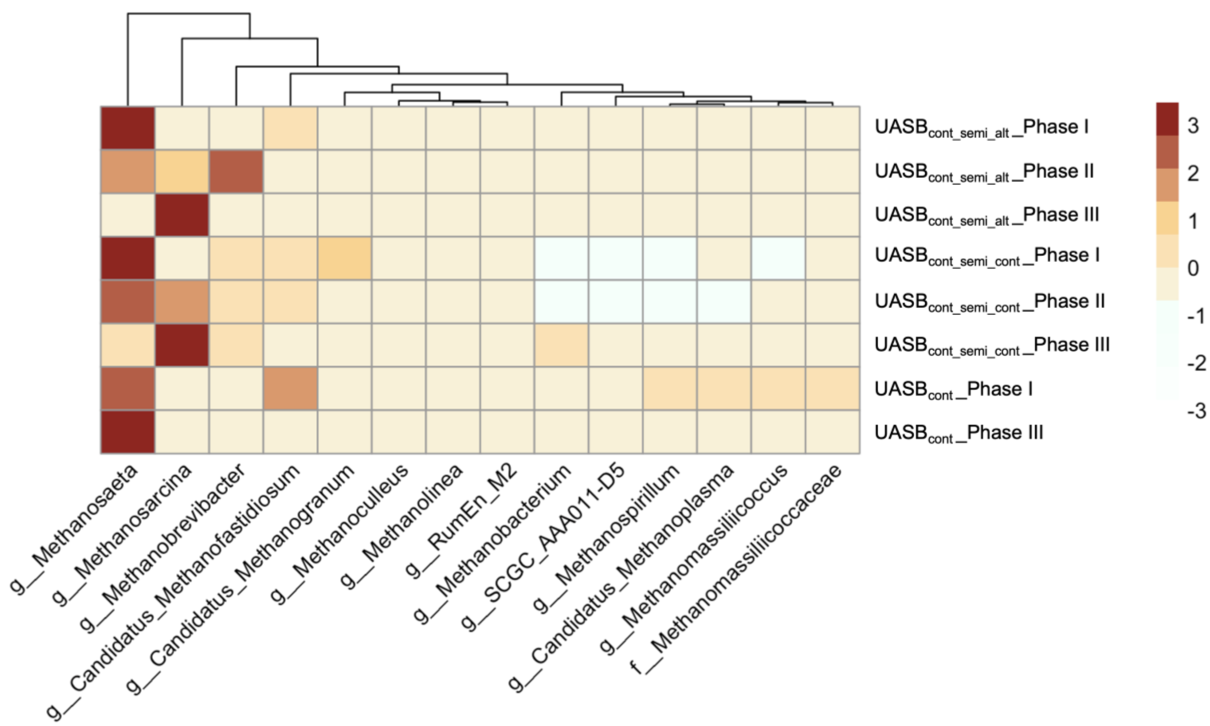


Fig. 11. Relative abundance of archaeal genera with abundances > 1% in UASB$_{cont-semi-alt}$, UASB$_{cont-semi-cont}$, and UASB$_{cont}$ at the end of each phase. Unidentified genera were named at family (f_).

3.3.2. Bacteria

The relative abundances of the 10 most abundant bacteria in all three reactors at the end of each phase are shown in Fig. 12 at the genus level. *Sporomusa* and an unclassified genus from the family *Anaerolineaceae* were predominant in UASB$_{cont-semi-alt}$ and UASB$_{cont-semi-cont}$ in the first two phases. *Anaerolineaceae*, an acetate-producing fermentative bacteria, has been reported to have strong synergistic interactions with *Methanosaeta* (McIlroy et al., 2017; Zamorano-Lopez et al., 2019), which also can be observed in $UASB_{cont}$ in phase III, the relative abundance of *Anaerolineaceae* reached 24.61%. The enriched population of the *Anaerolineaceae* in $UASB_{cont\text{-}semi\text{-}alt}$ and $UASB_{cont\text{-}semi\text{-}cont}$ suggests that this genus was sustained under various feeding patterns. However, the decreased abundance of *Methanosaeta* in phase III may have eventually led to the decreased *Anaerolineaceae* relative abundance in these two reactors. Meanwhile, the genus *Leucobacter* became dominant in phase III in $UASB_{cont\text{-}semi\text{-}alt}$ and $UASB_{cont\text{-}semi\text{-}cont}$. *Leucobacter* is a heterotrophic bacterium that belongs to the class *Actinobacteria*; it can live under both aerobic and anaerobic conditions (Nomoto et al., 2018). An increase in the phylum *Actinobacteria* was found to correlate with a shift from *Methanosaeta* to *Methanosarcina* (Jang et al., 2015), consistent with our observations in $UASB_{cont\text{-}semi\text{-}alt}$ and $UASB_{cont\text{-}semi\text{-}cont}$. Thus, a syntrophic relationship might exist between *Actinobacteria* and *Methanosarcina*. De Vrieze et al. (2013) found that semi-continuous feeding patterns can induce changes in bacterial evenness, dynamics, and diversity, providing them with higher functional stability in anaerobic digestions. The results presented in this study may indicate another possibility of establishing a robust microbial community structure by promoting the most efficient syntrophic metabolism through the manipulation of operational conditions.

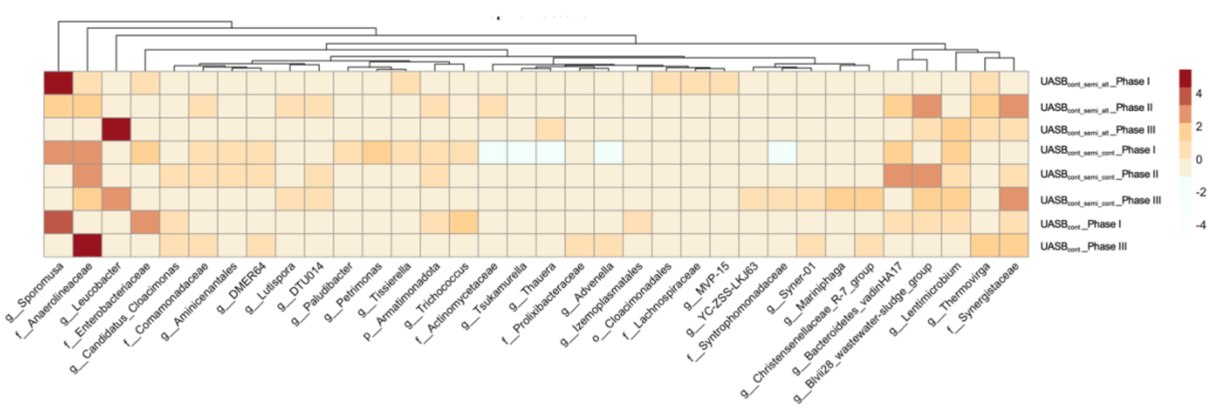
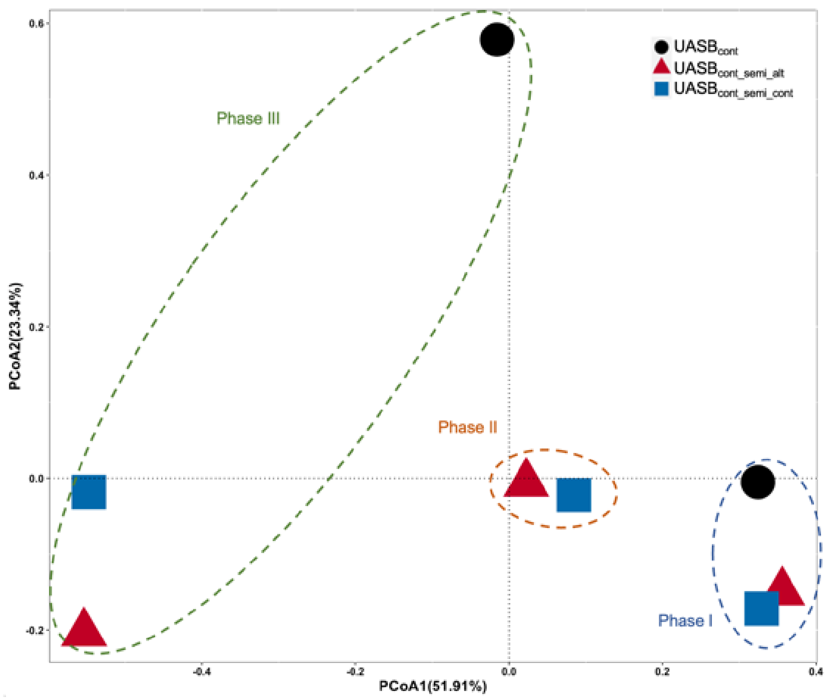


Fig. 12. Relative abundance of 10 most abundant bacteria in UASBcont-semi-alt, UASBcont-semi-cont, and UASBcont at the end of each phase. Unidentified genera were named at family (f_), or order level (o_).

3.4. Microbial community diversity and co-occurrence network analysis

Beta-diversity in the microbial community at the genus level was performed using Principal Coordinate Analysis (PCoA) with the distance calculated by the Bray-Curtis method (Fig. 13). Both the archaeal community (Fig. 13A) and the bacterial community (Fig. 13B) showed significant differences ($P<0.05$) between phases in $UASB_{cont-semi-alt}$ and $UASB_{cont-semi-cont}$. The differences between phases in $UASB_{cont}$ were not significant ($P>0.05$). This indicates that the feeding pattern played important roles in altering the microbial community; therefore, semi-continuous feeding or starvation/feast stress might play an important role in this process. The diversity of $UASB_{cont-semi-alt}$ and $UASB_{cont-semi-cont}$ are more similar to $UASB_{cont}$ in phase I than that in phase III; this is consistent with the enrichment of *Methanosarcina* and *Leucobacter* in phase III. As shown in Fig. 14, the phylum *Halobacterota*, which contains the genera *Methanosaeta*, *Methanosarcina*, *Methanolinea*, *Methanospirillum*, and *Methanoculleus*, exhibited a strong positive interconnection with the phylum *Actinobacteriota* (i.e., *Actinobacteria*). This result suggests that the syntrophic relationship between *Actinobacteria* and methanogens is important for efficient anaerobic digestion. The microbial communities in phase III were similar in $UASB_{cont-semi-alt}$ and $UASB_{cont-semi-cont}$ (Fig. 13), suggesting that an alternating high/low feeding pattern can maintain a stable microbial composition and, consequently, maintain a stable reactor operation as a continuous feeding pattern.

(A)



(B)

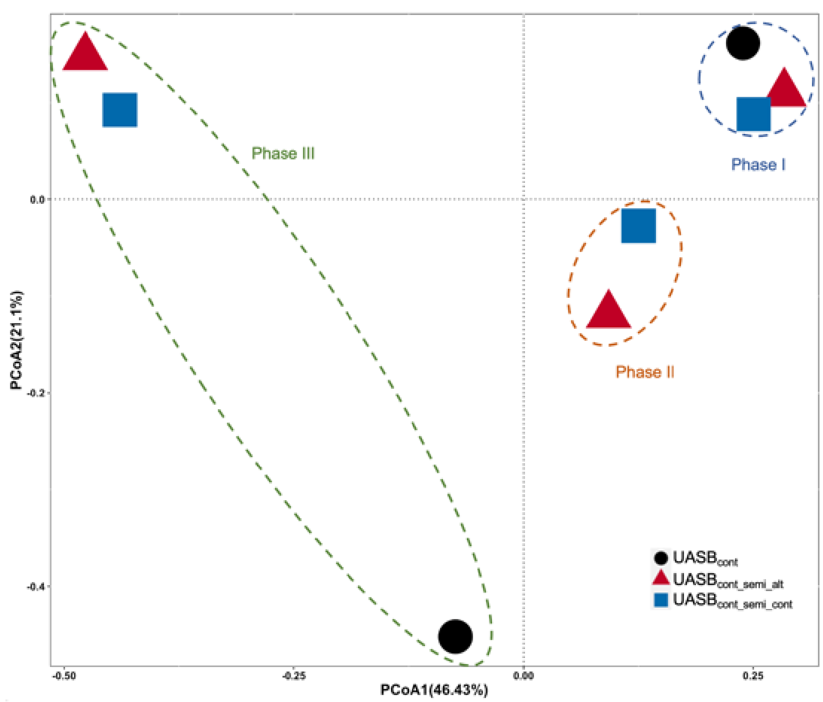


Fig. 13. Principal Coordinate Analysis (PCoA) of (A) archaea, (B) bacteria.

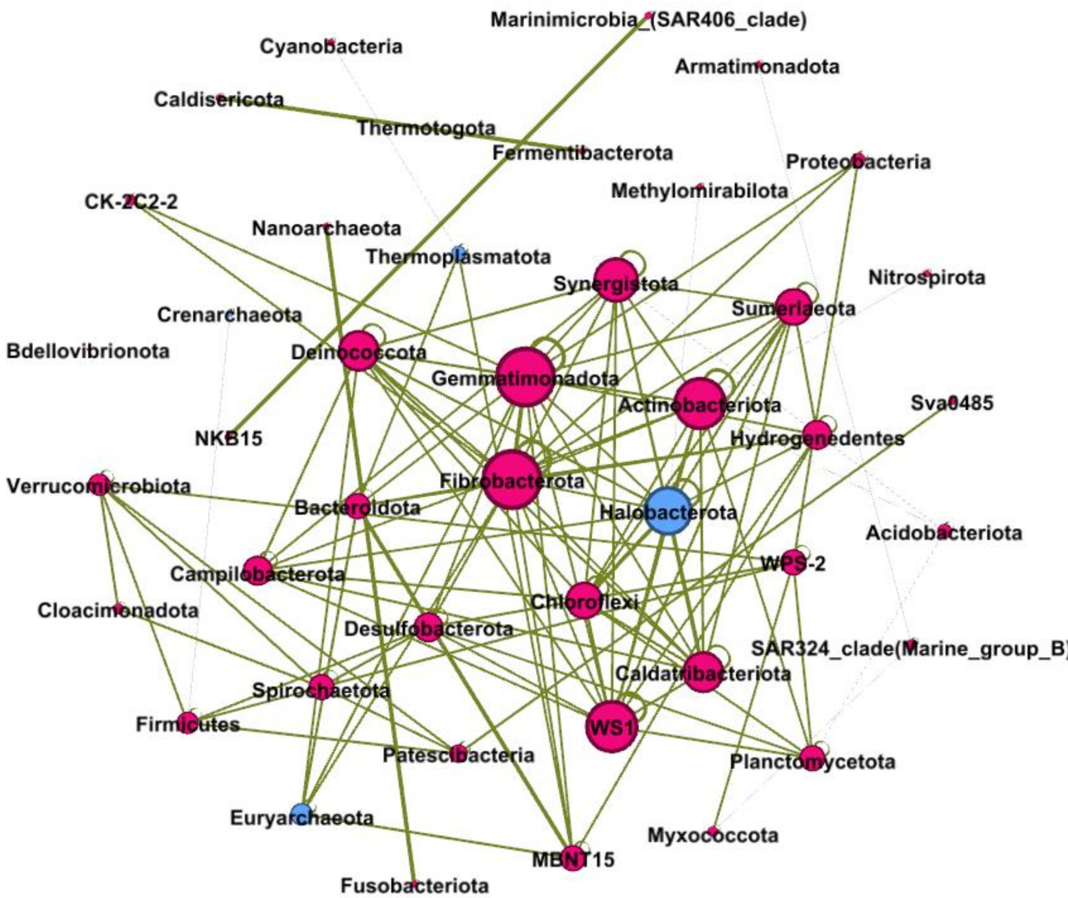


Fig. 14. Co-occurrence network analysis of phylum. Edges colour indicates positive interaction (green) and negative interaction (purple). Node colour refers to the domain: blue for Archaea and red for Bacteria

3.5 Correlations

Fig. 15 shows the correlation between putative functions, key bacteria/methanogens, and specific methanogenic activities. Four of the parameters including hydrogenotrophic methanogenesis, specific methanogenic activity for H_2 and CO_2, *Methanosarcina*, and *Leucobacter* were found to be significantly positively correlated. Given that higher methanogenic activities were observed in $UASB_{cont\text{-}semi\text{-}alt}$ and $UASB_{cont\text{-}semi\text{-}cont}$ than in $UASB_{cont}$ in phase III, and that microbial community analysis showed remarkably different key archaea and bacteria, it is likely that the cooperation between *Methanosarcina* and *Leucobacter* enhanced methane production in the hydrogenotrophic pathway. Acetate can be used to produce methane by (1) direct acetate degradation carried out by acetoclastic methanogens such as *Methanosaeta*, and (2) an indirect pathway where acetate is oxidized to hydrogen and carbon dioxide by syntrophic acetate oxidizing bacteria and the hydrogen is utilized by hydrogen-scavenging bacteria (Hattori, 2008). Stress conditions, such as high ammonium concentration (Hao et al., 2021), can cause a dominant metabolism of acetoclastic methanogenesis to change to syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis, the latter of which would contribute to an enhanced methane yield (Gao et al., 2019a). *Actinobacteria* (genus *Leucobacter*) is a potential acetate utilizing oxidizing bacteria (Wang et al., 2018a). The generation time of syntrophic acetate utilizing bacteria is relatively long; this could explain the lower sludge concentration in phase III (Westerholm et al., 2019). Thus, the alternation of the dominant metabolism pathway with hydrogenotrophic methanogenesis induced by semi-continuous feeding might enhance the methane-producing capacity in $UASB_{cont\text{-}semi\text{-}alt}$ and $UASB_{cont\text{-}semi\text{-}cont}$.

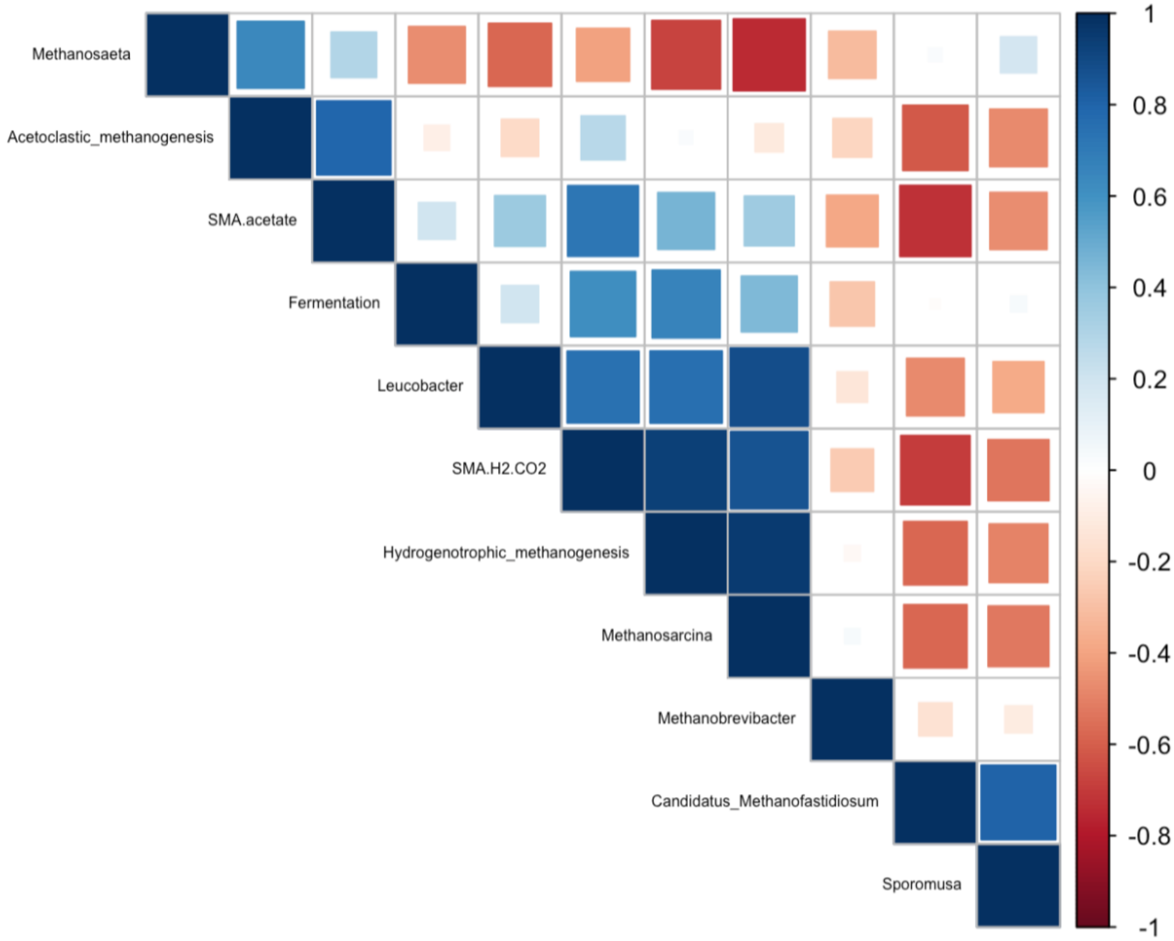


Fig. 15. The correlation of dominant bacteria and archaea with sludge activity and putative functions.

Chapter 5 - Conclusions and directions for future work

5.1 Conclusions

The aim of this study is to investigate the impact of UASB reactor feeding strategies on the anaerobic digestion of synthetic wastewater and methane yield. Three laboratory-scale UASB reactors were used to assess and compare three feeding strategies: continuous feeding, semi-continuous feeding (with starvation-feast cycles), and alternate feeding (with high loading-low loading cycles). A long-term starvation/feast stress provided by a semi-continuous feeding pattern eventually caused a deterioration of the reactor performance regardless of the frequency of feeding events. However, a shift in the microbial community was observed after the stress condition, which helped the selection of efficient microbiomes by re-establishing the metabolic pathway. The predominant *Methanosaeta* was replaced by *Methanosarcina* and the bacterial community showed a significantly increased *Leucobactor*. As a result of the microbial community changes induced by the application of a semi-continuous feeding mode, a significantly higher specific methanogenic activity was noticed. Interestingly, an alternating high/low feeding pattern successfully maintained a stable operation and produced a comparable methane yield with continuous feeding. Therefore, a new feeding strategy, alternating high/low feeding patterns is recommended if a flexible feeding regime is necessary when a continuous supply of feeding cannot be ensured. Brewery and dairy industries, for example, can maintain stable operations by reducing the pressure caused by the varied characteristics of industrial wastewater using the alternate feeding strategy.

5.2 Future work

This study solely focused on the three feeding strategies including continuous, semi-continuous and alternate feeding patterns on UASB reactors. However, future work should be conducted based on the current progress.

- Although the stress condition resulting from the semi-continuous feeding pattern promoted a significantly better performance and higher methanogenic activity compared with values at start-up, both reactors took a very long time to recover from the deterioration. Future studies can focus on the methods for speeding up the recovery process.
- The alternate high/low feeding pattern was successfully implemented on the UASB reactors, However, the application of this new feeding pattern on other types of reactors should be evaluated. In addition, a test on pilot-scale UASB reactors should be also considered in future studies.
- Synthetic wastewater is used to better understand the mechanism of driving the changes found in this study. In the future, real wastewater collected either from industries or municipalities should be used to simulate real situations.
- The changes in microbial communities observed in this study may induce higher functional stability when facing loading shocks. Thus, relative studies should be performed to investigate the impact of loading shocks on newly formed microbiomes.

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