Bio-methanation Of Fine Sieved Fraction Sequestered From Raw Municipal Sewage

Dara S.M. Ghasimi

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Proefschrift

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To my beloved father Seyed Jafar Ghasimi and mother Ameneh Farshgar…

پێشکەش بە ڕۆحی پاک و پەپوولە ئاسای دایک و باوکی ئازیزم...

ئەی تەمەنی پڕ ئەسرینم ئەڕۆی ئاوڕ نادەیتەوە

لەگەڵ هەمو هەنگاوێکا لە عومری من ئەسڕیتەوە

خۆزگە منیش وەك پەپوولە گشت تەمەنم وەرزێك بوایە

دوور لە خەم و تاڵی ژیان ، تەنیا گوڵم بناسیایە

ڕێگام سەودای تەنیاییە و کەسێك نایە بۆ سەردانم

ئێترچیبکەم لەم تەمەنە ، کە لە هەمووی پەشیمانم

لە گریانی منالیما پێکەنینم بە خەم ئەهات

کەچی ئێستا خەم دەریایەو بۆ کەنارم شەپۆل ئەدات

لە سەدجارا تەنیا جارێ زەردە خەنە دێتە میوان

وەك سێبەری لای ئێوارەی خۆرنشینی لای ئەرخەوان

ئەی هاورێیان چاوم نوقمی نیگارێکی پڕ بەهارە

تاسەم وەری، بەڵام هێشتا خۆشەویستیم هەر بێدارە

ئێستایش گەڵای عومری گەنجیم وەکوو پاییز هەڵوەریوە

کێ وەکوو من؛ تەم و خومار، پەنجەرەکەی داپۆشیوە

" تەمەن -هۆنراوە : ئەحمەد محەمەد"

A Kurdish poem entitled "Age" from Ahmad Mohammad.

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Chapter 1. Introduction

1.1 Part 1 - Energy use in municipal sewage treatment

The availability of clean fresh water and electric energy are indispensable for any modern society. In USA, water and wastewater treatment consumes about 35% of the total energy consumption of the municipal utility services (Cao, 2011; WERF, 2009a). Moreover, it has been estimated that municipal wastewater treatment plants (WWTPs) consume about 1-2% of the total electric energy in the United States (Stinson and Schroedel, 2009). The huge consumption of fossil fuel-based energy has its impact on the society's carbon footprint (CF) and greenhouse gas (GHG) emissions in the form of carbon dioxide (CO_2). The criticism in recent years on the non-sustainable energy use of traditional wastewater treatment is rising, leading to a call for a strategic paradigm shift of municipal wastewater treatment from solely waste removal and disposal to resource recovery, covering water, nutrients and energy (Cao, 2011;WERF, 2009b; STOWA, 2010).

Although municipal wastewater is negatively valued, it contains many different resources that could be recovered, contributing to improved sustainability in the water sector. One cubic meter of domestic wastewater is daily produced by 5-10 people and contains about 7.2 MJ equivalent of energy (or 2 kWh theoretical) , and sufficient nutrients for at least one square meter of agricultural production area per year (Jurg Keller, 2008). Yet, current conventional WWTPs based on activated sludge technology use fossil energy mainly to eliminate the chemical energy stored in the organic pollutants, as well as to eliminate the nutrients nitrogen and phosphorus. Estimations based on the concentration of organic pollutants in the raw wastewater indicate that only 18% of the influent bound chemical energy is needed to operate most conventional activated sludge type WWTPs (Cao, 2011). Some estimations even claim that the energy contained in wastewater and biosolids is up to 10 times the energy requirement for treatment (GWRC, 2008) and can potentially meet up to 12% of the electrical energy demand in the United States (Reinhardt, G., & Filmore, 2009). Nonetheless, in the UK, conventional technology allows already recovery of approximately 11% of the influent energy via electrical co-generation operating on methane gas produced by anaerobic digestion (AD) of the biosolids, which corresponds to about half the energy required for operation of the WWTP (Jonasson, 2009).

Along with the growth in environmental awareness, public perception, concerns on climate change, and expected rising oil prices on the long term, the increase in energy efficiency in municipal WWTPs has become increasingly important in recent years, especially in Europe and the United States. The efforts made are focused on two aspects:

(i) Savings in energy demand for aerators. Approximately 60% of the energy used at conventional WWTPs is currently needed for aeration (WERF, 2011). Therefore, significant energy savings can be made by optimised control and operation of the aerators and/or by the use of highly efficient aerators, such as fine bubble aerators.

(ii) Energy generation via bio-methanation of excess sewage sludge and biosolids. Most of the influent chemically bound energy is enclosed in the excess sludge. Research is focused on enhanced bio-methanation processes as well as increased electricity generation by applying more efficient combined heat and power (CHP) generators, or fuel-cells, and thermal technologies for biosolids treatment (Cao, 2011).

1.1.1 Energy recovery from sewage sludge

During the last 2 decades, developments in municipal wastewater treatment are mainly characterized by improving the quality of the effluent. Existing treatment plants were upgraded and new and more cost-effective treatment technologies, as Anammox and Nereda, have been developed and implemented (Rulkens, 2008; Rulkens and Bien, 2004). Parallel to the developments for improving the effluent quality, there is an increasing concern regarding excess sewage sludge production, which is related to the high costs for treatment and the potentials risks on the environment and human health (Rulkens, 2008). In The Netherlands, where the only outlet for excess sewage sludge is incineration, the costs for sewage sludge treatment are estimated at about 450 euro's per ton dewatered sludge, which is more than 50% of the total wastewater treatment costs (STOWA 2010-19, 2010). Considering the above, a strong need is observed to develop and apply more sustainable sludge management systems, which are primarily focused on the recovery of valuable products rather than on treating a waste product. Potential valuable products include energy in the form of organic carbon and inorganics as phosphates, silicates and aluminates. In addition, technology development is focused on the decrease in treatment costs in combination with the need to eliminate toxic pollutants (Englande and Reimers, 2001; Guibelin, 2004).

There are many sludge treatment and management options in which production of energy (heat, electricity, or biofuel) is one of the key treatment steps. The most important options are anaerobic digestion (AD) and (co-)incineration in combination with energy recovery. The energy efficiency of incineration and co-incineration strongly depends on the dewatering and drying steps. AD for biogas production is already applied for decades, whilst pre-hydrolysis techniques for additional energy recovery are gaining more interest in the last years. Sewage sludge is also frequently used as an energy source in the production of cement or building materials. Pyrolysis, gasification and supercritical (wet) oxidation are promising new techniques for direct energy production, however, still in the development or demonstration phase, and only a limited number of full-scale installations have been built. Anaerobic fermentation for production of hydrogen, acetone, butanol, or ethanol as alternative to biogas generation are in the research phase, but considered as upcoming methods (Rulkens, 2008). Direct generation of electrical energy by means of specific micro-organisms is investigated but not applied yet (Appels et al., 2011; Cao and Pawłowski, 2012; Rulkens, 2008; Rulkens and Bien, 2004; Tyagi and Lo, 2013).

1.1.2 Potential of increasing energy efficiency of WWTP

New policies have been promulgated mainly in Europe and United States to encourage and regulate the water industry to save energy and use renewable energy in municipal wastewater treatment processes (Cao, 2011). Europe is currently the global leader in energy recovery in municipal WWTPs most likely due to its land and resources constraints and strong environmental consciousness. About 63% of the WWTPs in UK employ AD with electricity generation (Jonasson, 2009), whereas in the United States less than 10% of the WWTPs use anaerobic digesters, of which about 19% of these WTTPs generate power from biogas and the other 81% of WWTPs just flare the biogas (WERF, 2009a). Dutch water authorities agreed to reduce their net energy consumption by 30% before 2020 leading to investments in energy efficiency and energy recovery at the Dutch WWTPs (Wade et al., 2011). Also the water industry in UK has agreed to a voluntary energy consumption reduction target: at least 20% of all energy used by the UK water industry should come from renewable sources by 2020 (UKWIR, 2009).

Many WWTPs in Europe have achieved up to 50% overall energy reduction (Jonasson, 2009) and there are many successful examples showing the enormous potential of increasing energy efficiency. For example, a 10 year period of energy auditing and benchmarking has led to a

reduction in energy consumption of 38% in Switzerland, 50% in 344 WWTPs in Germany and about 30% in Austria (Wett et al., 2007). Some cases show that a WWTP can be self-sufficient or even a net energy producer rather than being a consumer .

For a conventional activated sludge plant equipped with mesophilic digesters, which are characterised by 40% volatile solids (VS) destruction and an electricity generator with 35% conversion efficiency, about 20-50% of the required energy can be recovered from the excess sludge (Stinson and Schroedel, 2009; UKWIR, 2009). Applying pre-treatment of biosolids, or thermal digestion, or co-digestion of fat, oil, and grease (FOG), and effective energy saving processes, the energy recovery can increase to up to 80% of the energy demand, or even more, as illustrated by the central WWTP in Prague (Zabranska et al., 2009) and the Werdhölzli WWTP in Zurich. The Strass municipal sewage treatment plant in Austria even reaches an energy efficiency of 108% (Wett et al., 2007), meaning the electricity produced on-site is sufficient to operate the entire plant while an additional 8% of its generated energy is sent to the public grid for external use.

The percentage of energy self-sufficiency was steadily improved starting from 49% in 1996 to 108% in 2005 by many individual measures. A big step forward in energy production was the installation of a new 8 cylinder CHP unit (38% electrical efficiency), which provided 340 kW of power in 2001 (Wett et al., 2007). The most important strategies to reach a high level of energy efficiency include: (i) dynamic control of aeration; (ii) increased biogas production by maximizing the amount of COD that is sent to the anaerobic digester, e.g. by employing enhanced primary treatment or another pre-concentration process; (iii) adoption of high efficiency electricity generation; and (iv) reducing the aeration requirement by applying autotrophic nitrogen removal using Anammox-based technologies for treating sludge reject water (Wett, 2007; van Loosdrecht, 2008). Strategy (i) and (iv) reduce energy consumption and strategy (ii) and (iii) increase electricity generation. In all cases one should take care that suppressing aeration and thus increasing nitrite levels when N removal is pursued, is not inducing additional N_2O emission, a greenhouse gas 300 times stronger than CO_2 (Kampschreur et al., 2008).

1.1.3 Water environment research foundation (WERF) roadmap to sustainable wastewater treatment

The exploratory team of WERF focused on a new research plan for energy management to address the research gaps and information needs, aligned with the goal to transition to energy neutral wastewater treatment. In this research, simple energy balance depictions were used of conceptual wastewater treatment levels to illustrate the areas in the wastewater process and energy balance, which have the largest potential to contribute to energy neutral operations (WERF, 2011). Figure 1.1 shows a conventional WWTP, which is considered the reference or base line. Figure 1.2 depicts the current best practices, which are the next steps in the progression to energy neutral WWTPs. In the latter scenario, there are more efficient processes and units but also more tools need optimization. Figure 1.2 is consistent with the research goals and outcomes of the WERF's optimization challenge.

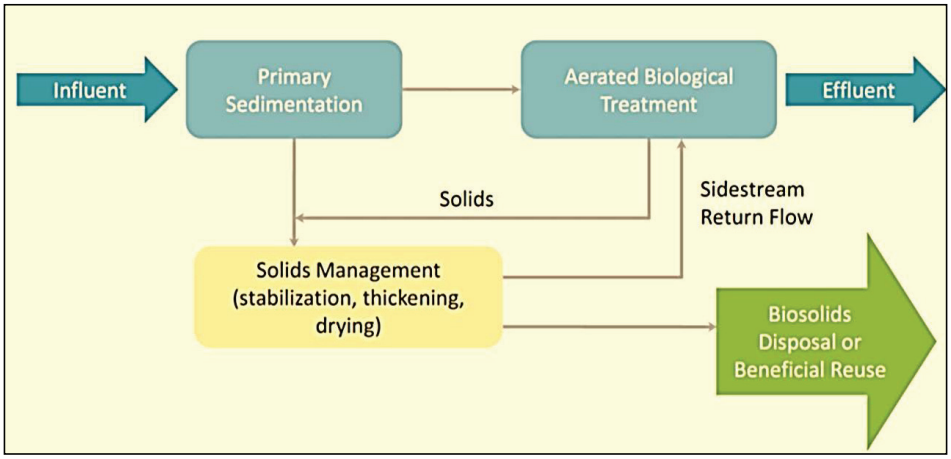

Figure 1.1 Conceptual conventional wastewater treatment. Adapted from (WERF, 2011)

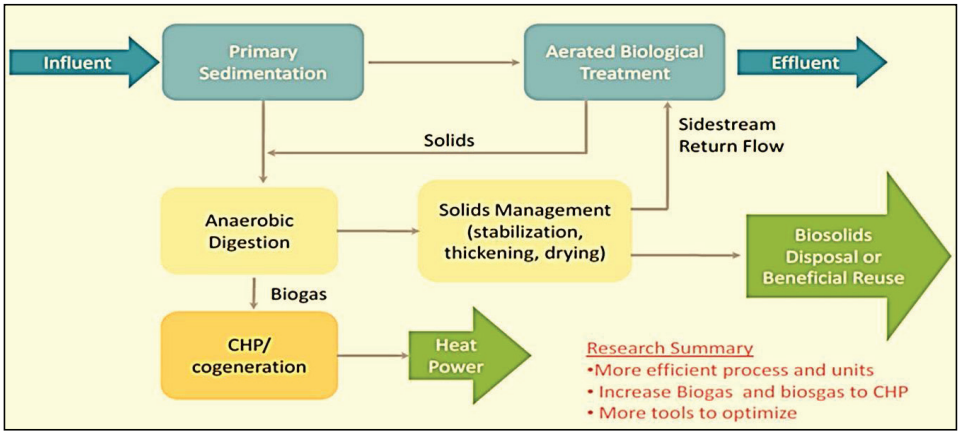

Figure 1.2 Conceptual diagram of wastewater, best practices for energy recovery (WERF, 2011)

In this work, certain research gaps emerged in critical areas to support the transition to energy neutral wastewater treatment facilities (WERF, 2011).The research needs are reflected in Figure 1.3 and include enhanced primary treatment, more co-digestion and more efficient CHP technologies as well as low energy side-stream treatment based on autotropic N removal, such as the Demon and Anammox nitrogen removal systems already operating at plants in Europe.

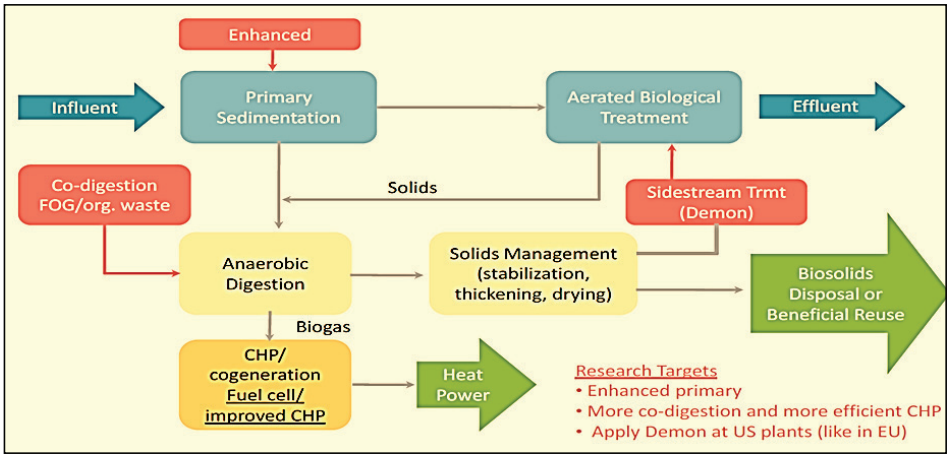

Figure 1.3 Conceptual diagram of wastewater processes with proven technologies for energy neutral wastewater treatment, presently demonstrated at few full-scale facilities. Adapted from (WERF, 2011)

The final step in the transition to energy neutral or energy producing WWTPs is to investigate the gaps that can lead to other energy recovery opportunities, such as heat pumps for low level

thermal energy recovery, solids pretreatment technologies to increase the energy recovery from wastewater residuals, fuel cell technology for electricity and heat production, energy recovery from residuals, and the development of low energy secondary treatment and other emerging processes, as illustrated in Figure 1.4.

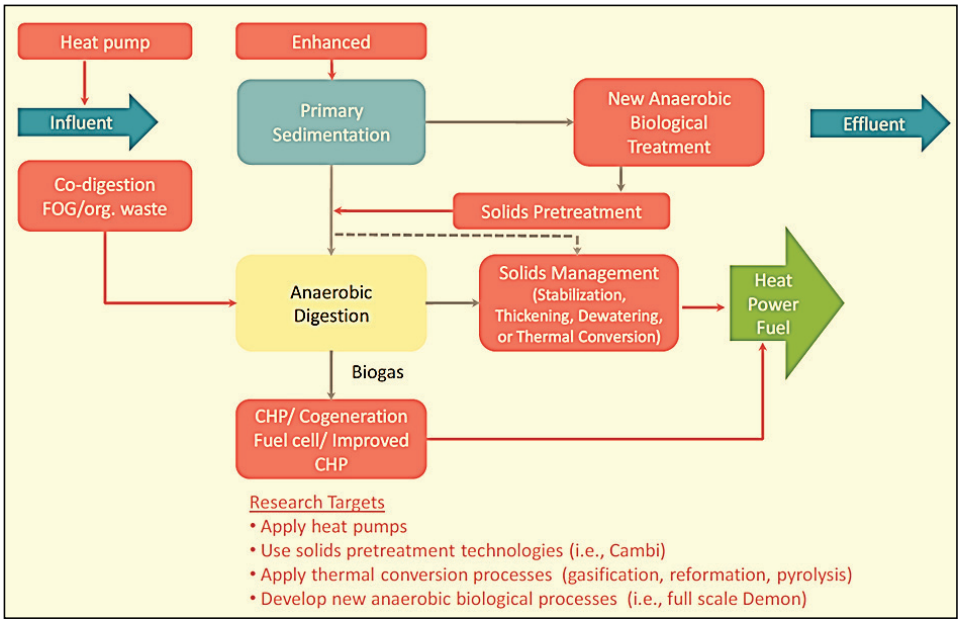

Figure 1.4 Conceptual energy neutral wastewater treatment with research gaps for energy neutral treatment. Adapted from (WERF, 2011)

1.1.4 Dutch roadmap for the WWTP of 2030

In 2008, the global water research coalition (GWRC) took the initiative to reflect on the future of municipal wastewater treatment, consisting of collection, transport and treatment, aiming at energy neutrality. Next to this, the global impact of the economic crises, the energy crises and the climate changes, caused countries to re-think their energy use and emission of greenhouse gases. Within the context of this global research program the Dutch road map for the WWTP of 2030 was initiated. In The Netherlands, the water sector has set limits to energy use and has proposed various large projects on energy efficiency in wastewater treatment facilities (STOWA, 2010). The main objective of the Dutch program was to elaborate and design the outlines of the municipal WWTP of 2030, focusing on three resources: water, energy and nutrient recovery.

In general, municipal wastewater treatment can be schematized into six process steps (Figure 1.5). For each process step different techniques are either applied, newly available, or under development. It is to be expected that before 2030 current techniques become abandoned, while newly available techniques become operational, and new techniques are developed. Adjustment of the different process steps with focus on water, energy and nutrient recovery can lead to a more sustainable sewage treatment plant. For instance, having the focus on energy recovery, the most important gains will be derived from i) adjustments in the pre-treatment, such as separation of fine sieved fractions or enhanced primary sludge separation, ii) changes in conventional (biological) treatment, such as reduced aeration, and implementation of autotrophic N removal (Anammox) or fast heterotrophic growth for biosolids production, and iii) sludge (pre)treatment and energy conversion. The latter will be discussed in further details in Chapter 4.

Focusing on water recovery, emphasis should be put on the post treatment of effluent in order to achieve high quality water for reuse, e.g. applying enhanced filtration processes using high rate filters and membranes as well as chemical adsorption or oxidative processes. Considering nutrient recovery, optimized pre-treatment combined with sludge reject water treatment should be considered, e.g. including struvite precipitation or other N and P recovery techniques, as well as changes in the basic treatment steps in the water line.

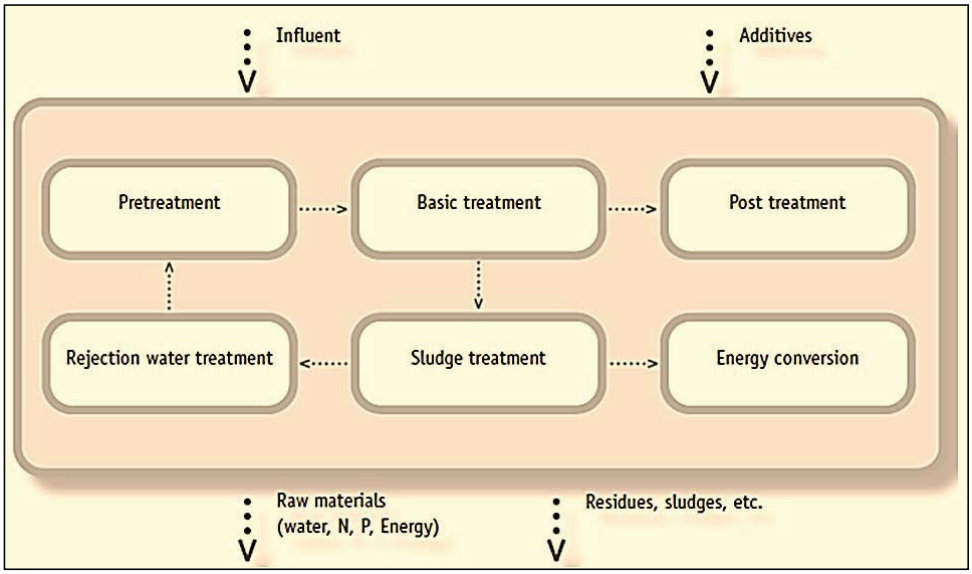

Figure 1.5 Scheme of various process steps of a WWTP, adapted from (STOWA, 2010)

1.1.5 Energy recovery

Traditionally, energy consumption was considered a given fact in wastewater treatment. However, energy is becoming more and more a spearhead for new developments, illustrated by the fact that all 22 Water Authorities in The Netherlands currently cooperate in transforming sewage treatment plants into 'Energy Factories', i.e. energy neutral or energy producing WWTPs. For achieving energy neutrality, minimization of energy consumption of the plant itself is required, followed by maximization of its energy recovery. An important characteristic of the "Energy Factory" is that COD is upfront concentrated instead of aerobically degraded, which will lead to less aeration energy consumption and increased biogas production in the digester (Figure 1.6). Another characteristic, in terms of energy optimisation, is the removal of N via autotrophic conversion routes, such as Anammox in the sludge reject water line or even in the main process line. Finally, enhanced or alternative techniques for converting COD to useful energy are considered, maximising energy recovery from pollutants. It was noted that supercritical gasification is not yet applied on a technical scale in The Netherlands (STOWA, 2010).

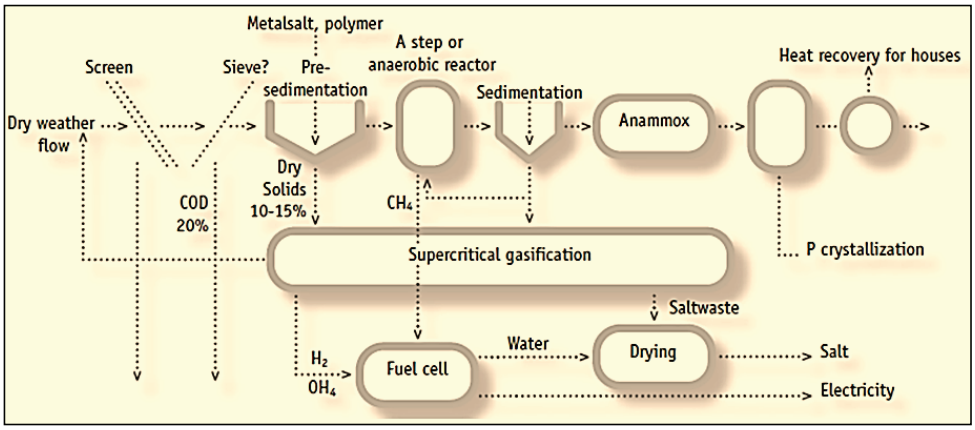

Figure 1.6 Building blocks of the Energy Factory, schematically presented by expert group (STOWA, 2010)

1.2 Part 2 - The role of fine sieves in the WWTP 2030

Toilet paper or toilet tissue is one of the most used hygiene products in industrialized countries, whereas it is less used in India and large parts of Asia and Africa (http://www.worldwatch.org/node/5142, Accessed on 22 December 2015).The major component of all hygienic papers is toilet tissue, which is the biggest single product made from cellulose-based tissue. Toilet paper should be smooth and can be embossed, unprinted or patterned, tinted, purely white or off-white (Holik, 2006), soft or more rigid as very cheap toilet papers might lack softness. Figure 1.7 shows the tissue consumption in 16 European countries for the period between 2009-2013. Germany, UK, France, Italy and Spain are the top five countries that are using most of the tissue papers.

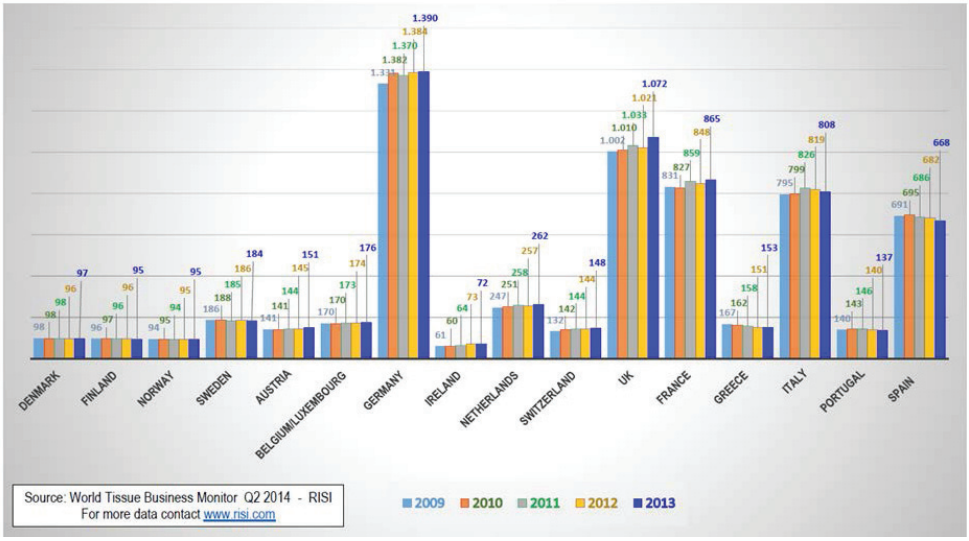

Figure 1.7 Tissue consumption in 16 European countries for the period between 2009-2013 (adapted from www.risi.com)

Cellulose makes up about 30-50% of the suspended solids (SS) in the sewage of western countries, mainly originating from the use of toilet paper which is estimated to be 10-14 kg per person per year (STOWA 2010-19, 2010). This material can enter the aerobic sewage treatment plant, adding significant costs to sewage treatment due to energy input for aerobic degradation and incineration costs of the non-degraded fibers that end up in wet waste sludge after digestion. To degrade all the cellulose aerobically, retention times of 30 to 40 days in an aerated basin are required (Breuer, 2009). Consequently, incomplete cellulose degradation (30 to 70%) occurs in conventional aerobic wastewater treatments where normally sludge

retention times between 10 to 15 days are applied, when denitrification is required (Metcalf & Eddy, 2003). Most WWTPs apply primary clarifiers to partly separate SS before aerobic treatment. The resulted primary sludge, with a dry solids (DS) content of about 5% after a thickening step, is in most cases anaerobically treated together with the secondary sludge to produce renewable energy. However, the cellulose fiber has poor settling properties and, therefore, the cellulose separation efficiency of a primary clarifier is relatively low, maximally up to 50% (STOWA 2010-19, 2010).

1.2.1 Norway

The Norwegian State Pollution Control Agency (SFT) took an initiative to evaluate and test several different technologies for primary treatment (Rusten and Ødegaard, 2006). In Norway, pre-treatment is the only treatment for small wastewater flows in the low populated areas. The goal of this study was to find reliable and cost efficient technologies that agree with the stringent EU criteria for sewage treatment. Most primary clarifiers only fulfil EU requirements if converted to enhanced primary treatment plants by adding chemicals (Rusten and Ødegaard, 2006). Primary treatment plants are required to remove at least 20% of organic matter (measured as biochemical oxygen demand (BOD_5)) and 50% SS. For this purpose, several types of sieves, such as rotating drum sieves, rotating disc sieves and rotating belt sieves were initially evaluated, as well as large septic tanks, clarifiers, dissolved air flotation (DAF) and deep bed filtration (Rusten and Ødegaard, 2006). Of the technologies that were considered fully developed, clarifiers and different types of fine mesh sieves were found most suitable for primary treatment. These technologies have been tested at full scale with sieving rates ranging from 20 $m^3/(m^2.h)$ to about 300 $m^3/(m^2.h)$ depending on wastewater characteristics and required removal efficiencies for both primary treatment and chemically enhanced primary treatment (Rusten and Ødegaard, 2006).

Results of the experiments showed that only rotating belt sieves fulfilled the EU treatment requirements (Rusten and Ødegaard, 2006), reaching 90% and 80% average removal efficiency for SS and BOD5, respectively. These results were obtained by operating the sieves with a thick filter cake on a rotating belt sieve with a mesh size of 350 microns at a sieve rate of only 25 $m^3/(m^2.h)$ (Rusten and Ødegaard, 2006). A cost comparison of primary treatment including sludge dewatering was carried out for rotating belt sieves and clarifiers. A dry weather flow of 200 m^3/h and an influent concentration of 250 mg SS/L were used.

The maximum wet weather flow was set at 400 m^3/h. The sieve rate was 100 $m^3/(m^2.h)$ at an average dry weather flow and 200 $m^3/(m^2.h)$ at the maximum dry weather flow. The costs of land was set at zero and the clarifiers were not covered. A 7% annual interest rate and 15 years depreciation was used to calculate annual capital costs. Both investment costs and total annual costs (annual capital costs plus operation and maintenance costs) for the rotating belt sieves turned out to be about 50% of the costs for the primary clarifiers (Rusten and Ødegaard, 2006).

1.2.2 The Netherlands

Waternet, the watercycle company of Amsterdam and surrounding areas, investigates the applicability of fine sieves on influent water since 2008. Waternet aims to elucidate the share of toilet paper in the influent, and to determine if fine sieves are feasible replacements for primary clarifiers. They are also evaluating the possible impact of fine sieves on the subsequent biological treatment process, e.g. whether or not the reduction of hairs and fibers in the influent reduces clutter formation (STOWA 2010-19, 2010). Ruiken et al.(2013) suggested the use of a fine sieve (mesh size 350 μm) instead of conventional primary clarifiers to separate SS from sewage before entering the biological treatment. Poor settling particle capturing was shown to be a major advantage of sieving. The efficiency of suspended solid removal was found to be comparable with a primary clarifier (up to 50% removal).

At the WWTP Blaricum, The Netherlands, a fine sieve (Salsnes Filter, Norway, mesh size of 350 μm) for raw sewage pretreatment was installed after the coarse screen (6 mm). This sieve is implemented as a compact alternative to primary clarification. The cake layer produced contains mainly cellulose originating from toilet paper and thus comprises a high cellulose fraction (Ruiken et al., 2013). This heterogeneous material is called fine sieved fraction (FSF) and can be used as a resource for further processing. Based on thermographic measurements, the cellulose fraction found in the FSF was 79% of the total mass and 84% of the organic mass; the inorganic matter fraction was 6%. In comparison, the cellulose fraction of primary sludge only reaches a maximum of 32-38% of the organic mass (Ruiken et al., 2013). Moreover, the total solids content in the FSF is higher than that of primary sludge, i.e. 20 to 30% (as shown in Chapter 2) compared to 4-12% of primary sludge (Tchobanoglous et al., 2003), resulting in a lower excess sludge flow.

The FSF can be used as fiber additive in several processes where nowadays recycled paper is used; however, the origin of these fibers hampers the opportunities (STOWA and Grondstoffenfabriek, 2013). A more straightforward method to valorise the FSF on site is by (dry) anaerobic digestion as discussed further in chapter 4. The produced bioenergy (methane) can contribute to the goal of realising an energy neutral or energy producing sewage treatment plant (STOWA, 2010). The different processing routes of FSF for energy recovery are described below.

1.2.3 Thermal conversion of FSF

The potential utilization of the FSF (with moisture content of ~70%) was studied by ECN, the Energy research Centre of The Netherlands. The lower heating value (LHV) and higher heating value (HHV) of the sludge was found to be 3.4 MJ/kg DS and 17.5MJ/kg DS respectively (ECN, 2009). After pressing (50% moisture) and drying (15% Moisture) the LHV is around 13.7MJ/kg DS, and thus this material can be used as an energy source through thermal conversion (ECN, 2009). Based on this study, STOWA has conducted a feasibility study of the thermal conversion of the sieving fraction as compared to the current applied scenarios in the WWTPs (no fine sieving). The comparison criteria were capital exploitation costs (CAPEX) or investment payback time, and overall energy scenario. The CAPEX analysis revealed that the investments for a pre-clarifier and sieve are comparable. The energy scenario study shows that up to 55% reduction in energy consumption of the existing WWTP can be achieved by substituting the pre-clarifier by fine sieves (STOWA 2010-19, 2010).

1.3 Anaerobic digestion (AD)

AD consists of a series of microbial processes that convert organic materials to methane and carbon dioxide in the absence of oxygen. It can take place under psychrophilic (10-20°C), mesophilic (25-40°C) or thermophilic (50-60°C) conditions, where biodegradation under mesophilic conditions is most common (Gujer and Zehnder, 1983; Speece, 1983). The valorization of the produced biogas, consisting of about 65% CH_4, 35% CO_2 and trace gases such as H_2S, H_2 and N_2, is energy efficient and environmentally friendly because of the low emission of hazardous pollutants (Appels et al., 2011). For conventional mesophilic AD of primary sludge, the total suspended solids (TSS) destruction ratio lies between 45 and 50%. The gas mixture has a heating value of approximately 20.5 MJ/m^3, which is about 60% of the heating value of natural gas (EPA&NREL, 1995). Thermophilic digestion increases volatile suspended solids (VSS) destruction (Metcalf & Eddy, 2003) and biogas production by more than 25% compared to the mesophilic digestion at a set digestion time of about 20-25 days

(Zabranska et al., 2009). Thermophilic processes are also more efficient in destroying pathogens.

AD is a robust process and its application for the treatment of organic waste has been emerging spectacularly with an annual growth rate of 25% during past years (Appels et al., 2011). Its main beneficial properties include (i) its ability to treat high moisture containing biomass, (ii) a very easy conversion of biomass into biogas, which can be incinerated/used with a very limited generation of pollutants, and (iii) its robustness and applicability on small scale (Appels et al., 2011). Various types of biomass and wastes are suitable for AD, such as solid wastes, slurries, industrial and domestic wastes, whereas co-digestion often leads to superior digestion efficiencies (Forster-Carneiro et al., 2007; Hartmann and Ahring, 2005; Mata-Alvarez, 2003). Although AD is a mature and widely well-applied technology, the digestion mechanisms of heterogeneous wastes is not yet completely understood because of the high complexity of the process (De Baere, 2006).

The AD process can be divided into four major microbial steps, i.e. hydrolysis, acidogenesis, acetogenesis, and methanogenesis, as shown in Figure 1.8 (Gujer and Zehnder, 1983). Hydrolysis is the first AD step and is performed by membrane bound and extracellular enzymes, which are produced by hydrolytic microbes. Hydrolytic enzymes decompose complex organic polymers to simple soluble monomers. Proteins, lipids, and carbohydrates are hydrolyzed to amino acids, long-chain fatty acids, and sugars, respectively. These small molecules are then converted by fermentative bacteria (acidogens) to a mixture of volatile fatty acids (VFAs), such as acetic, propionic, formic, lactic, and butyric acids, as well as to other products such as ethanol, carbon dioxide and hydrogen. Acetogenic bacteria further convert the VFAs to acetate, carbon dioxide, and/or hydrogen, being the precursors for methanogenesis, the last step of the AD process (Gujer and Zehnder, 1983).

In AD, the acid forming and the methane forming microorganisms differ widely in terms of biochemical composition, physiology, nutritional needs, growth kinetics and sensitivity to environmental conditions (Demirel and Yenigün, 2002).Failure to maintain the balance between these two groups of microorganisms is often the primary cause of reactor instability (Demirel and Yenigün, 2002; van Lier et al., 2008). In addition, inhibitory substances, which are sometimes present at considerable concentrations in wastewaters and sludges, may result

in reactor perturbation, reactor upset and/or complete failure. Inhibition is usually accompanied by a decrease in the methane gas production rate and the accumulation of organic acids (Kroeker et al., 1979).

As AD is a biological process, it is strongly influenced by environmental factors. Temperature, pH, acidity and alkalinity, carbon to nitrogen ratio (C/N) and toxicity are primary control factors (Ahring et al., 1995; Appels et al., 2008; de Mes et al., 2003; Kaparaju and Rintala, 2005; Killilea et al., 2000; Lopes et al., 2004). A wide variety of substances have been reported to be inhibitory to the AD processes. A material may be judged inhibitory when it affects bacterial growth, supresses the catabolic conversion, and/or causes an adverse shift in the microbial population (Speece, 1983).

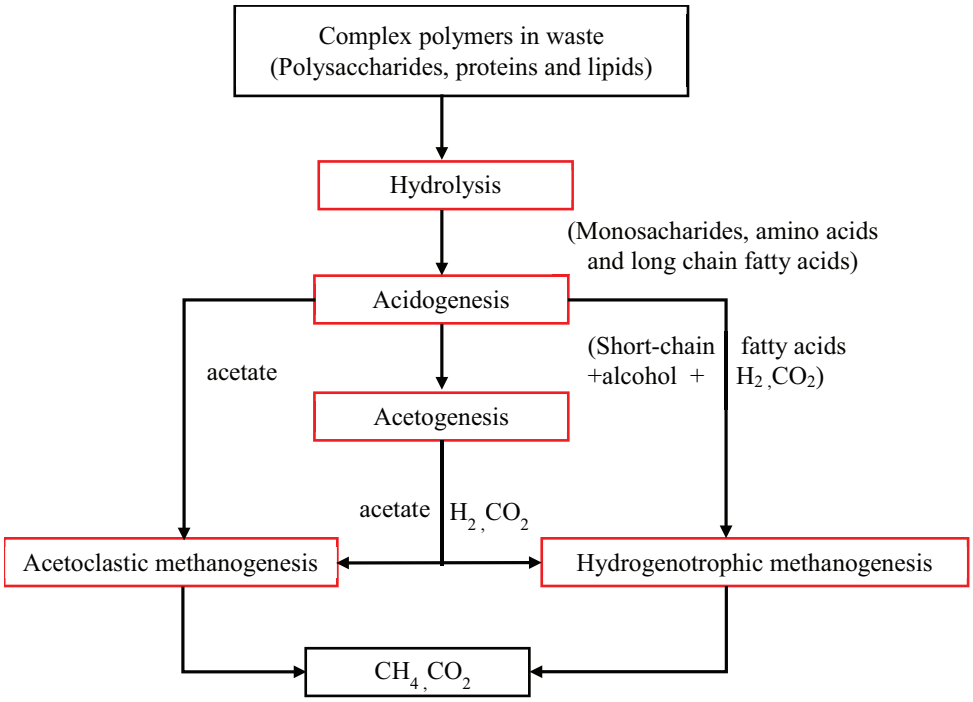

Figure 1.8 Schematic representation of the main conversion processes in AD, adapted from (Gujer and Zehnder, 1983)

1.3.1 Systems for the anaerobic treatment of solid waste and slurries

Systems used to digest solid waste are classified according to the percentage of total solids (TS) in the waste stream (de Mes et al., 2003):

- 15-25% low solids AD: wet fermentation;
- >30% high solids AD: dry fermentation.

In the year 2010, there were more than 200 full scale anaerobic waste treatment plants in Europe that handle an amount of 6,000,000 ton municipal solid waste (MSW) per year (De Baere and Mattheeuws, 2010). Of these plants about 63% are handling dry material. About 32% of the installed total amount of MSW reactors is operated under thermophilic conditions. Only 5% of the installed reactors are two-phase systems (De Baere and Mattheeuws, 2010; De Baere, 2000).

1.3.2 Wet versus dry digestion

The choice of dry or wet digestion generally depends on the solids concentration of the waste. Wet systems are mainly used for more dilute wet slurries such as pig or cow manure, but also for the co-digestion of MSW with these streams. The main advantages of dry over wet digestion are the limited required reactor space, the limited required amount of heating energy, and generally no additional treatment system is required for treating the sludge reject water. A drawback is that more robust equipment is required, such as pumps and mixers. This results in comparable investment cost for building a wet or dry digester when treating similar organic loads (Mata-Alvarez, 2003).

1.3.3 Anaerobic solid waste digesters

For wet digestion, completely stirred tank reactor (CSTR) systems are generally applied. In a CSTR, the substrate and biomass is ideally mixed, preventing short-circuiting and the possible decrease in active volume by preventing sedimentation of more heavy particles creating dead zones. Often a pre-treatment step is needed to avoid large particles from entering the CSTR (Monnet, 2003). Removing the large fraction can be a challenging task particularly for mechanical-sorted organic fraction of municipal solid waste (OFMSW). Such pre-treatment may result in a loss of 15 to 25 % of volatile solids and thus a decreased biogas yield (Monnet, 2003).

Dry digesters are hampered by a more problematic transportation of the substrate into the reactors. Feed inlet is performed by conveyor belts, screws and powerful pumps. Plug-flow

reactors are mostly applied for single stage dry digestion in order to prevent short-circuiting and to handle the high viscous flows. Examples of single-stage dry digesters are the continuously fed DRANCO (DRy ANaerobic COmposting), Kompogas, Linde-KCA, and Valorga reactors (Fig.1.9) and the batch-fed BIOCEL reactors (Fig.1.13).

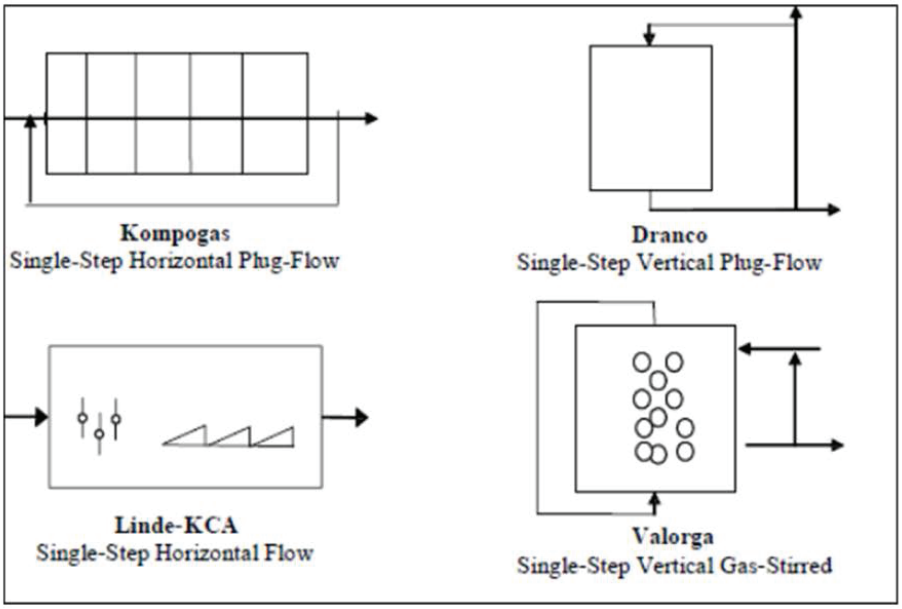

Figure 1.9 Schematics of commercial single stage dry solid digesters. Adopted from **(**Beck**,** 2004**)**

The DRANCO reactor, marketed by organic waste systems (OWS) of Belgium, is a vertical top to bottom plug flow system, developed in Ghent, Belgium, that relies on a recycle flow of a large proportion of the outgoing digestate to inoculate the incoming raw feedstock and, thereby, achieving good mixing (Fig.1.10). The process can be operated under both mesophilic and thermophilic conditions (De Baere and Mattheeuws, 2010).

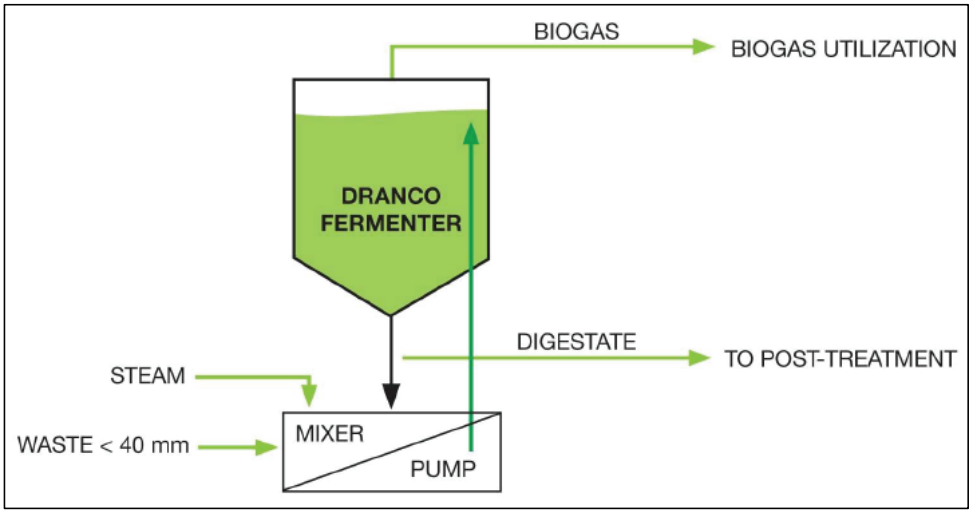

Figure 1.10 Basic Dranco process scheme adapted from (De Baere and Mattheeuws, 2010)

The reactor is designed to handle particles below 40 mm. Before entering the reactor the substrate is heated with steam and mixed with recycled digestate. The substrate/inoculum mixture is fed to the system at the top of the reactor. In 2 to 4 days the substrate will reach the bottom of the reactor by the force of gravity only. At the bottom the larger part will be recycled again, resulting in a retention time of around 20 days (De Baere and Mattheeuws, 2010). The DRANCO plant in Brecht, Belgium (12,000 t/y), treating the OFMSW, stably operates at an organic loading rate (OLR) of 15 kg VS/m^3.d, under thermophilic conditions, without any signs of inhibition. Input TS concentration is 35% with a retention time of 15 days and up to 65% VS destruction (De Baere, 2000). A gas production rate of up to 10 m^3/m^3.d achievable (De Baere and Mattheeuws, 2010). Other full-scale Dranco plants include Bassum, Germany (13,500 t/y), Kaiserslautern, Germany (20,000 t/y), and Salzburg, Austria (20,000 t/y) (De Baere, 2000).

The KOMPOGAS digester (Figure 1.11) is a horizontal plug-flow digester, operating under thermophilic conditions, developed in Switzerland (Kothari et al., 2014). The feedstock is heated in a tubular heat exchanger alongside the digester. Part of the digestate is recycled and mixed with the fresh material to assure inoculation. A gas production rate of up to 7.5m^3/m^3.d can be realized in KOMPOGAS reactors (Beck, 2004). The schematic flow sheet of a KOMPOGAS system is given in Figure 1.11.

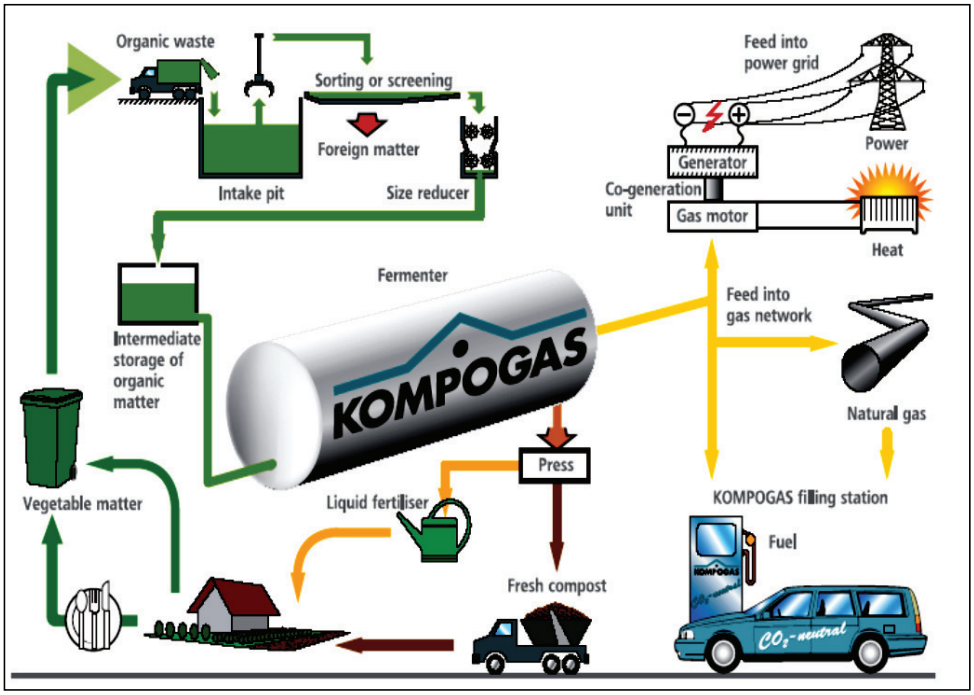

Figure 1.11 Flow sheet of a KOMPOGAS system. Adopted from (Hannes, 2007)

The LINDE-BRV digester is similar to the KOMPOGAS digester in flow arrangement. Heating of the feedstock primarily takes place inside the reactor through the digester wall with preheating taking place with an external heat exchanger. Inoculation takes place by recycling the liquid fraction after solids separation, which results in lower inoculation rates therefore requiring longer solids retention times (SRTs). Feedstock mixing takes place by transversal paddles resulting in more pronounced mixing, resulting in semi-plug-flow behavior. Schematic set-up of the reactor is given in Figure 1.12 (left).

The VALOGRA reactor, a semi-continuous one-step process, is also a vertical flow system but with direct steam injection for heating purpose, developed in France (Kothari et al., 2014) (Figure 1.12, right). It operates at a mesophilic temperature, with a high dry solid content of 25-35%. The mixing is done by recycling the biogas in the reactor intermittently at high pressure (6-7 bars). Full-scale Valorga plants include Grenoble, France (16,000 t/y), Amiens, France (85,000 t/y), Papeete, Tahiti (90,000 t/y), Tilburg, The Netherlands (52,000 t/y) and Tamara in French Polynesia (92,000 t/y) (Kothari et al., 2014). The maximum OLR reported for a VALOGRA treatment plant in Tilburg, The Netherlands, is about 5 kg VS/m^3.d, which

is a comparable loading rate of wet processes (Beck, 2004). The specific methane yield is between 220-250 m^3/tone of total volatile solids (TVS) fed to the digester or between 80-160 m^3/ton of waste fed (36-64% VS), depending on waste characteristics (de Mes et al., 2003; Saint-Joly et al., 2000).

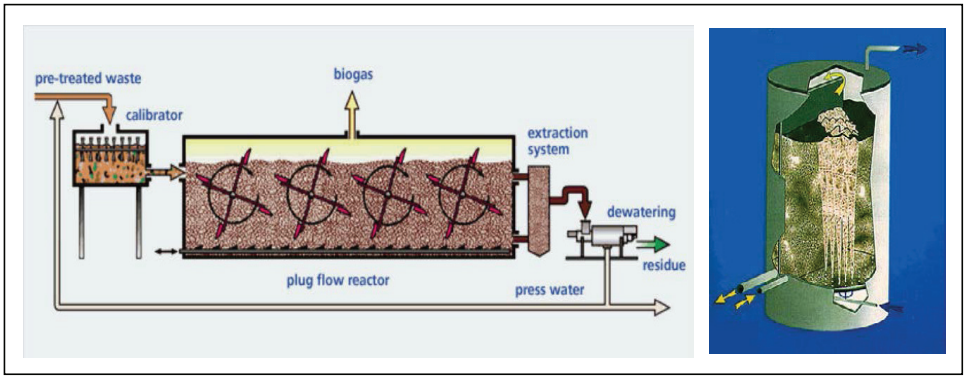

Figure 1.12 LINDE-BRV reactor (left), cross section of VALOGRA reactor (right). Adopted from (Beck, 2004)

A simpler batch process for dry mesophilic AD of organic solid wastes at high DS concentrations (30-40%) is called BIOCEL, of which the first full scale plant was started-up in 1997 in Lelystad, The Netherlands (Ten Brummeler, 2000). This plant is processing 50,000 tons of OFMSW, per year. The BIOCEL process can be considered a low-tech system although the achieved conversion rates in the BIOCEL process are similar to those achieved in advanced continuous dry digestion systems (ten Brummeler, 1993). At the start, the solid waste is mixed with the methanogenic inoculum from the previous batch feeding as a static pile. Hereafter, the leachate solution from a former digestion run is brought into the reactor and recycled from top to bottom. At full-scale, the reactor volume obviously consists of several units, which have to be loaded separately. During the batch digestion, leachate is recycled and biogas is extracted from the reactor. The residue needs to be dewatered to produce a stabilized compost-like end product (ten Brummeler, 1993). A schematic diagram of the process is shown in Figure 1.13.

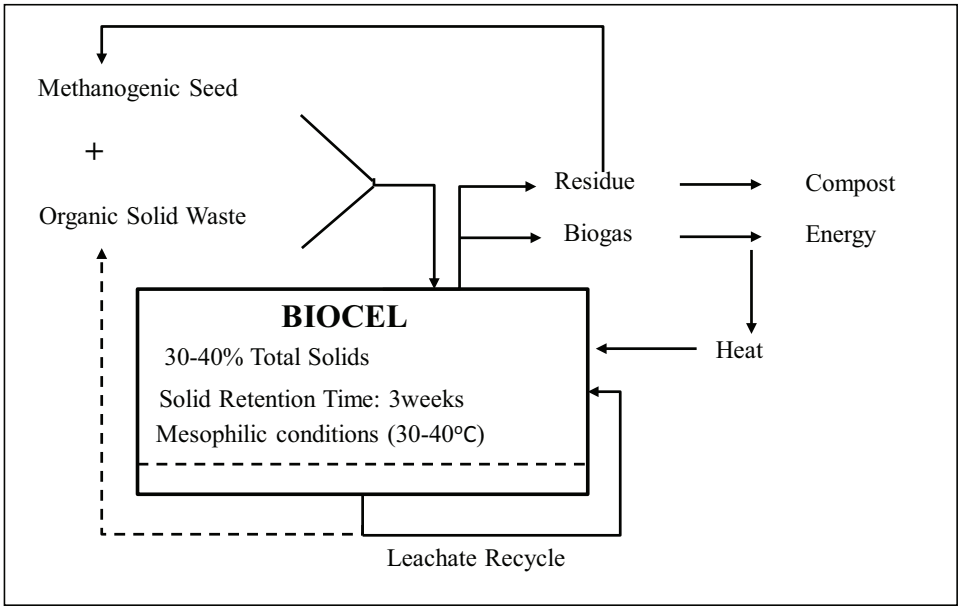

Figure 1.13 Flow sheet of a BIOCEL digester for dry anaerobic batch digestion of organic solid wastes. Adapted from (ten Brummeler, 1993)

1.4 Scope and outline of the thesis

1.4.1 Problem statement

Cellulose makes up about 30%-50% of the SS in the sewage of Western countries. It originates from the use of toilet paper which is estimated to be 10-14 kg per person per year (Ruiken et al., 2013). However, passing the primary clarifiers, a large part of the BOD from sewage SS will be oxidised in the subsequent aeration tanks and is thus unavailable for energy recovery, while adding to energy consumption. A very compact and efficient solution to minimise oxidation of filterable matter in aeration tanks is the recovery of cellulose-rich slurries from raw sewage with a fine-mesh sieve (e.g. Salsnes Filter, Norway, mesh size of 350 μm). The main advantage of fine sieving is that the filter cake, which is called fine sieved fraction (FSF), usually has a high DS content (20%-30%) without any chemical additions. Dewatering of FSF to 40%-50% DS content is simply possible by applying mechanical pressure (Ruiken et al., 2013).Therefore, FSF could be a very suitable substrate to be methanised in anaerobic digester systems, which may lead to efficient on-site energy recovery. On-site FSF digestion could contribute to the objective of realizing energy neutral or energy producing WWTPs, in line with the Dutch roadmap for the WWTP of 2030.

1.4.2 Objectives of the research

Resource recovery and energy neutral sewage treatment is nowadays the focus of many water authorities. This novel concept led to a new focus on optimisation of digestion processes and enhanced biogas production. In this scope, energy production via FSF digestion can also be used for energy generation at centralised WWTPs. Therefore, the main objective of this research was to investigate the bio-methane potential (BMP) and maximum methane production rates of FSF, sequestered from raw municipal sewage, for onsite energy recovery towards energy neutrality at WWTPs.

1.4.2.1 Research questions

In order to meet the aforementioned objective, the following research questions are formulated:

1. Can a high loaded stable mesophilic or thermophilic digestion process be developed for FSF?
    - What adaptation periods are required in the process of FSF digestion?

- Does accumulation of intermediates occur and if so how do they impact hydrolysis and methanogenesis under both conditions?
- Could data of microbial community changes be used to interpret the digestion process adaptation to FSF?

2. What are the main FSF characteristics, impacting its biodegradability and conversion rates?
3. Does thermophilic digestion of FSF result in a higher biogas production rate and/or a smaller required reactor volume compared to conventional mesophilic digestion of FSF?
4. Are energy neutral WWTPs feasible by applying an influent fine sieve?
   - How much energy can be recovered applying on-site FSF digestion?
   - To what extent can the aeration capacity be diminished in the aeration tank in dependence to fine sieve efficiency?

1.4.3 Outline of the thesis

In this thesis, physicochemical characteristics of FSF and its digestion characteristics under both thermophilic and mesophilic conditions are described. Experiments were performed using sequencing batch laboratory scale anaerobic digesters and an automated methane potential test system (AMPTS_II, Sweden) (Figure 1.14).

In **Chapter 2**, digester performance and microbial community changes in the aforementioned thermophilic and mesophilic sequencing batch reactors (SBRs) fed with the FSF of municipal sewage are described. A seven months adaptation time was allowed for the thermophilic and mesophilic digesters in order to adapt to FSF as the sole substrate. Microbial population dynamics during long term sludge adaptation (up to one year) of thermophilic and mesophilic SBRs treating FSF at varying OLRs are addressed in **Chapter 3**. In this chapter, the viscosity characteristics of both thermophilic and mesophilic sludges are also given.

Energy recovery from municipal raw sewage is expected to lead to energy neutral and more sustainable sewage treatment plants. FSF from municipal raw sewage is an energy rich material. Therefore, the potential of on-site energy recovery using high-rate thermophilic FSF bio-methanation is discussed in detail in **Chapter 4**.

Since the biodegradation characteristics of toilet paper tissue fibers in AD processes are unknown, different types of cellulosic fibers-based toilet papers and microcrystalline cellulose (MCC) as a kind of fibreless reference material were digested, which is described in **Chapter 5**. The cellulosic substrates studied included virgin pulp for paper production (VPPP), virgin fibers based toilet paper (VTP) and recycled fiber based toilet paper (RTP) and MCC. These results are compared to the biodegradation of FSF.

Accumulating intermediates of the AD fermentation process may have inhibitory effects on the overall conversion process and process performance. In **Chapter 6**, the impact of accumulating solid waste fermentation intermediates on hydrolysis and methanogenesis under both mesophilic and thermophilic conditions is discussed. Furfural, hydroxymethylfurfural (HMF) and vanillin were selected as intermediates and humic acid sodium salt as a recalcitrant compound.

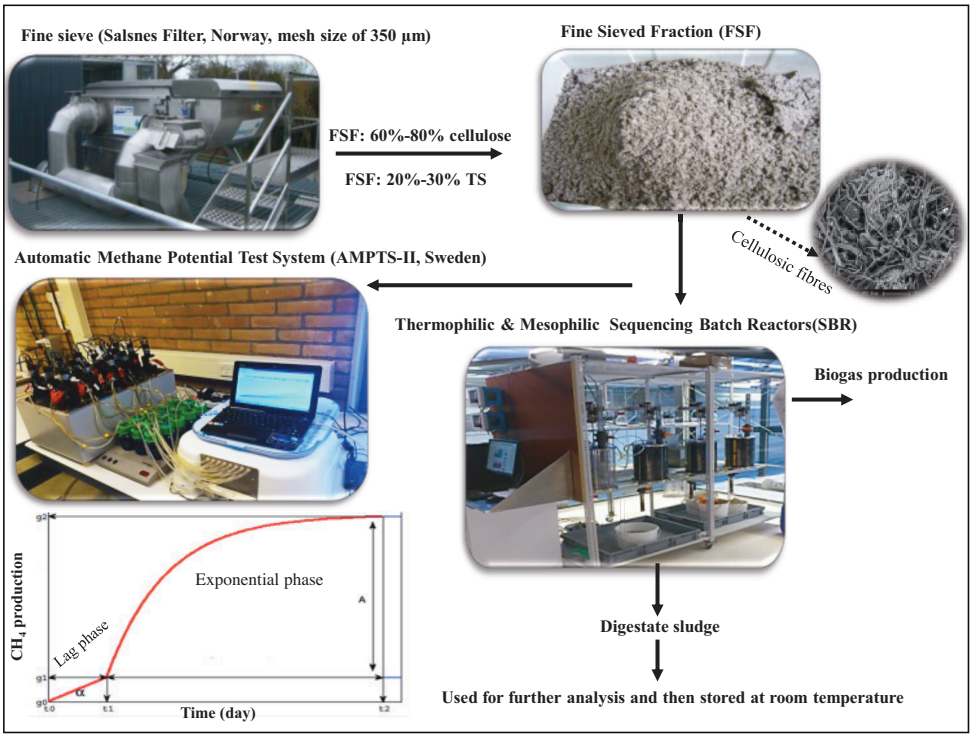

Figure 1.14 Overview of FSF collection, biomethane potential (BMP) test and anaerobic digestion of FSF using AMPTS system and SBR digesters

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Chapter 2. Digester performance and microbial community changes in thermophilic and mesophilic sequencing batch reactors fed with the fine sieved fraction of municipal sewage

Abstract

This study investigates the start-up and operation of bench-scale mesophilic (35°C) and thermophilic (55°C) anaerobic sequencing batch reactor (SBR) digesters treating the fine sieved fraction (FSF) from raw municipal sewage. FSF was sequestered from raw municipal wastewater, in The Netherlands, using a rotating belt filter equipped with a 350 micron mesh. For the given wastewater, the major component of FSF was toilet paper, which is estimated to be 10-14 kg per year per average person in the western European countries. A seven months adaptation time was allowed for the thermophilic and mesophilic digesters in order to adapt to FSF as the sole substrate with varying dry solids content of 10-25%. Different SBR cycle durations (14, 9 and 2 days) were applied for both temperature conditions to study methane production rates, volatile fatty acids (VFAs) dynamics, lag phases, as well as changes in microbial communities. The prevailing sludge in the two digesters consisted of very different bacterial and archaeal communities, with *OP9* lineage and *Methanothermobacter* being predominant in the thermophilic digester and *Bacteroides* and *Methanosaeta* dominating the mesophilic one. Eventually, decreasing the SBR cycle period, thus increasing the FSF load, resulted in improved digester performances, particularly with regard to the thermophilic digester, i.e. shortened lag phases following the batch feedings, and reduced VFA peaks. Over time, the thermophilic digester outperformed the mesophilic one with 15% increased volatile solids (VS) destruction, while applying an SRT of 64 days in both reactors, irrespective to the lower species diversity found at high temperature.

This chapter is based on:

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2.1 Introduction

Cellulose makes up about 30-50% of the suspended solids in the sewage of western countries, mainly originating from the use of toilet paper, which is estimated to be 10-14 kg per person per year (STOWA 2010). This material can enter the aerobic sewage treatment, adding significant costs to sewage treatment due to energy input for aerobic degradation and incineration costs of the non-degraded fibres that end up in wet waste sludge after digestion (Ruiken et al., 2013). Ruiken et al. (2013) suggested the use of a fine sieve (mesh size 350 μm) to separate suspended solids from sewage before entering the biological treatment, instead of using conventional primary clarifiers. Based on thermographic measurements, the cellulose fraction found in the FSF was 79% of the total mass and 84% of the organic mass; the inorganic matter fraction was 6%. In comparison, the cellulose fraction of primary sludge only reaches a maximum of 32-38% of organic mass (Ruiken et al., 2013). Also, the total solids content in the FSF without additional dewatering is higher than that of primary sludge, i.e. 10 to 25% as found in our present study, compared to 4-12% as indicated for primary sludge (Inc et al., 2003, Tchobanoglous et al., 2003), resulting in a lower total sludge volume production. FSF can be reused as fibres in several processes where, nowadays, recycled paper is used; however, the origin of these fibres could limit these opportunities (STOWA and Grondstoffenfabriek, 2013).

A more straightforward method to valorise the FSF on site is by anaerobic (dry) digestion. Anaerobic digestion is a carbon-neutral technology to produce biogas that can be used for heating, generating electricity, mechanical energy, or for supplementing the natural gas supply. The produced bioenergy such as methane can contribute to the goal of realising an energy-neutral or energy producing wastewater treatment plants (WWTPs) (Roeleveld et al., 2010).

In nature, hydrolysis and fermentation of lignocellulosic biomass is done by cellulolytic microorganisms belonging to the phyla *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, *Thermotogae* and *OP9* (Peacock et al., 2013, Kaoutari et al., 2013).These microorganisms can release fermentation products, such as various types of fatty acids, into natural environments and complete the carbon cycle via methane and/or CO_2 under anaerobic conditions (Minty et al., 2013, De Angelis et al., 2012). Lignocellulosic biomass, which has similar characteristics to FSF, has been widely used for bio-methanation by coupling

cellulolytic microorganisms, fermenting bacteria and methanogenic archaea in one or two-stage anaerobic bioreactors (Zhang et al., 2013, Merlino et al., 2013). Such process can be operated under mesophilic (35°C) and thermophilic (55°C) conditions.

Mesophilic anaerobic digestion of organic solids is often reported as the most convenient, stable and reliable form of substrate conversion leading to stable methane production rates. However, mesophilic hydrolysis rates are lower compared to thermophilic conversion rates (Lu et al., 2013). On the other hand, thermophilic digestion requires higher energy input, and is regarded more sensitive to changes in operational conditions, such as changes in temperature and the organic loading rate, as well as to changes in substrate characteristics (Kim et al., 2002, van Lier, 1996). The perceived poor process stability as well as the lack of experience in operating thermophilic processes are probably the main reasons that have prevented its wide-scale application. The higher vulnerability could be due to a less diverse microbial community (Raskin et al., 1994), persistence of propionate (Wilson et al., 2008) and increased toxicity of intermediates at the thermophilic temperature range (van Lier, 1996).

Thermophilic anaerobic digestion of lignocellulosic biomass, such as FSF, might be more effective than mesophilic digestion (De Baere, 2000). The hydrolysis of complex polysaccharides by thermophilic microorganisms establish higher rates compared to mesophiles; each 10 ^{o}C increase in temperature can increase enzymatic rates by two- to three-fold (Mozhaev, 1993). High temperatures can also increase substrate solubility (Mozhaev, 1993) and decrease the bulk liquid viscosity (Eshtiaghi et al., 2013), leading to improved mixing performance and thus an increased hydrolysis of (hemi-)cellulose to monomers (Eichorst et al., 2013).

At present, anaerobic digestion at the mesophilic temperature range is widely applied and well described in many publications, whereas the application of thermophilic digestion is still limited. With regard to lignocellulosic wastes, such as FSF, comparative studies conducted in parallel under both thermophilic and mesophilic conditions (Golkowska and Greger, 2013) are difficult to find. In this research, the feasibility and efficiency of one-step anaerobic digestion of FSF under thermophilic and mesophilic conditions in laboratory batch fed reactors (8 L) was compared. Digestion performance and microbial dynamics were followed in time under both conditions during reactor start-up and after extended adaptation times.

2.2 Materials and methods

2.2.1 Digester

Four water jacketed laboratory mixed digesters with a working volume of 8 L were used in duplicate to conduct the digestion of FSF under both thermophilic and mesophilic conditions, at 55°C and 35°C, respectively applying sequencing batch feeding conditions. The reactors were continuously mixed by stirring (60-80RPM, Maxon motor Benelux B.V., Switzerland) to achieve a more homogenized matrix. The system was equipped with a pH and temperature probe (CPS41D, Endress+Hauser B.V., Switzerland) and an on-line biogas measuring device (RITTER MilliGascounter MGC-1 PMMA, Germany). The temperature was controlled by circulating water from a programmable water bath (TC16, PMT TAMSON, The Netherlands). Temperature, pH, biogas flow rate were continuously monitored using Labview software.

2.2.2 Substrate

A rotating belt filter (Salsnes Filter, Norway) equipped with a 350 μm pore size fine sieve, was operated to treat the screened (mesh size 6 mm) sewage at WWTP Blaricum, The Netherlands (plant size: 30,000 pe, maximum hydraulic capacity 1600 m^3/h). The FSF coming from this sieve was collected once every four months and stored at 4 °C prior to use. The FSF contained mainly paper fibres, some sand, hair, leaves and some undefined materials. The key characteristics of FSF are listed in Table 2.1. The FSF was fed manually and batch wise in a way that first the corresponding mass to be fed was extracted from the reactor where after the reactor was fed with FSF. Sequencing batch feeding periods of 14, 9, and 2 days were applied. It is noted that no additional water or nutrients were supplied.

Table 2.1 Characterization of the raw FSF used in this study

Components	Unit	Values
Total solids (TS)	g/kg	100~250
Volatile solids (VS)	g/kg	90~225
COD	g/kg	130~400
VS/TS ratio	%	~90
COD/VS ratio	g/g	1.4~1.8
SCOD/VS ratio	g/g	0.16~0.18
TN/VS ratio	mg N/g	8.4~15.5
TP/VS ratio	mg P/g	3.4~7.8

2.2.3 Inoculum

In the first stage, the thermophilic inoculum was obtained from a plug flow dry anaerobic composting (DRANCO, OWS, Brecht, Belgium) digester (De Baere, 2000), operated at a solid retention time (SRT) of 15 days and treating mainly vegetable, fruit and yard wastes with a dry matter content of about 35% and a heterogeneous appearance. The thermophilic inoculum was sieved (4 mm mesh) prior to use. Mesophilic inoculum was taken from an anaerobic digester of a WWTP (Harnaschpolder, Delft, The Netherlands) that treats both primary and secondary sludge with a maximum solid content of 5% and which was operated at an SRT of 22 days.

In the second stage of this study, both adapted thermophilic and mesophilic sludge were taken directly from the FSF-fed laboratory scale anaerobic digesters that were operated at a dry solids content in the range of 4%~7%.

2.2.4 Analytical methods

Total solids (TS) and volatile solids (VS) were determined on weight base (g/kg) according to the standard methods for the examination of water and wastewater (APHA AWWA WEF 1998). Chemical oxygen demand (COD, 500~10000 mg/L), soluble chemical oxygen demand (sCOD, 25~1500 mg/L), total nitrogen (TN, 10~150 mg/L) and total phosphorous (TP, 0.5~25 mg/L) were measured spectrophotometrically using photometric cell tests (Merck, Germany). SCOD was measured after filtering the supernatant through syringe membrane filter (0.45 µm, Whatman, Germany). The substrate samples and digestates for analyses of COD, SCOD, TP and TN were diluted according to the used cell test range. All analysis were done in triplicate.

Volatile fatty acids (VFAs) were quantified by Gas Chromatography (GC, Agilent Technology 7890A), using a flame ionization detector (FID) and a capillary column type HP-FFAP Polyethylene Glycol (25 m x 320 µm x 0.5 µm) with helium as the carrier gas at a total flow of 67 ml min^{-1} and a split ratio of 25:1. The GC oven temperature was programmed to increase from 80 min to 180 °C in 10.5 min. The temperatures of injector and detector were 80 °C and 240 °C, respectively, and the injected volume was 1 µL. Prior to GC analysis, 10 ml of digested samples was first centrifuged at 15000 rpm for about 15~20 minutes. Then the

supernatant was filtered by syringe membrane filter (0.45 µm, Whatman, Germany). The filtrated liquid was diluted 2 and 3 times with pentanol as internal solution (300 ppm) for mesophilic and thermophilic digestion samples, respectively. Finally, 10 µL of formic acid (purity >99%) was added into the 1.5 mL vials.

2.2.5 Biomethane potential (BMP) test

The biomethane potential test (BMP) determines the quantity of methane (mL CH_4/ gVS_{fed}) at standardised temperature and pressure (STP: 0 °C and 1 atm.) that a waste can potentially produce under anaerobic condition. In this work, the BMP of the substrate was determined in-situ in two weeks batch digestion periods using the four laboratory reactors (working volume 8 L), with an inoculum to substrate ratio of 3 (VS basis). In this experiment two of the four digesters were used as blank, meaning one thermophilic and one mesophilic blank digester. The other two reactors were used to assess the BMP of FSF. Biogas mainly consists of methane and CO_2. To determine the composition of biogas in the digesters, biogas was led through four bottles (2L) filled with 3M NaOH. CO_2 gas is absorbed in the NaOH solution and pure CH_4 was recorded at STP.

2.2.6 BMP assays using AMPTS system (ex-situ BMP tests)

The anaerobic biodegradability of FSF during the adaptation period was performed ex-situ, using an Automated Methane Potential Test System (AMPTS II, Bioprocess Control, Lund, Sweden), according to an adapted protocol for BMP tests (Angelidaki et al., 2006, 2009). Each bottle was filled with the required amounts of inoculum and substrate, using an inoculum to substrate ratio ($R_{I/S}$) of 3 gVS_I/gVS_S, a macro- and micro-nutrients medium and buffer solution (Angelidaki and Sanders, 2004) to fill the bottle to the designated volume (400 mL). These 500 mL bottles were continuously stirred and incubated for at least 30 days in a temperature controlled water bath. CO_2 and H_2S were stripped from the biogas by leading the produced biogas through 100 mL 3M NaOH solution. Hereafter, the remaining methane containing biogas, was lead into a gas flow cell with a calibrated volume. When the gas volume equalled the calibrated volume of the flow cell, the gas was released and recorded as one normalized volume at time t. The BMP value is reached when the gas production was lower than 1% of the accumulated production for 3 consecutive days Microcrystalline cellulose (MCC) was used as a positive control. All batch tests including blank, MCC and FSF were conducted in triplicate.

2.2.7 Operation of the reactors

In this study, four different operational periods (I, II, III, IV) were applied (Table 2.2). The reactors were operated as a sequencing batch reactor with different feeding frequencies, ranging from once per 9 days (on average) to once per 2 days. The start-up lasted from Day 1 to Day 211 (Period I). Two extended non-fed periods were applied from Day 211 to Day 243 (Period II), with one feeding per 14 days. Periods III and IV were characterized by the increased OLR, under both thermophilic (55 ^{o}C) and mesophilic (35 ^{o}C) conditions. Methane production rates, VFAs accumulation, lag phase durations, as well as changes in microbial communities were investigated. It is noted that the increase in OLR in Period III from 1 (Day 243~284) to 2.5 (Day 284~333) was mainly brought about by the change in the FSF characteristics from WWTP Blaricum, whereas in the meantime, it was also decided to increase the OLR. The first 211 days of operation (Period I) were used for biomass adaptation, BMP tests, monitoring specific methanogenic activity (SMA) and simultaneously, regular analytical measurement of both FSF and inoculum.

Table 2.2 Operational conditions of each period

Period	Operational days	Feeding mode	Cycle duration (day)	OLR (kgCOD/m^3·d)
I	1~211	Irrigular batch fed	2~7	----
II	211~243	Slug dose (2x)*	14	----
III	243~333	Sequenced batch (SBR)	6*7+2*15+1*9	1-2.5
IV	333~393	Sequenced batch (SBR)	2	5.5

*Slug dose feeding was used to assess in situ BMP

2.2.8 Environmental Scanning Electron Microscopy (ESEM) & Energy Dispersive X-ray (EDX) element analysis

The structure of the substrate before degradation was studied using an ESEM & EDX element analyzing system. FSF was sampled from the storage container and pretreated immediately. Firstly, triplicated samples were washed for three times using 1×PBS. Then the samples were fixed by 2% (v/v) glutar-aldehyde for 4 hours and washed again using 1×PBS. All the samples were air dried before ESEM analysis. For ESEM analysis, samples were mounted on a 1 cm^2 metal support and kept in place with adhesive tape and observed with a Philips XL30 Series ESEM. The EDAM 3 EDS system (SUTW 3.3 EDX window and 128.0 eV EDX resolution) was applied to analyze the key elements of the substrates.

2.2.9 Metagenomic analysis

2.2.9.1 Sample preparation for pyrosequencing. Fresh biomass samples were washed by 1 X PBS and then centrifuged under 7000 *g* for 7 minutes. The supernatant was removed and the pellet was washed by PBS for a second time and centrifuged under 17000 X *g* for 20 minutes. The supernatant was removed and the pellet was stored (less than one month) at -25 ^{o}C for DNA extraction.

*2.2.9.2 DNA extraction***.** DNA extraction was performed using the MoBio UltraClean microbial DNA isolation kit (MoBio Laboratories, Inc., CA, USA) A minor modification of the manufacturers protocol was that twice bead-beating (5 min) and heating (5 min) were applied in sequence in order to enhance the lysis of microbial cells. DNA isolation was confirmed by agarose gel electrophoresis. The quality of DNA was verified by Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA).

2.2.9.3 454 Pyrosequencing. The amplification and sequencing of the 16S rDNA gene was performed by Research and Testing Laboratory (Lubbock, TX, USA) with following primers: (1) U515F ('5-GTG YCA GCM GCC GCG GTA A-3') and U1071R ('5-GAR CTG RCG RCR RCC ATG CA-3')(Wang and Qian 2009) were used for bacteria and archaea with a high coverage over 90% for each domain; (2) Arch341F ('5-CCC TAY GGG GYG CAS CAG-3') and Arch958R ('5-YCC GGC GTT GAM TCC AAT T-3') were used for archaea. The pyrosequencing was done using a Roche 454 GS-FLX system (454 Life Science, Branford, CT, USA) with titanium chemistry.

*2.2.9.4 Post analysis of pyrosequencing data***.** The post analysis of pyrosequencing data was performed by combining different programs from the Quantitative insights into microbial ecology (QIIME) pipeline, version 1.6.0 (Caporaso et al., 2010).

2.2.10 Real-time qPCR

Real-time qPCR was performed using an ABI 7500 instrument (Foster City, CA, USA) with three primer sets, including Bac516-F-Bac805-R (for all bacteria), ARC787-F-ARC1059-R for all archaea, FTHFS-F-FTHFS-R for syntrophic acetate-oxidizing bacteria (Yu et al., 2005). qPCR amplification was done in a 20 μL reaction volume. Each reaction tube contained 10 μL 2×SGExcel FastSYBR Mixture (With ROX, Sangong Biotech, Shanghai, China), 8.6 μL dH2O, 0.2 μL of each forward and reverse primer (1 pmol/ μL), and 1 μL of DNA template. Molecular grade water was used as a negative control. Triplicate PCR reactions were carried out for all samples and negative controls. The thermal cycling program consisted of 2 min at 50 ^{o}C, 1 min at 95 ^{o}C, followed by 40 cycles of 10 s at 95 ^{o}C, 35 s at

X ^{o}C (X=56 ^{o}C for Bac516-F/Bac805-R, X=61 ^{o}C for ARC787-F/ARC1059-R, X=55 ^{o}C for FTHFS-F/FTHFS-R). Finally, a melting curve analysis was performed for verifying the specificity of PCR products; denaturation of 1 min at 95 ^{o}C, cooling of 1 min at 55 ^{o}C and then heat till 95 ^{o}C again, at a rate of 0.5 ^{o}C per cycle. The standard curves for the above primer sets were constructed using all strains of the samples. The target 16S rDNA gene sequences were amplified from each strain by PCR with the corresponding primer sets and cloned into pGEM-T Easy vectors (Sangong Biotech, Shanghai, China). For each plasmid, a 10-fold serial dilution series ranging from 10^{10} to 10^{4} copies/mL was generated. The slopes of the plasmid standard curves were between -4.411 and -2.955, with a mean value about -3.313. The threshold cycle (C_T) values determined were plotted against the logarithm of their initial copy concentrations. All standard plasmids and 16S rDNA samples were amplified in triplicate.

2.3 Results

2.3.1 Characteristics of FSF

To understand the hydrolysis of FSF, characterization of the material is important. Therefore, the main characteristics of the different used FSFs batches were determined after every sampling event at WWTP Blaricum. A large variation in the measured components was found (Table 2.1). The heterogeneous appearance of FSF might be attributed to seasonal fluctuations (e.g. resulting in more leaves in the FSF in autumn), functioning of the fine sieve system, and FSF storage time and temperature in the on-site container. The maximum period that the FSF was stored in the on-site container was 2 weeks, and the temperature varied from >25 ^{o}C in summer to below 0 ^{o}C in winter. The raw heterogeneous FSF samples mainly consisted of intake fibrous material of different sizes (Figure 2.1). The EDX analysis revealed carbon (60%-64%) and oxygen (20%-27%) as major elements in the raw FSF materials.

Figure 2.1 Environmental scanning electron microscopic (ESEM) photograph of raw fine sieved fraction (FSF) materials

2.3.2 Adaptation of FSF to inoculum (Day 0-Day 211)

The first 211 days of reactor operation were used to adapt the mesophilic and thermophilic inoculum to FSF. The feeding sequence of the SBR digesters was once per 2-7 days. At day 0-32 and day 40-72, two BMP tests were performed under thermophilic and mesophilic conditions using FSF as the (heterogenic) substrate and MCC as the positive control (Figure 2.2). The mesophilic digestion of FSF showed a regular methane production rate, whereas a much more irregular methane production rate could be observed during thermophilic digestion.

The lag phase observed during the thermophilic batch tests of FSF was longer than under mesophilic conditions (Figure 2.2). The feedstock of the DRANCO digester, where the thermophilic inoculum was sampled from, was VFY waste, whereas the mesophilic inoculum was sampled from a digester at WWTP Harnaschpolder. The mesophilic sludge was therefore considered to be much more adapted to FSF than the thermophilic sludge, since the primary and secondary sludge digested in this full scale mesophilic digester also partly consists of the components that can be found in FSF. Because of the differences in methane production pattern between thermophilic and mesophilic digestion, it was decided to extent the adaptation period (phase I).

Another two series of BMP tests at day 118-165 and day 170-203 (Period I) under both mesophilic and thermophilic conditions, respectively, were performed to observe the changes in methane production from FSF after a long adaptation period (Figure 2.3). The biomethane

production rate under mesophilic conditions was higher than observed during the experiment at day 40-72, and the incubation time to reach the ultimate methane production was considerably lower for both MCC and FSF. Under thermophilic conditions, the lag phase increased for both MCC and FSF. The rate of biogas production increased as well, indicating adaptation of the thermophilic sludge to the FSF. The still existing lag phase, however, was presumed to indicate that further adaptation of the sludge was still necessary. The BMP values for MCC and FSF under both mesophilic and thermophilic conditions were found similar; respectively 350 ± 3 and 368 ± 3 for MCC and 332 ± 4 and 338 ± 8 for FSF.

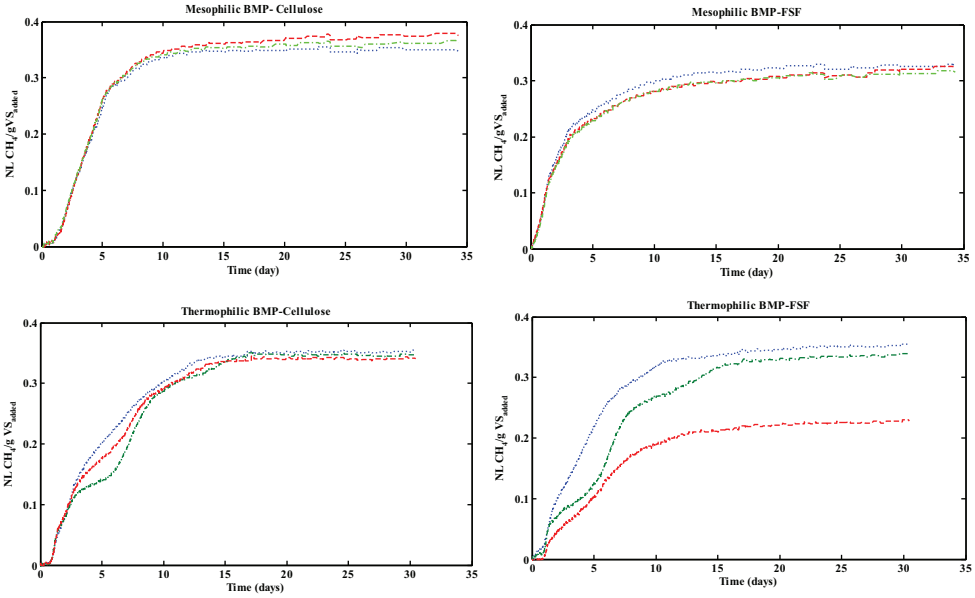

Figure 2.2 Mesophilic (top-right) and thermophilic (bottom-right) BMP tests of FSF. Mesophilic (top-left) and thermophilic (bottom-left) BMP tests of MCC (positive control). Batch tests were performed in triplicate at day 0-32 and day 40-72 (Period I) under thermophilic and mesophilic conditions, respectively

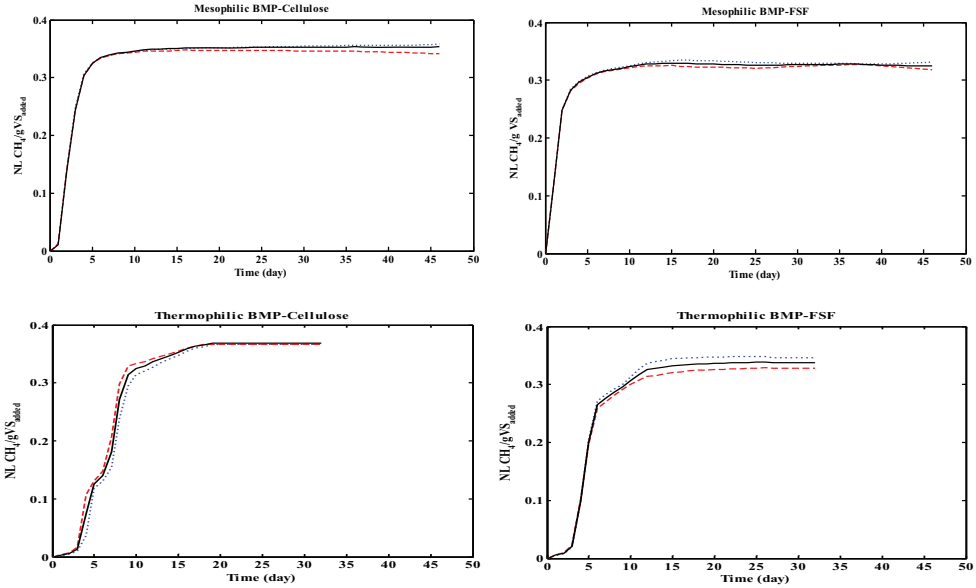

Figure 2.3 Mesophilic (top-right) and thermophilic (bottom-right) BMP tests of FSF. Mesophilic (top-left) and thermophilic (bottom-left) BMP tests of MCC (positive control). Batch tests were performed in triplicate at day 118-165 and day 170-203 (Period I) under mesophilic and thermophilic conditions, respectively

2.3.3 14-day batch feeding

From Day 211 to Day 243 (Period II) two extended non-fed periods, 14 days without feeding, were conducted to study the in-situ BMP of the FSF. In this paper, only one series of the extended non-fed periods is shown since the BMP and biogas trends were almost the same. An inoculum to substrate ratio of 3 was applied based on gram VS of the FSF. Some physicochemical characteristics of FSF substrate as well as the thermophilic and mesophilic sludge before and after BMP assessment have been summarized in Table 2.3.

Table 2.3 Physicochemical characteristics of raw FSF as substrate (mean values ± standard deviations of triplicates), the thermophilic (T) and mesophilic (M) sludge based on wet weight

Parameters	Unit	Raw FSF	T-digester	M-digester
pH	-	-	7.6±0.15	7.2±0.1
TS_{start}	g/kg	109±3	35±1.5	31±2.0
VS_{start}	g/kg	98±3	27±1.5	23±2.0
TS_{finish}	g/kg		27±1.0	28±1.5
VS_{finish}	g/kg		21±1.0	20±1.5
FSF COD	g/kg	140±10	-	-

The methane fraction in the biogas was 53 and 57% for thermophilic and mesophilic conditions, respectively. The results of the average BMP and biodegradability tests of the FSF in the thermophilic and mesophilic digesters were 335 and 325 $NmLCH_4/gVS_{added}$, and 66 and 64%, respectively (Figure 2.4A). These results are comparable to the results obtained by Paulsrud et al. (2014) who found values of 323 to 366 $NmLCH_4/gVS$ for sieved sludge. An inoculum to substrate ratio of 3 was applied based on gram VS of the FSF by dosing 63.7 gram FSF-VS to the thermophilic and 54.9 gram FSF-VS to the mesophilic reactor, corresponding to respectively 91 and 78.4 gCOD. Biodegradability was assessed as the experimental ultimate methane production (expressed in COD) over the initial tCOD of the substrate (Raposo et al., 2011). It is noted that this theoretical approach does not take into account the needs for bacterial cell growth and their maintenance, which has been reported typically 5%~10% of organic material degraded (Angelidaki and Sanders 2004; Symons and Buswell et al., 1933), meaning that not all biodegraded COD is transformed into methane. Moreover, accumulating biodegradable non-methanised intermediates are not reflected in this calculation and may create another discrepancy.

A clear lag phase of 4 days in gas production was observed directly after feeding the thermophilic digester (Figure 2.3A) while the mesophilic reactor did not show any lag phase. During the BMP tests, samples were taken from both digesters to measure the VFA concentration (Figure 2.4B and 2.4C). Higher concentrations of acetic and propionic acid in the thermophilic digester were observed compared to the mesophilic digester during the incubation period. Maximum acetic and propionic acid concentrations in the thermophilic digester were 620 and 415 mg/L, measured on Day 215 and 217, respectively.

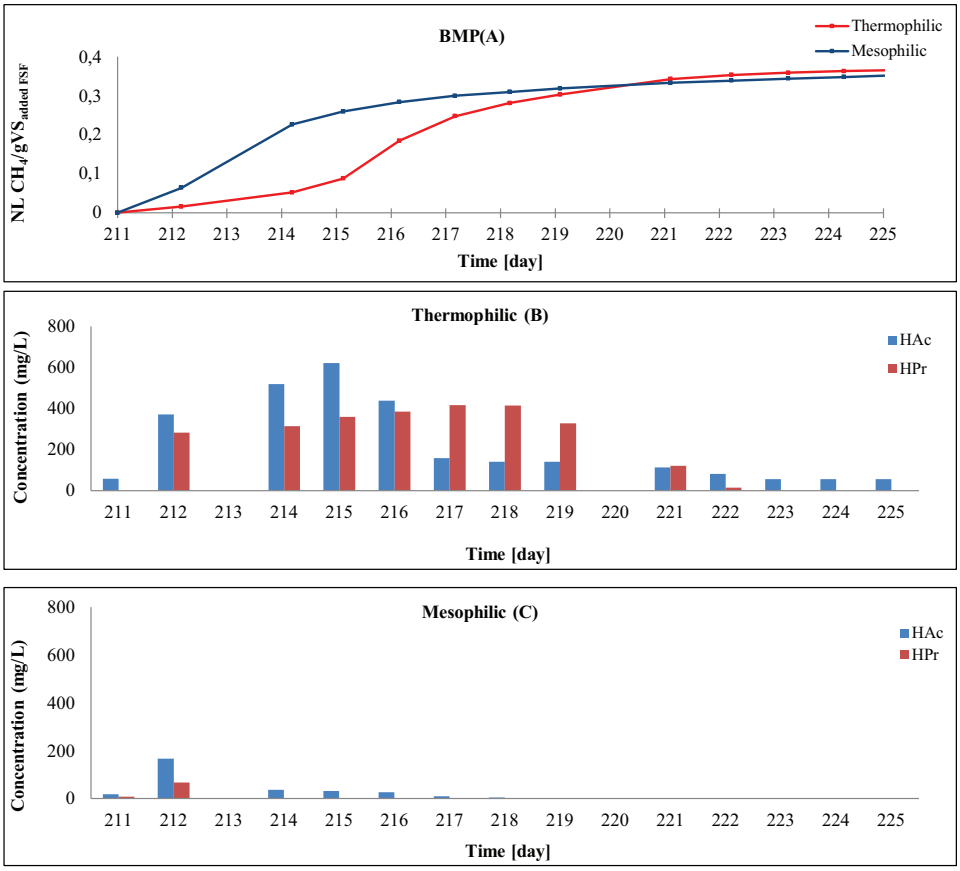

Figure 2.4 Methane production and VFAs accumulation during Period II. (A) Thermophilic and mesophilic BMP test at inoculum to substrate ratio of 3; (B) thermophilic acetate and propionate concentrations; (C) mesophilic acetate and propionate concentrations

2.3.4 9-day sequencing batch feeding (on average)

After assessing the in-situ BMP during the 14 days batch cycles in Period II, the three-month Period III commenced applying sequencing batch feedings of 9 days (on average) to all four digesters (two thermophilic and two mesophilic). In Period III, the TS of the FSF substrate increased from 10% to 27% and COD concentrations from 135±25 g/kg to 385±8 g/kg, applying an average solids retention time (SRT) of 144 days in the reactors. The imposed increase in OLR from 1 to 2.5 kgCOD/(m^3·d) was likely due to the change in FSF characteristics received from WWTP Blaricum. In addition, between Day 285 to 327, the OLR was increased by increasing the amount of substrate added. The increase in OLR led to a higher biogas production at both temperature conditions, which resulted in three big peaks

(Figure 2.5). The highest peaks were registered for the thermophilic digester, reaching values of 2100, 1970, and 1740 ml/h, on Day 291, 307, 320, respectively. In the mesophilic reactor, the methane production peaked to 1290, 1400 and 1640 mL/h, respectively. Strikingly, in the thermophilic reactor, methane generation was typically delayed after adding the new feedings. Apparently, non-fed conditions negatively impacted the maximum methane production in the thermophilic digester (Figure 2.5).

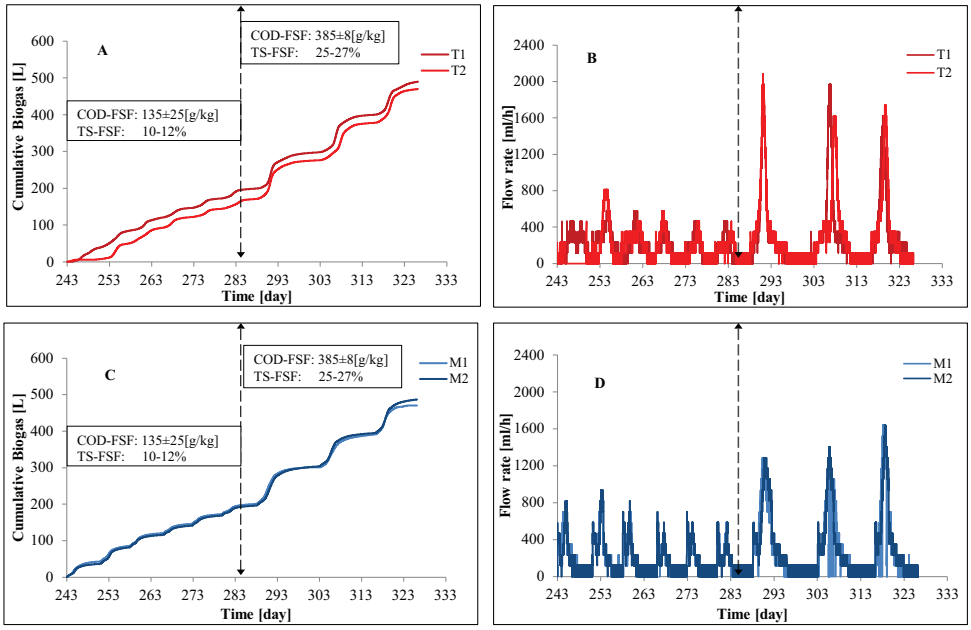

Figure 2.5 Cumulative biogas production and biogas flow rate for thermophilic (A, B) and mesophilic digestion (C, D) in Period III

Biogas flow rates of about 0.25 $L/L_{reactor}.h$ at peak level were found for the two thermophilic digesters, whereas the mesophilic ones reached a maximum of 0.2 $L/L_{reactor}.h$ (Figure 2.5). The last series of the 9-day batch feeding is displayed in more detail in Figure 2.6. Increasing the OLR led to a decreased lag phase in the thermophilic digester. A high peak of acetate (2.25 g/L) was obtained in the thermophilic digester at Day 319, which was five times higher than the acetate concentration measured in the mesophilic digester (0.45 g/L) (Figure 2.6). Despite this acetate accumulation, there was no significant pH drop in both mesophilic and thermophilic conditions (data now shown).

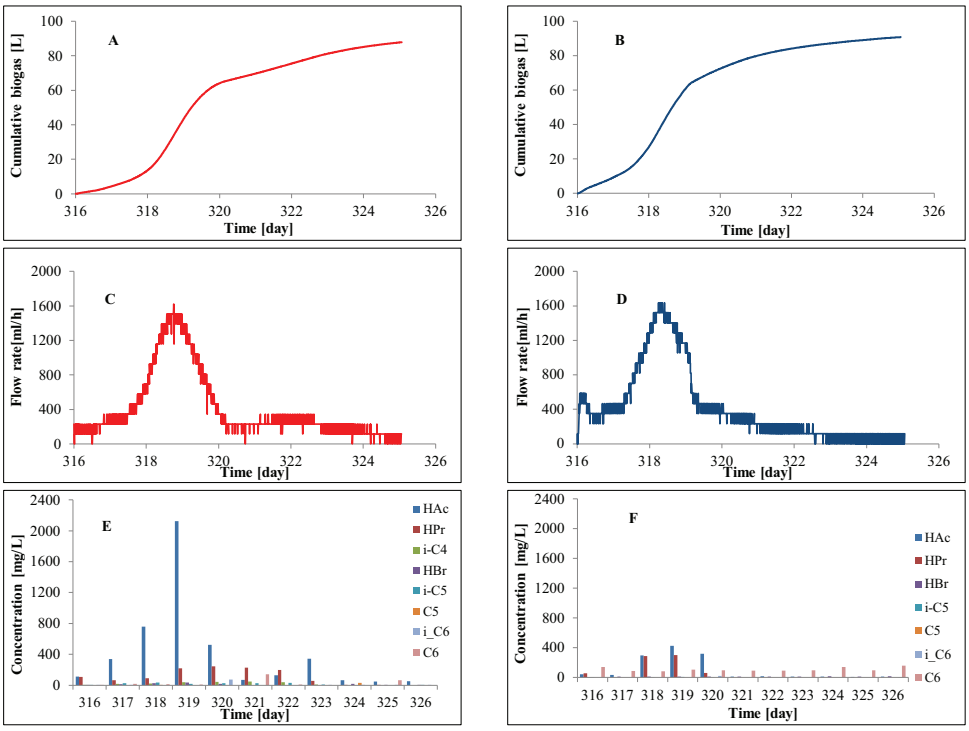

Figure 2.6 Cumulative biogas, biogas flow rate and VFAs in the batch thermophilic (A, C, E) and mesophilic (B, D, F) digesters

2.3.5 2-day sequencing batch feeding

The average OLR of the four reactors was increased at Day 333 from 2.5 to 5.5 kgCOD/(m^3·d), using FSF substrate with the following characteristics: 250 g FSF, COD 350±15 g/kg, TS 25% with a sequencing batch feeding every two days. An average biogas flow rate of 0.1 $L/L_{reactor}$.h (20 L/d) was obtained at both temperatures. At the beginning of each new batch feeding, a decrease in biogas flow rate is recorded (Figure 2.7) for both the thermophilic and mesophilic digesters, resulting from pressure drop in the system. Increased biogas production rates were observed for both thermophilic and mesophilic digesters from Day 378 to Day 390 (Figure 2.7 & Table 2.4). Average VS concentration in the thermophilic and mesophilic digester was determined at 30 and 34 g/kg (wet weight), respectively. It is speculated that the observed increase in biogas production rates from Day 378 to Day 390 (Figure 2.7) might be related to a further increase in loading rate, either related to a change in dry solids content of the FSF in the storage container (stored at 4 °C) or *in situ* degradation of more difficult biodegradable compounds that previously cumulated in the digester.

The thermophilic reactor showed a direct high peak biogas production, while the gas production in the mesophilic reactor increased at a lower rate (Figure 2.7).

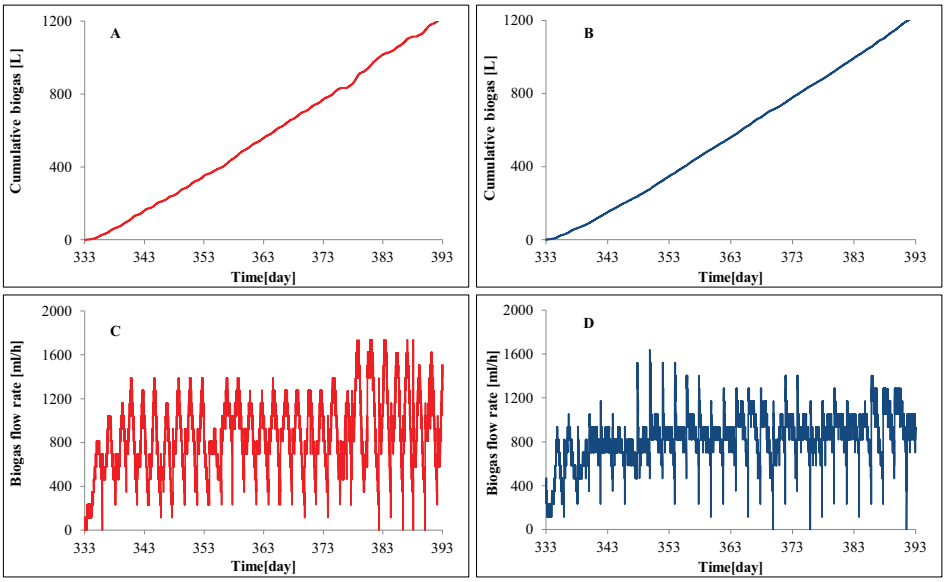

Figure 2.7 Cumulative biogas and biogas flow rate for the thermophilic (A, C) and mesophilic (B, D) digestion in Period IV

Table 2.4 Results of methane yield, SMPR, biodegradation and VS destruction percentages of FSF digestion (mean values ± standard deviations) under thermophilic and mesophilic conditions from day 378 to day 392 (period IV)

Parameters	Thermophilic (55ºC)	Mesophilic (35ºC)
Methane yield ($mLCH_4$, STP/$gVS_{substrate}$)	347±10	325±7
SMPR($mLCH_4$, STP/(gVS_{inoc}·d))	46±3	39±2
Biodegradation (%)	62±3	57±3
VS destruction (%)	46±4	40±5

The cumulative biogas, produced during the 2-day interval period, after 3 different batch feedings in both the thermophilic and mesophilic digester were followed (Figure 2.8). There was an increase in the biogas production rate over time in the thermophilic digester, whereas under mesophilic conditions the biogas production rate stayed the same (Figure 2.8). Apparently, the thermophilic biomass was still adapting to the increased substrate loading to the reactor, whereas the mesophilic biomass reached its maximum conversion capacity. Table 2.4, presents the average methane yields ($mLCH_4/gVS$), specific methane production rates (SMPR: $mLCH_4/gVS_{inoc}$·d), biodegradability and VS destruction percentages of FSF

digestion from Day 378-392 under both thermophilic and mesophilic conditions. SMPR was obtained by dividing the daily produced methane volume by the grams of inoculum VS added.

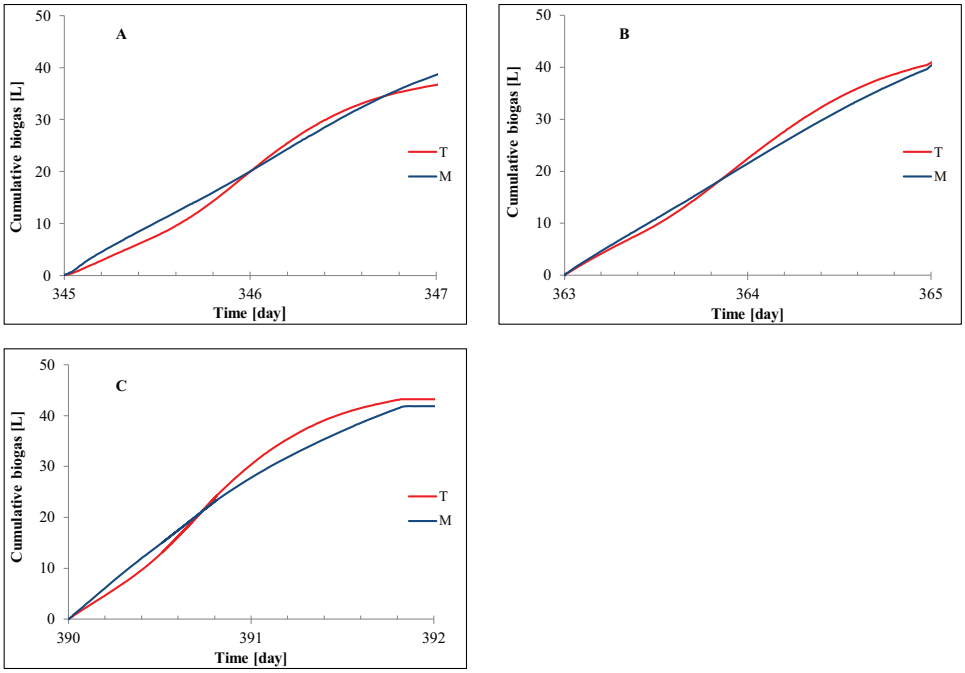

Figure 2.8 Cumulative biogas production for the thermophilic (T) and mesophilic (M) at the 2-day batch feeding within period IV: started period (A), middle (B), and end (C) while biogas production was increasing throughout the reactor (T) and staying stable

VFA measurements were periodically performed during the entire experimental period. Interestingly, only little VFA accumulation was observed in the thermophilic reactor (138±39 mg/L) when the more frequent feeding regime of once per two days was applied instead of feeding once per 9 days (on average). Under the latter condition, the acetate cumulated to 2250 mg/L at peak level. The acetate measured in the thermophilic reactors in the first 24 hours following the batch-feeding was about 9 times higher than that in the mesophilic reactor, whereas after 48 hours this ratio decreased to 3 times. The acetate concentration in mesophilic digester remained at a very low level during the whole operational period, i.e. 15± 1.5 mg/L. Noteworthy, propionate concentration remained at a lower value of 30±15 mg/L in the thermophilic digester.

2.3.6 Bacterial population dynamics

Periodically, biomass samples were taken in order to get some insights in the bacterial and archaeal population dynamics during the high loading conditions of both the mesophilic and thermophilic reactors. In the thermophilic reactor, *OP9* was the most dominant phylum, i.e. 43% and 85% in Period III and IV (green bars in Figure 2.9), followed by phylum *Firmicutes*, which accounted for 49% and 10%, respectively. There was a shift in bacterial genera in the thermophilic reactor after switching from the 9-day (on average) to 2-day batch feeding cycle from the end of Period III (Day 315) towards the end of Period IV (Day 375). After shortening the feeding cycle and thus increasing the OLR, the initially dominant bacterial genera, i.e. *Clostridium* (26%), *Garciella* (8%) and *Coprothermobacter* (13%) lost their dominancy and the OP9 bacterial linage became the sole dominant one (85%) (Figure 2.10). *OP9* is a fermentative bacterial lineage that is able to degrade (hemi)cellulose into H_2, acetate and ethanol under thermophilic conditions (Dodsworth et al., 2013).The relative abundance of another important bacterial genus, growing in syntrophic associations, i.e. *Syntrophomonas*, increased by more than three times from ~0.5% to ~1.6% (Day 315 to Day 375) in the thermophilic reactor (Figure 2.10).

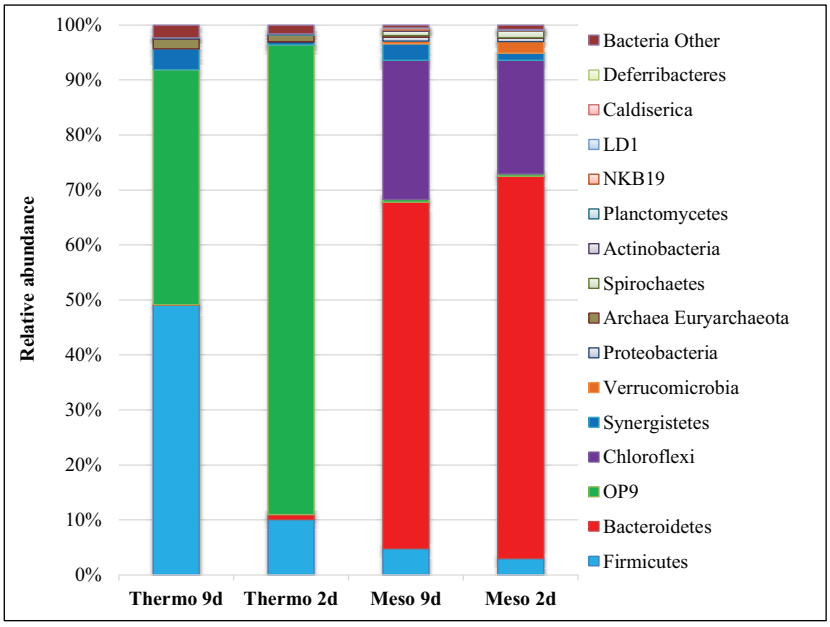

Figure 2.9 All Bacterial phyla and their relative abundances in the thermophilic and mesophilic digesters during sequencing batch feeding with FSF in cycle periods of 9 days (on average) and 2 days

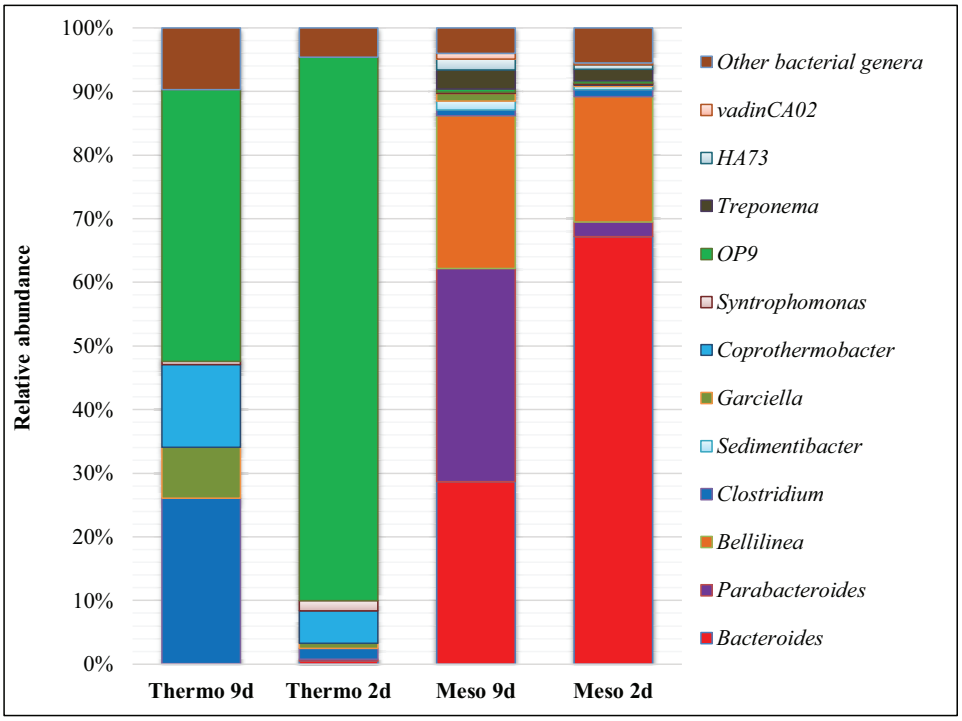

Figure 2.10 Major bacterial genera and their relative abundances in the thermophilic and mesophilic digesters during sequencing batch feeding with FSF in cycle periods of 9 days (on average) and 2 days

In the mesophilic reactor, both *OP9* and *Firmicutes* accounted for low abundance, i.e. only <5% in total. Two different phyla, namely *Bacteroidetes* (63%-70%) and *Chloroflexi* (17%-25%), had the highest abundance in the mesophilic reactor (Figure 2.9). There was also an enriching trend of genus *Bacteroidetes* in the mesophilic reactor from Period III to IV (Figure 2.10). *Bacteroidetes* are frequently found to be dominant in cellulose- and long chain fatty acid-fed anaerobic reactors, likely having a major role in the degradation of protein, fat, cellulose, and other polysaccharides.

2.3.7 Archaeal population dynamics

It is reported in literature that thermophilic conditions can favour the hydrogenotrophic methanogenesis pathway (van Lier 1996, Demirel and Scherer, 2008, Krakat et al., 2010). In this study, the difference in temperature and inoculum caused complete different archaeal communities in each reactor. The genus *Methanothermobacter* was the most dominant one in the thermophilic reactor (abundance over 90%), while *Methanosaeta* was top dominant

(abundance over 97%) in the mesophilic communities (Figure 2.11). The relative abundance of the total mass of methanogens in the thermophilic reactor was 1%~2%, in the mesophilic reactor this was lower than 0.3% (Figure 2.11). *Methanothermobacter* are typical hydrogenotrophic methanogens (methanogenesis from H_2 and CO_2) under thermophilic conditions. Species of *Methanosaeta* are typical acetoclastic methanogens in mesophilic anaerobic digestion systems.

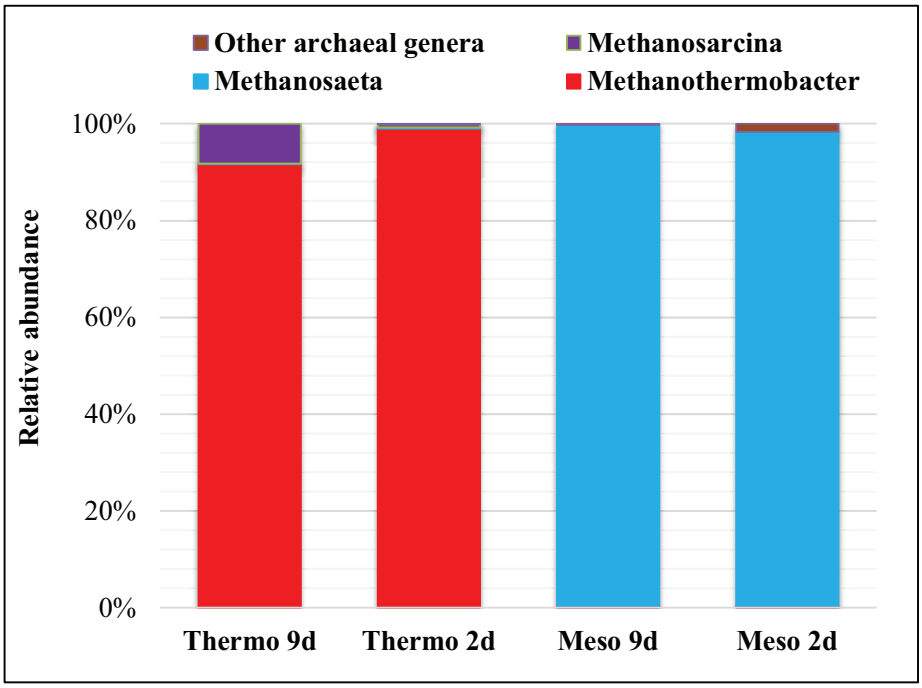

Figure 2.11 Archaeal genera and their relative abundances in thermophilic and mesophilic digesters during sequencing batch feeding with FSF in cycle periods of 9 days (on average) and 2 days

2.3.8 Phylogenetic diversity

We used several indexes to describe the phylogenetic diversity of microbial communities in each reactor. The terms α-diversity and β-diversity are commonly used to characterize mean species diversity within a given (microbial) habitat (α-diversity) and among different habitats (β-diversity) (Whittaker, 1972). Our results show that, for both thermophilic and mesophilic communities, the α-diversity of archaeal communities were much lower and more stable than that of bacterial communities (Figure 2.10). We used the Shannon-Wiener index, a popular ecological index (Shannon 1948) to characterize total α-diversity (Figure 2.12).

Notably, samples taken on Day 315 showed similar indexes for thermophilic and mesophilic bacterial communities. However, meanwhile applying higher loading conditions, the thermophilic index dropped by half after two months of sequencing batch feedings and the mesophilic index decreased only by 20% (Figure 2.12). The Shannon-Wiener index decreased by 20% in the thermophilic archaeal communities, whereas the mesophilic index increased by 27%-40% (Figure 2.12). Apparently, there was an enriching trend to several species in the thermophilic reactor, i.e. phylum *OP9*. The β-diversity clearly shows that the phylogenetic distance between the two thermophilic communities (Day 315 to Day 375) were far from each other, whereas that of the two mesophilic ones were much closer (Figure 2.13). This means that the mesophilic communities were more stable than the thermophilic ones, indicating a quite resilient community at mesophilic temperatures.

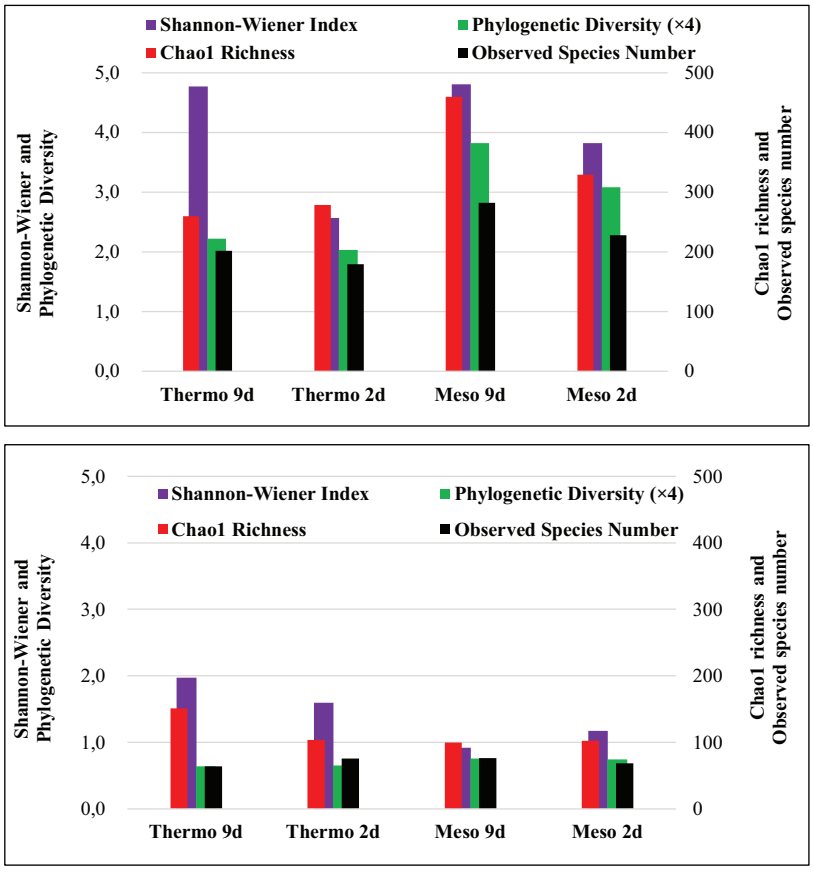

Figure 2.12 α-diversities of the microbial communities of each reactor. Bacterial communities (top); archaeal communities (bottom)

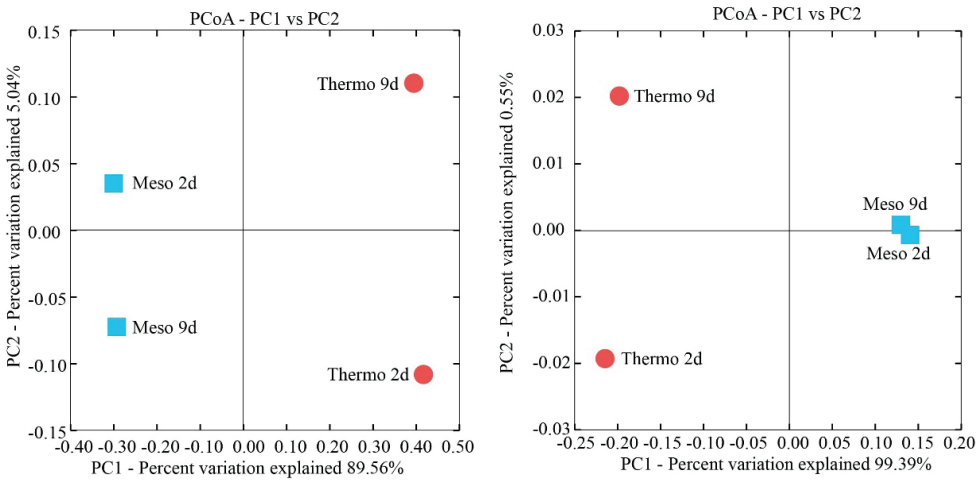

Figure 2.13 Principal Co-ordinate Analysis plots of sample fractions determined using the weighted Unifrac distance metric. Left, bacterial communities; Right, archaeal communities

2.3.9 Real-time quantification

Pyrosequencing reveals the relative abundance of each group of microorganisms, without having any quantitative information. We used real-time qPCR to quantify bacterial, archaeal and syntrophic acetate-oxidizing (SAO) bacterial amounts in each reactor (Figure 2.14). For the thermophilic reactor, the total amount of bacteria, archaea and SAO bacteria increased by 25%, 40% and 2%, respectively, from end of Period III towards end of Period IV (Day 315 to Day 375, Figure 2.14). For the mesophilic reactor, the amount of total bacteria and SAO bacteria increased by 55% and 220%, whereas the total archaeal amount decreased by 47% (Figure 2.14).

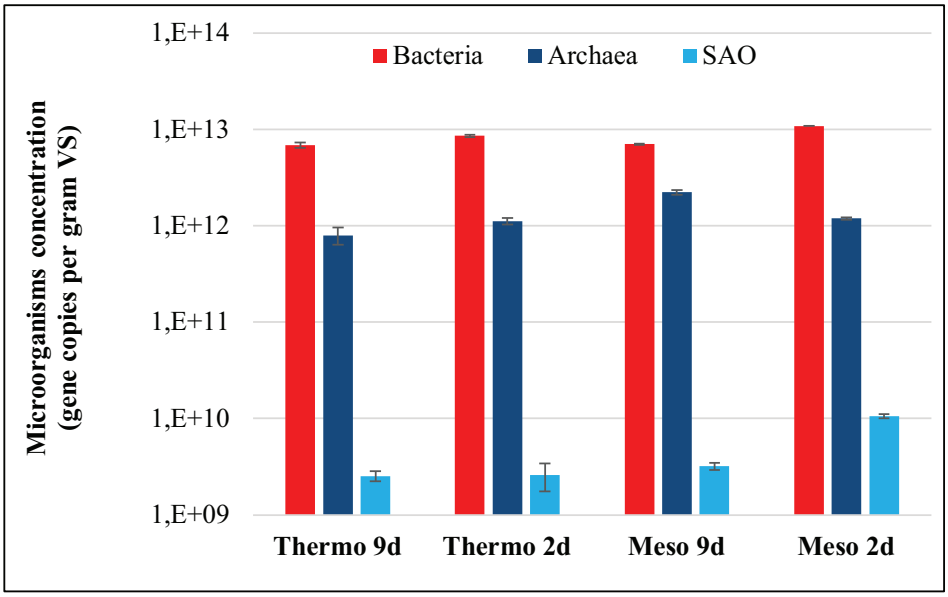

Figure 2.14 Bacterial, Archaeal, and SAO copy numbers in each reactor in period (III) and (IV) revealed by real-time qPCR

2.4 Discussion

In this research, we studied progressive feeding regimes in a sequencing batch mode, i.e. 14-day batch feeding, 9-day batch feeding (on average), and 2-day batch feeding, agreeing with a high increase in the reactor's organic loading rate, on the thermophilic and mesophilic digestion of FSF materials. A clear lag phase of 3-4 days was observed for the thermophilic batch digesters during the 14-day batch feeding cycle when performing the in-situ BMP tests (Figure 2.4A). The observed lag phase became shorter when the feeding cycles were shortened. Concomitantly, a considerable change occurred in the microbial communities of the thermophilic digester when the feeding cycles were intensified.

Our 454-pyrosequencing results revealed that the bacteria lineage belonging to *OP9* became exclusively dominant after intensifying the feeding cycles. Species of phylum *OP9* are fermentative bacteria that widely exists in anaerobic environments, such as petroleum reservoirs and engineered facilities (Dodsworth et al., 2013, He et al., 2012). The members of phylum *OP9* are able to degrade (hemi)cellulose via the Embden-Meyerhof glycolysis

pathway, while producing H_2, acetate and ethanol under thermophilic conditions (Dodsworth et al., 2013). Recent studies suggested that their existence in anaerobic digesters (Dodsworth et al., 2013, Riviere et al., 2009) play an important role in the thermophilic fermentation. Interestingly, in our thermophilic digesters, the hydrolysis efficiency was greatly improved after shortening the batch feeding cycles, meanwhile the abundance of *OP9* bacteria increased significantly.

It is notable that the relative abundance of the syntrophic genus *Syntrophomonas* increased from 0.5% to 1.6% from the end of Period III towards the end of Period IV (from Day 315 to Day 375) in the thermophilic reactor, while its abundance kept low (0.2%) in the mesophilic reactor. *Syntrophomonas* is an important fermenting bacterial genus (Hatamoto et al., 2007) that is closely involved in butyrate oxidation into acetate and H_2 (Zhang et al., 2013) in co-culture with hydrogenotrophic methanogens such as *Methanospirillum* (Li et al., 2013), *Methanosarcina* (Palatsi et al., 2011) and *Methanothermobacter* (Sieber et al., 2012). It is confusing that our qPCR data show only a slight increase (Figure 2.14) in SAO bacterial amount (*Syntrophomonas* included, theoretically). Likely this can be attributed to the weak specificity of the FTHFS primer sets (targeting formyltetrahydrofolate synthetases) that were used in our real-time qPCR study, whose full-length sequence match more than 30 species of acetogens, nonacetogens, homoacetogens and sulphate reducers (Sieber et al., 2012).

An important mutualistic partner of *Syntrophomonas* is *Methanothermobacter*, which was identified the sole dominant methanogen. This genus is one of the typical hydrogenotrophic methanogens (methanogenesis from H_2 and CO_2) under thermophilic conditions. *Methanothermobacter* consumes hydrogen (and formate), keeping a H_2 partial pressure low enough so the entire digestion process is thermodynamically favourable (Sieber et al., 2012). They have a relatively fast growth rate with doubling times of 2~5 h and are easily cultivable. It has been reported that the methanogen *Methanothermobacter* was predominant in thermophilic cellulose-degrading environments (Sasaki et al., 2012, Luo et al., 2013, Rademacher et al., 2012), and they are highly related to syntrophic processes. For example, a syntrophic acetate oxidation process, carried out by *Caldicellulosiruptor sp* and *Clostridium thermocellum*, coupled to hydrogenotrophic methanogenesis by strains of the genus *Methanothermobacter*, was also discovered during cellulose methanisation using office paper as the substrate, under thermophilic conditions (Lu et al., 2013).

Since the FSF consists for approximately 80% of cellulose, the abundance of this species in our experiments agrees with reported literature findings. In the mesophilic reactor, the abundance of *Bacteroidetes* increased from 29% in Period III to 67% in Period IV. The phylum *Bacteroidetes* is one of the most common bacteria in mesophilic anaerobic digesters (Li et al., 2013) and it was dominant in some cellulose- and long chain fatty acid-fed anaerobic reactors (Li et al., 2009), having a major role in the degradation of protein, fat, cellulose, and other polysaccharides. *Methanosaeta* was the only dominant archaeal genus in the mesophilic reactors (Figure 2.11). Previous studies have shown the predominance of the acetoclastic methanogen *Methanosaeta* in mesophilic anaerobic digestion systems (Williams et al., 2013), where acetoclastic methanogenesis was the major route of biomethane production.

It is notable that the biogas production rates (Figure 2.5, 2.7) were different between the two systems. The thermophilic rates increased faster to their peak value, after which they decreased until the next batch feeding, while the mesophilic digesters had a relatively stable biogas production throughout the cycle. This observation indicates that the thermophilic digester was not operated yet at its maximum conversion capacity, in contrast to the mesophilic reactor. The progressive increase in the methanogenic capacity of the thermophilic reactor is also clearly shown in Figure 2.6. The thermophilic biogas production rate increased over time, whereas the mesophilic rate remained stable, despite the consecutive sequenced feedings. The rapid increase in the biogas production rate in the thermophilic digester is only possible with an incrementally enhanced hydrolysis process, which is generally considered the rate limiting step. Remarkably, the accumulation of acetate to over 2 g/L in the thermophilic reactor shows that methanogenesis was the rate limiting step in this case. It was also observed that during the whole operation period of FSF digestion, the levels of propionate was kept decreasing form Period II (415 mg/L) to III (245 mg/L) and to IV (30 mg/L), while the substrate loading conditions were increasing. The stable thermophilic reactor performance might be linked to the more abundant presence of propionate oxidizing bacteria, such as *Firmicutes* (Zamanzadeh et al., 2013).

Finally, the results of our work clearly show that the thermophilic digester is sensitive to periods of non-fed conditions (Figure 2.5B). Each cycle starts with a lag phase and ends with a non-fed or starvation period, in which hardly any biogas is produced. Thermophilic digesters, due to the higher temperature, are characterized by higher substrate conversion

rates, but at the same time they suffer from higher decay rates (Duran and Speece, 1997). It is therefore expected that the application of a continuous feeding system for the thermophilic digestion of FSF will increase the stability of the digestion process. Nebot et al. (1995) studied the comparison between continuous feeding and different semi-continuous modes of feeding and concluded that the optimum feed frequency range is 24 doses/day or more. Therefore their conclusion is in agreement with this study: a continuously fed system has likely both a greater stability and higher conversion efficiency.

2.5 Conclusion

In this study, four bench-scale thermophilic (55°C) and mesophilic (35°C) anaerobic digesters were applied to treat the FSF obtained from the influent of a municipal WWTP. The start-up and operation of the anaerobic digestion processes were investigated with the following conclusions:

- Anaerobic digestion was proven feasible and efficient to convert the FSF of municipal sewage into methane.

- Decreasing the batch cycle period of the SBR resulted in improved digester performances, particularly with regard to the thermophilic digester, i.e. shortened lag phases and reduced VFA peaks. Over time, the thermophilic digester outperformed the mesophilic one.

- As expected, the two digesters harboured very different bacterial and archaeal communities, with OP9 lineage and *Methanothermobacter* being pre-dominant in the thermophilic digester and *Bacteroides* and *Methanosaeta* dominating the mesophilic one.

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Chapter 3. Microbial population dynamics during long term sludge adaptation of thermophilic and mesophilic sequencing batch digesters treating sewage fine sieved fraction at varying organic loading rates

Abstract

In this research, the feasibility of, and population dynamics in, one-step anaerobic sequencing batch reactor (SBR) systems treating the fine sieved fraction (FSF) from raw municipal wastewater was studied under thermophilic (55°C) and mesophilic (35°C) conditions. FSF was sequestered from raw municipal wastewater, in The Netherlands, using a rotating belt filter (mesh size 350 micron). FSF is a heterogeneous substrate that mainly consists of fibres originating from toilet paper and thus contains a high cellulosic fraction (60%-80% of total solids content), regarded as an energy rich material. Results of the 656-day fed-batch operation clearly showed that thermophilic digestion was relatively stable, applying high organic loading rates (OLR) up to 22 kg COD/(m^3·day). In contrast, the mesophilic digester already failed applying an OLR of 5.5 kg COD/(m^3·day), indicated by a drop in pH and increase in volatile fatty acids (VFAs). The observed viscosity values of the mesophilic sludge were more than tenfold higher than the thermophilic sludge. 454-pyrosequencing of eight mesophilic and eight thermophilic biomass samples revealed that *Bacteroides* and aceticlastic methanogen *Methanosaeta* were the dominant genera in the mesophilic digester, whereas OP9 lineages, *Clostridium* and the hydrogenotrophic methanogen *Methanothermobacter* dominated the thermophilic one. Our study suggests that applying thermophilic conditions for FSF digestion would result in a higher biogas production rate and/or a smaller required reactor volume, comparing to mesophilic conditions.

This chapter is based on:

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3.1 Introduction

Cellulose makes up about 30-50% of the suspended solids in the sewage of western countries (STOWA 2010-19, 2010) and it usually enters aerobic sewage treatment plants, adding significant costs due to difficulties in aerobic degradation (Ruiken et al., 2013). At wastewater treatment plant (WWTP) Blaricum (The Netherlands), the sewage flow is directed through a fine sieve with a mesh size of 350 μm. This sieve is implemented as a compact alternative to primary clarification to separate suspended solids from sewage prior to biological nutrient removal. The fine sieved fraction (FSF) is a heterogeneous substrate with high bio-energy potential. It mainly consists of partly disrupted toilet paper and cellulose accounts for 79% of the total mass and 84% of the organic mass according to a thermographic analysis (Ruiken et al., 2013). Because FSF has a high dry solids concentration (20%-30%), a straightforward method to stabilize it is by dry anaerobic digestion (AD) (Chapter 2).

Efficient AD of solids can proceed either under mesophilic (30-40 °C) or thermophilic (50-60 °C) conditions (Labatut et al., 2014; Tezel et al., 2014; Yu et al., 2014). Many previous studies indicate that, compared to mesophilic AD processes, thermophilic AD generally accepts a higher organic loading rate, more efficient degradation of organic matters (Ahring, 2003; Zábranská et al., 2000), higher biogas production efficiency (Goberna et al., 2010; Levén et al., 2007; McHugh et al., 2004; Siddique, 2014) and better sludge dewaterability (Rubia et al., 2002). It is hypothesized that microbial-ecology-driven factors play vital roles differentiating the performance in the above-mentioned aspects between thermophilic and mesophilic dry digesters. However, it still remains unknown about how and to which extent temperature can shape microbial communities during long-term dry anaerobic digestion.

In this study, two parallel anaerobic digesters were operated for over two years under 55 °C and 35 °C. The two digesters were fed with the FSF from WWTP Blaricum as a sole substrate in batch mode. The overall aim was to seek for insights into how the thermophilic and mesophilic communities will adapt and respond to increasing organic loading rates (OLRs) of FSF. The digesters performance such as biogas production, volatile fatty acids (VFAs) and sludge viscosity were followed to indicate the possible functional dynamics of the communities along with their structural variation.

3.2 Materials and methods

3.2.1 Digester

Four water-jacketed laboratory mixed digesters with a working volume of 8L were used in duplicate to conduct the digestion of FSF under both thermophilic and mesophilic conditions for more than 650 days at 55 °C and 35 °C, respectively. The reactors were continuously mixed by stirring (60-80RPM, Maxon motor Benelux B.V., Switzerland) to create a homogenized matrix. The system was equipped with a pH and temperature probe (CPS41D, Endress+Hauser B.V., Switzerland) and an on-line biogas measuring device (RITTER MilliGascounter MGC-1 PMMA, Germany). The temperature was controlled by circulating water from a programmable water bath (TC16, PMT TAMSON, The Netherlands). The pH, temperature, biogas flow rate were continuously monitored using Labview software.

3.2.2 Substrate

A rotating belt filter (Salsnes Filter, Norway) equipped with a 350 μm pore size fine sieve was operated to treat the screened (mesh size 6 mm) sewage at WWTP Blaricum, The Netherlands (plant size: 30,000 pe, maximum hydraulic capacity 1600 m^3/h). The fine sieved fraction (FSF) coming from this sieve was collected once every four months and stored at 4 °C prior to use. The FSF contained mainly paper fibers, some sand, hair, leaves and undefined materials. The main physicochemical characteristics of the different used FSFs batches were determined after every sampling event at WWTP Blaricum and prior to use (Chapter 2). This heterogenetic appearance of FSF (Figure 3.1) was likely due to seasonal fluctuations (e.g. more leaves in the FSF in autumn), functioning of the fine sieve system, FSF storage time and temperature in the on-site container (Chapter 2). The maximum period that the FSF was stored in the container was two weeks and the temperature varied from maximum 25 °C in summer to minimum 0 °C in winter. FSF was fed manually and batch wise in a way that first the corresponding mass to be fed was extracted from the reactor where after the reactor was fed with FSF.

Figure 3.1 Photos of raw FSF at different sampling time: dry weather (left) and wet weather (right).The heterogenetic appearance of the raw FSF can be attributed to several reasons, such as seasonal fluctuations (e.g. more leaves in the FSF in autumn), changes of functioning of the fine sieve system, storage time of FSF, and temperature in the on-site container for storing FSF.

3.2.3 Inoculum

It would be difficult and inefficient to inoculate both thermophilic and mesophilic digester using the same seed sludge, hence, two origins of seed sludge were chosen in this study. In the first stage (start-up of the experiment), the thermophilic inoculum was obtained from a plug flow dry anaerobic composting (DRANCO, OWS, Brecht, Belgium) digester (De Baere, 2000), operated at a solids retention time (SRT) of 15 days and treating mainly vegetable, fruit and yard wastes with a dry matter content of about 35% and a heterogeneous appearance. The thermophilic inoculum was sieved (4 mm mesh) prior to use. Mesophilic inoculum was taken from an anaerobic digester of a WWTP (Harnaschpolder, Delft, The Netherlands) that treats both primary and secondary sludge with a maximum solid content of 5% and which was operated at an SRT of 22 days. In the second stage of this study, both adapted thermophilic and mesophilic sludges were taken directly from the FSF-fed laboratory scale anaerobic digesters that were operated at a dry solids content in the range of 4% to 7%. Due to reactor instability, half of the biomass (about 120 gVS) in the mesophilic digester was replaced on day 402 by excess sludge that was collected from the same reactor. This excess sludge was collected each feeding period and stored for a maximum of two months at room temperature and no feeding applied. The biogas produced in the excess sludge container was released every two days.

3.2.4 Analytical methods

Total solids (TS) and volatile solids (VS) were determined on weight base (g/kg) according to the standard methods for the examination of water and wastewater (APHA, 2005). Chemical oxygen demand (COD) was measured in the range 500-10,000 mg/L. All analysis were done in triplicate.

Volatile fatty acids (VFAs) were quantified by Gas Chromatograph (GC, Agilent Technology 7890A), using a flame ionization detector (FID) and a capillary column type HP-FFAP Polyethylene Glycol (25 m × 320 μm × 0.5 μm) with helium as the carrier gas at a total flow of 67 ml min^{-1} and a split ratio of 25 :1. The GC oven temperature was programmed to increase from 80 °C to 180 °C in 10.5 min. The temperatures of injector and detector were 80 °C and 240 °C, respectively, and the injected volume was 1 μl. Prior to GC analysis 10 ml of digested samples was first centrifuged at 15,000 rpm for about 15-20 minutes. Then the supernatant was filtrated over 0.45 μm filter paper. The filtrated liquid was diluted 2 and 3 times with pentanol as internal solution (300 ppm) for mesophilic and thermophilic digestion samples, respectively. Finally, 10 μL of formic acid (purity >99%) was added into the 1.5 mL vials.

Gas composition (CH_4, CO_2) during reactor experiments was measured by using a GC (Agilent 7890A, with an Agilent 19095P-MS6 + 19095P-UO4 (60 m×530 μm×20 μm) column) equipped with a thermal conductivity detector (TCD). Helium was used as a carrier gas with a split flow of 10 ml/min and operation conditions were: Oven 100 °C (45 °C for 6 min then 25 °C/min to 100 °C for 1.8 min), detector 200 °C and injection port 45 °C. The biogas production volume was measured using a Ritter Milligascounter (MGC-1 PMMA, Germany). The pH, temperature, biogas flow rate were continuously monitored using Labview software. Biogas results were then converted to standard temperature and pressure conditions (T=0°C and P=1atm).

3.2.5 Specific methanogenic activity (SMA)

Specific methanogenic activity (SMA) was used to determine the conversion rate of acetate into CH_4 in the anaerobic system. In this study, the SMA of the mesophilic and thermophilic sludge was determined using an Automated Methane Potential Test System (AMPTS II, Bioprocess Control, Lund, Sweden). The SMA was conducted using sodium acetate COD

(2 g/L) as substrate and supplemented by a medium consisted of a mixture of macronutrients, trace elements and phosphate buffer solution (Angelidaki and Sanders, 2004). The inoculum amount was determined by setting an inoculum VS to substrate COD ratio on weight base (I/S) of 2:1. SMA was calculated by the slope of the accumulated methane production curve (mL/d) divided by the grams of VS introduced in the bottle (inoculum). The final values were expressed in: gCH_4-COD/(gVS. d). Experiments were conducted in triplicate.

3.2.6 Anaerobic biodegradability

Anaerobic biodegradability (AnBD) was assessed as the experimental ultimate methane production (expressed in COD) over the initial tCOD of the substrate (Raposo et al., 2011). Giving the conversion 1 $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$, 1 g COD equals 350 mL of CH_4 at 273 K and 1 bar. It is noted that this theoretical approach does not take into account the needs for bacterial cell growth and their maintenance, which has been reported typically 5-10% of organic material degraded (Angelidaki and Sanders, 2004), meaning that not all the COD is methanised. Moreover, during bioconversion, non-methanised biodegradable or non-biodegradable intermediates may occur, lowering the actual methane yield of the substrate.

3.2.7 Reactor operation

Four water-jacketed laboratory mixed digesters with a working volume of 8 L were used in duplicate to conduct the digestion of FSF under both thermophilic and mesophilic conditions for more than 650 days at 55 °C and 35 °C, respectively. The first 333 days of the experiment were mainly used to adapt the sludges to the FSF, especially the thermophilic sludge (Chapter 2). At the same period, several biomethane potential (BMP) tests and SMA tests were conducted to monitor the conversion capacity and activity of the biomass at both conditions. In the first stage of the study (day 333-393), it was decided to operate both thermophilic and mesophilic digesters at an organic loading rate (OLR) of 5.5 kg COD/(m^3·day), applying an FSF COD and TS of 350±15 g/kg and of 25%, respectively. In the second stage of this study (day 393 till day 656), the OLR was increased stepwise from 2.5 kg COD/(m^3·day) and 5.5 kg COD/(m^3·day) to a maximum of 22 kg COD/(m^3·day) for both the mesophilic and thermophilic digesters, resulting in decreasing solids retention times (SRT). Table 3.1 presents the digesters' operation performance within the extended research period and batch cycle duration of two days. It is noted that the AnBD percentage was calculated

after achieving a relatively stable process and not over the whole period of the experiment. The experiments were carried out in duplicate and the error from average values for all different tested OLRs was less than 5%.

Table 3.1 Average organic loading rates (OLR) and SRTs at different periods and the corresponding biodegradation percentage for both mesophilic and thermophilic conditions

Conditions and operational days	OLR kg COD/(m^3·day)	Duration of operation (day)	Calculated SRT (day)	AnBD (%)
Mesophilic				
333-393	5.5	60	64	N.D*
393-550	2.5	157	128	53
550-608	3.4	58	107	54
608-624	6.7	16	53	45
624-644	10.0	20	36	45
644-650	13.5	6	27	44
650-656	22.0	6	16	14
Thermophilic				
333-550	5.5	217	64	60
550-608	6.7	58	53	57
608-624	10.0	16	36	58
624-644	13.5	20	27	47
644-656	22.0	12	16	34

*N.D: Not detected

3.2.8 Environmental scan electron microscopy and energy dispersive X-ray element analysis (ESEM-EDX)

The samples for ESEM and EDX analysis were collected from both mesophilic and thermophilic digesters that were operated at an OLR of 2.5 and 5.5 kg COD/(m^3·day), respectively. The structure of the substrate before degradation was studied using an ESEM equipped with an EDX elemental analysis system. Raw FSF and sludge samples were pretreated immediately after collection. Triplicated samples were washed for three times using phosphate-buffered saline (PBS) (Sigma Aldrich). Then the samples were air dried and ready for analysis. Before ESEM analysis, the samples were mounted on a 1 cm^2 metal support and kept in place with adhesive tape and observed with a Philips XL30 Series ESEM. An EDAM 3 EDS system (SUTW 3.3 EDX window and 128.0 eV EDX resolution) was applied to analyze the key elements.

3.2.9 Viscosity analysis

Fresh thermophilic and mesophilic sludge were sampled for rheology tests on day 526 when the operating OLRs were 5.5 and 2.5 kg COD/(m^3·day), respectively. Rheological characteristics were determined with a universal dynamic rheometer (Paar Physica UDS 200, Stuttgart, Germany) equipped with a waterbath for temperature adjustment. The software US200/32 (V2.30) was applied for programming and data logging. For this purpose, it was attempted to conduct the test at two different temperatures, i.e. 35 ^{o}C and 55 ^{o}C, for both types of sludge.

3.2.10 DNA extraction

Fresh biomass samples were washed by 1 × PBS and then centrifuged under 7000×*g* for 7 minutes. The supernatant was removed and the pellet was washed by PBS for a second time and centrifuged under 17000×*g* for 20 minutes. The supernatant was removed and the pellet was stored (less than one month) at -25 ^{o}C for DNA extraction. DNA extraction was performed using the MoBio UltraClean microbial DNA isolation kit (MoBio Laboratories, CA, USA). A minor modification of the manufacturer's protocol was that twice bead-beating (5 min) and heating (5 min) were applied in sequence in order to enhance the lysis of microbial cells. DNA isolation was confirmed by agarose gel electrophoresis. The quality of DNA was verified by Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA).

3.2.11 454 Pyrosequencing

The amplification and sequencing of the 16S rDNA gene was performed by Research and Testing Laboratory (Lubbock, TX, USA) with following primers: (1) U515F ('5-GTG YCA GCM GCC GCG GTA A-3') and U1071R ('5-GAR CTG RCG RCR RCC ATG CA-3') (Wang and Qian, 2009) were used for bacteria and archaea with a high coverage over 90% for each domain; (2) Arch341F ('5-CCC TAY GGG GYG CAS CAG-3') and Arch958R ('5-YCC GGC GTT GAM TCC AAT T-3') were used for archaea. The pyrosequencing was done using a Roche 454 GS-FLX system (454 Life Science, Branford, CT, USA) with titanium chemistry.

3.2.12 Post analysis of pyrosequencing data

The post analysis of pyrosequencing data was performed by combining different programs from the Quantitative insights into microbial ecology (QIIME) pipeline, version 1.6.0 (Caporaso et al., 2010).

3.2.13 Real-time qPCR

Real-time qPCR was performed using an ABI 7500 instrument (Foster City, CA, USA) with three primer sets, including Bac516-F-Bac805-R (for all bacteria), ARC787-F-ARC1059-R for all archaea, FTHFS-F-FTHFS-R for syntrophic acetate-oxidizing bacteria (Yu et al., 2005). qPCR amplification was done in a 20-μL reaction volume. Each reaction tube contained 10 μL 2×SGExcel FastSYBR Mixture (With ROX, Sangong Biotech, Shanghai, China), 8.6 μL dH_2O, 0.2 μL of each forward and reverse primer (1 pmol/ μL), and 1 μL of DNA template. Molecular grade water was used as a negative control. Triplicate PCR reactions were carried out for all samples and negative controls. The thermal cycling program consisted of 2 min at 50^{o}C, 1 min at 95 ^{o}C, followed by 40 cycles of 10 s at 95 ^{o}C, 35 s at X ^{o}C (X=56 for Bac516-F/Bac805-R, X=61 for ARC787-F/ARC1059-R, X=55 for FTHFS-F/FTHFS-R). Finally, a melting curve analysis was performed for verifying the specificity of PCR products; denaturation of 1 min at 95 ^{o}C, cooling of 1 min at 55 ^{o}C and then heat till 95 ^{o}C again, at a rate of 0.5 ^{o}C per cycle. The standard curves for the above primer sets were constructed using all strains of the samples. The target 16S rDNA gene sequences were amplified from each strain by PCR with the corresponding primer sets and cloned into pGEM-T Easy vectors (Sangong Biotech, Shanghai, China).

For each plasmid, a 10-fold serial dilution series ranging from 10^{10} to 10^{4} copies/mL was generated. The slopes of the plasmid standard curves were between -4.411 and -2.955, with a mean value about -3.313. The threshold cycle (C_T) values determined were plotted against the logarithm of their initial copy concentrations. All standard plasmids and 16S rDNA samples were amplified in triplicate.

3.2.14 SDS–polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the NuPage® electrophoresis system instructions (InvitrogenTM Life Technologies, Carlsbad, CA, USA). Protein separation was performed using a precast NuPAGE 4-12% Bis-Tris Gel (Novex® Life Technologies, CA, USA). Protein concentrations were determined with a Bradford assay using bovine serum albumin (BSA) as a reference. Each sample was mixed with lithium dodecyl sulphate (LDS) sample buffer with an additional 5 mM DTT and heated at 95 oC for 5 minutes. After heating, the mixtures were centrifuged at 13,300 g for 5 minutes and the supernatant was immediately applied to the gel. For samples 1 to 8, 25 µl sample was loaded, for samples 9 to 17, 5 µl was loaded. The electrophoresis was done in MES SDS running buffer (Novex® Life Technologies, CA, USA) at a constant voltage of 200 V for 35 minutes. Gels were subsequently stained with the Colloidal Blue Staining Kit (Novex® Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

3.3 Results and discussion

3.3.1 Composition and surface architecture of FSF

Composition and surface morphology of solid wastes are often considered determining factors to hydrolyzing processes (Sanders et al., 2000). Therefore, it is important to characterize the FSF in detail. First of all, environmental scan electron microscopy and energy dispersive X-ray (ESEM-EDX) analysis revealed carbon (60%-64%) and oxygen (20%-27%) as major elements in raw FSF materials (Chapter 2), while a previous thermographic test showed that cellulose accounted for 79% of the total mass and 84% of the organic dry mass of raw FSF (Ruiken et al., 2013). Secondly, the FSF that were collected at the different times showed a variation in dry matter concentration and composition dependent on seasonal influences (e.g. resulting in more leaves in the FSF in autumn), functioning of the fine sieve system, and FSF storage time and temperature in the on-site container (Chapter 2). In addition, all the FSF samples showed a high heterogeneity (Figure 3.2a). Based on the above and our experimental BMP data (Chapter 2 and Chapter 4), we estimate a varying cellulosic content between 60% and 80%, depending on mentioned seasonal and influent fluctuations.

Smooth fibres can be seen in the raw FSF (Figure 3.2b) and a partially hydrolysed fibre after 36 hours in the mesophilic digester can be also seen (Figure 3.2c), in which a lot of cracks were present. A series of step-by-step magnified figures were captured to show the hydrolysing process in the mesophilic digester after 24 hours, from which gel-like materials and irregular pores can be observed on the surface (Figure 3.2d-3.2f). The EDX analysis showed that more than 90% of the hydrolysed fibre surface was composed of carbon and oxygen (Table 3.2). A microbial consortium that was hidden inside the digested FSF matrix was captured in the sample taken from the thermophilic digester after 48 hours digestion and very clear coccoid-shape microorganisms can be seen (Figure 3.2g-i). The ESEM-EDX results also showed that calcium accounted for almost 2% in weight on the surface of those coccoid-shape microorganisms (Figure 3.2i, Table 3.2), which might be derived from the scavenged toilet paper, or from possible inorganic precipitations occurring near the microbial consortia.

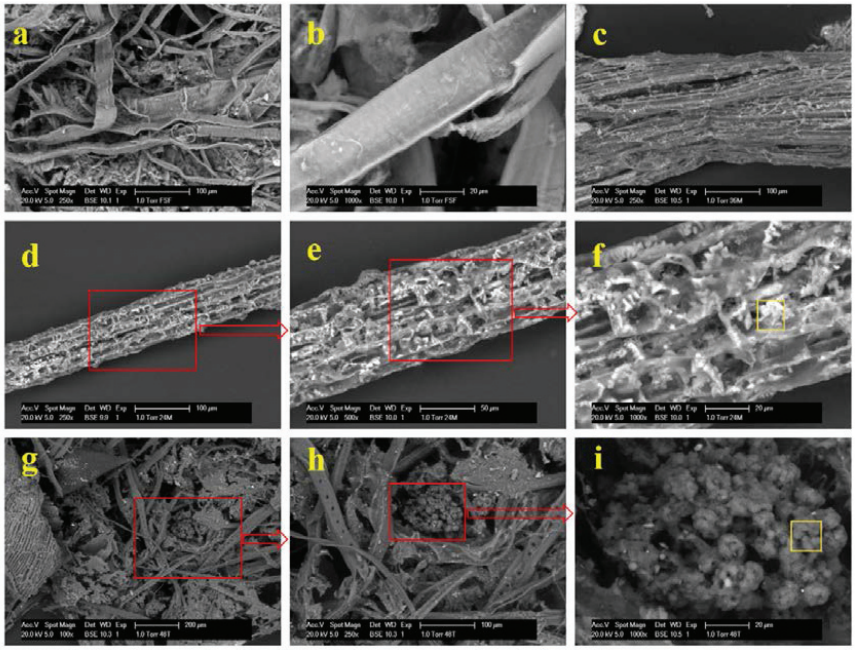

Figure 3.2 Scanning electron microscopic photograph of raw FSF matrix (a), smooth fibre in the raw FSF (b), partially fractured FSF after 36 hours in the mesophilic digester (c), step-by-step magnified sample from the mesophilic digester after 24 hours digestion (d-f), bacterial clusters within the digested FSF matrix sampled from the thermophilic digester after 48 hours digestion (g-i). The areas in red rectangles (d, g) represent the zoom-in areas of the subsequent figures (e and h), respectively. The areas in yellow rectangles (f and i) were the scanning location for the EDX analysis (results shown in Table 3.2)

Table 3.2 ESEM-EDX results revealing element content in the targeting spots of Fig. 1f and 1i

Elements	Fig. 1f		Fig. 1i	
	Weight (%)	**Atom (%)**	**Weight (%)**	**Atom (%)**
C	71.88	79.77	64.42	77.04
O	19.99	16.65	15.50	13.91
Na	2.10	1.22	3.26	2.04
Al	0.37	0.18	0	0
Si	0.94	0.45	0.40	0.21
P	0.69	0.30	0.78	0.40
S	0.75	0.31	1.79	0.83
Cl	1.67	0.63	1.85	0.83
Ca	1.16	0.39	1.95	0.70
Fe	0.45	0.11	0.56	0.14
Mg	0	0	0.27	0.16
K	0	0	0.27	0.10
Total	100.00	100.00	100.00	100.00

3.3.2 Biogas production and VFA accumulation

The start-up and adaptation of the mesophilic and thermophilic seed sludge to the FSF has been reported in comprehensively studied in our previous work (Chapter 2). A stable process was observed after almost a year of operation. Maximum daily accumulated biogas production rate for the thermophilic and mesophilic digesters was 2.5 L/($L_{reactor}$·day), from day 333 till day 393 at an OLR of 5.5 kg COD/(m^3·day) with FSF chemical oxygen demand (COD) of 350±15 g/kg and total solids (TS) of 25%, respectively (Figure 3.3a). The mesophilic digester became instable from day 393 (Figure 3.3a, 3.3b), indicated by a rapid decrease in pH and biogas production rate and increased TS from 4.2% on day 333 to 6% on day 396. The lowest registered pH of the digester was 6.3 on day 396 when the FSF digestion process was failing. In order to recover the reactor, feeding was stopped on day 396 and half of the mesophilic sludge in the digester was replaced with excess mesophilic sludge that was collected from the same digester before and stored at room temperature. Mixing both sludges led to a gradually increase in pH and to a drop in VFA concentrations (Figure 3.3b). Feeding of the mesophilic digester was restarted on day 407 and the OLR was reduced from 5.5 (SRT of 64 days) to 2.5 kg COD/(m^3·day) (SRT of 128 days) in order to avoid recurrence of the process instability.

In order to investigate the limits of the loading capacity of both digester systems (Figure 3.3c and Table 3.1), the OLR was increased stepwise from 2.5 to 22 kg COD/(m^3·day), leading to a decreased SRT from 128 to 16 days under mesophilic conditions. Therefore, the average biodegradation of the FSF slightly decreased from 53% at OLR of 2.5 kg COD/(m^3·day) to 44% at OLR of 13.5 kg COD/(m^3·day). Further increase in OLR to 22 kg COD/(m^3·day) led to an immediate process failure in the mesophilic digester, with the biodegradation decreasing to 14% (Table 3.1), acetate and propionate accumulating to 3.0 g/L and 2.6 g/L, respectively, and pH dropping to 5.7. It was notable that in this stage, at the applied SRTs, complete conversion of the FSF was not attained in both digesters, which was more strikingly for the mesophilic reactor.

For the thermophilic digester, the OLRs were continuously increased from 5.5 kg COD/(m^3·day) (day 333-550) to 22 kg COD/(m^3·day) (day 644-656), decreasing the SRT from 64 to 16 days (Figure 3.3c and Table 3.1). When the OLR was increased to 13.5 kg COD/(m^3·day) the biodegradation reduced from 60% to 47% (Table 3.1). A further increase to 22 kg COD/(m^3·day), further reduced biodegradation to 34%; at this stage acetate

and propionate accumulated to 0.17 g/L and 1.10 g/L, respectively. Nevertheless, the thermophilic digester could be operated without process failure during this period, showing a relatively stable biogas production. It is notable that, when the feeding to the reactor was paused for several days in the period of applying the highest OLR, the thermophilic reactor produced almost 100 L of CH_4, which relates to the conversion of about 0.82 kg FSF, assuming a COD content of FSF at 350 g/kg. The observed methane peak could be due to the further degradation of accumulated FSF in the digester. When the feeding was restarted, the biogas production rate was fully restored.

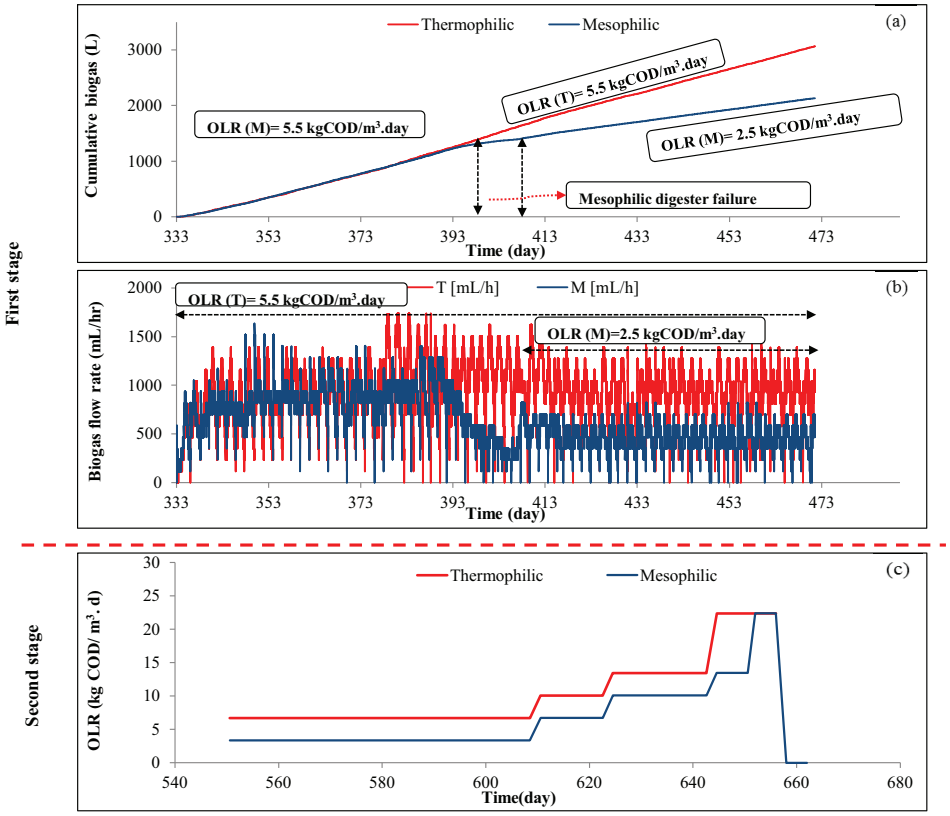

Figure 3.3 Cumulative biogas production (in L, a), biogas flow rate in the thermophilic (T) and mesophilic (M) digesters (in mL/hr, b) and OLR in the thermophilic and mesophilic conditions (in kg COD/(m^3·day), c)

Figure 3.4 shows the VFA concentrations in both thermophilic and mesophilic digesters during recovery of the mesophilic sludge (day 402-day 424). Restarting the batch wise feeding regime of the mesophilic digester at day 407, resulted in an increased acetate

concentration at the end of first two cycles. The reduced OLR of 2.5 kg COD/(m^3·day), however, was sufficient for stabilising the mesophilic reactor, indicated by decreased acetate concentrations and stable biogas production (Figure 3.3b). Within the VFA spectrum produced, acetate and propionate were observed in the highest concentrations under both conditions (Figure 3.4).Under mesophilic conditions a gradual pH decrease to 6.3 was observed from day 396 to 402, whereafter the VFA concentrations were measured from day 402 to day 424. Decrease in VFA concentrations (day 402 to day 407) is mainly due to: (1) replacing half of content of the mesophilic digester with excess mesophilic sludge collected and stored from the same digester (2) no feeding from day 402 to 407 in order to stabilize the conversion and to recover the sludge (Figure 3.3a & 3.b). After 15 days (day 424) the anaerobic digestion was considered stable again.

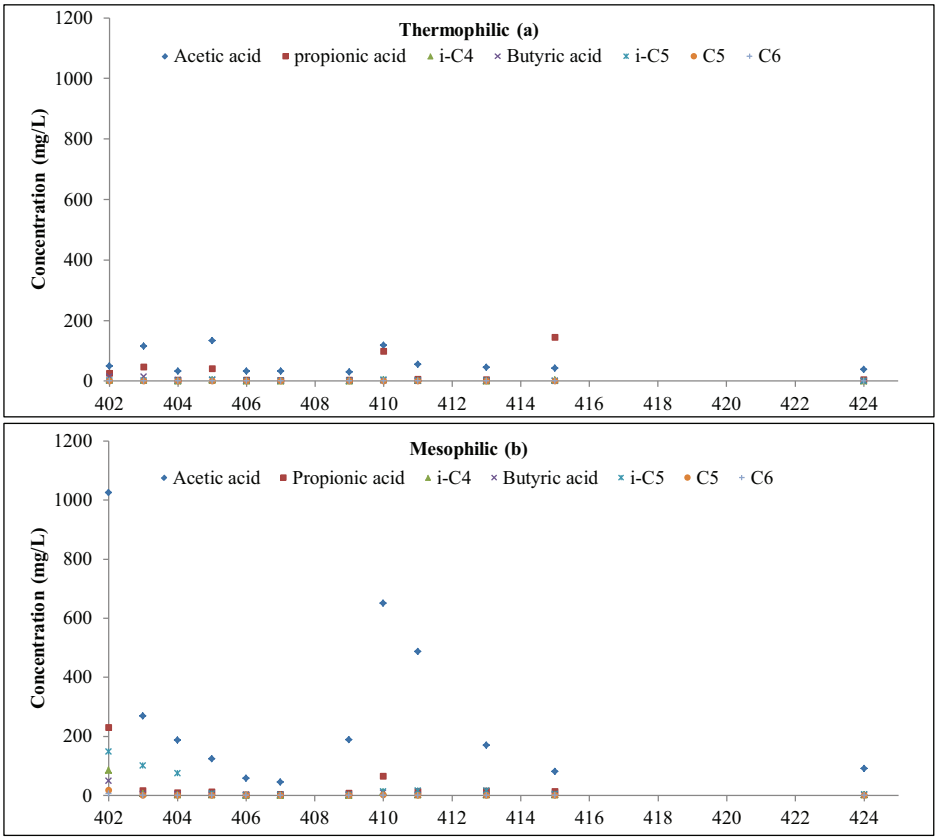

Figure 3.4 VFA concentrations in the thermophilic reactor (a): batch wise feeding occurred at the even days: odd numbered days represent 24h after feeding, while even days represent 48h after feeding. The VFA concentrations in the mesophilic reactor (b) were measured during recovery and after the batches of FSF at day 407; hereafter even numbered days represent 24h after feeding, while odd days represent 48h after feeding

3.3.3 Specific methanogenic activity (SMA) of mesophilic and thermophilic biomass

To investigate the activity of the methanogens after the mesophilic reactor recovered, four SMA tests were conducted at day 407 (Figure 3.5a), day 416 (Figure 3.5b), and day 462 (Figure 3.5c), after which the reactor was considered to be fully stable again. The SMA of the thermophilic sludge was determined at day 407 only (Figure 3.5d). The results showed that the activity of the methanogenic organisms was indeed low at reactor failure, while a long period without VFA accumulation resulted in an increased acetate consumption rate, however not exceeding 0.1 g COD-CH_4/(gVS·day) at day 462. This value was considerably lower than the conversion rate measured from the thermophilic sludges, which was 0.6 g COD-CH_4/(gVS·day) at day 407, higher than most of previously reported values (Table 3.3). This activity remained the same at the fixed OLR of 5.5 kgCOD/(m^3·day). The measured high SMA values of the thermophilic sludge are congruent with the observed high reactor stability at increased OLRs, whereas the observed low SMA values of the mesophilic digester agreed with instability and recovery period between day 402 to day 424.

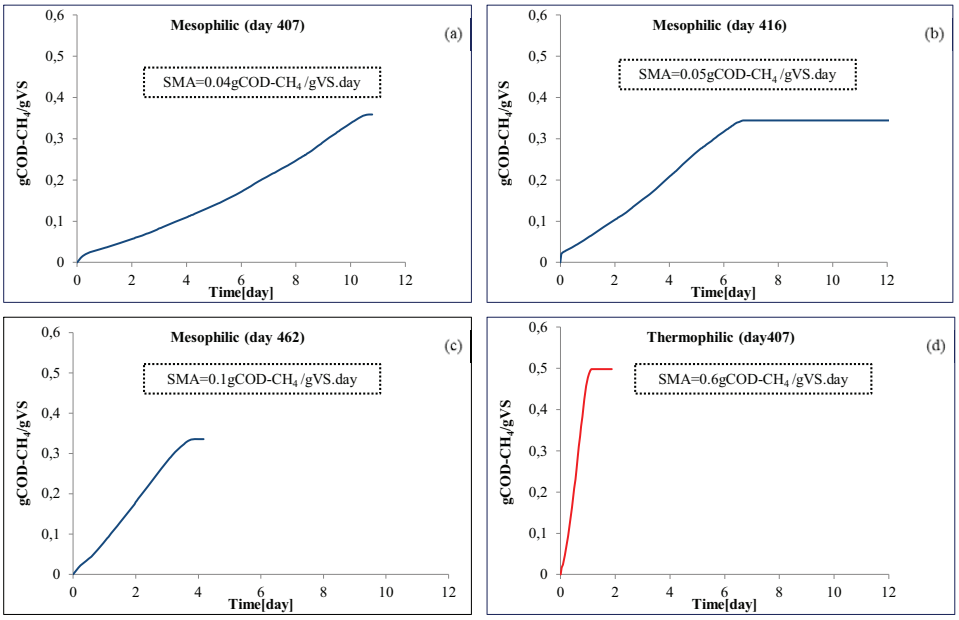

Figure 3.5 Specific methanogenic activity (SMA) conducted during recovery of the mesophilic digester at OLR of 2.5 kg COD/(m^3·day) (a,b,c) and of the thermophilic digester as comparison (d) at OLR of 5.5 kg COD/(m^3·day)

Table 3.3 Comparison of SMA values of this study with previous studies done on (co-)digestion of lignocellulosic wastes

Substrate type	Reactor	Temperature °C	SMA gCOD-CH_4/(gVS·day)	OLR[a] kg COD/(m^3·day)	Reference
Pre-hydrolyzed brewers' spent grain	EGSB	35	0.49[d]	10	(Wang et al., 2015b)
Cellulose powder	Batch digestion assays	35	0.18	N.A[b].	(Jensen et al., 2011)
Pig manure and rice straw	Batch digestion assays	35	1.31[d]	N.A[b].	(Jiménez et al., 2015)
Pig manure and rice straw	Batch digestion assays	55	1.38[d]	N.A[b].	(Jiménez et al., 2015)
Corn straw	Full-scale CSTR	37	0.2[d]	1.2[c]	(Qiao et al., 2013)
Agro-industrial wastes	Batch digestion assays	55	0.13	N.A[b].	(Pagés Díaz et al., 2011)
FSF	SBR Digester	35	0.1	2.5	This study
FSF	SBR Digester	55	0.6	5.5	This study

[a] The same period when the SMA of biomass was tested.
[b] N.A., data not available
[c] the unit here is kg TS/(m^3·day)
[d] gCOD-CH_4/(gVSS·day)

3.3.4 Rheology of mesophilic and thermophilic sludge

Sludge rheology determines to a large extend the prevailing mixing in reactor systems and the contact between bacteria and its substrate. Rheology of sludge is defined by its viscosity, which is a function of applied shear rate and shear stress. Sewage sludges are often non-Newtonian fluids, because the shear rate is not linearly proportional to the applied shear stress (Dentel, 1997).

Fresh thermophilic and mesophilic sludge were sampled for a rheology test when thermophilic and mesophilic digesters were operated stable (day 526) at an OLR of 5.5 and 2.5 kg COD/(m^3·day), respectively. The rheogram of both mesophilic and thermophilic sludge was plotted at 35 °C and 55 °C (Figure 3.6). The thermophilic sludge showed a viscoplastic fluids type at 35 ^oC, while it changed to a Bingham fluids type at 55 ^oC (Figure 3.6). The mesophilic sludge showed a pseudoplastic fluids type at both temperatures (Figure 3.6). Both viscoplastic and pseudoplastic fluids have shear thinning effect. An Ostwald de Waele Power law model (Figure 3.6) can be used to describe the rheology behaviour of mesophilic sludge and the thermophilic one that was tested at 35 ^oC (Eshtiaghi et al., 2013). It is notable that the thermophilic sludge behaved like Newtonian fluids at 55 ^oC, which meant that the shear thinning effect disappeared when the operational temperature increased from 35 ^oC to 55 ^oC. Meanwhile, the viscosity value of the thermophilic sludge at 55 ^oC was lower than at 35 ^oC, which was only 1/18 and 1/34 of the values of the mesophilic sludge tested at 55 ^oC and 35 ^oC, respectively, based on the consistency behaviour (0.006 and 0.0931 for thermophilic sludge at 55 ^oC and 35 ^oC, 1.7347 and 3.1694 for mesophilic sludge at 55 ^oC and 35 ^oC) from the Ostwald de Waele Power law model (Figure 3.6). The thermophilic sludge was characterised by a lower viscosity compared to the mesophilic one.

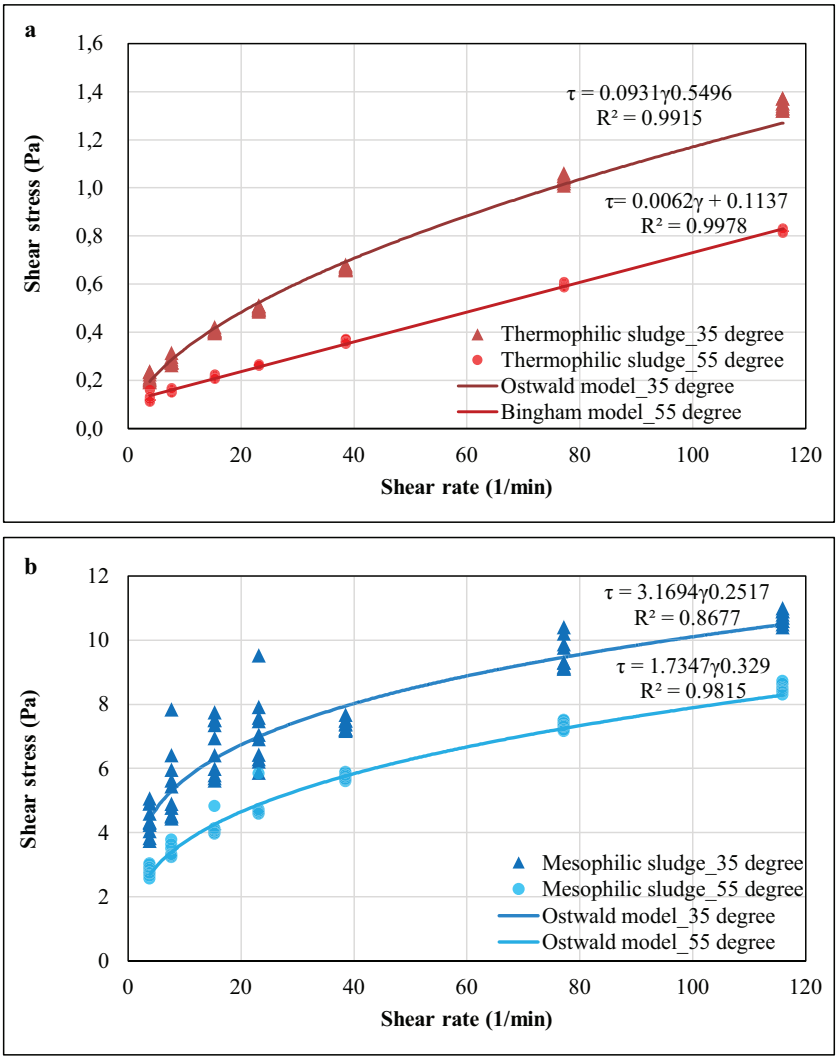

Figure 3.6 Rheogram of the thermophilic (a) and mesophilic (b) sludge

3.3.5 Microbial population dynamics in the mesophilic reactor

454 pyrosequencing was applied in order to investigate the dynamics of bacterial and archaeal populations under both temperature conditions. The changes in microbial communities in the mesophilic reactor before process failure and during recovery were followed closely as well. As expected, the microbial taxa analysed based on a total of about 78,000 sequences showed differences in population between the thermophilic and mesophilic digester over time.

Bacteroides was the sole dominant genus in the mesophilic digester after acclimation (Figure 3.7), with variation in its relative abundance from the highest 90% (sample day 456) to the lowest 46% (sample day 608). The second predominant genus in the mesophilic digester is *Anaerolinea*, which accounts for 5%-24% in relative abundance. *Parabacteroides* owned 34% of abundance in the sample of day 327 and 1%-12% in the other samples from the other periods, suggesting its dominant position in the bacterial community as well. *Parabacteroides* are obligatory anaerobic, short rods that were frequently discovered in anaerobic environments with capability of producing various of acids such as lactic acid, propionic acid, formic acid and acetic acid from carbohydrates (Tan et al., 2012). For archaeal lineages in the mesophilic digester, the genus of *Methanosaeta* owned the substantial proportion from 81% to 94% followed by *Methanobacterium* (2%-11%). *Methanosaeta* is a typical aceticlastic methanogen that prevails in many anaerobic biogas systems (Vanwonterghem et al., 2014). *Methanobacterium* belongs to group of hydrogenotrophic methanogens (Stantscheff et al., 2014).

About half of the biomass in the mesophilic digester was replaced by stored excess sludge on day 402. Although the exact composition of this excess sludge was unknown, it was anticipated that this microbial community was alike the reactor content before the disturbance at day 393, given the fact that excess sludge was stored without feeding, at room temperature and anaerobic conditions. Every two days, fresh excess sludge was added to the storage and a new storage container was started every two months.

The OLRs of the mesophilic digester were firstly increased to 5.5 kg COD/(m^3·day) during days of 333-393 and then decreased to 2.5 kg COD/(m^3·day) from day 393 to day 550, due to VFA accumulation and failure in biogas production. Hereafter, the OLR was increased again, stepwise from 2.5 kg COD/(m^3·day) to a maximum of 22 kg COD/(m^3·day). The variation in OLRs directly altered the SRTs of the reactor, which was anticipated to influence the microbial community as well. The abundance of *Bacteroides*, a key genus responsible for hydrolysing and fermenting polysaccharides (Li et al., 2013), increased by more than two times when the duration of the batch feeding cycle was shortened from 9 days, on average, (day 327, SRT 128 days, OLR 2.5 kg COD/(m^3·day)) to 2 days (day 375, SRT 64 days, OLR 5.5 kg COD/(m^3·day)). The resulting low pH could further favour the growth of *Bacteroides* (Feng et al., 2009), which experienced a sudden increase during day 394 to 398 (data not shown). Moreover, in the same period, the archaeal community quantity decreased by 60%

from day 327 to 375, while the homo-acetogenic, syntrophic acetate oxidising (SAO) bacteria increased more than three times. SAO bacteria are specialized to oxidize acetate into H_2 and CO_2. It was noticed that the thrived growth of the acetate oxidising bacteria could not prevent VFA accumulation, possibly because of an even higher productivity of VFAs from fermenting genera such as *Bacteroides*.

3.3.6 Microbial population dynamics in the thermophilic reactor

The thermophilic reactor harboured a substantially different microbial community assembly. The predominant bacterial genus altered from OP9 lineage in the samples taken during days 456-474 (relative abundance about 56%-62%) to *Clostridium* in the samples taken after day 594 (relative abundance about 32%-47%), while OP9 was dominant again (relative abundance of 50%) in the sample of day 628. Members of OP9 have been occasionally observed in anaerobic digestion reactors. For example, they were found to be able to hydrolyse complex carbohydrates such as cellulose under thermophilic conditions (Dodsworth et al., 2013; Zhang et al., 2013). In line with the hydrolytic community, the methanogenic community of the thermophilic digester was also completely different from the mesophilic one. *Methanosaeta* was found to be the absolutely dominant archaeal genus (91%) until day 273 of the thermophilic digestion, while the hydrogenotrophic methanogen *Methanothermobacter* was only 7% in relative abundance in the sample of day 273. However, *Methanothermobacter* became the most dominant methanogen afterwards in the thermophilic reactor until the end of operation, having a relative abundance of 60%-98% (Figure 3.7).

In the thermophilic reactor, the OLR was stepwise increased from 5.5 kg COD/(m^3·day) to 22 kg COD/(m^3·day). *Bacteroides*, OP9 and *Clostridium* were three dominant bacterial genera with total abundance over 50% under OLR lower than 5.5 kg COD/(m^3·day) and thus at higher SRTs (samples of day 273 and day 327).By increasing the OLR to 5.5 kg COD/(m^3·day), OP9 was the sole dominant genera with abundance over 50% (Figure 3.7). When the OLR was further increased to 6.7 kg COD/(m^3·day) (day 550-608) the dominancy of OP9 was replaced by *Clostridium*, but OP9 again dominated the bacterial community after OLR was increased to 13.5 kg COD/(m^3·day). *Bacteroides* was dominant once during the start-up period (before day 333) but its abundance decreased and varied in a range between 0.1% and 14% after the start-up (Figure 3.7).

It is interesting that *Methanosarcina*, a group of versatile methanogens that are capable of both aceticlastic methanogenesis and hydrogenotrophic methanogenesis, became dominant (13%-39%) during the same period that *Clostridium* replaced OP9 as the most dominant genera based on the samples of day 594 and 608 (Figure 3.7). Then the *Methanosarcina*'s abundance decreased to 4% on day 628 when *Clostridium* was no longer dominant. *Clostridium* are also versatile microorganisms capable of fermenting cellulose and various carbohydrates into acetate, butyrate, carbon dioxide, and hydrogen (Park et al., 2015; Valdez-Vazquez and Poggi-Varaldo, 2009). In line with our observations, it has been reported before that *Clostridium* co-exists with *Methanosarcina* in anaerobic digestion under thermophilic conditions (Palatsi et al., 2010).

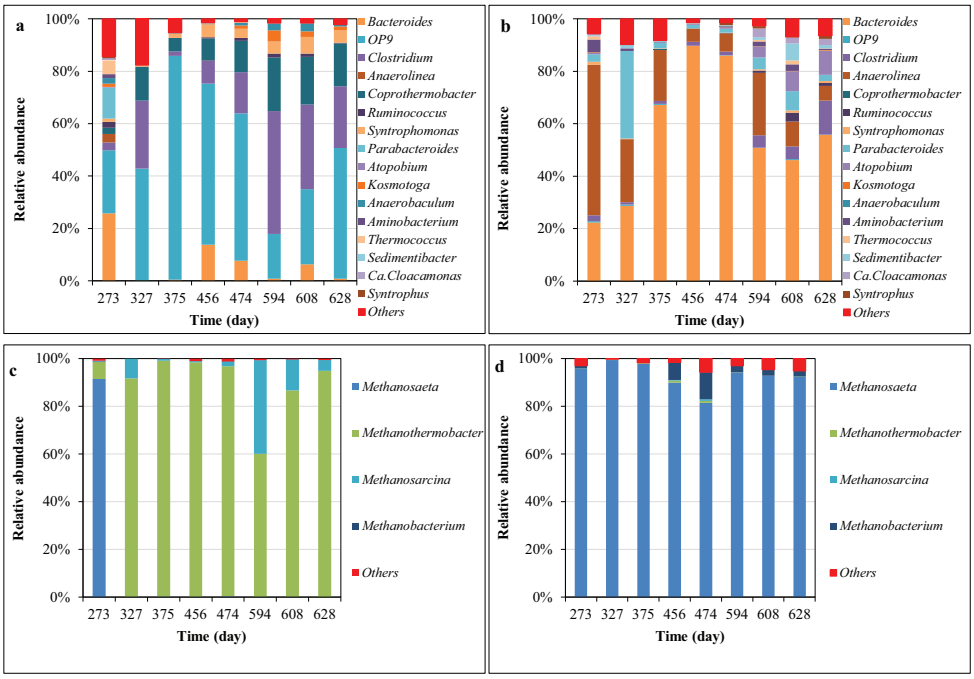

Figure 3.7 The dynamics and relative abundance of the dominant genera in function of time. a, bacterial community in the 55°C digester; b, bacterial community in the 35°C digester; c, methanogenic community in the 55°C digester; d, methanogenic community in the 35°C

3.3.7 Quantity and activity of microorganisms

Pyrosequencing data provide taxa information and their relative abundance, but will not give insight into quantities. In this study, qPCR tests based on bacterial, archaeal and SAO bacterial 16S rRNA genes were employed to give insight in specific quantities of bacteria and archaea in general, and SAO bacteria specifically. This analysis showed that the thermophilic reactor harboured marginally higher level of bacterial, archaeal and SAO bacterial 16S rRNA gene copies than the mesophilic reactor (Figure 3.8).

For the mesophilic reactor, bacteria had a higher quantity than the archaea and SAO bacteria (Figure 3.8). The quantity of bacterial 16S rRNA gene copies had small perturbation around 10^{12} to 10^{13} gene copies per gram sludge, except for a decrease in the sample of day 608 (Figure 3.8), during which period the OLR was increased from 3.4 to 6.7 kg COD/(m^3·day). The archaeal quantity also fluctuated within the magnitude from 10^{10} to 10^{11} gene copies per gram sludge. The changing trend of archaeal amount was similar to that of bacteria. The SAO bacterial quantity was considerably lower than the other two and fluctuated from 10^9 to 10^{10} gene copies per gram sludge. It is notable that the concentrations of both bacterial and archaeal gene copies decreased from day 375 to day 456. This is possibly linked to the pH drop on day 396 and the re-inoculation of stored mesophilic biomass on day 402.

For the thermophilic digester, the quantity distribution was quite similar to the mesophilic reactor, with bacteria having the largest quantity. SAO bacterial amount was stable around 8×10^9 gene copies per gram sludge, while the bacterial and archaeal quantity substantially dropped from day 474 to 594, which coincided to a sudden shift in the dominant bacteria from OP9 to Clostridium. Seasonal differences, changing the raw FSF characterises might have contributed to this change in microbial community. The archaeal quantity varied nearly two magnitudes between the samples taken from day 327 and day 628.

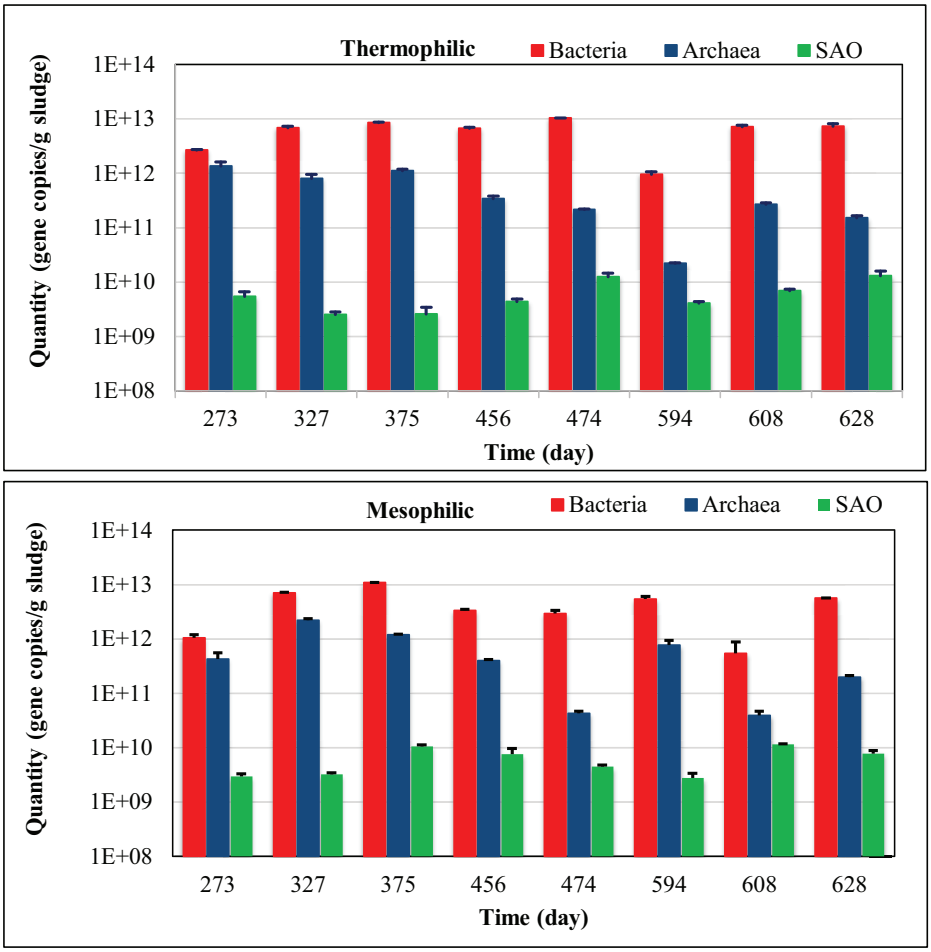

Figure 3.8 qPCR revealing the quantity of bacteria, archaea and SAO bacteria in the thermophilic reactor (top) and mesophilic reactor (bottom) at different stages of operating period. Some error bars are too small to be invisible in above logarithmic co-ordinates

Variation in community structure and microorganisms quantity was observed in both the mesophilic and thermophilic digester, however, the thermophilic digester performed more stably and robust than the mesophilic one. Considering the high percentage of cellulose inside FSF, there likely were more hydrolytic enzymes generated by the thermophilic sludge than by the mesophilic one. The protein analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and coommassie staining showed that the thermophilic sludge contained more protein than the mesophilic sludge (p<0.01, Student test) (Table 3.3, Figure 3.9). The protein concentration of the mesophilic sludge fluctuated within the range of 14-72 μg/mL without an obvious trend, while the protein concentration of the

thermophilic sludge almost increased by three times from day 356 (OLR=5.5 kg COD/(m^3·day)) to day 624 (OLR=10 kg COD/(m^3·day)).

The quantity of microorganisms and their released proteins together can indicate the efficiency of an anaerobic digester, which is generally, a direct response to operational parameter(s). From an engineering retrospective, the food to mass ratio (F/M ratio) or OLR, highly determines the design and applicability of a process. The higher the applicable OLR, the compacter the reactor and thus the more biogas can be produced per reactor volume. However, overloading a digester could lead to process instability due to accumulated VFA and decreased pH (as on day 396). In our study, both reactors were applied with varying OLRs, which has been strongly indicated as a driving force altering the digesters' microbiome (Jang et al., 2014; Wang et al., 2015a).Our results indicated that the applicable OLRs to a mesophilic digester was usually lower than that to a thermophilic process, possibly due to a limitation of methanogenic capacity (Figure 3.5) and an instable methanogenic community (Figure 3.7d) under mesophilic conditions. However, a very high OLR of 22 kg COD/(m^3·day) could be applied to the thermophilic digester where hydrolytic bacteria were abundantly present (Figure 3.7a) and large amount of hydrolytic enzymes could be observed (Table 3.4, Figure 3.9).

Table 3.4 Samples for SDS-PAGE & coommassie staining and protein concentrations for mesophilic and thermophilic sludges

Sample No.	Day	OLR kg COD/(m^3·day)	Protein concentration µg /mL
Mesophilic sludge			
1	273	1-2.5	64.4
2	306	1-2.5	52.4
3	356	5.5	34.4
4	540	2.5	52.4
5	571	3.4	14.4
6	600	3.4	48.4
7	624	10	72.4
Thermophilic sludge			
8	273	1-2.5	182.4
9	306	1-2.5	152.4
10	356	5.5	148.4
11	540	5.5	238.4
12	571	6.7	256.4
13	600	6.7	292.4
14	624	10	434.4

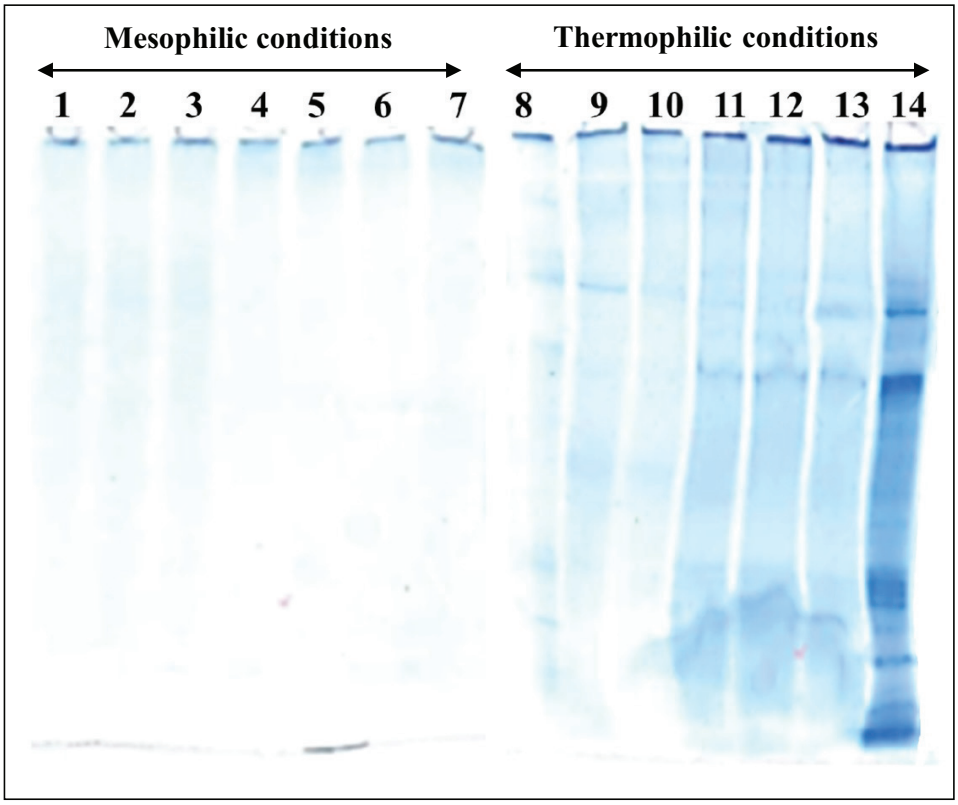

Figure 3.9 SDS-PAGE & coommassie staining of mesophilic (1-7) and thermophilic (8-14) sludge that were sampled at different time of operation (Table 3.3)

3.3.8 Microbial diversity

Both α- and β-diversity were analysed based on 454-pyrosequencing raw data. The α-diversity is defined as the diversity of organisms in one sample or environment and the β-diversity is the difference in diversities across samples or environments. We employed phylogenetic distance (PD), the observed number of operational taxonomic units (OTUs), Chao1 and Shannon index to fully characterize the α-diversity of each community, all of which are commonly used in recent years (Navas-Molina et al., 2013). For β-diversity, UniFrac method is considered to be very useful in revealing biologically meaningful patterns (Navas-Molina et al., 2013) and so was applied in this study (Figure 3.10).

Our results showed that the bacterial communities, for both thermophilic and mesophilic digesters, owned higher richness and evenness than the archaeal communities (Figure 3.11).

Both thermophilic and mesophilic bacterial community shared similar level of evenness (Shannon index, Figure 3.11a, 3.11b), while the archaeal community of the mesophilic reactor (Shannon index, Figure 3.11d) showed a lower level of evenness than the thermophilic reactor (Shannon index, Figure 3.11c). The observed species number of the thermophilic community (both bacterial, Figure 3.11a, and archaeal, Figure 3.11c) was more stable than the mesophilic one (Figure 3.11b, 3.11d). However, the β-diversity results proved that there was a stable archaeal community in the mesophilic digester as all blue dots were close to each other (Figure 3.10a). Also, the bacterial community in the mesophilic digester was more stable across day 273 through day 628 compared to the thermophilic reactor (Figure 3.10b). In other words, the dynamics of the thermophilic community was more intense than that of the mesophilic community.

Vanwonterghem et al. (2014) suggested that a deterministic process was important to microbial community dynamics in long-term operated anaerobic digesters, when a selective pressure imposed by the operational conditions existed (Vanwonterghem et al., 2014). In this study, the thermophilic reactor reveals a clear evolution of the bacterial community (Figure 3.10a), whereas the archaeal community in the same reactor showed a weaker pattern of evolution. The performance of the thermophilic reactor was primarily disclosed by the structure and functionality of bacterial community. Two important facts played a role: (1) the hydrolytic populations were consistently and strongly adapted during the OLR increase and (2) an efficient syntrophic relationship was eventually established at high OLRs. From this point of view, our study indicated that a deterministic process also guided microbial community dynamics in a thermophilic digester. For the mesophilic reactor, it is difficult to conclude a deterministic or a stochastic process of population dynamics because of the re-inoculation in the middle stage of the operation.

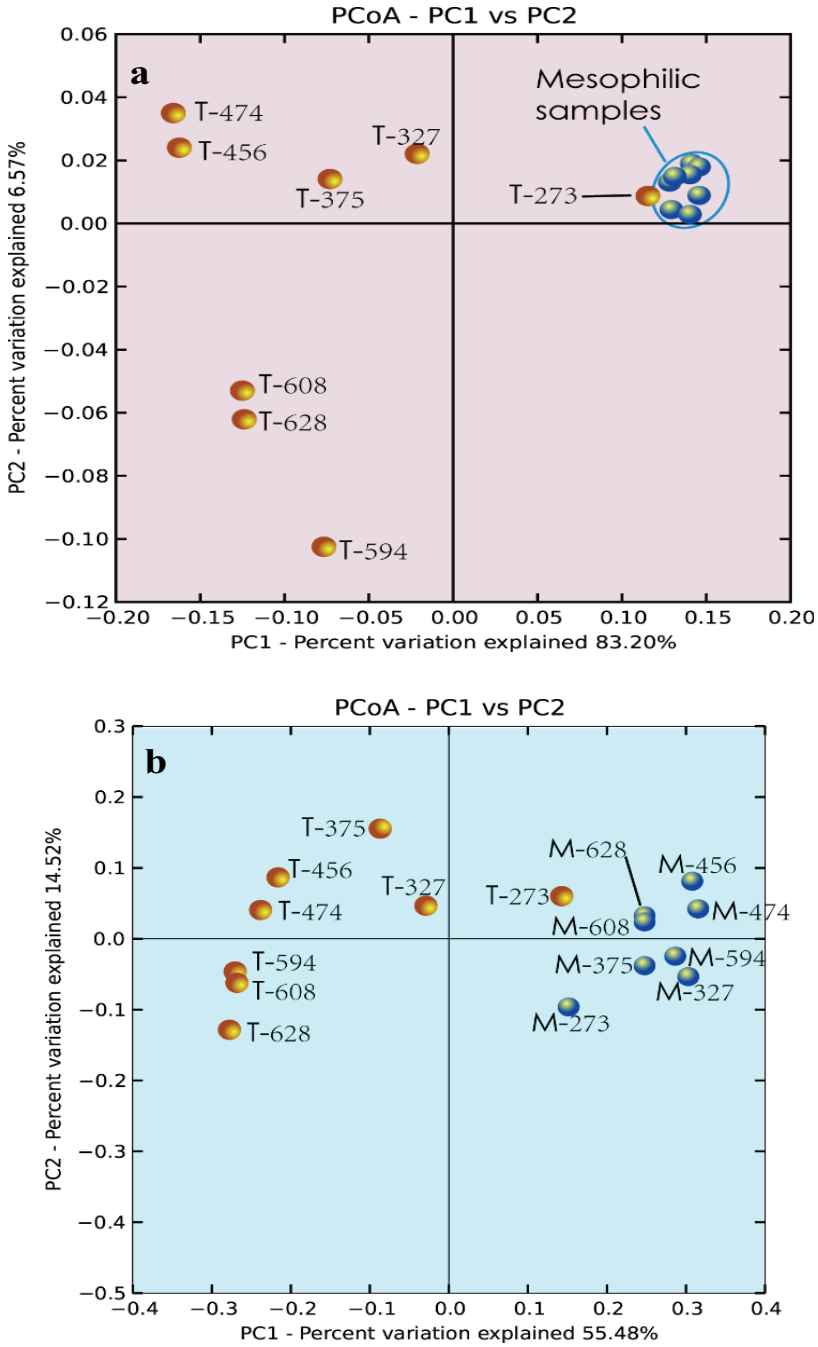

Figure 3.10 Principal coordinate analysis (PCoA) of bacterial (a) and archaeal (b) communities of the biomass that were sampled at different times from the thermophilic (marked with T) and mesophilic (marked with M) reactors

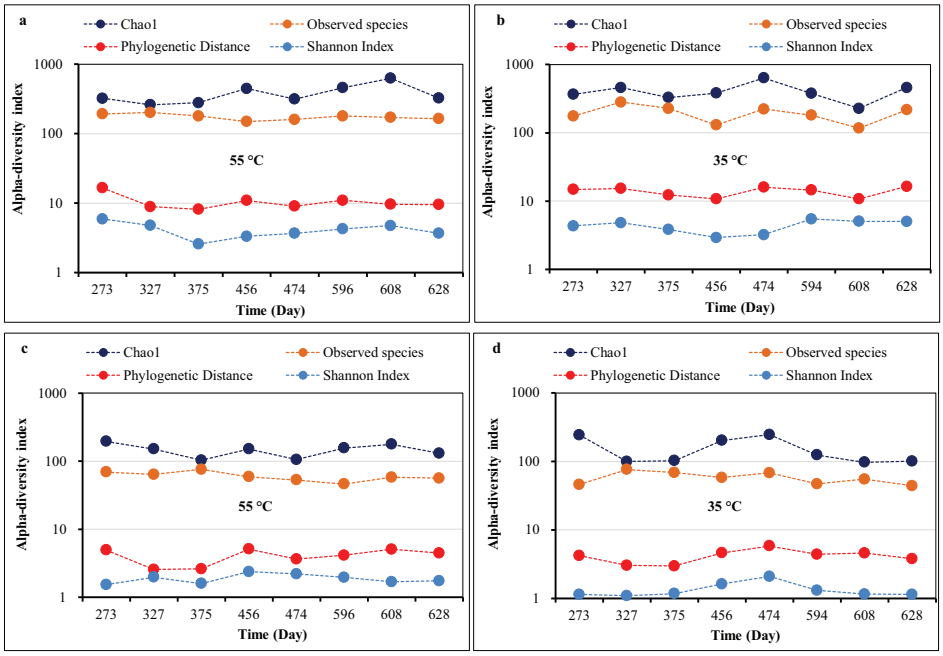

Figure 3.11 Alpha-diversity of bacterial and archaeal community at different stages in the thermophilic and mesophilic reactors. a, bacterial community in the thermophilic digester; b, bacterial community in the mesophilic digester; c, archaeal community in the thermophilic digester; d, archaeal community in the mesophilic digester

3.4 Conclusions

FSF as a new type of concentrated substrate sequestered from raw municipal sewage contains a high fraction of cellulosic fibres, originating mainly from toilet paper, hence FSF is rich in chemically bound energy. Our study demonstrates that FSF from a domestic sewage treatment plant that has 23% dry solids content can be digested either under thermophilic (55 °C) or mesophilic (35 °C) conditions. The long-term adapted microbial communities at 55 ^{o}C and 35 ^{o}C were distinctly different in composition and population dynamics. In the thermophilic community, OP9, *Clostridium,* and *Methanothermobacter* were the dominant species whereas in the mesophilic community *Bacteroides* and *Methanosaeta* were predominant. Eventually, the thermophilic digester produced biogas stably at an extreme loading rate of 22 kg COD/(m^3·day). In addition, the thermophilic sludge had a considerably lower viscosity than the mesophilic sludge. Results clearly show the high rate potentials of thermophilic conditions for the digestion of sewage FSF under extreme loading conditions.

3.5 References

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Chapter 4. High-rate thermophilic bio-methanation of the fine sieved fraction from Dutch municipal raw sewage: Cost-effective potentials for on-site energy recovery

Abstract

Sieving of Dutch raw sewage over a 350 μm screen, produces a cake layer called fine sieved fraction (FSF), an energy-rich material that contains mainly cellulosic fibres originating from toilet paper. The FSF biomethane potential (BMP) was studied under both mesophilic (35°C) and thermophilic (55°C) conditions, whereas the stability of the fed-batch digesters at both 35°C and 55°C was researched by varying the inoculum to substrate ratios ($R_{I/S}$: 0.5-15). Results clearly showed advantages of thermophilic conditions over mesophilic conditions at all tested $R_{I/S}$. Stable digestion was even possible at an $R_{I/S}$ of 0.5 at 55°C.

Following the results of the batch tests, a compact high loaded thermophilic digester for on-site energy recovery from FSF was proposed. Based on the results of the study, biogas production rates of 9.3 m^3/m^3.d (with 53% CH_4 in the biogas) at organic loading rates (OLRs) reaching 22.8 kgCOD/m^3.d, are predicted. In the energy balance calculations, surplus heat production from combined heat and power (CHP) was utilized to dry the digestate sludge before transportation to an incineration plant or for use in pyrolysis or gasification processes. Overall results showed the potential of generating 46% of the required energy for wastewater treatment via high rate FSF digestion and subsequent conversion of the bio-methane into electricity and heat. The net recoverable energy from fine sieving, anaerobic digestion of FSF, dewatering of digestate sludge and drying of dewatered digestate sludge, amounted 287 MJ/ton FSF and 237 kWh electric/ton FSF at 23% TS.

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4.1 Introduction

Energy recovery from raw municipal sewage for on-site use will minimize the fossil energy demand and contribute to the development towards energy neutral wastewater treatment plants (WWTPs). In principle this should be possible since the energy content of sewage is several times higher than the energy required for its treatment (Cao, 2011; Jenicek et al., 2013). For indeed achieving energy neutrality, or even energy production, at WWTPs, the energy balance should be optimized which requires a dual approach. Firstly, the total energy consumption during wastewater treatment requires optimization, such as more energy-efficient aeration and less energy losses in pumping and sludge dewatering. Moreover, implementing enhanced primary sludge production and alternative routes for nitrogen removal can drastically reduce the use of fossil fuels for aeration. Secondly, the recovery of chemically bound energy should be maximized, requiring an upgraded anaerobic digestion (AD) technology as well as the implementation of AD at those WWTPs that so far are not served by AD, such as extended aerated biological nutrient removal plants (Cao, 2011; Cavinato et al., 2013; Chudoba et al., 2011; Jenicek et al., 2013; Yazdani et al., 2012).

The biggest energy gains per m^3 of sewage can be made in small WWTPs that are not equipped with primary clarifiers and that apply low sludge loading rates. In these systems, all incoming biochemical oxygen demand (BOD), as well as a large extent of the newly grown sludge is converted aerobically. With a biomass growth yield of 0.6 g volatile suspended solids (VSS)/g BOD and a sludge degradation efficiency of 30-50% during digestion, one can easily reason that a large part of influent BOD is lost for energy recovery during the activated sludge treatment process (Metcalf & Eddy, 2003). In Western industrialized countries, a significant part of the sewage BOD consists of cellulose (60%-80% of total solids content), originating from the use of toilet paper (Chapter 2; Ruiken et al., 2013). Conventionally, a significant part of this cellulose fraction is removed in large conventional primary settlers. If primary settlers are absent, or only part of the cellulose is retained, the cellulose BOD is (partly) oxidized in the aeration tanks (Ruiken et al., 2013). A very compact and efficient solution to minimize oxidation of filterable matter in extended aeration tanks is the recovery of cellulose-rich slurries from raw sewage with a fine-mesh (< 500 μm) sieve. The derived fine sieved fraction (FSF) can then be used for on-site energy recovery through anaerobic digestion, instead of oxidation in the aeration tank. However, care should be taken that the required nutrient removal capacity remains unaffected.

At the WWTP Blaricum, The Netherlands, a 350 µm mesh size fine sieve (Salsnes Filter, Norway) for raw sewage mechanical pretreatment is installed after the coarse screen (6 mm) as a pilot study. This sieve was implemented as a compact alternative to primary clarification taking into account that the composition of the material coming from the fine sieve deviates from conventional primary sludge (Ruiken et al., 2013). At present, application of fine sieves receives growing interest in countries like The Netherlands, and water authorities are even exploring the recovery of cellulosic fibers for reuse. On the other hand, onsite bio-methanation of FSF at high dry solids contents, could contribute to the objective of drastically minimizing the fossil energy requirements at conventional WWTPs, eventually leading to energy neutral WWTPs (STOWA, 2010).

For anaerobic digestion, thermophilic (50-60°C) or mesophilic (30-40°C) conditions can be chosen (Golkowska and Greger, 2013; Kim et al., 2002; Yu et al., 2014). The rate of many, if not most, (bio)chemical reactions double as the reaction temperature increases by 10°C (Guo et al., 2014). Therefore, thermophilic anaerobic digestion processes are characterized by high metabolic rates (Fernández-Rodríguez et al., 2013). A filter cake containing very high dry solids concentrations (20%-30%) without any chemical additions is one of the main advantages of fine sieving (Chapter 2). For comparison, primary and secondary sludge reach only 6% after thickening or 20% when polymer dosage is applied (WEF, 2009). It is noteworthy that dewatering of FSF to 40%-50% dry solids content is simply possible by applying mechanical pressure (Ruiken et al., 2013).

The high dry solids concentrations of FSF makes the use of (semi) dry digesters possible, a technique that is usually applied in the digestion of the organic fraction of municipal solid waste (OFMSW) or food and yard waste digestion (Baere, 2000; ten Brummeler et al., 1992). During past research in fed batch laboratory scale systems, digestion of FSF under thermophilic conditions has been shown to be more efficient and reliable than under mesophilic conditions (Chapter 2, 3). Higher substrate doses could be applied and the measured higher reaction rate is expected to lead to a more efficient process with a lower retention time, thus leading to smaller reactor volumes (Hartmann and Ahring, 2006, 2005; Parkin and Owen, 1986). The additional amount of required heat for thermophilic operation might be offset if higher biogas production yields are attained under these conditions (Parkin and Owen, 1986). In general, at a fixed solids retention time, a thermophilic digester indeed produces more methane per weight of biomass than the mesophilic counterpart

(Ahring, 2003; McHugh et al., 2004; Siddique, 2014).

The substrate loading potentials and bio-methanation rates, as well as the maximum substrate conversion rates are parameters required for the design and operation of a biogas plant (Koch and Drewes, 2014; Lesteur et al., 2010). Besides, the optimal inoculum to substrate ratio ($R_{I/S}$) is considered a crucial parameter for design of batch-wise or plug flow operated solid state anaerobic digestion processes (Neves et al., 2004). Therefore, the energy potential, the hydrolysis kinetics, and optimal $R_{I/S}$ were assessed using biochemical methane potential (BMP) tests. The BMP tests were conducted with well-adapted sludges at different $R_{I/S}$ under both mesophilic (35°C) and thermophilic (55°C) conditions. Based on the results, the feasibility of FSF digestion process for onsite energy recovery towards energy neutrality at WWTPs is evaluated.

4.2 Materials and Methods

4.2.1. Substrate

FSF was collected from a 350 µm mesh fine sieve (Salsnes Filter, Norway) at WWTP Blaricum, The Netherlands and was stored at 4°C prior to conduct the BMP tests. Total solids (TS) and volatile solids (VS) were measured on weight base (g/L) according to the standard methods for the examination of water and wastewater (APHA, 2005). Chemical oxygen demand (COD) was measured using Merck photometric cell tests (500-10,000 mg/L, Merck, Germany). All analyses were done in triplicate.

4.2.2. *Inoculum*

The inoculum consisted of well-adapted sludges, directly taken from mesophilic and thermophilic laboratory mixed fed-batch digesters. The laboratory reactors were at steady state and operated for 480 days either at 35°C or 55°C, at the time of inoculum sampling. The characterization of both inoculates was similar to the characterisation of substrate, using the same methodology. The pH of the mesophilic and thermophilic sludge, prior to the experiments, were 7.0±0.1 and 7.6±0.2, under mesophilic and thermophilic conditions, respectively.

4.2.3. Volatile fatty acid (VFA)

Volatile fatty acids (VFAs) were quantified by Gas Chromatography (GC, Agilent Technology 7890A), using a flame ionization detector (FID) and a capillary column type HP-PLOT/U

(25 m x 320 µm x 0.5 µm) with helium as the carrier gas at a flow rate of 67 mL/min and a split ratio of 25:1. The GC oven temperature was programmed to increase from 80 to 180 °C in 10.5 min. The temperatures of injector and detector were 80 and 240 °C, respectively, and the injected volume was 1 µl. Prior to GC analysis, 10 mL of digested samples was first centrifuged at 13,000 rpm for 15 min and then the supernatant was filtered over 0.45µm filter paper. The filtered liquid was diluted 2 and 3 times with pentanol as internal solution (300 ppm) for mesophilic and thermophilic digestion samples, respectively. Finally, 10 µL of formic acid (purity >99%) was added to the 1.5 mL vials.

4.2.4. Specific Methanogenic Activity (SMA)

Specific methanogenic activity (SMA) assays were used to determine the rate capacity of methanogenic microorganisms to convert acetate into CH_4 in the anaerobic system. In this study, the SMA of the mesophilic and thermophilic sludge was determined using an Automated Methane Potential Test System (AMPTS_II) from Bioprocess Control (Sweden). The SMA was conducted using sodium acetate COD at a concentration of 2 g/L as the substrate, supplemented by a medium consisting of a mixture of macronutrients, trace elements and phosphate buffer solution (Angelidaki and Sanders, 2004). The inoculum amount was determined by setting an inoculum VS to substrate COD ratio (I/S) of 2:1. SMA was calculated by using the steepest slope of the accumulating methane production curve (mL/d) divided by the amount of VS introduced in the bottle (inoculum), using the proper conversion factor from CH_4 to COD to express the final values in gCOD-CH_4/gVS.d. Experiments were conducted in triplicate.

4.2.5. Biomethane potential (BMP) assays

The anaerobic biodegradability of the FSF was performed using the same AMPTS-II, applying adopted protocols suggested by Angelidaki et al. (2009, 2006). The 250 mL batch flasks containing inoculum and substrate were incubated in a temperature controlled rotational shaker (New Brunswick™ Biological Shakers Innova® 44/44R, USA) at 150 rpm, instead of using the AMPTS-II individual stirrers and waterbath. CO_2 and H_2S gas were stripped from the biogas by leading the biogas through 100 mL bottles containing a 3M NaOH solution. Hereafter the remaining gas, containing methane, flows into a gas flow cell with a calibrated volume. When the gas volume equals the calibrated volume of the flow cell, the gas was released and recorded as one normalized volume at time t. The test is finished at the moment gas production stops.

Biodegradation experiments were performed in duplicate for all $R_{I/S}$ values. In each test, a blank for the inoculum was included in triplicate. Every batch flask contained the same amount of inoculum, meanwhile the desired $R_{I/S}$ was obtained using different amounts of substrate (duplicate measurements). After adding the required amounts of inoculum and substrate, each bottle was filled with a medium including macro-and micro-nutrients and buffer solution to maintain the designated volume of 0.2 L, according to the mentioned protocols above (Angelidaki and Sanders, 2004). All batch tests including SMA and BMP blank were conducted in triplicate and tests with different $R_{I/S}$ were carried out in duplicate. It is noted that standard deviation for SMA and BMP blank and the error from average values for all assessed $R_{I/S}$ ratios of BMP tests under both thermophilic and mesophilic conditions were calculated to be less than 5%.

4.2.6. BMP analysis

The BMP is the net methane production per gram substrate VS added during the entire incubation period (subtracting the blank methane production) at standardised temperature and pressure (273K, 100 kPa), which has the unit of $mLCH_4/gVS_{added}$.

4.2.7. Specific methane production rate (SMPR)

The SMPR ($mLCH_4/gVS_{inoc.}.d$) was obtained by dividing the daily produced methane volume by the grams of inoculum VS added.

4.2.8. Anaerobic biodegradability (AnBD)

Anaerobic biodegradability (AnBD) was assessed as the measured ultimate methane production (expressed in COD) over the initial total COD of the substrate (Raposo et al., 2011). The relationship between AnBD and BMP (Buffiere et al., 2006) is expressed by Eq.(4.1)

$$\mathrm{AnBD} = \frac{BMP(mLCH_4 / gVS)}{350 \times COD_{substrate}(gCOD / gVS)} \tag{4.1}$$

Giving the conversion 1 $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$, 1 g COD equals 350 mL of CH_4 at standard temperature and pressure. It is noted that this theoretical approach does not take into account the needs for bacterial cell growth and their maintenance, which has been reported typically 5-10% of organic material degraded (Angelidaki and Sanders, 2004), meaning that not all biodegraded COD is transformed into methane. Moreover, during bioconversion non-methanised biodegradable or non-biodegradable intermediates may occur, lowering the actual

methane yield of the substrate.

4.2.9. Apparent hydrolysis rate (K_h)

Calculation of apparent K_h was done according to the protocol published by Angelidaki et al. (2009). The apparent K_h describes the velocity of degradation and typically follows first-order kinetics, assuming non-limited growth, meaning no inhibition, and no lack of macro or micro-nutrients (Koch and Drewes, 2014; Pfeffer, 1974; Tong et al., 1990). By assuming that hydrolysis was the rate-limiting step, a first-order kinetic model was used for calculating the apparent K_h, Eq.(4.2):

$$P=P_{max}.[1-\exp(-K_h.t)] \quad (4.2)$$

where, P= cumulative methane production from the BMP assay at time t (mL), P_{max}= ultimate methane yield from BMP assay at the end of the incubation time (mL), K_h= first-order apparent hydrolysis rate (1/d). The apparent K_h can be derived from the slope of the linear regression line plotted for the net accumulated methane production against time at all $R_{I/S}$ ratios.

4.2.10. Dissolved organic carbon (DOC)

All samples were stored at 4ºC after 0.45 µm filtration (Whatman, Dassel, Germany) to prevent biodegradation of organic matter and were characterized within 3 days after that the BMP experiments were finished. The concentration of bulk organic matter was determined as DOC by a total organic carbon analyzer (TOC-V_{CPN}, Shimadzu, Japan) for both mesophilic and thermophilic digested samples.

4.2.11. F-EEM

Fluorescence spectra were collected using a Perkin-Elmer LS-50B luminescence spectrophotometer, which uses a 450W xenon lamp. All samples were diluted with carbon-free electrolyte solution at pH about 7.5. Fluorescence excitation-emission matrix (F-EEM) spectroscopy was carried out at a concentration of 1 mg C/L to minimize the inner-filter effect. The acquisition interval and integration time were maintained at 0.5 nm and 0.1 sec., respectively. Right-angle geometry was used for liquid samples in a 10 mm fused-quartz cuvette. Three dimensional spectra were obtained by repeatedly measuring the emission spectra within the range of 280-600 nm, with excitation wavelengths from 200 to 400 nm, spaced at 10 nm intervals in the excitation domain. Spectra were then concentrated into an excitation-emission matrix (EEM).

4.2.12. Physicochemical inoculum and FSF characteristics

Table 4.1 and 4.2 describes the physicochemical characteristics of the mesophilic and thermophilic inoculum as well as FSF, used as the sole substrate during all experiments. Information on the added FSF and inoculum amounts during the BMP assays under both temperature conditions is presented in Table 4.2. The tested $R_{I/S}$ were 0.5, 1, 3, 5, 10 and 15, which were calculated by keeping a constant inoculum concentration at 27.6 and 30 gVS/L for mesophilic and thermophilic conditions, respectively. The substrate concentrations ranged from 55 ($R_{I/S}$=0.5) to 1.9 gVS/L ($R_{I/S}$=15) for the mesophilic conditions and from 60 ($R_{I/S}$=0.5) to 2 gVS/L ($R_{I/S}$=15) for the thermophilic conditions. Working volume of the batch digested bottles was 0.2 L and inoculum used in each bottle was 0.14 L.

Table 4.1 Physicochemical characteristics of the mesophilic and thermophilic inoculum (mean values ± standard deviations of triplicates), as well as FSF used as the sole substrate

Component	COD [g/L]	TS[g/L]	VS[g/L]	VS/TS	COD/VS
Mesophilic_Inoculum	65.0±6.6	50.0±0.4	39.5±0.4	0.79	1.64
Thermophilic_Inoculum	67.0±7.3	52.0±0.3	43.0±0.3	0.82	1.56
FSF	342.0±15	233.0±1	220.0±1.5	0.94	1.56

Table 4.2 Mesophilic BMP experiment set-up (M) and thermophilic BMP experiment set-up (T)

Components	$R_{I/S}$=0.5		$R_{I/S}$=1		$R_{I/S}$=3		$R_{I/S}$=5		$R_{I/S}$=10		$R_{I/S}$=15	
	M	T	M	T	M	T	M	T	M	T	M	T
FSF (g/bottle)	50.3	54.6	25.1	27.3	8.4	9.1	5.0	5.5	2.5	2.7	1.7	1.8
gVS/L (FSF)	55.0	60.0	27.5	30.0	9.0	10.0	5.5	6.0	2.8	3.0	1.9	2.0
gCOD/L (FSF)	86.0	93.5	43.0	46.8	14.4	15.6	8.6	9.4	4.3	4.7	2.9	3.0

4.2.13. Calculations for energy recovery potential at full scale

4.2.13.1 Plug flow reactor

For assessing the energy recovery potential at full scale, a horizontal plug flow reactor was considered with recirculation of the digested waste. Plug-flow operation was designed as a pulse fed batch reactor. Contact time (C_T) in a plug flow reactor depends on the recirculation factor (R). R can be defined from Eq.(4.3):

$$R = Q_R/Q_W \quad (4.3)$$

where Q_R (m^3/day) is the amount of recirculated inoculum and Q_W (m^3/day) is the amount of the waste fed to the reactor.

For a reactor with recirculation, C_T is defined according to Eq. (4.4):

$$C_T = V/(Q_w+Q_R) \quad (4.4)$$

where V is the reactor volume (m^3). The solids retention time (SRT) for a reactor, which is operated with or without recirculation of digested waste, is defined as:

$$SRT = V/Q_W \quad (4.5)$$

The solids retention time can be calculated by substituting Eq. 4.3 and 4.4 into Eq. 4.5, leading to:

$$SRT= C_T.(1+R) \quad (4.6)$$

4.2.13.2 Energy calculations

Specific heat capacity

The heating requirement of the incoming FSF was calculated based on the measured dry solids content of FSF (23% TS). FSF consisted of about 60-80% cellulose and the rest included hair, sands and clay (Chapter 3). It is noted that the sand trap at WWTP Blaricum is by passed when the fine sieve was in operation, explaining the relatively high fraction of inorganics and sand in the collected FSF (Figure 4.5). However, in the below calculations it was assumed that FSF contained 80% cellulose and 20% clay. The specific heat capacity of cellulose (C_C), water (C_W) and clay (C_{Cl}) is 1.55 kJ/kg°C, 4.20 kJ/kg°C and 0.92 kJ/kg°C, respectively, resulting in a specific heat capacity of FSF of 3.56 kJ/kg°C (23% TS). However, for the sludge entering the digester, i.e the sum of incoming FSF sludge and return sludge at 50% recirculation with 9% dry solids content, an average specific heat capacity of 3.78 kJ/kg°C (C_F) was calculated. It is noted that the heat capacity of solids in sludge (Cp_{sludge}) was assumed to be 1.95 kJ/kg°C and was determined based on the range of heat capacities of general organic and inorganic substances (Kim and Parker, 2008). This value is close to the value of 2.1 kJ/kg°C reported by Annadurai et al.(2003).

Heating and temperature control

The heat requirement of sludge digesters generally depends on: (i) the temperature difference between incoming sludge flow and digester; (ii) heat losses through reactor walls, floor and roof (Metcalf & Eddy, 2003) (iii) heat losses that might occur through piping and (iv) biogas production . By appropriate construction, the heat losses in the piping can be minimized to the point where such losses can be neglected (Zupancic and Ros, 2003). The measures and calculations for these heat losses are discussed one by one below.

Heat exchanger

To minimize heat losses through the incoming sludge, conventional counter current flow heat exchangers can be used to pre-heat the incoming sludge flow with the treated digestate (Zupancic and Ros, 2003). The temperature of incoming sludge after applying heat exchanger can be calculated using Eq. (4.7):

$$T_F = (T_{OUT} - T_{IN}) \cdot \varepsilon_H + T_{IN} \quad (4.7)$$

where T_F (°C) is the temperature of feed sludge to digester, T_{IN} (°C) is the temperature of incoming sludge, T_{OUT} (°C) is the temperature of the digestate and ε_H is the efficiency of the heat exchanger, which was assumed at 70% (McGraw-Hill, 2007). Average temperature of incoming sludge was estimated at 15°C.

Heat requirement

The required heat for increasing the temperature of the incoming sludge flow can be calculated using Eq. (4.8):

$$Q_H = Q_{(W+R)}.\ C_F\ (T_{AD} - T_F) \quad (4.8)$$

where Q_H is the amount of heat required (kJ/d), $Q_{(W+R)}$ the amount of waste fed to the reactor + the amount of digestate recycled to the entrance (kg/d), C_F the specific sludge heat transfer coefficient, T_{AD} the operating temperature of the digester which is 55 °C in this study, and T_F is the temperature of feed sludge (°C).

Heat loss

The amount of heat required to compensate the heat losses from the digester surface area is given by Eq. (4.9):

$$Q_L = U \cdot A \cdot (T_{AD} - T_a) \quad (4.9)$$

where Q_L is the reactor heat loss (J/s), U the heat transfer coefficient of the digester wall ($W/m^2.°C$), A the Digester surface area (m^2), T_{AD} the operating temperature of the digester (°C), and T_a the average ambient temperature outside of digester. For the heat loss due to heat transfer by the digester wall, it is assumed that the average ambient Dutch temperature is 15°C. Furthermore a reactor wall of 10 cm concrete and 10 cm Styrofoam insulation has a calculated heat transfer coefficient of 0.39 $W/m^2.°C$ (Persson et al., 1979). The area of the reactor wall is calculated by assuming a length (L) to diameter (D) ratio of 5 for the plug flow reactor.

Electric energy requirement for mixing and pumping

The electric energy requirement of the proposed anaerobic sludge digester consists of slow turning of agitators inside the digester (as e.g. proposed in the KOMPOGAS plug flow digester (http://www.axpo.com/kompogas, Accessed on 22 December 2015), pumping and mixing of the recycle flow with the incoming sludge. The material moves horizontally through the digester before it is discharged. A slowly turning agitator ensures that the digestate is optimally mixed and the biogas is released. The minimum power required for mixing in the anaerobic digester is 5–8 W/m^3 of digester volume and may be higher, if friction losses in the heat exchanger are high (Appels et al., 2008). To be on the safe side, in this study, we assumed 16 W/m^3. Based on the further-on calculated required digester volume of 52 m^3 (section 3.6), an amount of 20 kWh/d is consumed. It is also assumed that the return sludge is externally mixed with the feed sludge (FSF) inside a mixing tank with a maximum volume of 20 m^3, having a similar energy consumption for mixing, i.e. 16 W/m^3, resulting in an energy consumption of 7.7 kWh/d. The mixed sludges are conveyed by gravity or pumped into the digester. Energy consumption for pumping can be calculated from Eq. (4.10):

$$E_P = 24 \cdot \frac{Q_{(R+W)} \cdot \rho . g . h}{(3.6 \cdot 10^6) . \varepsilon_P} \tag{4.10}$$

where $Q_{(R+W)}$ is the flow of incoming FSF sludge and returned sludge (m^3/d), ρ the density of sludge (kg/m^3), g the gravity acceleration (9.8 m/s^2), h the differential head (height of reactor, m), the Ɛp is the pumping efficiency (75%) and E_p the required pumping energy (kWh/day). The efficiencies for centrifugal pumps normally range between 50% to 85% (McGraw-Hill, 2007). Values of 0.45 kWh/d and 0.9 kWh/d have been calculated for pumping the recirculated sludge to the mixing tank and also to pump the sum of incoming feed and returned sludges to the digester, respectively.

Energy consumption for FSF digestate dewatering and sludge drying

Dewatering technologies vary between plants and the energy consumed is highly variable. A value of 0.11 kWh per kg dried matter was used for dewatering the digested sludge (9% TS) to 20% TS (STOWA 2010-19, 2010). The energy consumption for drying (Q_{drying}) of dewatered sludge was calculated using Eq. (4.11) (Kim and Parker, 2008). The temperature difference employed in this equation was 90°C because the collected digested sludge was assumed to be kept at15°C after dewatering and were dried at 105°C .

$$Q_{drying} = M_{ws} . W . [(Cp_{water} . \Delta T) + \Delta H_{vap}] + [M_{ws} . (1-W)] . Cp_{sludge} . \Delta T \tag{4.11}$$

where M_{ws} is unit mass of wet sludge using a basis of 1 kg, W the water fraction in the sludge, Cp_{water} the heat capacity of water, ΔT the temperature difference between initial temperature of 15°C and the temperature of drying at 105 °C, ΔH_{vap} the latent heat for vaporization of water (2090 kJ/kg) and Cp_{sludge} the heat capacity of solids in sludge (1.95 kJ/kg. °C). The energy consumption for drying (Q_{drying}) was calculated to be 2008 kJ/kg as an average value.

4.3. Results and Discussion

4.3.1. Biomethane potential (BMP)

The BMP or ultimate methane yield tests giving the maximum amount of mL CH_4/gVS_{added}, were conducted under mesophilic and thermophilic conditions. Under mesophilic conditions, FSF digestion at $R_{I/S}$=0.5 failed (Figure 4.1). The high substrate concentrations, reaching 55 gVS/L or 86 gCOD/L, resulted in an imbalance between hydrolysis/acidogenesis on the one hand, and acetogenesis and methanogenesis on the other hand. The batch reactor pH dropped to 5.2, and acetate and propionate accumulated to 8.1 g/L, which equaled to about 78% of the total VFA (10.38 g/L) at the end of this batch test, indicating acidification of the medium (Figure 4.2). Based on the COD equivalents of acetate and propionate, 1.07 and 1.51 g COD/g, respectively, this equals 12 % of total influent FSF-COD (86 gCOD/L). An $R_{I/S}$=1 at 35°C resulted in a long lag phase of almost 10 days. At the end of the batch tests only low VFA concentrations remained, whereas at increased $R_{I/S}$ hardly any VFA could be detected (Figure 4.2, Table 4.3). Results indicate that mesophilic digestion of FSF at the ratio $R_{I/S}$=1 requires digestion times exceeding the 10 days that were standardized in our experimental set-up (Figure 4.1). With the increase in $R_{I/S}$ to 3, 5, 10 and 15, BMP values of 309, 291, 284 and 297 $mLCH_4/gVS_{added}$, respectively, were obtained (Table 4.3). In fact, all observed BMP values were more or less similar. The small fluctuations in BMP at $R_{I/S}$ ratios > 3 might be attributed to irregularities in the heterogeneous substrate. Previous studies have shown that decreasing the $R_{I/S}$, may have a negative impact on the ultimate methane potential of the substrate (Liu et al., 2009; Maya-Altamira et al., 2008; Zeng et al., 2010). As mentioned, in our tests a clear deviation was only found at $R_{I/S}$=1. Under thermophilic conditions, all applied $R_{I/S}$ ratios resulted in a good degradation of the FSF. Following BMPs were obtained: 334, 329, 338, 316, 297 and 299 $mLCH_4/gVS_{added}$ at $R_{I/S}$ 0.5, 1, 3, 5, 10 and 15 (Table 4.3) with initial substrate concentrations of 60, 30, 10, 6, 3 and 2 g VS/L, respectively (Table 4.2). Contrary to the mesophilic conditions, there was only a slight VFA

accumulation measured, at the end of the batch tests, at all ratios (Figure 4.2, Table 4.3), apparently there was neither substrate inhibition nor a pH drop, even at the lowest $R_{I/S}$ during thermophilic digestion. Our results indicate a better balance between hydrolysis and acidification and the activity of the methanogens under thermophilic conditions. Furthermore, the SMA tests indicated higher methanogenic activity in the thermophilic digester sludge prior to the BMP experiment, reaching values of 0.5±0.05 g COD-CH_4/gVS.d compared to an SMA of 0.2±0.03 g COD-CH_4/gVS.d of the mesophilic sludge. In fact, thermophilic digestion showed higher BMP values than mesophilic digestion under all tested conditions. This indicates that under thermophilic conditions, a higher biogas production per gram substrate can be expected. The fact that the thermophilic digester remained stable even at the lowest $R_{I/S}$ value means that higher substrate loading rates can be applied under thermophilic conditions compared to mesophilic conditions.

4.3.2. Specific methane production rate (SMPR)

The SMPR varied over time following the batch degradation of the substrate at the different $R_{I/S}$ under both mesophilic and thermophilic conditions (Figure 4.1). At $R_{I/S}$=0.5 and 1 under mesophilic and thermophilic conditions, fluctuating methane production rates were recorded over time. The SMPR under thermophilic conditions was always higher than during mesophilic digestion. The increased SMPR reflects increased apparent hydrolysis rates at higher temperatures, considering SMA was not rate limiting at the lower $R_{I/S}$ values. The observed fluctuations in SMPR might be caused by the occurrence of different hydrolyses steps in the degradation of FSF, which could be attributed to its heterogeneous nature. In addition, the characteristic drop in the SMPR between days 1.5 and 2 at the low $R_{I/S}$ of 0.5 and 1, observed at 35°C and particularly 55°C, may result from substrate inhibition as experienced by Hashimoto (1989) and Raposo et al. (2006), or from depletion of readily degradable substrate after 1.5 days and 'delayed hydrolysis' of less readily degradable substrates. The higher the $R_{I/S}$ the lower the SMPR peaks and the shorter the time interval between the different methane production rates, finally resulting in a stabilized SMPR at the highest $R_{I/S}$. At higher $R_{I/S}$ ratios, the relative low amount of substrate is certainly limiting the SMPR. Prashanth et al. (2006) explained the stable SMPR at high $R_{I/S}$ by the presence of a large pool of the different required enzymes that are necessary for complete biodegradation of the substrate. The highest observed SMPR values amounted 67 and 189 mLCH_4/gVS_{inoc}.d (≈ 0.19 and 0.54 gCOD/gVS_{inoc}.d) for mesophilic and thermophilic conditions, respectively, and were found at the lowest $R_{I/S}$=0.5. Table 4.3, presents SMPR values under both conditions,

showing higher values under thermophilic conditions at all $R_{I/S}$.

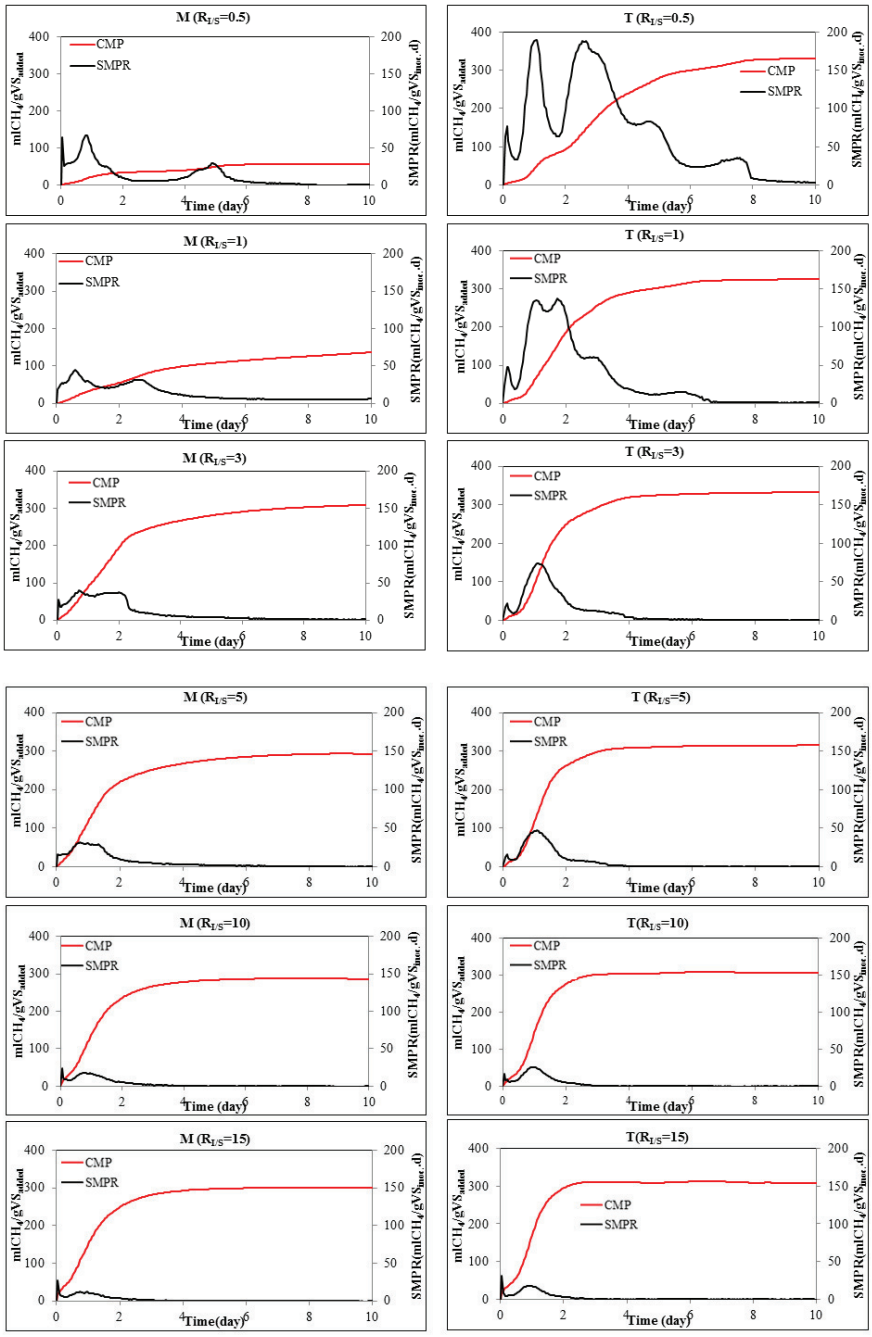

Figure 4.1 Cumulative methane production (CMP) and SMPR at different $R_{I/S}$ ratios at the mesophilic (M) (from left, row: 1, 2, 3, 4, 5, 6) and thermophilic (T) (from right, row:1, 2, 3, 4, 5, 6) conditions (error in duplicate ≤5%)

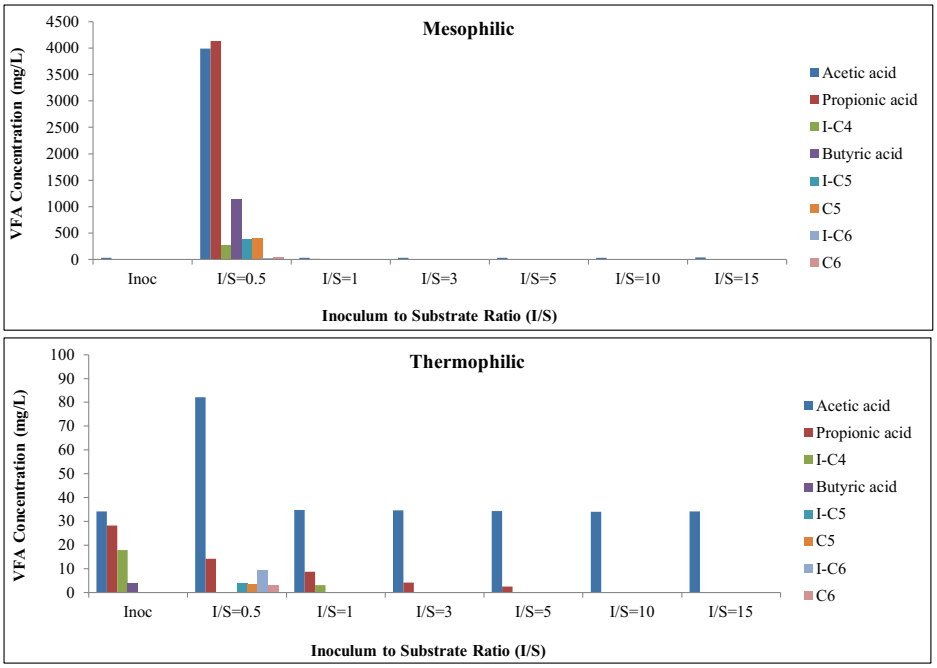

Figure 4.2 VFA production at different I/S ratios under mesophilic (top) and thermophilic (bottom) conditions (error in duplicate ≤5%) at the end of the batch tests; note difference in scale Y axis

The net cumulative methane production and the different $R_{I/S}$ applied, are plotted against the added FSF expressed in g VS, for both mesophilic and thermophilic conditions in Figure 4.3. Under mesophilic conditions the relation between methane production and the batch-fed substrate load (in g VS) is linear for $R_{I/S}$ ≥3. While under thermophilic conditions, there is a linear relationship (R^2 = 0.999) between the net produced methane and gVS added until the lowest $R_{I/S}$ of 0.5. The slope of the line gives an average methane yield coefficient (BMP) of 333 $mLCH_4/gVS_{added}$.

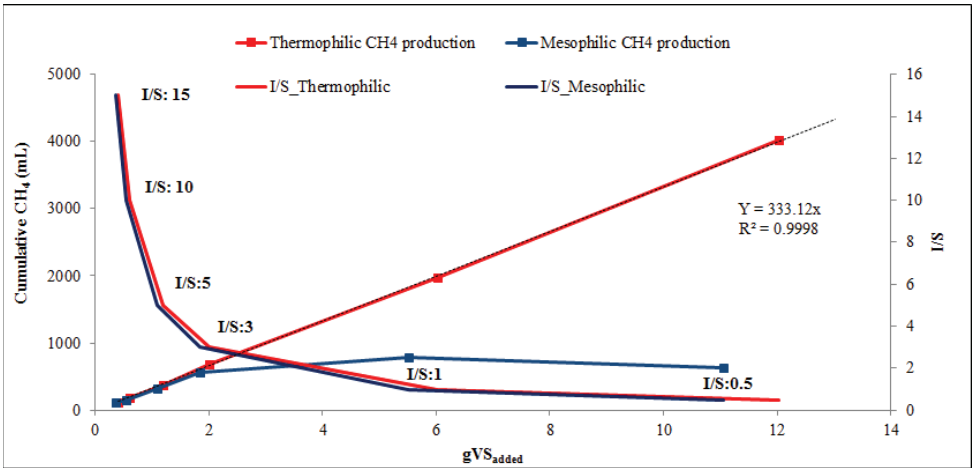

Figure 4.3 Net cumulative CH_4 production vs. gVS added per bottle at different I/S ratios (error in duplicate ≤5%)

4.3.3. Apparent hydrolysis rate (K_h)

By assuming that hydrolysis is the rate-limiting step, the apparent hydrolysis rate (K_h) was calculated using a first order kinetic model as described by Angelidaki et al. (2009). The apparent K_h has been derived from the slope of the linear regression line plotted for the net accumulated methane production against time for all $R_{I/S}$ ratios under both mesophilic and thermophilic conditions. Initial observed lag phases were disregarded, which were between 0.5-1 day for all conditions.

The observed apparent K_h at all $R_{I/S}$ was higher under thermophilic conditions compared to mesophilic conditions (Table 4.3). A gradual increase in apparent K_h was observed with increasing $R_{I/S}$ ratios under thermophilic conditions, whereas the maximum apparent K_h was observed at $R_{I/S}$ of 3 and 5, for the mesophilic digesters. The observed maximum apparent K_h was 0.60 (1/d) at $R_{I/S}$ of 3 and 1.80 (1/d) at $R_{I/S}$ of 15 under mesophilic and thermophilic conditions, respectively. Considering the possible increased accumulation of VFA intermediates during the first days of FSF digestion at $R_{I/S}$ of 0.5 and 1 under thermophilic conditions, the calculated apparent K_h value might be an underestimate of the maximum possible values. A clear inhibition by VFA was shown during mesophilic digestion of FSF at $R_{I/S}$ of 0.5 (Figure 4.1 & 4.2, Table 4.3). Therefore, only the obtained apparent K_h values at $R_{I/S}$>1 should be used for process evaluation. From above-mentioned results, it is concluded that the apparent K_h is a test dependent parameter as our results have shown different K_h values at different $R_{I/S}$ under mesophilic and thermophilic conditions (Table 4.3).

The changes in apparent K_h under both conditions might be linked to the presence of large differences in concentrations of hydrolytic enzymes (Figure 3.9 & Figure 4.4, Table 3.2) and differences in SMA (this chapter and Figure 3.5), which were both considerably higher under the thermophilic conditions.

4.3.4. Anaerobic biodegradability (AnBD)

The biodegradation efficiency was calculated for both mesophilic and thermophilic conditions at all $R_{I/S}$ (Table 4.3). Highest efficiencies were found at an $R_{I/S}$ of 3 for both conditions, whil e at all ratios the thermophilic batches revealed the highest efficiency.

Table 4.3 also shows the required incubation time to achieve 90% of the maximum cumulative methane production ($t_{90\%}CH_4$), which is another factor characterizing the bioavailability of organic matter (Parameswaran and Rittmann, 2012). Conform expectations, the $t_{90\%}CH_4$ was shorter under thermophilic conditions compared to mesophilic conditions, likely because of higher metabolic rates at higher temperatures as was also indicated with overall SMPR in the thermophilic batches. The BMP, SMPR, AnBD, apparent K_h, $t_{90\%}CH_4$, TVFA (at the end of batch tests), DOC and pH values at $R_{I/S}$ of 0.5 under mesophilic conditions indicated digestion failure owing to the high substrate dose (Table 4.3). In general, the required incubation period for our BMP experiments was considerably shorter than that described in the conventional BMP methodology (30-50days) (Hansen et al., 2004; Lesteur et al., 2010; Owen et al., 1979). Very likely, the use of well-adapted inoculum for FSF digestion (Chapter 2) resulted in rapid and stable substrate conversion.

Table 4.3 Variation of BMP, SMPRmax, AnBD, apparent K_h, t90%CH_4, $TVFA_{end}$, DOC and pH with changes in RI/S at mesophilic (top) and thermophilic (bottom) conditions (error in duplicate ≤5%)

$R_{I/S}$	BMP $\frac{mLCH_4}{gVS_{sub}}$	$SMPR_{max}$ $\frac{mLCH_4}{gVS_{inoc.} \cdot d}$	AnBD (%)	K_h (1/d)	$t_{90\%}CH_4$ (day)	$TVFA_{end}$ (g/L)	DOC (mg/L)	pH_{end}
Mesophilic conditions (35°C)								
0.5	-*	67	10.4	-**	5.2	10.38	6045	5.2
1	-*	43	26.1	-***	8.4	0.24	888	7.0
3	309	39	56.7	0.60	5.0	0.05	531	7.0
5	291	31	53.5	0.60	3.8	0.04	499	7.1
10	284	18	52.1	0.55	2.7	0.03	559	7.1
15	297	11	54.5	0.45	2.5	0.05	564	7.2
Thermophilic conditions (55°C)								
0.5	334	189	61.4	-***	6.1	0.12	1495	7.4
1	329	134	60.5	-***	4.4	0.22	1209	7.5
3	338	73	62.1	0.85	3.3	0.18	1046	7.5
5	316	46	58.2	1.20	2.6	0.13	1014	7.5
10	297	25	54.6	1.60	2.0	0.09	987	7.5
15	299	17	55.0	1.80	1.8	0.07	990	7.5

* No BMP value could be found,** K_h could not be calculated due to accumulation of intermediates ,*** K_h could not be calculated due to possible accumulation of intermediates

4.3.5. Protein matter and humic-like substances

It was hypothesized that the difference in BMP and conversion rates at the two conditions could be due to a different enzyme (protein) production rate by the microorganisms, or due to a change in the bioavailability of organic components such as humic-bound biodegradable compounds. Therefore F-EEM spectroscopy (Figure 4.3) was used to determine differences in protein-like (aromatic and tryptophan-like) and humic-like substances, which are considered the main fluorophores in sludge (Chen et al., 2003; Yamashita and Tanoue, 2003). In this study the observed peaks were identified by comparing their fluorescence properties (excitation/emission (Ex/Em)) with those of pure compounds, such as aromatic protein, tryptophan protein and humic and fulvic acids. The main intensities in four region peaks determined by F-EEM were tryptophan protein-like (Ex/Em =270-280/320-350 nm), aromatic protein-like (Ex/Em=220-240/320-350 nm), humic-like (Ex/Em=330-350/420-480 nm), and fulvic-like (Ex/Em=250-260/380-480 nm), respectively (Chen et al., 2003; Mobed et al., 1996; Yamashita and Tanoue, 2003).

F-EEM measurements were conducted with the supernatant of fresh mesophilic and thermophilic inoculum (Figure 4.4, first row), as well as all $R_{I/S}$ ratios, at the beginning and end of the experiment, to observe changes in protein and humic-like substances. At both temperatures, measured spectra for $R_{I/S}$ of 15, 10 and 5 were similar, therefore only F-EEM results of $R_{I/S}$=15 are presented. F-EEM spectroscopy revealed the presence of more fluorescent organic matter, especially protein-like substances under thermophilic conditions. It also indicates that humic-like and fulvic-like substances gradually decreased relative to the protein amount, during thermophilic digestion, while during mesophilic digestion hardly any change can be seen. The changes in fluorescence intensity became more apparent during digestion at low $R_{I/S}$. The fluorescence intensity of protein-like substances slightly increased with a decrease in $R_{I/S}$ in the thermophilic digestion (Figure 4.4, third row).

In contrast, there was no significant formation of protein-like substances observed under mesophilic conditions and the relative intensity of fulvic-like substances was higher except at $R_{I/S}$ of 0.5, which failed due to VFAs accumulation. Very likely, the high intensity of protein-like substances observed at the ratio $R_{I/S}$ = 0.5 was related to cell lysis of decaying biomass (Figure 4.4, second row). Although not very visible, the fulvic-like substances may also be present in the thermophilic assays, but if so, they are masked by the high intensity of the protein like substances. Results from the F-EEM analysis indicate that protein-like substances

are more produced under thermophilic conditions. Considering that these proteins may relate to hydrolytic enzymes than these results agree with the higher activity at thermophilic temperatures.

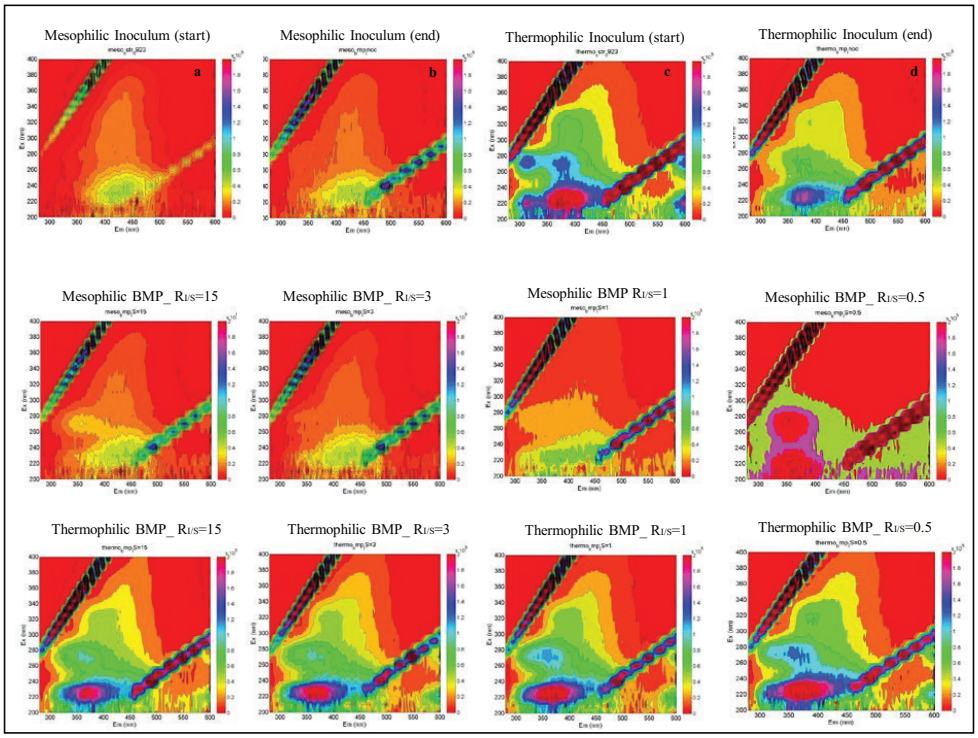

Figure 4.4 F-EEM images of the mesophilic inoculum at the start and the end of the incubation time (first row, a and b), the thermophilic inoculum at the start and the end (first row, c and d), mesophilic digested FSF at $R_{I/S}$ =15, 3, 1, 0.5 at the end of digestion (second row, left to right) and thermophilic digested FSF at $R_{I/S}$= 15, 3, 1, 0.5 at the end of digestion (third row, left to right)

4.3.6. *Energy recovery from municipal raw sewage*

4.3.6.1 Biogas production and electricity recovery by AD of FSF

Energy recovery from raw sewage by using fine sieves coupled to FSF digestion could be a feasible alternative to primary sludge digestion in conventional extended aeration WWTPs, avoiding the construction of large primary clarifiers. The results obtained with the BMP tests were used to quantify the potential energy recovery that could be gained from FSF digestion (Table 4.4) .

For calculating the energy recovery from FSF, the methane production rate per reactor volume ($m^3/m^3.d$), the lower heating value (LHV) of methane (50 MJ/kg) and its density at standardized temperature and pressure (0.716 kg/m^3, T=0°C and P=1 atm), as well as the required incubation time to achieve $t_{90\%}CH_4$, were taken into account for each assessed $R_{I/S}$. The biogas production rate was calculated based on an average methane composition of 53% and 57% for both thermophilic and mesophilic conditions, respectively (Chapter 2). Table 4.4 presents the normalized methane and biogas production per ton of FSF (wet weight basis at 23%TS)(Nm^3/t-FSF) translated to energy production as heat (MJ/t-FSF) and electricity generation (kWh), taking an electric and heat conversion efficiency of 40% and 50% using a modern combined heat and power (CHP) unit, heat losses are about 10% (Appels et al., 2008; Cavinato et al., 2013; van Lier, 2008). Furthermore, the rate of normalized methane and biogas production per reactor (working) volume ($m^3/m^3.d$) were calculated.

Table 4.4 Average energy recovery and electricity generation estimation (error from average values ≤5%) from mesophilic (top, 35°C) and thermophilic (bottom, 55°C) digestion of FSF

$R_{I/S}$	Methane (Nm^3/t-FSF)	Biogas (Nm^3/t-FSF)	MJ/t-FSF (CHP gross)	kWh/t-FSF (Eff. 40%)	Methane ($m^3/m^3.d$)	Biogas ($m^3/m^3.d$)
Mesophilic conditions (35°C)						
0.5	12	22	444	49	0.6	1.1 (Failed)
1	31	55	1121	125	0.5	0.8
3	68	119	2430	270	0.6	1.0
5	64	112	2293	255	0.4	0.7
10	62	110	2236	248	0.3	0.5
15	65	115	2337	260	0.2	0.4
Thermophilic conditions (55°C)						
0.5	74	139	2635	293	3.3	6.2
1	72	137	2595	288	2.2	4.2
3	74	140	2664	296	1.0	1.9
5	70	131	2495	277	0.7	1.4
10	65	124	2343	260	0.4	0.8
15	66	124	2360	262	0.3	0.6

As shown in Table 4.4, thermophilic digestion of FSF presents higher values for all parameters compared to the mesophilic conditions. Maximum values of biogas production rates were 1 and 6.2 m^3/m^3.d for mesophilic and thermophilic FSF digestion at $R_{I/S}$ ratio of 3 and 0.5, respectively. Typical values obtained for mesophilic sludge digestion at WWTPs are in the range of 0.5-1.0 m^3/m^3.d (Chudoba et al., 2011). Mesophilic digestion under high substrate loading ($R_{I/S}$=0.5) was shown to be impossible due to VFA accumulation and mixing problems owing to higher viscosity of the reactor broth (Chapter 3).

Semi-dry or dry thermophilic digestion using plug flow reactors could be of interest for FSF digestion, since dewatering (mechanically pressing) of FSF to 40-50% dry solids is possible (Ruiken et al., 2013). Batch and plug flow reactors also have a significant potential to produce biogas with low capital costs and high efficiencies (Sharma et al., 2000). Based on the batch tests results (Table 4.3) the applicable $R_{I/S}$ ratio for FSF digestion in such reactor could be as low as 1-0.5 for thermophilic conditions. Substrate loading rates of 60 kgVS/m^3 or 93.5 kgCOD/m^3 could be feasible based on the relatively short retention times needed for 90% conversion (3 to 6 days, Table 4.3 and 4.5). Such operational conditions would result in a very small and compact thermophilic reactor design (Table 4.5). Especially when compared to the conventional anaerobic digestion systems treating sludge from wastewater with maximum dry solids content of 9% and a long retention time (12-30 days). Therefore, the possible on-site energy recovery with thermophilic digestion of FSF was evaluated for the situation at WWTP Blaricum, The Netherlands. The average data used for this WWTP were: a dry weather flow (DWF) of 8000 m^3/d; influent wastewater COD of 424 mg/L; FSF COD of 342 g/kg at TS of 23% dry solids, and 35% COD removal efficiency by fine sieving (Ruiken et al., 2013).

The total mass flow of COD and flow rate of FSF that would enter the plug flow digester were calculated at 1187 kg COD/d and 3.47 m^3/d, respectively. Safety margin should be provided when selecting the designed SRT and, in practice, a multiplication factor of about minimum 2.5 is recommended (Metcalf & Eddy, 2003). This would result in a design based on 15 days SRT considering the batch retention time of 6.2 days at $R_{I/S}$ of 0.5 (Table 4.5) and thus a flow rate of FSF of 3.47 m^3/d. As a result, the volume of the digester would become 52 m^3 (e.g. L:D=5; L=11.85m and D=2.35m). During the batch experiments, an FSF biodegradability of 61% was found, leading to a calculated methane production of 255.5 m^3/d with the proposed digester. The biogas production rate would become 9.3 m^3/m^3.d (53% CH_4) under an OLR of 22.8 kg COD/m^3.d.

The estimated minimal recirculation rate for the thermophilic digestion of FSF would be approximately 50% with C_T of 7.5 days (Q_R=3.47 m^3/d and R=1). Using higher recirculation rates could result in incomplete substrate digestion, leading to a lower methane production per kg of FSF. It is recommended to study this assumption in a continuously operated pilot experiment. If such pilot digester could be operated with even shorter retention times or lower recycle ratios as were derived from the batch tests ($R_{I/S}$=0.5), overall dimensions could be significantly reduced.

Table 4.5 Operational conditions and biogas production from mesophilic and thermophilic digestion of FSF

Parameters	BMP results (Mesophilic)	BMP results (Thermophilic)
Operating temperature (°C)	35±1	55±1
Dry matter (%)	23	23
pH (after BMP)	7.0±0.1	7.4±0.1
Retention time (day)	2.5-5 ($R_{I/S}$=3-15)	1.8-6.1($R_{I/S}$=0.5-15)
Gas yield (Nm^3/ton)	119 ($R_{I/S}$=3)	139-140 ($R_{I/S}$=0.5-3)
Maximum gas production rate (m^3/m^3reactor.day)	1 ($R_{I/S}$=3)	6.2 ($R_{I/S}$=0.5)
Organic loading	9 kgVS/m^3 or 14.4 kgCOD/m^3 ($R_{I/S}$ =3)(58% conversion)	60 kgVS/m^3 or 93.5 kgCOD/m^3 ($R_{I/S}$=0.5)(61% conversion)

The calculated overall daily energy balance of FSF digestion in the proposed plug flow digester is presented in Table 4.6. Calculations were based on Eq. (4.7), (4.8), (4.9), (4.10) as well as suggested values to calculate, for instance, the mixing energy, efficiency of pumps and heat exchanger. The heat requirement of the digester (MJ/d) is composed of the total heat required to heat the incoming FSF and the heat loss from the digester surface areas. Electricity consumption (kWh/d) for mixing and pumping is presented as electrical requirement of digester in Table 4.6.

The total electricity consumption at WWTP Blaricum for the situations without fine sieve (reference), with fine sieve as well as combination of fine sieving and anaerobic digestion of FSF are presented in Table 4.7. It was estimated that the installation of a fine sieve would reduce the aeration energy in the WWTP Blaricum by 30% (Ruiken et al., 2013). The total electricity consumption of the WWTP including the fine sieve, aerators and other consumptions (Table 4.7) were obtained from previous studies (STOWA 2010-19, 2010).

Table 4.6 Calculated overall energy balance on FSF digestion in the plug flow digester

Parameter	Unit	Value
CHP gross energy (heat and electricity)	MJ/d	9146
CHP gross electrical output (40% efficiency)	kWh/d	1016
Electrical requirement of digester	kWh/d	-29
Net energy output as electricity	kWh/d	987
CHP gross heat output (50% efficiency)	MJ/d	4573
Heat requirement of digester	MJ/d	-445
Net energy output as heat	MJ/d	4128
Lost CHP energy (10%)	MJ/d	-915

Table 4.7 Total electricity consumption at WWTP Blaricum (STOWA 2010-19, 2010) and integration of FSF and anaerobic digestion (FSFAD) as proposed in this study

Components	Unit	Reference	Fine sieve	FSFAD
Fine sieving	kWh/d	0	132	132
Aeration	kWh/d	1655	1159	1159
Other consumptions	kWh/d	1340	1307	1307
Digestion of FSF	kWh/d	0	0	-987
Total	kWh/d	2995	2598	1611

The total electricity consumption at the WWTP Blaricum could be reduced with about 13% by the use of a FSF, without digestion of the FSF. The net electricity production from the anaerobic digestion of the FSF in combination with the reduced energy consumption by installing a fine sieve, would lead to a total of 46% lower (fossil) energy consumption at the WWTP Blaricum. In this calculation the reduction of sludge volume to be transported from WWTP Blaricum to the incineration plant (approximately 45 km) is not taken into account.

At WWTP Blaricum the activated sludge is only thickened upon transport. Therefore energy requirement for excess activated sludge dewatering is not included in this balance. However, removal of FSF from the sludge matrix might impact the energy consumption for dewatering. STOWA (The Foundation for Applied Water Research) in The Netherlands (STOWA 2010-19, 2010), reported an energy consumption of 108 kWh/d and 49 kWh/d for secondary sludge dewatering to 20% TS in absence of fine sieve (reference) and with one fine sieve, respectively.Different values have been reported on WWTP electricity consumption (kWh/m^3 of wastewater treated) showing the high variety among different facilities and countries (Cano et al., 2015). Average values reported varied from 0.30 to 0.78 kWh/m^3 (Cano et al., 2015; Hernández-Sancho et al., 2011). For WWTP Blaricum 0.37 kWh/m^3, 0.32 kWh/m^3 and 0.20 kWh/m^3 were calculated for the reference, fine sieve and FSFAD respectively. These numbers excluded secondary sludge treatment that results in energy consumption for dewatering that might be off-set when the sludge is digested.

4.3.6.2 Heat production by AD of FSF

Heat production of the CHP exceeds the amount of heat required to heat up the mixed sludges (incoming FSF sludge and returned sludge) to thermophilic conditions and the heat loss from digester surface area. For WWTP Blaricum, the net heat production was calculated for the proposed thermophilic digester according to Eq. (4.7), (4.8) and (4.9). The extra available heat production from FSF digestion (4128 MJ/d, Table 4.6) by the CHP unit can be utilized at the WWTP facility or its surroundings for e.g. heating water and buildings. In addition, also the heat requirement to maintain the mixing tank temperature where returned sludge is mixed with inflow FSF sludge can be covered. Currently, WWTP Blaricum consumes, on average, 26 GJ/y net heat energy (71 MJ/d), which can be supplied from the heat recovery of the CHP.
To reduce the volume of sludge to be transported and/or to create a possibility for more advanced technology for digestate processing, excess heat can be used for sludge drying (Appels et al., 2011; Kim and Parker, 2008). The required amount of heat to dry the flow of dewatered digestate (1.56 m^3/d) at 20% dry solids content at 15°C (assumed minimum temperature after dewatering) to 95% dry solids content at 105°C, was calculated to be 3133 MJ/d using Eq. (4.11). This amounts equals about 76% of the net heat production (4128 MJ/d) by the CHP.

By applying a drying process, the weight of transported biosolids to a gasification or pyrolysis plant amounts 120 ton/y, which is considerably lower compared to the amount of undigested FSF (1267 ton/y at TS≈23%) (Figure 4.5). Current practice for utilization of FSF from WWTP Blaricum is transporting FSF at 23% TS to waste incineration plant (AEB) in Amsterdam (distance approx. 45 km) to generate heat and electricity off-site.

Since costs for transportation, dewatering and incineration approximately amounts 60-100 euros per ton of solids cake in The Netherlands (Ringoot et al., 2014), therefore, the final cost of sludge treatment could be reduced over 76,000-127,000 euros per year. In the case of combining fine sieving and digestion, on-site dewatering of the digestate and drying the dewatered digestate, the costs of fuel consumption for 120 ton/y biosolids could reach €150/year (4277 MJ/year) if the biosolids are transported to gasification or pyrolysis plant (assumed the same distance as incineration plant). By this means, transport fuel for 38 trucks (30 tonnes) per year can be saved as well. In the calculation of fuel consumption cost, the capacity of a trucks (30 tonnes or 30 m^3), diesel fuel consumption (0.33 L/km), net heating

value of diesel fuel (36 MJ/L), approximate distance of transportation (45 km), and current price of diesel fuel in The Netherlands (€ 1.27/L) were taken into account.

Summarizing the overall energy (heat and electricity) use in a combination of fine sieving (TS of the FSF 23%), anaerobic digestion of FSF, dewatering of digestate sludge and drying of the dewatered digestate sludge, a net energy recovery can be calculated. Fine sieving (132 kWh/d), anaerobic digestion (29 kWh/d and 445 MJ/d), digestate dewatering from 9% to 20% TS (34 kWh/d or 0.11 kWh per kg dry solids) and sludge drying (3133 MJ/d), leads to a net recoverable heat energy of 287 MJ/ton FSF and 237 kWh electric/ton FSF.

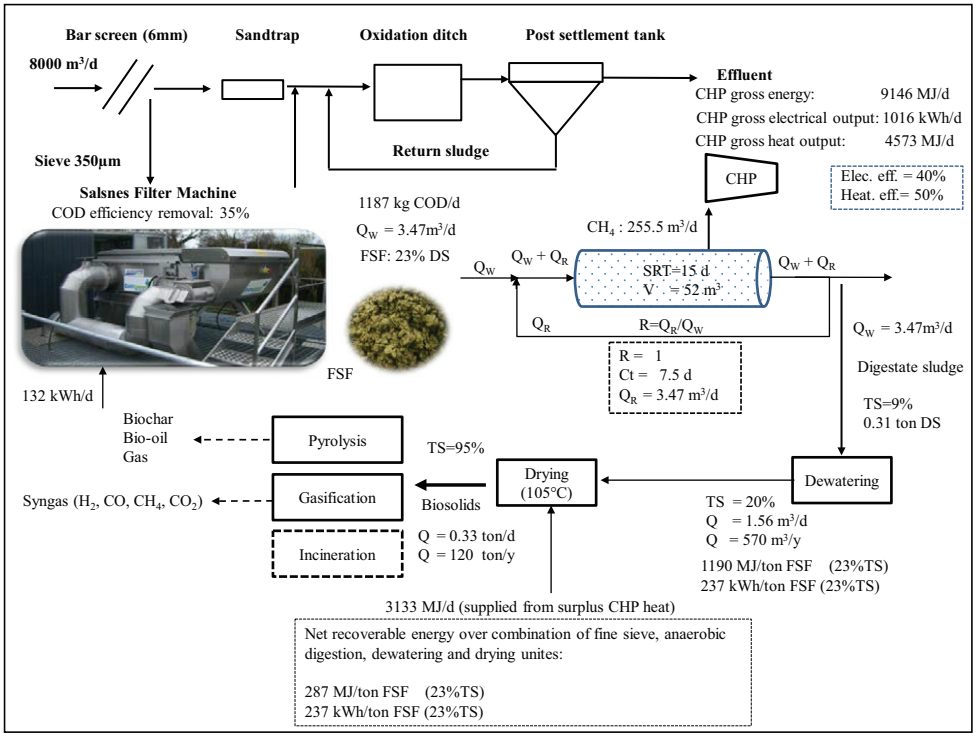

Figure 4.5 Schematic view of WWTP Blaricum combined with fine sieve, plug flow digester, CHP, dewatering and drying units and final destination of biosolids. Figure also contains further details about the mass flow rate of FSF, volume of digester, gross energy (heat and electricity) and net recoverable energy per ton of FSF at 23% TS

4.4 Other routes for energy recovery

In recent years, several valorisation routes have been practiced to find a more sustainable use of digestate sludge, such as the potential use of solids digestate as solid fuel (Kratzeisen et al., 2010) and production of bioethanol (Yue et al., 2010). These routes are more promising by means of energy and resource recovery than the conventional management and valorisation routes like landfilling, composting and incineration. Currently, there is growing attention towards the application of thermal processing techniques such as pyrolysis and gasification to treat sewage sludge (Bianchini et al., 2015; Cao and Pawłowski, 2012; Kim and Parker, 2008; Tabasová et al., 2012) or coupling anaerobic digestion to pyrolysis (Monlau et al., 2015) or gasification processes to generate syngas (H_2, CO, CH_4, CO_2) (Li et al., 2015).

Monlau et al. (2015) investigated the feasibility of combining anaerobic digestion and pyrolysis processes in order to increase the energy recovery from agricultural residues, which could be applicable to FSF too. It was reported that excess heat production from CHP could cover the drying needs for the solid fraction of the digestate, whereas pyrolysis of this fraction at 500 °C resulted in 8.8 wt.%, 58.4 wt.% and 32.8 wt.% of syngas, oil and char, respectively. The LHV of syngas was 15.7 MJ/Nm^3, whereas pyrolysis oil exhibited a higher heating value (HHV) of 23.5 MJ/kg after water extraction. Integrating these two processes and by using the heat production for sludge drying, could increase the production of electricity by 42% compared to anaerobic digestion as stand-alone plant (Monlau et al., 2015).

Since digestion of FSF does not need any pre-treatment process and dewatering of FSF to 40-50% dry solids is simply possible (Ruiken et al., 2013), it is speculated that a very compact high loaded system can be applied for semi-dry or dry thermophilic digestion of FSF in plug flow reactors. Moreover, it is predicated that coupling anaerobic digestion and gasification/pyrolysis systems, considering the economic analysis, legislation and incentives, would further increase the on-site energy recovery approaching to the level of energy neutrality or even energy positive WWTPs.

4.5 Conclusions

The outcomes of this study revealed promising biogas production rates from FSF digestion under low $R_{I/S}$, which were translated to a design of a compact thermophilic plug flow digester with high OLR. It was calculated that 46% reduction in electricity use could be reached when on-site digestion of FSF at WWTP Blaricum would be applied. Surplus heat production from the CHP would be enough to dry the digestate before transport to the final utilization unit. The net recoverable heat energy from FSF (23% TS) was estimated at 287 MJ/ton FSF and 237 kWh electric/ton FSF.

Based on the results of the batch tests, it can be concluded that thermophilic adapted biomass is more appropriate for FSF degradation than mesophilic adapted sludge. Higher SMA, BMP, SMPR, apparent K_h and AnBD values were found under thermophilic conditions for all $R_{I/S}$ ratios compared to mesophilic digestion. Physicochemical analysis of the reactor broth showed that protein-like substances were present in higher concentrations under thermophilic conditions than under mesophilic conditions at all applied $R_{I/S}$, indicating an increased amount of enzymes and thus higher substrate conversion rates at high temperatures.

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Chapter 5. Comparative analysis of digestibility of sewage fine sieved fraction and virgin and recycled hygiene paper

Abstract

Sewage fine sieved fraction (FSF) is a heterogeneous substrate consisting of mainly toilet paper fibers sequestered from municipal raw sewage by a fine screen. In earlier studies, a maximum biodegradation of 62% and 57% of the sewage FSF was found under thermophilic (55°C) and mesophilic (35°C) conditions, respectively. In order to research this limited biodegradability of sewage FSF, this study investigates the biodegradation of different types of cellulosic fibers-based hygiene papers including virgin fibers based toilet paper (VTP), recycled fiber based toilet paper (RTP), virgin pulp for paper production (VPPP) as a raw material, as well as microcrystalline cellulose (MCC) as a kind of fiberless reference material. The anaerobic biodegradation or digestibility tests were conducted under thermophilic and mesophilic conditions.

Results of the experiments showed different biomethane potential (BMP) values for each tested cellulose fiber-based substrate, which might be associated with the physical characteristics of the fibers, type of pulping, presence of lignin encrusted fibers, and/or the presence of additive chemicals and refractory compounds. Higher apparent hydrolysis rates (K_h), higher specific methane production rates (SMPR) and shorter required incubation times to achieve 90% of the BMP ($t_{90\%}CH_4$), were achieved under thermophilic conditions for all examined substrates compared to the mesophilic ones. Furthermore, the biodegradability of all employed cellulose fiber-based substrates was in the same range, 38%-45%, under both conditions and less than the observed FSF biodegradability, i.e. 57%-62%. MCC achieved the highest BMP and biodegradability, 86%-91%, among all cellulosic substrates.

This chapter is based on:

Ghasimi, D.S.M., M., Zandvoort, M.H., Adriaanse, M., van Lier, J.B., de Kreuk., 2015. Comparative analysis of digestibility of sewage fine sieved fraction and virgin and recycled hygiene paper. Journal of Waste Management (in press).

5.1 Introduction

At the sewage treatment plant (STP) Blaricum, the Netherlands, a 350 µm mesh size fine sieve (Salsnes Filter, Norway) for raw sewage pretreatment is installed, immediately after the 6 mm coarse screen. The fine sieve is implemented as a compact alternative to primary clarification to separate suspended solids from sewage prior to biological nutrient removal. The produced cake layer or fine sieved fraction (FSF) has a very heterogeneous composition but is presumed to contain mainly cellulosic fibers originating from toilet paper (Ruiken et al., 2013). Considering its nature and high energy content, FSF receives growing interest in countries like the Netherlands, either for cellulose fiber recovery or as feedstock for energy recovery (STOWA, 2010). Regarding the latter, increasing effort is put on onsite energy recovery for closing the energy balance, eventually realizing an energy neutral or energy producing STP.

Toilet paper or toilet tissue is one of the mostly used hygiene products, particularly in Northern Americas, and European countries, whereas it is less used in large parts of Asia and Africa (http://www.worldwatch.org/node/5142). The major component of all hygiene papers is fibrous cellulose, mostly from tree origin. Toilet papers are available in different qualities; they are generally smooth and can be embossed, unprinted or patterned, tinted, purely white or off-white (Holik, 2006).

Toilet paper is either made from virgin pulp, which is mainly extracted from wood and partly from non-wood cellulose (e.g., bamboo) and is called virgin fibers based toilet paper (VTP), or it is made from recycled paper fibers, which is known as recycled fibers based toilet paper (RTP). The type of pulp and paper chemicals used has an influence on the final quality of the tissue paper, e.g. softness, strength, absorbency and appearance. In the process of making virgin pulp as a raw material for paper production (VPPP), one type of wood is generally usually used, i.e. either soft or hard wood. However, in the production of VTP a combination of soft (long fiber for strength) and hard wood (short fiber for softness) is employed. Depending on the required specifications, paper makers choose their fiber source (long fibers, short fibers and combinations). RTP, which completely or partially consists of recycled fibers, may originate from different sources, such as mixed office waste, or old newsprints. Paper production using recycled fibers in the paper mill follows various process steps such as pulping, screening and de-inking stages (Kamali and Khodaparast, 2014). The majority of paper tissue used in the Netherlands is recycled fibers based. The ratio virgin fibers relative to recycled fibers determines the level of softness of the end product. However, application of

specific chemicals and process steps can improve the strength, softness, brightness, etc., of any tissue product, regardless the fibers used (WRAP, 2005). During pulp making, pulp processing and paper-making, certain types of chemicals are used as presented in Table 5.1. However, every papermaking factory deviates according to their applied raw materials, desired products and process optimization. Generally speaking, these additives can be divided in two categories: (1) additives used during the process (2) additives for product improvement (Table 5.1). Theoretically, both could end up within the product, which however, is more likely for the 'product additives' (Bos et al., 1995). Therefore, there is no standard composition of toilet paper and very likely, also the biodegradability will vary with its composition.

Cellulose is the main constituent of toilet paper and its biodegradability likely depends on its fibrous content and its crystallinity. Maximum biodegradability is expected when no fibers are present, i.e. when the cellulose consists of powdered cellulose (PC) or microcrystalline cellulose (MCC). The chemical composition and physical structure of MCC fully depend on the characteristics of the virgin material from which the cellulose is obtained as well as on the manufacturing conditions (Landin et al., 1993). As a result, several grades of MCC are available on the market with different physicochemical and thermal properties, exhibiting different functional parameters and applications (Azubuike and Okhamafe, 2012). MCCs are prepared by acid hydrolysis under mild conditions of native cellulose to a critical degree of polymerization (DP) (Shcherbakova et al., 2012).

Fibers originating from tissue paper can be screened from the waterline before biological sewage treatment, in order to reduce aeration energy requirements and to generate possibilities to (re-)use these fibers or its energy content. One of the processing routes of the FSF of sewage influent is digestion (Ghasimi et al., 2015). Although the exact composition of our FSF substrate was not measured, an approximate composition can be deduced from Appliedcleantech (www.appliedcleantech.com, accessed on 22 December 2015): 60-80% of cellulose, 5-10% of hemi-cellulose, 5-10% of lignin, 5-10% of oil and the rest accounted for inorganic salts (5-10%) ".

The FSF biodegradability was investigated in our previous researches in batch reactors, applying mesophilic and thermophilic conditions. Results of our previous study revealed a maximum biodegradability of 57% and 62% for mesophilic and thermophilic FSF digestion, respectively (Ghasimi et al., 2016). These low biodegradabilities raised the question about the

actual biodegradability of the source materials used in the different toilet papers and the contribution of other organic matter to FSF digestibility. Therefore, series of batch anaerobic digestion tests were conducted under both thermophilic and mesophilic conditions to investigate the ultimate methane potential yield (BMP), specific methane production rate (SMPR), apparent hydrolysis rate (K_h), incubation time needed to achieve 90% of the BMP ($t_{90\%}CH_4$) as well as anaerobic biodegradability (AnBD) of designated cellulose fiber-based substrates including VPPP, VTP, RTP and MCC as a fiberless reference material. The results were compared with FSF digestion results from previous studies.

5.2 Materials and Methods

5.2.1. Cellulose fibers-based substrates

VPPP, VTP and RTP samples were supplied from Dutch paper factories and were considered the cellulose fiber-based substrates in our experiments, whereas MCC was purchased from Sigma Aldrich (98% purity, Germany). Prior to conducting the experiments, VPPP, VTP and RTP were cut into 1-2 mm pieces. These pieces were mixed with demineralized water and blended for about 15 minutes to form a soft bulky substrate (Figure 5.1). Table 5.3 presents the characteristics of these substances.

5.2.2 Fine sieved fraction (FSF)

FSF was collected from the 350 µm mesh fine sieve (Salsnes. Norway) at the sewage treatment plant (STP) Blaricum, the Netherlands, and was stored at 4°C prior to conduct the BMP tests. Total solids (TS) and volatile solids (VS) were measured on weight base (g/L) according to the standard methods for the examination of water and wastewater (APHA, 2005). Chemical oxygen demand (COD) was measured using Merck photometric cell tests (500-10,000mg/L, Merck, Germany). All analyses were done in triplicate.

5.2.3 Inoculum

As inoculum for the batch tests, well-adapted and highly active sludge was used. Fresh inoculums were sampled from thermophilic and mesophilic mixed FSF fed-batch digesters (working volume of 8L), which were operated for over 500 days. The characterization of both inoculates was done according to the methods described in the previous paragraph. Initial pH of the thermophilic and mesophilic inoculum sludge were 7.4± 0.2 and 7.0± 0.1, respectively. Characteristics of the used substrates are given in Table 5.2.

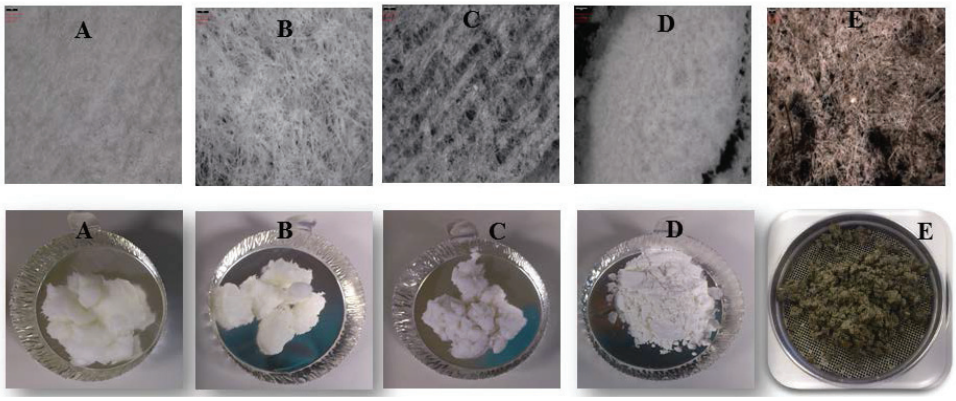

Figure 5.1 Microscopy images of VPPP (A), VTP (B), RTP (C), MCC (D) and FSF (E) in dried form using Leica Stereo Explorer 3D Microscope at 200 µm magnitude (first row: A-E) and after blending and mixing with water (except MCC and FSF) before conducting the BMP (second row: A-E)

Table 5.1 Types of additive compounds used in the papermaking process (Bos et al., 1995)

Kind/sort	Example	Purpose	Main effect
Defoamers	Alcohol derivatives	Process	Suppress foaming during processing and in the paper itself
Binders	Starch, Carboxymethylcellulose	product	Increase of the strength of paper
Bleaching	Sodium peroxide	product	Increase whiteness of the paper
Dispersants	Alcohol ethoxylate	Process	Prevention of coagulation or precipitation of pigments
Fixers	Various polymers	Process	Adhesion of several additives to the fibers
Dyes	Methyl red, violet	product	Colouring or shading of the paper
Adhesives	Resin Adhesive	product	Reduction of water absorption of paper
Wet strength agents	Urea formaldehyde resin	product	Improving the wet strength of paper
pH-regulators	Caustic soda	Process	Changing the acidity of pulp or paper
Cleaning agents	Solvents, acid, base	Process	Cleaning of machinery, piping, sieves and such during process interruption
Retention means	Polyamidoamide	Process	Reduction of fiber and filler fall-through in the sheet forming process
Slimicides	Methylene bis(thiocyanate)	Process	Inhibition of bacterial growth in pulp and process water
Felt detergents	Ethylene oxide	Process	Cleaning of machine clothing
Flocculants	Poly acrylate	Process	Promoting dewatering of rejects and sludge
Fillers	China clay	product	Opacities to improve printability of paper
Water treatment	Polyphosphate	Process	Preventing deposition of dissolved salts

5.2.4 Biomethane potential (BMP) assays

The anaerobic biodegradation of the FSF was performed using the anaerobic methane potential test (AMPTS-II), (Lund, Sweden), applying adopted protocols as suggested by Angelidaki et al. (2006, 2009). The 250 and 650mL batch flasks containing thermophilic and mesophilic inoculum, respectively, and designated substrates were incubated in a temperature controlled rotational shaker (New Brunswick™ Biological Shakers Innova® 44/44R, USA) at 150 rpm, instead of using the AMPTS-II individual mixers. The gases CO_2 and H_2S were stripped from the biogas by leading the biogas through 100 mL bottles containing a 3M NaOH solution. Hereafter the remaining gas, containing methane, flows into a gas flow cell with a calibrated volume. When the gas volume equals the calibrated volume of the flow cell, the gas was released and recorded as one normalized volume at time t. The test is finished at the moment gas production stops. Biodegradation experiments were performed in triplicate for all inoculum to substrate ratios ($R_{I/S}$) and every batch flask contained the same amount of inoculum. After adding the required amounts of inoculum and substrate, each bottle was filled with a medium including macro-nutrients, micro-nutrients and buffer solution following the protocols of Angelidaki et al. (2006, 2009), and liquid volumes were adjusted accordingly. The BMP is the net methane production per gram substrate VS added during the entire incubation period (subtracting the blank methane production) at standard temperature and pressure, which has the unit of mL CH_4/gVS_{added}.

The BMP tests were conducted at an inoculum to substrate ratio ($R_{I/S}$) of 3 under both conditions. Table 5.2 shows the dosed inoculum and substrate concentrations for the BMP tests at thermophilic and mesophilic conditions, as well as its VS content per sample. Working volumes of the digestion bottles were 0.2L and 0.4L for the thermophilic and mesophilic digestion series, respectively. The final inoculum concentration in the batch digestion bottles was 21.9 and 7.7 g VS/L and the substrate concentration (VS basis) was 7.3 and 2.6 g VS/L, both for the thermophilic and mesophilic conditions, respectively. It is noted that the TS and VS values of examined substrates were different under both conditions since the experiments were not performed simultaneously and new substrates were made for each condition. Owing to the used different volumes of the serum bottles, the amounts of TS and VS were higher under thermophilic conditions for all substrates except MCC (Table 5.3), however, the COD/VS ratio was constant under both conditions. The results of the BMP assays using different cellulosic fiber-substances and MCC were compared to the BMP of FSF under both

conditions as presented elsewhere (Ghasimi et al., 2016).

Table 5.2 Experimental set-up of the thermophilic (T) and mesophilic (M) BMP assays

Components	Substrate-wet basis (g/bottle=0.2L) (T, 55°C)	gCOD/L (T, 55°C)	Substrate-wet basis(g/bottle=0.4L) (M, 35°C)	gCOD/L (M, 35°C)
VPPP	10.6	12.0	12.2	4.8
VTP	8.9	11.0	9.9	3.8
RTP	9.9	11.8	12.6	5.1
MCC	1.5	8.5	1.1	3.0
FSF	9.1 (V_w=0.2L)	15.6	8.4 (V_w=0.4L)	14.3

5.2.5 Specific methane potential rate (SMPR)

Specific methane production rate (SMPR) (expressed in ml CH_4/g VS_{inoc}.d) was obtained by dividing the daily methane volume per gram added VS of inoculum.

5.2.6 Apparent hydrolysis rate (Kh)

Calculation of apparent K_h was performed according to the protocol published by Angelidaki et al. (2009). The apparent K_h describes the hydrolysis rate and typically follows first-order kinetics assuming normal growth (no inhibition, no lack of macro-nutrients or micro-nutrients) (Koch and Drewes, 2014; Pfeffer, 1974; Tong et al., 1990). When no intermediates accumulate, substrate hydrolysis can be regarded the rate-limiting step.

The K_h can then be derived from the accumulating methane production curve using a first-order kinetic model as expressed in Eq.(5.1):

$$P=P_{max}[1-\exp(-K_h.t)] \quad (5.1)$$

Where, P=cumulative methane production from the BMP assay at time t (mL), P_{max}= ultimate methane yield from BMP assay at the end of the incubation time (mL), K_h= first-order hydrolysis rate (1/d). The apparent K_h can be derived from the slope of the linear regression line plotted for the net accumulated methane production against time for each substrate at $R_{I/S}$ of 3.

5.2.7 Anaerobic biodegradability (AnBD)

The relationship between anaerobic biodegradability (AnBD) and BMP is given in Eq.(5.2) (Buffiere et al., 2006):

$$\text{AnBD} = \frac{BMP(mLCH_4 / gVS)}{350 \times COD_{substrate}(gCOD / gVS)} \quad (5.2)$$

Giving the conversion 1 $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$, 1 g COD equals 350 mL of CH_4 at standard temperature (273 K) and pressure (100 kPa). It is noted that this theoretical approach does not take into account the needs for bacterial cell growth and their maintenance, which has been reported typically 5-10% of organic material degraded (Angelidaki and Sanders, 2004), meaning that not all biodegraded COD is transformed into methane. Moreover, during bioconversion non-methanised biodegradable or non-biodegradable intermediates may occur, lowering the actual methane yield of the substrate. In the latter case apparent K_h must be calculated taking the accumulating intermediates into account.

5.3 Results and Discussion

Dry weight and ash content of the inoculum and substrates that were used in the experiments are presented in Table 5.3. Lowest and highest COD/VS ratios were found for MCC and VPPP, with values of 1.17 and 1.84, respectively. The high COD/VS ratio of VTTP, was rather surprising and possibly can be explained by the use of reduced chemicals during the paper production process. The Danish EPA conducted a survey on the possible chemical substances used in the paper making process, with handkerchiefs and toilet paper as end products (Abildgaard et al., 2003). They reported that, in general, up to 800 different chemical substances are used in the paper manufacturing. However, in the toilet paper and paper handkerchiefs production the variety of the chemicals used is somewhat narrower. The exact composition differs per factory and is unknown.

TS and VS concentrations of the cellulose-based substrates, except cellulose, differ between the mesophilic and thermophilic experiment since the thermophilic and mesophilic experiments were not performed at the same time and thus fresh substrates were made for each experiment.

Table 5.3 Characteristics of thermophilic (T) and mesophilic (M) inoculum and different cellulose-based substrates (VPPP, VTP, RTP , MCC and FSF)

Component	Appearance	COD/VS	TS[g/L]		VS[g/L]		VS/TS[%]
			T	M	T	M	
Inoculum (T)	Brown-darkish	1.54	30.0±0.0	-	24.0±0.0	-	79.6
Inoculum (M)	Brown-darkish	1.58	-	13.0±0.1	-	8.2±0.0	63.1
VPPP	Multi-layer compacted sheet, white	1.84	125.9±1.8	86.5±0.5	124.6±1.7	85.7±1.5	99.0
VTP	Very soft and white, 2-ply	1.50	168.8±3.5	115.0±0.9	166.8±2.0	113.9±1.8	99.0
RTP	Soft with some black spots, white-grey	1.43	168.7±0.9	115.0±1.0	166.0±1.8	112.7±2.0	98.0
MCC	Powder, white	1.17	960.0±1.2	960.0±1.2	960.0±1.2	960.0±1.2	100.0
FSF	Bulky, brownish	1.56	233.0±10.0	233.0±10.0	220.0±1.5	220.0±1.5	94.0

5.3.1 Biomethane potential (BMP)

The BMP, or ultimate methane yield tests, giving the maximum amount of mL CH_4/g VS_{added}, were conducted under mesophilic and thermophilic conditions for all substrates. Thermophilic and mesophilic digestion presented different substrate degradation characteristics (Figure 5.2, Table 5.4). With respect to the assessed BMP, the values for RTP, MCC and FSF were higher under thermophilic conditions compared to the mesophilic digesters, whereas VPPP and VTP obtained higher BMP values under mesophilic conditions. As expected, the highest BMP was found for MCC (369±5 mL CH_4/g VS) and the lowest for VTP (200±10 mL CH_4/g VS), both under thermophilic conditions. The second highest BMP was found for FSF with values reaching 338±8 and 309±5 mL CH_4/g VS under thermophilic and mesophilic conditions, respectively (Ghasimi et al., 2016) . FSF is more heterogeneous than the tested papers and virgin materials, since other particulate matter originating from the raw sewage, e.g. lipids and proteins will stay behind on the fine sieve. These compounds might have contributed to the overall higher BMP values for FSF (Table 5.4).

The reasons for the observed differences in BMP between the 2 temperature conditions are not (yet) clear and might be related to the added process chemicals (Table 5.1). During digestion, paper additives might be released, possibly impacting the methanogenic consortia differently. Various researchers showed a higher sensitivity of thermophilic methanogenic consortia compared to mesophilic ones (dos Santos et al., 2005; Kalyuzhnyi et al., 2000). Strikingly, the BMP values for VPPP and VTP were lower under the applied thermophilic condition, which is generally regarded more effective for anaerobic digestion of lignocellulosic biomass (De Baere, 2000). However, possibly more additives are released under thermophilic conditions, limiting bioconversion. In addition, it should be noted that the substrate doses on COD basis for VPPP, VTP, RTP, MCC and FSF were 2.5, 2.9, 2.3, 2.8 and 1.1 times higher for the thermophilic digesters compared to the mesophilic digesters, respectively (Table 5.2). Thus, the total quantity of possibly released additives and/or intermediate compounds might have been higher under thermophilic conditions, affecting the results.

Initial lag phases of almost 0.5 day and 1.2-2.0 days were found for all cellulose fiber-based substrates under thermophilic and mesophilic conditions, respectively, followed by a rapid methane production, which was higher in thermophilic assays compared to the mesophilic ones. However, no lag phase was observed during digestion of FSF, likely because of: (1) the

long adaptation period of the inoculum to FSF substrate (over 500 days) and (2) the presence of readily degradable matter in the FSF, like fat and proteins, that may have resulted in a steady methane generation from the start, masking any possible lag phase related to refractory fiber degradation. Previous studies achieved varying BMP values under mesophilic conditions for different types of paper: Paper and cardboard ranged between 109-128 mL CH_4/g VS (Pommier et al., 2010), whereas paper bags were reported to have a BMP of 250 mL CH_4/g VS (Hansen et al., 2004), office printer paper and newsprint paper gave a BMP of 340 and 58 mLCH_4/gVS, respectively (Jokela et al., 2005), newspaper (shredded) 92 mLCH_4/gVS (Tong et al., 1990) and magazine paper 203 mLCH_4/gVS (Owens and Chynoweth, 1993). For the commercial paper or cardboard, the range of lignin content is very wide: between 2% (office paper) and 24% (newspaper) according to Barlaz et al. (1990).

Since lignin is known to be persistent to anaerobic conversion, the variations in lignin content might partly explain the variations in reported BMP. Possibly, the low methane yield of lignin-rich substrates are rather related to lignin encrustation than to inhibitors like resin acids and sulphur-containing substances. A negative effect of possible inhibitors is found less plausible, since the substrates are highly diluted during the BMP test applying $R_{I/S}$ ratios of 3 (VS basis). Given the fact that well-adapted inoculates were used, it is assumed that hydrolytic enzymes are sufficiently available, agreeing with literature observations (Hagelqvist, 2013). In general, the BMP values found for the tested virgin hygiene papers in this study are in the high range, which might be attributed to the relatively low lignin content and limited accumulation of inhibitory additives.

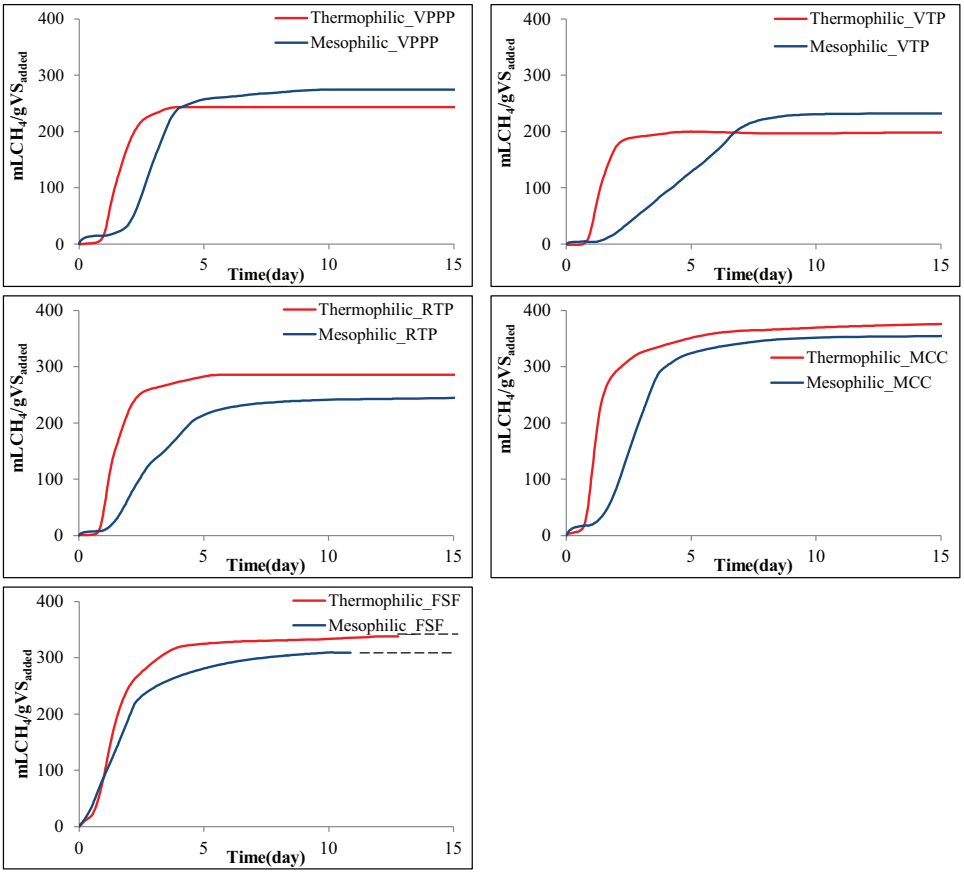

Figure 5.2 Biomethane potential (BMP) tests of VPPP, VTP, RTP, MCC and FSF under thermophilic and mesophilic conditions at $R_{I/S}$=3

5.3.2 Specific methane potential rate (SMPR)

The methane production rate varied over time, following the batch degradation of the substrate. The variation in SMPR, expressed in (mL CH_4/g $VS_{inoc.}$.d), during the digestion of the cellulose fiber-based substrates under both mesophilic and thermophilic conditions was further investigated (Figure 5.3). SMPR showed similar behaviour for all substrates under thermophilic conditions (Figure 5.3): very high rates were observed at the start of the BMP assay compared to the same substrates tested under mesophilic condition (indicated by arrow A) and they decreased rapidly after reaching their maximum values (indicated by arrow B). Under mesophilic conditions, the assessed SMPRs varied more over time and were different for the different substrates. They were always lower than the thermophilic rates and showed lag phases after an initial peak at the start of the experiment. These first peaks are probably

due to the degradation of easily biodegradable compounds in the substrate, whereafter a lag phase is observed due to a delay in degradation of the fibrous material. As it was mentioned earlier, FSF did not show any lag phase, likely due to the long adaptation period of the inoculum to FSF substrate and presence of easily degradable matters in the FSF, like fat and proteins. The high SMPR under the thermophilic conditions compared to the mesophilic conditions are likely associated with the more rapid hydrolysis of cellulose fibers and probably more rapid digestion of readily degradable compounds such as filling materials (e.g., starch) at elevated temperatures. The observed fluctuations in the methane production rate might indicate hydrolyses of different types of biopolymers in the degradation of substrates. Maximum and minimum amount of SMPR for all components under both conditions are presented in Table 5.4.

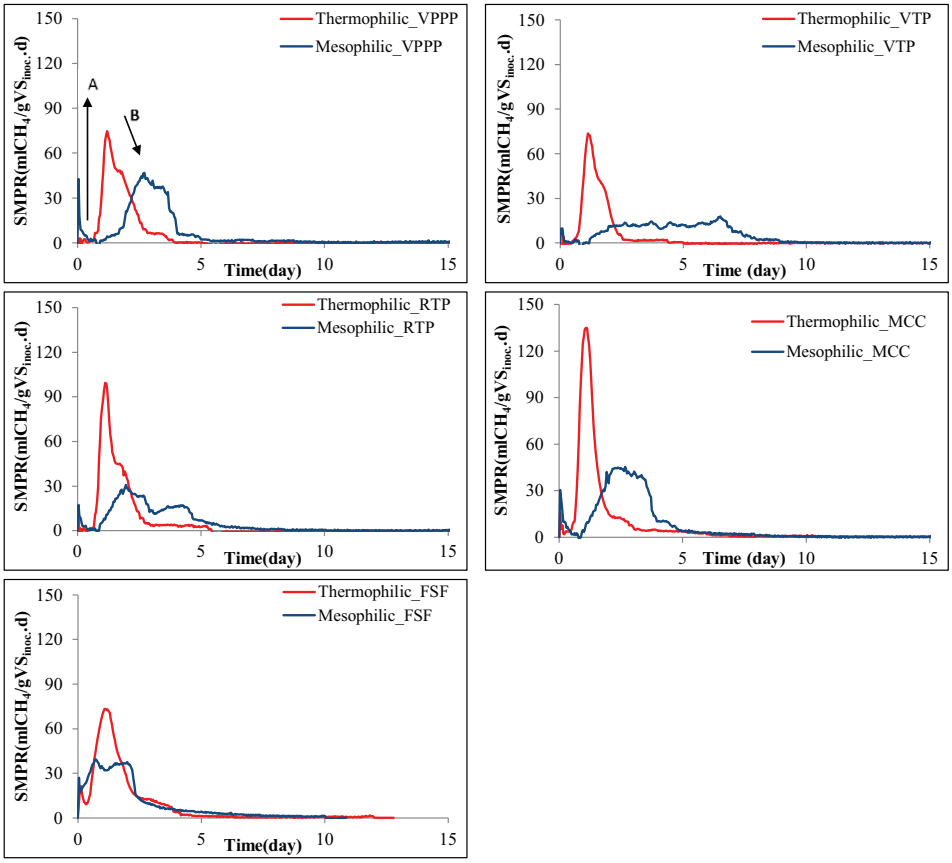

Figure 5.3 Specific methane production rate (SMPR) for VPPP, VTP, RTP, MCC and FSF under thermophilic and mesophilic conditions at $R_{I/S}$=3

5.3.3 Apparent hydrolysis rate (K_h)

Apparent hydrolysis rates (K_h) were calculated using the cumulative methane production curves from the BMP tests. Such mathematical approach is only warranted when no intermediates accumulate (see also section 5.2.6), thus, when acetogenesis and methanogenesis is not rate limiting. Owing to the set-up of the BMP batch assays, daily VFA measurements were not performed. However, by employing well-adapted inoculums and applying $R_{I/S}$ ratios of 3 in the BMP tests, we assumed that intermediates were not accumulating during the BMP tests. The applied $R_{I/S}$ of 3 in the BMP tests coincides with most literature values as reviewed by (Raposo et al., 2012). At this ratio, high amounts of active inoculum generally avoids any VFA accumulation. Similar to the SMPR results, higher apparent hydrolysis rates were found under thermophilic conditions compared to mesophilic conditions for all tested substrates (Table 5.4). Maximum and minimum apparent K_h values were found for VTP, i.e. 1.90±0.03 and 0.19±0.03 (1/d), under thermophilic and mesophilic conditions, respectively. The reason for this order of magnitude difference is not fully clear. Considering the relatively stable SMPR (Figure 5.3), the accumulation of (inhibitory) intermediates is not very likely. Speculatively, VTP may contain a higher amount of inhibitory paper chemicals. However, in the latter case, also the thermophilic batch test would have been impacted. Nonetheless, it is of interest to note that VTP obtained the lowest $SMPR_{max}$ value compared to other fiber-based cellulose, four times less than that under the thermophilic condition (Table 5.4). Unexpected inhibition phenomena have been previously observed with paper and pulp wastewaters (van Ginkel et al., 2007).

Although the inoculum was highly adapted to the FSF, resulting in absence of lag phases, the apparent K_h under thermophilic conditions was still the lowest for this material compared to the other substrates (0.85±0.05 1/d). Under mesophilic conditions the apparent K_h for FSF was comparable to the other substrates, except for the lower value of VTP.

Another factor characterizing the substrate biodegradability (Parameswaran and Rittmann, 2012) is the time required for achieving 90% of the BMP ($t_{90\%}CH_4$); results are shown in Table 5.4 as well. Shortest and longest $t_{90\%}CH_4$ under the thermophilic conditions were recorded at 2 and 4.3 days for VTP and MCC, whereas under mesophilic conditions FSF and MCC achieved the shortest $t_{90\%}CH_4$ of 5 days and VPPP obtained the longest $t_{90\%}CH_4$ of 7.6 days.

In general, the required incubation periods observed in our BMP experiments were considerably shorter than the ones described in the literature, which may range between 30-50 days (Owen et al., 1979; Hansen et al., 2004; Lesteur et al., 2010). Very likely, the use of well adapted inoculum is crucial for these substrates (Ghasimi et al., 2015), resulting in an extremely rapid conversion.

Table 5.4 Biomethane potential (BMP), maximum specific methane production rate ($SMPR_{max}$), apparent hydrolysis rate (K_h) and time to achieve 90% of maximum BMP ($t_{90\%}CH_4$) at $R_{I/S}$ of 3 under mesophilic and thermophilic conditions

Items	BMP (mL CH_4/gVS)		$SMPR_{max}$ (mL CH_4/(gVS_{in}·d)		apparent K_h (1/d)		$t_{90\%}CH_4$ (day)	
	35ºC	55ºC	35ºC	55ºC	35ºC	55ºC	35ºC	55ºC
VPPP	274±2	244±4	46.7±3.9	74.5±1.5	0.77±0.01	1.54±0.04	7.6	2.5
VTP	230±15	200±10	17.9±5.0	73.7±9.0	0.19±0.03	1.90±0.03	7.0	2.0
RTP	254±10	285±15	30.8±1.5	99.5±2.0	0.41±0.02	1.34±0.04	6.0	2.6
FSF	309±5	338±8	39.0±2.0	73.0±4.0	0.60±0.05	0.85±0.05	5.0	3.3
MCC	351±5	369±5	45.3±1.0	135.0±1.0	0.77±0.02	1.54±0.02	5.0	4.3

5.3.4 Anaerobic biodegradability (AnBD) of the different substrates

Figure 5.4 shows a similar anaerobic biodegradation for the tested substrates under both temperature conditions. Degradation of easily biodegradable compounds (e.g., lipids and proteins) might have directly contributed to the higher AnBD (>50%) for FSF under both conditions compared to VPPP, VTP and RTP that mainly consist of cellulose fibers. However, MCC, probably due to its physical and chemical structure and manufacturing conditions (Landin et al., 1993), obtained the highest biodegradation percentage of 91% and 86% under thermophilic and mesophilic conditions, respectively, also resulting in the highest BMP values among the tested substrates.

The observed differences possibly reflect the influence of physicochemical properties, used paper chemicals, and applied processing conditions, such as pretreatment and delignification, for the cellulolosic fibers and MCC. Pommier et al. (2010) showed a high heterogeneity in degree of biodegradation of different types of paper and cardboards (28-58%), which was ascribed to the differences in lignin content. In general, none of the employed cellulose fiber-based substrates had a higher biodegradation percentage than the 50% observed in our experiments. The aerobic biodegradation (45 days controlled aeration) of different paper wastes, including tissue paper (paper handkerchiefs, serviettes 50%, table cloths) were studied by Alvarez et al. (2009).

Results of their experiments indicated 50% biodegradation for the tissue paper compared to the theoretical biodegradable fraction of the paper volatile solids (≈ 63 %.), excluding 7 % of lignin content. Firstly, the observed low biodegradability could have been related to the organic additives dosed in the manufacturing or finishing process. Secondly, the particles of the tissue paper tended to form "balls" in the test containers due to absorption of humidity and swelling of fibers. This likely reduced the surface contact with enzymes lowering the final biodegradability determined (Alvarez et al., 2009).

Poor biodegradation of toilet paper during anaerobic digestion (<50%) might be due to the characteristics of the employed fibers (short or long), degree of crystallinity of the fibers, types of pulping and presence of poor lignin material, as well as formation of toxic and refractory compounds that are hardly biodegradable by anaerobic microorganism. Therefore, more detailed research is needed to evaluate the complex biodegradation process of toilet paper in terms of additive chemicals (i.e., resins, binders, wax, anti-foaming agents, cleaning agents, creping chemicals, dyes, etc.) and lignin compounds.

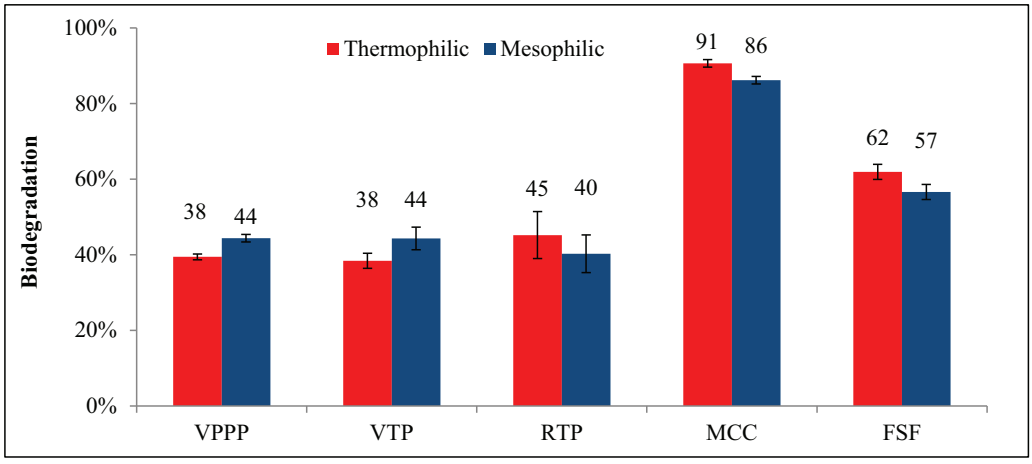

Figure 5.4 Biodegradation percentage of VPPP, VTP, RTP, MCC and FSF under thermophilic and mesophilic conditions at $R_{I/S}$ of 3

5.4 Conclusions

Based on the results of this study the following conclusions were drawn:

- Thermophilic and mesophilic digestion of different cellulose fiber-based substrates (VTP, VPPP and RTP) showed different conversion characteristics, as characterised by BMP, SMPR, AnBD, apparent K_h as well as $t_{90\%}CH_4$. However, the variations in BMP ranged from 5% to 12% and their anaerobic biodegradation percentage was, more or less, in the same range (<50%),

- The non-fibrous MCC obtained the highest BMP and biodegradation percentage under both thermophilic and mesophilic conditions compared to all employed substrates.

- The second most biodegradable substrate was FSF. The applied long adaptation period of the used inoculates and the assumed presence of more readily biodegradable compounds (e.g., proteins and lipids) in the FSF might have contributed to the higher BMP and biodegradation percentage compared to the fiber-based substrates.

5.5 References

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Chapter 6. Impact of lignocellulosic-waste intermediates on hydrolysis and methanogenesis under thermophilic and mesophilic conditions

Abstract

Intermediates of anaerobic conversion processes have been identified to inhibit methanogenic biomass and to decrease process performance. The used concentrations of model intermediates furfural, 5-hydroxymethylfurfural (HMF) and vanillin, as well as the recalcitrant humic acid were 0.4, 0.8 and 2.0 g/L. These compounds were used to determine their impact on methanogenesis by specific methanogenic activity (SMA) assays and hydrolysis by cumulative methane production (CMP) tests under thermophilic and mesophilic conditions at a concentrations of 0.8 g/L, using lignocellulosic biomass as the substrate.

HMF showed inhibitory effects at a concentration of 0.8 g/L under thermophilic conditions during SMA tests. HMF and furfural completely inhibited the methanogenic activity at 2.0 g/L under both thermophilic and mesophilic conditions. The inhibitory effect was absent with vanillin and humic acid at concentrations ≤ 2.0 g/L and 0.8 g/L, during SMA and CMP tests, respectively. The thermophilic microbial consortia were found to be more sensitive to increased concentrations of the intermediates than mesophilic consortia, determined by the methane production rates and quantity in CMP tests.

This chapter is based on:

Ghasimi, D.S.M., Aboudi, K., de Kreuk, M., Abbas, B., Zandvoort, M.H., van Lier, J.B., 2015. Impact of lignocellulosic-waste intermediates on hydrolysis and methanogenesis under thermophilic and mesophilic conditions. Chem. Eng. J. 295, 181-191.
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6.1 Introduction

At wastewater treatment plants (WWTPs) in The Netherlands, coarsely screened (6 mm) sewage is directed through a fine sieve (Salsnes Filter, Norway) with a mesh size of 350 μm. These sieves can be implemented as a compact alternative to primary clarification. The fine sieved fraction (FSF) is a heterogeneous substrate, sequestered from raw sewage, which mainly consists of partly dissolved toilet paper (with a high cellulose fraction), hair, lignin-rich compounds such as leaves and shell of fruits, sands and undefined materials. Although the exact composition of our FSF substrate was not measured, an approximate composition can be deduced from Appliedcleantech (www.appliedcleantech.com, accessed on 22 December 2015): 60-80% of cellulose, 5-10% of hemi-cellulose, 5-10% of lignin, 5-10% of oil and the rest accounted for inorganic salts (5-10%).

Anaerobic digestion (AD) is an attractive sludge treatment practice in which both waste control and energy recovery can be achieved (Abdelgadir et al., 2014; Chen et al., 2008a). Many agricultural and industrial wastes are ideal candidates for anaerobic digestion because they contain high levels of easily biodegradable materials (Chen et al., 2008b). Despite the vast knowledge on AD processes, unexpected low methane yields and process instability are frequently observed. Waste based inhibitory compounds and/or accumulating intermediates can be responsible for reactor perturbation and instabilities in the digestion process (Benjamin et al., 1984; Chen et al., 2008a; den Camp et al., 1988; Rajagopal et al., 2013). The nature and degree of inhibition fully depends on the type of inhibitor present, slowing down or even blocking the enzymatic activity (Brons et al., 1985; Fernandes et al., 2015; Palmqvist and Hahn-Hägerdal, 2000a).

During the physicochemical pretreatment or microbial hydrolysis of lignocellulosic biomass, soluble sugars (mainly xylose) are produced but by-products such as furan and lignin derivatives (phenolic compounds) are also generated in the biomass hydrolysate (Kumar et al., 2009; Zhao et al., 2009). The main furan compounds are furfural and 5-hydroxymethylfurfural (HMF) from the degradation of glucose (C_6) and xylose (C_5) moieties, derived from (hemi-)cellulose conversion, whereas the main inhibiting phenols and polyphenols originate from lignin polymers and/or lignin oligomers, vanillin and syringaldehyde, resulting from partial lignin degradation (Barakat et al., 2012; Klinke et al., 2002). Phenolic compounds as vanillin have a significant impact on the fermentation of hydrolysates and could be toxic at certain concentrations because they compromise the

integrity of biological membranes (Heipieper et al., 1994). HMF can be further degraded, forming levulinic acid. In addition, formic acid can be formed from furfural under acidic conditions at elevated temperatures (Clark and Mackie, 1984; Ulbricht et al., 1984). It has been reported that at concentrations of 4.6 g/L, formic acid was more inhibitory than levulinic acid which, in turn, was more inhibitory than acetic acid in the process of bioethanol production (Larsson et al., 1999). Hence, furfural was found to be more inhibiting than HMF, due to lower molecular weight of furfural compared with HMF, which facilitates its diffusion into microbial cells (Quéméneur et al., 2012). On the other hand, it was reported that when furfural is present alone at lower concentrations, it can be efficiently converted and metabolised, however, if it is present with HMF, the conversion rates of both decreased significantly and HMF degradation only proceeded when the complete degradation of furfural occurred (Taherzadeh et al., 1999).

Furthermore, furanic compounds (i.e. furfural and HMF) were reported to have detrimental effects on microorganisms by inhibiting cell growth, inducing DNA damage and inhibiting several enzymes of the glycolysis pathway (Almeida et al., 2009; Palmqvist and Hahn-Hägerdal, 2000b). Phenolic compounds damage microbial cells by altering selectively the membrane permeability, causing leakage of intracellular components and inactivation of essential enzymatic systems (Campos et al., 2009; Hierholtzer et al., 2013).

Humic substances, including humic acids and fulvic acids, are the main components of sludge organic substances (sewage sludge and compost) (Ayuso et al., 1997) that are recognized to be recalcitrant compounds and hardly degradable in biological treatment processes (Feng et al., 2008). They become enriched in oxygen functional groups and aromatic rings in the digestion chamber (Bartoszek et al., 2008) and probably no two humic acid molecules will be identical. Humic acid can be formed also from the phenolic compounds released during lignin decomposition (residues) as well as from reducing sugars and amino acids formed as results of microbial metabolism. At certain concentrations, humic compounds can have inhibitory effects on the methane production during the anaerobic digestion of organic waste (Azman et al., 2015; Brons et al., 1985; Fernandes et al., 2015; Monlau et al., 2014).

Fernandes et al. (2015) showed that humic acid like and fulvic acid like compounds may seriously impact the hydrolytic enzymatic activity. Azman et al. (2015) did not only find lower biomethane production (BMP) of the tested microcrystalline cellulose (Avicel) substrate at presence of humic acids, but also additional VFA accumulation, indicating the

inhibition of methanogens rather than hydrolysis as suggested earlier by others (Brons et al., 1985; Fernandes et al., 2015). There are plenty of researches conducted towards the effect of these inhibitor compounds on the fermentation of lignocellulosic materials for bioethanol production (Almeida et al., 2007; Mills et al., 2009; Palmqvist and Hahn-Hägerdal, 2000a). However, their effects on the anaerobic digestion under both thermophilic (55°C) and mesophilic (35°C) conditions hardly have been studied. Figure 6.1 schematically presents possible FSF (as a lignocellulosic material) conversion during (pre-)treatment, producing recalcitrant humic matter, furans (furfural and HMF) and aromatic compounds (Barakat et al., 2012; Monlau et al., 2014). Hence, the purpose of this study was to assess the impact of representative intermediates such as furfural, HMF, vanillin and humic acid sodium salt on anaerobic consortia from digesters operated under both thermophilic and mesophilic conditions. Influence of these potentially inhibiting intermediates on the hydrolysis and methanogenesis was studied employing specific methanogenic activity (SMA) assays and cumulative methane production (CMP) tests.

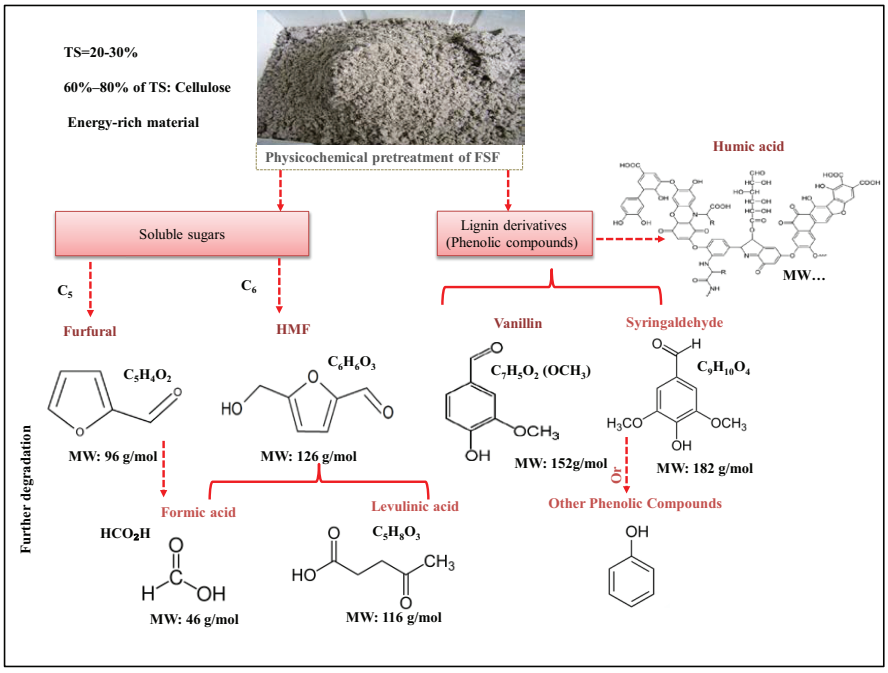

Figure 6.1 Schematic view of possible FSF conversion during (pre-)treatment, producing recalcitrant humic compounds, furans (furfural and HMF), and aromatic compounds (Barakat et al., 2012; Monlau et al., 2014).

6.2 Materials and Methods

6.2.1 Substrate

FSF was collected from a 350 μm mesh fine sieve (Salsnes, Norway) at wastewater treatment plant (WWTP) Loenen, The Netherlands, and stored at 4°C prior to conduct the CMP tests. Total solids (TS) and volatile solids (VS) were measured on weight base (g/L) according to the standard methods for the examination of water and wastewater (APHA, 2005). Chemical oxygen demand (COD) was measured using Merck photometric cell tests (500-10,000 mg/L, Merck, Germany). All analyses were done in triplicate. Furfural and hydroxymethyfurfural (HMF), Vanillin and humic acid sodium salt were purchased from Sigma Aldrich (98% purity, Germany).

6.2.2 Inoculum

Four water jacketed laboratory mixed fed-batch digesters operated as a fed-batch reactor (FBR) with a working volume of 8L were operated in duplicate to digest FSF under both thermophilic (55 ^{o}C) and mesophilic (35 ^{o}C) conditions over a period of 718 days, prior to harvest the inoculates. The inoculum was directly taken from the digesters. At the time of sampling, thermophilic and mesophilic digesters were operated at organic loading rates (OLR) of 5.5 and 2.5 kgCOD/m^3.d, respectively. The inoculates were characterized in the same way as the substrate. Prior to the experiments, sludge pH was determined at 7.4±0.2 and 7±0.1 for thermophilic and mesophilic sludge, respectively.

6.2.3 Volatile fatty acid (VFA)

Volatile fatty acids (VFAs) were quantified by Gas Chromatograph (GC, Agilent Technology 7890A), using a flame ionization detector (FID) and a capillary column type HP-FFAP Polyethylene Glycol (25 m × 320 μm × 0.5 μm) with helium as the carrier gas at a total flow of 67 ml/min and a split ratio of 25:1. The GC oven temperature was programmed to increase from 80 min to 180 °C in 10.5 min. The temperatures of injector and detector were 80 °C and 240 °C, respectively, and the injected volume was 1 μl. Prior to GC analysis 10 ml of digested sample was first centrifuged at 15,000 rpm for about 15-20 minutes. Then, the supernatant was filtrated over 0.45 μm filter paper. The filtrated liquid was diluted 2 and 3 times with pentanol as internal solution (300 ppm) for mesophilic and thermophilic digestion samples, respectively. Finally, 10 μL of formic acid (purity >99%) was added into the 1.5 mL vials.

6.2.4 Specific Methanogenic Activity (SMA)

Specific methanogenic activity (SMA) assays were used to determine the maximum methane production rate of methanogenic sludge using acetate as the substrate. SMA tests in the presence of intermediates were performed to determine possible inhibition caused by these compounds. In this study, the SMA of the mesophilic and thermophilic sludge was determined using an Automated Methane Potential Test System (AMPTS_II) from Bioprocess Control (Sweden). The SMA was conducted using sodium acetate COD (SA_{COD}) concentrations of 1.0 and 0.5 g/L as the substrate and a medium consisting of a mixture of macronutrients, trace elements and phosphate buffer solution (Angelidaki and Sanders, 2004). SMA was calculated by dividing the maximum slope of the accumulated methane production curve (mL/d) by grams volatile solids (VS) introduced in the bottle (inoculum). The final values were expressed in: gCH_4-COD/(gVS.d). SMA tests were conducted in triplicate, using a liquid working volume of 0.2 L under both thermophilic and mesophilic conditions. The impact of intermediate compounds as potential inhibitors was studied using concentrations of 0.4, 0.8, and 2.0 g/L, in the presence of 1 g SA_{COD}/L (first stage, Table 6.1) at both 35°C and 55°C. In addition, tests were performed using a concentration of the potential inhibitors of 0.4g/L and 0.5 g SA_{COD}/L, incubated at the same temperatures. (second stage; Table 6.1). It is noted that during the calculation of the SMA, methane production from the control bottles containing sodium acetate or sodium acetate with intermediates was not subtracted from the methane production of the inoculums.

6.2.5 Cumulative methane production (CMP) assays

The CMP is the net methane production per gram substrate VS added during the entire incubation period (subtracting the blank methane production) at standard temperature and pressure (T=0°C and P=1atm), which has the unit of mL CH_4/gVS_{added}.

The CMP tests were stopped under thermophilic and mesophilic conditions when the daily methane production was less than 5% of the CMP_t using the following equation (6.1):

$$\frac{CMP_t - CMP_{t-1}}{CMP_t} \leq 0.05 \qquad (6.1)$$

where CMP_t is the average cumulative methane production at time t (mL CH_4/gVS_{added}) and CMP_{t-1} is the average cumulative methane production one day before t (mL CH_4/gVS_{added}).

The anaerobic biodegradation tests of the FSF was performed using (AMPTS-II), applying adapted protocols suggested by (Angelidaki et al., 2009, 2006). The 250 mL batch flasks

containing inoculum and substrate were incubated in a temperature controlled rotational shaker (New Brunswick™ Biological Shakers Innova® 44/44R, USA) at 150 rpm. CO_2 and H_2S gas were stripped from the biogas by leading the biogas through 100 mL bottles containing a 3M NaOH solution. Hereafter the remaining gas, containing methane, flows into a gas flow cell with a calibrated volume. When the gas volume equals the calibrated volume of the flow cell, the gas was released and recorded as one normalized volume at time t. The test is finished at the moment gas production stops. After adding the required amounts of inoculum and substrate, using an inoculum to substrate ratio ($R_{I/S}$) of 3 gVS_I/gVS_S, each bottle was filled with a medium including macro-and micro-nutrients and buffer solution to maintain the designated volume (0.2L) according to the mentioned protocols above (Angelidaki and Sanders, 2004). The influence of intermediates on FSF degradation was studied by adding above-mentioned potential inhibitors at a fixed concentration of 0.8 g/L, under both conditions (third stage; Table 6.1).

All batch tests including blank (inoculum), SMA control (inoculum and sodium acetate) and CMP control (inoculum and FSF) were conducted in triplicate and tests with inhibitors in duplicate. It is noted that standard deviation for SMA and CMP controls and the error from average values for all inhibitor compounds during SMA and CMP tests under both thermophilic and mesophilic conditions were calculated at less than 5%.

6.2.6 Anaerobic biodegradability

Anaerobic biodegradation (AnBD) was assessed as the experimental ultimate methane production (expressed in g COD) over the initial amount of COD (tCOD) of the substrate (and intermediates) (Raposo et al., 2011).

Giving the conversion 1 $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$, 1 g COD equals 350 mL of CH_4 at standard temperature (273 K) and pressure (100 kPa). It is noted that the methane produced will be lower than this theoretical value, as it does not take into account the COD required for bacterial cell growth and their maintenance, which has been reported typically 5-10% of organic material degraded, depending on the type of substrate (Angelidaki and Sanders, 2004). Moreover, during bioconversion, non-methanised biodegradable or non-biodegradable intermediates may occur, lowering the actual methane yield of the substrate.

6.2.7 Physicochemical characteristics of thermophilic and mesophilic sludge, inhibitor concentrations and FSF used

Tables 6.1-6.5 present the details of the experimental stages of the SMA and CMP tests, applying different concentrations (0.4, 0.8, and 2.0 g COD/L) of intermediates and the potential inhibitors at a specific concentration. They also include characteristics of the thermophilic and mesophilic inoculum and the used FSF as well as the final pH of the SMA and CMP tests.

Table 6.1 Experimental stages conducted for SMA and CMP tests applying potential inhibitors and FSF

Stages	Type of experiment	Potential inhibitor concentration (g/L)
First	thermophilic and mesophilic SMA using SA_{COD} of 1 g/L	0.4, 0.8 and 2.0
Second	thermophilic and mesophilic SMA using SA_{COD} of 0.5 g/L	0.4
Third	thermophilic and mesophilic CMP	0.8

Table 6.2 COD of intermediates (or potential inhibitors) at different applied concentrations of 0.4, 0.8 and 2.0 g/L during thermophilic and mesophilic SMA and CMP tests

Components	gCOD/g	gCOD/L at [0.4 g/L]	gCOD/L at [0.8 g/L]	gCOD/L at [2.0 g/L]
Furfural ($C_5H_4O_2$)	1.67	0.67	1.33	3.33
HMF ($C_6H_6O_3$)	1.52	0.61	1.22	3.05
Vanillin ($C_8H_8O_3$)	1.79	0.72	1.43	3.58
Humic acid salt	1.87	0.75	1.50	3.75

Table 6.3 Thermophilic and mesophilic inoculum concentrations (gVS/L) at different SA_{COD} and intermediates (or potential inhibitors) concentration (I) applied during SMA tests

Inoculum	gVS/L [SA_{COD}=1g/L,I=0.4g/L]	gVS/L [SA_{COD}=1g/L,I=0.8g/L]	gVS/L [SA_{COD}=1g/L,I=2g/L]	gVS/L [SA_{COD}=0.5g/L, I= 0.4g/L]
Thermophilic	13.0	14.6	14.8	11.4
Mesophilic	11.1	11.0	11.0	14.2

Table 6.4 Physiochemical characteristics of thermophilic and mesophilic inoculum and FSF used

Component	COD[g/L]	TS[g/L]	VS[g/L]	VS/TS	gVS_{FSF}/bottle
Thermophilic_Inoculum	85.0±5	54.0±2	46.7±2	0.86	----
Mesophilic_Inoculum	68.3±3	60.0±2	48.6±9	0.81	----
FSF_T	410.0±20	293.0±3	277.0±2	0.95	2.50
FSF_M	448.0±10	301.0±3	283.0±2	0.94	2.60

Table 6.5 Final pH of thermophilic (T) and mesophilic (M) CMP and SMA tests at all applied intermediates (or potential inhibitors) (I) concentrations

Components	CMP-T	CMP-M	SMA-T	SMA-M	SMA-T	SMA-M	SMA-T	SMA-M
	pH (I=0.8g/L)	pH (I=0.8g/L)	pH (I=0.4g/L)	pH (I=0.4g/L)	pH (I=0.8g/L)	pH (I=0.8g/L)	pH (I=2g/L)	pH (I=2g/L)
Blank	7.3	6.9	7.3	6.9	7.3	6.9	7.3	6.9
Control	7.3	6.9	7.3	7.0	7.3	7.0	7.0	7.3
Furfural	7.3	6.8	7.1	7.0	7.1	6.9	6.3	5.6
HMF	7.3	6.9	7.3	7.3	7.0	7.0	6.6	6.4
Vanillin	7.2	6.9	7.2	7.0	7.2	7.0	6.9	6.9
Humic acid	7.3	6.9	7.5	7.2	7.6	7.3	7.1	7.2

6.3 Results and Discussion

The effects of four, possibly inhibiting, intermediate compounds, which occur during lignocellulosic biomass fermentation, were studied in batch tests. Intermediates selected were furfural, HMF, vanillin and humic acid, since their presence or formation is to be expected during the degradation of the lignocellulosic FSF. Their presence in higher concentrations may slow down, disorganize or possibly stop the activity of the microbial communities in anaerobic digestion.

SMA tests using a SA_{COD} concentration of 1.0 g/L and inhibitor compounds at concentrations of 0.4, 0.8 and 2.0 g/L (first stage) were performed under both thermophilic and mesophilic conditions. For the inhibitor concentration of 0.4 g/L an additional experiment with a SA_{COD} concentration of 0.5 g/L was performed (second stage). Hydrolysis inhibition was further studied by CMP tests with FSF, as the substrate, under thermophilic and mesophilic conditions (third stage).

6.3.1 First stage : Thermophilic conditions (SMA: SA_{COD} of 1.0 g/L & I=0.4, 0.8 and 2.0 g/L)

6.3.1.1 Inhibitors at 0.4 g/L:

All SMAs had approximately the same methane production rate as the SMA control, in which no inhibitors were added (Figure 6.2). In fact, no inhibitory effect was found. Furfural and vanillin were respectively 29% and 12% degraded (methanised). Degradation of HMF and humic acids were determined to be only 4% and 3%, respectively. However, since the errors from average values were reported <5 %, these values were considered inconclusive.

6.3.1.2 Inhibitors at 0.8 g/L:

SMA with Vanillin and humic acid addition had the same methane formation rate as the SMA control. Vanillin and humic acid biodegradation was 16 and 2 % , respectively (Figure 6.2). The SMA test with furfural addition moderately impacted the rate of methane formation. However, the furfural added batch digesters produced more methane than the SMA control indicating an estimated furfural biodegradability of about 20%. Metabolic conversion of furfural by a methanogenic Archaea, *Methanococcus* sp., strain B was studied by Boopathy (2009). The organism was grown on H_2-CO_2 in the presence of various concentrations of furfural, i.e. 0.48, 0.96, 1.44, 1.92, 2.40 and 2.88 g/L. Results of the experiment showed that

there was no inhibition for furfural at low concentrations of 0.48, 0.96 and 1.44g/L, and furfural was completely (100%) metabolized in the cultures within five days of incubation. The end product observed during furfural metabolism was furfuryl alcohol.

Presence of HMF at 0.8 g/L considerably decreased the methane production while the observed methane production rate during the first 1.3 days was very comparable to the blank; hereafter it was slightly higher. Results indicate that HMF affected the acetotrophic methanogens at this concentration (moderate inhibitory effect).

6.3.1.3 Inhibitors at 2.0 g/L:

Presence of furfural and HMF at a concentration of 2.0 g/L completely halted methanogenesis (Figure 6.2). Only furfural showed an initial methane production that was similar to the blank. The final pH values of the assay dropped to 6.3 and 6.6, respectively, possibly indicating formation of formic (p*K*a = 3.75) and levulinic acid (p*K*a = 4.59) from the inhibitors, which have p*K*a values below acetic and propionic acid (p*K*a = 4.76 and 4.87, respectively). Wirtz and Dague (1993) reported that anaerobic treatment of wastewater containing high concentrations of furfural resulted in a low reactor pH, which was not the case when no furfural was present. Boopathy (2009) have noticed that cultures exposed to 2.40 and 2.88 g/L of furfural were severely inhibited, while moderate inhibition was observed at 1.92 g/L. Vanillin affected the SMA rate but eventually produced the same methane quantity as the SMA control. Humic acid addition at this concentration did not affect the SMA and had the same rate of methane production as the SMA control. Delgenes et al. (1996) have found that vanillin inhibited the xylose fermentation process at a concentration of only 1.0 g/L, affecting the microbial strains studied by the author. It should be noted that depending on the activity of the bacterial consortium, intermediate compounds could selectively inhibit some microbial species more than others.

From the above discussed results, it can be concluded that at concentrations of 0.4 to 2.0 g/L, no inhibitory effects of humic acid on the methanogenic activity was observed under thermophilic conditions. However, vanillin slightly affected the SMA rate at a concentration of 2.0 g/L. Furfural was found to moderately and completely inhibit the SMA assays at 0.8 and 2.0 g/L, respectively, whereas HMF was inhibitory at 0.8 g/L. Several authors have demonstrated that the strongest inhibiting furan derivatives in the lignocellulosic hydrolysates are furfural and HMF affecting the cell growth (Liu et al., 2004; Quéméneur et al., 2012;

Taherzadeh et al., 1999). Similarly, Siqueira and Reginatto (2015) reported that furans derivatives (Furfural and HMF) are more strong inhibitors than vanillin.

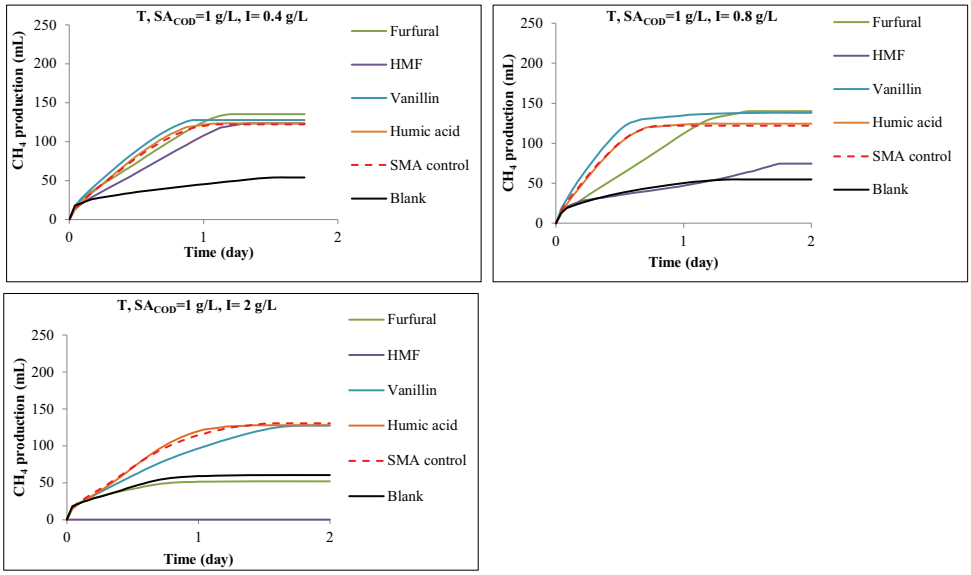

Figure 6.2 Effect of selected potential inhibitors (or intermediates) (I) on thermophilic (T) SMA at concentrations of 0.4, 0.8 and 2.0 g/L and a SA_{COD} concentration of 1.0 g/L

6.3.2 First stage : Mesophilic conditions (SMA: SA_{COD} of 1g/L & I=0.4, 0.8 and 2.0 g/L)

6.3.2.1 Inhibitors at 0.4 g/L: All incubations gave the same SMA values as the control. The amount of methane produced compared to the control was higher for all incubations, indicating partial conversion of the potential inhibitor, amounting 12%, 17%, 33%, and 22% for furfural, HMF, vanillin and humic acid, respectively (Figure 6.3).

6.3.2.2 Inhibitors at 0.8 g/L:

Batches with humic acid and vanillin addition had the same SMA values as the SMA control. Following the mass balance, vanillin was apparently degraded by 9%, whereas humic acid produced slightly less CH_4 than the SMA control. The result of the vanillin is in line with literature. Degradation of lignin-derived by-products, phenolic compounds, such as vanillin and syringaldehyde at a concentration of 1 g/L in presence of xylose at 1 g/L was investigated by Barakat et al. (2012) under mesophilic conditions. It was reported that the final methane

yields were also not reduced by addition of vanillin. Addition of HMF and furfural led to comparable methane production rates as the blank for almost 0.8 and 1.0 days for HMF and furfural, respectively (Figure 6.3). Siqueira and Reginatto (2015) studied the effect of furfural and HMF on H_2 production and reported that addition of furfural at 0.5 and 1g/L increased the lag-phase compared to the control, however, and differently to our results, HMF addition did not induce a lag-phase. Nevertheless, the system in which HMF was dosed eventually produced the same amount of methane as the SMA control, whereas the furfural-added incubation stayed below the SMA control (Figure 6.3).

6.3.2.3 Inhibitors at 2.0 g/L:

Adding humic acid to the SMA test with mesophilic sludge resulted in the same rate of methane production as the SMA control, but the total methane production was lower than the SMA control (Figure 6.3). Vanillin slightly affected the rate of methane production, but the same amount of produced methane was observed as in the control, which means that there was no degradation of vanillin at this elevated concentration. Apparently, furfural and HMF severely inhibited the methanogens at this concentration and the final pH measured after the experiment was measured at 5.6 and 6.4, respectively (Table 6.5, Figure 6.3). This suggests an additional acid formation, and therefore it is suspected that both furfural and HMF's have been acidified under mesophilic conditions. Shanmugam et al. (2014) reported that 2.0 g/L of furfural fed to a microbial culture significantly affected the methanogenesis step, inhibiting several metabolic pathways.

Overall, addition of 0.8 g/L of both furfural and HMF resulted in a similar methane production rate as the SMA control, except for the first day of incubation. However, furfural lowered the total amount of produced methane compared to the SMA control, whereas HMF produced the same amount of CH_4. There was no observed inhibitory effect of adding vanillin at lower concentrations of 0.4 and 0.8 g/L, however, it slightly affected the SMA rate at 2.0 g/L. Humic acid slightly affected the amount of methane production at concentrations of 0.8 and 2.0 g/L. At a concentration of 2.0 g/L, all components except vanillin and humic acid, completely inhibited the methane production.

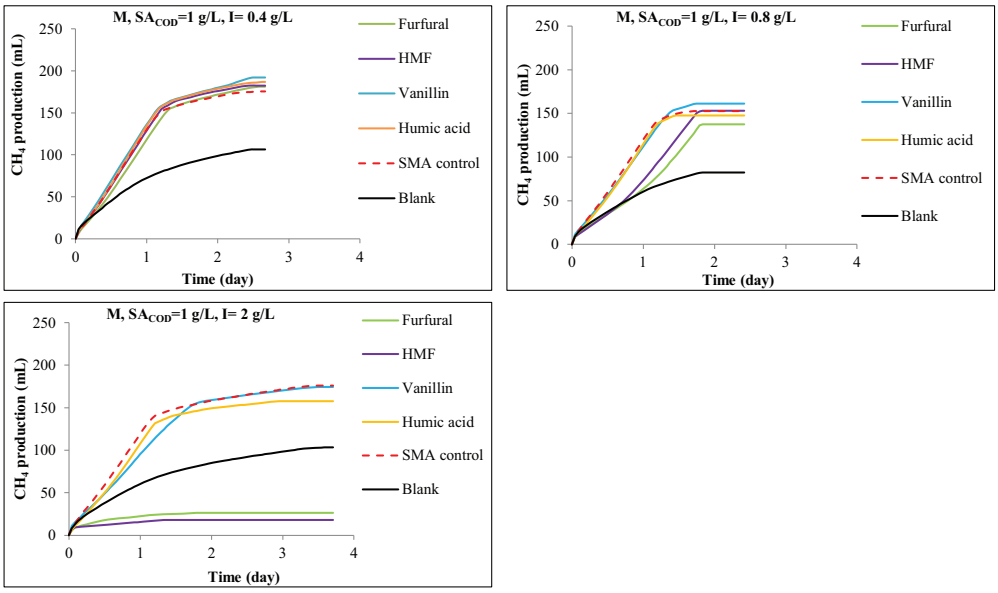

Figure 6.3 Effect of selected potential inhibitors (or intermediates) (I) on mesophilic (M) SMA at concentrations of 0.4, 0.8 and 2.0 g/L and a SA_{COD} concentration of 1.0 g/L

6.3.3 Second stage : Thermophilic and mesophilic conditions (SMA: SA_{COD} of 0.5 g/L & I=0.4 g/L)

In this experiment (second stage), it was aimed to study the degradation or inhibition effect of all employed inhibitors at a concentration of 0.4 g/L applying a reduced substrate concentration of 0.5 g/L SA_{COD}. As shown in Figure 6.4 (T, top), furfural and HMF significantly affected the rate of methane conversion under thermophilic conditions, but for both, eventually, the amount of produced methane was higher than in the SMA control, indicating HMF and furfural degradation in addition to the acetate conversion. Based on the mass balance, furfural and HMF were degraded by 97% and 20%, respectively.

Vanillin had the same rate of methane production as the SMA control, whereas humic acid slightly affected the rate of methane production. However, both components contributed to higher CH_4 production compared to the SMA control. Remarkably, at the higher SA_{COD} concentration of 1.0 g/L (Figure 6.2), the intermediates did not affect the rate of methane generation. Apparently, the methanogenic consortium is more affected at an initial low acetate concentration.

The mesophilic sludge SMA was hardly affected by the potential inhibitors at the low SA_{COD} (Figure 6.4, M, bottom). In fact, all methane production rates were very similar. Only furfural addition clearly declined the rate of methane generation to a rate similar as the blank, within the first 1.3 days of the assays. The additional methane production for all added intermediates under thermophilic conditions suggests higher degradation efficiencies with decreasing SA_{COD}. Surprisingly, the extra methane production with addition of furfural, HMF, vanillin and humic acids were 3.3, 5, 1 and 3.7 times higher than their correspondents at SA_{COD} of 1g/L (Figure 6.2, Figure 6.4). These results remarkably contrast to the ones obtained under mesophilic conditions, where intermediate compounds were more degraded at the higher SA_{COD}. There is no clear explanation for the observed phenomenon, and supplementary investigations are needed to further elucidate this effect. Possibly, the long adaptation period of the biomass to FSF that releases mentioned components during thermophilic degradation might have played an important role. Results suggest that the presence of high intermediate acetate concentrations negatively impact the conversion of the mentioned potential inhibitors. Vanillin degradation was not influenced by applying different SA_{COD} concentrations.

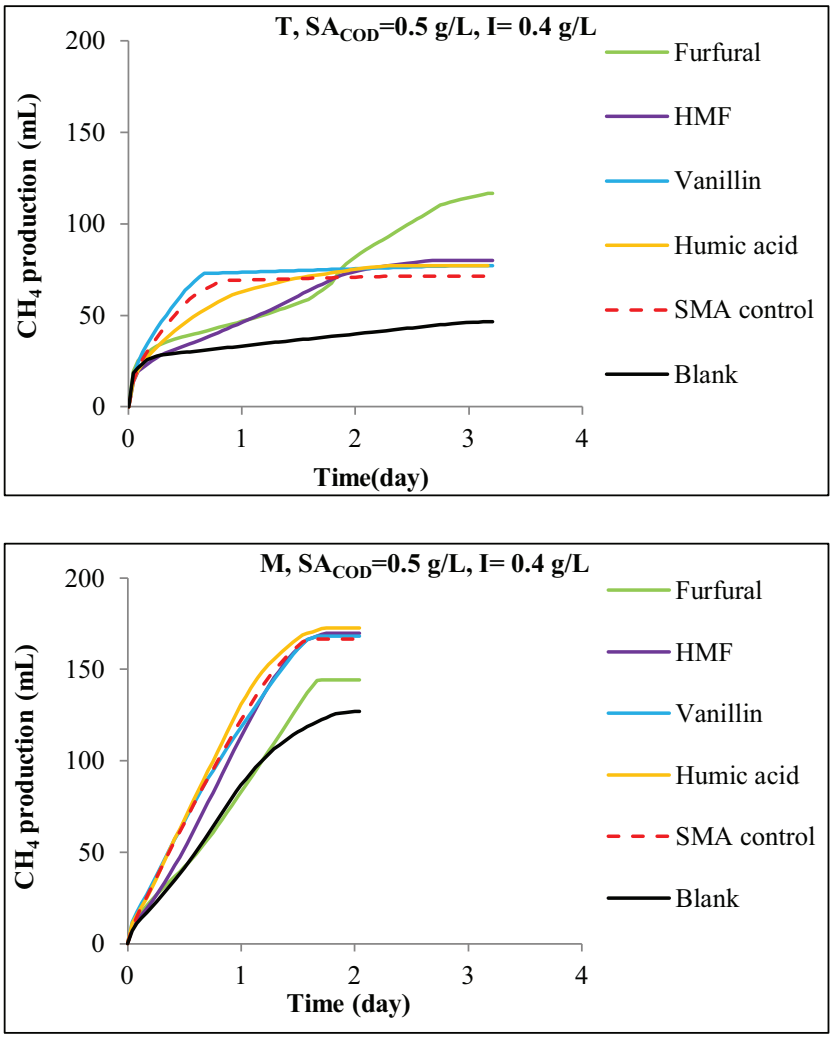

Figure 6.4 Effect of selected intermediates (I) (or potential inhibitors) on thermophilic (T, top) and mesophilic (M, bottom) SMA at concentration of 0.4 g/L and a SA_{COD} of 0.5 g/L

6.3.4 Third stage: Thermophilic conditions (CMP, I=0.8 g/L)

During the third stage of this study, the effect of the mentioned intermediate compounds on FSF digestion was investigated using CMP tests. The tests were conducted at an inhibitor concentration of 0.8 g/L under both thermophilic and mesophilic conditions (Figure 6.5 and Figure 6.6). Under thermophilic conditions, addition of furfural and HMF

resulted in a lower overall CMP compared to the control (only FSF) and to the assays with addition of vanillin and humic acid. In the presence of furfural and HMF, the CMPs were about 250 mL CH_4/gVS_{added}, which was about 77% of the control CMP, whereas the CMP of the batch with vanillin was unaffected. Surprisingly, the addition of humic acid even resulted in a higher CMP than the control, indicating a partial conversion of humic acids. Relatively low concentrations of acetate were found at the end of all CMPs, except for the one with vanillin dosage, which had a final concentration of 400 mg/L.

The inoculum of the thermophilic batch tests was taken directly from the laboratory scale FBR digesters fed with FSF at an OLR of 5.5 $kgCOD/m^3.d$. At the time of sampling, the biomass still contained high concentrations of VFA, resulting in relatively high concentrations of VFA at the start of the CMP test, i.e. propionate concentration was 1.3 g/L. Propionate was converted during most of the CMP tests (Figure 6.5), however, the final concentration of propionate in the bottles with added furfural and HMF increased with 58% and 56%, respectively. Based on COD, the FSF conversion into biogas in the uninhibited batches was similar to the sum of propionate and the amount of methane expressed in g COD, in the inhibited batches.

It was calculated that in the case of complete propionate degradation at concentrations of 2.08 and 2.05 g/L in the furfural and HMF added batches, respectively, the final CMP value would have increased from 238 and 242, to 326 and 329, respectively, considering the stoichiometric conversion factor of propionate of 1.51 g COD/g propionate and 2.49 g FSF-VS added. The incomplete propionate conversion might indicate that the hydrolysis was not or less affected by the furfural and HMF compared to the acetogens and/or hydrogenotrophic methanogens. Hence it could be concluded that furfural and HMF at a concentration of 0.8 g/L plus possible intermediates released from the FSF degradation (including furfural and HMF), affected the methanogenic consortium in the CMP assay.

The thermophilic CMP of FSF showed an irregular methane formation pattern with the existence of different subsequent hydrolysis rates. The observed methane production pattern strongly deviated from the generally applied first order model giving a single apparent hydrolysis rate. A similar observation was made during the adaptation of the inoculum to the FSF feed in the laboratory scale digester (Chapter 2). It is worthwhile to notice that just before the inoculates for our current batch experiments were taken from the lab scale FBR

digesters, the origin of the FSF that was fed to the digesters was changed, coming from a fine sieve located at a different WWTP, i.e. Loenen instead of Blaricum, both in The Netherlands. After the change of feed, the sludge probably had to re-adapt to some of the components of this FSF, as was shown in previous studies (Chapter 2). Nevertheless, the staggered methane production rates indicate subsequent hydrolysis of different fractions of the highly heterogeneous FSF.

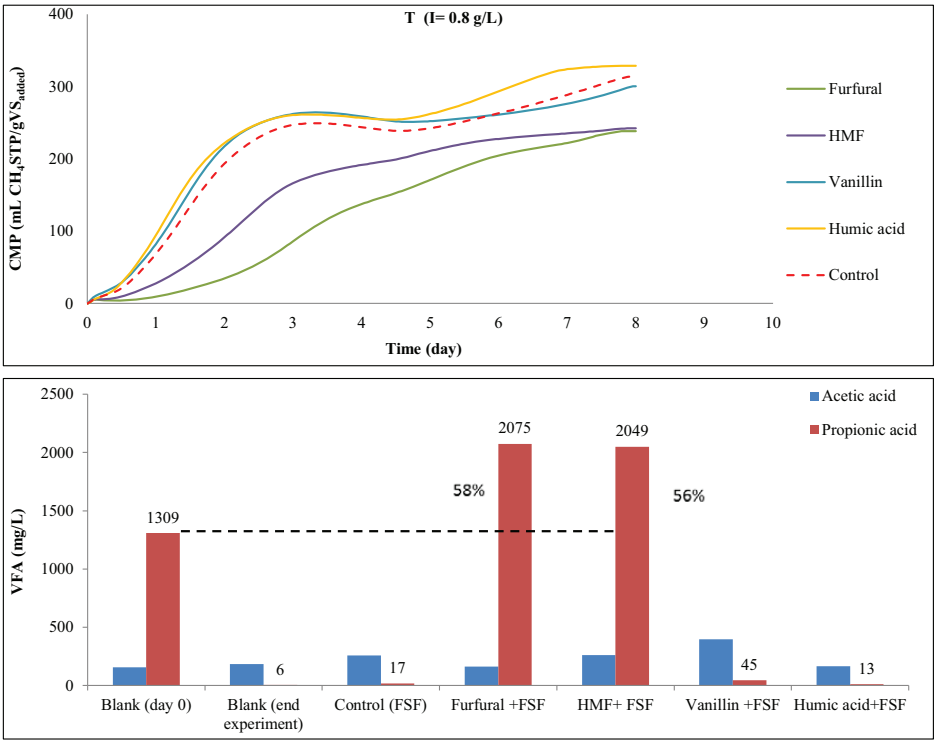

Figure 6.5 Thermophilic (T) CMP at I=0.8 g/L (top) and VFA concentrations before conducting CMP (blank at day zero) and after CMP assessment for all components including control (bottom)

6.3.5 Third stage: Mesophilic conditions (CMP, I=0.8 g/L)

The same CMP experiment was performed with mesophilic inoculum and under mesophilic conditions. In this assay, all components contributed to a higher methane production than the CMP control (Figure 6.6). Acetate and propionate concentrations in the inoculum as well as at the end of the experiment were below 80 mg/L. Only the addition of furfural showed a

modest impact on the methane production rate during the first days of incubation. Other potential inhibitors had no effect on hydrolysis or methanogenesis.

Possibly, under mesophilic conditions, the more recalcitrant intermediates were simply co-digested with the FSF substrate. This co-digestion was much more apparent under mesophilic than under thermophilic conditions. Similar observations were reported for bio-hydrogen production in the presence of by-products. Authors hypothesized that synergistic effects may occur that reduce the threshold value for inhibition compared to the situation when these by-products are converted separately (Bellido et al., 2011; Larsson et al., 1999; Li et al., 2013; Monlau et al., 2014; Mussatto and Roberto, 2004). Possibly, because of reduced species richness under thermophilic conditions, such synergistic co-digestion effects are less apparent at high temperatures (Gagliano et al., 2014; Lapara et al., 2000). This hypothesis is in line with our previous observations showing a more stable bacterial and archaeal community in the mesophilic digester compared to the thermophilic digester (Chapter 3).

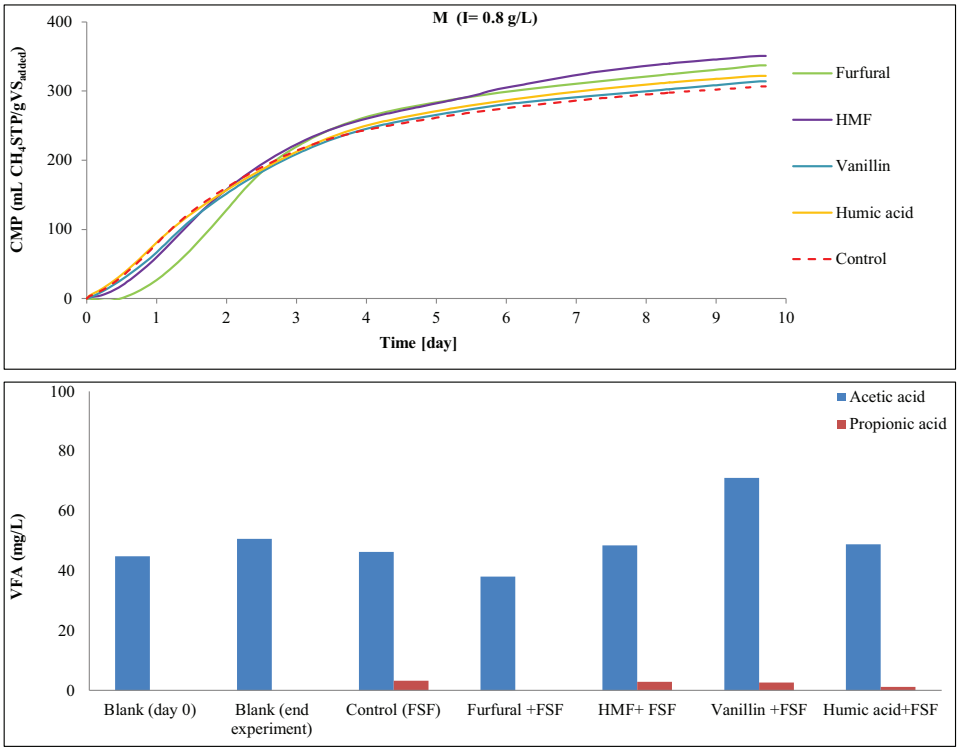

Figure 6.6 Mesophilic (M) CMP at I= 0.8 g/L (top) and VFA concentrations before CMP (blank at day zero) and after CMP assessment for all components including control (bottom)

Figure 6.7 summarizes the results of thermophilic and mesophilic CMP assays. Higher CMP values were measured when furfural, HMF and vanillin were added to the mesophilic batches, indicating bio-conversion (Figure 6.7). CMP of the FSF control bottles at 35°C and 55°C were similar, giving an overall biodegradability of 55%. Similar and increased CMP was also observed for the humic acid added FSF bottles, indicating a remarkable humic acid bio-degradation of about 37% based on observed increased CH_4 production. Both furfural and HMF were degraded by 85% and 100%, respectively, in the mesophilic CMP tests whereas, for instance, during the SMA tests at 0.8 g/L, furfural addition generated less methane production than the SMA control, and HMF addition gave the same amount of methane as the SMA control. Vanillin was only limitedly converted showing a biodegradability of only 18% in the mesophilic CMP tests compared to the other potential inhibitors. In the thermophilic CMP tests, vanillin only had a slightly negative effect (Figure 6.7).

The final VFA concentrations in the mesophilic CMP tests were much lower compared to the thermophilic batches. Nonetheless, addition of vanillin to the mesophilic CMP test resulted in the highest concentration of acetic acid in this series, i.e. about 70mg/L, which might be linked to the lower bio-transformation of vanillin, compared to the other added intermediates. Anaerobic degradation of vanillin at 2 g/L as sole carbon sources was investigated by Barakat et al. (2012). Vanillin was found to be recalcitrant to microbial degradation with a measured CMP of only 17% of the theoretical value. Considerable decrease in biomethane production was observed by Fedorak and Hrudey (1984) when phenol concentration exceeded about 1.2 g/L. Under mesophilic conditions, a higher conversion efficiency of different phenols has been demonstrated compared to thermophilic conditions (Levén and Schnürer, 2005; Levén et al., 2012).

A possible explanation could be related to the differences in microbial diversity, particularly the presence of phenol degrading bacteria in the ecosystem and/or the presence of temperature-sensitive enzymes (Levén et al., 2012). These results are in line with our findings. The lower methane production of vanillin in the mesophilic CMP test might be further explained by a possible surplus production of vanillin derived from FSF digestion, which is subsequently converted into phenol, which can increase the inhibitory potential of this compound in the anaerobic digestion process.

Recent results showed that increased humic acid concentrations (5-10g/L) adversely affected the methanogenic activity and resulted in reduced methane productions (Azman et al., 2015; Fernandes et al., 2015; Ho and Ho, 2012). However, Ho and Ho (2012) showed that lower humic acid concentrations, i.e. between 1.0 and 5.0 g/L, had a somewhat stimulatory effect on methane production in conjunction with enhanced VFAs degradation. Particularly at 1.0 g/L in pH-reduced piggery wastewater, low concentrations of humic acids were demonstrated to serve as electron acceptor in the anaerobic degradation of volatile organic acids (Ho and Ho, 2012). In this study, humic acid neither inhibited the SMA nor the CMP assays under both thermophilic and mesophilic conditions at all examined concentrations.

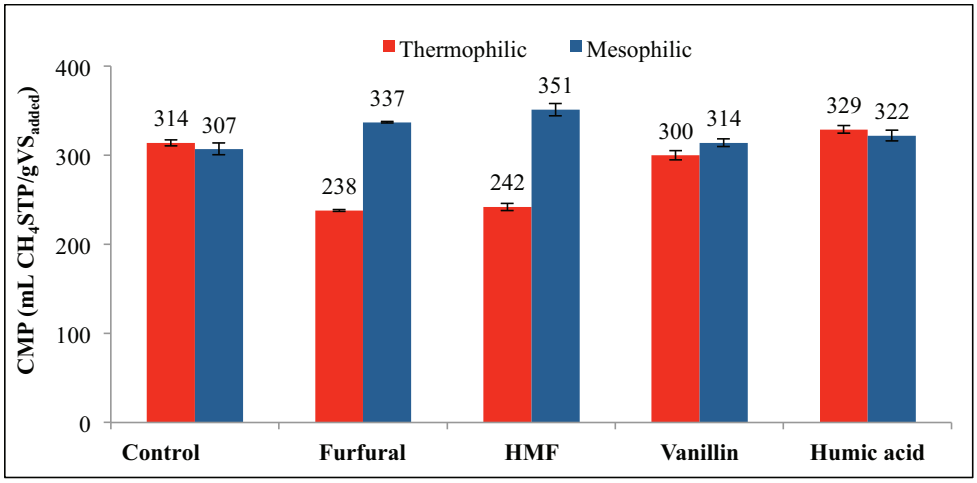

Figure 6.7 Comparison of thermophilic and mesophilic CMPs results for all intermediate components (error bars indicate the standard deviation for CMP controls and maximum and minimum value from average point for the intermediates (or potential inhibitors)

6.4. Conclusion

- Results of SMA and CMP tests showed that neither vanillin nor humic acid are considered as potential inhibitors at concentrations ≤ 2.0 g/L and 0.8 g/L, respectively, under both thermophilic and mesophilic conditions using the FSF adapted inoculates.

- Detrimental effects of furfural and HMF were found on FSF adapted methanogenic sludge at concentration of 2.0 g/L, under both conditions.

- Results of inhibitor additions to CMP tests with FSF as the substrate, indicated that thermophilic mixed culture consortia adapted to FSF conversion are more susceptible and sensitive to the presence of high inhibitors concentrations.

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Chapter 7. Recommendations and future perspectives

Resource recovery and energy neutral sewage treatment is nowadays focus of many water authorities. This novel concept led to a new focus on optimisation of digestion processes and enhanced biogas production. In this scope, the main objective of this research was to investigate the bio-methane potential and maximum methane production rates of FSF, sequestered from raw municipal sewage, for onsite energy recovery towards energy neutrality at WWTPs.

7.1 Recommendations

From this research, recommendations for further study were obtained:

i. In any test, evaluating the methane production rate of FSF, care should be taken to use well-adapted seed material. For thermophilic tests, the proper inoculum might not be available and should be cultivated, allowing long adaptation times before the inoculum can be used. Unlike our expectation, a period of seven months was needed for the thermophilic sludge treating VFY wastes (DRANCO, OWS, Brecht, Belgium) to fully adapt to FSF as the sole substrate.

ii. In most of the performed BMP tests, the apparent hydrolysis rate could be calculated from the cumulative methane production within the first 3-5 days of the test, assuming no inhibition, no intermediates accumulation and no lack of macro-micronutrients are prevailing.

iii. Thermophilic digestion was found more sensitive to periods of non-fed conditions than mesophilic digestion. Each feed cycle in the thermophilic digesters started with a lag phase following a period when the substrate of the previous feeding was depleted. Apparently, thermophilic digesters are on one hand characterized by higher substrate conversion rates, but at the other hand suffer from activity loss when the substrate is depleted. Therefore, it is expected that the application of a continuous feeding system for the thermophilic digestion of FSF will increase the stability of the digestion process. Ideally, this hypothesis should be studied at pilot scale, where clogging of the feeding pumps plays a smaller role than at laboratory scale.

iv. The assessed biodegradability of virgin paper and recycled paper, as well as virgin pulp for paper production, was found less than 50% (Chapter 5). The g COD/g mass of these compounds was higher than the COD value of cellulose, i.e. 1.18 g COD/g dry weight. It was hypothesized that the more reduced carbon fraction of FSF might originate from the presence of low lignin content and/or chemical additives used during manufacturing of the toilet paper. Therefore, more detailed research is needed to evaluate the complex biodegradation process of toilet paper in relation to the presence of additive chemicals, i.e., resins, binders, wax, anti-foaming agents, cleaning agents, creping chemicals, dyes, etc. The new finding might help to further understand the complex biodegradation process of toilet paper leading to an increase in potential energy recovery at the WWTPs.

v. In this thesis, the valorization of FSF via biogas production was researched in all depth. However, there are various alternative routes available for FSF utilization in addition to biogas production and thermal energy recovery processes. Such alternative routes include production of bioethanol, VFAs and other value-added chemical building blocks from FSF. Further researches need to be conducted in order to determine the most economic valorization routes for FSF in dependence to the local economic conditions.

7.2 Alternative routes for FSF valorisation

Recently, various valorisation routes for FSF have been identified, ranging from raw material use for the paper and cardboard industry, to insulation material for houses, polylactic acid production, polyhydroxyalkanoates production, bioethanol production, filler for the cat's litter box, burial casket, adhesion binders for asphalt construction, and as an ingredient for a dust preventing application in horticulture (STOWA, 2012). The identified applications have been well-evaluated using the following criteria: technological feasibility, economic perspective, time period for realization of valorisation and finally the amount of FSF that can be marketed. For most of the identified routes for FSF valorisation, it appears from a technological perspective that there are no barriers, which make that valorisation is impossible and all identified routes have been judged as 'positive' on the technological feasibility criteria. However, the same studied showed that the image of FSF is a considerable obstacle when FSF is used as raw material in the paper and cardboard industry. The same is true for the

production of polylactic acid. Moreover the odor and hygienic safety of FSF is frequently mentioned as an obstacle to consider replacing existing raw materials by FSF, even in applications such as road construction, as for producing insulation material and funeral caskets. Therefore, for these applications, it is a prerequisite that the material is odourless and hygienically safe and consequently is being washed and possibly sterilised before it can serve as raw material. This implies that the FSF needs a pretreatment by means of a washing step, which will add to the costs and likely creates a wastewater stream. When the FSF serves as an ingredient for the production of either polylactic acid or bioethanol it is expected that no pretreatment is needed. However more research is needed for using FSF in the latter two applications.

Summary

In this thesis, the anaerobic digestion of fine sieved fraction (FSF) is described and discussed, as well as its potential to contribute to onsite energy recovery towards closing the energy balance at conventional WWTPs. FSF is a heterogeneous substrate, mainly consisting of cellulose, and was sequestered from raw municipal wastewater at WWTP Blaricum, The Netherlands, by using a rotating belt filter (Salsnes filter, Norway), equipped with a 350 micron mesh. For the given wastewater, the major component of FSF was toilet paper. Fine sieving is implemented as a compact alternative to primary clarification to separate suspended solids from sewage prior to biological nutrient removal. The dry solids content of FSF produced from the effluent of the fine sieve could easily reach 25%-30% without using any chemicals. Applying optimized mechanical pressure would result in achieving an FSF with a dry solids content of 40%-50% (Ruiken et al., 2013). Since the components of FSF are only partly degradable in conventional WWTPs and excess sludge is poorly dewaterable, the application of fine sieving means significant reduction in sludge transportation costs compared to the reference condition when no fine sieving is applied.

The application of fine sieves receives growing interest as a simple and compact pre-treatment technique in densely populated countries like The Netherlands. In addition, the Dutch Water Authorities currently investigate the use of reclaimed cellulose fibres as a resource. However, to gain high value reuse opportunities, large quantities are often needed to gain interest by the processing industry. In a transition phase before these quantities are available, or at small-scale WWTPs where low quantities are produced, onsite bio-methanation of FSF at high dry solids content might contribute to the objective of realizing energy neutral or energy producing wastewater treatment plants (STOWA, 2010).

In this PhD project, the digestion efficiency of FSF under both thermophilic (55°C) and mesophilic (35°C) conditions was studied. The two thermophilic digesters were inoculated with biomass from a plug flow dry anaerobic composting digester (DRANCO, OWS, Brecht, Belgium; De Baere, 2000), treating mainly vegetable, fruit and yard (VFY) wastes. The two mesophilic digesters were inocculated with biomass from an anaerobic digester at WWTP Harnaschpolder, Delft, The Netherlands.

A long seven months adaptation time was needed for the thermophilic sludge in order to fully adapt to FSF as the sole substrate. Different SBR cycle durations of 14, 9 (on average) and 2

days were applied for both temperature conditions to study methane production rates, VFAs dynamics, lag phases, as well as changes in microbial communities. However, after successfully passing the adaptation period and by decreasing the batch cycle period from 9-day feedings on average to 2-day feedings, thermophilic digesters showed better performance, i.e. shortened lag phases, reduced VFA peaks and increased biogas production rate. Eventually, over time, the thermophilic digester outperformed the mesophilic one, as was discussed in **Chapter 2**.

In order to seek for the limits of the thermophilic process, the OLR was increased from 5.5 kg COD/(m^3·day) to 22 kg COD/(m^3·day), at which a relatively stable performance was still observed. In contrast, the mesophilic digester already failed at an applied OLR of 5.5 kg COD/(m^3·day), indicated by a drop in pH and increase in VFA concentrations. The optimum OLR for FSF digestion under mesophilic and thermophilic conditions was found between 2-2.5 kg COD/(m^3·day) and 5.5-6.7 kg COD/(m^3·day), respectively. The observed viscosity values of the mesophilic sludge were more than tenfold higher than the thermophilic sludge. The 454 pyrosequencing technique was used in order to investigate the dynamics of bacterial and archaeal populations under both applied conditions. The changes in microbial communities in the mesophilic reactor before process failure and during recovery were followed closely as well.

The long-term adapted microbial communities at 55 °C and 35 °C were distinctly different in composition and population dynamics. Results of 454-pyrosequencing of eight mesophilic and eight thermophilic biomass samples revealed that *Bacteroides* and the aceticlastic methanogen *Methanosaeta* were the dominant genera in the mesophilic digester, whereas OP9 lineages, *Clostridium* and the hydrogenotrophic methanogen *Methanothermobacter* dominated the thermophilic one **(Chapter 3)**.

Variation in community structure and microorganisms' relative quantity was observed in both the mesophilic and thermophilic digester, however, the thermophilic digester performed more stable and robust than the mesophilic one. Considering the high percentage of cellulose inside FSF, there likely were more hydrolytic enzymes generated by the thermophilic sludge than by the mesophilic one. The protein analysis by sodium dodecyl sulphate-polyacrylamide gel

electrophoresis (SDS-PAGE) and coommassie staining showed that the thermophilic sludge contained significantly more protein than the mesophilic sludge ($p<0.01$, Student test). These outcomes suggest that applying thermophilic conditions for FSF digestion would result in higher biogas production rates and/or a smaller required reactor volume, compared to mesophilic conditions (**Chapter 3**).

In **Chapter 4**, the maximum applicable substrate batch loadings by varying the inoculum to substrate ratios ($R_{I/S}$: 0.5-15) have been studied to further identify differences in robustness between well-adapted mesophilic and thermophilic sludge. Again, the latter turned out to be most robust: VFA accumulation did not occur at all ratios, indicating the possibility for higher loading rates and thus possible compact designs for on-site biogas production from FSF. Currently, there is a high interest for increased energy recovery from sewage sludge, which is crucial for achieving energy neutral WWTPs (STOWA, 2010; WERF, 2010, 2009a, 2009b). So far, only large WWTPs (>100.000 population equivalents) were targeted for reaching energy neutrality. However, considering the calculated energy potential and high applicable substrate load under thermophilic conditions, as well as the relatively short retention times needed for 90% conversion (3 to 6 days), thermophilic digestion using plug flow reactors could add to enhance energy recovery especially at small WWTPs, such as WWTP Blaricum. To verify the feasibility and contribution towards energy neutrality at small WWTPs, results obtained from batch experiments and data sets from WWTP Blaricum were taken into account for the energy balance estimates including FSF digestion.

Calculations showed a potential significant energy recovery at WWTP Blaricum when FSF digestion would be applied (**Chapter 4**). Calculations include the overall energy use in terms of heat and electricity, fine sieving until a total solids (TS) concentration of 23% is reached, anaerobic digestion of FSF, dewatering of digestate sludge and drying the dewatered digestate sludge. The required electric energy for fine sieving amounts 132 kWh/d, whereas electricity and heat requirement for anaerobic digestion of FSF requires 29 kWh/d and 445 MJ/d, respectively, digestate dewatering from 9% to 20% TS accounts for 34 kWh/d or 0.11 kWh per kg dry solids and sludge drying demands 3133 MJ/d. Application of high-rate FSF digestion would lead to a net recoverable heat energy of 287 MJ/ton FSF and 237 kWh electric /ton FSF at a TS content of 23%. Energy calculations based on the experimental digestion results from our study showed that the small scale WWTP Blaricum

(30,000 p.e.) could reach 46% energy coverage when their FSF is onsite treated in the proposed plug-flow reactor.

The low biodegradability of FSF under thermophilic (62%) and mesophilic (57%) conditions (as presented in **Chapter 4**) raised the question about the actual biodegradability of pure fibres in the different toilet papers and the contribution of other organic matter to FSF digestibility. Therefore, series of batch anaerobic digestion tests were conducted under both conditions to investigate the biomethane potential (BMP), specific methane production rate (SMPR), apparent hydrolysis rate (K_h), required incubation time to achieve 90% of the maximum cumulative methane production ($t_{90\%}CH_4$), as well as anaerobic biodegradability (AnBD) of designated cellulose fiber-based substrates including virgin pulp for paper production (VPPP), virgin toilet paper (VTP), recycled toilet paper (RTP) and microcrystalline cellulose (MCC) as a fibreless reference material (**Chapter 5)**.

Results of the experiments indicated that the biodegradability of all employed cellulose fiber-based substrates (VTP, VPPP and RTP) was in the same range under thermophilic and mesophilic conditions and was less than 50%. Poor biodegradability of toilet paper in anaerobic digestion (<50%) might be due to the characteristics of employed fibres (short or long), crystallinity of the fibres, types of pulping that has been applied in the paper making process and the presence of poorly degradable lignin material, as well as addition of paper chemicals and formation of toxic and refractory compounds during paper making. MCC achieved the highest BMP and biodegradability among all cellulosic substrates. FSF did not show any lag phase during substrate degradation under both thermophilic and mesophilic conditions. These observations are most probably due to presence of more readily biodegradable compounds (e.g., proteins and lipids) in the FSF, in combination with full adaptation of the used inoculates.

Intermediates of the fermentation process of cellulosic biomass during anaerobic digestion may have inhibitory effects on the microbial culture and thus on the process performance. For this purpose, the impact of several intermediates and by-products derived from the hydrolysis of lignocellulosic biomass, e.g., furfural, HMF, vanillin, and recalcitrant humic acid salt at different concentrations, was researched on hydrolysis and methanogenesis, under thermophilic and mesophilic conditions in **Chapter 6**. The impact of these compounds on methanogens was studied by conducting SMA tests using a sodium acetate COD (SA_{COD})

concentration of 1.0 g/L and inhibitor compounds at concentrations of 0.4, 0.8 and 2.0 g/L. Results of the experiments showed that at low inhibitor concentration (0.4 g/L) and SA_{COD} of 1.0 g/L, none of the intermediates showed inhibitory effect on the SMA under both thermophilic and mesophilic conditions. However, HMF showed inhibitory effect at a concentration of 0.8 g/L under thermophilic conditions. At a high concentration of 2.0 g/L, furfural and HMF completely inhibited the SMA tests under both temperature conditions. However, the inhibitory effect was absent with vanillin and humic acid at concentrations ≤ 2.0 g/L and 0.8 g/L, during the SMA and BMP tests, respectively (as shown in **Chapter 6**). Degradation of inhibitor compounds during SMA tests at a lower acetate concentration (0.5 gCOD/L) was compared to a higher acetate concentration (1.0 gCOD/L). In this experiment, a higher degree of conversion of the potential inhibitors was found under thermophilic conditions except for vanillin (no change observed). Surprisingly, the vice-versa behaviour was observed under mesophilic conditions, where intermediate compounds were more degraded at the higher SA_{COD}.

There is no clear explanation for the observed phenomenon, and additional investigations are needed to further elucidate this effect. Possibly, the long adaptation period of the biomass to FSF that releases mentioned components during thermophilic degradation might have played an important role. Vanillin degradation was not influenced by applying different SA_{COD} concentrations. The influence of intermediates on FSF degradation was studied by adding above-mentioned potential inhibitors at a fixed concentration of 0.8 g/L, under both conditions. Results of inhibitor additions to cumulative methane production (CMP) tests with FSF as the substrate indicated that thermophilic mixed culture consortia are more susceptible and sensitive to the presence of high inhibitors concentrations. It was also hypothesised that more recalcitrant intermediates were probably co-digested with the FSF substrate under mesophilic conditions.

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List of abbreviations

ABAI task group	anaerobic biodegradation, activity and inhibition
AD	anaerobic digestion
AMPTS	automatic methane potential test system
AnBD	anaerobic biodegradability
BMP	biochemical methane potential
BOD	biochemical oxygen demand
C/N	carbon to nitrogen ratio
CAPEX	capital exploitation costs
C_C	specific heat capacity of cellulose
C_{Cl}	specific heat capacity of clay
C_F	specific heat capacity of FSF
CHP	combined heat and power
CMP	cumulative methane production
COD	chemical oxygen demand
Cp_{sludge}	specific heat capacity of solids in sludge
CSTR	continuous-stirred tank reactor
CT	contact time
C_W	specific heat capacity of water
DAF	dissolved air flotation

DOC	dissolved organic carbon
DRANCO	dry anaerobic composting
DS	dried solids
ECN	energy research centre of The Netherlands
EDX	energy-dispersive X-ray
EGSB	expanded granular sludge bed
EPA	environment protection agency
ESEM	environmental scan electron microscopy
Ex/Em	excitation/emission
FBR	fed-batch reactor
F-EEM	fluorescence excitation-emission matrix
FID	flame ionization detector
FOG	fat, oil, and grease
FSF	fine sieved fraction
GC	gas chromatograph
GHG	greenhouse gas
GWRC	global water research coalition
HHV	higher heating value
HMF	hydroxymethylfurfural
I/S	inoculum to substrate ratio

K_h	apparent hydrolysis rate
KWR	watercycle research institute in The Netherlands
L:D	length to diameter
LHV	lower heating value
MCC	microcrystalline cellulose
MSW	municipal solid waste
NREL	national renewable energy laboratory
OFMSW	organic fraction of municipal solid waste
OLR	organic loading rate
OUT	operational taxonomic unit
OWS	organic waste systems
PC	powdered cellulose
PCoA	principal coordinate analysis
PD	phylogenetic distance
qPCR	quantitative polymerase chain reaction
R	recirculation factor
$R_{I/S}$	inoculum to substrate ratio
RTP	recycled fiber based toilet paper
SA_{COD}	sodium acetate COD
SAO	syntrophic acetate oxidising

SBR	sequencing batch reactor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SMA	specific methanogenic activity
SMPR	specific methane production rates
SRT	solid retention time
SS	suspended solids
STOWA	foundation for applied water research in The Netherlands
STP	standardised temperature and pressure
$t_{90\%}CH_4$	90% of the maximum cumulative methane production
tCOD	total chemical oxygen demand
TN	total nitrogen
TP	total phosphorous
TS	total solids
TSS	total suspended solids
TVFA	total volatile fatty acids
VDI 4630	protocol for fermentation of organic materials-characterisation of the substrate, sampling, collection of material data, fermentation tests
VFA	volatile fatty acid
VFY	vegetable, fruit and yard

VPPP	virgin pulp for paper production
VS	volatile solids
VSS	volatile suspended solids
VTP	virgin fibers based toilet paper
WERF	water environment research foundation
WWTP	wastewater treatment plant

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List of publication

Peer reviewed journal papers

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Curriculum vitae

Dara S.M. Ghasimi was born on 10 June 1978 in Marivan, Kurdistan province, Iran. He completed his BSc degree in Chemical Engineering from Iran University of Science and Technology (IUST) in Tehran, in January 2003. After graduation, he started working at Environment Protection Agency in Sannadaj, Kurdistan province, until 2005.

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