Norwegian University of Life Sciences
Faculty of Chemistry, Biotechnology and Food Science

Philosophiae Doctor (PhD)
Thesis 2018:53

Improving food waste anaerobic digestion: effects of digestate recirculation, post-treatment and methane-methane serial digestion

Forbedring av biogassproduksjon fra matavfall: Effekt av resirkulering av råtnerest, etterbehandling og metan-metan serieutråtning

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SUMMARY

Anaerobic digestion of food waste generates two valuable products: digestate and biogas. Today, most food waste in Norway is incinerated because it is collected as mixed waste. Hence, a shift from incineration to anaerobic digestion of food waste will produce more value and be a contribution to the emerging circular economy. As more food waste becomes available for anaerobic digestion, optimization of the process to allow higher substrate loading rates to existing biogas plants is needed.

In this thesis, several strategies for improved food waste anaerobic digestion was investigated. This included recirculation of digestate, post-treatment with thermal hydrolysis, and methane-methane serial digestion. Moreover, different approaches for organic matter determination was compared, and the relevance of small-scale experiments as a model system for large scale biogas plants was investigated. Comparing different approaches for organic matter determination showed that BMP data expressed on the basis of different VS methods varied more than BMP results expressed on the basis of different COD methods. Using COD for organic matter determination was therefore considered more reliable, and was recommended as the preferred method for comparing process performance between experiments. Mesophilic anaerobic digestion in CSTRs with 20 days retention time, and with and without digestate recirculation, resulted in methane yields of 280 mL/gCOD_{added}, which corresponds to 80% COD reduction. Thermophilic digestion of food waste under the same conditions resulted in accumulation of acids accompanied with reduced methane yields. In these digesters, acetic acid was accumulated when fresh water was added to the feed whereas propionate, butyrate and iso-valerate accumulated when digestate was recirculated. This was further linked to lower relative abundance of acetate oxidizers (*Thermoacetogenium*) and β-oxidizers (*Syntrophomonas*) respectively. Thermophilic co-

digestion of food waste and primary sludge also resulted in transitory propionate accumulation accompanied with transitory lower relative abundance of *Syntrophomonas*. Moreover, low feeding frequency resulted in temporary propionate accumulation and LCFA accumulation in laboratory digesters operated at high organic loading rates (21 $gCOD_{added}/L/d$) with 10 days hydraulic retention time, whereas the digester fed 10 times a day did not accumulate organic acids and had a methane yield of 305 $mL/gCOD_{added}$. Finally, post-treatment with thermal hydrolysis yielded more methane and improved dewaterability compared to conventional pre-treatment at 70 °C for mesophilic co-digestion of food waste and sewage sludge.

Overall, the experiments presented in this thesis showed that stable performance of anaerobic digesters treating food waste can be achieved at low hydraulic retention times and high organic loading rates. The observation of methane yields of 280-305 $mL/gCOD_{added}$ in the mesophilic digesters treating food waste as the sole substrates corresponds to 80-90% COD reduction, hence little of the substrate was recalcitrant and the hydrolysis was complete. Accumulation of VFAs and LCFAs in the thermophilic and high loaded mesophilic digesters indicates that the latter steps in the AD process, namely acetogenesis and methanogenesis, are the limiting steps in food waste anaerobic digestion. Hence, through implementing technologies accommodating the success of β-oxidizers, acetogens and methanogens, such as methane-methane serial digestion and a frequent and stable feeding regime, food waste anaerobic digestion efficiency can be improved.

SAMMENDRAG

Anaerob utråtning av matavfall danner to verdifulle produkter: råtnerest og biogass. I dag forbrennes mesteparten av matavfallet i Norge fordi det samles inn som blandet avfall. Et skifte fra forbrenning til anaerob utråtning av matavfall vil derfor produsere merverdi og bidra til en mer sirkulær økonomi. Når mer matavfall blir tilgjengelig for anaerob utråtning vil optimalisering av prosessen være nødvending slik at eksisterende biogassanlegg kan håndtere en høyere belastning.

I denne avhandlingen har flere strategier for forbedring av anaerob utråtning av matavfall blitt undersøkt. Dette inkluderte resirkulering av råtnerest, etter-behandling med termisk hydrolyse, og metan-metan serieutråtning. I tillegg ble nøyaktigheten til forskjellige tilnærminger til bestemmelse av organisk innhold sammenlignet og relevansen av små-skala forsøk som et modellsystem for storskala biogassanlegg undersøkt. Sammenligningen av forskjellige tilnærminger til bestemmelse av organisk innhold viste at BMP-data uttrykt på basis av forskjellige VS-metoder varierte mer enn BMP-resultater uttrykt på basis av forskjellige COD-metoder. Å bruke COD for å bestemme organisk innhold ble derfor ansett som mer pålitelig og anbefalt for sammenligning av prosessytelse mellom forsøk. Mesofil anaerob utråtning i CSTR-er med 20 dagers oppholdstid og med og uten råtnerest-resirkulering resulterte i metanutbytter på 280 mL/g$COD_{tilsatt}$ tilsvarende 80% COD reduksjon. Termofil utråtning av matavfall under de samme betingelsene førte til akkumulering av syrer fulgt av redusert metanutbytte. I disse biogassreaktorene akkumulerte eddiksyre når ferskvann ble tilsatt føden, mens propionat, smørsyre og iso-valerinsyre akkumulerte når råtneresten ble resirkulert. Dette ble videre koblet til lavere mengde eddiksyreoksiderende bakterier (*Thermoacetogenium)* og β-oksiderende bakterier (*Syntrophomonas)* i de respektive reaktorene. Termofil sambehandling av matavfall og

primærslam førte også til forbigående propionat-akkumulering fulgt av en forbigående lavere forekomst av *Syntrophomonas.* Videre førte lav fôringsfrekvens til forbigående propionat-akkumulering og akkumulering av langkjedede fettsyrer i laboratoriereaktorer driftet med høy organisk belastning (21 $gCOD_{tilsatt}/L/d$) og 10 dagers oppholdstid, mens reaktorer fôret 10 ganger om dagen, med ellers like betingelser, ikke akkumulerte noen syrer og hadde et metanutbytte på 305 $mL/gCOD_{tilsatt}$. Avsluttende viste etterbehandlingsforsøkene med termisk hydrolyse høyere metanutbytte og bedret avvanning sammenlignet med konvensjonell forbehandling ved 70 °C for mesofil sambehandling av matavfall og avløpsslam.

Oppsummert viste forsøkene i denne avhandlingen at stabilt ytende biogassreaktorer som behandler matavfall kan oppnås ved lav hydraulisk oppholdstid og høy organisk belastning. Observasjonen av metanutbytter i området 280-305 $mL/gCOD_{tilsatt}$ i mesofile biogassreaktorer som utelukkende behandlet matavfall tilsvarer 80-90% COD-reduksjon. Lite av substratet kan derfor tilskrives å være tungt nedbrytbart og hydrolysen var komplett. Akkumulering av kort- og langkjedede fettsyrer i de termofile reaktorene og i den mesofile med høy organisk belastning indikerer at de siste stegene i prosessen, nemlig eddiksyredannelsen og metandannelsen, er de begrensende stegene i anaerob utråtning av matavfall. Ved å iverksette teknologier som legger til rette for at β-oksiderende, eddiksyredannende og metanogene mikroorganismer lykkes, slik som metan-metan serieutråtning og en frekvent og stabil tilførsel av substrat, kan effektiviteten til anlegg som utråtner matavfall forbedres.

LIST OF PAPERS

Paper I

Svensson, K., Kjørlaug, O., Horn, S.J., Agger, J.W., 2017. Comparison of approaches for organic matter determination in relation to expression of bio-methane potentials. Biomass and Bioenergy 100, 31–38. doi:10.1016/j.biombioe.2017.03.005

Paper II

Zamanzadeh, M., Hagen, L.H., Svensson, K., Linjordet, R., Horn, S.J., 2016. Anaerobic digestion of food waste – Effect of recirculation and temperature on performance and microbiology. Water Res. 96, 246–254. doi:10.1016/j.watres.2016.03.058

Paper III

Svensson, K., Kjørlaug, O., Higgins, M.J., Linjordet, R., Horn, S.J., 2018. Post-anaerobic digestion thermal hydrolysis of sewage sludge and food waste: Effect on methane yields, dewaterability and solids reduction. Water Res. doi:10.1016/j.watres.2018.01.008

Paper IV

Svensson, K., Morken, J., Paruch, L., Linjordet, R., Horn, S., 2018. Stable performance of laboratory and full-scale anaerobic co-digesters treating food waste and sewage sludge at 62° C. (Draft manuscript)

Paper V

Svensson, K., Paruch, L., Gaby, J. C., Linjordet, R., 2018. Feeding frequency influences process performance and microbial community composition in anaerobic digesters treating food waste. (Draft manuscript)

In addition to Papers I-V, the author contributed to the following paper within the timeframe of this thesis work:

Zamanzadeh, M., Heldal, L., **Svensson, K.,** Linjordet, R., 2017. Biogas production from food waste via co-digestion and digestion – effects on performance and microbial ecology 1–12. doi:10.1038/s41598-017-15784-w

ABBREVIATIONS

AD	Anaerobic digestion
BMP	Biochemical methane potential
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
EU	European Union
HRT	Hydraulic retention time
LCFA	Long-chain fatty acids
PAD-THP	Post-anaerobic digestion thermal hydrolysis process
SRT	Solids retention time
TS	Total solids
UASB	Upflow anaerobic sludge blanket
VFA	Volatile fatty acids
VS	Volatile solids

1. INTRODUCTION

1.1 ANAEROBIC DIGESTION – AN ORGANIC WASTE MANAGEMENT STRATEGY FOR THE CIRCULAR ECONOMY

In 2015, the European Commission launched a circular economy action plan aiming to keep materials and resources in the economy for as long as possible, and minimizing waste generation (European Commission, 2015). EU's waste framework defines waste as "any substance or object which the holder discards or intends or is required to discard"(The European Parliament and the Council of the European Union, 2008). One of the action plan's targeted areas is food waste.

Currently, three organic waste management technologies dominates the European food waste market: composting, incineration and anaerobic digestion (Bartl, 2015). All three technologies produce a solid or slurry residue and energy. Because all three waste management technologies transform energy from organic material to heat or methane, they support a waste-to-energy supply chain. However, heat, produced during incineration and composting, has a low value compared to methane (van Gool, 1987). The energy carrier produced during anaerobic digestion, may be used for more than just heat generation. Its applications include upgrading to vehicle fuel, injection into existing natural gas infrastructure or conversion to heat and electricity with a combined heat and power (CHP) unit. Moreover, whereas fly and bottom ash from incineration is unsuitable for land application, the solid residues from composting and anaerobic digestion, compost and digestate, are valuable fertilizers and soil amendments. Hence, anaerobic digestion produces two valuable products, methane and digestate. To minimize the anaerobic digestion's environmental footprint, recovering methane within the reactor system and limiting emissions from digestate storage is important (Sambusiti et al., 2015).

1.2 AD - A MICROBIAL PROCESS

Anaerobic digestion (AD) of complex organic material may be divided into four major steps (Figure 1). The first step, hydrolysis, includes all processes transforming large biomolecules into soluble substances. Specifically, hydrolysis produces soluble carbohydrates, amino acids and ammonium, glycerol and long-chain fatty acids. In the second step, acidogenesis further converts these compounds to volatile fatty acids, hydrogen, CO_2 and acetate. Then, in the third step, acetogenesis converts fatty acids to hydrogen, CO_2 and acetate. Finally, methanogenesis produces methane from CO_2, H_2 and acetate. A complex microbial community performs these steps, and mutual metabolic interactions between the microorganisms regulates them. Balanced process rates therefore prevent accumulation of intermediate products and ensure complete transformation of complex organic material into methane and CO_2.

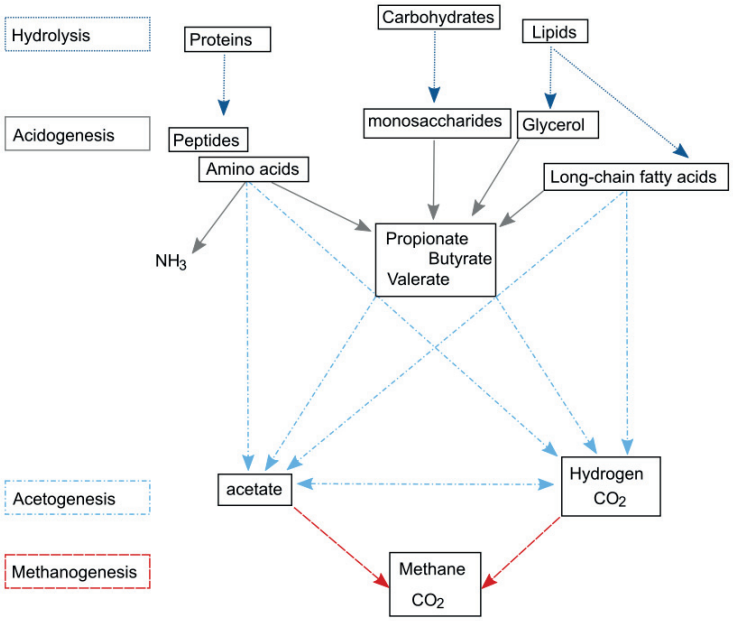


FIGURE 1: THE FOUR MAJOR STEPS IN ANAEROBIC DIGESTION OF COMPLEX ORGANIC MATERIAL.

Both bacteria and archaea constitute the anaerobic digestion microbial community, where the methanogenesis step is carried out by archaea while bacteria perform the hydrolysis, acidogenesis and acetogenesis steps. Such microbial communities are diverse and often redundant, with several populations occupying the same niche (Werner et al., 2011). For example, acidogenic processes such as sugar and glycol fermentation are phylogenetically widespread in bacteria. However, other metabolic traits are phylogenetically restricted to specialized syntrophic bacteria. For instance, bacteria that converts long-chain fatty acids (LCFA) to acetate and formate/hydrogen have been isolated from only two families, *Syntrophomonadaceae* and *Syntrophaceae* (Ziels et al., 2016). Moreover, acetoclastic methanogenesis is only performed by members of the order *Methanosarcinales* (Ferry, 1992). Hence, a successful anaerobic digestion process relies on specialist's success, syntrophic relationships and redundant metabolism through diverse and parallel pathways.

The syntrophic relationships and redundant metabolism microbial communities make identification of bottlenecks challenging. For example, in a reactor where methane production is the rate limiting process, it can slow down even further

if intermediate products reach inhibiting concentrations. Accumulation of volatile fatty acids (VFAs) may inhibit methanogens, resulting in more VFA accumulation because hydrogen consumption rates by methanogens slow down and thereby inhibits propionic acid degradation. The hydrolysis step can also be the rate limiting process, or very high hydrolysis rate may inhibit downstream processes. For instance, the hydrolysis products long-chain fatty acids inhibit methanogens and propionic acid oxidizers. Hence, increased lipid hydrolysis rates eventually decrease methane production rates, although overall methane yields may increase because more substrate is available (Meng et al., 2015; Sayed et al., 1988). Another process factor to consider is nutrient requirements, which may differ between metabolic pathways. For example, syntrophic hydrogenotrophic methanogenesis requires Se, Mo and W, while acetoclastic metanogenesis requires Co, Ni and Fe (Banks et al., 2012). Hence, identifying a process' bottleneck requires knowledge of syntrophic relationships, metabolic pathways and inhibition mechanisms.

1.3 Parameters influencing process-rates

1.3.3 Nutrients

Since anaerobic digestion is a biological process, macro- and micronutrients are required. Macronutrients include carbon, nitrogen and phosphorous, while micronutrients include trace metals. Since the aim of anaerobic digestion is to convert as much carbon as possible to methane, anaerobic digestion systems should never be carbon limited. Addition of readily available carbon such as glucose may, however, lead to accumulation of fatty acids,

but can also alleviate ammonium inhibition through increased microbial growth, which capture ammonium for amino-acid synthesis (Wang et al., 2016).

Domestic sludge usually contains sufficient quantities of nitrogen, while energy crops such as maize and wood residue requires nitrogen supplementation. However, micronutrients such as Fe, Co, Ni, Se, Mo and W are commonly supplemented to both energy crops and waste based anaerobic digesters to improve digester performance (Browne et al., 2014). Iron is often suggested as a trace metal needed for optimal process performance (Zhang and Jahng, 2012). It is also used to precipitate sulfides that otherwise can result in poor gas quality (Hilton and Archer, 1988). Although adding iron is beneficial both for methane production and for producing a cleaner biogas, it binds phosphate and reduces plant availability (Kahiluoto et al., 2015). Hence, adding iron to anaerobic digesters is therefore a trade-off between methane yields and digestate quality.

Another strategy to overcome macro-and micronutrient limitation is co-digestion. Many studies have been published on anaerobic co-digestion in the past decade (Mata-alvarez et al., 2014). Observed synergistic effects of co-digestion on biogas yields have been attributed to improved trace element availability (Moraes et al., 2015; Zhang et al., 2011), balanced C/N ratios (Moraes et al., 2015), higher energy density and biodegradability of the co-substrate compared to the main substrate (Silvestre et al., 2015) as well as fibers neutralizing long-chain fatty acids through adsorption of LCFAs onto the fibers (Labatut et al., 2014).

1.3.1 Control parameters

Operators manipulate process parameters to influence process-rates and optimize the anaerobic digestion process. Temperature might be the easiest process parameter to

manipulate, however, the consequences of changing the operating temperature is far reaching. For example, temperature influences biochemical reaction rates, which usually increase with increasing temperatures. However, temperature also influences equilibriums. For example, the equilibrium $NH_4^+ \leftrightarrow NH_3$ shifts towards NH_3 and the equilibrium $CO_{2(aq)} \leftrightarrow CO_{2(g)}$ shifts towards $CO_{2(g)}$ when the temperature is increased. Together this results in higher pH at higher temperatures in anaerobic digesters. Metabolic pathways are also influenced, for instance, acetate oxidation becomes more thermodynamically favorable at higher temperatures (Figure 2). Additionally, all microorganisms have a temperature range for growth. Because of the temperature limitations of the AD microbial community most anaerobic digesters operate in the mesophilic (30-40 °C) or thermophilic (50-60 °C) ranges, although some also operate at psychrophilic (<20°C) and extreme thermophilic (60-70°C) temperatures.

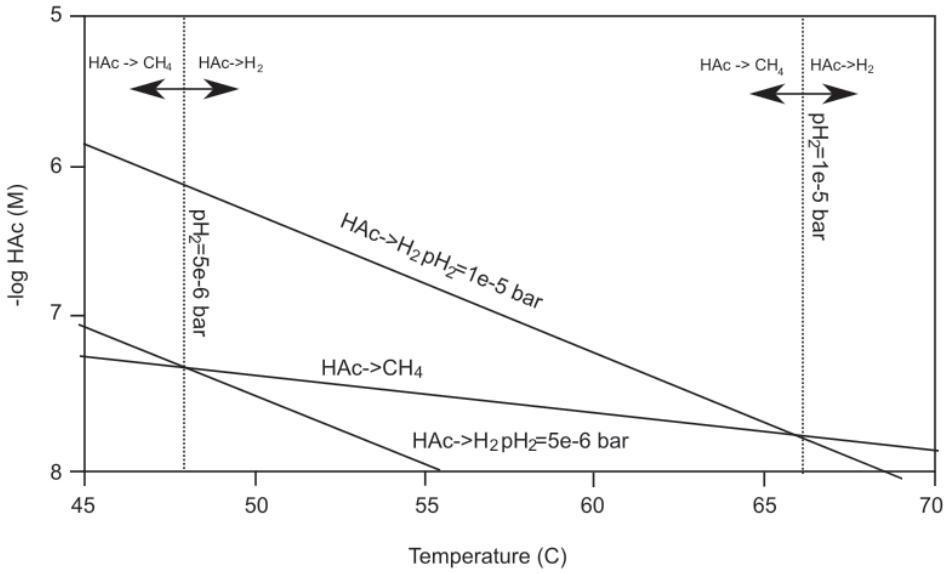


FIGURE 2: LINES SHOW CONSTANT ΔG'= 0, AT TWO DIFFERENT HYDROGEN PARTIAL PRESSURES. THE DOTTED LINES SHOW WHERE BOTH REACTIONS ARE EQUALLY POSSIBLE. ADOPTED FROM (BATSTONE ET AL., 2002).

The solids retention time, sludge retention time and hydraulic retention time are other design parameters. Here, solids retention time denotes the average retention time of any solids in an AD, sludge retention time denotes the average retention time of the microbial biomass and the hydraulic retention time denotes the average retention time of liquids. Lowering the sludge retention time may wash out slow growing organisms. For example, *Methanosarcina* has a higher growth rate compared to *Methanosaeta*, and through lowering the retention time of mesophilic digesters to 10 days or less, *Methanosarcina* will dominate the archaeal community (Conklin et al., 2006). On the other hand, lowering the solids retention time reduces the contact time between substrate and microorganisms and thereby influence the performance.

Another adjustable process parameter is pH. Microorganisms generally have a narrow optimum and a broad acceptable pH growth range. As a rule of thumb, optimal pH for VFA production is 6.0-6.5, while optimal methanogenesis-pH is 7.0-7.5 (McCarty, 1964; Wang et al., 2014). Many methanogens are severely inhibited by VFAs at pH below 6.5 (Horn et al., 2003). Hence, optimizing pH for hydrolysis and VFA production in a one-stage process has been considered impossible. pH also indirectly influences anaerobic digestion through modifying the process metabolites' equilibrium. Specifically, when pH increase, the ammonium-ammonia equilibrium shifts towards ammonia and the VFAs shift towards the dissociated form. Ammonia inhibits methanogenesis more than ammonium (Chen et al., 2014), while associated acids inhibit more compared to dissociated acids (Horn et al., 2003). Hence, the total inhibition from acids and ammonia due to pH change is difficult to predict.

1.3.2 METABOLITES THAT INHIBITS

The concentrations of metabolites in a digester is related to the set operational conditions, such as temperature, pH and feed composition. Their concentration is difficult to

alter directly, and the accumulation of one metabolite often interacts with the degradation of other metabolites. For example, when ammonium and ammonia inhibits methanogens this cascades into VFA and H_2 accumulation, which again cause long-chain fatty acid accumulation. Then, remembering that long-chain fatty acids inhibits methanogenesis, this can become a reinforcing process resulting in process failure (Tian et al., 2018).

Ammonium and ammonia

For substrates rich in protein, ammonia inhibition is a concern (Parkin and Owen, 1987). Ammonium and ammonia is well-studied inhibitors of the anaerobic digestion processes. A wide range of inhibitory total ammonia nitrogen concentrations have been reported, ranging from 1.7 to 14 NH_3-N g/L causing a 50% reduction in methane production rate (reviewed by Chen et al., 2008). Studies have shown that through allowing the microbial community to adapt to elevated ammonium/ammonia concentrations higher concentrations can be tolerated before inhibition occurs (Liu and Sung, 2002). Increasing ammonium concentrations induces a shift in the methanogenic pathway from acetoclastic to syntrophic acetate oxidation and hydrogenotropic methanogenesis (Schnürer and Nordberg, 2008). Different archaeal genera have different ammonium/ammonia tolerance, where *Methanosarcina* is found to generally be more tolerant than *Methanosaeta* (Calli et al., 2005).

The best correlation between concentration and inhibition is found when using free ammonia concentrations. The ammonium-ammonia equilibrium is shifted with pH and temperature. At higher temperature, the equilibrium is shifted towards ammonia, and mesophilic process temperatures are therefore preferred for nitrogen rich substrates.

Short-chain fatty acids (VFAs)

Short-chain fatty acids are products of acidogenesis and acetogenesis: butyric acid, propionic acid, acetic acid, lactic acid and formic acid. These acids can inhibit methanogens and syntrophic β-oxidizing bacteria (Aguilar et al., 1995; Lier et al., 1993). Accumulation of one acid can therefore cascade into accumulation of other acids. One example is the conversion of propionate by syntrophic β-oxidizing bacteria that is severely inhibited by acetate and butyrate (Lier et al., 1993). *Syntrophomonas wolfei's* ability to degrade butyrate is also inhibited by acetate and depends on the presence of a methanogen or syntrophic acetate oxidizer to remove the acetate that is produced from butyrate degradation (Beaty and Mcinerney, 1989).

Long-chain fatty acids (LCFAs)

LCFAs are believed to inhibit the anaerobic digestion process through adsorbing onto the cell wall and membrane, thereby hindering metabolic transport. LCFA inhibition has been the subject of many studies with the aim of improving anaerobic digestion of lipid rich substrates. Labatut et al. (2014) found that β-oxidation was inhibited in digesters treating dog food, probably due to accumulated LCFAs and propionic acid and increased hydrogen partial pressure. Summarized, LCFAs inhibit β-oxidation, propionic acid degradation and the methanogenesis steps of AD (Angelidaki and Ahring, 1992; Labatut et al., 2014; Ma et al., 2015).

There has been some discussion regarding which microbial groups are most inhibited by LCFAs. Ma et al. (2015) suggested that syntrophic acetogens were most sensitive to high lipid concentrations, while Kim et al. (2007) found that acetoclastic methanogens were more sensitive than acetogens. Moreover, acetoclastic methanogens are more inhibited than

hydrogenotrophic methanogens (Lalman and Bagley, 2001; Lalman and M, 2000). However, the inhibition effect has been reported to be reduced by acclimation (Alves et al., 2001). Hwu and Lettinga (1997) observed that oleate was toxic at lower doses at thermophilic temperatures compared to mesophilic temperatures, and therefore recommended treating substrates rich in fat at mesophilic temperatures.

Combined inhibitors effect

Ammonium inhibition have also been shown to increase with increasing concentrations of acetate, however, this only occurred at ammonium concentrations above 6 g-N/L (Lü et al., 2013). Moreover, recent studies have also found combined inhibitory effects of ammonia and LCFA (Tian et al., 2018; Wang et al., 2016). This co-inhibition from acetate and LCFAs may explain why inhibitory concentrations of ammonia varies between studies.

1.4 Food waste anaerobic digestion

The EU's circular economy action plan (European Commission, 2015) states that "food waste takes place all along the value chain: during production and distribution, in shops, restaurants, catering facilities and at home." The waste from different origins of the value chain vary in composition and thereby methane potential (Browne et al., 2014). Additionally, economic development, culture, geography, energy sources and climate influences food waste quality and quantity, inducing differences in waste composition between countries (Hoornweg and Bhada-Tata, 2012). For example, European food waste contains more organics of animal origin than Asian food waste, which contains more vegetable waste. However, some characteristics are universal, such as a moisture content of 74-90%, a high organic fraction of 85 ± 5% and a low pH of 5.1 ± 0.7 (Reviewed by

Braguglia et al., 2018). Moreover, methanogenesis is often considered the rate-limiting step in food waste anaerobic digestion (Braguglia et al., 2018).

1.5 FOOD WASTE AD IN NORWAY

Norway is among the "frontrunners" when it comes to recycling of solid waste with less than 10 % disposal (Bartl, 2015). However, when comparing Norway to the other frontrunner countries, it has the lowest recycling and composting rate (~ 40%). This is a consequence of the high level of waste incineration in Norway. However, food waste has a high water content, making the net calorific value low or negative, hence motivating a shift towards more utilization of food waste in anaerobic digestion plants.

TABLE 1: WASTE FRACTIONS IN NORWAY IN 2015.

Waste fractions		Treatment		
Origin	Collected (million kg)	AD	Composting	Incineration
Food waste				
- households	181			
- service industries	134			
- other industries	117			
- Sum	432	233	116	50
Mixed waste				
- households	1020			
- service industries	1050			
- other industries	n.d.			
- Sum	2232	16	0	2405
Estimated food waste in mixed waste				
- households	408			
- service industries	367.5			
- other industries	n.d.			
- Sum	776	16	0	760

Waste amounts in million kg. Numbers for Food waste and mixed waste were found in "Waste Accounts (Avfallsregnskapet)" (2017), while the estimated food waste in mixed waste was calculated using 40 % and 35 % food waste in household and service industries, respectively. This was based on the reported amount of wet organic waste in these fractions (Marthinsen et al., (2006)).

A large amount of food waste generated in Norway in 2015 ended up as mixed waste and was incinerated (Table 1). Although only 12 % of the source separated food waste was incinerated, an estimated 760 million kg wet organic waste from households and service industries were not source separated and therefore incinerated as part of the mixed waste. In a circular economy, households and industries should reduce food waste generation and governments should ensure waste separation. Complete waste separation will result in 4 times the amount of food waste that was available for AD treatment in 2015. However, Norway's ambitions are to reduce food waste generation by 50 % within 2030 ("Bransjeavtale om reduksjon av matsvinn," 2017) reducing the increase in separated food waste to double the amount of food waste currently available for AD treatment. Hence, to treat all generated food waste with AD, AD technology development to achieve higher treatment rates or investments in new digesters are needed.

1.6 AD REACTOR TECHNOLOGIES

Different reactor technologies are used for anaerobic digestion of organic wastes. Commonly used reactor technologies include, upflow anaerobic sludge blanket (UASB), continuous stirred tank reactors (CSTR), two-phase digesters and plug-flow digesters. In addition, pre-treatment and post-treatment technologies are frequently applied. The reactor technologies suitability depends on both substrate characteristics and scale.

UASB reactors are extensively used for industrial wastewater. The biomass is retained in the digester as granules, uncoupling hydraulic retention time and sludge retention time. This uncoupling makes the reactor type efficient for dilute warm wastewaters, however, it is

not suitable for wastes with high concentration of particulates. For food waste, the technology is applied for palm oil mill-, slaughterhouse- and dairy wastewater (Borja et al., 1996; Gavala et al., 1999; Ruiz et al., 1997) while municipal food waste must be separated into a liquid and solid fraction before the liquid fraction can be treated in an UASB digester (Xu et al., 2002). A number of other technologies based on the same principle, retaining the microbial biomass, are available (Rajeshwari et al., 2000), however, neither is suitable for wastes with high particulates content.

CSTRs are perhaps the most applied reactor technology for municipal food waste. The digesters are continuously fed, and the hydraulic and sludge retention time is equal. Because CSTRs are mixed with continuous feed flow, not all substrate units will have the same retention time. Some of the substrate is not retained in the reactor while other parts of the substrate is retained for a very long time (Figure 3). This results in that the hydraulic retention time must be very long in order to achieve high treatment rates.

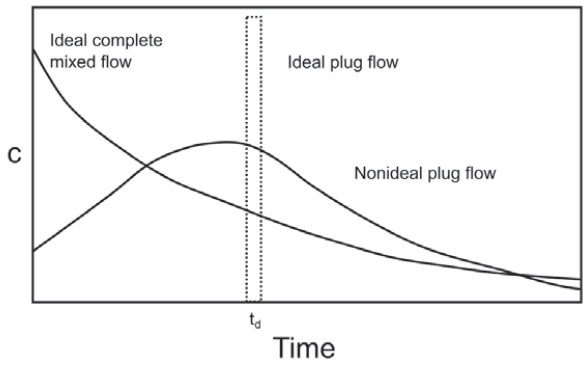


FIGURE 3: EFFLUENT TRACER CONCENTRATIONS FOR DIFFERENT REACTOR TECHNOLOGIES. ADOPTED FROM DROSTE (1997).

Two-stage anaerobic digestion of food waste has recently re-gained interest (Ren et al., 2018; Shen et al., 2013). Usually a two-stage system spatially separates the hydrolysis and

acidogenesis from acetogenesis and methanogenesis by operating two CSTR digesters in series. In this “acid-methane” configuration, the first digester has a lower pH and retention time, to optimize the first steps of anaerobic digestion. Others have suggested to separate propionate degradation by applying a two-stage “methane-methane” digester (Wiegant et al., 1986). While an acid-methane configuration is advantageous when hydrolysis is rate limiting, the methane-methane configuration is more relevant when the final steps limits the process. Regardless, two-stage digesters have been shown to improve stability and volumetric methane yields (Bouallagui et al., 2004; Wiegant et al., 1986).

Plug-flow digesters takes two-phase anaerobic digestion to the next level. It uses either more than two digesters in series, a compartmentalized reactor or a long pipe. Standard reactor technology principles deduce that for chemical reactions with reaction kinetics larger than zero, plug-flow reactors are more efficient compared to CSTRs (Droste, 1997). This follows from the fact that these reactions transpire more rapidly when reactant concentrations are high. In a plug-flow or serial reactor system, reactant concentrations can be remained at high concentrations in the inflow side of the system (or in the first reactors), improving reaction rates. In addition, less substrate will have very short retention times, as illustrated by non-ideal plug flow and ideal plug flow in Figure 3. However, for biological processes retention times must be sufficient to avoid microbial washout. One solution proposed by van Lier et al. (1994) is a compartmentalized thermophilic UASB where the active biomass is retained in several compartments. This reactor design generally achieve very low concentrations of VFAs in the effluent and stable reactor performance at thermophilic temperatures.

Pre-treatment is often applied to improve the accessibility to recalcitrant substrates. However, for food waste, where methanogenesis is the limiting step, pre-treatment could cause more problems than improvements (Meng et al., 2015; Sayed et al., 1988). Recently,

post-treatment has been suggested as an alternative (Sambusiti et al., 2015; Thygesen et al., 2014). Post-treatment can be applied in both singular and serial systems. By dewatering the effluent from the digester, the volumes that needs treatment is reduced, while the end-products hygiene for land application is assured. The treated effluent can be added as substrate in a second digester, or dewatered and returned to the first digester such as in Cambi's SolidStream configuration (Kolovos et al., 2016).

1.7 Digestate Utilization

The reason anaerobic digestion is considered the preferred waste management technology for solid organic wastes is that it has two useful products: methane and digestate. However, for the digestate to be suited for land application certain criteria must be met. The regulations for treatment of organic waste for the use of digestate on arable land is currently being revised in Norway (June 2018). The revisions are made in order to increase the use of organic (non-mineral) fertilizer products and encourage bioenergy production. Today different regulations are applicable depending on the type of substrate used to produce the organic fertilizer. In this thesis, only food waste and sewage sludge are used as substrates and only the regulations for these two substrates will briefly be presented (Lovdata, 2007, 2003).

Both regulations consist of quality requirements including hygiene requirements. The quality requirements include concentration limits for heavy metals and demands that producers and traders of organic fertilizer products are cautious to avoid harm on health and environment. The hygienic requirements are different for sludge and food waste, where food waste must be pretreated at 70 °C for 1h or equivalent, while sewage sludge digestate must be documented to be salmonella free and only contain a low concentration of total coliform bacteria (below 2500 per gram TS). The concentration of heavy metals on the basis of TS dictates if the digestate can be used on arable land or is restricted to other green areas, such as

landfill cover. The digestate must also be stable, and not cause odor issues after spreading on land. Moreover, any digestate that is produced from sewage sludge cannot be spread on areas where vegetables, potatoes, berries or fruit are grown.

Digestate is added to soil as an alternative to mineral fertilizer or in addition to mineral fertilizer. It also adds organic matter to the soil which can improve its water holding capacity. Because of uncertainty among farmers regarding how to best utilize digestate, it is generally an under-valued product, and usually carries a disposal cost for the anaerobic digestion plant operator (Riding et al., 2015). In addition, digestate cannot be spread on frozen or snow covered land, resulting in need for storage during the winter. AD plants therefore often dewaters the digestate, and disposes the nitrogen rich liquid to the sewer system. Reducing the mass of the solid fraction, the digestate cake, also help reducing the AD plants' final disposal cost. This can be achieved through improved solids destruction (improved methane yields) and improved dewatering.

2. THESIS AIM AND OUTLINE OF THE WORK

The overall aim of this thesis was to improve anaerobic digestion of food waste. To achieve this, the following secondary objectives were set: 1) Compare different methods for organic matter determination to test their reliability, 2) Investigate different processing technologies that may improve food waste digestion, and 3) Investigate if laboratory small scale experiments are relevant for large scale biogas plants.

Food waste anaerobic digestion is already established in Norway. However, in the emerging circular economy more food waste needs to be treated and treatment efficiencies must be improved. When evaluating process efficiency, it is important to use a reliable method for determination of the organic content of the biogas substrate and the digestate. Thus, this study was initiated by comparing different methods for organic matter determination (**Paper I)**. The main part of this work focused on technologies that can increase treatment efficiency in already operating CSTR digester systems. Specifically, recirculation of digestate, post-treatment with thermal hydrolysis and "methane-methane" serial digestion were investigated in **Papers II, III and IV**. Moreover, the relevance of laboratory experiments for full scale plants was addressed in **Papers IV and V,** where scales and different feeding frequencies were compared in terms of process performance and microbial community structures. These five papers constitute this thesis, and they have the following outline:

In Paper I, different approaches for organic matter determination in relation to expressing bio-methane potentials were compared. Anaerobic digestion efficiency is usually reported in methane per gram volatile solids (VS) fed or methane per gram chemical oxygen demand (COD) fed. Different approaches to measure both VS and COD were therefore compared for a range of substrates.

In Paper II, food waste anaerobic digestion at thermophilic and mesophilic temperatures with and without recirculation of digestate were compared. The laboratory CSTR digesters were operated with 20-days SRT, which is the same retention-time as in several full-scale food waste anaerobic digesters in Norway. The main objective was to investigate recirculation of digestate as a strategy for feed dilution instead of fresh water. The effect of recirculation and temperature on process performance and microbial community structure were investigated.

In Paper III, conventional pre-treatment at 70 °C and post-treatment using thermal hydrolysis were compared. The temperature and residence time of thermal hydrolysis of food waste-digestate and sludge-digestate were invetigated and evaluated in terms of solubilization of COD and BMP tests. Then CSTR digester performance was evaluated by comparing the conventional pre-treatment to post-treatment with thermal hydrolysis.

In Paper IV, a laboratory model of a full-scale plant co-digesting food waste and primary sludge at 62 °C was established. The full-scale plant applied two-digesters in series, in a "methane-methane" configuration. Process performance and microbial community structure were analyzed and compared between full-scale and lab-scale digesters.

In Paper V, the feeding of laboratory digesters once-daily or 10-times a day were compared. The study was inspired by the observed daily fluctuations in gas-production and pH observed during the work with **Paper II and IV**. Two digesters were operated in parallel and fed food waste with 10 days SRT. Process performance and microbial community structures were compared.

3. Main results and Discussion

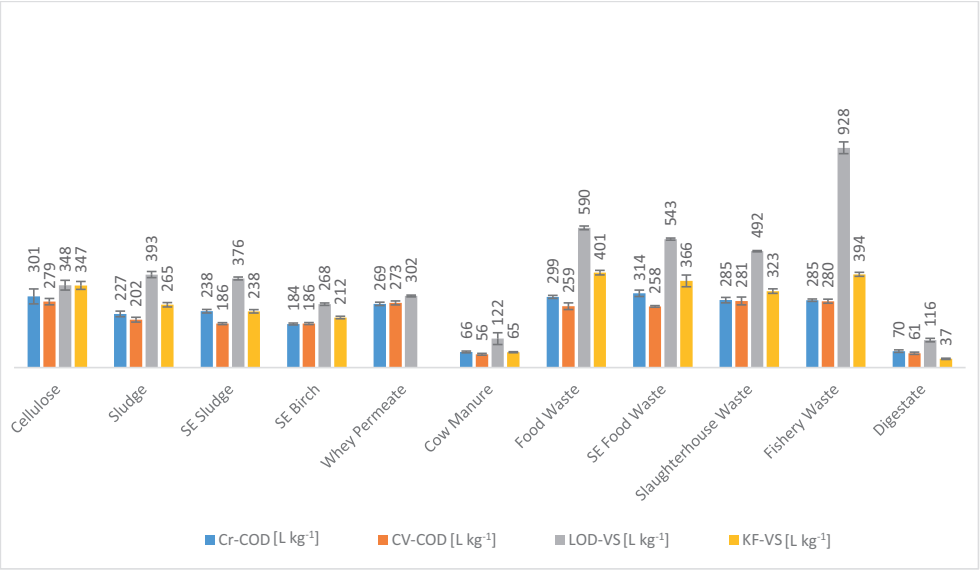
3.1 Comparison of approaches for organic matter determination (Paper I)

In this paper, four different approaches for organic matter determination were tested on 11 different substrates. Two methods for chemical oxygen demand (COD) determination were used: bomb calorimetry (denoted CV-COD) and wet-oxidation with di-chromate (denoted Cr-COD). Likewise, two methods for volatile solids (VS) determination were used: Karl Fischer titration (denoted KF-VS) and loss on ignition (denoted LOD-VS). Additionally, methane production from all the samples were determined using a BMP test.

Overall, the COD determined by wet-oxidation was in the range of 72-100% of the bomb-calorimetry measurements. It was observed that the greatest difference between the two COD methods were for biomass samples with high dry matter content, while they were not significantly different for whey permeate. Hence, we concluded that bomb calorimetry COD is more accurate for samples with high dry matter contents. BMPs for cellulose, whey, food waste, steam exploded food waste, slaughterhouse waste, and fishery waste were all in the range of 269 – 314 mL/g Cr-COD_{added} and 258 – 281 mL/g CV-COD_{added}. These substrates were expected to be completely degradable under the BMP test-conditions, and hence, their BMPs on the basis of COD was expected to be similar. However, the BMPs expressed on the basis of Cr-COD was generally higher, and they also showed more variability compared to those on the basis of CV-COD. Hence, CV-COD should be the preferred method, however, for COD-measurements results presented on the basis of CV-COD should not be compared with results based on Cr-COD. Therefore, to allow comparisons with results from other studies the most used method (Cr-COD) should be included.

Volatile solids were determined by two methods: water loss on drying and Karl Fischer titration. For most biomass samples, the differences between the two methods were

large, with the most extreme being fishery waste. For this biomass, VS determined using Karl Fischer titration was two-fold higher than that found using the loss on drying method. The consequences of using different methods for organic content determination on bio-methane potential are illustrated in Figure 4.



FIGUR 5: BIO-METHANE POTENTIALS PRESENTED ON THE BASIS OF WET CHEMISTRY COD (CR-COD), BOMB CALORIMETRY COD (CV-COD), LOSS ON DRYING VS (LOD-VS) AND KARL FISCHER VS (KF-VS).

Biased VS determination with the loss on drying method has previously been described and discussed (Agger et al., 2014). In this study we found that VS determination with Karl Fischer titration also is biased. For example, we were unable to determine water content in the powdered whey permeate because its chemical composition was unstable at the analysis temperature. Moreover, the analysis of the digestate sample underestimated the water content. Biased VS determination for digestate is particularly concerning when VS is used for mass-balance in anaerobic digestion systems.

3.2 Effect of digestate recirculation on food waste anaerobic digestion performance (Paper II)

Anaerobic digestion performance was evaluated in four lab-scale CSTR digesters treating food waste. Two digesters were operated in the mesophilic temperature range (37 ± 1 °C) and two digesters were operated in the thermophilic temperature range (55 ± 1 °C). Moreover, digestate was recirculated in one mesophilic and one thermophilic digester. In the digesters without recirculation, the food waste was diluted with water.

Overall, the mesophilic digesters' performance were similar. They achieved a specific methane yield of approximately 280 mL/gCOD$_{added}$, which corresponds to 80 % of the maximum theoretical methane yield of 350 mL/gCOD$_{added}$ and 93 % of the BMP measured for this substrate in **Paper I** (299 mL/gCOD$_{added}$). However, the mesophilic digester with recirculation had two times higher total ammonia nitrogen concentration than the reactor without recirculation, and four times higher free ammonia nitrogen concentration. Moreover, soluble COD concentrations were three times higher in the digester with digestate recirculation.

For the thermophilic digesters, methane yields were lower compared to the mesophilic digesters, being in the range 240 to 260 mL/gCOD$_{added}$. Again, total ammonia nitrogen and free ammonia nitrogen concentrations were higher in the digester with recirculation. Moreover, soluble COD concentrations were higher in the digester with recirculation, and the soluble COD concentrations were around 10 times higher in the thermophilic digesters compared to the respective mesophilic digesters. More notably, the thermophilic digester without recirculation suffered acetate accumulation, while that with recirculation suffered accumulation of propionate, iso-butyrate and valerate.

The acetate accumulation in the thermophilic digester without recirculation was probably linked to inhibition of the acetate oxidizers. It was a lower abundance of acetate oxidizers belonging to *Thermoacetogenium* in the digester without recirculation compared to that with recirculation (3 % vs. 6 %). In the thermophilic digester with recirculation, accumulation of propionate, butyrate and iso-valerate was related to lower abundance of β-oxidizers (*Syntrophomonas*) with 2 % relative abundance in this digester compared to 6 % in that without recirculation.

3.3 Post-anaerobic digestion thermal hydrolysis (Paper III)

In this paper, the effect of post treatment on the biogas process was compared to conventional pre-treatment. We tested the effect of different thermal hydrolysis conditions (time and temperature) on digestate cake in terms of solubilization of COD and resulting bio-methane potential. Two digestate cakes were selected, one from a plant treating food waste and the other from a plant treating sewage sludge. Moreover, we tested the effects of post-treatment on digester performance using semi-continuous lab-scale anaerobic digesters where one digester was fed conventional pre-treated food waste and sewage sludge, while the other was fed untreated food waste and sewage sludge in addition to centrate from post-treatment.

The digestate cake from the plant treating waste activated sludge was more efficiently thermally hydrolyzed at all conditions tested in relation to methane yields and extent of dewaterability, than the cake from a plant treating source separated food waste (SSFW). The cake from the plant treating food waste was determined to contain more fiber and have a higher C:N ratio, indicative of higher plant material content compared to the cake from the wastewater treatment plant. Previous studies have found that thermal hydrolysis of ligno-cellulosic materials are more efficient at temperatures above 170 °C, while 165 °C often is reported as optimal for sludge. This indicates that the applied temperature for thermal hydrolysis was too low to be efficient on food waste cake.

In the semi-continuous lab-scale digesters, post-treatment improved volumetric methane yields by 7 % and the COD-reduction increased from 68% to 74% despite lowering the solid retention time (from 17 to 14 days) compared to a conventional system with pre-treatment of feed substrates at 70 °C. Moreover, results from thermogravimetric analysis showed an expected increase in maximum TS content of dewatered digestate cake from 34% up to 46% for the SSFW digestate cake, and from 17% up to 43% in the sludge digestate

cake, after the PAD thermal hydrolysis process (PAD-THP). The increased dewatering alone would account for a reduction in wet mass of cake leaving a plant by 60% in the case of sludge digestate cake (Figure 6). Additionaly, the increased VS-reduction will contribute to further reduce the mass of wet cake.

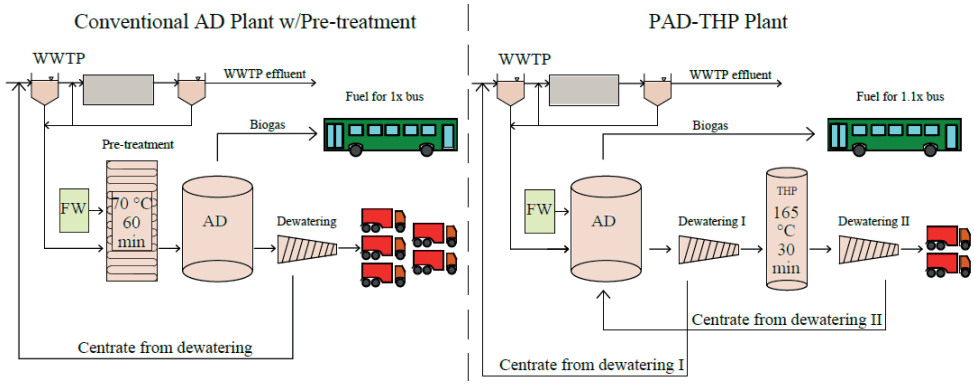


FIGURE 7: SCEMATIC ILLUSTRATION OF THE DIFFERENCE BETWEEN A CONVENTIONAL PLANT WITH PRE-TREATMENT AT 70 °C AND A POST-ANAEROBIC DIGESTION THERMAL HYDROLYSIS (PAD-THP) PLANT.

3.4 Stable anaerobic serial digestion at temperatures above 60 °C – a comparison of a full-scale and lab-scale digester system (Paper IV)

A full-scale anaerobic digestion plant was modelled using duplicate 6 L laboratory digesters. The digester system treated primary sludge and food waste at 62 °C with a methane-methane serial digestion configuration. The digester system had a total retention time of 19 days, divided equally between two digesters (Figure 8).

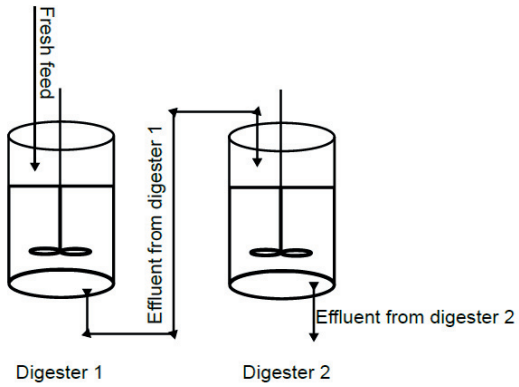


FIGURE 9: DIGESTER CONFIGURATION FOR BOTH FULL-SCALE AND LAB-SCALE DIGESTERS. ALL FRESH FEED IS ADDED IN DIGESTER 1, WHILE DIGESTER 2 RECIEVES ONLY THE EFFLUENT FROM DIGESTER 1.

The lab-scale digesters were fed with a 50:50 ratio of food waste and primary sludge on the basis of LOD-VS and the feed was diluted with water to meet the measured ammonium concentrations and hydraulic retention time in the full-scale digester. The full-scale digesters had stable performance with low residual VFA concentrations below the quantification limit of 10 mg/L. We operated the laboratory digesters until similar results were achieved, for a total of 252 days.

In the lab-scale digesters, low residual VFA concentrations was first reached in the second digester in each serial configuration. In fact, acetate concentrations in the second digesters were below 100 mg/L from day 56 and 106 in the two laboratory systems. Propionate concentrations were below 200 mg/L after 126 and 77 days. However, the first digesters in the laboratory methane-methane serial digestion system did not reach propionate concentrations below 200mg/L until 211 days after startup. Although the laboratory digesters had to be operated for more than 22 retention times before reaching low propionate concentrations, the experiment demonstrated that low residual propionate concentrations can be reached at higher thermophilic temperatures (62 °C).

TS, pH and biogas yields in the laboratory digesters differed from full-scale. TS concentrations were higher, while pH and biogas yields were lower in the full-scale digesters. The volumetric methane yield for the whole laboratory system was 1383 mL/L/d and the specific methane yield was 210 mL/gCOD_{added}. In comparison, the volumetric methane yield was 914 mL/L/d in the full-scale system. The exact feed composition in the full-scale plant was unknown, although the ratio between food waste and sludge was assumed to be 50:50 on VS basis, hence differences in process performance could be due to differences in feed composition. Moreover, HRT and OLR varied in the full-scale digester as a response to variation in sludge generation in the waste-water treatment plant, and the storage of substrates used in the laboratory experiments may have altered their composition.

The microbial community in the laboratory digesters were different from the full-scale digesters (Figure 11). Most surprising was the difference in the methanogenic community in the lab-scale and full-scale digesters. The community in the lab-scale digesters where dominated by hydrogenotrophic methanogens, while the full-scale digesters were dominated by the acetoclastic methanogens *Methanosaeta*. This finding in the full-scale community contrasted previous findings, where hydrogenotrophic methanogens typically dominate the

archaeal community in digesters operated at temperatures above 60 °C. Moreover, despite the differences in archaeal community structure, both full-scale and lab-scale digesters eventually showed stable performance with low residual VFA concentrations in the effluent.

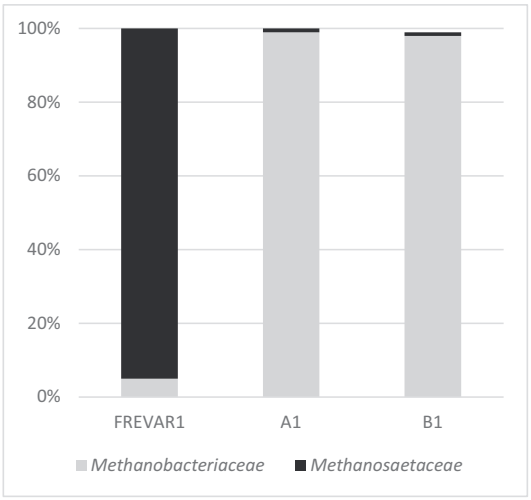


FIGURE 10: RELATIVE ABUNDANCE OF *METHANOBACTERIACEAE* (HYDROGENOTROPHIC METHANOGENS) AND *METHANOSAETACEAE* (ACETOCLASTIC METHANOGENS) IN THE ARCHAEAL COMMUNITY ON DAY 217 IN THE FULL-SCALE DIGESTER (FREVAR1) AND DAY 231 IN LABORATORY DIGESTERS A1 AND B1.

3.5 Feeding Frequency in laboratory scale digesters (Paper V)

In this study, the performance of two laboratory digesters were compared. One digester was fed once per day, while the other was fed 10 times a day. Both digesters were fed steam exploded food waste with an organic loading rate of 21 gCOD/L/d with a 10 days hydraulic retention time. Overall, the digester with the most frequent feeding (FFD) performed better with a high methane yield of 305 mL/gCOD$_{added}$, as compared to 236 mL/gCOD$_{added}$ in the digester fed once per day (DFD). This difference in methane yields was linked to improved digestion of long-chain fatty acids in the frequently fed digester. The microbial community structure in the two digesters was also different.

Biogas production rates between feedings varied significantly more in the daily fed digester compared to the frequently fed digester (Figure 11). Moreover, acetic acid concentrations in the daily fed digester varied between feedings, but not in the frequently fed digester. In the frequently fed digester, acetic acid concentrations remained below 500 mg/L at all sampling times. Elevated VFA concentrations are unfavorable for β-oxidation, and initial inhibition of methanogens have been suggested to initiate and enhance LCFA inhibition (Tian et al., 2018). Hence, the fluctuations in acetic acid caused by the daily feeding regime may also have initiated and enhanced LCFA inhibition, and thereby caused the reduced methane yields.

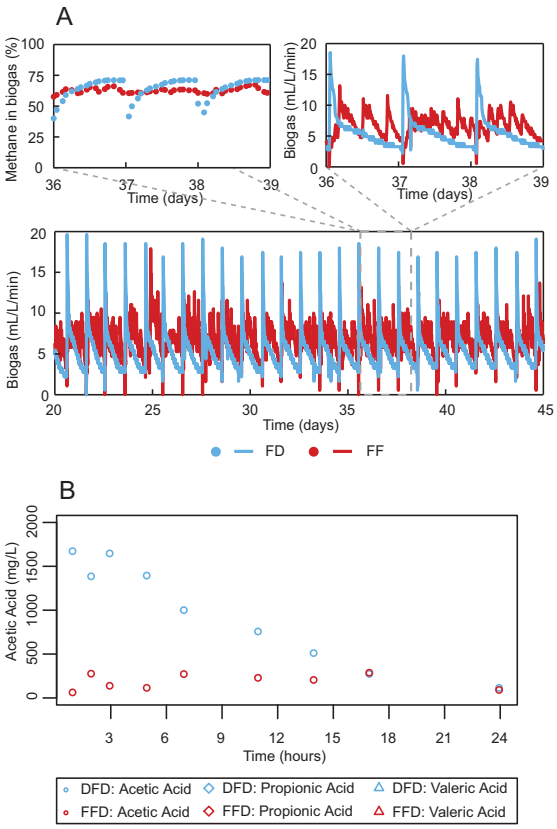


FIGURE 12: A) VARIATION IN GAS-PRODUCTION RATE AND METHANE CONTENT IN THE BIOGAS IN THE DAILY FED DIGESTER (DFD) AND THE FREQUENTLY FED DIGESTER (FFD). B) VARIATION IN ACETIC ACID CONCENTRATION BETWEEN FEEDINGS IN THE TWO DIGESTERS.

3.6 SUMMARY OF METHANE YIELDS DETERMINED IN PAPERS I-V

In **Papers I-V** we determined the methane yields in each system (summarized in Table 2). The same food waste source (Norwegain food recycling) was used in **Paper I, II, IV and V**, allowing a direct comparison of the results from these studies. Paper III was performed in the U.S., hence, a different source of food waste was used in this study.

TABLE 3: SUMMARY OF SPECIFIC METHANE YIELD IN THE 5 ARTICLES.

Paper	Substrate	Digester configuration	HRT	Temperature (°C)	Specific methane yield ($mL/gCOD_{added}$)	Rank	% of BMP
I	Food waste	Batch	-	37	299 ± 7	3	100
	SE Food waste	Batch	-	37	314 ± 14	1	100
	Sewage sludge (primary)	Batch	-	37	227 ± 12	10	100
	SE sewage sludge (primary)	Batch	-	37	238 ± 7	8	100
II	Food waste	Semi-CSTR[1]	20	37	283 ± 34	4	95
	Food waste	Semi-CSTR[1] + recirculation	60	37	280 ± 47	5	95
	Food waste	Semi-CSTR[1]	20	55	257 ± 44	6	86
	Food waste	Semi-CSTR[1] + recirculation	60	55	242 ± 45	7	81
III	Food waste + sludge (primary +waste activated)	Semi-CSTR[1]	17	37	186 ± 9.9	13	-
	Food waste + sludge (primary +waste activated)	Semi-CSTR[1]-PAD-THP	14	37	197 ± 9.4	12	-
IV	Food waste + sludge (primary)	Serial-semi-CSTR[1]	19	62	212 ± 1.4	11	81
V	SE food waste	Semi-CSTR[1]	10	37	236 ± 49	9	75
	SE food waste	CSTR	10	37	305 ± 35	2	97

[1]Semi-CSTR refers to semi-continuous stirred tank reactors, meaning that these reactors were fed once daily.

The digesters treating food waste as the sole substrate performed best in terms of methane yields based on Cr-COD. This shows that food waste is more easily digested

compared to sewage sludge. Overall, the batch digesters showed the highest methane yield for food waste and steam exploded food waste (SE food waste). This was expected, as the bio-methane potential tests are designed to find the substrates' maximum methane yields. The next best performance was for the frequently fed laboratory digester treating steam exploded food waste (**Paper V**). This digester reached 97 % of the BMP with only 10 days HRT. 3rd was the BMP of food waste, followed by the mesophilic semi-continuous digestion of food waste at 37 °C with 20 and 60 days HRT with around 94 % of the BMP (**Paper II**). Finally followed the thermophilic digesters from the same study which had the lowest methane yield. Interestingly, the semi-CSTR (the digester fed once daily) with steam exploded food waste and 10 days HRT reached only 75 % of the BMP of SE food waste (**Paper V**). Hence, by only varying the feeding frequency the treatment of steam exploded food waste at 10 days HRT changed from being the best to the worst performing food waste digestion system.

The methane yields in the co-digestion scenarios was lower compared to those with food waste as the sole substrate. A low methane yield for sewage sludge was also determined by the BMP tests carried out in **Paper I**. However, this sludge was mainly iron precipitated primary sludge, and primary sludge generally have higher BMPs compared to waste activated sludge (used in **Paper III**). The best performing co-digestion scenario was also that with primary sludge (**Paper IV**). In this study, approximately half of the COD_{added} came from food waste and the other half from primary sludge, hence, the methane yield equaled 80 % of the BMP from **Paper I**. Although the digesters in **Paper III** had the lowest methane yields, the methane yield in the digester with post-anaerobic digestion thermal hydrolysis was close to that found in **Paper IV**. In addition, the feed used in the digesters in **Paper III** only contained 30 % of the COD from food waste, while the remaining came from primary and secondary sludge. Hence, in terms of food waste digestion, the post-treatment may have yielded better results compared to the thermophilic serial digesters.

4. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The main objective of this thesis was to improve the anaerobic digestion of food waste. Different technologies were tested for their potential to improve food waste anaerobic digestion. Based on the results from these studies, the following conclusions can be made:

In single stage anaerobic digestion of food waste, mesophilic treatment is more efficient compared to thermophilic. Anaerobic digesters treating food waste at mesophilic temperatures, can also apply recirculation of digestate instead of diluting the feed with water without impeding the process. Thermophilic anaerobic co-digestion of food waste and sewage sludge is improved when a second stage digester is added. This second stage efficiently removes VFAs that has accumulated in the first stage, and therefore allow for higher loading of the first stage digester. Mesophilic co-digestion of sewage sludge and food waste is improved by thermal post-treatment. Although the post-treatment is more efficient for sewage sludge digestate cake, the improved sludge digestion increases the overall capacity of anaerobic digesters so they can treat more food waste as a co-substrate.

Overall, the observed methane yields of 280-305 mL/gCOD_{added} in the mesophilic digesters treating food waste as the sole substrate, corresponding to 80-90% COD reduction, show that the hydrolysis was complete. However, accumulation of VFAs and LCFAs in the thermophilic and high loaded mesophilic digesters indicates that the latter steps in the AD process, namely acetogenesis and methanogenesis, are the limiting steps in food waste anaerobic digestion. Hence, to improve food waste anaerobic digestion, technologies facilitating the success of β-oxidizers, acetogens and methanogens must be implemented. In this thesis, methane-methane serial digestion and a frequent and stable feeding regime was found to benefit β-oxidation, acetogenesis and methanogenesis, and thereby improve the anaerobic digestion of food waste. Other technologies that may facilitate these processes and

their respective microorganisms includes adding conductive carbon materials to promote direct interspecies electron transfer (Lin et al., 2017), plug-flow digestion (van Lier et al., 1994), lipid removal from the food waste prior to digestion, and co-digestion with fiber rich materials that adsorbs LCFAs (Labatut et al., 2014). However, Paper IV and V questioned how well small-scale laboratory experiments can model full-scale digestion plants. Thus, more research is needed to exploit the full potential of food waste as a substrate for anaerobic digestion.

In the introduction of this thesis, a doubling of source separated food waste was foreseen in the coming years resulting from the shift towards a circular economy. This is equivalent to 250 million kg more food waste available each year, which corresponds to about 70 million kg COD. Based on the methane yields achieved for single stage mesophilic digestion of food waste (ranging from 236-305 mL/gCOD_{added}), the estimated increase in yearly methane production is 16-22 million m^3. This corresponds to 180-240 GWh. In comparison, statistics from 2015 showed that 361 GWh of biogas was used for energy (in the form of district heating, electricity sold to the grid or vehicle fuel) in Norway that year (SSB, 2016). Hence, improved source separation of food waste and treatment with AD can significantly increase the amount of biogas produced in Norway. Although twice the amount of digestate will be produced if twice the amount of food waste is digested with today's technologies, the increase in wet digestate volumes can be limited through recirculation of digestate instead of using fresh water. Additionally, digestate cake production can be reduced by implementing post-treatment thermal hydrolysis that may double digestate cake TS from today's 20-25% up to 45%. Moreover, twice the amount of TAN will be available for nutrient recycling, corresponding to 500 000 kg-N (based on nitrogen concentrations reported in Paper II).

In summary, the findings reported in this thesis suggest that existing AD plants can increase their food waste loadings. Hence, existing infrastructure can be exploited and further developed, reducing the need for new plants. Overall, improved utilization of food waste in Norway would significantly contribute to the national biogas production and nutrient recycling, and thereby be an important contributor to the transition to a circular economy.

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Research paper

Comparison of approaches for organic matter determination in relation to expression of bio-methane potentials

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ABSTRACT

Bio-Methane Potential (BMP) tests are used to evaluate the suitability of a biomass for anaerobic digestion. BMP data are usually presented as the amount of methane produced from a kilogram of volatile solids (VS) or chemical oxygen demand (COD) of the substrate. However, the most used methods for determination of VS and COD are not always accurate. Oven drying may underestimate VS content due to loss of volatile organic compounds, and incomplete chemical oxidation may lead to underestimation of COD content. Bomb calorimetry is an attractive alternative to COD measurements, because the physical state of the biomass sample does not influence the measurement, and because sample preparation is limited. In this study, 11 biomass samples, wet and dry, were analyzed with different methods for organic content determination. COD (determined by bomb calorimetry and by wet chemistry) and VS (by Karl Fischer titration and loss on drying; LOD) were compared, and used for determination of BMP. In general, the BMP estimated on a VS basis were higher than those estimated on COD basis. For certain biomass samples the method for VS determination also greatly influenced the results; for fishery waste the BMP was estimated as 928 L kg^{-1} based on LOD-VS compared to 394 L kg^{-1} based on KF-LOD. Thus, this study shows that determination of organic content is not trivial and the method of choice strongly influences the estimation of bio-methane potentials. Bomb calorimetry offers a possibility to measure energy content directly, independent of biomass composition and physical state.

1. Introduction

Biochemical Methane Potential (BMP) tests are applied to evaluate the anaerobic biodegradability of a biomass and the effect of pretreatment technologies. For this purpose standard protocols have been proposed in an attempt to limit the variations between laboratories [1,2]. The protocols suggest that bio-methane yields are presented either on the basis of volatile solids (VS) or Chemical Oxygen Demand (COD), and most studies report either one or both of these values. In relation to this, it is acknowledged that VS and COD determinations in biomass samples are challenging, but few studies have investigated this in detail [1,3–5].

VS refer to the amount of organic compounds in a biomass sample and are defined as the difference between dry matter (DM) and the ash content. VS is usually determined gravimetrically as the difference in the sample weight after drying at 105 °C (Loss on Drying; LOD) and subsequent burning at 550 °C (Loss on Ignition; LOI). The method assumes no evaporation of organic material at 105 °C, which is not the case for many volatile compounds. To overcome this problem Agger et al. [3] suggested using Karl Fisher titration which specifically measures water content in biomass as an alternative to LOD for DM determination. The study demonstrated that Karl Fischer titration could give accurate measures for water content in various biomass samples and thus a more accurate determination of DM and VS, in particular for samples containing true volatile compounds. Both methods uses LOI for ash determination.

Abbreviations: BCV, net biological calorific value for biological degradation; BMP, bio-methane potential; CEP, chemical energy potential; COD, chemical oxygen demand; Cr-COD, chemical oxygen demand estimated with the closed reflux method; CV-COD, chemical oxygen demand calculated from the net calorific value; DM, dry matter; GCV, gross calorific value; KF, Karl Fischer; LOD, loss on drying; RSD, relative standard deviation; SE, steam exploded; SIR, substrate to inoculum ratio; ThCOD, theoretical chemical oxygen demand; VFA, volatile fatty acids; VS, volatile solids.

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COD is a term for the experimental determination of chemical oxidation where a strong oxidizing agent is used to oxidize organic material, followed by colorimetric or titrimetric determination of the reduction of the oxidizing agent. The result from such determination is often used as an estimate of Theoretical Chemical Oxygen Demand (ThCOD). ThCOD is the stoichiometric amount of oxygen required to oxidize an organic compound to minerals, H_2O, CO_2, NH_3, $H_2PO_4^-$ and H_2SO_4. However, due to the complex composition of real biomass samples, exact elemental composition is difficult to determine, thus ThCOD can often not be calculated. COD of biomass samples is commonly determined with commercial kits by chemical wet oxidation using potassium dichromate as the oxidant. Samples containing substances that are partially or completely recalcitrant to chemical oxidation by dichromate, e.g samples containing particulate materials, make COD measurements difficult and open to uncertainty [4,5]. In addition, real inhomogeneous biomass samples are exceptionally challenging to handle for COD determinations with commercial kits, because the analysis requires highly dilute and homogeneous sample preparation, as these kits were originally conceived for liquid samples.

Another empirical method to estimate ThCOD is by oxygen bomb calorimetry, where the gross calorific value (GCV) of a sample is determined by incineration in a bomb (an oxygen pressurized chamber). Bomb calorimetry can be considered a perfectly closed system and hence the GCV can be calculated from the temperature rise in the closed chamber surrounding the bomb [6].The energy released from the incineration is a result of the oxidation of the biomass, and the breaking and formation of chemical bonds. This is referred to as chemical energy potential (CEP). The amount of heat released depends on the type of chemical bond broken and formed, hence, the oxygen consumed and the energy released are not exactly proportional. On the other hand, oxygen consumption and released heat are correlated and a calorific-determined COD value can be derived, via the experimentally established correlation between CEP and COD (14 MJ kg^{-1} of COD) [7].

In this study we evaluate and compare the four methods mentioned here: VS calculated from DM determination by the Karl Fisher method, VS calculated from DM determination by LOD, wet oxidation COD, and bomb calorimetry determined COD for their applicability in describing the organic content in various biomass samples. Moreover, we carried out biogas batch tests of the same samples and investigated how the method for organic matter content determination affected the calculation of bio-methane yields. The general hypothesis was that the bomb calorimetrically determined COD method is the most generic method for inhomogeneous biomass samples, due to the completeness and unspecificity of the measurement, and hence should yield the most correct description for bio-methane potential estimations.

2. Materials and methods

2.1. Biomass samples

Biomass samples were selected to obtain a wide range of organic matter and energy content. An overview of the biomasses and relevant pretreatments is provided in Table 1, and includes untreated and pretreated sewage sludge, digestate, pretreated birch, cellulose, whey permeate, cow manure, food waste, pretreated food waste, slaughterhouse waste and fish waste. Pretreatment (steam explosion) was performed using the steam explosion unit (designed by Cambi AS, Asker, Norway) [8] situated at the Norwegian University of Life Sciences, in cases where the supplier did not already perform a similar kind of pretreatment. The pretreatment was performed on biomass samples that are commonly pretreated before anaerobic digestion in large scale application (sludge and food waste), or where such treatment is needed if the biomass should be used in large scale in the future (birch). Pretreatment with steam explosion increases the amount of volatiles in the biomass, making accurate organic matter determinations challenging. As water is added in the process, some kind of organic matter determination is needed in order to evaluate whether and how much the methane yield improves due to the treatment. Both un-pretreated and pretreated biomass samples were included for the possibility of evaluating the effect of pretreatment on the organic matter determination.

2.2. BMP-test

The BMP-test was performed as previously described by Horn et al. [9], with some modifications. In brief, inoculum from a full-scale biogas plant treating cow manure (Biowaz, Tomb, Norway) was used, diluted with water, and 0.7 L added to 1-L batch bottles to approximately 8 kg m^{-3} of COD. The substrate to inoculum ratio (SIR) on a Cr-COD basis was 1:3 for most biomasses, but a lower ratio was used for substrates where the production rate was expected to be too high for daily pressure measurements, hence slaughterhouse waste and whey was dosed as 1:6 on a COD basis. Biogas production was monitored regularly until the production in all flasks was less than 0.5 L kg^{-1} d^{-1} on the basis of COD. This monitoring was carried out for 49 days by measuring pressure with a digital manometer (GMH 3161 Greisinger Electronic, Germany). The pressure in the flasks was not allowed to exceed 100 kPa measured at incubation temperature: 310.15 K. The composition of the wet biogas was analyzed by gas chromatography (3000 Micro GC, Agilent technologies, USA) equipped with a thermal conductivity detector (TCD) using helium as a carrier gas. For calibration a standard mixture of methane (650 L m^{-3}) and carbon dioxide (350 L m^{-3}) was used. Methane production was calculated using normalized methane concentrations and subtracting the endogenous methane production from the blank controls, which was in all cases lower than the bottles containing substrate. Cellulose was used as positive control. All substrates were evaluated in triplicate. All gas volumes are reported at 273.15 K and 101.3 kPa.

2.3. Chemical oxygen demand by chemical wet oxidation

Analysis of COD was performed by use of Merck Spectroquant® COD Cell Test with measuring range 0.5–10 kg m^{-3}, where the reaction takes place in a closed glass vial. The method corresponds to DIN ISO 15705:2002 [10]. The samples were diluted with water prior to analysis to ensure that the measured COD was within the dynamic range of the test-cell. One exception was SE birch where 0.01 g of sample was added directly to the digestion cell followed by 1 cm^3 of water. Homogenization of food waste, sewage sludge and manure samples was performed using a homogenizer (IKA T18 basic ULTRA-TORRAX) prior to analysis. Diluted samples were mixed vigorously with a magnetic stir bar while the sample for analysis was extracted. All measurements were performed in triplicate. Commercial kits were used despite the inaccuracy of such kits for some solid samples, as this method is commonly used for such samples. The abbreviation used in the following sections for the results of this assay is Cr-COD.

2.4. Chemical oxygen demand by oxygen bomb calorimetry

The gross calorific value (GCV) of the biomass was analyzed using an IKA C200 oxygen bomb calorimeter. The thermal capacity of the bomb calorimeter was found to be 10 090 J K^{-1} by analyzing five individual standards of benzoic acid. Before, during and after the testing period, standards were measured to verify that the

Table 1
Biomass samples, origin and pretreatment method.

Biomass	Source	Pretreatment
Sewage Sludge	Norwegian Biogas Plant, Hias[a] Collected February 2015 and stored at 5 °C. Mix of primary, chemically precipitated and waste activated sludge.	Dewatered at AD plant with dewatering drum and screw press.
SE Sewage Sludge	Norwegian Biogas Plant, Hias[a] Collected February 2015. Sampled from recirculation circuit on buffer tank downstream SE unit and stored at 5 °C.	Steam explosion, 160 °C for 30 min[b]
Digestate	Norwegian Biogas Plant, Hias[a] Collected February 2015. Sampled from the recirculation circuit on the AD and stored at 5 °C.	SE sewage sludge after AD treatment
SE Birch	Norwegian birch (*Betula pubescens*) wood from a 30 year old three growing 95 m above sea level in Norway (59°N 10°E). Harvested in June 2014. Stored at ambient temperature prior to steam explosion. After steam explosion stored at −20 °C.	Debarked wood. The trunk was cut with a chain saw, split in 3–4 cm pieces, chopped in a garden grinder, freeze dried and milled on a Retch cutter down to <0.5 mm. Steam explosion, 225 °C for 10 min.[c]
Cellulose	Sigma-Aldrich, microcrystalline, powder. Stored dark at ambient temperature.	No pretreatment
Whey Permeate	Norwegian dairy producer, Tine AS.[d] Received April 2014 and stored dark at ambient temperature.	Dried powder. Dried by producer
Cow Manure	NMBU farm, collected October 2014, stored at 5 °C.	No pretreatment
Food waste	Norwegian food recycling, Norsk matretur AS[e] Received September 2014 Stored at 5 °C.	Particle size reduction < 7 mm Pasteurized, 70 °C, 1 h by producer.
SE Food waste	Norwegian food recycling, Norsk matretur AS[e] Received September 2014 and stored at 5 °C, before and after SE.	Steam explosion, 170 °C for 30 min[c] February 2015.
Slaughterhouse waste[g] (blood)	Sampled at the regional waste and water treatment facility, Frevar[f] Collected June 2014 and stored at −20 °C.	No pretreatment.
Fish waste (Fish waste from fish processing factory[h])	Sampled at regional waste and water treatment facility, Frevar[f] Collected June 2014 and stored at −20 °C	No pretreatment.

[a] Hias AD plant, Hamar, Norway.
[b] Steam explosion performed at Hias AD plant, Hamar, Norway.
[c] Steam explosion performed at NMBU facility [22].
[d] Tine AS, Jæren, Norway.
[e] Norsk matretur AS, Finstadjordet, Norway.
[f] Frevar, Fredrikstad, Norway. Collected June 2014.
[g] Nortura SA, Tønsberg, Norway. Pig slaughterhouse and producer of processed pork products.
[h] ABBA, Orkla Foods Sverige AB, Eslöv, Sweden. Producer of canned seafood including soups, caviar, fish balls, mussels and herring.

bomb calorimeters heat capacity did not change within the experimental period. The thermal capacity is sensitive to physical changes such as water and room temperature. The standards analyzed during and after the test period showed less than 0.5% deviation from the thermal capacity determined initially.

The measurements were performed in triplicates according to EN-ISO 9831:2003 [11] with some modifications: The GCV was determined on samples with their original moisture content. Standard paraffin oil (0.1–0.3 g) was used in all samples either to ensure complete combustion of the samples with high water content or to avoid splashing from powder substrates. Samples were placed in a glass crucible including a cotton thread for ignition and standard parafilm was used to seal the crucible to avoid escape of sample or burning aid. Paraffin oil (46 300 J g^{-1}), parafilm (46 630 J g^{-1}) and cotton thread (50 J per cotton twist) were all of standardized quality with a known calorific value and provided by IKA, Germany.

Equation (1) describes the major reactions contributing to temperature rise in the bomb:

$$\text{Organic material} + N_{2(g)} + NH_4^+{}_{(aq)} + H_2O_{(l)} + O_{2(g)} \rightarrow CO_{2(g)} + CO_{2(aq)} + H_2O_{(l)} + H_2O_{(g)} + N_{2(g)} + HNO_{3(aq)} + H_2SO_{4(aq)} + H_2PO_{4(aq)} \quad (1)$$

The equation show that oxygen bomb calorimetry has different end products ($CO_{2(aq)}$, $H_2O_{(g)}$, $N_{2(g)}$ and $HNO_{3(aq)}$) than those for ThCOD ($CO_{2(g)}$, $H_2O_{(l)}$, $NH_{3(aq)}$). Not all energy from the oxygen bomb calorimetry measurement is biologically available and in order to calculate a Biological net calorific value (BCV) describing the energy maximally available for biological digestion with the same end products as for ThCOD, corrections must be made. Equation (2) can be used to calculate the necessary corrections (e_1-e_4) to convert GCV into BCV.

$$BCV = GCV - e_1 - e_2 - e_3 - e_4 \quad (2)$$

where BCV (net calorific value) is the energy available for biological degradation if degradation is complete and GCV (gross calorific value) is the energy measured in the bomb calorimeter.

And the four corrections are as following:

1) e1 is the energy from the combustion of burning aids.
2) e_2 is the energy from the oxidation of organic-N + $NH_3 \rightarrow N_2$ (e_2 = 25 MJ kg^{-1} of N).
3) e_3 is the energy from the oxidation of $N_2 \rightarrow HNO_3$ (e_3 = 3.8 kJ kg^{-1} of HNO_3).
4) e_4 is the energy required for the vaporization of water $H_2O_{(l)} \rightarrow H_2O_{(g)}$ and from dissolvation of CO_2 in water (e_4 = max. 8 kJ m^{-3} bomb volume).

In the combustion all organic nitrogen and ammonium is oxidized to nitrogen gas, and e_2 is therefore proportional to the total Kjeldahl Nitrogen of the biomass [12]. The oxidation of nitrogen gas to nitric acid is incomplete (Equation (1)), and e_3 must be calculated from the actual nitric acid formed during each combustion reaction and this was determined according to EN-ISO 9831:2003: The bomb was washed with distilled water and the washings were brought to boil to expel CO_2 prior to titration with NaOH to pH 4.4 using a pH probe. The amount of nitric acid in the sample was then calculated based on the consumption of NaOH.

e_4 was neglected for all samples based on the assumption that in the most extreme case e_4 will be 2 J in a 0.26 L bomb [6]. With a

Table 2
Comparison of organic content in biomass determined by Karl Fischer titration, Loss on drying at 105 °C, bomb calorimetry and chemical oxidation.

Sample	Ash (g kg^{-1})	KF				LOD			Bomb calorimetry			Chemical oxidation		CV-COD/KF-VS (kg kg^{-1})	CV-COD/LOD-VS (kg kg^{-1})
		H_2O[a] (g kg^{-1})	RSD H_2O	VS (g kg^{-1})	RSD VS	TS (g kg^{-1})	VS (g kg^{-1})	RSD VS	kJ g^{-1}[a]	CV-COD (g kg^{-1})[b]	RSD	Cr-COD (g kg^{-1})[a]	RSD		
Cellulose	n.d.	32.2	3.3%	967.8	0.11%	965.1	965.1	3.4%	16.9	1210	0.1%	1120	9.3%	1.2	1.2
Whey Permeate	50.0	n.d.	n.d.	n.d.	n.d.	946.3	886.3	0.1%	13.7	981	9.1%	996	2.3%	n.d.	1.1
SE Birch	0.6	666.5	0.91%	332.9	1.8%	263.5	263.0	0.8%	5.30	379	3.6%	382	1.4%	1.1	1.4
Food Waste	17.3	774.7	1.5%	208.0	5.6%	158.7	141.4	0.3%	4.51	322	2.0%	279	1.8%	1.5	2.3
SE Food Waste	12.1	835.4	3.0%	152.5	16%	115.0	102.9	0.6%	3.03	216	1.4%	178	4.3%	1.4	2.1
Sludge	31.5	777.9	0.53%	190.6	2.2%	160.2	128.7	1.0%	3.50	250	8.5%	223	4.2%	1.3	1.9
SE Sludge	17.5	869.0	1.6%	113.5	12%	89.2	71.6	0.2%	2.03	145	3.7%	113	2.7%	1.3	2.0
Digestate	20.7	848.4	2.1%	122.5	14%	57.8	37.0	0.5%	0.99	71.1	4.0%	62	4.2%	0.6	1.9
Cow Manure	7.7	860.9	1.3%	131.7	8.4%	69.4	62.0	4.4%	2.12	152	3.4%	129	5.6%	1.2	2.5
Fish Waste	7.7	796.0	3.1%	196.3	13%	91	83.3	3.2%	3.86	276	6.9%	272	0.6%	1.4	3.3
Slaughterhouse Waste	6.3	842.6	0.97%	151.1	5.4%	105.6	99.3	0.2%	2.43	173	4.6%	171	4.0%	1.1	1.7

[a] Measured value.
[b] Calculated value. Based on a theoretical estimation that the correlation between COD and energy content is described by a factor known as CEP (chemical energy potential) which has been determined to be 14 MJ kg^{-1} of COD [7]. CEP is the total energy potential of a given biomass, when this is fully oxidized to CO_2 and H_2O.

sample containing an absolute energy content of 200 J, e_4 will contribute less than 1% of the measured GCV. All samples in this study had higher energy content than 200 J with the exception of digestate; e_4 was in all cases neglected despite the potential of introducing a small bias for digestate.

BCV was converted to a COD value based on an experimentally determined correlation between COD and CEP (chemical energy potential) of (14 MJ kg^{-1} of COD) as described by Owen 1982 [7]. The abbreviation used in the following text to describe the COD value from the bomb calorimetry assay is CV-COD.

2.5. Nitrogen

Total Kjeldahl Nitrogen (TKN) was analyzed by the institute for Animal and Aquacultural sciences at the Norwegian University of Life Sciences (NMBU, Norway) according to the official AOAC method 2001.11 as described by Thiex et al. [13] with the following modifications: 15 cm^3 of H_2SO_4 (>950 g kg^{-1}) instead of 12 cm^3 was added to all samples, and the digestion time was accordingly reduced from 60 min to 45 min.

2.6. DM, ash and VS

Dry matter (DM) was determined with two methods. DM based on loss on drying (LOD) was analyzed by drying samples at 105 °C overnight. DM based on measurement of water content with Karl-Fisher titration was analyzed as described by Ref. [3] using 160 °C as extraction temperature and running the extraction for approx. 1.4 h. All samples were compatible with this method except from whey, which was not stable under the extraction conditions. Therefore, no Karl Fischer determined water content is given for whey. The ash content was determined by burning samples at 550 °C. The volatile solid (VS) was calculated by subtracting the ash mass from the DM mass. All determinations were performed in triplicates.

3. Results and discussion

11 different biomass samples were analyzed with respect to chemical oxygen demand (determined with chemical wet oxidation and oxygen bomb calorimetry) and VS content (determined by both LOD and KF-titration; Table 2).

3.1. Comparison of Cr-COD and CV-COD

Generally, the results show that Cr-COD gives lower or equal values compared to bomb calorimetry. For non-uniform samples with high particulates content (e.g. food waste, sludge, cow manure, cellulose), the oxygen demand determined by bomb calorimetry was higher compared to the oxygen demand determined by wet oxidation. For the more uniform materials with low particulates content (e.g. whey permeate, fish waste, slaughterhouse waste), the oxygen demand determined by bomb calorimetry and chemical wet oxidation was similar. Steam exploded birch also showed similar results for the two methods, despite the fact that this is an insoluble particulate biomass.

For all the tested biomass samples, Cr-COD values were within the range of 80–100% of the CV-COD. Previous studies have found that the suspended solids content of sludge reduces the measured Cr-COD to 90–95% of that measured with an improved empirical method [4], while the Cr-COD for a defined class of organic chemicals consisting of amino acids, carbohydrates, carboxylic acids and ketones, was found to be 92–98% of ThCOD [5]. For real biomass samples, it is not possible to calculate the theoretical COD as the exact molecular composition is most often unknown, and thus COD-recovery cannot be estimated. Although elementary

composition can be analyzed, the method for carbon, hydrogen and nitrogen measurement is influenced by the same biases as VS-measurements, since the sample must be dried before analysis. COD calculated from such analysis is therefore not the theoretical COD of the biomass sample. The COD recovery found in this study, by comparing Cr-COD to CV-COD, was within the range (72–100%) of the earlier observations of the difference between measured Cr-COD and ThCOD and the improved empirical method [4,5]. This opens for the possibility that the difference seen between Cr-COD and CV-COD in our measurements are due to the same limitations previously described for Cr-COD, e.g. incomplete oxidation of particulate material in Cr-COD.

Both COD determination methods (chemical and bomb) have some limitations. The chemical determination is dependent on complete oxidation, which can be difficult for particulate samples. Also, sample handling including a high level of dilution is prone to mistakes. For the bomb calorimetric measurements, the nitrogen correction procedures are susceptible to biases and the amount of biomass used in the analysis must be adjusted to obtain complete incineration while the temperature rise in surrounding water chamber stays below 4 K. In addition to this, the relationship between CEP and COD of 14 MJ kg^{-1} of COD is empirically determined, and an incorrect factor will cause the CV-COD to be an incorrect estimate of ThCOD. The relationship was determined by Owen [7] by using the average of a range of standard substrates with a known GCV, and the value holds true for methane, carbohydrates, VFAs and alcohols, while for example for fat and cellulose, the relationship is closer to 15 MJ kg^{-1} of COD rather than 14 MJ kg^{-1} of COD, and will result in an expected overestimation of ThCOD by CV-COD for biomass samples rich in fats and cellulosic material. None of the biomasses used in this study was very fat rich, considering both the nature of the samples and the calculated COD:VS ratios in Table 2 (we discuss this further in section 3.2). However, biomass samples such as SE birch and cow manure are cellulose rich, and expected to be overestimated. The theoretical bias of CV-COD for cellulose is 7% higher than thCOD, which is exactly what is observed. However, for SE birch, the difference between Cr-COD and CV-COD is only 1% and for cow manure CV-COD is 18% higher than Cr-COD. Clearly, the uncertainty in the relationship between bomb calorimetry measurements and Cr-COD is not sufficient to explain these observations. We consider the bomb calorimetry derived CV-COD to be a good estimate of ThCOD for the biomass samples analyzed here.

Errors related to incomplete incineration in the oxygen bomb can be avoided by careful inspection of the resulting residue after the reaction [11]. The extent of nitrogen corrections varies between biomass samples and the largest corrections in this study were needed for the slaughterhouse waste samples (Table 3). The nitrogen correction is a combined effect of the total Kjeldahl nitrogen determination (18% of the final CV-COD for slaughterhouse waste), and the formation of nitric acid (6% of the final CV-COD). The slaughterhouse waste used here consisted of blood, which is easily soluble in water, and was therefore theoretically an unproblematic biomass for Cr-COD determination. The results from Cr-COD analysis of this biomass was therefore expected to be 92–98% of ThCOD [5]. When COD content determined for slaughterhouse waste by wet oxidation and bomb calorimetry was compared (Table 2) they showed similar results (Cr-COD was 99% of CV-COD), indicating that the N-corrections are reasonable. The apparent underestimation of chemical oxygen demand was only observed for substrates containing particulates, which indicates that errors are related to particle size distribution and sampling procedures. Interestingly, underestimation was not observed for SE birch, where the sampling was performed differently compared to the other substrates. Here the biomass sample was added directly into the cell test whereas all other samples were diluted first. This suggests that the bias in Cr-COD determination of particulate substrates was caused by the sampling procedure that included dilution of solid samples, rather than insufficient contact between oxidizing agent and biomass. No particles were visually observed in any of the test vials after digestion.

The relative standard deviations of the two COD-methods (Table 2) show that the intermediate precision of the bomb calorimetry method and the COD method is in the same range, having RSD (relative standard deviation) values for most of the samples between 0.1% and 5%. Both methods occasionally yielded measurements with higher RSD (whey permeate, sludge and fish waste for CV-COD and cellulose and cow manure for Cr-COD), but all values are below 10%. The bomb calorimetry generally had higher intermediate precision for samples that are poorly soluble in water (e.g. cellulose, cow manure), while Cr-COD generally yielded higher reproducibility for samples that are easily soluble in water (e.g. whey, fish waste). For samples with high water content, the sample size in the bomb calorimeter must be reduced and the relative amount of paraffin oil increased, both influencing the accuracy of the method negatively. However, the observed RSD for CV-COD and Cr-COD for the biomass samples with the highest water content (digestate and SE sludge) are all in the range 2.7–4.2%. This indicates that sample size and amount of paraffin oil are not major contributors to errors in the CV-COD method, and the water content of the biomass sample is therefore of lesser impact on the accuracy of the results.

Table 3
Nitrogen corrections for calculation of net biological calorific value (BCV). The energy released by formation of HNO_3 and N_2 contribute to 5–20% of the GCV measured by bomb calorimetry. The corrections were especially high for slaughterhouse waste and digestate and are necessary when converting an energy value obtained by bomb calorimetry to a COD value. Although a large correction was applied to calculate the CV-COD for slaughterhouse waste, the CV-COD and Cr-COD for the biomass was similar (Table 2), indicating no large errors in the estimation of the nitrogen correction factors.

Sample	GCV (J g^{-1})	HNO_3 (J g^{-1})	N_2 (J g^{-1})
Cellulose	n.d.	n.d.	n.d.
Whey Permeate	13 851	37	73
SE Birch	n.d.	n.d.	n.d.
Food Waste	4776	118	153
SE Food Waste	3240	115	100
Sludge	3827	123	204
SE Sludge	2275	125	118
Digestate	1279	154	130
Cow Manure	2281	113	44
Fish Waste	4042	140	41
Slaughterhouse waste	2876	133	415

3.2. Comparison of KF-VS and LOD-VS

Several alternative methods for correction of LOD results making up for the loss of volatiles during drying has been suggested in literature and are either based on pH adjustment [14] or direct measurement of volatile compounds followed by a correction of LOD results [15–17]. The choice of method is determined by the type of biomass. The pH adjustment method is limited to the types of biomass where the volatiles are volatilized at a certain pH, hence these methods cannot be used on types of biomass containing large amounts of e.g. alcohols. The methods based on quantification of volatile compounds that are lost during drying are also dependent on the pH of the sample (which influences how much of the acids and ammonia is lost during drying). In addition, the quantification of all volatile compounds requires a large array of analytical capacity and each analytical step contributes to the uncertainty of the

result. Because of this, our scope was to test if Karl Fischer titration could be used for a wider range of biomass samples, as this method does not have any of the abovementioned drawbacks.

Since LOD-VS is a calculated value based on water and ash content, and because LOD measures organic volatiles as water, KF titration generally gives higher estimates of DM and thereby higher estimates of VS (Table 2). This is expected and reported previously [3,18]. For a biomass with a low content of truly volatile organic components like cellulose, both methods yielded similar VS. For the remaining biomass samples tested, the difference between the two methods ranged from 41.9 g kg^{-1} (SE sludge) to 113 g kg^{-1} (fish waste). Previous studies have found that up to 68.2 g kg^{-1} of the mass of ensiled crops consisted of lactic acid, ethanol, acetic acid and butyrate [15]. The food waste, cow manure, fish waste and slaughterhouse waste was stored at ambient temperature prior to collection. This storage could facilitate fermentation, which increases the amounts of volatile fatty acids and alcohols in the biomass. The pretreated biomass (SE birch, SE food waste and SE sludge) was steam exploded yielding an increase in the amount of volatile fatty acids. It is not possible to determine if the difference between LOD-VS and KF-VS is solely due to volatiles lost during drying or also errors related to the KF method when comparing the results from the two methods alone. Hence, results from COD analysis or analysis of specific volatile compounds can be used to find out if the differences between the methods are related to the known errors of the LOD method or from unknown errors in the Karl Fischer method.

In order to evaluate which of the methods for determination of VS is the most accurate, the CV-COD:VS ratio was calculated (Table 2) and evaluated against theoretical COD:VS ratios. For standardized substrates, a range of COD:VS ratios can be calculated based on stoichiometry, and can be used to evaluate the COD:VS ratios found in this study. The theoretical range of COD:VS ratios are calculated based on four groups of organic molecules with an assumed average composition; carbohydrates and lignin ($C_6H_{10}O_5$), protein ($C_5H_7O_2N$), lipids ($C_{57}H_{104}O_6$) and VFAs ($C_2H_4O_2$) [19]. The theoretical COD:VS ratios for each of these four groups of compounds are 1.19 kg kg^{-1}, 1.42 kg kg^{-1}, 2.90 kg kg^{-1} and 1.07 kg kg^{-1}, respectively. The CV-COD was used to determine the COD:VS ratio, and the possibility of a bias in the COD measurement was evaluated when the COD:VS ratio deviated from the theoretical COD:VS ratio (below 1.1 kg kg^{-1} or above 2.9 kg kg^{-1}).

For VS calculated from DM values determined with LOD, the CV-COD:LOD-VS ratio for cellulose and whey permeate is similar to that of carbohydrates and lignin. SE birch is close to the average for protein, while fish waste has a CV-COD:LOD-VS ratio higher than the average of lipids. Birch is a tree, and consists of mainly cellulose and lignin. Fish waste should have a similar substrate characterization of fish, consisting of mainly protein and lipids. Both results for the CV-COD:LOD-VS ratios are therefore implausible. CV-COD and Cr-COD results for both SE birch and fish waste were very similar, and since all bias related to Cr-COD are negative, biased COD determinations would have resulted in underestimation of the COD:VS ratio. Hence, it is clear that the high CV-COD:LOD-VS ratios is a result of underestimated VS. For VS calculated from DM values determined with Karl Fischer titration, the CV-COD:KF-VS ratio for cellulose, SE birch, cow manure and slaughterhouse waste is close to that of carbohydrates and the CV-COD:KF-VS ratio of food waste, SE food waste, sludge, SE sludge and fish waste is close to that of protein. The CV-COD:KF-VS ratio for digestate is only 0.6 kg kg^{-1}. Even if the CV-COD determination of digestate was underestimated by 60%, the COD:KF-VS ratio would have been below the theoretical range. Thus, for most biomass samples VS calculated from KF measurements yields the most reasonable COD:VS ratios, probably because oven drying (LOD) tends to overestimate DM content. However, the dataset generally illustrates a high level of complexity in determining VS.

KF-VS did not give more reasonable results compared to LOD-VS in the case of digestate. Digestate has a very low CV-COD:KF-VS ratio indicating some form of analytical error. Digestate was also the biomass sample with the largest observed difference between LOD-VS and KF-VS. Together, this made us suspect a gross analytical error related to the Karl Fischer instrument or the sampling. However, after a thorough troubleshooting of the instrument and several re-analysis, the results remained similar and we did not find any instrument error. A possible reason for underestimating water content with Karl Fischer analysis could be that the initial water extraction is not sufficient however the titration curves for digestate did not stand out from the titration curves of the other substrates, and reached a plateau well before the extraction period had ended. We have not been able to find any explanation why the water content was most probably underestimated by KF determination for this particular substrate, and the results exemplifies the difficulty of finding one method for VS determination that is applicable to all biomass samples.

VS determination is a routine method in most laboratories to measure the mass of organic matter in a biomass sample. However, our results show that this analysis is far from trivial, and can deviate largely from the true VS (the mass of organic compounds in the sample). Although our results indicated that VS calculated from KF analysis were more reasonable for most biomass samples, the RSD observed from the method was higher compared to VS calculated from LOD results. Additionally, VS measurements gives no information about the biodegradability or the energy content of a biomass sample, which is a disadvantage when the energy conversion efficiency from waste to bio-methane is evaluated (e.g. for energy production).

3.3. Results of BMP tests

The theoretical maximum bio-methane potential is 350 L kg^{-1} of COD and was not reached for any of the substrates in the BMP results presented here (Fig. 1). Cellulose (often used as a positive control in BMP assays) and whey permeate, assumed to be 100% degradable under the test conditions, reached 80–85% of the theoretical bio-methane potential based on Cr-COD and CV-COD measurements. On a g VS basis, the observed methane production for cellulose was 84% of the theoretical maximum of 414 L kg^{-1} of VS (350 L kg^{-1} of COD). BMP results are expected to be maximum 90–95% of the theoretical maximum, as some of the organic material is used for growth [20].

The BMP results are within the range of results from previous studies on similar substrates (SE Birch [21], Cow manure [22], Food Waste [23], Sludge [24]). Because of the variation in composition of substrates like cow manure and sludge, a wide range of BMP-values can be found in the literature.

The BMP results observed here on VS basis varies greatly between substrates (Fig. 1), while for BMP results on a COD basis substrates with similar biodegradability have similar BMP (e.g. slaughterhouse waste, fishery waste and whey permeate). Biodegradability in this context refers to how much of the organic material that can be degraded anaerobically. BMP will be a measure of the anaerobic biodegradability in the same manner as BOD (Biological Oxygen Demand) is a measure of aerobic biodegradability. By expressing the BMP results on a COD or VS basis, the interpretation of the results changes. COD is an indirect measure for the energy content in a sample while VS only measures the mass of the organic matter. Results based on VS will depend on both the biodegradability of the biomass and the energy density. On a COD basis, energy density is already taken into account, and the results

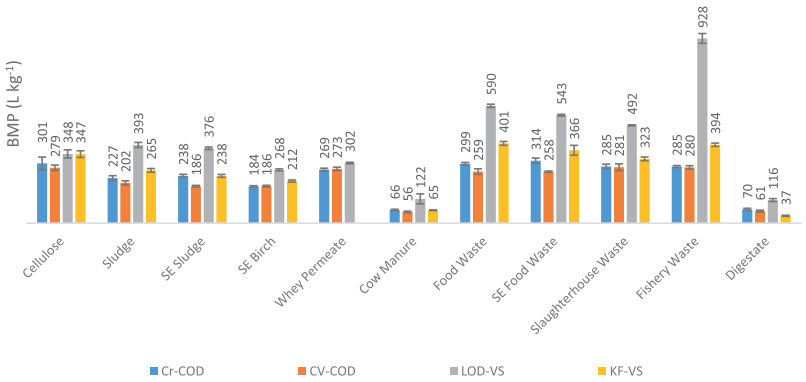

Fig. 1. Bio-methane potentials. BMPs were calculated based on the measured methane production in the anaerobic batch-tests, and the measured Cr-COD, CV-COD, LOD-VS and KF-VS values. All results are at standard temperature and pressure. BMPs on a COD basis are similar for biomass with similar expected degradability (e.g. slaughterhouse waste and cellulose); this is because COD measures energy content in the biomass. The data show large variations in BMPs on LOD-VS basis caused both by the bias of LOD-VS determinations and the nature of VS measurements, which does not include the energy content of the biomass. No BMP result for whey permeate based on KF-VS is shown because no KF-VS data was obtained (see Table 2).

will only depend on the biodegradability of the sample. BMP on a COD basis is the anaerobic equivalent of the aerobic BOD:COD ratio.

BMP on LOD-VS basis varies more than BMP results based on KF-VS. This can be illustrated by the bio-methane potential of the substrate having the highest CV-COD/LOD-VS ratio, namely fish waste (Fig. 1). The bio-methane potential on a LOD-VS basis for fish waste is much higher than for any of the other substrates, giving a BMP value of 928 L CH_4 kg^{-1} of VS, which is higher than the theoretical maximum for lipids and clearly wrong. Overestimation of BMP is a general trend observed for bio-methane potentials based on LOD-VS (Fig. 1), and reinforces the notion that determination of organic content by LOD can severely underestimate the VS content. The BMP for fish waste on LOD-VS basis is also approximately twice as high as the bio-methane potential for other substrates expected to have the same energy density and degradability, e.g. food waste and slaughterhouse waste (Fig. 1). The difference in bio-methane potential based on either of the two parameters for COD are smaller compared to the difference between LOD-VS and KF-VS. Statistically, using ANOVA followed by Tukey pairwise comparison with alpha = 0.05, all BMP values based on VS except one (cellulose) are significantly different, while based on COD all except SE birch are significantly different. BMP values presented on a Cr-COD basis give in all cases except two (SE birch and whey permeate) a larger average value than CV-COD.

3.4. Practical implications

One of the main advantages of steam explosion of wastes is a more rapid degradation during anaerobic digestion. Thus, evaluation of the effect of pretreatment needs to be done by studying the early phase of the BMP test before the test reaches its plateau. Table 4 presents the increase in bio-methane potential caused by steam explosion for two substrates, food waste and sludge on day 5 of the BMP test. The BMP increase was calculated for two methods of organic matter determination using equation (3):

$$BMP\ increase = \frac{BMP_5(\text{pretreated}) - BMP_5(\text{untreated})}{BMP_5(\text{untreated})} \times 100\,\% \quad (3)$$

The results show that the calculated increase in BMP, due to pretreatment, is dependent on the method for organic matter determination. For sludge, LOD gives about twice as high increase compared to CV-COD. For Food Waste CV-COD gives about twice as high increase compared to LOD.

Steam explosion leads to formation of volatiles, but the amount and type of volatiles depend on the biomass pretreated [25,26]. Sludge is known to release a relatively high concentration of volatiles during pretreatment [26], while food waste contains many volatiles prior to pretreatment. The difference in increased BMP as a result of pretreatment (Table 4) reveals exactly the weakness of having inaccurate determination of organic content. During LOD-measurements the VS-content is underestimated for sludge because the relatively high content of volatiles after pretreatment result in significantly higher effect of pretreatment than by using CV-COD for organic measurement (13.2% compared to 8.5%). Vice versa, underestimation of VS-content by LOD-VS in the un-pretreated material, as would be the case for food waste, causes the effect of pretreatment to diminish (6.1% compared to 15.0%). The volatiles from food waste will to some extent disappear from the substrate during pretreatment, as a result of the increased temperature and pressure. In the particular examples illustrated in Table 4, the effects of pretreatment will have opposite interpretations depending on the method of choice for organic matter determination. LOD-VS is especially difficult to use when evaluating the effect of pretreatment because the pretreatment affects the amount of volatiles in the samples and thus the VS determination.

Table 4
Pretreatment effects estimated by various methods for determination of organic matter. The table shows how different methods for organic matter determination have an effect when evaluating the effect of steam explosion on biogas potential. BMP tests on day 5 for untreated and steam exploded materials are compared, and the differences relative to the untreated materials' BMPs are given as percent increased bio-methane potential. Calculations according to equation (3).

Biomass	Increased BMP CV-COD	Increased BMP LOD-VS
Sludge	8.5%	13.2%
Food waste	15.0%	6.1%

4. Conclusion

Results from Bio-methane potential tests are more accurately

expressed based on COD compared to VS. CV-COD gives an accurate measure of the energy content of a large range of biomass samples and is superior for samples with high dry matter content. Cr-COD is suitable for biomass samples that are soluble in water. Special caution is needed when bio-methane potential test results are preferably presented on a VS basis. VS determined by loss on drying followed by loss on ignition is not reliable and VS determined based on indirect dry matter determinations by Karl Fischer titration are also questionable for some biomass samples.

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Paper II

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Anaerobic digestion of food waste – Effect of recirculation and temperature on performance and microbiology

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ABSTRACT

Recirculation of digestate was investigated as a strategy to dilute the food waste before feeding to anaerobic digesters, and its effects on microbial community structure and performance were studied. Two anaerobic digesters with digestate recirculation were operated at 37 °C (MD + R) and 55 °C (TD + R) and compared to two additional digesters without digestate recirculation operated at the same temperatures (MD and TD). The MD + R digester demonstrated quite stable and similar performance to the MD digester in terms of the methane yield (around 480 mL CH_4 per gVS_{added}). In both MD and MD + R *Methanosaeta* was the dominant archaea. However, the bacterial community structure was significantly different in the two digesters. *Firmicutes* dominated in the MD + R, while *Chloroflexi* was the dominant phylum in the MD. Regarding the thermophilic digesters, the TD + R showed the lowest methane yield (401 mL CH_4 per gVS_{added}) and accumulation of VFAs. In contrast to the mesophilic digesters, the microbial communities in the thermophilic digesters were rather similar, consisting mainly of the phyla *Firmicutes*, *Thermotoga*, *Synergistetes* and the hydrogenotrophic methanogen *Methanothermobacter*. The impact of ammonia inhibition was different depending on the digesters configurations and operating temperatures.

1. Introduction

During the last decade, energy recovery from various sources of organic materials has gained increased interest. Bio-methane is a renewable fuel that can be produced from biomass via anaerobic digestion. Internationally, there is a trend to recover the energy content of municipal food wastes through anaerobic digestion instead of landfilling, which has the risk of watershed pollution and greenhouse gas emissions. Food waste (FW), which has a high biogas potential, can make operation of anaerobic digesters and co-digesters more economical through enhanced methane production (Hartmann and Ahring, 2005).

Typically, FW has high solids content and thus it needs to be diluted before feeding to anaerobic digesters. Water can be used to dilute FW before feeding to an anaerobic digester. However, access to water may be limited and costly in some locations. It is also not a sustainable option to use clean water for dilution of food waste. Thus, processed water and/or digestate may be used to dilute the FW, reducing the water consumption in biogas plants.

Even though FW has a high potential for production of renewable energy, it may inhibit certain microbial processes of anaerobic digestion due to its high content of nitrogen-bearing materials or too much acidification (Ganesh et al., 2014; Mata-Alvarez et al., 1992). During the digestion process, nitrogen is released into the bulk liquid and, depending on pH, organic loading and temperature, this may lead to high concentrations of free ammonia in the

Abbreviations: ADM, anaerobic digestion model; AM, acetoclastic methanogens; bp, base pair; COD, chemical oxygen demand; CODCH4, COD equivalent of methane; FAN, free ammonia nitrogen; FW, food waste; HM, hydrogenotrophic methanogens; HPLC, high performance liquid chromatography; KI,NH3, inhibitory ammonia coefficient; MD, mesophilic digester; MD + R, mesophilic digester with recirculation; OUT, operational taxonomic unit; PCOD, particulate chemical oxygen demand; PCR, polymerase chain reaction; rpm, round per minute; SAB, syntrophic acetogenic bacteria; SAOB, syntrophic acetate oxidizing bacteria; SCOD, soluble chemical oxygen demand; SCODe, soluble COD in effluent; SCODin, soluble COD in influent; SNH3, free ammonia concentration; T, temperature; TAN, total ammonia nitrogen; TCD, thermal conductivity detector; TCOD, total chemical oxygen demand; TD, thermophilic digester; TD + R, thermophilic digester with recirculation; TS, total solids; VFA, volatile fatty acids; VS, volatile solids.

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digester. Inhibition due to high nitrogen content of the substrate has been reported previously (Sheng et al., 2013; Procházka et al., 2012). Therefore, concern of ammonia inhibition on the methanogenesis process should be taken into account when using FW or recirculating digestate bearing a high content of ammonia back to a digester (Wilson et al., 2012; Gallert et al., 1998).

Anaerobic digestion is a complex bioprocess, in which microorganisms belonging to different functional groups degrade various organic compounds in a concerted effort into methane and carbon dioxide. However, our understanding of the function and metabolic capabilities of microbial communities in anaerobic digestion is limited (Vanwonterghem et al., 2014). Application of culture-independent molecular techniques have provided some information on the complex and diverse microbial communities in anaerobic digesters (Vanwonterghem et al., 2015; De Vrieze et al., 2015). Various parameters may influence microbial community structures, including digester configuration, feedstock, temperature and other operational parameters. Accordingly, several researchers have investigated the microbial ecology in FW-fed anaerobic digesters ran under various operational conditions using molecular techniques (Cardinali-Rezende et al., 2009; Kim et al., 2014, 2015). However, information about the effect of digestate recirculation on microbial community structure of FW-fed anaerobic digesters and its correlation with performance is lacking in the literature.

The primary objective of this study was to characterize and compare the performance of four anaerobic digesters fed with food waste at mesophilic (37 °C) and thermophilic (55 °C) conditions with and without digestate recirculation. The microbial community structures of the four digesters were analyzed to evaluate the influence of temperature and recirculation. In addition, possible correlations between the function of microbial groups and the performance of the mesophilic and thermophilic digesters were investigated.

2. Materials and methods

2.1. Operation of lab-scale digesters

Four 10-L laboratory-scale continuously stirred tank reactors (BelachBioteknik, Sweden) were used in this study and all were fed with food waste (FW). Two digesters were operated as flow-through reactors at mesophilic (MD) and thermophilic (TD) temperatures, and two with digestate recirculation at mesophilic (MD + R) and thermophilic (TD + R) conditions. The mesophilic and thermophilic temperatures were set to 37 °C and 55 °C, respectively. The operational conditions of the digesters are summarized in Table 1. The digesters operated at 37 °C were initially seeded with 3 L of inoculum taken from a full-scale biogas plant digesting food waste at mesophilic conditions (Romerike biogas plant, RBA; Esval, Norway). The digesters were then fed with food waste, gradually increasing the organic loading rate (OLR) from 1 to 3 g VS L^{-1} d^{-1} over a period of 3 weeks until a total volume of 6 L was reached. The other two digesters at 55 °C were seeded with 3 L inoculum from a thermophilic biogas plant digesting food waste (FREVAR; Fredrikstad, Norway), and then fed food waste in the same way as the mesophilic digesters. The working volume of the digesters was 6 L and hydraulic retention time (HRT) was maintained at 20 days through withdrawing 300 mL per day of digested waste and adding the same amount of pretreated food waste after dilution with either tap water or sieved digestate. However, due to the recirculation of the digestate, the actual HRTs in the MD + R and TD + R digesters were longer (approximately 60 days). Prior to the experiments, the food waste was milled to pass a 10-mm sieve and pasteurized at 70 °C for 1 h (in accordance with Norwegian regulations on the use of food waste in biogas plants). The characteristics of the FW used are shown in Table 2. For the digesters with recirculation, the digestate was manually screened using a 2-mm sieve (through which the digestate almost entirely passed) and then used to dilute the feed. The return ratio was approximately 2 (200 mL digestate to 100 mL feed), with some variation over time due to small differences in the volatile solids content of the food waste. The organic loading rate was set at 3 gVS L^{-1} d^{-1}. Stirrer speed (100 rpm), pH, temperature, gas flow and gas volume was monitored in real time using BIOPHANTOM software (Belach Bioteknik, Sweden). Produced biogas in each anaerobic digester was measured by a water displacement gas-meter and recorded by the software. The digesters were run for 152 days and samples for various analyses were collected throughout the experimental period.

Table 1
Operational condition of digesters.

Parameter	Digester			
	MD	TD	MD + R	TD + R
Average OLR, gVS/d	18.5	18.5	18.5	18.5
HRT, d	20	20	60	60
Total Solids, %	16.6 ± 0.8	14.0 ± 1.2	29.5 ± 1.1	26.2 ± 1.7
Temperature, °C	37	55	37	55
Inoculum source	Romerike	Frevar	Romerike	Frevar
Digestate recirculation	NO	NO	YES	YES

2.2. Analysis of chemical parameters

Samples were taken from the food waste and digesters on a regular basis for analysis of total solids (TS), volatile solids (VS), and total chemical oxygen demand (TCOD). A fraction of the samples was centrifuged for analysis of pH, NH_3 and alkalinity, and filtered (0.45 μm pore size) for analysis of soluble COD and volatile fatty acids (VFAs). The COD, TS and VS analyses were carried out following standard methods (APHA, 1998). Ammonium measurement was done using a probe according to the company's manual (Orion 93; Thermoscientific, USA). In addition to on-line monitoring of the pH, liquid samples were regularly taken to also measure pH by a separate pH instrument (Orion, Thermoscientific, USA). Samples for VFAs were stored at −20 °C. Before VFA analysis, the samples were thawed and the pH of the samples was adjusted to less than 2.5. After centrifugation at 14,000 rpm, the samples were filtered using 0.45-μm syringe filter. VFAs (formate, acetate, propionate, butyrate and valerate) were quantified by a high performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) equipped with a UV detector. The column used was a Zorbax Eclipse Plus C18 (Agilent, USA; 150 × 2.1 mm column; 3.5 μm particles) equipped with a guard column (12.5 × 2.1 mm; 5 μm particles). The column was

Table 2
Food waste characteristics (average ± standard deviation).

Parameters	Unit	Food waste
Total solids	%	17.8 ± 1.2
Volatile Solids	% vs	16.1 ± 1.2
VS/TS		0.90
TCOD	g/L	271 ± 57.5
TCOD/VS		1.7 ± 0.3
SCOD	g/L	95 ± 12
Ammonia	mg/L	504 ± 153
pH		3.9 ± 0.1
Acetate	mg/L	44,642 ± 16,576
Propionate	mg/L	1251 ± 547
i-Butyrate	mg/L	212 ± 14
n-Butyrate	mg/L	244 ± 57

operated at 40 °C at 0.3 mL/min and 1 μl sample was injected. A gradient flow was applied using the eluents methanol and 2.5 mM H_2SO_4. The biogas composition was monitored on-line with an SRI gas chromatograph (Model 8610C) equipped with a thermal conductivity detector (TCD) and a 2 m Haysep-D column. The injector, detector, and column were operated at 41, 153 and 81 °C, respectively. Helium was used as a carrier gas at 20 mL min^{-1}.

2.3. Microbial analysis

At day152 of the experiments, samples were taken from all the digesters for microbial analysis. The samples were frozen immediately and stored at −20 °C. For DNA extraction, the samples were thawed and centrifuged at 14,000 rpm for 7 min to remove the liquid. The pellet was then resuspended in S.T.A.R buffer (Roche Diagnostics Corporation, USA) to stabilize nucleic acid and prevent bacterial growth. Cells were dissociated from large particles by vortex followed by slow spin. Larger particles precipitated to the lower phase, while the upper phase containing cells was transferred to a FastPrep24 tube with acidic washed glass beads. The cells were then mechanically lysed. DNA was extracted using an automated DNA magnetic bead-based method (LGC Genomics, UK) with minor modifications. DNA concentration was measured with Qubit™ fluorometer and the Quant-iT™ dsDNA BR Assay Kit (Invitrogen, USA), and solutions were kept at −20 °C until 16S rRNA sequencing.

For 16S rRNA gene sequencing amplification of V3–V4 hyper variable regions of bacterial and archaeal 16S rRNA genes were carried out using the Pro 341F/Pro805R primer set selected from Takahashi et al. (2014): 5′-CCTACGGGNBGCASCAG -3′/5′- GACTACNVGGGTATCTAATCC -3'. Illumina adaptor overhang was added to the primer pair in addition to the region specific sequences. The amplicon PCR reaction mixture (25 μl) consisted of 12.5 ng microbial gDNA, iProof HF DNA polymerase (BioRad, USA) and 0.2 μM of each primer. The PCR reaction was performed with an initial denaturation step at 98 °C for 30 s, followed by 25 cycles of denaturation at 98 °C in 30 s, annealing at 55 °C in 30 s, extension at 72 °C in 30 s, and completed by a final elongation at 72 °C in 5 min. A PCR clean-up step of the 16S V3–V4 amplicon was conducted with AgencourtAMPure XP beads (Beckman Coulter, USA). An index PCR reaction was carried out to attach unique 6–bp indices (Nextera XT Index Kit) to the Illumina sequencing adaptors to allow multiplexing of samples. The PCR conditions were as followed: 98 °C in 3 min, 8 cycles of 95 °C in 30sec, 55 °C for 30 s, 72 °C for 30 °C, completed by a final elongation step at 72 °C for 5 min. The indexing step was finalized with an additional AMPure XP PCR clean-up. The 16S rRNA amplicons were quantified (Quant-IT™ dsDNA HSAssay Kit and Qubit™ fluorometer, Invitrogen, USA), normalized and pooled in equimolar concentrations. The mulitiplexed library pool was then spiked with 30% PhiX control to improve base calling during sequencing of low complexity libraries. A final concentration of 8 p.m. denatureated DNA was sequenced on an Illumina MiSeq instrument using the MiSeq reagent kit V3.

Sequence analysis was conducted using the Quantitative Insight Into Microbial Ecology (QIIME) version 1.8.0 (Caporaso et al., 2010). Single-end reads were quality filtered (at Phred ≥ Q20) and trimmed to 200 bp before proceeding with downstream analysis. USEARCH61 was used for detection of chimeric sequences, followed by clustering (at 97% sequence identity) of non-chimera sequences and denovo picking of OTUs (Edgar, 2010; Edgar et al., 2011). OTUs were then assigned to taxonomy with QIIME's uclust-based taxonomy assigner. The OTUs observed fewer times than two times and OTUs with a total observation count less than 0.005% were filtered out to remove singletons and reduce the complexity.

2.4. Data accessibility

Sequence data are available at NCBI Short Read Archive under accession number SRP066159.

3. Results and discussion

3.1. Performance of digesters

Table 2 shows the characteristics of the food waste (FW) that was used to feed the digesters. The organic fraction of FW was quite high with a VS/TS ratio of 0.90 ± 0.01. The pH was relatively low, while SCOD, VFAs and NH_3 concentrations were higher than the typical FW values reported previously (Zhang et al., 2011). This was most likely due to the pretreatment and storage of the FW before shipping to the biogas laboratory. Although the FW used had a relatively low pH, the pH within the digesters were quite stable and showed average values of 7.7 ± 0.1, 8.0 ± 0.1, 7.8 ± 0.1 and 8.0 ± 0.1 in the MD, MD + R, TD and TD + R, respectively. The presence of the high alkalinity in the digesters (Table 3) was important in maintaining stable pH.

The average methane yields for the four digesters are shown in Table 3. For the mesophilic digesters the methane yields were quite similar (480 and 475 mL CH_4/gVS_{added}), and higher than the yields in the thermophilic reactors (7% and 18% higher than the TD and TD + R digesters, respectively). A wide range of methane yields of about 350–480 mL CH_4 per gVS_{added} has been reported in the literature for FW digestion (Zhang et al., 2011; El-Mashad and Zhang, 2010; Cho et al., 1995). Thus, the methane yields obtained in this study were in the higher end of values reported earlier. This could be attributed to high degradability of the pretreated FW used in this study.

While recirculation did not affect the methane yield in the mesophilic digesters, recirculation had a clear detrimental effect under thermophilic conditions (Table 3). Therefore, using recirculation for on-site clean water conservation is most applicable under mesophilic condition.

3.2. Solubilization of organic matter

Solubilization of the food waste were compared among the digesters using Equation (1) (Ge et al., 2011). This equation estimates the fraction of particulate COD that is solubilized into soluble material (SCOD). Since part of the SCOD is converted into methane, the COD equivalent of methane needs to be included to estimate the total extent of solubilization.

$$Extent\ of\ solubilization\ (\%) = \frac{COD_{CH4} + SCOD_e - SCOD_{in}}{PCOD_{in}} \quad (1)$$

Where COD_{CH4} is the COD equivalent of the CH_4 produced; $SCOD_e$ is soluble COD in effluent; $SCOD_{in}$ is soluble COD in influent and $PCOD_{in}$ is the particulate COD in influent.

The extent of solubilization of the substrate in the four digesters are presented in Fig. 1. It clearly shows that the highest solubilization was achieved at the thermophilic conditions without recirculation (TD). The TD digester and its mesophilic counterpart (i.e., MD digester) showed 62.5% and 56.6% solubilization, respectively. For the reactors with recirculation, the solubilization extent in the MD + R and TD + R reactors were 57.2% and 52.2%, respectively. Although the TD reactor showed a higher solubilization extent than the mesophilic digesters, its methane production was lower (Table 3). Partly, this might be explained by the ammonia inhibition and a shift in methane production pathway at the higher temperatures (discussed below). Digestate recirculation had a clear

Table 3
Average performance parameters during the stable operation of the digesters.

Parameters	Unit	MD	MD + R	TD	TD + R
Biogas composition	% CH4	63	62	62	58
	% CO2	37	37	38	42
Methane yield	mL CH4/g VS_{added}	480 ± 33	475 ± 29	448 ± 44	401 ± 45
	mL CH4/g COD_{added}	283 ± 34	280 ± 47	257 ± 61	242 ± 27
Alkalinity	mg/L as $CaCO_3$	5329 ± 145	11,267 ± 425	4200 ± 358	8319 ± 335
pH		7.7 ± 0.1	8 ± 0.1	7.8 ± 0.2	8 ± 0.1
Ammonia	mg N–NH_4/L	1109 ± 139	2150 ± 204	1258 ± 167	2258 ± 187
Free ammonia[a]	mg N–NH_3/L	49	200	198	597
SCOD	mg/L	932 ± 151	3167 ± 540	9413 ± 1915	20,932 ± 1990

[a] Calculated from Equation (3).

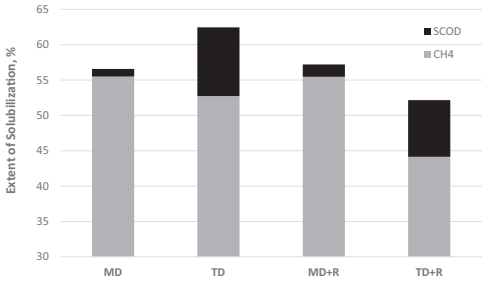

Fig. 1. Average extent of solubilization in the four digesters.

negative effect on solubilization at thermophilic conditions.

A closer analysis of the solubilization data (Fig. 1) revealed an imbalance between hydrolysis and methanogenesis processes in the thermophilic digesters (TD and TD + R), which had higher soluble COD fractions as compared to the mesophilic digesters. Increase in the soluble COD fraction as compared to the methane fraction has been reported previously for anaerobic digestion of waste sludge at elevated temperatures (Ge et al., 2011). The soluble COD fraction accounted for 10% and 8% of the solubilization for the TD and TD + R, respectively, indicating that solubilized products were not removed in these digesters as effectively as in the mesophilic counterparts. This observation agreed with the higher levels of VFAs in the thermophilic digesters as compared to the values obtained for the mesophilic ones (Fig. 2). Acetate and propionate levels were, on average, 175 ± 55 and 10 ± 6 mg/L in the MD and 278 ± 93 and 12 ± 7 mg/L in the MD + R, respectively. The VFA profiles in the thermophilic digesters TD and TD + R were very different. Acetate and propionate concentrations increased over time in the TD and were, on average, 2028 ± 864 and 833 ± 280 mg/L, and the longer VFAs ranged between 230 and 84 mg/L (Fig. 2C). For the TD + R, the main VFA was propionate (2300 ± 1250), followed by iso-valerate, iso-butyrate, *n*-butyrate and n-valerate (Fig. 2D). Interestingly, the acetate concentration was very low in the TD + R and averaged 29 ± 7 mg/L.

3.3. Ammonia inhibition

Anaerobic digestion model. No 1 (ADM 1) considers free ammonia inhibition on methanogenesis process in anaerobic digesters (Batstone et al., 2002). Based on the ammonium concentrations and pH values obtained for each digester, the free ammonia fraction was calculated using Equation (2) (Anthonisen et al., 1976; see Table 3).

$$\frac{FAN}{TAN} = \frac{10^{pH}}{10^{pH} + e^{6344/(273+T)}} \quad (2)$$

Where FAN is free ammonia nitrogen in mg/L as N; TAN is total ammonia nitrogen in mg/L as N; T is temperature in °C.

To estimate possible inhibition on the digesters' performance, a non-competitive inhibition (Equation (3)) model was used to evaluate the inhibition effect of free ammonia on methanogenesis (Batstone et al., 2002).

$$Inhibition\ factor\ (I_{nh3}) = \frac{1}{1 + \frac{S_{NH3}}{K_{I,NH3}}} \quad (3)$$

Where, S_{NH3} is the free ammonia concentration (see Table 3) and $K_{I,\ NH3}$ is the inhibitory ammonia coefficient where 50% reduction happens in methane production. The $K_{I,\ NH3}$ values for thermophilic and mesophilic temperatures were taken from Gallertand Winter (1998) and were 251 and 92 mg/L NH_3–N, respectively. The inhibition factors (I_{nh3}) computed for the MD, TD, MD + R and TD + R were 0.65, 0.56, 0.32 and 0.30, respectively. High inhibition factor (I_{nh3}) indicates low inhibitory effect on methanogenesis process. Comparison of the digester sets, that is, MD vs. TD and MD + R vs. TD + R, showed 17% and 7.5% greater ammonia inhibition effect on methane production under the thermophilic conditions. In addition, the model estimated a severe inhibition of methanogenesis in the digesters with digestate recirculation, regardless of the operating temperature. The lower performance of the thermophilic digesters (i.e., TD and TD + R) in terms of methane production might partially be explained by ammonia inhibition. However, the methane yields (Table 3) indicated comparable results for the MD and MD + R. It should be noted that the concentration of active microbial biomass might be different at the two temperatures. The microbial decay rates are higher at elevated temperatures, potentially yielding lower active microbial biomass in the thermophilic digesters. Consequently, the overall effect of ammonia inhibition on the acetoclastic methanogenesis pathway was potentially more profound for the thermophilic digesters.

3.4. Microbial analysis

To investigate the effect of digesters configurations (with and without digestate recirculation) operated at mesophilic and thermophilic temperatures on bacterial and archaeal communities, total DNA was isolated from the MD, MD + R, TD and TD + R digesters after 152 days of operation, and analyzed for 16S rRNA sequences. Due to the high microbial diversity that was observed in the digesters, only the phyla with higher than 1% relative abundancy were considered for discussion and comparison.

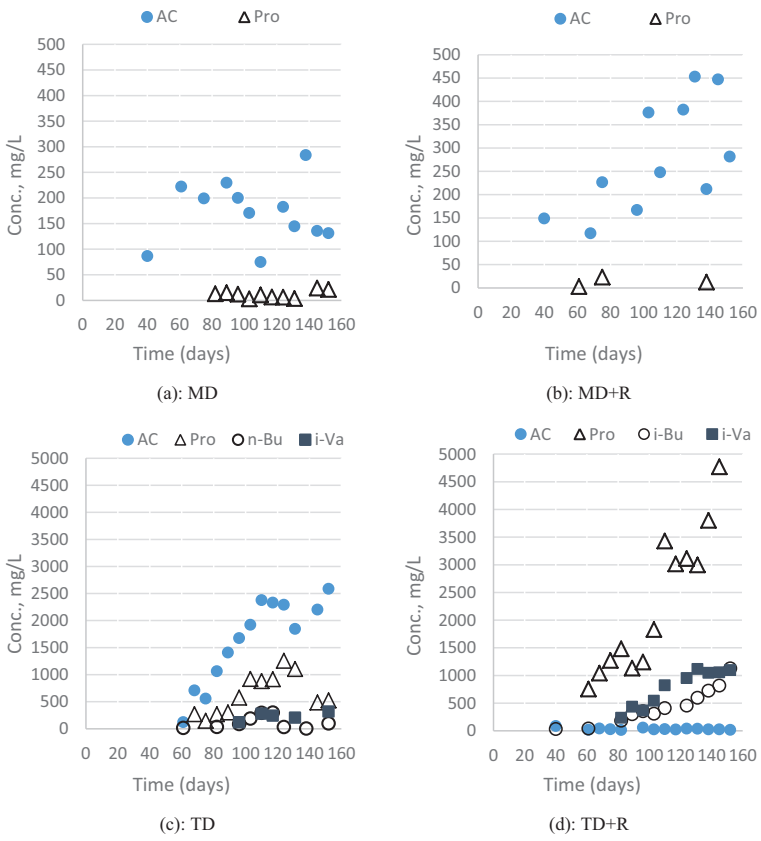

Fig. 2. Concentration of volatile fatty acids in the digesters.

3.4.1. Microbial community structure in mesophilic digesters

The results of the microbial community analysis under stable operation of the MD and MD + R are presented in Fig. 3. The dominant bacterial phyla in both of the mesophilic digesters were *Firmicutes* (25% in the MD and 75% in the MD + *R*), *Chloroflexi* (54% in the MD and 6% in the MD +R), *Bacteroidetes* (16% in the MD and 7% in the MD + R) and *Actinobacteria* (2% in the MD and 3% in the MD + R). A meta-analysis by Nelson et al. (2011) and an extensive analysis of various full-scale digesters by Sundberg et al. (2013) showed that *Firmicutes*, *Chloroflexi*, *Bacteroidetes* as well as *Proteobacteria* have typically been found as predominant bacterial groups in anaerobic digesters operated under various conditions. *Actinobacteria* was found as the fourth dominant phylum in the MD and MD + R, while the relative abundance of *Proteobacteria* was less than 1% of the total reads in this study. However, this agreed with the previous findings indicating the dominance of either *Actinobacteria* or *Proteobacteria* depending on the digesters operating conditions (Cardinali-Rezende et al., 2012).

Although the bacteria comprising the four most prominent phyla in both mesophilic digesters were the same during the steady-state operation of the mesophilic digesters, the recirculation of the digestate significantly influenced the relative abundance of each of these four bacterial groups. While phyla *Chloroflexi* (mostly represented by the candidate division T78 of the family *Anaerolinaceae*) dominated in the MD, *Firmicutes* was by far the most dominant phyla in the MD + R. In total 75% of the reads in the MD + R was affiliated to *Firmicutes*, represented by a major fraction of *Clostridium* (48% of total reads). In strong contrast, only 1% of the total reads of the MD affiliated to this genus. *Firmicutes* in general and the genus *Clostridium* represent members that are versatile in metabolic capabilities and include proteolytic and saccharolytic bacteria, as well as syntrophic species involved in VFA degradation (Riviere et al., 2009; Vanwonterghem et al., 2014; Hippe et al., 1992).

It was also found that 53% of all 16S rRNA gene reads in the MD were affiliated to candidate division T78, while only 5% was observed in the MD + R. Presence of *Anaerolinaceae* in anaerobic digesters fed with various organic wastes has been widely reported in the literature (St-Pierre and Wright, 2014; Kim et al., 2014). Many of the genera that have been identified in this family are strictly anaerobic bacteria and fermentatively use carbohydrates as substrate for growth (Yamada et al., 2006; Sekiguchi et al., 2003). Thus, it would appear that the members of *Anaerolineaceae*, Candidate division T78, contributed to the degradation of carbohydrate fraction of the food waste. Additionally, the remarkable difference observed in the dominance of *Chloroflexi* in the MD and *Firmicutes* in the MD + R might reflect the less tolerance of the *Chloroflexi* members to the high levels of ammonia, which was 2.2 times

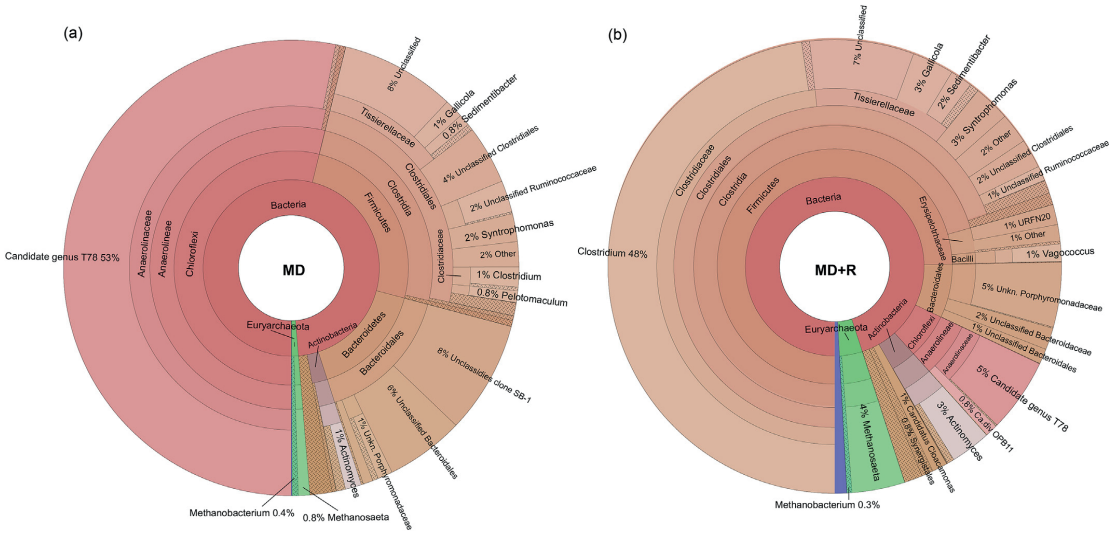

Fig. 3. Microbial structure in MD (a) and MD + R (b) after 153 days of AD, illustrated by simplified Krona plots based on 16S rRNA gene sequencing. The abundance of each taxonomic group corresponds to the percentage of the total number of reads. The shaded areas represent the presence of two or more low abundant taxa.

higher in the MD + R than MD. Yi et al. (2014) reported a reduction of *Anaerolineae* (of *Chloroflexi*) by increasing TS concentration in the anaerobic digesters used for FW treatment. The increase in TS content was accompanied with a severe increase in total ammonia level (from 400 to 1920 mg/L). The results agreed with our observation that the elevated ammonia concentrations led to a decrease in *Chloroflexi* abundance.

Interestingly, analysis of *Firmicutes* revealed a significant difference in distribution of bacterial groups at the family level within the mesophilic digesters. *Tissierellaceaea* accounted for 43% of all *Firmicutes*' reads in the MD, while *Clostridiaceaea* was the dominant family in the MD + R accounted for 65% of the phylum reads. The family *Clostridiaceaea* only constituted 7% of the *Firmicutes* in the MD.

The phyla *Bacteroidetes* and *Actinobacteria* represented respectively 16% and 2% of the readings in the MD, and 7% and 3% in the MD + R. However, the majority (98% in the MD + R and 72% in the MD) of the reads were not affiliated to a known genus of the *Bacteroidetes*. Regarding the *Actinobacteria*, the genus *Actinomyces* was the main member of the phylum found in both mesophilic digesters. The relative abundancies were, respectively, 3% and 1% (of the total reads) in the MD + R and MD. The higher relative abundance of the *Actinomyces* in the MD + R was likely due to the effluent recirculation of recalcitrant fiber materials to the digester, since it was previously reported the probable involvement of *Actinomycetes* in hydrolysis of cellulose (Ziganshin et al., 2011, 2013).

Overall, based on the performance results (Table 3), both MD and MD + R showed stable and comparable performance with high methane production. Thus, the differences in abundance of the predominant phyla did not influence biogas production. This observation support a possible functional redundancy of the *Chloroflexi* and *Firmicutes* members (Allison and Martiny, 2008).

The phylogenetic analysis of archaea demonstrated that almost all sequences were affiliated with the *Euryarchaeota* phylum (Fig. 3), comprising 4% and 1% of the microbial community in the mesophilic digesters MD + R and MD, respectively. A notable difference in the relative abundance of methanogens was observed for the mesophilic digesters. At the genus level, *Methanosaeta* and *Methanobacterium* were dominant genera and accounted for 65% and 32% of all *Euryarchaeota*'s reads in MD, respectively. On the other hand, *Methanosaeta* accounted for 91% of all *Euryarchaeota*'s reads for the MD + R, while *Methanobacterium* constituted 8% of the phylum. Thus, it appeared that the recirculation of the digestate in the MD + R resulted in a high prevalence of *Methanosaeta* species. The prevalence of acetoclastic methanogens (i.e., *Methanosaeta*) over hydrogenotrophic methanogens (*Methanobacterium*) in the MD and MD + R probably demonstrated the acetate cleavage as the main pathway for methane production. Low acetate concentrations in the mesophilic digesters (Fig. 2), which were, on average, 175 mg/L in the MD and 278 mg/L in the MD + R, supported the efficient conversion of acetate into methane by the acetoclastic methanogensis pathway.

Additionally, as described earlier, the use of an inhibition model demonstrated a potential severe free ammonia inhibition on the acetoclastic methanogenesis in the MD + R due to high NH_3 levels within the digester, which averaged 198 mg/L. However, the performance and microbial data showed a stable and comparable performance to the MD. Therefore, it interestingly appeared that the recirculation of digestate attenuated the effect of free ammonia on acetoclastic methanogens, since it is well documented that *Methanosaetaceae* are sensitive to high ammonia concentrations (Ho et al., 2013; Karakashev et al., 2005).

3.4.2. Microbial community structure in thermophilic digesters

The dominant bacterial phyla found in the thermophilic digesters included *Firmicutes*, *Thermotoga*, and *Synergistales* (Fig. 4). In contrast to the mesophilic digesters where a clear difference in the distribution of prominent groups was observed, the overall community structure was similar in the TD and TD + R. Thus, the effect of recirculation on the microbial community structure was more noticeable under mesophilic conditions. In both thermophilic digesters, *Firmicutes* and *Thermotoga* made up the majority of the reads. *Firmicutes* and *Thermotoga* accounted respectively for 35% and 40% of all reads for the TD and 41% and 37% for the TD + R. As

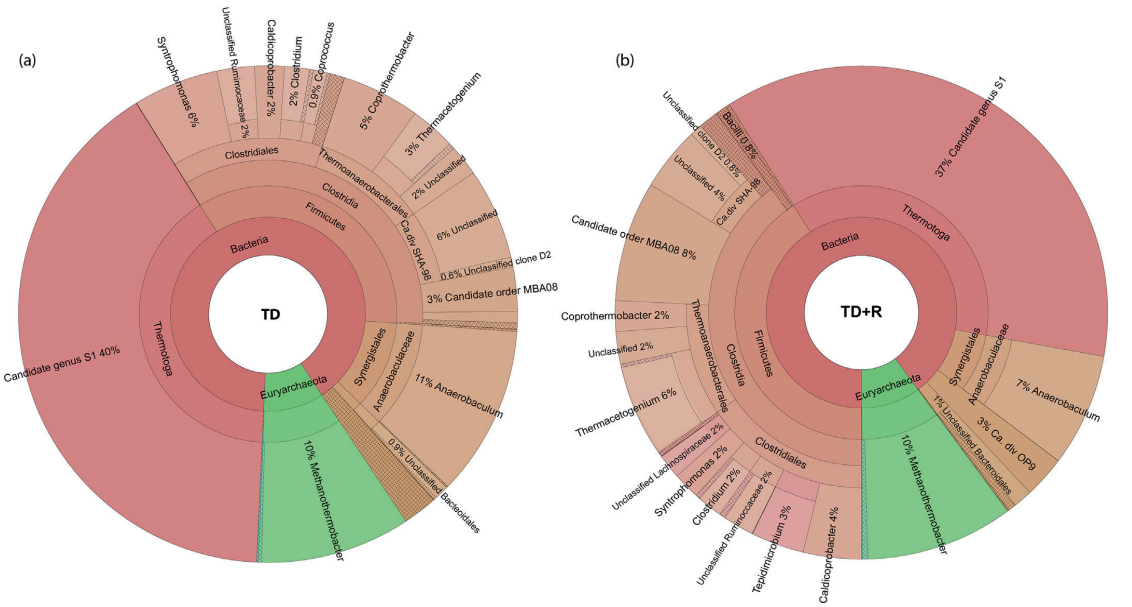

Fig. 4. Microbial community structure in TD (a) and TD + R (b) after 153 days of AD, illustrated by simplified Krona plots based on 16S rRNA gene sequencing. The abundance of each taxonomic group corresponds to the percentage of the total number of reads. The shaded areas represent the presence of two or more low abundant taxa.

with the mesophilic digesters, the diversity within *Firmicutes* was high, comprising several genera mainly within the orders *Clostridiales*, *Thermoanaerobacterales*, candidate division SHA-98 and the candidate order MBA08. Uncultured representatives from candidate order MBA08 have been observed to dominate in thermophilic (and mesophilic) digesters with high ammonia content (De Vrieze et al., 2015). Additionally, MBA08 has previously been reported to dominate in cellulolytic communities of anaerobic digesters (Sun et al., 2015), thus suggesting that this group was probably responsible for the hydrolysis of cellulosic materials in the food waste.

The genera *Coprothermobacter* and *Thermacetogenium* were dominant members of the order *Thermoanaerobacterales* in both TD and TD + R. Even though the relative abundance of *Thermoanaerobacterales* was quite similar in both digesters, the distribution of *Coprothermobacter* and *Thermacetogenium* genera within the order was notably influenced by the digestate recirculation. *Coprothermobacter* was the main genus (49%) of the order *Thermoanaerobacterales* in the TD, which was followed by *Thermacetogenium* (28%). For TD + R *Thermacetogenium* constituted 60% of the order, followed by *Coprothermobacter* (19%). The genus *Thermacetogenium* grows acetogenically on various hydrolysis products including amino acids, organic acids and H_2/CO_2. They are also able to oxidize acetate in co-culture with hydrogenotrophic methanoges (Hattori et al., 2000). This might explain the low concentration of acetate in the TD + R digester (Fig. 2D). Presence of relatively high abundance of *Coprothermobacter* (5% and 2% in TD and TD + R, respectively) was likely due to their contribution to the degradation of proteinaceous fraction of the food waste (Sasaki et al., 2011). The *Coprothermobacter* members are proteolytic bacteria that degrade proteins into acetate, H_2 and CO_2. In the presence of hydrogenotrophic methanogens, an increased amount of propionate and butyrate production has been reported (Sasaki et al., 2011). Interestingly, the observation of the relatively high abundance of *Thermoanaerobacterales* was concomitant with high concentrations of NH_3, propionate, butyrate and isovalerate (Table 3 and Fig. 2). This was likely due to their high ammonium tolerance (up to 6 g/L), which was reported by Ollivier et al., 1985.

A significant difference was observed in the relative abundances of the genera within the *Clostridiales* when the bacterial community structure was compared in the TD and TD + R. *Syntrophomonas* accounted for 40% of the genera found in the order *Clostridiales* in the TD, while this value for the TD + R was only 12%. *Caldicoprobacter* and *Tepidimicrobium* (of the family *Tissierellaceae*) were the predominant genera in the TD + R, accounting for 25% and 23% of the order. *Caldicoprobacter* and *Tepidimicrobium* are both fermentative microorganisms, where the former ferments sugars and the latter grows on a number of proteinaceous substrates (Slobodkin et al., 2006; Yokoyama et al., 2010). As reported previously, the majority of the members of the *Syntrophomonas* are extreme anaerobes, so called syntrophic acetogenic bacteria (SAB), that use β-oxidation process to break down long chain organic acids (C_4–C_{18}) to acetate, propionate and H_2 in a syntrophic cooperation with hydrogenotrophic methanogens (Zhao et al., 1993). Consequently, it may be inferred that the relatively greater fraction of *Syntrophomonas* in the TD helped the formation of an enhanced syntrophic degradation of the organic acids and resulted in a better performance. The recirculation of digestate in the TD + R seemed to negatively influence this syntrophic reaction and resulted in the accumulation of the propionate, butyrate and iso-valerate within the digester (Fig. 2). Additionally, the relatively lower abundance of *Anaerobaculum* (phylum *Synergistetes*) in the TD + R (7% of the reads) as compared to the TD (11% of the reads) might account for the accumulation of VFAs in the TD + R. *Anaerobaculum* members are capable of converting organics acids, peptides and a limited number of carbohydrates to acetate, CO_2 and H_2 (Menes and Muxí, 2002).

Thermotoga was solely represented by the candidate division S1

within *Thermotoga* (40% and 37% of the total reads in the TD and TD + R, respectively), a phylum that contains known syntrophic acetate oxidizers (Balk et al., 2002). Additionally, an enhanced growth of *Thermotoga* has been reported in co-culture with a hydrogen consumer (Frock et al., 2012; Conners et al., 2006). Because of its high relative abundance, it is tempting to suggest candidate division S1 as a possible candidate of syntrophic acetate oxidizer.

Analysis of the archaeal composition in the thermophilic digesters revealed that both of the digesters were almost completely dominated by the hydrogenotrophic methanogens (Fig. 4). The genus *Methanothermobacter* (of the order *Methanobacteriales*) accounted for 97% and 96% of the archaea in the TD and TD + R, respectively. Only a small fraction (~1%) of archaeal readings in both digesters was identified as the genus *Methanosarcina*, which is able to use either hydrogenotrophic or acetoclastic methanogenesis pathway. Due to the presence of the various syntrophic members (as discussed above), it was expected to find high abundance of hydrogenotrophic methanogens in the thermophilic digesters. The dominance of H_2 utilizing methanogens has previously been reported in digesters fed with food waste and operated at thermophilic conditions (Guo et al., 2014; Giuliano et al., 2014). The hydrogenotrophic methanogens constituted 10% of the total reads in both TD and TD + R. Based on these observations, it may be inferred that the prevalent pathway for methane production in the thermophilic digesters was hydrogentrophic methanogensis.

3.4.3. Dominant microbial pathways for methane production

Based on process data and microbial community data a schematic diagram of probable dominant pathways in the four digesters can be made (Fig. 5). The very low levels of longer VFAs (propionate and butyrate) in both MD and MD + R (Fig. 2a,b) demonstrated an efficient removal of these intermediates into acetate by syntrophic acetogenic bacteria (SAB). These digesters also had relatively low levels of acetate, demonstrating efficient conversion of acetate to methane. Based on microbial data, the main pathway for methane production seemed to be carried out by acetoclastic methanogens (i.e., *Methanosaeta*) in both MD and MD + R (Fig. 5, grey arrows).

Microbial data showed that the dominating pathway for methane production in the thermophilic digesters was hydrogenotrophic methanogenesis, probably due to free ammonia inhibition of acetoclastic methanogens (Fig. 5, dotted arrows). This means that acetate was metabolized by syntrophic acetate oxidizing bacteria (SAOBs) in the thermophilic digesters. This is supported by the high abundance of *Thermotoga* and *Thermacetogenium* in these digesters, which has been suggested to be syntrophic acetate oxidizers (Hattori et al., 2000). Syntrophic acetate oxidation has been reported to occur under elevated temperatures and high ammonia concentrations (Karakashev et al., 2005). In TD, acetate was accumulating in the digester, while propionate, butyrate and iso-valerate concentrations were relatively low (but higher than in the mesophilic digesters; Fig. 2c). This acetate accumulation demonstrated that the SAOB pathway was more inhibited than the SAB pathway in the TD digester. The accumulation of propionate and longer VFAs in the TD + R digester indicated a much stronger inhibition of the SAB pathway in this digester, probably due to the high concentration of free ammonia (Siegrist et al., 2002). The presence of higher relative abundances of *Syntrophomonas* and *Synergistales* in the TD as compared to the TD + R supported a more effective syntrophic acetogenesis in the TD digester.

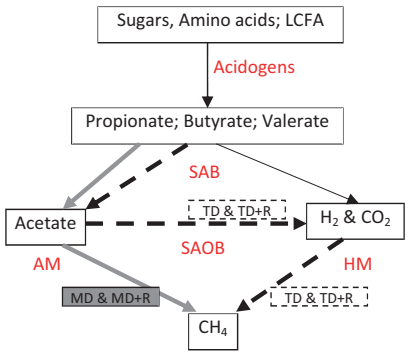

Fig. 5. Dominant methane production pathways in the various digesters. Solid grey arrows indicate a pathway through syntrophic acetogenesis and acetoclastic methanogenesis which dominated in MD and MD + R. Dashed arows indicate a pathway through syntrophic acetogenesis, acetate oxidation and hydrogenotrophic methanogenesis which dominated in TD and TD + R. SAB: syntrophic acetogenic bacteria; SAOB: syntrophic acetate oxidizing bacteria; AM: acetoclastic methanogens; HM: hydrogenotrophic methanogens.

4. Conclusion

Regardless of the digestate recirculation, anaerobic digestion of the pretreated FW under mesophilic conditions outperformed the thermophilic digesters in terms of the methane production. Accumulation of VFAs in the thermophilic digesters indicated an imbalance between solubilization of the substrate and the methane production process.

Recirculation of digestate, as a strategy to reduce water consumption, worked very well under mesophilic conditions despite resulting in relatively high levels of ammonia.

In both mesophilic digesters *Methanosaeta* was the dominant archaea, but the bacterial community structure was significantly different. *Firmicutes* dominated in the MD + R, while *Chloroflexi* was the dominant phylum in the MD. In contrast to the mesophilic digesters, the microbial communities in the thermophilic digesters were rather similar, consisting mainly of the phyla *Firmicutes*, *Thermotoga*, *Synergistetes* and the hydrogenotrophic methanogen *Methanothermobacter*. Thus, a combination of digesters configurations, operating temperatures and ammonia concentrations resulted in different dominant pathways for methane production. A conventional acetoclastic methanogenesis appeared to be the main pathway in the mesophilic digesters, while syntrophic acetate oxidation and hydrogenotrophic methanogenesis seemed to be the dominant pathway in the thermophilic digesters. Practically, a mesophilic temperature may be recommended in cases where digestate is to be used for dilution of food waste.

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Post-anaerobic digestion thermal hydrolysis of sewage sludge and food waste: Effect on methane yields, dewaterability and solids reduction

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ABSTRACT

Post-anaerobic digestion (PAD) treatment technologies have been suggested for anaerobic digestion (AD) to improve process efficiency and assure hygenization of organic waste. Because AD reduces the amount of organic waste, PAD can be applied to a much smaller volume of waste compared to pre-digestion treatment, thereby improving efficiency. In this study, dewatered digestate cakes from two different AD plants were thermally hydrolyzed and dewatered, and the liquid fraction was recirculated to a semi-continuous AD reactor. The thermal hydrolysis was more efficient in relation to methane yields and extent of dewaterability for the cake from a plant treating waste activated sludge, than the cake from a plant treating source separated food waste (SSFW). Temperatures above 165 °C yielded the best results. Post-treatment improved volumetric methane yields by 7% and the COD-reduction increased from 68% to 74% in a mesophilic (37 °C) semi-continuous system despite lowering the solid retention time (from 17 to 14 days) compared to a conventional system with pre-treatment of feed substrates at 70 °C. Results from thermogravimetric analysis showed an expected increase in maximum TS content of dewatered digestate cake from 34% up to 46% for the SSFW digestate cake, and from 17% up to 43% in the sludge digestate cake, after the PAD thermal hydrolysis process (PAD-THP). The increased dewatering alone accounts for a reduction in wet mass of cake leaving the plant of 60% in the case of sludge digestate cake. Additionaly, the increased VS-reduction will contribute to further reduce the mass of wet cake.

1. Introduction

Anaerobic digestion (AD) is commonly used in waste management for treatment of organic wastes such as sewage sludge and food waste, with the aim of waste stabilization, methane generation and production of a digestate that can be used as a fertilizer. AD processes typically have long retention times, meaning large digesters and thus large plant footprints. The waste stabilization efficiency and digestate quality depends on the characteristics of the waste and the AD technology. Technologies assuring a high hygienic quality of the digestate as well as high waste stabilization rates are key for a successful AD plant, and pre-treatment technologies such as the thermal hydrolysis process (THP) has been extensively used to improve process performance (Barber, 2016; Carrere et al., 2016; Neyens and Baeyens, 2003).

THP has increased degradation rates and biogas yields for a wide range of wastes, including sewage sludge and lignocellulosic biomasses (Bauer et al., 2014; Dereix et al., 2006; Estevez et al., 2012; Horn et al., 2011b; Lizasoain et al., 2016; Vivekanand et al., 2013; Wilson and Novak, 2009). The optimum temperature and time combination during THP pretreatment depends on the type of substrate. THP treatment has resulted in reduced capillary suction time (CST) and filtration time of sludge, both parameters important for the rate of the dewatering process (Dereix et al., 2006; Everett, 1972; Haug et al., 1978). However, CST and filtration methods are not necessarily correlated with maximum cake solids (Kopp and Dichtl, 2001). Technologies that increases the total solids (TS) in dewatered digestate have a large potential for reducing the storage silo footprint as well as transportation costs for disposal of the

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digestate cake. Although improved dewaterability is well documented in sludge after THP (Everett, 1972; Haug et al., 1978; Neyens and Baeyens, 2003; Skinner et al., 2015), the mechanism is not well understood, and the optimum THP treatment conditions for different wastes are largely unknown.

THP-based technologies result in solubilization of organic material and thus release of readily degradable organic matter to the liquid fraction (Dereix et al., 2006). In Norway, pre-treatment of waste is mainly applied to meet health regulations where the minimum requirement is heating for 1 h at 70 °C for sludge and waste of animal origin (Nærings- og fiskeridepartementet, 2007). Although pre-treatment results in a reduction of pathogenic bacteria in the digestate and improved process performance (Bagge et al., 2005; Lang and Smith, 2008; Wang et al., 1997), large fractions of the waste are readily bio-degradable and do not benefit from such treatment. Post-anaerobic digestion treatment (PAD) has recently been suggested as an alternative (Sambusiti et al., 2015; Thygesen et al., 2014). This means that only a fraction of the original waste needs to be treated, while still ensuring a hygienic end-product for land application. In a typical PAD-THP setup, the digestate would be dewatered, treated with THP and then after THP undergo a subsequent dewatering, where the liquid fraction is recirculated to the anaerobic digester. The patented Cambi Solid-Stream™ (Kjorlaug et al., 2015; Kolovos et al., 2016; Solheim and Nilsen, 2014) is based on this idea, and involves post-treatment of digestate cake using THP.

So far, only one full-scale plant has installed a PAD-THP process, which is the Cambi SolidStream™ (Amperverband in Olching, Germany). No laboratory scale studies have been published on this topic. Thus, many of the mechanisms of the technology are not well documented and understood. For example, recirculation of the centrate from the post-treated digestate can result in a reduction of sludge retention time (SRT), which could reduce the efficiency of the AD process (Jang et al., 2014), possibly counteracting the beneficial effect of post-treatment. In addition, optimal THP conditions found for other substrates will not necessarily apply to biogas digestates, and studies of how digestate cakes of different origin respond to THP are lacking. A third unknown factor of THP is the effect on digestate dewaterability. Up to now, the effect of THP treatment on different digestate cakes is not described in the literature.

The objectives of this study were to evaluate the effect of:

1) thermal hydrolysis conditions (time and temperature) on the solubilization of COD and resulting biogas production from digestate cakes using biochemical methane potential (BMP) tests; and
2) PAD-THP on digester performance and overall solids reduction using semi-continuous anaerobic digesters.

2. Materials and methods

The experimental work was in part performed at the Biogas Laboratory at the Norwegian University of Life Sciences (Ås, Norway) and at the Environmental Engineering Laboratory at Bucknell University (Lewisburg, PA, USA). Due to differences in the laboratory equipment at the two locations, it was not possible to use the same methods for all analyses; however, we consider the methods used compatible.

2.1. Experimental design

This study is based on two experiments. The first experiment was designed to find the optimal THP conditions for two digestate cakes using the biochemical methane potential (BMP) test. The second experiment was designed to investigate how the Solid Stream approach affects the performance of semi-continuous anaerobic digesters operated until steady state conditions were achieved.

2.2. Materials for THP conditions experiment

We obtained centrifuged digestate cake from two different full-scale AD plants. One cake was from a food waste anaerobic digester operating in the thermophilic range (52–53 °C; Hadeland and Ringerike waste company (HRA), Ringerike, Norway), and source separated food waste (SSFW) was its sole substrate. HRA pretreats the SSFW according to Norwegian regulations at 70 °C for 1 h. The second cake was from an anaerobic digester operating in the mesophilic range (35 °C) treating sludge and collected at Hampton Roads Sanitation District's (HRSD) Nansemond Treatment Plant (Suffolk, Virginia, USA). HRSD's plant treats a mix of primary and waste activated sludge (WAS) from a Bio-P process. Both plants use high solids centrifuges for dewatering.

2.3. THP conditions experiment

The digestate cakes (HRA and HRSD) were used for testing different post-treatment conditions. The post treatment of HRA digestate cake was performed in Norway, using a small Cambi mini test steam explosion unit with a reactor volume of 1 L (CAMBI GROUP AS, Asker, Norway), while the HRSD digestate cake was post-treated in a larger Cambi mini test steam explosion unit at Bucknell University with a reactor volume of 5 L (CAMBI GROUP AS, Asker, Norway). The characteristics of the two cakes prior to post-treatment are presented in Table 1.

To examine the effect of different THP conditions on BMP and dewatering properties of digestate cakes, a set of seven different pre-incubation times and temperatures, spanning from 134 °C to 175 °C and from 20 min to 30 min, was applied. The lowest temperature was not tested with the 20 min treatment because this combination of time and temperature does not fulfill the current regulations for sanitation. Pre-incubation time was measured from the time the desired temperature in the reactor was reached. The post-treated digestate cakes were separated in a centrifuge at 2000 relative centrifugal force (RCF) for 30 min and the liquid and solid fractions were used in BMP and dewatering tests. The BMP results for the liquid fraction is presented on the basis of COD and the solid fraction on the basis of TS, because much of the liquid COD was volatiles that would result in falsely low TS measurements, and the solid fraction contained particulates making COD-measurements

Table 1
Characteristics of digestate cakes from HRA and HRSD. Standard deviations are listed in parenthesis. All percentages are on the basis of TS with the exception of Ash which is on the basis of wet weight.

	Unit	HRA	HRSD
TS	%	18.5 (0.3)	21.8 (0.1)
VS	%	73.1 (0.4)	68.4 (0.4)
Ash	%	5.0 (0.7)	6.9 (0.1)
COD	g/L	226 (2.6)	183 (17)
COD:VS		1.7	1.2
C	%	42.7 (0.3)	32.7 (0.2)
H	%	5.45 (0.09)	5.47 (0.08)
N	%	2.43 (0.05)	4.70 (0.1)
S	%	0.49 (0.02)	1.95 (0.01)
C:N		17.6 (0.5)	7.0 (0.2)
ADF[a]	%	54.6 (0.3)	31.4 (1.3)

[a] ADF = Acid detergent fiber.

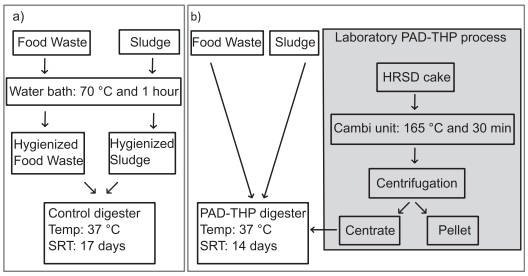

Fig. 1. Experimental setup for CSTR experiment. a) In the control digester, substrates were heated at 70 °C for 1 h in a water bath prior to feeding. b) In the PAD-THP digester, untreated food waste and sludge was used as substrate. Centrate was produced by thermal hydrolysis of HRSD cake and separated using a laboratory centrifuge. The centrate was fed to the digester together with food waste and sludge.

less reliable. BMP results of the solid fraction is given on the basis of TS instead of VS to directly relate the methane yield of the cake to predicted cake solids.

2.4. Semi-continuous stirred tank reactors experiment – PAD-THP

For the semi-continuous stirred tank reactor (CSTR) experiments, feed sludge (primary sludge and waste activated sludge (WAS)) was shipped overnight from HRSD weekly and stored at 5 °C until use. Waste used for the digester fed with conventional pre-treated waste was pretreated upon arrival by heating in closed containers in a water bath for one hour at 70 °C. The initial heating of the waste to reach 70 °C took approximately 1 h. A preprocessed food waste called an Engineered BioSlurry (EBS) was supplied by Waste Management, Inc. The food waste was a homogenized product of different commercial food wastes, including pre-consumer and post-consumer organic waste streams such as waste from restaurants, grocery stores, expired packaged goods, food processors and residential organics. The food waste samples were stored in a freezer until use. In order to get enough centrate for the PAD-THP digester, it was decided to only use centrate from post-treatment of HRSD cake from the full-scale digester and not from the laboratory digesters. This decision was based on the results from the THP-conditions experiment on dewatering properties which revealed that we were not able to dewater the post-treated cake to the final cake solids achieved by a full scale centrifuge with a laboratory centrifuge, hence, much of the centrate would be left in the pellet and not recirculated to the laboratory digester. Another reason for the decision was that digestate is lost both in the initial dewatering before THP to reach a TS of 16%, but even more in the Cambi unit because some of the digestate is deposited on the inside walls of the THP reactor. The treated digestate cake was separated by centrifugation while still hot at 3000 RCF for 30 min in the laboratory centrifuge, and this centrate was used in the PAD-THP digester (Fig. 1).

Two, 10 L CSTRs were used in the experiment, one control-digester using conventional technology fed with hygenized sludge and food waste (70 °C for 1 h) and one PAD-THP digester fed with untreated sludge, food waste, and centrate from THP-treated HRSD cake (treated for 30 min at 165 °C). The feed characteristics are presented in Table 2. The ratio of food waste, sludge and centrate was 15:100:10 on a wet weight basis and approximately 10:20:1 on the basis of COD (Table 3). The digesters were fed once a day, immediately after digester wasting. Biogas was transported through a tube in the headspace of the digester to a PF-8000 respirometer from Respirometer Systems and Applications (Springdale, Arkansas, USA) which recorded total gas volume and gas production rates. In addition, a second tube was connected to a gas bag that collects or supplies gas during the brief periods of wasting and feeding in order to maintain a stable concentration of gases in the headspace and not create a vacuum or pressure in the digester. The bag has a valve that is opened only during the feeding and wasting operation. A sealed septum port in the top of each reactor allows sampling of the headspace for gas composition. Both reactors were inoculated with digestate from two lab-scale digesters digesting sewage sludge from HRSD at day 0 of the experiment.

A co-digestion scenario with mixing of food waste and sewage sludge for digester feed was chosen in order to test the effect of PAD-THP on a digester already operating under high organic loading rates, and low SRT. Concentrated food waste have 2–3 times the COD concentration of sewage sludge (Table 2), and can be used to improve volumetric biogas yields of sewage sludge digesters. Organic loading rate (OLR) and sludge retention time (SRT) is presented in Table 3. The SRT is lowered as a consequence of the recirculation-stream. A low SRT (17 and 14 d), compared to conventional plants, was used in the experiment in order to be able to observe a possible reduced efficiency due to the lowering of SRT.

2.5. Analyses

Total solids (TS) and volatile solids (VS) were determined gravimetrically by drying at 105 °C and subsequent burning at 550 °C. Volatile fatty acids (VFAs) in the CSTR effluent were measured using an Agilent 5890 (Agilent Technologies, Santa Clara, California, USA) gas chromatograph (GC) with a flame ionizing detector (FID) equipped with a 30 m × 0.53 mm × 1 μm film thickness Supelco Nukol Fused Silica capillary column (Catalog # 25357). Samples were first centrifuged at 3000 × *g* for 15 min, and then

Table 2
Feedstock characteristics used for CSTR experiment. Standard deviations are listed in parenthesis.

	Unit	Untreated sludge	Hygenized sludge	Untreated food waste	Hygenized food waste	PAD-THP centrate
COD	g/L	92.2 (6.9)	102 (8.3)	270 (29.7)	261 (13.2)	38.7 (4.9)
S-COD	g/L	3.0 (0.7) *	12.6 (1.6) *	96.8 (11.4)	96.5 (7.5)	35.7 (3.3)
TS	%	6.3 (0.4)	6.9 (0.8)	13.4 (0.2)	13.4 (0.5)	2.8 (0.2)
VS	% of TS	79 (0.7)	79 (1.0)	91 (1.0)	90 (1.4)	88 (1.9)
TAN[a]	mg/L	221 (45) *	363 (117) *	396 (66)	388 (82)	1248 (318)
PO4-P	mg/L	541 (394)	781 (474)	493 (202)	477 (226)	712 (505)
Acetic acid	mg/L	578 (258)	592 (133)	3383 (1833)	3157 (1950)	934 (202)
Propionic acid	mg/L	408 (164)	277 (148)	132 (23)	127 (20)	666 (113)
tVFAs	mg/L	1211 (567)	1022 (333)	3677 (1873)	3540 (1837)	2202 (411)
pH		5.7 (0.2) *	6.0 (0.3) *	3.9 (0.1)	3.9 (0.1)	6.9 (0.2)

* values of untreated and hygenized substrates are significantly different (P-value < .05).
[a] TAN = total ammonia nitrogen.

Table 3
Experimental design of CSTR experiment. The organic content of the centrate is not included in the organic loading rate (OLR) for the PAD-THP digester, because this is a recirculation-stream and not fresh substrate added to the digester feed. The difference in OLR is caused by the difference between the pasteurized and non-pasteurized feed. Standard deviations are listed in parenthesis.

	Unit	Control	PAD-THP
FW	g/day	75	75
Sludge	g/day	500	500
Centrate	g/day	0	150
Digester volume	L	10	10
Feed mass	g/day	575	725
SRT	day	17	14
OLR on COD basis	g/L*d	7.1 (0.48)	6.8 (0.52)
OLR on VS basis	g/L*d	3.6 (0.27)	3.4 (0.14)

supernatant was filtered through a 0.45-μm filter. Next, 0.5 mL of sample was placed in a gas chromatography vial and diluted with 0.5 mL of deionized water. Then 50 μL of methanesulfonic acid was added to the vial, and the vial was capped. Samples were auto-injected into the gas chromatograph at a volume of 1 μL. The injector temperature was 238 °C, and the oven was first held at 105 °C for 4.00 min, followed by a 5 °C/min ramp to 145 °C, followed by a 10 °C/min ramp to 190 °C and a hold of 5.50 min. The detector temperature was 200 °C.

COD was determined using commercial kits (Merck in Norway and CHEMetrics in the USA). For determination of soluble fraction of COD, the samples were prepared by centrifugation (RCF of 23,907 for 10 min) prior to filtration. A 0.2 μm syringe filter was used for THP conditions experiments, as this excludes more particulates than the 0.45 μm filter and will give a more correct measure of true solubles. For the CSTR experiment 0.45 μm filters were used, as this filter pore size is more common when evaluating effluent quality from anaerobic digesters.

CHNS analysis was performed on a Vario El Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) by combusting the dried samples at 1150 °C under a constant flow of oxygen gas.

Acid Detergent Fiber (ADF) was analyzed with an Ankom^{200} Fiber Analyzer (ANKOM Technology, Macedon, New York, USA) according to manufacturer's description using F58 filter bags to retain more of the fiber fraction.

Biochemical methane potential tests were performed as previously described by Horn et al. (2011a,b). In brief: inoculum and substrate was mixed in 500 mL bottles with rubber septa sealed with aluminum screw caps. Pressure and biogas composition was measured using an electronic manometer (GMH 3161 Greisinger Electronic, Regenstauf, Germany) and an Agilent 3000A GC (Agilent Technologies, Santa Clara, California, USA) for 30 days. All BMP tests were performed in triplicate, including blanks with only inoculum and positive controls containing cellulose. Substrate to inoculum ratio was 1:3 on the basis of VS.

Prediction of cake solids were measured with a thermogravimetric method using the conditions described by Kopp and Dichtl (2001). In brief: A Netzsch Simultaneous TG-DTA/DSC Apparatus STA 449 F1 Jupiter® (NETZSCH-Geräutebau GmbH, Selb, Germany) was used with drying at 35 °C and a constant flow of nitrogen (20 mL/h). The drying curve was analyzed to find the amount of free water. This amount of water was assumed to correspond to the maximum water mass possible to remove from the digestate by high solids centrifuges, and hence a theoretical maximum TS of the sludge cake was estimated. The setup was calibrated using monodisperse silica particles of diameters 1.86 μm, 4.08 μm, 7.75 μm (Cospheric LCC, Santa Barbara, California, USA).

2.6. Calculations

COD and VS reduction, cake reduction and the volume of centrate recirculated to the PAD-THP CSTR was calculated based on mass balance. Equations are formulated in the supplemental material.

Statistical analysis were performed with the software R. For the THP conditions experiment a 2- way analysis of variance (ANOVA) was used with the parameters time, temperature and cake origin. A paired *t*-test was conducted to test if the daily methane production of the two CSTRs were significantly different, and if the pre-treatment of the substrates in the CSTR experiment influenced the measured parameters. All statistical tests were performed at the significance level of 95%.

3. Results and discussion

3.1. Testing of different THP conditions for post-treatment

Digestate cakes from two AD plants were thermally hydrolyzed with different combinations of time and temperature, ranging from 20 to 30 min and 134–175 °C, respectively. The aim was to investigate how different post-treatment conditions affected the two cakes with regards to solubilization, specific methane yield and dewaterability. The results from these experiments are presented and discussed in this section.

3.1.1. Solubilization and methane potential in centrate

The amount of soluble COD relative to the total COD can be used as a measure of solubilization during thermal hydrolysis. The effect of THP treatment time and temperature on digestate cakes from HRA and HRSD are presented in Fig. 2a. ANOVA showed that THP temperature and cake origin had a significant effect on the solubilization of the digested cake (p-value < .001 and < .01, respectively). The ANOVA also showed that the response to THP temperature was significantly different for the two cakes (p-value < .01), where soluble COD increased more in HRSD cake compared to HRA cake.

The highest solubilization of the HRSD cake was 187% higher than the lowest; for HRA cake the highest solubilization was 50% higher than the lowest. Additionally, the highest solubilization of HRSD cake was 113% higher than the highest solubilization of the HRA cake. For the HRSD cake (digested sludge) the highest solubilization was achieved for the 30 min treatment at 175 °C (32%), while the lowest solubilization for the 30 min treatment at 134 °C (10%). The increase in solubilization is greater between 152 °C and 165 °C compared to 165 °C and 175 °C for both the 20 min and the 30 min treatment time. For the HRA cake (digested food waste), the increase in solubilization over the temperature range (134 °C–175 °C) is small. Here the 30 min treatment at 175 °C gave the highest solubilization (15%), while 30 min at 134 °C gave the lowest solubilization (9%).

Several authors have observed that solubilization increased with increasing temperature and time of the THP (Haug et al., 1978; Li and Noike, 1992; Wilson and Novak, 2009). Haug et al. (1984) found that solubilization of WAS was highest for 30 min at 175–200 °C. Li and Noike (1992) tested WAS at temperatures up to 175 °C for 30 min and found that, generally, the solubilization increased with increasing temperature. Wilson and Novak (2009) tested WAS and primary sludge with pretreatment temperatures up to 220 °C for 2 h, and also found that the solubilization increased with increasing temperature. The results presented here agree with the previous findings in published literature on WAS and primary sludge. However, the degree of solubilization seems to be feedstock dependent.

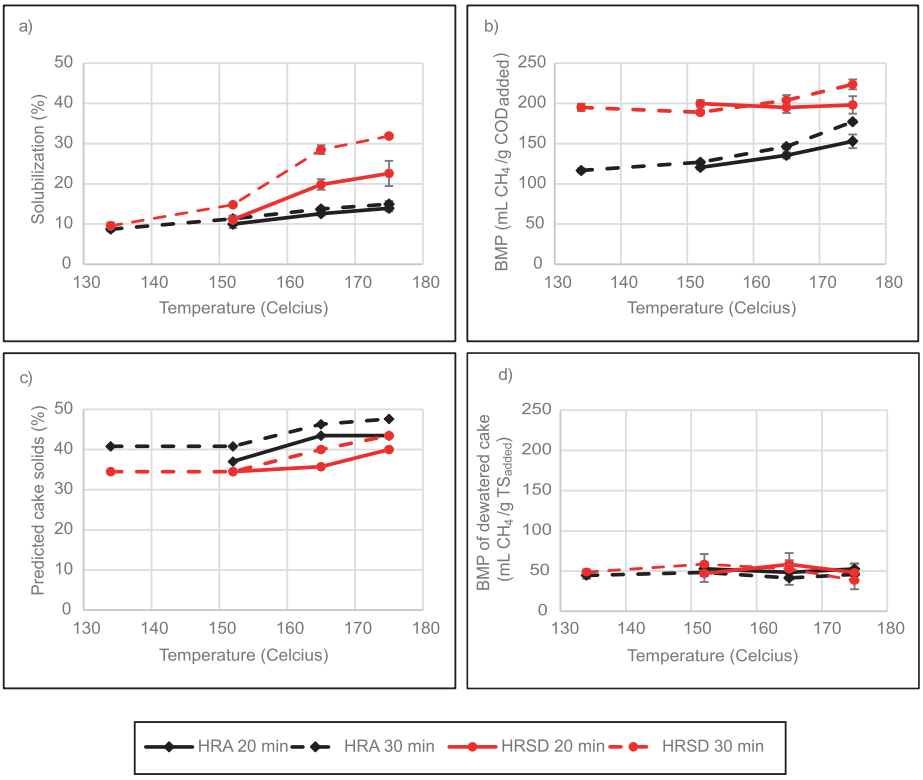

Fig. 2. a) Degree of solubilization achieved by thermal hydrolysis of cake from HRA and HRSD as soluble COD per total COD. Standard deviation of the COD measurements are presented as error bars. b) BMP of centrate from thermally hydrolyzed cake on the basis of COD added. Standard deviations of the COD measurements and methane measurements are presented as error bars. c) Predicted maximum TS in dewatered sludge cake. No replicates was made for this analysis, with the exception of the least uniform sample: untreated HRA, which had a RSD of 4% between triplicates. d) BMP in dewatered cake. Standard deviations of the TS measurements and methane measurements are presented as error bars.

In a PAD-THP, the centrate after post-treatment will be recirculated to the AD for additional biogas production. Therefore, in this study, the BMP of the centrate produced from the different post-treatment conditions was determined. ANOVA showed that the thermal treatment resulted in significantly different specific methane yields of the centrate from the two post-treated cakes (p-value < .001). The different centrates originating from HRSD cake in all cases yielded more methane compared to the centrates from the HRA cake (Fig. 2b). Temperature of the THP treatment also had a significant impact on methane yields of the centrates (p-value < .01), and the response to the temperature of the THP treatment was significantly different for the two cakes (p-value < .05). Increasing temperatures made the centrate from PAD-THP of HRA cake more available for conversion to methane. For centrates from PAD-THP of HRSD cake the response to higher temperature was smaller than for HRA cake, but the overall conversion to methane was significantly higher for centrates from HRSD cake.

Several authors have observed that methane yields increase with increasing temperature and time up to a certain level as a result of the treatment, before the effect levels off or decreases (Li and Noike, 1992; Stuckey and McCarty, 1984). Stuckey and McCarty (1984) found the optimum temperature for WAS to be 175 °C. Li and Noike (1992) found that the methane yield of WAS leveled off at a temperature of around 150 °C. Our results show increase in methane yields for the centrate from both cakes for temperatures up to 175 °C and treatment time 30 min.

The differences in solubilization and specific methane yield between the HRSD and HRA cakes can have several explanations; first, the pretreatment was performed with two different THP pilots and with different water-content in the incoming sludge cake (14.2% TS in HRSD cake and 18.5% TS in HRA cake; data not shown). This could have had an effect on the mixing of steam and digestate cake in the mini Cambi SE test unit, giving a lower treatment efficiency for the HRA compared to HRSD.

Second, it has been shown that the effect of THP depends on waste characteristics (Bougrier et al., 2008; Wilson and Novak, 2009), and the characteristics of the two cakes used in this experiment were different (Table 1). The HRSD cake, coming from an anaerobic digester treating sludge from a Bio-P plant, had a higher ash content compared to the HRA cake from the food waste plant and a lower COD:VS ratio. The HRSD cake also had a lower carbon content and higher nitrogen and sulfur contents. Plant material and lipids generally have a high C:N ratio, while protein have lower C:N ratios. A high COD:VS ratio is an indication of a more energy dense material, containing for instance more lipids. Further, analysis of acid detergent fiber (ADF), confirmed a larger fiber fraction (74% higher ADF) in the digestate cake from HRA compared to the

digestate cake from HRSD. The larger fiber fraction in HRA digestate cake indicates that this waste contains more plant material compared to the HRSD digestate cake.

Primary sludge and WAS has previously been reported to be efficiently solubilized at 165 °C (Wilson and Novak, 2009), and the maximum increase in methane yield have also been found around 30 min treatment time at 165 °C (Haug et al., 1978; Li and Noike, 1992; Stuckey and McCarty, 1984). Bougrier et al. (2008) found that at temperatures lower than 150 °C, carbohydrate solubilization was more important than protein solubilization in activated sludge. Several authors have found that lignocellulosic biomass solubilizes more at treatment temperatures higher than 175 °C (Horn et al., 2011a, 2011b; Vivekanand et al., 2013). Bauer et al. (2014) found only small increases in methane yields when treating late harvested straw at temperatures between 160 °C and 220 °C and treatment times of 15 min, while Lizasoain et al. (2016) tested treatment temperatures between 160 °C and 220 °C and treatment times of 5–20 min, and found that the methane yield of reed was highest for the treatment at 200 °C for 15 min, while only a small increase in methane yield was observed at 160 °C. Hence, a possible explanation of the lower solubilization and lower increase in methane yield of digestate from HRA compared to HRSD could be that the HRA digestate cake has more resemblance to lignocellulosic wastes (i.e. high fiber content) while the HRSD digestate cake may have more resemblance to primary sludge and WAS (i.e. higher protein content). The larger increase in solubilization observed for HRSD cake between temperatures 152 °C and 165 °C compared to the increase in solubilization of HRA cake, could also be explained by a lower protein content in the HRA cake (Bougrier et al., 2008).

3.1.2. Dewatering properties

The costs associated with the transport and disposal of the digestate cake are significant for many AD plants, and with a typical water content of around 80% in the cake, improved dewatering would be beneficial. We determined the dewatering properties as predicted maximum solids for the digestate cakes after post-treatment (Fig. 2c) according to the thermogravimetric method described by Kopp and Dichtl (2001). ANOVA showed that both temperature and cake origin had a significant effect on the maximum cake solids (p-value < .001 and < .01, respectively), where higher temperature resulted in better dewaterability. Above a temperature of 152 °C, the cake solids increased with increasing temperature and treatment time for both digestate cakes. Maximum predicted TS for the treated cakes were 43 and 46% for the HRSD and HRA cakes, respectively.

The dewatering properties of the original untreated cakes were very different: the HRA cake showed the ability to be dewatered to the predicted TS of 34.0% (data not shown), while the HRSD cake only achieved the predicted TS of 17.0% (data not shown) Thus, the post-treatment was clearly most efficient for the HRSD cake.

The observed difference in efficiency of the thermal hydrolysis could again be explained by the origin of the two digestate cakes. In sludge, water is bound inside viable cells (Vesilind, 1994) and between microbial cells in flocs (Higgins and Novak, 1997; Li and Yang, 2007). The amount of bound water is effected by high concentrations of phosphate, disturbing the cation-bridging of the sludge-flocs, as well as the concentration of extracellular polymeric substances (EPS). If the amount of intracellular and floc-bound water is less in food waste cakes (such as the HRA cake), the potential for improving the dewaterability of these types of digestate cakes will also be lower.

The results presented here demonstrate that post-treatment could increase the maximum cake solids in digestate cakes, and thereby cut the transportation costs of AD plants significantly. The predicted increase in maximum cake solids was from 34% up to 46% for the HRA digestate cake, and from 17% up to 43% for the HRSD digestate cake (Fig. 2c). Alone, the increase in maximum cake solids contributes to the reduction of final wet cake mass after post treatment by 26% of the original untreated cake for the HRA digestate cake and 60% of the original untreated cake for the HRSD digestate cake. Hence, the practical implication for plants similar to HRSD of implementation of PAD-THP technology will be that for every ten trucks that is needed for digestate cake transportation today, only four will be needed if thermal hydrolysis post-treatment is implemented. In addition, the relative reduction in wet cake mass will depend on the digestate cake treated. A digestate cake that is already dewatered to 30% before PAD-THP will have less potential for reduced wet cake mass, compared to a digestate cake that only dewaters to 15%. It will therefore be of paramount importance to take several considerations into account when making decisions on post-treatment.

3.1.3. Residual methane potential in cake

The residual methane potential of the cake fraction is of interest when evaluating to what extent the remaining methane potential in the digestate cake has been exploited by PAD-THP. Because of the small volume of post-treated digestate available, and the limitations of the laboratory equipment, it was not possible to produce a cake with equal properties as full-scale cake. The separation in the laboratory centrifuge resulted in a pellet with higher water content compared to the predictions of water content after full scale dewatering presented in section 3.1.2. To overcome this challenge, BMP was measured on the pellet and the results were then corrected by subtracting the BMP coming from the centrate which would be removed during dewatering in a full scale plant. The BMP based on these calculations are presented in Fig. 2d.

ANOVA showed no significant difference in BMP of the two cakes at different THP treatment times or temperatures. BMP of the untreated HRA digestate cake and the HRSD digestate cake treated for 60 min at 70 °C and centrifuged was found to be 75 and 63 mL CH_4/g TS_{added}, respectively (data not shown). The BMP of the treated HRA cakes was in all cases lower compared to the untreated cake, and ranged from 41 to 53 mL/g TS_{added}. The BMP of the treated HRSD cakes was in all cases lower than the cake treated at 70 °C for 60 min, and ranged from 38 to 59 mL/g TS_{added}. The BMP of the centrates was observed to be much higher (Fig. 2b) compared to the BMP of the cake presented here, and improved separation of the liquid fraction will lower the methane yield from the cake. Although there still is some methane potential left in the digestate cakes, it is not evident that this will be emitted as methane during storage or after land application. BMP-tests are designed to give the maximum methane yield from a test material, and will therefore give a worst-case scenario; other parameter such as oxygen and moisture access will influence the methane production from the cake during storage and after land application.

3.2. CSTR experiment

Based on the batch testing, the treatment of digestate cake for 30 min at 165–175 °C gave the best results in regard to solubilization, specific methane yields of the centrate, and dewaterability. In a full scale system, the centrate after post-treatment will be recirculated to a continuous digester. Hence, results from batch experiments do not give the full picture of the impact of solid stream on an AD system. In order to evaluate the impact of the recirculation on continuous processes, two semi-continuous CSTRs were run in parallel, co-digesting food waste and sewage sludge, with a low SRT and high OLR (Table 2). The control digester received hygenized feed which was a mix of sewage sludge and food waste, to meet the health regulations (heat treated at 70 °C for 60 min), while the PAD-

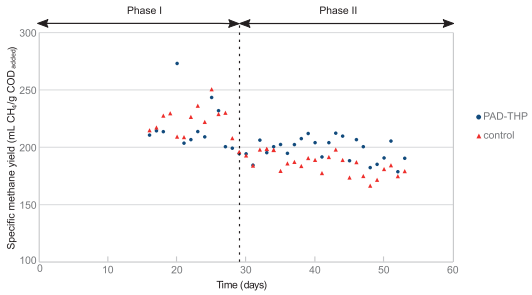

Fig. 3. Volumetric methane yield during the time of the experiment. The first 28 days (Phase I), the PAD-THP reactor did not receive any centrate.

THP digester received unhygenized feed and centrate from the PAD-THP (30 min at 165 °C) HRSD-cake.

During the first phase of the experiment (the first two SRTs), the PAD-THP digester was operated without adding centrate. In this period, the control digester had a higher volumetric methane yield compared to the PAD-THP digester (Fig. 3). From day 28, the second phase of the experiment, centrate was added with the feed to the PAD-THP digester every day. During the second phase, both reactors performed well, achieving COD-reduction of around 70% with low residual VFA-concentrations in the effluent and a close to neutral pH (Table 4). The PAD-THP digester performed better than the control in regard to volumetric methane yield and COD-reduction, with an average increase in volumetric methane yield of 7%. It also had marginally higher concentration of acetic-, propionic- and total volatile fatty acids as well as higher TAN, PO_4^--P and S-COD in the effluent. After the PAD-THP centrate was added, the volumetric yield of the PAD-THP digester was higher compared to the control digester for all days. Both the daily volumetric methane yield and specific methane yield was significantly higher in the PAD-THP digester (p-value < .001 and < .01, respectively) compared to the control digester.

The relative moderate increase in volumetric methane yield (7%) compared to the observed 25% (Kjorlaug et al., 2015) and 50% (Kolovos et al., 2016) increase in volumetric biogas yield in the full scale plant in Germany could be a result of a higher methane yield in the control digester in this study due to the pretreatment of the feed substrates. The hygenization of the feed for the control digester resulted in a different feed sludge composition for the two digesters. Based on the paired *t*-test, three of the measured parameters were significantly different for untreated and hygenized sludge; S-COD (p-value < .001), TAN (p-value < .01) and pH (p-value < .05), while none of the parameters were significantly different for untreated and hygenized food waste. This difference indicates that the pre-treatment solubilized some of the feed sludge and degraded some of the proteins in the sludge (Table 2).

The feed for the PAD-THP digester included centrate. In order to get enough and consistent centrate, it was decided to only use centrate from post-treatment of HRSD cake and not from the laboratory digesters. The COD content of the centrate was to a large degree soluble, with a ratio of S-COD:COD of 92%, had a strength of 39 g COD/L and the TS was 88% organic (Table 2). In comparison, analyses of samples from the full scale Cambi plant in Germany showed a centrate strength of 40 g COD/L and a VS of 85–90% (Kjorlaug et al., 2015). A high conversion rate of food waste to methane could explain why a smaller difference in methane yields was observed in this study compared to the results from the full scale plant (Kjorlaug et al., 2015; Kolovos et al., 2016). The full scale plant does not receive food waste, and the higher the conversion rate in the control digester, the less will the potential of improvement of methane yields be.

In addition to feed composition, the SRT of the two digesters were different. The SRT was set to be 17 days for the control digester, resulting in a SRT of 14 days for the PAD-THP digester. Both SRTs are low compared to the SRTs of conventional full-scale plants, which commonly operates with average SRTs of 20–25 days in the US. We chose a low SRT in order to evaluate the suitability of PAD-THP for digesters that is already operating close to their limit. As many sewage plants implement co-digestion of sludge and food waste, SRTs are also lowered, as long as the digester volume remains unchanged. However, the low SRT in this study could have contributed to the smaller difference between methane yields of the control digester and the PAD-THP digester, compared to what has been observed in the full-scale plant in Germany (Kjorlaug et al., 2015; Kolovos et al., 2016).

Differences in laboratory scale and full-scale configuration may also have influenced the results presented in this section. In a full scale PAD-THP system the solids content of the influent can be higher than in a pre-hygienization system and because of this the SRT can be higher. The centrate will be continuously produced from the effluent and will respond to changes in solids reduction and effluent characteristics. Another difference in full-scale digesters is that they receive feed continuously contrary to the laboratory reactors that was fed once per day. The effects of feeding frequency on anaerobic digesters is not well documented. We think that by using the adaptations described in section 2.4, the results presented in section 3.2 is a conservative estimate of what can be achieved in full-scale.

3.3. Major differences between pre-treatment and post-treatment

Researchers have shown that pre-pasteurization processes can

Table 4
Results from CSTR experiment. Standard deviations are listed in parenthesis.

	Unit	Control Digester	PAD-THP Digester
COD reduction	%	68 (2)	74 (1)
VS reduction	%	63 (1)	72 (1)
TAN[a]	mg/L	2110 (253)	2161 (466)
PO_4-P	mg/L	499 (38)	542 (64)
Acetic acid	mg/L	73 (14)	84 (19)
Propionic acid	mg/L	16 (7)	23 (9)
tVFAs	mg/L	98 (15)	120 (31)
S-COD	g/L	5 (1.6)	8 (0.9)
pH		7.3 (0.1)	7.3 (0.1)
Specific methane yield on COD basis	mL/g COD_{added}	186 (9.9)	197 (9.4)
Specific methane yield on VS basis	mL/g VS_{added}	365 (17)	415 (20)
Volumetric methane yield	L/L*d	1.32 (0.060)	1.41 (0.067)

[a] TAN = total ammonia nitrogen.

experience reactivation and regrowth of indicator organisms such as fecal coliforms and *E. coli* (Chen et al., 2011; Higgins et al., 2007). This is thought to be due to inadequate time-temperature treatment associated with pre-pasteurization which does not completely inactivate the organisms. With PAD-THP, all digestate cake is treated at 165 °C for 30 min, which effectively inactivates pathogens, minimizing the risk of reactivation and regrowth. Pre-THP would also achieve the same effect and minimize the risk of reactivation and regrowth.

Post-digestion treatment will improve the overall energy balance. The amount of solids that are heat treated is less with PAD-THP compared to pre-AD hygienization or pre-THP because of the solids reduction that occurs during digestion. For example, in comparing pre-THP to PAD-THP, the heat treatment is applied at the same solids concentration, around 16%, but with PAD-THP, the total solids to be treated could be reduced by 50% or more due to biodegradation that occurs in the digester. Increased solids reduction and conversion to biogas influences the energy balance both directly through methane production and indirectly through lowering the heat loss of the PAD-THP system. With PAD-THP most of the energy used for sterilization is returned to the digester for heating by returning the centrate.

Another advantage of PAD-THP compared to pre-THP and pre-hygenization is the improvement in cake solids during final dewatering which can have a large beneficial impact on economics. Pre-THP has been shown to improve final dewaterability after digestion, however, digestion reduces the extent of dewaterability compared to the solids immediately after pre-THP. Hasan et al. (2017) reported that the cake solids after dewatering of a mixed primary and secondary sludge that had undergone pre-THP was around 43%, but after subsequent anaerobic digestion the cake solids decreased to around 31%. In the PAD-THP scheme, the solids are thermally hydrolyzed and dewatered immediately, which improves cake solids. In addition, the solids are dewatered at higher temperatures immediately following PAD-THP which further improves cake solids.

4. Conclusion

This study obtained novel insights into the differences in the effect of PAD-THP on digestate cakes from a food waste plant and a wastewater treatment plant.

Post-treatment improved methane yields both in batch and in a semi-continuous system and improved the extent of dewaterability of the digestate cakes. The effect of the post-treatment was influenced by digestate cake characteristics, and the treatment was more efficient for the cake from a plant treating sewage sludge compared to the cake from a plant treating source separated food waste. Improved VS reduction in the anaerobic digester and improved dewatering of the treated digestate cake assured that the final cake product was stable, with low residual methane potential. Our estimates suggests a reduction in final wet cake mass due to improved dewatering from PAD-THP of 60% of the original wet cake mass for digestate cake from an anaerobic digester treating sewage sludge from a Bio-P plant.

Our results indicate that thermal hydrolysis of digestate cake is an efficient technology for improving methane production and dewaterability in conventional anaerobic digesters, and performs better compared to the conventional pre-treatment technology at 70 °C. Practically, the technology will improve methane yields and the extent of digestate dewaterability, increase the AD plants income through higher methane production and reduce the AD plants transportation and disposal costs through reduction of final wet cake mass.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.01.008.

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Paper IV

Stable performance of laboratory and full-scale anaerobic co-digesters treating food waste and sewage sludge at 62 °C

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Abstract

Thermophilic anaerobic digestion at temperatures above 60 °C have recently gained interest as a means of in situ sludge sanitation. However, propionate accumulation is a persisting concern in thermophilic digesters. In this study, we mimicked a full-scale anaerobic serial digestion plant successfully operating in the temperature range 60-64°C with low effluent propionate concentrations using 6L laboratory digesters. Our results demonstrated that low residual propionate concentrations could indeed be achieved. Moreover, we found that acetoclastic methanogens dominated the full-scale archaeal communities, while hydrogenotrophic methanogens dominated in the laboratory digesters. Hence, although the laboratory digesters showed similar low VFA concentrations as the full-scale digesters, process performance and microbial community structure were different.

1. Introduction

Anaerobic digestion (AD) is commonly used to stabilize and sanitize organic waste and simultaneously produce the valuable biofuel methane. The sanitation is either performed by pretreatment at temperatures >70 °C in combination with AD at mesophilic (20-40 °C) temperatures or by operating the digester at thermophilic (50-70 °C) temperatures. Thermophilic operation removes the need for pretreatment or heat exchange before AD. Additionally, higher process temperatures allows treatment at lower retention times, improving AD plant efficiency. Although thermophilic process efficiency has been studied in lab-scale over several decades (Ahring, 1994; Labatut et al., 2014; Ryan et al., 2010; Zinder et al., 1984), unstable process performance with high concentrations of soluble chemical oxygen demand (SCOD) and volatile fatty acids (VFAs) in the effluent is still a challenge. The suggested reasons for the observed problems with stability in thermophilic AD are the relatively low microbial diversity and higher microbial sensitivity towards inhibitors (e.g. ammonia and long-chain fatty acids) at high temperatures.

Most research on thermophilic AD has been performed at 55 °C, and Ahring (1994) has suggested that in cow manure digesters operational temperatures should be kept below 60 °C. However, previous studies have also found that elevating temperatures improves solubilization of particulate material (Lee et al., 2009). Recently, new studies on thermophilic anaerobic digestion have suggested that temperatures above 60 °C are more efficient. Chen et al. (2017) investigated the optimal temperature for AD of sludge with the aim of in-situ sludge sanitation, and found that the optimal temperature for AD of the sludge tested was 65 °C. In their study, the AD treated a mix of primary and secondary sludge dry solids < 2%) and the authors requested more research, in particular on substrates with higher dry solids and energy density, to improve the thermophilic AD energy balance. Such substrates with higher

dry solids and energy density are for example primary sludge and food waste. In fact, in Norway, a full-scale plant operating in the higher thermophilic range (60-64 °C) already treats primary sludge and food waste. The full-scale plant consists of two digesters operated in series. Moreover, the digester effluent has low VFA and SCOD concentrations (Hagen et al., 2017).

The microbial community of this thermophilic full-scale plant has been studied by Hagen et al. (2017), but more studies are needed to characterize and better understand this stable thermophilic AD system. We therefore designed a laboratory experiment operated at 62 °C with the same substrates as the full-scale digester: primary sludge and food waste. The main objective of this work was to establish and investigate the process performance of a laboratory scale AD system mimicking the full-scale plant, and achieve low residual VFA concentrations in the digesters effluent. Moreover, another objective was to compare the microbial community structures in the laboratory scale digesters with the full-scale system. Based on the scale comparison it was investigated if the laboratory scale digester data were suitable for predicting full-scale performance.

2. Materials and methods

In this study, the efficiency and microbial community structure of two serial laboratory anaerobic digestion systems operating at 62 °C were investigated. To study process performance, laboratory continuous stirred tank reactors (CSTRs) fed once daily were used. Microbial community structure in laboratory and full scale digesters was studied by sequencing of the V3-V4 hypervariable region of the 16S rRNA gene.

2.1 Laboratory digester configuration

Four laboratory digesters (BELACH BIOTEKNIK AB, Stockholm, Sweden) were operated with a working volume of 6 L at 62 °C and a stirrer speed of 100 rpm. Gas production volume was measured with water displacement, and gas composition was measured every hour using gas chromatography as previously described (Zamanzadeh et al., 2016). Initially, two of the digesters (A1 and B1) were filled with 6 L of inoculum from a laboratory CSTR which had been operating for 6 months digesting undiluted food waste and sewage sludge with a hydraulic retention time (HRT) of 20 days. This laboratory digester was originally started with inoculum from the full-scale FREVAR plant. The inoculum reactor was operated at 62°C with an organic loading rate (OLR) of 3 gVS/L/d.

To model the full-scale plant, the four digesters were operated as two serial digestion systems, A and B, where in each system the first reactor, A1 and B1, received fresh feed every day and the second reactor, A2 and B2, received the effluent from the first reactor to mimic the full-scale biogas plant (Figure 1). When the experiments started, the first reactor in each serial digestion system (A1 and B1) received fresh feed with an OLR of 3 gVS/L/d and an HRT of 20 days. The OLR and HRT was gradually ramped up to 6 gVS/L/d and 10 days, respectively, over a 2 week period to reach the average organic loading rate and hydraulic retention time

(9-10 days) of the full scale plant. The working volume was maintained at 6L throughout the ramp up. The effluents from the first stage reactors were then added to the second stage reactors (A2 and B2) until the volume of the second stage reactors also reached a volume of 6 L. After the second stage reactors had reached a volume 6 L, effluents were taken from these reactor every day to maintain the same volume. After 63 days, reactor A1, which had low activity, was emptied and inoculated with effluent from A2. From this day the feed recipe was also changed, and the first reactors in both systems was fed a more dilute feed at the same OLR, reducing the HRT from 10 to 9 days in each reactor. This adjustment was made since measurements showed that the total ammonia concentrations in the laboratory digesters were higher than in the full-scale plant. The exact feed composition of the full-scale plant in unknown, however, plant operators assume a 50:50 ratio on VS basis for food waste and sludge and the effluent volume over the course of one year operation corresponded to a hydraulic retention time of 9.1-9.3 days in each digester. Throughout the experiment, the digesters were fed 6 days a week. The experiment lasted for 252 days.

During the time-period of the laboratory experiment, samples for microbial community analysis was taken twice from the first and second full-scale digesters, FS1 and FS2, to compare the microbial community in the laboratory and full-scale digestion systems.

2.2 Materials

Food waste and sludge was used as the substrate blend for anaerobic digestion in both full scale and laboratory scale digesters. The food waste originated from health institutions and restaurants, and was collected from Norwegian Food Recycling (Norsk Matretur AS, Lørenskog Norway) after hygienization at 70 °C for 1 hour. Sewage sludge was collected from the wastewater plant FREVAR (Fredrikstad, Norway) after hygienization at 70 °C for 1

hour. The wastewater plant uses chemical treatment with iron precipitation, hence, the sludge can be characterized as primary sludge. Both sewage sludge and food waste was frozen, and a new batch was thawed and stored at 5 °C approximately once per month. The characteristics of the sludge and food waste are shown in table 1.

2.3 Sampling and chemical analysis

Samples for total solids (TS), volatile solids (VS), ash, pH, chemical oxygen demand (COD), total ammonia nitrogen (TAN) and volatile fatty acids (VFAs) were taken immediately after the daily discharge of effluent approximately once per week. Samples from the full-scale reactors were taken twice during the experimental period, on day 41 and 217.

TS, VS and ash were determined gravimetrically by drying at 105 °C and subsequent burning at 550 °C. pH was measured using a pH electrode (Orion, Thermo Scientific, MA, USA) and pH/ISE meter (Orion Dua Star, Thermo Scientific). COD was determined using Merck Spectroquant® commercial kits (Merck KGaA, Darmstadt, Germany). TAN was determined using an ion selective electrode (Orion 93, Thermo Scientific). VFAs in the effluent were analyzed as previously described by (Estevez et al., 2014). Total VFAs (FOS) and total alkaline carbonate (TAC) were measured by titration (Nordmann, 1977).

2.4 DNA extraction and purification

Samples for microbial community analysis were collected by withdrawing effluent at the start of the experiment and on day 7, 63, 109 and 231. The samples were collected in 15 mL centrifuge tubes and immediately stored at -20 °C until DNA extraction. Genomic DNA from each sample was extracted in triplicate using the Power-Soil DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). The entire process was carried out according to the

manufacturer's protocol, with minor modification of the bead beating whereby a Precellys®24 homogenizer was used at 5000 rpm for 20 seconds. The extracted and purified DNA was pooled for each individual sample and measure fluorometrically on a Qubit™ fluorometer (Life Technologies, Eugene, OR, USA) using the Quant-IT™ dsDNA HSAssay Kit (Invitrogen, Carlsbad, CA, USA). On average, a concentration of 172 ± 146 ng/µL DNA was extracted from each digester sample.

2.5 Sequence library preparation

We selected the V3-V4 hypervariable region of the 16S rRNA gene for construction of amplicon libraries to be sequenced on Illumina MiSeq. To do this, two steps of PCR were sequentially performed. In the first PCR, 16S primers as described by Takahashi et al. (2014), Pro341F (5'-CCTACGGGNBGCASCAG -3') and Pro805R (5' GACTACNVGGGTATCTAATCC -3)'were modified by appending a sequence complementary to the binding site of the Illumina flow cell. In the second PCR, a unique 12 bp index was integrated to the reverse site of each sample library using NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0 (Bioo Scientific Corporation, Austin, TX, USA).

The first PCR was carried out in a reaction mixture (50 µl) consisting of 100 ng genomic DNA, 1.0 unit of Platinum® Pfx DNA polymerase (Invitrogen), 1mM $MgSO_4$ and 0.3 µM of each primer. The PCR reaction was started with an initial denaturation at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 15 sec, annealing at 55 °C for 30 sec, extension at 68 °C for 45 sec, and completed by a final elongation at 68 °C for 10 min. The first PCR products were cleaned up using Agencourt AMPure XP beads (Agencourt Bioscience Corporation, Beverly, MA, USA) following the protocol of the NEXTflex™ kit. In the second PCR twelve amplification cycles were used, following the NEXTflex™

protocol. The PCR products were purified with AMPure beads, and DNA concentrations were measured on Qubit. Amplicon libraries were normalized and pooled in equimolar concentrations to create a multiplexed library pool. This resulting library pool was furtherly purified by gel extraction (E-Gel 1% agarose, Invitrogen, and MinElute Gel extraction Kit, Qiagen, Hilden, Germany). The final quality of the library was verified by agarose gel electrophoresis in addition to spectrophotometry with Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA). The multiplexed library pool was sequenced on an Illumina MiSeq with paired-end 300 bp cycle run using MiSeq reagent kit V3 (Illumina Inc., San Diego, CA, USA) at the Norwegian sequencing center (Oslo, Norway).

2.6 Sequence analysis

Sequence analysis was conducted using the Quantitative Insight Into Microbial Ecology (QIIME) version 1.9.1 (Caporaso et al., 2010). Single-end reads were merged using PEAR (Zhang et al., 2014) before quality filtering with PRINSEQ (Schmieder and Edwards, 2011) using minimum quality score 20, average quality score 30, minimum length 300 and maximum length 500, before downstream analysis. VSEARCH (Rognes et al., 2016) was used for detection of chimeric sequences (uchime_denovo with default parameters), followed by open reference clustering with USEARCH61 (at 97% sequence identity) of non-chimera sequences and denovo picking of operational taxonomic units (OTUs). The OTUs were assigned to taxonomy with QIIME's uclust-based taxonomy assigner with the Greengenes database and singletons were removed as they tend to contain disproportionate errors. Finally, the arithmetic mean of values from the PCR triplicates was used in data analysis.

2.6 Data accessibility

Sequence data are available at NCBI Short Read Archive under accessions SRR7458332 to SRR7458398 as part of BioProject PRJNA478694.

2.7 Data exploration and statistical analysis

Statistical testing of the process parameters was done using the two-sided paired student t-test at 0.05 significance. Principal component analysis of the correlation matrix of the process parameters was done in PAST version 3.17 (Hammer et al., 2001). β-diversity was calculated with the UniFrac distance metric (Lozupone and Knight, 2005), with a cutoff at 15 000 sequences per sample.

205 **3. Results and discussion**

206 3.1 Reactor performance in laboratory scale

207 The digesters reached steady state conditions for methane yields, pH and alkalinity after about
208 100 operating days (Figure 2). After 210 days, all laboratory digesters reached low residual
209 VFA concentrations with propionate concentrations below 200 mg/L and acetate
210 concentrations below 50 mg/L. Hence, the experiment confirmed that stable operation of
211 anaerobic digesters at temperatures above 60 °C with high effluent quality is possible when
212 treating food waste and primary sludge.

213

214 During steady state performance, the first stage digesters in each series, A1 and B1, produced
215 on average 2600 ± 58 mL/L/d methane (Figure 2A). In comparison, the second stage digesters
216 in each series, A2 and B2, produced 166 ± 9 mL/L/d accounting for only 6 % of the total
217 methane produced in each serial system. This low production of methane from the second
218 stage digester indicates that most of the feed's methane potential was recovered in the first
219 digesters. A higher loading rate in the first digester is therefore possible and can improve the
220 overall digester system's performance.

221

222 The process performance prior to steady state conditions included large differences between
223 the parallel digesters and process failure indications. Specifically, digester A1's performance
224 deteriorated rapidly upon startup, and it only produced on average 57 ±13 mL/L/d methane
225 during the first eight weeks. This coincided with pH and alkalinity depletion, and a spike in
226 acetic acid and FOS concentrations. Meanwhile, its replicate digester, B1, produced more than
227 2000 mL/L/d every week from the second operating week. Simultaneously, methane
228 production also increased in A2, eventually approaching B1's performance, and thereby
229 demonstrating resilience and flexibility in the serial system. However, because our aim was to

establish replicate steady state systems, we replaced the content of A1 with effluent from digester A2 on day 63 and recovered A1's methane production. Eventually, the process parameters in A1 and B1 during steady state performance were not significantly different.

Before all digesters reached low VFA concentrations in the effluent, we observed propionic acid concentrations above 200 mg/L in digester A1 and B1. Propionate accumulation is common in thermophilic systems and Ahring et al. (2001) reported severe inhibition of propionate oxidation at 65 °C. Specifically, they observed 700 mg/L of propionate after operating a CSTR for 90 days combined with no propionate degradation in specific methanogenic activity tests at 65 °C. In contrast, our system reached propionate concentrations below 200 mg/L after 210 days. Hence, the trend from the initial high propionate levels to very low levels reported here, illustrates that short-term studies of thermophilic anaerobic digestion processes might wrongfully conclude that propionate accumulation is inevitable in AD systems operated at temperatures above 60 °C. Moreover, our observations of final propionic acid concentrations below 100 mg/L in the first stage and below the detection limit in the second stage are consistent with the findings of Chen et al. (2017) and Hagen et al. (2017), who reported 70-140 mg/L and 32 mg/L propionate in their studies, respectively. It should be noted that from 70 days to 150 days it was observed simultaneous high propionic acid concentration in A1/B1 and low propionic acid concentration in A2/B2, proving propionic acid degradation in the stage 2 reactors. Hence, we conclude that anaerobic digesters can be operated at temperatures above 60 °C without propionic acid accumulation, even with feed concentrations of 4-5 % VS.

3.2 Comparison of full-scale and lab-scale process performance

The full-scale plant operators regularly measures pH, FOS, TAC, TS and VS in the effluent, and gas volume produced is also recorded. However, they do not measure the TS in the influent or the methane content in the biogas. We compared the average values for the abovementioned parameters in the full-scale and lab-scale systems (Table 2).

TAN was one of the design parameters, and its concentration was similar in the second stage laboratory and full-scale digesters. However, in the first stage digester TAN concentrations were lower in the full-scale plant. Moreover, the alkalinity was similar in both stages and for both laboratory and full scale digesters. FOS was on average higher in the first stage laboratory digesters, while in the second stage, the laboratory and full-scale digesters performed similarly well. However, the pH in the laboratory digesters was significantly higher. Moreover, the TS concentration was more than twice as high in the full-scale digester. The estimated methane yield was lower in the full-scale system.

Thus, there are both similarities and differences between the process parameters in the full-scale digesters and the lab-scale digesters. More concentrated feed, and lower degradation in the full-scale digester would contribute to the higher effluent TS. However, more concentrated feed would normally contribute to elevated TAN and TAC concentrations, unless the proteolysis and methanogenesis is severely reduced. Another, more likely explanation is therefore that the plant was treating more sewage sludge compared to food waste, which will normally result in lower TAN concentrations and lower methane yields. Moreover, feed volumes, hydraulic retention time and gas production rates varies in the full scale digesters because weather conditions influences the sludge generation in the wastewater treatment plant. Finally, from the fact that the effluent TS concentration is higher in FS2 than the feed

concentration in the lab-scale digesters, it can be deduced that the VS loading rate in FS1 was higher than that in A1 and B1.

3.3 Microbial community diversity and composition

Although the laboratory and full-scale digesters had similar operational conditions in terms of pH, VFAs, TAN and TAC, the microbial community in the laboratory and full-scale digesters were different. Specifically, phylogenetic diversity and richness in laboratory digesters and full-scale digesters differed, where full-scale reactors had significantly higher diversity (Figure 3). PCoA analysis of the weighted UniFrac distance metric showed that the microbial community structure clearly differed between laboratory and full-scale digesters (Figure 4). Among the laboratory digester samples, one stands out; digester A1 on day 7. The performance of this digester was different compared to other laboratory digesters (previously described in section 3.1) and thus not representative, and was therefore not included in the following discussions. On day 63, A1 was inoculated with the effluent from A2, hence, the microbial community in A1 and A2 was the same.

To explore the differences in microbial community structure, we first determined the dominating phyla in the bacterial and archaeal kingdoms. For *Bacteria*, the dominating phylum was *Firmicutes* in all digesters (Figure 5). For *Archaea*, we observed that *Methanosaeta* dominated the communities in the full-scale reactors, whereas an unassigned genus of *Methanobacteriaceae* dominated the laboratory-scale reactors. Because *Methanosaeta* and *Methanobacteriaceae* exploit different methanogenic pathways, this finding demonstrates a fundamental difference in the microbial community functioning under laboratory and full-scale conditions. Specifically, *Methanosaeta* produce methane through acetate cleavage, while *Methanobacteriaceae* produce methane through CO_2 reduction with

H_2. Moreover, such differences in methanogenic communities are linked to differences in affinity and growth rate as well as syntrophic partners, and many studies have therefore attempted to identify the selective forces that determine the dominating methanogenic pathway (De Vrieze et al., 2012; Hattori, 2008).

3.3.1 Methanogenic pathways in full-scale and lab-scale reactors

The obligate acetoclastic genus *Methanosaeta*'s dominance in the full-scale archaeal communities contrasts other studies on thermophilic anaerobic digesters operated at temperatures above 60 °C. Most studies report a shift from acetoclastic methanogenesis to the syntrophic acetate oxidation - hydrogenotrophic methanogenesis (SAO-HM) pathway at temperatures above 60°C (Chen et al., 2017; Ho et al., 2016; Watanabe et al., 2017) and at elevated free ammonia concentrations (Kato et al., 2014). However, Hagen et al. (2017) observed that FS2 contained a strain belonging to *Methanosaeta* which was highly metabolically active. Yet, their results from the 16S rRNA gene sequencing showed that hydrogenotrophic methanogens belonging to *Methanobacteriaceae* dominated during this earlier sampling time.

In addition to the acetoclastic methanogens, some hydrogenotrophic methanogens were present in the full-scale digesters. In fact, 5 % of *Archaea* belonged to obligate hydrogenotrophic methanogens and 2 % to *Methanosarcina*, a genera known to use both methanogenic pathways. This presence of hydrogenotrophic methanogens indicates that both methanogenic pathways were utilized in the full-scale system. Thus, in anaerobic digesters operated at temperatures above 60 °C both methanogenic pathways can contribute to methane production

328 The dominance of *Methanobacteriaceae* in the lab-scale digesters, however, is consistent with
329 other authors' observations (Chen et al., 2017; Hagen et al., 2017; Ho et al., 2016; Watanabe
330 et al., 2017). Temperature, ammonium concentrations, organic loading rate and hydraulic
331 retention time are considered the parameters determining the methanogenic pathway in
332 anaerobic digesters (De Vrieze et al., 2012; Hattori, 2008). Yet, temperature, ammonium
333 concentrations, and hydraulic retention time in our laboratory digesters were similar to the
334 full-scale digesters by design. Moreover, the organic loading in the FS digesters was higher,
335 which is more favorable for hydrogenotrophic methanogens. Hence, our results imply that
336 other selective forces determines the dominating methanogenic pathway in this case. Another
337 difference between the full-scale and lab-scale digesters is feeding frequency, which has been
338 shown to influence fluctuations in VFA concentrations (Mauky et al., 2015; Mulat et al.,
339 2016) and the methanogenic community (Conklin et al., 2006). The storage of the feed was
340 also different, which for the laboratory digesters were stored for several weeks at 5°C. The
341 finding of *Archaeal* communities dominated by *Methanosaeta* in full-scale and
342 *Methanotermobacteriaceae* in lab-scale, although process conditions were fairly similar,
343 further indicates that these digesters were operated close to conditions where both acetoclastic
344 methanogenesis and syntrophic acetate oxidation is possible.
345
346 3.3.2 Propionic acid accumulation in lab-digesters and its link to the microbial community
347 structure
348 Despite the differences in methanogenic community structures, low propionate and acetate
349 concentrations were observed in both lab-scale and full-scale digesters. Rapid and extensive
350 acetate removal is key for obtaining low effluent propionate concentrations (Beaty and
351 Mcinerney, 1989; Fukuzaki et al., 1990). Hence, the low concentrations demonstrates the
352 success of both the acetoclastic and the SAO-HM pathway, as well as propionate oxidation. In

353 the laboratory digesters, the syntrophic acetate oxidizer, *Thermoacetogenium,* had a relatively
354 high abundance (Figure 5). Moreover, Hagen et al. (2017) found that *Thermoacetogenium*
355 *phaeum* was the most numerically abundant phylotype inferred in acetate oxidation in their
356 sample from FS2.
357
358 Propionate was the VFA that reached the highest concentrations and persisted at elevated
359 concentrations for the longest time in the laboratory digesters. Propionate oxidation has been
360 identified in species belonging to the four genera *Syntrophobacter*, *Smithella*, *Pelotomaculum*
361 and *Desulfotomaculum*. Hagen et al. (2017) found that *Pelotomaculum Thermopropionicum*
362 was the only organism detected in the full-scale digester that potentially could produce the
363 enzymes needed for oxidizing propionate to acetate and CO_2. In our study, *Pelotomaculum*
364 was the only genera of the four aforementioned genera represented in all samples. This
365 indicates that *Pelotomaculum* oxidized most of the propionate in the digesters we studied.
366
367 Propionate oxidation is in addition to acetate, inhibited by long-chain fatty acids (Lier et al.,
368 1993). This links propionic acid accumulation and β-oxidation. Taxa belonging to
369 *Syntrophomonadaceae* and *Syntrophomonas* are central in β-oxidation in anaerobic digesters
370 (Ziels et al., 2017) and we observed an increase of *Syntrophomonas'* relative abundances in
371 the first stage reactors in the laboratory experiment throughout the experimental period
372 (Figure 6). This increase in *Syntrophomonas* can indirectly be one of the reasons for the
373 consumption of propionate towards the end of the laboratory experiment.
374
375 3.3.3 Coprothermobacter and syntrophic acetate oxidation
376 The most abundant bacterial genus both in the laboratory and full-scale digesters was
377 *Coprothermobacter*. This is consistent with previous findings for anaerobic digesters operated

at temperatures above 60 °C (Chen et al., 2017; Gaby et al., 2017; Hagen et al., 2017; Ho et al., 2016). In previous studies, Coprothermobacter have been suggested to grow syntrophically with acetate oxidizers, *Methanosarcina* and *Methanothermobacter* (Gagliano et al., 2015) or even be directly responsible for syntrophic acetate oxidation (Ho et al., 2016). Because the growth of Coprothermobacter spp. on protein rich substrates are closely associated with hydrogen production, Gagliano et al. (2015) argued that Coprothermobacter spp. should benefit from the activities of the hydrogenotrophic methanogens in anaerobic digesters. It is therefore interesting that they also dominated the microbial community in the full-scale digesters. However, since the archaeal community in the full-scale digesters were not exclusively acetoclastic, and 5 % assigned to *Methanobacteriaceae*, there could still be a syntrophic association between *Coprothermobacter* and *Methanobacteriaceae* in the full-scale digesters. Moreover, although Coprothermobacter benefits from Methanobacteriaceae, they are not dependent on hydrogenotrophic methanogens to grow (Sasaki et al., 2011). And as one of few proteolytic thermophiles (Gagliano et al., 2015) it is expected to be present in thermophilic digesters treating protein rich substrates such as sewage sludge and food waste.

3.3 Practical implications

Full-scale anaerobic digestion plants that have more than one reactor usually operating these in parallel. Parallel operation can be advantageous when operators must perform maintenance work, such as removing sand and grit, in one reactor. For startup of the maintained digester, operators use effluent from other digesters as inoculum. The results presented here demonstrates that a serial digestion system also has this flexibility. When A1 did not produce methane, A2 produced similar amounts as B1. When A1 needed to be restarted, inoculum from A2 was successfully used and the methane production from the A system soon became similar to that of the B system. It should be noted that the second reactor in each system has a

large unused capacity, as it only produced 6 % of the total methane yield of each serial system, indicating that the plant performance can be improved through treating more waste.

Conclusion

The laboratory digesters' TS, pH and biogas yields differed from the full-scale system, while VFA, TAC and FOS concentrations were similar. Specifically, low effluent propionate concentrations was demonstrated in the laboratory digesters. However, the methanogenic communities differed between the full scale and lab scale systems, and demonstrated that both acetoclastic and SAO-HM pathways can dominate anaerobic digesters operated at temperatures above 60 °C. Consequently, the laboratory-scale digesters cannot predict all performance parameters of the full-scale digesters. Our results support that *Coprothermobacter* dominates thermophilic anaerobic digestion in the temperature range 60-64°C regardless of the methanogenic community and scaling effects, and that low residual propionate concentrations are achievable under these conditions.

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527
528

529 ## Tables

530 **Table 1:** Feed characteristics. mean ± standard deviation.

	Sewage Sludge	Food Waste
TCOD (g/L)	120 ± 22	278 ± 45
Total ammonia nitrogen (mg/L)	257 ± 98	619 ± 76
TS (%)	8 ± 2	17 ± 2
VS (% of TS)	74 ± 4	90 ± 1
pH	6.0 ± 0.5	3.83 ± 0.07

531

532

533 **Table 2:** Comparison of process parameters in the laboratory digester during steady state and the full-scale
534 digesters. Averages ± std.

	A1 and B1	FS1	A2 and B2	FS2
pH	8.0 ± 0.08	7.4 ± 0.2	8.3 ± 0.07	7.6 ± 0.2
FOS (mg HAc^-/L)	821 ± 212	561 ± 216	484 ± 345	597 ± 243
TAC (mg HCO_3^-/L)	3121 ± 101	3100 ± 666	3983 ± 146	3854 ± 668
TS (%)	-	n.d.	1.9 ± 0.1	4.6 ± 1.8
VS/TS (%)	-	n.d.	58 ± 0.7	49 ± 5
TAN (g/L)	901 ± 88	677	1000 ± 80	1140
Methane (mL/L/d)			1359 ± 78[1]	914 ± 361[1]

535 Averages for the laboratory digesters are based on both A and B systems after steady state conditions were
536 reached. For the full-scale digesters, averages was calculated based on one year continuous operation, With the
537 exception of TAN, which was only measured for the samples taken for microbial community analysis. Methane
538 yield for the full-scale digester was calculated assuming 60% of the biogas was methane. [1]Total methane
539 production for first and second stage digesters.
540

541

Figure captions:

Figure 1: Digester setup. Laboratory digesters were operated in series, where all fresh feed was added to digester 1, and the effluent from digester 1 was fed to digester 2.

Figure 2: Digester process performance. The dashed line marks day 63, when digester A1 was reinoculated and the hydraulic retention time of all digesters were reduced to 7 days. A) Volumetric methane yields, B) pH, C) Total inorganic carbon (TAC), D) Volatile fatty acids determined titrimetrically, E) Acetic acid, F) propionic acid, G) Total ammoniacal nitrogen (TAN). The arrows in A), B) and C) indicates the day when this parameter reached steady state conditions.

Figure 3: Phylogenetic alpha diversity in the laboratory and full-scale digesters.

Figure 4: PCoA plot of the weighted unifrac metric on 16-S rRNA gene sequencing results of samples from laboratory and full-scale digesters.

Figure 5: Barplot of genera relative abundances in samples from the laboratory and full-scale digesters.

Figure 6: Relative abundance of *Syntrophomonas* vs. sampling day in laboratory digesters. A) digester A1, B) digester B1, C) digester A2, D) digester B2.

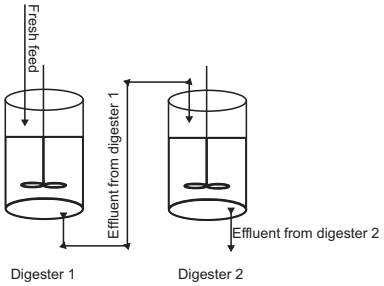

Fresh feed
Effluent from digester 1
Effluent from digester 2
Digester 1
Digester 2

Figure 1

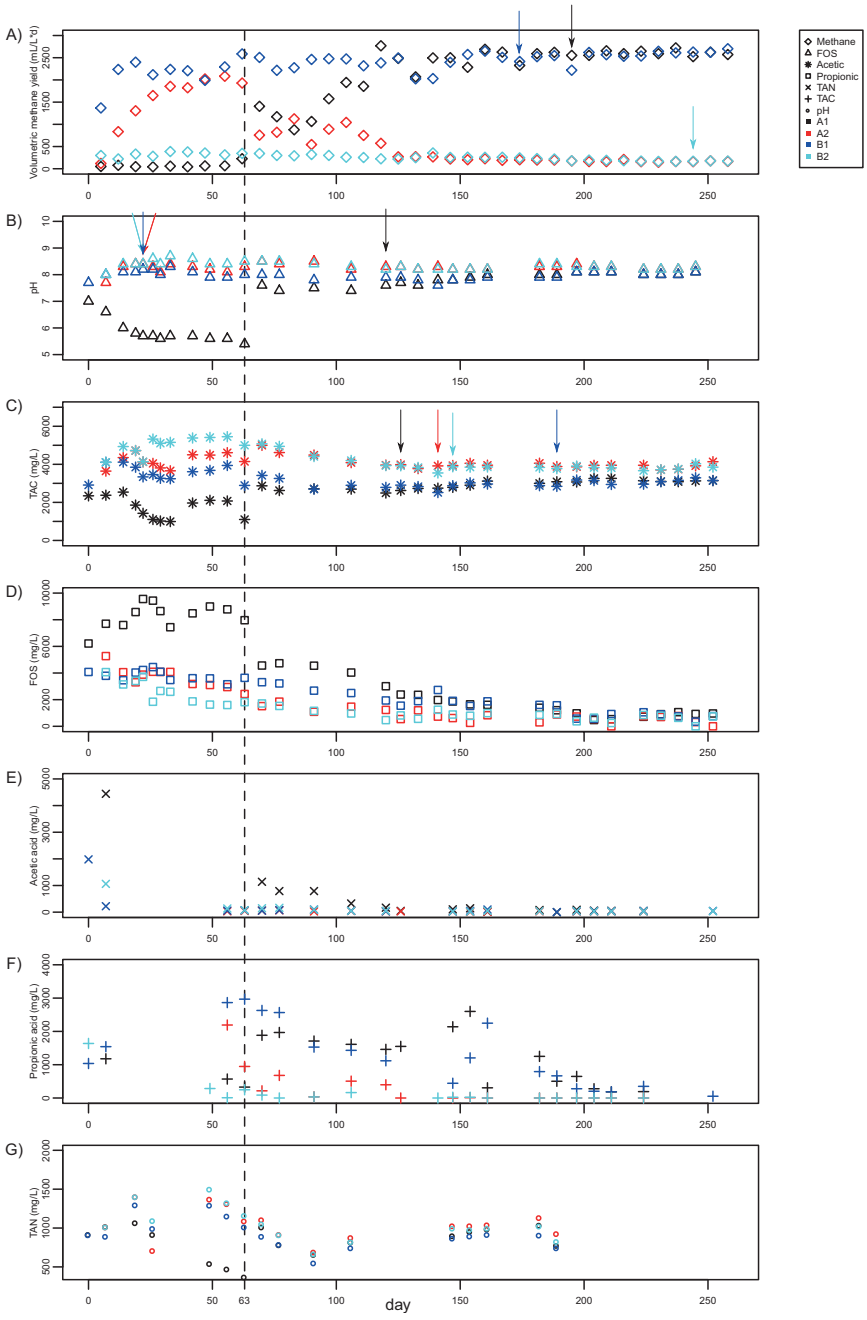

A)
Volumetric methane yield (mL/L*d)
B)
pH
C)
TAC (mg/L)
D)
FOS (mg/L)
E)
Acetic acid (mg/L)
F)
Propionic acid (mg/L)
G)
TAN (mg/L)
day
Methane
FOS
Acetic
Propionic
TAN
TAC
pH
A1
A2
B1
B2

Figure 2

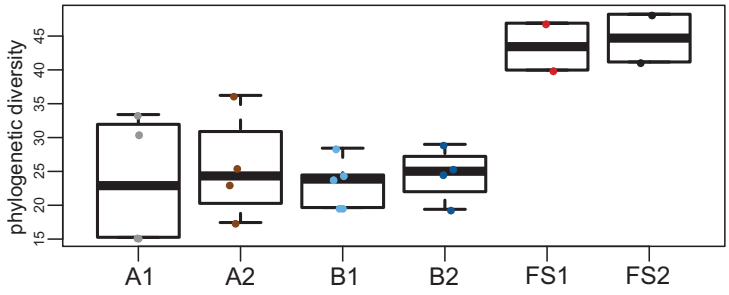
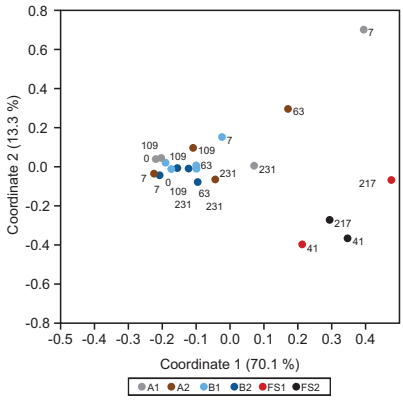


Figure 3


0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Coordinate 2 (13.3 %)
-0.5 -0.4 -0.3 -0.2 -0.1 0.0 0.1 0.2 0.3 0.4
Coordinate 1 (70.1 %)
A1 A2 B1 B2 FS1 FS2
7
63
109
0
109
7
109
63
231
7
0
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63
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231
217
217
41
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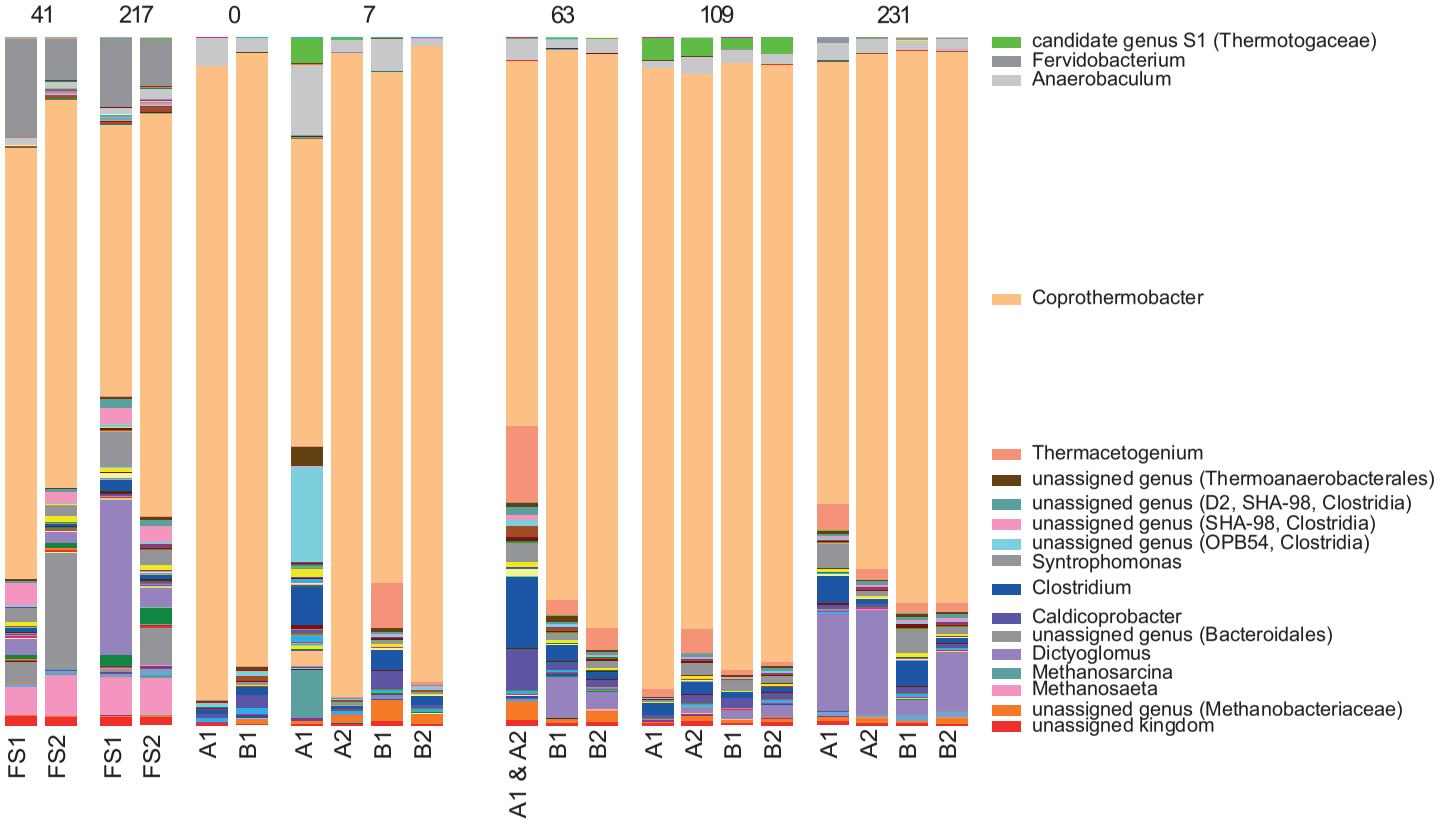
Figure 4


41
217
0
7
63
109
231
FS1
FS2
FS1
FS2
A1
B1
A1
A2
B1
B2
A1 & A2
B1
B2
A1
A2
B1
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B2
candidate genus S1 (Thermotogaceae)
Fervidobacterium
Anaerobaculum
Coprothermobacter
Thermacetogenium
unassigned genus (Thermoanaerobacterales)
unassigned genus (D2, SHA-98, Clostridia)
unassigned genus (SHA-98, Clostridia)
unassigned genus (OPB54, Clostridia)
Syntrophomonas
Clostridium
Caldicoprobacter
unassigned genus (Bacteroidales)
Dictyoglomus
Methanosarcina
Methanosaeta
unassigned genus (Methanobacteriaceae)
unassigned kingdom

Figure 5

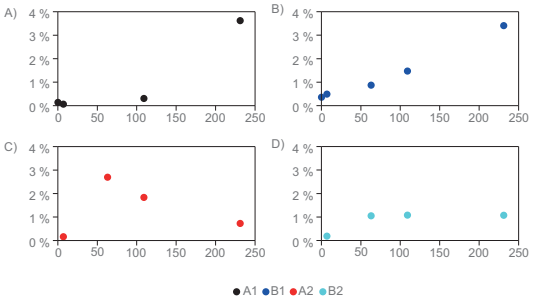

Figure 6

Paper V

Feeding Frequency Influences Process Performance and Microbial Community Composition in Anaerobic Digesters Treating Steam Exploded Food Waste

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Abstract:

In anaerobic digestion, studies of feeding frequency have produced conflicting results. Hence, we investigated the effect of feeding frequency on process variables and microbial community structure by comparing a laboratory-scale digester fed steam exploded food waste 10 times daily (FFD) vs. one fed an equivalent amount once daily. FFD produced on average 20% more methane and had lower effluent concentrations of long-chain fatty acids. Greater daily fluctuations in acetate, pH and biogas production rate could explain the lower specific methane yield and β-oxidation. Feeding frequency also influenced the microbial community whereby *Tenericutes* (42%) dominated in FFD but *Firmicutes* (31%) was most abundant in DFD. We postulate that feeding frequency effects are more likely to occur in digesters fed high organic loading rates of a labile feedstock.

Keywords: beta-oxidation, thermal hydrolysis, diversity, biogas, CSTR, stability, methane

1. Introduction:

Increasing interest in food waste anaerobic digestion (AD) has resulted in a number of laboratory-scale studies (Braguglia et al., 2018; Ren et al., 2018). However, laboratory-scale and full-scale anaerobic digesters often differ in physical characteristics such as size, stirring speed and feeding frequency. For example, laboratory continuously stirred tank reactors (CSTRs) are commonly fed once daily although full-scale AD plants are fed continuously throughout the day. Furthermore, AD systems often operate at high organic loading rates as a strategy to increase methane yields from food waste. Hence, when translating results from semi-continuous laboratory studies to continuously fed full-scale AD-systems, the feeding frequency's influence on digester performance is often critically overlooked.

Recently, some researchers suggested that feeding frequency influences methane yields in anaerobic digesters, but results are conflicting (Conklin et al., 2006; De Vrieze et al., 2013; Lv et al., 2014; Mulat et al., 2016; Ziels et al., 2017). Most tests omitted continuous feeding, which best represents operating practices for full-scale food waste plants. Only two studies investigated approximately continuous feeding frequencies (Mulat et al., 2016; Ziels et al., 2017), but both used fiber based substrates. Hence, research on how feeding frequency influences food waste AD is lacking.

Food waste AD is a complex process whereby multiple sub-processes influence the outcome and require active monitoring to ensure stable and efficient digester performance. Four trophic levels are defined in the anaerobic decomposition of organic matter by microbes. First, particulate material is solubilized, followed by production of organic acids.

Next, acetate is generated and oxidized, and finally methane is produced. When feeding digesters once daily, researchers report that the aforementioned product concentrations fluctuate (De Vrieze et al., 2013; Lv et al., 2014; Mauky et al., 2015; Mulat et al., 2016). Moreover, changes in product concentrations influence process thermodynamics (Fukuzaki et al., 1990) and modify the environment to select new microbial taxa (Conklin et al., 2006). Therefore, we expect that feeding frequency will affect process performance and microbial community composition in anaerobic digesters receiving food waste.

In this study, we compare feeding once-per-day vs. 10-times-per-day at a high organic loading rate (21 gCOD/L/d) during the AD of thermally hydrolyzed food waste. We monitored process performance at two time interval resolutions; that is, we made observations every 24 hours immediately before feeding, herein referred to as "daily process performance", and we also made observations at multiple time-points within a 24-hour period, herein referred to as "within-day process performance". Finally, we characterized microbial community diversity and identified taxa whose abundance changed due to the different feeding frequencies.

2. Materials and Methods:

We compared the effect of feeding frequencies by using laboratory CSTRs fed thermally hydrolyzed food waste. We measured biogas production with an automated system for continuous sampling, and effluent samples were taken for analysis of process parameters and microbial community structure.

2.1 Digester configuration

Two laboratory digesters (BELACH BIOTEKNIK AB, Stockholm, Sweden), a Daily-Fed Digester (DFD) and a Frequently-Fed Digester (FFD), were operated with a maximum working volume of 6 L at 37 °C and a stirrer speed of 100 rpm. Gas volume was measured with water displacement, and gas composition was measured every hour using gas chromatography as previously described (Zamanzadeh et al., 2016). To begin, we filled the digesters with 3 L of identical inoculum from a laboratory CSTR, D0, which had been operating for 8 months digesting hygienized (pretreated at 70 °C for 1 hour) food waste. The inoculum digester was fed once daily, and was operated with an organic loading rate (OLR) of 3 gVS/L/d and a hydraulic retention time (HRT) of 60 days. Digester operation of DFD and FFD began by feeding with an OLR of 11 gVS/L/d, corresponding to 21 gCOD/L/d, and they were operated as batch digesters until the full digester volume of 6 L was reached after 7 days. The average HRT of both digesters was 10 days in continuous mode.

Digester feeding and discharge occurred daily. DFD was fed semi-continuously, i.e. once daily, whereas FFD was fed by a continuous feeding system. The continuous feeding system automatically delivered feed to the digester at 2.4 hour intervals and was refilled daily. When the full digester volume was reached, discharge was taken from both digesters immediately before feeding or refilling of the continuous feeding system.

Digester C1 operated in parallel with the experimental reactors, and served as a control to DFD in that C1 received the same food waste, but without the steam explosion pre-treatment, and with the same OLR on a VS basis. The inoculum source for C1 also

differed from the experimental digesters, and came from a digester operated at high organic loading rates. The process performance data from C1 is available in supplementary material (S2 and S4).

2.2 Food Waste Characteristics

The substrate in DFD and FFD was food waste that originated from health facilities and restaurants and was obtained from Norwegian Food Recycling (Norsk Matretur AS, Lørenskog, Norway) after hygienization at 70 °C for 1 hour. We steam exploded the food waste at the Biogas Laboratory at the Norwegian University of Life Sciences (Ås, Norway) at 135 °C for 20 min in order to make it more easily degradable. Both the untreated and steam exploded food waste were stored at 5 °C until we fed it to the digesters.

The characteristics of the hygienized versus the steam exploded food waste differed (Table 1). Mainly, the thermally hydrolyzed and steam exploded food waste was more dilute due to water from the steam that is added to the substrate when it is steam exploded. The steam exploded food waste also contained less concentrated acetic acid and lactic acid compared to the hygienized food waste. The lactic acid concentration was the highest of the solubles measured and the four compounds lactic acid, acetic acid, propionic acid and glucose together summed to 56.7 gCOD/L corresponding to 83% of the soluble COD in the feed substrate.

2.3 Sampling and chemical analysis

We measured total solids (TS), volatile solids (VS), ash, pH, chemical oxygen demand (COD), total ammonia nitrogen (TAN), VFAs, and long-chain fatty acids (LCFAs) in samples taken after the daily discharge on days 38, 44 and 46, and we took additional VFA samples on days 18, 19, 25 and 29.

For within-day process performance, we sampled the digesters for analysis of acetic acid at nine different time points: 1, 2, 3, 5, 7, 11, 14, 17 and 24 hours after feeding. To minimize process disturbance caused by sampling, we sampled different time points on different days. For example, the 5^{th} hour sample was collected on day 27 while the 7^{th} hour sample was collected on day 28. Because the acetic acid concentration in the 24^{th} hour sample (immediately before feeding) remained low (< 300 mg/L) in the time period for the within-day process performance sampling, we consider the use of different sampling days for within-day acetic acid variation justified. Samples from time points 1, 2 and 24 hours after feeding were sampled on several days, and average values were used for further analysis. An overview of the sampling time points and days is presented in supplementary materials (S1).

TS, VS and ash were determined gravimetrically by drying at 105 °C and subsequent burning at 550 °C. The pH was measured using a pH electrode (Orion GD9156BNWP, Thermo Scientific, MA, USA) and pH/ISE meter (Orion Dual Star, Thermo Scientific). Chemical oxygen demand (COD) and total ammonia nitrogen (TAN) were determined using Merck Spectroquant® commercial kits (Merck KGaA, Darmstadt, Germany). Soluble COD (SCOD) was analyzed after filtering the sample through a 0.45 µm cellulose acetate syringe filter. Volatile fatty acids (VFAs) in the effluent were analyzed with a Dionex 3000 HPLC as previously described by Estevez et al. (2014). Substrate VFAs were analyzed using the same instrument equipped with an Aminex® HPX-87H column, 300x7.8 mm and a Micro-guard cation H+ guard column (Cat.No.: 125-0129, Bio-Rad Laboratories Inc., Hercules, CA, USA). The analysis was done isocratic

with 0.4 mM aqueous H_2SO_4 with a flow of 6 mL/min at 50 °C. VFAs were detected with a UV detector at 210 nm, while glucose was detected with refractor index (RI) (RefractoMax521, ERC Inc., Saitama, Japan).

The samples taken at the daily discharge were also analyzed for long-chain fatty acids (LCFA) with gas chromatography mass spectrometry (GC-MS). The GC used was an Agilent 6890 GC equipped with a Gerstel PTV injector with solvent evaporation and the MS was an Agilent 5973 MSD in Selected Ion Monitoring (SIM) mode. A CP-SIL 8 CB column (Varian) with inner diameter 250 µm, length 50 m and 0.25 µm film thickness was used for separation of the fatty acid esters. The carrier gas was Helium with a flow of 29 cm/L. The injector temperature was initially 50 °C and the solvent was evaporated with a flow of 50 mL/min for 1.89 min. The injector temperature then increased by 270 °C/min until 320 °C was reached and held for 1.2 min. The column oven was first held at 40 °C for 1.89 min, followed by a 20 °C/min ramp to 160 °C and a hold for 2 min, followed by an 80 °C/min ramp to 270 °C, followed by a 50 °C/min ramp to 325 °C, which was held for 2 minutes. Prior to injection, an internal standard of 50 µg of nonadecanic acid-ester (C19:0) was added to the samples, and then the samples were extracted and methanolyzed. 0.1 g digestate was added to a 20 ml centrifuge tube, followed by 4 mL DCM/MeOH (2:1), 0.1 mL 1M HCl and 5 mL 0.9% aqueous NaCl. The centrifuge tube was then mixed by hand for 1 minute and then centrifuged at 2500 rpm for 5 minutes. After centrifugation, the water phase was removed and the DCM phase dried under N_2 at 60 °C. We added 200 µL BCl_3-methanol 14% w/w (Aldrich B1252 -100 mL) to the dried extract, sealed and heated it at 60 °C for 20 min for the methanolysis to occur. The derivatized extract was cooled to room

168 temperature, and 1 mL milliQ water was added with 1 mL hexan and vortexed for 20 s. The
169 hexan phase was then transferred to a GC-vial for analysis.

170 **2.4 DNA extraction and purification**

171 Samples for microbial community analysis were collected by withdrawing effluent
172 at the start of the experiment and after 38, 44 and 46 days of digester operation. The
173 samples were collected in 15 mL centrifuge tubes and immediately stored at -20 °C until
174 DNA extraction. Genomic DNA from each sample was extracted in triplicate using the
175 Power-Soil DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). The entire
176 process was carried out according to the manufacturer's protocol, with minor modification
177 of the bead beating whereby a Precellys®24 homogenizer was used at 5000 rpm for 20
178 seconds. The extracted and purified DNA was pooled for each individual sample and
179 measured fluorometrically on a Qubit™ fluorometer (Life Technologies, Eugene, OR,
180 USA) using the Quant-IT™ dsDNA HSAssay Kit (Invitrogen, Carlsbad, CA, USA). On
181 average, we obtained a concentration of 45 ± 15 ng/μL DNA from each digester sample.

182 **2.4.1 Sequence library preparation**

183 We selected the V3-V4 hypervariable region of the 16S rRNA gene to produce
184 amplicon libraries for Illumina MiSeq sequencing. We prepared indexed amplicons in two
185 sequential PCR steps whereby in the first we amplified using the 16S-specific primers from
186 Takahashi et al. (2014), Pro341F (5'-CCTACGGGNBGCASCAG -3') and Pro805R (5'
187 GACTACNVGGGTATCTAATCC -3'), onto which the Illumina adapter sequence was
188 included. In the second PCR, a unique 12 bp index was integrated into the reverse site of

each sample library using NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0 (Bioo Scientific Corporation, Austin, TX, USA).

The first PCR was carried out in a 50 µl reaction volume consisting of 46 ng digester DNA, 1.0 unit of Platinum® *Pfx* DNA polymerase (Invitrogen), 1mM $MgSO_4$ and 0.3 µM of each primer. The PCR reaction began with an initial denaturation at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 15 sec, annealing at 55 °C for 30 sec, extension at 68 °C for 45 sec, and a final elongation at 68 °C for 10 min. The first PCR products were purified using Agencourt AMPure XP beads (Agencourt Bioscience Corporation, Beverly, MA, USA) following the protocol of the NEXTflex™ kit. In the second PCR, twelve amplification cycles were used, following the NEXTflex™ protocol. The PCR products were purified with AMPure beads, and DNA concentrations were measured on Qubit. Amplicon libraries were normalized and pooled in equimolar concentrations to create the multiplexed library pool. We further purified this resulting library pool by gel extraction (E-Gel 1% agarose, Invitrogen, and MinElute Gel extraction Kit, Qiagen, Hilden, Germany). The final quality of the library was verified by agarose gel electrophoresis in addition to spectrophotometry with Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA). The multiplexed library pool was sequenced on an Illumina MiSeq with paired-end 300 bp cycle run using MiSeq reagent kit V3 (Illumina Inc., San Diego, CA, USA) at the Norwegian Sequencing Center in Oslo, Norway.

2.4.2 Sequence analysis

We performed sequence analysis with Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 (Caporaso et al., 2010) and other stand-alone programs. The paired-

end reads were merged using PEAR (Zhang et al., 2014) before quality filtering with PRINSEQ (Schmieder and Edwards, 2011) using a minimum quality score 20, average quality score 30, minimum length 300 and maximum length 500, before downstream analysis. VSEARCH (Rognes et al., 2016) was used for detection of chimeric sequences (uchime_denovo with default parameters), followed by open reference clustering with USEARCH61 (at 97% sequence identity) of non-chimera sequences and denovo picking of operational taxonomic units (OTUs). The OTUs were assigned to taxonomy with QIIME's uclust-based taxonomy assigner with the Greengenes database, and singletons were removed as they tend to contain disproportionate errors. The arithmetic mean of values from the PCR triplicates was used in the data analysis.

After quality filtering with PRINSEQ, 822 859 sequences remained for downstream analysis. 135 376 of these sequences were identified as chimeras (among which 70 070 were singletons) and removed resulting in a final 687 483 sequences used in the analysis of the microbial community. The lowest number of sequences in any sample was 20 175. A total of 179 genera and 9382 OTUs were identified.

2.4.3 Data accessibility

Sequence data are available at NCBI Short Read Archive under accessions SRR6484246 to SRR6484281 as part of BioProject PRJNA430711.

2.5 Data exploration and statistical analysis

Statistical testing of the process parameters was done using the two-sided paired student t-test at 0.05 significance.

We examined β-diversity by calculating the Bray-Curtis, Jaccard, and UniFrac distance metrics (Lozupone and Knight, 2005) at a cutoff at 15 000 sequences per sample. We conducted ANalysis Of SIMilarity (ANOSIM) via the compare_categories.py script in QIIME with 1000 permutations.

We performed differential abundance analysis with the LefSe algorithm (Segata et al., 2011). In LefSe, pairwise comparisons were done only between samples from the same day, and the threshold logarithmic LDA score was set to 4.5 to limit complexity. The alpha value was set to 0.05 for all statistical tests.

3 Results and discussion

3.1 Daily process performance

We observed significantly higher biogas and methane yields in FFD, which produced on average 20% more methane (Table 2). This contrasts previous findings. For example, Mulat et al. (2016) found lower methane yields with frequent feeding, while Ziels et al. (2017) found no difference. However, their substrates and organic loading rates differed from ours. For instance, Mulat et al. (2016) used a lignocellulosic substrate and Ziels et al. (2017) co-digested cow manure and oleate. Furthermore, hydrolysis limits AD of both cow manure and lignocellulosic materials (Shrestha et al., 2017), whereas methanogenesis limits food waste AD (Braguglia et al., 2018). Since hydrolysis and methanogenesis have different optimal conditions (Kumanowska et al., 2017), the substrate could explain why we observed improved methane yields with higher feeding frequency contrary to previous findings.

253 Lower methane yields accompanied 14 g/L higher LCFA concentration in the daily-
254 fed digester when compared to the frequently fed digester (Table 3). This equals a
255 stoichiometric methane potential of 490 mL/L/d. Hence, it explains one third of the
256 difference in methane yields and accounts for more than the difference in TCOD between
257 the two digesters (Table 2).

258 Propionic acid was the dominant VFA in the digester effluent and displayed the
259 greatest difference between the two digesters (Figure 1A). Specifically, DFD propionate
260 concentrations peaked at 2500 mg/L while the FFD propionate concentrations never
261 exceeded 40 mg/L. Because propionate and LCFA accumulation follow LCFA inhibition
262 (Labatut et al., 2014; Ma et al., 2015), we infer that lower feeding frequency may have
263 caused fluctuations in metabolites that synergized with the inhibition mechanism to lower
264 methane yields as well as increase concentrations of particulate COD, LCFA, and propionic
265 acid in DFD.

266 **3.2 Within-day process performance**

267 Biogas production rate, biogas methane concentration, pH, temperature and acetic
268 acid concentrations fluctuated within the 24-hour feeding intervals (Table 4, Figure 1B and
269 Figure 2). In DFD, the apparent gas production rate spiked immediately after feeding,
270 before rapidly decreasing (Figure 2). When the gas production rate increased, the biogas
271 methane concentration dropped. We also observed that gas production rate and methane
272 concentration in FFD varied, but significantly less. The pH and temperature also varied
273 significantly more in DFD (Table 4). Further, DFD's acetic acid concentration increased
274 immediately after feeding, to 1500 mg/L before declining below 300 mg/L, which was the

concentration immediately before feeding (Figure 1B). Observing these fluctuations are paramount because acetate inhibits β-oxidation and propionate oxidation (Beaty and Mcinerney, 1989; Fukuzaki et al., 1990; Lier et al., 1993), and pH fluctuations impair methanogens (Sowers et al., 1984). Hence, they could explain why LCFA accumulate in the daily-fed digester and not in the frequently fed one.

Studies usually disregard within-day process parameter fluctuations because they collect samples immediately before each feeding event. Prior studies of feeding frequency corroborate our observation of fluctuation, and there are studies that demonstrate even larger fluctuations in the less frequently fed digesters (Mauky et al., 2015; Mountfort and Asher, 1978; Mulat et al., 2016). These fluctuations result from changes in the physical, chemical and biological conditions of the reactor. For example, the labile substrate influx results in increased microbial acidogenesis. Furthermore, acetic acid's within-day variation indicates that acetogenesis proceeded at a higher rate than methanogenesis in DFD. Moreover, the decrease in biogas methane concentration immediately after feeding can be explained by: 1) low feed temperature causing digester heating after feeding (Table 4), releasing CO_2 more rapidly than CH_4, 2) feed acidity (Table 1) reducing digester pH (Table 3) and leading to more rapid release of CO_2 to the gas phase and inhibition of methanogens or 3) increased microbial acidogenesis resulting in pH reduction combined with CO_2 production.

3.3 Microbial community diversity and structure

DFD and FFD developed microbial communities of divergent composition, and this result is consistent for three common distance metrics (Figure 3). Both FFD and DFD

297 started from the same inoculum, D0, and thus began with the same microbial community
298 composition, but then diverged during operation of the reactors under different feeding
299 regimes as is consistent with previous results (Ferguson et al., 2016). This divergence is
300 evident along the main ordination axis of the β-diversity plots (Figure 3), which explains 45
301 to 50% of the variation for either the weighted UNIFRAC, Bray-Curtis, or binary Jaccard
302 distances. The microbial community in the semi-control, C1, whose feeding regime and
303 operation most closely resembles DFD, overlapped with DFD in ordination plots for the
304 Bray-Curtis and the binary Jaccard distances; however, for the Weighted UNIFRAC metric,
305 reactor C1 separated to a minor extent along the second ordination axis explaining 30% of
306 the variation (Figure 3). Weighted UNIFRAC considers the phylogenetic tree of taxa
307 present in two samples and weights branch lengths according to the relative abundance of
308 those taxa present in one sample vs. the other (Lozupone et al., 2007). Two dominant taxa,
309 *Acholeplasma* and candidatus Cloacamonas, differ in abundance between DFD and C1
310 (Figure 4 and S5), and differences in how weighted UNIFRAC and Bray-Curtis metrics
311 weight dominant taxa could account for the separation of C1 and DFD in the weighted
312 UNIFRAC ordination. Regardless, clustering of C1 and DFD demonstrates that feeding
313 frequency is a stronger influence on microbial community composition than inoculum
314 source or pre-treatment method (the food waste input to DFD was steam-exploded whereas
315 that to C1 was not).

316 Alpha-diversity, evenness and richness were significantly different in the two
317 digesters (Table 5), with a richer, more diverse and even community in DFD, which is
318 consistent with the findings of De Vrieze et al. (2013). The main taxon whose abundance

differed between FFD and DFD was an uncharacterized genus in the *Acholeplasmataceae* (Figure 4). This genus had a relative abundance of 38% in FFD and consisted of a single OTU. Moreover, its abundance was a mere 0.003 % in DFD (Table 6).

Tenericutes' dominance in FFD and relatively high abundance in DFD (16%) contrasts previous findings that report it as a minor phylum in anaerobic digesters (Nelson et al., 2011). The *Tenericutes* observed in this study belong to the *Acholeplasmataceae* and comprise two OTUs, one associated with the genus *Acholeplasma*, and another that is an uncharacterized lineage within the *Acholeplasmataceae*. *Acholeplasma* are predominantly associated with animals and have been isolated from mammalian fluids. The phytoplasmas, a candidatus genus of uncultured, plant-associated bacteria, group phylogenetically into the *Acholeplasmataceae*. Hence, one can reason that bacteria within the *Acholeplasmataceae* would grow on food waste comprised of plant and animal matter in a 37°C digester. Furthermore, an isolate from a laboratory biogas reactor was shown to have a 16S sequence 92% similar to *A. morum* and to produce acetic acid, suggesting that this organism may play a role as an acetogen fermenting amino acids (Cibis et al., 2016). Previously, increase in *Tenericutes* abundance has been observed after increasing the OLR of food waste and chicken waste digesters (Fitzgerald et al., 2015; Guo et al., 2014; Li et al., 2016; Yi et al., 2014; Ziganshina et al., 2015). However, the functionality of these species in anaerobic digesters is largely unknown. Nevertheless, it appears from our results that in addition to high OLR, high feeding frequency stimulates *Tenericutes*' abundance and selects for specific *Tenericutes* species.

340 In addition to *Tenericutes*, LefSe analysis showed that taxa belonging to four other
341 phyla were differentially abundant in DFD and FFD (Table 6). For example, *Bacteriodales*
342 and *Clostridiales* were more abundant in DFD. For *Bacteriodales,* an unknown genus
343 belonging to an unknown family was differentially abundant, hence the functioning of these
344 organisms are unknown. However, the *Bacteriodales* includes proteolytic bacteria (Rivière
345 et al., 2009), and the higher TAN concentration in DFD indicates higher proteolytic activity
346 in this digester. Moreover, *Clostridiales* abundance has been shown to positively correlate
347 with several parameters related to lower methane production values, including VFAs
348 (Vrieze et al., 2015). Another differentially abundant taxa within the phylum *Firmicutes*
349 was the RFN20 genus of the family *Erysipelotrichaceae,* which was more abundant in DFD
350 (6.3% vs. 0.08%). *Erisypelotrichaceae* have previously been observed in anaerobic
351 digesters, for example, *Erisypelotrichaceae* comprised 12.8% of the microbial community
352 in rice straw fermentation liquor (Zhao et al., 2012). In other studies of microbial
353 communities in anaerobic digesters, the genus RFN20 comprised between 0.2 and 2.1% of
354 microbes digesting petrochemical oil refinery waste activated sludge (Wang et al., 2016)
355 and 0.04-5.49% of those digesting marine macroalgae (Zhang et al., 2017). However, since
356 little is known of the ecophysiology of these taxa in AD, we are unable to infer why feeding
357 frequency influenced their abundance.

358 Furthermore, Candidatus Cloacamonas, which fall within the candidate division
359 WWE1, was also differentially abundant in DFD. However, W22 belonging to WWE1 was
360 almost as abundant as Candidatus Cloacamonas in DFD (6.6% vs. 4.7%). Organisms within
361 the WWE1 phylum are commonly found in AD systems and may contribute to the

362 breakdown of cellulose and the fermentation of sugars and proteins found in food waste (Ju
363 and Zhang, 2014; Pelletier et al., 2008). Moreover, experiments have suggested that WWE1
364 organisms can ferment cellulose hydrolysis intermediates (Limam et al., 2014).
365 Metagenomic recovery of the genome sequence of Candidatus Cloacamonas
366 acidaminovorans, suggests it is a hydrogen producing syntroph (Pelletier et al., 2008). On
367 the other hand, the genus W22 (more abundant in FFD), has been observed at 25%
368 abundance in a 37 °C, phenol-degrading digester (Ju and Zhang, 2014). The same study
369 found W22-affiliated, shotgun metagenomic sequences closely related to candidatus
370 Cloacamonas acidaminovorans. Hence, although there is limited knowledge of the
371 functioning of W22 and candidatus Cloacamonas in anaerobic digesters, it suggests that the
372 two genera share similar roles and that an unknown environmental factor favors one over
373 the other in our digesters.

374 Last, SR1 was differentially abundant, with higher abundance in FFD. Davis et al.
375 (2009) hypothesized that bacteria belonging to SR1 have a sulfur-based metabolism and
376 have a competitive advantage over other sulfur-metabolizing bacteria, such as
377 *Proteobacteria*, when there is a constant supply of *fairly high* levels of sulfur and sulfide.
378 High feeding frequency may have supplied sulfate and sulfide consistently as compared to
379 feeding once per day and thus favored SR1. Although we found no significant difference in
380 the relative abundance of *Proteobacteria* using LefSe, the relative abundance in DFD was
381 approximately 0.1% while in FFD it was approximately 0.01% (S5).

382

383 **5. Conclusion**

Our study demonstrates that feeding frequency can affect both process performance and microbial community composition in AD of food waste. By contrasting daily vs. within-day process performance, we show that a daily-fed digester exhibits greater fluctuation in variables such as pH and acetic acid. These variables characterize the digester environment and thus could account for differences in the microbial community composition. In addition, we found that once-per-day feeding led to inhibition of β-oxidation and propionic acid degradation. The inhibition in turn results in reduced overall methane yield. We further postulate that the difference between studies which demonstrate an effect of feeding frequency vs. those that do not is due to a combination of feedstock and OLR, whereby high OLR in combination with a labile feedstock like steam exploded food waste result in process instability under once-per-day feeding.

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551 **Figure captions**

552 Figure 1: VFA concentrations in digester effluent. A) Concentration of major VFAs in
553 digester effluent immediately before feeding and refilling of automatic feeding device. B)
554 Within-day concentration of acetic acid. Time-points are hours post-feeding of DFD.

555

556 Figure 2: Variation in biogas production rate and methane concentration in biogas.

557

558 Figure 3: Multivariate analysis of process parameters and microbial community. A) PCA of
559 process parameters. B) PCoA of the weighted UniFrac distance metric.

560

561 Figure 4: Stacked bar plot of relative abundance of taxa in each sample. Each sample is the
562 mean of three PCR replicates. Colors indicate taxa as displayed in the figure legend. Only
563 taxa with a relative abundance above 2% are labeled.

564

TABLES

Table 1: Food waste (FW) characteristics (average ± standard deviation). Concentrations of acids and glucose on the basis of gCOD/L are given in parenthesis.

	Unit	Steam Exploded FW	Hygienized FW
TCOD	g/L	208.3 ± 4.7	292 ± 9.7
SCOD	g/L	68.7 ± 5.3	97.1 ± 0.9
TS	%	11.8 ± 0.2	17.6 ± 0.7
VS/TS	%	89.5	89.6 ± 0.5
TCOD/VS		1.97 ± 0.05	1.85 ± 0.03
pH		4.3 ± 0.1	3.9 ± 0.08
TAN	mg/L	283 ± 21	378 ± 23
Lactic acid	g/L (COD)	31.3 ± 0.4 (33.4 ± 0.4)	61.3 ± 0.2 (65.3 ± 0.2)
Acetic acid	g/L (COD)	2.20 ± 0.04 (2.34 ± 0.04)	3.646 ± 0.005 (3.886 ± 0.005)
Propionic Acid	g/L (COD)	2.71 ± 0.05 (4.10 ± 0.08)	4.40 ± 0.01 (6.65 ± 0.02)
Glucose	g/L (COD)	0.18 ± 0.02 (0.19 ± 0.02)	0.15 ± 0.06 (0.15 ± 0.06)

570

571 Table 2: Process parameters (average ± standard deviation) measured on samples from day 38, 44 and 46.
572 Acid concentrations on the basis of mgCOD/L is given in parenthesis.

	unit	DFD	FFD	p-value
pH		7.80 ± 0.17	7.80 ± 0.09	1
TS	% of ww[1]	5.5 ± 0.6	5.4 ± 0.4	0.4
VS/TS	%	77 ± 2	76 ± 2	0.1
ash	% of ww[1]	1.29 ± 0.08	1.29 ± 0.01	0.9
TCOD	g/kg	72 ±10	61 ± 4	0.1
TCOD/VS		1.69 ± 0.05	1.51 ± 0.01	0.1
SCOD	g/L	13.3 ± 1	12.7 ± 0.8	0.5
PCOD	g/kg	58 ± 9	48 ± 4	0.2
TAN	g/L	1.8 ± 0.3	1.52 ± 0.03	0.2
FAN	mg/L	140 ± 44	116 ± 19	0.3
Acetic Acid	mg/L (COD)	95 ± 98 (101 ± 104)	33 ± 58 (35 ± 62)	0.2
Propionic Acid	mg/L (COD)	636 ± 813 (962 ± 1229)	56 ± 67 (85 ±101)	0.3
$COD_{reduction}$	%	65 ± 6	71 ± 2	0.2
Volumetric biogas yield	L/L/d	8 ± 2	10 ± 1	< .05
Specific CH_4 yield	$mL/gCOD_{added}$	236 ± 49	305 ± 35	< .05
Specific CH_4 yield	mL/gVS_{added}	465 ± 86	601 ± 56	< .05
$COD_{CH4}/COD_{reduced}$	%	109 ± 20	121 ± 14	0.1
Volumetric methane yield	L/L/d	4.9 ± 0.9	6.3 ± 0.6	< .05

573

574 [1]ww= wet weight

575

576 Table 3: Concentration of long-chain fatty acids in reactor effluent.

	DFD (g/kg)	(gCOD/kg)	FFD (g/kg)	(gCOD/kg)	p-value
Lauric acid	0.026 ± 0.005	0.07 ± 0.01	0.009 ± 0.005	0.03 ± 0.02	< .05
Myristic acid	0.29 ± 0.04	0.8 ± 0.1	0.09 ± 0.03	0.3 ± 0.1	< .01
Pentadecanoic acid	0.030 ± 0.004	0.09 ±0.01	0.012 ± 0.005	0.04 ± 0.1	< .005
Palmitoleic acid	0.032 ± 0.004	0.09 ± 0.01	0.017 ± 0.004	0.05 ± 0.01	< .05
Palmitic acid	3.3 ± 0.5	9.4 ± 1	0.8 ± 0.3	2.2 ± 0.9	< .01
Linoleic acid	0.30 ± 0.03	0.86 ± 0.07	0.23 ± 0.03	0.66 ± 0.09	< .05
Oleic acid	1.5 ± 0.2	4.4 ± 0.5	0.7 ± 0.2	1.9 ± 0.7	< .01
Stearic acid	1.7 ± 0.2	4.9 ± 0.5	0.7 ± 0.3	2.1 ± 1	< .05
Sum LCFAs	7.1 ± 0.9	21 ± 3	3 ± 1	7 ± 3	< .01

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579

580 Table 4: Mean ± standard deviation of within-day ranges for select process parameter values, which indicates
581 greater stability in FFD.

Variation over 24 hours	unit	DFD	FFD	p-value
CH_4	%-points	28 ± 2	6 ± 2	< .001
Gas flow	mL/min	91 ± 16	64 ± 12	< .001
pH		0.50 ± 0.09	0.04 ± 0.01	< .001
Temp	°C	2.2 ± 0.9	0.11 ± 0.04	< .001

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584

585 Table 5: Mean ± standard deviation of alpha diversity indices in DFD and FFD

	DFD	FFD	p-value
Simpson	0.94 ± 0.01	0.83 ± 0.05	< .05
Shannon	5.9 ± 0.2	4.5 ± 0.4	< .01
Gini	0.971 ± 0.005	0.985 ± 0.003	< .05
Richness (observed OTUs)	1009 ± 108	716 ± 69	< .05
Phylogenetic diversity	90 ± 6	71 ± 4	< .05

586

587

588

589 Table 6: Taxa that are more abundant in DFD or FFD, LDA-score in parenthesis. The mean relative
590 abundance ± standard deviation for differentially abundant taxa in each digester is presented in separate
591 columns to the right of the taxa name.

Taxa more abundant in DFD	Abundance in DFD (%)	Abundance in FFD (%)	Taxa more abundant in FFD	Abundance in DFD (%)	Abundance in FFD (%)
***Firmicutes* (4.79)**	31 ± 1	17 ± 3	***Tenericutes* (5.14)**	16 ± 4	42 ± 7
Clostridia (4.61)	24 ± 2	16 ± 3	*Mollicutes* (5.13)	15 ± 4	42 ± 7
Clostridiales (4.75)	20 ± 2	9.5 ± 0.4	*Acholeplasmatales* (5.14)	15 ± 4	42 ± 7
			Acholeplasmataceae (5.11)	15 ± 4	42 ± 7
Erysipelotrichichales (4.52)	6.3 ± 0.7	0.08 ± 0.03	unassigned genus (5.26)	0.003 ± 0.002	38 ± 7
RFN20 (4.50)	6.3 ± 0.7	0.08 ± 0.03			
			SR1		
***Bacteroidetes* (4.80)**	26 ± 3	14 ± 6	unassigned family (4.51)	0.4 ± 0.5	7 ± 2
Bacteroidia (4.78)	26 ± 3	14 ± 6			
Bacteroidales (4.80)	26 ± 3	14 ± 6			
unassigned family (4.78)	13 ± 4	2.3 ± 0.7			
unassigned genus (4.76)	13 ± 4	2.3 ± 0.7			
WWE1					
Candidatus Cloacamonas (4.53)	6.6 ± 0.4	0.03 ± 0.8			
Tenericutes					
Acholeplasma (4.79)	15.2 ± 4	3.3 ± 0.9			

592
593

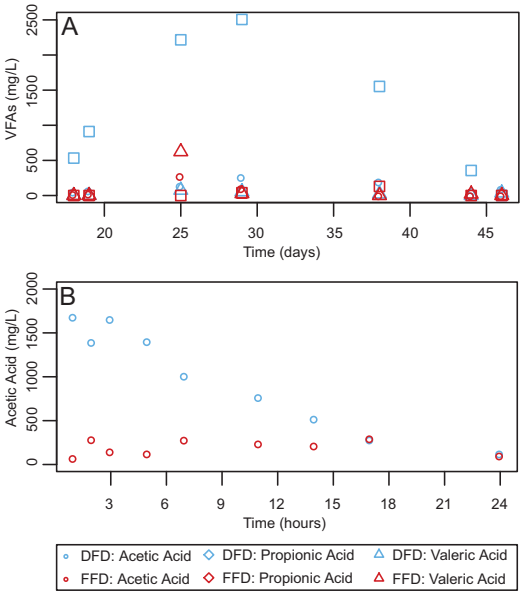

A
VFAs (mg/L)
0
500
1500
2500
20
25
30
35
40
45
Time (days)
B
Acetic Acid (mg/L)
0
500
1000
1500
2000
3
6
9
12
15
18
21
24
Time (hours)
DFD: Acetic Acid
DFD: Propionic Acid
DFD: Valeric Acid
FFD: Acetic Acid
FFD: Propionic Acid
FFD: Valeric Acid

Figure 1

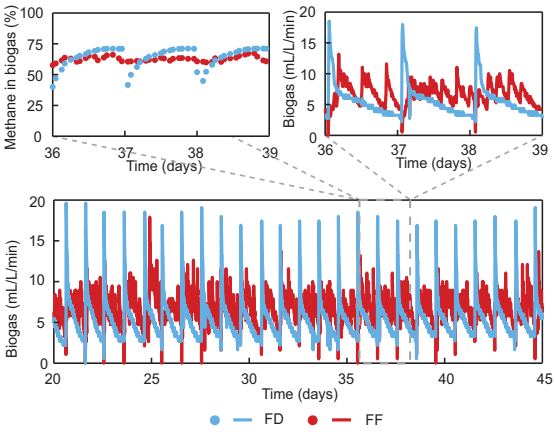

Methane in biogas (%)
100
75
50
25
0
36
37
38
39
Time (days)
Biogas (mL/L/min)
20
15
10
5
0
36
37
38
39
Time (days)
Biogas (mL/L/min)
20
15
10
5
0
20
25
30
35
40
45
Time (days)
FD
FF

Figure 2

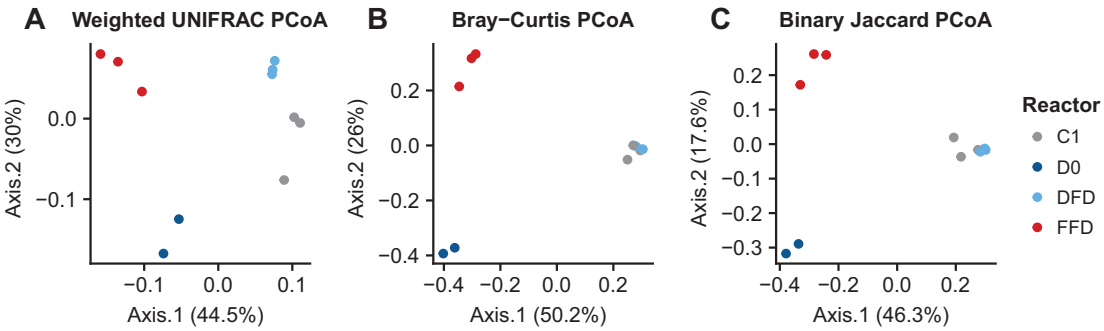

A
Weighted UNIFRAC PCoA
Axis.2 (30%)
Axis.1 (44.5%)
B
Bray−Curtis PCoA
Axis.2 (26%)
Axis.1 (50.2%)
C
Binary Jaccard PCoA
Axis.2 (17.6%)
Axis.1 (46.3%)
Reactor
C1
D0
DFD
FFD

Figure 3

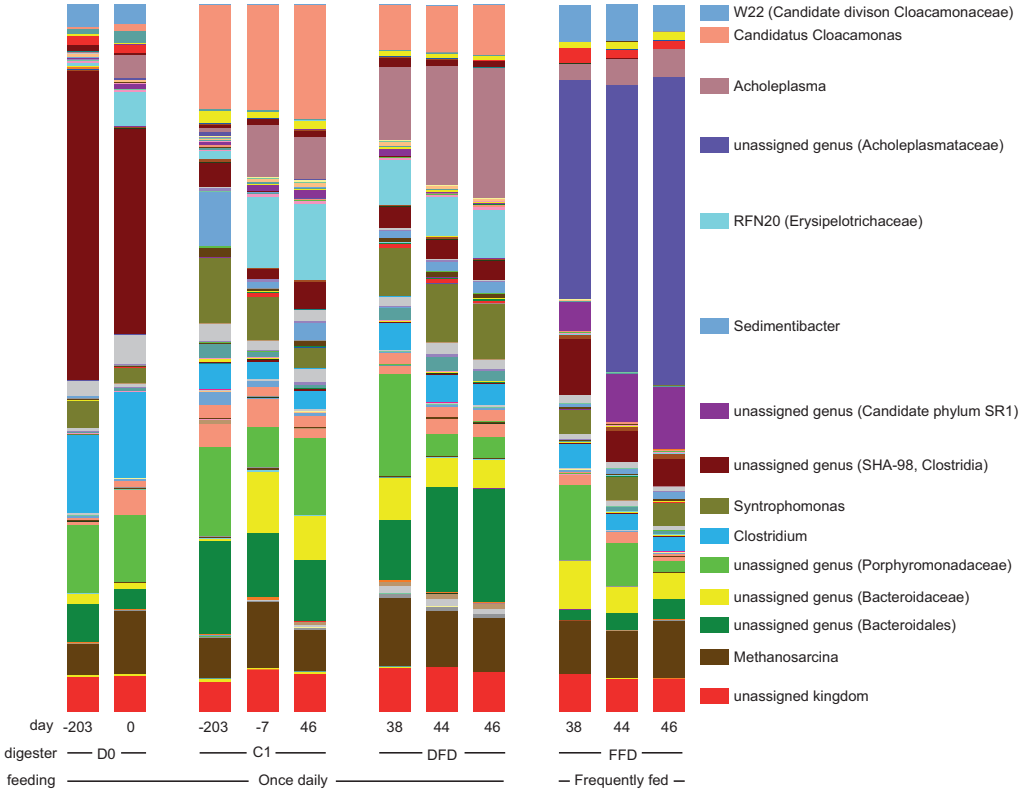
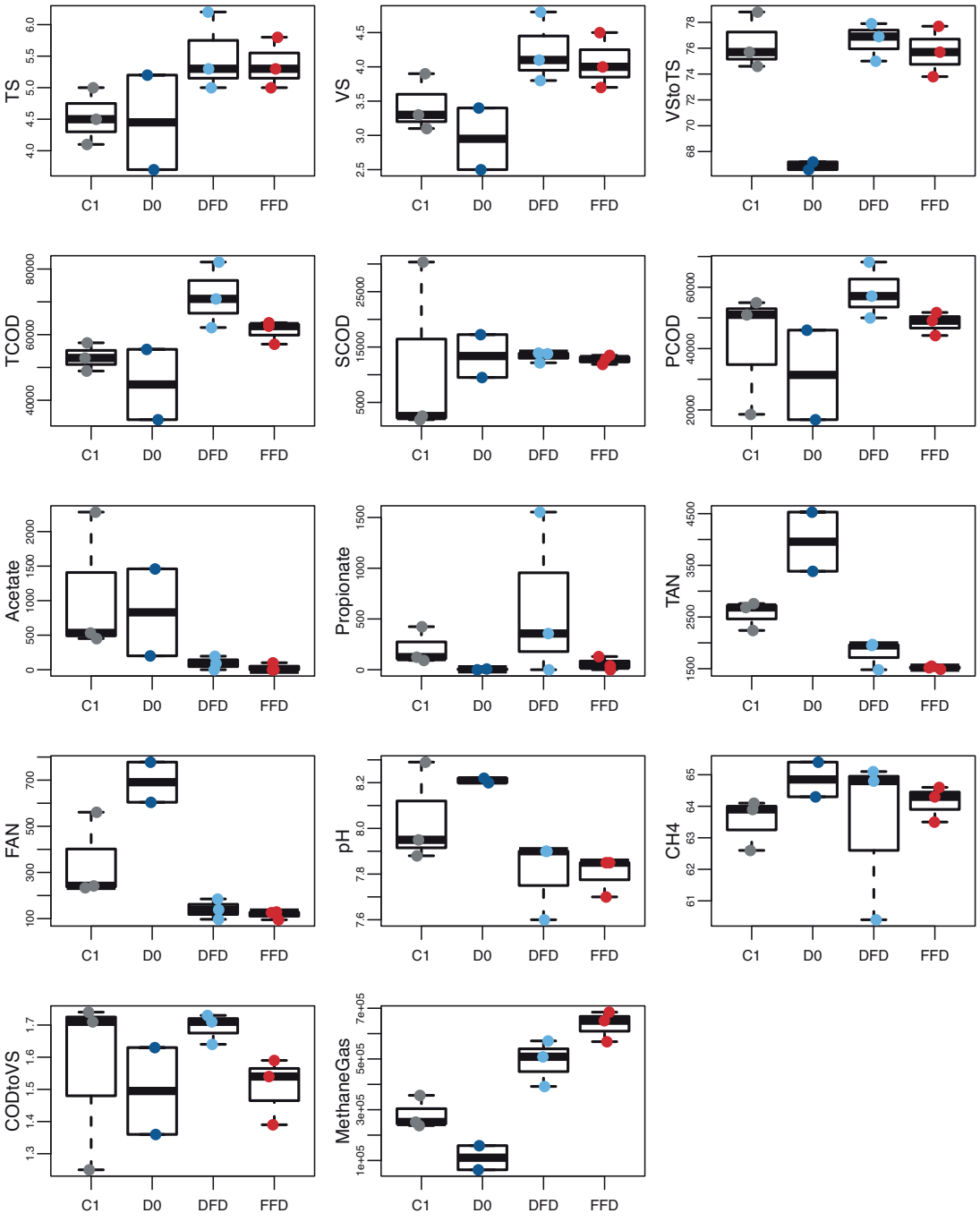

W22 (Candidate divison Cloacamonaceae)
Candidatus Cloacamonas
Acholeplasma
unassigned genus (Acholeplasmataceae)
RFN20 (Erysipelotrichaceae)
Sedimentibacter
unassigned genus (Candidate phylum SR1)
unassigned genus (SHA-98, Clostridia)
Syntrophomonas
Clostridium
unassigned genus (Porphyromonadaceae)
unassigned genus (Bacteroidaceae)
unassigned genus (Bacteroidales)
Methanosarcina
unassigned kingdom
day -203 0 -203 -7 46 38 44 46 38 44 46
digester D0 C1 DFD FFD
feeding Once daily Frequently fed

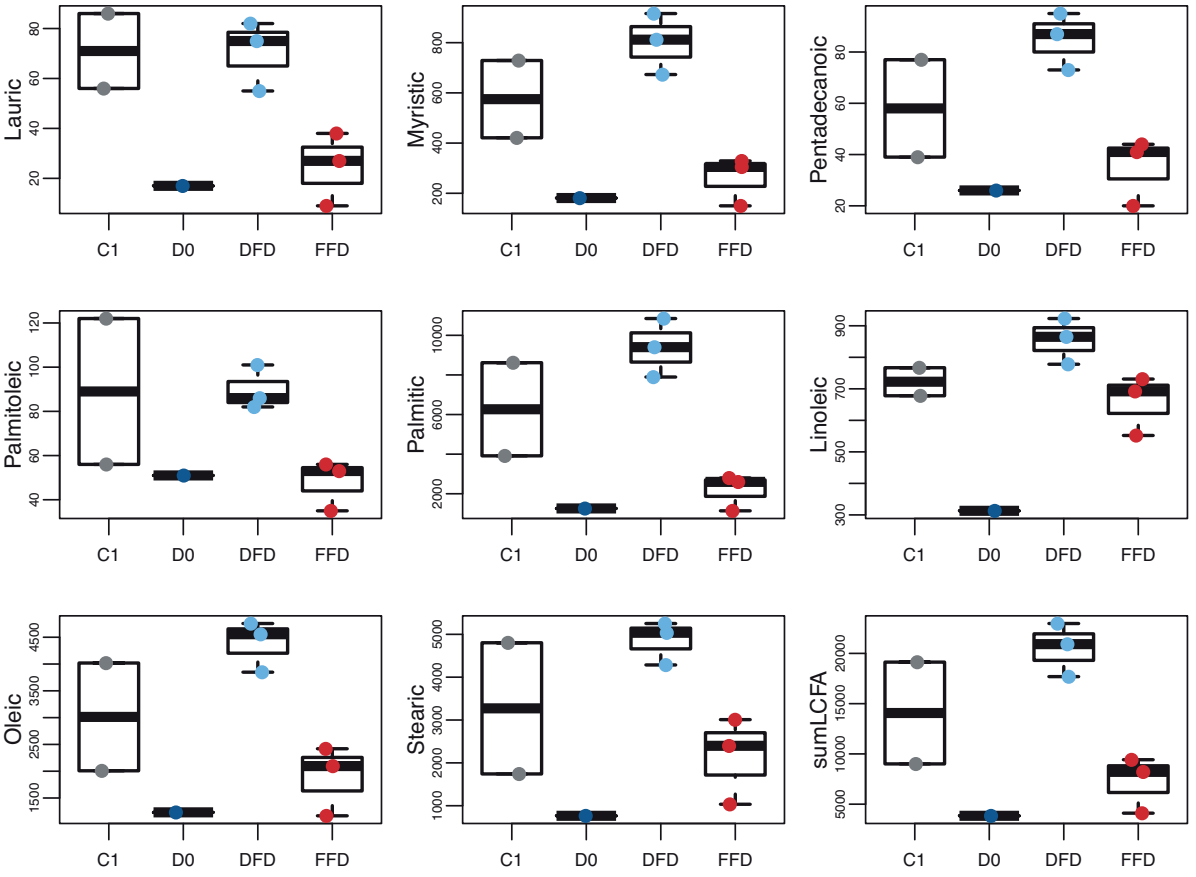
Figure 4

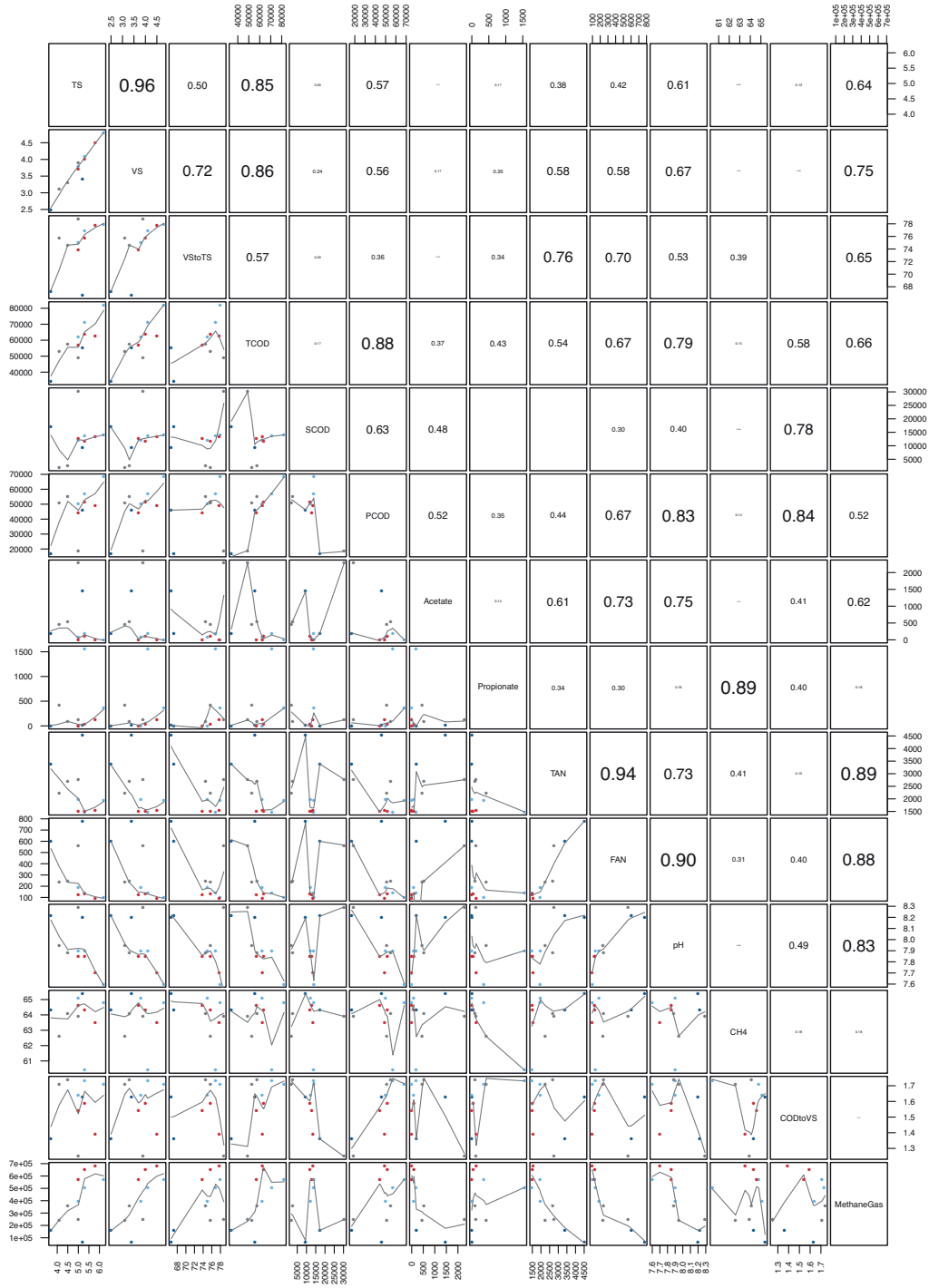
SUPPLEMENTARY MATERIALS

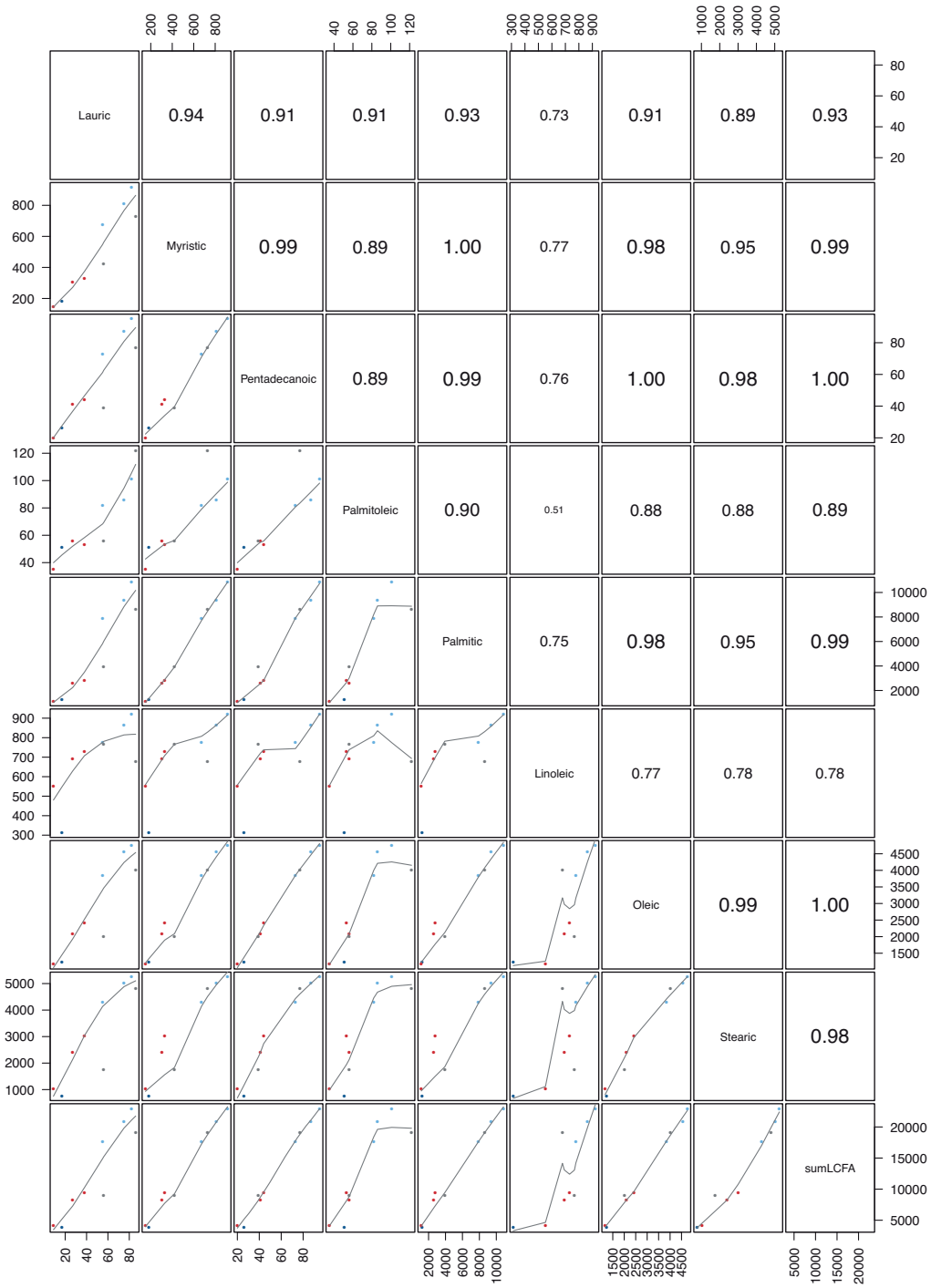
S1: Overview of sampling days for VFA measurements over 24 hour period

Hours after feeding	Day
1	18,26,39
2	18,26
3	18
5	27
7	28
11	34
14	34
17	33
24	19,25,38,46


TS
4.0
4.5
5.0
5.5
6.0
C1
D0
DFD
FFD
VS
2.5
3.0
3.5
4.0
4.5
VStoTS
68
70
72
74
76
78
TCOD
40000
60000
80000
SCOD
5000
15000
25000
PCOD
20000
40000
60000
Acetate
0
500
1000
1500
2000
Propionate
0
500
1000
1500
TAN
1500
2500
3500
4500
FAN
100
300
500
700
pH
7.6
7.8
8.0
8.2
CH4
61
62
63
64
65
CODtoVS
1.3
1.4
1.5
1.6
1.7
MethaneGas
1e+05
3e+05
5e+05
7e+05


Lauric
20
40
60
80
C1
D0
DFD
FFD
Myristic
200
400
600
800
C1
D0
DFD
FFD
Pentadecanoic
20
40
60
80
C1
D0
DFD
FFD
Palmitoleic
40
60
80
100
120
C1
D0
DFD
FFD
Palmitic
2000
6000
10000
C1
D0
DFD
FFD
Linoleic
300
500
700
900
C1
D0
DFD
FFD
Oleic
1500
2500
3500
4500
C1
D0
DFD
FFD
Stearic
1000
2000
3000
4000
5000
C1
D0
DFD
FFD
sumLCFA
5000
10000
15000
20000
C1
D0
DFD
FFD


TS
VS
VStoTS
TCOD
SCOD
PCOD
Acetate
Propionate
TAN
FAN
pH
CH4
CODtoVS
MethaneGas
0.96
0.50
0.85
0.57
0.38
0.42
0.61
0.64
0.72
0.86
0.56
0.58
0.58
0.67
0.75
0.57
0.36
0.34
0.76
0.70
0.53
0.39
0.65
0.88
0.37
0.43
0.54
0.67
0.79
0.58
0.66
0.63
0.48
0.30
0.40
0.78
0.52
0.44
0.67
0.83
0.84
0.52
0.61
0.73
0.75
0.41
0.62
0.34
0.30
0.89
0.40
0.94
0.73
0.41
0.89
0.90
0.31
0.40
0.88
0.49
0.83

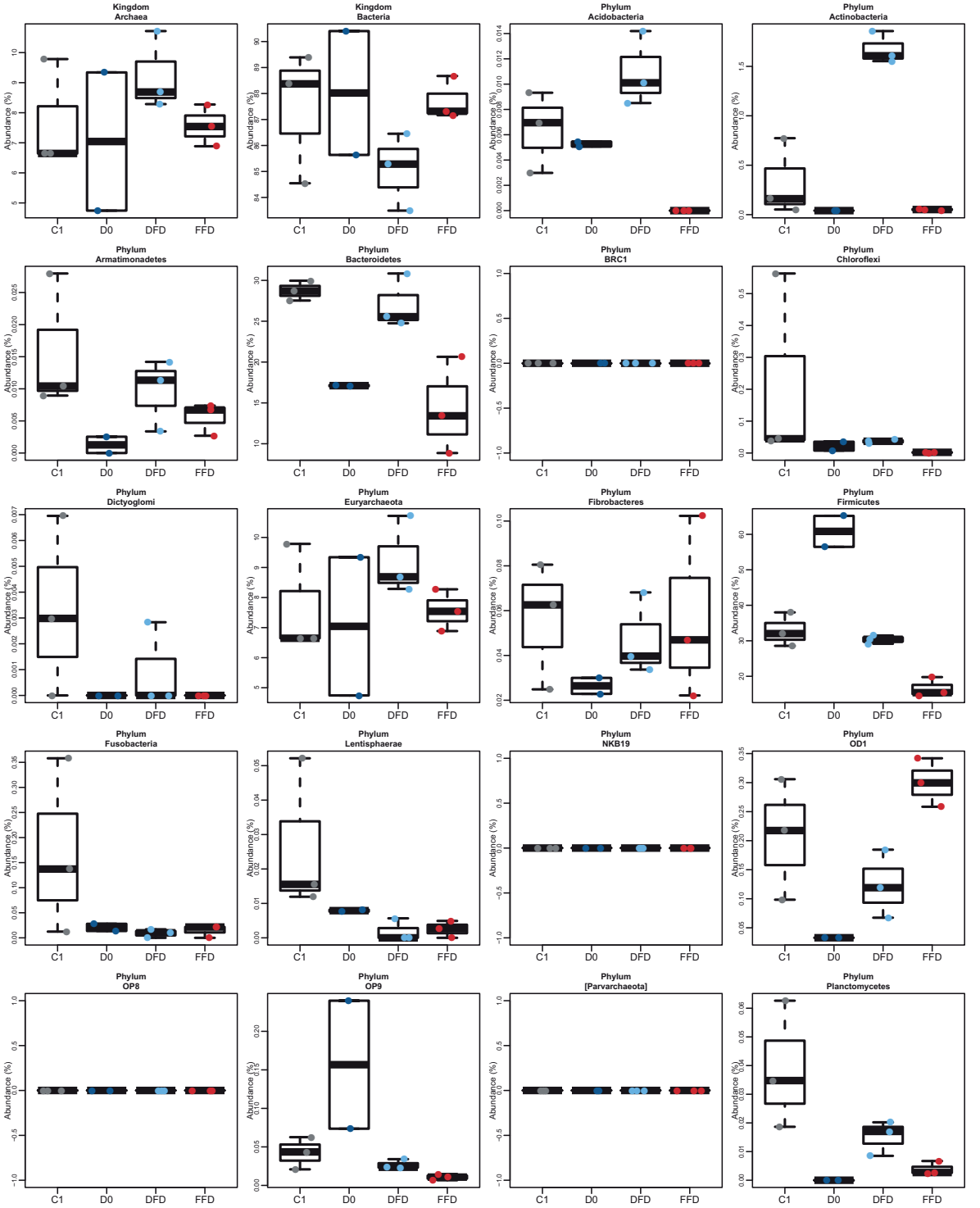

Lauric
Myristic
Pentadecanoic
Palmitoleic
Palmitic
Linoleic
Oleic
Stearic
sumLCFA
0.94
0.91
0.91
0.93
0.73
0.91
0.89
0.93
0.99
0.89
1.00
0.77
0.98
0.95
0.99
0.89
0.99
0.76
1.00
0.98
1.00
0.90
0.51
0.88
0.88
0.89
0.75
0.98
0.95
0.99
0.77
0.78
0.78
0.99
1.00
0.98

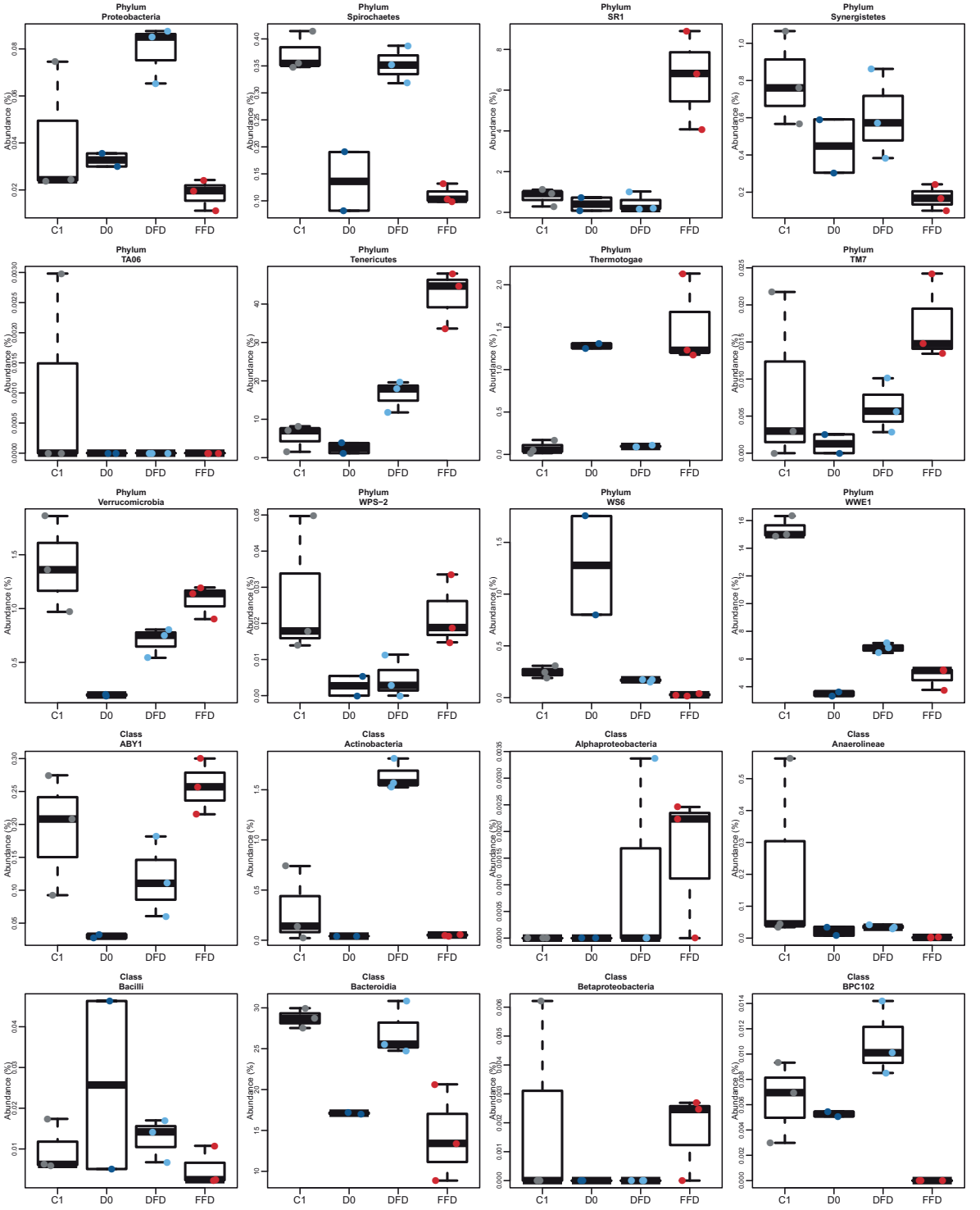
S4: Tables of all metadata taken at the observed timepoints including measured process variables and digester operating characteristics. Continues on the next pages.

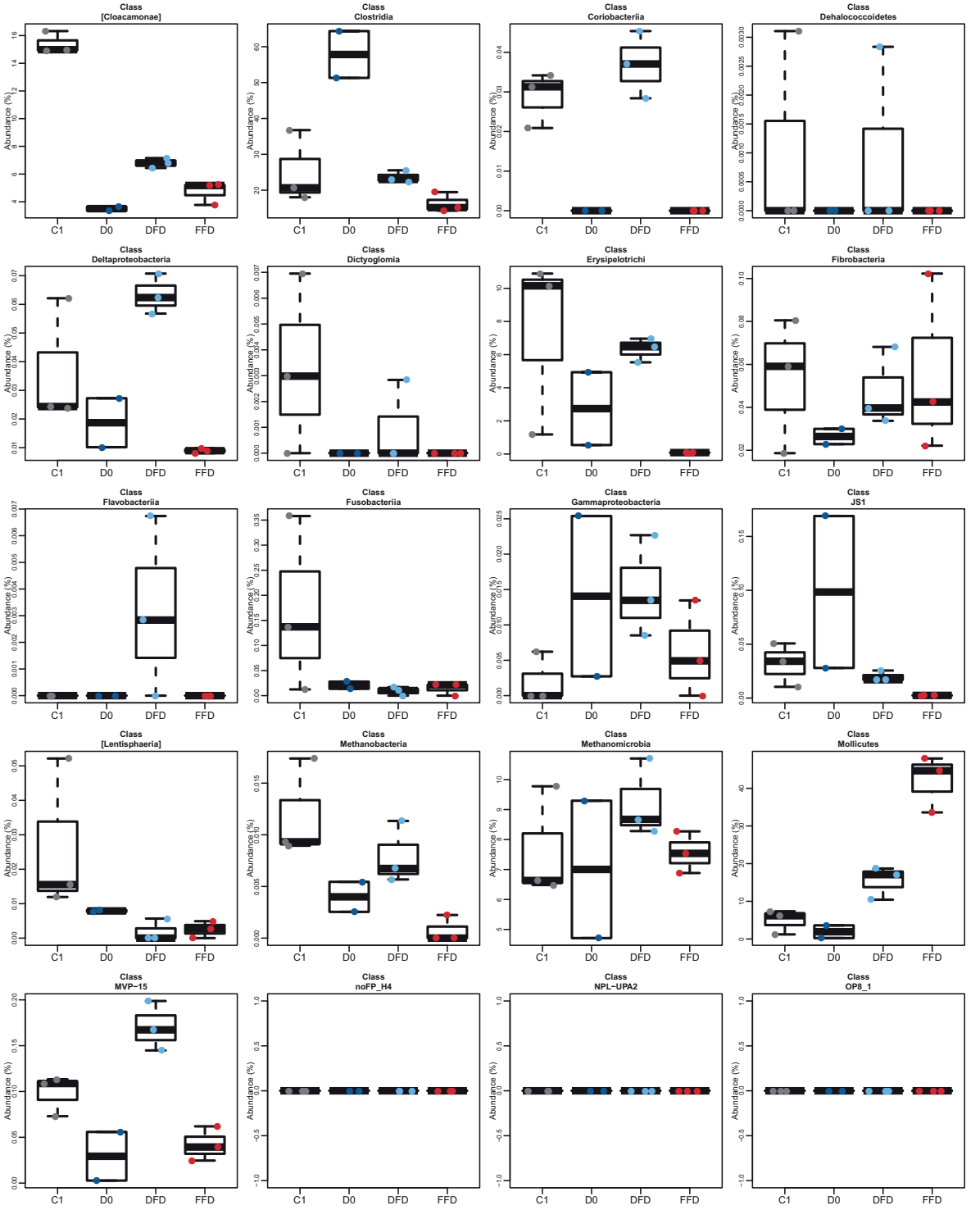
SampleName	SampDate	Reactor	day	TS	VS	VstoTS	TCOD	SCOD	PCOD	Ac	Prop	TKN	TAN	FAN	pH	Biogas
D0-0	2016-08-25	D0	0	5.2	3.4	66.6	55522	9505	46017	1460	8	6.48	4530	778	8.2	968
D1-38	2016-10-02	DFD	38	5.3	4.1	76.9	70900	13800	57100	196	1553	4.1	1480	139	7.9	8423
D1-44	2016-10-08	DFD	44	6.2	4.8	77.9	82125	13920	68205	0	357	4.49	1950	97	7.6	8815
D1-46	2016-10-10	DFD	46	5	3.8	75	62160	12155	50005	91	0	3.33	1970	185	7.9	6018
D2-38	2016-10-02	FFD	38	5.3	4	75.7	63640	11870	51770	101	40	4.88	1520	129	7.85	10124
D2-44	2016-10-08	FFD	44	5.8	4.5	77.7	62600	13510	49090	0	130	5.23	1550	95	7.7	10779
D2-46	2016-10-10	FFD	46	5	3.7	73.8	57096	12830	44266	0	0	4.63	1490	126	7.85	8796
D0-m203	2016-02-04	D0	-203	3.7	2.5	67.2	34080	17250	16830	201	0	NA	3385	604	8.22	2467
C1-m203	2016-02-04	C1	-203	5	3.9	78.8	48915	30350	18565	2282	124	NA	2760	561	8.29	3937
C1-m7	2016-08-18	C1	-7	4.1	3.1	75.7	52895	1880	51015	451	424	NA	2240	234	7.95	3796
C1-46	2016-10-10	C1	46	4.5	3.3	74.6	57505	2540	54965	533	92	NA	2685	242	7.88	5565

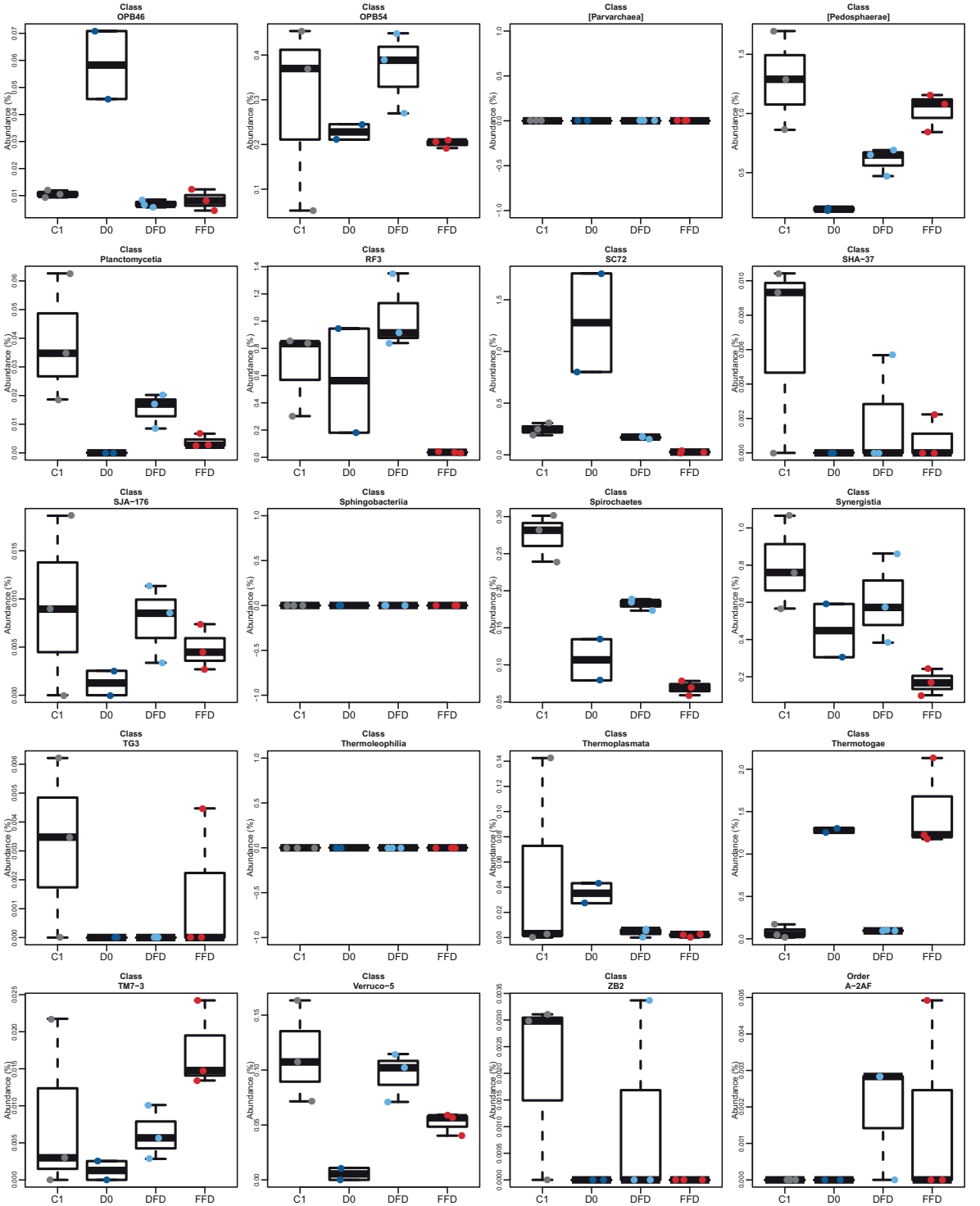
SampleName	CH4	CODtoVS	OLR	SRT	Temp	SerialReactor	feedstock	FeedFreqPerDay	FeedFreqPerWeek	methanegas
D0-0	65.4	1.63	3	60	37	1	FW	1	5	63307.2
D1-38	60.4	1.73	10	10	37	1	SEFW	1	7	508749.2
D1-44	64.8	1.71	10	10	37	1	SEFW	1	7	571212
D1-46	65.1	1.64	10	10	37	1	SEFW	1	7	391771.8
D2-38	64.3	1.59	10	10	37	1	SEFW	10	70	650973.2
D2-44	63.5	1.39	10	10	37	1	SEFW	10	70	684466.5
D2-46	64.6	1.54	10	10	37	1	SEFW	10	70	568221.6
D0-m203	64.3	1.36	3	60	37	1	FW	1	5	158628.1
C1-m203	63.9	1.25	10	14	37	1	FW	1	5	251574.3
C1-m7	62.6	1.71	10	14	37	1	FW	1	5	237629.6
C1-46	64.1	1.74	10	10	37	1	FW	1	7	356716.5

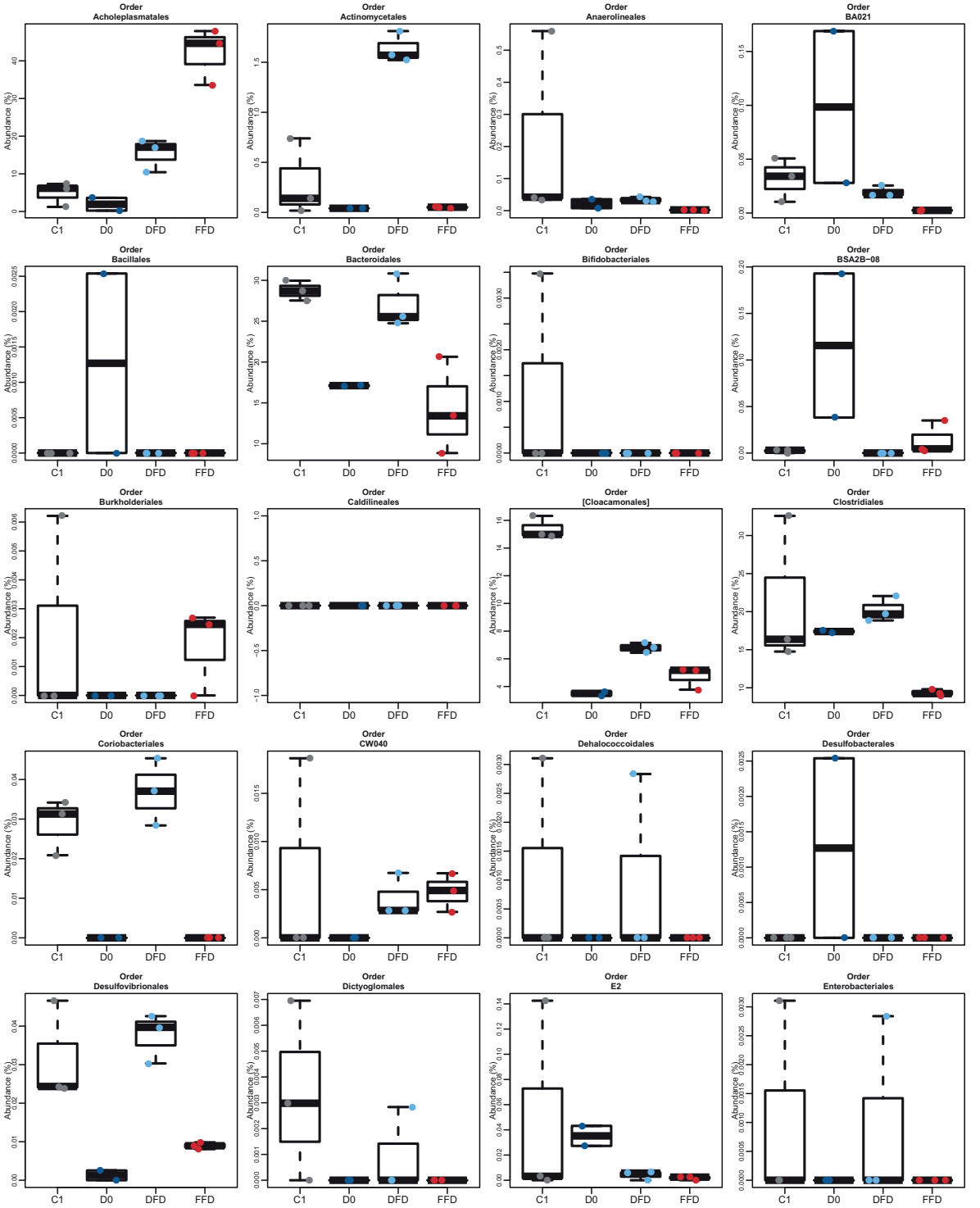
SampleName	Lauric	Myristic	Pentadecanoic	Palmitoleic	Palmitic	Linoleic	Oleic	Stearic	ΣLCFA
D0-0	17	181	26	51	1258	313	1232	765	3844
D1-38	75	812	87	86	9403	865	4556	5036	20920
D1-44	82	916	95	101	10847	923	4756	5252	22971
D1-46	55	673	73	82	7894	778	3848	4285	17688
D2-38	38	329	44	53	2799	731	2419	3009	9422
D2-44	27	305	41	56	2599	692	2096	2398	8215
D2-46	9	150	20	35	1139	552	1168	1034	4106
D0-m203	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C1-m203	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C1-m7	56	421	39	56	3915	767	2008	1742	9005
C1-46	86	729	77	122	8618	678	4019	4803	19132


Kingdom
Archaea
Kingdom
Bacteria
Phylum
Acidobacteria
Phylum
Actinobacteria
Phylum
Armatimonadetes
Phylum
Bacteroidetes
Phylum
BRC1
Phylum
Chloroflexi
Phylum
Dictyoglomi
Phylum
Euryarchaeota
Phylum
Fibrobacteres
Phylum
Firmicutes
Phylum
Fusobacteria
Phylum
Lentisphaerae
Phylum
NKB19
Phylum
OD1
Phylum
OP8
Phylum
OP9
Phylum
[Parvarchaeota]
Phylum
Planctomycetes
Abundance (%)
C1
D0
DFD
FFD

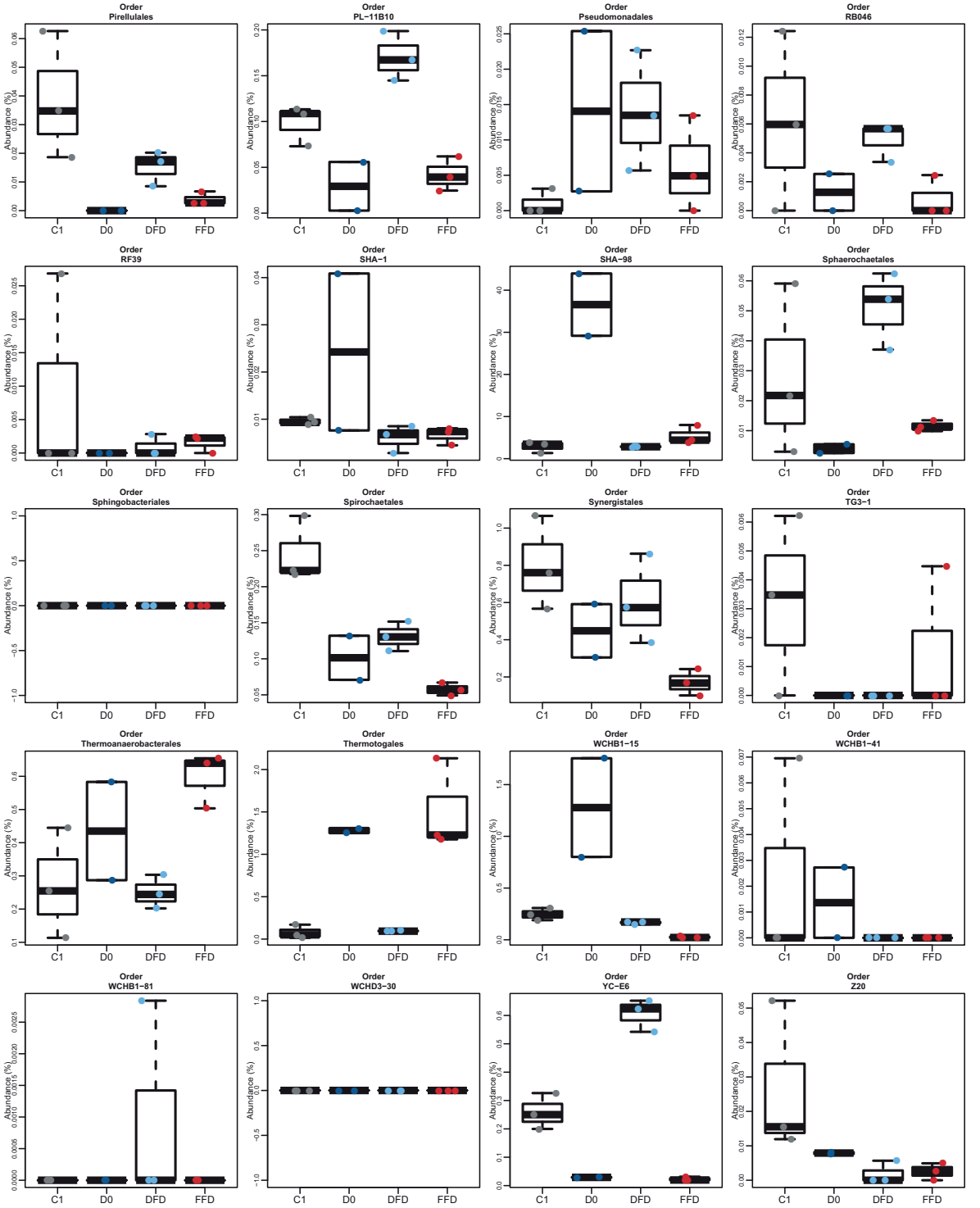

Phylum
Proteobacteria
Phylum
Spirochaetes
Phylum
SR1
Phylum
Synergistetes
Phylum
TA06
Phylum
Tenericutes
Phylum
Thermotogae
Phylum
TM7
Phylum
Verrucomicrobia
Phylum
WPS−2
Phylum
WS6
Phylum
WWE1
Class
ABY1
Class
Actinobacteria
Class
Alphaproteobacteria
Class
Anaerolineae
Class
Bacilli
Class
Bacteroidia
Class
Betaproteobacteria
Class
BPC102
Abundance (%)
C1
D0
DFD
FFD

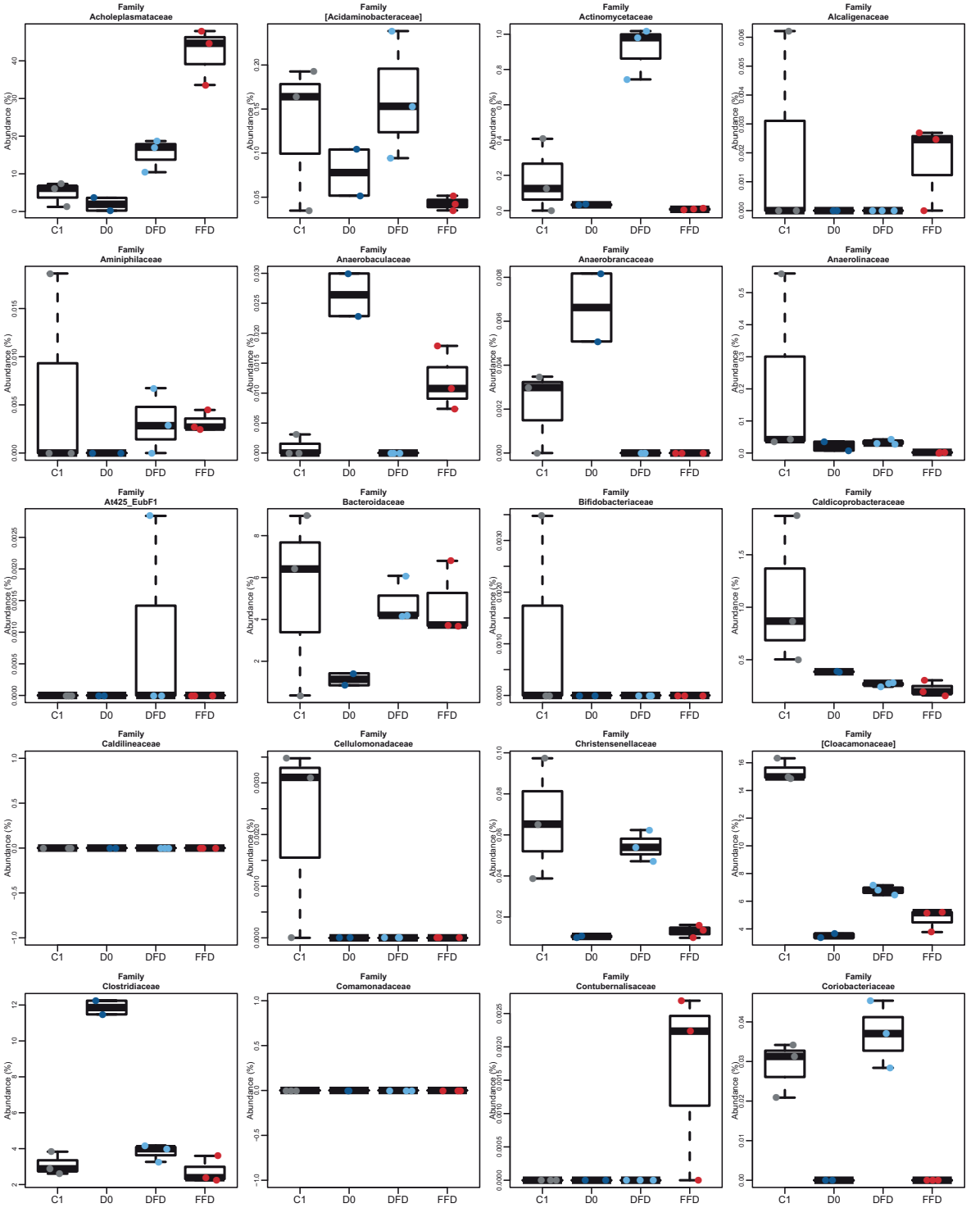

Class [Cloacamonae]
Class Clostridia
Class Coriobacteriia
Class Dehalococcoidetes
Class Deltaproteobacteria
Class Dictyoglomia
Class Erysipelotrichi
Class Fibrobacteria
Class Flavobacteriia
Class Fusobacteriia
Class Gammaproteobacteria
Class JS1
Class [Lentisphaeria]
Class Methanobacteria
Class Methanomicrobia
Class Mollicutes
Class MVP-15
Class noFP_H4
Class NPL-UPA2
Class OP8_1
Abundance (%)
C1
D0
DFD
FFD


Class
OPB46
Class
OPB54
Class
[Parvarchaea]
Class
[Pedosphaerae]
Class
Planctomycetia
Class
RF3
Class
SC72
Class
SHA−37
Class
SJA−176
Class
Sphingobacteriia
Class
Spirochaetes
Class
Synergistia
Class
TG3
Class
Thermoleophilia
Class
Thermoplasmata
Class
Thermotogae
Class
TM7−3
Class
Verruco−5
Class
ZB2
Order
A−2AF
Abundance (%)
C1
D0
DFD
FFD


Order
Acholeplasmatales
Order
Actinomycetales
Order
Anaerolineales
Order
BA021
Order
Bacillales
Order
Bacteroidales
Order
Bifidobacteriales
Order
BSA2B−08
Order
Burkholderiales
Order
Caldilineales
Order
[Cloacamonales]
Order
Clostridiales
Order
Coriobacteriales
Order
CW040
Order
Dehalococcoidales
Order
Desulfobacterales
Order
Desulfovibrionales
Order
Dictyoglomales
Order
E2
Order
Enterobacteriales
Abundance (%)
C1
D0
DFD
FFD

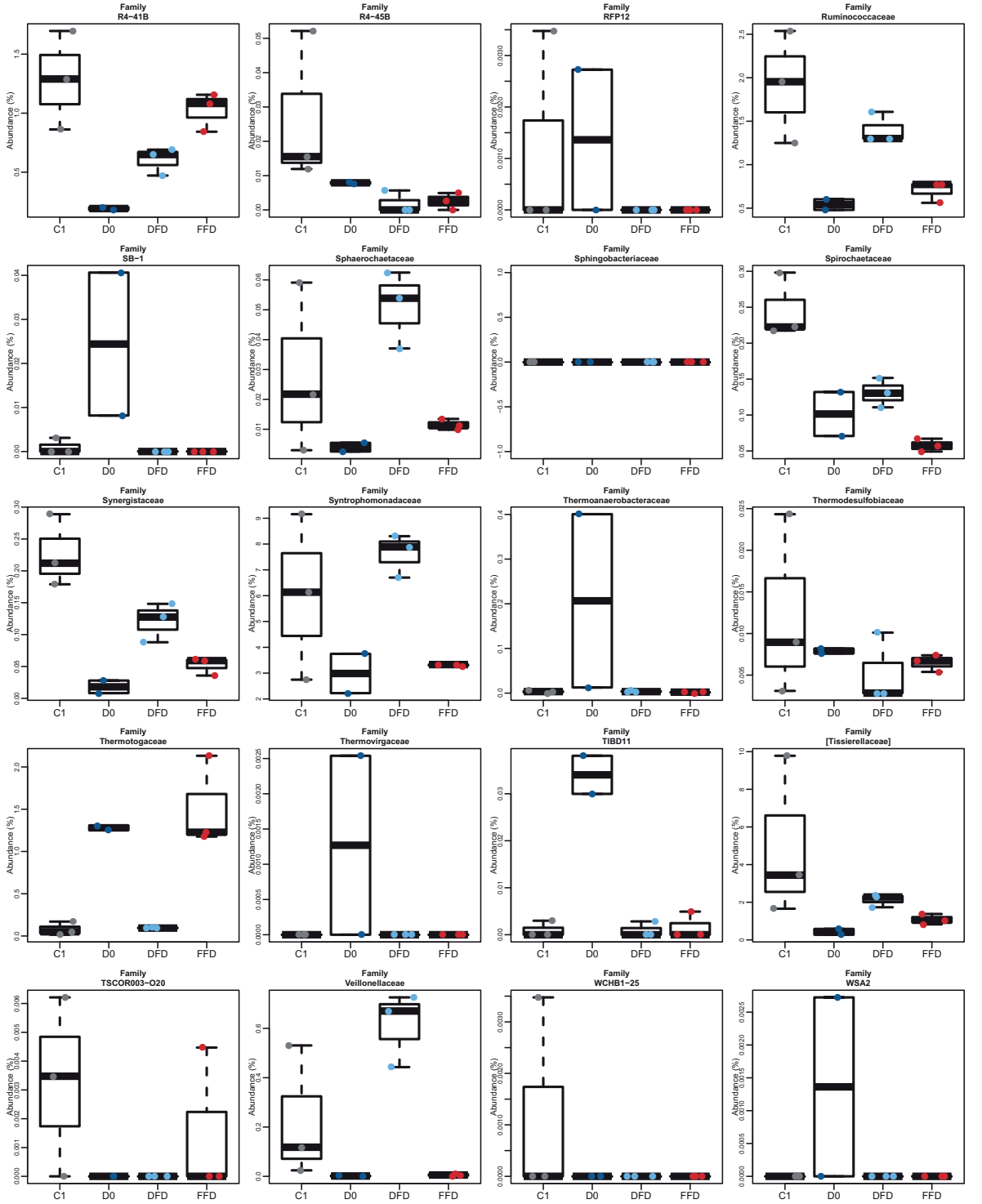

Order
Erysipelotrichales
Order
Fibrobacterales
Order
Flavobacteriales
Order
Fusobacteriales
Order
GAB−B06
Order
GMD14H09
Order
Halanaerobiales
Order
Lactobacillales
Order
LD1−PB3
Order
M2PT2−76
Order
MBA08
Order
Methanobacteriales
Order
Methanomicrobiales
Order
Methanosarcinales
Order
ML615J−28
Order
MVS−40
Order
Natranaerobiales
Order
OPB11
Order
OPB72
Order
[Pedosphaerales]
Abundance (%)
C1
D0
DFD
FFD


Order Pirellulales
Order PL−11B10
Order Pseudomonadales
Order RB046
Order RF39
Order SHA−1
Order SHA−98
Order Sphaerochaetales
Order Sphingobacteriales
Order Spirochaetales
Order Synergistales
Order TG3−1
Order Thermoanaerobacterales
Order Thermotogales
Order WCHB1−15
Order WCHB1−41
Order WCHB1−81
Order WCHD3−30
Order YC−E6
Order Z20
Abundance (%)
C1
D0
DFD
FFD

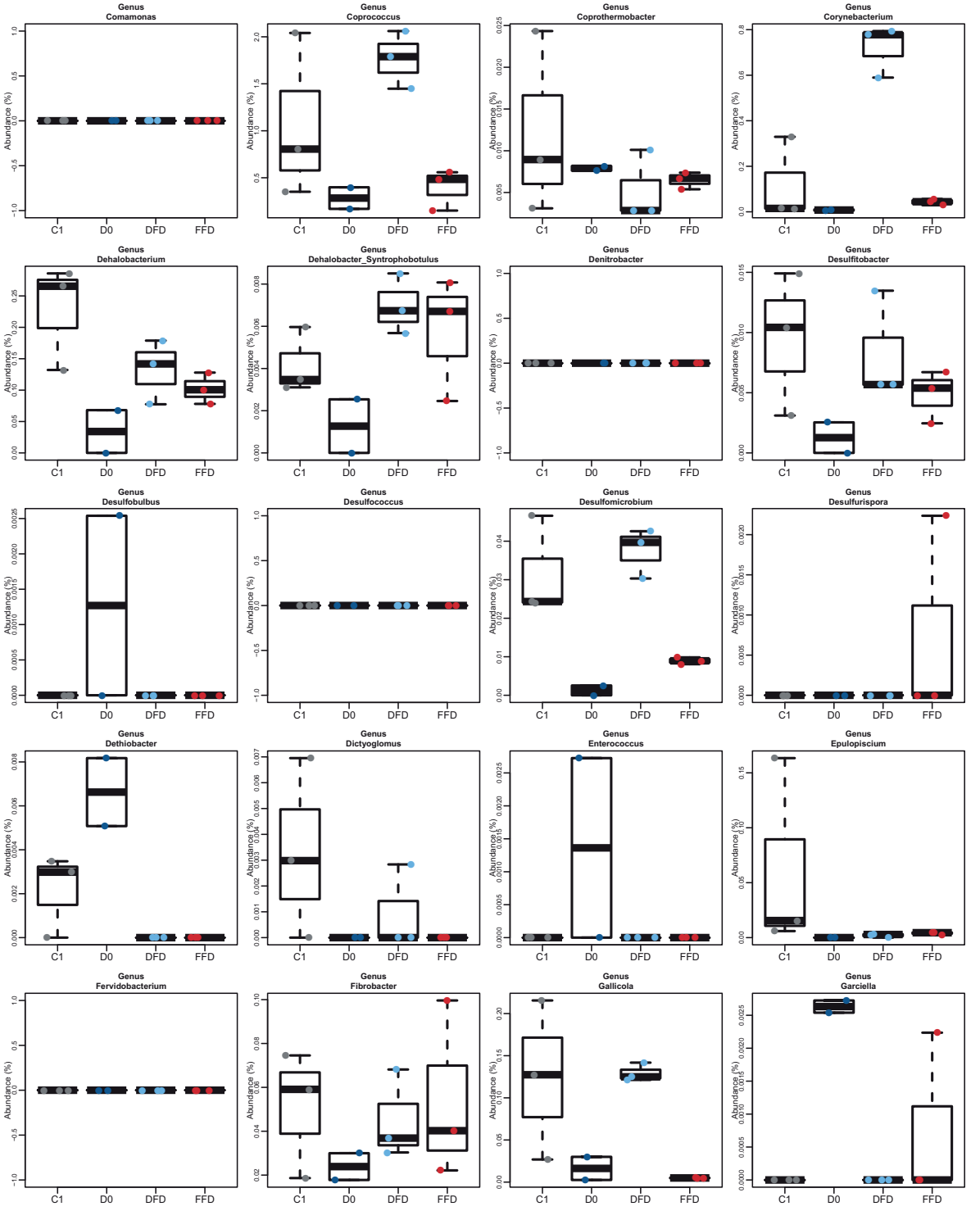

Family
Acholeplasmataceae
Family
[Acidaminobacteraceae]
Family
Actinomycetaceae
Family
Alcaligenaceae
Family
Aminiphilaceae
Family
Anaerobaculaceae
Family
Anaerobrancaceae
Family
Anaerolinaceae
Family
At425_EubF1
Family
Bacteroidaceae
Family
Bifidobacteriaceae
Family
Caldicoprobacteraceae
Family
Caldilineaceae
Family
Cellulomonadaceae
Family
Christensenellaceae
Family
[Cloacamonaceae]
Family
Clostridiaceae
Family
Comamonadaceae
Family
Contubernalisaceae
Family
Coriobacteriaceae
Abundance (%)
C1
D0
DFD
FFD


Family
Corynebacteriaceae
Family
CW−1
Family
D2
Family
Dehalobacteriaceae
Family
Dehalococcoidaceae
Family
Desulfobacteraceae
Family
Desulfobulbaceae
Family
Desulfomicrobiaceae
Family
Desulfovibrionaceae
Family
Dethiosulfovibrionaceae
Family
Dictyoglomaceae
Family
Enterobacteriaceae
Family
Enterococcaceae
Family
Erysipelotrichaceae
Family
EtOH8
Family
Eubacteriaceae
Family
F16
Family
Fibrobacteraceae
Family
Flavobacteriaceae
Family
Gracilibacteraceae
Abundance (%)
C1
D0
DFD
FFD

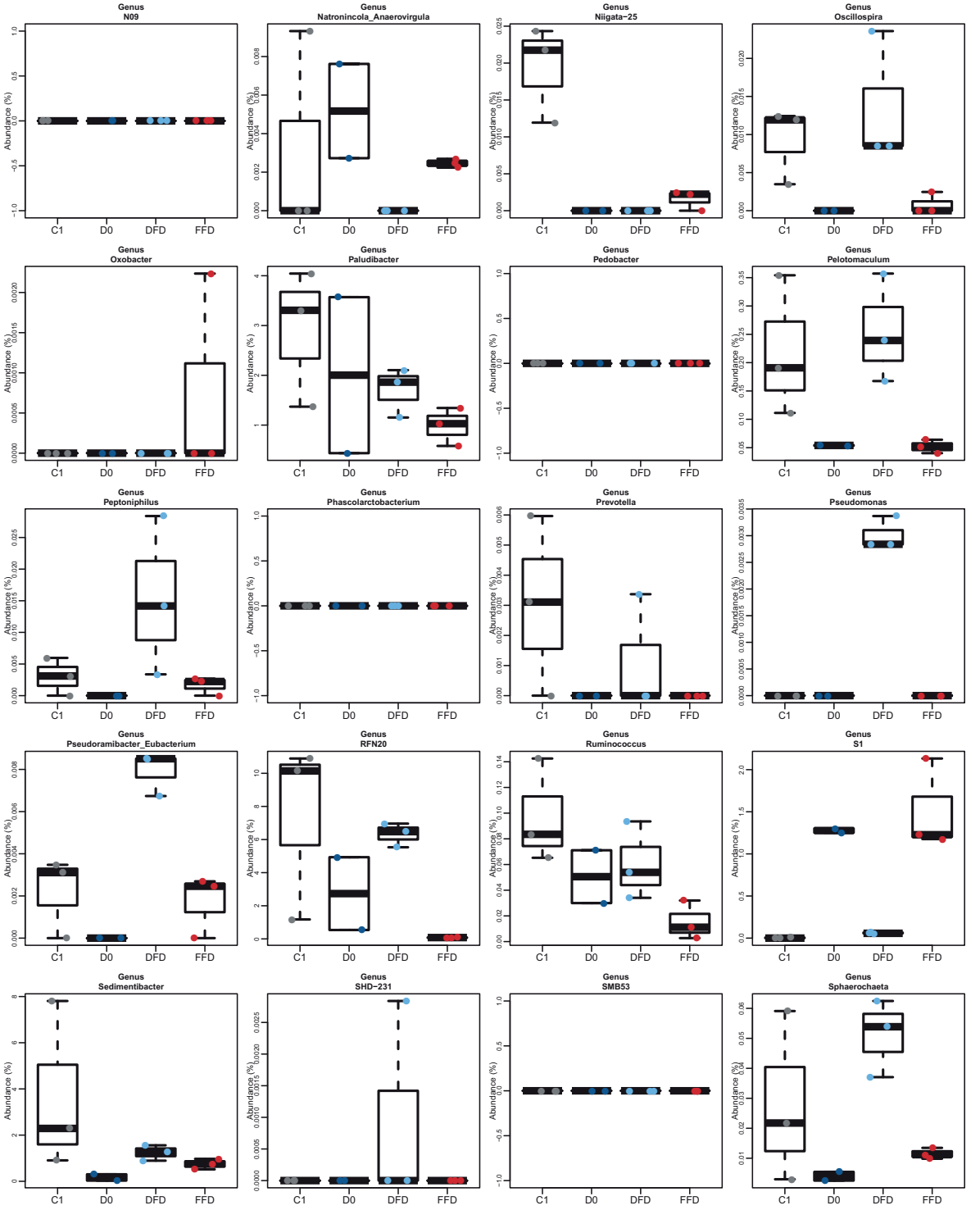

Family GZKB119
Family Halanaerobiaceae
Family Lachnospiraceae
Family Lactobacillaceae
Family Marinilabiaceae
Family Methanobacteriaceae
Family [Methanomassiliicoccaceae]
Family Methanomicrobiaceae
Family Methanosaetaceae
Family Methanosarcinaceae
Family Methanospirillaceae
Family ML1228J−1
Family [Mogibacteriaceae]
Family Moraxellaceae
Family Mycobacteriaceae
Family Peptococcaceae
Family Pirellulaceae
Family Porphyromonadaceae
Family Prevotellaceae
Family Pseudomonadaceae
Abundance (%)
C1
D0
DFD
FFD

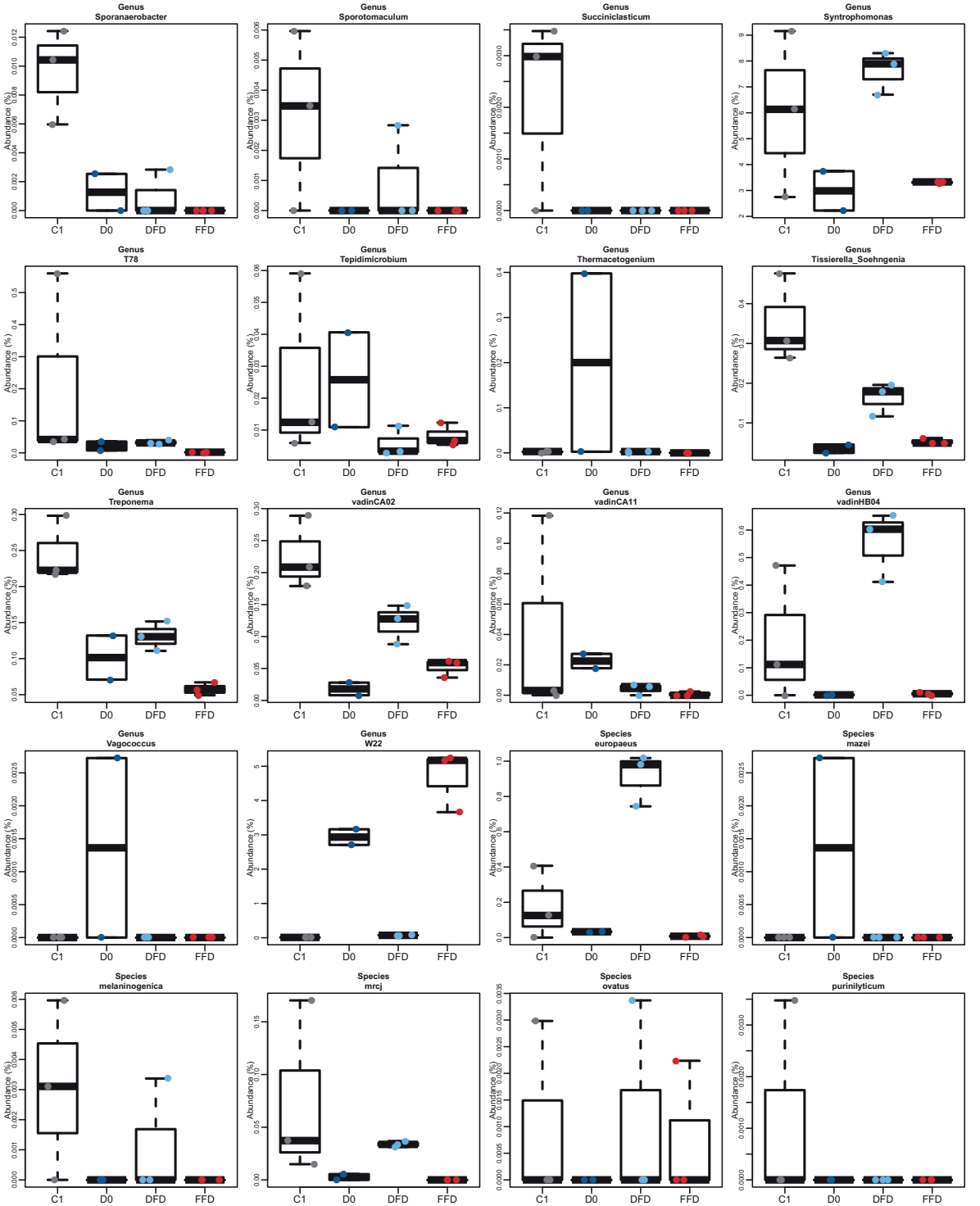

Family
R4−41B
Family
R4−45B
Family
RFP12
Family
Ruminococcaceae
Family
SB−1
Family
Sphaerochaetaceae
Family
Sphingobacteriaceae
Family
Spirochaetaceae
Family
Synergistaceae
Family
Syntrophomonadaceae
Family
Thermoanaerobacteraceae
Family
Thermodesulfobiaceae
Family
Thermotogaceae
Family
Thermovirgaceae
Family
TIBD11
Family
[Tissierellaceae]
Family
TSCOR003−O20
Family
Veillonellaceae
Family
WCHB1−25
Family
WSA2
Abundance (%)
C1
D0
DFD
FFD

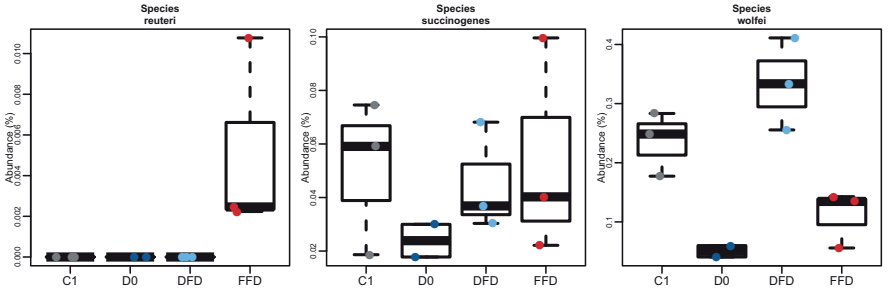

Family
YAB3B13
Genus
Acholeplasma
Genus
Acidaminobacter
Genus
Acidaminococcus
Genus
Acinetobacter
Genus
Actinomyces
Genus
Actinotalea
Genus
Alkaliphilus
Genus
Aminobacterium
Genus
Anaerobaculum
Genus
Anaerotruncus
Genus
Anaerovorax
Genus
Bacteroides
Genus
BHB21
Genus
Bifidobacterium
Genus
Caldicoprobacter
Genus
Candidatus Cloacamonas
Genus
Candidatus Contubernalis
Genus
Christensenella
Genus
Clostridium
Abundance (%)
C1
D0
DFD
FFD


Genus
Comamonas
Genus
Coprococcus
Genus
Coprothermobacter
Genus
Corynebacterium
Genus
Dehalobacterium
Genus
Dehalobacter_Syntrophobotulus
Genus
Denitrobacter
Genus
Desulfitobacter
Genus
Desulfobulbus
Genus
Desulfococcus
Genus
Desulfomicrobium
Genus
Desulfurispora
Genus
Dethiobacter
Genus
Dictyoglomus
Genus
Enterococcus
Genus
Epulopiscium
Genus
Fervidobacterium
Genus
Fibrobacter
Genus
Gallicola
Genus
Garciella
Abundance (%)
C1
D0
DFD
FFD


Genus
Geosporobacter_Thermotalea
Genus
Gracilibacter
Genus
Guggenheimella
Genus
GW−34
Genus
HA73
Genus
Kosmotoga
Genus
L7A_E11
Genus
Lactobacillus
Genus
Lutispora
Genus
Megasphaera
Genus
Methanobacterium
Genus
Methanoculleus
Genus
Methanofollis
Genus
Methanomassiliicoccus
Genus
Methanosaeta
Genus
Methanosarcina
Genus
Methanospirillum
Genus
Mogibacterium
Genus
Moorella
Genus
Mycobacterium
Abundance (%)
C1
D0
DFD
FFD


Genus
N09
Genus
Natronincola_Anaerovirgula
Genus
Niigata−25
Genus
Oscillospira
Genus
Oxobacter
Genus
Paludibacter
Genus
Pedobacter
Genus
Pelotomaculum
Genus
Peptoniphilus
Genus
Phascolarctobacterium
Genus
Prevotella
Genus
Pseudomonas
Genus
Pseudoramibacter_Eubacterium
Genus
RFN20
Genus
Ruminococcus
Genus
S1
Genus
Sedimentibacter
Genus
SHD−231
Genus
SMB53
Genus
Sphaerochaeta
Abundance (%)
C1
D0
DFD
FFD


Genus
Sporanaerobacter
Genus
Sporotomaculum
Genus
Succiniclasticum
Genus
Syntrophomonas
Genus
T78
Genus
Tepidimicrobium
Genus
Thermacetogenium
Genus
Tissierella_Soehngenia
Genus
Treponema
Genus
vadinCA02
Genus
vadinCA11
Genus
vadinHB04
Genus
Vagococcus
Genus
W22
Species
europaeus
Species
mazei
Species
melaninogenica
Species
mrcj
Species
ovatus
Species
purinilyticum
Abundance (%)
C1
D0
DFD
FFD


Species
reuteri
Abundance (%)
0.000
0.002
0.004
0.006
0.008
0.010
C1
D0
DFD
FFD
Species
succinogenes
Abundance (%)
0.02
0.04
0.06
0.08
0.10
C1
D0
DFD
FFD
Species
wolfei
Abundance (%)
0.1
0.2
0.3
0.4
C1
D0
DFD
FFD

Errata list

P. nr.	Paragraph	Changed from	Changed to
viii	Sammendrag	«hydrauliske»	«hydraulisk»
X	List of papers	Short dash	long dash
7	1.3.2	«pH»	«pH»
11	Table1	Variable font size in table text	Font size 10
17	2	«... more food waste need be treated ...»	«... more food waste needs to be treated ...»
19	3.1	«... should be used wrestults presented on the basis of CV-COD should not be compared with results based on Cr-COD.»	«... results presented on the basis of CV-COD should not be compared with results based on Cr-COD.»
23	3.3	«effect»	«effects»
23	3.3	« .»	«.»
30	3.4	«Figure 12»	«Figure 11»
30	3.4	No page number	Page number inserted
30	3.4	Page layout	ISO A4
32	3.5	«(SE Food waste)»	«(SE food waste)»

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