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Abstract

The increasing need for alternatives to fossil fuels and traditional waste management systems can be simultaneously addressed by the production of biogas using organic waste as input material. The biogas produced can be used directly for energy production or upgraded to increase methane purity for use in the engines of motor vehicles. Furthermore, the volume of waste sent to landfills greatly reduced in the process and, when a hygienization step is included, the nutrient-rich output digestate can be used as agricultural fertilizer. Not only does the use of digestate as fertilizer reduce emissions of greenhouse gases by avoiding landfilling, it also decreases the use of fossil fuels associated with the production of chemical fertilizer.

Several hindrances to biogas production exist, however, and at different levels. Economic interests demand as high efficiency as possible which includes operating a system close to its limits regarding organic loading rate (OLR), fatty acid content, ammonia content, sulfide content and trace element content. Such operation presents challenges on a technical level. The presence of trace elements may counteract certain forms of inhibition that arise but are an expense biogas plants would gladly do without. Meanwhile political will may lie with other energy sources or waste management systems. The mass balance of trace elements on agricultural land from biogas derived fertilizer must also be considered.

In this study, the effects of trace elements in thermophilic biogas production (52°C) were investigated through the operation of two 5-litre CSTR reactors. Surveillance parameters were tracked over the 184 day experiment period with microbiological analysis performed on 9 digester liquid samples taken during three key periods: startup, 3 hydraulic retention times after startup and 3 hydraulic retention times after an increase in OLR. Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis was used to analyze the dynamics of microbial community members carrying the functional gene, *fhs*, encoding the enzyme formyltetrahydrofolate synthetase (FTHFS) active in the Wood-Ljungdahl pathway and present in syntrophic acetate oxidizing bacteria. These acetogens are especially important given their role in helping to define the balance between the two possible pathways of methanogenesis, acetoclastic and hydrogenotrophic. A clone library of variants of the *fhs* gene was constructed to identify dominant members of this population. A phylogenetic analysis was also carried out to place gene variants found in this analysis in the greater context of variation of the *fhs* gene from previous sequencing of other biogas reactors.

This study revealed a lack of differences between biogas production with or without trace element additions which was in contrast to previous studies. While gaps in knowledge in thermophilic systems limited more extensive analysis and thus more definitive conclusions, the variation in composition of food waste must be considered a main variable when considering differences between seemingly similar systems.

Keywords: Biogas, thermophilic, trace elements, ammonia, SAO, *fhs*

Popular Summary

People produce a lot of garbage. Every time a dinner is cooked, a part of the food ends up in the waste bin rather than on the plate. At the same time, fossil fuels are used to make the fertilizer used on the farm that grew that food, in the tractor used to spread that fertilizer, in the trucks that brought the food to the store where the food was bought and even in the car or bus that brought the consumer to and from the store. The use of fossil fuels release carbon dioxide, a greenhouse gas, into the atmosphere and contributes to climate change.

Biogas production is a way to use the food you throw away that also produces a fuel that can be used instead of fossil fuels and after all that, the leftovers can even be used as fertilizer on farms. Biogas is essentially a combination of carbon dioxide and methane. As in the fossil fuel, natural gas, methane is the part of biogas that acts as the fuel. The difference being that, unlike fossil fuels, biogas only takes in and rereleases the carbon already present in the atmosphere. In this way, it does not hurt the environment by introducing new greenhouse gases to the atmosphere.

Biogas is produced in tanks called digesters. They're called that because they are like a stomach. If you mix food waste with the right kind of microorganisms, like bacteria, they will breakdown (digest) that waste and what comes out is biogas and digestate.

Unfortunately it is not that simple. They say moderation is the key to success and the same is true for the bacteria that make biogas. Like humans, too much fat, protein or carbohydrate will make bacteria sick and prevent them from making biogas. What makes it more complicated is that it isn't just what is added but the compounds that get released during the breakdown process that can cause problems. Compounds like fatty acids from fat, ammonia and hydrogen sulfide from protein and acetate from carbs can inhibit the biogas process. For a long time now biogas producers have known about these compounds and measure them as much as possible to make sure they don't accumulate.

With newer technology, these inhibitory compounds can not only be measured more accurately but the bacteria themselves can even be checked on. Microbiological methods like Terminal Restriction Fragment Length Polymorphism (TRFLP) and sequencing DNA from a clone library of a relevant gene may sound complicated but they're really just ways of getting a picture of which bacteria and how much of each of them are present in the digesters. That picture is called the microbial community structure. If a certain bacterium is always around when biogas is produced most efficiently, it probably means it is good to have during digestion. On the other hand if a certain bacterium is always around right before problems arise, knowing that bacterium is present would be a good hint that something needs to be done to avoid those problems.

Trace elements are metals that bacteria need in small amounts. Commonly important ones are iron, nickel, cobalt etc. If there's not enough of these in the food that is fed into the digesters then the bacteria won't be able to efficiently break it down and make biogas. For this reason, a lot of biogas plants add trace elements just in case, even if they're not sure if it is necessary. The problem is that trace elements are expensive. Plants could save money if they know they don't really need to add trace elements in certain cases.

In this study, the microbial community structure was analyzed to try to see what differences exist if you add trace elements or not to the kind of food and slaughterhouse waste that is used at Uppsala Vatten's biogas plant. And if so could those differences be linked to better or worse amounts and quality of biogas. It turns out there wasn't a big difference and it's not clear why because a number of other researchers were able to see differences when they digested local food waste. A good guess why there was no difference here is that there was already a lot of trace elements in the food waste and maybe there wasn't much in those other places. Different temperatures may also play a role. But even a good guess is just a guess. The only way to find out for sure is to do more research and try to get a better idea of what's going on inside the biogas digester.

Abbreviations

CSTR	Continuously stirred tank reactor
FW	Food waste
HRT	Hydraulic Retention Time
OLR	Organic Loading Rate
TE	Trace Element
TS	Total solids
VS	Volatile Solids
FA	Fatty Acid
VFA	Volatile Fatty Acid
LCFA	Long Chain Fatty Acid
RA	Relative Abundance
SAO	Syntrophic Acetate Oxidation
SAOB	Syntrophic Acetate Oxidizing Bacteria
SRB	Sulfate Reducing Bacteria
TRFLP	Terminal Restriction Fragment Length Polymorphism
TRF	Terminal Restriction Fragment
fhs	formyltetrahydrofolate synthetase gene
FTFHS	formyltetrahydrofolate synthetase enzyme

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1. Introduction

1.1 Literature Study

Biogas

Issues of sustainability and environmental concern in recent years have seen the rise in the use of renewable, lower polluting energy sources (Table 1). In 2013, more than 25% of the electricity consumed in the European Union was produced from renewable sources with hydro, wind and solar power the major contributors (EurObserv'ER, 2014). Biogas is one energy source with potential to address the need for alternates to fossil fuels and a reduction in greenhouse gas emissions. Yet despite recent steady growth, with room to continue, it remains little used in Europe. It is especially attractive in that, along with being an energy source to be used for heat or electricity production, or refined to be used as a natural gas substitute or biofuel, the process also serves as a low-energy waste management or waste water treatment system, converting waste into agricultural fertilizer (Figure 1) (Schnürer & Jarvis, 2009; Weiland, 2010; Zheng, et al., 2014)

Biogas is the product of the anaerobic digestion of a given substrate resulting in a gaseous mixture containing methane (CH_4) (55-70%), carbon dioxide (CO_2) (30-45%) and small proportions (<1%) of nitrogen (N_2), nitrogen oxides (NOx), hydrogen (H_2), ammonia (NH_3), hydrogen sulfide (H_2S) and other volatile compounds (Angelidaki & Sanders, 2004; Weiland, 2010).

Table 1. Relative consumption of electricity (%) of various energy sources in EU27.

Energy Source	2013	2010	2005	2000
Oil	41	42	43	44
Gas	27	29	28	26
Nuclear	14	13	14	14
Coal	13	11	13	14
Other Renewables	3	2	1	0
Hydroelectric	2	2	1	2
Biomass and Waste (incl. Biogas)	0.81	0.6	0.43	0

Source: Breakdown of Energy Consumption Statistics, via: www.tsp-data-portal.org, accessed Oct. 2015

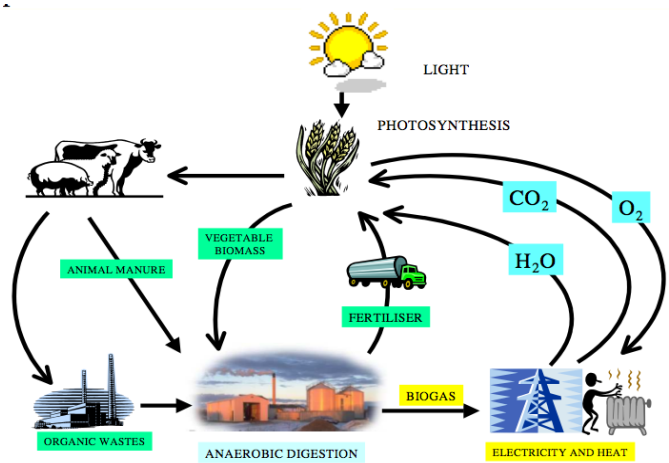

Figure 1. Schematic flow diagram of inputs and outputs of biogas production (Al Seadi, et al., 2008).

Biogas Production

The production of methane is a natural process occurring in environments deplete of oxygen, such as lake bottoms and swamps. Methane is the product of the anaerobic degradation of organic compounds (Claassen, et al., 1999). Harnessing this process allows for the production of biogas and its use as a fuel (Energigas Sverige, 2014)

Biogas can be produced in a system consisting of any of a number of technologies, using various substrates or a combination of substrates (i.e. co-digestion) (Al Seadi, et al., 2008). The energy potential of a given system is defined by the method of production, the substrate(s) used and loading rate and retention time (Weiland, 2010). The two main groups of reactor, or digester, types in terms of substrate input and output are batch and continuous, though any reactor must be airtight with substrate input and biogas and digestate, or sludge, output systems in place (Al Seadi, et al., 2008).

Batch digestion consists of a repeated cycle of loading, digestion and unloading of substrate. That is, a "batch" of material is loaded into the reactor and allowed time to digest. Once digested, the old batch is removed and replaced with a new batch. Continuous digestion, meanwhile, is defined by the regular addition of substrate to, and withdrawal of biogas and digestate from, the reactor. The total solids content (TS) often dictates the type of reactor to be used with continuous digestion more suited to wet substrates (<15% TS) thanks to greater ease in pumping and stirring. Water, however, may be added to a dry substrate (20%-40% TS) to enable such digestion as well (Al Seadi, et al., 2008).

Numerous variations of each main type exist, each designed to address the needs or capacities of the system in which it is used (Figure 2) (Luostarinen, et al., 2011). The smallest scales of production consists of household digesters which are simple in design, typically smaller than 10 m^3 relating to a few kW. On this scale, household and animal waste tends to be the source of substrate with the produced biogas used for lighting and cooking. These digesters are most common in warmer climates, making temperature control unnecessary. Agricultural plants allow farmers to close their nutrient cycle by using animal manure, which would otherwise emit methane directly to the environment, and crop biomass as substrate while employing the digestate as fertilizer and the biogas in electricity and heat production. Individual farms tend to generate less than 70 kW whereas multi-farm cooperatives, by pooling resources, can reach an electrical capacity in the hundreds of kW (Al Seadi, et al., 2008; Luostarinen, et al., 2011). Centralized biogas plants tend to use the most advanced and product-specific technologies, including pre- and post-treatment steps to digest an array of substrates at the largest scale to produce the highest quality product possible. Typical substrates include one or a combination of organic fractions of municipal household, restaurant or industry solid waste, agricultural waste, energy crops, or sludge from wastewater treatments. The primary aim of production of such large scale plants includes direct energy generation, natural gas standard methane, vehicle fuel, fertilizer production, waste material stabilization and environmental load reduction. Depending on the aim of production, plant output can range from hundreds of kW of electrical power to tens of MW of thermal power (Al Seadi, et al., 2008; Luostarinen, et al., 2011).

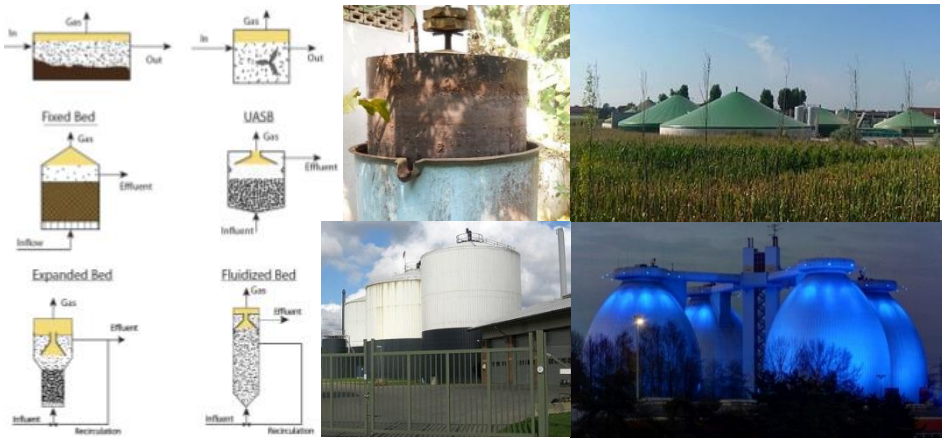

Figure 2. (left) Schematic examples of various biogas digester technologies (Luostarinen, et al., 2011) (right) clockwise from top left: household scale digester, single farm digester, waste water treatment plant-associated digesters, municipality scale centralized digesters (including agriculture cooperative). (Photos from public domain (creative commons))

The energy potential of a substrate is related to the amount of methane that can be produced from it which is in turn determined largely by the composition of the substrate. Approximate methane yields of simple compounds such as fat, protein and carbohydrates have long been established empirically which led to the derivation of the Buswell Formula to enable the calculation of the yields of other compounds from their C, H, N, O and S composition (Buswell & Neave, 1930). Calculating energy potential based on such figures is difficult however as they presume 100% biodegradation. A biodegradation coefficient of less than 100% implies not all of the energy present in the substance is accessible, leading to a lower energy potential (Berglund & Börjesson, 2003). Furthermore, excesses of certain compounds may lead to inhibition, discussed below, which can contribute to lower production and thus lower energy potential (Chen, et al., 2008). Table 2 highlights certain key attributes of several simple and more complex substrates.

Table 2. Specific Methane Production and Energy Potentials of common substrates

Substrate	Biogas Production (NmL gVS^{-1})	Methane Content (%)	Methane Production (NmL gVS^{-1})	Energy Potential** (kJ $NmLBiogas^{-1}$)
Carbohydrate	830	50	415	19
Fat	1449	70	1014	33
Protein	775	64	496	18
Food Waste	400	85	340	9
WWTP sludge*	333	65	217	8
Slaughterhouse Waste*	639	63	403	15
Straw*	87	70	61	2
Liquid Pig Slurry*	361	65	235	8
Lignin*	0	-	0	0

Sources: (Berglund & Börjesson, u.d.; Luostarinen, et al., 2011; Weiland, 2010)
*VS was assumed to be 90% if not otherwise reported
**Energy potential calculated using methane production multiplied by the lower calorific value of methane (Swedish Gas Centre, 2012)

Steps of Production

Biogas production is a multistage process consisting of four main steps, carried out by interdependent communities of microorganisms. These steps describe the successive breakdown of complex polymers through monomers and other metabolic intermediates to the products, CH_4 and CO_4 (Figure 3) (Demirel & Schere, 2008; Mara & Horan, 2003).

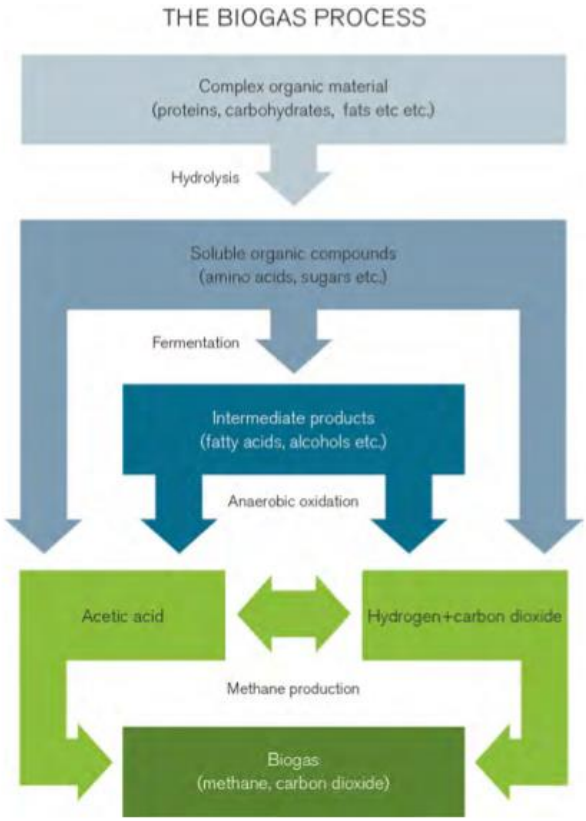

Figure 3. The biogas process. Figure from Swedish Gas Centre (2012)

Hydrolysis

Hydrolysis is the first of the four steps and consists of the initial degradation of complex polymers such as polysaccharides, proteins, lipids and nucleic acids in solution (Mara & Horan, 2003). Extracellular enzymes produced by hydrolytic bacteria perform this initial breakdown of polymers to the monomers, sugars, amino acids, long-chain fatty acids, nucleotides and glycerol (Zieminski & Frac, 2012). In some cases, hydrolysis may be the rate limiting step in the overall process depending on the biodegradability of the polymers present in the influent substrate. Lignocellulose, for example, is one such difficult to degrade compound that can slow the entire process (Claassen, et al., 1999).

Acidogenesis & Acetogenesis

The monomers formed in the hydrolysis step are further degraded by acidogenic or acetogenic bacteria in the proceeding steps (Zieminski & Frac, 2012). Two pathways, acidogenesis and acetogenesis, connect hydrolysis and methanogenesis.

In the case of acidogenesis, which can be considered the second of the four major steps, short chain fatty acids (>2C), such as propionate and lactate, and alcohols, such as ethanol are produced through different fermentation reactions.

In the third step, acetogenesis, breakdown continues from the products of acidogenesis to acetate, H_2 and CO_2 via anaerobic respiration. Acetogenesis also includes the direct conversion of some hydrolysis-produced monomers to these same products (Demirel & Schere, 2008).

Syntrophic Acetate Oxidation and Methanogenesis

The fourth step of the process consists of the production of methane which can follow two different pathways, acetoclastic and hydrogenotrophic methanogenesis. Acetoclastic methanogenic archaea convert acetate to CH_4 and CO_2 whereas hydrogenotrophic

methanogenic archaea convert, H_2 and CO_2 or formate to CH_4 and CO_2 (Table 3, Reactions 1 and 3) (Demirel & Schere, 2008).

Syntrophic acetate oxidative bacteria (SAOB) are able to carry out syntrophic acetate oxidation (SAO). This pathway includes the *fhs* gene (previously found in the Wood-Ljungdahl pathway) and allows the conversion of acetate to H_2 and CO_2 or formate to CH_4 in syntrophy with hydrogenotrophic methanogens (Müller, et al., 2013). SAO is energetically unfavourable unless hydrogenotrophic methanogens consume H_2 or formate, products of SAO, to a great enough extent that SAO is driven forward (Table 3, Reactions 2-4). Otherwise, acetate production by SAOB may result as per the Wood-Ljungdahl pathway. In this way, SAOB act as a fulcrum upon which the balance between acetoclastic and hydrogenotrophic methanogenesis rests (Schink, 1997).

The partial pressure of H_2 then gains critical importance as such hydrogenotrophy requires $P_{H_2} \geqq 10^{-6}$ atm whereas SAO requires $P_{H_2} \leqq 10^{-3}$ atm (approximate values for 55°C) (Hattori, 2008; Schnürer, 2015). P_{H_2} must lie within this range for hydrogenotrophic methanogenesis to proceed. When SAO and hydrogenotrophic methanogenesis are coupled, the same change in Gibbs free energy is produced as with acetoclastic methanogenesis (-31 kJ mol^{-1}). In the case of SAO-linked hydrogenotrophic methanogenesis, however, this energy must be shared between two bacteria which is not the case for acetoclastic methanogenesis. Along with typically high levels of acetate, this energy dynamic is a major reason why acetoclastic methanogenesis is more often the dominant pathway. In certain conditions, alternatively, such as at high levels of NH_3, in which acetoclastic methanogens are more severely inhibited than hydrogenotrophic methanogens, the latter tend to dominate (Sun, et al., 2014; Yenigün & Demirel, 2013). Hydraulic retention time and temperature also play roles in the balance between methanogenesis pathways (Westerholm, et al., 2011a)

Table 3. Reactions and change in Gibbs free energy of methanogenesis pathways

Process	Reaction	$\Delta G^{0,}$ (kJ mol^{-1})
1. Acetoclastic Methanogenesis	$*CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.0
2. Syntrophic Acetate Oxidation	$CH_3*COO^- + 4H_2O \rightarrow HCO_3^- + 4H_2 + H*CO_3^- + H^+$	+104.6
3. Hydrogenotrophic Methanogenesis	$4H_2 + H*CO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.6
4. Coupled SAO-Hydrogenotrophy	$CH_3*COO^- + H_2O \rightarrow HCO_3^- + CH_4$	-31.0

Table adapted from Hattori, 2008

* Indicates carbon destined to become C in resulting methane molecule

Process Parameters and Inhibition

Certain process parameters are under direct control of the reactor operator and can be altered to affect production and the microbial community.

OLR, HRT and Substrate Quality

The organic loading rate (OLR) is defined as the amount of organic material (VS) (as a proportion of total solids (TS)) that is fed into the reactor per day (Equation 1). The hydraulic retention time (HRT) is inversely proportional to the OLR in that it is defined as the reactor volume divided by the volume of daily digestate withdrawal, although substrate addition is more often used in practice (Equation 2) (Schnürer & Jarvis, 2009). As high an OLR as possible will maximize the gas production per unit substrate as more substrate will be present for conversion to methane assuming the degree of degradation is not limited by too short of a HRT. As low an HRT as possible will maximize the gas production per unit volume of the reactor used as the material will travel through that volume more quickly, again, as long as degree of degradation is maintained. However, an OLR in excess of a certain threshold limit, specific to a given system, will lead to process failure due to an accumulation of inhibitory substances (Kim & Lee, 2015). Conversely, in continusously stirred reactors, microorganisms will be washed out when the HRT is less than their doubling time (Weiland, 2010).

Equation 1 $$OLR = \frac{Daily\ Substrate\ Volume\ (g\ Day^{-1}) * TS * VS}{Reactor\ Volume\ (L)}$$

Equation 2 $$HRT = \frac{Reactor\ Volume\ (L)}{Daily\ Substrate\ Volume\ (\ L\ Day^{-1})}$$

FAs

Fatty acids (FAs) comprise one class of inhibitory compound that may accumulate as a result of excessively fatty substrate or a combination of overfeeding and insufficient withdrawal of H_2 by methanogens (van Nevel, et al., 1971). Among them, long chain fatty acids (LCFAs) (C>=13) pose the threat of direct inhibition of gram positive bacteria and methanogens via the disruption of cell membrane integrity with downstream effects on the pH gradient, metabolic transport and energy utilization (Demeyer & Henderickx; 1966, Galbrath, et al., 1971).

Short chain, or volatile, fatty acids (VFAs) (C<=5) may also accumulate from overfeeding if acetogens and methanogens fail to utilize them at a sufficient rate with propionate especially dangerous (Weiland, 2010). High levels (< 1 g L^{-1}) may lead to product inhibition of the fermentative bacteria or a decrease in alkalinity followed by pH, to the detriment of the biogas process in general (Pind, et al., 2003). Undissociated acetic acid has also been found to be inhibitory via the depletion of cellular methionine pools and the accumulation of the toxic intermediate, homocysteine (Roe, et al., 2002).

Ammonia

The degradation of protein-rich material may also lead to an accumulation of the inhibitory compound ammonia (NH_3), released from the amine group present in the constituent amino acids (Koster & Lettinga, 1988). Although ammonia is required for growth, large amounts (>3.0–3.3 g NH_4^+-N/L; 0.14–0.28 g NH_3/L) can inhibit important microorganisms acting at various steps in the process, with acetoclastic methanogens especially prone to this type of inhibition (Sprott, et al., 1984; Westerholm, et al., 2015).

The hydrophobicity of ammonia enables its passive entry into the cell. Inhibition occurs when, after cell entry, ammonia takes up a proton from the cytoplasm to become

ammonium, thereby dissipating the pH gradient and thus the proton motive force across the cell membrane (Sprott, et al., 1984).

Ammonia inhibition is considered of particular concern in high pH and/or thermophilic systems due the shift in equilibrium that occurs (Figure 4, Equation 3); the higher the pH or temperature, the more the equilibrium between ammonium and ammonia shifts towards the latter, more toxic, form (Rajagopal, et al., 2013).

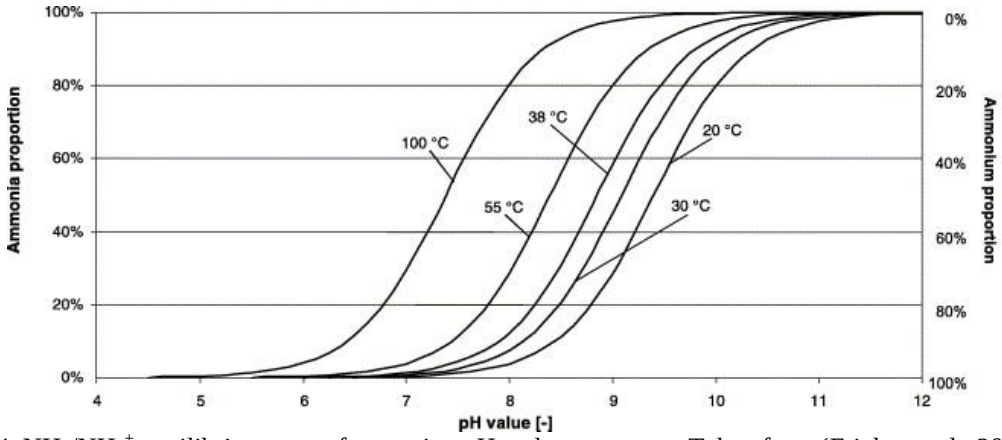

Figure 4. NH_3/NH_4^+ equilibrium curve for varying pH and temperature. Taken from (Fricke, et al., 2007 with calculations from Kollbach, et al., 1996)

Equation 3 $$NH3 = \frac{(\mathrm{NH4^+ - Nt})}{\left((10^{\mathrm{pKa-pH}}) + 1\right)}$$

Sulfur: Sulfates, Sulfides

As with nitrogen, the presence of an excessive amount of sulfur will affect the amount and quality of biogas produced. Similarly, sulfur is most often introduced into a biogas system by way of its release from sulfur-containing amino acids, such as cysteine and methionine, during protein degradation (Abatzoglou & Biovin, 2009). Petrochemical plant and tannery waste streams are other common contributing sources of sulfur rich substrate (Cai, et al., 2008).

In biogas reactors, sulfur exists most commonly as sulfates (e.g. SO_4^-) and sulfides (e.g. H_2S), each with a corresponding pathway of inhibition. Sulfate reduction (to H_2S) by sulfate reducing bacteria (SRB) is more energetically favourable than methanogenesis (ΔG = - 43 kJ mol^{-1}, compare with Table 3) (Gerardi, 2006). For this reason, SRBs outcompete methanogens for available carbon and hydrogen leading to a reduced rate of methanogenesis. Subsequently, H_2S can have a direct negative impact on the cellular functions of microorganisms if concentrations exceed 200 ppm. It can passively enter cells with inhibitory effects including the denaturing of proteins via crosslinking of peptides and the disturbance of cellular pH control due to interference of sulfur assimilation (Chen, et al., 2014). A further, indirect impact of H_2S is the sequestration of important trace elements as metal-sulfide precipitates (e.g. FeS), discussed below (Dhar, et al., 2012).

Moreover, even fairly low levels of H_2S (>350 ppm) in the resulting biogas are undesirable due to the corrosion of engines caused by products of the combustion of the gas (e.g. H_2SO_3) (Wellinger & Lindberg, 1999).

Temperature

Biogas production can operate at temperatures from just above 4°C to over 75°C but in practice, mesophilic (30-40°C) and thermophilic (40-60°C) are the two most common ranges of operation (Nordberg, 2006). Compared to the mesophilic range, biogas production operating at thermophilic temperatures offers the advantages of higher rates of

methane production, greater degradation of substrate, higher OLR and lower HRT, low viscosity and the possibility of auto-hygienization (Ostrem, 2004). Disadvantages exist as well, however, as a higher temperature consumes more energy and the process is often less stable (Al Seadi, et al., 2008). The reason for this instability is a combination of factors. As touched on previously, an increased production rate will release inhibitory compounds at a higher rate from the substrate, such as NH_3, VFAs and H_2S (Kim & Lee, 2015). The NH_3/NH_4^+ ratio will also increase with temperature (Rajagopal, et al., 2013). Furthermore, and crucially, abundance and diversity of the microbial community is diminished at higher temperatures (Levén, et al., 2007)).

Trace Elements

The addition of trace elements such as iron, nickel, cobalt, zinc, molybdenum, selenium and others is common in most large-scale biogas plants (Schattauer, et al., 2010) because of the positive effects they are thought to provide despite specific mechanisms of action of these metals being poorly understood (Osuna, et al., 2003; Wilkie, et al., 1986).

Known or proposed mechanisms include the addition of iron, e.g. as $FeCl_2$, which counteracts inhibition by H_2S, described above, through the precipitation of harmless FeS (Dhar, et al., 2012). This process serves multiple purposes in that such inorganic precipitates decrease the concentration of toxic sulfides, avoid the sequestration of other important metals and can provide support for adhesion of bacteria by stabilizing bacterial aggregates within granules (Osuna, et al., 2003). Ferric iron compounds have also been shown to counteract inhibition by LCFAs (Galbrath & Miller, 1973).

The benefits of other metals seem to lie in their roles as cofactors to process-related enzymes. For example, in methanogens, cobalt is required for the activity of methyltransferase, nickel is needed for efficient dehydrogenation and zinc is present in carbonic anhydrase (Ferry, 1999).

Importantly, concentrations of trace elements in biogas digestate above local guidelines may limit its use as fertilizer (e.g. $Ni \leq 50$ mg/kgTS, $Zn \leq 800$ mg/kgTS) (Afvall Sverige, 2016).

Surveillance Parameters

Surveillance parameters provide insight into the status of the biogas production process. CH_4 and CO_2 contents are two of the simpler parameters to measure but can vary across systems making general guideline values of little use. However, these parameters tend to remain fairly constant within a system as long as alterations in process parameters are minimal. Consistent monitoring of surveillance parameters therefore enables the establishment of a "normal" state from which deviations, indicative of process disturbances, can be observed (Drosg, 2013)

Total gas production per unit time is a key surveillance parameter in production as it is directly observable. When combined with CH_4 content and OLR, total gas production can be used to calculate specific methane production (SMP), i.e. volume of methane production per unit time and g VS of substrate, indicative of the efficiency of the use of substrate (Equation 4).

Degree of substrate degradation (DoD) is another measure of the efficiency of use of the substrate. It can be measured either by calculating the ratio of the difference between the TS and VS of the substrate and digestate to that of the substrate alone with the assumption that the volume difference between substrate and digestate is negligible (Equation 5) or by a flow dependent calculation which takes into account the organic fraction remaining in the digestate and is built on the conservation of ash (Equation 6) (Schnürer & Jarvis, 2009).

Equation 4 $$SMP = \frac{Daily\ Gas\ Production\ (NmL\ Day^{-1})\ *\ Methane\ Content\ (\%)}{OLR\ (gVS\ Day^{-1}\ L^{-1}) * Reactor\ Volume\ (L)}$$

Equation 5 $$DoD1 = \frac{(\ TSsubstrate\ *\ VSsubstrate\ -\ TSdigestate\ *\ VSdigestate\ )}{TSsubstrate\ *\ VSsubstrate} * 100$$

Equation 6 $$DoD2 = \frac{(VSsubstrate\ *\ VSdigestate)}{(1 - \big((1\ -\ VSdigestate)\ *\ VSsubstrate\big)\ )} * 100$$

Optimal pH for biogas production lies between 6 and 8 (Luostarinen, et al., 2011). Higher pH is often a result of an accumulation of NH_3, while low pH often stems from an accumulation of fatty acids with H_2S also affecting pH. pH within the optimal range usually indicates process stability and is commonly used because it is faster and easier to measure than the compounds that influence it. Certain cases arise, however, in which accumulations of these compounds exist without the manifestation of a change in pH. High alkalinity of the substrate may act as a buffering system or a balance may be struck between acidic and basic components leading to pseudostability, whereby production is inhibited despite an "optimal" pH (Al Seadi, et al., 2008). For these reasons, along with those described above (see Process Parameters), the regular measurement of NH_3, VFAs and H_2S must also be included as a part of effective biogas production surveillance.

1.2 Purpose

The purpose of this study was to investigate the effects of the addition of trace elements on the microbial communities active in certain thermophilic biogas processes operating at high ammonia levels.

1.3 Hypothesis

The addition of trace elements was expected to improve biogas production by promoting stability of the syntrophic acetate oxidizing microbial community.

2. Materials and Methods

2.1 Reactor Experiment

A reactor experiments was performed consisting of a pair of reactors, GP1 and GP2. Each reactor was a laboratory scale continuously stirred tank reactor (CSTR) operating under anaerobic conditions with a total volume of 8-L and an active volume of 5-L. All reactors operated at 52°C with stirring of 90 rpm.

Operation of the reactors began on 27 January 2015 with 5-L of inoculum in each reactor. Inoculum and substrate were obtained from Uppsala Vatten's Biogas Plant, located at Kungsängen, Uppsala, which operates at 52°C. Substrate consisted of source-sorted organic food waste (FW) from households and companies (Table 4) in combination with slaughterhouse waste from within the Uppsala Municipality (Kommun).

The total solids contents (TS) of the inoculum and substrate were calculated as ratios of dry to wet weights with dry weight measured after incubation of 40-80 g wet weight samples overnight at 105°C. Volatile solids contents (VS) were calculated as the ratios of combustible to dry weight with combustible weight measured after 1 hour at 350°C then 6 hours at 550°C of the dried samples.

Initial and final operational parameters of the biogas processes are presented in Table 5. OLR and HRT values represent weekly averages, which take into account a feeding routine of approximately 6 days per week, in place for social reasons.

Starting 28 January (day 1), a commercially available TE mixture (BDP-865, 9% iron (Fe^{2+}) with cobalt, nickel, selenium, tungsten, and hydrochloric acid) purchased from Kemira AB, was added to the substrate of both reactors, to establish similar starting conditions, at a concentration of 2.7g L^{-1}, equating to 0.26 g gVS^{-1}. From 6 February (day 10), feeding of reactor GP2 (+TE) continued with substrate containing this same concentration of trace elements whereas GP1 (-TE) was fed with substrate without trace elements. The concentration mentioned is used in the large-scale plant and was maintained for both reactors for the first weeks of the experiment period to avoid shocking the system immediately upon transition to the lab-scale reactors.

In an effort to observe a greater effect of TE, the OLR of each reactor was increased by 1 gVS L^{-1} Day^{-1} after 3 HRT had eclipsed (ensuring a complete turnover of reactor liquid) on April 24 (day 87). The increase occurred over an 8 day period, from 29 April (day 92) to 6 May (day 99), in two increments of 0.5 gVS L^{-1} Day^{-1}. A seven day intermediate step at 3.7 gVS L^{-1} Day^{-1} was included to allow for a less stressful transition.

Operation of the reactors continued for more than 3 HRT following the increase in OLR before the collection of data to be used in this analysis was ended on 30 July 2015, after 184 days of total operation.

The software, Dolly (™) v 2.03 (Belach Bioteknik) was used to continuously measure total gas production over time from each reactor. Immediately prior to each daily feeding event, the volume of total accumulated gas production since the previous feeding event was noted.

Biogas CH_4 content was measured weekly by taking a 2 ml gas sample of the headspace of each reactor followed by GC analysis (described below, Gas Analysis).

Digester liquid samples from each reactor were taken weekly. pH was measured immediately upon taking the samples whereas the remaining volumes of samples were stored at -20°C for later VFA and microbiological analysis.

H_2S content was measured weekly over a period spanning approximately one month prior to and one month following the change in OLR (described below, Gas Analysis)

Table 4. Composition of food waste from Uppsala Municipality as a percentage (%) of organic matter

Substrate	Cellulose	Crude Fat	Starch	Lignin	Hemicellulose	Sugar	Metals	Plastics
Food Waste	15.6	15.0	13.2	9.9	3.2	1.6	1.2	7.6

Values derived from Eklind & et. al. , 1997
Metals and plastics removed from waste before treatment

Table 5. Operation Parameters of Reactor Experiment

Material	Substrate Type	TS (%)	VS* (%)	OLR (gVS L^{-1} Day^{-1}) Pre-OLR	Post-OLR	HRT (Days) Pre-OLR	Post-OLR	TE mixture BDP-865, 9% $FeCl_2$) (g gVS^{-1})
Inoculum	Food waste + Slaughterhouse waste	3.2	66.0	3.2	4.2	30	23	0.26
Substrate		10.9	89.7					

*VS presented as a proportion of TS
Pre-OLR: weeks 1-13, Post-OL: weeks 14-27

Degree of Degradation

A measure of efficiency of a biogas system is the degree of degradation of substrate. Both equations to determine this value were used for both reactors. Samples of digester liquid from GP1 and GP2 were taken in triplicate during weeks 13 and 14, before and after three retention times had eclipsed. TS and VS were measured from the triplicate samples and degree of degradation was calculated as described above (Equations 4 and 5).

Kinetics Monitoring

The kinetics of production was measured on two occasions during the experiment period by taking 2 ml gas samples from the headspace of each of the two reactors at time intervals over a 24 hour period following feeding. In both cases feeding occurred at approximately 10:00 with samples taken at hourly intervals although more frequently initially after feeding and not at all overnight. Gas sampling and analysis is described in more detail below (Gas Analysis).

2.2 Laboratory and Analytical Techniques

Sample storage and selection

The microbiological analysis was carried out using samples of reactor digester liquid collected weekly and stored at -20°C in 15 ml centrifuge tubes. Nine relevant time points were selected for TRFLP analysis (Table 6). The three earliest time points represented startup conditions, which were similar for both reactors. Three middle time points were taken during the weeks leading up to the OLR increase and just before the completion of three HRT from startup. The final three selected time points follow the completion of three HRT after the increase in OLR.

Due to time constraints, the clone library was constructed using samples obtained from a previous reactor experiment under similar conditions to this study: the same source of inoculum and substrate, 52°C or 37°C operation temperature, 90 rpm stirring, OLR = 3 gVS L^{-1} Day^{-1}, HRT = 28 Days (Isaksson, 2015).

Table 6. Dates of sampling points for microbiological analysis

Sampling Points	Dates of Sampling		
Similar Starting Conditions	17/02/2015	20/02/2015	03/03/2015
Pre-OLR Increase (3 HRT*)	08/04/2015	14/04/2015	21/04/2015
Post-OLR Increase (3 HRT*)	09/07/2015	23/07/2015	30/07/2015

*Represents the number of hydraulic retention times from previous sampling group
Pre-OLR: weeks 1-13
Post-OL: weeks 14-27

DNA extraction

Weekly samples previously stored at -20°C were thawed in a water bath at room temperature. The FastDNA SPIN Kit for Soil (MP Biomedicals) was used to isolate PCR-ready genomic DNA starting with 200 µl per extraction with each sample extracted in triplicate. The provided protocol was largely followed with the exception of the prolongation of the first centrifugation step to 15 minutes; the addition of a washing step using 500 µl per sample of humic acid wash including 2.75 M guanidine thiocyanate; and elution with between 60-70 µl of the provided elution buffer.

TRFLP

Terminal restriction fragment length polymorphism analysis (Liu, et al., 1997) was performed using the mentioned extracted DNA. DNA from the functional gene, *fhs*, encoding the enzyme formyltetrahydrofolate synthetase (FTHFS), active in the Wood-Ljungdahl pathway of acetogenic bacteria, including SAOB. Analysis of this gene allows for the tracking of changes in population dynamics of these acetogens which play an important role in high ammonia systems such as this one. The *fhs* gene was amplified by PCR using IQ™ Supermix (Biorad) and the fluorescent-labelled forward primer, 3SAOfhs-famfw (fam-CCNACNCCNGCHGGNGARGG) and the reverse primer, fthfs-HP-br (TGVGCRATRTTNGCRAANGGNCC). Triplicates of amplification products of each time point were pooled prior to gel electrophoresis, after which, the expected band size of 664 kb was cut from the gel on a Chromato_VUE ® Transilluminator (UltraViolet Products Inc.) and purified using MinElute Gel Extraction Kit (Qiagen) according to the provided protocol.

The resulting DNA of each time point was divided into two groups, each to be digested with either the restriction enzyme AluI (Thermo Scientific) or Hpy188III (NEBiolabs). Restriction occurred at 37°C for 1 hour before heat inactivation at 65°C for 20 minutes.

Digested DNA was sent to NGI Uppsala (SciLife Genome Centre) for the final steps of the analysis. These steps consisted of the separation of the restriction fragments by length, and the reading of the strength of the fluorescent signal emitted by each fragment to be later used to calculate the relative abundance (RA) of each fragment.

Clone Library of the *fhs* gene

The *fhs* gene was amplified, gel extracted and purified from the extracted DNA as described above but with the Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific). The forward primer, 3SAOfhs (CCNACNCCNGCHGGNGARGG) was paired with the same reverse primer used in the TRFLP protocol. The amplified DNA was ligated into the pJet1.2 cloning vector using T4 DNA ligase (CloneJET PCR Cloning Kit, Thermo Scientific) with incubation at room temperature for one hour. Following incubation, the ligation mixture was transformed into JM109 High-Efficiency Competent Cell (Promega) and spread onto LB agar plates with 100ug/l ampicillin to create a clone library whereby each colony contained a plasmid with one gene variant of the *fhs* gene. Plates were incubated at 25°C over one night, or two nights if necessary for sufficient growth.

The resulting colonies were picked with pipette tips for colony PCR using Dreamtaq Master Mix (2x) (Thermo Scientific) and the primers, pJet1.1fwd (CGACTCA-CTATAGGGAGAGCGGC) and pJet1.2rev (AAGAACATCGATTTTCCATGGCAG). Picked colonies were also restreaked on similar LB + ampicillin plates in case of the need of further use. The amplified DNA was sent for sequencing to Macrogen Europe (Amsterdam, Netherlands).

Gas Analysis
Carbon dioxide content of the reactor headspace was measured by titrating a 5 ml sample of the headspace gas, taken with a needle and syringe through a rubber stopper, in 5 ml of 7M NaOH in a graduated curved buret. CO_2, but not any other component of the gas mixture, dissolves completely in the NaOH solution upon addition. The remaining gas mixture accumulates at the top of the buret thereby displacing the NaOH solution downwards. The volume of displacement subtracted from 5 ml provides the volume of CO_2 in the reactor headspace and from that a percentage of CO_2 can be calculated.

Gas chromatography was used to measure the methane content of the head space of the CSTR reactors just before feeding during regular surveillance and the kinetic experiment as described in Westerholm, et al. (2010). In short, 2 ml gas samples were taken from the reactors with a needle and syringe and transferred to airtight vials for analysis in a PerkinElmerARNEL 500 gas chromatograph.

The Biogas 5000 (Geotech) was used to measure the H_2S content of the reactors. A syringe and needle was attached to the end of tubes connected, respectively, to the in- and outflows the machine. The needles were inserted into the reactors through rubber stoppers. In this way, the reactor gas was circulated out of and back into the reactor via the Biogas 5000 which measured H_2S content via an electrochemical sensor in the process.

VFA Analysis
Approximately 2 ml of the weekly digester liquid sample was transferred to 2 ml microcentrifuge tubes for VFA extraction followed by analysis by high-performance liquid chromatography (HPLC) in an Agilent 1100 series HPLC as described previously (Westerholm, et al., 2015).

Carbon and Nitrogen Analysis
Digestate samples from each reactor were collected in separate plastic sampling containers on consecutive days at approximately 200 ml per day. Containers were stored at 4°C until a volume of approximately 500 mL was reached at which point containers were stored at -20 until being sent for analysis. Samples were taken during the week of 27 April 2015, following the completion of 3 HRTs but before the OLR had been increased. Samples were sent to Agrilab AB, Uppsala for analysis. Total nitrogen (Tot-N) and total carbon (Tot-C) were analyzed using a LECO CHN-600 elemental analyzer (LECO Corporation, St. Joseph, MI, USA (IOS, 1998, IOS, 1995).

2.3 Statistical and Computational Procedures

Standard Deviation
All standard deviation values presented represent sample standard deviation (Equation 7).

Equation 7 $$SD = \frac{\sqrt{\sum(x - \bar{x})^2}}{(n - 1)}$$

TRFLP Data Analysis
Raw TRFLP data was prepared for further analysis as described in Westerholm, et al. (2011b). In brief, for each sample point's TRFLP profile, generated from Peak Scanner software (Applied Biosystems), lengths of TRFs were rounded to integers and duplicates were removed along with those of lengths outside the range 50-664 bp with longer TRFs assumed to be uncut sequences. Fluorescence of each TRF was divided by total fluorescence to obtain relative abundance values. TRFs with a relative abundance below 0.5% was also removed. Relative abundances were recalculated after a final manual

binning step to merge TRFs similar in length (+/- 3 bp) and representing a single gene variant. Relative abundances of these binned TRFs were summed and the average length of binned TRFs was used for further notation.

PCA

Using the prepared TRFLP data, principle component analysis (PCA) was employed as a means of identifying the TRFs for which the greatest differences in relative abundance existed between treatments for each restriction enzyme digestion. Means of the pre-OLR and post-OLR increase time points were analyzed separately. Principal component 1 (x-axis) represents the variation from average relative abundance while principal component 2 (y-axis) represents the variation between treatments.

TRF Identification and Clone Library Analysis

The DNA sequences resulting from the clone library were compared to eliminate redundancy (>97% similarity between sequences) and produce unique sequences, or operational taxonomic units (OTUs) (Blaxter, et al., 2005). OTUs were digested *in silico* with the same restriction enzymes as in the TRFLP analysis (AluI, Hpy188III) using the CLC workbench software (Qiaqen). TRFs of similar length (+/- 5 bp) in both the *in vitro* and *in silico* digestions were considered to represent the same gene variant of *fhs* (Clement, et al., 1998). The clone library derived sequences and their corresponding amino acid sequences were queried in the nBlast or pBlast databases (National Library of Medicine) to identify previously sequenced TRFs and their environments of isolation. Matches were considered of high certainty if % similarity ≥ 99% for DNA sequences or ≥ 89% for amino acid sequences, as previously described (Westerholm, et al., 2015)

Phylogenetic Analysis

fhs gene OTUs derived from the clone library were incorporated into a previously constructed maximum likelihood tree including *fhs* gene OTUs obtained from previous projects. A phylogenetic tree was constructed using PhyML v3.0 based on 100 bootstraps (Müller, et al., 2015).

3. Results

3.1 Surveillance Parameters

Specific Methane Potential

The weekly average SMP values for both reactors were similar for the first three weeks of the experiment with values of 420 NmL gVS^{-1} Day^{-1} and 413 NmL gVS^{-1} Day^{-1} for GP1 and GP2, respectively (Figure 5). Typical for startup periods (Schnürer & Jarvis, 2009), variation in weekly SMP of each reactor was high during these first three weeks with sample standard deviations of 153 and 154 NmL gVS^{-1} Day^{-1} for GP1 and GP2, respectively

Addition of TE to GP1 was stopped in week 3 to establish the two treatments of interest (i.e. GP1 (-TE) and GP2 (+TE)). For the period from weeks 4 to 13, within-treatment variation was low as values stabilized with averages of 497 (SD = 28) NmL gVS^{-1} Day^{-1} and 464 (SD = 29) NmL gVS^{-1} Day^{-1} for GP1 and GP2, respectively. Surprisingly, GP1 (-TE) seemed to perform slightly better than GP2 (+TE), even after 3 HRT.

In order to test if the presence of TE would enable GP2 to better cope with the stresses of a higher organic loading rate, the OLR of each reactor was increased to 4.2 gVS L^{-1} Day^{-1} (from 3.2 gVS L^{-1} Day^{-1}) in week 14, with a resulting decrease in HRT from 30 to 23 days.

In the period following the OLR increase (weeks 14-27), mean SMP for GP1 decreased to 451 (SD = 37) NmL gVS^{-1} Day^{-1} (from 497 NmL gVS^{-1} Day^{-1}). Such a decrease was not seen for GP2 which had SMP of 457 (SD = 51) NmL gVS^{-1} Day^{-1} during that latter period.

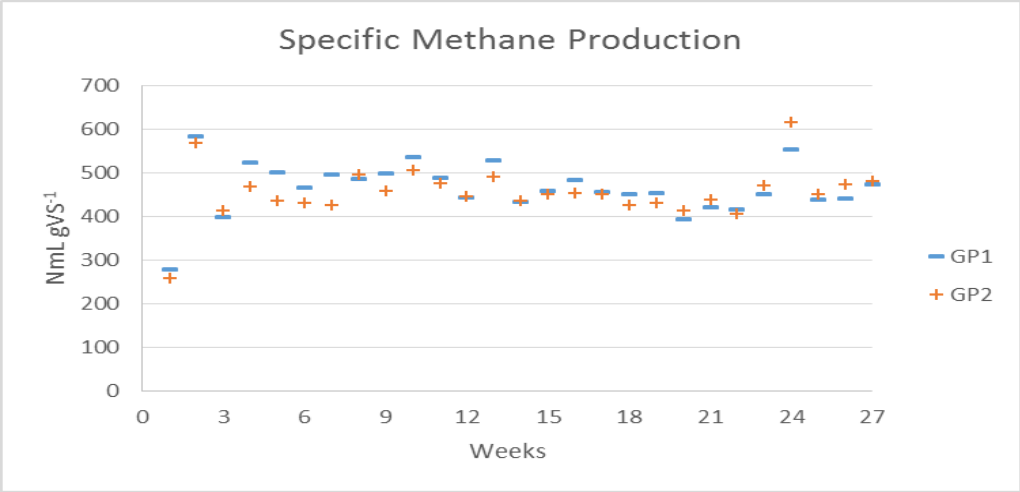

Figure 5. Weekly mean Specific Methane Production (NmL) of reactors GP1 (-TE) and GP2 (+TE)

pH

Variation in pH was low within (i.e. before and after the OLR increase) and across treatments with no significant differences found (Figure 6). Excluding the first three weeks, pH ranged from 7.6 to 8.0 for GP1 and 7.6 to 78.0 for GP2. A decreasing trend was observed, however, over the first three retention times, reaching the lowest point in week 11 for both reactors with pH = 7.6. In the weeks following the OLR increase pH returned to slightly higher pH for both reactors and ranged between 7.7 and 8.0 for the remainder of the experiment period.

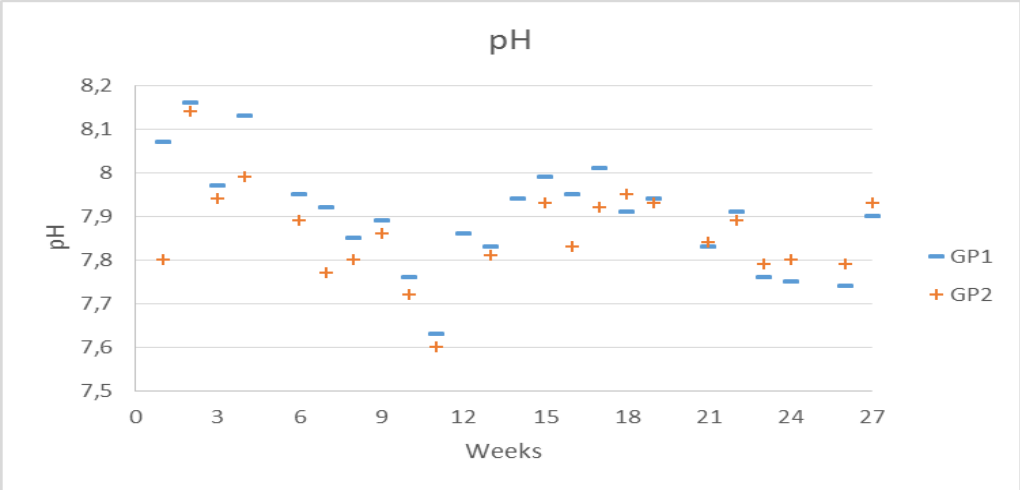

Figure 6. Weekly pH of reactors GP1 (-TE) and GP2 (+TE)

Hydrogen Sulfide

H_2S concentrations were only measured between weeks 6 and 21 because of the intention of monitoring changes in H_2S concentration leading up to and following the increase in OLR (Figure 7). From the first measurement, and prior to the OLR increase, a large difference between reactors was observed. Average concentration for GP1 for weeks 6 - 13 was 943 (SD = 99) ppm whereas the average concentration during that time for GP2 was 148 (SD = 49) ppm. Following the OLR increase (weeks 14-21), H_2S concentrations for GP1 increased to 1406 (SD = 331) ppm. Equivalent values for GP2 remained similar at 138 (SD = 59) ppm. H_2S concentrations for GP1 continued to increase from week 13 but at the final measurement, in week 21, at 1036 ppm, was similar to values observed before the increase in OLR. A dip in concentration for GP2 over the weeks preceding, during and following the OLR increase (weeks 12-14) was the only variation from the average value.

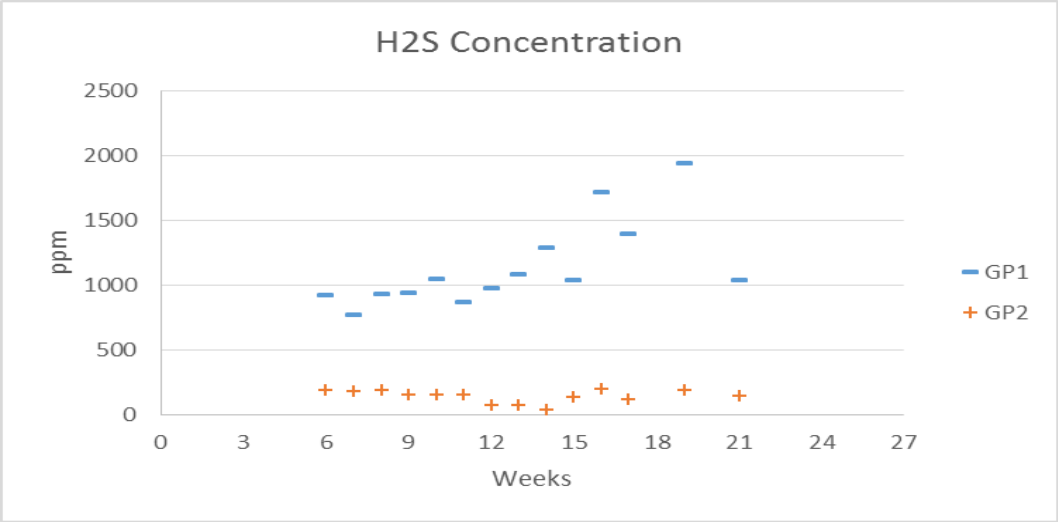

Figure 7. Weekly H2S concentrations (ppm) of reactors GP1 (-TE) and GP2 (+TE) between weeks 6 - 21

VFAs

The level of total volatile fatty acid concentrations was highest for both reactors during startup (VFA_{GP1} = 2.0 g L^{-1}, VFA_{GP2}= 2.4 g L^{-1}) when acetate was the main contributor ($acetate_{GP1}$ = 1.6 g L^{-1}, $acetate_{GP2}$ = 2.0 g L^{-1}) (Figure 8). VFA concentration for both reactors decreased during the first few weeks of the experiment, settling at low levels (< 1 g L^{-1}) prior to the OLR increase (weeks 4-13), with values ranging from 0.1 to 0.5 g L^{-1} for GP1 and 0.0 to 1.4 g L^{-1} for GP2. Exceptional spikes in both acetate (0.70 g L^{-1}) and

propionate (0.60 g L^{-1}) concentrations occurred in GP2 in week 13 but otherwise concentrations remained low.

Total VFA concentration for GP1 increased following the increase in OLR (from week 14), reaching a peak of 1.9 g L^{-1} in week 21 before dropping slightly to around 1.4 g L^{-1}. Again, acetate and propionate were the main contributors but I-butyrate and I-valerate were both consistently detected from about week 18. The steady increase seen in GP1 was not present in GP2 in which total VFA concentration quickly returned to low levels after the peak in week 13. An isolated jump to 1.1 g L^{-1} for acetate in week 23 was the only remarkable observation during this time period.

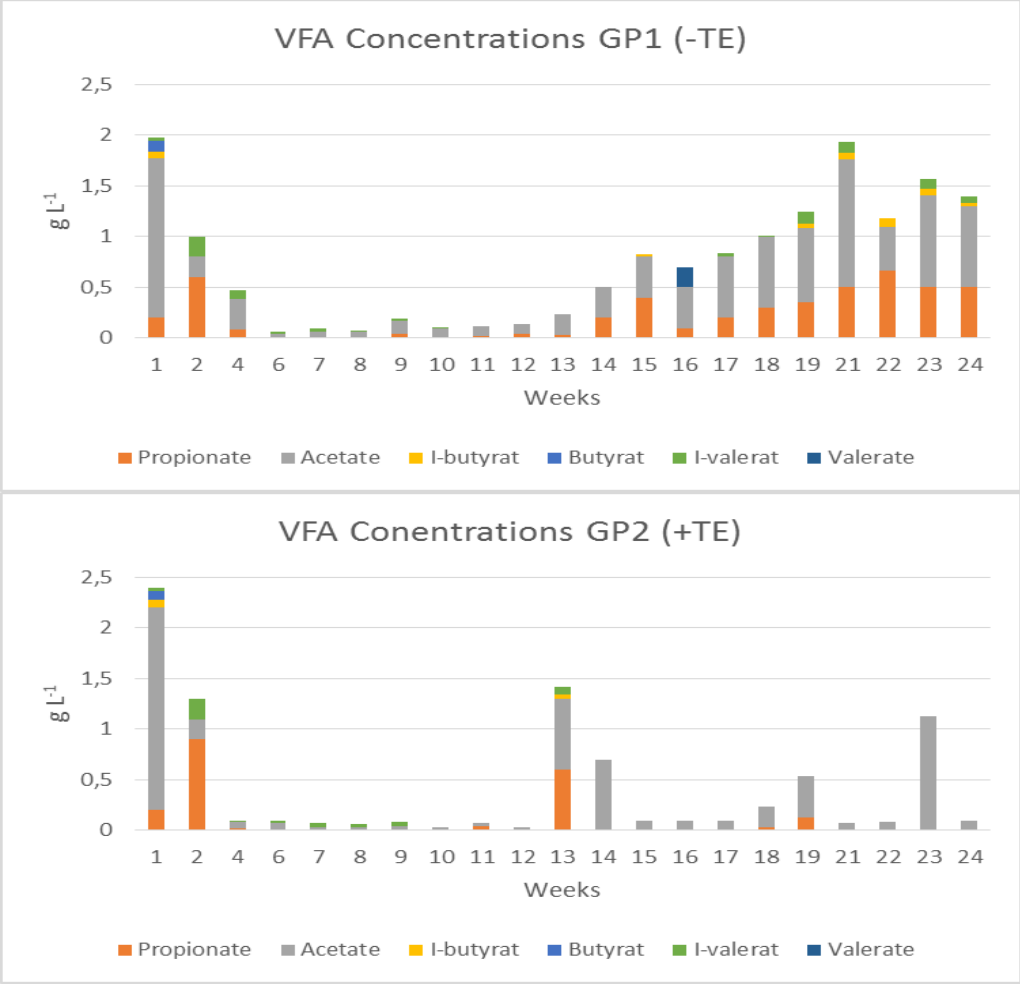

Figure 8. Weekly levels of acetate, propionate, butyrate, I-butyrate, valerate and I-valerate in concentration (g/L^{-1}) of reactors (above) GP1 (-TE) and (below) GP2 (+TE)

Degrees of Degradation

Two methods exist to calculate degree of degradation and results from both are presented in Table 7. Though the difference is small, the degree of degradation of GP1 from both methods (method 1 = 76%, method 2 = 69%) is lower than the corresponding values for GP2 (method 1 = 77%, method 2 = 75%).

Table 7. Mean (SD) degree of degradation

Calculation Method	-TE	+TE
1	76% (4%)	77% (5%)
2	69% (7%)	75% (6%)

Degrees of degradation (% of total VS degraded) represent means of calculation based on samples from weeks 13 and 14

Kinetic Experiment

Natural variation precludes direct comparison of curves on different sampling days but a difference had clearly developed across treatments in the time between sampling days. The curves of both reactors preceding the OLR increase were virtually identical whereas after the increase GP1 production lagged behind GP2 production over the first several hours after feeding. GP1 accumulated production caught up overnight and eventually surpassed GP2 accumulated production implying that the rate of production for GP2 must have been lower than for GP1 during the unmeasured, overnight period.

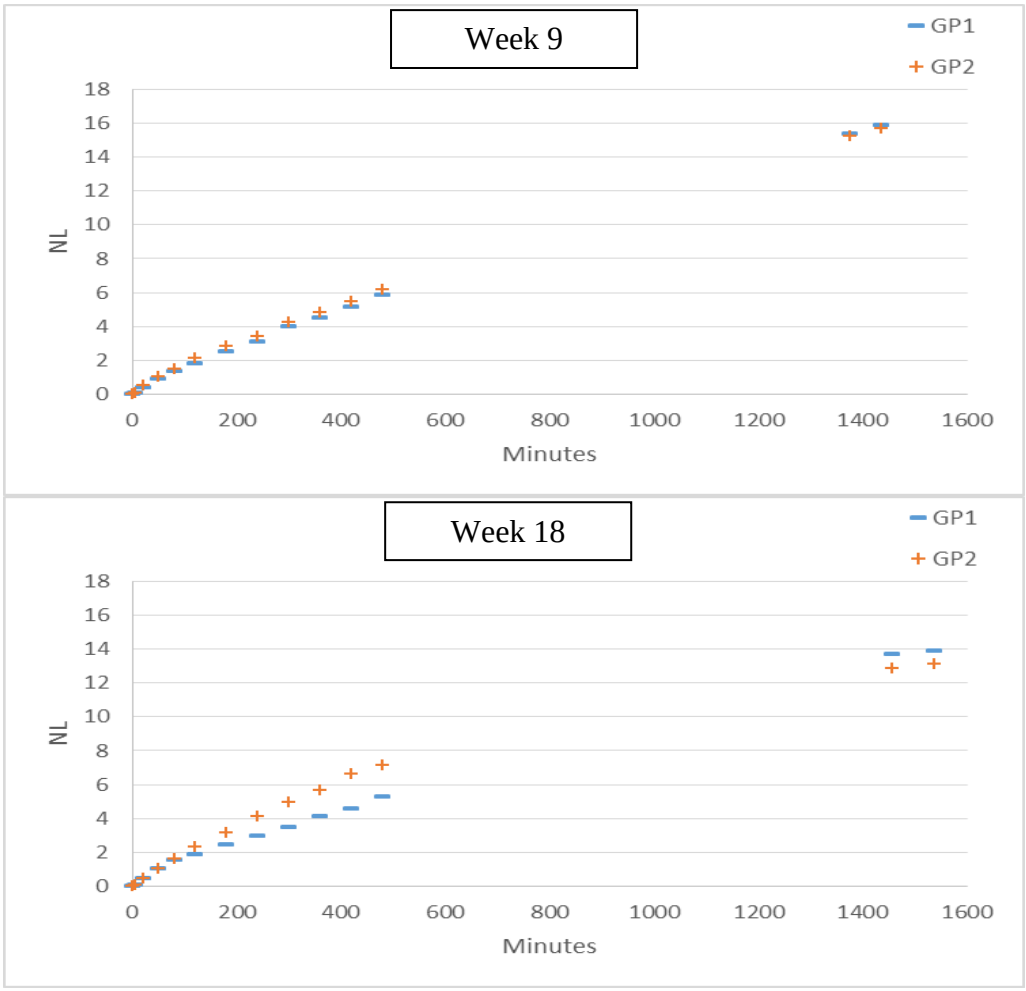

Figure 9. Biogas production kinetics i.e. accumulated methane production (NL) over a 24-hr period. (above) 24 March 2015, week 9 (below) 28 May 2015, week 18

Carbon and Nitrogen Analysis

Digester liquid samples analysed after 3 HRTs showed essentially no difference between treatments (Table 8). High concentrations of NH_4^+-N (total) and NH_3 confirmed the status of these reactors as high ammonia systems.

Table 8. Carbon and Nitrogen Analysis

Treatment	Total-N	Organic-N	NH_4^+-N_t	NH_3*	Total-C	C/N
TE (-)	4.7	1.6 (33.2)	3.1 (66.8)	0.4 - 1.0	16.5	3.5
TE (+)	4.9	1.7 (33.5)	3.3 (66.5)	0.4 - 0.8	17.8	3.6

Unit of values not in parentheses: g kg^{-1}
Unit of values in parentheses: percent of Total-N
*Values reflecting range of observed pH values and assuming constant NH_4^+-N_t (values presented in this table)

3.2 Microbiological Analysis

TRFLP

A TRFLP analysis was carried out by amplifying the *fhs* gene from digester liquid samples of 9 key time points followed by restriction digestion by the enzymes, AluI and Hpy188III, separately.

AluI-Digested

Based on principal component analysis of the AluI-digested TRFLP analysis the TRFs, 58, 81, 131 were identified as both dominant in the microbial community and differing between treatments (Figure 10, 11 and Table 9).

Over the experiment period, a decreasing trend was observed for relative abundances (RA) of TRFs 58, 131 and 268, while an increasing trend was seen for 81. The presence of TRF 548 was notable in week 1, 11 and 12 but otherwise its RA remained low. All of these trends, however, were present in both reactors pointing to natural community temporal dynamics as the cause rather than the experimental treatment. No

No differences in species richness or evenness were found.Of the dominant TRFs, 58 was the only TRF with consistently lower RA values in GP1 than GP2. The opposite was true for TRFs 81 and 131.

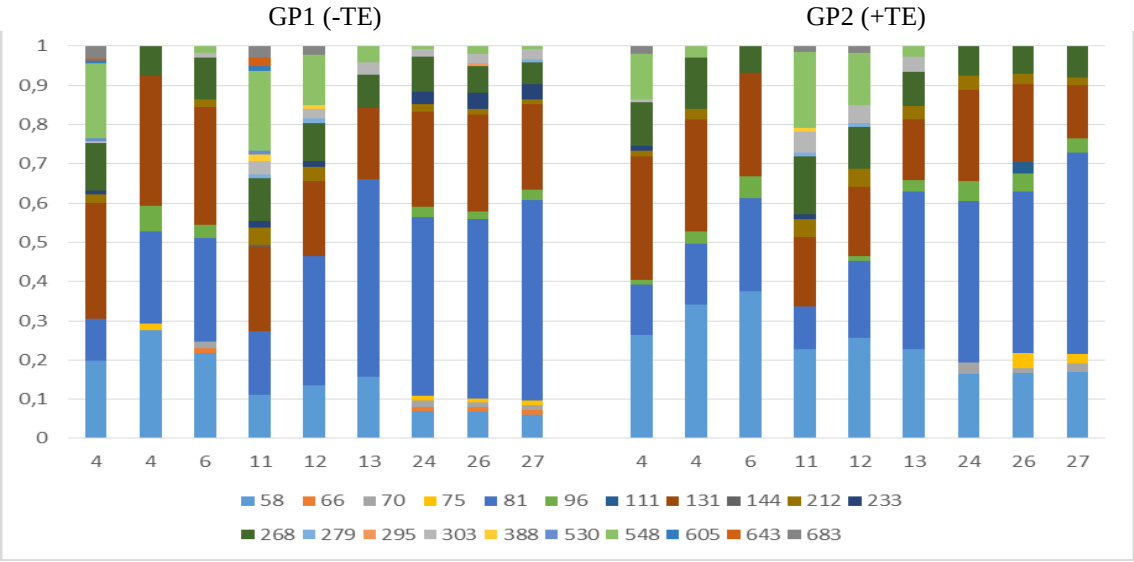

Figure 10. Relative abundance of AluI-digested TRFs from reactors without (-TE) or with (+TE) trace elements throughout the experiment period. The x-axis represents weeks of sampling.

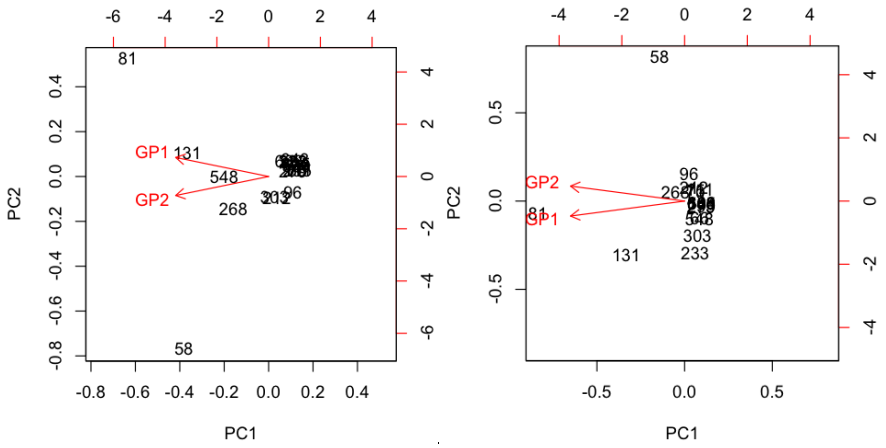

Figure 11. Principal component analysis of AluI-digested TRFs (left) mean of pre-OLR increase time points (weeks 11-13) and (right) mean of post-OLR increase (weeks 24, 26, 27) with PC1 (distance from average) explaining between 95% and 98% of variation for individual time points and PC2 (distance between treatments) explaining the remainder. Note difference in scales of axes.

Table 9. Mean relative abundances of dominant AluI-Digested TRFs for the entire experiment period

TRFs	GP1 % (SD)	GP2 % (SD)
58	14 (8)	24 (8)
81	34 (15)	28 (15)
131	25 (5)	22 (6)
268	9 (2)	10 (3)
548	7 (8)	6 (7)

Hpy188III-Digested

PCA analysis of the TRFLP analysis from Hpy188III-digested sequences revealed TRF 286 was the single most dominant TRF but this was true for both treatments (Figure 12, 13 and Table 10). Other noteworthy TRFs were 73, 295 and 310 because of the difference in relative abundance between treatments, even if their RAs were much lower than TRF 286.

RA of TRF 295 was lower in GP1 than GP2 for all time points while RA of 310 was consistently higher in GP1 (Figure 14 and Table 10). RA of TRF 73 was lower in GP1 than GP2 in weeks 11 and 12 but otherwise even between reactors. Again, no difference in species richness or evenness were found.

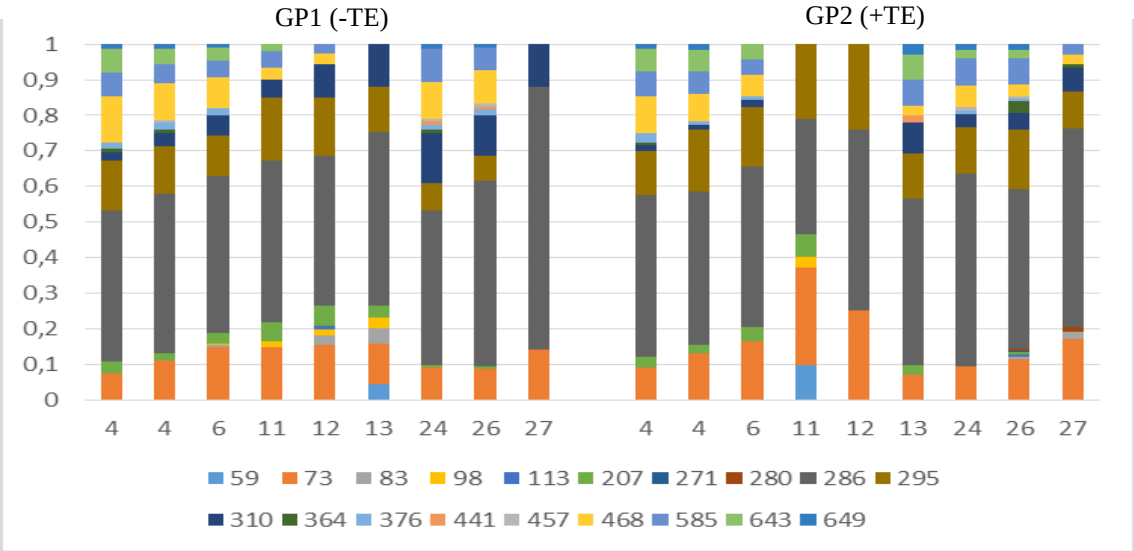

Figure 12. Relative abundance of Hpy188III-digested TRFs from reactors without (-TE) or with (+TE) trace elements at time points throughout the experiment period. TRFs increase in length from bottom to top of each bar.

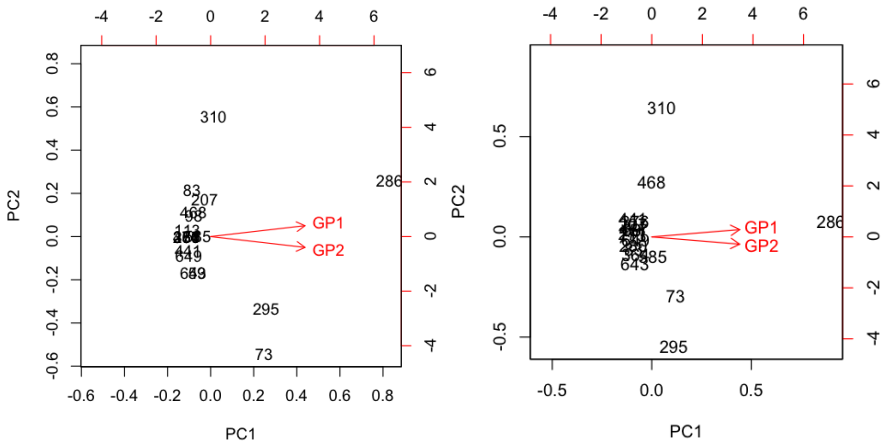

Figure 13. Principal component analysis of Hpy188III-digested TRFs (left) mean of three time points prior to OLR increase (weeks 11-13) and (right) mean of three time points 3 HRT after the OLR increase (weeks 24, 26, 27) with PC1 (distance from average) explaining between 95% and 98% of variation for individual time points and PC2 (distance between treatments) explaining the remainder. Note difference in scales of axes.

Table 10. Mean relative abundances of dominant Hpy188III-Digested TRFs for the entire experiment period

TRFs	GP1 % (SD)	GP2 % (SD)
73	12 (3)	15 (7)
286	49 (10)	47 (7)
295	11 (5)	16 (4)
310	8 (4)	3 (3)
468	6 (5)	4 (4)
585	4 (3)	5 (3)

TRF Identification and Clone Library Sequence Analysis

OTUs of the *fhs* gene, derived from the clone library based on samples from a previous but similar experiment, were used to identify TRFs from the TRFLP analysis and contextualize their role in biogas production (Table 11).

Of the 26 operational taxonomic units resulting from the clone library procedure, only OTUs 6, 17 and 19 could not be matched to TRFs from the TRFLP analysis even at low levels of certainty. All but two sequences (OTUs 8 and 22) were identified through either querying in nBlast or pBlast databases with 8 OTUs showing a match of high certainty in one and/or the other database. Based on previous reports of likely matches to the OTUs found in this study, the most common environments of isolation were anaerobic lab-scale digesters. Most were operated at mesophilic temperatures and several with high ammonia levels (Table 11).

AluI-Digested

TRFs 131 and 268 were the only dominant AluI-digested TRFs which could be matched to OTUs with high certainty (OTUs 22 and 13, respectively). No matches in either Blast database were found for OTU 22 (TRF 131). nBlast revealed a match of low certainty (79% similarity) for OTU 13 (TRF 268) with the sulfate-reducing *Desulfobacterium oleovorans* Hxd3 (Copeland & et al., 2007). pBlast pointed to the Human gut-derived *Firmicutes bacterium* CAG:170 (Nielsen & et al., 2012) as a high certainty match for the *in silico* translation of OTU13 with 89% similarity. TRF 111 was the third and only other TRF to match to an OTU with high certainty (OTU11). The DNA and protein sequences of OTU11 (TRF 111) show a perfect match (100%) to *Aminobacterium colombiense*, isolated from the anaerobic lagoon of a dairy wastewater treatment plant (Lucas & et al., 2010). OTUs 2, 5, 21, 24 and 25 lacked restriction sites for AluI and can thus be assumed to be represented by TRF 683.

Hpy188III-Digested

In silico digestion of OTUs with Hpy188III did not produce a match to the lone dominant TRF 286. A TRF of length 283 bp was, however, found in a previous study relating to an uncultured bacterium clone isolated under conditions similar to this studies', excluding temperature (Westerholm, et al., 2015).

OTUs could be matched to the lesser dominant TRFs 73, 295 and 310 and were able to then be matched to previously sequenced OTUs in either pBlast or nBlast. OTU9 (TRF 73) matched to the amino acid sequence of an uncultured bacterium clone isolated from a high ammonia system (Moestedt, et al., 2014) with 84% similarity. Also based on its amino acid sequence, OTU2 (TRF 295) might relate to *Phycisphaerae bacterium* SM1_79 (71% similarity) while OTU23 (TRF 310) might relate to *Anaerolineae bacterium* SM23_63 (64% similarity) with both isolated in a project investigating the sulfate-methane transition zone of estuary sediment (Baker, et al., 2015). In the case of Hpy188III only OTUs 1 and 5 were without restriction sites meaning they were likely represented by TRFs 643 and/or 649.

Table 11. Clone library derived unique *fhs* sequences with likely nBlast, pBlast and TRF identification

OTU	nBlast Match	pBlast Match	AluI	Hpy188III	Previous System*
Mesophilic					
1	FP929046	AIE39691	303	x	Mesophilic anaerobic digester
2	-	KPL22996	x	295	Estuary sediments (*Phycisphaerae*)
3	JQ082254	AFD97647	-	271	Mesophilic anaerobic digester
4	KP184587	AKA87401	-	310	High ammonia anaerobic digester
5	-	ABS80941	x	x	Anaerobic sludge
6	-	AKA87400	-	-	High ammonia anaerobic digester
7	-	AIE39691	268	98	Mesophilic anaerobic digester
8	-	-	81	-	-
9	-	AKA87400	-	73	High ammonia anaerobic digester
10	-	AIE39691	303	-	Mesophilic anaerobic digester
11	CP001997	ADE57663	111	364	Mesophilic Anaerobic dairy WWTP2, *Aminobacterium colombiense* DSM 12261
12	-	CAJ70914	303	207	Anammox bioreactor (Anoxic)
13	CP000859	CDB88042	268	-	Human gut (*Firmicutes bacterium*)
14	-	KPK74796	x	207	Estuary sediments (*Phycisphaerae*)
Thermophilic					
15	-	WP_044993384	-	98	*Lachnospiraceae bacterium* JC7
16	-	AFD97650	-	271	Mesophilic anaerobic digester
17	JQ979074	WP_044665140	-	-	Mesophilic anaerobic digester, *Syntrophaceticus schinkii*
18	KC256780	WP_028264064	303	-	*Atopobium fossor*
19	JQ082239	AFD97663	-	-	Mesophilic anaerobic digester
20	-	KKO19470	-	207	Bioreactor enrichment culture (*Candidatus Brocadia fulgida*)
21	-	CAJ70914	x	207	Anammox bioreactor (Anoxic)
22	-	-	131	113	-
23	-	KPK88675	x	310	Estuary sediments (*Anaerolineae*)
24	CP002106	WP_002563432	303	-	*Atopobium*
25	-	KPL22996	x	310	Estuary sediments (*Phycisphaerae*)
26	KP184580	AKA87394	96	73	High ammonia anaerobic digester

Only the top n- or pBlast matches are presented in this table
Green: matches of high certainty, ≥ 99% nBlast, ≥ 89% pBlast, +/- 5 bp between TRFS from in vitro/in silico digestions
*System descriptions represent findings from nBlast and/or pBlast (in that order if separated by commas)

Phylogenetic Analysis

OTUs from this study were incorporated into a phylogenetic tree constructed from deduced FTFHS amino acid sequences of previously isolated OTUs (Müller, et al., 2015) (Figure 14). OTUs 2, 12, 13, 14, 17, 21, 23 and 25 grouped in the cluster at the top of the tree that is clearly distinguished from the rest of the tree. Surrounding sequences relate to both sulfate-reducing bacteria (e.g. *Desulfovibro disulfuricans*, *Desulfotomaculum carboxidivorans* and *Desulfosporosinus orientis*) and more devoted syntrophic acetate-oxidizing bacteria (e.g. *Thermacetogenium phaeum* and *Syntrophaceticus schinkii*). OTUs 1, 6, 9, 16 and 19 group together in a cluster containing several previously sequenced uncultured bacterium clones (e.g. JQ082241-43) isolated from a mesophilic anaerobic digester (Muller & Schnurer, 2011). OTUs 18 and 24 showed high similarity and were clustered with *Tepidanaerobacter acetatoxydans* and *Thermacetogenium kivui*. Interestingly a fungus was identified (OTU20) and related to a Hpy188III-digested TRF (TRF 207) shared with other bacterial OTUs (12, 14 and 21).

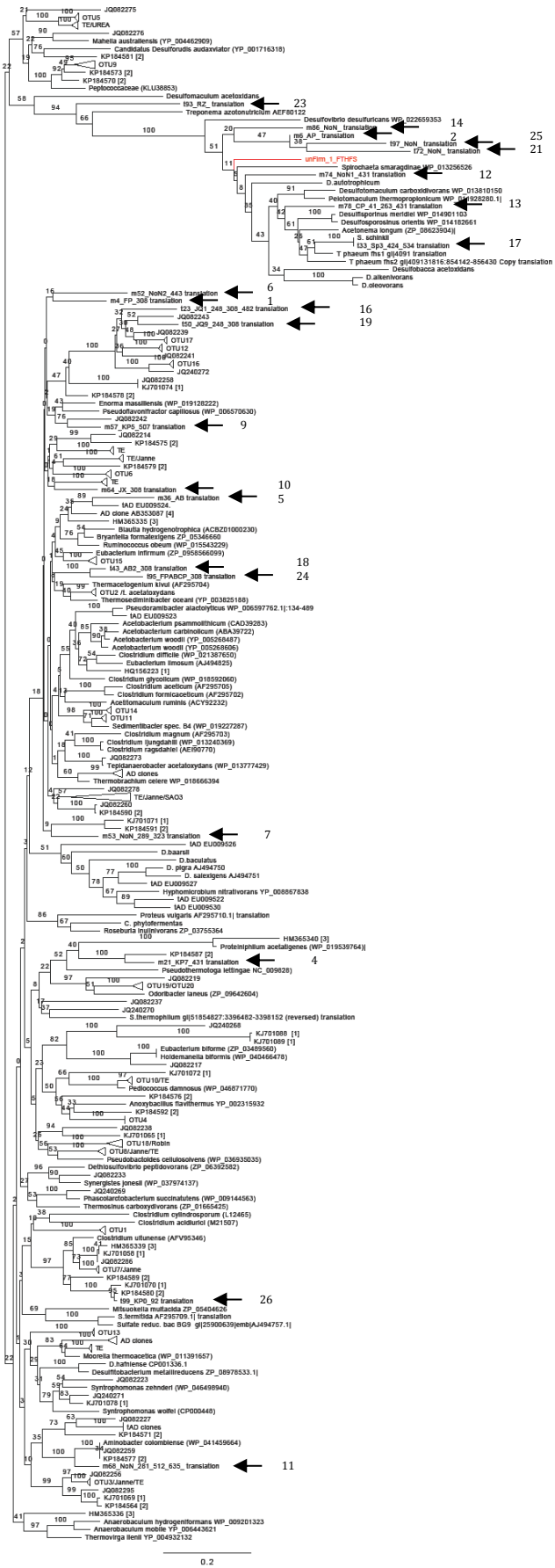

Figure 14. Phylogenetic tree constructed from deduced FTFHS amino acid sequences of the *fhs* gene. OTUs sequenced in this study identified with arrow and OTU number.

4. Discussion

The lack of differences between reactors observed for SMP, pH, the carbon and nitrogen analysis, degrees of degradation and the microbiological analyses point to little effect of trace element additions in this thermophilic, high ammonia biogas system with respect to promotion of stability in the syntrophic acetate oxidizing bacterial community or otherwise. Some differences were observed, such as production kinetics after the OLR increase but this difference apparently had little impact on other parameters. H_2S concentrations were consistently higher in GP1 than GP2 with this difference possibly related to the difference in kinetics but without an impact on total production. The decreased solubility of H_2S at elevated temperatures may explain the lack of inhibition in this thermophilic system (Al Seadi, et al., 2008). By the end of the experiment period, VFA concentrations were also higher in GP1 than GP2 but again seemingly without inhibitory effects. SMP had decreased for GP1 compared to the period before the increase in OLR but only to a level similar to GP2. That SMP decrease for GP1 to a level similar to that for GP2 after the OLR increase may indicate the presence of a threshold OLR value in this system below which TE additions have a negative effect and above which they have a positive, or at least neutral, effect. Conversely, the difference in the first half of the experiment may just as well be caused by natural variation due to startup-related disturbances that were eventually dampened out over time (Schnürer & Jarvis, 2009).

The microbiological analyses of the *fhs* gene were expected to reveal differences in reactors that may not have been observable form surveillance parameters alone but they too showed little difference. Only two dominant AluI-digested TRFs (58 and 81) showed consistent differences between reactors and the single dominant Hpy188III-digested TRF (286) could not be matched to an OTU. The importance of the *fhs* gene lies in its presence in syntrophic acetate oxidizing bacteria which can be part of hydrogenotrophic methanogenesis, as opposed to acetoclastic methanogenesis. The former can take over from the latter as the dominant pathway of methanogenesis in certain instances, such as high ammonia levels as found in this study. These findings point to no appreciable difference in the balance between these pathways across treatments.

Further microbiological analysis was difficult due to the mentioned lack of matches between the most dominant TRFs and clone library derived OTUs. These TRFs could not be identified and therefore their prevalence and roles could not be contextualized within the wider *fhs* gene-possessing SAO community of biogas production. Matching of other TRFs to OTUs that could in turn be matched to previously published *fhs* sequences showed that the thermophilic temperature of this system did not exclude the growth of bacteria known to exist in mesophilic reactors. The reason for this finding, however, may be an underrepresentation of thermophilic isolates in the databases due to little previous research in thermophilic reactors. The inability to identify the most dominant TRFs from this analysis impeded the drawing of definitive conclusions. The shortcomings of TRFLP, for example the unclear relationship between the abundance of a given DNA sequence and its level of expression or the activity of the associated enzyme, can also be considered an impediment.

The general finding of this study, that no appreciable effect of TE additions on the anaerobic degradation of food waste, was in contrast with previous studies (Banks, et al., 2012; Karlsson, et al., 2012; Wei, et al. 2014; Westerholm et al., 2015; Zhang & Jahng, 2012). These previous studies reported higher SMP and lower VFA and H_2S concentrations with TE additions compared to without. The differences in VFA and H_2S in this study were not seen to the same extent as in previous studies nor did VFA accumulation lead to eventual system failure in the absence of TE.

While previous microbiological analyses have showed greater species richness and eveness at mesophilic than thermophilic temperatures (Carballa, et al., 2011; Levén, et al.,

2007) and with TE than without (Ünal, et al., 2012) none have compared these variables with and without trace elements at thermophilic temperatures. Furthermore, 16S rRNA or the methanogenesis gene *mcrA* are most often the basis for micriobiological analysis rathger than the SAO-relevant *fhs* gene used here. The lack of comparable studies, therefore, again limits the ability to draw a conclusion from the findings in this study at this time.

Beyond the lack of truly similar studies for direct comparison, the variability in the composition of what is considered food waste may underlie some of the differences in otherwise similar systems. The inclusion of slaughterhouse waste, for example, in this study's substrate may have provided the elements that would otherwise have been lacking. This kind of variation is one example of the uniqueness of different systems and therefore the necessity of individual assessment in imposing certain operational or mitigation treatment options.

5. Conclusions

Contrary to the hypothesis and expectations based on the findings of previous studies, the presence of trace elements did not improve the production of biogas at thermophilic temperature in this study. Few differences were observed between reactors with and without TE and those that did exist did not seem to affect the amount or quality of biogas produced. Especially surprising was the lack of difference between treatments at the microbial community level based on analysis of the *fhs* gene relevant in syntrophic acetate oxidation; microbial community structure dynamics showed little effect of TE additions.

A possible explanation for these findings may be the high variability in composition of food waste which can range from low (Zhang & Jahng, 2012) to high (Schattauer, et al., 2010) TE content. TE may simply not have been limiting in the substrate used in this study. The necessity of TE additions in biogas production may not be as widespread as their current use would imply. The decision to supplement reactors with TE may need to be made on a case by case basis rather than accepted as a general rule.

The findings of this study also highlight a gap in knowledge in the field of the microbiology of thermophilic biogas systems both in general and specifically regarding the effects of trace elements. Many of the OTUs from this study could not be matched with high certainty to previous sequences in Blast databases and sources of the previous sequences which could be matched were often mesophilic anaerobic digesters. More direct analytical methods than TRFLP need to be put into use as they become available. While the study of the impact of trace elements on biogas processes seems to be a growing field, few other studies have investigated this impact at thermophilic temperatures. This area of research requires further investigation to address the uncertainties of production in this temperature range.

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