UNIVERSITY OF SOUTHAMPTON

FACULTY OF CIVIL ENGINEERING AND THE ENVIRONMENT

Thermophilic Anaerobic Digestion of Food Waste

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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THERMOPHILIC ANAEROBIC DIGESTION OF FOOD WASTE

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There is a requirement in the European Union to divert organic wastes from landfill because of the risk of methane emissions. One alternative is anaerobic digestion of organic wastes, such as food waste, to stabilise them whilst at the same time recovering the energy from them. One problem with this approach is that the high nitrogen content of food waste may lead to ammonia inhibition. A solution to this has been found for mesophilic digestion but had not been attempted in thermophilic digestion where ammonia toxicity is known to be more acute.

The work was carried out in laboratory-scale semi-continuous digesters operated over long time periods to provide maximum opportunity for acclimatisation, and in duplicate to give an indication of reproducibility. A series of experimental runs were undertaken at thermophilic temperatures to assess the influence of trace element (TE) addition on the digestion process. These were carried out at organic loading rates (OLR) of 2, 3 and 4 g volatile solids (VS) l^{-1} day^{-1} against unsupplemented controls at OLR 2 g VS l^{-1} day^{-1}. Although TE addition could offset the accumulation of VFA which occurred in response to an increasing concentration of total ammonical nitrogen (TAN), it could not prevent this. The high alkalinity resulting from ammonia, however, allowed the digesters to continue to produce methane until VFA had accumulated to high concentrations before eventual failure due to a rapid drop in pH.

To determine the threshold inhibitory ammonia concentration in thermophilic digestion, one pair of digesters was run on synthetic low nitrogen food waste (low-N food waste) at an OLR 2 g VS l^{-1} day^{-1} and compared to a control pair running on domestic food waste at the same loading. All four digesters received TE supplementation. The digesters fed with low-N food waste showed consistently stable performance with pH ~7.4, IA/PA ratio ~0.4-0.5, SMP 0.39 l CH_4 g^{-1} VS, 52-55% CH_4, total VFA <500 mg l^{-1} and 88% VS destruction whereas the controls showed signs of failure after 112 days and irreversible VFA accumulation at a TAN concentration >3.5 g N l^{-1}. One of the low N digesters was supplemented with urea slowly and one by a shock dose: both showed signs of VFA accumulation at TAN >2.5 gl^{-1} and, again, an irreversible trend in propionic acid build-up when TAN >4 g N l^{-1}. Long term operation showed meta-stable conditions when the digesters were operated at TAN between 2.5 - 3.5g l^{-1} with oscillations in VFA (especially propionic acid) concentration.

Mesophilic digestion at 37^{o}C with TE addition showed very stable performance with pH ~8, IA/PA $\leq$ 0.3, SMP ~0.48 l CH_4 g^{-1} VS, 55-60% CH_4, total VFA < 300 mg l^{-1} and VS destruction ~75-78% with a final total ammonia nitrogen (TAN) concentration of 4.5 g N l^{-1}. As the temperature in digesters was raised from 35 to 43 ^{o}C in 1^{o}C steps a change in performance was noted when the temperature reached 40 ^{o}C. Above this temperature VFA concentrations rose above 4000 mg l^{-1} and biogas and methane production fluctuated. It is probable that the higher temperature increased the concentration of free ammonia nitrogen (FAN) to ~800 g N l^{-1} at the measured TAN concentration ~5.5-6.0 g N l^{-1} and this was sufficient be inhibitory even with TE dosing.

Fluorescent in situ hybridisation (FISH) was used to identify the methanogenic populations in some of the trials over selected time periods. This showed changes in population structure both in relation to temperature (mesophilic or thermophilic) and also in response to increasing concentrations of TAN. At high TAN concentrations *Methanomicrobiales* was the dominant methanogenic group and under mesophilic conditions this proved to be extremely ammonia tolerant. A ^{14}C radio-labelling assay confirmed the dominant pathway to methane formation was by the hydrogenotrophic route which reflected the known metabolic pathway of this methanogen.

It was concluded that thermophilic digestion of source segregated domestic food waste would lead to instability and failure of the process unless measures were introduced to reduce the TAN concentration to < 3.5 g N l^{-1}, and preferably to < 2.5 g N l^{-1}.

Keywords: Anaerobic digestion, food waste, biogas, VFA accumulation, ammonia inhibition, Fluorescent in situ Hybridisation (FISH)

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DECLARATION OF AUTHORSHIP

I, Chaowana YIRONG declare that this thesis entitled:

Thermophilic anaerobic digestion of food waste

and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:

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Signed: .....

Date:.....

Chapter 1 Introduction

1.1 Overview

The European Landfill directive (99/31/EC) EU (1999), which requires the diversion of biodegradable municipal waste from landfill, has focused attention on source segregation of kitchen waste from domestic and commercial properties. Quested et al. (2012) noted that in the UK in year 2012, 4.2 million tonnes of food waste were thrown away from a total of 7.0 (100%) million tonnes of household waste. Food waste is organic matter, consisting mainly of carbohydrates, proteins and fats, and has the potential for further usage such as animal feed (subject to safety and regulatory requirements), organic fertilizer (compost or digestate), and energy production via anaerobic digestion or combustion. One of the most promising technologies available to treat this waste is anaerobic digestion as it can recover more of the available energy than combustion, and retains the nutrients for reuse in agriculture through land application of the digestate. Moreover, controlled anaerobic digestion can be an effective method to reduce greenhouse gas emissions.

Anaerobic digestion is generally carried out in one of two main temperature ranges: mesophilic or thermophilic. Some researchers have suggested that thermophilic anaerobic digestion provides a better performance than mesophilic (Cecchi et al., 1991, Cecchi et al., 1993, Gallert and Winter, 1997, Kim et al., 2002, Kim et al., 2003, Cavinato et al., 2013).Thermophilic conditions, however, have some disadvantages for anaerobic digestion: for example increased ammonia toxicity from nitrogen-rich waste, as discussed in a number of reviews (Koster and Lettinga, 1984, Zeeman et al., 1985, Sprott and Patel, 1986, Robbins et al., 1989, Angelidaki and Ahring, 1993, Kayhanian, 1994, Borja et al., 1996, Hansen et al., 1998, Sung and Liu, 2003, Zhang et al., 2012a, Liu et al., 2012). Initial digestion trials with digestion of food waste showed accumulation of volatile fatty acids (VFA) over extended run times in both mesophilic and thermophilic digestion systems (Banks et al., 2008). Similar observations were made in a full-scale thermophilic digester treating restaurant and catering waste (Neiva Correia et al., 2008), and in the UK Government's demonstration food waste digestion plant in Ludlow, UK (Banks et al., 2011); and have also been shown in laboratory-scale digesters (Park et al., 2008, Zhang and Jahng, 2012). It is also recognised that higher

temperatures lead to more serious ammonia toxicity at the same pH (Angelidaki and Ellegaard, 2003). The accumulation of VFA in mesophilic digesters, however, can be prevented by supplementing with selected trace elements (Zhang et al., 2011, Banks et al., 2012, Karlsson et al., 2012, Zhang and Jahng, 2012, Facchin et al., 2013, Qiang et al., 2013). More recently, digesters have been shown to operate with good gas production and low VFA concentrations at ammonia concentrations in excess of 6000 mg l^{-1} but with a purely hydrogenotrophic methanogenic population, and a mechanism by which trace element addition can prevent VFA accumulation at these high ammonia concentrations in mesophilic anaerobic digesters was also put forward (Banks et al., 2011). With the same strategy, it may be helpful to apply this scheme in thermophilic anaerobic digestion.

In recent years tools and techniques have been developed that allow identification of both the microbial populations and the metabolic pathways in anaerobic systems: these include FISH and 14C labelling (Calli et al., 2005, Hori et al., 2006, Ariesyady et al., 2007, Montero et al., 2008). Both the population structure and the pathways are closely interlinked with digester environmental conditions (Xia et al., 2011, Xia et al., 2012b). A knowledge of these may provide insights into the factors determining digestion performance, and thus to potential solutions for and limitations on the stable thermophilic digestion of high nitrogen feedstocks.

1.2 Statement of Research Aims and Objectives

The overall aim of this research was to determine the feasibility or otherwise of stable long-term anaerobic digestion of pure undiluted source separated domestic food waste in thermophilic conditions. To achieve this aim a number of specific objectives were set, which developed sequentially during the course of the research. These objectives were:

- to acclimate a low-nitrogen mesophilic inoculum to thermophilic conditions by a single-step rise in temperature

- to establish the process stability of thermophilic digestion of normal source segregated food waste with and without trace element supplementation at different organic loading rates, through constant monitoring of stability and performance parameters

- to compare the performance of thermophilic digesters to that of mesophilic digesters fed on the same food waste, and to thermophilic digesters fed on a low-nitrogen food waste at the same organic loading rate

- to determine the critical threshold(s) for ammonia toxicity in thermophilic conditions by adding urea to digesters fed on low-nitrogen food waste, until VFA accumulation occurred

- to assess the effects of increasing the temperature in mesophilic digestion of source segregated domestic food waste, with a view to identifying the main factor(s) causing instability

- Using fluorescent in situ hybridisation (FISH) and ^{14}C labelling techniques, to elucidate changes in the microbial population and metabolic pathway linked to changes in ammonia concentration and digestion performance and stability

Chapter 2 Literature Review

2.1 Principles of anaerobic digestion

Anaerobic digestion occurs by the action of micro-organisms in the absence of oxygen, and breaks down organic matter to form biogas, a mixture of methane and carbon dioxide. There are several groups of microbes involved in this decomposition, which takes place in 4 stages:

Hydrolysis

Acidogenesis

Acetogenesis

Methanogenesis

These are inter-linked as shown in Figure 2.1.

Each step is described in detail in the following sections

2.1.1 Hydrolysis

This is the breakdown of organic polymers including carbohydrates, lipids and proteins into simpler monomers (sugars, fatty acids and amino acids). This process converts the particulate material to simpler dissolved substrates that can pass the cell wall of the microorganisms (Chernicharo, 2007). The process is facilitated by the action of extra-cellular enzymes produced by bacteria such as *Clostridia* (hydrolyses protein to amino acids) (Khanal, 2008).

2.1.2 Acidogenesis

Fermentative bacteria convert the products from hydrolysis (sugars, fatty acids, and amino acids) to volatile fatty acids (VFA), alcohols, lactic acid, carbon dioxide, ammonia and also to the energy used for new cell synthesis. The most common products of acidification are acetate and mixed isomers of C3-C8 chain length volatile fatty acids and, to a lesser extent, ethanol and lactic acid. The fermentation results in a carbon loss

in the form of carbon dioxide and the excess electrons pass to hydrogen gas (Rodriguez et al., 2006). Examples of microorganisms which undertake acidogenesis are members of the family of *Bacteroidaceaea* (Chernicharo, 2007).

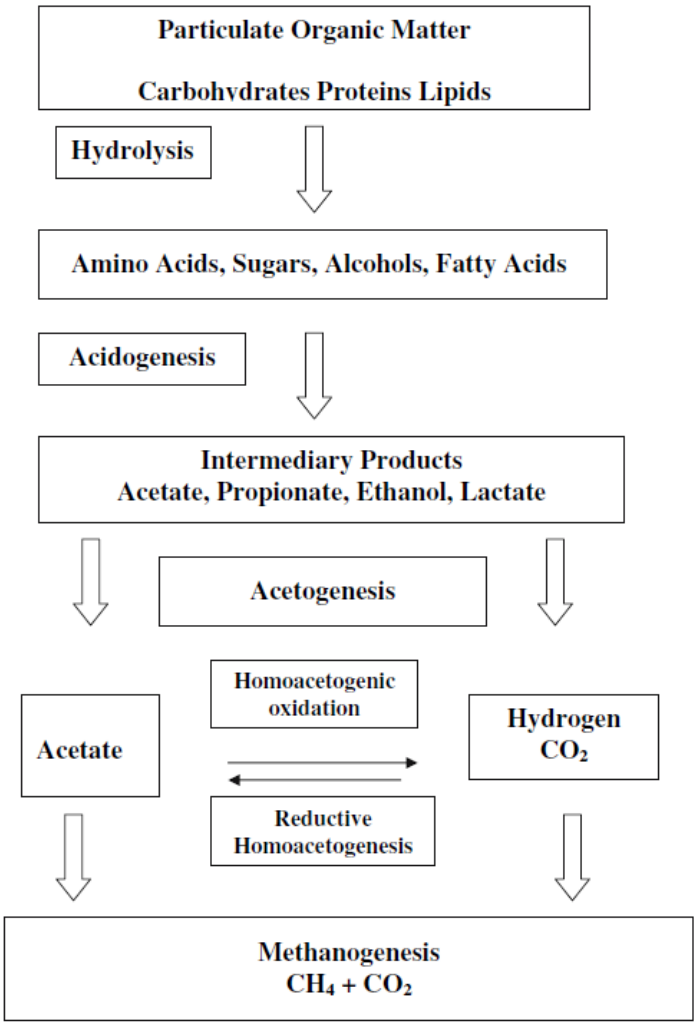


Figure 2.1 Anaerobic degradation of organic matter

Source: Demirel and Scherer (2008)

2.1.3 Acetogenesis

The acid products with a C chain length of 3 or more can be further converted to acetate, formate, hydrogen and carbon dioxide by acetogens. All of these products are precursors for methane formation in the process of methanogenesis (Chernicharo, 2007). Examples of microorganisms in this group are: *Syntrobacter wolinii* and *Syntrophomonas wolfei* (McInerney et al., 1981)

2.1.4 Methanogenesis

In this process, the methanogenic archaea convert a limited number of substrates to methane and carbon dioxide, the main components of biogas. Known substrates are: acetic acid, hydrogen, carbon dioxide, formic acid, methanol, methylamines, and carbon monoxide. (Chernicharo, 2007). The methanogens are divided into 2 main groups according to the substrate they consume.

a: acetoclastic methanogens (acetate-using microorganisms). This group uses acetic acid or methanol as substrates. Acetoclastic methanogens are often reported to account for 60-70% of all methane production in systems such as sewage sludge digestion (Chernicharo, 2007). The methanogens able to cleave acetic acid belong to the order *Methanosarcinales* which has two families; *Methanosaetaceae* (Figure 2.2) and *Methanosarcinaceae* (Figure 2.3).

Methanosaetaceae mainly grows on acetic acid, with a doubling time of 4-9 days whilst *Methanosarcinaceae* consumes several sources of organic carbon such as acetic acid, methanol, methylamines, and can also utilise H_2/CO_2 (Zinder, 1990, Garcia et al., 2000, De Vrieze et al., 2012). Typically, *Methanosarcinaceae* has a doubling time of about 1-2 days on acetic acid. Therefore, if a digester has a retention time less than the doubling time of *Methanosaetaceae*, it could be washed out from the digester leaving *Methanosarcinaceae* as the dominant group (Zinder, 1990).

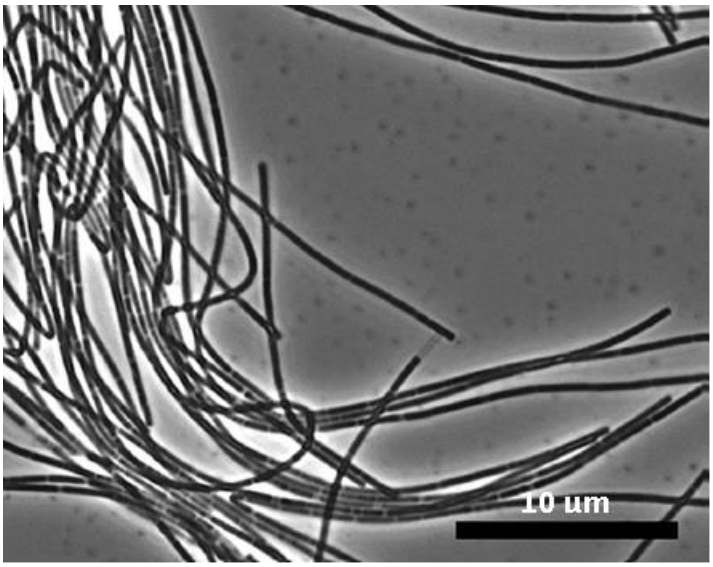


Figure 2.2 *Methanosaeta pelagica*, an example species of *Methanosaetaceae* (Mori et al., 2012)

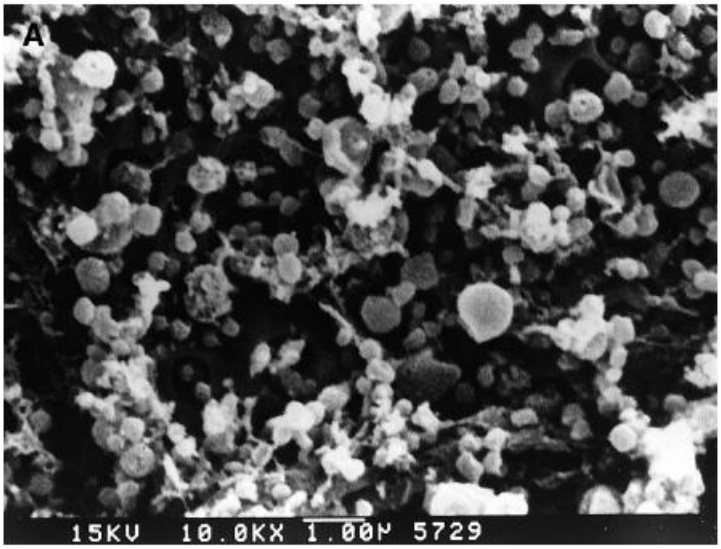


Figure 2.3 *Methanosarcina mazei,* an example species of *Methanosarcinaceae* (Lai et al., 2000)

b: hydrogenotrophic methanogens (hydrogen-using microorganisms). Hydrogen and carbon dioxide are used by this group to form methane. Methanogens of this group are *Methanobacteriales* (Figure 2.4), *Methanococcales* (Figure 2.5), and *Methanomicrobiales* (Figure 2.6).

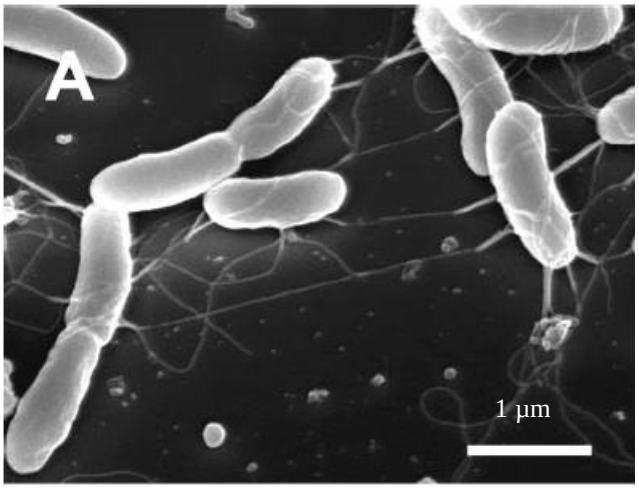


Figure 2.4 *Methanothermobacter thermautotrophicus*, an example species of *Methanobacteriales* (Ishii et al., 2005)

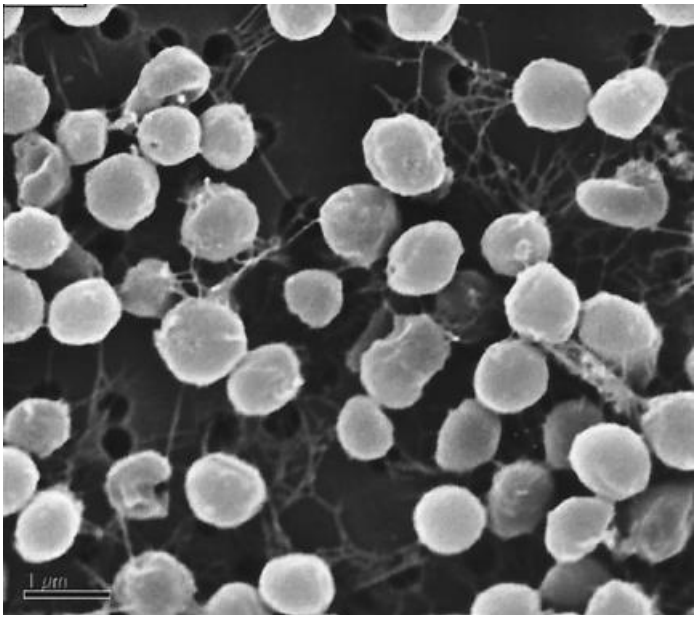


Figure 2.5 *Methanococcus jannaschii*, an example species of *Methanococcales* (Park and Clark, 2002)

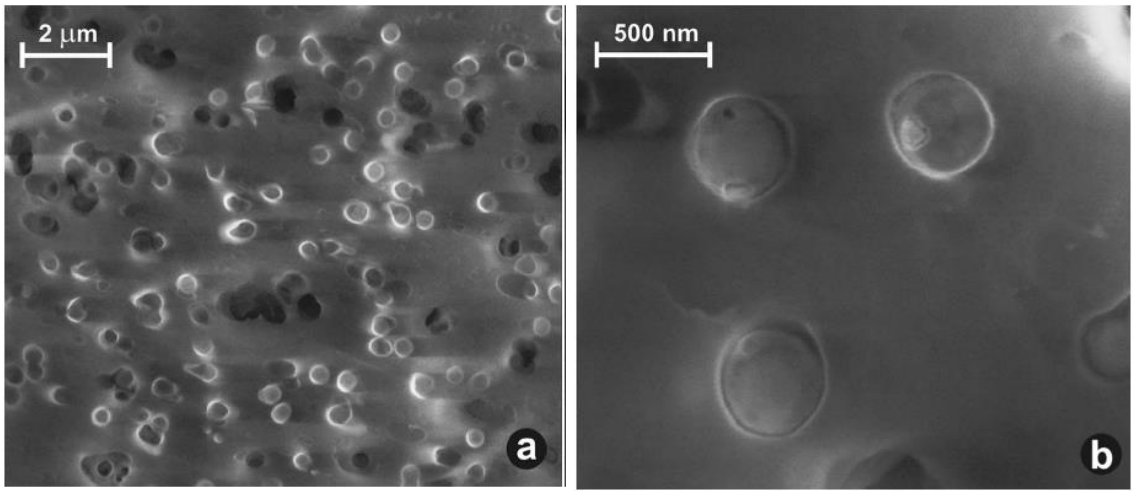


Figure 2.6 *Methanocorpusculum parvum,* an example species of *Methanomicrobiales* (Strąpoć et al., 2008)

In some circumstances, such as ammonia inhibition of acetoclastic methanogenesis, the conversion pathway may shift to hydrogenotrophic methanogenesis by coupling with syntrophic acetate oxidation. The acetate oxidising bacteria can convert acetic acid to H_2 and CO_2 which are later consumed by hydrogenotrophic methanogens. Moreover, the acetate oxidising bacteria can also combine the H_2 and CO_2 to form acetate (Demirel and Scherer, 2008).

2.2 Biochemistry of anaerobic digestion

Anaerobic digestion depends on a very complex set of reactions and a balance between all of the microorganisms involved is needed to maintain the stability of the system. As the key intermediates, VFA are food for methanogens; on the other hand, high VFA concentrations can cause the pH to drop to a level that is unsuitable for methanogens. Acetic and propionic acids are the main supplies for methane formation. Acetic acid is the main acid product formed in acidogenesis; to a lesser extent longer carbon chain length acids such as butyric and propionic are also formed. When acetoclastic methanogens are dominant in systems such as sewage sludge digesters acetic acid is the major precursor of the methane formed, and together with propionic acid, accounts for about 85% of the total methane production (Chernicharo, 2007). Hence, conversion of acetic and propionic acid to methane plays a significant role in a stable anaerobic

digestion system. The efficiency of the conversion depends on how easily the required reactions occur.

Thermodynamics is an important aspect for the understanding of anaerobic metabolism. The net free energy change (ΔG) shows the possibility of a reaction. A positive value means the reaction is not possible and a negative value shows it is possible. For example, in Table 2.1 the net free energy change of propionic acid to acetic acid and hydrogen gas is positive; the degradation is therefore unfavourable under standard conditions. If, however, the hydrogen concentration in the reactors is low, degradation might occur (Chernicharo, 2007, Mata-Alvarez, 2003). From Table 2.2, it can be seen that methane formation is possible in most cases with net free energy change < 0.

Table 2.1 Free energy changes for anaerobic oxidation by hydrogen-producing acetogenic bacteria in pure cultures under standard conditions (pH 7, 1 atm, and 25 ^{o}C)

Reactions	$\Delta G^{\circ\prime}$ (kJ/reaction)
Propionate → acetate (i) $CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2$	+76.1
Butyrate → acetate (ii) $CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$	+48.1
Benzoate → acetate (iii) $C_7H_5CO_2^- + 7H_2O \rightarrow 3CH_3COO^- + 3H^+ + HCO_3^- + 3H_2$	+53
Ethanol → acetate (iv) $CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	+9.6

Source: Khanal (2008)

Table 2.2 Energy yields for anaerobic reactions

Reaction	**ΔG_0, (kJ/mol)**
(A) $4H_2 + CO_2 \rightarrow CH_4+2H_2O$	-139
(B) $4CHOO^- + 2H^+ \rightarrow CH_4+ CO_2 + 2HCO_3^-$	-127
(C) $CH_3COO^- + H_2O \rightarrow CH_4+ HCO_3^-$	-28
(D) $4CH_3OH \rightarrow 3CH_4+2H_2O + CO_2$	-103
(E) $4CH_3NH_2 + 2H_2O + 4H^+ \rightarrow 3CH_4+ CO_2 + 4NH_4^+$	-102
(F) $2(CH_3)_2S + 2H_2O \rightarrow 3CH_4 + CO_2 + 2H_2S$	-74

Source: modified from Khanal (2008)

2.3 Stoichiometry

The theoretical methane yield from anaerobic digestion of a substrate can be calculated if the chemical composition (CHON) is known, by using the well-known (modified) Buswell equation (Buswell and Mueller, 1952) which was originated by Symons and Buswell (1933), as follows.

$$CaHbOc+(a-b/4 -c/2)H_2O \rightarrow (a/2–b/8+c/4)CO_2+(a/2+b/8–c/4)CH_4 \quad \textbf{Equation 2.1}$$

Boyle (1976) modified this equation by inclusion of nitrogen and sulphur to obtain the proportion of ammonia and hydrogen sulphide gases, as shown below.

$$CaHbOcNdSe + (a – b/4 – c/2 + 3d/4 + e/2)\ H_2O \rightarrow (a/2 + b/8 – c/4 - 3d/8 – e/4)\ CH_4 + (a/2 - b/8 + c/4 + 3d/8 + e/4)\ CO_2 + dNH_3 + eH_2S \quad \textbf{Equation 2.2}$$

Where CaHbOc and CaHbOcNdSe are the empirical chemical formula of the biodegradable organic substrates. The methane yield derived from the Buswell equation is the maximum possible, assuming total conversion and no microbial uptake.

2.4 Environmental Factors

An appropriate physical and chemical environment enhances degradation by anaerobes. Since degradation requires the interaction of fermentative bacteria and methanogens, a successful process depends on a good balance between the microbial groups in the digester. Suitable conditions for thermophilic anaerobic digestion are summarised in the following sections:

2.4.1 Temperature

Optimum conditions for thermophilic anaerobic organisms occur in the temperature range of 45-70 ^{o}C. Temperature directly affects microorganisms, but stable conditions are required. It is recommended that the variation of temperature in the digesters should not be more than 0.6-1.2 ^{o}C (Khanal, 2008).

Some authors have suggested that the benefits of thermophilic anaerobic digestion do not outweigh the disadvantages, such as the additional energy required for heating, poor quality of supernatant and the instability of the system (Chernicharo, 2007). Others have disagreed, however: Khanal (2008) and Mata-Alvarez (2003) reported an increase in methane generation rates of 25-50% over mesophilic anaerobic digestion. Khanal (2008) cited the work of Zinder (1988) who reported that the hydraulic retention time of thermophilic anaerobic digestion could be reduced compared to mesophilic digestion because of the higher growth rate. In contrast, Khanal (2008) mentioned obstacles to the process such as slow start-up, and susceptibility to load variation, substrate changes, or toxicity, as noted by Mata-Alvarez (2003). Thermophilic temperatures may make digestion more sensitive to ammonia inhibition (Angelidaki and Ahring, 1994).

2.4.2 pH

pH directly affects both acidogens and methanogens. The appropriate pH for acidogens is 5.5-6.5, and for methanogens 7.8-8.2. The optimum pH for both groups is 6.8-7.4. Unstable pH affects methanogens more than acidogens. If low pH occurs in a digester, the activity of methanogens may reduce and cause acid accumulation and high concentrations of hydrogen. If the digester is not recovered in time, this leads to failure (Khanal, 2008).

Changes in pH values in a digester occur in different circumstances such as increasing pH through ammonification, and decreasing pH from VFA accumulation. Adequate buffer and reduced feeding are important to recover the damaged system (Khanal, 2008).

2.4.3 Alkalinity

Alkalinity provides the capacity to buffer the system against changes in pH in reactors. Alkalinity, or buffer solution, consists of weak acid and its correlated salts. Alkalinity prevents or avoids a sudden change of pH from increasing VFA and ammonia accumulation. The equation below shows the reaction of alkalinity (HCO_3^-) with acetic acid.

$$HCO_3^- + HAc \leftrightarrow H_2O + CO_2 \uparrow + Ac- \qquad \textbf{Equation 2.3}$$

In the case of excess acetic acid, buffer is used to maintain the system, leading to a higher concentration of carbon dioxide and decreased pH (Khanal, 2008). Protein degradation directly increases alkalinity in the form of NH_4CO_3. A higher degree of protein degradation, for example due to longer SRT, results in higher alkalinity (Speece, 2008a).

A bicarbonate alkalinity of 1,000 mg l^{-1} as $CaCO_3$ is needed to maintain pH over 6.8. In the case of low alkalinity in digesters, chemical supplementation is a strategy to smooth the system. Examples of suitable chemicals are sodium bicarbonate, sodium carbonate, ammonium hydroxide, gaseous ammonia, and lime. Sodium bicarbonate is the best because of its high solubility, long lasting impact, and low toxicity (Khanal, 2008). Alkalinity is divided into 2 types:

Partial alkalinity (PA): normally equal to bicarbonate alkalinity. In analytical terms this is defined as the value for titration to pH 5.75.

Intermediate alkalinity (IA): in practical terms, equal to alkalinity of volatile acids. Defined by titration from 5.75 to pH 4.3

2.4.4 Nutrients

Microorganisms need nutrients for cell growth and to maintain metabolic functionality. Nutrients can be divided into 2 types: macronutrients and micronutrients. The chemical compositions of methanogens in terms of both types are shown in Table 2.3 (Chernicharo, 2007).

Based on an empirical formula of $C_5H_7O_2N$ microbial cells are composed of 12% nitrogen. The theoretical minimum ratio of carbon:nitrogen:phosphorus is 350:7:1 for operation at high loadings where a high cell growth rate is maintained. Nitrogen is provided in the form of urea, aqueous ammonia, or ammonium chloride. For phosphorus, phosphoric acid or phosphate salt is consumed (Khanal, 2008).

Table 2.3 Nutrient composition of methanogens

Macronutrients		**Micronutrients**	
Element	**Concentration (g/kg TSS)**	**Element**	**Concentration (mg/kg TSS)**
Nitrogen	65	Iron	1800
Phosphorus	15	Nickel	100
Potassium	10	Cobalt	75
Sulphur	10	Molybdenum	60
Calcium	4	Zinc	60
Magnesium	3	Manganese	20
		Copper	10

Source: Chernicharo (2007)

2.4.5 Toxic substances

Toxic substances in anaerobic digestion come from both outside and inside the reactors. External toxic substances include pesticides and solvents. Internal toxic materials are mostly from degradation, for instance, excess ammonia and VFA accumulation. In this research, internal toxicity associated with degradation is considered.

2.4.6 Ammonia

Ammonia can be beneficial or toxic to digestion, depending on its concentration and form. As a macronutrient, ammonia is the form of nitrogen that is needed for new cell synthesis in microorganisms. Excess ammonia, however, may cause digesters to fail. High concentrations, above 4,000 mg l^{-1}, are reported as inhibitory (Angelidaki and Ahring, 1994). Hansen et al. (1998) found that free ammonia concentrations over 1,100 mg l^{-1} caused inhibition in batch anaerobic digestion at pH 8.0 and at a temperature of 55 ^{o}C. Regardless of pH, ammonia nitrogen in excess of 3000 N mg l^{-1} may itself be toxic, as shown in Table 2.4 (McCarty, 1964). The concentration at which ammonia becomes inhibitory is uncertain, however, because it depends on many factors such as pH, temperature and microorganism adaptation (Angelidaki et al., 2003).

Table 2.4 Effect of ammonia nitrogen on anaerobic digestion

Ammonia (mg l^{-1})	**Effect**
50-100	Beneficial
200-1000	No adverse effect
1500-3000	Inhibitory effect (for higher pH value)
>3000	toxic

Source: Khanal (2008)

Ammonia also provides buffering in anaerobic digestion systems. If it combines with carbonic acid, the product is ammonium bicarbonate (NH_4HCO_3), a buffer solution, as seen in the following equation (Chernicharo, 2007):

$$NH_4HCO_3 \rightarrow NH_4^+ + HCO_3^- \qquad \textbf{Equation 2.4}$$

Besides acting as a buffer in digestion, ammonia causes higher pH, which results directly from the cation form (soluble) in the digestate. The soluble form is less toxic than free ammonia (non-ionized form). The free ammonia concentration is strongly affected by the pH and temperature of the digesters (Figure 2.7): increasing pH and temperature increases the free ammonia percentage (Angelidaki et al., 2003). From Figure 2.7, it can be seen that lower pH results in a lower concentration of the free ammonia form, and therefore is better for stable digestion. When the pH rises, it results in a higher concentration of free ammonia and a stronger toxic or inhibitory effect. This

can result in acid accumulation as methanogenesis fails; this in turn will cause a decrease in pH, leading to the less toxic form of ammonia which could help in recovery. Hence, ammonia and VFA are buffers for each other in stable conditions: this is known as a self-stabilizing mechanism (Angelidaki et al., 2003).

Free ammonia can be calculated using the equation below:

$$[NH_3] = [T\text{-}NH_3]/(1 + [H^+]/k_a,) \qquad \textbf{Equation 2. 5}$$

Where $[NH_3]$ and $[T\text{-}NH_3]$ are the free and total ammonia concentrations, respectively, and k_a is the dissociation constant of 38.3×10^{-10} at 55°C (Borja et al., 1996)

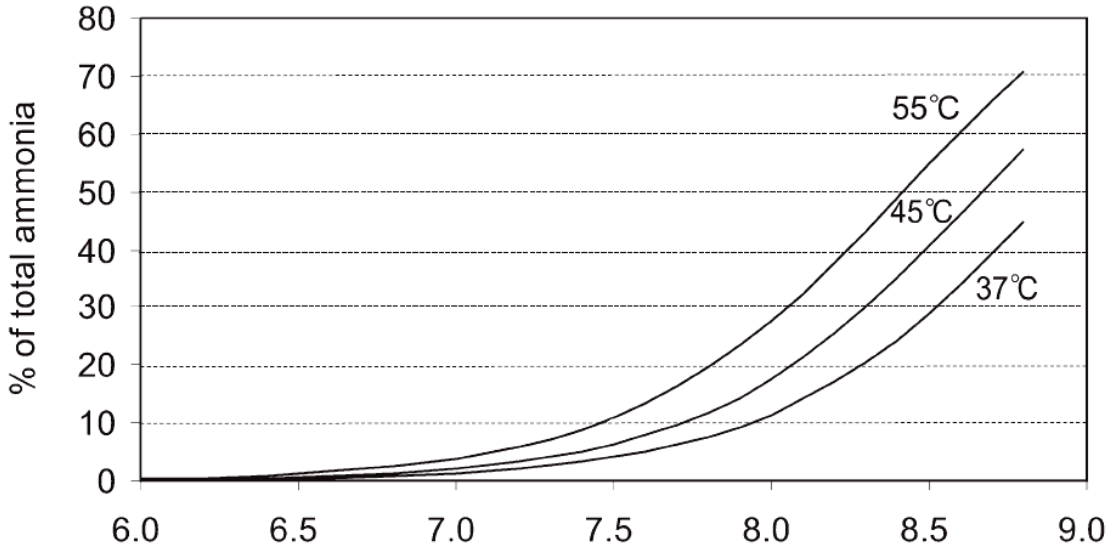


Figure 2.7 Percentage of free ammonia concentration correlates with pH and temperature

Source: Angelidaki et al. (2003)

2.4.7 Volatile fatty acids (VFAs)

As products of acidogenesis, VFA provide both the benefit of being substrates for methanogens and the problem of being toxic in case of excess accumulation. The inhibition caused by VFA may be indirectly linked to many other factors such as the presence of sulphide, ammonia, heavy metals, and also to environmental changes (pH, temperature, limiting nutrients).

Table 2.5 presents information on the toxicity of VFA to anaerobic digestion. It can be seen that VFA are toxic in themselves and proper operation is needed to allow stable digestion. Excess hydrogen concentrations in reactors also affect VFA degradation, especially degradation of propionic acid Khanal (2008).

Table 2.5 Concentrations of VFA species effect anaerobic digestion

Species	Concentration ($mg\ l^{-1}$)	Effect	References
VFA (total)	2000	Inhibitor	Khanal (2008)
Acetic acid	10000	Inhibitor at neutral pH	Khanal (2008)
Butyric acid	10000	Inhibitor at neutral pH	Khanal (2008)
Propionic acid	6000	Inhibitor at neutral pH	Khanal (2008)
	3000	Definitely toxic, digestion fails	Stronach et al. (1986)

2.4.8 Heavy metals

Some soluble heavy metals affect anaerobic digestion more than the insoluble form (Hayes and Theis, 1978). In order of toxicity, these are Ni>Cu>Pb>Cr>Zn, respectively (Lettinga, 1995).

2.4.9 Salts

Some salts inhibit anaerobes. These are usually in the form of cations (+ form), as shown in Table 2.6. Sometimes, however, anaerobes can adapt to these salts.

Table 2.6 Inhibitors from salts

Cation	Concentration ($mg\ l^{-1}$)		
	Stimulating	Moderate inhibition	Strong inhibition
Calcium	100-200	2500-4500	8000
Magnesium	75-150	1000-1500	3000
Potassium	200-400	2500-4500	12000
Sodium	100-200	3500-5500	8000

Source: McCarty (1964)

2.4.10 Others

Other toxicants may include xenobiotics such as solvents, alcohols, organic acids, ketones, phenols, pesticides etc. These chemicals inhibit degradation by anaerobic microorganisms. Hydrogen sulphide, formed by anaerobic digestion, is one of the most significant toxic compounds with a threshold value of 200-1500 mg l^{-1} (Mata-Alvarez, 2003).

For successful anaerobic digestion, with no VFA accumulation and stable pH and gas production, the feedstock characteristics must also be considered. These help to determine a suitable choice of system (e.g. one or two stage) and conditions (e.g. temperature range). For example, if the feedstock has a high protein content, ammonia toxicity may be an issue. Mesophilic conditions with trace element supplementation, co-digestion and/or in-situ treatment (ammonia stripping) may be considered in order to provide stable and effective operation without stress or failure in the long term.

2.5 Fluorescent in Situ Hybridisation

Fluorescent in-situ hybridisation (FISH) is a valuable molecular technique to observe microbial cells in real conditions or in the environment (Amann et al., 2001, Daims et al., 1999, Sanz and Köchling, 2007). It is a very useful means of identification for microorganisms that are difficult to culture, such as biofilm microbes and those in granular sludge. The principle of FISH is to detect nucleic acid sequences by a fluorescent probe that hybridizes with specific target sequences in the microbial cells. The probes are hybridized with RNA of 15-30 nucleotides length, mostly 16S RNA. The process for FISH comprises the following steps (Moter and Göbel, 2000, Daims et al., 1999);

- Fixation
- Preparation
- Hybridisation
- Visualisation

1) Fixation

Fixation is a preliminary process that stops the degradation of RNA and enhances the penetration of probes into the gene sequences. Better fixation results in better probe penetration. Generally, 3-4% (v/v) formaldehyde or paraformaldehyde (PFA) is sufficient for gram-negative microbes. In case of gram-positive ones, ethanol/formalin (9:1 v/v) or heat treatment is suggested.

2) Preparation

This step is carried out on glass slides, coated with agent. The cells are fixed, air dried and dehydrated (by various concentrations of ethanol) on the slides.

3) Hybridisation

The specific bonding between fluorescence labelled oligonucleotide probes to the ribosomal-RNA in microbial cells is controlled by the stringency of the hybridisation buffer. The correct hybridisation stringency is usually achieved by adding formamide to the hybridisation buffer at the right concentration. Formamide interferes with the hydrogen bonds that stabilise nucleic acid duplexes. The right formamide concentration should ensure the specific binding of probes to target organisms and prevent the non-specific binding of probes to non-target organisms. The hybridisation step should be carried out in a dark humid chamber, with the temperature between 37-50 ^{o}C, and an incubation time of 30 minutes to several hours. Afterwards, the slides are rinsed with deionised water to remove the unbound probes. Common fluorophors used are fluorescein, tetramethylrhodamine, Texas Red, and carbocyanine dye (Cy 3 or Cy 5). Full details of the hybridisation step are given in Chapter 3 (section 3.2.14.2 Hybridisation).

4) Visualisation

A conventional epifluorescence microscope with narrow-band-pass filters can be used for work on multi-coloured FISH. Another option is the confocal laser scanning microscope. The images seen from these microscopes are fluorescent coloured objects with the shape of the relevant microorganisms. Figure 2.8 is an example of visualizing

FISH, of bacterial cells in a filter sterilized (0.2 μm) anaerobic digester (Gruden et al., 2003)

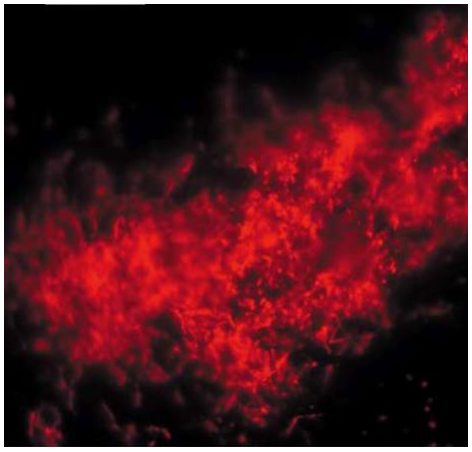


Figure 2.8 Bacterial cells in a filter sterilized (0.2 micron) anaerobic digester

Source: Gruden et al., 2003

The applications of FISH are widespread and include identifying microbial groups both in the environment and in treatment plants. FISH is also applied in medicine, for instance, to analyse the complex microbial communities in the oral cavity, gastro-intestinal flora, respiratory tract infections, and to detect pathogens within tissues.

2.6 Related research

2.6.1 Ammonia

As noted in section 2.4.6 above, ammonia is an important nutrient for anaerobes but is toxic at high concentration. Zeeman et al. (1985) reported problems in thermophilic AD (50^{o}C) of cattle manure with an ammonia nitrogen concentration of around 3000 mg N l^{-1} and with trace elements addition. Hashimoto (1986) reported that, without acclimatisation, both mesophilic and thermophilic AD were inhibited by 2500 mg N l^{-1}. With acclimatisation, the inhibition began when the ammonia nitrogen was around 4000 mg N l^{-1}. All trials were supplemented with trace elements.

Angelidaki and Ahring (1993) stated that ammonia inhibition of thermophilic AD of cattle manure started at 4000 mg N l^{-1} of total ammonia nitrogen. To prevent strong

inhibition, gradually increasing the ammonia in the digester should be considered. It was also found that acetoclastic methanogens were more sensitive to ammonia toxicity than the hydrogenotrophic groups.

Angelidaki and Ahring (1994) studied the effects of ammonia nitrogen and temperature on thermophilic AD of cattle slurry. One reactor was used as a control at 55 ^{o}C, with a digestate ammonia concentration of 2500 mg N l^{-1} from a cattle slurry feedstock. In two reactors the ammonia concentration was increased to 6000 mg N l^{-1} by supplementation with ammonium chloride, while two more reactors were left with the same ammonia concentration as the control. Once steady state was reached, the temperature in one of each pair of high and low ammonia reactors was increased from 55 ^{o}C to 58, 61 and 64 ^{o}C; while in the other reactor of each pair the temperature was decreased from 55 to 51, 46 and 40 ^{o}C. At 55 ^{o}C, the biogas yield in the control and low ammonia reactors was about 0.30 l g VS^{-1} while for the high ammonia reactors, yield was significantly lower at about 0.18 l g VS^{-1}. For the low ammonia load, decreasing the temperature caused only a brief fall in biogas production which soon returned to its previous value. Increasing the temperature led to an immediate fall and required more time to recover; a small increase in VFA concentration with temperature was also noted. In the high ammonia reactor, increasing the temperature led to an immediate fall in biogas yield and increase in VFA concentrations, especially acetic acid. Reduction of temperature in the high ammonia reactors from 55 to 51 ^{o}C improved the gas yield from 0.18 to 0.25 l g VS^{-1}. A further increase in biogas production to 0.30 l g VS^{-1} was also found with the temperature decrease from 51 to 46 ^{o}C. Reducing the temperature from 46 to 41 ^{o}C decreased the gas yield slightly, however. In addition, the reduction in temperature had a positive effect on VFA degradation. Acetic acid reduced from 2402 to 901 mg l^{-1} with the change from 55 to 40 ^{o}C, while increasing the temperature in the high ammonia reactors led to corresponding higher VFA at 6606 mg l^{-1} (acetic acid). The authors concluded that free ammonia nitrogen above 700 mg l^{-1} affected the stability of reactors. At the low ammonia concentration, biogas production was not influenced by temperature change between 44-55 ^{o}C. Increasing the temperature from 55 to 64 ^{o}C negatively affected digester performance in both low and high ammonia reactors. When high ammonia concentrations occur in the digester, decreasing the temperature may reduce the inhibitory effect of ammonia. It can be concluded that the higher

concentration of ammonia made the anaerobic digestion process more sensitive to change in comparison with the lower concentration.

Borja et al. (1996) set up batch experiments to study the effect of ammonia on thermophilic digestion of cattle manure in UASB reactors at ammonia concentrations of 5000 and 7000 mg N l^{-1}. The experiments were set up in 2 groups. Experiment I studied the effect of a sudden increase in ammonia from 2000 mg N l^{-1} (from cattle manure feedstock) to 5000 mg N l^{-1} and to 7000 mg N l^{-1}. The control reactors were operated at 2000 mg N l^{-1}, a second set of reactors was supplemented to 5000 mg N l^{-1} and a third set to 7000 mg N l^{-1} by addition of NH_4Cl. It was found that the methane yield decreased for both sets after 48 days of operation. For the reactors at 5000 mg N l^{-1} production was 25% lower than in the control set, or from 0.2 to 0.05 l CH_4 g^{-1} VS. For the set of reactors at 7000 mg N l^{-1}, the decrease in methane yield was even more severe. While the methane yields decreased, VFA concentrations rose in both sets. In Experiment II, the effect of increasing ammonia was studied in 3 sets of reactors: a set of controls at 3000 mg N l^{-1} ammonia, a set with the ammonia concentration suddenly increased to 7000 mg N l^{-1} and a set with ammonia gradually increased from 3000 to 4000, 5000, and finally to 6000 mg N l^{-1} on day 8, 38 and 68, respectively. The results showed the same ammonia and VFA concentrations as in Experiment I. There was no significant effect from the different ammonia concentrations between 2000 and 3000 mg N l^{-1}. The addition of ammonia to 7000 mg N l^{-1} decreased the methane yield to 0.06 l CH_4 g^{-1} VS whereas VFA rose from about 1000 to above 5000 mg l^{-1}. After 2 hydraulic retention time (HRT) (30 days), however, there were signs of adaptation as shown by an increase in methane yield (to 0.1 l CH_4 g^{-1} VS) and a decrease in VFA to 3000 mg l^{-1}. In the set where the ammonia concentration was increased gradually, there was no significant change in the methane yield and VFA concentration compared with the controls. After reaching an ammonia concentration of 5000 mg N l^{-1}, a lower methane yield and higher VFA concentration was observed. It was also found that after 2 HRT, the methane yield recovered again whereas VFA was still high. After raising the ammonia concentration to 6000 mg N l^{-1}, serious failure occurred with low methane yields (0.1 l CH_4 g^{-1} VS). After day 180, at the end of experiment, the stabilisation of both systems (gradual and sudden increase) showed a methane yield of 0.15 l CH_4 g^{-1} VS and VFA of 3000 mg l^{-1}.

The researchers also studied the activity of the acetoclastic and the hydrogenotrophic methanogens. The results indicated significantly lower activity of both groups at an ammonia concentration of 7,000 mg N l^{-1}: this was 72% lower for acetoclastic groups and 56% for hydrogenotrophic groups compared to the control set. From this research it can be concluded that high ammonia concentrations were inhibitory to anaerobic digestion, starting from 5000 mg N l^{-1}. Moreover, ammonia affected acetoclastic methanogens more severely than the hydrogenotrophic ones.

Hansen et al. (1998) reported the stable anaerobic digestion of swine manure with a total ammonia nitrogen (TAN) concentration of 6000 mg N l^{-1} in continuously stirred tank reactors (CSTR). The experiments were set at temperatures of 37, 45, 55, and 60°C and the methane yields obtained were 188, 141, 67 and 22 ml-CH_4/g-VS, respectively. It was said that the higher temperature led to the higher inhibition. They also studied the ammonia toxicity in batch tests, and found that the inhibition occurred with a free ammonia nitrogen (FAN) concentration of 1100 mg N l^{-1} at pH of 8 (TAN = 4100 mg N l^{-1}).

Sung and Liu (2003) also studied the toxicity of ammonia to thermophilic anaerobic digestion. Two 14-litre CSTR were maintained at 55 ^{o}C and fed with soluble non-fat dry milk at a loading rate of 4 g COD l^{-1} day^{-1}. The study was carried out at TAN concentrations of 400, 1200, 3050, 4920, and 5770 mg N l^{-1} which were raised sequentially by addition of ammonium chloride solution. It was found that ammonia concentrations of 4920 and 5770 mg N l^{-1} led to a decrease in methane production of 39% and 64%, respectively, compared to the control. Increasing the ammonia from 3050 to 5770 mg N l^{-1} caused an increase in VFA to 2730 mg l^{-1}, with a fall in pH and alkalinity. The specific methanogenic activity was similar to that at an ammonia concentration around 0-4000 mg N l^{-1} with microorganisms acclimatized to 1200 mg N l^{-1} ammonia nitrogen before testing and with a pH of around 7.5. Higher activities were found at the lower ammonia concentration at the neutral pH. Ammonia concentrations lower than 1500 mg N l^{-1} did not affect the specific methanogenic activity. On the other hand, higher ammonia concentrations (> 4000 mg N l^{-1}) resulted in noticeable inhibition.

Calli et al. (2005a) reported that FAN of 200 mg N l^{-1} significantly inhibited propionate degrading acetogenic bacteria (by FISH) in mesophilic UASB digesters fed with synthetic wastewater. Observations were made to determine the syntrophic change in reactors by adding NH_4OH from 1000 to 2500 mg N l^{-1} (Calli et al., 2005b). It was found that the population changed from *Methanosaeta* to *Methanosarcina*.

Siles et al. (2010) studied the effect of increasing concentrations of ammonia on thermophilic AD (52 ^{o}C). It was found that a FAN of 620 mg l^{-1} inhibited digestion, with 21% lower gas production.

Fernandes et al. (2012) found no inhibition from ammonia to the hydrolysis of tributyrin or cellulose in the range of 2.4–7.8 g NH_4^+–N l^{-1} (283–957 mg NH_3–N l^{-1}). The experiment was set up in batch mesophilic anaerobic digesters with inoculum from a pig manure co-digester in mesophilic conditions.

Liu et al. (2012) studied the inhibitory effect of high-strength NH_3-N on mesophilic anaerobic biodegradation of landfill waste. It was found that NH_3-N at concentration of 1500–3000 mg N l^{-1} inhibited the biodegradation.

Fotidis et al. (2013) investigated the effect of acetate and ammonia on the methanogenic pathway and on the methanogenic communities. Only the effect of ammonia is considered in this review. Two experiments were set up in which inoculum was 1) gradually exposed to increasing ammonia, and 2) directly exposed to different ammonia concentrations. It was found from FISH that thermophilic microbes clearly shifted their acetate bioconversion pathway from syntrophic acetate oxidisation (SAO) coupled with *Methanobacteriales spp.* and/or *Methanomicrobiales spp.* to *Methanosarcinaceae spp*. In contrast, acclimatisation resulted in no pathway shift with the mesophilic acclimatised culture which was SAO with hydrogenotrophic methanogenesis. When nonacclimatised thermophiles were exposed to high ammonia levels (7 g NH_4^+-N l^{-1}), the *Methanosarcinaceae spp*. was found to be the dominant methanogen. Table 2.7 summarises the critical ammonia concentration and conditions in the above research.

Table 2.7 Summaries of critical ammonia concentration with various conditions

Item	Substrate	Reactor type	Temp (^oC)	TAN* (mg l^{-1})	FAN* (mg l^{-1})	Microbial study	Affected group	References
1	Cattle manure	batch	50	3000		no		Zeeman et al. (1985)
2	Cattle manure	batch	55	2500		no		Hashimoto (1986)
			35	2500		no		
			55	4000		no		
3	Cattle manure	UASB	55	5000		yes	Acetoclastic	Borja et al. (1996)
4	Swine manure	batch	55	4100	1100	no		Hansen et al. (1998)
5	Soluble non-fat dry milk	CSTR	55	4000		no		Sung and Liu (2003)
6	Synthetic	CSTR (batch)	52		620	no		Siles et al. (2010)
7	Leachate	EGSB+	34-36		1500	no		Liu et al. (2012)
8	Growth medium	batch	37	7000	210	Yes	Methanosarcinaceae	Fotidis et al. (2013)
			52.5	7000	440	Yes	Methanosarcinaceae	

Note: * represented the critical concentration , + expanded granular sludge bed

2.6.2 Nutrients

As previously noted, nutrients are important to anaerobic microorganisms and many researchers have investigated their effects on anaerobic digestion. Kayhanian and Rich (1995) studied the performance of high solids thermophilic AD of biodegradable municipal waste (BMW) to identify nutrient rich co-substrates for optimum performance, these were wastewater treatment sludge (activated sludge), dairy manure and chemicals. The experiment was carried out in 4 sets of digesters operated for 3 months at an organic loading rate of 7.8 g VS kg^{-1} day^{-1} in each set.

Experiment I: BMW was mixed with water to give a solids content of 30-35%. The results showed that this feedstock by itself was sensitive to upsets and the pH ranged from 7.0 to 6.6 with gas production from 0.62 to 0.48 m^3 kg^{-1} VS day^{-1}. It was concluded that long term operation without nutrient addition was not possible.

Experiment II: mixtures of BMW and activated sludge at a ratio of 1:1 on dry weight basis. The output showed better process stability compared to Experiment I. Stable pH was observed with a fluctuation of only 0.1 pH unit. Ammonia and VFA increased (1040 and 12000 mg l^{-1}, respectively) without disturbance to gas production. The biogas production rate was 0.62-0.72 m^3 kg^{-1} VS day^{-1}, with an average of 0.63 m^3 kg^{-1} VS day^{-1}.

Experiment III: mixtures of BMW, activated sludge and dairy manure at a ratio of 7:1:0.5 on a dry weight basis. Stable operation and high gas production were observed. High alkalinity (16000 mg l^{-1}) resulted in a pH of 7.4, higher than at the beginning. Ammonia and VFA were typically 1000 mg l^{-1} and 11000 mg l^{-1}, respectively and biogas production was 0.75 m^3 kg^{-1} VS day^{-1} on average (range 0.65-0.85 m^3 kg^{-1} VS day^{-1})

Experiment IV: This used similar conditions to experiment III except that chemical supplements were used instead of dairy manure. The chemicals used were potassium phosphate, cobalt, chloride, nickel, sulphate, and sodium molybdate. Sodium bicarbonate was used as buffer solution. The results showed that chemical nutrients addition stabilized the system effectively; however, it could not enhance the gas production to the level of Experiment III. Alkalinity, ammonia, and VFA concentrations were 16000, 1050, and 12400 mg l^{-1}, respectively. Gas production ranged from 0.35-0.70 m^3 kg^{-1} VS day^{-1}, with an average of 0.67 m^3 kg^{-1} VS day^{-1}. After two months of feeding, the effluent became slimy and the digestate solids and liquid separated, and the system became inhibited. To recover the system, pH buffering was added on day 65 and gave better results. The authors suggested that highly soluble chemicals were more than the system could cope with due to their increased bio-availability. The slime was considered to be phosphorus bound with digested solids.

It was found that the combination of biodegradable municipal solid waste, wastewater treatment sludge and dairy manure at a ratio of 7:1:0.5 provided the best results in terms of both stability and gas production. It was suggested that this was due to the high levels of nutrients from both sources, especially nitrogen, phosphorus, potassium and nickel.

Jarvis et al. (1997) considered the influence of iron, nickel, cobalt and molybdenum with the aim of improving the performance of mesophilic AD. Laboratory-scale experiments were carried out on digesters fed with grass-clover silage. The selected trace elements were iron, nickel, cobalt and molybdenum. These were added either individually or as mixed trace elements at the concentrations shown in Table 2.8.

The results showed a clear increase in methane production after adding cobalt at a concentration of 0.2 and 2.2 mg l^{-1}. Molybdenum and nickel did not improve methane production compared to the control digesters. Moreover, high concentrations of nickel inhibited methane production. Four days after the addition of iron, a slight improvement in methane production was detected. Acetic acid decreased after adding Co. Since Co is a composition of corrinoids, a derivative of vitamin B12, it is concerned in methyl transfers in methanogenesis. Increasing the OLR to 7.0 g VS l^{-1} day^{-1} with cobalt addition led to higher methane production, but also to foaming and failure after 80 weeks of operation.

Table 2.8 Final concentration (mg l^{-1}) of trace elements (iron, nickel, cobalt and molybdenum) in 30 ml in batch assays.

Addition	Level	Fe	Ni	Co	Mo
Single	low	720	2.3	0.2	0.5
Single	high	3600	23	2.2	5.0
Mixture	low	109	0.7	0.2	0.5
Mixture	high	132	1.7	0.8	1.6
Control	-	99	0.4	0.02	0.07

Source: Jarvis et al. (1997)

Kim et al. (2002) conducted a batch experiment on hydrolysis and acidogenesis to evaluate the effect of pH and inorganic nutrient supplementation (Ca, Fe, Co and Ni) on degradation of suspended organic materials under mesophilic (35 ^{o}C) and thermophilic (55 ^{o}C) temperature conditions at different pH values (uncontrolled, 4.5, 5.5 and 6.54). The results showed that pH 6.5 best to support hydrolysis reactions. Thermophilic temperatures increased the rate of reaction but were more sensitive to pH change than mesophilic. The trial showed greater acid production in the reactors with nutrient

supplementation at both temperatures. Therefore, the nutrients enhanced the microbial hydrolysis and acidogenesis.

Zitomer et al. (2008) carried out an experiment on metal stimulation (Ni, Co, and Fe) of methanogenesis. The results showed a statistically significant increase in acetic and/or propionic acid degradation for both the mesophilic and thermophilic samples. Research by Pobeheim et al. (2010) showed similar results. They studied the influence of various trace elements on methane production in mesophilic anaerobic digestion with real and synthetic maize silage composed of 50% xylan, 46% starch, and 4% urea mixtures. Trace elements used in this study were nickel, cobalt and molybdenum, applied individually. A trace elements solution of Fe^{2+} 7.5 μM (0.419 mg l^{-1}), Zn^{2+} 0.5 μM (0.033 mg l^{-1}), Mn^{2+} 0.5 μM (0.027 mg l^{-1}), B^{3+} 0.1 μM (0.001 mg l^{-1}), Co^{2+} 0.8 μM (0.047 mg l^{-1}), Cu^{2+} 0.01 μM (0.001 mg l^{-1}), Ni^{2+} 0.1 μM (0.006 mg l^{-1}), Se^{6+} 1.0 μM (0.079 mg l^{-1}), Mo^{6+} 0.15 μM (0.014 mg l^{-1}), and W^{6+} 0.1 μM (0.018 mg l^{-1}) was used to support anaerobic digestion. An increasing methane yield was observed when trace elements were added to the reactor for both feedstocks at a ratio of 1 ml trace elements to 1 litre of digestate. By increasing the ratio to 5:1, the methane yield was only 10-13% higher than in the control with no trace element added. With no nickel addition, the methane yield was 18% lower compared to the reactor with trace elements. The explanation is that nickel is a component in the enzyme for carbon dioxide and hydrogen conversion (Kayhanian and Rich, 1995). Without cobalt, the effect was similar to lacking nickel, with a decrease in methane yield (~10%) and impaired process stability. Without molybdenum there was little effect on the methane yield and only slightly decreased productivity. The results for individual additions of nickel, cobalt and molybdenum are shown in Table 2.9.

Table 2.9 Methane production from adding nickel, cobalt and molybdenum

Nickel		Cobalt		Molybdenum	
(mg l^{-1})	**Methane yield (l kg^{-1} VS at STP)**	**(mg l^{-1})**	**Methane yield (l kg^{-1} VS at STP)**	**(mg l^{-1})**	**Methane yield (l kg^{-1} VS at STP)**
Control	342	Control	335	Control	370
0.02	399	0.02	341	0.16	375
0.08	386	0.03	344	0.22	378
0.62	407	0.12	371	0.61	374
1.02	245	1.00	320	1.11	383
10.04	188	10.02	288	5.08	390
				49.98	371
				99.97	345

Source: Pobeheim et al. (2010)

From Table 2.9, it can be concluded that trace elements must be present at the correct concentration and not in excess or deficient. The appropriate concentration of nickel, cobalt and molybdenum from this study was 0.62, 0.12, and 5.08 mg l^{-1}, respectively

Feng et al. (2010) set up laboratory-scale mesophilic reactors to treat food industry waste with trace metal additions. Groups of cobalt (Co), nickel/molybdenum/boron (Ni/Mo/B) and selenium/tungsten (Se/W) were added to investigate the biogas production and the microbial community by Quantitative Real-time PCR. The highest methane production (predicted value: 860 ml g^{-1} VS) was found in the sets of high Se/W and a low level of Co. There was a higher diversity amongst the bacteria than there was amongst the archaea in all treatments. The two dominant archaeal groups were *Methanosarcina siciliae* and *Methanoculleus bourgensis*, respectively. The combination of Ni/Mo/B limited the bacterial community, while the addition of Ni/Mo/B and/or Se/W benefited the archaeal populations.

Uemura (2010) investigated the mineral requirement for mesophilic (37 ^{o}C) and thermophilic (55 ^{o}C) anaerobic digestion of synthetic organic solid waste at an OLR of 3.0-3.5 g TS l^{-1} day^{-1}. A methane yield of 0.30 and 0.38 l CH_4 g VS^{-1} was obtained from the thermophilic and mesophilic digesters, respectively. Without trace element addition,

both digesters failed after 51 days. Ni, Co, and Fe were injected to both groups and the mesophilic digester recovered whereas the thermophilic reactor did not. It was suggested that thermophilic digestion required a higher concentration of these elements than mesophilic. Addition of trace elements at the beginning of the experiment led to better performance in the digesters. Supplementation saved the thermophilic digester from failure, with a similar biogas yield (0.38 l CH4 g VS^{-1}) to the mesophilic one. In addition, the author suggested that of the three trace elements Ni was the most important for AD of kitchen waste.

2.6.3 Volatile fatty acids (VFAs)

Öztürk (1991) studied the conversion of acetic, propionic and butyric acids to methane under thermophilic conditions in batch tests using a synthetic VFA mixture composed of acetic: propionic: butyric in the ratio of 100: 100: 100 g kg^{-1}. VFA and gas composition were measured daily. For the first experiment, it was reported that butyric acid was degraded rapidly (about 40 hours for complete degradation), while acetic acid was not degraded. The concentration of acetic and valeric acid continuously increased at the same time as butyric degradation. Acetic acid degradation started once butyric acid had been completely converted. Then, after the complete conversion of acetic acid, propionic acid was decomposed to acetic acid and then converted to methane and carbon dioxide. Hence, it could be concluded that the preferred order of degradation of VFA species was butyric, acetic, and propionic acid, respectively. In a second test butyric and propionic acid were degraded to acetic acid simultaneously which was then converted to methane and carbon dioxide. It was observed that the degradation rate in the second run was 2.5 times faster with a greater volume of biogas production. This was attributed to adaptation of the microbial population.

The order of VFA degradation in mesophilic conditions (36^{o}C) was investigated (Wang et al., 1999). The initial concentration of acetic acid, propionic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid, iso-hexanoic acid, and n-hexanoic acid was about 700 mg l^{-1} each. Under the same conditions, VFA degradation was considered as 4 groups: A) n- butyric acid; B) acetic acid, n-hexanoic acid, n-valeric, and iso-butyric acid C) propionic acid and D) iso-hexanoic acid. The degradation rate was in order of A>B>C>D, respectively. In addition, the iso- forms were degraded more slowly than

the normal forms. Acetic acid was always the predominant acid as this was a breakdown product from the longer chain length acids. Propionic acid also was found at high concentration since it is also a final VFA product and has a slow degradation rate. It was also found that acetic acid inhibited propionic acid degradation when the acetic acid concentration was higher than 1400 mg l^{-1}.

A comparison of the performance of mesophilic and thermophilic systems for AD of food waste was carried out in pilot-scale CSTRs (Banks et al., 2008). Two reactors were set up in parallel with an initial OLR of 4 kg VS m^{-3} day^{-1}. After 20 days of operation, VFA accumulated as a result of increasing the OLR from 4.1 to 5.72 kg VS m^{-3} day^{-1} in the mesophilic reactor. The biogas yield was 0.63 m^3 kg^{-1} VS_{added} with 59% methane content and 67% VS destruction. Very high VFA concentrations of 27400 mg l^{-1} appeared to have no effect on gas production. Ammonia and alkalinity was 5200 and 13900 mg l^{-1}, respectively. VFA concentrations were reduced to 7000-12000 mg l^{-1} by mixing the feedstock with water instead of recycled digestate, resulting in lower ammonia concentrations (3000 mg l^{-1}). For the thermophilic anaerobic digester, gas production was 0.67 m^3 kg^{-1} VS_{added} and 58% methane content and 70% VS destruction. In the thermophilic reactor, however, the VFA accumulated to 45000 mg l^{-1}, and then caused a pH drop and instability. The reason why VFA accumulation was detrimental to the performance appears to be that it is associated with a failure in autotrophic methanogenesis. The lack of consumers of hydrogen and carbon dioxide affects the hydrogen partial pressure and the propionic acid concentration in the reactors increases. Other explanations include high ammonia concentration (> 3500 mg l^{-1}) inhibited butyric acid degradation to acetic acid, and also the carbon to nitrogen ratio of 14:1 which was less than optimum (30:1).

Zhang et al. (2009) studied hydrolysis and accumulation of short-chain fatty acids under mesophilic and thermophilic conditions at different pH values. A feedstock of activated sludge from the secondary sedimentation tank of a municipal wastewater treatment plant in Shanghai, China was used, with pH values of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and uncontrolled (initial pH 6.64). Two groups of CSTRs were set up at mesophilic (35±2 ^{o}C) and thermophilic (55±2 ^{o}C) temperatures. It was found that hydrolysis in the thermophilic set was higher than in the mesophilic set at all pH values. Hydrolysis was higher in alkaline conditions than acidic, for both mesophilic and thermophilic sets. A

higher concentration of short-chain fatty acids was found for operation at pH 8.0 in both mesophilic and thermophilic set, with 9 days of fermentation time. Acetic acid was the main short-chain fatty acid found in thermophilic conditions and was at a higher concentration than under mesophilic conditions. A higher percentage of acetic acid was also observed under alkaline or acid pH compared to neutral in both mesophilic and thermophilic conditions. This was explained by the better degradation of acetic acid to methane, leading to lower results in the neutral pH range. For methane production, pH 7 gave the best results. Mesophilic digesters showed superior methane production compared to the thermophilic ones. Carbohydrates were better degraded in thermophilic conditions, while mesophilic provided better degradation of proteins. For methane yield, mesophilic digesters showed a better yield than thermophilic ones.

From this work, it could be concluded that thermophilic anaerobic digestion was better at performing acidogenesis than mesophilic digesters, however, mesophilic digesters could utilise the VFA better than thermophilic ones, perhaps because of VFA accumulation in thermophilic digestion.

Iron, cobalt and nickel addition were studied in thermophilic methane fermentation using high-solid food waste (Qiang et al., 2013). A mixed solution of iron, cobalt and nickel at 10, 1 and 1 mg l^{-1}, respectively was added to the reactor approximately every 45 days after day 57 due to a fall in gas production and rise in VFA. The gas production, methane content and pH resumed stable levels after routine trace element addition. The authors stated that the most suitable values for Fe/COD, Co/COD, Ni/COD were 276, 4.96 and 4.43 mg kg^{-1} $COD_{removed}$, respectively, for thermophilic digestion of food waste.

2.6.4 Operational function and performance

Cecchi et al., (1991) conducted mesophilic (37^{o}C) and thermophilic (55^{o}C) anaerobic digestion studies using the mechanically sorted organic fraction of municipal solid waste. At the same hydraulic retention time (14.7 days), thermophilic AD gave better volumetric gas production compared to mesophilic digestion (2.5 m^3 m^{-3} day^{-1} compared to 1.4 m^3 m^{-3} day^{-1}) and VFA profiles were similar (389 and 268 mg CH_3COOH l^{-1} for thermophilic and mesophilic AD, respectively). The mesophilic AD, however, ran at a higher OLR (7.5

kg VS m^{-3} day^{-1}) than the thermophilic AD (5.9 kg VS m^{-3} day^{-1}). When run at a similar OLR (7.5 for mesophilic AD and 6.9 for thermophilic AD), the thermophilic AD showed better performance with a biogas production rate of 2.8 m^3 m^{-3} day^{-1} compared to 1.4 m^3 m^{-3} day^{-1} for mesophilic reactors. The methane percentage was significantly higher in the thermophilic reactor: 62% compared to 52% for the mesophilic digester. The VFA of both digesters was similar (284 and 268 mg CH_3COOH l^{-1} for thermophilic and mesophilic AD, respectively).

Kim et al. (2002) evaluated the process stability and efficiency of thermophilic and mesophilic anaerobic digestion with different reactor configurations, microbial proximity, and nutrient supplementation. Only the results from the thermophilic experiments are discussed here. Four configurations were used: daily batch-fed single-stage CSTR, continuously fed single-stage CSTR, daily batch-fed two-stage CSTR and daily batch-fed non-mixed reactor with a temperature of 55 ^oC. The digesters were started using dog food as the substrate to mimic primary sludge. At a 4% solids concentration this resulted in a fall in pH and high VFA; the concentration was therefore reduced to 0-2% and then gradually increased back to 4%. Without nutrient supplement, thermophilic batch-fed single-stage CSTR was found to be less stable than the other reactors. During the start-up period the two-phase digesters had advantages over single-stage digesters, continuous feeding had advantages over batch feeding, and no-mixing had advantages over continuous mix. These advantages were expressed on the basis of how well the digesters reached stable operation in terms of pH and VFA. Except in the case of the two-phase CSTR digester the thermophilic digesters had high acetic acid concentrations which the authors attributed to the slower growth of methanogens compared to acidogens. After adding a trace element cocktail of Ca, Fe, Co, and Ni, gas production increased and VFA decreased, except propionic acid. The propionic acid, however, decreased in the thermophilic non-mixed reactor. The longer HRT, inorganic nutrients supplement, and proximity of the microbial consortia were the reasons given for this. The authors suggested that to improve VFA degradation, gas production, and VS removal, closer microbial consortia proximity should be considered.

Research evaluating the performance of thermophilic sludge digestion with decreasing sludge retention time (SRT) and increasing organic loading rate (OLR) was undertaken by Ferrer et al. (2010). The experiment used 5-litre active volume digesters at a temperature

of 55 ^{o}C. Inoculum was taken from a mesophilic digester and acclimated by a single step temperature change. Feedstock was primary and secondary wastewater sludge from the treatment plant in Barcelona, Spain. The experiment was divided into two periods: low- and high- solids content feeding. The SRT was set from >30, 30, 25, 20, 15, and 10 days (day 1-437) for low solids content feeding (TS < 40 g l^{-1}), and a second period (day 484-653) with high solid content feeding (TS > 40 g l^{-1}) and an SRT of 10 days which decreased to 6 days. It was, however, necessary to increase it again to 10 days because of VFA accumulation. For the low solids content period it was observed that the lower SRT (or the higher OLR) provided better gas production both in quantity and quality. At a 30-day SRT (0.69 kg VS m^{-3} day^{-1}) gas production was 0.22 m^3 CH_4 m^{-3} day^{-1}, and when SRT was reduced to 10 days (1.5-2.0 kg VS m^{-3} day^{-1}*)* gas production rose to 0.36 m^3 CH_4 m^{-3} day^{-1}. After the sludge feed was switched to a high solid content and a 10-day SRT (3-4 kg VS m^{-3} day^{-1}) gas production almost doubled to 0.62 m^3 CH_4 m^{-3} day^{-1}. A further reduction in SRT to 6 days (4.5-6. kg VS m^{-3} day^{-1}) further increased gas production to 0.86 m^3 CH_4 m^{-3} day^{-1}. Although productivity was initially higher there were signs of failure as VFA accumulation was observed, rising from 4000 to 10000 mg l^{-1} in 10 days. From long term observation it was suggested that the limiting value to prevent failure was: acetic acid < 600 mg l^{-1}, acetic to propionic acid ratio < 500, VFA < 3700 mg l^{-1}, intermediate alkalinity < 1800 mg $CaCO_3$ l^{-1}, IA/PA < 0.9, IA/TA < 0.5 and methane content (%CH_4 in biogas) > 55%. Increasing OLR appeared to affect methanogens more than a reduction in SRT. Acidogenic activity, however, was affected by both increasing OLR and decreasing SRT.

2.6.5 Anaerobic digestion for Food waste

Zhang et al. (2007) studied the anaerobic digestion of food waste from San Francisco, California in 1-L batch digesters (0.5 l effective volume) at OLR of 6.8 and 10.5 g VS l^{-1} and a temperature of 50 ^{o}C. Inoculum was taken from a thermophilic anaerobic digester at a municipal wastewater treatment plant. The specific methane yield was 0.425 and 0.445 l g^{-1} VS_{added} for OLRs of 6.8 and 10.5 g VS l^{-1}, respectively.

Stabnikova et al. (2008) studied the effect of freezing and thawing food compared to fresh food using a batch test in a two-stage anaerobic digester under mesophilic conditions (35 ^{o}C). It was observed that the frozen and then thawed food waste had

increased acidogenesis. Scanning electron microscopy indicated that the structure of the frozen and then thawed food waste was looser than the fresh material and improved its solubility. The maximum rates of COD, VFA, and methane production from frozen and then thawed food waste were 20%, 235%, and 41% higher compared to the fresh food waste.

Experiments on food waste anaerobic digestion were conducted to maximize VFA production as an electron donor for biological nitrogen and phosphorus removal (Lim et al., 2008). A 2-l semi-continuous mesophilic reactor was set up with pH control and fed once a day. Changes in HRT (4, 8 and 12 days), OLR (5, 9, 13 g l^{-1} day^{-1}), temperature (25, 35 and 45 ^{o}C), pH (5.0, 5.5, and 6.0) were investigated. Increasing HRT gave a higher total VFA, of 20500-22000 mg l^{-1} with 12-hour HRT. However, there was no significant difference in VFA yield between 8- and 12- hour HRT. Acetic and propionic acid were the dominant forms found throughout the experiment. The higher OLR gave the higher VFA, in both concentration and yield. The temperature giving the highest VFA yield was 35^{o}C. In the 45^{o}C experiment, little propionic acid was found (600-900 mg l^{-1}). Increases in VFA occurred with increasing pH; however, there was an insignificant difference between pH of 5.5 and 6.0. To obtain the highest VFA yield, the optimum conditions were 35 ^{o}C, pH 6.0, and HRT 8 days.

Banks and Zhang (2010) carried out digestion trials with source separated domestic food waste at different organic loading rates in 35-L semi-continuous mesophilic digesters at 36 ^{o}C. The specific biogas production (SBP), volumetric biogas production (VBP) and specific methane production were 0.695 m^3 kg^{-1} VS_{added}, 1.39 m^3 m^{-3} day^{-1}; and 0.425 m^3 kg^{-1} VS_{added}, respectively. TAN was 3,800 mg N l^{-1}, pH 7.7 and VS destruction 83.9%. VFA was between 9000-10000 mg l^{-1}. Propionic acid accumulation was found and caused a loss in SMP, as well as foaming. These conditions indicated the onset of failure. Reducing the OLR and even stopping feeding were unable to recover the digester. In a 4-L lab-scale experiment the digester failed as a result of VFA accumulation and feeding had to be stopped. In larger-scale experiments (75 L) operating at an OLR of 2 g VS l^{-1} day^{-1} a food waste feedstock was compared with a mixed feedstock of food waste co-digested with cattle slurry or with card packaging. The digesters fed on food waste alone suffered from VFA accumulation after 210 days of operation (rising from 200 to 1000 mg l^{-1} between days 180 and 210). After 3 HRTs

the VFA was above 4000 mg l^{-1}. The accumulation was probably as a result of trace metal deficiency, accompanied by a high TAN (> 4000 mg N l^{-1})

Zhang et al. (2012a) investigated the performance from source segregated domestic food waste (ss-FW) and the mechanically-recovered organic fraction of municipal solid waste (mr-OFMSW) in mesophilic CSTR (36 ^{o}C). The performance indicators of mr-OFMSW were; specific methane production (SMP) 0.304 STP m^3 CH_4 kg^{-1} VS_{added}; specific biogas production (SBP) 0.529 STP m^3 kg^{-1} VS_{added}; methane percentage 57.5%; VFA < 100 mg l^{-1}; TAN of 1400 mg N 1^{-1} and pH 7.5. It was found that the SMP was 86% of the biochemical methane potential (BMP), implying that most of the biodegradable part of mr-OFMSW had been converted to biogas. After 6 HRT (180 days), propionic acid rose rapidly and led to decreasing gas production at a TAN of 3500 mg l^{-1}, approximately. The reactor finally reached stressed conditions with VFA rising to 20,000 mg l^{-1} and pH falling to 7.1. Problems of foaming also occurred. No recovery method (for example reducing the loading rate, stopping liquor recirculation, and finally stopping feeding the digester) was able to rescue the digester. The results from the stable period showed a high specific methane yield of 0.425 STP m^3 CH_4 kg^{-1} VS_{added}. High ammonia nitrogen and VFA were claimed as the cause of the stressed digesters.

2.6.6 FISH

Hori et al. (2006) investigated dynamic transitions of methanogens in a thermophilic anaerobic digester (55 ^{o}C). The results indicated that archaeal community structure related closely to VFA concentration, whereas the bacterial community was influenced by pH. The two main species of hydrogenotrophic methanogen were *Methanoculleus sp*. and *Methanothermobacter sp.; Methanosarcina sp*. were the main acetoclastic methanogens. In stable and low VFA conditions, the *Methanoculleus sp*. was dominant. While there was an accumulation of VFA, the dynamic transition changed to the group of transition of hydrogenotrophic methanogens, *Methanothermobacter sp.*

Ariesyady et al. (2007) reported the functional bacteria and archaea community structures of a full-scale anaerobic digester (40 ^{o}C) treating wastewater biosolids from a domestic wastewater treatment facility. The bacteria phyla found were *Actinobacteria, Firmicutes,* and

Chloroflexi. For methanogens, it was noted that *Methanosaeta* (7.67±0.8%) was the majority group followed by *Methanomicrobiales* (2.87±0.6%) and *Methanobacteriaceae* (2.77±0.4%), respectively.

Banks et al. (2012) performed research on the trace element requirements for mesophilic anaerobic digesters treating domestic food waste at high ammonia concentrations. Flask tests showed that Se and Mo supplementation decreased the degradation time for acetic and propionic acids. In semi-continuous digestion both Se and Co addition maintained VFA concentrations below 500 mg l^{-1}, even when the loading rate was raised to 5 g VS l^{-1} day^{-1}. Digesters receiving mixed TE supplementation maintained VFA concentrations of less than 500 mg l^{-1}. The main group of methanogens found in the digestate was *Methanomicrobiales*, the hydrogenotropic methanogens.

Xia et al. (2012a) identified the diversity of archaeal communities during anaerobic co-digestion of chicken feathers and other animal wastes. The digesters inoculated with adapted swine manure, slaughterhouse sludge or dairy manure was fed with raw feathers. The results from FISH revealed that methanogens from the *Methanosarcinales*, *Methanomicrobiales*, and *Methanobacteriales* comprised a major fraction (78%) of all observed archaea. A high correlation was seen between the distribution of functional archaeal groups and the TAN levels of digester mixed liquors. The substrates significantly affected the archaeal communities. The authors found that only the hydrogenotrophic methanogens (*Methanomicrobiales* and *Methanobacteriales*) were present in the communities with the highest TAN levels (upto 7 g l^{-1}). Moreover, the communities with lower TAN (up to 4 g N l^{-1}) consisted of hydrogenotrophic/acetotrophic *Methanosarcina.* Acetotrophic *Methanosaeta* was found in the digester with the lowest TAN level (upto 3 g N l^{-1}).

Regueiro et al. (2012) studied the relationship between microbial activity and microbial community structure in six full-scale mesophilic anaerobic digesters from varied wastes such as sewage sludge, brewery, dairy, sugar, yeast, and mixed dairy and fish waste. The pH, TAN and VFA varied from 6.5-7.9, 430-1900 mg l^{-1} and 0.06-5.41 g l^{-1}, respectively. The different substrates reflected the different values of hydrolytic (0.6–3.5 g COD g^{-1} VSS d^{-1}) and methanogenic (0.01–0.84 g COD g^{-1} VSS d^{-1}) activities,

whereas no significant differences were observed among the acidogenic activities (1.5–2.2 g COD g^{-1} VSS d^{-1}). Mostly, the higher the hydrolytic and the methanogenic activity were, the higher the bacteroidetes and archaea percentages presented. It was stated that hydrogenotrophic methanogenic activity was always higher than acetoclastic methanogenic activity. The highest values were found in those substrates with lower percentages of *Methanosaeta*.

2.6.7 Conclusion

Based on the literature review it is clear that there are recognised issues with high nitrogen wastes leading to high ammonia concentrations in the digestion process. It is also clear that operating temperature plays an important role in digestion performance and stability, due both to the increase in ammonia toxicity at higher temperatures and to likely changes in the microbial population. There is growing understanding of the role and importance of trace elements in contributing to stable and effective digestion; while the development of new tools for analysing microbial communities and metabolic pathways provides improved insights into the relationships between environmental factors and microbially-mediated reaction. In the current research, these techniques were therefore applied in a programme of laboratory digestion trials aimed at determining the feasibility or otherwise of food waste digestion at thermophilic temperatures and the key factors that determine this.

Chapter 3 Materials and Methods

3.1 General

3.1.1 Reagents

Except where otherwise stated all chemicals used were of laboratory grade and obtained from Fisher Scientific (Loughborough, UK)

3.1.2 Water

Solutions and standards were prepared using ultra-pure deionised (DI) water obtained from a Barnstead Nanopure ultrapure water purification system (Thermo Scientific, UK)

3.1.1. Laboratory practice

All laboratory operations were carried out using good laboratory practice, after first carrying out the appropriate risk assessments and, where necessary, COSSH assessments. All equipment, laboratory apparatus, and analytical instruments were operated in accordance with the manufacturer's instructions. All glassware was washed using washing detergent followed by rinsing with tap water and deionised water. The glassware used for the acid digestion was soaked in a 10% nitric acid bath for a 24 hour period after which the glassware was rinsed with Milli-Q water.

3.2 Analytical methods

3.2.1 pH

pH was measured using a Jenway 3010 meter (Bibby Scientific Ltd, UK) with a combination glass electrode, calibrated in buffers at pH 4, 7 and 9.2. The pH meter was temperature compensated and had a sensitivity of ±0.01 pH unit and accuracy of 0.01±0.005 pH units. Buffer solution used for calibration was prepared from buffer tablets (Fisher Scientific, UK) prepared according to the supplier's instructions. During measurements, the sample was stirred to ensure homogeneity. In addition, the pH probe

was rinsed with DI water in between measurements and placed into a mild acid solution to avoid cross-contamination. Digestate samples were measured immediately after sampling to prevent changes in pH due to the loss of dissolved CO_2.

3.2.2 Total solids (TS) and volatile solids (VS)

TS and VS determination was based on Standard Method 2540 G (APHA, 2005). After thorough agitation, approximately 10 g of sample was transferred into a weighed crucible. Samples were weighed to the range of 210±0.0001 g (Sartorius BP210S balance, Sartorius AG, Gottingen Germany) and placed in an oven (LTE Scientific Ltd., Oldham UK) for drying overnight at 105 ^{o}C ± 1 ^{o}C. After drying the samples were transferred to a desiccator to cool for at least 40 minutes. Samples were then weighed again with the same balance, transferred to a muffle furnace (Carbolite 201, Carbolite, UK) and heated to 550 ± 10 ^{o}C for two hours. After this ashing step, samples were again cooled in a desiccator for at least one hour before weighing a third time.

After all analyses, crucibles were washed with detergent, rinsed with DI water, and dried in an oven at least an hour and then transferred from the oven to a desiccator for cooling to room temperature and stored there for the next analysis. Total and volatile solids were calculated according to the following formulae:

$$\%TS = \frac{W_3 - W_1}{W_2 - W_1} \times 100 \qquad \textbf{Equation 3.1}$$

$$\%VS(based \cdot on \cdot total \cdot weight) = \frac{W_3 - W_4}{W_2 - W_1} \times 100 \qquad \textbf{Equation 3.2}$$

$$\%VS(based \cdot on \cdot total \cdot solids) = \frac{W_3 - W_4}{W_3 - W_1} \times 100 \qquad \textbf{Equation 3.3}$$

Where

W_1 is the weight of the empty crucible, g;
W_2 is the weight of the crucible containing fresh sample, g;
W_3 is the weight of the crucible and sample after drying at 105 ^{o}C, g;
W_4 is the weight of the crucible and sample after heating to 550 ^{o}C, g.

3.2.3 Alkalinity

Alkalinity was measured by titration based on Standard Method 2320B for Alkalinity (APHA, 2005). Digestate was sieved to obtain a homogenous sample and 2-5 g of this was added to 40 ml of DI water. Titration was done using a Schott Titroline Easy automatic digital titration burette system (Schott, Mainz, Germany), with the samples being magnetically stirred while the titration was carried out. A 0.25 N H_2SO_4 titrant was used to determine endpoints of pH 5.7 and 4.3, allowing calculation of total (TA), partial (PA) and intermediate alkalinity (IA) (Ripley et al., 1986). PA is a measurement of bicarbonate buffering while IA is attributed to the buffering capacity of volatile fatty acids (VFA).

The pH probe was calibrated before titration using buffers as described above, and washed with DI water between subsequent samples to avoid cross contamination. Alkalinity was calculated according to equations 3.4-3.6.

$$\mathrm{TA} = \frac{(\mathrm{V}_{4.0} + V_{4.3} + V_{5.7}) \times N \times 50000}{\mathrm{V}} \qquad \textbf{Equation 3.4}$$

$$\mathrm{PA} = \frac{\mathrm{V}_{5.75} \times N \times 50000}{\mathrm{V}} \qquad \textbf{Equation 3.5}$$

$$\mathrm{IA} = \frac{\mathrm{V}_{\mathrm{pH4.3}} \times N \times 50000}{\mathrm{V}} \qquad \textbf{Equation 3.6}$$

Where

TA is the total alkalinity, mg $CaCO_3$ l^{-1};

PA is the partial alkalinity or bicarbonate alkalinity, mg $CaCO_3$ l^{-1};

IA is the intermediate alkalinity or volatile fatty acid alkalinity, mg $CaCO_3$ l^{-1};

N is the normality of H_2SO_4;

V is the volume of sample, ml.

3.2.4 Volatile fatty acid (VFA) by gas chromatography

The method used was based on SCA (1979): Determination of Volatile Fatty Acids in Sewage sludge (1979). Samples were prepared for analysis by centrifugation at 14000 g (micro-centrifuge, various manufacturers) for 15 minutes. 0.9 ml of the supernatant was transferred by pipette to vials with 0.1 ml formic acid to give a final concentration of 10% formic acid. Where dilution was necessary, DI water was used and formic acid was added to give a concentration of 10% of the total volume for analysis. If the samples at this point were turbid they were centrifuged again at 14000 rpm to obtain a clearer supernatant. The supernatant after acidification and centrifugation was transferred into the vials and loaded onto the GC auto-sampler ready for the VFA measurement.

A standard solution containing acetic, propionic, iso-butyric, n-butyric, iso-valeric, valeric, hexanoic and heptanoic acids, at three dilutions to give individual acid concentrations of 50, 250 and 500 mg l^{-1} respectively, was used for calibration and also loaded onto the GC. Quantification of the VFA was by a Shimazdu GC-2010 gas chromatograph (Shimadzu, Milton Keynes, UK), using a flame ionization detector and a capillary column type SGE BP-21. The carrier gas was helium at a flow of 190.8 ml min^{-1} and a split ratio of 100 to give a flow rate of 1.86 ml min^{-1} in the column and a 3.0 ml min^{-1} purge. The GC oven temperature was programmed to increase from 60 to 210 ^{o}C in 15 minutes with a final hold time of 3 minutes. The temperatures of injector and detector were 200 and 250 ^{o}C, respectively.

3.2.5 Gas composition by gas chromatography

Biogas composition was quantified using a Varian Star 3400 CX gas chromatograph (GC), (Varian Ltd, Oxford, UK). The device was fitted with a Hayesep C column with argon as the carrier gas at a flow of 50 ml min^{-1} and a thermal conductivity detector. The biogas composition was compared with a standard gas sample containing 65% CH_4 and 35 CO_2 (v/v) (BOC, UK) for calibration. A sample of 5 ml was taken from the gas impermeable bag or gas collection cylinder used for sample collection and was injected into a gas sampling loop.

3.2.6 Ammonia (distillation)

Total ammonia nitrogen (TAN) analysis was based on Standard Method 4500-NH3 B and C (APHA, 2005). A sample aliquot of between 2-4 g was weighed (i201, My Weigh Europe, Huckelhoven Germany) into a digestion tube and 40 ml of DI water added. Blanks (40 ml DI water) and standards (containing 10 ml of 1000 mg l^{-1} NH_4Cl with 30 ml DI water) were also prepared in digestion tubes. 5 ml of 10 M sodium hydroxide (NaOH) was added to each digestion tube to raise the pH above 9.5 and the samples were distilled using either a Foss Tecator Kjeltec system 1002 distillation unit (Foss Tecator A-B, Hoganas, Sweden) or a or a Büchi K-350 Distillation Unit (Büchi, UK) Erlenmeyer flasks previously filled with 25 ml of boric acid as an indicator were used to collect the distillate and progress of the distillation was indicated by a colour change from purple to green. The distillate was titrated manually with 0.25 N H_2SO_4 using a digital titration system (Schott Titroline, Gerhardt UK Ltd) until an endpoint was reached as indicated by a colour change to purple at which point the volume of titrant added was recorded. Standards and blanks were distilled in the same way. The TAN concentration was calculated using equation 3.7.

$$\text{TAN mg N } l^{-1} = \frac{(A-B)\times 14.0\times N\times 1000}{V_{sample}} \qquad \textbf{Equation 3.7}$$

Where

A is the volume of 0.25 N H_2SO_4 used to titrate the sample, ml;
B is the volume of 0.25 N H_2SO_4 used to titrate the blank, ml;
N is the normality of standardised sulphuric acid, or the theoretical Normality multiplied by a factor (F) for the actual normality of the solution.
V_{sample} is the volume of sample, ml.

To obtain the free ammonia nitrogen (FAN), the equation from Østergaard (1985) was used to calculate the FAN as shown in equation 3.8.

$$\frac{FAN}{TAN} = \left(1 + \frac{10^{-pH}}{10^{-\left(0.09018+\frac{2729.92}{T(K)}\right)}}\right) \qquad \textbf{Equation 3.8}$$

Where

FAN is the concentration of free ammonia nitrogen, mg N l^{-1};

TAN is the concentration of total ammonia nitrogen, mg N l^{-1};

pH is the pH of the samples

T is the temperature of the samples, K.

3.2.7 Acid digestion for Total Kjeldahl Nitrogen extraction (TKN)

Total Kjeldhal Nitrogen (TKN) analysis was carried out on duplicate samples alongside blanks and controls as follows: 1-3 g (weighed to ± 1 mg) of sample was placed in a glass digestion tube. Two Kjeltab Cu 3.5 catalyst tablets were added to facilitate acid digestion by lowering the activation energy of the reaction. 12 ml of low-nitrogen concentrated H_2SO_4 was added carefully to each digestion tube and agitated gently to ensure that the entire sample was completely exposed to acid. The digestion tubes were then placed into the heating block with exhaust system using either a Foss Tecator 1007 Digestion System 6 (Foss Analytical, Hoganas Sweden) or a Büchi K-435 Digestion Unit (Büchi, UK) for approximately two hours until the solution colour became a clear blue-green. Both systems operated at 420 ± 5 ^{o}C and once the reaction was completed the tubes were cooled to around 50 ^{o}C and 40 ml of DI water slowly added to the digestion tube to prevent later crystallisation on further cooling. Samples, blanks and standards were then distilled and titrated as described in section 0.

TKN was calculated using the equation below:

$$TKN = \frac{(A-B)\times 14.01\times N\times 1000}{mg\,(wet\cdot weight\cdot sample)} \qquad \textbf{Equation 3.9}$$

where:

A = volume of H_2SO_4 titrated for sample, ml;

B = volume of H_2SO_4 titrated for blank, ml;

N is the normality of standardised sulphuric acid, or the theoretical Normality multiplied by a factor (F) for the actual normality of the solution.

The results from TKN analysis also can be used to estimate the crude protein for substrate characterisation by multiply with 6.25 base on the assumption of 16%N in animal protein (cited from Salo-väänänen and Koivistoinen (1996)).

3.2.8 Elemental composition

A sample of around 10 kg was taken from the whole batch of around 200 kg and remixed once more to ensure homogenisation. 1 kg of well-mixed sample was air-dried and then ground with a centrifugal mill (Glen Creston Ltd, England) to a particle size of < 0.5 mm. The ground samples were kept in a desiccator and used for analysis of lipids, carbohydrates, fibre, calorific value and acid digestion for metal extraction.

Carbon, hydrogen and nitrogen contents of samples were determined using a FlashEA 1112 Elemental Analyser (Thermo Finnigan, Italy). Sub-samples of approximately 3-4 mg were weighed into standard weight tin disks using a five decimal place analytical scale (Radwig, XA110/X, Poland) which were placed in draft shield. These were placed in a combustion/reduction reactor held at 900 °C then flash combusted in a gas flow temporarily enriched with oxygen resulting in a temperature greater than 1700 ^{o}C and the release of N_xO_x, CO_2, H_2O and SO_2 (depending on the composition of the sample). The gas mixture was then analysed by GC with the different components are measured by appropriate detectors. The working conditions of the elemental analyser were as described in the manufacturer's technical literature and method sheets. Standards used in this analysis were atropine, nicotinamide and birch leaf. The TS and VS of dried and milled samples were also analysed to obtain the most accuracy values. The results of the elemental composition (CHON) were also used for calculation the theoretical biogas production by (modified) Buswell equation (Buswell and Mueller, 1952) equation and the Dulong equation according to the method in Combustion file 24 (IFRF, 2013).

3.2.9 Acid digestion for metals extraction

1 - 3 g of sample was added to the digestion tube, with blanks prepared in parallel. 7.5 ml of 35-36% w/v HCl (hydrochloric acid) was added, then after ~5 minutes 2.5 ml of 70% w/v HNO_3 (Nitric acid) was added, and the tubes were gently agitated. The tubes were placed into the digestion block (Gerhardt Kheldatherm), connected to the

condenser system and left for 24 to 48 hours prior to heating. The acid digestion involved gradually increasing the temperature first to 100 ^{o}C and then to the final temperature of ~180 ^{o}C which was maintained for about 2 hours ± 10 min. After cooling, the mixtures were filtered (Filter paper No. 1 Qualitative 11 cm, Whatman, UK) into a 50-ml volumetric flask. Any remaining residue in the tube was washed out with ~5 ml of warm 12.5% v/v HNO_3 and transferred to the 50 ml flask, with up to 5 washes being performed. The volume was then made up to 50 ml with HNO_3 (12.5% v/v). The filtrate was then transferred into a PET bottle and sent for analysis by ICP-MS (Severn Trent Services, Coventry, UK).

3.2.10 Fibre

Neutral detergent fibre (NDF), Acid detergent fibre (ADF) and Acid detergent lignin (ADL) were analysed using the Fibertec™ 2021/2023 FiberCap™ system (FOSS Analytical AB, Sweden) according to the manufacturer's instruction with modifications adapted from Goering and Soest (1970) and Kitcherside et al. (2000). These acid solubilisation and gravimetric analysis were used for determination of cellulose, hemicellulose and lignin of SBP. The formulae for calculation are presented in Appendix A.

3.2.11 Carbohydrates

Carbohydrates determination consisted of 2 steps: hydrolysis to convert the carbohydrates to sugars and sugar determination by Dionex. The air-dried and ground samples were hydrolysed using 72% sulphuric acid in an orbital shaker (1 hour at 150-200 rpm) (Sluiter et al., 2012). The hydrolysate was autoclaved at 121^{o}C for 1 hour and analysed using a Dionex HPLC with an EC-400 detector and fitted with a Carbopac PA1 column (250 x 4 mm) in combination with a Carbopac guard column (25 x 4 mm) (Dionex corp, Sunnyvale, USA)

3.2.12 Lipids

The air-dried and ground samples were weighed with a balance with an accuracy of 0.1 mg and then measured after Soxhlet extraction using n-hexane (USEPA, 1998). The samples were also analysed for TS and VS to allow reporting as %VS.

3.2.13 Calorific value

CV was measured using a ballistic bomb calorimeter (CAL2k, Digital Data Systems Ltd, South Africa) according to the manufacturer's instructions. Benzoic acid was used as standard, with a higher heating value (HHV) of 26.454 kJ g^{-1}. Samples of around 1 g were weighed to an accuracy of 0.1 mg and then placed in the vessel.

3.2.14 Fluorescent In-situ Hybridisation (FISH)

The method for FISH analysis was obtained mainly from Daims et al. (2005). The FISH process involved 3 steps: fixation, hybridisation and microscopy. The procedure used was as follows:

3.2.14.1 Fixation

Reagents and equipment

Equipment

- 2 ml plastic tubes, autoclaved
- Disposal pipette
- Pipette tips, autoclaved
- rack
- Laboratory balance
- Gloves, dust mask
- 0.2 µm filter, syringe, and needle
- Waring blender
- Centrifuge at 20000 g
- Fridge (4°C) and freezer (-20 °C)

- Magnetic stirrer with heater (60-70 °C)
- Fume hood

Preparation of reagents

- Phosphate buffer: 20:80 (v/v) mixture of 200 mM NaH_2PO_4 and 200 mM Na_2HPO_4, pH of the buffer mixture was between 7.2-7.4
- 3x phosphate buffered saline (PBS): 390 mM NaCl, 15% (v/v) phosphate buffer, pH of the buffer was between 7.2-7.4
- 1xPBS: 130 mM NaCl, 5% (v/v) phosphate buffer, pH of the buffer was between 7.2-7.4
- 4% paraformaldehyde (PFA) solution: to make 50 ml of PFA solution 33 ml distilled water was heated to 65 °C and 2 g PFA (P6148, Sigma-Aldrich) added while stirring. NaOH was then added until the paraformaldehyde was dissolved and 16.6 ml of 3xPBS was added and the solution left to cool to room temperature. The pH was adjusted to 7.2-7.4 and the solution filtered using a syringe and a 0.2 μm filter cartridge. (Note: the solution could be stored at -20 °C. but could only be thawed once as a repeat freeze and thaw cycle will damage its function.) (**CAUTION:** PFA is toxic and is only handled when wearing gloves, a dust mask, and working using a fume hood.)
- Ethanol, absolute;
- Nycodenz solution: 6 g Nycodenz (D2158 Histodenz, Sigma-Aldrich) was dissolved into 10 g of deionised water;

Procedures

1. **Fixing.** This was carried out as soon as possible. Around 10 g of the fresh sample were weighed and mixed with a minimum of 100 g of 1xPBS. The mixture was blended for about 1 minute while flushing with compressed CO_2/N_2 gas to maintain anaerobic conditions. 1 ml of the blended fluid was aseptically pipetted into a 2-ml autoclaved centrifuge tube.

2. 1 ml nycodenz solution was gently injected and the mix centrifuged for 30 minutes at 14000 rpm. This allowed separation of the mix into 3 layers, with the microbial layer mostly suspended in the middle.

3. A narrow bulb pipette was then used to transfer the fluid in the centrifuge tube into a small glass beaker.

4. This was then mixed with at least 10 ml of 1xPBS before a portion was transferred to a new 2-ml autoclaved centrifuge tube to ensure that the solid was well resuspended.

5. The tube was centrifuged for 10 minutes at 14000 rpm.

6. **Washing of the cells.** The supernatant was removed and the solids from the bottom of tubes mixed together into a single tube which was then topped up to 2 ml with 1xPBS and centrifuged again for 10 minutes at 14000 rpm.

7. After washing, the supernatant is removed, 0.3 ml 1xPBS was added to the tube and the cells were resuspended. 0.9 ml of PFA was then added to the tube, mixed well and then incubated in the fridge (4 ^{o}C) for 3 hours. (Caution: for the unused PFA, pour it to the waste container labelled with "PFA" and the "toxic" sign.)

8. After 3 hours of incubation the tube was centrifuged for 10 minutes at 14000 rpm. The sample was washed with 1xPBS at least 3 times.

9. The cells are then resuspended with 1 ml 1xPBS and 1 ml of absolute ethanol added to the tube and mixed well.

10. The fixed sample can be stored for up to one year at -20 ^{o}C.

3.2.14.2 Hybridisation

Reagents and equipment

Equipment

- 1.5 ml plastic tubes, autoclaved
- 50 ml screw-cap tubes
- Disposal pipettes
- 0.2 μm filters and syringes
- Laboratory balance
- Gloves, dust mask
- Fridge (4 °C) and freezer (-20°C)
- Hybridization incubator (46°C)
- Microscope slides (Teflon-coated slides partitioned into 6-10 fields) and suitable cover slips

- Glass or plastic containers and a slide holder for immersing microscope slides
- Tissue paper
- Rack to hold 50 ml screw-cap tubes in a horizontal position
- Water bath (48°C)
- Tweezers
- Fume hood
- Oil-free compressed air pump
- Epifluorescence or confocal laser scanning microscope, filters and lasers that match the excitation and emission wavelengths of the fluorochromes
- Image analysis software capable to detect objects in images and to measure their area (in pixels) and brightness

Reagents

- 50%, 80%, and 96% (v/v) ethanol
- autoclaved deionized water (dH_2O)
- 5 M NaCl
- Tris buffer (1 M Tris/HCl, pH 8.0)
- Formamide (molecular biology grade)
- 10% (w/v) sodium dodecyl sulphate (SDS)
- 0.5 M ethylenediaminetetraacetate (EDTA), pH 8.0
- Hybridisation buffer [180 µl 5 M NaCl, 20 µl Tris buffer, formamide and dH_2O (both varied depending on stringency), 1 µl 10% SDS]
- Washing buffer [1 ml Tris buffer (8.88 g Tris/HCl+5.30 g TrisBase made up to a volume 100 ml), 5 M NaCl and 0.5 M EDTA, made up with with dH_2O to 50 ml]
- Fluorescently labelled rRNA-targeted oligonucleotide probes.
- Ice-cold dH_2O
- Antifadent (Citifluor AF1, Citifluor Ltd., London, UK)
- Hand-warm 0.5-1% (w/v) agarose
- Nail polish

Procedure

Dehydration of fixed samples

1. 5-30 µl of PFA fixed sample was applied to one well of a teflon coated microscope slide.

2. The slide was dried in the hybridization incubator at 46 °C.

3. The slide was then dipped for 3 min each into 50%, 80%, and 96% (v/v) ethanol, respectively (these were typically prepared the previous day as this gives better dehydration and less bubbles on the slides). The dehydrating effect of the ethanol concentration series disintegrated cytoplasmic membranes which thus became permeable to oligonucleotide probes.

4. The slides were dried for a couple of minutes at 46 °C.

5. An agrose solution at the hand warm temperature was used to coat the dry slides and then allowed to set in the fume hood.

In situ hybridisation

1. 1 ml of fresh hybridisation buffer was prepared to give 20% hybridisation stringency all over the experiment.

2. The oligonucleotide probe solutions were thawed and kept on ice and protected from light. Note: small volumes of oligonucleotide probe solutions were thawed because fluorescently labelled oligonucleotides were damaged if they were repeatedly frozen and thawed. The oligonucleotide probes used in this experiment were showed in Table 3.1.

Table 3.1 Oligonucleotide probes used in FISH

Probe name	Target group	Probe sequence (5'–3')	Fluoro-chrome
EUB338	Bacteria (most)	GCTGCCTCCCGTAGGAGT	Cy5
EUB338+	Bacteria (remaining)	GCWGCCACCCGTAGGTGT	Cy5
ARC915	Archaea	GTGCTCCCCCGCCAATTCCT	6-Fam
MX825	*Methanosaetaceae*	TCGCACCGTGGCCGACACCTAGC	Cy3
MS1414	*Methanosarcinaceae*	CTCACCCATACCTCACTCGGG	Cy3
hMS1395	MS1414-helper	GGTTTGACGGGCGGTGTG	–
hMS1480	MS1414-helper	CGACTTAACCCCCCTTGC	–
MG1200	*Methanomicrobiales*	CGGATAATTCGGGGCATGCTG	Cy3
MB1174	*Methanobacteriales*	TACCGTCGTCCACTCCTTCCTC	Cy3
MC1109	*Methanococcales*	GCAACATAGGGCACGGGTCT	Cy3

Source: Banks et al. (2012)

3. 1 µl of each probe was added to 10 µl of hybridisation buffer, mixed well and applied to the dehydrated sample on a microscope slide (or onto the fields of a Teflon-coated slide which contain a sample).

4. A piece of tissue paper was placed into a 50 ml screw-top plastic tube and the remaining hybridisation buffer was poured onto this tissue paper.

5. The slide was then immediately placed horizontally into the tube and the tube closed. The tube was then place in a horizontal position and incubated at 46°C for 3 hours. The tightly sealed plastic tube functioned as a moisture chamber preventing the evaporation of hybridisation solution from the slide. In particular, the evaporation of formamide could cause non-specific probe binding to non-target cells.

6. 50 ml of washing buffer was prepared in a 50 ml tube and preheated to 48°C in a water bath. Ice-cold dH_2O was also prepared.

7. The slide when removed from the incubator was immediately washed to remove the hybridisation buffer using a small volume of pre-warmed washing buffer. The slide was then transferred into the remaining washing buffer. This should be done quickly to minimise cooling of the hybridisation buffer on the slide.

8. The tube containing the washing buffer and the slide were returned to the water bath and incubate for a further 10-15 min at 48 °C.

9. The slide was then removed from the tube and dipped for 2-3 seconds into ice-cold dH_2O in order to remove residual washing buffer from the slide. This was necessary because fluorescent salt crystals hampered the microscopic observation of FISH results. However, the low ionic strength of dH_2O destabilises nucleic acid duplexes and therefore the water must be cold in order to minimise dissociation of probes from the ribosomes of their target organisms.

10. The slide was then air dried as quickly as possible. Fast drying also reduces probe dissociation.

11. Dried slides could then be stored in the dark at -20°C for several weeks without significant loss of probe-conferred fluorescence signal.

3.2.14.3 Microscopy

1. Two drops of antifadent were placed on the slide close to the left and right ends (frozen slides should be warmed and dried to room temperature prior to this step).

2. A microscope cover slip was placed on the slide which allowed the antifadent to spread over the whole slide. Caution: too much antifadent can blur the microscope image.

3. The slide was sealed by applying nail varnish to the edge of the cover slip to 'glue' it to the slide.

4. The sample was observed under an epifluorescence microscope (Nikon Eclipse E400, Japan) or confocal laser scanning microscope (Leica TCS SP2, Germany) equipped with suitable filters or lasers, respectively.

5. Slides embedded in antifadent could be stored at 4 °C (without freezing) for several days before the probe-conferred fluorescence began to decline. Alternatively, the antifadent could be removed with dH_2O and the dried slides could then be stored at -20°C for a longer period.

3.2.15 Carbon-14 Testing

Carbon-14 testing was carried out to determine the metabolic pathway for methane production. Tests were run in duplicate. Each sample was mixed with anaerobic medium in the ratio of 1:2. 2.5 KBq of $14CH_3COONa$ was added into 45 ml of the sample/medium mixture and incubated in 119 ml crimp top serum bottles under 37 ^oC for 24 hours. The CO_2 and CH_4 produced in the headspace were separately collected in alkali traps containing 20 ml 1 M NaOH solution. After absorption, 1 ml NaOH solution in each alkali trap and 1 ml of the sample/medium mixture (after centrifugation) were added into 15 ml Gold Star multi-purpose liquid scintillation cocktail (Meridian

Biotechnologies Ltd, Surry, UK) and counted in a Beckman Coulter LS6500 scintillation counter (Beckman Coulter, Inc., UK).

3.3 Food waste

During the course of the research food waste was obtained from 2 sources and a synthetic food waste was also prepared.

3.3.1 Collected food waste

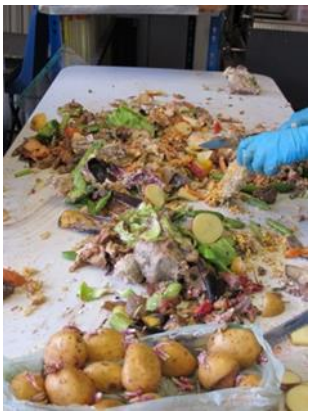


Figure 3.1 Preparing food waste before grinding

The first batch of food waste was collected from the Biocycle plant in Ludlow (Biocycle Ltd, Ludlow, Shropshire). The second came from a source segregated food waste collection round at Eastleigh, Hampshire, and was picked up at Otterbourne waste transfer station (Veolia Environmental, Hampshire). The food waste was manually separated (Figure 3.1) to take out unwanted and undegradable pieces such as. plastic bags, bones, garden rubbish, paper, seeds, as seen in Figure 3.2. The waste was then macerated using a food waste disposal unit (S52/010 Waste Disposer, Imperial Machine Company (IMC) Limited, Hertfordshire, UK), mixed thoroughly and packed into 4-litre plastic storage containers, and frozen at -20 ^{o}C. The same batch of food waste was used to feed both mesophilic and thermophilic digesters

a) Bones

b) Plastic bags

c) Garden refuse

e) Paper

f) Plastic

g) Seeds

Figure 3.2 Contaminants found in food waste

(Image courtesy of Dr. Sonia Heaven)

3.3.2 Synthetic low nitrogen food waste

This was mainly composed of vegetables and fruits but with some inclusion of meat, milk and bread to provide a nominal amount of nitrogen in the feedstock. The fresh ingredients were cut, mixed and then ground twice with a macerating grinder (S52/010 Waste Disposer, IMC Limited, UK), and then packed into 4-litre plastic storage containers. The weight of each box was recorded and kept in a cold room (-20 ^{o}C). Before using the feedstock was thawed and mixed with cellulose (α-cellulose, product no. C8002-5KG, CAS. No. 9004346, Sigma-Aldrich) to increase the TS/VS to a concentration similar to that of collected food waste.

The composition is shown in Table 3.2.

Table 3.2 Ingredients used in the preparation of synthetic low nitrogen food waste

Item	Contribution to the substrate		Weight (kg)
	TS (%)	N (mg kg^{-1})	
Potatoes	2.6	400	2.49
Apples	3.8	109	8.01
White sliced bread	3.3	637	1.62
Barbecue mix (sausages, burgers, chicken drumsticks	0.3	162	0.16
Lettuce	0.2	75	0.80
Bananas	1.3	91	1.76
Oranges	1.4	156	4.13
Milk	0.0	14	0.08
Courgette	0.2	54	3.14
Onion	0.6	92	2.06
Pear	0.8	32	2.02
Carrots	0.6	78	2.09
Celery	0.2	58	1.62
Eggs	0.1	52	0.08
Cabbage	0.2	75	1.80
Total	15.6	2085	31.85

3.3.3 Trace element mix

The trace element mix used in the initial thermophilic digestion trial contained (g l^{-1}): Aluminium (Al) 0.1, Boron (B) 0.1, Cobalt (Co) 1.0, Copper (Cu) 0.1, Iron (Fe) 10.0, Manganese (Mn) 1.0, Nickel (Ni) 1.0, Zinc (Zn) 1.0, Molybdenum (Mo) 0.1, Selenium (Se) 0.1, Tungsten (W) 0.1. Cation and anion mixes were prepared separately to prevent precipitation, as detailed in Table 3.3.

Table 3.3 Elements used in preparation of trace element mixes for the initial trial of the thermophilic anaerobic digestion

Trace element as cation	Compound used	Element concentration in the working condition (after diluted by 1000 times), mg l^{-1}	Compound concentration in stock solution, g l^{-1}
Aluminium (Al)	$AlCl_3 \cdot 6H_2O$	0.1	0.895
Boron (B)	H_3BO_3	0.1	0.572
Cobalt (Co)	$CoCl_2 \cdot 6H_2O$	1	4.038
Copper (Cu)	$CuCl_2 \cdot 2H_2O$	0.1	0.268
Iron (Fe)	$FeCl_2 \cdot 4H_2O$	10	35.597
Manganese (Mn)	$MnCl_2 \cdot 4H_2O$	1	3.602
Nickel (Ni)	$NiCl_2 \cdot 6H_2O$	1	4.050
Zinc (Zn)	$ZnCl_2$	1	2.084
Trace element as oxyanion	Compound used	Element concentration in the working condition (after diluted by 1000 times), mg l^{-1}	Compound concentration in stock solution, g l^{-1}
Molybdenum (Mo)	$(NH_4)_6Mo7O_{24}\ 4H_2O$	0.1	0.184
Selenium (Se)	Na_2SeO_3	0.1	0.219
Tungsten (W)	$Na_2WO_4 \cdot 2H_2O$	0.1	0.179

The trace element mix used in all subsequent mesophilic and thermophilic anaerobic digestion trials was prepared as shown in Table 3.4. This revised formula was based on Banks et al. (2012) who studied the amount of TE required in anaerobic digestion of high nitrogen food waste, making it more suitable for this experiment.

Table 3.4 Elements used in preparation of trace element mixes for the trial of mesophilic and thermophilic anaerobic digestions

Element	mg l^{-1}after dilution to 1000 ml	g l^{-1}in stock solution	Atomic weight (g)	Molecular Weight (g $mole^{-1}$)	final volume (ml)	final chemical weight (g)
Co^{2+} ($CoCl_2.6H_2O$),	1	10	58.933	237.93	100	4.037
Ni^{2+} ($NiCl_2.6H_2O$),	1	10	58.693	237.69	100	4.050
Se^{6+} (Na_2SeO_3)	0.2	2	78.96	172.94	100	0.438
Mo^{6+} $(NH_4)6Mo_7O_4.4H_2O$)	0.2	2	95.942	1235.87	100	0.368
W^{6+} ($Na_2WO_4.2H_2O$).	0.2	2	183.84	329.86	100	0.359

3.3.4 Anaerobic digesters

3.3.4.1 Digester construction

The eight digesters used had a 5-litre capacity with a 4-litre working volume and were constructed of PVC tube with gas-tight top and bottom plates (Figure 3.3). The top plate was fitted with a gas outlet, a feed port sealed with a rubber bung, and a draught tube liquid seal through which an asymmetric bar stirrer was inserted with a 40 rpm motor mounted directly on the top plate. Temperature was controlled at either 37 or 55 ^oC by circulating water from a thermostatically-controlled bath through a heating coil around the digesters. Semi-continuous operation was achieved by removing digestate through an outlet port in the base plate before adding feed via the hole in the top plate.

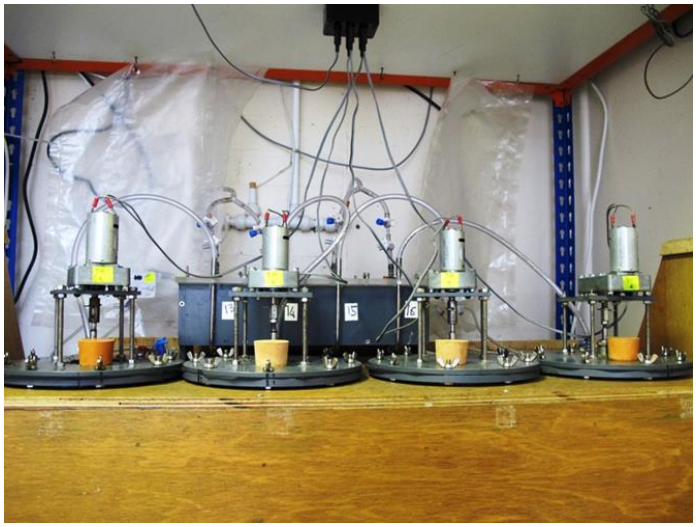


Figure 3.3 CSTR reactors used in experiment

3.3.4.2 Digester operation and monitoring.

The digesters were inoculated using digestate taken from a mesophilic anaerobic digester treating wastewater biosolids at Millbrook Wastewater Treatment plant, Southampton (Southern Water). The digesters were fed daily and digestate was removed weekly to maintain an approximately constant working volume. All digestate removed was weighed. All weights of food added to the digesters were recorded together with an addition such as trace elements (TE). Digestate TS and VS, VFA, pH, ammonia, and alkalinity were analysed at least weekly and more frequently in most cases. Samples for FISH analysis were taken weekly. Since FISH observation depends to a degree on evaluation by the observer, for quality assurance purposes a second independent observer (Dr Louise Byfield) carried out a check on the same samples.

Biogas production was recorded using tipping-bucket gas counters with continuous datalogging (Walker et al., 2009). Gas composition and gas counter calibration were carried out weekly by collecting the gas that had passed through the gas counter and measuring its volume. The biogas volume was measured using a weight-type water displacement gasometer (Walker et al., 2009). The measurement procedure included: the initial height of solution in the gasometer (h_1) was recorded before the gas collected with the gas bag was introduced into the column through the top valve. After the bag was empty, the final height (h_2) and the weight of water (m) were recorded, as well as the temperature (T) and pressure (P) in the room, and the measurement time. All gas

volumes reported are corrected to standard temperature and pressure of 0 ^{o}C, 101.325 kPa.

3.4 Biochemical Methane Potential (BMP) Test

The BMP test in this work was performed in 1.5-litre stirred digesters placed in a temperature controlled water bath at 35 ^{o}C (Figure 3.4). The inoculum was taken from Millbrook wastewater treatment plant, Southampton and was sieved before use to remove large particles such as grit and hair. The inoculum-to-substrate (I/S) ratio used was 5:1 on a VS basis and the test was run over a period of 28 days. No supplements were added, as the inoculum used was known to be sufficiently rich in the required nutrients. Biogas was collected in glass cylinders by displacement of a 75% saturated sodium chloride solution acidified to pH 2, in order to reduced losses of methane by dissolution. The height of the solution in the collection cylinder was recorded manually. Vapour pressure and salt solution density were taken into account in correction of gas volumes to a standard temperature and pressure (STP) of 0 ^{o}C and 101.325 kPa (Walker et al., 2009).Samples for gas composition analysis were taken from the cylinders each time they were refilled, at intervals of no more than 7 days to avoid the risk of overfilling or losses of methane. Samples were run alongside blanks (inoculums only) and positive controls (cellulose powder from Sigma-Aldrich, Dorset-UK), all in triplicate. The water level was recorded, also time, ambient temperature and pressure.

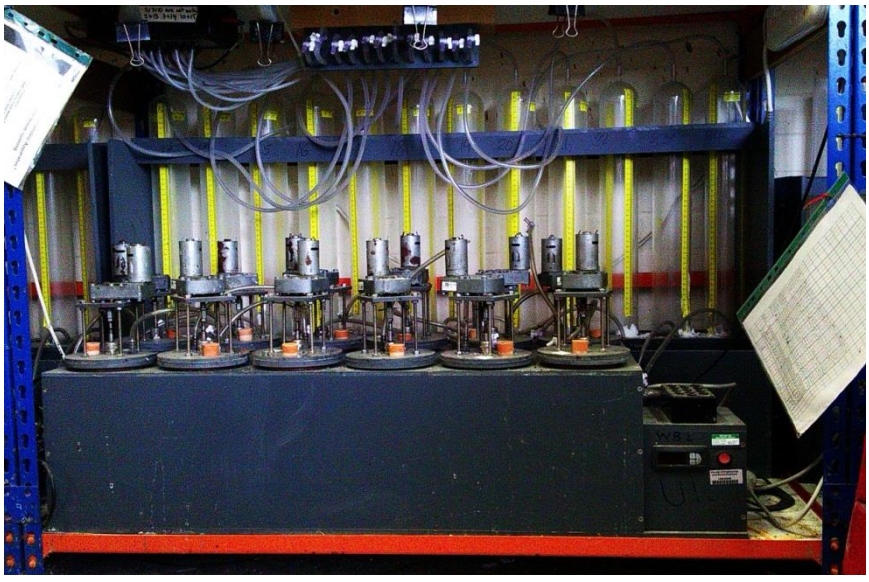


Figure 3.4 The BMP stirred reactors

Chapter 4 Digestion studies

This chapter reports on the results of laboratory-scale thermophilic and mesophilic digestion trials to investigate the effects of operating temperature, nitrogen content and trace element supplementation on operational performance and stability.

4.1 Initial Trial of Thermophilic Digestion with and without TE Supplementation

4.1.1 Aim

The main purpose of this experiment was to determine whether a trace element (TE) supplementation strategy that had proved effective in mesophilic digestion of food waste would allow stable digestion of the same feedstock in thermophilic conditions.

4.1.2 Method

Eight digesters were seeded with mesophilic digestate from Millbrook Wastewater Treatment Plant, Southampton, UK and acclimatised to 55 ^{o}C by raising the temperature in a single step followed by no feeding for 14 days. The digesters were then fed at an incremental loading starting at 0.01 g VS l^{-1} day^{-1} and increasing to 0.23 g VS l^{-1} day^{-1} over 20 days. On day -1 the contents of all 8 digesters were mixed and redistributed to ensure a common start point. Feeding started on day 0 at an organic loading rate (OLR) of 1 g VS l^{-1} day^{-1} which was gradually increased to 2 g VS l^{-1} day^{-1} by day 32. In two pairs of digesters the OLR was then gradually increased to 3 (by day 82) and 4 g VS l^{-1} day^{-1} (by day 103). The remaining two pairs continued at an OLR of 2 g VS l^{-1} day^{-1}. All of the digesters apart from one pair at 2 g VS l^{-1} day^{-1} were supplemented with TE stock solution as described in section 3.3. TE supplemented digesters received an initial addition of 1 ml l^{-1}, then a weekly addition of 1 ml of this solution for every litre of digestate removed to maintain the TE concentration. The operating conditions are summarised in Table 4.1. Frequency of sampling and analysis are shown in Table 4.2. The experiment ran from May 2010 to January 2011.

Table 4.1 Digester operating conditions

Reactor	Unit	TE-2-A & TE-2-B	TE-3-A & TE-3-B	TE-4-A & TE-4-B	noTE-2-A & noTE-2-B
OLR	g VS l^{-1} day^{-1}	2	3	4	2 (no TE)
Food waste VS	g VS kg^{-1} wet weight	230	230	230	230
Feeding rate	g WW day^{-1}	34.8	52.2	69.5	34.8
HRT*	days	115	77	58	115

* For calculation of HRT it is assumed that 1 litre of digestate = 1 kg

Note: Digester names indicate the operational conditions: for example TE-2-A means the digester with OLR of 2 g VS l^{-1} day^{-1} and supplemented with TE. A and B represent the duplicate reactors A and B, respectively.

Table 4.2 Frequency of analysis in the initial trial of thermophilic anaerobic digestion

Parameter	Frequency
pH, ammonia, alkalinity, TS, VS	Weekly
VFA	every 2 days
Gas bag calibration	Twice a week
Gas composition	Twice a week

4.1.3 Food waste characteristics

The characteristics of the batch of food waste used throughout this trial are shown in Table 4.3. The TKN of this food waste was similar to values reported in the literature (VALORGAS, 2011). It also quite similar to the nitrogen content (presented in Table 4.4) for the batch used in the experiment described in section 4.2.

Table 4.3 Food waste characteristics

Basic characteristics for anaerobic digestion	Value
TS (% fresh matter)	24.8±0.19
VS (% fresh matter)	23.6±0.22
COD (g kg^{-1} TS)	998±71
TKN (g kg^{-1} TS)	30.0±0.1
Essential trace elements (mg kg^{-1} TS)[a]	
Cobalt (Co)	0.10
Iron (Fe)	88.97
Manganese (Mn)	92.08
Molybdenum (Mo)	0.37
Selenium (Se)	0.17
Elemental composition (%TS)	
N	3.00±0.01
C	52.32±1.13
H	6.89±0.16

Note: [a] Results for essential trace elements are reported as single value from an external laboratory (Severn Trent Services Analytical, UK).

4.1.4 Initial acclimatisation

Figure 4.1 shows the increase in OLR applied to the digesters from day 1-50. When feeding began on day 1 at an OLR of 1 g VS l^{-1} day^{-1}, all of the digesters receiving TE supplementation responded in a similar way. There was a small but distinct peak in acetic acid of 600-900 mg l^{-1} in the first 2-3 days which declined by day 10 (Figure 4.2). This was followed by a second peak in propionic acid of ~1000 mg l^{-1} which in turn declined to give a total VFA concentration ≤ 500 mg l^{-1} by day 35. By this time the OLR had reached 2 g VS l^{-1} day^{-1}, with a specific methane production of around 0.42 l CH_4 g^{-1} VS day^{-1} in all digesters. Only one digester (TE-2-A) showed slightly different behaviour in terms of its VFA profile (Figure 4.2a), with a higher propionic acid peak of around 1600 mg l^{-1} which by day 38 had only fallen to around 400 mg l^{-1}, after which the concentration began to rise again.

It is also worth noting that during this period the pH in all digesters fell from ~8.3 to ~7.8 by day 17 (Figure 4.3a), and then recovered, eventually reaching ~8.5. The total ammonia nitrogen (TAN) concentration also fell slightly from an initial value of ~1.8 g N l^{-1} to 1.5 g N l^{-1} by day 17 (Figure 4.3b), then began to rise. This change may reflect a period of rapid growth in the microbial population, during which the rate of nitrogen uptake into cell biomass temporarily exceeds the rate of breakdown of organic nitrogen in the feedstock to ammonia. The increase in TAN and fall in VFA concentration were reflected in the IA/PA ratio which fell from ~0.9 to ~0.5 (Figure 4.3c). By day 40 these digesters, with the possible exception of TE-2-A, were considered to be in a stable state at the base OLR of 2 g VS l^{-1} day^{-1} and to have successfully acclimated to thermophilic conditions with a specific methane production of around 0.40 l CH_4 g^{-1} VS day^{-1} (Figure 4.3d).

In the two digesters without TE addition the pattern was slightly different, as the early increase in propionic acid did not return to a baseline value. This was especially pronounced in one of the pair of digesters, which showed no fall in VFA concentration (Figure 4.2g and h); in the second digester of the pair there was a reduction in propionic acid to ~800 mg l^{-1} between days 30-40, after which the increase in concentration resumed. In all other respects, including specific methane production, acclimatisation of these digesters also appeared to be successful (Figure 4.3).

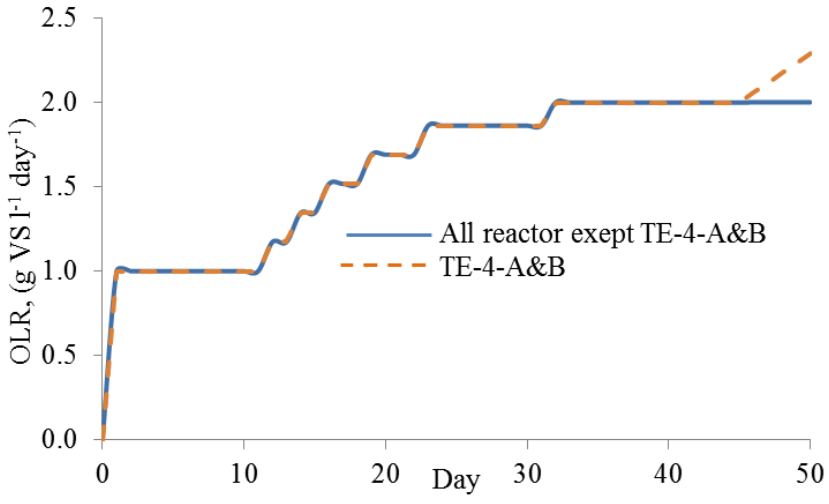


Figure 4.1 Increase in OLR for all digesters from day 1-50

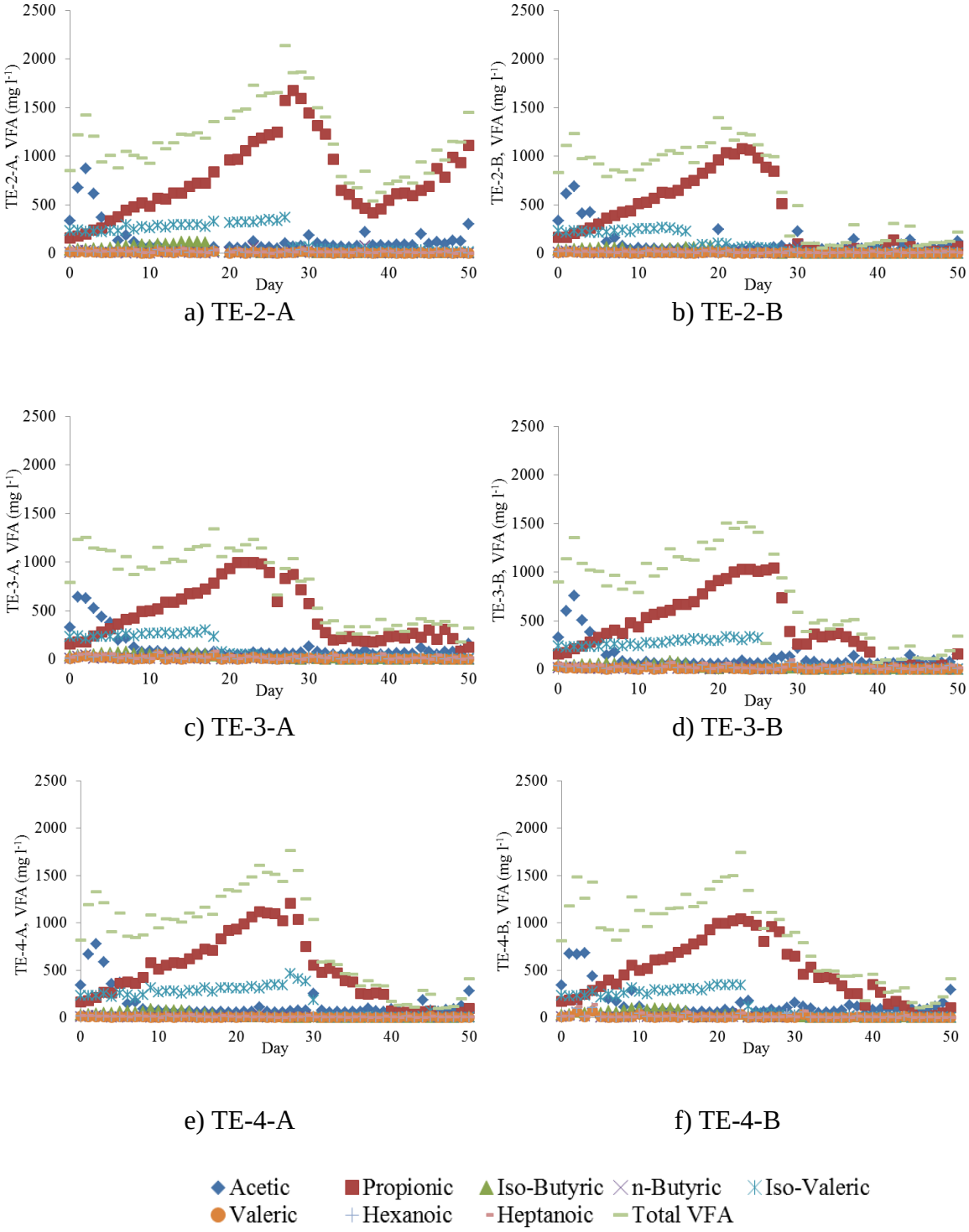
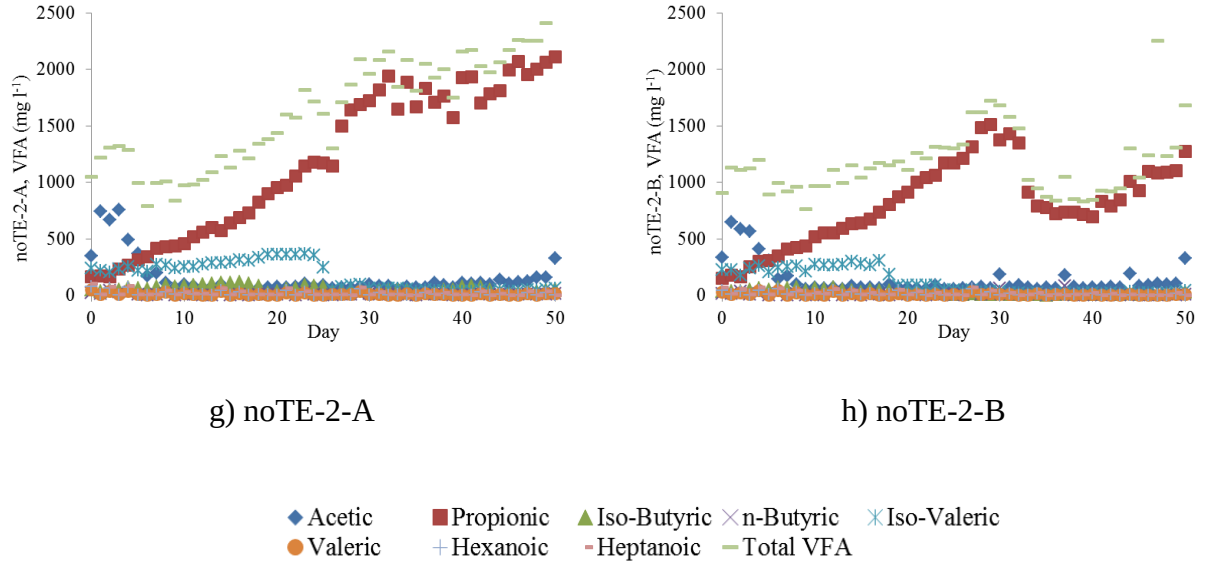
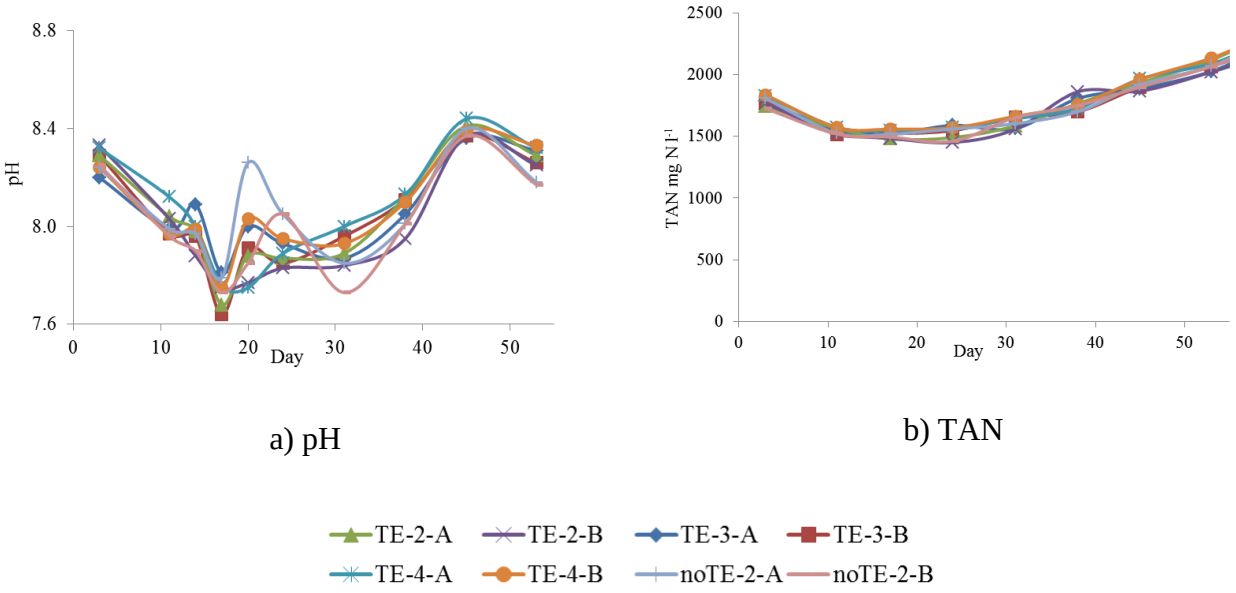


Figure 4.2 VFA profiles in TE-2-A&B, TE-3-A&B, TE-4-A&B (with TE supplement) and noTE-2-A&B (without TE supplement) during increase in OLR from day 1-50



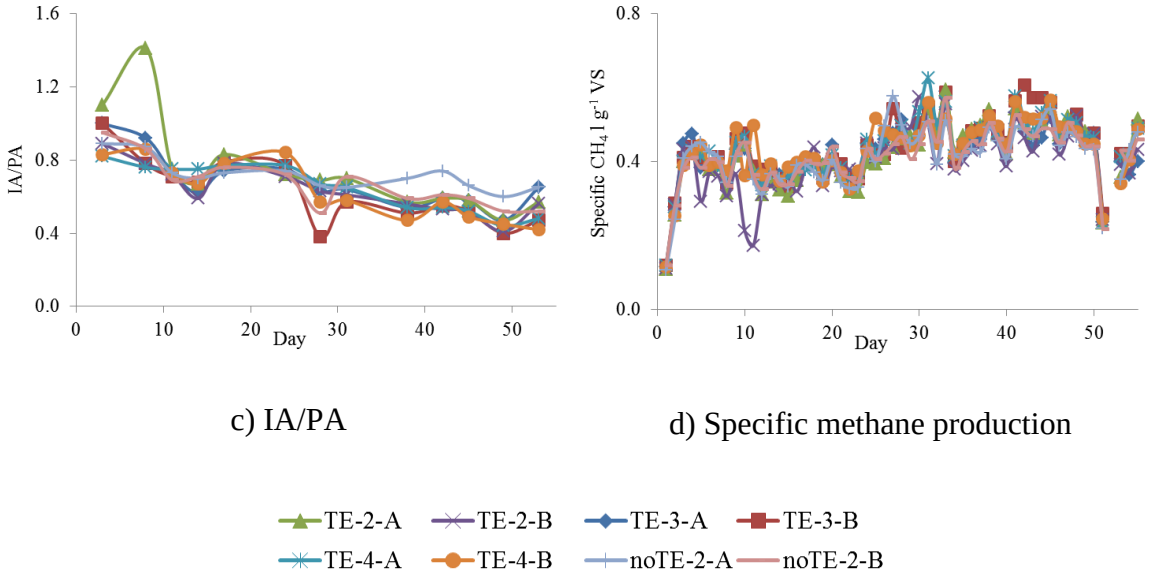
g) noTE-2-A h) noTE-2-B

Figure 4.2 VFA profiles in TE-2-A&B, TE-3-A&B, TE-4-A&B (with TE supplement) and noTE-2-A&B (without TE supplement) during increase in OLR from day 1-50 (continued)



a) pH b) TAN

Figure 4.3 pH, TAN, IA/PA ratio and specific methane production in all reactors between day 1-55



c) IA/PA

d) Specific methane production

Figure 4.3 pH, TAN, IA/PA ratio and specific methane production in all reactors between day 1-55 (continued)

The rate of increase in propionic acid from day 1-20 was very similar in all digesters at around 36 mg l^{-1} day^{-1}, with the TE supplemented digesters distinguished from the unsupplemented ones only by the marked fall in concentration after day ~25 (Figure 4.4). Iso-valeric acid concentrations, initially around 220 mg l^{-1}, fell sharply after day 20-30 to <10 mg l^{-1} in all digesters (Figure 4.2).

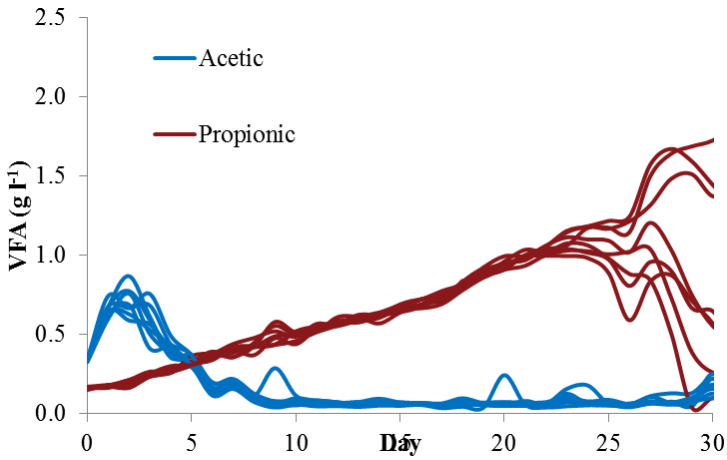


Figure 4.4 Acetic and propionic acid concentrations in all digesters with and without TE addition from day 0-30

The above results indicated that trace element supplementation had a beneficial effect during the start-up phase of digestion.

4.1.5 Main experimental period

In the following period TAN concentrations in all digesters increased from the initial value of ~1.8 g N l^{-1} to between 5-6 g N l^{-1} (Figure 4.5a), as expected due to the high nitrogen content of the incoming feedstock. Changes in alkalinity reflected this, with TA increasing from ~8 g $CaCO_3$ l^{-1} to ~23.5 and ~19.7 g $CaCO_3$ l^{-1} in digesters with and without TE supplementation, respectively (Figure 4.5b). The VS concentration rose fairly steadily from 2.6 to 3.8 g VS l^{-1} between day ~40-90 and VS content stabilised at around 75% of TS (Figure 4.5c and d) in all digesters.

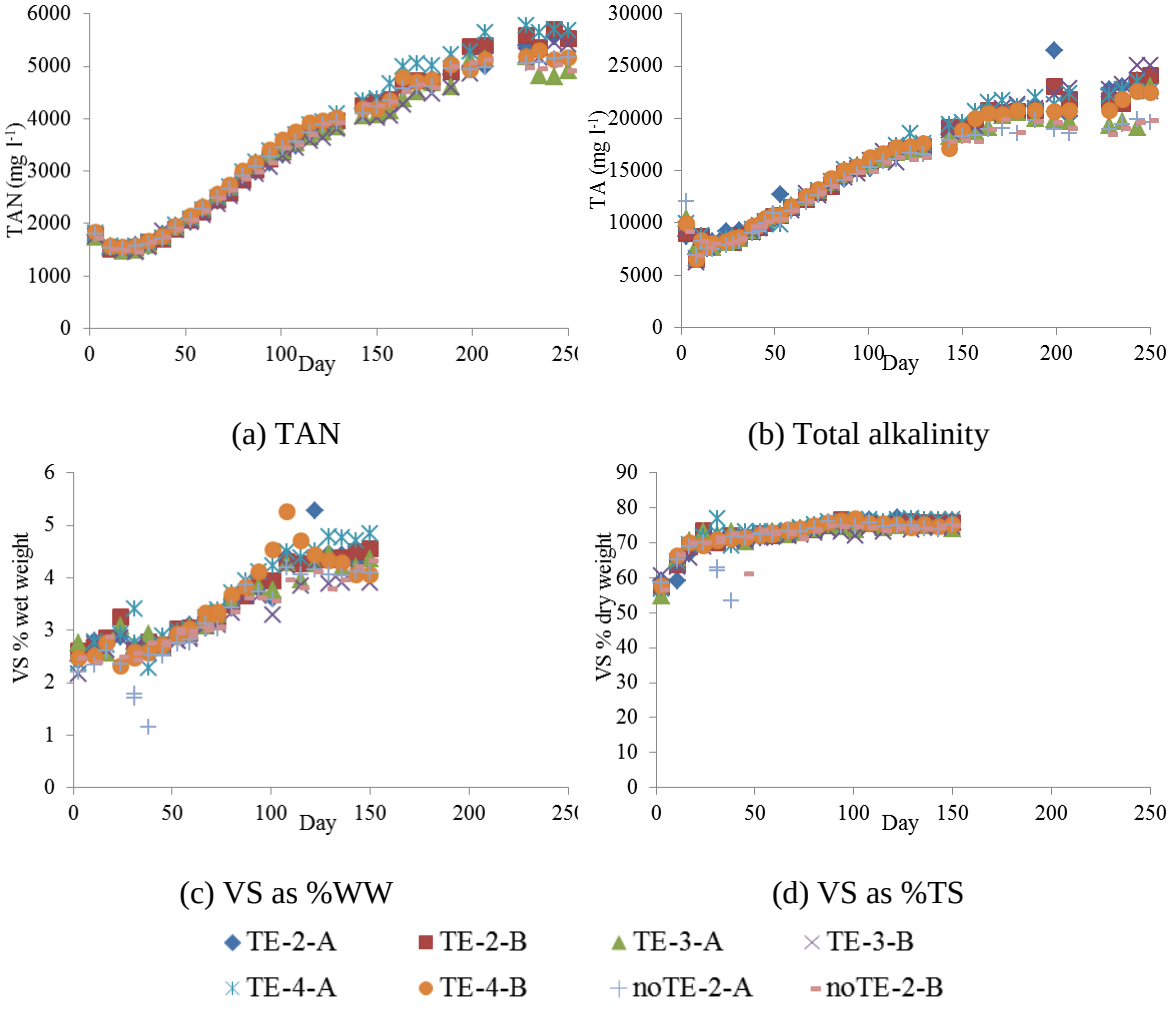
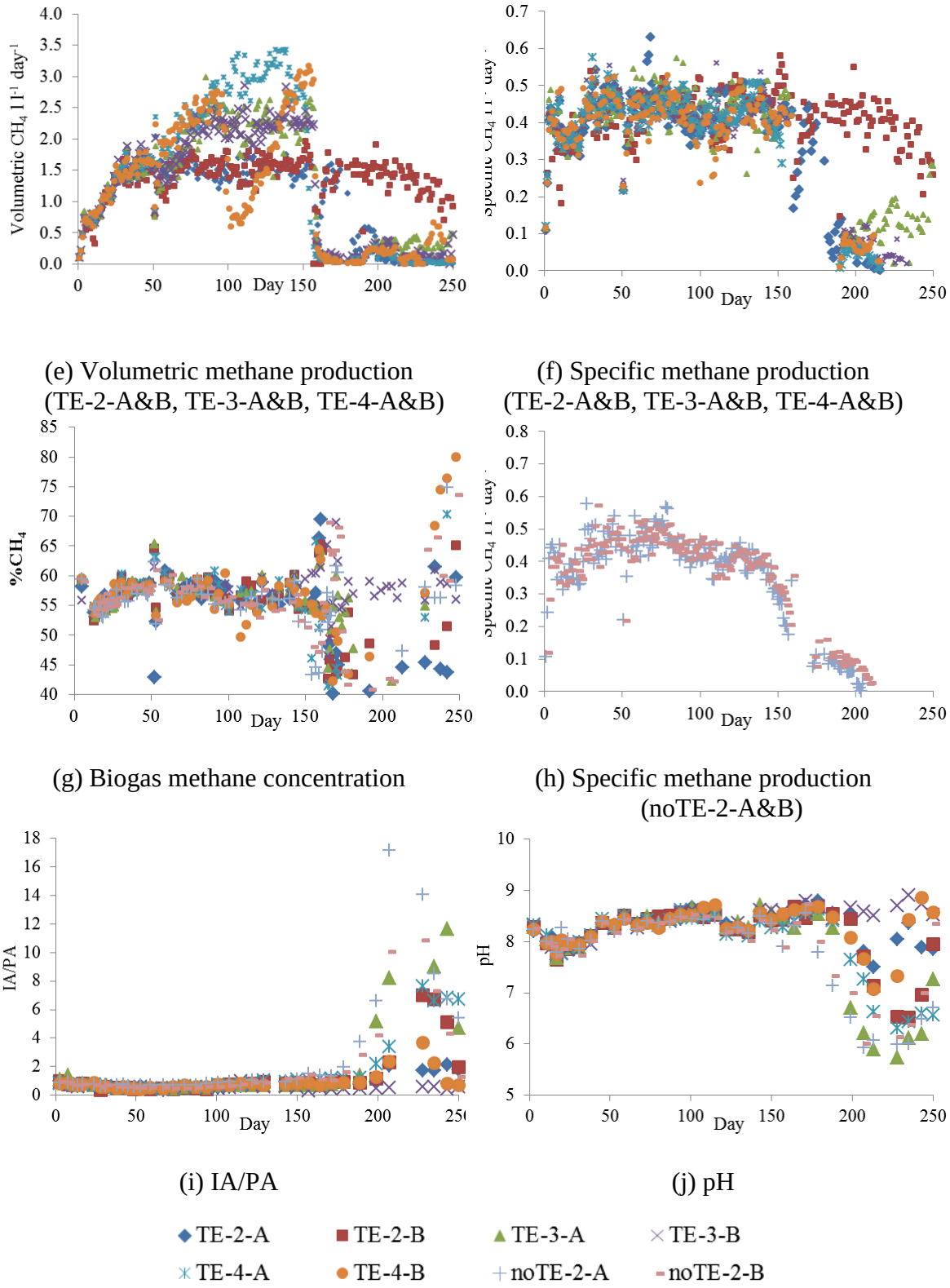


Figure 4.5 Monitoring parameters in all digesters from day 0-250



(e) Volumetric methane production (TE-2-A&B, TE-3-A&B, TE-4-A&B)

(f) Specific methane production (TE-2-A&B, TE-3-A&B, TE-4-A&B)

(g) Biogas methane concentration

(h) Specific methane production (noTE-2-A&B)

(i) IA/PA

(j) pH

Figure 4.5 Monitoring parameters in all digesters from day 0-250 (continued)

Note: data shown is for all digesters unless otherwise stated

4.1.5.1 TE supplemented digesters

Up until day 158, when there was a change in feeding regime, all of the TE supplemented digesters continued to operate well, with volumetric biogas production increasing in proportion to sequential load increases and specific methane production remaining fairly constant at around 0.45 l CH_4 g^{-1} VS day^{-1} (Figure 4.5e and f).

VFA profiles for each digester during the whole experimental run are shown in Figure 4.6. As can be seen, there was some variability between paired digesters as well as between those at different loadings: this often occurs in stressed biological systems, where many different factors can affect the exact onset and mode of failure, and may be regarded as a form of the Anna Karenina principle (Moore, 2001). In all of the TE supplemented digesters, however, there was a rise in total VFA to above 2000 mg l^{-1} from around day 70, with increasing concentrations of both acetic and propionic acid. At this point the TAN (Figure 4.5a) in the digesters had reached 2500 mg N l^{-1}. By day 150 the digesters at OLR 3 and 4 g VS l^{-1} day^{-1} (TE-3-A&B and TE-4-A&B, respectively) had TAN concentrations of around 4.2 g N l^{-1} and an accumulated total VFA of 15-20 g l^{-1}, with propionic acid accounting for about 10 g l^{-1} of this (Figure 4.6a-d) . It was clear from this that the TE supplementation strategy adopted had not been successful in preventing VFA accumulation in thermophilic conditions.

Earlier studies by Banks et al. (2008) and Neiva Correia et al. (2008) had shown that thermophilic food waste digesters could operate for long periods at even higher VFA concentrations, but may be liable to sudden failure if a change in conditions results in the buffering capacity of the system being exceeded, with a subsequent fall in pH, leading to cessation of methanogenesis and 'souring' of the digester. At day 158 therefore, feeding of all digesters was stopped to see whether they were able to reduce the total VFA concentration and how they would respond to a cessation of loading. In response to this acetic acid concentrations fell, but there was no reduction in VFA of higher carbon chain lengths, indicating some blockage in this metabolic route, and the total VFA concentration was largely unaffected (Figure 4.6a-d).

Feeding of these four digesters began again on day 190 at a reduced OLR of 1 g VS l^{-1} day^{-1} and immediately resulted in an accumulation of acetic acid, taking the total VFA

to 30-35 g l^{-1} by day 210. At the same time the IA/PA ratio rose sharply (Figure 4.5i) and in the following days the pH in three of the digesters fell to below ~7 (TE-3-B, TE-4-A&B, Figure 4.5j), and feeding of them was stopped. During the period of feeding there had been no significant resumption of volumetric gas productivity or specific methane yield (Figure 4.5e and f). After cessation of feeding the second time one of the digesters showed some reduction in acetic acid and total VFA (TE-4-B, Figure 4.5d), but concentrations in the others remained high (Figure 4.6b and c). These results may indicate inhibition of the acetoclastic methanogens by ammonia which was present in concentrations > 5000 mg N l^{-1}; and/or by VFA (Borja et al., 1996, Sung and Liu, 2003, Zhang et al., 2012a). The pH in the fourth digester remained at ~7.5 and feeding continued at 1 g VS l^{-1} day^{-1} which allowed a short term reduction in acetic acid concentration (TE-3-A, Figure 4.6a) and some recovery in gas production and methane concentration (Figure 4.5e, f and g).

Feeding of the digesters at OLR 2 g VS l^{-1} day^{-1} was also stopped on day 158 but was re-started after 3 days, and both digesters initially responded by resuming gas production as before. In the digester with the higher VFA accumulation (TE-2-A, Figure 4.6e) this was not sustained, with a rapid fall in gas volumes between days 179 to 182 (Figure 4.5e and f) accompanied by a sharp increase in acetic acid concentration and a step change in IA/PA ratio (Figure 4.5i). In the digester with the lower VFA accumulation (Figure 4.6e) gas production continued for a longer period, but began to decline from around day 220 (Figure 4.5e and f).

It is interesting to note that until day 158 these digesters were continuing to function well in terms of specific and volumetric gas production despite IA/PA ratios of around 1. This supports the observation by Ferrer et al. (2010), who suggested an IA/PA ratio of 0.9 as the upper limit for stability rather than the 0.3 originally proposed by Ripley et al. (1986). This ratio will depend on the buffering capacity in a digester, and it is likely that the high ammonia concentrations are themselves able to buffer a higher VFA concentration before the pH falls to a level at which methanogenesis is inhibited. It appears, however, that the digesters are in a meta-stable condition rather than in a fully stable operational state. Despite the slow accumulation of VFA, it is clear that there is a steady flow of carbon through to gaseous products, equivalent to a typical specific methane yield for the feedstock.

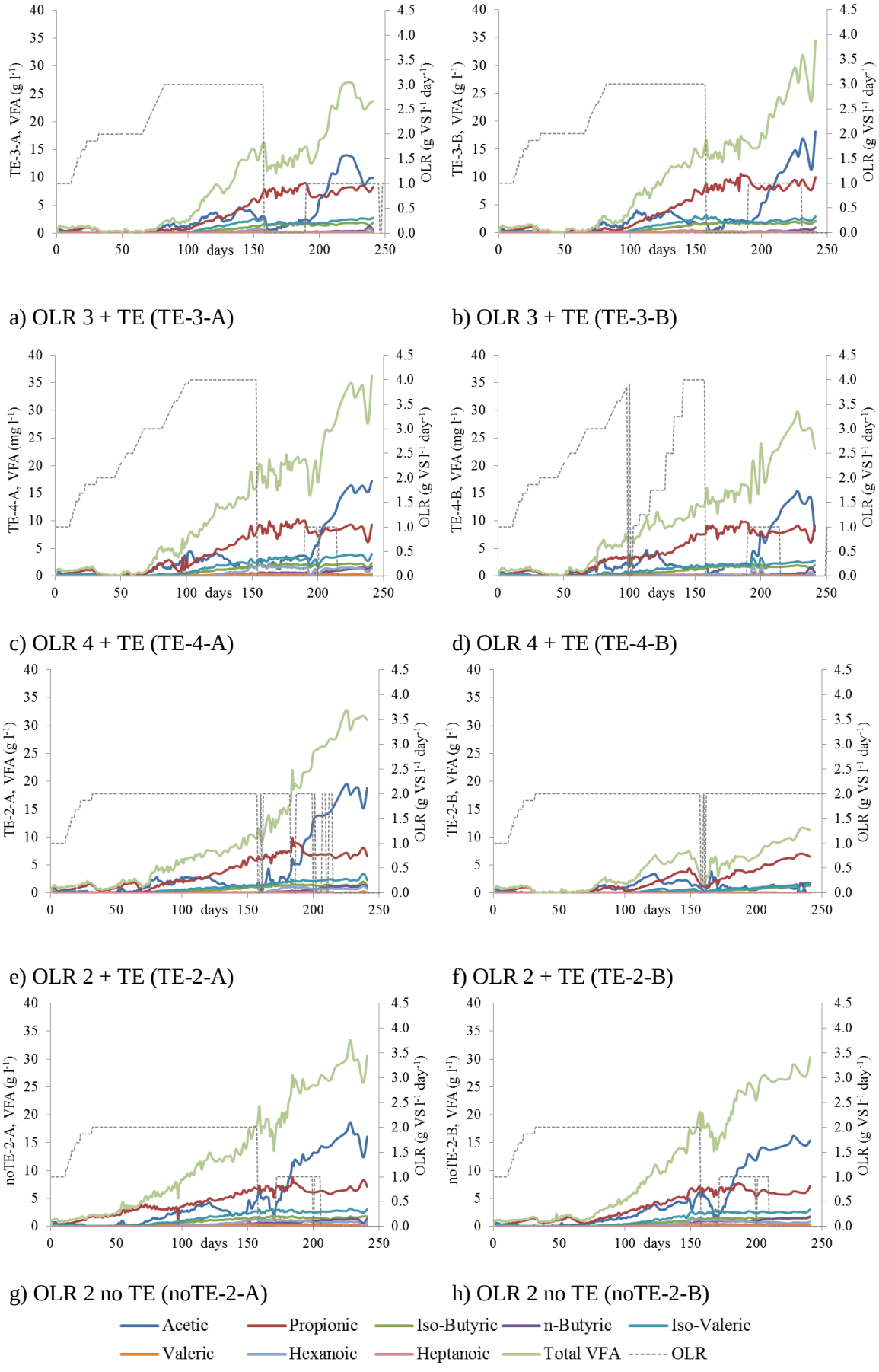


Figure 4.6 Digester OLR and VFA profile from day 0-250

4.1.5.2 Digesters without TE supplementation

In contrast to the TE supplemented digesters, specific methane production started to decrease from about day 70, and by day ~140 was falling rapidly (Figure 4.5f), with an IA/PA ratio above 1. Digester noTE-2-A showed an almost continuous rise in VFA concentration from the start of feeding, while digester noTE-2-B again showed an intermediate profile similar to that of the TE supplemented digesters (Figure 4.5h). In both cases, however, the rates of VFA accumulation of noTE-2-A and noTE-2-B (136.4 and 172.5 mg l^{-1} day^{-1}, respectively) were higher than in the TE supplemented digesters, TE-2-A and TE-2-B (90.5 and 86.7 mg l^{-1} day^{-1}, respectively) at the same OLR (Figure 4.7).

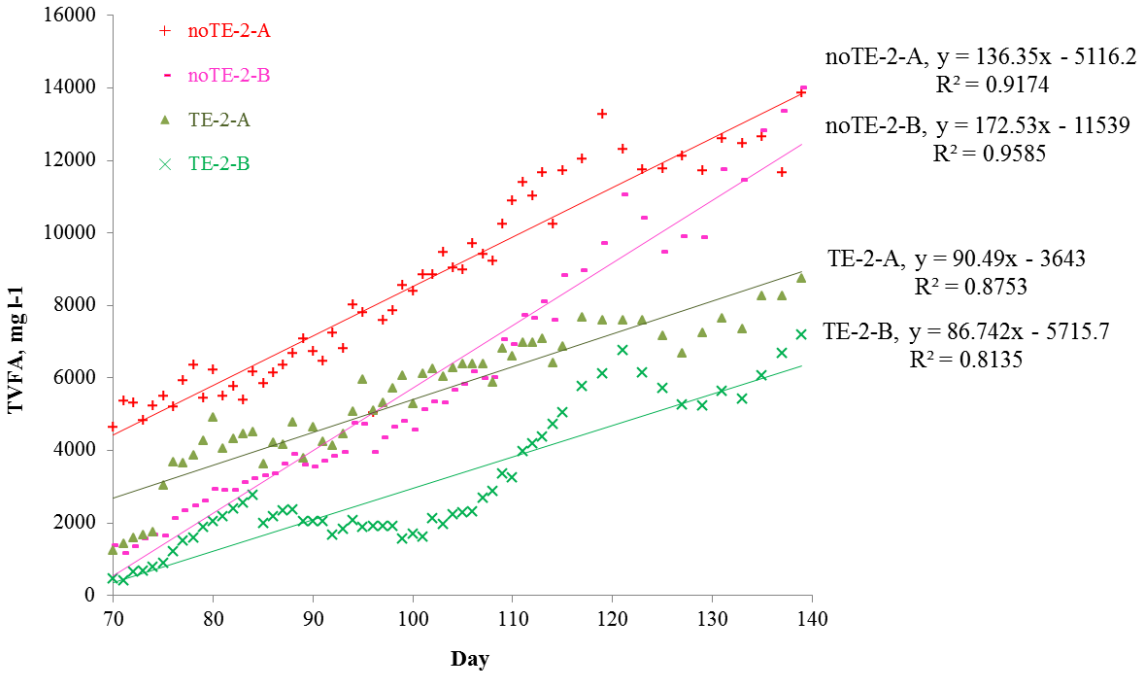


Figure 4.7 VFA concentrations in TE supplemented and unsupplemented digesters at OLR 2 g VS l^{-1} day^{-1} between day 70-140

On cessation of feeding on day 158 the acetic acid concentration in both reactors fell, causing a fall in total VFA. The response to resumption of feeding at a reduced OLR of 1 g VS l^{-1} day^{-1} was the same as in the TE supplemented digesters, however, with a sharp rise in acetic acid concentration (Figure 4.6g and h) and IA/PA ratio and a dramatic fall in pH (Figure 4.5i and j). After feeding stopped around day 210 no reduction in VFA concentrations occurred.

4.1.6 Discussion

It is clear from the above results that while the addition of TE did delay the onset of propionic acid accumulation, it was unable to prevent it at the trace element concentrations used. It is therefore unlikely that a population change occurs within the digester that allows the system to operate at elevated concentrations of ammonia, as appears to be the case in mesophilic systems. This is supported by the work of Uemura (2010) who found that mesophilic anaerobic digestion recovered from acidification after adding Ni, Co, and Fe of 0.04, 0.16, and 5.2 mg per litre organic solid waste; whereas this did not work with thermophilic anaerobic digestion. The bioavailability of the trace elements present was not checked in the current work, but this may be critical (Speece, 2008b), and may differ under mesophilic and thermophilic conditions. The detrimental effects of ammonia appeared to become evident at around 2500 mg N l^{-1} which is lower than the inhibitory concentration observed in mesophilic digesters operating on the same substrate (Banks et al., 2008). This threshold may also reflect the degree of acclimation of the inoculum to high ammonia concentrations: Hashimoto (1986) observed that ammonia inhibition began at about 2.5 g N l^{-1} and 4.0 g N l^{-1} for unacclimated and acclimated thermophilic methanogens, respectively. The results support previous findings that mesophilic and thermophilic digester populations behave in different ways under conditions of high ammonia concentration.

4.1.7 Conclusions from initial trial

Digestion of food waste results in high concentrations of ammonia that can lead to the accumulation of volatile fatty acids, but this problem can be resolved under mesophilic conditions by the addition of a specific combination of trace elements. In thermophilic digestion this did not appear to be the case, and apart from a slight delay in the onset of VFA accumulation little difference was observed between digesters receiving or not receiving TE supplementation. Good volumetric and specific gas production were observed for a period of ~70 days in non-supplemented and ≥ 158 days in TE supplemented digesters, despite the increasing VFA concentration: all of the digesters appeared to be working in a meta-stable state, however, and were sensitive to small changes in operational conditions (in this case an interruption in feeding), which led to a rapid change in the IA/PA ratio, pH reduction and cessation in gas production.

4.2 Effect of operating temperature and nitrogen content on AD of food waste

4.2.1 Aim

The aims of this work were firstly to confirm the issues associated with thermophilic digestion for food waste treatment and energy production; and secondly to determine the TAN concentration at which VFA accumulation occurred.

The following specific objectives were associated with these aims: i) to acclimate a low-nitrogen inoculum to thermophilic conditions; ii) to raise the organic loading on digesters fed with a high-nitrogen food waste (normal food waste) without dilution, with constant monitoring of stability and performance parameters; iii) to compare the performance of these digesters to that of mesophilic digesters fed on the same food waste, and to thermophilic digesters fed on a low-nitrogen food waste (low-N food waste) at the same organic loading.

4.2.2 Method

Eight digesters were inoculated with mesophilic digestate taken from a digester treating municipal wastewater biosolids (Millbrook WWTW, Southampton). An initial dose of 1 ml l^{-1} of the cation (cobalt and nickel) and oxyanion (molybdenum, tungsten, and selenium) stock solutions was added to the inoculum and mixed thoroughly, then 4 litres of the inoculum/TE mix were added to each digester. The digesters were acclimated to food waste in mesophilic (M1-M4) and thermophilic (T1-T4) conditions and operated for periods of up to 874 days. During operation all digesters were supplemented with the TE stock solutions at the rate of 1 ml kg^{-1} of feedstock added, unless otherwise noted.

Operation of the mesophilic digesters: Four digesters were started at an OLR of 2 g VS l^{-1} day^{-1} and then run for 97 days to ensure stable operation before increasing the OLR for M1 and M2. Two of the digesters (M3 and M4) continued at 2 g VS l^{-1} day^{-1} whilst the OLR to the other 2 digesters (M1&2) was increased to 3 g VS l^{-1} day^{-1}. This was achieved over a 3-week period by raising the loading by 0.05 g VS l^{-1} day^{-1} every two

days (Figure 4.8). On day 177, the OLR was increased to 3 and 4 g VS l^{-1} day^{-1} for M3&4 and M1&2 respectively. This OLR was maintained until the end of the experiment on day 636.

Operation of the thermophilic digesters: The digesters were acclimatised to thermophilic conditions (55 ^{o}C) by increasing the temperature in one step and then not feeding for 6 days: this slight modification to the protocol used in the previous trial was made in response to suggestions that the initial period at a low OLR might have led to poor adaptation of the consortium, and thus contributed to the failure of the digestion. Digesters T1 and T2 were fed with low nitrogen food (low-N food waste) waste whilst digesters T3 and T4 were fed with Eastleigh food waste (normal food waste). Feeding was started at an OLR of 0.5 g VS l^{-1} day^{-1} and this was increased slowly by 0.05 g VS l^{-1} day^{-1} until 2 g VS l^{-1} day^{-1} was reached after 33 days (Figure 4.8). All of the digesters were then run at OLR 2 g VS l^{-1} day^{-1} for the duration of the experiment, except when failure conditions were apparent, at which point feeding was stopped to allow recovery. The experimental period of T1&2 was done for 874 days whereas it was shorter for T3 (433 days) and T4 (309 days) due to failure. .

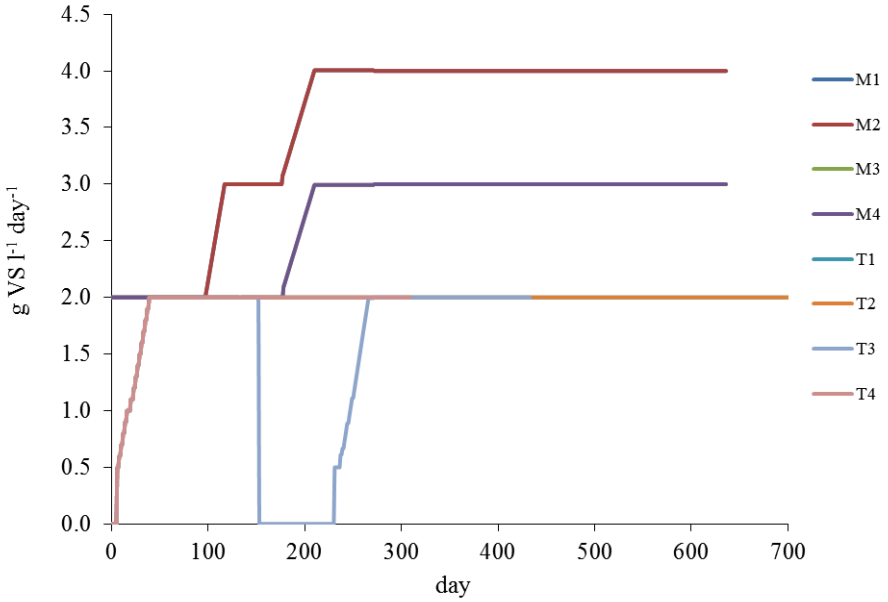


Figure 4.8 Feeding rate as OLR (g VS l^{-1} day^{-1})

4.2.3 Food waste and inoculum characteristics

Food waste. The characteristics of the two food wastes used in the experiments are shown in Table 4.4, and are similar to those previously reported for material from source separated domestic food waste collections (VALORGAS, 2011). It can be seen that the %VS (wet weight) for both types of food waste (normal food waste and low-N food waste mixed with alpha cellulose) was closely similar. After mixing with cellulose, however, the low -N food waste had half the nitrogen content of the normal food waste.

Table 4.4 Characteristics of food waste

Parameter	Normal food waste	Low-N food waste
Basic characteristics for anaerobic digestion		
TS (% wet weight)	23.65±0.38	22.52±0.37
VS (% wet weight)	21.99±0.49	21.94±0.39
VS (% TS)	92.96±1.00	97.41±0.26
Nutrient value		
TKN (% dry basis)	3.09±0.9	1.45±0.03
Essential trace elements (mg kg^{-1} TS)		
Cobalt (Co)	0.086±0.006	<0.002
Iron (Fe)	121.1±9.1	38.2
Manganese (Mn)	90.05±1.51	-
Molybdenum (Mo)	0.506±0.016	0.23
Selenium (Se)	0.127±0.006	<0.002
Tungsten (W)	<0.015	-
Biochemical composition (g kg^{-1} VS)		
Carbohydrates	525±18	555±9
Lipids	151±1	13±0.4
Crude proteins	213±6	91
Hemicellulose	66	49
Cellulose	68	324
Lignin	16	5
Elemental analysis (%VS)		
C	54.89	47.52±0.34
H	6.89	6.94±0.06
N	3.32	1.49±0.03
S	0.25	-

Inoculum. From Table 4.5 it can be seen that the inoculum had: a neutral pH; moderate ammonia nitrogen and alkalinity; an acceptable IA/PA ratio; and low VFA. The inoculum could therefore be considered as being in good condition.

Table 4.5 Inoculum characteristics from Millbrook Wastewater Treatment plant

Parameters	**Concentration**
pH	7.26
Total ammonia nitrogen (mg l^{-1})	1393
Total alkalinity (mg l^{-1}as $CaCO_3$)	7027
Intermediate alkalinity (mg l^{-1}as $CaCO_3$): IA	2238
Partial alkalinity (mg l^{-1}as $CaCO_3$) :PA	4789
IA/PA ratio	0.47
Total solids (TS) (%)	3.46
Volatile Solid (% wet weight)	2.31
Volatile Solid (% TS)	67
Total VFA	45
• Acetic acid (mg l^{-1})	33
• Propionic acid (mg l^{-1})	<5
• Iso-butyric acid (mg l^{-1})	<5
• n-butyric acid (mg l^{-1})	<5
• Iso-valeric acid (mg l^{-1})	<5
• Valeric acid (mg l^{-1})	<5
• Hexanoic acid (mg l^{-1})	5
• Heptanoic acid (mg l^{-1})	7

4.2.4 Mesophilic digestion

The experiment ran for 296 days, equivalent to a total of 4.0 and 3.1 HRTs in digesters M1&2 and M3&4 respectively (Table 4.6). Results for digestion performance are presented in Figure 4.9 and described below.

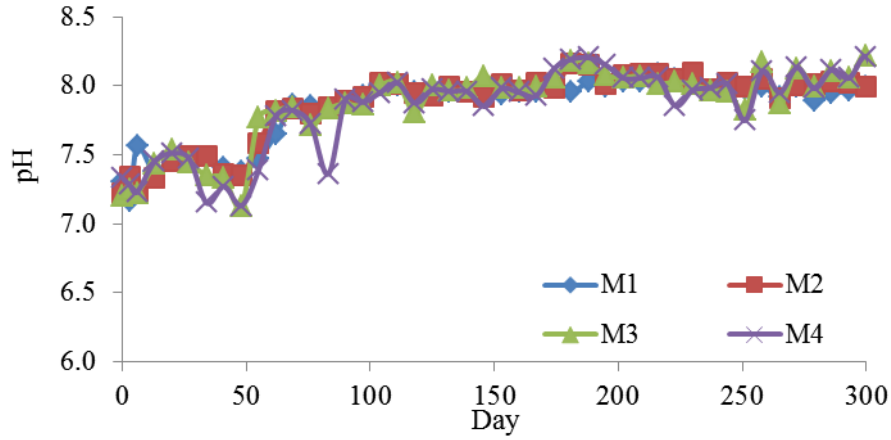
Table 4.6 OLR and HRT in mesophilic digestion trial

	M1&2		M3&4	
	OLR	Equivalent HRT [a]	OLR	Equivalent HRT [a]
Day 0-97	2	0.9	2	0.9
Day 98-116	2-3	0.2	2	0.2
Day 117-176	3	0.8	2	0.5
Day 177 – 209	3-4	0.5	2-3	0.4
Day 210 - 296	4	1.6	3	1.2
Total equivalent HRT [a]		4.0		3.1

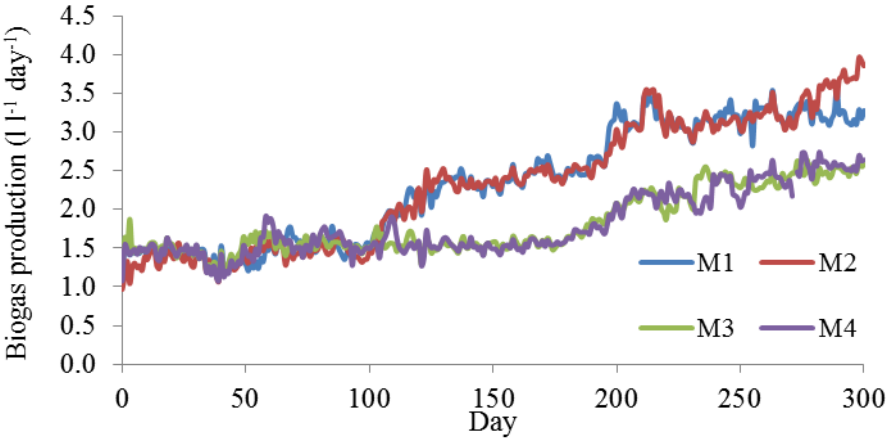
[a] Equivalent HRT calculated from mass of feedstock divided by volume of digester (i.e. assuming feedstock density = 1 kg l^{-1})

pH. The initial pH in all reactors was ~7.2. This gradually increased to between 7.9 - 8.0 (Figure 4.9a) Although the pH was higher than values often quoted as optimum (Chernicharo, 2007, Khanal, 2008) performance was satisfactory, as shown by stable gas production (both volumetric biogas production and methane concentration (Figure 4.9b and Figure 4.9c), low VFA (Figure 4.9d), and low IA/PA ratio (Figure 4.9e).

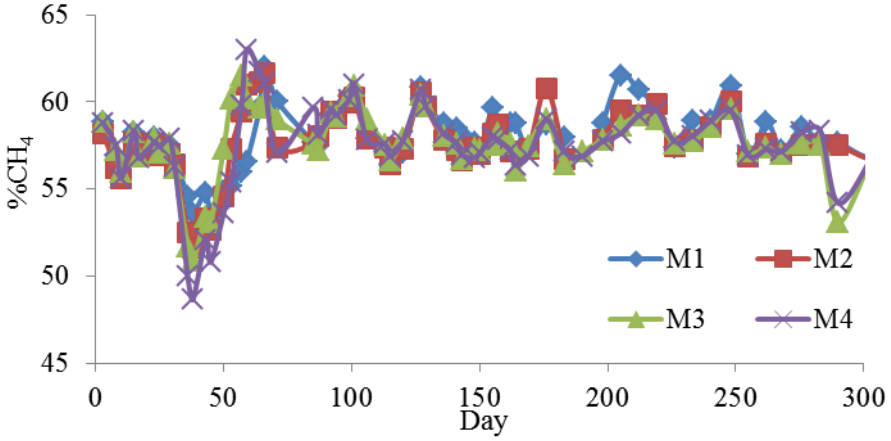
Ammonia nitrogen. Total ammonia nitrogen (TAN) in the inoculum was approximately 1.4 g N l^{-1} (Figure 4.9f), in the acceptable range for operation of a mesophilic anaerobic digester without known toxic effects (Khanal, 2008). On feeding with food waste, the digestate TAN increased to a value of around 4.5 g N l^{-1} by day 290. This increase probably accounted for the rise in pH, and did not appear to affect the performance of the digesters. Increasing the OLR to 2 and 3 g VS l^{-1} day^{-1} (M3&4), or 3 and 4 g VS l^{-1} day^{-1} g VS l^{-1} day^{-1} (M1&2) did not significantly affect the TAN concentration. This was as expected, as the concentration mainly reflects the degree of hydrolysis of the TKN in the feedstock, and provided that hydrolysis is not inhibited a steady state concentration will be reached. It is possible that a lower ammonia concentration might be observed at higher loading rates if a significant amount of nitrogen released from the hydrolysis in the form of ammonia is then used for cell synthesis by the microorganisms.



a) pH

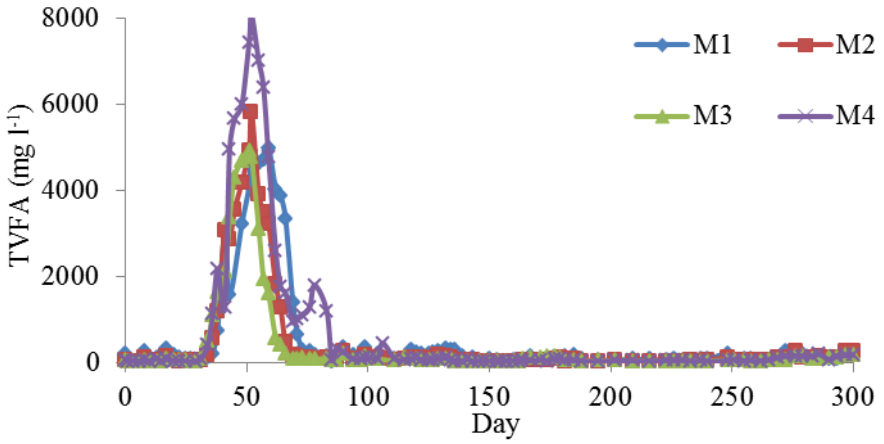


b) Volumetric biogas production

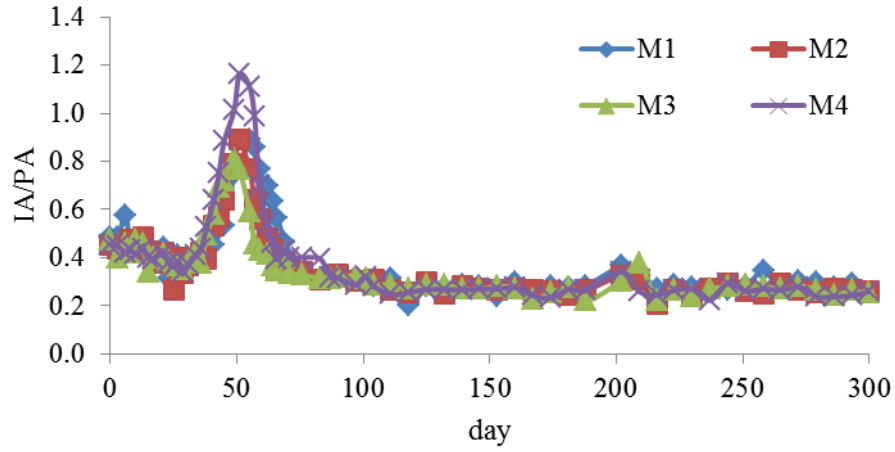


c) Methane percentage

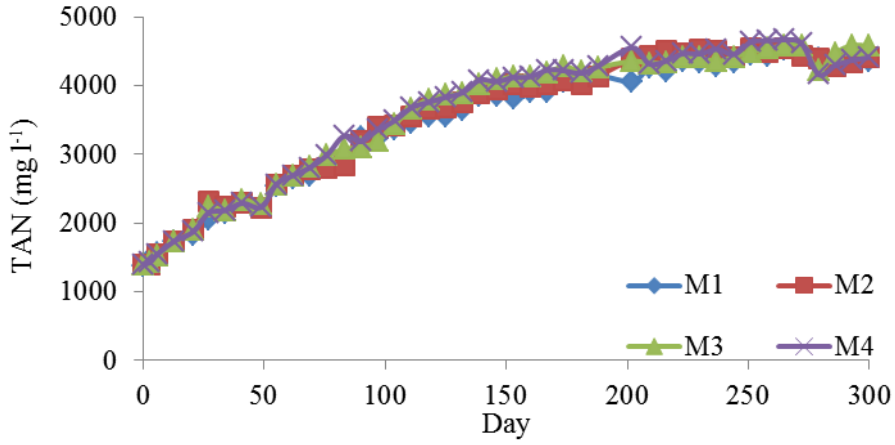
Figure 4.9 Monitoring parameters for mesophilic anaerobic digestion of normal food waste at OLR 2-4 g VS l^{-1} day^{-1}



d) total VFA

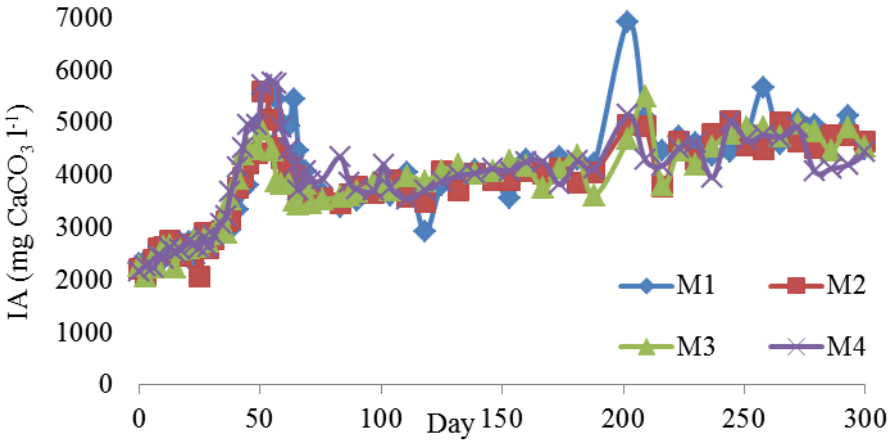


e) IA/PA ratio

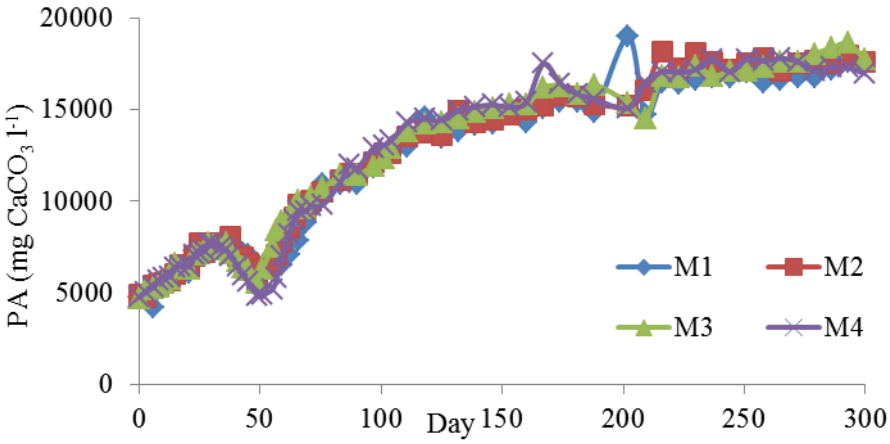


f) TAN

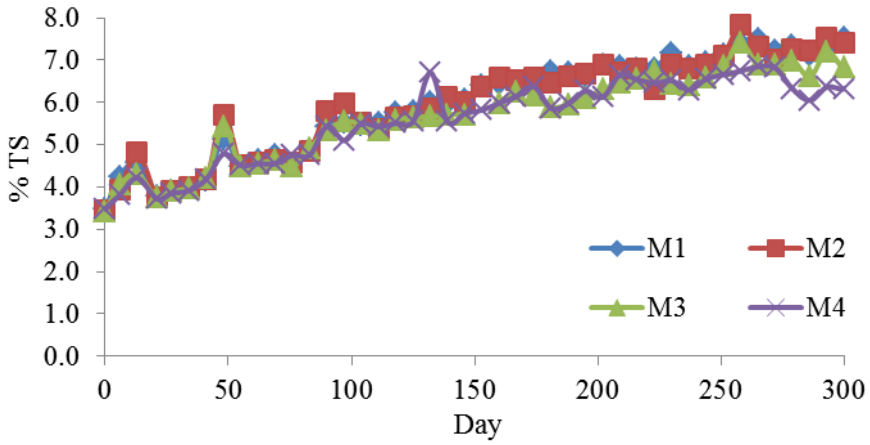
Figure 4.9 Monitoring parameters for mesophilic anaerobic digestion of normal food waste at OLR 2-4 g VS l^{-1} day^{-1} (continued)



g) IA

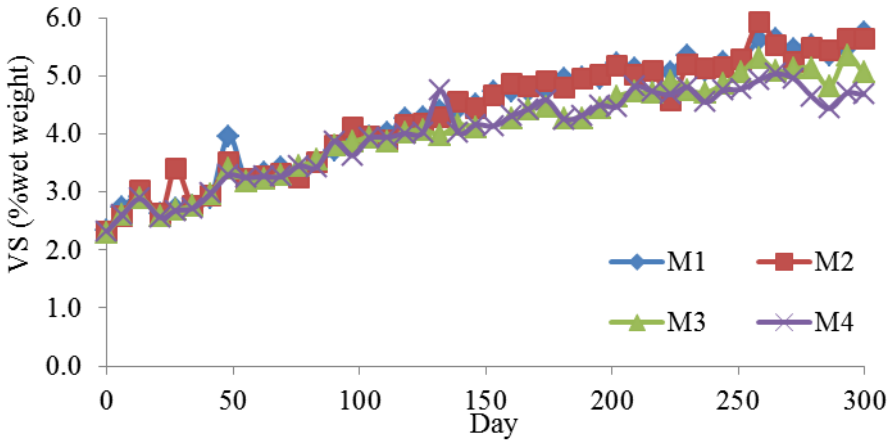


h) PA

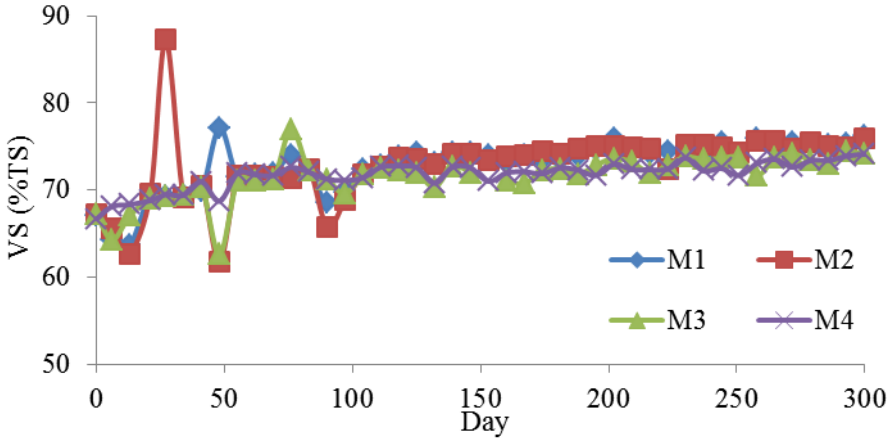


i) Total solids

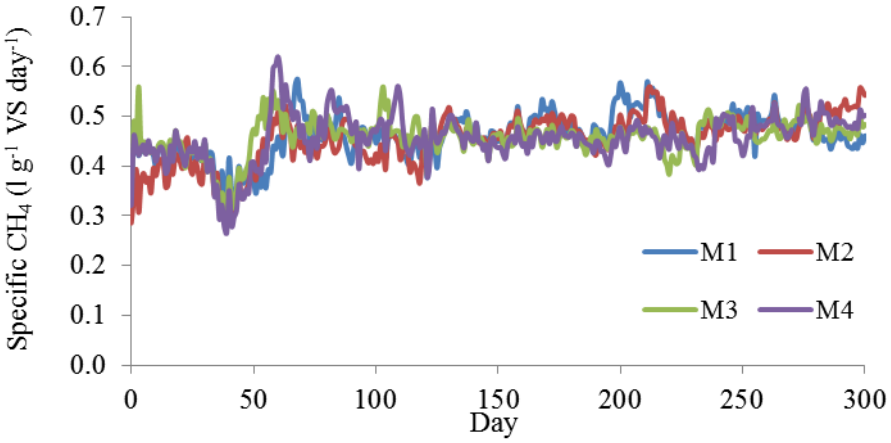
Figure 4.9 Monitoring parameters for mesophilic anaerobic digestion of normal food waste at OLR 2-4 g VS l^{-1} day^{-1} (continued)



j) Volatile solids (%wet weight)



k) Volatile solids (%TS)



l) Specific methane production

Figure 4.9 Monitoring parameters for mesophilic anaerobic digestion of normal food waste at OLR 2-4 g VS l^{-1} day^{-1} (continued)

Alkalinity. From Figure 4.9g, it can be seen that the initial IA was 2.2 g $CaCO_3$ l^{-1} and this did not change much apart from a period of disturbance around day 40 to 70. On day 177 the IA in M1&2 rose to about 7 g $CaCO_3$ l^{-1} after the OLR was increased to 4 g VS l^{-1} day^{-1}, and to 5 g $CaCO_3$ l^{-1} in M3&4 when the OLR was increased to 3 g VS l^{-1} day^{-1}. A few weeks after these loading increases, the IA returned to a value of approximately 4 g $CaCO_3$ l^{-1}.

PA gradually increased from 4 to above 15 g $CaCO_3$ l^{-1} from the beginning of the experiment to day around 130 (Figure 4.9h), corresponding to the increase in TAN concentration. The higher PA gave improved digester stability: the IA/PA ratio (Figure 4.9e) is an important parameter reflecting the stability of the system and it has been suggested this should not be higher than 0.3. At the beginning of the experiment the IA/PA ratio was 0.45-0.48 and it increased to a peak value of ~0.75-1.16 between days 40 and 70, then decreased to ≤ 0.3 even when the OLR was increased to 3 and 4 g VS l^{-1} day^{-1}, well within the value suggested by Ripley et al. (1986).

TS and VS. The solid content of the inoculum was 3.46 %TS and 2.31 % VS on a wet weight basis, meaning that 67 % of the TS was VS. Figure 4.9i, j and k show a general increase in the TS and VS where normal food waste was used, and a general increase in the VS as a proportion of the TS in the digestate. It can also be seen from Figure 4.9i that the % TS in M1&2 was higher than in M3&4, as a result of the higher OLR applied. There was no observable difference in the proportion of VS in the TS at the different OLRs applied. The VS destructions were 75% for M1&2, and 76% and 78% for M3 and M4, respectively.

Gas composition and production. Figure 4.9c shows the percentage of methane in the biogas. Biogas composition did not change greatly over the duration of the experiment: methane content varied between 55-60 %, with the lower percentage associated with the period of disturbance between days 40-70. Changes in OLR did not affect the methane percentage, indicating stable digestion over the range of loadings applied.

Figure 4.9b and l show the gas production from digesters M1-M4. The inoculum responded to the food waste input almost immediately, with the biogas volume increasing to above 1.3 l l^{-1} day^{-1} after two days' feeding (Figure 4.9b). Although the

response in digester M2 was slower, the gas production was similar to the others after about two weeks of operation. Volumetric biogas production was stable at the loading of 2 g VS l^{-1} day^{-1} and increased with each successive increase in OLR. For digesters M1&2, the volumetric gas production doubled between OLR of 2 and 4 g VS l^{-1} day^{-1}. The specific methane production (SMP) was 0.47-0.50 l CH_4 g VS^{-1} day^{-1} for OLR 4 g VS l^{-1} day^{-1}. As can be seen from Table 4.7, there was some variability in the SMP and a slight upward trend as the loading increased, which was unexpected. For the SMP to increase with increasing loading, the proportion of substrate being degraded must be increasing and this may be as a result of longer term acclimatisation of the anaerobic microbial population to the food waste substrate: it should be noted that the digesters were not operated for a full 3 HRT between each rise in OLR (see Table 4.6). It is clear, however, that mesophilic anaerobic digestion with TE addition coped very well with OLR of 2, 3 and 4 g VS l^{-1} day^{-1}.

VFA. Figure 4.10 shows the VFA profiles in M1-M4. Values were very low for most of the time, at less than 300 mg l^{-1}. High concentrations were only observed between days 40-70 when the total VFA concentration increased to approximately 5000 mg l^{-1} in M1, M2, and M3, and 8000 mg l^{-1} in M4. Similar VFA peaks have previously been observed during acclimatisation of this inoculum to source separated food waste (unpublished data, University of Southampton). The main acid present was acetic, with iso-valeric at the second highest in concentration. The rise in VFA corresponded to a small decrease in gas production (both volume and $\%CH_4$) and changes in pH and the IA/PA ratio.

From day 70 operation of all digesters continued with high and stable gas production, low VFA and a stable IA/PA ratio. The above results confirmed that with suitable trace element supplementation mesophilic anaerobic digestion of normal food waste gave a satisfactory performance, despite the relatively high nitrogen content of this feedstock which agreed with the results from Banks et al. (2012), Zhang et al. (2012b) and Zhang and Jahng (2012).

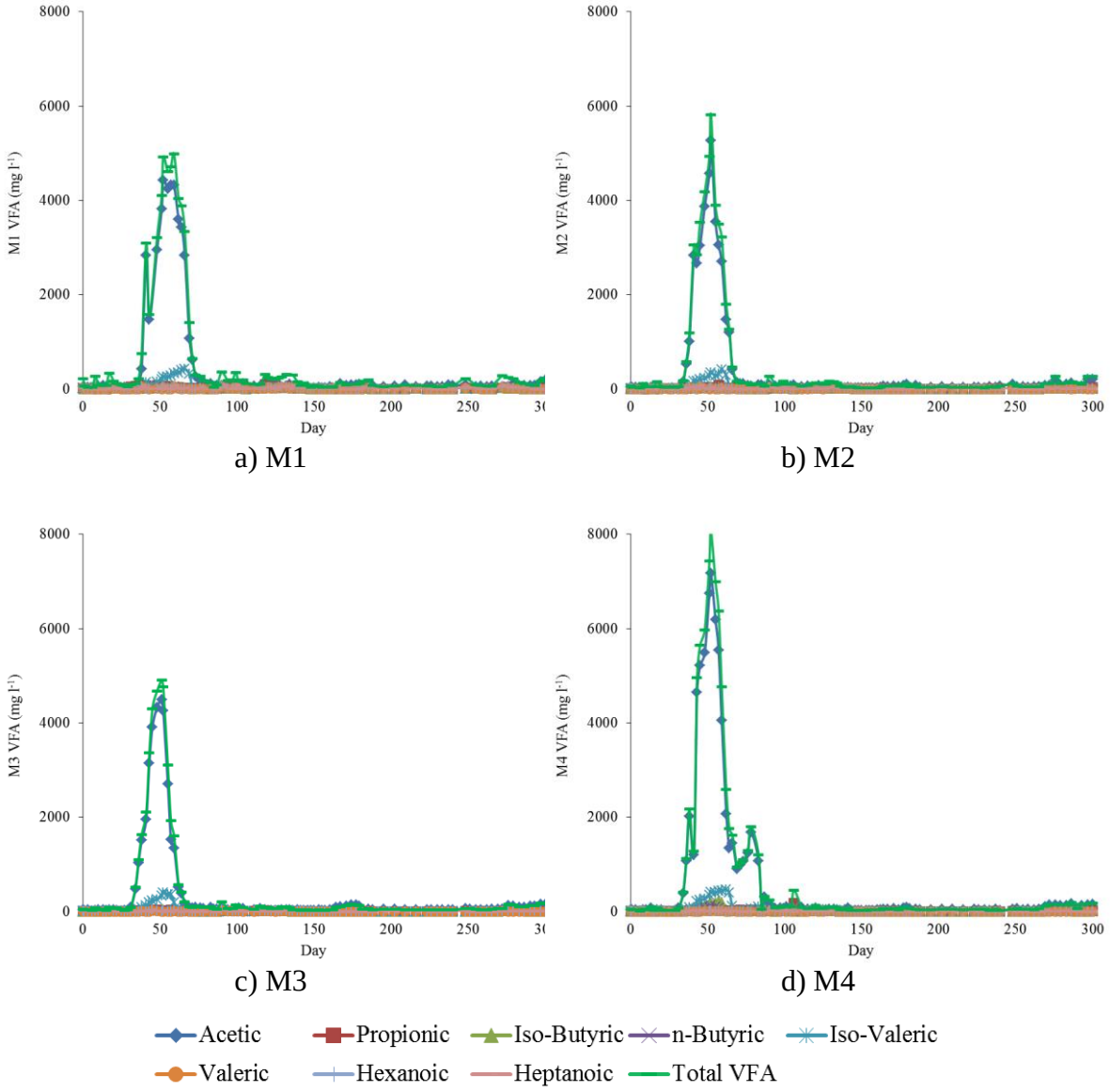


Figure 4.10 VFA in mesophilic anaerobic digesters during the experimental period

Table 4.7 SMP of M1&2 and M3&4 at each OLR (average for period shown)

Days	OLR[a]	SMP (l CH_4 g VS^{-1} day^{-1})							
		M1		M2		M3		M4	
		Average	SD	Average	SD	Average	SD	Average	SD
67-97	2	0.482	0.038	0.448	0.023	0.471	0.015	0.481	0.037
146-176	2	-	-	-	-	0.458	0.016	0.450	0.017
146-176	3	0.486	0.024	0.480	0.018	-	-	-	-
266-296	3	-	-	-	-	0.477	0.017	0.491	0.022
266-297	4	0.469	0.025	0.496	0.025	-	-	-	-

Note: [a] unit of OLR = g VS l^{-1} day^{-1}

4.2.5 Thermophilic digestion

4.2.5.1 Acclimatisation to thermophilic conditions

As expected from the previous trial (section 4.1) some instability was observed immediately after the sharp increase in temperature, but this was limited and transitory. There was an initial accumulation in total VFA (Figure 4.11a) but by day 20 this had begun to decline, falling to below 1000 mg l^{-1} by day 60. The accumulation and subsequent reduction of VFA was reflected in changes in IA/PA ratio (Figure 4.11b) which stabilised at around 0.4-0.5 after the VFA had been removed. By day 60 TAN concentrations in the low and high-nitrogen digesters had diverged to around 1.3 and 2.5 g N l^{-1} respectively (Figure 4.11c), and this was reflected in a small difference in pH (Figure 4.11d). Specific methane production (SMP) increased as the digesters acclimated to the temperature increase and the gradually incremented organic loading rate (OLR), and by day 60 had stabilised at around 0.4 l CH_4 g^{-1} VS day^{-1} in all digesters (Figure 4.11e). Methane content of the biogas fell to between 20-35% around day 8-10 but recovered to over 50% in all digesters by day 23 (Figure 4.11f). Apart from the differences in TAN and pH there were no apparent differences between the normal and low-N digesters, suggesting that the behaviour in this period primarily reflected the acclimatisation to the change in temperature, feedstock and OLR.

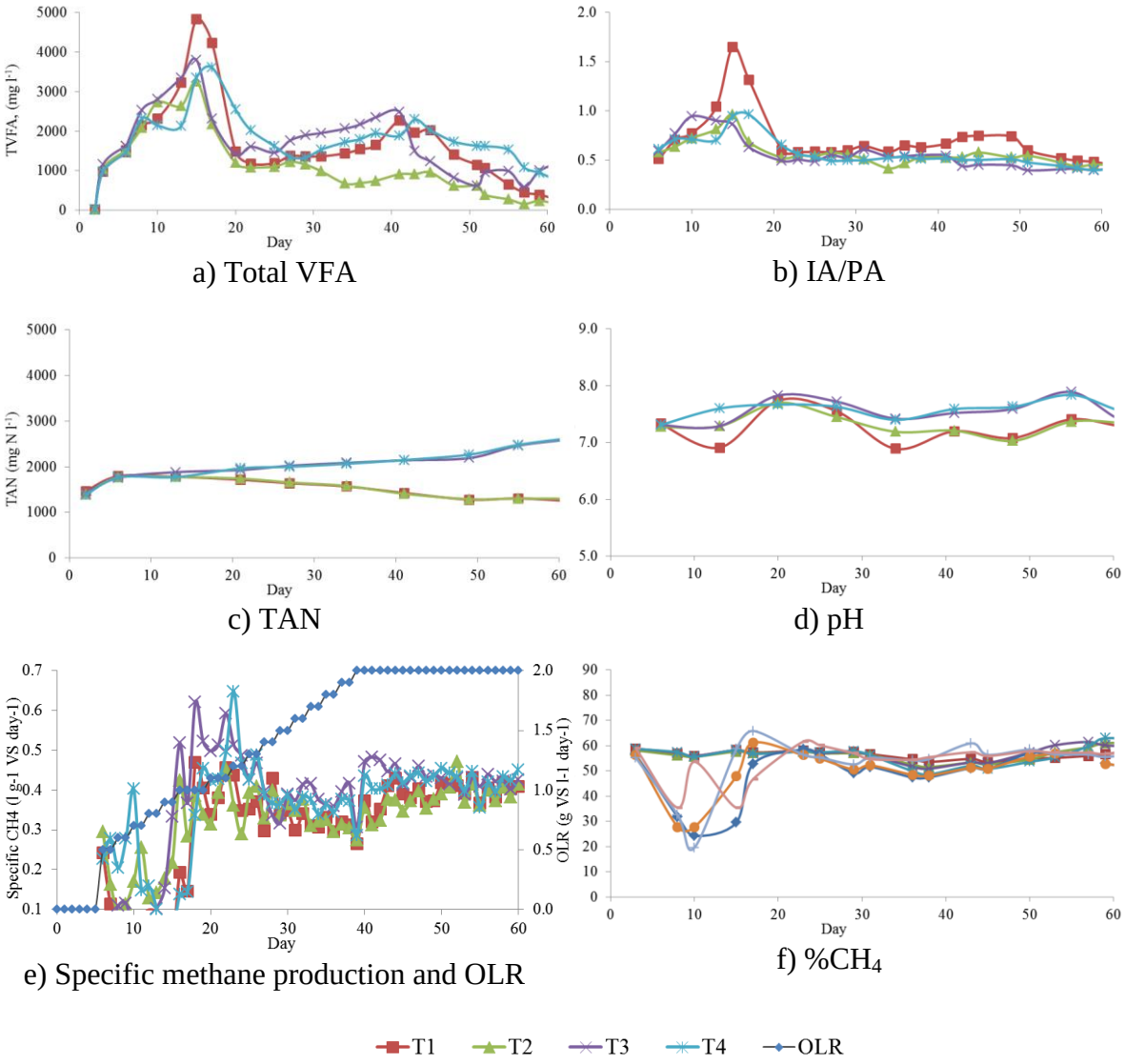


Figure 4.11 Digestion parameters during the first 60 days after temperature increase to thermophilic conditions

VFA profiles for T1 – T4 in the first 60 days are shown in Figure 4.12. The pattern of an initial peak in acetic acid followed by a propionic peak that subsequently reduces was very similar to that in TE supplemented digesters in the first thermophilic trial (Figure 4.2), although the peak concentrations, especially for acetic acid, were generally higher and the reduction slightly delayed. This may reflect the slightly more rapid increase in OLR compared to that in the previous trial. Again, there were no apparent differences between the low-N and normal food waste digesters. The influence of trace element addition seen in the early stages of the previous trial (section 4.1.4) was

therefore probably due to a beneficial effect on acclimatisation to thermophilic conditions, rather than to a high-nitrogen feedstock.

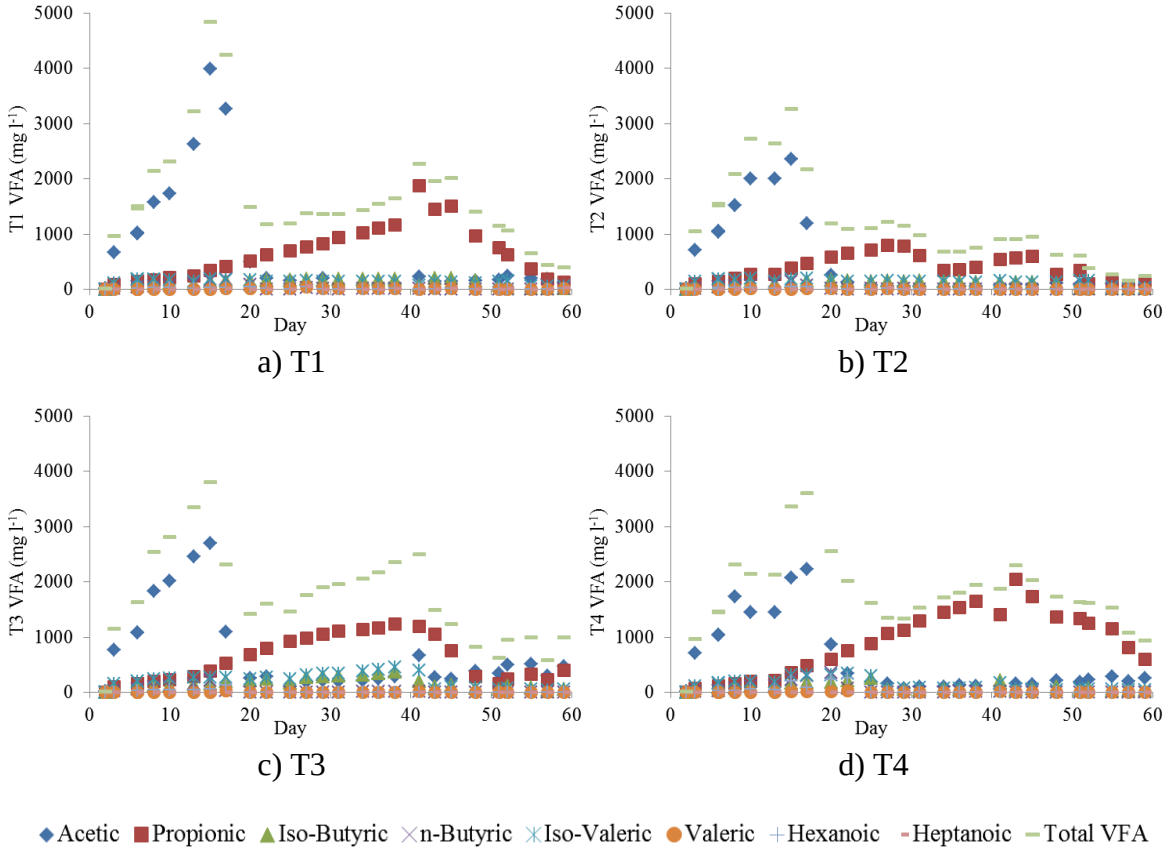


Figure 4.12 VFA profiles in T1-4 from day 1-60

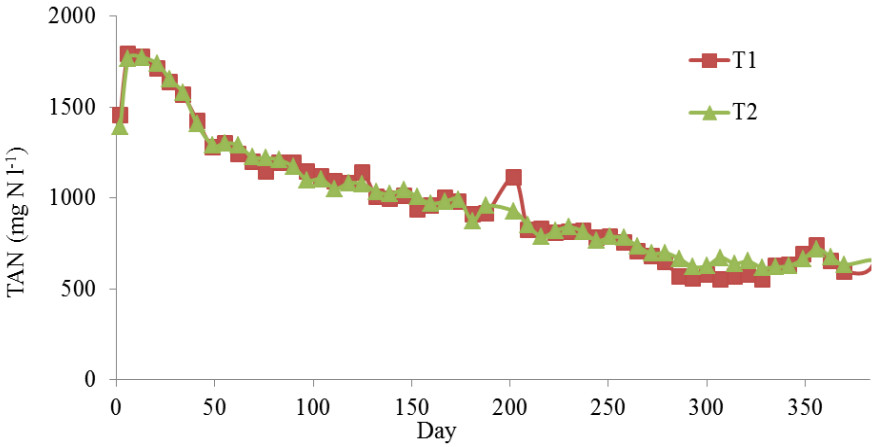
The gradual increase in loading thus allowed successful acclimatisation of all four digesters over the first ~60 days of operation. This simple method of a single step temperature change provided an effective alternative to the step-wise changes in temperature that are sometimes recommended (Boušková et al., 2005). Trace element addition appeared to have a beneficial effect during this process.

4.2.5.2 Effect of different N content of feedstocks on digestion

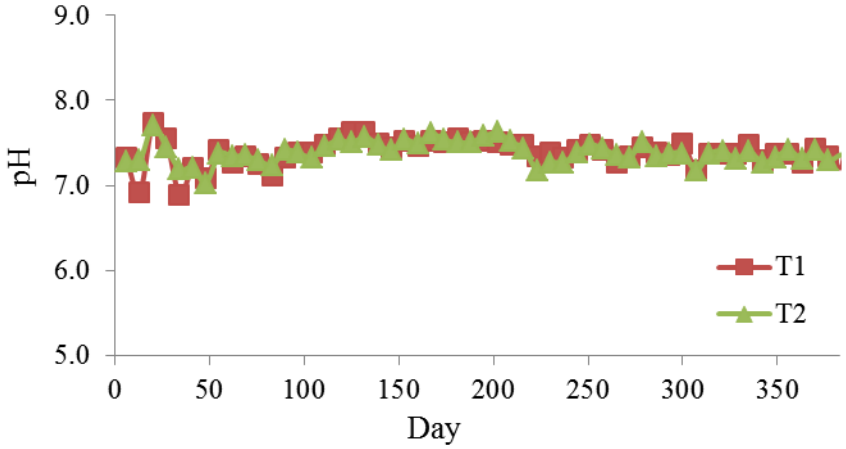
As noted above, T1 and T2 were fed with low-N food waste as a control to ensure that acclimatisation to thermophilic conditions could be achieved, and also to demonstrate that long-term stable operation could be maintained. The OLR was increased from 0.5 to 2 g VS l^{-1} day^{-1} between days 6-39 and then maintained at 2 g VS l^{-1} day^{-1}. These digesters ran on low-nitrogen food waste for 383 days (equivalent to around 3.3 HRT

based on quantity of added feed) and reached a final TAN concentration of around 600 mg N l^{-1} (Figure 4.13a).

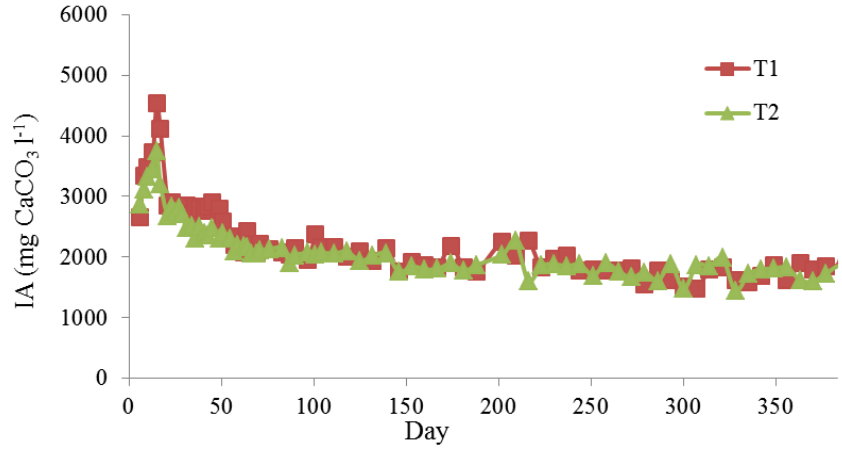
By day 50 digesters T1 and T2 were operating very stably, with pH around 7.4 (Figure 4.13b). IA, PA, and TA were between 1.25-2.0, 4.0-4.5, and 5.5-6.5 g $CaCO_3$ l^{-1}, respectively (Figure 4.13c, d, and e), IA/PA ratio between 0.4-0.5 (Figure 4.13f), and total VFA generally below 500 mg l^{-1} (Figure 4.13g). These values are within the limits suggested by Ferrer et al. (2010) who proposed the following parameter values to prevent failure: acetic acid < 600 mg l^{-1}, total VFA < 3700 mg l^{-1}, intermediate alkalinity < 1.8 g $CaCO_3$ l^{-1}, IA/PA < 0.9, and methane content (% CH_4 in biogas) >55%. Gas production appeared to be less stable for short periods around days 133 and 297 (Figure 4.13h and i), but this was due to short-term heater failures causing temporary cooling of the digesters and production rapidly recovered when the operating temperature was restored. The average SMP of T1 and T2 between days 283-383 was 0.39±0.04 and 0.39±0.05 l CH_4 g^{-1} VS, respectively. The digestate VS concentration was around 2.5% (Figure 4.13j), corresponding to around 88% VS destruction of the low-N food waste which resulted in low FAN<200 mg N l^{-1} (Figure 4.13k). It can be seen that the low N feed allowed successful and stable long term operation.



a) TAN

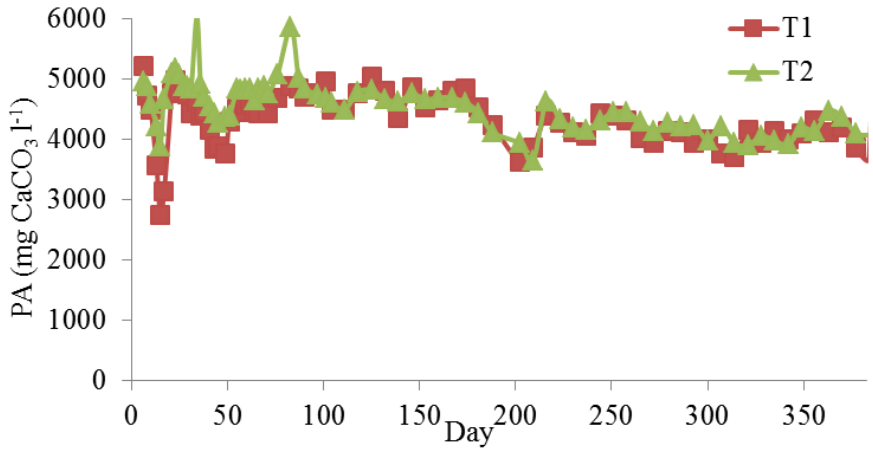


b) pH

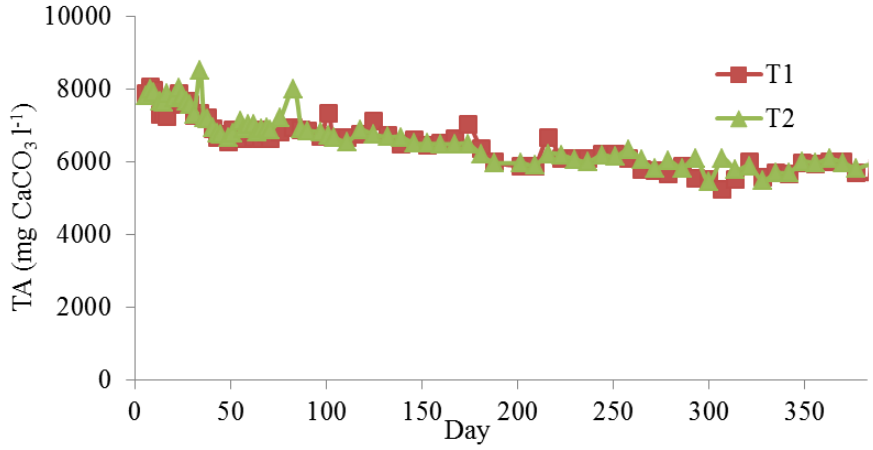


c) IA

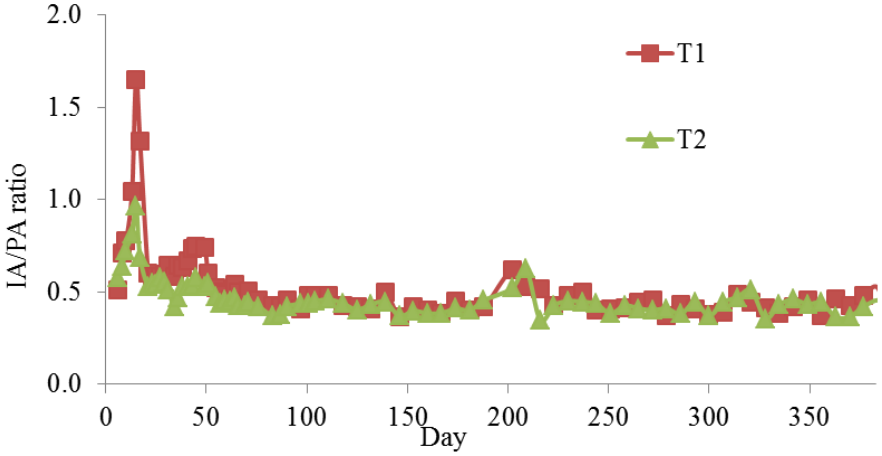
Figure 4.13 Monitoring parameters for thermophilic anaerobic digestion in T1 and T2 from day 0-383



d) PA

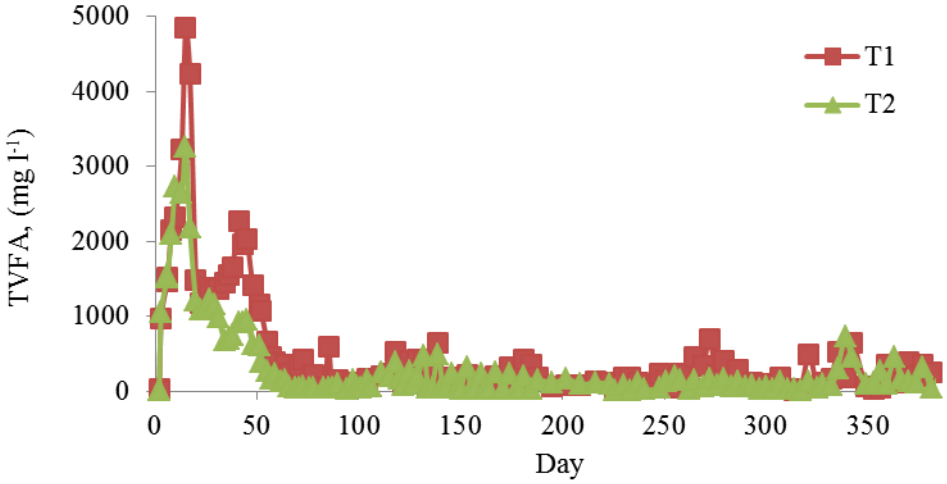


e) TA

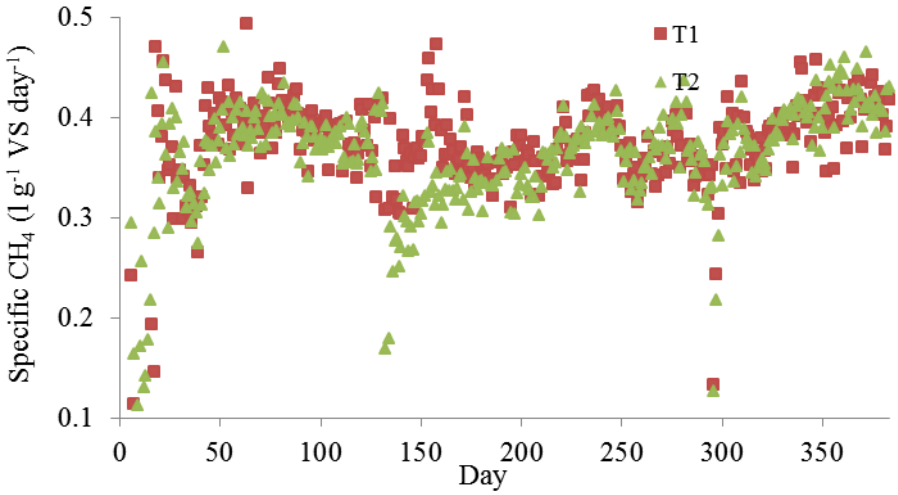


f) IA/PA

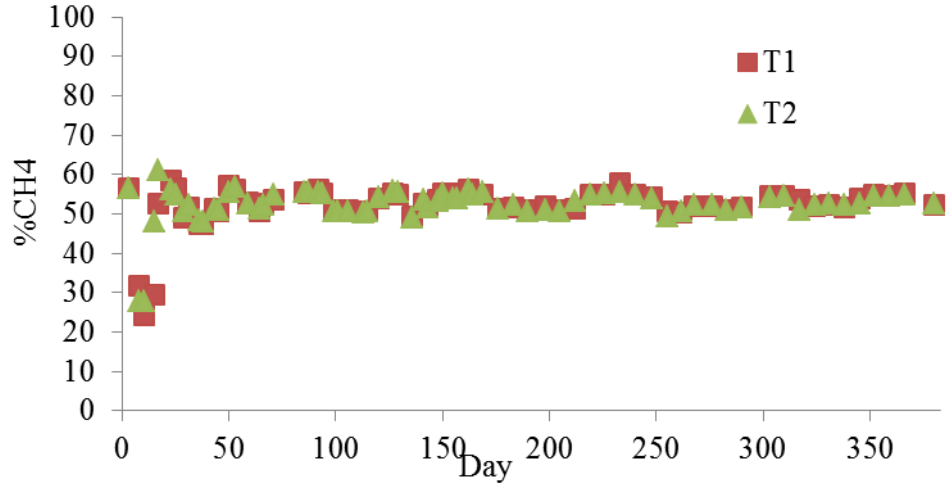
Figure 4.13 Monitoring parameters for thermophilic anaerobic digestion in T1 and T2 from day 0-383 (continued)



g) Total VFA

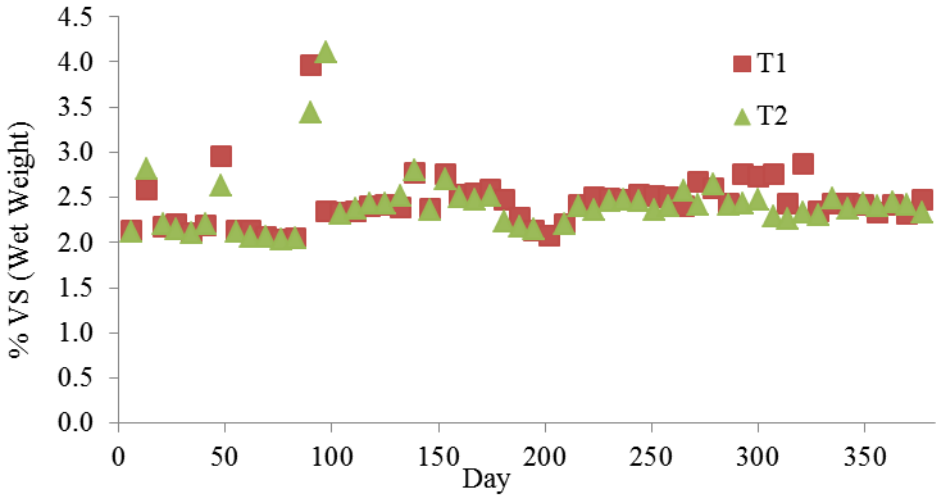


h) Specific methane production

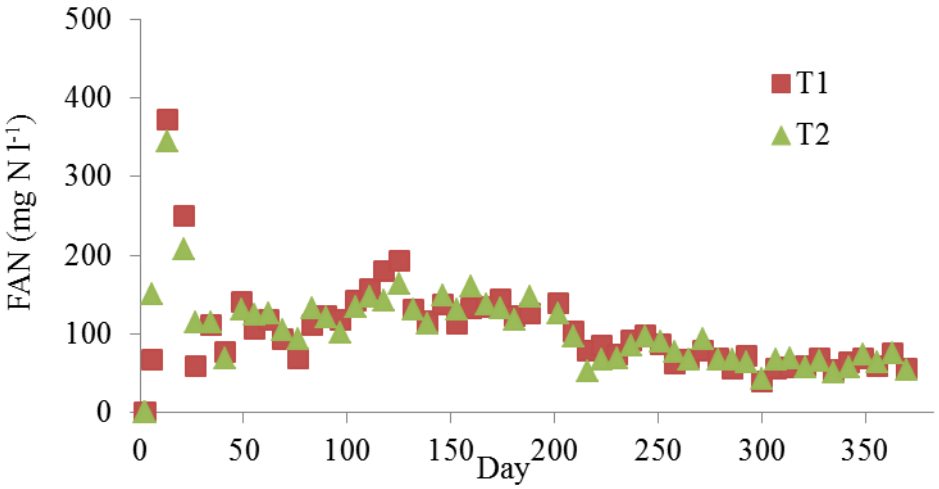


i) Methane percentage

Figure 4.13 Monitoring parameters for thermophilic anaerobic digestion in T1 and T2 from day 0-383 (continued)



j) Volatile solids (% wet weight)



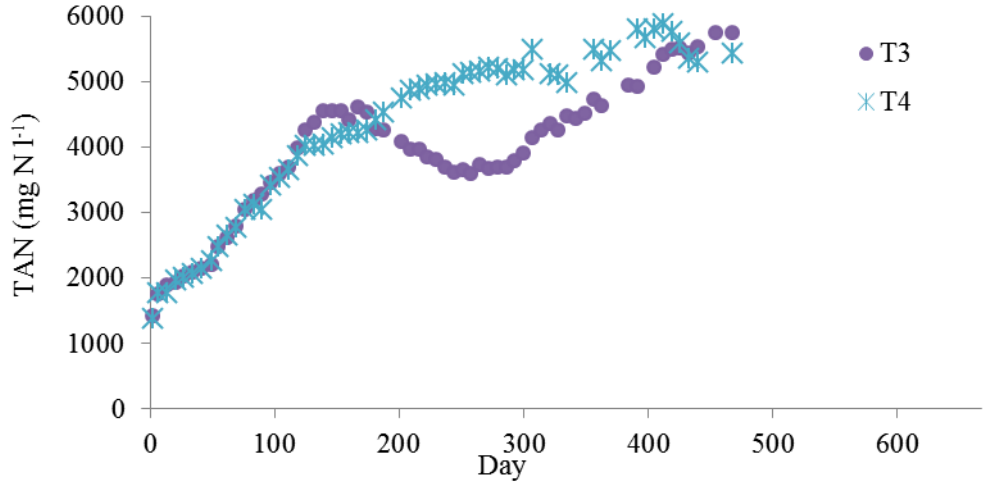
k) Free ammonia nitrogen

Figure 4.13 Monitoring parameters for thermophilic anaerobic digestion in T1 and T2 from day 0-383 (continued)

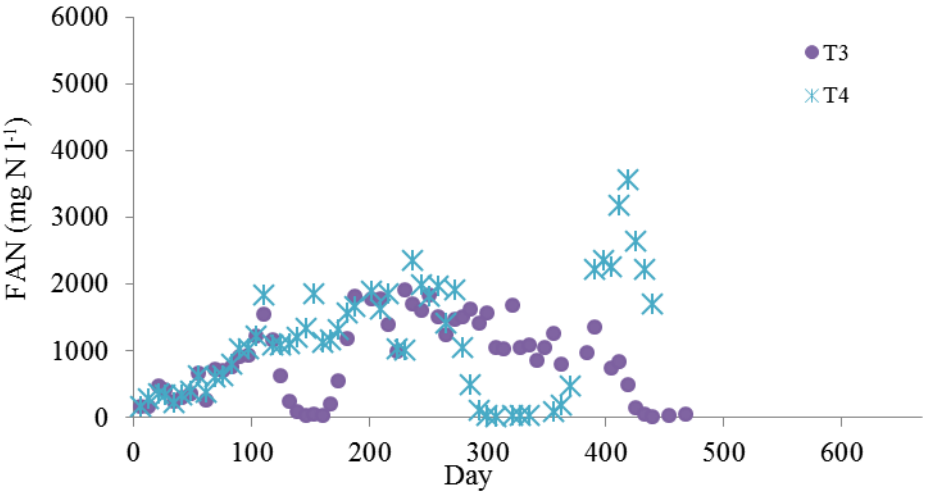
In the pair of digesters fed on normal food waste (T3 and T4) successful acclimatisation to thermophilic conditions also took place (Figure 4.11 and Figure 4.12). Not long after, however, these digesters began to show signs of stress as the TAN concentration rose above ~2.5 g N l^{-1} from around day 60 onwards (Figure 4.14a), corresponding of ~600 mg N l^{-1} FAN (Figure 4.14b). Both digesters showed a very rapid increase in total VFA (Figure 4.14c) and a fall in specific methane production and biogas methane content (Figure 4.14d and e). In digester T3 there was also a sharp rise in IA/PA ratio (Figure 4.14f and g), which was less severe in T4.

In an attempt at stabilisation and recovery a one-off dose of the original TE solution was given to T3 on day 119 to double the baseline concentration. This higher concentration was maintained by regular supplementation until day 132, when the 11-element TE formulation was introduced in both T3 and T4. This gave immediate digester concentrations of (mg l^{-1}) Aluminium (Al) 0.4, Boron (B) 0.4, Cobalt (Co) 4.0, Copper (Cu) 0.4, Iron (Fe) 40.0, Manganese (Mn) 4.0, Nickel (Ni) 4.0, Zinc (Zn) 0.8, Molybdenum (Mo) 0.8, Selenium (Se) 0.8, Tungsten (W) 0.8, which were then maintained by weekly addition in proportion to the feed. These measures were taken based on the report by Uemura (2010) that thermophilic digestion has a higher trace elements requirement than mesophilic. The total VFA concentration in digester T3 continued to rise, however, and feeding was stopped on day 156.

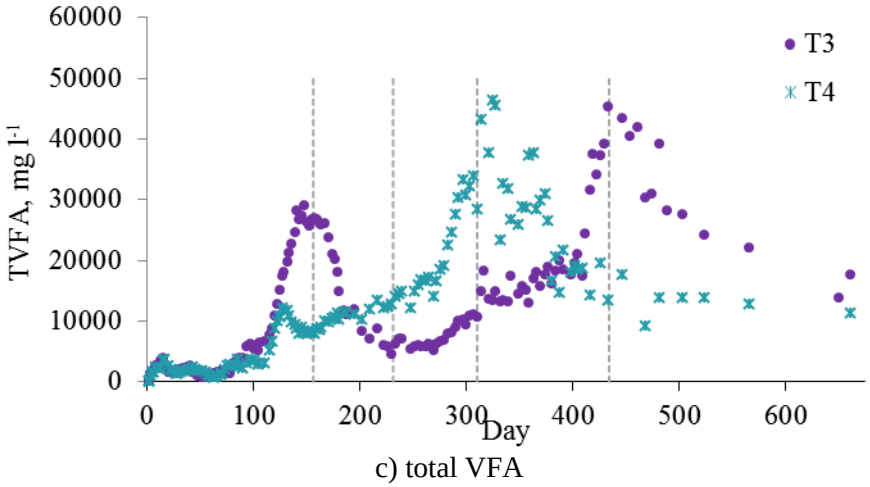
As the pH in digester T3 had fallen to < 6.0 and the biogas methane content to < 20% (Figure 4.14h and e), it was decided to re-seed the digester to dilute out the accumulated VFA and enrich the failed methanogenic population. This was achieved on a gradual basis by adding the waste digestate from digesters T1, T2 and T4 which resulted in the lower VS content in T3 (Figure 4.14i). Feeding of T3 started again on day 231 after a 78-day pause, at which point the IA/PA ratio was around 0.5.



a) TAN



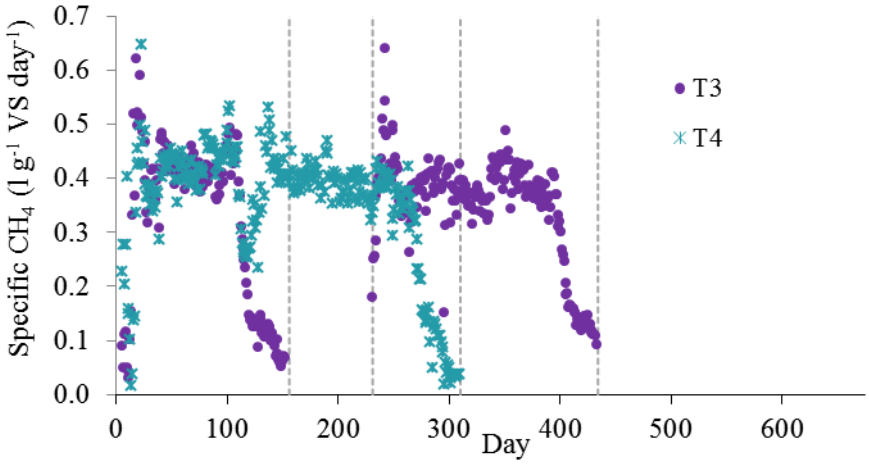
b) FAN



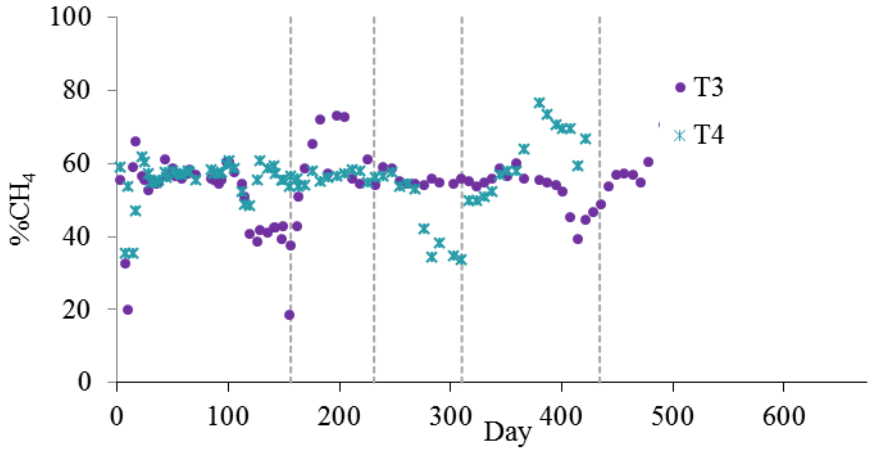
c) total VFA

Figure 4.14 Monitoring parameters in T3 and T4 (normal food waste) from days 0-662.

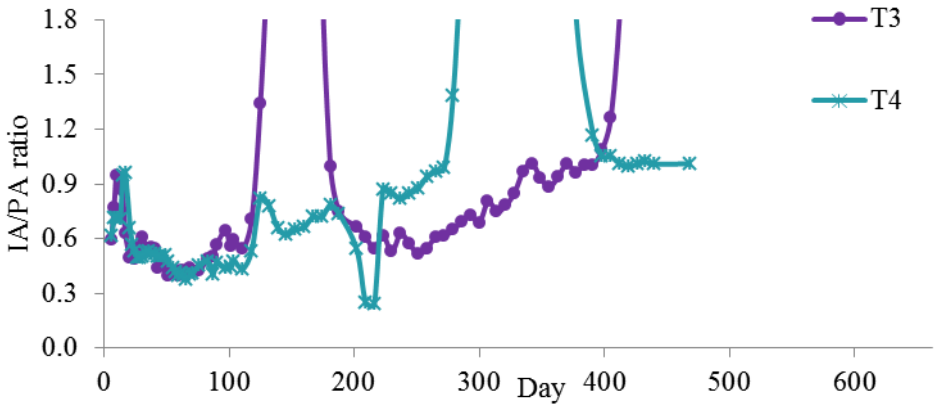
Note: Vertical dotted lines indicate change in feeding: day 156 feed to T3 stopped, day 231 feed to T3 restarted after partial re-seeding; day 310 feed to T4 stopped; day 434 feed to T3 stopped



d) Specific methane production



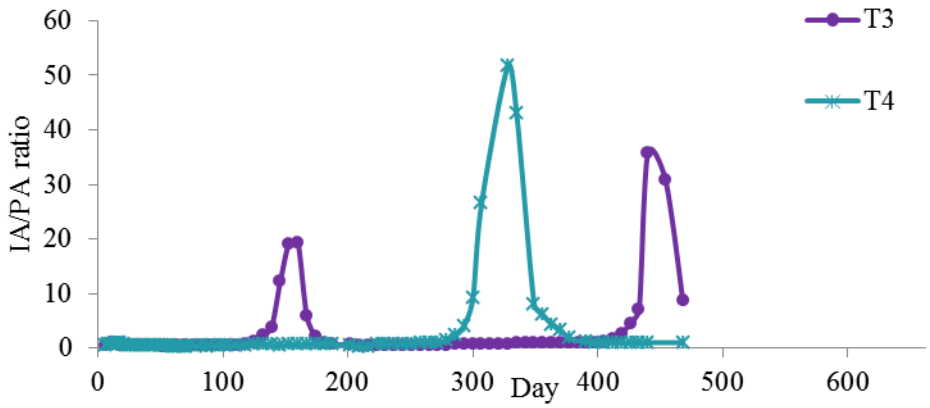
e) Biogas methane percentage



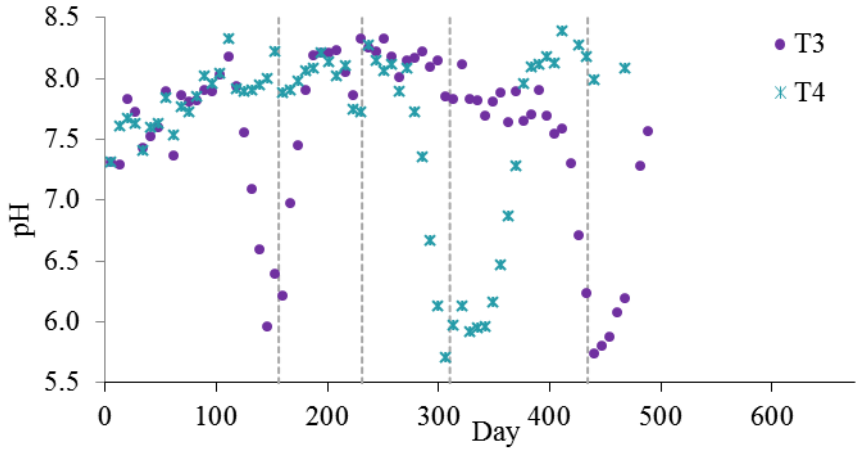
f) IA/PA (detail)

Figure 4.14 Monitoring parameters in T3 and T4 (normal food waste) from days 0-662 (continued)

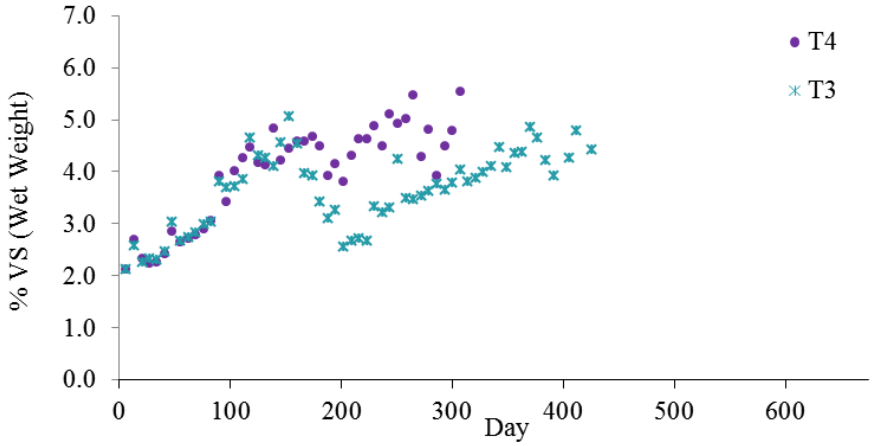
Note: Vertical dotted lines indicate change in feeding: day 156 feed to T3 stopped, day 231 feed to T3 restarted after partial re-seeding; day 310 feed to T4 stopped; day 434 feed to T3 stopped.



g) IA/PA (all data)



h) pH



i) Volatile solids (%wet weight)

Figure 4.14 Monitoring parameters in T3 and T4 (normal food waste) from days 0-662 (continued)

Note: Vertical dotted lines indicate change in feeding: day 156 feed to T3 stopped, day 231 feed to T3 restarted after partial re-seeding; day 310 feed to T4 stopped; day 434 feed to T3 stopped.

In T4 the rapid increase in total VFA stopped at around 12 g l^{-1} on day 132, and the concentration fell slightly over the next several days, then started to increase again but more gradually. The rise in TAN concentration also slowed during this period (Figure 4.14a), possibly indicating incorporation into new biomass. The recovery in T4 may have been aided by the modified TE supplementation. Even if this measure did help to overcome the rapid acidification, however, it was unable to prevent the longer term accumulation of VFA. The feed to T4 continued uninterrupted, while pH actually increased to around 8, despite the observed increase in total VFA (Figure 4.14c). Total VFA concentrations rose to ~19 g l^{-1} by day 279 when there was again a very sharp increase in the rate of accumulation, with the concentration reaching > 33 g l^{-1} on day 307. This increase in total VFA was sufficient to overcome the buffering capacity of the digestate and the pH dropped sharply to < 6 by day 307 (Figure 4.14h) while the IA/PA ratio rose to 26.5. At this point the TAN in T4 had risen to > 5 g N l^{-1} and feeding to digester T4 was stopped on day 310. Total VFA concentrations continued to rise to a peak of > 46 g l^{-1} on day 325 then fell for the next 56 days to around 18 g l^{-1} which was sufficient for the pH to return to > 8 and the IA/PA ratio to fall to 1.3. At the end of the experiment on day 662, however, the total VFA concentration was still ~11 g l^{-1}. The VFA profile in Figure 4.15 shows the final rapid VFA accumulation to be in the form of acetic acid. This was subsequently removed, but the 'fixed' VFA was propionic acid and its rate of removal was only very slow even under starvation conditions.

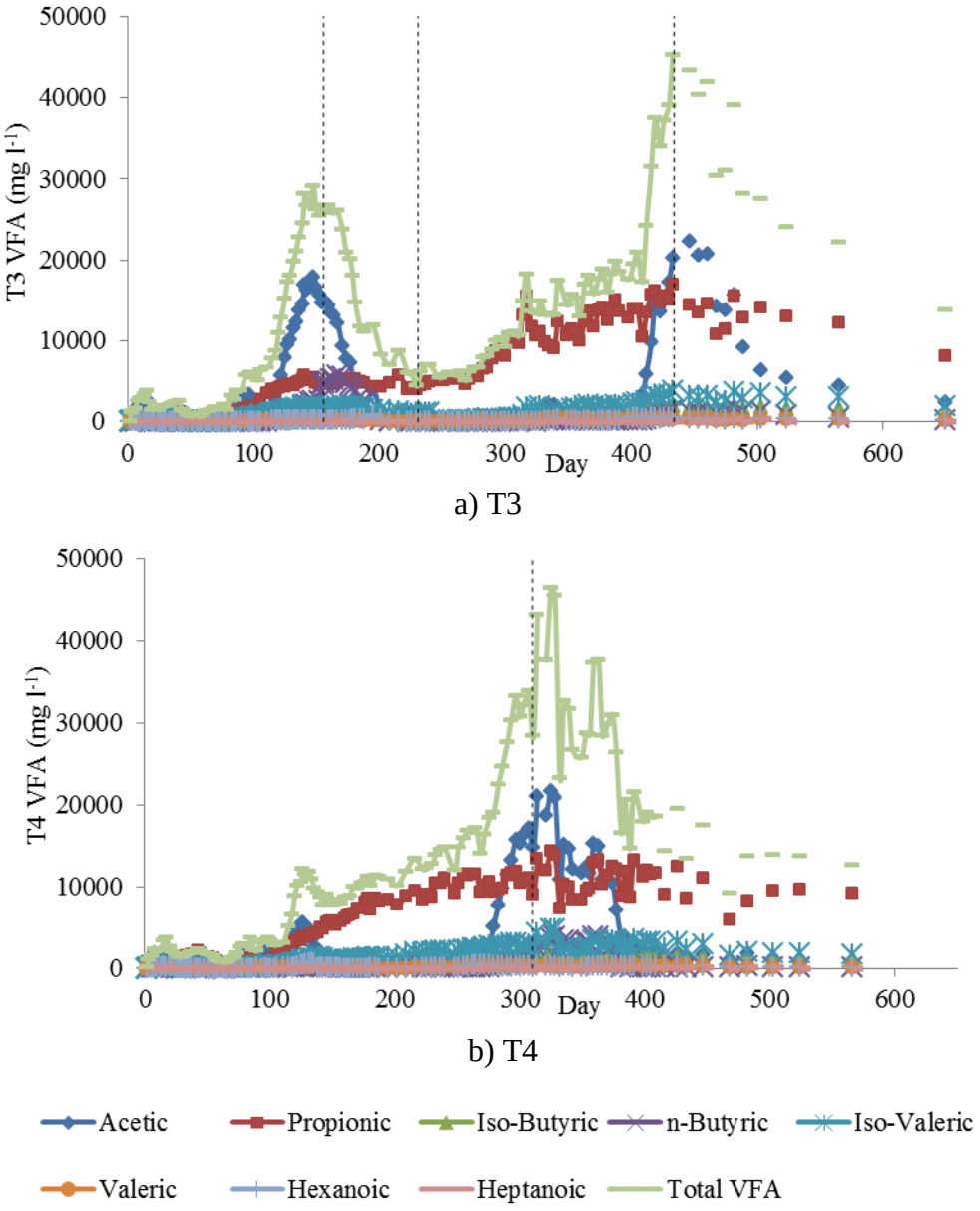


Figure 4.15 VFA profiles in digesters T3 and T4 (normal food waste) from day 0-662. Note: Vertical dotted lines indicate change in feeding: day 156 feed to T3 stopped, day 231 feed to T3 restarted after partial re-seeding; day 310 feed to T4 stopped; day 434 feed to T3 stopped.

A similar pattern was seen in T3 after day 231, although this lagged behind T4 as a result of the cessation of feeding for 78 days and the re-seeding of the digester. The second sharp increase in total VFA again occurred when the total VFA concentration had reached about 18 g l^{-1} (Figure 4.14c), and was due to acetic acid accumulation (Figure 4.15a). Total VFA peaked at 45 g l^{-1}, and the IA/PA ratio reached 51.7 (Figure 4.14g). Feeding was stopped on day 434 and the same pattern of partial recovery was seen as in T4, with a drop in acetic acid and a return to a pH 7 with the IA/PA ratio falling to 0.9.

The results suggest that irreversible VFA accumulation occurs in thermophilic digestion when the TAN concentration exceeds 3.5 g N l^{-1}. This value is similar to that reported by Hendriksen and Ahring (1991) who showed that initial inhibition between 3000-4000 mg N l^{-1}. TAN itself may not be the most critical parameter, as the toxicity is due mainly to free ammonia and the equilibrium is pH and temperature dependent. In the current trial the pH in digesters T3 and T4 was about 8.0, and the free ammonia (Figure 4.14b) was thus in the same range as the 700-1100 mg N l^{-1} inhibitory concentration suggested by Angelidaki and Ahring (1994).

4.2.5.3 Effect of urea addition to low-N digesters

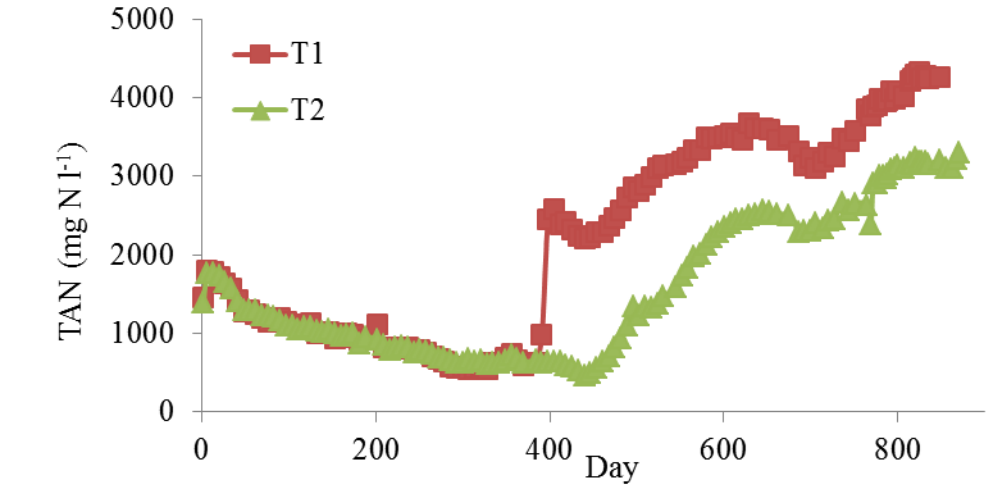
Aim. The aim of this experiment was to identify the critical threshold(s) for ammonia toxicity by adding urea to digesters fed on low-N food waste, until VFA accumulation occurred.

Method. This experiment used digesters T1&2 and followed on immediately from the study in section 4.2.5.2 above. To facilitate comparison between the periods before and after urea addition, the entire operational period is shown (i.e. day numbering is continuous from the previous trial). The same batch of low-N food waste was used and the OLR was maintained at 2 g VS l^{-1} day^{-1}. Nitrogen concentrations in the digesters were increased by the addition of urea (Fisher Scientific, UK). This was added to T1 on day 384 as a single dose of 20.0 g aimed at raising the TAN concentration to ~2.5 g N l^{-1}. T1 was then left for 80 days without further urea addition to allow acclimatisation, after which the TAN content was raised gradually to a final value of ~3.5 g N l^{-1} by adding urea to the daily feed to give this target concentration. The TAN concentration was further increased to a final value of around ~4.2 g N l^{-1} between day 750 and 875.

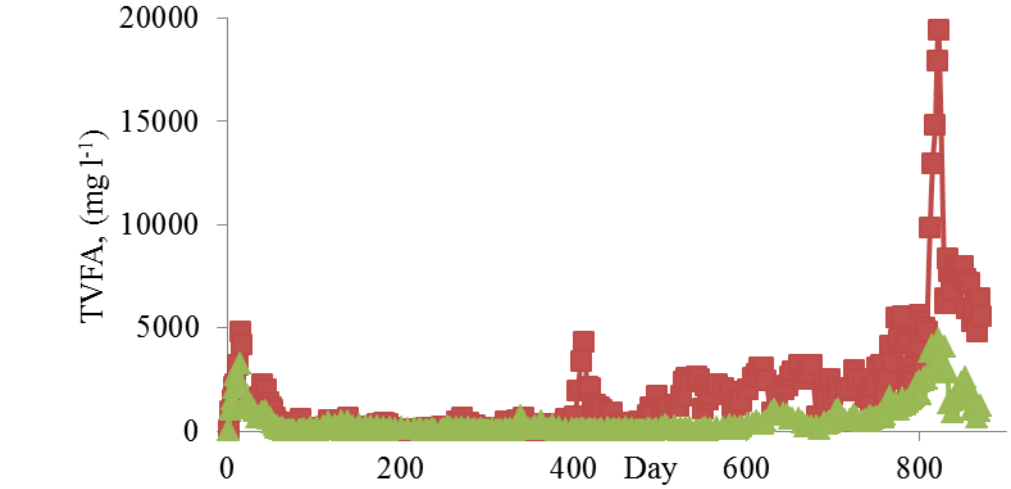
The TAN concentration in T2 was increased to ~2.5 g N l^{-1}, but this was done more gradually by adding an initial dose of 7.7 g of urea to the digester on day 441 then adding urea to the daily feed to reach the target concentration, in order to allow comparison with the effect of the shock urea load applied to T1. Between day 750-875 the TAN concentration in T2 was raised to ~3.0 g N l^{-1}. Monitoring of digester pH, ammonia nitrogen, alkalinity, TS and VS, gas production and gas composition continued as before. TE was still added as same scheme as usual.

Results

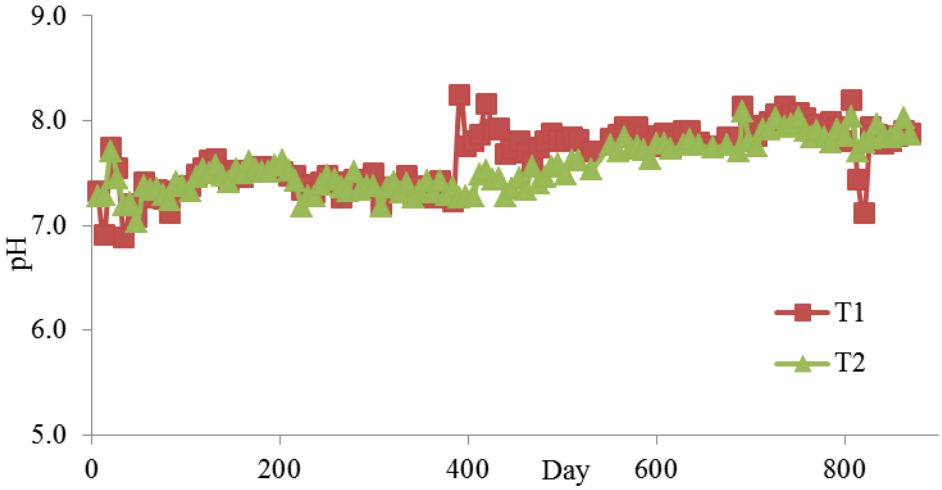
The experiment to assess the impact of increasing the TAN concentration in the low-N digesters T1 and T2 by urea addition began on day 384, with a single dose added to T1 to raise the TAN concentration to ~2.5 g N l^{-1} (Figure 4.16a). During the acclimatisation period no further urea was added, and the TAN concentration reduced slightly due to washout; then started to rise again with the addition of urea in the low-N food waste from day 464. After day 750 the concentration in T1 rose again to a final value of around 4.2 g N l^{-1}. In digester T2 the TAN rose more gradually from day 441 over a period of about 200 days to reach its target of 2.5 g N l^{-1} (Figure 4.16a), followed by a further increase to around 3 g N l^{-1}.



a) TAN

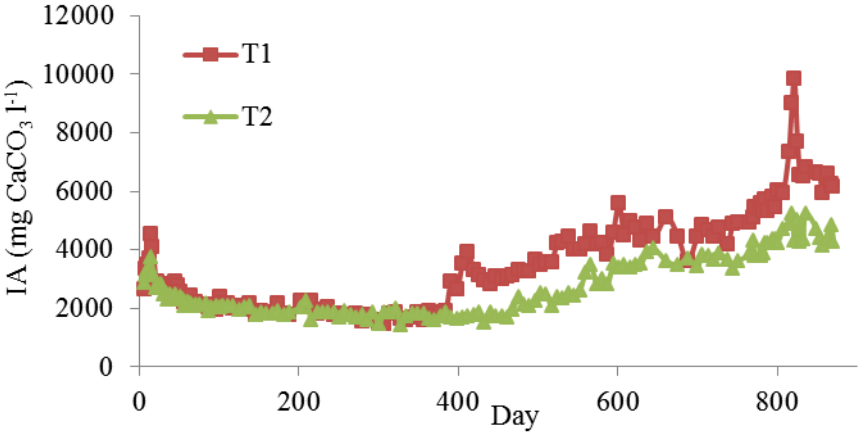


b) Total VFA

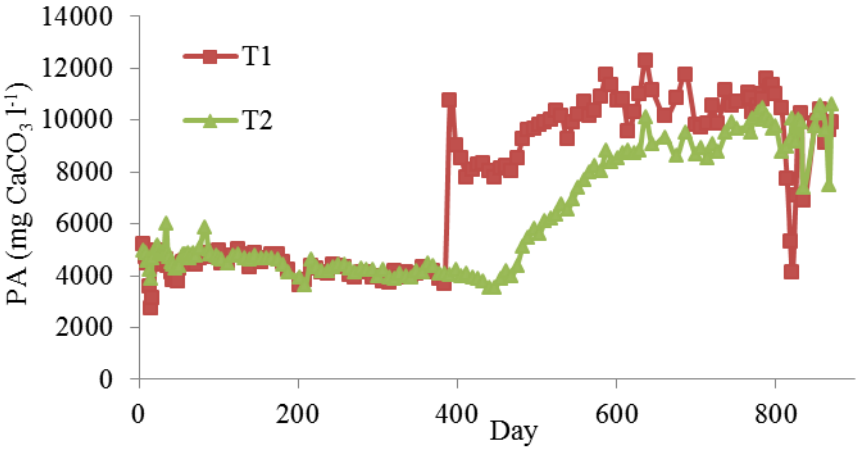


c) pH

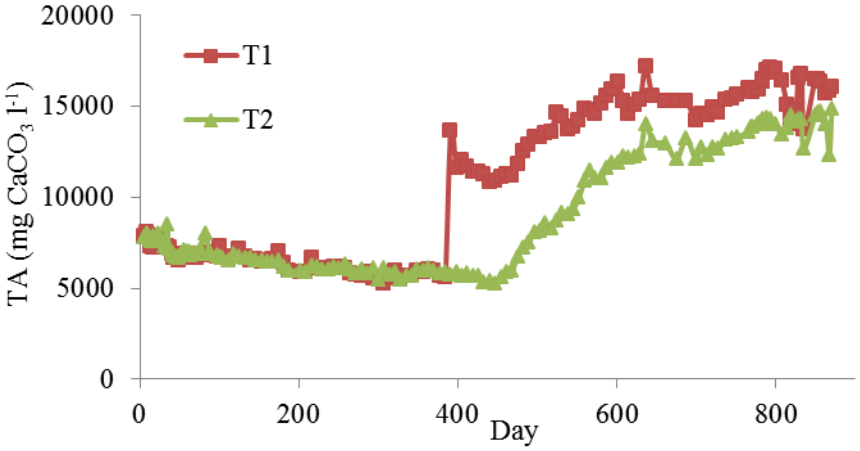
Figure 4.16 Monitoring parameters in T1 and T2 (low-N food waste) throughout the experimental period



d) IA

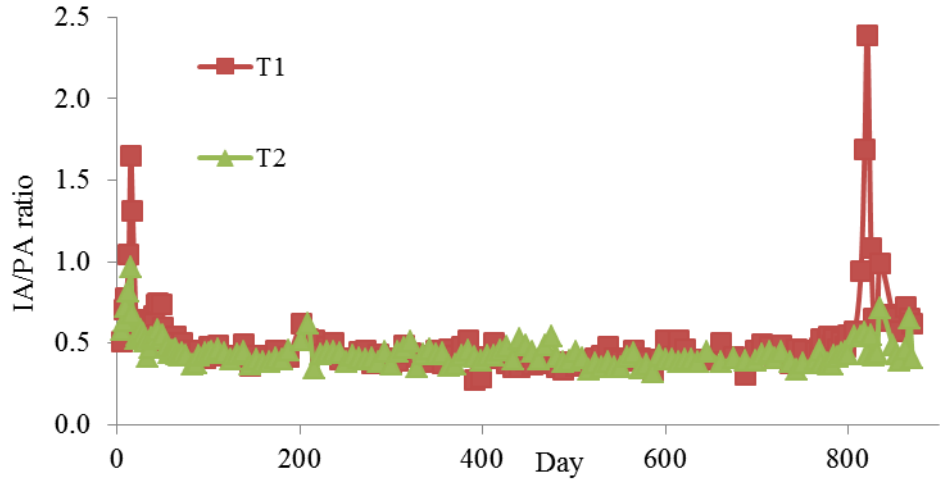


e) PA

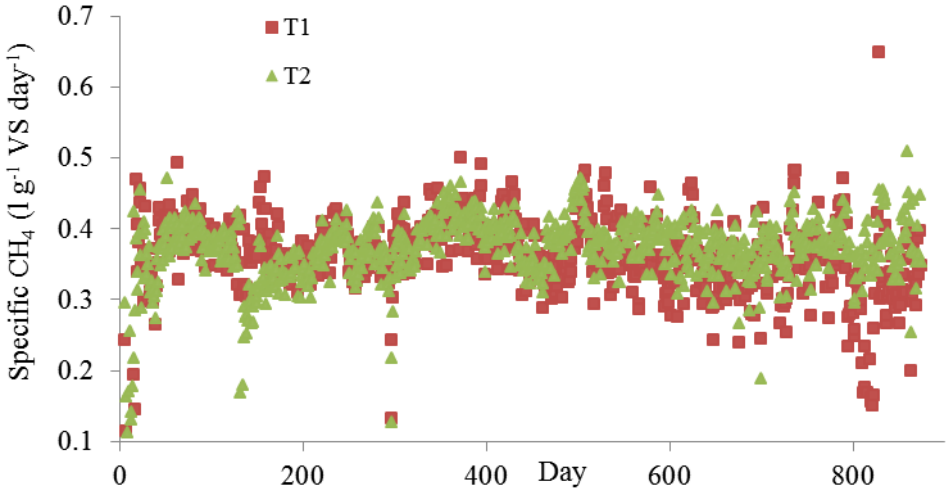


f) TA

Figure 4.16 Monitoring parameters in T1 and T2 (low-N food waste) throughout the experimental period (continued)



g) IA/PA ratio



h) Specific methane production

Figure 4.16 Monitoring parameters in T1 and T2 (low-N food waste) throughout the experimental period (continued)

The response to these increases in TAN in both digesters can be seen in the total VFA in Figure 4.16b and Figure 4.17. In T1 there was an immediate increase in total VFA to around 4000 mg l^{-1} when the initial urea spike was added, but this declined to <500 mg l^{-1} as the TAN concentration fell to ~2.2 g N l^{-1}. This VFA peak was buffered by the increased TAN, resulting in a rise in pH (Figure 4.16c). IA continuously rose from around 2 g $CaCO_3$ l^{-1} and peaked to around 10 g $CaCO_3$ l^{-1} and then remained at ~6 g $CaCO_3$ l^{-1} (Figure 4.16d). PA also increased from ~4 g $CaCO_3$ l^{-1} and rose above 10 g $CaCO_3$ l^{-1} (Figure 4.16e). TA, as same as IA and PA, rose from ~6 to ~14-16 g $CaCO_3$ l^{-}

[1] (Figure 4.16f). There was little or no change in the IA/PA ratio (Figure 4.16g) which remained below 0.5, or in the SMP which continued at ~0.40 l CH_4 g^{-1} VS (Figure 4.16h). Addition of urea to the feed from day 464 led to a gradual increase in TAN and once it reached around 2.5 g N l^{-1} VFA peaks of up to 3300 mg l^{-1} again began to appear. These were characterised by sharp increases in propionic acid, followed by even sharper falls (Figure 4.17a); acetic acid was also slightly elevated but the fluctuations in concentration were less pronounced. A slight reduction in TAN to below 3.5 g N l^{-1} between day 687-737 was accompanied by a fall in propionic acid concentration. When the TAN rose above ~3.5 g N l^{-1} after day 750 the magnitude of the VFA peaks increased and at a concentration of 4 g N l^{-1} there was no further decline in propionic acid, and peaks in n- and iso-butyric appeared along with a rise in the iso-valeric concentration to around 800 mg l^{-1}.

The more gradual increase in TAN in digester T2 had no effect on total VFA until the concentration rose to about 2.5 g N l^{-1} when total VFA peaks of around 1000 mg l^{-1} began to appear (Figure 4.17b), which were mainly composed of acetic acid. A further increase in TAN concentration to around 3.0 g N l^{-1} showed an increase in total VFA and the appearance of propionic acid peaks which showed the characteristic saw-tooth fluctuation observed in T1. Digestate pH increased in response to the urea addition (Figure 4.16c) and the average SMP showed little change with urea addition, from 0.39 to 0.36 CH_4 kg^{-1} VS. Likewise, digestate VS concentration remained at ~2.5%, corresponding to 88% VS destruction.

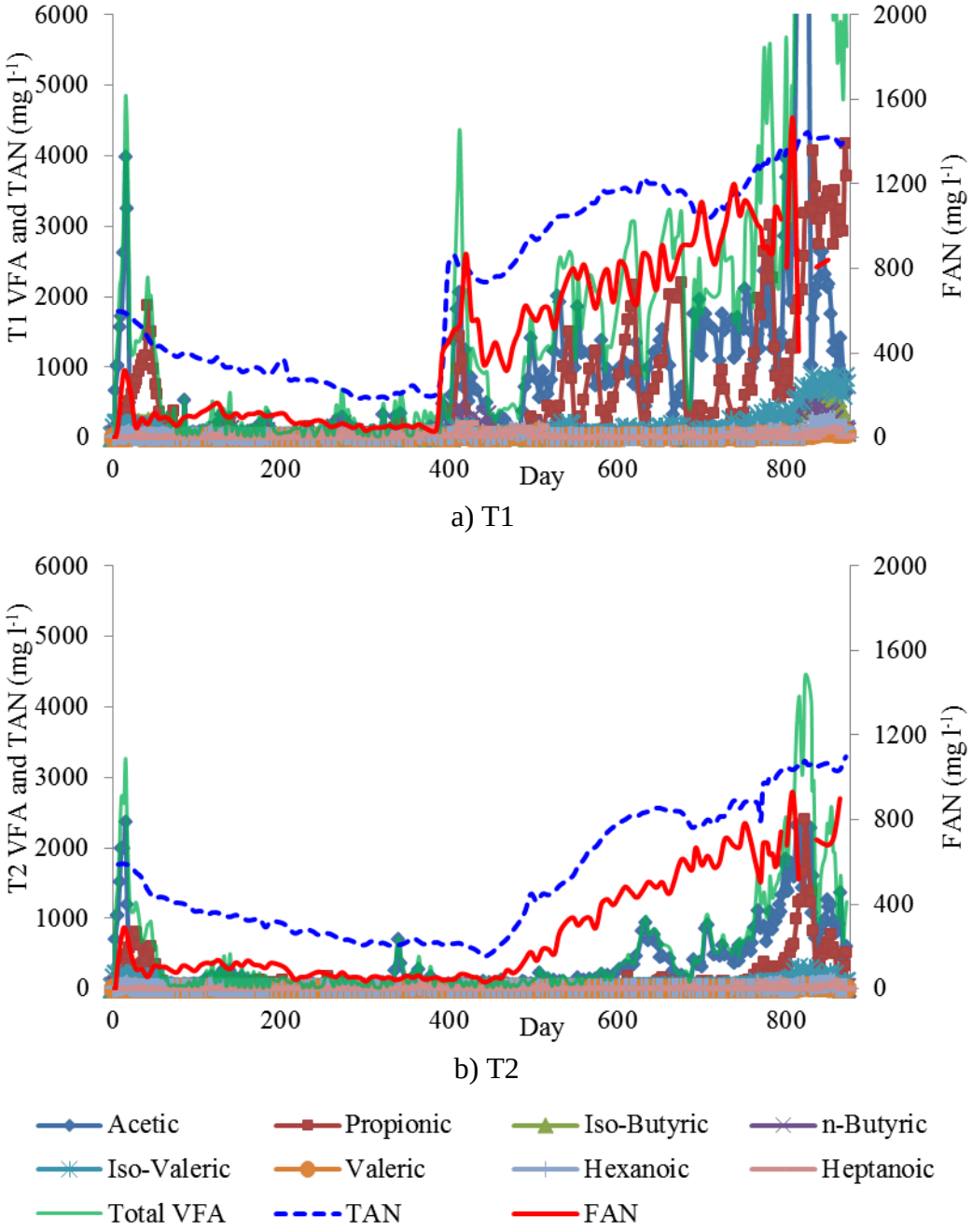


Figure 4.17 VFA profile and TAN / FAN concentration in digesters T1&2 (low N food waste) throughout the experimental period

Discussion

A step change in temperature from mesophilic to thermophilic temperatures followed by a period of starvation and then a gradual increase in load appeared to be an effective way of acclimating digester inoculum to thermophilic conditions. Running the digesters on high-nitrogen food waste without dilution was not possible in the long term, however. Although gas production continued at high total VFA concentration, the increase in VFA eventually overcame the buffering capacity of the digesters, resulting in a rapid decrease in pH and catastrophic failure. Very sharp increases in total VFA concentration were observed as the TAN concentration exceeded 3.5 g N l^{-1} and, although this could be partially overcome in the short term, there was no longer term solution to the accumulation of propionic acid. It was possible to run the digesters long term on a low-N food waste with good methane production and VS destruction. Raising the TAN of this feed material with urea provided a method of establishing the critical TAN concentration. It was possible to run a digester without significant accumulation of VFA at a TAN concentration of $\leq$ 2.5 g N l^{-1} but propionic acid started to accumulate at a TAN concentration above 3.0 g N l^{-1} and had become persistent at a concentration of 3.5 g N l^{-1}. Hashimoto (1986) observed that ammonia inhibition began at about 2.5 g N l^{-1} and 4 g N l^{-1} for unacclimatised and acclimatised thermophilic methanogens, respectively; while previous studies have shown that acclimatisation to high ammonia concentrations is possible (Angelidaki and Ahring, 1994, Borja et al., 1996, Gallert and Winter, 1997, Sung and Liu, 2003). In the current study, however, there was no real evidence of adaptation despite the prolonged operating periods. The results suggested that acclimatisation to thermophilic conditions is possible on a far shorter timescale than to high TAN concentrations. As inoculum for seeding large-scale digesters is generally taken from mesophilic digesters treating municipal wastewater biosolids, this presents a significant issue for start-up of thermophilic AD plants processing source separated domestic food waste and other high-nitrogen feedstocks.

The experiment also compared the results of mesophilic and thermophilic AD of normal food waste. Both sets of digesters fed on normal food waste had similar TAN concentrations under the different temperature conditions, indicating hydrolysis of proteins was equally effective in both cases while the digesters were operating 'normally' i.e. in a stable or meta-stable state. The performance indicators: pH, IA/PA

ratio, gas compositions, and specific methane productions between the two sets of digesters were, however, very different. The mesophilic AD digesters had consistent pH, a low IA/PA and smooth uninterrupted gas production with a constant proportion of methane. The thermophilic digesters showed early signs of failure as indicated by the rising acetic acid at day 112 which led to complete failure in T3 although T4 recovered for a period of time. The higher temperature makes the process more sensitive to ammonia concentrations (Angelidaki and Ahring, 1994) because of the higher proportion of free ammonia present within the total. It had been reported that under thermophilic conditions, the requirements for TE might be higher (Uemura, 2010) and increasing the concentration was used as part of the recovery strategy, but with only a limited success. It cannot, therefore be concluded that the reason for the recovery was due to the TE addition as this did not lead to a reduction in concentration of all the accumulated acids. Even if the TE had played a role in the recovery of digester T4 in the long term additional TE supplementation cannot prevent acid accumulation and eventual digester failure. It was clearly seen that the composition of food waste influenced the performance of the AD, especially the nitrogen content by the comparison of the kerbside collected food waste with the low nitrogen synthetic food waste under thermophilic conditions. The VFA profile of thermophilic AD with low-N food waste was quite similar to the mesophilic digesters, with a slight difference in that the thermophilic system showed a small but recoverable build-up of propionic acid at the beginning of the trial. After the initial acclimatisation, the VFAs remained lower than 500 mg l^{-1} throughout the experiment. Thermophilic anaerobic digestion can therefore acclimate using the conditions applied and provide a stable means of treating biodegradable waste at an influent TS content of around 23% provided that it has low nitrogen content.

4.3 Effect of temperature increment in mesophilic anaerobic digestion

The previous experiments had shown that long-term stable operation of thermophilic digestion was not possible on a feedstock of normal food waste. The objective of the current experiment was to determine at what temperature instability arose by gradually increasing the temperature until VFA accumulation was observed.

4.3.1 Aim

The aim of this work was to assess the effect of increasing the digestion temperature, with a view to identifying the main factors causing instability.

4.3.2 Method

This experiment used digesters M1-4 and followed on immediately from the comparative study in section 4.2.4 above. The same batch of normal food waste was used as feedstock, and the OLR was maintained at 4 and 3 g VS l^{-1} day^{-1} for M1&2 and M3&4, respectively. Digester temperature was gradually increased from 35 ^{o}C to 43 ^{o}C by raising the set point of the thermostatic controller: actual temperature was recorded manually at intervals using a thermistor thermometer (model 2046T, Digitron, UK) submerged to a fixed depth of around 3 cm below the digestate surface, immediately before feeding or digestate removal. On day 297 the temperature was raised from 35 to 36 ^{o}C and for the next 6 weeks it was increased in steps of 1 ^{o}C per fortnight. The timing of temperature rises was then adjusted according to the monitoring results before reducing the temperature again to 35 ^{o}C in a single step on day 582. Monitoring of digester pH, ammonia nitrogen, alkalinity, TS and VS, gas production and gas composition continued as before. The trial ran for a total of 340 days.

4.3.3 Results

Figure 4.18 shows the measured digestate temperatures during the days 297-582 of the experimental period. It can be seen that there were some day-to-day variations depending on the ambient temperature as well as small differences between digesters, with M3 consistently around 0.5-1.0 ^{o}C higher than the others.

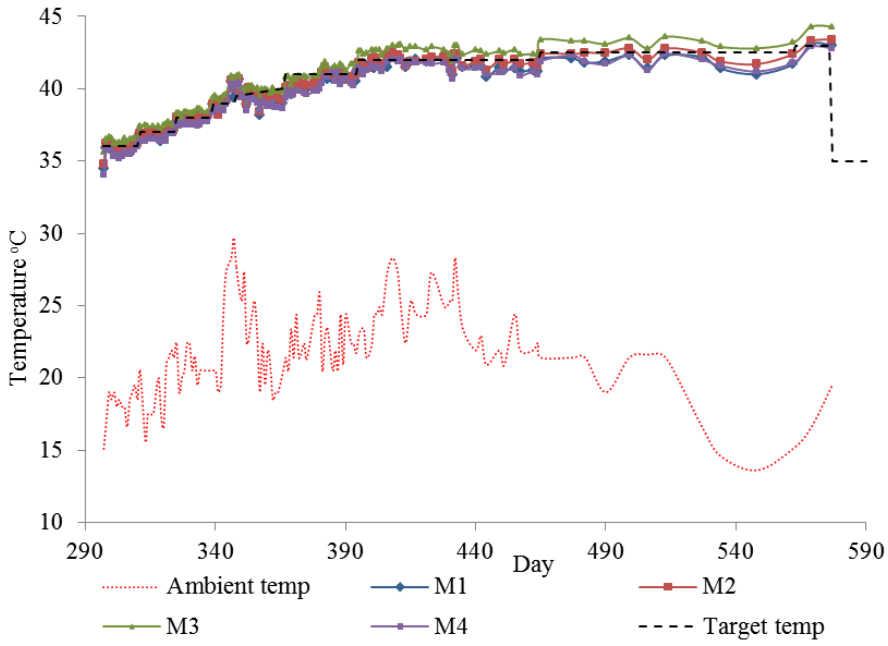


Figure 4.18 Temperature profiles of M1-M4 with ambient temperature from day 297-636

The main effect of the temperature rise was a rapid increase in VFA concentrations at around 40 ^{o}C (Figure 4.19). This zone of instability has been noted by other researchers; 42 ^{o}C by Agler et al. (2010), 47 ^{o}C by Boušková et al. (2005). In this trial total VFA concentrations rose from below 500 mg l^{-1} to peak values of 5742, 10093, 4857, and 4335 mg l^{-1} for M1, M2, M3, and M4, respectively. The rise in VFA was reflected in the intermediate alkalinity (Figure 4.20a), but this was buffered by the increasing partial and total alkalinity (Figure 4.20b and c) with the result that the IA/PA ratio did not exceed 0.6 (Figure 4.20d), and pH remained relatively stable (Figure 4.20e). Biogas and methane production (Figure 4.20f-h) showed some fluctuations, with a fall corresponding to the period of VFA accumulation followed by a rise in volume and an increase in methane content associated with degradation of the accumulated VFA. TAN concentrations in all four digesters increased slightly with the rise in temperature from 35 to 40 ^{o}C, before stabilising at around 5.5 – 6.0 g N l^{-1} (Figure 4.20i). This accounted for the increase in alkalinity, and may have been due to enhanced hydrolysis: support for this view is given by a slight decrease in VS in the same period (Figure 4.20j and k). Similar results were reported by Komemoto et al. (2009) who found that increasing the temperature from 35 to 45 ^{o}C in the anaerobic digestion of food waste led to an increase in solubilisation (hydrolysis) from 70.0% to 72.7%. In

section 4.2.4 and 4.2.5 of the current study, before the onset of failure the VS content in thermophilic digesters was slightly lower than that in mesophilic digesters fed on the same feedstock and at the same OLR (see Figure 4.14i and Figure 4.9j). The increasing temperature also affected the FAN concentration in the digesters which peaked at 1.4-1.8 g N l^{-1} around day 419-426 (Figure 4.20l).

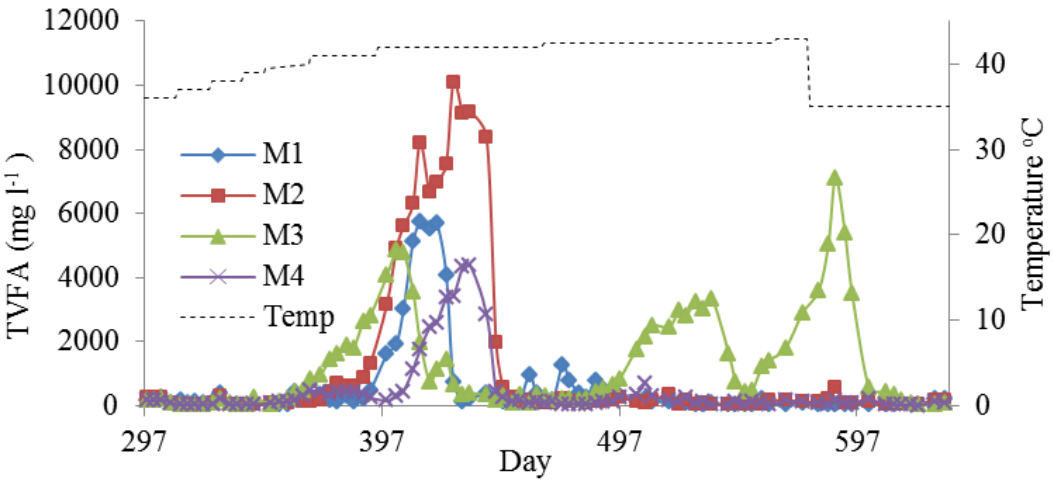
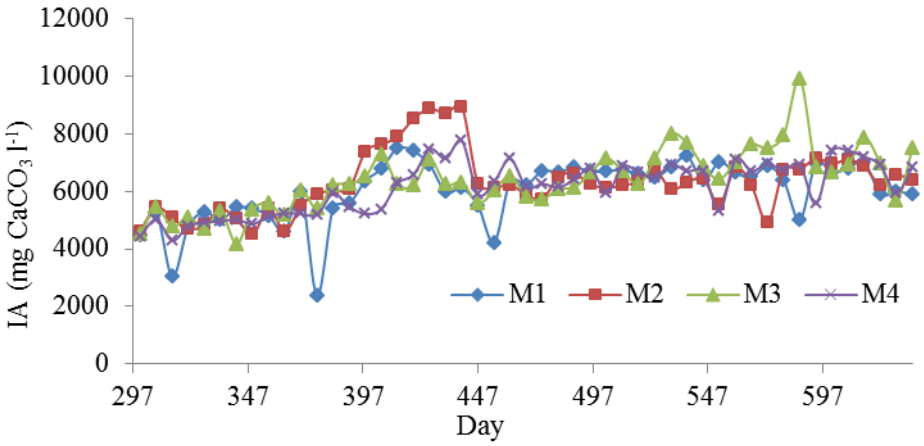
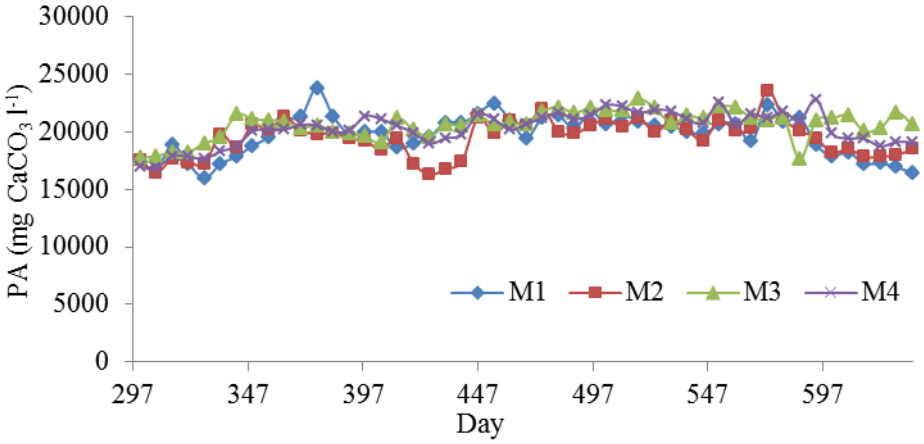


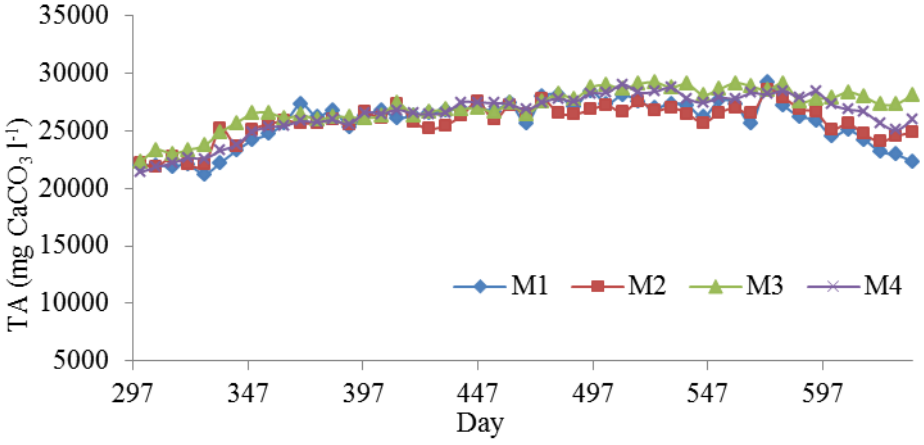
Figure 4.19 Total VFA and target temperature during experimental period



a) IA

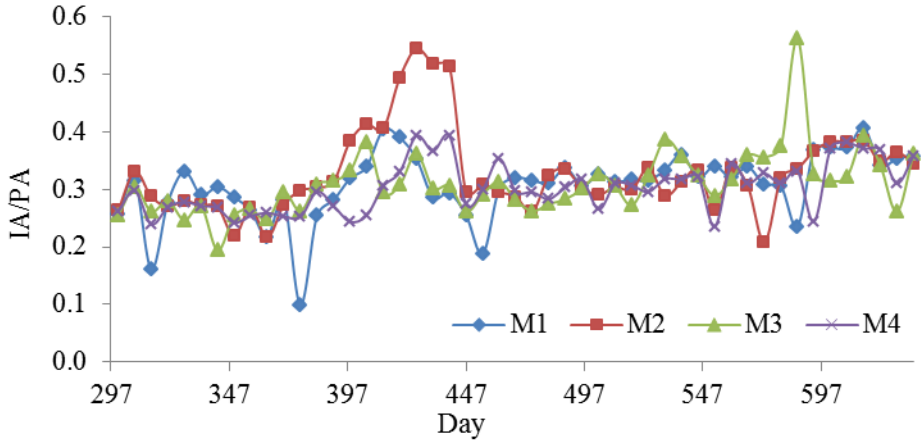


b) PA

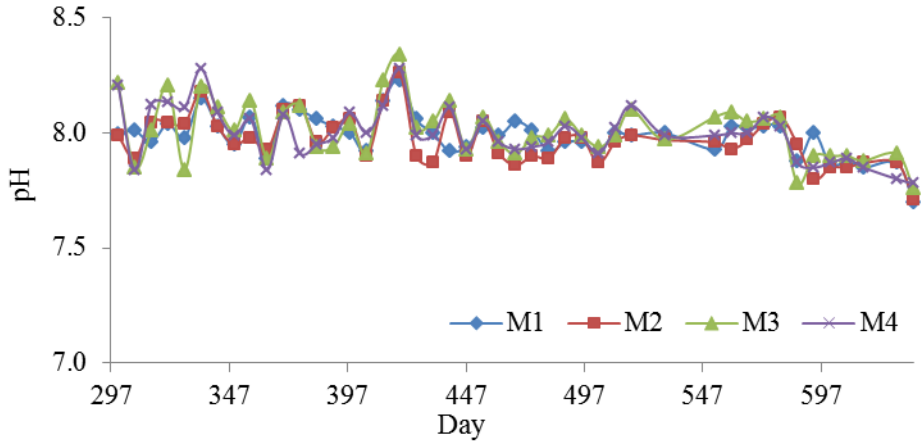


c) TA

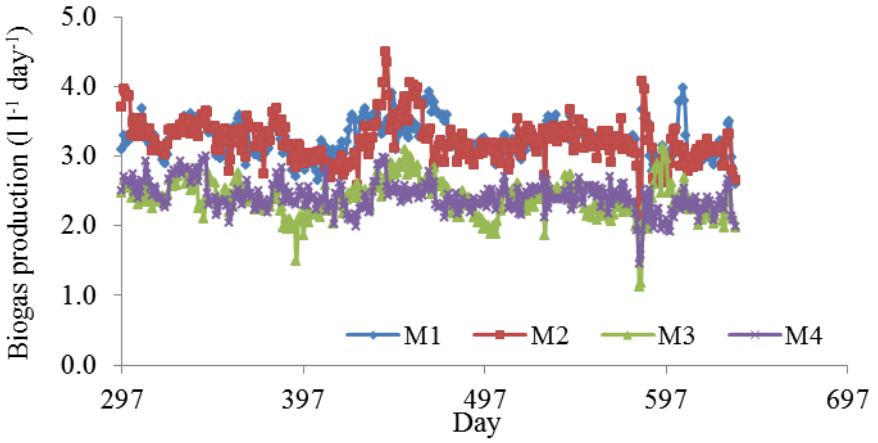
Figure 4.20 Monitoring parameters for digesters M1-M4 during experimental period



d) IA/PA ratio

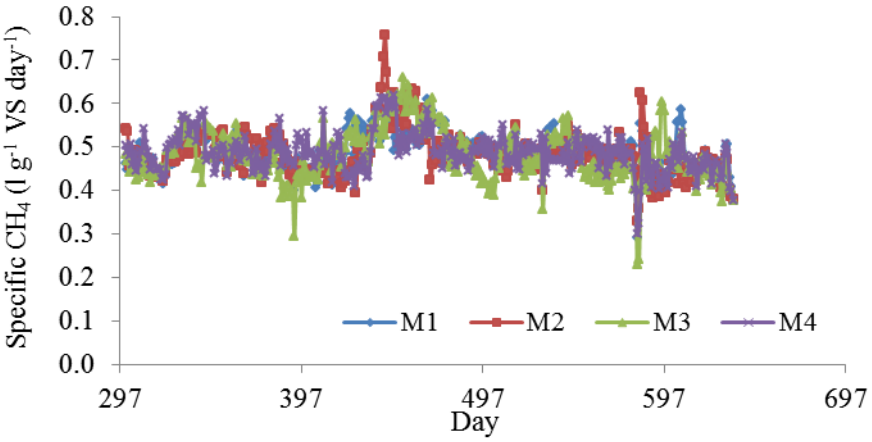


e) pH

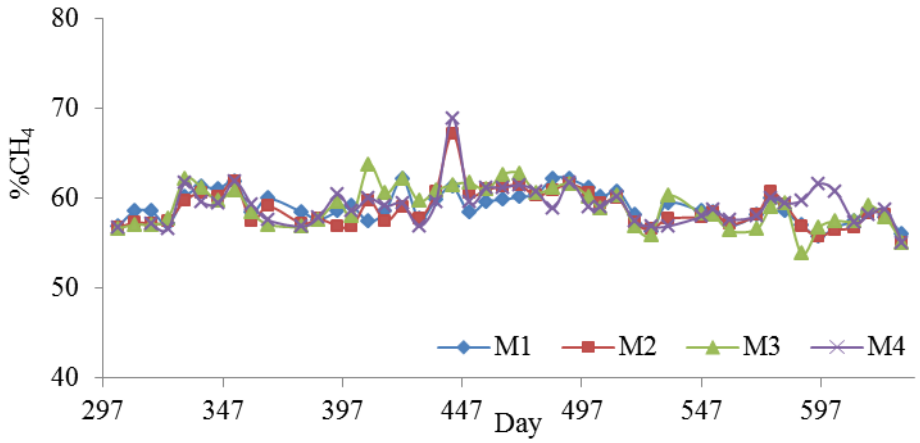


f) Volumetric biogas production

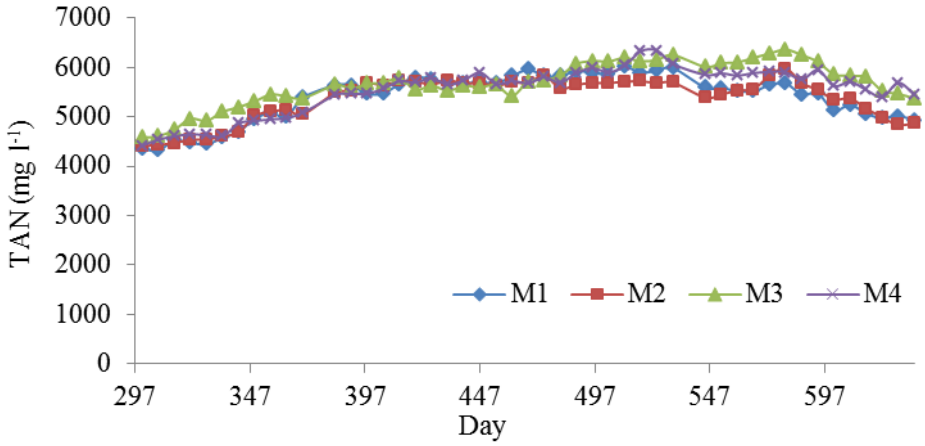
Figure 4.20 Monitoring parameters for digesters M1-M4 during experimental period (continued)



g) Specific methane production

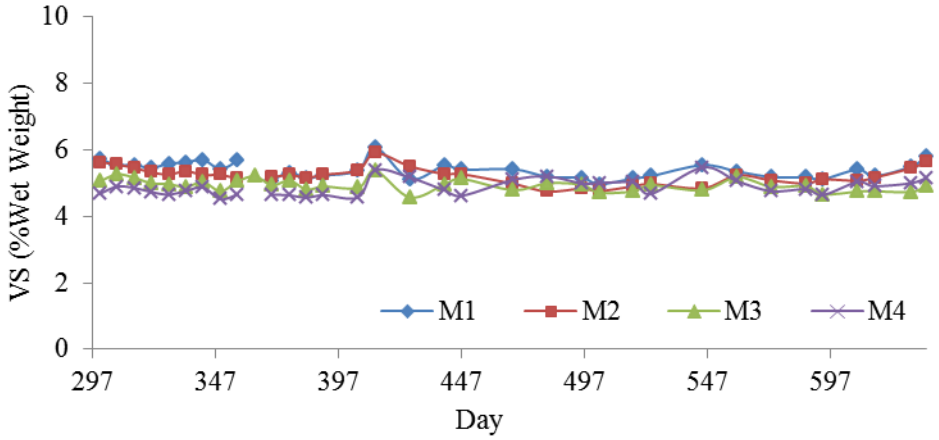


h) Methane percentage

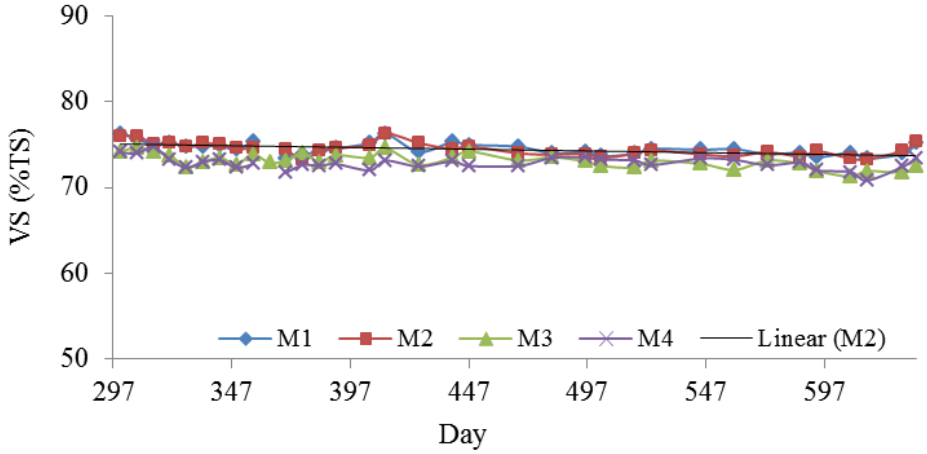


i) TAN

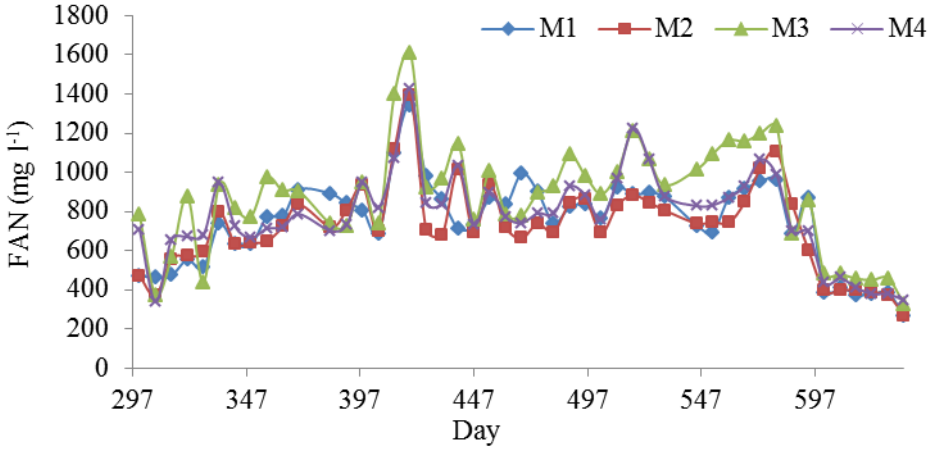
Figure 4.20 Monitoring parameters for digesters M1-M4 during experimental period (continued)



j) VS (%wet weight)



k) VS (%TS)



l) FAN

Figure 4.20 Monitoring parameters for digesters M1-M4 during experimental period (continued)

VFA profiles for each digester are shown in Figure 4.21 and it can be seen that the predominant acid causing the main VFA peak of was propionic, with smaller peaks in acetic acid associated with periods of rapid propionic degradation. After reaching the peak, VFA concentrations fell sharply to average values of < 200 mg l^{-1}. From day 395 to day 464 the target temperature was maintained at 42 ^{o}C, by which time the VFA peak had passed in all four digesters. An increase of around 0.5 – 1 ^{o}C on day 464 had little or no effect on three of the digesters, but in M3 which was operating at a slightly higher temperature (Figure 4.18) a second VFA peak occurred then a third, with an increase in acetic acid concentration. It can be seen that in each case VFA accumulation occurs when the free ammonia concentration exceeds ~800 mg N l^{-1} (Figure 4.20l), as proposed by Calli et al. (2005a). VFA concentrations in digesters M1, M2 and M4 reduced and stabilised when FAN remained close to this critical value, but in M3 the higher temperature led to a longer period when the free ammonia was consistently higher, producing the characteristic saw-tooth pattern of VFA peaks seen in the previous thermophilic digestion trial (Figure 4.15 and Figure 4.17a). It should also be considered, however, that several authors have suggested the upper boundary of optimal mesophilic temperature is around 40 ^{o}C (Chernicharo, 2007, Khanal, 2008, Batstone and Jensen, 2011). Without any stress, mesophilic digesters could possibly be operated optimally at higher temperatures. For example, Boušková et al. (2005) showed stable performance of mesophilic anaerobic digesters fed with mixed primary and activated sludge up to 47 ^{o}C, when signs of VFA accumulation occurred. Clear effects from temperature increase in the presence of ammonia were shown in the research of Angelidaki and Ahring (1994), who ran digesters at 2.5 and 6.0 g N l^{-1} and decreased the temperature from 55 to 40 ^{o}C in steps of 5 ^{o}C. The 2.5 g N l^{-1} digester showed smooth and stable performance without any symptoms of stress. In contrast, the 6.0 g N l^{-1} digester showed obvious stress with high VFA accumulation. Therefore, it can be said that in unstressed conditions, mesophilic anaerobic digestion can operate in the critical temperature range (40-47 ^{o}C). In this research, however, the combination of the raised ammonia concentration and the critical temperature range caused signs of stress in the digesters.

It can be said that the higher temperature affected the digesters by changing the conditions for the microbes and also by increasing the free ammonia nitrogen. Further

temperature increase beyond 43 ^{o}C would lead to instability of the type seen in the thermophilic trials, despite the relatively long acclimation periods.

After the temperature was reduced to 35 ^{o}C on day 582, there was a sharp fall in free ammonia in all 4 digesters and a rapid drop in VFA in M3 (Figure 4.21c). The effect of the sudden change could also be seen in a fall in pH, gas production and methane content (Figure 4.20e-h) and a rise in IA/PA ratio and VS on a % wet weight and % TS basis (Figure 4.20d, j-k). The effects of this disturbance were still evident at the end of the run on day 636. To have a clarifying result from the effects of temperature change, it is recommended that the same experiment is carried out with digesters fed on low-nitrogen foodwaste: this may be considered as future work.

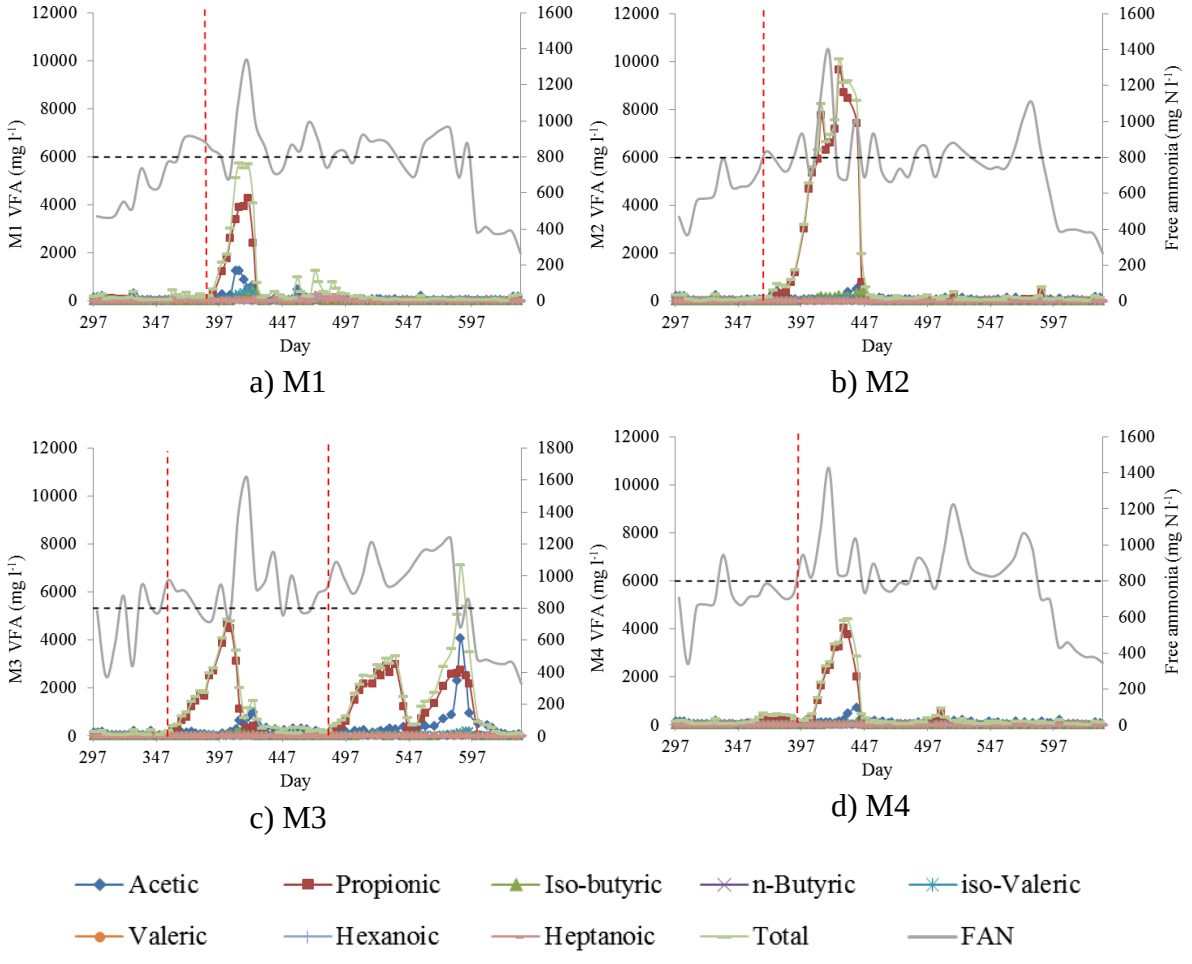


Figure 4.21 VFA profiles and FAN in digesters M1-4 during the experimental period
Note: horizontal grey dotted line corresponds to FAN concentration of 800 mg N l^{-1}, vertical red dotted line to onset of rapid VFA accumulation

4.3.4 Conclusion

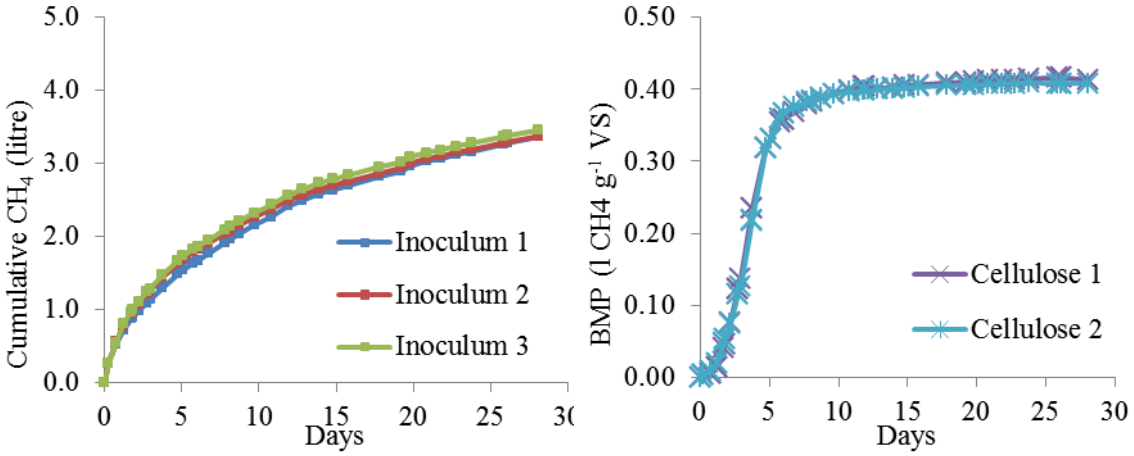
The results of the experiments described above confirmed that the onset of instability (as shown by rapid VFA accumulation) occurred at around 40 ^{o}C, as reported by previous researchers. In the case of food waste, it seems likely that this is driven by increasing temperature which also increased FAN to around 800 mg N l^{-1} and that therefore acclimatisation to thermophilic temperature is unlikely to be achieved by this means.

4.4 Energy comparisons

4.4.1 Biochemical Methane Potential (BMP) test

Biogas generated in the reactors during the BMP test was collected and its composition was analysed each time the collection cylinders were emptied. Although steps were taken to minimise dissolution of gases by using acidified saline water, a proportion of the CO_2 is lost in this way resulting in an apparently higher methane concentration than that actually generated. Methane concentration in this study reached around 70%. Methane is relatively insoluble, and therefore the results are expressed as methane yield.

Quality assurance. Figure 4.22 shows the cumulative specific methane yield for the inoculum-only blanks and the positive controls. By day 28, the methane production for the three inoculum-only controls was 0.109, 0.110 and 0.112 l CH_4 g^{-1} VS, quite similar to results previously obtained for this inoculum (unpublished data, University of Southampton). The specific methane yield of the two positive controls was 0.416 and 0.408 l CH_4 g^{-1} VS_{added} respectively, very close to theoretical value (from the Buswell equation) of 0.415 l CH_4 g^{-1} VS_{added}, indicating a healthy inoculum and good control. Taken together these results confirm the suitability of the assay conditions.



(a) Inoculum-only controls (b) Positive controls

Figure 4.22 Cumulative methane productions of inoculum-only controls and the specific methane production of positive controls (cellulose) in food waste BMP test

The BMP tests was set up in triplicate, but for each of the test substrates one digester showed signs of blockage and/or leaking, and these results were therefore not taken into consideration. The results of the test are shown in Figure 4.23 and

Table **4.8**. Both pairs showed good replication, with R^2 of 0.9910 and 0.9924 for normal food waste (Normal FW) and low-N food waste (Low-N FW) sets, respectively. The methane yields of normal food waste and low-N food waste were 0.471 and 0.379 l CH_4 g^{-1} VS_{added} (average for last 5 days), respectively. The BMP of normal food waste was slightly higher than that of 0.456 l CH_4 g^{-1} VS_{added} found by Zhang et al. (2012a) for similar source segregated domestic food waste. The BMP of normal food waste was higher than the low-N food waste one, reflecting the higher content of protein and lipid in substrate leading to a higher percentage of C and H (Table 4.4).

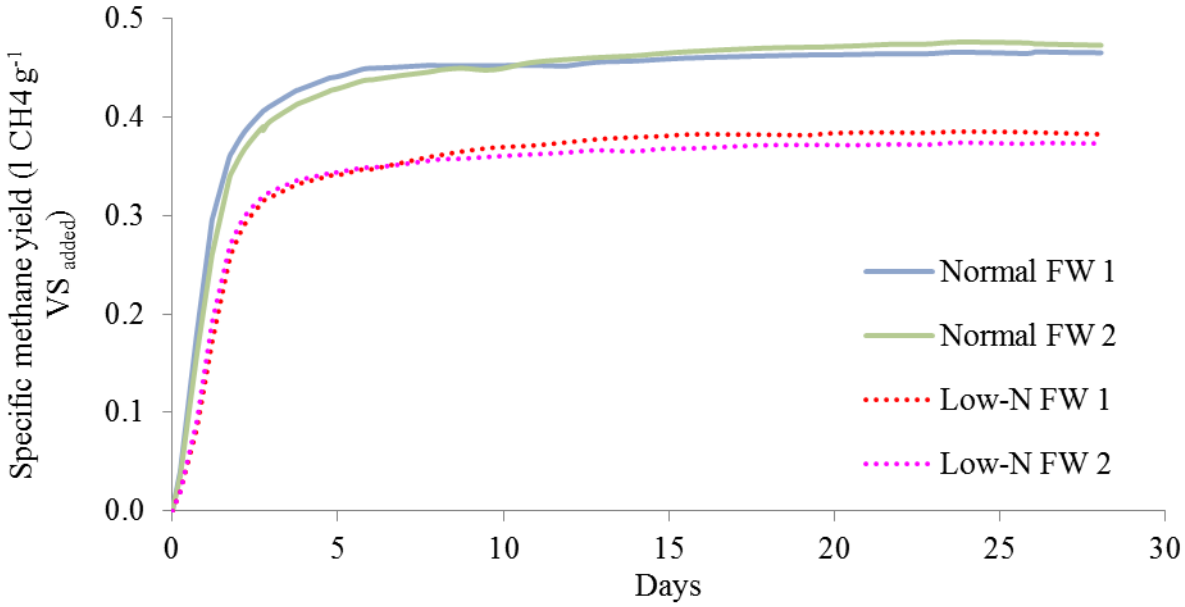


Figure 4.23 BMP of normal food waste (Normal FW 1, 2) and synthetic low nitrogen food waste (Low-N FW 1, 2)

Table 4.8 BMP values for normal and low nitrogen food waste

Substrates	Specific biogas ($l\ g^{-1}VS_{added}$)		Specific methane ($l\ CH_4\ g^{-1}VS_{added}$)	
	Average (last 5 days)	SD	Average (last 5 days)	SD
Normal FW 1	0.783	0.003	0.466	0.001
Normal FW 2	0.762	0.001	0.475	0.001
Average	**0.772**		**0.471**	
Low-N FW 1	0.667	0.001	0.385	0.000
Low-N FW 2	0.670	0.003	0.374	0.001
Average	**0.669**		**0.379**	

4.4.2 Energy consideration

The methane production in batch and semi-continuous trials was converted and compared with the calorific values (CV) of the feedstocks based on their elemental compositions (CHON) using the Buswell equation, the Dulong equation, their biochemical characteristics (carbohydrate, protein, lipid and fibre), and the calorific values from bomb calorimeter analysis. The results are shown in Table 4.9.

Table 4.9 Energy comparison between low-N and normal food waste

Analysis	Substrates	SMP ($l\ CH_4\ g^{-1}VS$)	CV* ($kJ\ g^{-1}\ VS$)
Calculated values from the Buswell equation			
Low-N FW		0.476	19.0
Normal FW		0.563	22.4
Calculated values from the Dulong equation			
Low-N FW	-	-	19.0
Normal FW	-	-	22.6
Calculated values from biochemical compositions			
Carbohydrates	Low-N FW	0.230	9.2
Lipids	Low-N FW	0.013	0.5
Crude proteins	Low-N FW	0.045	1.8
Hemicellulose	Low-N FW	0.021	0.8
Cellulose	Low-N FW	0.134	5.4
Lignin	Low-N FW	0.004	0.1
total		**0.448**	**17.8**
Carbohydrates	Normal FW	0.218	8.7
Lipids	Normal FW	0.153	6.1
Crude proteins	Normal FW	0.106	4.2
Hemicellulose	Normal FW	0.028	1.1
Cellulose	Normal FW	0.028	1.1
Lignin	Normal FW	0.012	0.5
total		**0.545**	**21.7**
Measured calorific values			
Low-N FW	-	-	18.0
Normal FW	-	-	23.2
Biochemical Methane Productions (BMP)			
Low-N FW 1	Low-N FW	0.379	15.1
Normal FW 1	Normal FW	0.471	18.8
Mesophilic AD (loading 2 g VS l^{-1} day^{-1})			
M3	Normal FW	0.458	18.2
M4	Normal FW	0.450	17.9
Thermophilic AD (loading 2 g VS l^{-1} day^{-1})			
T1	Low-N FW	0.398	15.9
T2	Low-N FW	0.392	15.6
T3	Normal FW	0.408	16.3
T4	Normal FW	0.436	17.4
%CV from SMP of mesophilic and thermophilic AD to the measured CV			
M3	Normal FW		78.6
M4	Normal FW		77.3
T1	Low-N FW		88.2
T2	Low-N FW		86.8
T3	Normal FW		70.1
T4	Normal FW		74.9
%SMP of mesophilic and thermophilic AD to the BMP			
M3	Normal FW		97.2
M4	Normal FW		95.5
T1	Low-N FW		88.2
T2	Low-N FW		86.8
T3	Normal FW		70.1
T4	Normal FW		74.9

Note: [a] the Dulong equation according to the method in Combustion file 24 (IFRF, 2013)

* Calorific value of CH_4 at 0 ^oC and 101.325 kPa = 39.84 MJ m^{-3} (BS EN ISO 6976, 2005).

[b] Values for mesophilic AD are the average for day 146-176 before increasing the OLR from 2 to 3 g VS l^{-1} day^{-1}, and for thermophilic AD from days 70-100 before VFA accumulation. Only values from M3 and M4 are shown as the OLR of M1 and M2 was increased to 3 g VS l^{-1} day^{-1} in this period.

The calorific values calculated from the Buswell and Dulong equations were in very good agreement (~100%), while those based on the biochemical composition were slightly lower (~5% lower than the Buswell and Dulong equations' values). For normal food waste the Buswell and Dulong values were in closest agreement with the measured CV, while for low-N food waste the biochemical composition was slightly closer. In all cases, however, the agreement was good enough to provide support for the accuracy of the analytical results.

In the BMP test, the calorific value of the methane produced by the low-N food waste was 15.1 kJ g^{-1} VS, equivalent to 84% of the measured CV; the equivalent values for normal food waste were 18.8 kJ g^{-1} VS and 81%, reflecting its higher protein and lipid content and slightly lower degradability. The specific methane production from normal food waste in semi-continuous mesophilic digestion trials was only slightly lower, equivalent to around 78% of the measured calorific value and 96% of the BMP value, again confirming the rapid degradability and good energy yield of this substrate. The value of 78% of CV also corresponds well to the measured VS destruction of 76-78% for M3 and M4. In meta-stable thermophilic conditions the specific methane production for normal food waste was lower and less consistent, at 70% of measured CV for T3 and 75% for T4. For the low-N food waste the specific methane production in semi-continuous thermophilic conditions was around 87% of the measured CV, in close correspondence to the VS destruction of ~88%; and at 15.9 and 15.6 kJ g^{-1} VS for T1 and T2 respectively was slightly higher than the BMP value of 15.1 kJ g^{-1} VS added. As the BMP test was conducted in mesophilic conditions this may confirm the slightly higher degree of conversion achieved at 55 ^{o}C. In general the results confirm the very high degree of conversion and energy recovery achievable with this type of substrate.

4.5 Conclusions

- In the initial trials in thermophilic conditions, anaerobic digestion of food waste led to VFA accumulation and eventually to failure, probably due to high TAN concentrations. A second attempt gave closely similar results.
- Thermophilic anaerobic digestion of low-nitrogen food waste showed smooth and stable performance with no sign of VFA accumulation, confirming that the high nitrogen content of normal food waste was a major cause of the digestion failure.
- Mesophilic anaerobic digestion of normal food waste with trace element addition

presented very stable performance with no sign of VFA accumulation even though the TAN was above 4 g N l^{-1}, a toxic level in thermophilic conditions. It can be concluded that thermophilic anaerobic digestion has less ammonia toxicity tolerance than mesophilic anaerobic digestion.

- Addition of trace elements in the concentrations successfully applied in mesophilic digestion of food waste could postpone the onset of VFA accumulation in thermophilic conditions, but could not prevent it. A higher TE dose (4 times that used in mesophilic anaerobic digestion) may have aided in recovery from an episode of rapid acidification, but was unable to prevent irreversible accumulation of VFA in the longer term.
- A temperature of 40 ^{o}C was the starting point for unstable performance as indicated by VFA accumulation. This appeared to be associated with higher FAN concentrations, and may be due to changes in the microbiological population which will be discussed in Chapter 5.

Chapter 5 FISH Analysis

5.1 Introduction

This chapter discusses changes in the methanogenic population of the experimental digesters in relation to the changing conditions described in Chapter 4: in particular with respect to increasing total ammonia nitrogen (TAN) for thermophilic AD and increasing temperature for mesophilic AD. The first section presents observations from the 3-HRT period before any changes were applied to the digesters. These results thus provide baseline data for comparison with later sections. The following section shows the results after increasing the temperature and compares them with the previous period in mesophilic AD. The comparison of mesophilic- and thermophilic- AD is then discussed. The last section considers the overall results for thermophilic AD.

Although samples were taken and fixed weekly, only selected samples for the first 3 HRT of operation were analysed, using the fluorescent labelled probes as described in Chapter 3 (Table 3.1).

5.2 FISH from Inoculum

Table 5.1 shows the population structure of the mesophilic inoculum: in each case the methanogenic population groups are reported as the percentage of each probe compared to ARC 915 (universal archaea). The main methanogenic groups were *Methanosaetaceae*, then *Methanococcales* and *Methanobacteriales*, respectively. *Methanococcales* and *Methanobacteriales* consume H_2/CO_2 whereas *Methanosaetaceae* (Figure 5.1) consume acetate. In conjunction with the chemical characteristics (also reported in section 4.2.3), it is clear that the inoculum was microbiologically healthy as reflected by the abundant variety of methanogens. This implied that substrate degradation could follow both the acetoclastic and the syntrophic routes. Although it was only present as a minority, *Methanomicrobiales* (as shown in Figure 5.2) later showed its significance.

Table 5.1 Proportion of archaea with chemical characteristics of inoculum

Archaea	Proportion	Chemical characteristics of inoculum	Value
Methanosaetaceae	>60%	pH	7.26
Methanosarcinaceae	<10%	TAN (mg N l^{-1})	1393
Methanobacteriales	<20%	Intermediate alkalinity (mg $CaCO_3$ l^{-1}): IA	2238
Methanomicrobiales	<10%	Partial alkalinity (mg $CaCO_3$ l^{-1}) :PA	4789
Methanococcales	<20%	IA/PA ratio	0.47
		Total VFA (mg l^{-1})	53

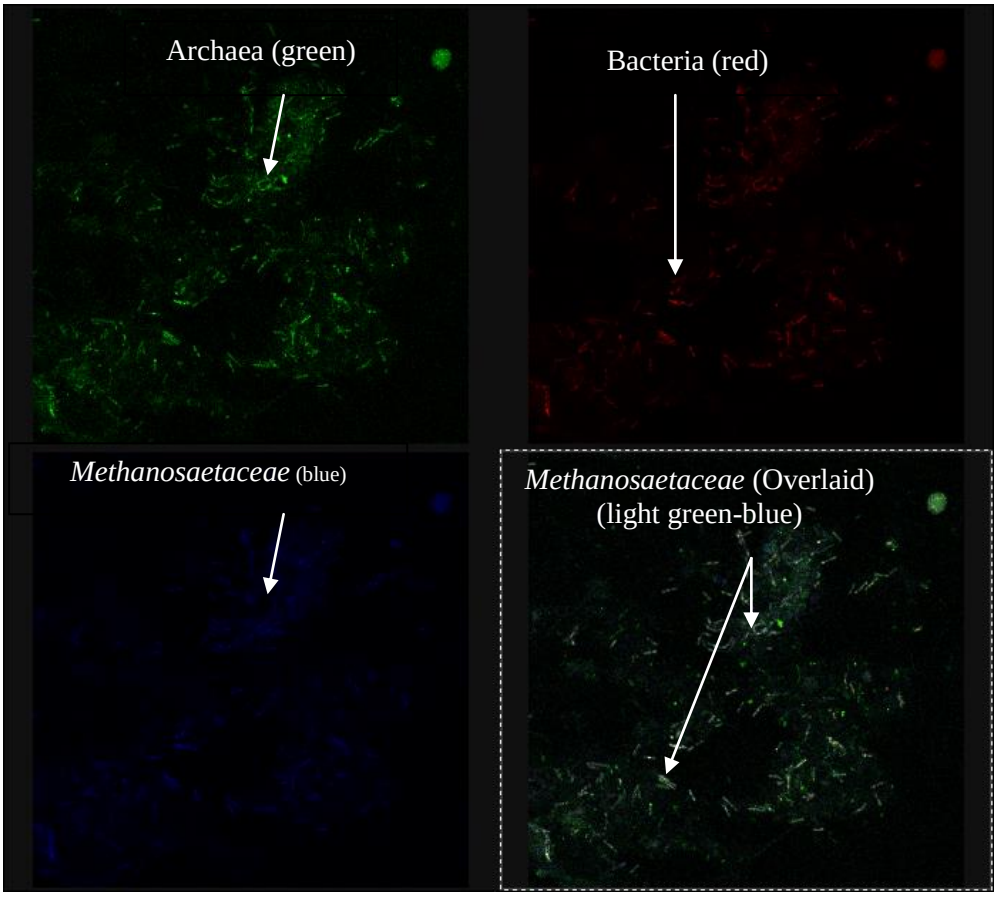


Figure 5.1 *Methanosaetaceae* in inoculum

Note: The inoculum sample from day 0 was hybridised with the probes ARC915 for archaea, EUB338 and EUB338+ for bacteria and MX825 for *Methanosaetaceae.* The images were taken by confocal laser scanning microscope (Leica TCS SP2, Germany)

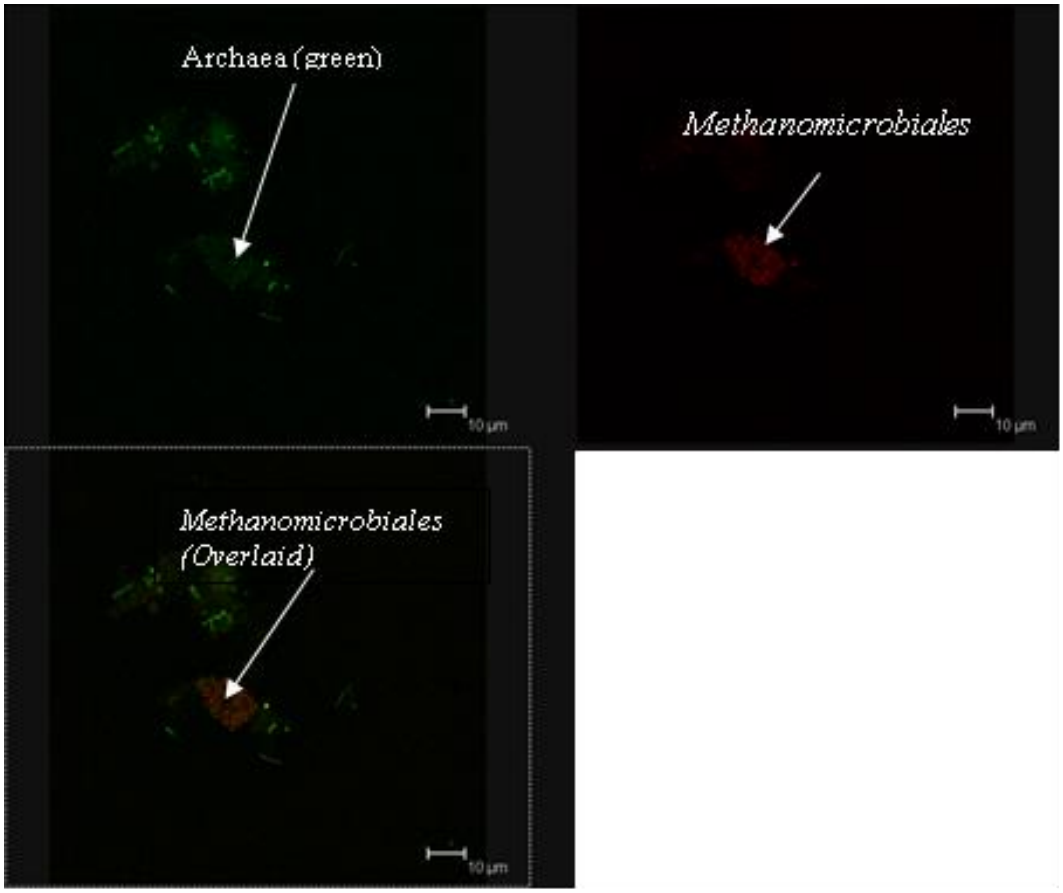


Figure 5.2 *Methanomicrobiales* in inoculum

Note: The inoculum sample from day 0 was hybridised with the probes ARC915 for universal archaea and MG1200 for *Methanomicrobiales.* The images were taken by confocal laser scanning microscope (Leica TCS SP2, Germany)

5.3 FISH from Mesophilic AD before temperature increment trial

This section presents the results for the baseline period from day 0 to day 209 for M1 (Table 5.2) and day 265 for M3 (Table 5.3), respectively. Starting with a healthy inoculum, the mesophilic reactors continued working well throughout the baseline period (for details see section 4.2.4). With increasing TAN concentration, however, the population changed. Initially, *Methanosaetaceae* with *Methanococcales* and *Methanobacteriales* were dominant, followed by *Methanosarcinaceae,* and *Methanomicrobiales*, respectively. This pattern persisted to day 55, with the methanogenic groups maintaining the same percentages. Interestingly, even during the 'disturbance' period around day 55 which was marked by rising VFA and IA/PA ratios, there was no change in the methanogen profiles in either reactor (Table 5.2 and Table

5.3), with *Methanosaetaceae* remaining dominant. The dominant population then changed to *Methanosarcinaceae* on day 97 and 118 for M1, and on day 73, 90 and 111 for M3, respectively. In M3 from day 146, *Methanomicrobiales* emerged as dominant with *Methanosaetaceae* falling until day 209 while the percentage of *Methanosarcinaceae* remained steady.

In M1 the OLR was raised from 2 to 3 g VS l^{-1} day^{-1} between day 117-176, and then from 3 to 4 g VS l^{-1} day^{-1} between day 177-209. Although an increase in *Methanomicrobiales* was also seen in M1 on day 174 and 209, the pattern of change in this reactor was less clear. From day 237-601 samples from M1 showed a very limited positive response in which the fluorescence emission could not be properly detected; whereas samples from M3, where the OLR lagged behind that in M1 (Table 5.3), still showed a positive response. Therefore, reporting and discussion from day 237 on is based on the results from M3 solely. This phenomenon of weak hybridisation signals, especially at high FAN concentrations, was also noted by Calli et al. (2005b) in mesophilic UASB treating synthetic wastewater at elevated TAN concentrations (from 1-6 g N l^{-1}) and at an OLR of 1.2 g COD l^{-1} day^{-1}.

From day 237 to 265, the dominant group in M3 remained *Methanomicrobiales* (>80%) followed by *Methanosarcinaceae* (<30%) and with *Methanosaetaceae*, *Methanococcales*, and *Methanobacteriales* (< 10%) present as minorities. Excluding the disturbance period, the performances of M1 and M3 were very stable in terms of IA/PA ratio and SMP with low VFA while TAN and FAN was increasing.

Table 5.2 Methanogen community structure profile and chemical characteristics in M1

Operation time (days)	*Methanosaetaceae (%)*	*Methanosarcinaceae (%)*	*Methanomicrobiales (%)*	*Methanobacteriales (%)*	*Methanococcales (%)*	IA/PA	SMP (l CH_4 g^{-1} VS day^{-1})	Acetic acid (mg l^{-1})	Propionic Acid (mg l^{-1})	Total VFA (mg l^{-1})	TAN (mg l^{-1})	FAN (mg N l^{-1})
0	> 60	< 10	< 10	< 20	< 20	0.49	-	39	3	216	1400	37
34	> 60	< 10	< 10	< 20	< 20	0.40	0.381	40	58	114	2140	84
55	> 60	< 10	< 10	< 20	< 20	0.89	0.356	4244	61	4608	2530	99
97	< 20	> 70	~ 20	< 10	< 10	0.32	0.454	114	34	189	3240	342
118	~ 20	> 70	< 20	< 10	< 10	0.20	0.417	88	104	301	3560	392
174	~ 50	< 30	< 30	< 10	< 10	0.28	0.490	81	10	92	4060	495
209	> 30	< 50	< 30	< 10	< 10	0.35	0.516	78	0	78	4260	556
237~ 601	Very limited positive response so that fluorescence emission was not detectable											

Table 5.3 Methanogen community structure profile and chemical characteristics in M3

Operation time (days)	*Methanosaetaceae (%)*	*Methanosarcinaceae (%)*	*Methanomicrobiales (%)*	*Methanobacteriales (%)*	*Methanococcales (%)*	IA/PA	SMP (l CH_4 g^{-1} VS day^{-1})	Acetic acid (mg l^{-1})	Propionic Acid (mg l^{-1})	Total VFA (mg l^{-1})	TAN (mg N l^{-1})	FAN (mg N l^{-1})
0	> 60	< 10	< 10	< 20	< 20	0.48	-	34	0	48	1400	30
34	> 60	< 10	< 10	< 20	< 20	0.42	0.348	478	14	510	2180	66
55	> 60	< 10	< 10	< 20	< 20	0.59	0.476	2698	16	3099	2560	193
73	< 20	> 70	~ 10	< 10	< 10	0.34	0.466	87	18	104	2900	198
90	< 20	> 70	~ 20	< 10	< 10	0.32	0.449	58	5	200	3100	289
111	< 20	> 70	< 10	< 10	< 10	0.29	0.439	38	2	46	3670	465
146	~ 30	< 30	> 50	< 10	< 10	0.27	0.412	25	0	28	4090	572
174	~ 20	< 30	> 70	< 10	< 10	0.25	0.437	136	9	155	4280	590
209	~ 10	< 30	> 80	< 10	< 10	0.38	0.455	37	0	37	4330	605
237	< 10	< 30	> 80	~ 10	< 10	0.27	0.500	36	0	36	4350	497
265	< 10	< 30	> 80	~ 10	< 10	0.27	0.455	40	0	44	4580	427
335	< 10	< 10	> 80	< 10	< 10	0.27	0.509	49	2	65	5100	1105

Table 5.3 Methanogen community structure profile and chemical characteristics in M3 (continued)

Operation time (days)	*Methanosaetaceae* (%)	*Methanosarcinaceae* (%)	*Methanomicrobiales* (%)	*Methanobacteriales* (%)	*Methanococcales (%)*	IA/PA	SMP (l CH_4 g^{-1} VS day^{-1})	Acetic acid (mg l^{-1})	Propionic Acid (mg l^{-1})	Total VFA (mg l^{-1})	TAN (mg N l^{-1})	FAN (mg N l^{-1})
363	< 10	< 10	> 80	< 10	< 10	0.25	0.482	129	328	474	5420	695
384	< 10	< 10	> 80	< 10	< 10	0.31	0.429	91	1674	1815	5650	883
419	< 10	< 10	> 80	< 10	< 10	0.31	0.455	782	315	1152	5550	1863
447	< 10	< 10	> 80	< 10	< 10	0.26	0.580	256	22	289	5600	1334
475	< 10	< 10	> 80	< 10	< 10	0.26	0.540	157	0	234	5750	1060
510	< 10	< 10	> 80	< 10	< 10	0.31	0.464	265	2180	2512	6200	1183
545	< 10	< 10	> 80	< 10	< 10	0.32	0.490	378	362	754	6020	1372
573	< 10	< 10	> 80	< 10	< 10	0.36	0.426	721	2072	2889	6290	1411
601	< 10	< 10	> 80	< 10	< 10	0.32	0.454	534	36	595	5860	582
615	< 10	< 10	> 80	< 10	< 10	0.39	0.398	172	3	175	5804	553

Note: The temperature trial phase started from day 279, the data from day 335 belonged to the temperature trial experiment.

From the results in Table 5.2 and Table 5.3, the methanogen profiles can be separated into 3 ranges according to the TAN concentration:

First range: TAN < 2500 mg N l^{-1}, *Methanosaetaceae* was dominant. As an acetoclastic methanogen, *Methanosaetaceae* may account for the low concentrations of VFA observed, especially acetic acid. Karakashev et al. (2005) reported similar results with *Methanosaetaceae* dominant at low concentrations of TAN and VFA.

Second range: 2500 < TAN < 3500 mg N l^{-1}, *Methanosarcinaceae* was dominant. *Methanosarcinaceae* consumes a variety of substrates (Garcia et al., 2000, Worm, 2010) and also tolerates higher ammonia concentrations than *Methanosaetaceae* (Karakashev et al., 2005). *Methanosarcinaceae* was found to be dominant at high ammonia concentrations while conversely *Methanosaetaceae* was in the majority at low

concentrations (Karakashev et al., 2005). *Methanosarcinaceae* has a higher specific growth rate than *Methanosaetaceae* (Schmidt and Ahring, 1999, Conklin et al., 2006) and this may explain its dominance in this range.

Third range: TAN > 4000 mg N l^{-1}, *Methanomicrobiales* was dominant. It is known that acetoclastic methanogens are more sensitive to ammonia toxicity than hydrogenotrophic methanogens (Koster and Lettinga, 1984, Sprott and Patel, 1986, Angelidaki and Ahring, 1993, Borja et al., 1996, Procházka et al., 2012). This may explain why the acetoclastic groups disappeared and the population was dominated by hydrogenotrophic methanogens. It may be said that acetoclastic methanogens were inhibited above 4000 mg N l^{-1} TAN, which is similar to the reports from Hobson and Shaw (1976), Hashimoto (1986) and Angelidaki and Ahring (1991). Moreover, it has been claimed that *Methanomicrobiales* is very resistant to ammonia toxicity (Angenent et al., 2002).

The next question was, without acetoclastic methanogens, why did the VFA remain low, especially the acetic acid? Why was methane production still normal? It is known that acetic acid is typically converted to biogas by the acetoclastic pathway, which is thermodynamically favourable with ΔG^0 = -31.0 kJ mol^{-1} (Zinder, 1990, Hattori, 2008). There is, however, another pathway that could convert acetic acid to methane; the coupling of syntrophic acetate oxidizing bacteria and hydrogenotrophic methanogenesis. Considering each step separately, syntrophic acetate oxidation is not thermodynamically favourable (ΔG^0 = +104.6 kJ mol^{-1}, Zinder, 1990; Hattori, 2008). But coupled with hydrogenotrophic methanogenesis, ΔG^0 = -135.6 kJ mol^{-1}, the process is feasible with ΔG^0 = -31.0 kJ mol^{-1} (Zinder, 1990; Hattori, 2008). Since the acetoclastic methanogens were almost absent because of ammonia inhibition, the coupling of syntrophic acetate oxidation and hydrogenotrophic methanogenesis is likely to have played a more important role converting both the acetic acid and the H_2/CO_2 to methane with a thermodynamically favourable overall outcome (Hattori, 2008). This occurrence has also been found in other studies (Angelidaki and Ahring, 1993, Schnürer et al., 1999, Shigematsu et al., 2004, Karakashev et al., 2006, Hattori, 2008). It can be noted that the population shift did not affected the overall performance of mesophilic AD as shown by the stable pH, IA/PA, SMP and low concentration of VFA, as seen in Table 5.3.

Another interesting issue is the presence of *Methanosaetaceae* above a TAN concentration of 4000 mg N l^{-1}. As mentioned before, acetoclastic methanogens, especially the *Methanosaetaceae*, are sensitive to ammonia toxicity. Various values have been reported for the maximum ammonia concentration (mg N l^{-1}) at which *Methanosaetaceae* could survive: 560 (40 mM NH_4Cl) (Sprott and Patel, 1986), 1500 (Karakashev et al., 2005), 3600 (2.2% of *Methanosaetaceae* was found, Angenent et al., 2002), and 4000 mg N l^{-1} (Calli et al., 2005b). The maximum reported concentration was thus 4000 mg N l^{-1}; however, the authors stated that this *Methanosaetaceae (Methanosaeta sp.* (CM27)) was an uncultured species that was not found in the inoculum and was represented by a faint band (Calli et al., 2005b). In the current study *Methanosaetaceae* represented 10-30% of the population at TAN concentrations between 4090-4330 mg N l^{-1}, indicating that this is a tolerable range. Ammonia tolerance in *Methanosaetaceae* may be an interesting topic for future study.

5.4 FISH from Thermophilic AD

The results of observations from days 0-650 on T1 (the low nitrogen digester) and day 0-90 on T3 (the high nitrogen digester) are shown in Table 5.4 and Table 5.5, respectively. The dominant methanogens at day 0 were *Methanosaetaceae* (>60%). The longer the experiment went on, however, the smaller the proportion of the general microbial population that could be identified as archaea. This reduction may have been due to a number of different factors in the changing environment. Xia et al. (2012a) stated that a statistically significant difference was found in the compositions of archaeal communities fed with different feedstocks. Moreover, the number of years of operation and the management of the digesters were hypothesised as possible factors accounting for the different methanogenic profiles (St-Pierre and Wright, 2013).

It is known that a low acetate environment is favourable to *Methanosaetaceae* whereas *Methanosarcinaceae* prefers high acetate conditions (Conklin et al., 2006, Smith and Ingram-Smith, 2007). From Table 4.5 it can be seen that the acetic acid concentration in the inoculum was very low, in line with the dominance of *Methanosaetaceae* in the inoculum (as shown in Table 5.1); similar results have been seen in inoculum from other sources of anaerobic sewage sludge (McMahon et al., 2004, Karakashev et al., 2005, Ariesyady et al., 2007, Williams et al., 2013). According to Table 5.4,

Methanosaetaceae remained dominant until around day 90, when dominance shifted to *Methanosarcinaceae*: the shift may have occurred gradually but was clearly seen in the sample from T1 on day 90. It can be noted that in the first few days after the start of thermophilic anaerobic digestion, concentrations of VFA and especially of acetic acid rose sharply. The increasing VFA concentration may have affected the growth of *Methanosaetaceae*. Conklin et al. (2006) also proposed an acetate upper bound of 1.9 mM (114 mg l^{-1}) for *Methanosaetaceae*. The acetic acid concentration in this trial rose to almost 4000 mg l^{-1}, then declined to below 100 mg l^{-1} by around day 70. In this period, *Methanosaetaceae* was alive and growing but probably quite limited due to unfavourable conditions; meanwhile, the new conditions benefited *Methanosarcinaceae* which has a higher growth rate and biomass yield than *Methanosaetaceae* (Schmidt and Ahring, 1999, Conklin et al., 2006). In these conditions *Methanosarcinaceae* therefore out-competed *Methanosaetaceae*. This, however, suggests the question: in the low acetic acid conditions after day 90, why did *Methanosaetaceae* not become dominant over *Methanosarcinaceae*? A possible explanation is that the unfavourable conditions due to rising acetic acid caused slower growth for *Methanosaetaceae* but faster for *Methanosarcinaceae*. Also the faster doubling time of 1-2 days for *Methanosarcinaceae* compared to 4-9 days for *Methanosaetaecae* (Zinder, 1990) and the versatility of *Methanosarcinaceae* with respect to substrate, may have played a role in its ability to outcompete *Methanosaetaceae*. *Methanosarcinaceae* has the higher growth rate and biomass yield and started to grow faster than *Methanosaetaceae*. The work of Kundu et al. (2012) also showed that *Methanosaetaceae* was dominant in a mesophilic (35 ^{o}C) anaerobic digester fed on a synthetic substrate while *Methanosarcinaceae* was dominant in a thermophilic (55 ^{o}C) digester with the same substrate.

It is noteworthy that there was an increase in the proportion of *Methanosaetaceae* in M1 and M3 after VFA peaked during the disturbance period (Table 5.2 and Table 5.3). *Methanosaetaceae* was <20% on day 97 but increased to around 50% on day 174 in M1, and <20% on day 73 to around 30% on day 146 in M3. Further recovery and stabilisation was impossible, however, due to inhibition by ammonia concentrations above 3000 mg l^{-1} (Zeeman et al., 1985, De Vrieze et al., 2012).

As mentioned in section 3.5.2 quality assurance for FISH was provided by independent analysis of samples from T1 by Dr Louise Byfield (15 samples). The results of both sets

of observations were similar, providing some confidence in the observation and interpretation.

In T3, the initial dominance of *Methanosaetaceae* (Figure 5.3 and Figure 5.4) shifted to *Methanosarcinaceae* on day 90 (Table 5.5). When the TAN and VFA were above 4 g N l^{-1} and 15 g l^{-1} respectively, however, the ability to detect fluorescent emissions from samples decreased. A general reduction in apparent density of the methanogenic population due to reducing emissions was also observed throughout the experimental period. The difficulties in signal detection may be due to low physiological activity (cell functioning) (Moter and Göbel, 2000) or more probably to other sources of interference making the results unrepresentative and unreliable. Hence, no more results are presented for samples taken after day 90.

Table 5.4 Methanogen community structure profile and chemical characteristics in T1

Operation time (days)	Overall archae (%)	*Methanosaetaceae* (%)	*Methanosarcinaceae* (%)	*Methanomicrobiales*(%)	*Methanobacteriales* (%)	*Methanococcales* (%)	IA/PA	SMP ($l\ CH_4\ g^{-1}\ VS\ day^{-1}$)	Acetic acid ($mg\ l^{-1}$)	Propionic Acid ($mg\ l^{-1}$)	Total VFA ($mg\ l^{-1}$)	TAN ($mg\ l^{-1}$)	FAN ($mg\ l^{-1}$)
0	100	> 60	< 10	< 10	< 20	< 20	0.47	-	33	0	53	1400	-
6	90-100	> 70	< 10	< 10	< 10	< 10	0.51	0.243	1015	136	1486	1790	167
62	80-90	~ 70	< 10	< 10	< 10	< 10	0.45	0.393	79	104	243	1240	102
90	70-80	< 10	> 70	< 10	< 10	< 10	0.46	0.367	58	31	142	1200	111
125	~50	< 10	> 50	~ 20	~ 10	< 10	0.42	0.351	210	68	395	1140	191
153	~50	< 10	> 70	< 10	< 10	< 10	0.42	0.437	80	64	213	930	127
181	~50	< 10	> 50	< 10	~ 20	< 10	0.40	0.346	197	79	421	910	134
216	~50	< 10	> 80	< 10	~ 10	< 10	0.51	0.333	70	40	129	830	103
251	~50	< 10	> 80	< 10	< 10	< 10	0.41	0.339	73	25	147	790	99
279	30-40	< 10	> 80	< 10	< 10	< 10	0.37	0.350	190	57	399	650	75
398	30-40	< 10	> 80	< 10	< 10	< 10	0.29	0.335	149	16	234	2453	531
454	~20	< 10	> 80	< 10	< 10	< 10	0.37	0.337	123	11	225	2285	532
531	~20	< 10	> 80	< 10	<10	< 10	0.42	0.414	1930	442	2565	3144	622
615	-	< 10	> 70	< 10	<10	< 10	0.52	0.293	999	1856	3062	3490	756
650	~20	< 10	<10	>80	<10	< 10	0.40	0.332	1373	1065	2660	3582	825

Note: [1] chemical characteristic was obtained from inoculum characteristics

[2] the ammonia increment trial started from day 384, showed below the dash line.

Table 5.5 Methanogen community structure profile and the chemical characteristics in T3

Operation time (days)	Overall archae (%)	*Methanosaetaceae* (%)	*Methanosarcinaceae* (%)	*Methanomicrobiales* (%)	*Methanobacteriales* (%)	*Methanococcales* (%)	IA/PA	SMP ($l\ CH_4\ g^{-1}$ VS day^{-1})	Acetic acid ($mg\ l^{-1}$)	Propionic Acid ($mg\ l^{-1}$)	Total VFA ($mg\ l^{-1}$)	TAN ($mg\ l^{-1}$)	FAN ($mg\ l^{-1}$)
0	100	> 60	< 10	< 10	< 20	< 20	0.47	-	33	0	53	1400	151
6	90-100	~ 30	~ 20	~ 40	< 10	< 10	0.59	0.091	1086	151	1629	1760	157
20	90-100	~ 60	~ 20	~ 20	< 10	< 10	0.49	0.498	258	678	1415	1930	473
62	80-90	~ 40	~ 20	~ 40	< 10	< 10	0.42	0.379	631	463	1246	2620	260
90	~50	< 10	~ 50	~ 30	< 10	< 10	0.57	0.365	2167	1084	3785	3270	903

Note: [1] chemical characteristic was obtained from inoculum characteristics

Figure 5.3 and Figure 5.4 show *Methanosaetaceae* from the thermophilic anaerobic digester (T3) after 49 days of operation. At this time the TAN concentration was around 2 g N l^{-1}, the digester had a low VFA of 800 mg l^{-1} and acetogenotrophic methanogens were still present.

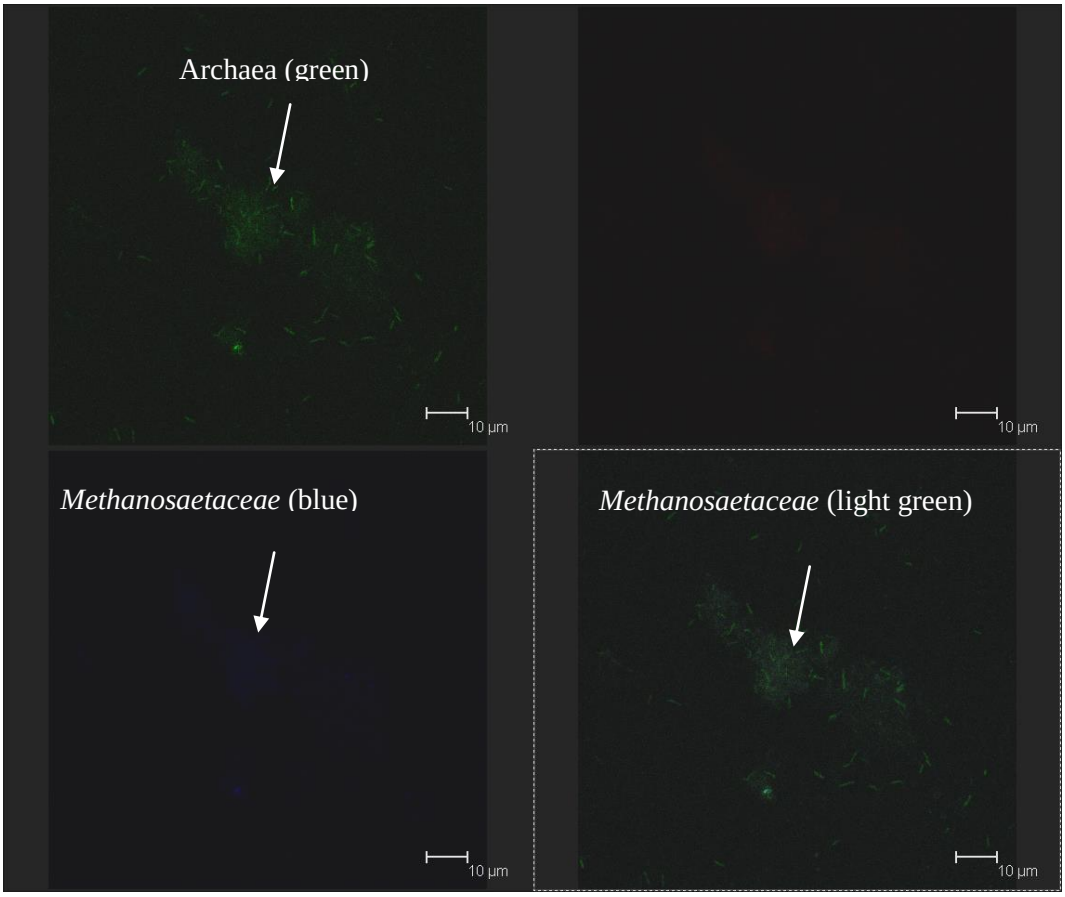


Figure 5.3 *Methanosaetaceae* in digester T3

Note: Thermophilic digester fed on normal food waste fed after 49 days of operation. The probes used in this examine were ARC 915 for archae and MX825 for *Methanosaetaceae.* The image was taken by from confocal laser scanning microscope (Leica TCS SP2, Germany).

It should be noted that the change of environment appeared to have an effect on the shape of *Methanosaetaceae*: in an unfavourable environment such as combined high ammonia nitrogen and temperature, *Methanosaetaceae* was observed as single rod cells instead of a filamentous floc.

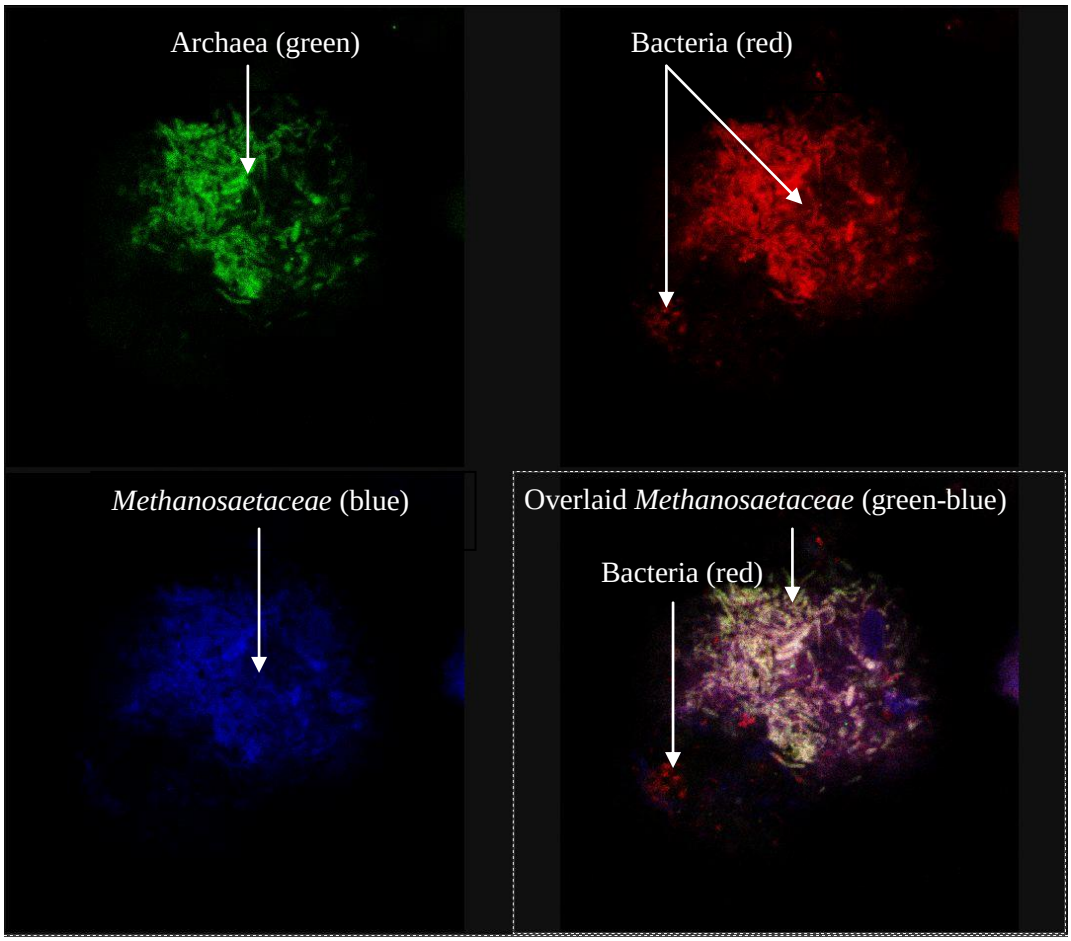


Figure 5.4 *Methanosaetaceae* (close up)

Note: This image shows a close-up of *Methanosaetaceae* from T3 digestate. The filamentous *Methanosaetaceae* are sometimes attached to bacteria, and/or somehow overlaid the bacteria and are reflected as white or pink/purple in this picture. The probes used were ARC 915 for archaea and MX825 for *Methanosaetaceae.* The image was taken by confocal laser scanning microscope (Leica TCS SP2, Germany)

It should be noted that the FISH images shown were mainly taken in the early period of the experiment. They showed some non-specific bonding such as the archaea probe attached to the bacteria cells. This phenomenon led to trials to adjust the method to suit the samples obtained from this experiment, e.g. by varying the stringency or incubating time. This suggests the need for future work to improve the methods for FISH analysis for the samples from anaerobic digestion of food waste.

5.5 FISH from Thermophilic AD with urea addition to low nitrogen food waste

In this section the effect of increasing the TAN concentration in T1 and T2 on the methanogenic population is considered. Prior to the addition of urea T1 and T2 showed good replication with similar performance (see section 4.2.5). During this baseline period the methanogen profile in T2 was assumed to be similar to that in T1. T1 was then given a shock load of urea to observe the effect of a sudden change in TAN on the methanogenic population, whilst the TAN concentration in T2 was increased more slowly. The results of FISH observations are shown in Table 5.4 and Table 5.6.

According to Table 5.4, the abrupt increase in TAN from around 600 to 2500 mg N l^{-1} did not cause a sudden change in the methanogenic population, which was still dominated by *Methanosarcinaceae* (>80% on day 398 and 454). The shock load only affected the overall percentage of methanogens, which reduced from 30-40 (day 398) to ~20% (day 454) (Table 5.4). *Methanosarcinaceae* (Figure 5.5) continued as dominant even though the TAN was above 3000 mg N l^{-1} (3144 mg N l^{-1} on day 531). The higher the TAN, the greater the percentage of *Methanomicrobiales* present: as mentioned before, *Methanomicrobiales* can tolerate TAN better than acetoclastic methanogens, in this case *Methanosarcinaceae.* From day 531, even though the VFA had started to rise, the performance was as good as in the previous period, with SMP of around 0.33 l CH_4 g^{-1} VS day^{-1} and an IA/PA ratio of ~0.4. When the TAN concentration rose a little higher to around 3600 mg N l^{-1}, the dominance shifted to *Methanomicrobiales* (>80%) on day 650. The shift was accompanied by a complete change in the pathway to hydrogenotrophic methanogenesis: the FISH observation was confirmed by ^{14}C testing on day 674 which gave a $^{14}CO_2/^{14}CH_4$ ratio of 1.98. It is likely that the shift in population started at around 3000 mg N l^{-1} of TAN, and completely shifted when it reached around 3500 mg N l^{-1}, or FAN above 800 mg N l^{-1}. Angelidaki and Ahring (1994) stated that FAN concentration of 700 mg N l^{-1} caused poorer process efficiency in thermophilic AD of cattle manure. The poor process might be a result of the shift or adaptation of methanogens in digesters.

To compare to the effect of the shock load in T1, TAN in T2 was slowly increased from around 600 to 1500 mg N l^{-1} and then to 2500 mg N l^{-1}. As reported in section 4.2.5.3,

up to a TAN concentration of 2500 mg N l^{-1} there was no major change in VFA (total VFA still < 500 mg l^{-1}) or in other parameters such as pH, IA/PA ratio, SMP. The sample on day 559 (TAN = 1837 mg N l^{-1}) was chosen for analysis as it had an intermediate TAN concentration between 1500 and 2000 mg N l^{-1}. As reactors T1 and T2 showed very good replication (see section 4.2.5.2 and Figure 4.12 and Figure 4.13), the structure of the methanogenic profile of T2 was assumed to be similar to T1 before the urea addition trial (day 0-279). Therefore Table 5.6 shows the chemical characteristics of T1 for day 0-279, then continues with the chemical characteristics and methanogenic profile of T2 on days 559 and 650.

According to Table 5.6, the population in T2 on day 559 was still dominated by *Methanosarcinaceae* (>80%), reflecting the TAN and FAN concentration of around 1800 and 360 mg N l^{-1}. The sample on day 650 with TAN of around 2500 mg N l^{-1} was chosen because of the rising acetic and propionic acid concentrations which were hypothesised to indicate some change in the methanogenic population. The FISH observation confirmed this, as the population shifted from *Methanosarcinaceae* (from >80 to ~20%) to *Methanomicrobiales* (from <10 to >60%). According to FISH analysis only, hydrogenotrophic methanogenesis was the main degradation pathway. The ^{14}C testing, however, gave a $^{14}CO_2/^{14}CH_4$ ratio of 0.32 which was considered to indicate acetoclastic methanogenesis, in contradiction to the observations from FISH. The likely explanation is that *Methanosarcinaceae* played a role as acetoclastic methanogens. At the sampling date, the population seemed to shift from *Methanosarcinaceae* to *Methanomicrobiales*. This may imply that, at this time, *Methanosarcinaceae* produced methane through the acetoclastic methanogenesis pathway.

The change from *Methanosarcinaceae* to *Methanomicrobiales* was similar to that seen in T1. The higher TAN or FAN resulted in a greater percentage of *Methanomicrobiales* due to the lower ammonia tolerance of *Methanosarcinaceae*.

Table 5.6 Methanogen community structure profile and the chemical characteristics in T2

Operation time (days)	Overall archae (%)	*Methanosaetaceae* (%)	*Methanosarcinaceae* (%)	*Methanomicrobiales* (%)	*Methanobacteriales* (%)	*Methanococcales* (%)	IA/PA	SMP (l CH_4 g^{-1} VS day^{-1})	Acetic acid (mg l^{-1})	Propionic Acid (mg l^{-1})	Total VFA (mg l^{-1})	TAN (mg l^{-1})	FAN (mg l^{-1})
0	100	> 60	< 10	< 10	< 20	< 20	0.47	-	33	0	53	1400	-
6	90-100	> 70	< 10	< 10	< 10	< 10	0.51	0.243	1015	136	1486	1790	167
62	80-90	~ 70	< 10	< 10	< 10	< 10	0.45	0.393	79	104	243	1240	102
90	70-80	< 10	> 70	< 10	< 10	< 10	0.46	0.367	58	31	142	1200	111
125	~50	< 10	> 50	~ 20	~ 10	< 10	0.42	0.351	210	68	395	1140	191
153	~50	< 10	> 70	< 10	< 10	< 10	0.42	0.437	80	64	213	930	127
181	~50	< 10	> 50	< 10	~ 20	< 10	0.40	0.346	197	79	421	910	134
216	~50	< 10	> 80	< 10	~ 10	< 10	0.51	0.333	70	40	129	830	103
251	~50	< 10	> 80	< 10	< 10	< 10	0.41	0.339	73	25	147	790	99
279	30-40	< 10	> 80	< 10	< 10	< 10	0.37	0.350	190	57	399	650	75
559	30	< 10	> 80	< 10	< 10	< 10	0.42	0.363	120	14	134	1837	363
650	30	< 10	~20	>60	<10	< 10	0.45	0.392	451	120	592	2556	566

Note: Data from day 0-279 was from T1. And the data of days 559 and 650 was from T2

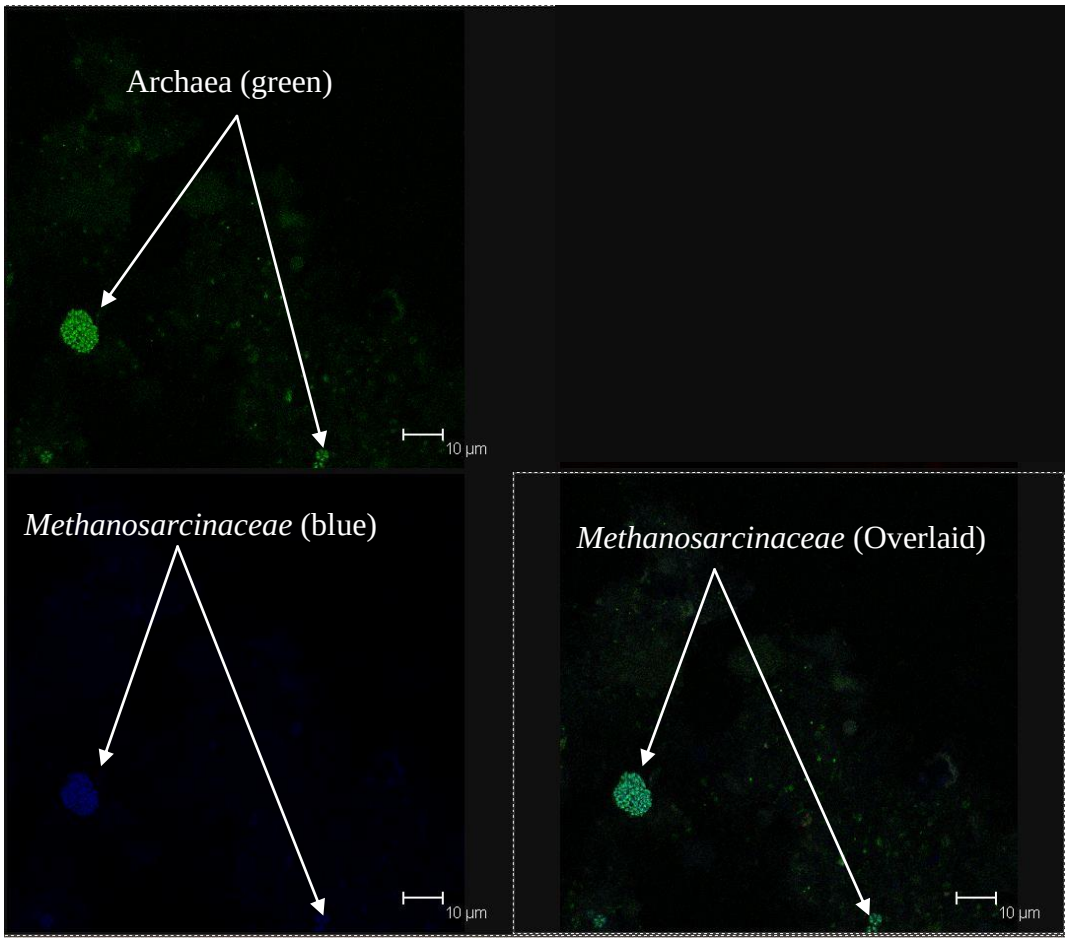


Figure 5.5 *Methanosarcinaceae* in T1

Note: The inoculum sample from day 153 was hybridised with the probes ARC915 for archae and MS1414 for *Methanosarcinaceae.* The images were taken by confocal laser scanning microscope (Leica TCS SP2, Germany)

Based on the above results, the critical level for thermophilic AD due to ammonia toxicity was considered to be 3.5 g N l^{-1}. T3 failed on day 112 (see section 4.2.5) with a TAN concentration of approximately 3.5 g N l^{-1}; this also coincided with no detectable samples from FISH observation after day 90 (TAN ~ 3.3 mg N l^{-1}).

Based on the results from T1, the 'risk zone' (when VFA started accumulating) was considered to be in the TAN concentration range of 3.0 to 3.5 g N l^{-1}. From the VFA profile in Figure 4.17 it can be seen that acetic and propionic acid concentrations fluctuated sharply after TAN rose above 2.5 g N l^{-1}. The digester performance continued as before with the same population profile until the TAN concentration reached approximately 3.5 g N l^{-1} (around day 615) when gas production declined due to

accumulation of VFA from < 0.5 to > 2.5 g l^{-1} (Figure 4.16). At this point, the methanogenic population shifted from *Methanosarcinaceae* to *Methanomicrobiales*. It could be said that a TAN concentration of 3.5 g N l^{-1} may be the boundary for the shift to mainly hydrogenotrophic methanogens. A critical TAN concentration of 3.5 g N l^{-1} obtained for T1 thus agrees with that for T3.

Based on the results from T2, the boundary of the 'risk zone' appeared to be slightly lower than in T1. The shift in dominance from *Methanosarcinaceae* to *Methanomicrobiales*, similar to that in T1, occurred at a TAN concentration of approximately 2.5 g N l^{-1}. This difference may be due to the difference in acclimatisation to the added urea: T1 was representative of a non-acclimatised inoculum while T2 underwent some acclimatisation. Fotidis et al. (2013) also found that FISH results showed a richer variety of population in a non-acclimatised digester than an acclimatised one. They stated that direct exposure to ammonia nitrogen was preferable to the gradual method because there was a greater diversity of methanogens to respond to the ammonia toxicity. In comparison, with gradual adaptation a less diverse group developed during the process. The results of the present research appear to support this. In conclusion, for stable thermophilic AD, it appears the 'safe zone' for ammonia toxicity should be lower than 2.5 g N $l^{-1}$of TAN. The system may be able to withstand TAN of up to 3.5 g N l^{-1}. But it is strongly recommended to avoid operation at or above this concentration for long periods, to prevent the onset of irreversible VFA accumulation and failure.

5.6 FISH from mesophilic AD with the temperature increment trial

As described in section 4.3, this experiment continued from the previous one by increasing the temperature in steps of 1^{o}C. The samples from M3 selected for FISH analysis were chosen mainly on the basis of the digester VFA profile. The first sample (day 335) was taken because of the low VFA concentration, and presented the same methanogenic profile as in the preceding period (day 265 in Table 5.3). The dominant group was still *Methanomicrobiales*.

During the series of VFA peaks on days 384, 510, and 573 the methanogenic groups did not show any great change. The rising temperature affected the system, as shown by

increasing VFA and ammonia contents (TAN and FAN); however, it did not change the methanogenic population which was still dominated by *Methanomicrobiales* and a rich array of archaea. *Methanomicrobiales* could survive even high TAN and FAN, above ~ 6.2 and 1.1 g N l^{-1} respectively as reported on day 510. Hansen et al. (1998) and Nielsen and Angelidaki (2008) stated that the inhibition level for FAN was 1100 and 1200 mg N l^{-1}, respectively. In the current study FAN sometimes was above 1500 mg N l^{-1}: the maximum value was 1863 mg N l^{-1} on day 419 when the temperature was 43 ^{o}C. In this study, *Methanomicrobiales* could survive at these FAN concentrations with similar gas production as in the previous period[1] before the rise in temperature, providing strong proof of its ammonia tolerance. After the VFA accumulation reduced, *Methanomicrobiales* remained the most abundant group. Therefore, it may be said that increasing the temperature from 35 to 43 ^{o}C did not change the methanogenic population.

These FISH observations were also confirmed by ^{14}C radio-labelling assay (analysis carried out by Dr Ying Jiang) based on samples taken on day 636. When acetoclastic methanogenesis is the only pathway, all ^{14}C is converted to methane ($^{14}CH_4$). Conversely, when syntrophic methanogenesis (syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis) takes place, there should be more $^{14}CO_2$ than $^{14}CH_4$. If the $^{14}CO_2/^{14}CH_4$ ratio is higher than 1, it is assumed that syntrophic methanogenesis is the main pathway (Fotidis et al., 2013). In the sample on day 636 the ratio of $^{14}CO_2/^{14}CH_4$ was 2.31, strongly indicating syntrophic methanogenesis as the route for M1. The radio-assay results also showed the same trend for M1 with a $^{14}CO_2/^{14}CH_4$ ratio of 2.86. Although FISH results were only available for M3, the similarity between the two digesters was confirmed by the results of the radio labelling assay. Full details of the ^{14}C testing results are given in Appendix B.

[1] SMP from last 30 days (day 267-296) before raising temperature was 0.478±0.017 l CH4 g^{-1} VS day^{-1}, and the SMP from 10 days (day 415-425) of the maximum range of 1863 mg N l^{-1} FAN on day 419 was 0.497 ±0.036 l CH4 g^{-1} VS day^{-1}.

5.7 Comparison of FISH Observations between Mesophilic and Thermophilic AD

M3 and T3 were fed with the same feedstock (normal food waste) at different operating temperatures. According to Table 5.3 and Table 5.5, the profiles of M3 and T3 at the beginning were quite similar: they were initially rich in *Methanosaetaceae* up to a TAN concentration of approximately 2.5 g N l^{-1}, then shifted to *Methanosarcinaceae*. The differences started after this. In M3 *Methanosarcinaceae* dominated until TAN rose to approximately 3.5 g N l^{-1} and after this the dominant population shifted permanently to *Methanomicrobiales*. T3 experienced failure at a TAN concentration of approximately 3.5 g N l^{-1} when gas production fell suddenly at around day 112. After reseeding, the dominant group of both reactors was the same, i.e. *Methanomicrobiales*. This may imply that *Methanomicrobiales* is a strongly ammonia-resistant methanogen whatever the operating temperature. Nettmann et al. (2010) also found *Methanomicrobiales* as the dominant group in five out of six full-scale anaerobic digesters with TAN ranging from 1.6-4.3 g N l^{-1}. It was confirmed that *Methanomicrobiales* was the active group in an anaerobic digester treating swine waste at a high ammonia concentration (>3.5 g N l^{-1}) (Angenent et al., 2002). Fotidis et al. (2013) found *Methanomicrobiales* as one of the dominant groups in a mesophilic batch test with TAN of 5 and 7 g N l^{-1}, and in a thermophilic test with TAN of 3 g N l^{-1}. Therefore, it can be said that *Methanomicrobiales* is one of the most ammonia tolerant methanogens. Moreover, it was confirmed that temperature had a significant effect on the methanogens due to the different FAN which was 465 mg N l^{-1} (TAN 3669 mg N l^{-1}, pH 8.02) and 1552 mg N l^{-1} (TAN 3686 mg N l^{-1}, pH 8.18) for M3 and T3, respectively (data from day 111).

5.8 Limitations of FISH

As a tool to reveal the population structure in digesters, FISH is very useful technique because it can be carried out on samples with no need to culture the microbes. In the current study, however, FISH showed limitations with feedstocks used, which are presented here based both on direct experience and from personal communication with Dr Louise Byfield.

5.8.1 Sample quality

There were two main issues related to sample quality. The first was the age of the fixed sample. Hybridisation of fixed samples was carried out intermittently. The first trial (for training and practice) was done on 13/10/2011 using samples fixed on 10/10/2011 and the results showed clear fluorescent signals. Hybridisation of the same samples carried out a year later showed weaker fluorescent emission, and in general the longer the time since the samples were fixed, the less effective was the hybridisation. This effect was also found in Dr Byfield's examination of the same samples. The samples with a longer storage period gave weaker fluorescence signals, as also found in the study by Cajulis et al. (1996).

The second issue was the effect of ammonia nitrogen in digestate on the identification of methanogens. It was found that higher ammonia nitrogen resulted in a poorer observed signal. Similarly, almost no fluorescent emission signal was found in samples from mesophilic anaerobic digestion of chicken manure and of food waste, in TAN was >3.0 g N l^{-1} (personal communication, Dr L Byfield). The higher TAN also affected the shape of methanogens. Based on observations in the current study, *Methanosarcinaceae* were typically smaller at higher TAN concentrations, while *Methanomicrobiales* changed from an oval or round plate shape to an irregular plate shape. Calli et al. (2005b) reported similar observations, and found that the best hybridisation signals from FISH were obtained from samples with low FAN, while few and weak hybridisation signals were observed from the sample with probes MB1174 (*Methanobacteriales*) and MG 1200 (*Methanomicrobiales*).

5.8.2 Interference from non-methanogens

During observation by epifluorescence microscope, inert particles that could emit fluorescent signals (self-emission fluorescence) were sometimes found. These particles, however, were not difficult to distinguish from methanogens due to their shapes and size. Most of the inert particles were larger than methanogens: for example *Methanococcales* is 1-3 μm in diameter (Liu, 2010). In practice this means that not only the positive signals from all probes but also the size and shape have to be considered. The probe ARC-915 emitted a green fluorescent signal whilst the other probes (MX

825, MS 1414, MG1200, MB1174, and MC1109) emitted red. Thus to confirm the *Methanosaetaceae*, for instance, the signals from probe ARC-915 (universal archaea) which was green and probe MX 825 (*Methanosaetaceae*) which was red should be both positive. The size and shape were then considered: *Methanosaetaceae* is normally rod-shaped filamentous. Therefore, for positive confirmation of *Methanosaetaceae*, the positive signals (both green and red) and shape of *Methanosaetaceae* (rod-shape filamentous) had to agree.

In addition to interference with the observed fluorescent emission from inert particles, auto-fluorescent microbes also sometimes caused problems. The observed signal was usually red which is the same as the Cy-3 signal (such as MX 825 for *Methanosaetaceae*). The signal from auto-fluorescent microbes, however, was much weaker than from the hybridised methanogens. To give positive confirmation of the methanogenic group, both the red and green signals must be found in the same spot. Auto-fluorescent microbes also were found during FISH observation by Montero et al. (2009) who used FISH to specify the methanogens in thermophilic-dry anaerobic digestion of synthetic organic fraction of municipal solid waste (OFMSW).

5.8.3 Method problem

FISH is a very useful tool to identify the methanogenic population groups in digesters. Sometimes, however, there are systematic difficulties. For example, different stringencies result in identification of different percentages of the population observed. In unpublished work by Dr Louise Byfield it was found that the optimal stringency for food waste samples was 20 and 25%. Higher stringency gave a smaller positive output (negative error) whereas lower stringency was reflected in a higher positive error.

Therefore, to obtain reliable results on the methanogenic population, FISH should be implemented in parallel with other methods, for instance ^{14}C radio-labelling assays or gene sequencing, and carried out in parallel with chemical analyses. More frequent sampling may also help to ensure the reliability of the results in terms of methanogenic population structure.

Chapter 6 Conclusions and Future Work

6.1 Conclusions

6.1.1 General

- Long-term stable operation of thermophilic digesters (55 ^{o}C) fed on undiluted source segregated domestic food waste was not possible due to problems associated with ammonia toxicity from the feedstock.
- Trace element supplementation strategies successful in allowing stable mesophilic digestion of source segregated food waste were not effective in thermophilic conditions, even at a 4 times higher dose.
- Monitoring of standard digester operating parameters (pH, VFA, specific methane production) confirmed that acclimatisation from mesophilic to thermophilic conditions was a rapid process, while in both mesophilic and thermophilic conditions changes in the population structure linked to increasing ammonia concentrations took place over a much longer period
- There was, however, no evidence of further acclimatisation to ammonia even after extended periods of operation at or near to threshold inhibitory concentrations; nor after periods of temporary removal of organic loading to encourage depletion of accumulated VFA at higher ammonia concentrations
- The response to temperature increases indicated that the main factor causing instability was FAN, and that acclimatisation to thermophilic conditions by this means was therefore unlikely to be successful for source segregated domestic food waste

6.1.2 Specific experimental findings

6.1.2.1 Acclimation to thermophilic conditions

- A single-step change in temperature from 35 ^{o}C to 55 ^{o}C, followed by ~5 days without feeding then a gradual increase in OLR, was an effective means of acclimating a low-nitrogen mesophilic inoculum from a municipal wastewater biosolids digester to digestion both of source separated domestic food waste (normal food waste) and of low nitrogen food waste (low-N food waste) in

thermophilic conditions. This approach is likely to offer the fastest and most effective strategy for start-up of large-scale thermophilic plant where acclimated inoculum is not available.

- During the early stages of acclimation to thermophilic digestion of normal food waste, trace element supplementation at concentrations used successfully in mesophilic digestion appeared to delay the onset of accumulation of VFA, principally due to a fall in propionic acid concentrations. Trace element supplementation is therefore likely to be a useful strategy during full-scale digester start-up.
- The results clearly show that acclimatisation to thermophilic conditions is possible on a far shorter timescale than to high TAN concentrations.

6.1.2.2 Meta-stable operation in thermophilic conditions

- In an initial trial, thermophilic digesters fed on normal food waste at OLR of 2, 3 and 4 g VS l^{-1} day^{-1} with TE supplementation were successfully operated for ~150 days with a specific methane production of ~0.45 l g^{-1} VS and volumetric gas production increasing in proportion to sequential loading increases. It can be concluded that the process was successful in providing a steady flow of carbon through to gaseous products during this period.
- Despite a rise in TAN concentrations to ~4.2 g N l^{-1} and in total VFA to 15-20 g l^{-1} during this period, gas production, pH and IA/PA ratio remained relatively stable, indicating that the digesters were operating in a meta-stable condition in which VFA are buffered by high ammonia concentrations.
- The thermophilic digesters without TE supplementation showed more severe and faster VFA accumulation even in the period of low TAN (< 2.5 g N^{-1}), indicating that the trace elements added were useful in thermophilic conditions.
- In this meta-stable state, the process may be highly vulnerable to small shocks. A deliberate disturbance in the form of a pause in then resumption of feeding led to a sharp rise in VFA concentration (primarily acetic acid), a rapid increase in the IA/PA ratio, a fall in pH and cessation of gas production, which could not be effectively recovered. This happened in all digesters irrespective of loading rate or operating status, indicating their vulnerability to sudden failure in these conditions.

6.1.2.3 Comparison of mesophilic, thermophilic and low-nitrogen operation

- In a second trial, thermophilic digestion of normal food waste with TE supplementation showed good methane production (~0.43 l CH_4 g^{-1} VS) for up to ~110 days before a decline in pH, increase in IA/PA ratio and eventual failure at a TAN concentration >4.5 g N l^{-1}. This confirmed the previous study and indicated that long-term stable digestion was not possible without some further process intervention.
- Increasing the TE supplementation by up to four times the value adopted in mesophilic conditions may have aided recovery from a period of rapid acidification, but was unable to prevent VFA accumulation in thermophilic conditions in the longer term if the TAN was still higher than 3.5 g N l^{-1}.
- Long-term operation (> 3 HRT) of trace element supplemented mesophilic anaerobic digesters on the same source segregated domestic food waste was successful at TAN concentrations of 4.5 g N l^{-1} without VFA accumulation or other signs of instability. Specific and volumetric methane production at OLR of 4 g VS l^{-1} day^{-1} was around 0.47-0.50 l CH_4 g^{-1} VS and 3.2-3.4 l l^{-1} day^{-1}, respectively, with a biogas methane content of around 57%. Gas production was slightly higher than reported by Zhang et al. (2012a) for typical source segregated domestic food waste, and this was attributed to the high carbon content of the feedstock which indicated a relatively high proportion of protein and lipids. The theoretical and measured calorific values showed good agreement at 22.6 (the Dulong equation) and 23.2 kJ g^{-1} VS for normal food waste, respectively, supporting the validity of the compositional analysis. The specific methane production was equivalent to about 96% of the BMP value and 78% of the measured calorific value. From these results it can be concluded that that single-stage mesophilic digestion of food waste with TE supplementation is a highly effective conversion process presenting no operational stability issues
- Thermophilic digesters acclimated to a low-nitrogen food waste (TKN 2.1 g N kg^{-1} WW or 14.5 g N kg^{-1} TS) showed stable operation for > 3 HRT with no VFA accumulation. Specific and volumetric methane production was around 0.40 l g^{-1} VS and 1.5 l l^{-1} day^{-1}, with a biogas methane content of around 54%. Theoretical and measured calorific values showed good agreement at 19.0 (Du Long equation) and 18.0 kJ g^{-1} VS for low-N food waste respectively,

supporting the validity of the compositional analysis. This result confirmed that protocol for acclimatisation to thermophilic conditions was successful, and that stability issues in the thermophilic digestion of normal food waste are almost certainly associated with its nitrogen content.

6.1.2.4 Ammonia toxicity thresholds

- Addition of a 'shock load' of urea to thermophilic digestion of low-nitrogen food waste at OLR 2 g VS l^{-1} day^{-1} to give a TAN concentration of around 2.5 g N l^{-1} caused a short-term increase in VFA concentration to 4000 mg l^{-1} but no significant process disturbance, as indicated by continuing stable values of IA/PA ratio (~0.4) and specific methane production.
- Gradual addition of urea confirmed that stable operation in thermophilic conditions with very low VFA concentrations (typically < 300 mg l^{-1}) was possible at TAN concentrations below 2.5 g N l^{-1}.
- At TAN concentrations around 2.5 g N l^{-1} thermophilic digestion was characterised by occasional short-term VFA peaks of up to 1000 mg l^{-1}, with the main component being acetic acid.
- From 2.5-3.5 g N l^{-1} of TAN the frequency of these peaks increased to give a saw-tooth profile characterised by a sharp increase and even sharper fall in propionic acid, while the magnitude of the VFA peaks rose to ~2500-5500 mg l^{-1}.
- The critical TAN concentration causing irreversible propionic acid accumulation at 55 ^{o}C was ~3.5 g N l^{-1}. In these conditions there was also an increase in other VFA, especially iso-butyric and iso-valeric acids.
- The critical FAN concentration causing the disturbance in anaerobic digesters started at around 0.8 g N l^{-1}. The disturbance showed by the sharply increasing of VFA, especially the acetic acid, however, it did not affect the IA/PA due to the good buffering from ammonia nitrogen.

6.1.2.5 Temperature increase

- A step-wise increase in temperature from 35 to 39 ^{o}C had no adverse effect on mesophilic digestion of normal food waste.

- The onset of instability (as indicated by rapid VFA accumulation) occurred at around 40 ^{o}C. In the case of food waste it seems likely that this is driven by the rising free ammonia concentration or the limits of the appropriate temperature range for mesophilic condition and that therefore acclimatisation to thermophilic temperature is unlikely to be achieved by this means.
- The increasing temperature slightly enhanced the VS destruction which also resulted in slightly higher SMP.
- Reducing the temperature from above 40 ^{o}C (43 ^{o}C in this study) to the optimal point of 35 ^{o}C may be an option to relieve the stress of VFA accumulation.

6.1.2.6 Methanogenic population structure

- During acclimation of an inoculum taken from a mesophilic anaerobic digester treating municipal wastewater biosolids to a feedstock of source segregated domestic food waste at an OLR of 2 g VS l^{-1} day^{-1}, in mesophilic conditions, a period of disturbance was observed between days ~40-70, indicated by a rise in VFA concentration and IA/PA ratio and a fall in biogas production. In this period, however, no change was observed in the methanogenic population profile, which remained dominated by *Methanosaetaceae.* This result suggests that the disturbance is not a result of population shift. The further explanation or finding for this occurrence may be more considered or studied in the future.
- In mesophilic anaerobic digestion of source segregated domestic food waste acetoclastic *Methanosaetaceae* were dominant at TAN concentrations of ≤ 2.5 g N l^{-1}. Between 2.5-4 g N l^{-1} TAN the dominant group was *Methanosarcinaceae*, capable of utilising acetate and H_2 + CO_2; while the hydrogenotrophic *Methanomicrobiales* were dominant at TAN concentrations above 4.0 g N l^{-1}. These FISH observations were also confirmed by ^{14}C radio-labelling assay.
- Increasing the temperature from 35 to 43 ^{o}C in a well-acclimated digester operating on source segregated domestic food waste did not affect the methanogenic population profile, with *Methanomicrobiales* remaining the dominant group.
- In thermophilic anaerobic digestion when ammonia concentrations were not near the toxicity threshold *Methanosarcinaceae* was dominant.
- *Methanomicrobiales* was generally dominant at TAN concentrations above 3.5 - 4.0 g N l^{-1}, probably because of its free ammonia tolerance.

- A single shock load of ammonia nitrogen did not show any rapid effect on the methanogenic population profile in thermophilic anaerobic digestion of low-nitrogen food waste, with *Methanosarcinaceae* remaining dominant.
- The population shift in ammonia-acclimatised thermophilic anaerobic digestion appeared to occur at a TAN concentration of ~2.5 mg N l^{-1} whereas it was 3.5 mg N l^{-1} for the non-acclimatised reactor. The pattern of change was the same, however, with *Methanosarcinaceae* shifting to *Methanomicrobiales*.
- The higher the TAN concentration, the greater the percentage of hydrogenotrophic methanogens present, especially *Methanomicrobiales*.

6.2 Future Work

- Further investigation of the effects of transient and shock loads on TAN in thermophilic anaerobic digestion may be useful for the development of operational strategies to prevent digester failure.
- *Methanosaetaceae* are believed to be sensitive to ammonia toxicity, but were found in digesters with high total ammonia nitrogen concentrations. Further study is needed to establish how far members of this group can develop group ammonia tolerance, and consider ways in which this could help scientists or operators to understand and run anaerobic digesters more efficiently.
- Methods are needed to allow FISH analysis of samples from digesters with high TAN concentration or other factors causing weak hybridisation signal strength
- Since stable long-term digestion of food waste is not possible digestion in thermophilic conditions, alternative strategies to allow effective operation must be identified and investigated: these could include ammonia stripping and feedstock dilution to reduce TAN and FAN concentrations down to the levels identified as acceptable in this work.

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Appendices

Appendix A: Determination of fibre content

Principle:

Fibre is an inhomogeneous mixture of various macromolecules. Most of these are structural polysaccharides (e.g. cellulose, hemicellulose and pectin), but also noncarbohydrates like the aromatic lignin, non-digestible proteins and others are normally counted as fibre constituents. The most commonly used terms, based on chemical analytical techniques, are Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF) and Acid Detergent Lignin (ADL). All of these methods are based on subsequent steps of chemical treatments to solubilise 'non-fibre' components and final determination of the residue obtained. Depending on determination approach various kinds and amounts of fibre constituents are achieved in the residues.

Neutral Detergent Fibre (NDF) is defined to be the residue after treatment with a neutral detergent solution. In this procedure, sample is boiled for one hour with neutral detergent (ND). Enzymatic incubation before, during and after the ND treatment helps to break down protein and starch. The residue is then dried and ashed. The weight reduction by ashing is the sample content of hemicellulose, cellulose and lignin.

Acid Detergent Fibre (ADF) is defined to be the residue after treatment with an acid detergent solution. Sample is boiled with acid detergent (AD) for one hour, and dried and ashed. The weight reduction by ashing is the sample content of cellulose and lignin.

Acid Detergent Lignin (ADL) is defined to be the residue after initial treatment by the ADF method followed by removal of the cellulose fraction through extraction using 72% H2SO4, and then dried and ashed. The weight reduction by ashing is the sample content of Lignin.

Apparatus:

1. FibreCap 2023 system;

2. Ashing crucibles (45 x 60 mm) x18;

3. Analytical balance with an accuracy of 0.1 mg.

Reagents:

1. Neutral Detergent Solution:

- Disodium ethylene diaminetetraacetate EDTA (dehydrate) 18.61g x2
- Sodium Borate 6.81g x2
- Sodium lauryl sulphate 30g x2
- 2-ethoxyethanol 10 ml x2
- Disodium hydrogen phosphate (anhydrous) 4.56g x2
- Alfa-Amylase solution – Termamyl 300L, type DX available from Foss Tecator x2
- Place 18.61g of EDTA (Disodium ethylene diaminetetraacetate, C10H14N2Na2O8x2H2O) and 6.81g of Sodium Borate decahydrate, (Na2B4O7x10H2O), in a beaker and add some distilled water and heat until dissolved. Add 30g Sodium Lauryl Sulphate,
- (C12H25OSO3Na), 10 ml of 2-ethoxyethanol, (C4H10O2) and 4.56g Disodium Hydrogen phosphate, (Na2HPO4). Add water and heat until dissolved. Mix and dilute to 1000 ml.

Check pH which should be in the range 6.9-7.1. Adjust by NaOH if necessary.

Repeat this step twice to produce two 1000 ml ND solutions.

2. Acid Detergent Fibre Solution: 1.00N H2SO4 with CTAB

- Concentrated sulphuric acid 49.04 x2

- Cetyl trimethylammonium bromide CTAB 20g x2

Weigh 49.04 g conc. H_2SO_4 into a 1000 ml volumetric flask containing 400 ml deionised water. Make up to volume with deionised water. Add 20 g of CTAB (Cetyl trimethylammonium bromide, $CH_3(CH_2)_{15}(CH_3)_3NBr$).

Repeat this step twice to produce two 1000 ml AD solutions.

3. Acid Detergent Lignin Solution: Sulphuric acid, 72%

- Concentrated sulphuric acid, 98%

Weigh 433 g of deionised water into 1000 ml volumetric flask and add 1201 g (or 653 ml) of conc. H_2SO_4 slowly with occasional swirling. The flask must be cooled in water in order to add the required weight of acid. Cool to 20°C and check if volume is right. If volume is too large, take out 5 ml solution and add 4.55 ml conc. H_2SO_4. If volume is too small, take out 1.5 ml solution and add 2.5 ml of deionised water. Repeat if necessary. Meniscus should be within 0.5 cm of the calibration mark at 20°C.

Sample preparation:

Solid samples are normally ground to less than 1.0 mm.

Semi-solid is difficult to handle particularly when where is a wide variation in particle size and / or hardness of constituents. Depending on the particular sample type, homogenizing, liquefying or ball milling may provide a suitable sample for analysis. if possible dry sample before milling.

Analytical Procedure for Neutral Detergent Fibre (NDF):

1. Label 18 capsules with an indelible pen and dry them with lids in the oven at 105°C for at least 30 minutes. Transfer to desiccator, cool for at least 5 minutes prior to weighing sample;

2. Weigh pre-dried capsule+lid (W1), tare and weigh around 1 g of ground sample to an accuracy of ±0.1 mg (W2) into each capsule, secure lids. Place the capsules in the trayholder, and place the tray in place in the carousel. Triplicate analysis for each sample (totally 5 samples can be treated in a run), and the rest three capsules and lids are the control;

3. If the fat content is above 5%, samples should be de-fatted prior to analysis: Add 1000 ml of ether to the extraction beaker. Place the try holder with the capsules in the solution and agitate for 30 seconds. Lift the tray holder out of the solution and drain the capsules from solvent. Repeat three times in three different containers with solvent. Remove tray holder and allow capsules to drain and air-dry in fume hood. For samples containing fatty substances that cannot be removed directly, the extraction shall be carried out after the detergent treatment using acetone (CH3COCH3);

4. Put 1000 ml of hot water (80°C) and 21-28 ml of 2% Amylase to the extraction beaker. Place the carousel with capsules into the beaker and gently agitate to mix well. Allow standing for 15 minutes at room temperature;

5. Drain the solution out of the capsules. Wash once with cold water and drain;

6. Place extraction beaker with 1000 ml of Neutral Detergent (ND) solution. Lower the carousel unit into the reagent sufficient to immerse the samples. Gently agitate to thoroughly disperse samples and then fully lower the carousel into the reagent.

7. Put the beaker on the hot plate and place condenser on top of the extraction beaker. Open cold water tap (0.4 l/min) for the reflux system. Let it boil gently for 30 minutes. Always measure boiling from the time when the solution has reached the boiling point (determined by the presence of small air bubbles breaking the surface of the liquid);

8. Remove the carousel from the beaker and dry the lid membrane with a piece of soft tissue. Discard half (500 ml) of the extraction solution. Add another 500 ml of fresh ND solution and 21-28 ml of 2% amylase solution;

9. Lower the carousel into the extraction beaker and agitate. Put the beaker back on the hot plate and fit the condenser on the top;

10. Bring the solution up to boiling and boil gently for 30 minutes again. Meanwhile, preheat ~3 liters of water to boiling;

11. Remove the condenser. Remove the extraction beaker from the hotplate. Remove the carousel from the beaker and empty the beaker and capsules of liquid. If solution is present on the lid membrane, it might be difficult to filter the capsule. Tap the whole extraction carousel against a hard surface and dry the lid membrane with soft tissue. Return the carousel to the empty extraction beaker and 'spin' rotate to remove all of the liquid from the capsules and discard;

12. Fill the beaker with 1000 ml of boiling water (to mark). Wash by partially lowering the extraction carousel into the water ensuring that the capsules refill, gently agitate the carousel and raise it to empty the capsules and lower to refill. Do not use so much water so that the lids are covered with water. Empty the capsules and extraction beaker. Dry the capsule lid with soft tissue if necessary. Repeat the washing procedure twice more;

13. Add 1000 ml of hot water (~80°C) and 21-28 ml of 2% amylase to the extraction beaker. Return carousel to the beaker and agitate. Allow standing for 15 minutes;

14. Wash the capsules twice with cold water following the above washing procedure;

15. De-fatting with acetone if necessary;

16. Put the capsule tray on the drying stand, and dry capsules in an oven at 105±2°C for 5 h;

17. Cool the capsules to room temperature in a desiccator and weigh with a precision of ±0.1 mg (W3);

18. Place the capsules in pre-dried and pre-weighed (W4) ashing crucibles. It is important that the crucible used is high enough so that all of the ash is retained inside the crucibles as a standing capsule can fall during ashing;

19. The pressure inside the capsule can increase during ashing. To avoid this, make a small slit in the capsule using a scalpel prior to ashing;

20. Ash the capsules in the ashing crucibles for 4 h at 600 ± 10°C. Do not place capsules in hot furnace. Always try to heat them slowly by having them in a cold furnace from the beginning and then increase the temperature;

21. Cool the ashing crucibles slowly, at ~200°C place them in a desiccator. When room temperature is reached, weigh with a precision of ±0.1 mg (W5);

$$\%NDF = Hemicellulose + Cellulose + Lignin = \frac{W_3 - (W_1 \times C) - (W_5 - W_4 - D)}{W_2} \times 100$$

22. Calculation:

where,
W1 = Initial capsule weight, g;
W2 = Sample weight, g;
W3 = Weight of capsule + residue sample after extracting and drying, g;
W4 = Weight of empty ashing crucible, g;
W5 = Weight of total ash and ashing crucible, g;
C = Blank correction for capsule solubility;
D = Capsule ash, g.

The capsules can lose a small amount of weight during reaction with the reagents. A correction factor (C) to compensate for this loss is used in the formula for calculation of analytical results. Typically the correction factor (C) is >0.9990, corresponding to ~3 mg weight loss of a capsule during processing:

C = *blank capsule weight after extractions*
blank capsule weight at start

During the final ashing step some ash weight is obtained from the capsule itself (D). It is recommended to make an ash evaluation in each batch of samples being analysed. The ash weight contribution from the capsule is typically < 3 mg.

Analytical Procedure for Acid Detergent Fibre (ADF):

The first three steps follow the instructions for NDF 1-3;

4. Put 1000 ml of AD solution to the extraction beaker. Gently lower the carousel into the beaker ensuring all capsules have been wetted and then raise the capsules out again;

5. Place the carousel with capsules back into the AD solution avoiding getting fluid on the lid of the capsules. Dryness of the lids is essential;

6. Rotate the carousel gently and make sure that there is fluid in each cap;

7. Put the beaker on the hot plate and place condenser on top of the extraction beaker. Open cold water tap (0.4 l/min) for the reflux system. Let it boil gently for 60 minutes and rotate occasionally if desired. Always measure boiling from the time when the solution has reached the boiling point (determined by the presence of small air bubbles breaking the surface of the liquid);

8. Carefully take carousel out of the AD solution and carefully dispose of the solution down the sink with plenty of running water;

9. Place the carousel back into the empty beaker and spin it to displace the fluid. Dry the lids with soft tissue;

10. Fill the beaker with 1000 ml of boiling water. Lower the carousel into the boiling water and ensure all capsules have water in them. Twist the carousel backwards and forwards to rinse the capsules;

11. Remove the carousel from the water.

12. Dispose of the water down to the sink.

13. Replace the carousel back into the beaker and spin off any excess water. Dry the capsule lids with soft tissue;

14. Wash the capsules up to 4 times more with hot water following the above washing procedure. On the last rinse wash the lids of the capsules;

15-21. Follow the instructions for NDF 15-21;

$$\%ADF = Cellulose + Lignin = \frac{W_3 - (W_1 \times C) - (W_5 - W_4 - D)}{W_2} \times 100$$

22. Calculation:

See the instruction for NDF 22 for the meaning of each symbol.

Analytical Procedure for Acid Detergent Lignin (ADL):

The first steps follow the instructions for ADF 1-14;

Note: Please do not fill the capsule with sample higher than half the capsule height. Otherwise, the acid is difficult to be washed out later and the capsules will burn in the oven when drying.

15. Place ~700 ml of 72% sulphuric acid into the beaker;

16. Place the capsule tray onto the drying stand. Lower the tray with capsules into 72% sulphuric acid for 4 hours in fume cupboard;

17. Wash the samples in cold water for times until wash off all acid (wash in warm water later if necessary), and make sure the pH of the washing solution is neutral at last;

18. Follow the instructions for NDF

19. Calculation:

$$\%ADL = Lignin = \frac{W_3 - (W_1 \times C) - (W_5 - W_4 - D)}{W_2} \times 100$$

See the instruction for NDF 22 for the meaning of each symbol.

Appendix B: ^{14}C radio-labelling assay results

Sample ID	DPM	Counting Eff.	Total DPM	DPM recovered	Kbq	recovery rate	CO2/CH4
M1 CO2	1203.5	95.31	24069.2				
M1 CH4	438.04	95.43	8760.8				**2.75**
M1 Sludge	2130.7	91.5	99075.225	131905.225	2.19842	88%	
M1 CO2	1258.6	95.3	25172				
M1 CH4	424.38	95.32	8487.6				**2.97**
M1 Sludge	2318.5	88.22	107811.18	141470.78	2.357846	94%	
average							2.86
M3 CO2	815.34	95.42	16306.8				
M3 CH4	358.53	95.39	7170.6				**2.27**
M3 Sludge	1911.6	92.7	88888.935	112366.335	1.872772	75%	
M3 CO2	835.37	95.35	16707.4				
M3 CH4	356.42	95.39	7128.4				**2.34**
M3 Sludge	2044.2	92.53	95054.37	118890.17	1.981503	79%	
average							2.31
T1 CO2	206.78	95.51	4135.6				
T1 CH4	122.7	95.36	2454				**1.69**
T1 Sludge	3447.7	85.75	160317.12	166906.72	2.781779	111%	
T1 CO2	133.74	95.33	2674.8			113%	
T1 CH4	58.76	95.26	1175.2				**2.28**
T1 Sludge	3624.6	85.24	168542.97	172392.97	2.873216	115%	
Average							1.98
T2 CO2	174.17	95.31	3483.4				
T2 CH4	557.96	95.3	11159.2				**0.31**
T2 Sludge	3327.9	83.81	154746.89	169389.485	2.823158	113%	
T2 CO2	183.55	95.35	3671			108%	
T2 CH4	560.55	95.39	11211				**0.33**
T2 Sludge	3025.6	80.7	140691.33	155573.33	2.592889	104%	
Average							0.32
T3 CO2	66.04	95.35	1320.8				
T3 CH4	16.79	95.27	335.8				**3.93**
T3 Sludge	3006.2	70.82	139785.98	141442.575	2.357376	94%	
T3 CO2	55.57	95.37	1111.4			94%	
T3 CH4	17.31	95.31	346.2				**3.21**
T3 Sludge	2960	70.83	137640.47	139098.065	2.318301	93%	
Average							**3.57**