UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING AND THE ENVIRONMENT

ANAEROBIC DIGESTION OF FOOD AND VEGETABLE WASTE

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ANAEROBIC DIGESTION OF FOOD AND VEGETABLE WASTE

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ABSTRACT

ANAEROBIC DIGESTION OF FOOD AND VEGETABLE WASTE

FACULTY OF FACULTY OF ENGINEERING AND THE ENVIRONMENT
Doctor of Philosophy
By Ying Jiang

Food and vegetable wastes contribute a large percentage of the organic fraction of municipal solid waste (OFMSW), and anaerobic digestion potentially offers an ideal method for their management. Their chemical composition can, however, lead to unstable operation and in extreme cases complete process failure has been reported with this type of substrate.

Semi-continuous trials on vegetable waste were carried out in laboratory-scale digesters with daily feed additions at different organic loading rates (OLR). At an OLR of 2 g volatile solids (VS) l^{-1} day^{-1} the methane yield was 0.345 l g^{-1} VS added, or 99% of that found in a biochemical methane potential (BMP) test. Higher OLRs led to reduced methane yield and energy conversion efficiency and to a drop in digester pH which could not be effectively controlled by alkali additions. To maintain digester stability it was necessary to supplement with additional trace elements including tungsten, which allowed OLR of up to 4 g VS l^{-1} day^{-1} to be achieved. Stability was also improved by the addition of yeast extract (YE). Co-digestion with card packaging and cattle slurry proved an effective means of restoring and maintaining stable operating conditions.

Digesters fed on source segregated domestic food waste showed a long term pattern of failure as a result of the build-up of ammonia in the digester and the accumulation of volatile fatty acids, in particular propionic acid. The research gathered evidence to link this to an enzyme deficiency which was caused by a lack of selenium, or possibly other trace elements.

Results from a fractional factorial designed batch experiment showed the importance of Se in the degradation of propionic acid, and indicated that Mo and Co might also play a role in this respect. Semi-continuous trials using laboratory-scale digesters also confirmed the requirement for Se which proved to be essential for both propionate oxidation and syntrophic hydrogenotrophic methanogenesis. Supplementation with Se allowed the digesters to operate at substantially higher OLR. At high loadings cobalt also becomes limiting, due to its role either in acetate oxidation in a reverse Wood-Ljungdahl pathway or in hydrogenotrophic methanogenesis. Critical Se and Co concentrations were established as 0.16 and 0.22 mg kg^{-1} fresh matter feed at moderate loading. At this dosage the OLR could be raised to 5 g VS l^{-1} day^{-1} with specific and volumetric biogas productions of 0.75 m^3 kg^{-1} VS_{added} and 3.75 m^3 m^{-3} day^{-1} at a standard temperature and pressure (STP) of 0 ^oC and 101.325 kPa, representing a significant increase in process performance and operational stability. A tracer experiment using a radio-labelled isotope [2-^{14}C] of acetate proved that under high ammonia concentrations, hydrogenotrophic methanogenesis was the dominant pathway leading to methane production in food waste digesters.

A rapid non-derivatisation gas chromatographic method for quantification of palmitic, stearic and oleic acids was developed. These long chain fatty acids (LCFA) can accumulate in anaerobic digesters, and a simple extraction method was also developed to permit a more rapid sample turn-around time. The method was tested on digestate from food waste digesters. It was observed that the concentrations of stearic and palmitic acid in digesters routinely supplemented with trace elements were proportionally lower with respect to their relative loading when compared to those without supplementation.

It was concluded that both Se and Co were deficient in source segregated domestic food waste collected in the UK, and that supplementation with these elements could improve digestion stability, allow higher OLR to be applied, and in doing so improve the energy yield of the digestion process.

Keywords: Anaerobic digestion, trace element, ammonia, food waste, vegetable waste, long chain fatty acid, selenium, cobalt, biogas

Contents

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NOMENCLATURE

ACS	Acetyl-CoA Synthase
ASBR	Anaerobic Sequencing Batch Reactor
AD	Anaerobic Digestion
ANOVA	Analysis of Variance
C/N	Carbon Nitrogen Ratio
COD	Chemical Oxygen Demand
CODH	Carbon Monoxide Dehydrogenase
CSTR	Continuous Stirred-Tank Reactor
DI	Degradation Index
DMSO	Dimethyl Sulphoxide
FA	Free Ammonia
FAD	Flavoprotein
FDH	Formate Dehydrogenase
FISH	Fluorescent in situ hybridisation
FM	Fresh Material
FVW	Fruit and Vegetable Waste
FW	Food Waste
HDR	Heterodisulphide Reductase
HRT	Hydraulic Retention Time
K_m	Michaelis Constant. It is the substrate concentration at which the enzyme catalysed biochemical reaction rate is half of maxium rate.
LCFA	Long Chain Fatty Acid
LHV	Lower Heat Calorific Value
MSW	Municipal Solid Waste
MV	Methyl viologen
ODM	Organic Dry Matter
OFMSW	Organic Fraction of Municipal Solid Waste
OHPA	Obligate hydrogen-producing acetogens
OLR	Organic Loading Rate
PS	Primary Sludge
PCR	Polymerase chain reaction
RCF	Relative Centrifugal Force (×g)
SBP	Specific Biogas Production
SS	Sum of Squares
STP	Standard Temperature and Pressure (0 ^{o}C, 101.325 kPa)
TAN	Total Ammonia Nitrogen
TE	Trace Element
TKN	Total Kjedahl Nitrogen

TS	Total Solids
UASB	Upflow Anaerobic Granular Sludge Bed
VBP	Volumetric biogas production
VFA	Volatile Fatty Acid
VS	Volatile Solids, also sometimes known as organic dry matter (ODM)
VSS	Volatile Suspended Solids
VW	Vegetable Waste
WW	Wet Weight
YE	Yeast Extract

CHAPTER 1

Introduction

Anaerobic digestion (AD) is the breakdown of organic material by micro-organisms in the absence of oxygen. Biological methanogenesis is one of the most important processes for the maintenance of the carbon cycle on earth because methanogenic organisms catalyse the terminal step in the mineralisation of organic material in many anaerobic environments (Garcia et al., 2000). CH_4 concentration in the atmosphere has, however, increased by 150% since 1750 (IPCC, 2001) as a result of anthrogenic activities. This is of great importance to the global ecology because methane is one of most the potent greenhouse gases and contributes significantly to global warming, and measures must be sought to eliminate or minimise these emissions. On the other hand, the controlled anaerobic digestion of organic matter produces biogas (mainly methane and carbon dioxide) that offers an alternative renewable energy source which can have a very favourable net energy balance.

AD thus offers an ideal way for the treatment of the organic fraction of municipal solid wastes (OFMSW). Using this process provides a means of diverting material away from landfill, helping to meet the objectives of the EU Landfill directive (Council Directive 99/31/EC), providing renewable energy and a stabilised residue. The growing popularity of AD in Europe has been reflected in the installed plant capacity, which has increased sharply in the past few years from 4 million tonnes/year in 2006 (De Baere, 2006) to about 6 million tonnes/year by the end of 2010; this capacity is shared between around 200 plants in 17 European countries (De Baere and Mattheeuws, 2010). When compared with other options for the treatment of OFMSW, i.e. landfill, incineration and composting, anaerobic digestion technology stands at a more advanced position that not only able to achieve most of the benefits other treatment methods offer but also some extra advantages as well.

In general, the benefits of anaerobic digestion can be found in 3 major aspects: energy benefits, environmental benefits and waste treatment benefits, which are summarised in Table 1.1.

Table 1.1. Summary of the benefits of AD (Adapted from Braber, 1995).

Energy benefits	1. Net production of energy, providing the biogas is used 2. Potential to gain energy from the 'wet' fraction of municipal solid waste (MSW) which itself may be endothermic when combusted. 3. Removing the wet fraction from waste improves the calorific value of the residual waste stream. 4. Biogas can be used for transport, local heat and power production, and injected into the gas grid. These options give flexibility to its use and energy benefit.
Environmental benefits	1. Environmentally benign waste treatment 2. Co-digestion with agricultural wastes can displace fugitive methane emissions from agriculture. 3. Reduced CO_2 emissions, by displacement of fossil fuels. 4. Recycling of nutrients and organic matter to land providing the digestate is used for agronomic purposes. 5. Digestion can be an odour free process. 6. Potential reduced salt content in digestate compared to aerobic compost.
Waste treatment benefits	1. Volume reduction of the waste for subsequent transport, reuse or disposal compared to landfill. 2. Potential for co-digestion with other organic waste streams (e.g. industrial wastes such as food processing waste and agricultural wastes such as manure). 3. A means of diverting OFMSW from landfill in EU countries where this is a requirement. 4. Potential for economic benefit from the sale of energy through feed in tariffs and the sale of carbon credits.

Although AD offers these benefits, its application to certain types of organic waste has been difficult to implement because of technical limitations and issues of process stability. It is possible that some of these limitations might be overcome by a better understanding of the microbiology and biochemistry of the methanogenic microbial consortium and adaptation of the engineering design of the process. Factors that need to be considered include: balancing the nutrient requirements, understanding critical process control parameters, overcoming issues of inherent toxicity, engineering to improve biogas productivity and minimise fugitive emissions, and producing a residue (effluent or digestate) of suitable quality for release to the environment (Speece, 1983).

1.1 Background and Overview

1.1.1 Food Waste as a Digestion Substrate

Food waste constitutes a large proportion of the OFMSW. It is estimated that globally 1.3 billion tonnes of food produced for human consumption is lost or wasted (Gustavsson et al., 2011). In the UK an extensive study carried out by Waste & Resources Action Programme (Johnson et al. 2008) estimated that 6.7 million tonnes food was thrown away by UK consumers each year. 2.68 million tonnes (40% by weight) of this food waste was made up of fruit and vegetables. Despite the fact that food waste is one of the largest single fractions of the organic fraction of the waste stream it is only recently that attention has been turned to its source segregation and separate treatment. In 2007, Hogg et al. (2007) estimated that in the UK only about 2% of food waste was collected separately for composting or anaerobic digestion. A recent survey (Climenhaga and Kapoor, 2012) has shown that now at least 50% of local authorities in England and Wales operate a separate source segregated collection scheme for household generated wet organics, including food waste.

Alongside this development in collection there has also been an associated development of AD facilities to recycle this material back to land and to recover its energy potential. This development has not been without technical difficulties, and the first demonstration plant built and operated in the UK (Arnold et al., 2010) exhibited a characteristic build-up of volatile fatty acids (VFAs) that has been observed on a number of occasions and at different scales of operation (Banks et al., 2008; Banks and Zhang, 2010; Climenhaga and Banks 2008; Neiva Correia et al., 2008). When starting from an inoculum of municipal wastewater biosolids digestate, performance is initially good and VFA accumulation only starts after a period of months. This takes the form of an initial increase in acetic acid concentration which reaches a peak then declines, and is then followed by a longer term accumulation of propionic acid. The time span over which these changes occur depends on the process loading, but typically it may be more than a year before the accumulation of acid products overcomes the digester buffering capacity leading to process failure. A theory was put forward to explain this observation (Banks and Zhang, 2010). It was proposed that the peak in acetic acid is a consequence of inhibition of the acetoclastic methanogens as the ammonia concentration reaches a threshold value (Karakashev et al., 2006; Schnürer and Nordberg, 2008). The subsequent decline in acetic acid concentration, despite increasing ammonia concentrations, and its stabilisation at a low value was thought to indicate a shift in the dominant methanogenic activity from acetoclastic to hydrogenotrophic. There is a growing amount of evidence which indicates that this latter group of methanogens have higher tolerance to ammonia (Angelidaki and Ahring, 1993;

Hansen et al., 1998; Schnürer and Nordberg, 2008). No study so far, however, has conclusively proved that this type of methanogenic pathway shift occurs in the food waste digestion process.

The non-reversible long-term accumulation of propionic acid was postulated to occur because of a deficiency of the trace elements required for synthesis of the enzymes needed in syntrophic hydrogenotrophic methane production. In particularly selenium (Se), molybdenum (Mo) and tungsten (W) have all been reported as important in formate oxidation because of the requirement for them in the enzyme formate dehydrogenase (Böck, 2006). An accumulation of formate, a breakdown product of propionic acid, had been reported as possibly triggering a feedback inhibition in propionic acid oxidation (Dong, 1994). Such an accumulation could possibly be prevented if sufficient enzyme co-factor (Se, Mo or W) was available to allow the formate dehydrogenase enzyme to be manufactured in sufficient quantity to meet the load of propionic acid being generated as a result of acidogenesis.

In source segregated food waste produced in the UK both selenium and cobalt are only found at very low concentrations (Banks and Zhang, 2010) and although typically present in the start-up inoculum for a food waste digester, which is usually taken from a municipal wastewater biosolids digester, these trace elements are in the course of time diluted out of an operational food waste digester.

The primary aim of the research undertaken was to provide supporting evidence for the theory proposed above.

1.1.2 Vegetable Waste as a Digestion Substrate

Source segregated food waste as described in section 1.1.1 above is typically a high calorific value material rich in carbohydrates, proteins and fats. In contrast vegetable waste (also a food waste) has very different characteristics. It has been included as part of the research as there are a growing number of anaerobic digestion facilities that are receiving single input material of this type. One such industry is the industrial mass production of pre-packed agricultural products which gives rise to a centralised availability of waste agricultural biomass. Thus, fruit and vegetable waste (FVW) is nowadays more likely to be

collected and treated as a single waste stream, and this itself may lead to operational problems in using digestion as a means of processing this type of waste.

Carbohydrate-rich substrates such as FVW are fast producers of VFAs (Mata-Alvarez et al., 2000), and may therefore have a tendency to accumulate VFA which can lead to acidity, low pH and process inhibition. The organic fraction of FVW typically includes about 75% sugars and hemicelluloses, 9% cellulose and 5% lignin (Raynal et al., 1998, Verrier et al., 1987). Although rich in energy-yielding compounds this may not be a well-balanced mix for anaerobic digestion. Maintaining a suitable carbon to nitrogen ratio is essential for sustainable digestion, with the optimum in the range of 25-30 (Hartmann and Ahring, 2006). In the case of substrates with an unfavourable C/N ratio, studies suggest that co-digestion with other substrates that have complementary nutrient characteristics can improve process performance (Bouallagui et al., 2009).

The waste studied in this research was derived from high-value products grown in Kenya for export to Europe, which are first sorted and trimmed before being crated for air freight. This process generates substantial amounts of waste, as any sub-standard items are also rejected, and cannot be re-distributed for consumption for reasons of hygiene. Value could be reclaimed from this material, however, by AD to provide a reliable source of heat and electricity for product refrigeration and factory operations. This would also help to reduce spoilage and losses, by reducing the factory's dependence on unreliable centralised power distribution systems, and would thus contribute to waste minimisation in the early stages of this product chain (Jiang et al., 2012).

The aim of this part of the research was therefore to assess the stability of the digestion process when processing a vegetable waste stream and to assess the potential net energy generation.

1.2 Aims and Objectives

This research has two major aims which were met by their dependent research objectives:

Aim 1. To provide supporting evidence to the theory that in the anaerobic digestion of food waste the formation of biogas is dominated by a hydrogenotrophic methanogenic

consortium and that the metabolic pathways are regulated by the supply of essential trace elements.

Objective 1: To identify and categorise the specific problems encountered in treating vegetable and food waste by means of a critical review of the studies undertaken on anaerobic digestion of these two substrates. This would include known inhibitors such as ammonia and long chain fatty acids (LCFA).
Objective 2: Through an in-depth literature review to present state-of-the-art knowledge of the biochemical pathways that underpins our understanding of the role of trace elements and their specific function in the anaerobic digestion process.
Objective 3: To develop a practical method using a radioactive carbon labelling technique to determine the predominant methanogenic route under high ammonia concentration in food waste digestion.
Objective 4: To develop a rapid and reliable method for the determination of LCFA in digesters treating food waste.
Objective 5: To carry out batch and semi-continuous fed experiments in order to determine the essential trace elements and their concentrations required to maintain stable digestion of food waste.
Objective 6: To determine the process limitations and optimum control procedures that will allow a maximum volumetric biogas productivity within a stable process

Aim 2. To assess the stability of the digestion process when processing a vegetable waste stream and to assess the potential for energy generation.
Objective 1: To carry out semi-continuous fed experiments in order to determine modes of operation that would allow stable operation of vegetable waste digesters. To consider the role of trace elements and the possibility of co-digestion to alter the carbon to nitrogen ratio.
Objective 2: To determine the energy yield from the digestion process and develop techniques to express this as a proportion of the potential energy in the substrate.

CHAPTER 2

Literature review

In this chapter the fundamental principles of anaerobic digestion are presented and reviewed in detail. This includes: a discussion of the biochemical pathways, the nutrient requirements of the process and the enzymology of methanogenesis. An attempt has also been made to highlight the relationships between enzymology and trace elements requirement. Common inhibitors in food and vegetable waste are also introduced.

2.1 Overview of Anaerobic Metabolism

Stable anaerobic digestion is achieved by several groups of microorganisms forming a syntrophic relationship. In the process, complex organic material (biomass) is broken down in four biologically-mediated stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2.1).

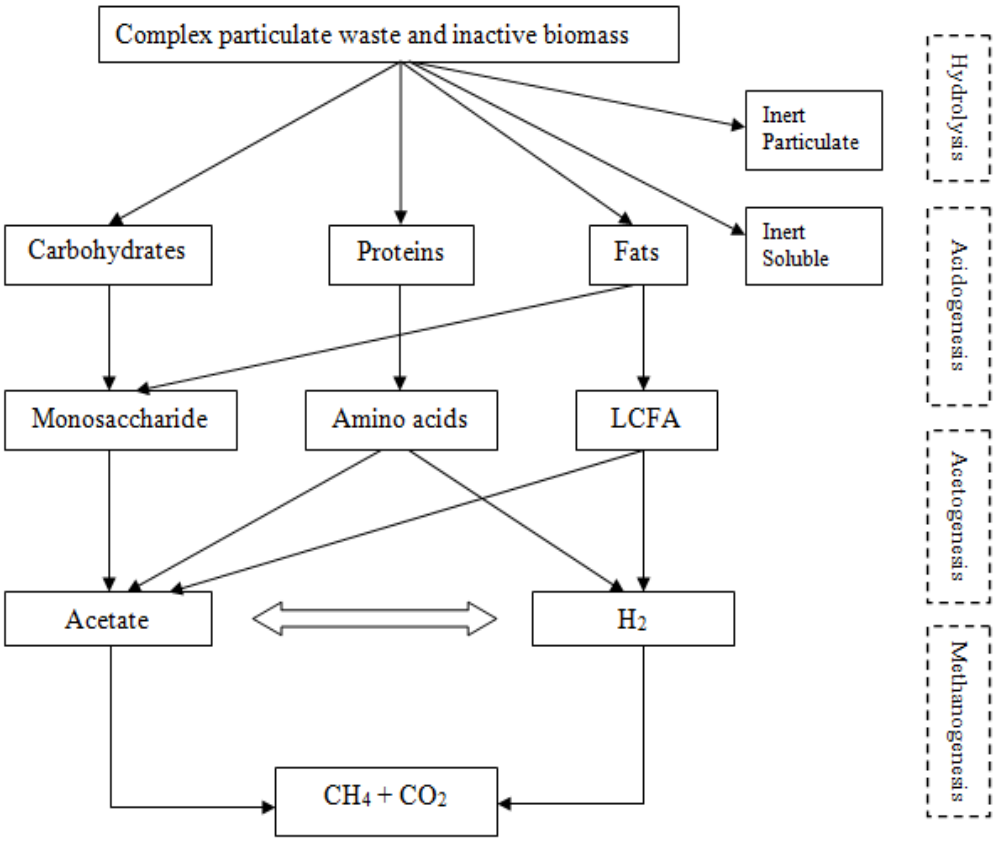


Figure 2.1. A schematic pathway of anaerobic conversion from biomass to methane (adapted from Demirel and Scherer, 2008)

2.1.1 Hydrolysis

Macromolecules which include protein, cellulose, hemicelluloses and lipids are firstly degraded and solubilised (equation 2.1 and 2.2) under the catalytic effects of extracellular enzymes (exoenzymes) produced by hydrolytic bacteria (Table 2.1). The products of the hydrolysis process are organic monomers, mainly amino acids, sugars, and fatty acids, which can be used as substrates by either anaerobic fermentative organisms (amino acids and sugar) or anaerobic oxidisers (fatty acids). (Demirel and Scherer, 2008)

$$\text{Cellulose} + H_2O \xrightarrow{Hydrolysis} \text{Soluble sugars} \quad [2.1]$$

$$\text{Proteins} + H_2O \xrightarrow{Hydrolysis} \text{Soluble amino acids} \quad [2.2]$$

The rate of hydrolysis is a function of factors such as pH, temperature, substrate composition, hydraulic retention time (HRT) and particle size (Veeken and Hamelers, 1999; Zeeman et al., 1999). A number of studies have stated that the hydrolysis stage can be the rate limiting factor in the anaerobic digestion of solid substrate material, such as food waste (Henze and Mladenovski, 1991; Parkin and Owen, 1986; Veeken and Hamelers, 2000). In order to achieve stable anaerobic digestion, it is important to keep a balance between the rate of hydrolysis and the rate of methanogenesis, as methanogenic bacteria are far more sensitive to accumulation of VFA and the corresponding pH drop than the other microorganisms involved. If the rate of methanogenesis cannot keep up with hydrolysis there will be an accumulation of VFA and hydrogen which may lead to irreversible acidification of the digester (Pavlostathis and Giraldo-Gomez, 1991).

Various studies have attempted to model the substrate hydrolysis rate during the anaerobic digestion process, and in these, first order kinetics is the simplest and most widely applied approach. According to Eastman and Ferguson (1981) a first-order hydrolysis function is purely an empirical expression that reflects the cumulative effect of many processes. The Monod equation is also sometimes used for degradation of particulate matter (Ghosh et al., 1980). Hobson (1983) proposed a model with two Monod equations that allows the distinction to be made between non-degradable, rapidly degradable, and slowly degradable fractions.

Subsequent studies have confirmed the conclusions of previous work but using different models for the more accurate simulation of the hydrolysis process. Vavilin et al. (1996) reviewed four types of kinetic models describing hydrolysis (first-order, Monod, Contois, and two-phase), taking into account colonisation of the particles by bacteria. It was concluded that all types of hydrolysis kinetics could fit a variety of experimental data comparatively well.

Several modifications of the simple first-order kinetics were proposed by Mata-Alvarez (1987) and Llabre´s-Luengo and Mata-Alvarez (1988), to take into account high concentrations of VFA accompanied by low pH which may inhibit hydrolysis. They tested the Monod, first-order, and Hashimoto models but these all showed a significant lack of fit to the experimental data. Llabre´s-Luengo and Mata-Alvarez (1988) thus proposed a kinetic model where the hydrolysis rate was proportional to the substrate volatile solids (VS), whilst the biomass concentrations was inversely proportional to the VFA concentration. The proposed model, together with fitted parameters, adequately represented the hydrolysis process under all conditions tested, and it was concluded that hydrolysis kinetics could be described by their VFA inhibition model.

Table 2.1. Exoenzymes that participate in the hydrolysis stage of anaerobic digestion (Gerardi, 2003)

Substrate to be degraded	Exoenzyme needed	Example	Representative product
Polysaccharides	Saccharolytic	Cellulase	Simple sugar
Proteins	Proteolytic	Protease	Amino acids
Lipids	Lipolytic	Lipase	Fatty acids

2.1.2 Acidogenesis

Acidogenesis refers to the metabolism of soluble monomers, such as amino acids, sugars and alcohols to produce a variety of organic acids (VFA and LCFA), hydrogen, and carbon dioxide without the requirement for an additional electron acceptor or donor (Gerardi, 2003; Mara and Horan, 2003). This process is carried out by acidogenic bacteria and provides the substrates for acetogens and methanogens in the later stages of anaerobic digestion.

The acidogenic bacteria include a large variety of fermentative genera and species, such as *Clostridium Barteroides, Rumminococcus, Butyribacterium, Propionicbacterium, Eubacterium, and lactobacillus* (Mara and Horan, 2003). The facultative members of this group also help protect the oxygen-sensitive methanogens by consuming traces of oxygen that may enter in the feed (Mara and Horan, 2003).

Acidogenesis is considered to be the fastest step in the anaerobic digestion process (Mata-Alvarez, 2003) and the microorganisms involved in acidogenesis are least susceptible to inhibition: as often observed in acidified digesters, although methane production ceases, the fatty acids present in the digestate can continue to increase. However, according to Zhang et al. (2005), acidogenesis works at its best at a pH neutral condition. The results of a batch experiment showed pH adjustment to 7 almost doubled acidogenesis rates of kitchen wastes, compared with pH at 5, 9 and 11.

2.1.3 Acetogenesis

Acetogenesis is carried out by acetogenic bacteria and produces acetate, carbon dioxide and hydrogen, which are substrates that can be used by methanogens. Acetogens can be classified, on the basis of their metabolism, into two distinct groups, i.e. the obligate hydrogen-producing acetogens (OHPA) and homoacetogens (Mara and Horan, 2003).

The OHPA, also termed proton-reducing acetogens, feed mainly on fatty acid intermediates (propionic acid, butyric acid and other longer chain fatty acids) produced in the acidogenic process, and convert these into acetic acid, hydrogen, and carbon dioxide via a β-oxidation pathway (Mara and Horan, 2003). In this process the carbon chain of the fatty acid is cleaved by coenzyme A to yield a acetyl-CoA and a CoA linked fatty acid chain 2 carbon atoms shorter than the previous one (Berg et al., 2006). In the final step, the CoA is split from the carbon chain to form acetic acid and fatty acid which is two carbons shorter (Figure 2.2).

$$\underset{Stearic\,acid}{CH_3(CH_2)_{15}CH_2COOH} \xrightarrow{\beta - oxidation} \underset{Palmitic\,acid}{CH_3(CH_2)_{13}CH_2COOH} + \underset{Acetic\,acid}{CH_3COOH} + 2H_2 \quad [2.3]$$

In case of a saturated fatty acid with an even number carbon chain, the oxidation process is simply repeated until the fatty acid is completely converted into acetyl-CoA (Berg et al.,

2006). Although most fatty acids that occur naturally have such a structure because of their synthesis mode (Berg et al., 2006) there are still many which have an odd number of carbons in the chain.

In the case of those fatty acids containing an odd number of carbons a more complicated process is involved. Firstly, they are oxidised via β-oxidation in the same way as the even carbon chain length fatty acids until in the final round of the degradation propionyl-CoA and acetyl CoA are the two product molecules rather than two molecules of acetyl-CoA. Therefore propionic acid is always produced in the last stage of oxidation of fatty acids with an odd number in carbon chain (Pereira, 2003).

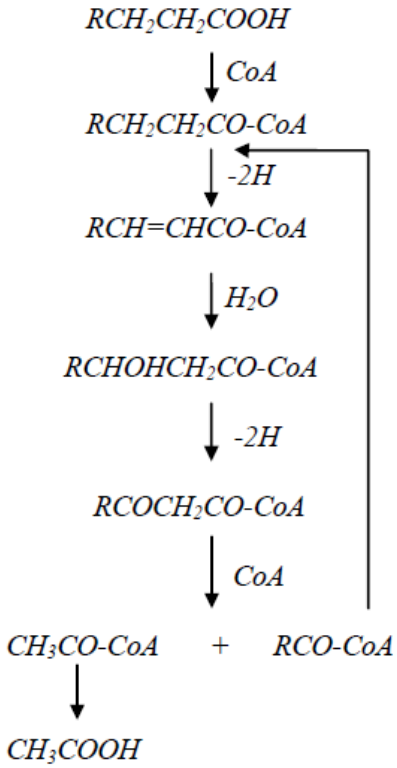


Figure 2.2. Schematic illustration of β-oxidation pathway (Pereira, 2003)

Oxidation of propionate requires an obligate syntrophic consortium of acetogenic proton- and bicarbonate reducing bacteria and methanogenic archaea (De Bok et al., 2004) for its degradation. Because of it key role as an intermedate in the anaerobic degradation of lipid and fatty acid a number of studies have explored the exact degradation pathway of propionate, and so far serveral possible pathways have been proposed.

One proposed route for propionate oxidation is via methyl-malonyl-CoA pathway, a route in which propionyl-CoA, methyl-malonyl-CoA, succinyl-CoA, succinate, furmarate, malate, pyruvate, and acetyl-CoA are intermediates, yielding acetate, CO_2, and H_2 or formate (de Bok et al. 2004; Houwen et al., 1987; Houwen et al., 1990; Koch et al., 1983; Schink, 1985; Schink, 1991). A second pathway was also discovered in which there is dimerisation to a six-carbon intermediate which is subsequently cleaved into an acetyl and a butylyl part followed by partial further oxidation (Figure 2.3) (De Bok et al., 2001; De Bok et al., 2004).

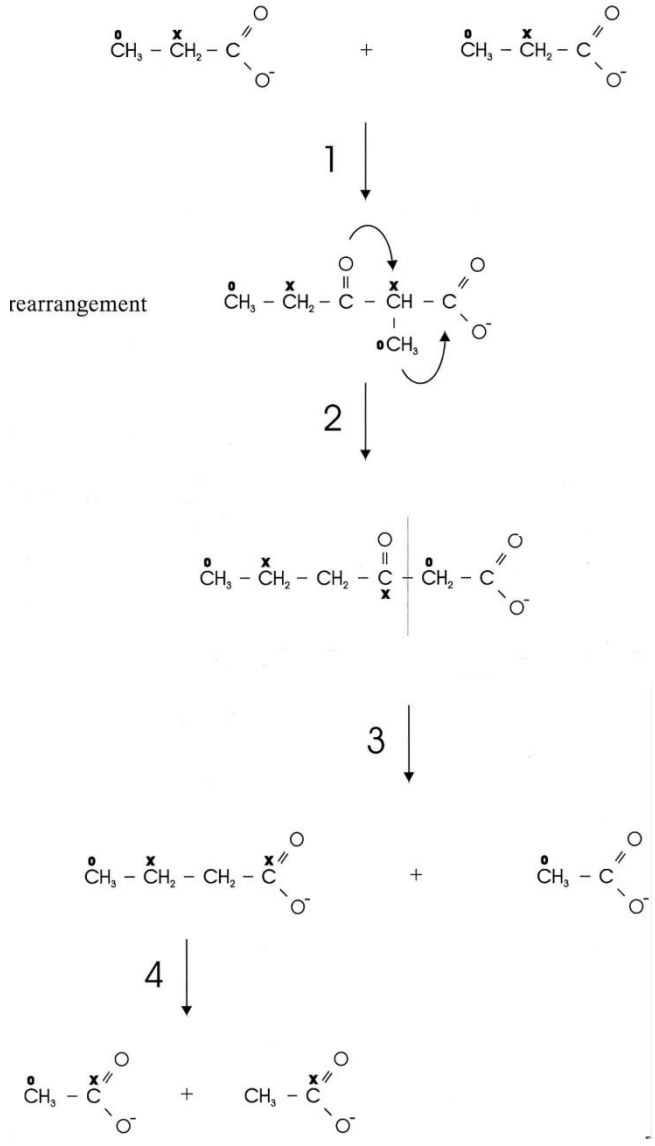


Figure 2.3. One of the pathways for propionate conversion proposed by De Bok et al. (2001)

The second group of acetogens, i.e. homoacetogens, are obligate anaerobes that use the unique reductive Wood-Ljungdahl pathway, this is also called the carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) metabolic pathway (Figure 2.4), because the main mechanism for energy conservation and for synthesis of acetyl-CoA and cell carbon is from CO_2 (Müller, 2003).

$$2CO_2+4H_2 \rightarrow CH_3COOH+2H_2O \quad [2.4]$$

$$4CO+2H_2O \rightarrow CH_3COOH+ 2CO_2 \quad [2.5]$$

$$4HCOOH \rightarrow CH_3COOH+2H_2O \quad [2.6]$$

$$4CH_3OH +2CO_2 \rightarrow 3CH_3COOH+2H_2O \quad [2.7]$$

$$2CO+2H_2 \rightarrow CH_3COOH \quad [2.8]$$

$$2HCOOH+2H_2 \rightarrow CH_3COOH + 2H_2O \quad [2.9]$$

It is this metabolic capability that differentiates homoacetogens from organisms that synthesize acetate by other metabolic pathways and enables them to grow by the conversion of C1 compounds such as H_2-CO_2, CO, formate and various other products (Reaction 2.4-9) from the biodegradation of most natural polymers like cellulose and hemisellulose: these includes sugars, alcohols, organic acids, aldehydes, and aromatic compounds which are converted into acetate (Diekert and Wohlfarth, 1994; Ragsdale and Pierce, 2008).

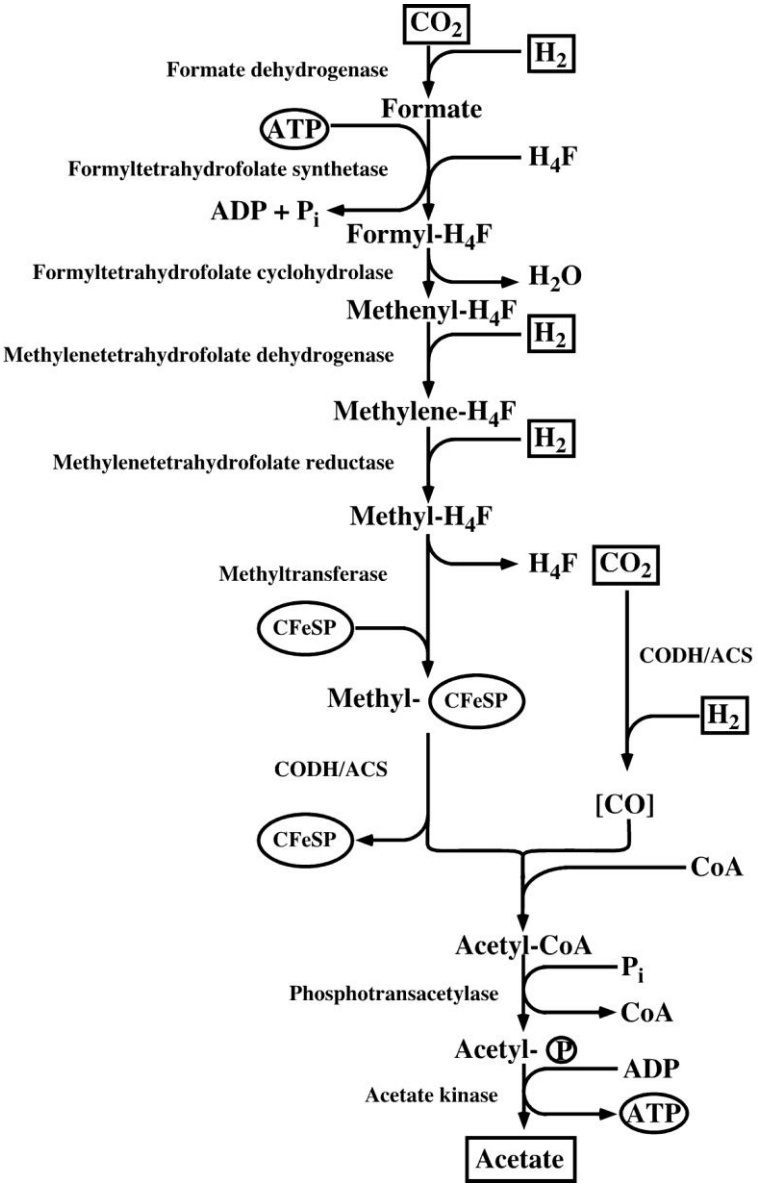


Figure 2.4. Wood-Ljungdahl pathway (Müller, 2003)

2.1.4 Methanogenesis

Methanogenesis is the final stage of anaerobic digestion which is performed by methanogenic archaea, namely methanogens, using a variety of one-carbon (C-1) compounds or acetic acid as a terminal electron acceptor (Ferry 1992a). Three metabolic pathways in methanogens have so far been discovered (Galagan et al., 2002): 1.) hydrogenotrophic pathway: in this methanogens reduce CO_2 to methane using electrons derived by oxidizing H_2; 2.) acetoclastic pathway: in this methanogens split acetate into a methyl group and an enzyme-bound CO, with the CO subsequently oxidized to provide electrons for the reduction of the methyl group to methane; and 3.) methylotrophic

pathway: in this the methanogens utilize C-1 compounds such as methanol or methylamines with one molecule of C-1 compound being oxidized to provide electrons for reducing three additional molecules to methane. In all cases, an electrochemical gradient is generated which is used in ATP synthesis and in all pathways the final step is the reduction of methyl-CoM to methane (Galagan et al., 2002). A schematic illustration of the metabolic route in methanogenesis is shown in figure 2.5.

The methanogens are thus a highly specialised group of microorganisms with a restricted feedstock and methanogenesis being the only way that they can obtain energy for growth (Thauer, 1998). Most methanogens possess only one of the three methanogenic pathways described; however, *Methanosarcina* species possess all three. (Galagan et al., 2002).

Table 2.2 Classification of methanogens (Kasapgil, 1994)

Order	Family	Genus	Species	Gram reaction	Morphology	Substrate
Methanobacteriales	Methanobacteriaceae	*Methanobacterium*	*M. fomcicum*	+	Long rods, filaments	H_2, CO_2, formate
			M. bryanti	+	Short/long rods	H_2, CO_2, formate
			M. thermoautotrophicum	+	Long rods, filaments	H_2, CO_2, formate
			M. wolfei	+	Rods	H_2, CO_2
			M. alcaliphilum	+	Rods	H_2, CO_2
			M. uliginosum	-	Rods	H_2, CO_2
			M. thermoformicium	+	Rods	H_2, CO_2, formate
		Methanobrevibacter	*M. arbophilius*	+	Short rods and short chains	H_2, CO_2, formate
			M. ruminantinum	+		H_2, CO_2, formate
			M. smithii	+		H_2, CO_2, formate
	Methanothermaceae	*Methanothermus*	*M. fervidus*		Short rods	H_2, CO_2
			M. sociabilis		Rods	H_2, CO_2
Methanococcales	Methanococcaceae	*Methanococcus*	*M. vannielli*	-	Irregular cocci	H_2, CO_2, formate
			M. voltae	-		H_2, CO_2, formate
			M. maripaludis	-	Single or pairs	H_2, CO_2, formate
			M. thermolithotrophicus	-		H_2, CO_2, formate
			M. halophilus	-		Methanol, methylamines
			M. jannaschi	-	Irregular cocci	H_2, CO_2, formate
			M. deltae	-		H_2, CO_2, formate
			M. frisisus	-	Irregular cocci	Methanol, methylamines
Methanomicrobiales	Methanomicrobiaceae	*Methanomicrobium*	*M. mobile*	-	Short rods single	H_2, CO_2, formate
			M. paynter	-	Short rods single	H_2, CO_2
		Methanogenium	*M. cariaci*	-	Irregular cocci,	H_2, CO_2, formate

		M. marisnigri	-	single or pairs	H_2, CO_2, formate
		M. olentagyi			H_2, CO_2, formate
		M. thermophilicum	+	Irregular cocci	H_2, CO_2
		M. aggregands	+	Irregular cocci	H_2, CO_2, formate
		M. bourgense	+	Irregular cocci	H_2, CO_2, formate
		M. tationis		Irregular cocci	H_2, CO_2, formate
	Methanospirillum	*M. hungatei*	-	Spirillum, regular rods and filaments	H_2, CO_2, formate
Methanoplanaceae	*Methanoplanus*			Plate shape	H_2, CO_2, formate
Methanosarcinaceae	*Methanosarcina*	*M. limicola*	-	Pseudosacina	H_2, CO_2, formate
		M. barkeri	+	Irregular cocci in large aggregates	
		M. Mazei	+	Pseudosacina	
		M. thermophila		Pseudosacina, coccoid	Acetate
		M. acetivorans	+	Pseudosacina	Methylamines
		M. vacuolate		Irregular cocci	
	Methanococcoides	*M. methylutents*	+	Irregular cocci sheath forming long filament	Methanol, methylamines
	*Methanothrix**	*M. soehngenii*	+		Acetate
		M. concilli		Sheathed rod	Acetate
	Methanolobus	*M. tindarius*	+	Irregular cocci single or loose	Methanol, methylamines

* The usage of genus name *Methanothrix* has been replaced by *Methanosaeta*, as the previous description of the type species, *Methanothrix soehngenii*, was based on a contaminated culture. (Patal and Sprott, 1990)

Taxonomically methanogens all belong to the archaeal kingdom of *Euryarchaeota*. Based on their distinct cell physiological and nutritional properties, Kasapgil (1994) made an attempt to organise the taxonomy of methanogens and the result of his work is summarised in Table 2.2.

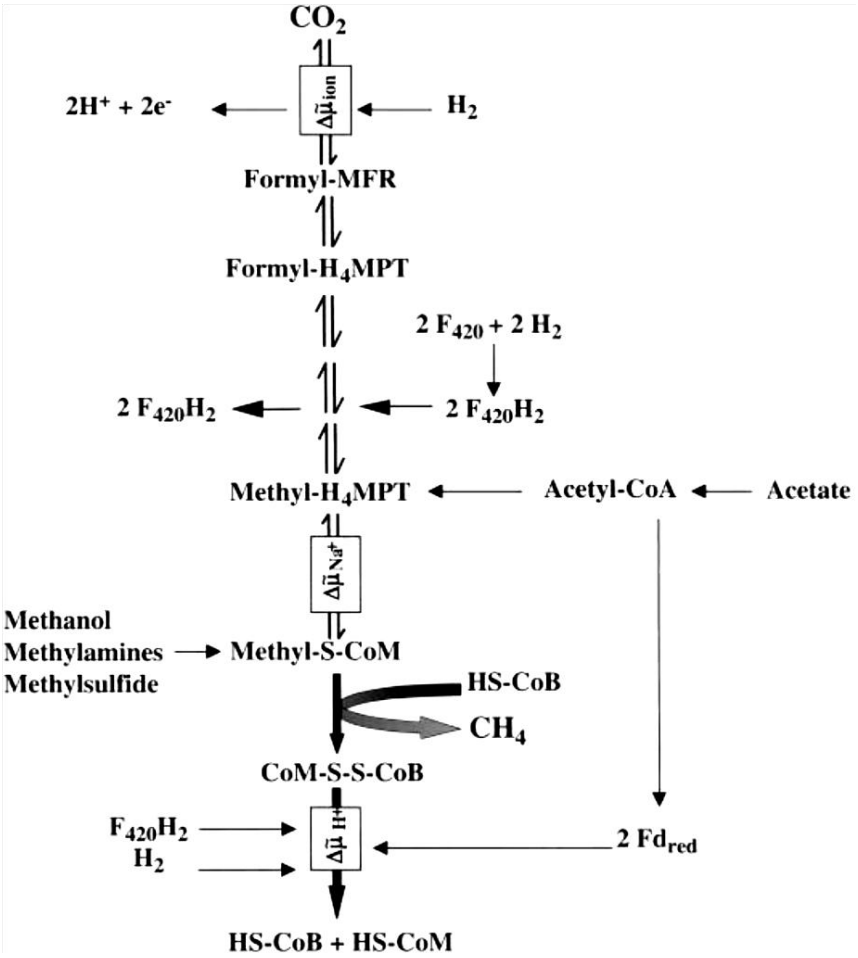


Figure 2.5. Schematic overview of the methanogenic pathways.

Notes: $\Delta\tilde{\mu} H^+$, electrochemical proton gradient; $\Delta\tilde{\mu} Na^+$, electrochemical sodium ion gradient; $\Delta\tilde{\mu}$ ion, electrochemical ion gradient (either Na^+ or H^+ are used as coupling ions). Reducing equivalents for the reduction of CO_2 are probably derived from the catalytic activity of the Ech hydrogenase that oxidizes molecular hydrogen (Deppenmeier, 2002).

Two mechanisms by which acetate can be converted into methane have been discovered. *Methanosarcina, Methanosaeta spp* and the thermophilic, acetate-utilizing methanogenic (TAM) organism (Ahring and Westermann, 1984, Ferry, 1992b) apply the acetoclastic pathway. The second mechanism involves syntrophic acetate oxidation in which the

acetate is converted to carbon dioxide and hydrogen which are subsequently converted to methane by hydrogenotrophic methanogens (Schnürer et al., 1994).
A number of important environmental factors influence the metabolic pathway from acetate. These are: temperature; organic acid concentrations; and ammonia concentration (Karakashev et al., 2006). At temperatures between 50°C and 65°C, syntrophic acetate oxidation is favoured at low acetate concentrations, while acetoclastic methanogenesis is favoured at high acetate concentrations (Zinder and Koch, 1984). The dominance of syntrophic acetate oxidation at lower concentrations increases with increased temperature (Karakashev et al., 2006). In the presence of inhibitors, particularly ammonium, syntrophic acetate oxidation is believed to be the main mechanism for acetate degradation (Schnürer et al., 1999).

Several studies have reported that acetoclastic methanogens are more sensitive to ammonia toxicity than the hydrogenotrophic methanogens (Sprott and Patel, 1986, Schnürer et al., 1999). Although it is often stated that two thirds of the methane formed in an anaerobic digester is derived from the acetoclastic pathway (Gujer and Zehnder., 1983) and that the syntrophic pathway was only observed under thermophilic condition (Zinder and Koch, 1984), this view is no longer valid. In mesophilic high ammonia environments the syntrophic acetate oxidation pathway has been observed (Schnürer et al., 1994, Schnürer et al., 1999, Schnürer et al., 1996) suggesting that dominant acetate oxidation pathway can shift under such conditions.

The acetoclastic pathway has generally been considered to be the dominant pathway, with either *Methanosarcinaceae* or *Methanosaetaceae* as the dominant methanogenic groups; therefore digester operation and optimisation are often based on maintaining acetoclastic methanogen populations (Karakashev et al., 2006). If a second pathway, such as syntrophic acetate oxidation can become dominant then it may become necessary to re-evaluate this strategy under certain conditions in commercial digesters.

2.1.4.1 Acetoclastic Pathway

In the acetoclastic pathway, acetate is cleaved to a methyl group and a carboxyl group. The methyl group is converted directly to methane, whereas the carboxyl group is oxidised to CO_2 (Reaction 2.11). Reduction of the methyl group to methane is made possible *in vivo*

by using electrons derived from the oxidation of the acetate carbonyl group catalysed by CODH. The requirement for an electron carrier such as ferredoxin has been established using cell extracts with hydrogen gas as the source of reducing equivalents (Ragsdale and Pierce, 2008). Carbonyl group oxidation to carbon dioxide was shown by experiments in which there was an isotope exchange between [1-14C] acetyl-CoA and CO, in extracts of *Methanosarcina barkeri* (Fischer and Thauer, 1990).

$$CH_3COOH = CH_4 + CO_2 \quad [2.11]$$

Reaction 2.11 is an exergonic reaction (ΔG= -31.0 kJ mol^{-1}), therefore is the favoured option and theoretically it can proceed in the absence of other methanogenic pathways. At present, two genera of methanogen, *Methanosarcina* and *Methanosaeta*, are known to perform this biological process (Hattori, 2008).

In retrospect to the Wood-Ljungdahl pathway discussed previously, the mechanism of acetate cleavage is analogous, but opposite in direction to, the autotrophic CO_2 fixation and acetate synthesis direction of the Wood-Ljungdahl pathway.

The acetoclastic pathway employs a set of highly specialised enzymes and coenzymes and it is achieved through biochemical reactions in a stepwise manner.

The central reaction in this pathway involves the enzyme carbon monoxide dehydrogenase (CODH). The CODH complex also oxidizes CO to CO_2 and, therefore, is referred to as a CODH enzyme complex; however, the primary function is cleavage of acetyl-CoA during growth on acetate. The CODH catalysed reaction involve excision of the carbonyl group of acetyl- CoA, the release of free CoA, and transfer of the methyl group to an acceptor corrinoid iron-sulphur protein (Grahame, 1991). Detailed reactions are discussed below:

First, the acetate binds with coenzyme A (CoA) to form acetyl-CoA ($CH_3COSCoA$) (Reaction 2.12). The enzymes involved in this step are acetate kinase and phosphotransacetylase in *Methanosarcina spp*. or acetate thiokinase in *Methanosaeta spp*.

$$CH_3COOH + CoA \longrightarrow CH_3COSCoA + H_2O \quad [2.12]$$

One high-energy phosphate bond is consumed for each acetate molecule which is bound with CoA: this represents a considerable investment considering the small amount of energy available for ATP synthesis (Ferry, 1992a).

Acetyl-coA has been identified as the activated form of acetate required for *in vitro* methanogenesis (Fischer and Thauer, 1988). The methyl group of acetyl-CoA is ultimately transferred to coenzyme M and then reduced to methane in a pathway in which methyltetrahydromethanopterin (CH_3-H_4SPT) is an intermediate (Fischer and Thauer, 1989); the latter is shown in reactions 2.13, 2.14 and 2.15.

After activation of acetate to acetyl- CoA the CODH enzyme complex plays an essential role in the cleavage of the C-C and C-S bonds of acetyl-CoA, as shown by the exchange of CO with the carbonyl group of acetyl-CoA (Raybuck et al., 1991). After cleavage of the acetyl-CoA, the enzyme-bound carbonyl group is oxidized to CO_2 and the methyl group is transferred to the cofactor tetrahydrosarcinapterin (H_4SPT), which is a one-carbon carrier found only in members of the archaea domain (Ferry, 1992a). (Reaction 2.13)

$$CH_3COSCoA + H_4SPT \longrightarrow CH_3\text{-}H_4SPT + CO_2 + CoA + 2[H] \qquad [2.13]$$

After cleavage of acetyl-CoA, the methyl group of CH_3-H_4SPT is subsequently transferred to coenzyme M (H-S-CoM) by substitution of the hydrogen atom in the thiol group (Reaction 2.14), involving Methyl-H_4SPT and coenzyme M methyltransferase as an intermediate.

$$CH_3\text{-}H_4SPT + H\text{-}S\text{-}CoM \longrightarrow CH_3\text{-}S\text{-}CoM + H_4SPT \qquad [2.14]$$

The final step in the utilisation, which is common for all methanogenic substrates, is the reductive demethylation of CH_3-S-CoM to CH_4 and this is catalysed by CH_3-S-CoM methylreductase. The two electrons required for the reduction are derived from the sulphur atoms of CH_3-S-CoM and H-S-HTP (7-mercaptoheptanoylthreonine phosphate), yielding the heterodisulphide CoM-S-S-HTP (Reaction 2- 15).

$$CH_3\text{-}S\text{-}CoM + H\text{-}S\text{-}HTP \longrightarrow CoM\text{-}S\text{-}S\text{-}HTP + CH_4 \qquad [2.15]$$

Figure 2.6 summarises the general route and enzymes involved in the acetoclastic pathway adopted by *Methanosarcina* and *Methanosaeta spp.*

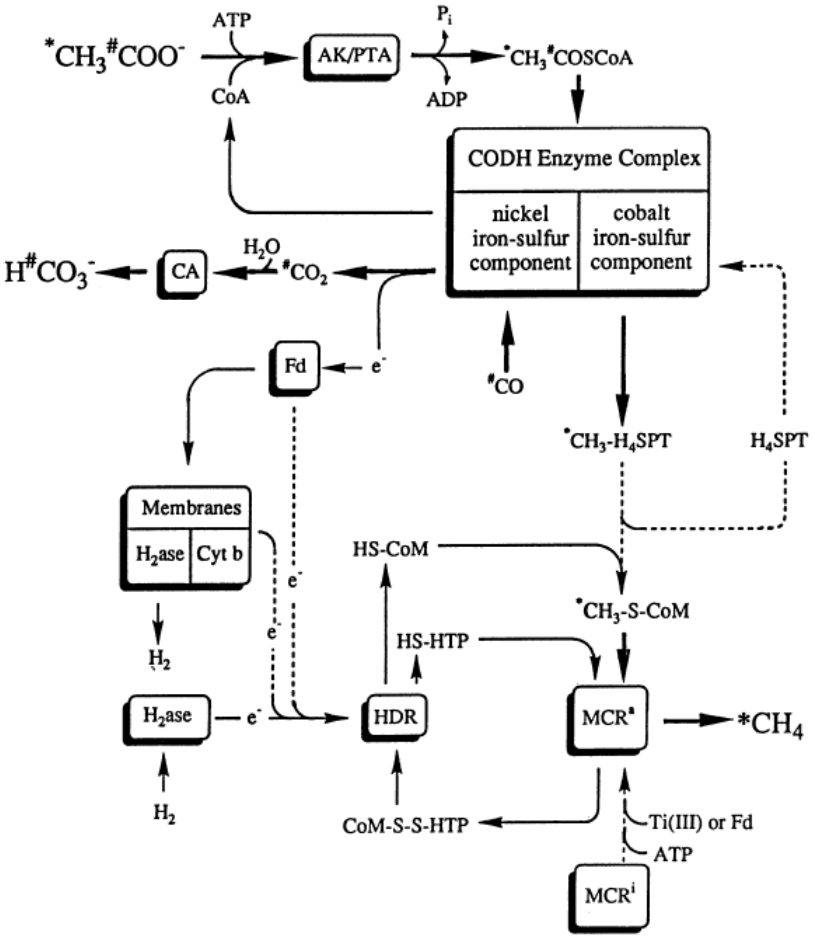


Figure 2.6. A schematic illustration of the pathway for the conversion of acetate to CO_2 and CH_4.

Note: AK= acetate kinase; PTA= phosphotransacetylase; CA=carbonic anhydrase; MCR^i= inactive methylreductase; MCR^a= active methylreductase; HDR= heterodisulphide (CoM-S-S-HTP) reductase; Fd= ferredoxin; Cyt b= cytochrome b; H_2ase= hydrogenase; e^-=electron. The carbon atoms are marked with * and # symbols to distinguish the two sources of carbons, the methyl and carboxyl groups, respectively (Ferry, 1992a).

2.1.4.2 Syntrophic Acetate Oxidation to Methane Pathway

Syntrophic acetate oxidation to methane consists of two major steps: syntrophic acetate oxidation to H_2 and CO_2 coupled to hydrogenotrophic methanogenesis (Barker, 1936). The pathway thus relies on two groups of microbes: syntrophic acetate oxidising bacteria and hydrogenotrophic methanogens, respectively. The two microbes have an obligatory requirement for each other because the acetate oxidising bacteria require a hydrogen

scavenger to reduce the hydrogen partial pressure which is necessary to allow the acetate oxidising reaction to proceed. On the other hand, the hydrogenotrophic methanogens require a hydrogen supply (Lee and Zinder, 1988c).

In syntrophic acetate oxidation, both methyl and carboxyl groups are oxidised to CO_2 along with the production of H_2 (Reaction 2.16)

$$CH_3COO^- + 4H_2O = 2HCO_3^- + 4H_2 + H^+ \quad [2.16]$$

This reaction is energetically unfavourable under standard conditions due to the high Gibbs free energy (ΔG=+104.6 kJ mol^{-1}); however the reaction can proceed if H_2 consuming methanogenesis eliminates the hydrogen (Reaction 2.17).

$$4H_2 + 2HCO_3^- = CH_4 + 3H_2O \quad [2.17]$$

Reaction 2.17 is an exergonic reaction (ΔG=-135.6 kJ mol^{-1}), therefore from the combined reactions 2.16 and 2.17, the overall reaction becomes exergonic with the same Gibbs free energy as from acetoclastic methanogenesis.

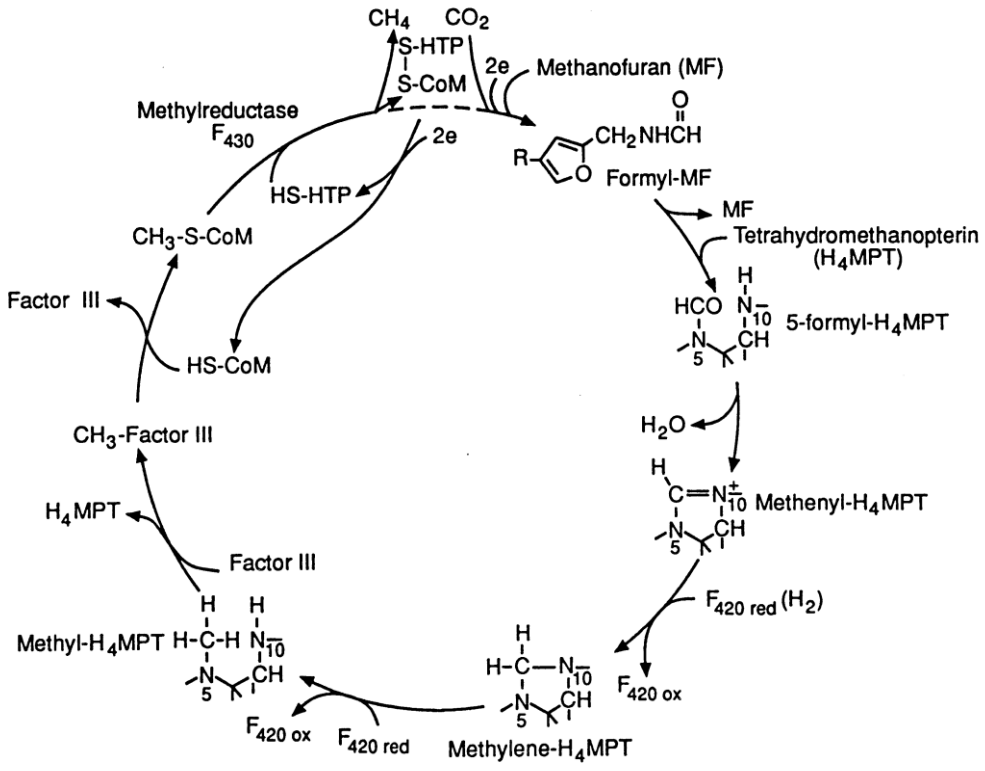


Figure 2.7. The pathway of CO_2 reduction to CH_4 (Ferry, 1992b)

To the best of the author's knowledge, four syntrophic acetate oxidising bacteria have been reported to date and are known to work syntrophically with hydrogenotrophic methanogens. These bacteria are thermophilic acetate-oxidising rod-shaped eubacterium (AOR) (Lee and Zinder, 1988a), *Clostridium ultunense* strain BS (Schnürer et al., 1996), *Thermacetogenium phaeum* strain PB (Hattori et al., 2000) and *Thermotoga lettingae* strain TMO (Balk et al., 2002). Apart from *Thermotoga lettingae*, the other three all have the ability to synthesis acetate from CO_2 and H_2. In addition, in these three bacteria the key enzymes of the Wood-Ljungdahl pathway (CO dehydrogenase, formate dehydrogenase, formyl-THF synthetase, methylene-THF dehydrogenase and methylene-THF reductase) have been detected when grown in pure culture. The evidence therefore strongly implies that the mode of operation of the oxidation is by the Wood-Ljungdahl pathway in these pure cultures. Similar observations have been made in the AOR/methanogen co-culture, *Clostridium ultunense/*methanogen co-culture and *Thermotoga lettingae/*methanogen co-culture.

The hydrogenotrophic methanogens reduce carbon dioxide using hydrogen as an electron donor. The metabolic pathway is illustrated schematically in Figure 2.7.

The biochemical reaction of this pathway involves several novel enzymes and cofactors.
First, carbon dioxide is reduced to the methyl level; the reaction is catalysed by formyl-methanofuran (Formyl- MF) dehydrogenase. The reaction involves bonding of CO_2 to a primary amine followed by a two-electron reduction (reaction 2.18) (Ferry, 1992b).

$$CO_2 + MF + H_2 \longrightarrow \text{formyl-MF} + H_2O \qquad [2.18]$$

It is noteworthy that the enzyme activity of Formyl-MF dehydrogenase is rapidly inhibited by cyanide, which is consistent with the presence of the cyanide-sensitive molybdenum site of the enzyme (Börner et al., 1989; Ferry, 1992b).

The formyl group of formyl-methanofuran is then transferred to tetrahydromethanopterin (H_4 MPT) catalysed by formyl-MF:H_4 MPT formyltransferase (FTR), as shown in Reaction 2-19, and subsequently converted to the methenyl derivative by a dehydrating cyclisation as shown in Reaction 2-20. Conversion of 5-formyl-H_4 MPT to the 5,10-methenyl derivative is catalysed by 5,10-metheny1-H_4 MPT cyclohydrolase.

Formyl-MF + H_4 MPT ⟶ 5-formyl-H_4 MPT + MF [2.19]

5-formyl-H_4 MPT + H^+⟶ 5, 10-methenyl-H_4 MPT $^+$ + H_2O [2.20]

The methenyl group is then further reduced to formaldehyde (carbon atom with 2 hydrogen bonds) by the reduced coenzyme F_{420} and catalysed by 5,10-methenyl-H_4MPT dehydrogenase (shown in Reaction 2-21). Similarly the methenyl group is reduced to the methyl level and this is catalysed by 5,10-methylene-H_4 MPT reductase that also utilizes reduced F_{420} ($F_{420}H_2$) as the physiological electron donor. (Reaction 2-22)

Coenzyme F_{420} is therefore an obligate two-electron carrier that donates or accepts a hydride ion. It therefore exists in two forms (reduced and oxidised), dependent on the hydride ion flow direction (Figure 2.8). The electrons are derived from reducing F_{420} to its reduced form ($F_{420}H_2$), which is catalysed by the F_{420}-reducing hydrogenase. Electron carriers other than F_{420} that could participate in electron transfer from H, or formate have not been identified (Ferry, 1992b).

5,10-methenyl-H_4 MPT $^+$ + $F_{420}H_2$⟶ 5,10-methylene-H_4 MPT + F_{420} + H^+ [2.21]

5,10-methylene-H_4 MPT + $F_{420}H_2$ ⟶ 5-methyl-H_4 MPT + F_{420} [2.22]

The methyl group of methyl-H_4 MPT is subsequently transferred to coenzyme M (HS-CoM) by the methyl-H_4 MPT:HS-CoM methyltransferase (Reaction 2-23).

5-methyl-H_4 MPT+HS-CoM⟶CH_3-S-CoM+H_4 MPT [2.23]

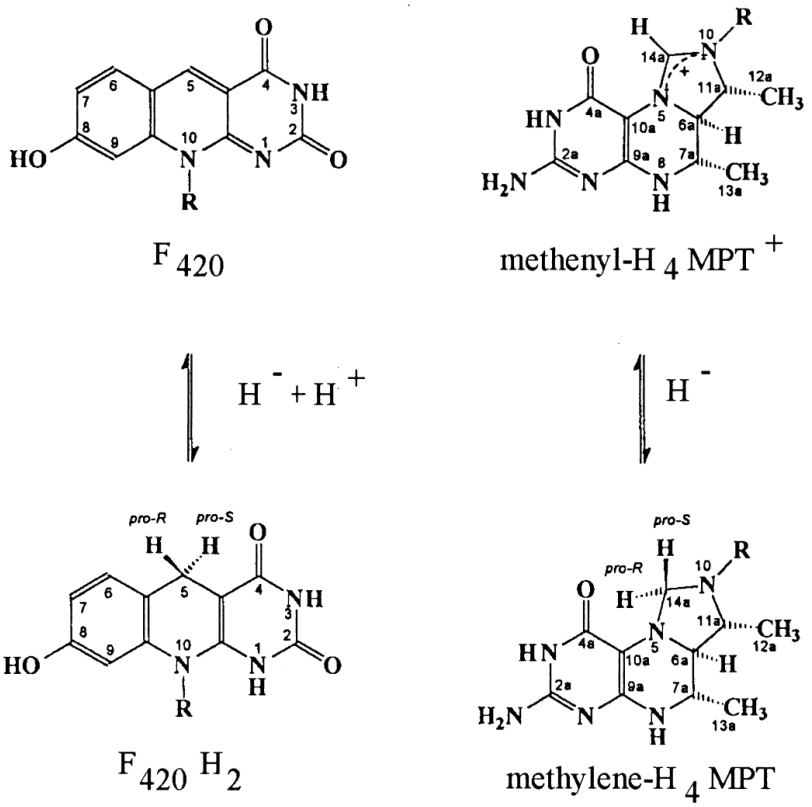


Figure 2.8. Structure of F_{420} (reduced and oxidised) and the conversion of methenyl-H_4 MPT^+ to methylene-H_4 MPT

This exergonic reaction ($\Delta G'_0$ = –29.7 kJ mol^{-1}) is coupled to the formation of an electrochemical sodium ion gradient (Deppenmeier, 2002). In the final reaction (2-24), catalysed by a soluble methyl-CoM reductase, methylcoenzyme M (CH_3-S-CoM) is reduced with N-7-mercaptoheptanoylthreonine phosphate (H-S-HTP = CoB) to yield the final product methane (Ellermann et al. 1988).

$$CH_3\text{-S-CoM} + \text{HS-HTP} \longrightarrow CH_4 + \text{CoM-S-S-HTP} \qquad [2.24]$$

2.1.5 Determination of the Dominant Methanogenic Pathway in Anaerobic Digesters

Our current knowledge about the metabolic profile of methanogens in anaerobic digesters is limited. Although attempts have been made by a number of researchers to study methanogen population profile (Hansen et al., 1999; Karakashev et al., 2005; Karakashev et al., 2006; McMahon et al., 2001; Raskin et al., 1994; Schnürer et al., 1994; Schnürer and Nordberg, 2008), there is still a broad range of systems that have not been surveyed. In

addition, the influence of environmental and operating conditions, such as temperature, organic acid concentrations, ammonia concentrations, loading rate, pH, and the presence of certain inhibitors, has not been assessed in conjunction with an analysis of the methanogenic community. Assessing these factors in relation to the process biochemistry and/or population structure may allow better optimisation of the process by identifying key factors that could account for enhanced or reduced process efficiency or stability.

Where community structure has been analysed this has usually employed molecular biology techniques such as Fluorescent *in situ* Hybridisation (FISH), Quantitative Polymerase Chain Reaction (q-PCR) and DNA sequencing to profile the methanogen phylogenetic groups (Daims et al. 2005, Karakashev et al., 2006, Schnürer and Nordberg, 2008). From studying the composition of the methanogenic population, information on pathways in methanogenesis can be gathered. There are, however, several shortcomings in using molecular biology techniques to determine the dominant methanogenic pathway. Firstly, all the methods require extensive training, complicated sample preparation and instrumentation. Secondly, it is possible that certain phylogenetic group of methanogens that are present in the system may not be actively involved in methanogenesis due inhibitors etc. Thirdly, *Methanosarcina* (a member of the *Methanosarcinaceae* family) is known to adopt both acetoclastic and hydrogenotrophic pathways (Galagan et al., 2002): it is therefore not possible to distinguish the dominant methanogenic pathway if *Methanosarcina* is dominant in the sample.

For practical reasons, it is much more desirable to have a direct method to determine the methanogenic pathway in anaerobic digesters without the need for molecular biology methods. Currently there are a number of experimental approaches that can achieve this, for example the stable C-13 or radioactive C-14 isotopic tracer and specific inhibitor methods. Within those, the most frequently reported method is to adopt radioactive isotopic tracers, e.g. addition of C-14 isotope labelled bicarbonate and/or acetate.

Isotope labelling experiments can provide important information regarding the carbon flow in complex biochemical reactions. Some pioneering works (Wood, 1952) in searching for the mechanism of CO_2 fixation and methanogenesis were reliant on the application of isotopic tracer techniques.

It is relatively straightforward to detect acetate oxidation activity by measuring the production of $^{14}CH_4$ and $^{14}CO_2$ from acetate labelled in the methyl group (C-2) (Karakashev et al., 2006). When acetoclastic methanogens degrade acetate, the labelled methyl group will form only labelled methane (Ferry, 1993). During syntrophic acetate oxidation, both carbon atoms of acetate are converted to carbon dioxide, and some of the carbon dioxide is subsequently reduced to methane (Schnürer et al., 1999). Therefore, significant concentrations of labelled carbon dioxide from [2-14C] acetate will be formed during the oxidation of acetate. By following the production of $^{14}CH_4$ and $^{14}CO_2$, the degree of acetate oxidation (measured as $^{14}CO_2$ /$^{14}CH_4$ ratio) can be used as indicator of the dominant methanogenesis route. A low $^{14}CO_2$ /$^{14}CH_4$ ratio is indicative of an acetoclastic dominated route and a high ratio indicative of high level of syntrophic acetate oxidation.

Several studies have proposed methods for acetate isotope labelling experiments, (Karakashev et al., 2006; Schnürer et al., 1994; Schnürer et al., 1996; Schnürer et al. 1998; Schnürer and Nordberg, 2008; Zehnder et al., 1979). Most of these have adopted a liquid scintillation counting technique for the determination of abundance of radioactive labelled CH_4 and CO_2. The method involves passing the biogas sample through an alkali (or in some cases ethanol-ethanolamine (1:2)) trap to absorb the CO_2 content. Both the CO_2 trap solution and the remaining CH_4 gas in the gas sample are subsequently added into their respective scintillation cocktail for radioactivity determination.

Nelson and Zeikus (1974) introduced an in-line GC-isotopic gas proportion counter technique. This gas chromatographic procedure offers simultaneous analysis of ^{14}C-labelled and unlabelled metabolic gases from microbial methanogenic systems. No special preparatory methods are required prior to the injection of gas samples into the gas chromatograph. The procedure for sampling is merely to withdraw gas from a reaction vial headspace and to inject this into the gas chromatograph-gas proportional counting system.
Such a system has great advantages over liquid scintillation counting in respect of preparation and handling of samples and has been used in several studies (Fey and Conrad, 2000; Fey et al., 2004; Zinder and Koch, 1984). According to Zehnder et al. (1979), however, the counting efficiency of this technique is low and variable if a high concentration of non-radioactive methane is present in the gas sample, due to its own quench effect.

2.2 Nutrient Requirement for Anaerobic Digestion

A well-balanced anaerobic digestion system requires a mix of nutrients to sustain all groups of anaerobic microbes. The elements that are essential fall into the categories of macro-nutrients – elements that are required in substantial amounts, for example, nitrogen and phosphorus; and micronutrients – elements that are required only in trace amounts, but that are essential for the continuation of the process: for example, cobalt, iron, nickel, tungsten, molybdenum and selenium. Many essential nutrients, however, can become inhibitory or toxic to the anaerobic digestion process when present in high concentrations. More details about inhibitory/toxic effects during anaerobic digestion are discussed in next section.

2.2.1 Macro-Nutrients

Macro-nutrient requirements for anaerobic processes are much lower than the requirements for aerobic processes due to the lower cell yield from the degradation of equal quantities of substrate. Apart from the carbon source, the two major macro-nutrients in anaerobic processes are nitrogen and phosphorus. These must be available in the digester and the quantity required can be determined from knowing the COD of the digester feedstock. For anaerobic treatment of wastewater, Henze and Harremoes (1983) recommended a COD: N ratio of 350:7 and 1000:7 for a highly loaded and low loaded system, respectively. Generally, the most commonly recommended COD:N ratio in anaerobic digestion is 100:2.5 (Mara and Horan, 2003). Alternatively, other researchers prefer to cite C/N ratio instead of COD:N ratio. In practice, when treating various feedstocks with different characteristics, the C/N ratio has proven to be critical in the consistent operation of the anaerobic digesters. If the C/N of the substrate is too high, the digester will be deficient in nitrogen, which is needed for build-up of the bacterial mass. If the C/N ratio is too low, the degradation of the substrate leads to increases in ammonia formation and this is toxic to the anaerobic microbes (Hartmann and Ahring, 2006). For food and yard (garden) waste, the C/N ratio, based on the biodegradable organic carbon, is below 20, whereas with mixed paper, the ratio can be over 100 (Kayhanian and Tchobanoglous, 1992). For the degradation of substrates in 'high solids' (dry) anaerobic digesters, the optimum C/N ratio was found to be in the range of 25 to 30 (Hartmann and Ahring, 2006). Since different waste fractions are characterised by different C/N ratios, the optimum C/N ratio can be

achieve by co-digestion, substrate with a higher C/N ratio (e.g. paper, wood and cardboard, etc.) can be mixed with those with a lower C/N ratio.

Phosphorus is required for biological metabolism of all organisms involved in anaerobic digestion. Based on the empirical formula for cell biomass ($C_5H_7O_2NP_{0.06}S_{0.1}$), Speece (1996) has suggested a N:P ratio of 7:1. In practice, it is common to adopt a COD:P ratio in the range of 80:1 to 200:1 (Mara and Horan, 2003).

In addition to the two major macronutrients, other elements such as sulphur, calcium, magnesium, although required to a lesser extent, also have important roles in anaerobic microorganisms. Table 2.3 summarises the optimal concentration and effects of these macro-nutrients in the anaerobic digestion process.

Table 2.3. Effects and required concentration in anaerobic digestion for some macronutrients

Nutrient	**Concentration required (mg l^{-1})**	**Effects on digestion**
Ca	100-200 (Mara and Horan, 2003)	Essential for cell growth (Murray and Zinder, 1985) Facilitate granulation in UASB reactors (Mara and Horan, 2003)
Mg	75-150 (Mara and Horan, 2003) 720 (Ahring et al. 1991)	Increase cell activity and facilitate granulation.
Na	100-200 (Mara and Horan, 2003)	Increase cell activity
S	0.001-1.0 (Speece, 1996)	Sulphur source for protein synthesis

2.2.2 Micro-Nutrients (Trace elements)

As discussed in the previous section, under anaerobic conditions, microorganisms utilise a set of unique enzyme systems. Trace elements are often present in these enzyme systems as part of a cofactor or they are of vital importance for the enzyme system. On the non-enzymatic level, trace elements are also involved in microbial respiration processes with an electron transfer bound to cell wall or extracellular electron acceptors (Zandvoort et al., 2006). Although required only in trace amounts, in the case of deficiency, the anaerobic metabolic pathway will be hindered. On the other hand, all the trace elements are potential

toxicants. Once over the tolerable concentrations, they also can be inhibitory to microbial activity.

The supplementation of trace elements into the anaerobic digester is necessary when the trace element contents in the substrates are not sufficient for the metabolic processes of anaerobic digestion under the operational conditions. The specific requirement, however, in terms of essentiality and optimum dosage, is strongly dependent upon the speciation and the bioavailability of the element to the microorganisms (Zandvoort et al., 2006, Romera et al., 2007). Therefore, it is impossible to propose a 'generic' trace element supplementation regime which applies universally to all anaerobic digesters treating different varieties of substrates, and the optimisation of trace element supplementation still has to be based on the quantitative experimental trials. Nevertheless, the fundamental biochemistry of how trace elements function in anaerobic metabolic process is essential for the interpretation of experimental results.

2.2.2.1 Trace Element Uptake and Bioavailability

In order to have a biological function in microorganisms, trace elements first have to be taken up into the cells. The uptake is assumed to proceed mainly via the transport of free ions across the cell membrane (Zandvoort et al., 2006). This occurs in two steps: a passive adsorption of onto the biomass surface, followed by an energy-dependent transport into the cell. The former step is usually unspecific, constitutively expressed, and driven by the chemiosmotic gradient across the cytoplasmatic membrane of microorganisms; whereas the latter has high substrate specificity, is slower and inducible, often uses adenosine triphosphate (ATP) hydrolysis as the energy source, and is only used by a cell in times of need, starvation, or a special metabolic situation (Aquino and Stuckey, 2007).

Before the essential elements actually reach the biomass present in a biofilm or sludge, they are subjected to complex (bio)chemical processes in the reactor liquid such as precipitation (e.g., as sulphides) and the formation of inorganic and organic complexes. These processes can reduce the free ion concentration in solution to extremely low values. A lot of research has been published on speciation and the effect of trace elements on organisms in natural systems (Filgueiras et al., 2002, Hullebusch et al., 2005, Mossop and Davidson, 2003, Tessier et al., 1979, Wright et al., 2003). Little knowledge is available, however, on the relation between ion speciation and ion bioavailability in anaerobic

bioreactors; therefore rendering extremely difficult the determination of the optimum trace ion concentrations range to maintain an optimised cell functions.

2.2.2.2 Essential Elements and the Enzymological Foundation for Their Requirement in Methanogenesis

As discussed previously, the requirement for trace elements is very specific and dependent largely on the phylogenetic groups and metabolic pathways that are adopted. In a review of the enzymes involved in acetoclastic and hydrogenotrophic methanogenesis, it is understood that the specific trace element requirement is usually because of their irreplaceable function in enzyme systems associated with each of these pathways. To date, trace elements including selenium, cobalt, nickel, iron, molybdenum and/or tungsten have been identified as a constituent of enzymes or are a required cofactor involved in methanogenesis.

Methanogenesis was regarded as the step most likely to be subject to trace element deficiency in the anaerobic digestion process (Takashima et al. 1990, White and Stuckey 2000). This was thought to be because of their requirement in elaborate enzyme system associated with the biochemical reactions of methanogenesis. According to several studies and reviews (Diekert et al., 1980; Fermoso et al., 2009; Gonzalez-Gil et al., 1999; Zhang et al., 2003) all methanogens appear to require nickel, cobalt, and iron for growth; however, most of these studies are qualitative regarding the trace element requirements.

Scherer et al. (1983) has quantitatively determined methanogen elemental composition in 10 methanogen species. The 10 species were representative of all three orders of the methanogens and were cultivated under defined conditions. Special emphasis was given to *Methanosarcina barkeri*, represented by 5 strains and cultivated on various substrates. Trace metal elements as well as macro nutrients (C, H, N, P, K, S, Na, Ca, etc.) were measured by ICP-MS and an elemental analyser. The results showed that the metal content varied considerably between the different species of methanogens even when they were from the same genus and converting the same substrate. The ranges of trace elements contents in these 10 methanogens are listed following: Mg (0.09–0.53%), Fe (0.07–0.28%), Ni (65–180 ppm), Co (10–120 ppm), Mo (10–70 ppm), Zn (50–630 ppm), Cu (<10–160 ppm), Mn (<5–25 ppm). All investigated species contained high zinc contents, whereas copper seemed to be present only in some species.

Additional trace element requirements, i.e. selenium and tungsten have been reported for some methanogens (Fermoso et al., 2009). Selenium is present in a variety of selenoproteins, e.g. in hydrogenase and formate dehydrogenases (*Methanococcus jannaschii*) (Jones et al., 1979), formylmethanofuran dehydrogenase (*Methanopyrus kandleri*) (Vorholt et al., 1997). According to Ching et al. (1984), in *Methanococcus vannielii,* selenium containing tRNAs account for 13 to 20% of the total tRNA.

The essential biological functions of tungsten have been discovered relatively late, compared to its chemically analogous element molybdenum (Mo) (Kletzin and Adams, 1996). A stimulatory effect of W on the growth of methanogens was first reported in the late 1970s with *Methanococcus vannielii* (Jones and Stadtman, 1977). Its growth was dramatically enhanced by the addition of W, but not by Mo, to the growth medium when formate was used as the carbon and energy source, but not when the organism was grown on H_2 and CO_2. This suggested the involvement of a W-containing formate dehydrogenase (FDH).

The stimulatory effect of W on cell growth to some methanogen species is very concentration dependent. Winter et al. (1984) reported an obligately autotrophic (H_2/CO_2-utilising) methanogen, *Methanobacterium wolfei* whose growth was dependent upon the addition of yeast extract, ash of yeast extract (showing that an organic cofactor was not involved), or W (the responsible moiety). A linear correlation of growth rate and W-content was found up to a concentration of 7.6µM W in the medium.

As reviewed previously (see Figure 2.5), methanogenesis shares several biochemical reactions in common due to the overlapping routes, and therefore various trace elements involved in enzymatic systems responsible for these reactions are ubiquitously required in all methanogenic pathways. On the other hand, certain reactions are unique to each methanogenic pathway, therefore special elements are required.

All methanogenic pathways converge to the enzymatic reduction of methyl coenzymeM to methane. This reduction is catalysed by the Methyl-coenzyme M reductase complex, which includes a nickel containing cofactor F_{430} (Friedmann et al., 1990, Thauer, 2003).

Another metallo-enzyme that is present in all methanogenic pathways is the cobalt / corrinoid containing methyl-H_4MPT: Coenzyme M methyltransferase complex (Thauer, 1998). The first step of methanogenesis from methanol is also catalysed by a specific cobalt dependent methyltransferase, methanol:coenzyme M. In addition to cobalt, this enzyme also contains zinc (Sauer and Thauer, 2000).

Heterodisulphide reductase (HDR) is found in a number of methanogenic archaea, particularly in *Methanosarcina* species, which are also involved in all methanogenic pathways. It is an iron-sulphur protein that catalyses the reversible reduction of the heterodisulphide (CoM-S-S-CoB) of the methanogenic thiol-coenzymes, coenzyme M (CoM-SH) and coenzyme B (CoB-SH) (Hedderich et al., 2005).

From enzyme purification studies carried out on *Methanosarcina thermophila* (Murakami et al., 2001), *Methanothermobacter marburgensis* (Duin et al., 2002), *Methanosarcina barkeri* (Heiden et al., 1994) *and Methanothermobacter thermautotrophicus* (Setzke et al., 1994), it is understand that Fe is found in all HDR purified from the 4 methanogens, and Ni was found in *Methanosarcina barkeri* (Heiden et al., 1993) *and Methanothermobacter thermautotrophicus* (Setzke et al., 1994).

Formate dehydrogenase (FDH) is a member of the dimethyl sulphoxide (DMSO) reductase family. It catalyses the reversible two-electron oxidation of formate or reduction of CO_2 which is the first step of the hydrogenotrophic pathway. In some anaerobic microorganisms, FDH was reported to have a molybdenum or tungsten *bis*-(pyranopterin guanidine dinucleotide) cofactor and iron-sulphur clusters (Hilli, 2002; Romão, 2009) and shows great variability in quaternary structure, physiological redox partner, and cellular location (da Silva et al., 2011). Jones and Staddtman (1981) also reported two types of FDH in *Methanococcus vannielii*: a molybdenum-tungsten-selenium-iron-sulphur protein and a molybdenum-iron-sulphur protein with neither selenium nor tungsten.

Hydrogenases play key roles in the metabolism of methanogenic archaea in the hydrogenotrophic pathway. These comprise of a group of iron-sulphur flavoprotein (FAD) which contain nickel or in some cases also contain selenocysteine (Thauer et al., 1993). Two distinct hydrogenases have been detected in all methanogens capable of chemolithoautotrophic growth (Baron and Ferry, 1989, Michel et al., 1995). One (F_{420}-

hydrogenase) reduces the physiological low potential two-electron acceptor coenzyme F_{420} and also, to a lesser extent, the artificial electron acceptor methyl viologen (MV), the riboflavin analogue of F_{420} and flavins. The other (MV-hydrogenase) reduces MV but not F_{420}. All the hydrogenases purified from methanogens so far form large aggregates with molecular weights of 720,000 to 1,300,000 and contain nickel and iron-sulphur centres. Selenocysteine, in particular, was discovered in hydrogenase purified from *Methanococcus vannielii* (Yamazaki, 1982) and *Methanosarcina barkeri* (Michel et al., 1995)

Formylmethanofuran dehydrogenase is a molybdenum-containing enzyme that catalyses the terminal step in the oxidation of methanol to CO_2 and the first step in CO_2 reduction to CH_4 in autotrophic CO_2 fixation (Bertram and Thauer, 1994). A study carried out by Hochheimer et al. (1995) on pure culture shows that *Methanobacterium thermoautotrophicum* contains two formylmethanofuran dehydrogenase iso-enzymes, a tungsten form and a molybdenum containing form. The molybdenum enzyme is synthesized only when molybdenum is available in the growth medium. The tungsten enzyme is synthesized when either tungsten or molybdenum is present. If the growth medium contains molybdenum, the tungsten enzyme will contain molybdenum instead of tungsten (Hochheimer et al., 1995).

The key enzyme complex in the acetoclastic pathway is carbon monoxide dehydrogenase (CODH). CODH cleaves the C-C and C-S bonds in the acetyl moiety of acetyl-CoA, oxidizes the carbonyl group to CO_2 and transfers the methyl group to Coenzyme M. The CODH complex is composed of two enzyme components: a nickel/iron-sulphur component and a corrinoid/iron-sulphur component (Ferry, 1999). This enzyme complex is also involved in the formation of acetate by acetogens from H_2/CO_2 and methanol (Zandvoort et al., 2006).

Table 2.4 summarises the essential trace elements in various enzymes catalyzing anaerobic reactions and transformations.

Table 2.4. Major metalloenzymes identified in methanogenesis pathway

Metalloproteins/metalloenzymes	**Pathway involved**	**Trace elements**
Formate dehydrogenase	Hyd	Se, Mo/W, Fe
Coenzyme F_{420} Hydrogenase	Hyd	Se, Ni, Fe
Formyl-methanofuran dehydrogenase	Hyd	Se, Mo/W, Fe

CO dehydrogenase/acetyl-CoA decarbonylase	Ace	Co, Ni, Fe
Methyl-H_4 MPT:HS-CoM methyltransferase	Ace and Hyd	Co, Ni, Fe, Zn
Heterodisulphide reductase	Ace and Hyd	Fe, Ni
Methyl-CoM reductase	Ace and Hyd	Ni

Note: Hyd- hydrogenotrophic pathway, Ace- acetoclastic pathway

2.2.2.3 Trace Metal Requirement by Other Anaerobes

Apart from being essential to methanogens, several trace element species are critically required by syntrophic acetate oxidising bacteria adopting the reverse Wood-Ljungdahl pathway (oxidative acetyl-CoA pathway) and the syntrophic propionate oxidising route.

In syntrophic acetate oxidising pathway, four key metalloproteins/metalloenzymes have been identified in the reverse Wood-Ljungdahl pathway: formate dehydrogenase (FDH), corrinoid- and Fe/S cluster-containing protein (CFeSP), Carbon monoxide dehydrogenase (CODH), and Acetyl-CoA synthase (ACS). The trace elements identified to be used in these protein/enzymes are listed in Table 2.5 (Zhu 2009).

Table 2.5. Metalloproteins/metalloenzymes identified in Wood-Ljungdahl pathway

Metalloproteins/metalloenzymes	**Trace elements**
Formate dehydrogenase	Se, Mo/W, Fe
Corrinoid- and Fe/S cluster-containing protein	Co, Fe
Carbon monoxide dehydrogenase	Ni, Fe
Acetyl-CoA synthase	Ni, Fe

The syntrophic propionate oxidising pathway is adopted by syntrophic propionate-oxidising bacteria (POB) present in the anaerobic digestion process. In a number of studies, *Syntrophobacter* spp., *Pelotomaculum* spp. and *Smithella* spp. have been recognised as the main stream of syntrophic propionate oxidising bacteria (McMahon et al. 2004, Worm et al. 2009, Müller et al. 2010).

Two formate dehydrogenases were isolated from the syntrophic propionate oxidising bacterium *Syntrophobacter fumaroxidans* (de Bok et al., 2003). Both enzymes were produced in axenic fumarate-grown cells as well as in cells which were grown syntrophically on propionate with *Methanospirillum hungatei* as the H_2 and formate scavenger. The purified enzymes exhibited extremely high formate oxidation and CO_2 reduction rates, and low K_m values for formate (0.01~0.04 mM). Both enzymes contained tungsten and selenium, while molybdenum was not detected. This matches with the

genome analysis of *Syntrophobacter fumaroxidans* which indicated that *S. fumaroxidans* could code for a cytoplasmic [NiFe]-hydrogenase, two cytoplasmic [NiFeSe]-hydrogenases, a [NiFe]-hydrogenase maturation protein, and two cytoplasmic formate dehydrogenases (Müller et al., 2010).

Several trace element supplementation/deficiency experiments were carried out to distinguish the real trace element requirement for syntrophic propionate oxidisation. Plugge et al. (2009) studied the effect of W and Mo on the growth of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei* co-culture and also in their individual pure strain cultures. The study concluded that the effect of W and Mo on the activity of formate dehydrogenase was considerable in both the organisms, whereas hydrogenase activity remained relatively constant. Depletion of tungsten and/or molybdenum, however, did not affect the growth of the pure culture of *S. fumaroxidans* on propionate plus fumarate significantly, although the specific activity of hydrogenases and especially formate dehydrogenase were influenced by the absence of Mo and W. The result suggests a more prominent role for H_2 as electron carrier in the syntrophic conversion of propionate, when the essential trace metals W and Mo for the functioning of formate dehydrogenase are depleted.

Worm et al. (2009) observed a propionate degrader shift from *Syntrophobacter* spp. to *Pelotomaculum* spp. and *Smithella* spp. in a UASB digester fed with synthetic propionate medium without molybdenum, tungsten and selenium. The authors argued that one of the reasons for the species shift was because *Pelotomaculum* spp. and *S. propionica* may need molybdenum for formate dehydrogenase activity whereas *Syntrophobacter* spp. needed tungsten for formate dehydrogenase activity and molybdenum can even have an antagonistic effect as was described for *S. fumaroxidans*. The medium, however, was deficient in both molybdenum and tungsten which could not support the authors' explanation, especially since *Syntrophobacter* spp. was the dominant propionate oxidising species at the beginning of the experiment.

2.2.2.4 The Effects of Trace Element Supplementation on Anaerobic Digester Performance

Although trace elements are essential for anaerobic treatment processes, the supply of these to bioreactors has received less attention than their toxicity. There are some literature reports, however, on the benefits of trace elements supplementation on anaerobic archaeal

growth and metabolic rates of conversion (mainly VFA), either in a pure culture or in anaerobic digesters.

Scherer and Sahm (1981) reported the growth of *Methanosarcina barkeri* on methanol as the energy source and found this to be dependent on cobalt and molybdenum. In the presence of 10^{-6} M Co and 5×10^{-7} M Mo optimal growth occurred. Furthermore it was demonstrated that nickel and selenium each in a concentration of 10^{-7} M stimulated the growth of this methanogenic bacterium while the other elements B, Cr, Cu, Mn, Pb tested in the range of 10^{-7} M to 10^{-3} had no influence. The requirements of Co and Ni for optimal growth were in agreement with the result that the cells contained a Co containing corrinoid Factor III (0.1 - 0.2 mg 5-hydroxylbenzimidazolylcyanocobamide per g wet cells) and the nickel containing cofactor F_{430}.

According to Osuna et al. (2003), the addition of trace elements to upflow anaerobic sludge blanket (UASB) reactors significantly stimulated the conversion of propionate in a mixture of VFAs (acetate, propionate, butyrate, in a ratio 3:1:1). The trace nutrient solution contained (in milligrams per litre) $FeCl_2.4H_2O$ (2000), H_3BO_3(50), $ZnCl_2$ (50), $MnCl_2.4H_2O$ (500), $CuCl_2.2H_2O$ (38), $(NH_4)_6$ $Mo_7O_{24}.H_2O$ (50), $CoCl_2.6H_2O$ (2000), $NiCl_2.6H_2O$ (142), $Na_2SeO_3.5H_2O$ (164) and 1 ml of the solution was added into every 1 litre of the influent. At the end of the experiment, the COD removal efficiencies were 99 and 77% for the trace elements supplemented and deprived reactors, respectively.

Florencio (1994) found that the specific methanogenic activity of a methanogenic sludge towards methanol was greatly stimulated by adding increasing concentrations of trace elements, especially cobalt. The maximum activity was found for a cobalt concentration of 0.1 mg l^{-1}. Gonzalez-Gil et al. (1999) confirmed that biomass from UASB and Expanded Granular Sludge Bed (EGSB) reactors can be nickel and cobalt limited during the degradation of methanol.

Speece et al. (1983) reported a significant stimulation of methane fermentation from acetate with addition of the trace metal nickel (10 mg I^{-1}). In the absence of nickel, the specific acetate utilisation rates were in the range of 2–4.6 g acetate g^{-1} VSS day^{-1}. In the presence of nickel, this rate was as high as 10. When nickel was supplemented with iron, cobalt and yeast extract in different combinations, the maximum acetate utilisation rate

was observed to be 51 g l^{-1} day^{-1}.

Worm et al. (2009) examined the effect of the long-term absence of molybdenum, tungsten and selenium from the feed to the UASB reactor on microbial community dynamics and activity. A substantial decrease in propionate degrading activity and minor changes within the propionate degrading community were later observed and it was reported that this could be as a result of the depletion of these trace elements.

Interestingly, Williams et al. (1986) observed that the stimulating effects of metals can occur even when high total concentrations of these metals are present in the reactor system: this implies that the metals can be present in a non-bioavailable form. In their study nickel (at 0.587 mg l^{-1}) stimulated the biogas production of a chicken manure digester, while nickel was present in the effluent (14.851 mg l^{-1}) before 'extra' nickel was added.

Since the addition of trace elements may increase the operating costs of the process, considerable attention has been given to the effect of the addition of different trace element concentrations on the performance of anaerobic bioreactor systems, in order to achieve a more economic and effective control of the process; but the minimum amount of trace elements required for optimal performance of the anaerobic system is still not well defined (Osuna et al., 2003).

Obtaining an ideal trace element concentration is not essential, but if insufficient trace element is present then process compensation must be made by either lowering the loading rate or accepting a lower treatment efficiency. In other words, the metabolic capacity of the digester is determined by the availability of trace elements which ultimately determine the enzyme activity which is available for the catabolic reactions required to deal with the organic load applied (Banks and Heaven, 2012). Thus, in the case of a reactor having sufficient mass transfer capacity of substrate gas (H_2:CO_2) from gas phase to the liquid and sufficient nitrogen and sulphur source for the growth of the acclimated-methanogens, the cell density and methane production rate were only dependent on the trace elements concentration added to the reactor (Zhang et al., 2003).

In the above review of the trace element requirements in the anaerobic digestion process, specific attention has been given to Co, Ni, Fe, Se, Mo, and W and their enzymological

roles. It is, however, noteworthy that other elements such as Cu, Mn and Zn also have an important biological function (Feng et al. 2010, White and Stuckey 2000). It is only their natural abundance in substrates and low level of demand in the process that have made them less prominent as controlling factors compared to the ones highlighted above. Table 2.6 summarises some other trace elements that can potentially be involved in anaerobic digestion and their possible functions.

Table 2.6. Roles of some essential trace elements involved in anaerobic reaction and transformation (Fermoso et al., 2009)

Metals	Functions
Cu	• Superoxide dismutase • Hydrogenase (Facultative anaerobes) • Nitrite reductase • Acetyl-CoA synthase
Mn	• Stabilise methyltransferase in methanogens • Redox reactions
Zn	• Hydrogenase • Formate dehydrogenase • Superoxide dismutase

2.3 Review of Anaerobic digestion of Food Waste and Fruit and Vegetable Waste

The above sections have reviewed extensively the fundamentals of anaerobic digestion process. The knowledge is essential for the optimisation of digestion process by satisfying the optimum growth condition and nutrient requirements for the anaerobic microorganism consortia. From a practical perspective, however, in order to operate successfully an anaerobic digestion process to treat food waste and vegetable waste, in-depth knowledge of the waste stream characteristics is of great importance as well as the knowledge of the state-of-art of work carried out with these two waste streams.

2.3.1 Anaerobic Digestion of Food Waste

2.3.1.1 Waste Stream Characterisation and Current Knowledge

Food waste makes up around 20% of the domestic waste stream in the UK (Hogg, 2006), with approximately equal quantities arising from food manufacture and catering outlets. If food wastes from agro- and food industries are included an estimated total of 200 million tonnes year^{-1} is available in the EU (Bio Intelligence Service, 2010).

Source segregated food wastes are very high in potential energy but because of the high water content of this material the only effective way to gain energy from it is through biochemical conversion, and it is potentially an excellent substrate for biogas production through anaerobic digestion (Liu et al., 2009; Zhang et al., 2007). Previous studies (Zhang et al. 2012) have shown that food wastes have a high recoverable energy content (0.425 STP m^3 kg^{-1} VS_{added}), equivalent to ~100 m^3 of methane for each tonne of wet weight added to the digester.

There are, however, many examples of food waste digestion where the AD process has suffered from high VFA concentration after extended periods of operation. These include a technical-scale trial using kitchen waste collected in the UK (Banks et al., 2008), and the digester failure in a 30,000 tonne per year food waste digester operated by Valorsul SA in Lisbon, Portugal (Neiva Correia et al., 2008). Similar problems were also observed repeatedly in smaller scale laboratory trials (Wang and Banks, 2003; Climenhaga and Banks 2008; Zhang and Banks 2008; Banks and Zhang, 2010) when source-segregated catering waste or domestic food waste was used as a sole substrate in the digestion process. In the study by Climenhaga and Banks (2008) source separated food residues (a varied mix of fruits, vegetables, meats and fried foods) were digested in bench-scale single-stage digesters at a constant OLR and different HRT (25, 50, 100 and 180 days). Initial problems with the digestion were overcome when the authors used a trace element solution containing Fe, Cu, Zn, Mn, Mo, Co, Al and Se (Gonzalez-Gil et al., 2001). This work showed the importance of micronutrients in digestion of a mixed food waste feedstock: reactors supplemented with trace elements showed a stable digestion, while non-supplemented reactors showed methanogenic failure. In a recent study the effect of trace element addition, namely Co, Ni, Mo, B, Se and W, was investigated in a lab-scale work on biogas production from food industry residues (Feng et al., 2010). The laboratory-scale batch reactors worked at mesophilic temperature and trace elements were added with the daily feed. High addition of Se/W resulted in the highest production of methane in combination with a low concentration of Co. The ranges of Se and W concentrations in the reactors after addition varied from 8 to 800 mg m^{-3}, and 18 mg m^{-3} to 1.80 g m^{-3}, respectively, while Co concentration ranged between 60 mg m^{-3} and 6 g m^{-3}. This study suggested that the archaea spp. in biogas reactors were more sensitive to trace element concentration when compared with the other members of the microbial community.

In another recent batch study, using OFMSW as substrate, the effects of the trace elements Cr, Ni, Zn, Co, Mo and W on mesophilic biogas production were investigated (Lo et al., 2012). In these tests all had the potential to enhance biogas production with the exception of Zn. The useful concentrations of Cr, Ni, Co, Mo and W were reported to vary between 2.2 to 21.2 mg m^{-3} (Cr), 801 to 5,362 mg m^{-3} (Ni), 148 to 580 mg m^{-3} (Co), 44 to 52.94 mg m^{-3} (Mo), and 658 to 40.39 mg m^{-3} (W), respectively. Metal concentrations higher than threshold values would result in adverse effect, leading to inhibition of biogas production.

Banks and Zhang (2010) carried out semi-continuous trial in CSTR reactors under mesophilic conditions, using food waste as substrate. The aim of this study was to explore the possibility of regulating the metabolic pathway leading to methane production by trace element addition. Mo, W, and Ni are present in food waste in sufficient quantities for moderate loadings, but may have to be supplemented in digestion at a high OLR (5 g VS l^{-1} day^{-1}).

The stimulatory effects of Ni, Co and Mo on biogas and methane production rates have also been previously studied in a methanogenic fixed-film reactor treating food residues (Murray and Berg, 1981). According to the study, when Ni and Co were tested in combination, they stimulated methanogenesis to a much greater extent than could have been expected when they were added individually; and molybdenum increased methanogenic activity only when added in combination with both nickel and cobalt. Total biogas and methane production increased approximately 42% with addition of 5.87×10^{-3} mg l^{-1} Ni (100 nM), 2.94×10^{-3} mg l^{-1} Co (50 nM) and 4.8×10^{-3} mg l^{-1} Mo (50 nM).

Zhang and Jahng (2012) also reported the positive effect of trace elements (Co, Mo, Ni and Fe) supplementation on the long-term anaerobic digestion of food waste in a semi-continuous single-stage reactor. Stable anaerobic digestion of food waste was achieved for 368 days by supplementing trace elements with OLR of 2.19-6.64 g VS l^{-1} day^{-1}, compared to process failure when digesting food waste without supplementing trace elements.

A study carried out by Lin et al. (2011) investigated the possibility of co-digestion of food waste with fruit and vegetable waste using mesophilic continuously stirred tank reactors (CSTR). It was found that at ratio of 1:1 on a VS basis, co-digestion of the 2 waste streams could improve both process stability and efficiency. Stability could not be achieved in

digesters fed with a higher ratio of food waste or on food waste alone, possibly due to the high nitrogen content in such a substrate.

2.3.1.2 Problems Encountered with Food Waste Digestion

The work of Banks and Zhang (2010) showed clearly that VFA accumulation in mesophilic food waste digesters is a common problem and starts with acetate accumulation, which may indicate inhibition of acetoclastic methanogenesis. This increase is followed by a decline in acetic acid concentrations and a subsequent increase in longer chain length VFA species, particularly propionic acid, probably due to product-induced feedback inhibition. Anaerobic digestion is a complex process and its metabolic routes are moderated and directed through syntrophic reactions between different groups of microorganisms (Pind et al., 2003; Speece 1983), and this feedback control is governed by the build-up of methane precursors, namely, hydrogen, formate, and acetate. It is now thought that this pattern is the result of ammonia inhibition of the acetoclastic methanogenic population followed by a shift in the population structure to give a predominantly hydrogenotrophic methanogenic population. Other work indicating such a shift in population at high ammonia concentrations has been shown on a number of occasions (De Bok et al., 2003; Plugge et al. 2009; Schnürer et al., 1994; Schnürer and Nordberg, 2008; Westerholm et al. 2011), and suggests the importance of the hydrogenotrophic methanogenic population under these conditions.

2.3.2 Anaerobic Digestion of Fruit and Vegetable Waste

2.3.2.1 Characteristics of Fruit and Vegetable Waste

Fruit and vegetable wastes (FVW) are characterised by a high percentages of moisture (> 80%), high organic content (volatile solids > 95% of total solids) and are readily biodegraded, which suggests the potential for anaerobic digestion (Arvanitoyannis and Varzakas, 2008). The organic fraction typically includes about 75% sugars and hemicelluloses, 9% cellulose and 5% lignin. Gunaseelan (1997) reviewed the data on anaerobic digestion of different types of fruit and vegetable waste, and concluded that the methane yield of FVW is very high compared to other municipal solid wastes (MSW). Some of the reviewed data are presented in Table 2.7.

Table 2.7. Summary of performance of semi-continuous CSTR digesters operated at 33 ^{o}C and fed with FVW at a HRT 32 days in previous studies

Feed	OLR (g VS l^{-1} day^{-1})	CH_4 yield (l g^{-1} VS)	VS reduction (%)
Spinach waste	0.83-1.18	0.316	70
Asparagus peels	0.74-1.06	0.219	40
French bean waste	0.96-1.15	0.343	70
Strawberry slurry	1.02-1.15	0.261	50
Apple pulp	1.02-1.60	0.308	40
Apple slurry	0.83-1.15	0.281	60
Carrot waste	0.80-0.90	0.417	75
Green pea slurry	0.87-1.25	0.310	75

(adapted from Gunaseelan, 1997)

Bouallagui et al. (2005) investigated the potential of anaerobic digestion for material recovery and energy production from FVW containing 8–18% TS, with a VS content of 86–92%. Using different operating conditions and bioreactor types it was concluded that the material was readily biodegradable with a conversion of 70–95% of the organic matter to methane at volumetric OLR of between 1- 6.8 g VS l^{-1} day^{-1}.

2.3.2.2 Problems Encountered with FVW Digestion

Although readily degradable, a number of studies have, however, indicated problems and limitations in anaerobic digestion of FVW. Knol *et al.* (1978) reported the results of a 90-day experiment using a mixed waste of apples, asparagus, carrots, green peas, French beans, spinach and strawberries from a canning factory. Digestion was at mesophilic temperatures at OLR between 0.80-1.60 g VS l^{-1} day^{-1} with a 32-day HRT, and produced average biogas yields of 0.30-0.58 l g^{-1} VS. Digestion of some of these materials showed instability, and in these cases remedial measures were introduced which included alkali addition, feed interruption and mixing with a nitrogen-rich supplement. In a similar study, Mata-Alvarez *et al.* (1992) reported on the performance of a mesophilic single-stage stirred digester for the treatment of a mixture of fruit and vegetable wastes from a large food market, and found the maximum OLR that could be achieved was <3 g VS l^{-1} day^{-1}. Lane (1979) also reported from experiments using completely mixed digesters with a FVW input that a loading above 4 g VS l^{-1} day^{-1} caused a fall in pH, lower gas yield and increased CO_2 content. These studies suggest that for the anaerobic digestion of FVW it is difficult to achieve a stable operation at high OLR.

2.3.2.3 Strategies for Optimising Performance and Stability of FVW Digestion

1.) Pre-Treatment of Substrates

Several pre-treatment techniques have been proposed to improve the amenability of FVW to anaerobic digestion. The most commonly reported is homogenisation of the feed material to reduce the fibre length. The best results showed that biogas production could be increased by about 20% if the fibre size was reduced to less than 0.35 mm (Mata-Alvarez et al., 2000). Some authors have suggested dilution of the feedstock to alleviate adverse impacts due to the acidity of the FVW and as a way of adjusting hydraulic retention time (HRT) (Bouallagui et al., 2003; Mata-Alvarez et al., 1992). This option is not favoured, however, as it reduces the volumetric loading rate. An alternative strategy is to buffer the low pH produced by FVW, for example by the addition of sodium hydroxide solutions (Mata-Alvarez et al., 1992, Srilatha et al., 1995).

Converti et al. (1999) pre-treated FVW at high temperature to improve the efficiency of digestion, while Srilatha et al. (1995) pre-treated orange processing waste by solid state fermentation using selected strains of *Sporotrichum*, *Aspergillus*, *Fusarium*, and *Penicillium* to obtain improved biogas and methane productivity at higher OLR.

2.) Co-digestion

Co-digestion can improve biogas yield and stability as a result of positive synergisms due to the supply of missing nutrients (Mata-Alvarez et al., 2000). In the particular case of FVW, which generally has low nitrogen and phosphorus but a very high carbohydrate content, co-digestion with nitrogen-rich co-substrates is widely recommended (Alatriste et al., 2006, Bouallagui et al., 2009, Callaghan et al., 1999, Poggi-Varaldo et al., 1997). Several authors have proposed an optimum C/N ratio between 20-25:1 for stable and effective anaerobic conversion of organic wastes (Parkin and Owen, 1986, Mshandete et al., 2004, Yen and Brune, 2007). Kayhanian and Hardy (1994) reported C/N ratios between 25 and 30 as being optimal. Kivaisi and Mtila (1998) argued, however, that a C/N ratio between 16-19:1 is optimal for methanogenic performance. Although the literature values are not entirely consistent they provide a general guideline applicable to the anaerobic digestion of FVW.

Bouallagui et al. (2009) investigated the effect of fish waste, abattoir wastewater (AW) and waste activated sludge (WAS) as co-substrates in FVW digestion under mesophilic

conditions using four anaerobic sequencing batch reactors (ASBR). The reactors were operated at an OLR of 2.46–2.51 g VS l^{-1} day^{-1}, of which approximately 90% was from FVW, and a HRT of 10 days. Using AW and WAS co-digestates at a ratio of 10% of the VS load enhanced biogas yield by 51.5% and 43.8% and VS removal by 10% and 11.7%, respectively. Fish waste addition led to improvement in process stability, as indicated by the low VFA/alkalinity ratio of 0.28, and allowed digestion of FVW without chemical alkali addition. Despite a considerable decrease in the C/N ratio from 34.2 to 27.6, the addition of fish waste slightly improved the gas production yield (8.1%) compared to anaerobic digestion of FVW alone.

Gómez et al. (2006) compared the digestion of primary sludge (PS) alone to co-digestion with the fruit and vegetable fraction of municipal solid wastes. The results for co-digestion were better than those obtained from the digestion of PS alone as a result of the higher feedstock VS content. Specific gas production for the two processes was, however, similar. The co-digestion process was also evaluated at different OLR, with reasonable performance at loadings of up to 3.8 g VS l^{-1} day^{-1} and recovery even after short periods of shock overloading.

Murto et al. (2004) reported two laboratory-scale co-digestion experiments. In the first, sewage sludge was co-digested with industrial waste from potato processing. This resulted in a poorly buffered system and when the fraction of starch-rich waste was increased the process became overloaded at a lower overall OLR. In the second trial, pig manure, slaughterhouse wastes, vegetable waste, and various kinds of industrial waste were co-digested. This gave a highly buffered system that worked well, with biogas yields of 0.8–1.0 m^3 kg^{-1} VS.

3.) Temperature

Bouallagui et al. (2004) compared the performance of anaerobic digestion of FVW in thermophilic (55 ^oC), mesophilic (35 ^oC) and psychrophilic (20 ^oC) conditions in a tubular digester at a laboratory scale. Biogas production from the thermophilic digester was higher on average than from psychrophilic and mesophilic digesters by 144 and 41%, respectively. Methane percentage in biogas from thermophic digestion is similar to mesophilic and higher than psychrophilic digesters by 4-5.5%. Under psychrophilic condition, methane content has been proved to be the lowest compared to other temperature conditions.

4.) Process Configuration

Due to the high biodegradability, hydrolysis rate and potential for VFA build-up during anaerobic digestion of FVW some studies have recommended the use of a two-stage anaerobic digestion process (Raynal et al. 1998, Poirrier et al., 1999). Mtz.-Viturtia et al. (1995) reported on a two-phase mesophilic (35°C) anaerobic digestion of FVW carried out at laboratory scale. A higher OLR could be applied to the simple two-phase system but this resulted in lower specific gas yields. It was concluded that this two-phase system was not appropriate unless equipped with some method of control of the hydrolytic phase.

5.) Trace Element Supplementation

In an early study by Lane (1984), performance of a laboratory-scale digester treating residuals from fruit and vegetable processing was improved after the addition of a trace elements solution containing Fe, Zn, Mn, Cu and Mo. The conversion percentage for VS increased from 88 to 96%, with a corresponding increase in CH_4 percentage in the biogas. Other studies on digestion of potato process waste (Kurmar et al., 2006) and on a mixture of fruits, vegetables, meats and fried foods (Climenhaga and Banks, 2008) have also suggested improved stability and performance after supplementation with trace elements (Fe, Cu, Zn, Mn, Mo, Co, Al and Se). Jarvis et al. (1997) operated digesters at an OLR of 3 g VS l^{-1} day^{-1} with grass-clover silage as feedstock and a HRT of 20 days: the digesters initially performed well, but gas production and pH started to drop after ~4 weeks, and could be recovered by cobalt supplementation. In a semi-continuous study using maize silage as substrate, Pobeheim *et al.* (2010) reported that nickel and cobalt concentrations below 0.06 mg kg^{-1} and 0.02 mg kg^{-1} respectively on a wet weight basis were limiting factors for digester performance. Addition of extra nickel to 0.6 and cobalt to 0.05 mg kg^{-1} fresh matter led to recovery of process stability and a fast metabolisation of acetic and propionic acid was detected.

2.5 Inhibitors in Anaerobic Digestion of Food and Vegetable Waste

Various substances and conditions may cause inhibitory effects on the anaerobic digestion process.

Anaerobic microbes require specific physical conditions to maintain enzyme activities to facilitate the biochemical reactions, therefore unfavourable conditions in anaerobic reactors, such as temporal overloading, a decreasing pH, and rapid temperature change will inhibit anaerobic processes. Inhibitory compounds may also impact on the system in a number of ways: interference with metabolic enzymes of one or more members of a trophic group; with energy metabolism by uncoupling of growth and ATP production; disruption of cell membranes creating intracellular changes of the pH or the salt concentration (Gallert et al., 1998).

In the case of anaerobic digestion of food waste, apart from the physical factors like temperature and pH, the effect of inhibitors such as ammonia and long chain fatty acids (LCFA) are a major concern.

2.5.1 Ammonia

In anaerobic digestion, ammonia is mainly produced by the degradation of the nitrogenous matter present in the feedstock, mostly in the form of protein (Kayhanian et al., 1999; Kotsyurbenko et al., 2004). In digesters treating high protein content substrates such as the yard and food waste fraction of MSW, animal manure and slaughterhouse waste, high ammonia concentration in digestate is a common problem. According to McCarty (1964) ammonia concentrations between 50 and 200 mg l^{-1} have a beneficial effect on anaerobic processes as nitrogen from ammonia is an essential element for synthesis of amino acids, proteins and nucleic acids, therefore essential for bacterial growth. Also ammonia, as a base, neutralises the volatile acids produced by fermentative bacteria, and thus helps maintain neutral pH conditions essential for cell growth.

If present at high concentrations, however, ammonia is inhibitory to methanogenesis (McCarty, 1964; Gallert et al., 1998). For anaerobic treatment of organic waste with high nitrogen content in typical continuous stirred tank reactors (CSTR), ammonia toxicity potentially poses the most challenging problem for various reasons. The most important of these are:

1.) Ammonia cannot be further degraded anaerobically; therefore once a high concentration of ammonia has been reached in digesters, the problem will normally persist unless ammonia can be physically or chemically removed.

2.) When operation has reached its stable state, ammonia concentration in digesters is defined only by nitrogen content in feedstock, and is not determined by OLR (i.e. reduced loading will not result in a lower ammonia concentration in digesters).

The quantity of ammonia that will be generated from an anaerobic biodegradation of organic substrate can be estimated using the following stoichiometric relationship (Nielsen and Angelidaki, 2008)

$$C_aH_bO_cN_d + (4a - b - 2c + 3d)/4\ H_2O = (4a + b - 2c - 3d)/8\ CH_4 + (4a - b + 2c + 3d)/8\ CO_2 + d\ NH_3 \quad [2\text{-}25]$$

In aqueous conditions, ammonia exists mainly in two forms, Ammonium ion NH_4^+ and free ammonia (NH_3). The fraction of free ammonia relative to total ammonia concentration is pH and temperature dependent, as shown in the following formula (Koster, 1986).

$$NH_3\ (Free) = TAN \times \left(1 + \frac{10^{-pH}}{10^{-(0.09018 + \frac{2729.92}{T(K)})}}\right)^{-1} \quad [2.26]$$

Where:

NH_3 (Free) = free ammonia nitrogen concentration (mg l^{-1})

TAN = total ammonia nitrogen concentration (mg l^{-1})

T(K)= temperature (Kelvin)

The free ammonia (FA) fraction is usually considered to be the main cause of inhibition (Kroeker et al., 1979, De Baere et al., 1984, Koster, 1986, Angelidaki and Ahring, 1993, Kayhanian, 1994, Kayhanian, 1999). Equation 2.26 implies that the free ammonia fraction increases with temperature and pH and therefore the TAN that could be tolerated at high pH and thermophilic temperatures would be lower than at mesophilic temperatures. Findings from several studies (Angenent et al., 2002, Koster, 1986) support this and have indicated that methane fermentation of high ammonia-containing wastes is more easily inhibited at thermophilic temperatures than at mesophilic temperatures. This is in agreement with the speculation that it is the free ammonia which causes toxicity.

The understanding of the variable concentration and toxicity of free ammonia under different environment conditions is of importance, particularly for digesters treating food waste or cattle waste which under normal operational conditions have a high pH value (often higher than 8) (Borja et al., 1996). In these reactors, an increase in pH from 7 to 8 will actually lead to an eight-fold increase in the free ammonia concentration (Koster, 1986). For digesters operating at thermophilic temperatures, the increase in free ammonia concentration is even more severe.

The inhibitory threshold of ammonia has been reported in a number of studies, but the value at which inhibition commences in these studies varies significantly. Koster and Lettinga (1984) indicated that under mesophilic conditions, the maximum methanogenic activity was found to be unaffected at a TAN concentration of 680 mg l^{-1} (Free ammonia = 26.5 mg l^{-1}). As the TAN concentration was increased to 1600 mg l^{-1} (Free ammonia = 60.3 mg l^{-1}), however, methanogenesis decreased to about 75%. The methanogenic activity declined further as the TAN increased. Kayhanian (1994) revealed that under thermophilic conditions, methane production decreased at total ammonia nitrogen concentration of 1000 mg l^{-1} (Free ammonia = 60 mg l^{-1}) when the digester was operated at pH of 7.5 or higher. A higher inhibitory threshold was reported at TAN concentration of 1500-2500 mg l^{-1} (Kleiner, 1993, Wiegant and Zeeman, 1986).

This discrepancy suggested the disadvantage of reporting the ammonia inhibitory concentration based on TAN rather than using free ammonia, i.e. the total ammonia inhibitory concentrations reported in different studies are not comparable unless the pH and temperature conditions are cited as well. In the two studies (Koster and Lettinga, 1984) and (Kayhanian, 1994) described above, although the inhibitory concentrations presented in terms of ammonia nitrogen are different, once converted to free ammonia according to their respective environment conditions they are consistent with each other.

Other factors such as microbial acclimation to the high ammonia concentration and cation antagonism effects (Kartal et al. 2010, Chen et al., 2008) could also contribute to the broad range of ammonia inhibitory thresholds reported in the literature. By acclimation of the anaerobic process to ammonia, higher tolerance can be achieved, as discovered by Van Velsen (1986). When digested sewage sludge and digested piggery manure, acclimated to 815 mg l^{-1} and 2420 mg l^{-1} ammonia nitrogen respectively, were used as inoculums in a

batch experiment, the digested sewage sludge showed a longer lag phase of methane production at increasing ammonia nitrogen concentrations in the range 730-4990 mg l^{-1}. On the other hand, with digested piggery manure methane formation started immediately without any lag phase. In both cases, however, the maximum methane formation rates slowly decreased with the increasing ammonia concentration.

Hansen et al. (1998) conducted a batch experiment to determine ammonia toxicity using inoculums acclimated to high ammonia. The study demonstrated that inhibition to the process started at a free ammonia concentration of 1.1 g N l^{-1} (TAN = 3.4 g l^{-1}). Below this value the specific apparent growth rate was found to be constant.

A number of other continuous studies have also reported adaptation of methanogenesis to ammonia concentrations far above those believed to be inhibitory. Hashimoto (1993) observed that ammonia inhibition began at about 2.5 g N $l^{-1}$and 4 g N l^{-1} for unacclimated and acclimated thermophilic methanogens, respectively. Parkin and Miller (1987) reported that TAN concentrations as high as 8–9 g l^{-1} could be tolerated with no significant decrease in methane production after acclimation. The experiments clearly demonstrated the possibility of obtaining stable digestion of manure with ammonia concentrations exceeding 5 g N l^{-1}after an initial adaptation period.

In general, the literature on anaerobic digestion shows considerable variations in the inhibition/toxicity concentrations reported for TAN and FA, as shown in Table 2.8 and 2.9.

Since methanogens are the most sensitive amongst the complex microbial population involved in anaerobic digestion (Koster and Koomen, 1988) and the resistance to ammonia toxicity within methanogen species varies significantly (Raynal et al., 1998, Koster and Koomen, 1988, Borja et al., 1996), the acclimation of anaerobic process to high concentration of ammonia may be the result of internal changes in the predominant species of methanogens, or of a shift in the methanogenic pathway (Raynal et al., 1998).

In order to gain further understanding of the digester behaviour and successful operation of anaerobic treatment of organic waste with high nitrogen content such as food waste, a review of the ammonia toxicity mechanism towards anaerobic digestion, in particular its

impact towards the methanogen population and potential for alteration of methanogenic pathway in anaerobic digesters is of great importance.

Table 2.8. Inhibition limit of FA and TAN in continuously fed reactors

Temp. ^{o}C	Substrate	Reactor	Inoculum	Inhibition limit FA mgN l^{-1}	Inhibition limit TAN g N l^{-1}	% reduction in CH_4 production	pH	References
55	Soluble non fat dry milk + NH_4Cl	CSTR	Acclim		5.77	64	6.40	Sung and Liu, 2003
55	Cattle manure	CSTR	Acclim	600-800	NR		7.4-7.9	Angelidaki et al., 1994
55	Cattle manure	UASB	Acclim	500	7.00	72		Borja et al., 1996
50	Cattle manure	CSTR	NR	NR	1.70	Initial inhibition	NR	Zeeman et al., 1985
55	Cattle manure	Continuously fed reactor	NR	900	4.00	25	NR	Angelidaki and Ahring, 1993
55	Swine manure	CSTR	NR	1600	NR	70	7.97	Hansen et al., 1998
60	Swine manure	CSTR	NR	2600	NR	96	8.15	Hansen et al., 1998
55	OFMSW	Complete-mix reactor	NR	45	1.2		7.20	Kayhanian et al., 1999
37	Food waste	CSTR	NR	> 1000	5.7	NR	>7.5	Climenhaga and Banks, 2008
55	OFMSW	CSTR	Acclim	680-690	3.4-3.5	50	7.60	Gallert and Winter, 1997
37	OFMSW	CSTR	Acclim	220-280	3.0-3.7	50	7.60	Gallert and Winter, 1997
55	OFMSW	CSTR with waste recirculation	Acclim	251	1.83	NR	NR	Gallert et al., 1998

Note: Acclim = acclimatised; FA= Free Ammonia; NR= Not reported

Table 2.9. Inhibition limit of FA and TAN in batch reactors

Temp. ^{o}C	Substrate	Inoculum	Inhibition limit FA mgN l^{-1}	Inhibition limit TAN gN l^{-1}	% reduction in CH_4 production	pH	References
52	Glucose+Na_2SO_4+ ammonia	Acclim	620	NR	21	NR	Siles et al., 2010
55	Soluble non fat dry milk + NH_4Cl	Acclim	NR	10.00	100	NR	Liu and Sung, 2002
55	Cattle manure + NH_4Cl	Acclim	NR	4.00	NR	7.2	Hashimoto et al., 1986
55	Swine manure	NR	1100	NR	NR	NR	Hansen et al., 1998
55	Poultry anure+NH_4Cl	NR	NR	2.6-8	80-90	NR	Krylova et al., 1997
55	Synthetic OFMSW	NR	468	6.07	50	7.5	Benabdallah El Hadi T. et al., 2009
37	Synthetic OFMSW	NR	215	4.08	50	7.5	Benabdallah El Hadi T. et al., 2009

Note: Acclim = acclimatised; FA= Free Ammonia; NR= Not reported

2.5.1.1 Mechanisms of Ammonia Inhibition to Methanogenesis

To understand the possible mechanisms of ammonia inhibition, it is important to consider the chemical interaction of ammonia and cells. For most of microorganisms, the energy equilibration in ATP is achieved by proton-translocating ATPases applying proton motive force ($\Delta_{\mu H}^{+}$) across the cell membrane. The energy in the $\Delta_{\mu H}^{+}$ is the sum of the energies in the trans-membrane pH gradient (ΔpH) and the trans-membrane electrical gradient (Δψ) (Wolin and Miller, 1982). For methanogens living in a slightly alkaline environment, studies indicated that the trans-membrane pH gradient (ΔpH) is small or even negative (Wolin and Miller, 1982); therefore those methanogens can grow with a near-neutral cytosol even when the external pH is above 7.

The mechanism of ammonia toxicity in methanogens is currently not clear; however a physical model (Figure 2.9) was proposed by Kayhanian et al. (1999). The model was based on the understanding of trans-membrane electrical and pH gradient theory and it convincingly described the entrance of the NH_3 molecule into cell and subsequently the internal accumulation of ammonia.

According the model, free ammonia molecules will diffuse readily through cell membranes into the cells of methanogens (De Baere et al., 1984, Kayhanian et al. 1999, Wolin and Miller, 1982), equilibrating the intracellular and extracellular concentrations of NH_3. On the other hand, ammonium (NH_4^+) does not readily diffuse through cell membranes. This in turn leads to a rapid increase of cytosolic concentration of un-ionized ammonia when a methanogen cell is exposed to an increased extracellular ammonia concentration (Kleiner, 1993). Under such a scenario, the intracellular and extracellular concentrations of NH_4^+ are dependent on NH_3 concentration and the local pH and temperature. Thus, cells with intracellular pH lower than the extracellular pH (i.e. negative ΔpH) would have an intracellular NH_4^+ concentration greater than that of their environment. In cells with a very negative ΔpH, cytosolic NH_4^+ may constitute a considerable fraction of the intracellular cations.

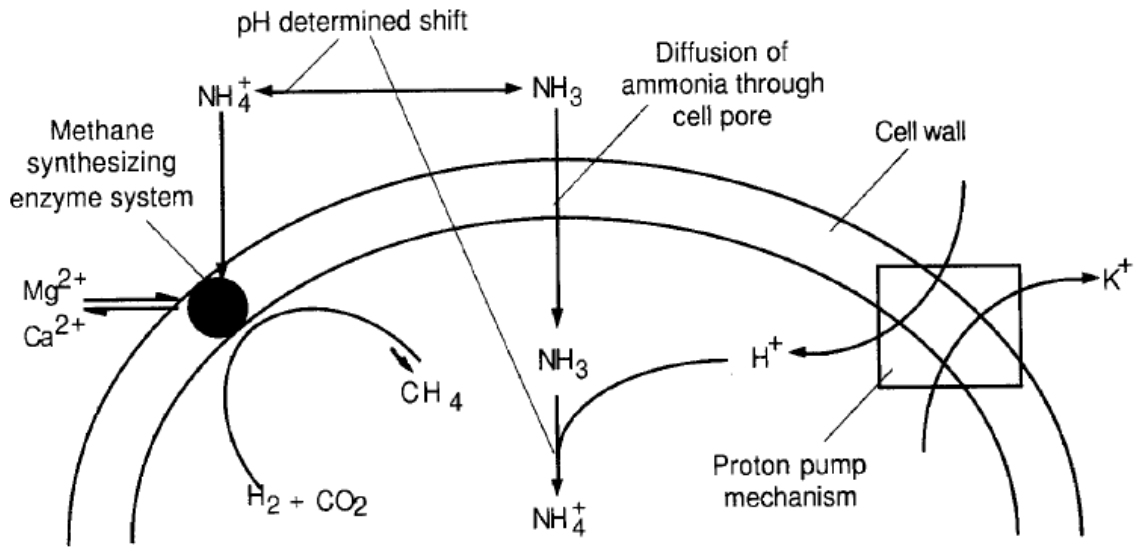


Figure 2.9. Proposed mechanism of ammonia inhibition in methanogenic bacteria (Kayhanian et al. 1999)

Due to the complicated procedures for measuring intracellular pH and cation concentrations, experimental work to support this hypothesis is very limited. A study carried out using the methanogen strain *Methanolobus taylorii* has shown that most of the energy in the $\Delta_{\mu H}^{+}$ of the methanogen cell is accounted for by its large $\Delta\psi$ (i.e., the outside of the cell is more positively charged); its ΔpH is therefore small or even negative (Jeris and McCarty, 1965). The study discovered that for the methanogen strain *Methanolobus taylorii,* the negative ΔpH allows it to grow with a near-neutral cytosol even when the external pH is above 7. The active extrusion of potassium by this methanogen was suggested as a mechanism by which it increases its $\Delta\psi$, thereby allowing it to grow with a low or negative ΔpH (Wolin and Miller, 1982, Koster, 1986).

Following the diffusion of ammonia into cell, at least two possible mechanisms of ammonia toxicity have been postulated (Kadam and Boone, 1996):

1. The direct inhibition of the activity of cytosolic enzymes by un-ionized ammonia. Kadam and Boone (1996) studied the active level of three ammonia-assimilating enzymes (glutamate dehydrogenase, glutamine synthetase, and alanine dehydrogenase) in three methanogen

species of the family *Methanosarcinaceae*. The results showed diversified enzymatic responses in different species towards a high concentration of ammonia in the growth media, which implied variable tolerances to ammonia between the three species.

2. Free NH_3 enters into the cells and the lower intracellular pH causes the conversion of part of this into ammonium (NH^{4+}), absorbing protons in the process. The cells must then consume energy in proton balancing, using a potassium (K^+) pump to maintain the intracellular pH, thus increasing maintenance energy requirements, and potentially causing inhibition of specific enzyme reactions (Gallert et al., 1998, Sprott et al., 1984, Whittmann et al., 1995). Also when the cell is exposed to high ammonia and the potassium (K^+) pump cannot keep up with the accumulated NH^{4+} inside cells, intracellular pH cannot be maintained; this therefore might be the cause of toxicity (Sprott and Patel, 1986). In a study conducted by Sprott et al. (1984) using pure culture *Methanospirillum hungatei* exposed to ammonia in a K^+ free buffer. It was observed that the methanogen lost up to 98% of the cytoplasmic K^+ through an ammonia/K^+ exchange reaction. The experiment also suggested that additions of NH_4OH or various NH_4^+ salts (or methylamine) were most effective in causing K^+ depletion in a media of alkaline pH (i.e. containing a higher proportion of the unionised form free ammonia), suggesting that NH_3 was the active chemical species crossing the cell membrane and causing toxicity. Other essential cytosolic cations such as Mg^{2+} and Na^+ have also been reported to be affected in the same way by ammonia (Kadam and Boone, 1996). Based on the findings of these studies, it is reasonable to speculate that high ammonia could also affect the uptake of essential trace elements required for cell function and thereby cause micro-nutrients deficiency.

Either way, it is understandable that high pH and high total ammonia concentration could exert their toxicities synergistically. At higher pH values, a larger fraction of total ammonia is unprotonated, i.e. in the form of free ammonia (at 35°C, about 1.1% at pH 7 but almost 53% at pH 9, calculated according to equation 2-26). Also, if bacteria growing at a higher pH establish a more negative ΔpH to maintain a near-neutral cytosol, then the potential toxicity due to NH^{4+} accumulation would also be greater.

2.5.1.2 Impact of Ammonia Toxicity to the Diversity of Methanogens and Methanogenesis Pathway in Anaerobic Digestion

It is generally acknowledged that of all the anaerobic microorganisms involved in anaerobic digestion, the methanogens are the least tolerant to environmental inhibitors and the most likely to be affected by ammonia inhibition (Kayhanian, 1994, McMahon et al., 2001, Kotsyurbenko et al., 2004).

Koster and Lettinga (1988) studied the microbial activity change of acidogenic and methanogenic population in granular sludge as ammonia nitrogen concentrations were increased in the range of 4051–5734 mg N l^{-1}. The experiment showed the methanogenic population lost 56.5% of its activity; while the acidogenic populations were hardly affected. The particular sensitivity of methanogens to ammonia toxicity will lead to the cessation of the methanogenesis stage of anaerobic digestion, whilst acid production remains unaffected. Consequently the build-up of organic acids resulting from inhibited methanogenesis will cause a rapid fall in pH and complete failure of the whole anaerobic process (McMahon et al., 2001).

Among the methanogenic strains, the tolerance towards ammonia toxicity varies significantly. In an early study (Wiegant and Zeeman, 1986), hydrogen-utilising methanogens were reported to be more susceptible to ammonia than acetoclastic methanogens under thermophilic conditions. In the same study, the author postulated that hydrogen accumulation due to the blockage of the hydrogenotrophic route caused a build-up of propionate which in turn, acts as an inhibitor of the acetoclastic methanogens. In a later study carried out by Angelidaki and Ahring (1993), however, specific methanogenic activity (SMA) was monitored for both acetoclastic and hydrogenotrophic methanogens under thermophilic conditions. It was observed in this study that the SMA of acetoclastic methanogens decreased more than hydrogenotrophic methanogens under ammonia stress. These experimental results therefore did not support the previous study by Wiegant and Zeeman (1986).

There is, in fact, increasing evidence in the literature indicating that acetoclastic methanogens are more sensitive to ammonia toxicity than hydrogenotrophic ones (Robbins et al., 1989, Schnürer and Nordberg, 2008, Sprott and Patel, 1986). Koster and Koomen (1988) studied

ammonia inhibition specifically of hydrogenotrophic methanogens using sludge that had never experienced ammonia inhibition before. The hydrogenotrophic population was found to grow well at an ammonia concentration as high as 6.3 g N l^{-1}. Interestingly, in this study using only hydrogenotrophic methanogens strains, the methanogenesis started without a requirement for acclimation. Whereas in other studies using unacclimatised mixed populations of acetoclastic and hydrogenotrophic methanogens (Van Velsen, 1979, Koster and Lettinga, 1984, Koster, 1986), temporary cessation of methanogenesis was always encountered after exposure to ammonia and recovered only after an adaptation period. This again is in accordance with earlier findings that the hydrogenotrophic methanogens are less susceptible to ammonia toxicity.

A few toxicity studies have been carried out studying the different inhibition thresholds of ammonia towards hydrogenotrophic and acetoclastic methanogens. Jarrell et al. (1987) used pure cultures to study the tolerance to ammonia in four methanogen strains commonly isolated from sludge digesters which can grow on H_2 and CO_2 (*Methanospirillum hungatei*, *Methanosarcina barkeri*, *Methanobacterium thermoautotrophicum*, and *Methanobacterium formicicum*). It has been observed that although being the most sensitive to ammonia of the four strains, *Methanospirillum hungatei* can tolerate ammonia concentrations up to 4.2 g N l^{-1}; whilst the other three strains tested were resistant to ammonia concentrations higher than 10 g N l^{-1}. Similar pure culture studies conducted on four strains of thermophilic hydrogenotrophic methanogens showed population growth could still be observed for some strains even at ammonia concentration of 9g N l^{-1} (Hendriksen and Ahring, 1991). The concentrations of ammonia that could be tolerated by methanogens in those studies were significantly higher than in other studies using mixed cultures (Poggi-Varaldo et al., 1997), further indicating that hydrogenotrophic methanogens have higher tolerance towards ammonia.

Although there is an apparent difference in ammonia tolerance between the two groups of methanogens, nothing in the scientific literature puts forward a possible mechanism, on a molecular microbiology level, to explain this difference e.g. effect on certain enzymes used exclusively by the acetoclastic pathway. Some investigations have been conducted on microbial behaviour under ammonia toxicity as in Borja et al. (1996) who observed that

inhibition of the acetoclastic populations showed a sigmoidal pattern. This finding is in agreement with the study conducted by Poggi-Varaldo et al. (1991) who found that the bacterial growth rate and the specific acetate-uptake rate were affected by the free ammonia concentration in a three-stage pattern: initial inhibition, plateau and final inhibition. This inhibition pattern could indicate that two inhibition mechanisms are involved which act at different concentration levels. The hydrogenotrophic populations, however, exhibited a more linear pattern of inhibition (Borja et al., 1996).

Other studies have focused on microbial diversity and morphology at a genus and species levels. Sprott and Patel (1986) found that methane formation from obligate acetotroph *Methanosaeta concilii* was completely inhibited at a TAN concentration of 560 mg l^{-1}, while methane formation from *Methanosarcina barkeri* was not inhibited at a TAN concentration of 2800 mg l^{-1}.

Amongst the 83 species of methanogens discovered, the majority (61 species) are hydrogenotrophs that oxidise H_2 and reduce CO_2 to form methane, while only 9 species of acetoclastic methanogens are known that utilise acetate to produce methane (Garcia et al., 2000). Some researchers have concluded that cell morphology plays an important role in resisting ammonia toxicity. Demirel and Scherer (2008) attributed the particular susceptibility of acetate-utilising methanogens *Methanosaetaceae* to the cell morphology which is one of thin filaments. Due to the cell shape, *Methanosaetaceae* seemed to offer more surface area than the hydrogenotrophic methanogens which grow as rods, or *Methanosarcinaceae* which grow as thick clumps. The diffusion of free ammonia into the cells would be faster in the filamentous cells when expressed on the basis of kilograms of NH_3 entering per kilogram cell mass per hour. Similar speculation has also been by made by Wiegant and Zeeman (1986). A study by Hendriksen and Ahring (1991) even suggested that for some thermophilic hydrogenotrophic methanogens, high ammonia concentration in the growth medium can induce formation of large cell aggregates, which implied a possible defence mechanism for those strains against ammonia toxicity.

The selective inhibition of ammonia towards methanogens will have a profound impact on the diversity of the methanogenic population in an anaerobic digester treating an ammonia/ nitrogen rich substrate, i.e. under the influence of ammonia, hydrogenotrophic methanogens will gradually become the predominant species and as the result the major methanogenesis pathway will shift to hydrogenotrophic (Calli et al., 2005, Westerholm et al. 2011).

The application of advanced molecular microbiology techniques such as Fluorescent *in situ* Hybridisation (FISH) and Quantitative Polymerase Chain Reaction (q-PCR) have made it possible to monitor changes in the methanogenic population in anaerobic digesters exposed to a high concentration of ammonia. Results from these studies (Calli et al., 2005, Goberna, 2010, Schnürer and Nordberg, 2008, Westerholm et al. 2011) have clearly indicated the obvious change of dominate methanogen population to hydrogenotrophic after the exposure to ammonia.

Changes in the methanogenic population and major methanogenic pathway were examined during start-up of a full-scale anaerobic sequencing batch reactor (ASBR) treating swine waste (Angenent et al., 2002). It was observed that after the increase in total ammonia concentration during the start-up process, acetate degradation increased; however the acetoclastic methanogens population decreased based on 16S ribosomal RNA (rRNA) concentrations.

Karakashev et al. (2005) studied the influence of environmental parameters on the diversity of methanogenic communities in 15 full-scale biogas plants treating either manure or sludge as substrates under different conditions. The findings of this study indicated that in plants operating in the mesophilic range, where the free ammonia concentration was lower than in thermophilic plants, the diversity of the methanogenic population was broader. Dominance of the acetoclastic phylogenetic group *Methanosaetaceae* was observed in digesters fed with sludge (ammonia concentration at 0.03-0.3 g N l^{-1}). However, *Methanosaetaceae* was never found to be dominant in digesters treating manure (ammonia concentration at 2.1-6.1 g N l^{-1}). The authors reported that the inoculum type and loading rate did not affect the diversity of methanogens in biogas reactors, but the concentrations of ammonia and VFA were influential.

The consequences of the change in methanogenic pathway for the apparent behaviour and maintenance strategies of the anaerobic digestion process are significant. Under normal, uninhibited conditions, 70% of methanogenesis is attributed to the acetoclastic route and only 30% of methane is formed via the hydrogenotrophic route (Jeris and McCarty, 1965, Gujer and Zehnder., 1983). During anaerobic digestion, hydrogenotrophic methanogens play a crucial role to keep the hydrogen partial pressure low enough to make it thermodynamically possible for propionate and butyrate to be converted into the methanogenic substrates acetate and hydrogen (Wolin and Miller, 1982). According to Gujer and Zehnder (1983), the hydrogen partial pressure should be maintained under 10^{-4} bar (0.1 kPa) in order to sustain a healthy digestion process; furthermore hydrogen partial pressure is the parameter that most promptly indicates a disturbance in the digestion process. Therefore if the digestion has been inhibited by ammonia and methanogenesis is reliant solely on the hydrogenotrophic pathway, any further disturbance to methanogenesis would cause accumulation of hydrogen which subsequently results in thermodynamic blockage of propionate degradation in a timescale of a second (Koster and Koomen 1988); whereas acetoclastic blockage affects anaerobic digestion by reducing pH, which normally takes about 1 day (Gujer and Zehnder, 1983). This could very likely be the reason why anaerobic digesters treating nitrogen-rich substrate such as food waste, are prone to rapid and irreversible operational failure (Neiva Correia et al., 2008; Banks and Zhang, 2010).

The above review of ammonia toxicity has established that acetoclastic methanogens are inhibited at high ammonia concentrations and that this inhibition threshold is lower than that for hydrogenotrophic methanogens, in both thermophilic and mesophilic digesters. It is therefore likely that at the high ammonia concentrations reached in food waste digestion the acetoclastic methanogenic population will be lost or severely inhibited, and the direct formation of methane and carbon dioxide by cleaving of acetic acid would then be no longer possible. For the autotrophic methanogens to continue to operate under these conditions the acetic acid must first be converted to hydrogen and carbon dioxide. If this happens the process of methane production does not stop, only the route through which it forms changes; but current literature on ammonia toxicity does not offer any good explanation as to why these systems are likely to run at elevated VFA concentrations.

2.5.2 Long Chain Fatty Acids

Long chain fatty acid (LCFAs) are hydrolysis products of fats and oils and can be degraded through anaerobic digestion process; however, LCFAs are acknowledged to be inhibitory to anaerobic process (Hanaki et al., 1981). In fact, the inhibitory effect of long-chain fatty acids means they have been widely applied as food preservatives and dietary supplements for ruminants to suppress methane production (Angelidaki and Ahring, 1992). The low degradation rates of LCFAs and their inhibitory effects are major problems in the development of suitable anaerobic treatment systems for municipal solid waste with high lipid content such as food waste.

Long-chain fatty acids have been reported to be inhibitory at low concentrations for gram-positive microorganisms (Sheu and Freese, 1973). The toxicity of LCFA has been attributed to their amphiphilic properties that allow them to be easily adsorbed onto a microbial surface, therefore impeding the passage of essential nutrients through the cell membrane (Henderson, 1973; Huw *et al.* 1998; Alves, 2001; Pereira *et al.*, 2005). Gram-negative microorganisms are far less sensitive to LCFA, however, due to the intact lipopolysaccharide layer of the cell envelop which prevents LCFA accumulating on the inner cell membrane, (Sheu and Freese, 1973).

There is some debate concerning the concentrations at which LCFA become inhibitory, and this may also depend on the digester operating mode and degree of acclimatisation. In batch experiments with granular sludge in which *Methanosaeta spp.* are the predominant acetoclastic methanogens, Koster and Cramer (1987) showed the maximum specific acetoclastic methanogenic activity had been reduced to 50% at concentrations of 0.32, 0.68, 0.45, 0.59 and 0.97 g l^{-1} for lauric, oleic, capric, myristic and caprylic acids respectively. The study concluded that the inhibition was correlated with concentration rather than with the amount per unit of biomass, although adsorption of the inhibitor on the cell wall might play an important role in the mechanism of inhibition. Also synergism of LCFAs can be expected to enhance the inhibition of methanogenesis.

Angelidaki and Ahring (1992) carried out thermophilic batch tests on cattle manure. The lag phase before which methanogens start to produce biogas increased when the concentrations of oleate and stearate were 0.2 g l^{-1} and 0.5 g l^{-1}respectively. Methanogenic activities ceased entirely at concentration of 0.5 g l^{-1} for oleate and 1.0 g l^{-1} for stearate. This supported the findings of Koster and Cramer (1987), who also suggested that inhibition was concentration-dependent.

Koster (1987) discovered that lauric acid had a strong affinity for methanogenic sludge and that 50% of the methanogenic potential of granular sludge was lost after only 5-7 minutes of exposure. The research also looked at the antagonistic effect of calcium ion to lauric acid. It was proved that the inhibition of methanogenesis caused by lauric acid can be reversed by the addition of calcium chloride, provided the addition is applied within a few minutes of an inhibitory lauric acid concentration being dosed. The antagonistic effect of calcium chloride is claimed to be caused by precipitation of the inhibitory LCFA as calcium salts. However, currently no other research has offered any other solutions (e.g. trace elements supplementation) to eliminate the inhibitory effects of LCFAs.

Lalman and Bagley (2000, 2002), using unacclimated batch cultures at 21°C, showed inhibition of acetoclastic methanogenesis by oleic and linoleic acids at 30.85 mg l^{-1} (0.11 mM) but not by stearic acid at concentrations up to 99.57 mg l^{-1} (0.35 mM); all three acids showed only slight inhibition of hydrogenotrophic methanogenesis.

Alves *et al.* (2001) tested for inhibition in a fixed bed digester at 35 ^{o}C continuously fed with 4.15 g l^{-1} sodium oleate at an OLR of 8-9 kg COD m^{-3} day^{-1}, and showed it was efficiently converted to methane. Using granular sludge from fixed and expanded bed digesters Pereira *et al.* (2003, 2004) reported that LCFA had adverse effects on functionality, but also that the effect was reversible under appropriate conditions and LCFA could be efficiently converted. Palatsi *et al.* (2009, 2010) have more recently shown that the tolerance of anaerobic consortia towards LCFA could be improved by proper acclimation.

The traditional gas chromatography method for LCFA determination requires free fatty acids to be derivatised to a methyl ester (FAME). This approach was introduced by Morrison and Smith (1964) and similar methods are still used (Masse *et al.* 2002; Palatsi *et al.*, 2009). A two-step procedure is required: firstly methylation of free fatty acids under high temperature with a suitable catalyst; then extraction of the derivatised fatty acids using a solvent. Methylation enhances the volatility and reduces activity of the free fatty acid. Morrison and Smith (1964), Angelidaki et al. (1990), Chou *et al.* (1998) and Masse *et al.* (2002) used a catalyst prepared by dissolving Boron Fluoride, a very toxic gas, into methanol. Other workers (Eras *et al.*, 2004; Palatsi *et al.*, 2009) have used the less toxic Clorotrimethylsilane (CTMS)-methanol, but CTMS reacts violently with water requiring lyophilisation of all samples before extraction, with a significant increase in sample preparation time. Two less dangerous reagents, HCl:1-propanol and methanolic HCl, are reported in Neves *et al.* (2009) and Sönnichsen and Müller (1999) respectively, and good methylation has been achieved.

Irrespective of the catalyst selected, the methylation step requires a long reaction time (from 1-16 hours) at high temperature (90-100 °C). For routine monitoring of LCFA where a high sample throughput and a short turnaround time are essential, these methods are therefore not very suitable. There is also a concern that with small sample sizes, a complicated procedure is likely to be less accurate (Sönnichsen and Müller, 1999).

CHAPTER 3

Materials and Methods

3.1 General Analytical Methods

3.1.1 Chemical Reagents and Glassware Used

All glassware used was washed with detergent and rinsed with tap water followed by deionised water before reuse. The deionised water was purified by a reverse osmosis system (Barnstead RO system, Thermo Scientific, UK).

All glassware and apparatus used in the analysis of trace concentrations of metals was washed with detergent, rinsed twice with tap water and twice with deionised water, then soaked in an acid bath of 10% hydrochloric acid for at least 12 hours. Water used in the acid bath, subsequent rinsing and all wet chemistry analyses (alkalinity, ammonia, Kjeldahl nitrogen and fibre analysis) was deionised water.

Both nitric and hydrochloric acids used in analysis were trace analysis grade. All reagents, standards and VFA samples were prepared using ultra-pure deionised water (Barnstead Nanopure ultrapure water purification system, Thermo Scientific, UK). Chemicals used were from Fisher Scientific (Loughborough UK) except where otherwise specified.

Glassware and apparatus used for lipid analysis was washed with detergent and heated to 550 ^{o}C to burn off any lipids or other organics that could affect analysis.

3.1.2 Total and Volatile Solids

Substrate and digestate samples were analysed according to the following procedure:
After thorough homogenisation of the sample, approximately 10 g of sample was transferred into a weighed crucible by pouring (digestate samples) or spatula (substrate samples). Samples were weighed using a balance with accuracy of ±0.001 g (Sartorius LC6215 balance, Sartorius AG, Gottingen Germany) and placed in an oven (Vulcan laboratory oven, LTE Scientific Ltd.,

Oldham UK) for drying overnight at 105°C ± 2°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 Furnace 201, Carbolite UK, Hope Valley UK) and heated to 550 °C ± 10 °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 deionised water, and stored in an oven until required for the next analysis. Crucibles were transferred from the oven to a desiccator for cooling to room temperature before each analysis. TS and VS were calculated according to the following formulae:

$$\%TS = \frac{W_3 - W_1}{W_2 - W_1} \times 100 \quad [3.1]$$

$$\%VS(based \cdot on \cdot total \cdot weight) = \frac{W_3 - W_4}{W_2 - W_1} \times 100 \quad [3.2]$$

$$\%VS(based \cdot on \cdot total \cdot solids) = \frac{W_3 - W_4}{W_3 - W_1} \times 100 \quad [3.3]$$

Where
W_1 is the weight of the empty crucible;
W_2 is the weight of the crucible containing fresh sample;
W_3 is the weight of the crucible and sample after drying at 105°C;
W_4 is the weight of the crucible and sample after heating to 550°C.

The volatile solids destraction rate is calculated using the formula:

$$\%VS\ destruction = \frac{VS_{Feedstock} \times W_1 - VS_{Digestate} \times W_2}{VS_{Feedstock} \times W_1} \times 100 \quad [3.4]$$

Where
$VS_{Feedstock}$ is the VS content of the feedstock,
$VS_{Digestate}$ is the VS content of digestate withdrawn from the digester,
W_1 is the weight of feedstock added to the digester
W_2 is the weight of digestate withdrawn from the digester.

3.1.3 pH

pH of digestate was measured using a Jenway 3310 pH meter (Jenway Ltd., Essex UK), immediately after sampling to avoid pH changes due to temperature drop and loss of CO_2. The meter has a sensitivity of 0.01 pH units and accuracy to 0.01 ± 0.005 units.

Calibration of the probe was carried out before each day's pH measurement, using buffer solutions of pH 4.0, 7.0 and 9.2, prepared from pH buffer tablets (Fisher Scientific) dissolved in 100 ml of deionised water. Fresh buffer solutions were prepared weekly and stored in closed containers between uses. Cross-contamination was avoided by thorough rinsing of the probe between each measurement, and storage of the probe in a pH 7 buffer solution between uses.

3.1.4 Alkalinity

A sample volume of 20 ml of digestate was made up to 50 ml with deionised water (to minimise foaming) and titrated with 0.25 N H_2SO_4, with constant stirring using a magnetic stirrer. To distinguish the different types of buffering, Partial Alkalinity (PA) was measured to pH 5.75 as a measure of bicarbonate buffering, while Intermediate Alkalinity (IA) was measured between pH 5.75 and 4.3, as the buffering capacity attributable primarily to the salts of VFA. The ratio of IA to PA (Ripley Ratio) reflects the VFA concentrations compared to the buffering capacity of the system (Ripley et al., 1986).

The pH probe was calibrated before each alkalinity titration using the buffer solutions noted above for pH measurement. Cross-contamination was avoided by thorough rinsing of the probe between each measurement, and storage of the probe in a pH 7 buffer solution between uses.

The method is based on the Standard Method 2320B for Alkalinity (APHA, 2005). Alkalinity in mg $CaCO_3$/L was calculated based on the equation below:

$$ALK = \frac{A \times N \times 50{,}000}{V_S} \quad [3.5]$$

Where:

A is the amount of acid used to titrate the sample, in ml;

N is the normality of titrant, in N;

50,000 is the conversion factor of g to mg and 50 mg $CaCO_3$ to 1 milliequivalent alkalinity;

Vs is the volume of the sample, in ml.

3.1.5 Total Kjeldahl Nitrogen (TKN)

Substrate and digestate samples were analysed for Total Kjeldahl nitrogen (TKN), which measures the nitrogen content in its reduced forms, i.e. organic and ammonia form.

For each sample, a mass of 3-5 g was weighed to an accuracy of 1 mg into a glass digestion tube, and two catalyst tablets added to facilitate acid digestion by lowering the activation energy of the reaction. 12 ml of concentrated sulphuric acid (H_2SO_4) was added to each digestion tube and the tubes were gently agitated to ensure contact of the entire sample with the acid. One tube contained the Kjeltabs and acid with no sample, as a blank. Tubes were then subjected to acid digestion on a heating block with exhaust system (Foss Tecator 1007 Digestion System 6, Foss Analytical, Hoganas Sweden) at 420^{o}C ± 5^{o}C until all tubes showed a clear blue-green solution, a time of at least two hours.

After cooling, the samples were subjected to steam distillation. For this step, a Foss Tecator Kjeltec System 1002 distillation unit (Foss Tecator AB, Hoganas Sweden) was used. Each cooled sample was distilled for a minimum of four minutes, after the addition of laboratory-grade concentrated sodium hydroxide (NaOH, ca. 10 m) to raise the pH beyond 9.5 and facilitate the volatilisation of ammonia. Distillate was collected in an Erlenmeyer flask containing indicating boric acid solution. The distillate was then titrated with H_2SO_4 (0.25 N), until a colour change from green to lavender was obtained. The nitrogen content of the sample was calculated according to the following formula:

a. Liquid samples: $$mgN/L = \frac{(A-B)\times 14.0\times N\times 1000}{mL(sample)} \quad [3.6]$$

b. Solid samples: $$\%N = \frac{(A-B)\times 14.0\times N\times 100}{mg(dry\cdot wt\cdot sample)} \quad [3.7]$$

where:
A = volume of H_2SO_4 titrated for sample, in ml;
B = volume of H_2SO_4 titrated for blank, in ml;
N = normality of standard sulphuric acid titrant, in N

3.1.6 Total Ammonia Nitrogen (TAN)

Total ammonia nitrogen was measured using a Kjeltec System 1002 distillation unit (Foss Tecator AB, Hoganas, Sweden) which distils off the ammonia into a boric acid solution which is then subsequently titrated with 0.25N H_2SO_4 until a colour change from green to pale lavender. Digestate samples were measured immediately after sampling to avoid any ammonia losses.

3.1.7 Volatile Fatty Acid (VFA)

The method is based on SCA (1979): Determination of Volatile Fatty Acids in Sewage sludge (1979). Digestate samples were prepared for VFA analysis by centrifugation (Eppendorf 5417 C/R, Eppendorf, Hamburg Germany) at 17,900 g (13,000 rpm) for 15 minutes and 0.9 ml of the supernatant was transferred by pipette (Finnpipette, Thermo Fisher Scientific, UK) to vials with 0.1 ml formic acid to give a concentration of 10% formic acid. Where dilution was necessary, deionised water was used and formic acid was added to a concentration of 10% of the total volume for analysis. VFA concentrations were quantified in a Shimazdu 2010 gas chromatograph using a flame ionisation detector and a capillary column type SGE BP 21 with helium as the carrier gas at a flow of 190.8 ml min^{-1}, with a split ratio of 100 giving 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. Three standard solutions containing 50, 250 and 500 mg l^{-1} of acetic, propionic, iso-butyric, n-butyric, iso-valeric, valeric, hexanoic and heptanoic acids were used for VFA calibration.

3.1.8 Gas Composition

Biogas composition was measured using a Varian CP-3800 gas chromatograph (Varian, Oxford, UK) with a gas sampling loop and thermal conductivity detector with argon as the carrier gas at a flow of 50 ml min^{-1}. The GC was fitted with a Hayesep C column and a molecular sieve 13 × (80-100 mesh) operating at a temperature of 50 ^{o}C. The GC was calibrated using a standard gas containing 35% CO_2 and 65% CH_4. The small amount of air in the sample normally caused by atmospheric entering digester headspace during feeding is corrected by excluding the volume of air from the total sample volume. For example, in a

sample with a% of CH_4, b% of CO_2 and C% of atmospheric air, the corrected percentage of CH_4 and CO_2 will be $a\%/(1-c\%)$ and $b\%/(1-c\%)$, respectively.

The theoretical gas composition based on elemental analysis was calculated using the Buswell equation (Symons and Buswell, 1933):

$$C_cH_hO_oN_nS_s + 1/4(4c - h - 2o + 3n + 2s)\ H_2O = 1/8(4c - h + 2o + 3n + 2s)\ CO_2 + 1/8(4c + h - 2o - 3n - 2s)\ CH_4 + nNH_3 + sH_2S \quad [3.8]$$

3.1.9 Calorific Value (CV)

CV was measured using a ballistic bomb calorimeter (CAL2k, Digital Data Systems Ltd, South Africa). Theoretical calorific value (TCV) was calculated in accordance with PD CEN/TR 13767 (2004) using the formula:

$$TCV = (34.1C + 102H + 6.3N + 19.1S - 9.85O) / 100 \quad [3.9]$$

3.1.10 Elemental Composition

C, H, N and O were analysed using a FlashEA 1112 Elemental Analyzer, (Thermo Finnigan, Italy) according to the manufacturer's instructions with methionine, L-cystine and sulphanilamide as standards.

3.1.11 Trace Metals Extraction and Analysis

Samples with higher solids contents such as food waste and vegetable waste substrates were first air dried and then ground into fine powder using a centrifuge mill with a 0.5 mm mesh sieve (Retsch ZM-1, Retsch GmbH, West Yorkshire, UK**)**, followed by acid digestion (SCA, 1986).

Anaerobic digestate samples were prepared for metals analysis using hydrochloric-nitric acid digestion, in accordance with EPA method 3010A (acid digestion of sediments, sludges, and soils).

After digestion, the digested samples were gravitationally filtered through an acid resistant cellulose filter paper and then the filter paper was rinsed 5 times using 12.5% nitric acid. The filtrate was quantitatively transferred to volumetric flasks and made up to 50 ml with ultra-pure deionised water.

Trace element concentrations were determined using ICP-MS or ICP-OES at a UKAS accredited commercial laboratory (Severn Trent Services, Coventry, UK).

3.1.12 Fibre Analysis

Neutral detergent fibre (NDF), Acid detergent fibre (ADF) and Acid detergent lignin (ADL) were analysed using FiberCap™ system (FOSS Analytical AB, Sweden) according to the manufacturer's instruction with modifications according to Goering and Soest (1970) and Kitcherside et al. (2000).

The methods for pH, TS and VS, gas composition, VFA determination, elemental analysis, calorific value, Kjeldahl Nitrogen, metals extraction and fibre analysis are given in more detail in Appendices 2-10.

3.2 Digesters

3.2.1 Digester Construction and Operation

Digesters used in the study were of a continuously stirred tank reactor (CSTR) design. Two volume capacities were used, depending on the study. The first type had a 5-litre capacity and a 4-litre working volume, and the second type a 2-litre capacity with a 1.5-litre working volume. Irrespective of size the digesters were constructed of PVC tube with gas-tight top and bottom plates. 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 (Fig.3.1). Temperature was controlled at 35 ^{o}C by circulating water from a thermostatically-controlled bath through a heating coil around the digesters. Biogas was measured using tipping bucket gas counters with continuous datalogging

(Walker et al., 2009) and all gas volumes reported are corrected to standard temperature and pressure (STP) of 0 ^{o}C and 101.325 kPa). Semi-continuous operation was achieved by removing digestate through an outlet port in the base before adding feed via the feed port. During this process a small amount of atmospheric air enters the headspace but in insufficient quantities to affect the redox conditions in the digester: any air detected in the gas composition is corrected for (as described in section 3.1.8), as this is not normally produced as a result of the digestion process. Except where otherwise stated feeding was done on a daily basis throughout the experimental period with regular checks to ensure a constant level was maintained in the digesters.

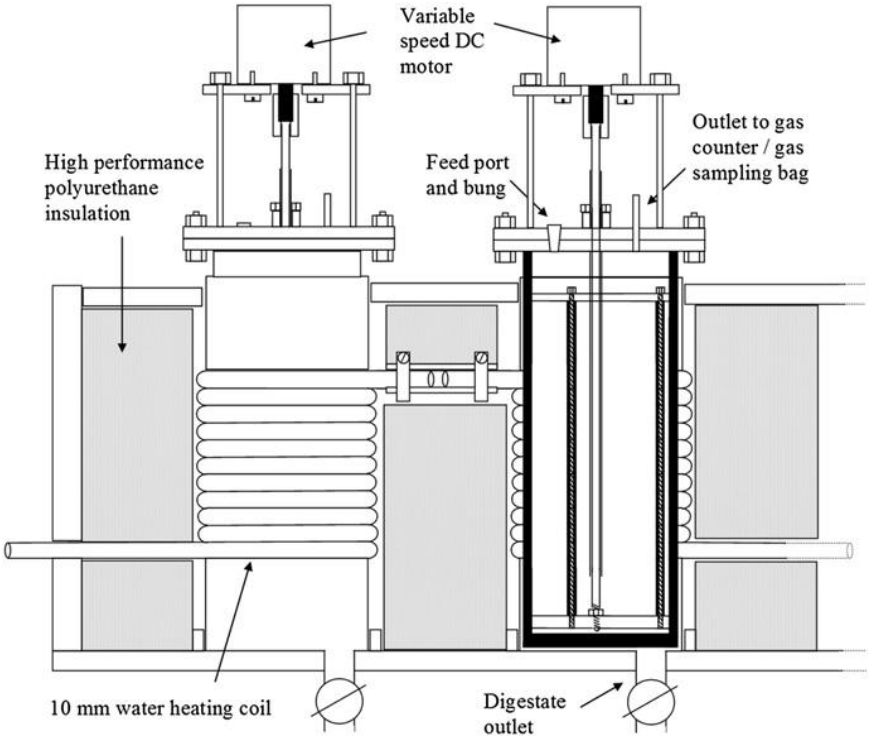


Figure 3.1 Typical schematic of the 5-litre and 2-litre CSTR digesters used with cross-section showing details of heating and stirring systems.

3.2.2 Digester Inoculums

The 5-litre digesters used for the semi-continuous vegetable waste trial were initially seeded with digestate from a digester treating municipal wastewater biosolids at Millbrook Wastewater Treatment plant, Southampton, UK. Acclimated digestate from previous experimental runs was used as the inoculum in the second vegetable digestion trial.

For food waste batch testing for determination of trace element requirements the inoculum was taken from a 5-litre food waste digester (OLR = 2 g VS l^{-1} day^{-1}) which had received no additional trace elements supplementation for its 112 days of operation. The inoculum used in the semi-continuous food waste digestion trial was taken from a 75-litre (working volume) food waste digester, the previous feedstock and loading history of which are given in detail in Zhang et al. (2012).

3.3 Digester Feedstocks

3.3.1 Vegetable Waste

The vegetable waste (VW) provide by Biojoule Ltd. is derived from high-value products grown in Kenya for export to Europe, which are first sorted and trimmed before being crated for air freight. This process generates considerable amounts of waste, as any sub-standard items are also rejected, and cannot be re-distributed for consumption for reasons of hygiene. The VW material consisted of extra fine beans 11.6%, fine beans 23.3%, runner beans 21.6%, stringless beans 18.2%, baby corn 18.8%, carrot 3.5%, and broccoli 3.0%, on a wet weight (WW) basis. These were macerated in a grinder (series 526, Imperial Machine Co Ltd, UK), then frozen in 4-litre batches and defrosted and stored at 4^{o}C before use.

3.3.2 Co-substrates

A number of co-substrates and supplements were used in attempt to achieve digestion stability, including: card packaging (wet ground with added tap water) obtained from Alton materials reclamation facility (Hampshire, UK) and cattle slurry from a dairy farm (Rownhams, Southampton, UK).

3.3.3 Food Waste

Source segregated food waste from domestic properties, as delivered to the South Shropshire digestion facility at Ludlow, UK was the sole substrate for the digesters in the food waste study. The material was first taken out of biodegradable collection bags and any non-biodegradable contaminants were removed. It was then homogenised using a macerating grinder (S52/010 Waste Disposer, IMC Limited, UK), packed into 4-litre plastic storage

containers, and frozen at -18^{o}C. Before use the frozen feedstock was thawed, and stored at 4^{o}C for no more than one week.

3.4 Digester supplements

3.4.1 Gonzalez-Gil Trace Element Mix

The recipe used was that of Gonzalez-Gil (2001) which consisted of the following (g l^{-1}): $FeCl_2.4H_2O$, 2; $CoCl_2.6H_2O$, 2; $MnCl_2$. $4H_2O$, 0.5; $AlCl_3.6H_2O$, 0.09; H_3BO_3, 0.05; $ZnCl_2$, 0.05; $(NH_4)_6 Mo_7O_{24}.4H_2O$, 0.050; $CuCl_2.2H_2O$, 0.038; $NiCl_2.2H_2O$, 0.142; Na_2SeO_3, 0.164, EDTA, 1.

3.4.2 Modified Trace Element Supplement

Based on results of initial experiment and literature survey, it was suspected that the concentration of certain trace elements supplemented using Gonzalez-Gil recipe was not sufficient to sustain efficient digestion. Therefore a modified TE recipe was devised with an adjusted concentration. This contained the following (g l^{-1}): $FeCl_2.4H_2O$, 35.5; $CoCl_2.6H_2O$, 4.034; $MnCl_2$. $4H_2O$, 0.36; $AlCl_3.6H_2O$, 0.894; H_3BO_3, 0.571; $ZnCl_2$, 20.833; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.184; $CuCl_2.2H_2O$, 0.268; $NiCl_2.2H_2O$, 2.825; Na_2SeO_3, 0.219; $Na_2WO_4.2H_2O$, 0.18. The concentration of each element in this recipe can be grouped into: 1.) high concentration elements, which includes Fe and Zn, both at 10 g l^{-1}, 2.) medium concentration, which includes Co and Ni at 1 g l^{-1}, and 3.) low concentration, which includes the rest of the elements (Mn, Al, B, Mo, Cu, Se and W) at 0.1 g l^{-1}.

During the second part of the semi-continuous digestion trials on vegetable waste, a mixed solution of all elements was prepared; it was noticed, however, that precipitation occurred during storage. Therefore in the further study, instead of preparing a mixed elements solution, each element solution was prepared and stored individually.

3.4.3 Yeast Extract

Yeast extract (Merck, Darmstadt, Germany) solution was prepared at 200 g TS l^{-1}.

3.5 Biochemical Methane Potential (BMP) Test

The test was carried out in duplicate with 3 positive controls using cellulose as a standard material and 3 inoculum-only controls. Anaerobic digester sludge from Millbrook Wastewater Treatment Works, Southampton was used as the inoculum for all reactors. The tests were carried out in static batch reactors with a total volume of 500 ml at a temperature of 35 +/-0.2 ^{o}C, maintained by a thermostatic water bath (Figure 3.2). Biogas generated was collected in perspex cylinders holding approximately 0.9 litres, filled with a 75 % saturated solution of sodium chloride acidified to pH 2 using hydrochloric acid to reduce carbon dioxide solubility. Gas production was measured by manual readings of the displacement of this solution. Samples of biogas were taken each time the collection cylinder was refilled and analysed for methane content by gas chromatography.

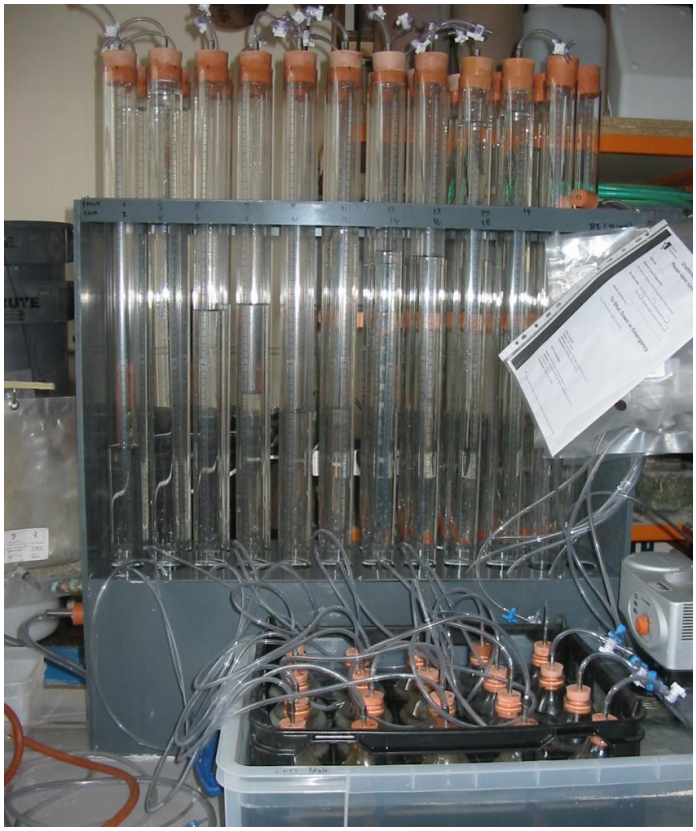


Figure 3.2. BMP reactors and test rig

3.6 Semi-Continuous Digestion Trials on Vegetable Waste

The study was divided into 2 parts. In the first part eight 5-litre digesters (D1-D8) seeded with digestate taken from Millbrook wastewater treatment plant (section 3.2.1) were used. These were initially intended to operate at OLRs of 2, 3, 4 and 5 g VS l^{-1} day^{-1}, equivalent to HRTs of around 40, 26, 19 and 15 days respectively when fed on the vegetable waste (section 3.3.1). Amendments had to be made to this plan during the experiment due to signs of stress in the digestion. Full details of the actual loadings and operating conditions applied are given in the results section in Table 5.3. The digesters were fed daily and biogas collected as described in section 3.2. The Gonzalez-Gil trace element solution (section 3.4.1) was added at the start of the experiment to each digester in the proportion of 1 ml l^{-1} digestate, and then weekly in the same proportion based on the volume of feedstock added.

In the second part of the semi-continuous digestion study, six 2-litre digesters were used (A1-A6). These were seeded with digestate taken from D1 and D2 (above). The digesters were fed daily and supplemented as previously, but using the modified TE supplement. Digesters were fed initially at an OLR of 2 g VS l^{-1} day^{-1} for 10 days. The loading on pairs of digesters A1-2 remained at 2 g VS l^{-1} day^{-1}, while the OLR on A3-4 was incrementally raised to 3 g VS l^{-1} day^{-1}, and on A5-6 to 4 g VS l^{-1} day^{-1}, equivalent to HRTs of 40, 25.6 and 19 days respectively.

3.7 Batch Screening Tests to Assess the Influence of Trace Elements

3.7.1 Experimental Design

This experiment was aimed to investigate the effects on VFA degradation of supplementation with 6 elements (Co, Ni, Mo, Se, Fe and W) using a 2-level factorial experimental design. Due to practical difficulties, it was not feasible to run a full 2-level factorial experiment, which requires 64 ($=2^6$) samples to be prepared for each single replicated experiment. Therefore a 2^{6-2} fractional factorial design, which is ¼ of the full factorial design with resolution of IV, was adopted. Such a design can clearly reveal the main effects, but cannot fully distinguish the 2-factor interaction due to effect aliasing (Wu & Hamada, 2009). The subset (fraction) of the

experimental runs of a full factorial design was generated using SAS JMP statistical suite (SAS Institute Inc. Cary, North Carolina, USA).

3.7.2 Experimental Method

32 conical flasks each with 250 ml capacity were inoculated with 200 ml of sieved digestate taken from a food waste digester. The inoculum digestate was also supplemented with a mix of sodium acetate and sodium propionate to make up the concentration of both to 10000 mg l^{-1}. D-glucose and starch were added as macro-nutrients to give concentrations of 4000 mg l^{-1} of each in the digestate. Individual working solutions of 6 trace elements (Fe, Co, Mo, Ni, Se, and W) were made up as follows (g l^{-1}): $FeCl_2.4H_2O$, 17.85; $CoCl_2.6H_2O$, 4.034; $(NH_4)_6 Mo_7O_{24}.4H_2O$, 0.184; $NiCl_2.2H_2O$, 4.11; Na_2SeO_3, 0.219; $Na_2WO_4.2H_2O$, 0.18. To each flask was added 0.2 ml as shown by the + signs in Table 3.1

Table 3.1. Experiment design matrix

Flasks	Co	Ni	Mo	Se	Fe	W
1, 2	-	-	-	-	-	-
3, 4	-	-	-	+	+	+
5, 6	-	-	+	-	+	+
7, 8	-	-	+	+	-	-
9, 10	-	+	-	-	+	-
11, 12	-	+	-	+	-	+
13, 14	-	+	+	-	-	+
15, 16	-	+	+	+	+	-
17, 18	+	-	-	-	-	+
19, 20	+	-	-	+	+	-
21, 22	+	-	+	-	+	-
23, 24	+	-	+	+	-	+
25, 26	+	+	-	-	+	+
27, 28	+	+	-	+	-	-
29, 30	+	+	+	-	-	-
31, 32	+	+	+	+	+	+

The headspace of each flask was flushed with a carbon dioxide and nitrogen (20:80) mixture (BOC, UK) before the flasks were sealed with rubber bungs with an outlet connection to a 1-litre gas sampling bag (Tedlar, SKC Ltd, UK) to collect generated gas and maintain the system

at ambient pressure. The flasks were then randomised and incubated in an orbital incubator (Weiss-Gallenkamp, UK) at a constant temperature of 36°C and agitated at 60 RPM.

Samples were regularly taken from the flasks to monitor the VFA degradation. After sampling, the flasks were flushed with carbon dioxide and nitrogen (20:80) and sealed again before being returned to the incubator. The experiment continued for about 90 days until all VFAs in flasks were depleted.

3.7.3 Data Analysis

To give a mathematical description of how effectively the VFAs were degraded an approach similar to that adopted by Olaisen et al. (2003) was used. In this the numerical integrals of each VFA degradation curve were assigned as the Degradation Index (*DI*), which is calculated using trapezoidal rule (Equation 3-9):

$$DI = \int_0^{\infty} f(t)\mathrm{d}t \approx \sum_{i=0}^{n}(t_{i+1} - t_i) \times (C_{i+1}+C_i)/2 \qquad [3.10]$$

Where: t is the time in days; C is the measured value of VFA concentration. A smaller *DI* indicates a more efficient degradation, as can be seen in the degradation curve in the later section (Section 6.2.2, Figure 6-1).

The *DI* numbers were then used as experimental responses in statistical and modelling analysis.

To analyse the results, main effects (*ME*) was calculated to evaluate and quantify average effects given by each factor. For any factor (*A*), the main effect is calculated by equation 3.10:

$$ME(A) = \bar{z}\,(A+) - \bar{z}\,(A-) \qquad [3.11]$$

Where $\bar{z}$ (*A*+) is the average value of all observations at the high (+) level of factor (*A*)
$\bar{z}$ (*A*-) is the average values of all observation at the low (-) level of factor (*A*).

Higher order factorial effects can be calculated in the similar way.

The factorial effects in the 2 level design can be estimated in the linear model (multiple regression model) (Wu and Hamada, 2009). For this experiment where *16* observations are collected, the model can be expressed as:

$$z_i = \beta_0 + \sum_{i=1}^{16} \beta_i x_i + \sum\sum_{j<i} \beta_{ij} x_i x_j \quad [3.12]$$

Where: β_0, β_i *and* β_{ij} are the regression coefficients which are estimated using Least squares method. z_i is the experiment response (*DI*). x_i and x_j are the variables (i.e. the factors in the experiment, coded with +/-).

3.8 Semi-Continuous Digestion Trials on Food Waste

6 pairs of 5-litre digesters were used in the trial. The digesters were initially inoculated with 3.5 litres of digestate from a 75-litre food waste digester (Zhang et al., 2012) and were fed on food waste initially at a loading (OLR) of 1.6 g VS l^{-1} day^{-1} without any digestate removal, until they reached a working volume of 4 litres.

Table 3.2. Additions and loadings applied to the food waste digesters

Digester ID	**Trace elements added**	**OLR (g VS l^{-1} day^{-1}) and comment**
F1 & F2	No TE addition (control)	2.0 g VS l^{-1} day^{-1} gradually decreasing as experiment progressed
F3 & F4	No TE addition (control)	Increased to 3.0 g VS l^{-1} d^{-1} on day 14, then decreased to 0 g VS l^{-1} d^{-1} when feeding stopped in an attempt to recover the digester. Digester recovered by TE additions
F5 & F6	Se and Mo	Increased successively to 3, 4, 5 and 5.5 g VS l^{-1} d^{-1} on day 14, 112, 203 and 626. Se concentration doubled on day 329 and Co added on day 427 and Se returned to initial concentration
F7 & F8	Se, Mo, Co and W	Increased successively to 3, 4, 5 and 5.5 g VS l^{-1} d^{-1} on day 14,112,203 and 626.
F9 & F10	Se, Mo, So, W, Fe and Ni	Increased to 5.5 g VS l^{-1} d^{-1}. TE supplementation stopped on day 112.
F11 & F12	Se, Mo, Co, W, Fe, Ni, Zn, Cu, Mn, Al, B.	Increased successively to 3, 4, 5 and 5.5 g VS l^{-1} d^{-1} on day 14, 112, 203 and 626.

When the digesters reached working volume, they were supplemented with trace elements (TE) and the OLRs were increased gradually as shown in Table 3.2. The concentration of TE supplemented is shown in Table 3.3, and this was then maintained by weekly additions. The amount added weekly was equal to that required for the wet weight of food waste added to the digester each week; calculation of this did not take into account any additional trace element input arising from the food waste itself.

Food waste was added each day to give the desired OLR and digestate was removed once per week to maintain a working volume of 4.0 litres. The retention time was therefore determined by the wet weight of the food waste, and was around 95, 63, 48, and 38 days at an OLR of 2, 3, 4, and 5.5 g VS l^{-1} day^{-1}, respectively.

Biogas production was measured daily and biogas composition and VFA determined twice weekly. Other digestate parameters such as pH, solids, TAN and alkalinity were analysed once per week. The process efficiency was estimated by calculating the specific biogas production (SBP) and volumetric biogas production (VBP). The stability of digester operation was evaluated by reference to other parameters such as pH, VFA, ammonia and alkalinity.

Table 3.3. Existing and additional concentration of trace elements added to the digesters.

		Element concentration (mg l^{-1})	
Element	**Compound Used**	**Measured trace element concentration in the inoculum digestate**	**Initial trace element additional made on day 1 per litre of digestate**
Aluminium (Al)	$AlCl_3.6H_2O$	63.3	0.1
Boron (B)	H_3BO_3	2.5	0.1
Cobalt (Co)	$CoCl_2.\ 6H_2O$	0.083	1
Copper (Cu)	$CuCl_2.\ 2H_2O$	5.75	0.1
Iron (Fe)	$FeCl_2.\ 4H_2O$	173.7	5
Manganese (Mn)	$MnCl_2.\ 4H_2O$	18.5	1
Molybdenum (Mo)	$(NH_4)_6\,Mo_7O_{24}.4H_2O$	0.29	0.2
Nickel (Ni)	$NiCl_2.2H_2O$	2.9	1
Selenium (Se)	Na_2SeO_3	0.05	0.2
Tungsten (W)	$Na_2WO_4.2H_2O$	<0.035	0.2
Zinc (Zn)	$ZnCl_2$	8.11	0.2

CHAPTER 4

Development of analytical techniques

4.1. Adaptation of ^{14}C Labelled Acetate Method to Determine the Methanogenic Pathway

As reviewed in the previous chapter (section 2.1.4), during the biochemical reaction of acetoclastic methanogenesis, the C-C bond between methyl and carboxyl group of the acetic acid is split with CH_4 being formed from methyl group while CO_2 is derived from carboxyl group (Ferry, 1992a). If methyl carbon labelled acetate ($^{14}CH_3COO^-$) is to react completely via the acetoclastic route, all ^{14}C atoms flow into CH_4, therefore none of ^{14}C isotope will be detected in the CO_2 produced through the reaction. When acetoclastic and hydrogenotrophic methanogenesis take place simultaneously, the radioactive isotope presented in CO_2 will rise as the reliance on hydrogenotrophic methanogenesis increases in the system.

4.1.1. Sampling and Culture Medium

Samples taken from digesters were put in 200 ml polyethylene bottles with screw caps. To minimise contamination from oxygen, each sample bottle was filled to its maximum capacity.

Table 4.1 Culture medium based on Zinder and Koch (1984)

Macro-Nutrients	**Concentration (g l^{-1})**
NH_4Cl	1
NaCl	0.1
$MgCl_2$. $6H_2O$	0.1
$CaCl_2$ $2H_2O$	0.05
$K_2HPO_4.3H_2O$	0.4
Sodium acetate	0.25
Trace element solutions	
$CoCl_2.6H_2O$	4.03
$(NH_4)_6 Mo_7O_{24}.4H_2O$	0.184
$NiCl_2.2H_2O$	4.11
Na_2SeO_3	0.219
$Na_2WO_4.2H_2O$	0.18

50 g of sample was then transferred into a 250 ml conical flask containing 100 ml of the culture medium as shown in Table 4.1 and thoroughly mixed.5 element solutions as shown in Table 4.1 were prepared individually and 0.1 ml of each trace element solution was added into 1 litre of the medium. The medium solution was boiled under N_2 and autoclaved (15 minutes, 120 ^{o}C) before use.

4.1.2 Preparation of $^{14}CH_3COONa$

1.85 ml of ultra-pure deionised water sterilised by microfiltration (0.2 µm pore size) was added to 1.85 MBq [2-^{14}C] of sodium acetate *($^{14}CH_3COONa$)* in the form of crystalline solid (MP biomedical, Solon, OH, United States) contained in the isotope container; this gave a stock solution with a specific activity of 1 MBq ml^{-1}. A working solution of 10 kBq ml^{-1} was then prepared by adding 0.5 ml of the stock solution into a 50 ml volumetric flask and making up to volume with deionised water.

4.1.3 Test Mixture

Duplicate 45 ml aliquots of the sample/culture medium mix were dispensed into a crimp top serum bottles with a capacity of 119 ml. 1 ml of the *$^{14}CH_3COONa$* working solution was added into each mix to give a 10 kBq ^{14}C dose. The headspace of the serum bottle was flushed with N_2/CO_2 (80:20) (BOC, UK) and sealed using a crimp cap PTFE coated silicon septa. The serum bottles were then incubated for 48 hours in an incubator (Hybaid Maxi 14, Thermo Scientific, UK) at 37^{o}C with orbital shaking at ~50 RPM.

4.1.4 Gas Sparging of the Mixture and Separated Collection of CO_2 and CH_4

A 10% mix of oxygen in nitrogen was prepared in a 3-litre Tedlar gas sampling bag and after incubation of the sample this gas mix was sparged into the liquid and headspace of the serum bottle for 45 minutes. This was achieved using a Watson Marlow 505U peristaltic pump fitted with a type 323 pump head and 3.2/1.6 mm (Bore/Wall) marprene tubing to give a flow rate of 30 ml min^{-1}. The pump had previously been calibrated using a water displacement method in which nitrogen was pumped at various speeds into a 100 ml glass graduated measuring cylinder filled with water and submerged upside-down in a water tank (See appendix 1 for calibration data). Before the sparge started, 0.2 ml of 1M HCl solution was added to the serum

bottle using a syringe with a needle, in order to shift the equilibrium from carbonate, bicarbonate to carbonic acid and CO_2 and therefore maximise the release of any dissolved CO_2 in the form of carbonate or bicarbonate.

As illustrated in Figure 4.1, the sparge gas from the serum bottle was first passed through 20 ml of 5 M NaOH to trap $^{14}CO_2$. The absorption efficiency of CO_2 by the alkali was tested during the preliminary method development trials by using two CO_2 traps connected in series. In these the sparge gas passed sequentially through the bottles and the radioactivity of each was determined. The $^{14}CO_2$ was only found in the first trap and therefore a single CO_2 trap was sufficient for effective CO_2 absorption.

The sparge gas then passed to a tube furnace consisting of a heating block within which was embedded a quartz tube (6.2 mm OD, 4 mm ID, 180 mm in length, H. Baumbach & Co Ltd, Suffolk, UK) packed with copper (II) oxide to facilitate methane oxidation (Figure 4.2). The operating temperature was regulated at 800±5^{o}C using a temperature controller (Omega DP7004, Manchester, UK). A schematic of the equipment is shown in Figure 4.2.

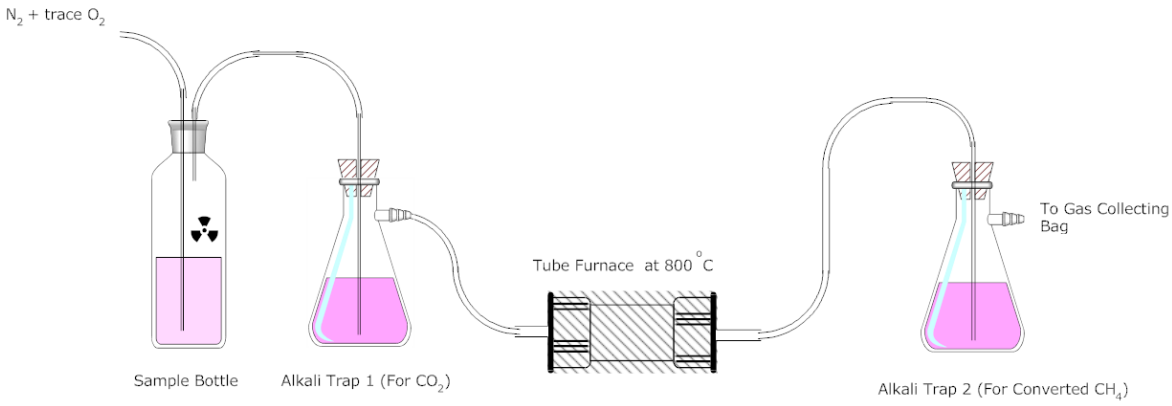


Figure 4.1 Schematic illustration of the radioactive ^{14}C labelling experiment

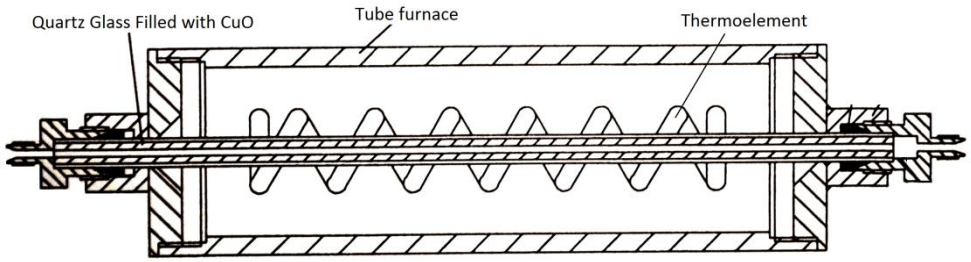


Figure 4.2 Tube furnace gas convertor for the conversion of methane to carbon dioxide

The CuO used was of a granular form with a particle size of ~0.5 mm. This size provided sufficient surface area for the catalytic reaction while not allowing the granule to be so densely packed that it produced a high back pressure. The copper oxide catalyst was dried by heating at 105^{o}C for 1 hour before packing into the quartz tube; quartz glass wool was positioned on both sides to stop the catalyst being blown out of the tube. It should be noted that during the oxidation process, the CuO is reduced to Cu_2O (and therefore is not strictly a catalyst). Once the majority of CuO has been reduced, it has to be regenerated. The regeneration procedure can be carried out either by passing oxygen though the heated furnace for 30 minutes after every sample, or alternatively by maintaining about 10 percent oxygen in the sparge gas flow so that the CuO is continuously regenerated. The latter practice was adopted in this study.

After oxidation of the methane in the furnace the carbon dioxide produced in the sparge gas was passed through another carbon dioxide trap filled with 20 ml of 5 M NaOH. The residual sparge gas was collected in a 3-litre Tedlar bag, and after the sparge process was completed, it was fed through the tube furnace a second time to ensure complete conversion of methane. This was done using a peristaltic pump at the same flow rate as before. During the preliminary method development, results indicated that 99.5% of the methane was converted if the second conversion cycle was applied.

4.1.5 Scintillation Counting

A specified volume (1-2 ml) of sample from the CO_2 and CH_4 targeted alkali traps were mixed with 15 ml Hionic-Fluor™ scintillation cocktail (PerkinElmer Inc., Buckinghamshire, UK). In addition the culture/medium mix in the serum bottle was centrifuged at 12,400 g (Beckman

Microfuge 12, Bechman Coulter, Inc. UK) and 0.5 ml of the supernatant was mixed with 15 ml Hionic-Fluor cocktail. A standard 20 ml scintillation vial made of high density polyethylene and a polypropylene, linerless screw cap was used for all the scintillation samples.

The scintillation cocktail used for this study was specially designed to accommodate high ionic and alkaline samples. This gave it a higher capacity to accept the alkaline trap sample by avoiding '2-phase' (separation of sample and scintillation cocktail) occurring in the sample, and more importantly by minimising chemiluminescence caused by single photons excited due to the exothermic reaction when an alkali mixes with the emulsifier in the scintillation cocktail (Thomson and Burns, 1995). All scintillation counting was carried out on a Beckman Coulter LS6500 scintillation counter (Beckman Coulter, Inc., UK). Each sample was counted for 2 minutes to ensure accuracy. The automatic quench compensation function was applied to every sample counted.

4.1.6 Scintillation Counting on Residual Culture Fluids

The mixture of digestate and culture medium left in the serum bottles was also added to scintillation fluid for determination of the remaining ^{14}C in the form of acetate. During the sample preparation for this stage it was noticed that centrifugation to remove particulate matter was necessary in order to achieve a reliable counting efficiency. Without the centrifugation step, the particles would gradually settle to the bottom in the scintillation fluid during the counting, therefore the counting efficiency varied over time. As shown in Figure 4.3, adding the digestate sample directly into scintillation fluid resulted in a heavily coloured sample. This substantially increased the colour quench of the sample, to the extent that was normally beyond the range of colour quench correction in scintillation counter. After centrifugation, the samples became much clearer as the particles were removed. When mixed with scintillation fluid, the sample emulsified into a homogeneous liquid with improved counting efficiency.

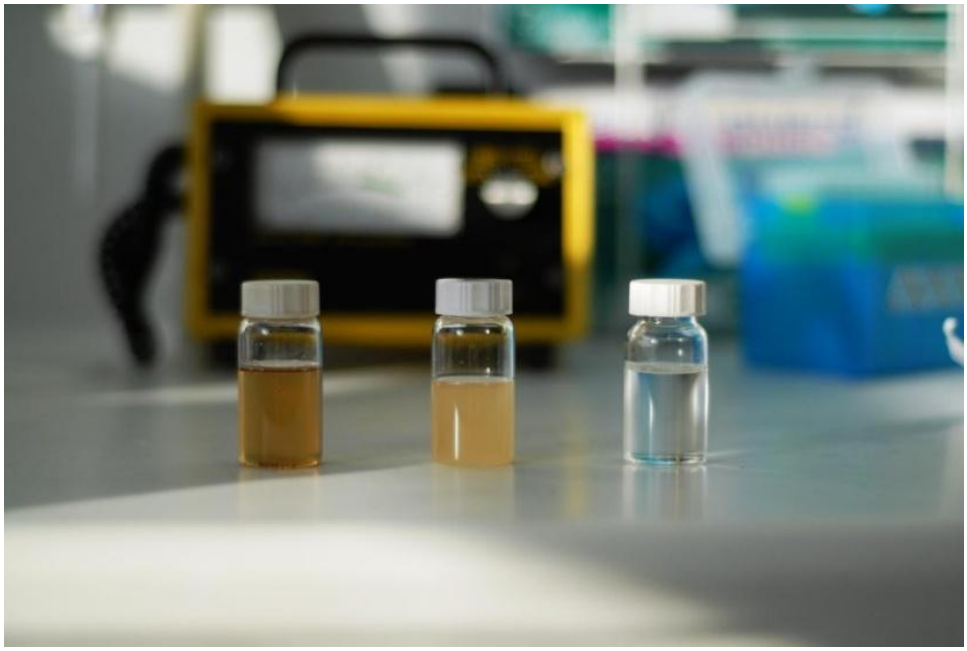


Figure 4.3. Appearance of sample-scintillation cocktail mix for different sample types and under different sample preparation procedure.

Note: From left: 1.) digestate culture/medium sample added into scintillation fluid without centrifugation; 2) digestate culture/medium sample added into scintillation fluid after centrifugation; 3) NaOH solution in CO_2 trap mixed with scintillation fluid.

4.2 Development of a Gas Chromatographic Method for the Determination of Long Chain Fatty Acids (LCFA) in Digestate

4.2.1 Food Waste Digestate

The digestate samples used in the method development were taken from two food waste digesters described in section 3.8. These were a control digester (F1) with no trace element addition operating at an OLR of 1.8 g VS l^{-1} day^{-1} and digester F5 operated at 5.5 g VS l^{-1} day^{-1} and supplemented with Se, Mo, Co. A digestate sample was also taken from another CSTR food waste digester operating in the laboratory which had been inoculated using the same inoculum and fed with the same food waste feedstock as described above at an OLR of 3 g VS l^{-1} day^{-1}).

4.2.2 Standards and Reagents

Analytical grade palmitic (C16:0) and oleic (C18:1) acids were obtained from Fisher Chemical, UK. GC grade Stearic acid (C18:0) of ≥98.5% purity was obtained from Sigma-Aldrich, UK. Hexane (HPLC grade), Methyl tertiary butyl ether (MTBE) (HPLC grade), sodium chloride (analytic grade) and sulphuric acid (analytic grade) were purchased from Fisher Chemical, UK. Each standard was prepared by dissolving the LCFA into a 1/1 hexane-MTBE mixture. These were prepared at 50, 100 and 250 mg l^{-1} and either kept in a sealed gas-tight bottle or prepared freshly before use.

4.2.3 LCFA Extraction from Digestate

The procedure was modified from that of Neves *et al.* (2006) and Lalman and Bagley (2000). A known weight of around 1.5 g of digestate was added to a 50 ml centrifuge tube, followed by 0.05 g NaCl, 0.2 ml of 50% H_2SO_4, and 5 ml of 1/1 Hexane- MTBE mixture. The centrifuge tube was closed and the contents mixed vigorously with a vortex mixer (FB15024, Fisher Scientific). The tube was then placed in an ultrasonic bath (Crest Ultrasonic CP1100, UK) for 20 minutes. The contents of the tube were allowed to separate and 2 ml of the upper layer was carefully transferred into a 2 ml tube and centrifuged for 5 minutes at RCF of 20,800 g (Eppendorf 5417C); the clear organic layer was used in gas chromatographic analysis.

4.2.4 GC Method.

The method was developed on a Shimazdu 2010 gas chromatograph (Shimadzu, UK) fitted with a flame ionisation detector (FID) using a highly polar capillary BP-21 (FFAP) column 0.25 mm × 30 m, 0.25 µm thickness (SGE Forte GC, UK). The optimum instrument parameters were found to be: FID 280°C with H_2 and air flows of 40 and 400 ml min^{-1} respectively; makeup flow: 30 ml min^{-1} (helium); column flow: 2.0 ml min^{-1} (helium); oven temperature: initial 160 °C, ramp rate 10 °C min^{-1}, final 225 °C, final hold 20 minutes; injection volume 1 µl.

4.2.5 Validation Procedure

Precision of the method was evaluated based on reproducibility and repeatability (Miller and Miller, 1993; Caulcutt and Boddy, 1983), indicated by relative standard deviation (RSD, %).

To check reproducibility over time, three mixed standard solutions containing palmitic, oleic, and stearic acids at individual acid concentrations of 50, 100 and 250 mg l^{-1} were injected 6 times over a one-month period.

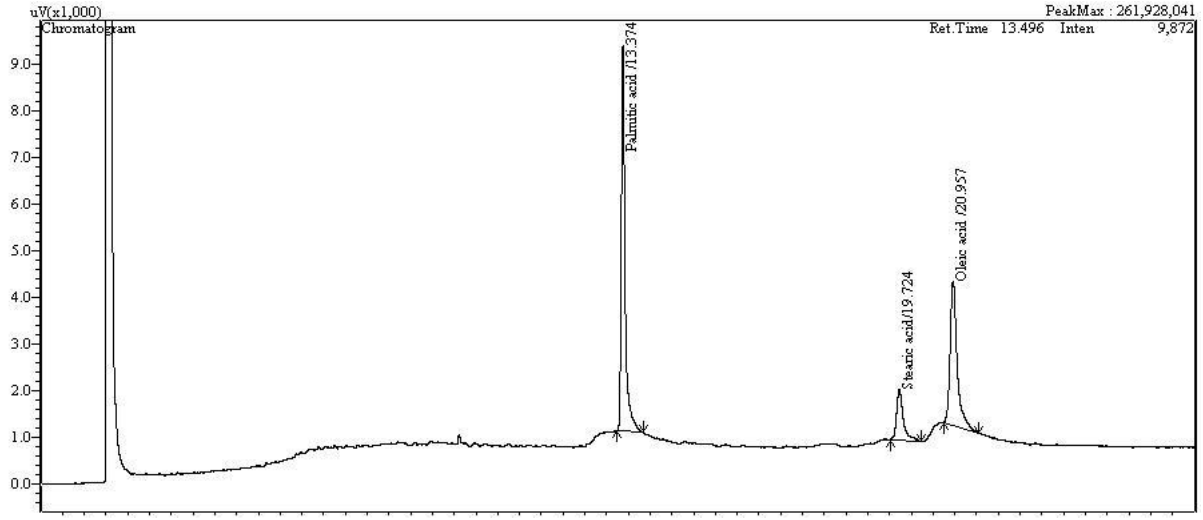
To confirm repeatability single samples were taken from three food waste digesters F1, F5 and R3 operating at different OLRs and different TE supplementation regimes. Each of the 3 samples was then subdivided into 6 sub-samples, each of which was extracted and each extract run in triplicate on the GC.

To validate the extraction efficiency, three digestate samples were prepared and each spiked with 0.1 mg palmitic, stearic and oleic acid; these were recovered and analysed using the above methods with percentage recovery based on the difference between spiked and unspiked samples.

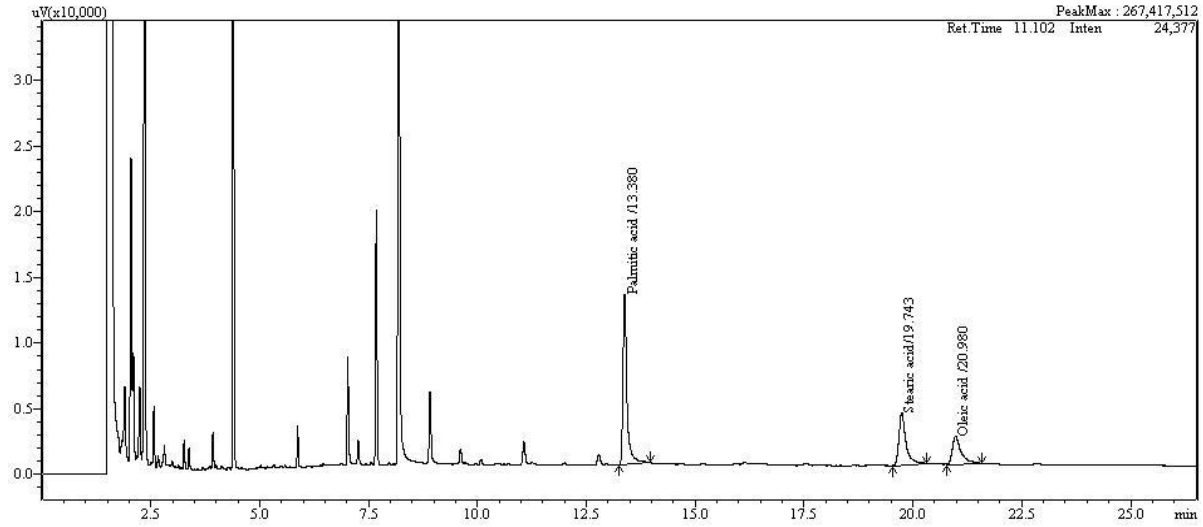
4.3 Results from LCFA Analysis of Digestates and Spiked Samples

4.3.1 GC Calibration and Validation

The GC analysis showed good reproducibility for peak amplitude and retention time for the three fatty acids used as standards. A typical chromatogram is shown in Figure 4.4 and the RSD of peak responses for the six runs conducted over a one-month period are given in Table 4.2. The RSD values obtained were low compared to the 20% which might be considered acceptable (Shah et al., 1992). Under the flow conditions used the variations in retention time windows were ±0.016, 0.017, and 0.018 minutes for palmitic, stearic and oleic acid respectively with mean values of 13.4, 19.7 and 21.0 minutes.



a) Representative chromatogram using a 50 mg l^{-1} standard LCFA mix



b) Chromatogram of identified LCFA from a digestate sample

Figure 4.4. Typical chromatograms for extracted LCFA

The calibration curves plotted for the three standards were linear over the concentration range studied, with correlation coefficients $R^2 \geq 0.99$ for all the analysed LCFA. The slopes of the regression equations obtained are shown in Table 4.2.

Table 4.2. Peak area shift in sequential injections of standards and calibration curve Parameters

	mg l^{-1}	Run1	Run2	Run 3	Run 4	Run 5	Run 6	Average	SD	%RSD
	50	52268	49481	49972	51130	46892	51154	50150	1873	3.74
	100	90426	108081	99801	110980	97601	109874	102794	8173	7.95
Palmitic	250	249226	253405	268318	245168	257543	278050	258618	12403	4.8
	Slope	1002	1008	1099	953	1056	1131	1042	67	6.40
	R^2	0.9965	0.9984	0.9995	0.9960	0.9999	0.9999	1.0000		
	50	13389	11486	15043	10210	15398	12561	13015	2017	15.5
	100	20233	23367	25481	21871	25298	25872	23687	2277	9.61
Stearic	250	58161	53420	59879	57348	53475	56045	56388	2595	4.6
	Slope	231	208	225	236	190	214	217	17	7.85
	R^2	0.9900	0.9987	0.9997	1.0000	0.9999	0.9963	1.0000		
	50	37540	34936	31971	35190	34578	37540	35293	2087	5.91
	100	68213	64066	65469	57543	67564	65423	64713	3828	5.92
Oleic	250	141673	136942	167305	123628	154432	142110	144348	15000	10.39
	Slope	514	504	677	442	595	520	542	82	15.17
	R^2	0.9977	0.9985	1.0000	1.0000	0.9993	0.9997	0.9996		

4.3.2 Extraction Procedure and Repeatability with Single Samples

In the LCFA extraction procedure a 1/1 hexane and MTBE mixture was chosen because this has a lower flash point than hexane and was found to give a better peak response than other potential solvents. Methanol and ethanol were also tested as alternative solvents, but neither gave a satisfactory peak response.

Table 4.3 shows the results for the LCFA subsamples from three digestate samples quantified using triplicate injections. The TE supplemented digester at a moderate OLR (R3, OLR = 3g VS l^{-1} day^{-1}) had lower LCFA concentration than those in samples from the unsupplemented control (F1, OLR = 1.8 g VS l^{-1} day^{-1}) and TE supplemented with high OLR (F5, OLR = 5.5g VS l^{-1} day^{-1}). In general, RSD values were reasonably low; although it was noticed oleic acid had a larger RSD value due to the relatively lower concentration in all samples. Subsample 4 of F1 digestate sample and subsample 2 of the digestate sample R3 were 2 outliers, probably caused by errors during the LCFA extraction stage. When the outliers were excluded from the calculation, the RDS value was reduced significantly. LFCA concentrations in TE supplemented digester F5 were higher than in the unsupplemented control; this mainly reflected the lipid loading rate, which was three times higher for the TE supplemented digester than for the control.

Table 4.3. LCFA concentration (mg l^{-1}) in subsamples from control and TE supplemented food waste digesters (3 injections)

		Unsupplemented control F1 (OLR=1.8 g VS l^{-1} day^{-1})				TE supplemented 1 F5 (OLR=5.5g VS l^{-1} day^{-1})				TE supplemented 2 R3 (OLR=3g VS l^{-1} day^{-1})			
Subsample	LCFA	1#	2#	3#	Ave.	1#	2#	3#	Ave.	1#	2#	3#	Ave.
1	Palmitic	133.3	130.6	129.1	131.0	205.2	201.3	197.5	201.3	49.6	49.0	48.5	49.0
	Stearic	292.6	290.9	289.4	291.0	402.5	394.7	388.3	395.2	114.5	111.6	112.1	112.8
	Oleic	75.5	71.6	69.1	72.1	134.1	129.9	126.6	130.2	11.0	11.0	10.7	10.9
2	Palmitic	110.6	110.4	111.1	110.7	253.7	240.5	257.4	250.5	45.4	49.2	45.5	47.3
	Stearic	271.2	271.2	272.6	271.6	487.9	466.8	501.5	485.4	113.0	115.2	115.0	114.1
	Oleic	50.6	48.4	48.5	49.2	181.2	161.0	179.4	173.9	19.6	20.1	18.5	19.9
3	Palmitic	117.4	116.9	118.7	117.7	346.8	272.6	302.7	307.4	48.8	48.8	49.2	48.8
	Stearic	281.8	281.7	285.3	282.9	667.8	527.8	590.2	595.3	119.5	118.6	118.3	119.0
	Oleic	51.4	54.2	54.4	53.3	248.1	182.7	206.8	212.5	9.6	7.9	7.7	8.7
4	Palmitic	96.0	95.5	95.6	95.7	249.2	241.6	281.7	257.5	46.1	46.7	46.2	46.4
	Stearic	235.5	233.9	232.8	234.1	489.2	476.3	555.6	507.0	116.2	117.6	117.7	116.9
	Oleic	35.9	34.0	34.4	34.8	162.4	157.3	190.2	170.0	7.2	5.9	5.6	6.5
5	Palmitic	138.5	136.8	136.8	137.4	219.1	247.2	243.6	236.6	52.1	53.0	52.2	52.5
	Stearic	323.3	318.9	316.3	319.5	430.0	491.9	484.1	468.6	107.7	109.8	109.1	108.7
	Oleic	55.4	54.5	53.3	54.4	151.9	178.3	174.9	168.4	13.7	13.8	15.0	13.7
6	Palmitic	118.9	107.8	104.4	110.4	335.4	377.4	325.2	346.0	49.6	47.3	56.7	48.4
	Stearic	281.1	255.7	247.3	261.4	772.8	867.8	752.4	797.7	120.2	113.0	112.1	116.6
	Oleic	68.9	61.5	59.8	63.4	281.0	316.5	272.3	289.9	8.2	8.1	7.6	8.2
Average		**average**	**stdev**	**% RSD**	**%RSD without the outlier**	**average**	**stdev**	**% RSD**		**average**	**stdev**	**% RSD**	**%RSD without the outlier**
	Palmitic	**117.1**	15.2	12.9	10.1	**266.6**	51.9	19.5		**48.7**	2.1	4.3	4.5
	Stearic	**276.8**	28.8	10.4	7.8	**541.5**	141.1	26.1		**114.7**	3.7	3.2	3.6
	Oleic	**54.5**	12.7	23.3	15.7	**190.8**	55.1	28.9		**11.3**	4.9	43.0	28.9

4.3.3 Recovery Efficiency

The average recovery from the LCFA spiked into digestate samples was 103.8%, 127.2% and 84.2%, for palmitic, stearic and oleic acid respectively (Table 4.4).

Table 4.4. Extraction recovery of the spiked samples

	Spike recovery		
	Palmitic	Stearic	Oleic
Replicate 1	108.9%	121.8%	64.7%
Replicate 2	110.2%	133.9%	75.8%
Replicate 3	92.4%	125.8%	112.2%
Average	103.83%	127.17%	84.23%

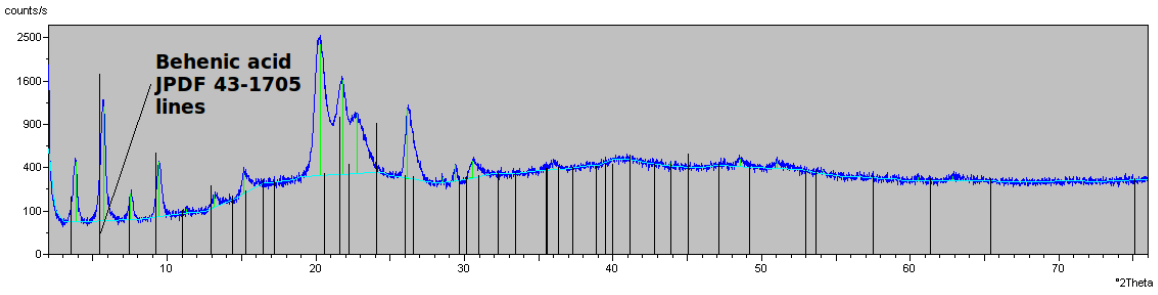
The method showed that a highly polar capillary column used at high temperature can give good peak separation and signal response without the need for methylation of the sample. The sample preparation time was significantly reduced (45 minutes on average), allowing a much higher sample throughput.

In the digesters studied the LCFA concentrations measured may not necessarily reflect the actual accumulation of these compounds in the digestate. LCFA have been observed to accumulate as discrete inclusions forming around inert material such as fruit pips. These inclusions appear to grow, as the core material is gradually coated in layers of material. An example from a 75-litre digester fed on food waste (Zhang et al., 2012) is shown in Figure 4.5. Large amounts of this material were also observed in samples taken from a full-scale commercial food waste digester with accumulated VFA (data not shown). The material itself was light and did not affect the buoyancy of the core and it therefore floated on the surface of digestate. The total solids content of this material was 75% of which 82% was volatile and the TKN was low at 2.2 g kg^{-1} TS.

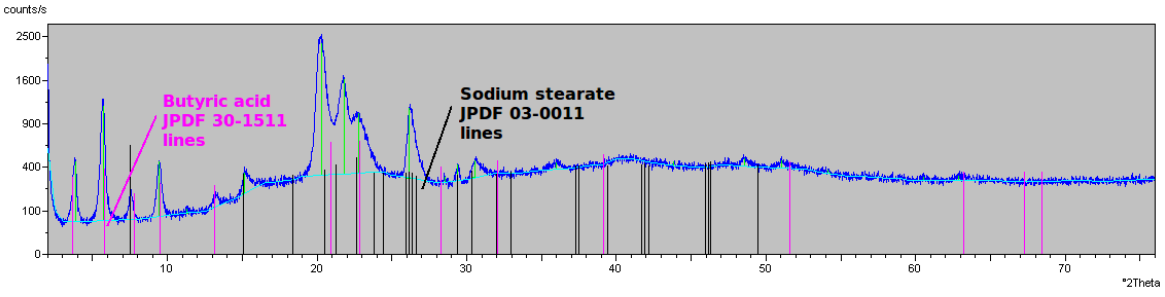
A preliminary identification was carried out by others, using X-ray diffraction (XRD) analysis on the whole granular inclusion and also on material from the shell of the granule. Both samples were manually ground using an Agate pestle and mortar although this was difficult as the material was 'soapy' and somewhat difficult to grind up to a fine powder. All X-ray diffraction patterns were recorded on a Philips X'Pert Pro diffractometer using *CuKα* radiation (35kV, 40mA) at angles from 2 to 76° with a step size of 0.02° and dwell time 1.2 s per step. Phases were qualitatively identified with the Philips XPert software, which is based on the pattern database from the JCPDS (Joint Committee on Powder

Diffraction Standards). No quantitative analysis was carried out. The X-ray diffraction traces of both samples were nearly identical (Figure 4.6). No database X-ray diffraction pattern fully matched the sample and it was thought to be made up of a mixture of compounds even though it was clearly crystalline. On matching fragments of the diffraction trace to known compounds it was deduced that the material consisted mainly of salt derivatives of fatty acids/carboxylic acids.

Figure 4.5. Samples of inclusion granules taken from a 75-litre digester operated on food waste (Zhang et al., 2012). (Image courtesy of Dr Yue Zhang).



a) XRD trace of powdered inclusion sample. The lines represent 'behenic acid' in the powder diffraction file database.



b) XRD diffraction trace of the powdered inclusion sample. Database butyric acid lines are overlaid on the sample trace.

Figure 4.6. x-ray diffraction analysis of inclusions discovered in food waste digesters (Figures courtsey of Dr Ludwig Gredmaier)

The measured LFCA values therefore reflect the proportion miscible in the digestate which had not been hydrolysed in the degradation process. Considering, however, that at the time of sampling the digesters had been receiving food waste for a period of almost 2 years at a lipid concentration of around 150 g kg^{-1} VS, it seems probable that the degree of degradation is quite high. This view is supported by the studies of Angelidaki and Ahring (1992) and Masse et al. (2002) who suggested that in an anaerobic environment the lipid load to the digester is readily hydrolysed to free LCFA and glycerol. Subsequently the free LCFA are oxidised by acidogenic bacteria through ß-oxidation (Masse et al., 2002) which leads to the final formation of simple VFAs and hydrogen. β-oxidation is thermodynamically unfavourable under standard conditions, however, due to its positive Gibbs free energy (equation 4.1), therefore requiring constant removal of the reaction products (Fox and Pohland, 1994).

n-carboxylic acid $\longrightarrow$ (n-2) carboxylic acid + CH_3COOH + 2 H_2 ΔG_0= + 48 kJ mol^{-1} [4.1]

Methanogenesis provides the syntrophic complement to the process by using acetate, formate and hydrogen. The concentration of LCFA found in the TE supplemented digester F5 was higher than that in the non-supplemented control which may reflect the difference in lipid loading between the two digesters. Proportionally to the load, however, the concentrations of palmitic and stearic acids in the non-supplemented digesters were higher as was the total VFA concentration, giving some further support to the view that TE supplementation was required to prevent an accumulation of intermediate products (Ferry, 1999; Ragsdale and Pierce, 2008). The concentrations of palmitic, stearic and oleic acid found in the TE supplemented digestate are below the values suggested as inhibitory in other studies (Koster and Cramer, 1987; Angelidaki and Ahring, 1992; Lalman and Bagley, 2002).

CHAPTER 5

Vegetable Waste Digestion

5.1 Biochemical Methane Potential and Semi-Continuous Digestion Trials on Vegetable Waste

5.1.1. Chapter Summary

The physical and chemical characteristics of a mixed vegetable waste were determined and the theoretical energy yield predicted. This was compared to experimentally-determined calorific values and to energy value of the methane generated from a batch biochemical methane potential test. A semi-continuous digestion trial was carried out at different OLR and performance of this was enhanced by trace element supplementation, chemical supplementation to maintain alkalinity and through co-digestion to recovery failing digesters and to achieve digestion stability and efficiency.

5.1.2 Feedstock Characteristics

TS and VS content of VW material were 77.4 and 72.4 g kg^{-1} WW, respectively. TKN was 3.46 g N kg^{-1} WW, phosphorus 0.86 g P kg^{-1} WW and potassium 2.44 g K kg^{-1} WW. Elemental composition was C 51.11%, N 4.63%, H 6.76% and O 37% on a VS basis, with a C/N ratio of 11:1. Fibre content was 13.16% cellulose, 2.56% hemicellulose and 8.16% lignin on a VS basis. The pH was 5.77, and the calorific value was 20.58 MJ kg^{-1} VS. The calorific value calculated from the elemental composition of the VW using equation [3.9] was 20.99 MJ kg^{-1}VS which is in good agreement with the value measured by bomb calorimetry. The result therefore supports the accuracy of the elemental composition analysis. TS and VS contents of the supplements and co-substrates were 883.0 and 667.0, 89.0 and 66.9, and 202.0 and 165.5 g kg^{-1} WW, for yeast extract, cattle slurry and card packaging respectively.

5.1.3 Biological Methane Potential Test on Vegetable Waste and Yeast Extract

Table 5.1 and Figure 5.1 show the cumulative net specific methane production over a 28-day test period for the VW feedstock and YE used in the first semi-continuous trial in 5-

litre digesters (section 3.6). The BMP results showed good agreement between duplicate samples, with a specific methane production of 0.352 l g^{-1} VS added. This is equivalent to 25.5 m^3 CH_4 per wet tonne, which reflects the high water content of the material and is fairly typical of literature values (Knol et al. 1978, Lane 1984, Viturita et al. 1989).

Table 5.1. Specific biogas and methane yields (BMP test value)

Samples	**Biogas** (m^3 kg^{-1} VS added)	**Methane** (m^3 kg^{-1} VS added)
VW 1	0.630	0.351
VW 2	0.622	0.353
VW average	**0.626**	**0.352**
YE 1	0.589	0.398
YE 2	0.597	0.405
YE average	**0.593**	**0.402**
Cellulose 1	0.695	0.430
Cellulose 2	0.692	0.433
Cellulose average	**0.694**	**0.432**

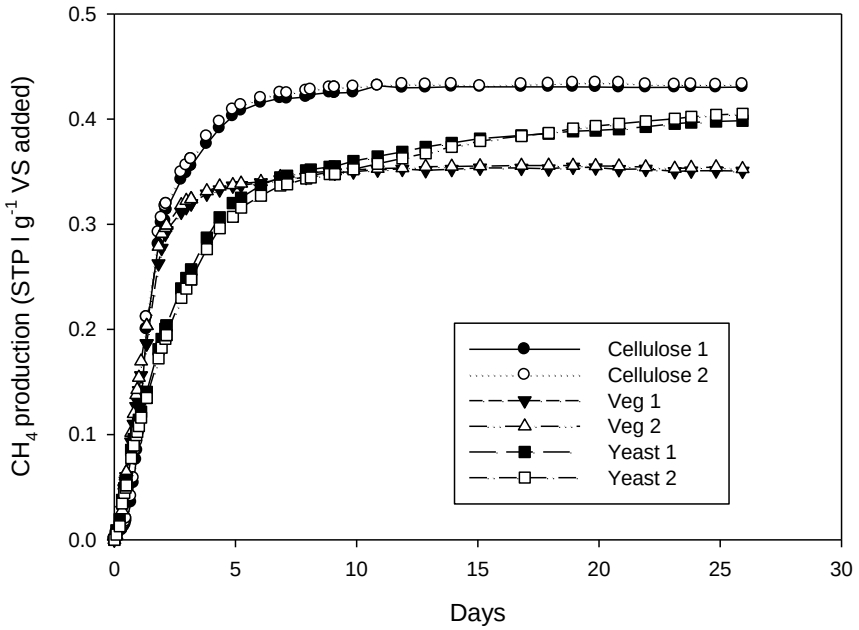


Figure 5.1. Cumulative net specific methane production of vegetable waste, yeast extract and the cellulose positive control.

5.1.4 BMP Kinetic Model

An attempt was made to relate the kinetics of the BMP test to the degradability and gas production of the material as seen in a continuous or semi-continuous anaerobic digestion system. This can only be done on a fairly qualitative basis, however, as the rate of reaction

in a BMP test is very dependent on the characteristics of the inoculum, including its degree of acclimatisation, the ratio of inoculum to substrate at the start of the test, and the methods used for preparation and homogenisation of the substrate. Consideration of the kinetics can, however, provide some insight into the potential behaviour of the material in a full-scale system.

To determine the kinetic constants, the specific methane production was modelled using two models: a simple first-order degradation (Model 1), and a pseudo-parallel first-order model (Model 2). For model 1 the methane production is given by

$$Y = Y_m (1 - e^{-kt}) \qquad [5\text{-}1]$$

where:

Y is the cumulative methane yield at time t

Y_m is the ultimate methane yield

k is the first order rate constant

Previous work at Southampton and by others (Rao et al., 2000) suggests that when using organic solid waste materials as the substrate, it may be better to consider that the gas production curve corresponds to the rapid breakdown of readily degradable components followed by a much slower degradation of the fibrous proportion of the material. The methane production is therefore governed by two rate constants k_1 and k_2 rather than a single constant:

$$Y = Y_m (1 - Pe^{-k_1 t} - (1-P) e^{-k_2 t}) \qquad [5\text{-}2]$$

where: Y is the cumulative methane yield at time t

Y_m is the ultimate methane yield

k_1 is the first order rate constant for the proportion of readily degradable material

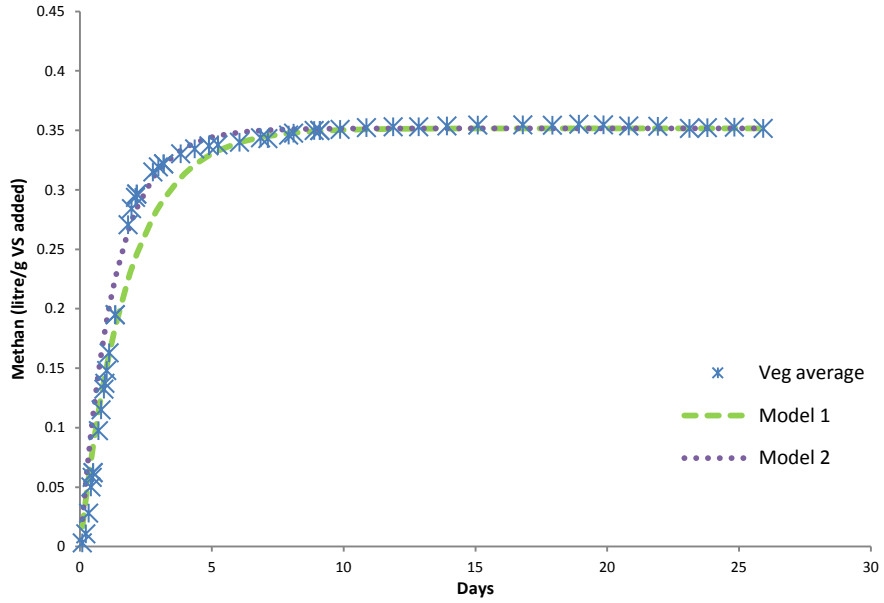
k_2 is the first order rate constant for the proportion of less readily degradable material

P is the proportion of readily degradable material

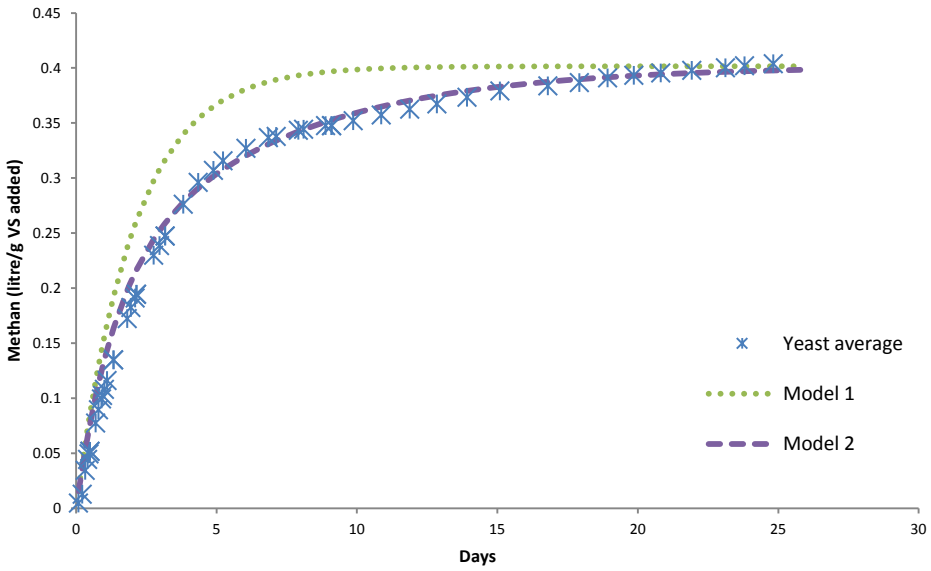
The results of these two modelling approaches are shown in Figure 5.2 and the kinetic constants obtained are given in Table 5.2.

Table 5.2. Kinetic constants from modelling

	Vegetable waste *model 1*	**Vegetable waste** *model 2*	**Yeast extract** *model 1*	**Yeast extract** *model 2*
$\mathbf{Y_m}$	0.352	0.352	0.402	0.402
P	1	0.33	1	0.52
$\mathbf{k_1}$	0.56	0.72	0.49	0.16
$\mathbf{k_2}$		0.80		0.77
$\mathbf{R^2}$	0.9867	0.9955	0.9889	0.9983



a) Vegetable waste



b) Yeast extract

Figure 5.2. Kinetic models showing methane production from vegetable waste and yeast extract

From Figure 5.2 and its associated R^2 values (Table 5.2), it can be seen that the simple first-order model (Model 1) gives only a moderately good fit to the experimental data (R^2 = 0.9867 and 0.9889 for vegetable waste and yeast extract respectively). A better fit is obtained by model 2 (R^2 = 0.9955 and 0.9983).

Around 90% of the methane is released after 9 days, suggesting that a continuous or semi-continuous digester operating with a short retention time of about 15 days might therefore be expected to yield a high proportion of the methane potential. These values are only indicative, however, as the real gas yield from a continuous system will depend on the digester type and operating parameters, including the loading rate. Reliable design values can only be obtained by carrying out a continuous or semi-continuous fed trial over a period of several retention times.

5.2 Semi-Continuous Digestion Trials (Part 1)

5.2.1 Summary Method

Eight 5-litre digesters (D1-D8) were used which were fed initially at an OLR of 0.7 g VS l^{-1} day^{-1} for 14 days. The initial experimental plan was to raise the loadings incrementally to give OLRs of 2, 3, 4 and 5 g VS l^{-1} day^{-1} in digester pairs D1-2, D3-4, D5-6 and D7-8, equivalent to HRTs of around 40, 26, 19 and 15 days respectively.

This plan was changed during the experiment when the digester performance showed signs of stress.

Table 5.3 summarises the major operational parameter changes during the course of the experiment. Gonzalez-Gil trace element solution was added at the start of the experiment to each digester in the proportion of 1 ml l^{-1} digestate, and then weekly in the same proportion based on the volume of feedstock added.

Table 5.3. Additions and loadings applied to the VW digester D1-D8

Digester ID	Supplements added	OLR and comments
D1	Gonzalez-Gil TE solution. Additional W was added on day 137	2 g VS l^{-1} d^{-1}
D2	Gonzalez-Gil TE solution. Additional W and yeast extract was added on day 137	2 g VS l^{-1} d^{-1}
D3	Gonzalez-Gil TE solution	3 g VS l^{-1} d^{-1}, operation terminated on day 133
D4	Gonzalez-Gil TE solution. NH_4HCO_3 was added on day 91	3 g VS l^{-1} d^{-1} , operation terminated on day 133
D5	Gonzalez-Gil TE solution. NH_4HCO_3 and $NaHCO_3$was added on day 94	Increased to 4 g VS l^{-1} d^{-1} on day 52, then showed digester stress. Feeding ceased on day 69. Reseeded with fresh inoculum on day 70 and started feeding at OLR=3 g VS l^{-1} d^{-1}. An attempt was made to increase loading to 3.4 g VS l^{-1} d^{-1} on day 92, but followed by digester stress. Reduced loading to 2.5 g VS l^{-1} d^{-1} on day 94.
D6	Gonzalez-Gil TE solution. $NaHCO_3$was added on day 94	
D7	Gonzalez-Gil TE solution. Co-digest with cattle slurry (CS) started on day 110.	Same loading history as D5 and D6 until day 92. Then only fed intermittently. Co-digestion started on day 110 (VW=2.3 g VS l^{-1} d^{-1}, CS=1.1 g VS l^{-1} d^{-1}).
D8	Gonzalez-Gil TE solution. Co-digest with card packaging (CP) started on day 110.	Same loading history as D5 and D6 until day 92. Then only fed intermittently. Co-digestion started on day 110 (VW=1.67 g VS l^{-1} d^{-1}, CP=0.83 g VS l^{-1} d^{-1}).

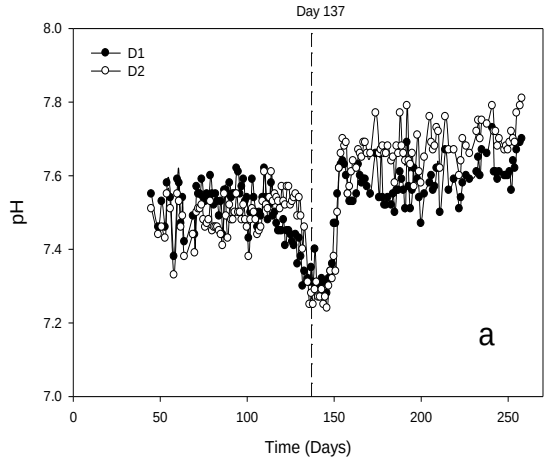
5.2.2 Experimental Results

The pair of digesters at OLR 2 g VS l^{-1} day^{-1} (D1 and D2) operated for 257 days. During this time they remained stable for 120 days and then showed a decrease in pH from 7.5 to 7.2, a reduction in gas production and an increase in VFA concentrations (Figure 5.3a, b and c). On day 137 additional supplementation of the trace element tungsten (not present in the Gonzalez-Gil formula) was added to D1 and D2 to give a concentration of 0.1 mg W l^{-1} above the existing concentration in the digester. This was achieved by initially adding 1 ml of a 0.18 g l^{-1} $Na_2WO_4 \cdot 2H_2O$ solution to each digester, followed by supplementation with 0.04 ml of this solution every 7 days to compensate for washout. Digester D2 also received a daily supplement of 4 ml of a 200 g TS l^{-1} yeast extract (YE) solution. In both cases gas production recovered and pH increased to ~7.6-7.7. The slightly higher biogas yield in D2 can be attributed to the yeast extract addition, as shown in the BMP test. Average parameter values for the stable operating period of 112 days at the end of the run were:

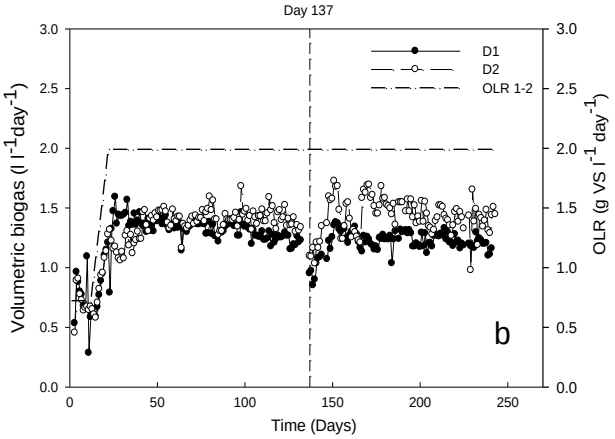
methane yield 0.345 l CH_4 g^{-1} VS_{added}; digestate VS 15.5 g kg^{-1}; VS destruction 81.3%; total ammonia nitrogen 1.73 and 2.29 g kg^{-1} in D1 and D2 respectively; biogas methane content 53.4% and Ripley ratio ~0.3. Based on a lower heat calorific value (LHV) of 35.82 MJ m^{-3} CH_4, the energy recovered in the form of methane represents 68.6% of the measured calorific value of the vegetable waste.

In the second pair of digesters (D3 and D4) the OLR was increased to 3 g VS l^{-1} day^{-1} after 30 days and remained stable with an average specific methane yield of 0.31 l CH_4 g^{-1} VS $_{added}$ until around day 80 at which point there was a drop in gas production, a rapid fall in pH and accumulation of VFA (Figure 5.3 d, e and f). Various attempts were made to recover this pair of digesters by stopping feeding and then resuming at a reduced loading; all these attempts failed. Digester D4 received additional buffering on day 91 by adding 3 g of ammonium bicarbonate. To maintain pH, however, a cycle of intermittent feeding and alkali additions was necessary which resulted in a series of pH oscillations and a generally decreasing performance. This run was terminated on day 133.

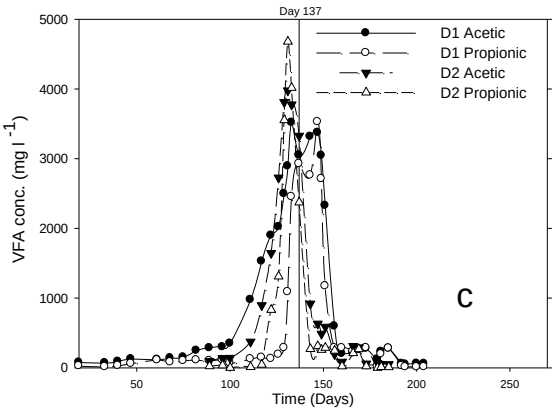
The OLR in D5-8 was increased from 0.7 to 4 g VS l^{-1} day^{-1} over a period of 52 days, after which time all four digesters were suffering from low pH values and poor gas production and were stopped on day 69. The four digesters D5-8 were emptied and re-seeded with fresh inoculum from the Millbrook digester (section 3.2.2) and feeding started at an OLR of 3 g VS l^{-1} day^{-1}. Between days 85-92 the OLR was gradually increased to 3.4 g VS l^{-1} day^{-1} but this resulted in a decline in gas production and pH decreased to below 7. From this and the previous results it appeared that operation at or above an OLR of 3 g VS l^{-1} day^{-1} would probably not be achievable without supplementation or the use of co-substrates. Digesters D5 and D6 were therefore given chemical supplements aimed at enhancing digester alkalinity, while in digesters D7 and D8 card packaging and cattle slurry were added as co-substrates to improve the C/N ratio and possibly provide additional trace elements.



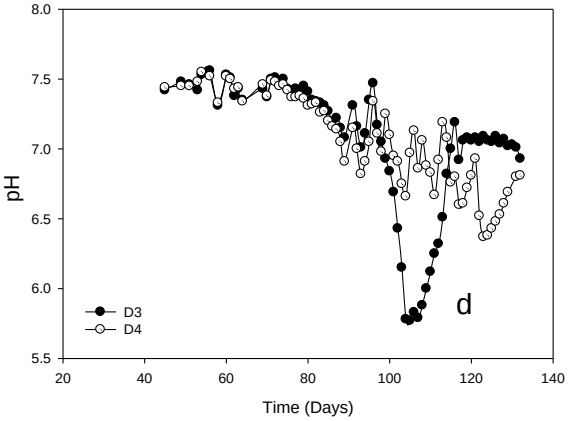
a) pH in D1 and D2



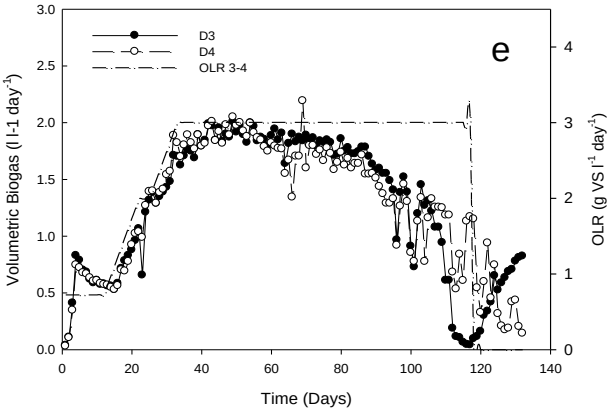
b) Daily volumetric biogas production in D1 and D2



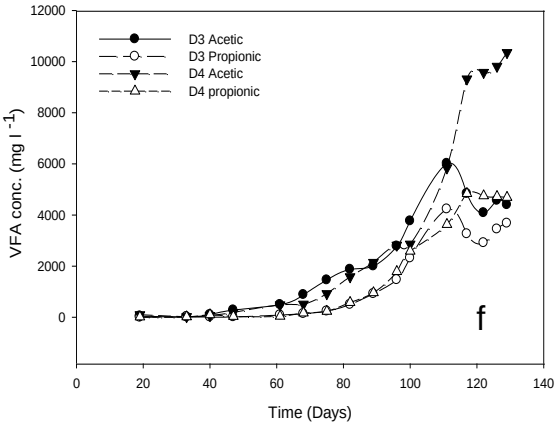
c) VFA profile in D1 and D2



d) pH in D3 and D4



e) Daily volumetric biogas production in D3 and D4



f) VFA profile in D3 and D4

Figure 5.3. Results from trace elements addition experiment

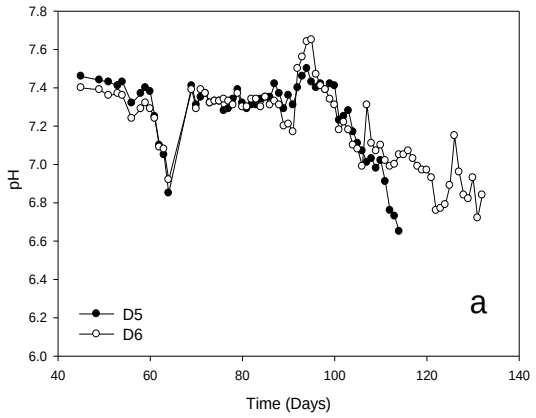
Digester D5 was given ammonium bicarbonate and sodium bicarbonate daily (2.5 g NH_4HCO_3 and 2.5 g $NaHCO_3$). Digester D6 received an additional daily 5 g of $NaHCO_3$. Digester D7 was fed on 127 g VW (OLR = 2.3 g VS l^{-1} day^{-1}) and 63 g cattle slurry (OLR

= 1.1 g VS l^{-1} day^{-1}) to give a combined OLR of 3.4 g VS l^{-1} day^{-1}. Digester D8 was fed on 92 g VW feedstock (1.67 g VS l^{-1} day^{-1}) to 20 g card packaging (0.83 g VS l^{-1} day^{-1}), giving a combined OLR of 2.5 g VS l^{-1} day^{-1}. Chemical supplementation by adding both NH_4HCO_3 and $NaHCO_3$ or $NaHCO_3$ alone to maintain alkalinity showed no sustainable beneficial effect, with pH and gas production continuing to decline rapidly as shown in Figure 5.4a and c, and VFA concentrations reaching 15-20 g l^{-1}. The chemical supplementation trial was terminated on day 133.

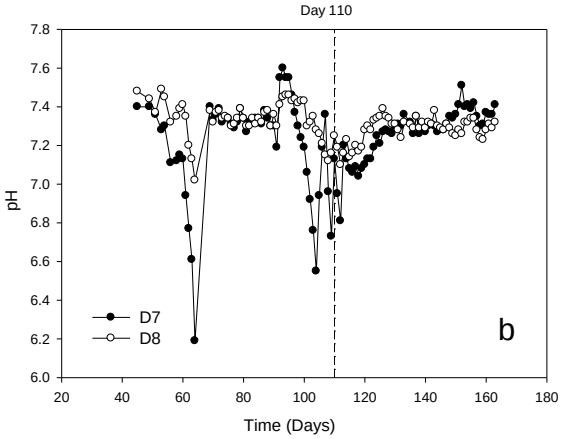
When D7 and D8 were fed on cattle slurry and card packaging as co-substrates the downward trends in performance were reversed, as shown in Figure 5.4b, d and f. The pH stabilised at around 7.4, accumulated VFA were consumed resulting in concentrations of <1000 mg l^{-1} by the end of the trial, and specific methane production stabilised at 0.258 and 0.277 l CH_4 g^{-1} VS added for the co-substrate mixes containing card packaging and cattle slurry respectively.

Results from trace element analysis on digestate from selected digesters on day 164 (approximately 3 HRT after re-seeding) are shown in Table 5.4. The data showed that in digesters D3 and D6, tungsten concentrations were below the detection limit of 0.01 mg l^{-1}. In D1 and D2 the measured tungsten concentrations of 0.10 and 0.14 mg l^{-1} were in good agreement with the intended supplementation value, indicating good analytical accuracy. It is also noteworthy that in D7 and D8, the two digesters with co-substrates, the tungsten concentrations were relatively high. Since no tungsten was supplemented, the extra tungsten can only have come from the co-substrates.

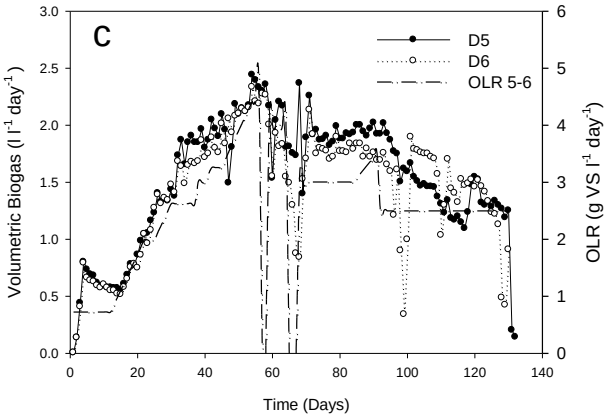
The above findings support the importance of tungsten in maintaining the anaerobic process. The requirement of methanogens growing on H_2 and CO_2 for tungsten has long been known (Zellner and Winter 1987), and this element has also been reported to play a significant role in propionate degradation (Plugge et al., 2009; Reda et al., 2008). Despite this, however, tungsten is not included in most of the commonly-used trace element recipes found in the literature. In digester D1 which received YE addition cobalt and selenium concentrations were found to be higher than in the other digesters, suggesting that YE was an additional source for these two trace elements.



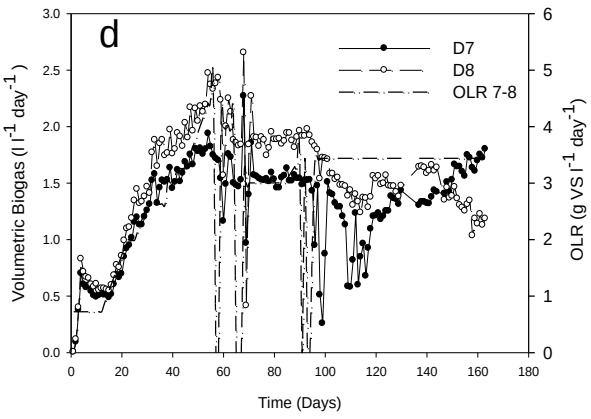
a) pH in D5 and D6 before and after chemical supplements were added from day 94



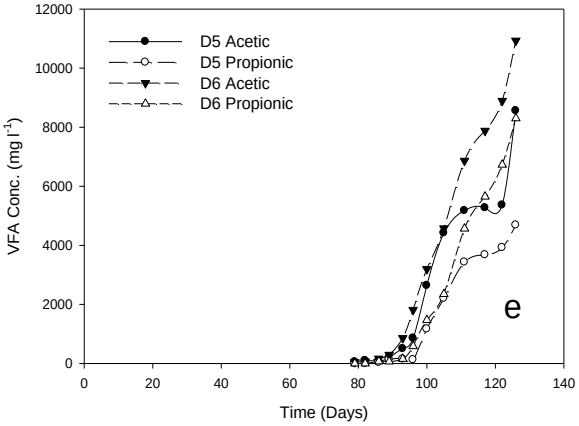
b) pH in D7 and D8 before and after the change to card packaging and cattle slurry on day 110



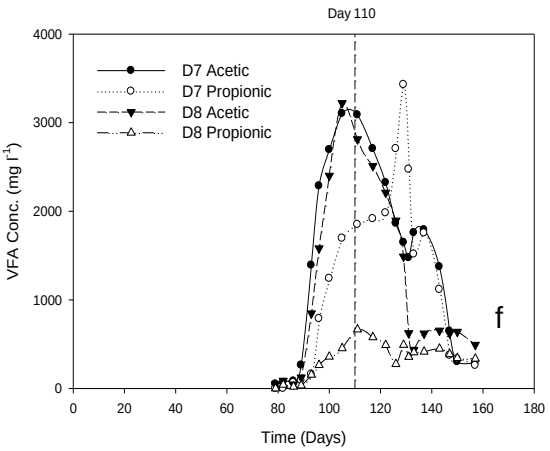
c) Daily volumetric biogas production in D5 and D6 before and after chemical supplements added from day 94



d) Daily volumetric biogas production in D7 and D8 before and after the change to co-substrates



e) VFA profiles in D5 and D6



f) VFA profiles in D7 and D8 before and after the change to co-substrates

Figure 5.4. Results from chemical supplementation and co-digestion experiments

Table 5.4. Trace elements concentration in representative digesters at the end of the trial

Element (mg l^{-1})	D1	D2	D3	D6	D7	D8
Al	3.58	5.50	15.61	15.14	NM	NM
B	1.94	2.35	1.53	1.68	NM	NM
Co	0.52	**1.13**	0.25	0.31	0.31	0.34
Cu	0.79	0.71	0.99	1.00	1.61	2.14
Fe	18.56	18.95	72.01	41.41	40.36	47.75
Mn	2.45	2.20	1.86	2.14	9.20	3.41
Mo	0.44	0.41	0.22	0.24	0.27	0.29
Ni	1.23	1.18	0.24	0.19	1.11	1.07
Se	0.08	**0.28**	0.05	0.05	0.04	0.04
Zn	8.23	7.50	2.31	2.50	5.36	5.26
W	**0.14**	**0.10**	NM	NM	**0.68**	**2.09**

Note: highlighted numbers are high values resulting from TE supplementation or co-substrates. NM=not measured.

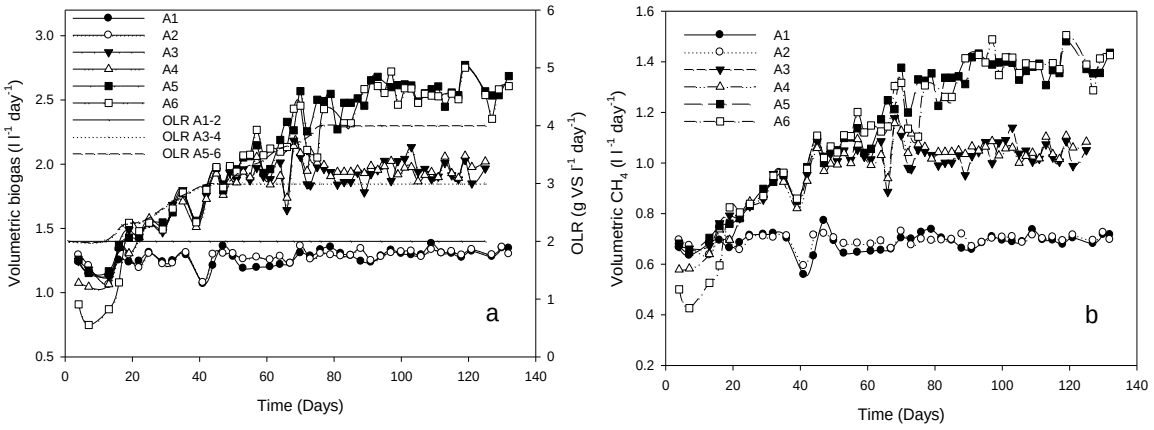
5.3 Semi-continuous Digestion Trial (Part 2)

5.3.1 Summary Method

Digestate taken from digesters D1 and D2 (section 4.1.2) was used as an inoculum for six digesters (A1-6). These were supplemented as previously but using the modified TE supplement (section 3.4.2). The digesters were fed initially at an OLR of 2 g VS l^{-1} day^{-1} for 10 days. The OLR on pairs of digesters A1-2 remained at 2 g VS l^{-1} day^{-1}, while the OLR on A3-4 was incrementally raised to 3 g VS l^{-1} day^{-1}, and on A5-6 to 4 g VS l^{-1} day^{-1}, equivalent to HRTs of around 40, 26 and 19 days respectively.

5.3.2 Experimental Results

All six digesters achieved their target OLRs, with digestion continuing stably for at least 3 HRT. Specific methane production was between 0.348 - 0.353 l g^{-1} VS, and volumetric biogas and methane productions are shown in Figure 5.5. The pH in all digesters remained at ~7.5 throughout the experimental period. Alkalinity in all digesters was sufficiently high with a Ripley ratio constantly below 0.4 indicating stable operation. There was no build-up of longer chain VFA such as iso-butyric, n-butyric, iso-valeric, valeric, hexanoic, and heptanoic acid but there was some accumulation in acetic acid at the start of the experimental period (Figure 5.6). The concentration fell as the experiment continued, however, and stabilised at <50 mg l^{-1} irrespective of the OLR. This initial elevation in acetic acid happened in all 6 digesters and was therefore possibly as a result of an initial adaption. Average values for operational parameters after each pair of reactors acheieved the targeted OLR are shown in Table 5.5.



a) Daily volumetric biogas production in A1-6 b) Daily CH_4 production in A1-6

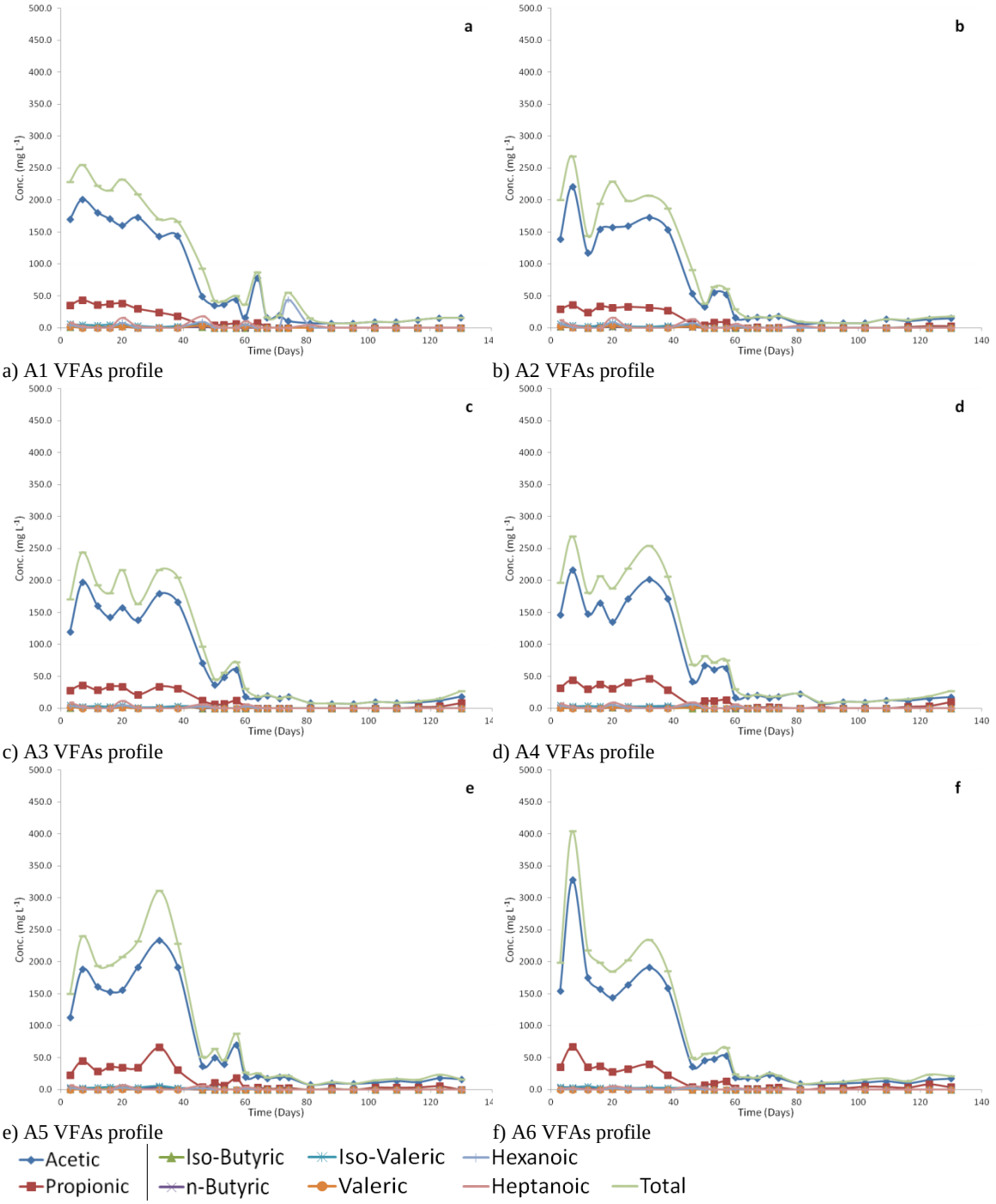
Figure 5.5 Results from 1.5-litre digester optimised TE supplementation experiments

Table 5.5 Average operational parameters in A1- A6

	OLR g VS l^{-1} day^{-1}	Ammonia N (g kg^{-1}) ±s.d.	Digestate VS (g VS kg^{-1}) ±s.d.	VS Destruction %	Volumetric biogas (l l^{-1} day^{-1}) ±s.d.	CH_4 %	TA* (mg l^{-1})	PA* (mg l^{-1})	IA* (mg l^{-1})	Specific CH_4 (l g^{-1} VS)
A1	2	2.39±0.21	12.9±0.48	85.3	1.27±0.06	54.9	12127	9122	2875	0.348±0.02
A2	2	2.31±0.13	13.1±0.30	85.0	1.28±0.06	54.9	12058	9040	2895	0.352±0.01
A3	3	2.26±0.09	14.6±0.40	83.6	1.93±0.09	54.7	11803	8830	2816	0.352±0.02
A4	3	2.22±0.21	14.3±0.43	83.9	1.94±0.9	54.6	11988	9149	2691	0.353±0.02
A5	4	2.22±0.06	15.0±0.70	83.5	2.57±0.12	54.7	11715	8966	2629	0.351±0.03
A6	4	2.21±0.07	14.7±0.78	83.7	2.55±0.13	55.0	11612	8830	2673	0.350±0.03

TA: Total Alkalinity, PA: Partial Alkalinity, IA: Intermediate Alkalinity

The use of the modified trace element recipe with the inclusion of tungsten and higher concentrations of other known essential elements allowed stable operation at OLR higher than those achieved in previous studies using similar FVW feedstocks (Knol et al., 1978, Viturtia et al., 1989, Yang et al., 1984, Stewart et al., 1984).



a) A1 VFAs profile

b) A2 VFAs profile

c) A3 VFAs profile

d) A4 VFAs profile

e) A5 VFAs profile

f) A6 VFAs profile

Figure 5.6. VFAs results from 1.5-litre digester experiment

5.4 Discussion of BMP and Semi-continuous Trials

5.4.1 Digester Stability during Vegetable Waste Semi-Continuous Trials

It has been suggested that failures in anaerobic digestion of vegetable wastes are due to the rapid hydrolysis and subsequent build-up of VFA (Bouallagui *et al.*, 2005; Misi and Forster, 2001). If the failures were simply due to rapid acidification as suggested, however, the effects should be apparent very rapidly; yet the digesters in the 5-litre trial only started to show stress after running stably for a relatively long period (approx 3 HRTs). It is possible that failure at a high loading rate could be a result of the washout of either the microbial population or of essential trace elements. The experiments carried out in 2.0-litre digesters achieved good stability and performance even at very high OLR, indicating that rather than washout of the microbial population, it is likely that a deficiency of trace elements resulted in interruption of the syntrophic metabolic pathways leading to methane production. This view is also supported by the behaviour of D1 and D2, where the addition of trace element supplement at the point of failure led to stabilisation. Similar findings have been reported in a number of studies as reviewed previously (Kurmar et al., 2006, Climenhaga & Banks, 2008, Jarvis *et al.*, 1997, Pobeheim *et al.*, 2010**).**

In the 5-litre digester study the trace element solution of Gonzalez-Gil *et al.* (2001) was used, so at least some trace elements were present. It is possible, however, that the concentration was not high enough to satisfy the enzyme demands for the increased metabolic activity needed to process the higher loadings applied. The vegetable waste itself has only a very low concentration of cobalt (0.04 mg kg^{-1}), and this could be one of the major limitations. Another possible explanation for the apparent trace elements deficiency is their presence in forms that are not readily available to the microorganisms. It should be noted that EDTA which is added to trace element solutions to facilitate dissolution has been demonstrated to be such a strong chelator that it can preclude bioavailability; because EDTA is not biodegradable aerobically or anaerobically, its adverse effect on metal bioavailability will be persistent (Speece, 1996). Also certain important elements were not present in the original trace element recipe, as demonstrated in this study by the quick recovery of the digesters D1 and D2 after supplementation with tungsten. The modified TE recipe was devised to address these problems by using higher concentrations, eliminating the use of EDTA and including the missing element W. The results in the 1.5-litre experiment have proved the success of these modifications.

5.4.2 Comparison of Theoretical Methane and Energy Yield with Experimental Results

Values for the elemental composition of the vegetable waste were used to calculate theoretical gas composition and theoretical calorific value using equation [3.8] and [3.9]. The calorific value (CV) of the dried material was also measured. The combined results were then compared using a mass balance approach (Table 5.6).

Table 5.6 Energy value of vegetable waste

Parameter	**Value**
Actual CH_4 yield, m^3 kg^{-1} VS	
- BMP	0.352
-Semi-continuous trial	0.345
Empirical formula	$C_{4.26}H_{6.76}O_{2.31}N_{0.33}$
Theoretical methane content of biogas (Buswell)	53.3%
Calculated CV MJ kg^{-1} VS	20.99
Measured CV MJ kg^{-1} VS	20.58
CV Recovery as methane in MJ kg^{-1} VS	12.57
% Recovery of measured CV	58.4%
Lower heat value MJ kg^{-1} VS	-0.74

The calorific value calculated from the elemental composition of the vegetable waste using equation [3.9] was in good agreement with the value measured by bomb calorimetry. The result therefore supports the accuracy of the elemental composition analysis. The recovery of energy in the form of methane during the semi-continuous trials represents 60% of the total energy potential of the material based on its higher heat value, and confirms the suitability of anaerobic digestion as an energy recovery technology. In contrast the lower heat value of the material is -0.74 MJ kg^{-1} wet weight, reflecting its high moisture content of around 92% and therefore suggesting anaerobic digestion is the only available technique to gain positive energy for the treatment of such material.

The semi-continuous trials on VW material achieved ~99% of the BMP value despite the relatively short HRT, confirming the rapid biodegradability of the substrate and the BMP results, which showed most of the gas production occurring in the first 10 days (Figure 5.1).

The relatively high BMP of yeast extract (0.402 m^3 kg^{-1} VS) indicated that the increased biogas production is contributed by the organic matter from yeast extract in the semi-continuous trial 1. Although Speece (1983) demonstrated the stimulatory effects of yeast

extract on methanogens he concluded that the trace metal constituents in yeast extract, especially significant amounts of nickel, are the cause of the stimulation. In the trace element analysis of the digestate, it is noticeable that the cobalt concentration in the yeast extract supplemented digester D2 was more than double that in D1, and it is therefore very likely that the yeast extract supplementation was responsible for this. Also according to Gonzalez-Gil et al. (2003), nickel and cobalt form organic complexes with yeast extract, therefore increasing the bioavailability of these essential metals in anaerobic reactors.

CHAPTER 6

Food Waste Digestion

6.1 Chapter Summary

The experiments undertaken were designed to determine which trace elements are important in the anaerobic digestion of domestic food waste. The first set of experiments looked at the degradation of VFA in batch tests using 6 trace elements which were chosen as factors to be investigated in this 2-level factorial experiment design. Semi-continuous digestion trials were undertaken over an extended period in which different trace element combinations were assessed in response to increases in OLR. A radioactive ^{14}C tracer experiment was used to identify the dominant methanogenic pathway, in order to support the postulated theory that a shift in population structure from acetoclastic to hydrogenotrophic occurs under high ammonia concentration.

6.2. Batch Screening Experiment for Trace Element Influence VFA Degradation in Food Waste Digesters

6.2.1. Summary Method

32 conical flasks were inoculated with 200 ml of sieved digestate taken from a food waste digester, and then supplemented with nutrients and carbon sources. The concentrations of trace elements in the inoculum are given in Table 6.1.

Individual working solutions of 6 trace elements (Fe, Co, Mo, Ni, Se, and W) were made and added to selected flasks so that the concentration was raised by 1 mg l^{-1}, 1 mg l^{-1}, 0.2 mg l^{-1}, 0.2 mg l^{-1}, 5 mg l^{-1} and 0.2 mg l^{-1} for Co, Ni, Mo, Se, Fe and W, respectively. The pattern of addition is shown in Table 6.2 and a + sign denotes where the element has been supplemented

The headspace of each flask was flushed with a mix of carbon dioxide and nitrogen (20:80), and the flasks were incubated at a constant temperature of 36°C, in an orbital shaker operating at 60 RPM. Samples were regularly taken from the flasks to monitor the VFA degradation. The experiment continued over 90 days until all VFAs in flasks were depleted.

Table 6.1. Background trace elements concentrations and other parameter of the digestate inoculum

Trace element (mg l^{-1})	
Fe	103
Co	0.035
Mn	14.2
Al	42.8
Zn	7.94
Mo	0.13
Cu	2.23
Ni	0.304
Se	0.061
W	<0.1
Other parameters	
pH	7.9
TAN (mgNH_3-N l^{-1})	5000
Total VFA (mg l^{-1})	5800
Acetic acid (mg l^{-1})	3500
Propionic acid (mg l^{-1})	1400

Table 6.2. Experiment design matrix

Flasks	**Co**	**Ni**	**Mo**	**Se**	**Fe**	**W**
1, 2	-	-	-	-	-	-
3, 4	-	-	-	+	+	+
5, 6	-	-	+	-	+	+
7, 8	-	-	+	+	-	-
9, 10	-	+	-	-	+	-
11, 12	-	+	-	+	-	+
13, 14	-	+	+	-	-	+
15, 16	-	+	+	+	+	-
17, 18	+	-	-	-	-	+
19, 20	+	-	-	+	+	-
21, 22	+	-	+	-	+	-
23, 24	+	-	+	+	-	+
25, 26	+	+	-	-	+	+
27, 28	+	+	-	+	-	-
29, 30	+	+	+	-	-	-
31, 32	+	+	+	+	+	+

6.2.2. Experimental Results

Only small variations can be observed between the replicates. The mean values of the duplicates with error bars showing the range of duplicate results are plotted in Figure 6.1 to illustrate the VFA degradation over the trial period. Clear divergences of degradation rate were observed amongst samples with different trace element supplementation combinations. This was particularly evident in samples where selenium was present and in

these cases both acetate and propionate degradation rate was significantly higher compared to selenium deprived groups.

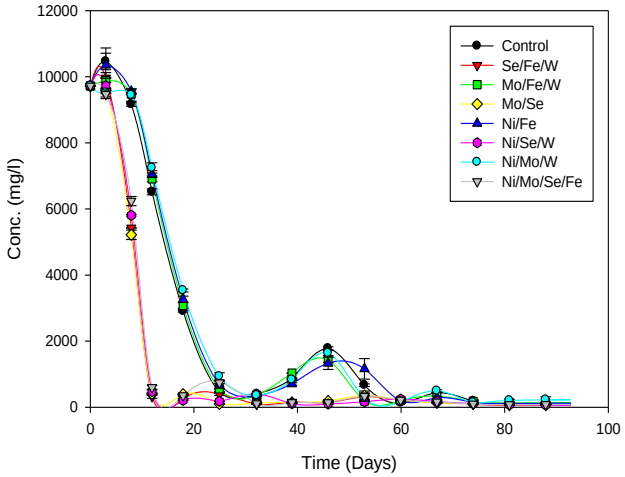
The VFA degradation indexes (*DI*) under different factor combinations were calculated using equation 3.9 (section 3.7.3). The results are summarised in Table 6.3.

Table 6.3. VFA Degradation Indexes

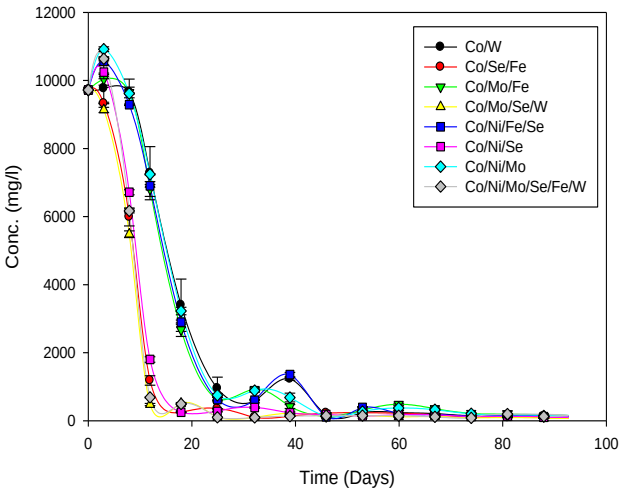
Runs	Combinations	*DI(Ave)*		
		Acetic	Propionic	Total
1, 2	Control	153014	267184	527562
3, 4	Se/Fe/W	84583	174707	315682
5, 6	Mo/Fe/W	155723	249938	488264
7, 8	Mo/Se	81555	127735	260954
9, 10	Ni/Fe	160765	277510	521616
11, 12	Ni/Se/W	85390	208527	349237
13, 14	Ni/Mo/W	161457	255408	498665
15, 16	Ni /Mo/Se/Fe	123414	156092	300108
17, 18	Co/W	163569	232874	462669
19, 20	Co/Se/Fe	88583	170724	314340
21, 22	Co/Mo/Fe	156647	212234	441349
23, 24	Co/Mo/Se/W	81935	117920	246362
25, 26	Co/Ni/Fe/Se	157778	231647	459108
27, 28	Co/Ni/Se	99000	209378	364809
29, 30	Co/Ni/Mo	166373	220501	454576
31, 32	Co/Ni/Mo/Se/Fe/W	91865	141395	278231

By day 28, acetic acid had been depleted to less than 100 mg l^{-1} in all samples. After day 30, however, the acetic acid concentration started to increase in some samples (Figure 6.1a and b).

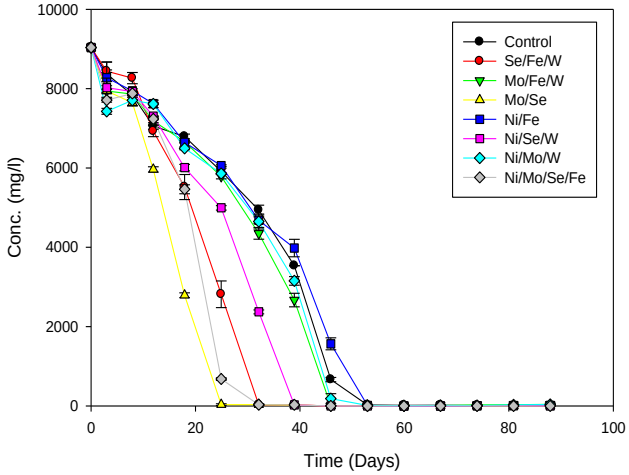
An increased propionate degradation rate could be observed at the same time (Figure 6.1c and d) in these samples. The increase of acetic acid was therefore attributed to the propionic acid degradation. To avoid this complication of secondary degradation the degradation indexes for acetic acid were calculated by integrating the numerical integrals to day 28.



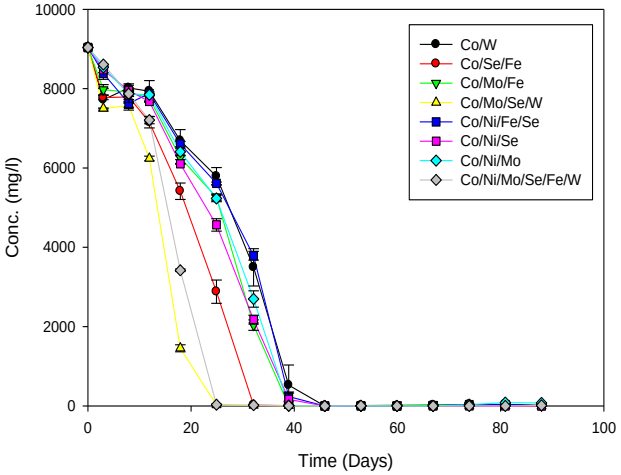
a) Sample 1-16 (Acetic acid)



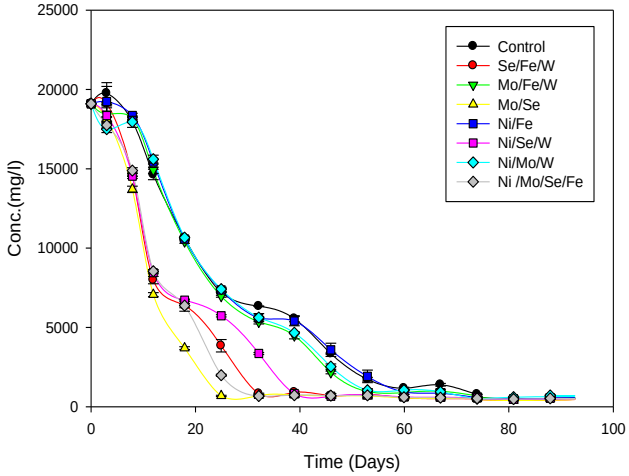
b) Sample 17-32 (Acetic acid)



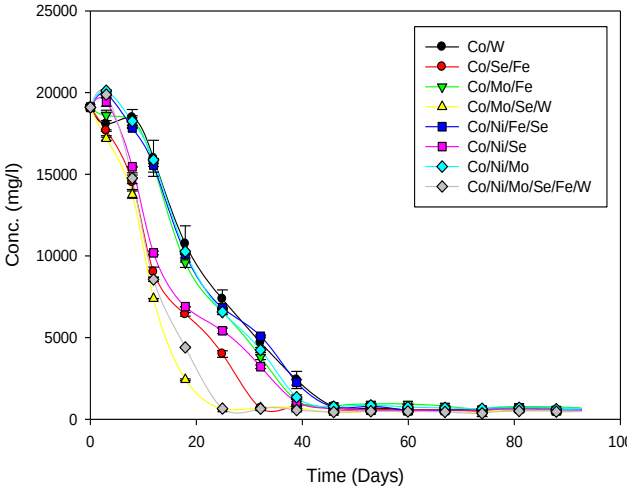
c) Sample 1-16 (Propionic acid)



d) Sample 17-32 (Propionic acid)



e) Sample 1-16 (Total VFA)



f) Sample 17-32 (Total VFA)

Figure 6.1. VFA degradation profiles

6.2.3. Analysis of Factorial Effects

The weighing of each individual factor on VFA degradation rate can be assessed by the main effect calculated according to equation 3.10. The results of all main effects are shown in Table 6.4. The '+/-' sign indicates whether an effect contributes positively or negatively to the DI: in other words, a positive effect increases the time required for degradation of VFA and vice versa.

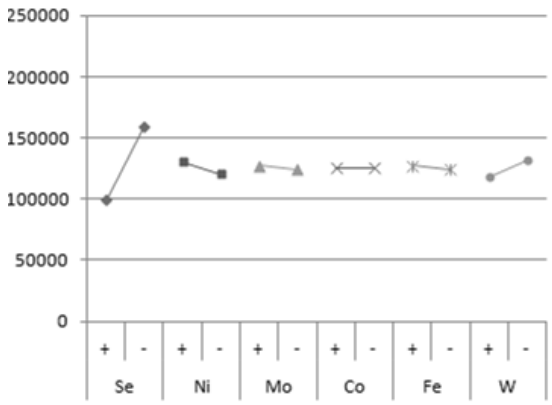
In the IV resolution fractional design, the main effects of all 6 factors are clear, and according to the effect hierarchy principle (Wu and Hamada, 2009) these are more likely to be important higher order effects. Therefore in this study, all the 3-factor combinations (which are aliases to the main effects) are ignored. All the factors / factor combinations with their corresponding aliases are listed in Table 6.4.

The main effects are displayed graphically in Figures 6.2a, 2c and 2e. Each factor was represented by a line connecting the average values of all observations at the high (+) level and low (-) level; the vertical height of each line indicates the main effect.

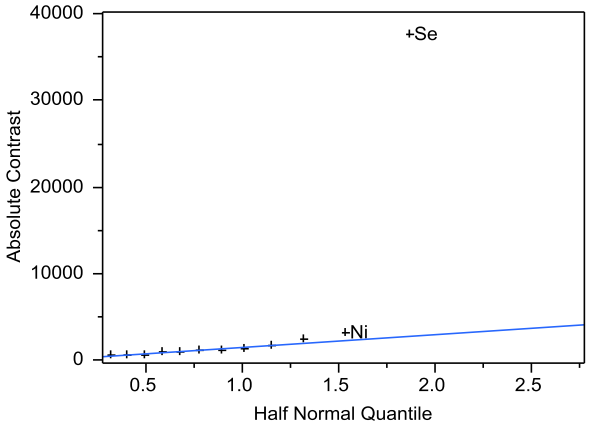
The main effects analysis suggested that selenium (Se) reduced the time for the degradation of both acetic and propionic acid. Molybdenum (Mo) and Cobalt (Co) showed a moderate influence on decreasing propionic degradation time, whereas nickel increased the degradation time for both acetic and propionic. High concentrations of nickel have been reported as being inhibitory to anaerobic digestion in several studies (Lin, 1993, Lin, 1992, Ahring and Westermann, 1983). Lin (1992) observed a moderate inhibitory effect from Ni in a batch experiment under mesophilic conditions: the concentrations of Ni to inhibit 50% of the acetic and propionic acid degradation are 89 and 226 mg l^{-1}, respectively. The concentration of Ni in current study was 1.3 mg l^{-1}, however, which is significantly lower than the reported value; therefore further experiments will be required to confirm the inhibitory effect of Ni at such a low concentration.

If the half-normal probability of the estimated effect is plotted (Figure 6.2b, d and f), it can be seen that all the large estimated effects appear in the upper right corner and fall above the straight line through the small estimated effects, and it is clear that selenium was the

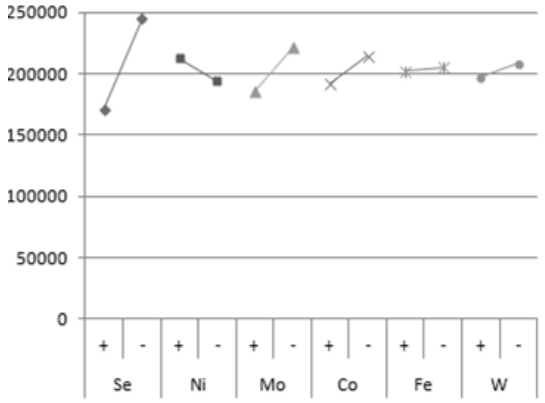
most pronounced factor that facilitates VFA degradation. As suggested by the main effects plot, Mo and Co exhibited some effect on propionic degradation.



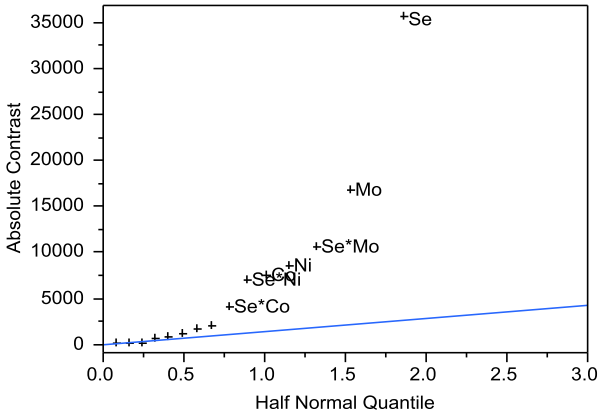
a) Main effects plot of acetic degradation



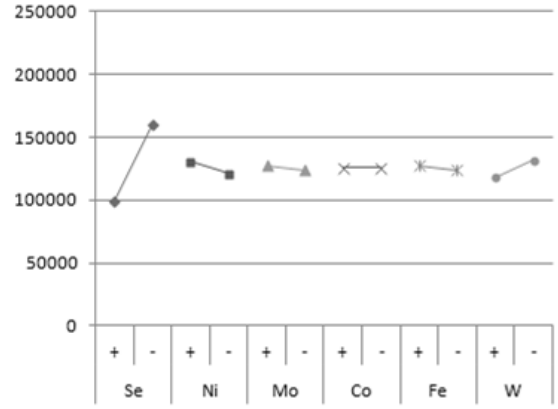
b) Half normal plot of effects to acetic degradation



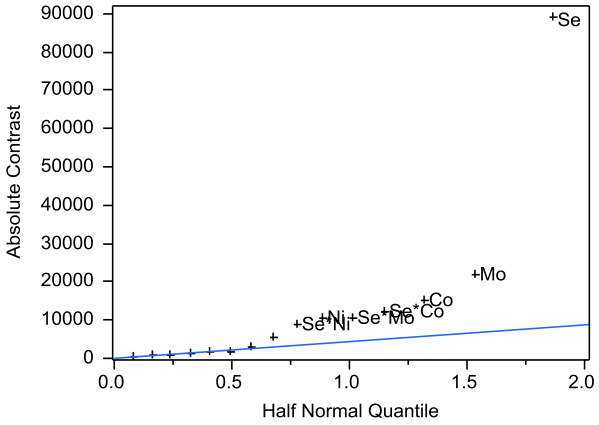
c) Main effects plot of propionic degradation



d) Half normal plot of effects to propionic degradation



e) Main effects plot of total VFA degradation



f) Half normal plot of effects to total VFA degradation

Figure 6.2. Main effects plot and Half-normal probability plot of standardised effects for VFA degradation

The two factors combination Se*Mo (confounded with Co*W and Ni*Fe due to the aliasing of effect of the fractional factorial design) and Mo*W (confounded with Se*Co) were also shown to have an influence on propionic degradation, indicating synergistic (reducing the degradation time) and antagonistic (increasing the degradation time) effects, respectively.

Aliasing of effects is an intrinsic inadequacy of a fractional design: in this case a 2^{6-2} has only 15 degrees of freedom and therefore cannot estimate all 63 factorial effects ($\binom{6}{1} + \binom{6}{2} + \binom{6}{3} + \binom{6}{4} + \binom{6}{5} + \binom{6}{6} = 6 + 15 + 20 + 15 + 6 + 1 = 63$) among the 6 factors. Although statistically ambiguous, the aliased effects can be untangled from prior knowledge and direct experimental observation. During the semi-continuous food waste digestion experiment reported by Banks et al. (2012) (from which the inoculum of this study was obtained), after an increase in OLR VFA started to accumulate to around 12000 mg l^{-1} in the Se and Mo supplemented digester. The supplement of Co was then added which resulted in a rapid fall in VFA concentrations to values of <500 mg l^{-1}. This clearly proved that cobalt was beneficial to VFA degradation in the presence of selenium, therefore the aliasing factorial effect of Se*Co can be dismissed in the current study and leaves the W*Mo effect clear. In fact, the antagonistic effect between W and Mo with some methanogens grow on H_2/CO_2 has been addressed in a number of studies (Kletzin and Adams, 1996, May et al., 1988, Zellner and Winter, 1987). It was suggested that W replaces Mo as the centre of formate dehydrogenase (FDH) which consequently deactivates the enzyme (May et al., 1988). It should be noted, however, that this experimental design was not intended to explore the 2 factor interaction. Therefore to confirm the antagonistic effect between W and Mo under current experiment conditions, a further experiment which is designed with this specific purpose in mind is required.

To express quantitatively the deviation of each estimated effect from the straight line in the half-normal plots (Figure 6.2b, d and f), Lenth's t-Ratio of each effect was calculated and shown in Table 6.5. The individual p-value and multiple-comparison adjusted p-value corresponding to each Lenth's t-Ratio are also reported in Table 6.5.

The strong reliance on selenium shown in VFA degradation in this study can be attributed to the intensive requirement for selenium in the methanogenic pathway. Since selenium

has not been reported to be required in the acetoclastic pathway, whilst a number of studies (Ferry 1990, Ferry 1999, Jones et al., 1979, Vorholt et al., 1997) reported the involvement of selenium in the hydrogenotrophic pathway, it is therefore reasonable to postulate that the hydrogenotrophic methanogenic pathway is responsible for the majority of acetic acid degradation in current study.

6.2.4. Regression Models for VFA Degradation Indexes (DI)

The regression coefficients of the main effects and second order effects were calculated and presented in table 6.5 together with their corresponding t-Ratios and Prob>|t| values. The coefficients can be applied to equation 3 and regression models which correlate 5 main effects and their second order interactions with degradation index (*DI*) are given as follow:

$$DI_{HAc} = 125728.25 - 33687.5X_{Se} - 1642.9X_{Mo} - 9.4X_{Co} + 5027.2X_{Ni} - 2940.6X_{W} + 3383X_{Fe} + 1008.8X_{Se}X_{Mo} - 1685.4X_{W}X_{Mo} - 3156.7X_{Mo}X_{Co} + 2849.6X_{Se}X_{Ni} + 3379.1X_{Mo}X_{Ni} - 1991.9X_{Co}X_{Ni} - 3692.1X_{Ni}X_{W} \quad [6.1]$$

$$DI_{HPr} = 203360.9 - 40051.1X_{Se} - 18208.0X_{Mo} - 11276.6X_{Co} + 9196.4X_{Ni} - 1808.9X_{W} - 1580.0X_{Fe} - 9316.2X_{Se}X_{Mo} + 7821.2X_{W}X_{Mo} - 863.7X_{Mo}X_{Co} + 6341.9X_{Se}X_{Ni} - 1000.2X_{Mo}X_{Ni} + 550.1X_{Co}X_{Ni} - 1504.0X_{Ni}X_{W} \quad [6.2]$$

$$DI_{Total} = 392720.7 - 89005.3X_{Se} - 21657.1X_{Mo} - 15040.2X_{Co} + 10573.0X_{Ni} - 5443.6X_{W} - 2883.7X_{Fe} - 10644.4X_{Se}X_{Mo} + 12260.4X_{W}X_{Mo} - 893.9X_{Mo}X_{Co} + 8807.8X_{Se}X_{Ni} + 1258.5X_{Mo}X_{Ni} + 927.7X_{Co}X_{Ni} - 1540.0X_{Ni}X_{W} \quad [6.3]$$

To simplify those equations, insignificant terms at 5% significance level can be neglected, i.e. adjusted p-value >0.05 (Table 6.5) and the equations become:

$$DI_{HAc} = 125728.25 - 33687.5X_{Se} \quad [6.4]$$

$$DI_{HPr} = 203360.9 - 40051.1X_{Se} - 18208.0X_{Mo} - 11276.6X_{Co} + 9196.4X_{Ni} - 9316.2X_{Se}X_{Mo} + 7821.2X_{W}X_{Mo} \quad [6.5]$$

$$DI_{Total} = 392720.7 - 89005.3X_{Se} - 21657.1X_{Mo} \quad [6.6]$$

Analysis of variance (ANOVA) has been used to evaluate the adequacy of the empirical models. The results showed coefficients of determination (R^2) of 0.9976, 0.9998 and 0.9998 for the acetic, propionic and total VFA model, respectively. F ratios are 65.27, 949.25 and 614.63 with p-value of 0.0152, 0.0011 and 0.0016 for acetic, propionic and total VFA respectively. Large F-ratios and low p-values indicate large values of model sum of squares (SS) as compared with small values of error SS; therefore the models are able to give a good estimate of response under the experimental conditions.

Table 6.4. Summary of factorial effects

		Acetic acid				Propionic acid				Total VFA			
Term	**Aliases**	**Factorial effect**	**Lenth t-Ratio**	**Individual p-Value**	**Adjusted p-Value**	**Factorial effect**	**Lenth t-Ratio**	**Individual p-Value**	**Adjusted p-Value**	**Factorial effect**	**Lenth t-Ratio**	**Individual p-Value**	**Adjusted p-Value**
Se	Mo*Co*W, Mo*Ni*Fe	-67375	-12.19	<0.0001*	<0.0008*	-80102.2	-26.69	<0.0001*	<0.0001*	-178010.6	-20.58	0.0001*	0.0002*
Mo	Se*Co*W, Se*Ni*Fe	-3285.8	0.59	0.5904	1.0000	-36416	-12.14	0.0002*	0.0008*	-43314.2	-5.01	0.0035*	0.0234*
Co	Se*Mo*W, Ni*W*Fe	-18.8	-0.00	0.9982	1.0000	-22553.2	-7.52	0.0005*	0.0052*	-30080.4	-3.48	0.0102*	0.0936
Ni	Se*Mo*Fe, Co*W*Fe	10054.4	1.82	0.0890	0.5926	18392.8	6.13	0.0019*	0.0110*	21146	2.44	0.0326*	0.2636
W	Se*Mo*Co, Co*Ni*Fe	-5881.2	-1.06	0.2782	0.9865	-3617.8	-1.21	0.2160	0.9517	-10887.2	-1.26	0.1986	0.9317
Fe	Se*Mo*Ni, Co*Ni*W	3383	0.61	0.5788	1.0000	-3160	-1.05	0.2735	0.9877	-5767.4	-0.67	0.5002	1.0000
Se*Mo	Co*W, Ni*Fe	2017.6	0.37	0.7363	1.0000	-18632.4	-6.21	0.0019*	0.0106*	-21288.8	-2.46	0.0317*	0.2560
Mo*W	Se*Co	-3370.8	-0.61	0.5800	1.0000	15642.4	5.21	0.0028*	0.0210*	24520.8	2.83	0.0227*	0.1986
Mo*Co	Se*W	-6313.4	-1.14	0.2465	0.9700	-1727.4	-0.58	0.5990	1.0000	-1787.8	-0.21	0.8474	1.0000
Se*Ni	Mo*Fe	5699.2	1.03	0.2896	0.9913	12683.8	4.23	0.0064*	0.0503	17615.6	2.04	0.0610	0.4518
Mo*Ni	Se*Fe	6758.2	1.22	0.2135	0.9454	-2000.4	-0.67	0.5002	1.0000	2517	0.29	0.7855	1.0000
Co*Ni	W*Fe	-3983.8	-0.72	0.4522	1.0000	1100.2	-0.37	0.7350	1.0000	1855.4	0.21	0.8421	1.0000
Ni*W	Co*Fe	-7384.2	-1.34	0.1769	0.8976	-3008	-1.00	0.2958	0.9934	-3080	-0.36	0.7413	1.0000

Note: p-Value marked with '*' denote the factor being significant (<0.05)

Table 6.5. Coefficients of the empirical regression models for acetic, propionic and total VFA degradation index (*DI*)

	Acetic Acid			**Propionic Acid**			**Total VFA**		
Term	**Coefficient**	**t Ratio**	**Prob>|t|**	**Coefficient**	**t Ratio**	**Prob>|t|**	**Coefficient**	**t Ratio**	**Prob>|t|**
Constant	125728.25	104.63	<0.0001	203360.9	466.45	<0.0001	392720.7	367.71	<0.0001
Se	-33687.5	28.03	0.0013	-40051.1	91.86	0.0001	-89005.3	83.34	0.0001
Mo	-1642.9	-1.37	0.3049	-18208.0	41.76	0.0006	-21657.1	20.28	0.0024
Co	-9.4	0.01	0.9945	-11276.6	25.87	0.0015	-15040.2	14.08	0.0050
Ni	5027.2	-4.18	0.0527	9196.4	-21.09	0.0022	10573.0	-9.90	0.0100
W	-2940.6	2.45	0.1342	-1808.9	4.15	0.0535	-5443.6	5.10	0.0364
Fe	1691.5	-1.41	0.2945	-1580.0	3.62	0.0684	-2883.7	2.70	0.1142
Se*Mo	1008.8	0.84	0.4895	-9316.2	-21.37	0.0022	-10644.4	-9.97	0.0099
Se*Co	-1685.4	-1.40	0.2958	7821.2	17.94	0.0031	12260.4	11.48	0.0075
Mo*Co	-3156.7	-2.63	0.1195	-863.7	-1.98	0.1861	-893.9	-0.84	0.4907
Se*Ni	2849.6	2.37	0.1411	6341.9	14.55	0.0047	8807.8	8.25	0.0144
Mo*Ni	3379.1	2.81	0.1066	-1000.2	-2.29	0.1487	1258.5	1.18	0.3599
Co*Ni	-1991.9	-1.66	0.2392	550.1	-1.26	0.3343	927.7	0.87	0.4766
Ni*W	-3692.1	-3.07	0.0916	-1504.0	-3.45	0.0747	-1540.0	-1.44	0.2861

Note: Prob>|t|: Probability value of getting an even greater t-statistic (in absolute value), less than 0.05 are considered as significant evidence that the parameter is not zero.

6.3. Semi-continuous Digestion Trials on Food Waste

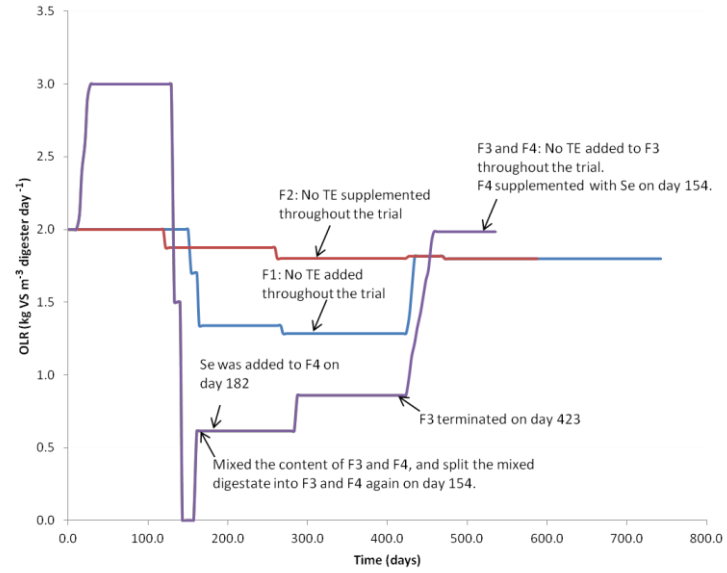
6.3.1 Summary Method

The semi-continuous digesters were fed over a period of 780 days on source segregated food waste. 8 digesters were run in pairs at different OLR and supplemented with different TE combinations within a selection of 11 trace elements (Se, Mo, Co, Ni, W, Fe, Al, B, Cu, Mn and Zn) to test the impact of TE addition on process stability and efficiency. Digester F1 and F2 were intended to be the controls without TE addition running at OLR of 2 g VS l^{-1} day^{-1}. Digester F3 and F4 were also TE deprived as in F1 and F2, but running at a higher OLR of 3 g VS l^{-1} day^{-1}. Digester pair F5 and F6 was given a TE combination of Se and Mo, and F7 and F8 were given Se, Mo, Co, and W at the concentrations shown in Table 3.2. The OLR in these two pairs of digesters were increased successively to 5.5 g VS l^{-1} day^{-1} through the course of the experiment.

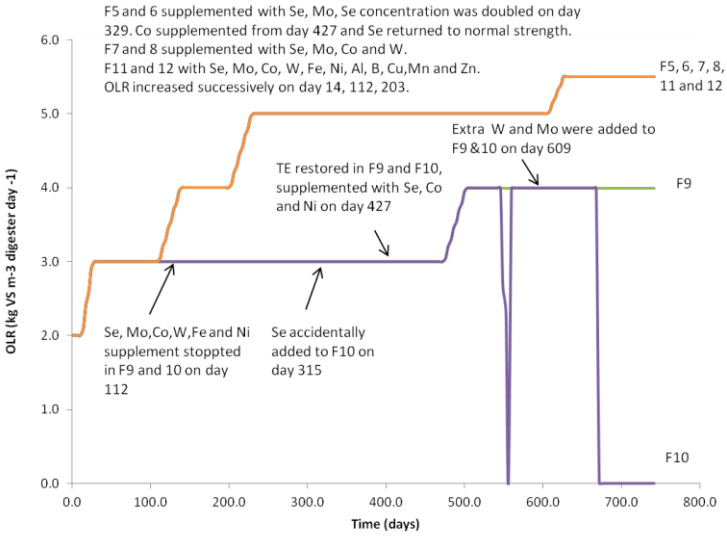
Both F9 and F10 were fed at OLR of 3 g VS l^{-1} day^{-1} and supplemented with Se, Mo, Co, W, Fe, and Ni from the start of the experiment. The weekly TE supplementation was removed from day 112, and therefore the existing TE in the digestate would have been gradually diluted out. This experiment was intended to explore the minimum threshold of TE concentration to sustain stable digestion.

F11 and F12 were supplemented with full suite of 11 TE and the OLR in these 2 digesters were increased successively to 5.5 g VS l^{-1} day^{-1}.

Due to performance deterioration in some digester, changes were made in an attempt to restore a normal digester performance. Figure 6.3 summaries the entire operational history of the 12 digesters in respect of OLR and TE supplementation regime.



a) Digesters F1-4



b) Digesters F5-12

Figure 6.3. Loading and TE addition changes made throughout the trial

6.3.2. Experimental Results

Feedstock and inoculum characteristics are shown in Table 6.6.

Table 6.6. Characteristics of the inoculum digestate at the start of the semi-continuous fed trial and the average composition of the food waste used over the trial period.

	Digestate	Food waste
pH	8.0	4.71 ± 0.01 (1:5)
TS (% of WW)	6.34	23.74 ± 0.08
VS (% of WW)	4.59	21.71 ± 0.09
VS (% of TS)	72.4	91.44 ± 0.39
Trace elements (mg kg^{-1} fresh matter)		
Aluminium (Al)	63.3	NM
Boron (B)	2.5	NM
Cobalt (Co)	0.083	<0.060
Copper (Cu)	5.75	1.7 ± 0.2
Iron (Fe)	173.7	54
Manganese (Mn)	18.5	20 ± 3
Molybdenum (Mo)	0.29	0.11 ± 0.01
Nickel (Ni)	2.9	1.7 ± 0.7
Selenium (Se)	0.050	<0.070
Tungsten (W)	<0.035	<0.25
Zinc (Zn)	8.11	7.8 ± 2.6
Potentially toxic element (mg kg^{-1} fresh matter)		
Cadmium (Cd)	0.038	<0.25
Chromium (Cr)	5.25	6.9 ± 0.3
Lead (Pb)	0.63	<2.5
Mercury (Hg)	<0.010	<0.003
Macro nutrients (g kg^{-1} fresh matter)		
Calcium (Ca)	2.16	NM
Magnesium (Mg)	0.168	NM
Potassium (K)	2.63	3.39 ± 0.19
Sodium (Na)	1.13	NM
Phosphorus (P)	0.700	1.28 ± 0.08
Total Kjeldahl Nitrogen (N)	8.47	8.12 ± 0.01
Other digestate parameters (g kg^{-1} fresh matter)		
Total ammoniacal nitrogen	4.7	NM
Total volatile fatty acid	4.4	NM
Acetic acid	4.1	NM
Propionic acid	0.1	NM

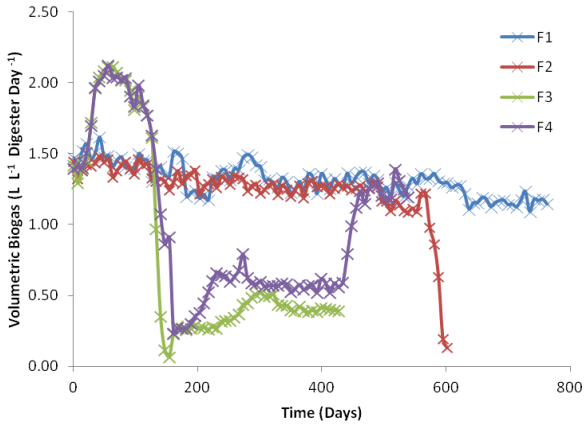
NM = not measured

Specific and volumetric biogas production (SBP and VBP) for all the digesters are shown in Figure 6.4. The control digesters F1 and F2 which were initially fed at an OLR of 2 g VS l^{-1} day^{-1} showed relatively consistent SBP over the trial period, fluctuating around 0.7 l g^{-1}

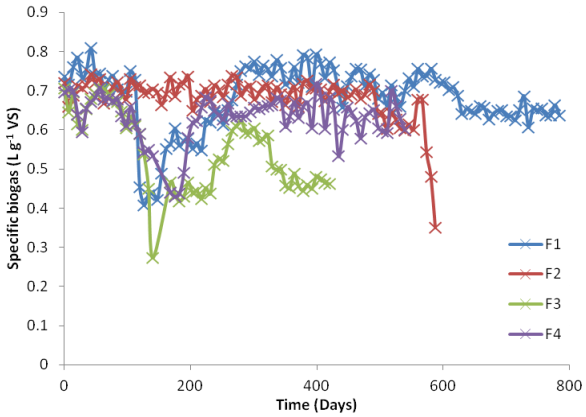
VS_{added}, although in order to maintain both this yield and the stability of the digestion as indicated by other monitoring parameters the OLR had to be gradually reduced (Figure 6.3a). The volumetric biogas production (VBP) reflected changes in the OLR applied to the digesters and was reduced as the loading was gradually decreased.

Under the initial OLR = 2 g VS $l^{-1}day^{-1}$, total VFA concentration in F1 rapidly accumulated to around 20000 mg l^{-1} within 200 days (Figure 6.5a). At this point, it appeared unlikely the digestion could be sustained and therefore the OLR was reduced to 1.28 g VS $l^{-1}day^{-1}$ (Figure 6.3a). As soon as the OLR had been reduced, the VFA concentration fell and stabilised at around 7500 mg l^{-1} (total VFA). An attempt to increase the loading to 1.8 g VS l^{-1} day^{-1} on day 430 caused an increase in VFA accumulation and after operating at this loading for another 130 days the VFA concentration started to decline (Figure 6.5a). It seemed at this point that the system had gradually adapted to the higher loading and stable digestion had been achieved. VFA started to increase again, however, from 5510 mg l^{-1} (total VFA) on day 670 to 15600 mg l^{-1} on day 760 when operation of the digesters was terminated (Figure 6.5a).

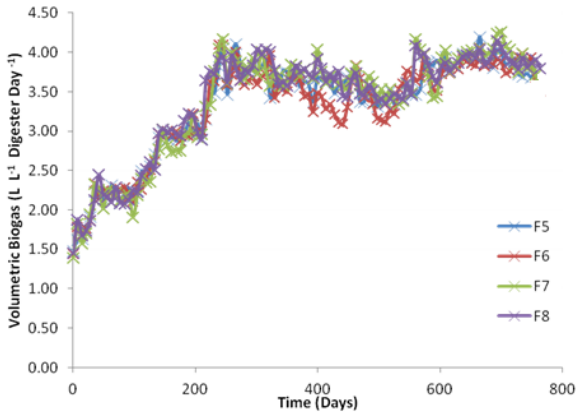
Digester F2 initially suffered from VFA accumulation, although at a slightly slower rate than had been seen in F1. The OLR was gradually reduced to 1.8 g VS $l^{-1}day^{-1}$ in attempt to achieve stability (Figure 6.3a). After operating for 350 days, the increasing trend in VFA concentration had reduced and the total VFA concentration stabilised at around 20000 mg l^{-1} . On day 570 the VFA rapidly increased to 31700 mg l^{-1} within 60 days and this was accompanied by a large fall in biogas production and caused a drop in pH indicating digestion failure (Figure 6.5b).



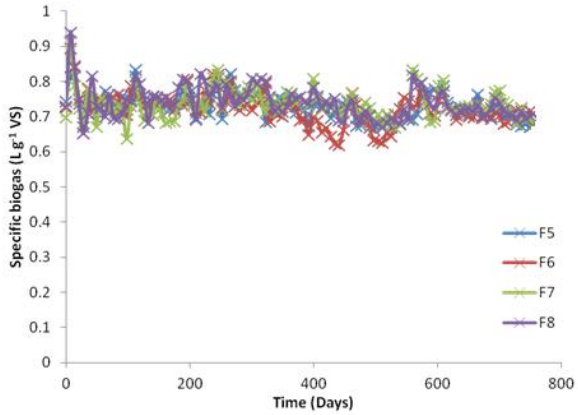
a) F1-4 VBP



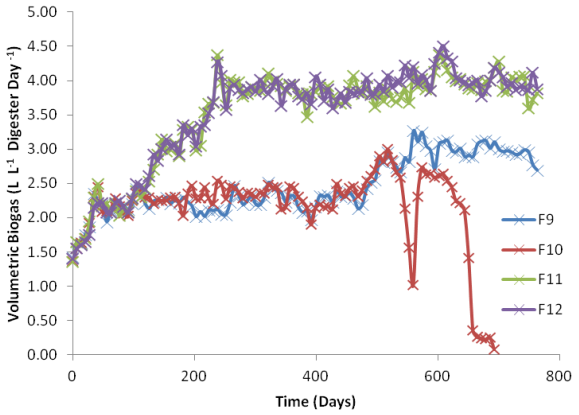
b) F1-4 SBP



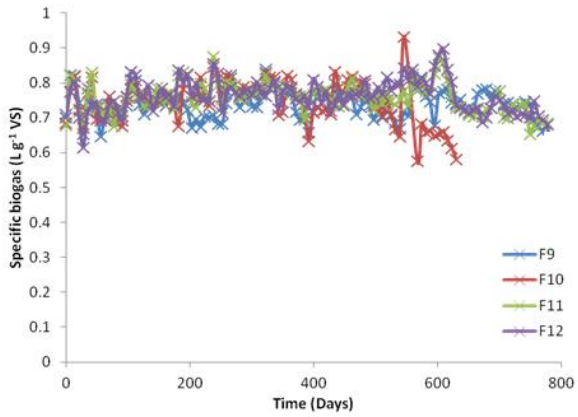
c) F5-8 VBP



d) F5-8 SBP



e) F9-12 VBP



f) F9-12 SBP

Figure 6.4. Specific and volumetric biogas production in control and TE supplemented digesters.

Digester F3 and F4 operated at a higher OLR which was increased to 3 g VS $l^{-1}day^{-1}$ by day 35 (Figure 6.2a). This pair of digesters showed a rapid decline in biogas production following the loading increase, and feeding was stopped on day 120. The VFA concentration, however, persistently increased to around 35000 mg l^{-1} in both digesters (Figure 6.5c and d). On day 154, the digestate from the pair was mixed and then returned back into the digesters, in order to avoid further divergence and to maintain them as a pair for the next step of the trial. In this F3 was then fed at an OLR of 0.6 g VS l^{-1} day^{-1} which was gradually increased to 0.9 g VS l^{-1} day^{-1} (Figure 6.2a). Even at this low OLR, there was no sign of improvement in digestion, i.e. total VFA remained over 30000 mg l^{-1} and propionic acid concentration kept on increasing (Figure 6.5c and d).

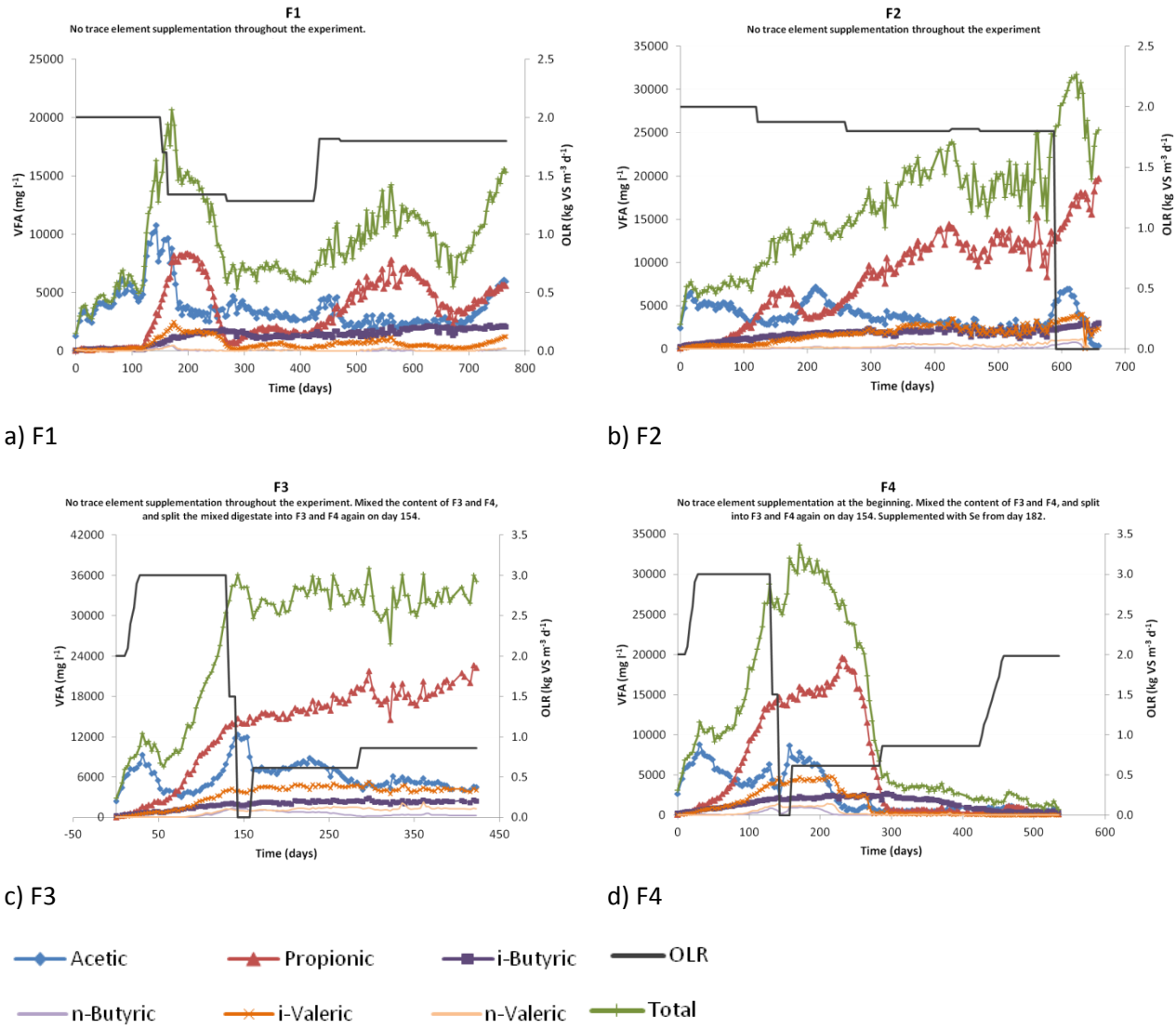
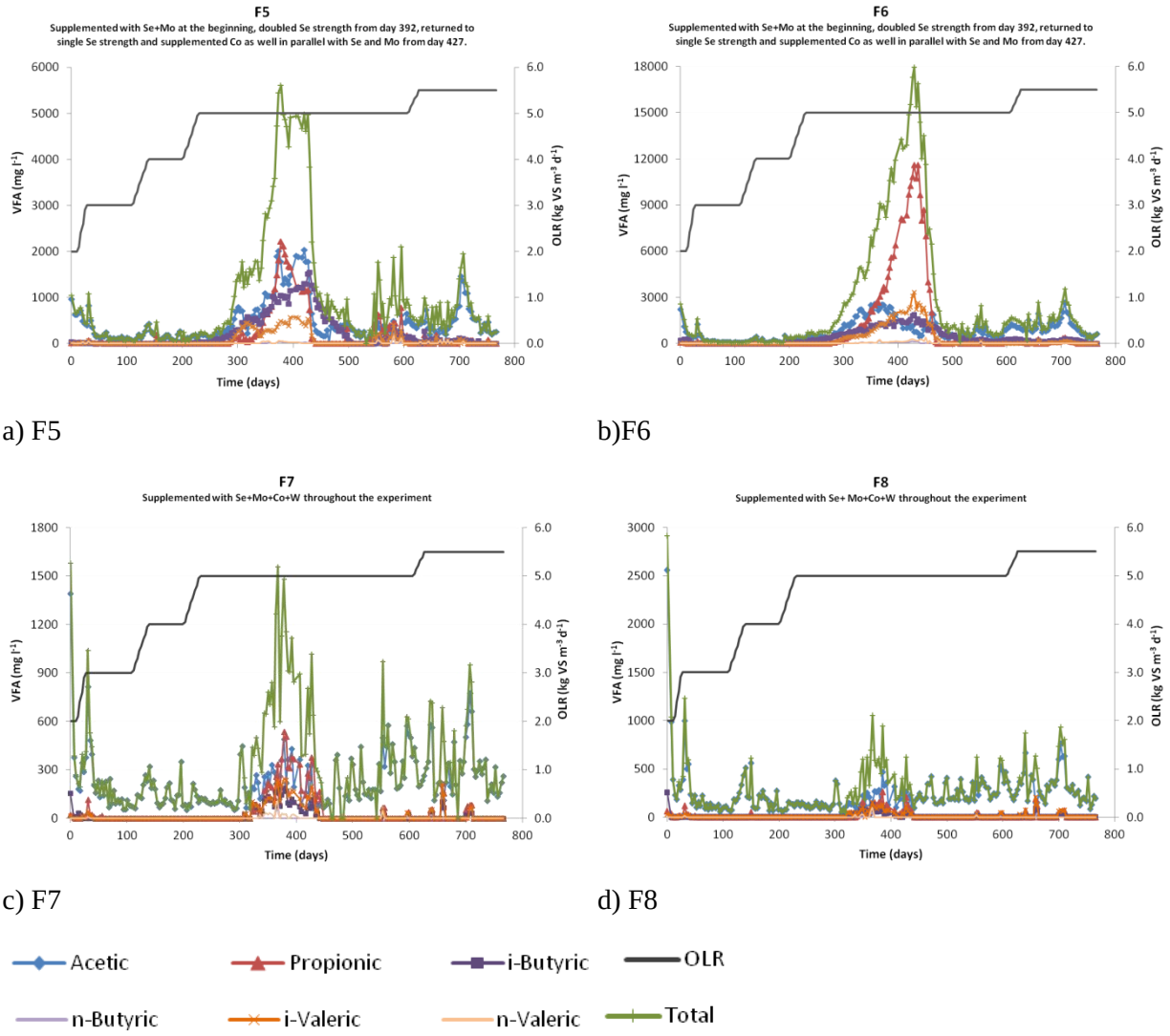


Figure 6.5. VFA profile in digester F1-4

Se was added into digester F4 on day 182 and showed an immediate and significant effect. The acetic acid concentration decreased immediately (Figure 6.5d) and this was followed later by a decrease in propionic acid. Gradually the OLR was raised to 2 g VS $l^{-1}day^{-1}$ (Figure 6.3a) and stable digestion was achieved with the total VFA concentration fluctuating around 1000 mg l^{-1}.

The SBP in digesters F5-12 receiving trace element supplementation fluctuated around 0.75 l g^{-1} VS_{added} (Figure 6.4d and f), with no apparent deterioration in this yield as the OLR gradually increased to 5.5 g VS l^{-1} day^{-1} (Figure 6.3b). In the TE supplemented digesters VBP

increased from around 1.5 l l^{-1} day^{-1} at OLR = 2 g VS $l^{-1}day^{-1}$ to around 4 l l^{-1} day^{-1} at the highest loading of 5.5 g VS l^{-1} day^{-1}.



a) F5 b)F6

c) F7 d) F8

Figure 6.6. VFA profile in digester F5- 8

Although it appeared at first that supplementing a combination of Se and Mo was sufficient to prevent VFA accumulation in digesters F5 and F6, it was not until shortly after an increase in OLR to 5 g VS l^{-1} day^{-1} that VFA started to accumulate (Figure 6.6a and b). A slight increase in VFA was also observed at the time in digester F7 and F8, although the extent was much less significant (Figure 6.6c and d). It is not uncommon to see a VFA increase following an increase in loading, and once the system has adapted to the higher loading the VFA concentration reduces again: this is what happened in digester F7 and F8. In the digester pair

F5 and F6, however, there was no reduction and the accumulation of VFA became more severe, especially in F6 where total VFA gradually reached 18000 mg l^{-1} (Figure 6.6b). The sequence of appearance of the VFA species and their proportion in the total VFA at this point was: acetic acid (30%), iso-butyric acid (35%), iso-valeric acid (25%), and propionic acid (10%). It was obvious that if no action was taken digestion would fail in this pair of digesters.

It was initially hypothesised that this unbalanced operation might be due to an increasing demand for Se-containing enzyme systems as the OLR was increased, resulting in increased growth rate and metabolic activity. To test this, the Se dose in digester F5 and F6 was increased on day 329 (Figure 6.3b) to double the original level; but no reduction in VFA was observed. This suggested that at the higher load some other component had become limiting. Total VFA concentrations in this digester were therefore allowed to accumulate to around 12000 mg l^{-1} before the Se dose was reduced to normal and a supplement of Co was added. This resulted in a rapid fall in VFA concentrations to values of < 500 mg l^{-1} (Figure 6.6a and b). This recovery was sustained for a further 4 HRT (around 160 days) and the OLR was increased to 5.5 g VS l^{-1} day^{-1} (Figure 6.3b). A slight elevation in VFA was observed shortly after the loading increase, but the total concentration never exceeded 3600 mg l^{-1} in either digester and quickly declined to under 1000 mg l^{-1} (Figure 6.6a and b).

Digester F7 and F8 were supplemented with a TE combination of Se, Mo, Co and W over the operational period. The OLR in this pair of digesters was increased successfully to 5.5 g VS l^{-1} day^{-1} without encountering any major operational difficulty.

Digester F9 and 10 were originally supplemented with Se, Mo, Co, W, Fe and Ni. From day 112, TE supplementation was stopped in this pair of digesters; feeding of both digesters continued at an OLR of 3 g VS l^{-1} day^{-1} (Figure 6.3b), in this way the effect of trace elements being diluted out of the digesters could be monitored. The suspension of TE supplementation in this pair of digesters continued for 210 days (around 3 HRT). The digester from which TE supplementation was withdrawn began to show deterioration in both SBP and VBP between days 350 and 400 (Figure 6.4e and f), corresponding to the time necessary for washout of the trace elements that had been previously supplemented. VFA also started accumulating around

day 280 (Figure 6.7a and b), and had reached a concentration of about 4000 mg l^{-1} when a one-off dose of 0.12 mg Se was accidently added, equivalent to increasing the concentration of Se in the digesters by 0.03 mg l^{-1}. The very rapid reduction in accumulated VFA observed after this event was confirmed by improvement in the IA:PA ratio. The improvement was short-lived, however, as the additional Se was gradually diluted out. On day 322, the total VFA concentration in both digesters had reached over 10,000 mg l^{-1}, of which acetic acid was the dominant VFA, and the proportion of other VFA species present (propionic acid, iso-butyric acid, and iso-valeric acid) was less than 10% of the total VFA concentration (Figure 6.7a and b). At this point, both the digesters were supplemented with Se, Co and Ni, which rapidly reduced the VFA to < 500 mg l^{-1} (Figure 6.7a and b). An attempt was made on day 476 to increase OLR in F9 and F10 to 4 g VS l^{-1} day^{-1} (Figure 6.3b). The higher OLR was achieved after 21 days in both digesters; however, there was a divergence of performance after about 40 days. Digester F10 started accumulating VFA rapidly and daily feeding had to be ceased on day 551 (Figure 6.7b), whilst F9 continued to operate normally without signs of upset. After feeding stopped in F10 for 10 days, it appeared that operational parameters such as pH and IA: PA ratio showed an improvement which was accompanied by a fall in acetic acid concentration from about 10000 mg l^{-1} to 2000 mg l^{-1}, propionic acid, however continued to increase (Figure 6.7b). Feeding was restored to F10 on day 560 and this was followed by an immediate deterioration in performance. Extra trace elements Mo and W were supplemented on day 609 into both F9 and F10 in attempt to recover the digestion balance, however no apparent effect was observed.

There was no obvious reason that could account for the divergence of digesters F9 and F10; however it seemed unlikely that this had been caused by a lack of any trace element component. Even after additional supplementation Mo and W no improvement was seen, despite this combination having successfully improved performance in digesters operating at even higher OLRs. It is possible that digestion failure in F10 was due to simple human error either as a result of an omission or inclusion of an essential or inhibitory material.

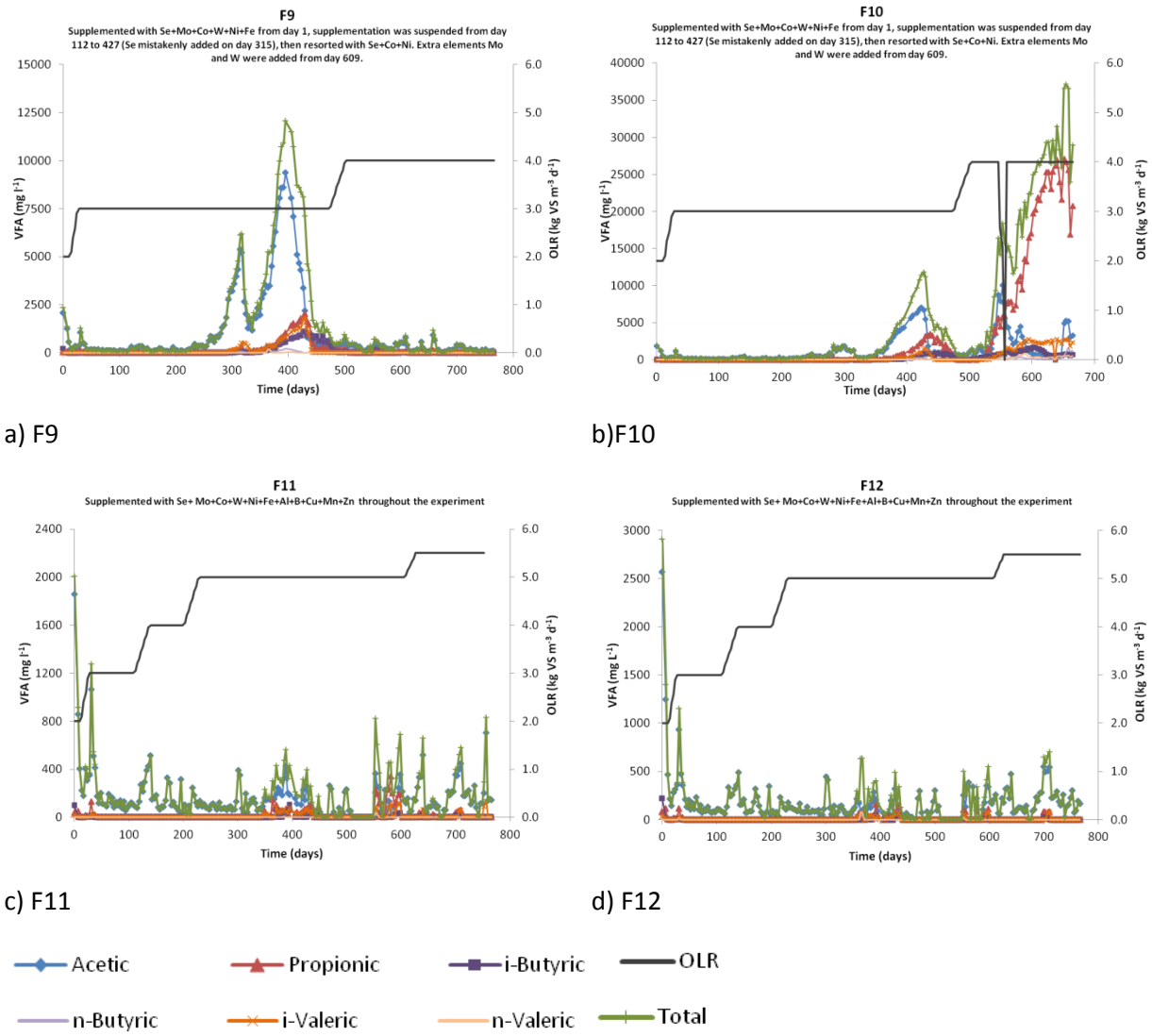
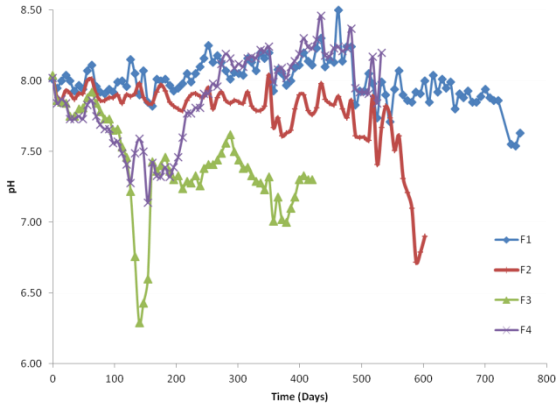
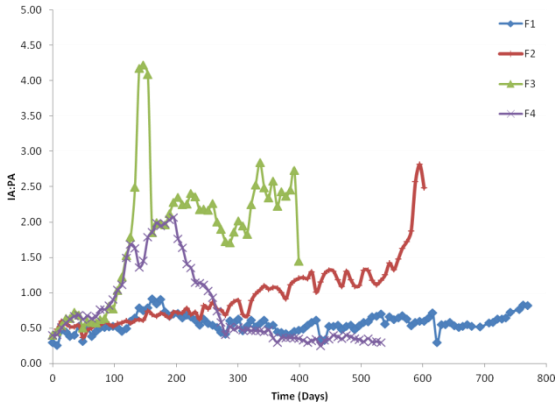


Figure 6.7. VFA profile in digester F9-12

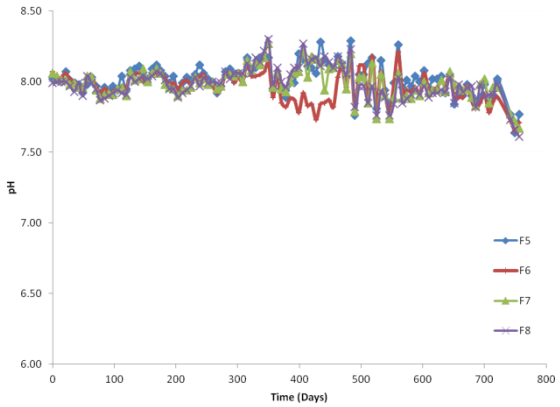
Digester F11 and F12 supplemented with full suite of TE performed optimally over the whole trial without encountering any digestion instability. The concentration of each VFA monitored never exceeded 200 mg l^{-1} in the pair of digesters supplemented with Se, Mo, Co and W (F7 and 8) and in the pair of digesters with full trace element supplementation (F11 and 12), as shown in Figure 6.6c and d; Figure 6.7 c and d.



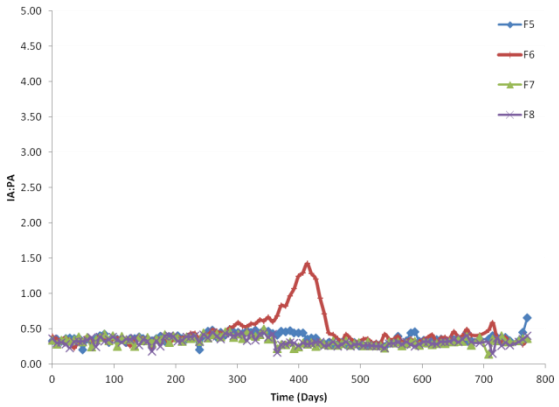
a) pH in F1-4



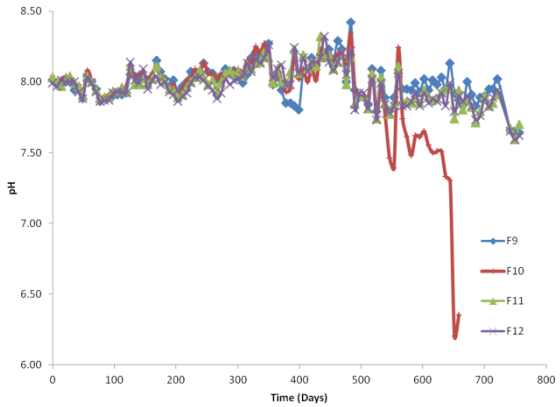
b) Intermediate : Partial alkalinity in F1-4



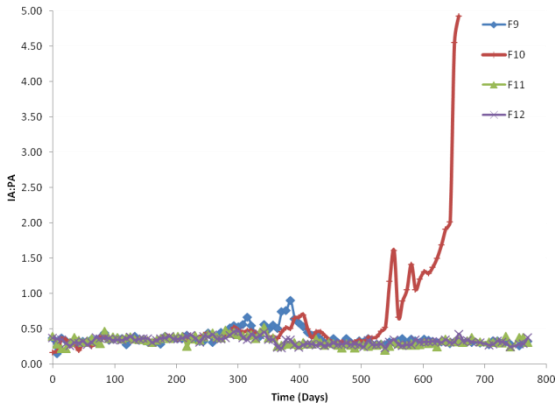
c) pH in F5-8



d) Intermediate : Partial alkalinity in F5-8



e) pH in F9-12



f) Intermediate : Partial alkalinity in F9-12

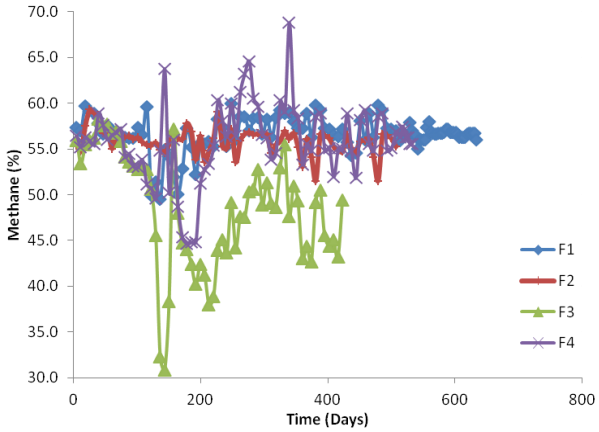
Figure 6.8. pH and alkalinity in control and TE supplemented digesters

Other digestion performance parameters are shown in Figure 6.8. The pH of the control digesters F3 and F4 in which the loading was increased had declined to less than 7 by the time that feeding ceased. This decline is reflected in the change in the ratio of intermediate

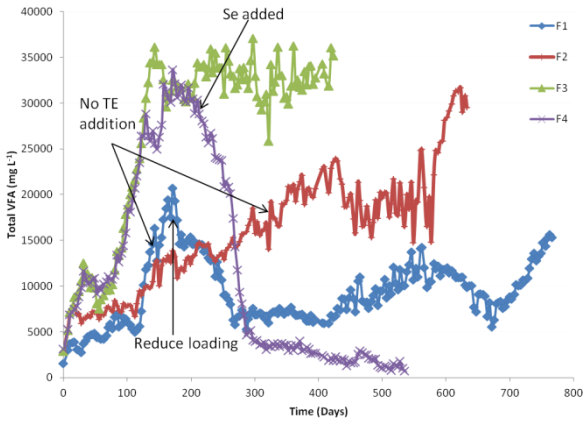
alkalinity: partial alkalinity (IA:PA), which climbed to around 2.75 (Figure 6.8b), indicating both the accumulation of VFA and the development of digestion instability (Ripley et al., 1986). The pH in the lower loaded controls showed a gradual decline over the trial period and a corresponding increase in the IA:PA ratio (Figure 6.8a and b), despite the reduction in loading. The pH in the digesters receiving TE supplements remained around 8 over the trial period (Figure 6.8c and e), and the IA:PA ratio in these digesters remained below 0.5 (Figure 6.8d and f). The exception was the digester from which trace element supplementation was removed. In this digester the IA:PA ratio was seen to increase slightly from day 300, and then reduced slightly when a single dose of Se was accidently added on day 315. The ratio rose after this event, peaking at about day 390; and then fell again when trace element supplementation to this digester was deliberately resumed on day 427 (Figure 6.8f).

6.3.3. Effects of Trace Element Supplementation on the Performance and Stability of Food Waste Digesters

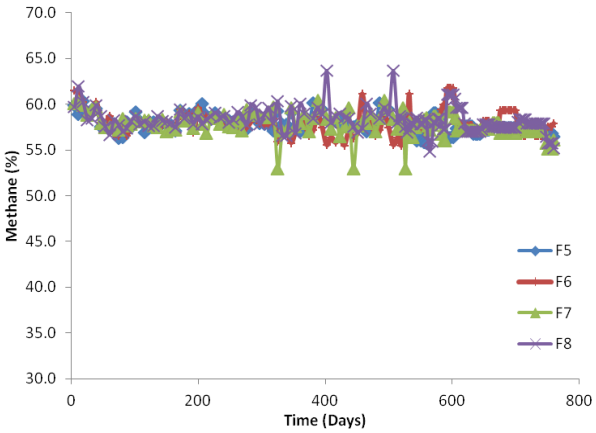
The trace element supplementation significantly improved the digestion stability and process efficiency. During the stable stage of the digestion (day 81 to 503), there was a 3% difference in the average biogas methane percentage in the control digesters at 55% compared to 58% for those supplemented with TE (Figure 6.9a, c and e).



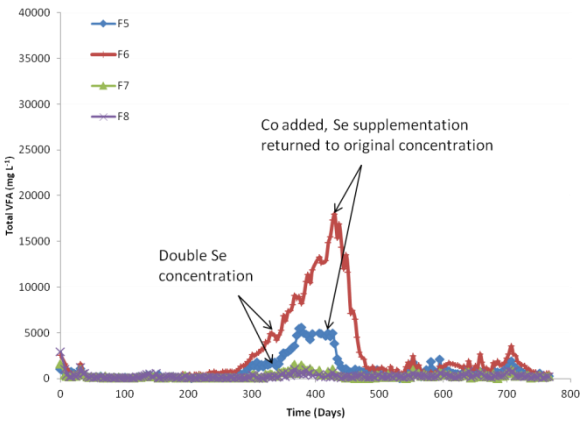
a) F1-4 methane percentage in biogas



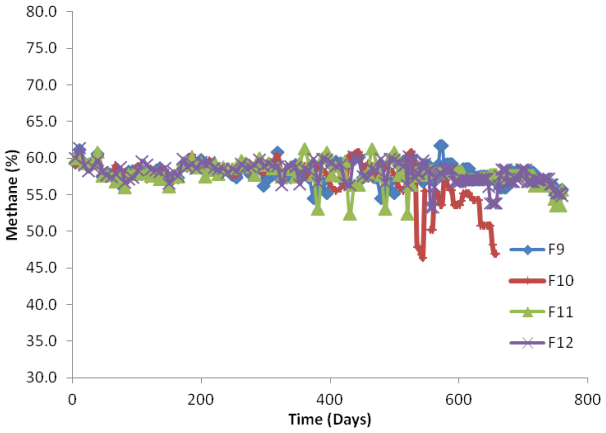
b) F1-4 total VFA profile



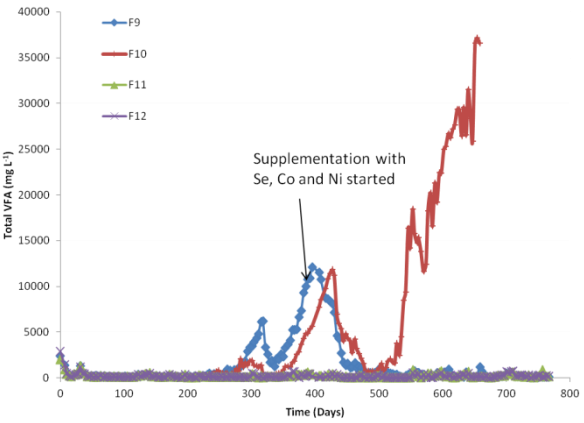
c) F5-8 methane percentage in biogas



d) F5-8 total VFA profile



e) F9-12 methane percentage in biogas



f) F9-12 total VFA profile

Figure 6.9. Biogas methane percentage and total VFA profiles in TE supplemented and control digesters

Figure 6.9b, d and f show the total VFA profile in the digesters and provide some explanations for changes seen in the other digestion performance parameters.

The inoculum used in the semi-continuous food waste digestion was already acclimated to food waste, having previously been fed on the same source segregated material in a 75-litre working capacity digester for a period of over a year, albeit at a low OLR. The digesters to which TE supplements were added showed a rapid reduction in the VFA that was already present in the inoculum digestate. The non-supplemented controls F1-4 showed an initial increase in VFA corresponding to the load increase. This was much more severe in the control in which the load was gradually increased to 3 g VS l^{-1} day^{-1}, and when feeding stopped on

day 120 the total VFA concentration in this digester had risen to more than 30000 mg l^{-1}. The lower-loaded control which maintained good SBP showed a gradual increase in total VFA concentration over a 400-day period, which reached 24000 mg l^{-1} before falling slightly and stabilising at around 20000 mg l^{-1}. In both cases the increase in VFA corresponded to changes in the IA:PA ratio, providing further support for this ratio as a rapid and reliable indicator of the onset of digester instability due to VFA accumulation.

The digesters receiving multiple TE supplements including Se and Co as part of the mix showed stable digestion throughout the trial period, with VFA concentrations never exceeding 500 mg l^{-1}. A trace element supplementation experiment by Feng et al. (2010) using a central composite design for digesters receiving industrial food wastes found that addition of Se and W increased methane yield as well as maintaining low VFA concentrations. No additional requirement for Co was found at the OLR used, and it may be that this is necessary only at higher loadings, as in the present study. Their research also indicated that supplementation with Ni, B or Mo was not essential; this is in agreement with the current work where food waste was considered to have sufficient concentration of these elements.

The concentration of trace elements in the digesters was analysed on day 112. This was at the time when trace element addition to digesters F9 and 10 ceased, and when the organic loading rate to the other digesters receiving trace element supplementation increased from 3 to 4 g VS $l^{-1}day^{-1}$. Samples were again taken for trace element analysis on day 301 when the digesters with trace element supplementation had been running at an OLR of 5 g VS l^{-1} $day^{-1}$for 74 days (around 2 HRT). The trace element concentration in the pair of digesters where supplementation of Se, Mo, Co, W, Fe and Ni ceased was monitored at shorter time intervals, and the duplicate digesters were sampled separately to investigate the repeatability of the experiment.

It can be seen from Table 6.7 that selenium and cobalt had a very low concentration in the control digesters (0.04~0.08 mg l^{-1}) and the supplementation significantly increased the quantity in the digestate. Molybdenum had a slightly higher concentration in the control digesters (0.08~0.17 mg l^{-1}), and the additional dosing doubled its concentration in the

digestate. No clear difference can be seen in Al, B, Cu, Fe, Mn, Ni, and Zn concentration in all digesters, mainly because food waste has a relatively high total concentration of these elements compared with the level of supplementation.

Table 6.7. Trace element concentration in TE supplemented and control digesters (mg l^{-1})

Digesters	Day	**Al**	**B**	**Co**	**Cu**	**Fe**	**Mn**	**Mo**	**Ni**	**Se**	**Zn**
F1-2 Average	112	26.4	2.01	0.038	2.23	79.7	10.4	0.168	1.10	0.044	3.62
	301	31.8	2.13	0.035	1.79	39.3	14.5	0.210	1.27	0.073	5.12
F3-4 Average	112	23.2	1.98	0.057	1.82	50.3	10.2	0.083	0.438	0.044	3.72
F3	301	33.5	2.06	0.033	2.02	41.2	15.5	0.200	0.855	0.064	5.39
F4 (Se supplemented)	301	53.1	2.29	0.033	1.89	40.5	13.5	0.184	1.11	0.298	4.99
F5-6 Average	112	32.3	2.81	0.048	2.58	56.1	13.9	0.339	1.08	0.297	5.26
	301	26.6	1.80	0.027	1.29	25.9	14.6	0.297	1.35	0.328	4.67
F7-8 Average	112	32.1	2.48	0.644	2.26	48.9	12.8	0.276	0.616	0.263	4.62
	301	27.3	2.08	0.765	1.36	23.8	16.0	0.279	0.536	0.341	4.92
F9 (TE diluted out period)	112	27.5	2.48	0.620	2.05	56.3	12.3	0.319	1.44	0.271	4.56
	154	30.5	1.93	0.522	1.77	39.8	12.4	0.305	1.46	0.251	4.92
	196	27.3	2.26	0.346	1.68	34.3	13.1	0.258	1.20	0.207	5.02
	245	30.3	2.05	0.212	1.53	30.7	13.6	0.222	0.873	0.150	4.94
	301	33.1	2.40	0.158	1.59	35.2	16.6	0.296	1.72	0.139	5.36
F10 (TE diluted out period)	112	29.4	2.33	0.614	2.02	50.6	11.4	0.299	1.39	0.269	4.16
	154	27.4	1.88	0.532	1.87	40.0	11.7	0.334	1.46	0.283	4.68
	196	29.4	1.98	0.374	1.71	35.1	13.0	0.289	1.21	0.191	4.97
	245	30.3	2.09	0.220	1.57	30.4	13.5	0.212	0.778	0.165	4.84
	301	31.3	2.24	0.161	1.58	33.2	15.8	0.278	1.46	0.165	5.08
F11-12 Average	112	29.3	2.21	0.605	2.26	59.3	12.5	0.324	1.62	0.297	4.34
	301	30.8	2.15	0.835	1.82	58.5	17.4	0.780	1.49	0.467	5.13

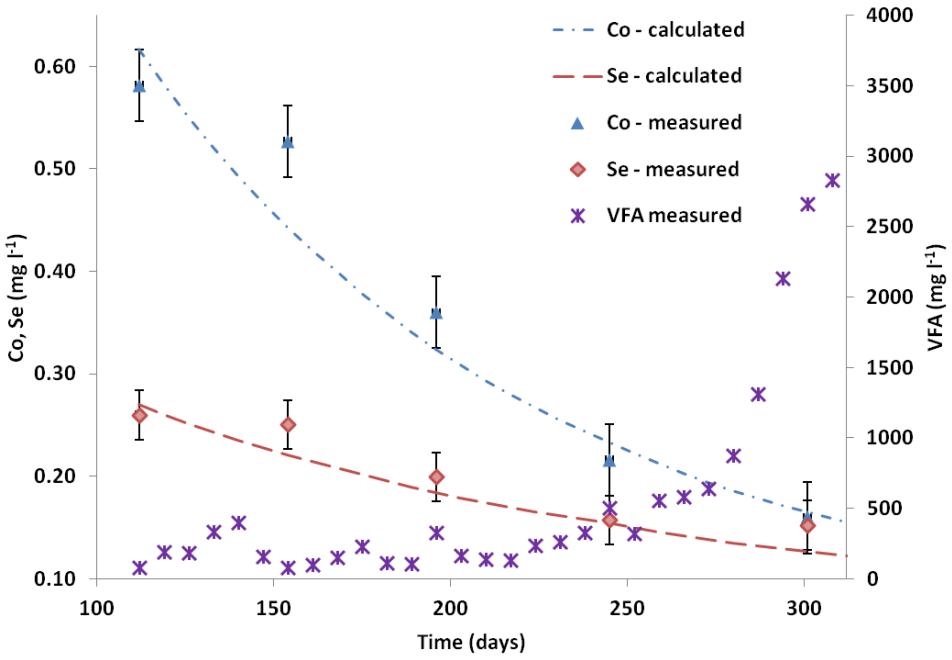


Figure 6.10. Measured and calculated Se and Co concentration and total VFA in digester F9 and F10 after cessation of TE supplementation (Measured values are average of duplicate digesters with error bars showing range of variation).

Figure 6.10 shows the dilute-out curve for the digester in which TE supplementation ceased on day 112. This was modelled assuming the digester to be a continuously stirred tank reactor (CSTR) with a hydraulic retention time calculated from the working volume and the wet weight of food waste added each day (assuming a density of 1 kg l^{-1}). Digestate samples were taken for TE analysis over this time and the results for Se and Co are plotted in Figure 6.10, showing good agreement with calculated values. The total VFA concentration is shown on the same graph, and the limiting concentrations of the two elements are taken to be at the point when VFA concentration curve started rising upwards. Based on this, the required concentrations of Se and Co were estimated to be 0.16 and 0.22 mg kg^{-1} (WW basis) feed material at moderate loading of 3 g VS $l^{-1}day^{-1}$; these concentrations may need to be increased at higher loadings. The concentrations of both Se and Co in the food waste used as substrate in the trials were below these critical thresholds. These values would also account for the high VFA concentrations that have been observed in a full-scale digester treating food waste from the same source (Zhang et al., 2012) and in earlier pilot-scale and laboratory trials.

The results from semi-continuous and batch trials show that Se is an essential trace element in food waste digestion. This requirement provides circumstantial evidence to support the original hypothesis that under high ammonia concentrations hydrogenotrophic methanogenesis may be the principle route to methane formation. This was confirmed by the results from FISH analysis that showed that by day 316 no acetoclastic methanogens could be identified in samples from any of the digesters; by this time the ammonia concentration was above 5000 mg l^{-1} in all digesters (Banks et al., 2012). The results also confirmed previous observations that in food waste digesters without trace element supplementation an accumulation of VFA occurred, and the main component in this accumulation was propionic acid, as can be seen in the VFA profile of the control digesters (Figure 6.5). The VFA profile showed that initially acetic acid was predominant, with propionic acid and longer chain length VFA at low concentrations as expected. The build-up of propionate can be seen from around day 100 and from that time becomes the predominant VFA.

The non-reversible accumulation of propionic acid was postulated to occur because of a deficiency of the trace elements required for synthesis of the enzymes needed in syntrophic hydrogenotrophic methane production, particularly the formate dehydrogenase required for formate oxidation. The experimental results do not, however, conclusively prove this original hypothesis as the oxidation of propionic acid, with its uneven carbon chain length, produces a mixed product of acetate, CO_2, H_2 and formate (Mueller et al., 2010), with side-reactions to produce butyrate and higher-chain fatty acids (De Bok et al., 2001; Stams et al., 1998). The enzymes required for propionic acid oxidation may themselves require the trace elements Se, Mo, and W (De Bok et al., 2003; Mueller et al., 2010; Worm et al., 2011). It has long been recognised, however, that the syntrophic degradation of propionate can be inhibited by a product-induced feedback inhibition (Dong et al., 1994; Fukuzaki et al., 1990; Kus and Wiesmann, 1995). The experimental results presented suggest that in this particular case it is unlikely to be triggered by H_2. The maximum partial pressures of H_2 under which syntrophic propionate and acetate oxidisation can take place are in the same range (Schink, 1997) and as there is no accumulation of acetate its oxidation does not appear to be inhibited. It is possible that formate is the trigger for inhibition, but the concentration range at which this occurs has

rarely been studied (Schink, 1997), whereas the partial pressure at which H_2 has an effect is well known (Cord-Ruwisch et al., 1997).

Although most microorganisms involved in interspecies electron transfer can exchange hydrogen with formate and vice versa, and the standard redox potential of both electron carrier systems (H^+/ H_2 and CO_2/formate) is nearly identical (Thauer et al., 2008), the conversion between them requires formate dehydrogenase (FDH) which has been reported to require Se, Mo and W (Andreesen and Ljungdahl, 1973; de Bok et al., 2003; Worm et al., 2011). The results presented cannot confirm that the Selenium was exclusively required for this enzyme system, but it is clearly vital to the proper functioning of this pathway. Oxyanions are also required for formyl-methanofuran dehydrogenase, and Se for hydrogenase and heterodisulphide reductase (Müller, 2003; Thauer et al., 2008; Stock and Rother, 2009; Zhu and Tan, 2009).

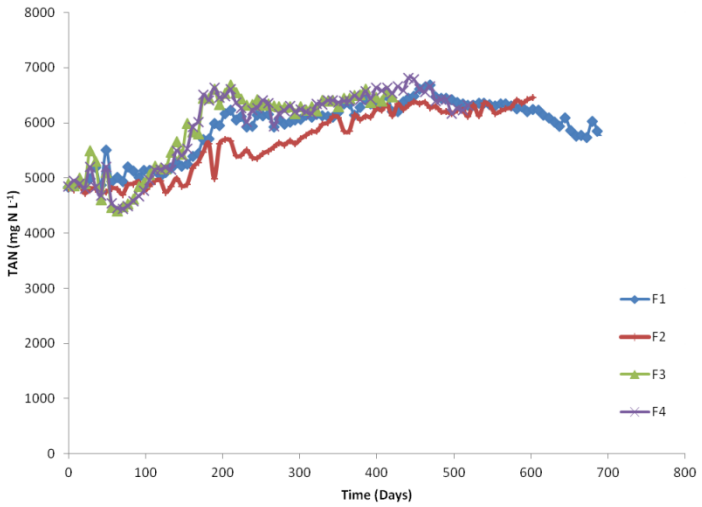
The trace elements requirement for syntrophic hydrogenotrophic methanogenesis is quite different to that required when the acetoclastic methanogenic pathway is predominant. When this is the case Co, Ni, Fe are essential in the formation of CO dehydrogenase, acetyl-CoA decarbonylase, methyl-H_4SPT:HS-CoM methyltransferase, methyl-CoM reductase and other enzymes (Ferry, 1992b; Kida et al., 2001). These three cations, however, remain essential in the syntrophic acetate oxidisation by the reverse Wood-Ljungdahl pathway and the hydrogenotrophic route (Thauer et al., 2008; Zhu and Tan, 2009). At the lower loadings there appear to be sufficient trace elements to supply the needs of the enzymes in this pathway; but it is clear that at the higher loading used in these experiments cobalt also becomes limiting, although this was not apparent until around day 280.

It can be seen that once coupled syntrophic acetate oxidisation and hydrogenotrophic methanogenesis are established in a non-competitive environment with the necessary trace element supplementation then concentrations of all species of VFA can remain low. Supplementation with Se and Co allowed the OLR on the system to be increased to 5 g VS l^{-1} day^{-1} resulting in a higher specific methane yield and almost three times the volumetric biogas production. In terms of a commercial digester treating food waste, this represents a significant

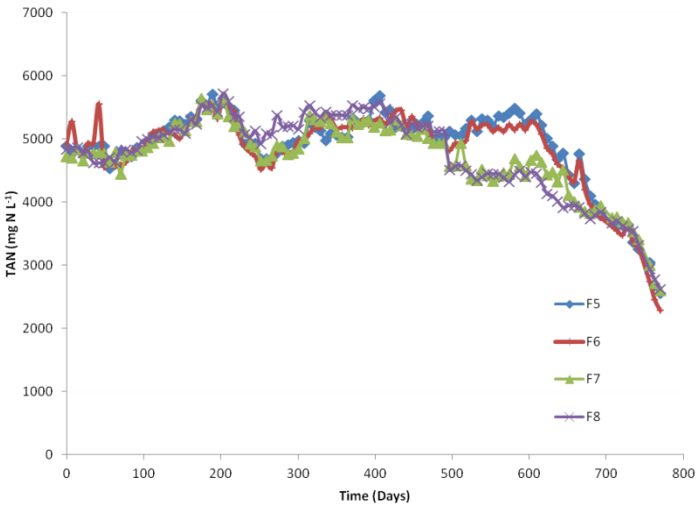
enhancement in performance as well as a reduced risk of process failure due to accumulation of VFA.

6.3.4. Ammonia Nitrogen Concentration Changes in Food Waste Digesters during the Prolonged Semi-Continuous Digestion Trial

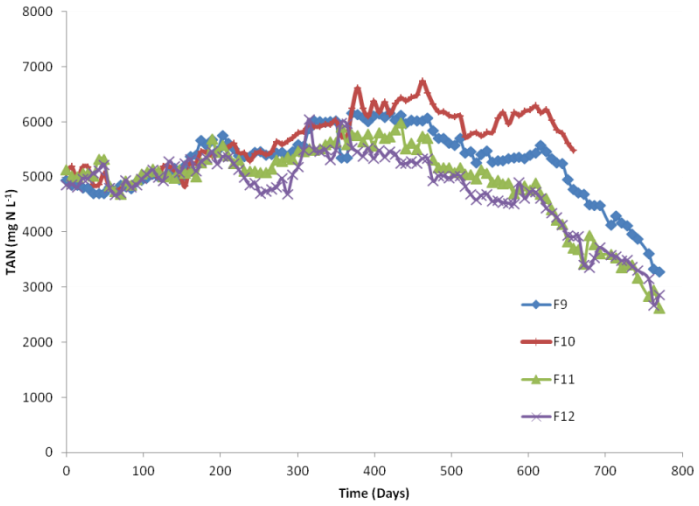
The ammonia nitrogen concentration in an anaerobic digester is of great importance with regard to the microbial population and operation stability, as discussed in section 2.3.1. When digestion is stable the TAN concentration is determined by degradable nitrogen content in the feedstock: in other words the ammonia nitrogen concentration in digestate is in equilibrium with the concentration of organic nitrogen in the substrate. This equilibrium is governed by the amount of nitrogen that can potentially be released in the form of ammonia (i.e. amino acid and protein), but under stable operation it will remain relatively stable. Over the prolonged period of semi-continuous food waste digestion, however, changes in the ammonia nitrogen concentration in the digester have been observed (Figure 6.11).



a) TAN in F1-4



b) TAN F5-8



c) TAN F9-12

Figure 6.11. Ammonia nitrogen changes over the semi-continuous trial period

The TAN concentration in the digesters at the start of the trial was around 5 g l^{-1} and in the control digester this gradually increased to a maximum of 6.1 g l^{-1}; a similar increase was seen in the digester from which TE supplementation was removed. TAN in the TE supplemented digesters showed an initial increase until the OLR increased from 4 to 5 g VS l^{-1} day^{-1}, at which point there was a small drop in concentration which then stabilised at around 5.4 g l^{-1}. Interestingly the TAN concentration in the digester F9 and F10 also dropped after TE supplementation was resumed. This decrease in the TAN concentration was unexpected as the concentration of TKN in the feedstock was relatively constant

There are no reports in the literature that describe a similar reduction in TAN, although Lindorfer et al. (2011) have recently shown a relationship between trace element supplementation and biological fixed nitrogen. It is therefore possible that the reduction in TAN may represent an increased uptake by the microbial biomass in the digestate, which would increase the TKN associated with that biomass. Considering the degree of change, however, where the TAN dropped from around 5.5 to below 3 g NH_3-N kg^{-1}, this biological fixation may contribute only limited amount to this reduction.

Miron et al. (2000) reported a similar TAN reduction in a CSTR design digester and explained this as being the result of a shortened HRT which affected hydrolysis of protein. In the same study the authors also postulated that precipitation of ammonium as struvite ($MgNH_4PO_4 \cdot 6H_2O$) may also contribute to the decrease in the NH_4^+–N concentrations. This is not likely in the current study, however, due to the limited concentration of magnesium presented in food waste. The reduction due to a washout of protein at the shorter HRT is also unlikely as even at an OLR of 5 g VS l^{-1} day^{-1} conversion of substrate through to methane was apparently uninhibited, as there was no accumulation of methanogenic precursors and the VS destruction remained high.

The TKN of all digestates was determined on day 616 (Table 6.8) and showed very similar values in the range of 10.45±0.3 g N kg^{-1} WW, in contrast to the significant TAN variations seen between non TE control digesters operating at low loadings and the high loading TE supplemented digesters.

Table 6.8. Comparison of TKN and TAN concentration between digesters

Digester	**TAN (g NH_3-N kg^{-1})**	**TKN (g N kg^{-1})**	**TAN / TKN**
F1	6.14	10.29	59.7%
F5	5.22	10.42	50.1%
F6	5.07	10.16	49.9%
F7	4.64	10.43	44.5%
F8	4.31	10.41	41.4%
F9	5.57	10.75	51.8%
F10	6.17	10.40	59.3%
F11	4.69	10.52	44.6%
F12	4.61	10.75	42.9%

The reasons for the drop in TAN therefore remain unanswered: the results from the current work do not allow a full nitrogen mass balance to be completed and therefore are unable to elucidate this further.

6.4. Radioactive Tracer Experiment to Determination the Methanogenic Pathway in Food Waste Digesters

The method followed was that developed and reported in Chapter 4. High ammonia digestate samples were taken from food waste digesters (F1, F5, F6, F7, F8, F9, F11 and F12) between days 760-780 (TAN in the range of 3.5-5.3 g NH_3-N kg^{-1} WW). A low nitrogen digestate sample (TAN = 1.58 g NH_3-N kg^{-1} WW) taken from a digester treating municipal wastewater biosolids (Millbrook Wastewater Treatment plant, Southampton, UK) was used for comparison.

For those digesters operating in duplicate, 50 g of the raw digestate from each one of the duplicate pair was mixed together as a representative sample. Where digesters had deviated in their behaviour these were treated separately. The isotope labelling experiment was therefore performed on samples from F1, F5+F6 (50:50), F7+F8 (50:50), F9, F11+12 (50:50) and Millbrook anaerobic digester sludge.

After incubation, carbon dioxide and methane labelled with radioactive isotope produced from each digestate sample were sequentially captured in NaOH solution. The radioactivity of each was subsequently determined by scintillation counting and despite very high ionic strength and alkalinity a transparent solution was formed and a counting efficiency above 90% was achieved in all samples. Any ^{14}C remaining in the digestate/culture medium mix in the culture bottles was also accounted for following a centrifugation step

Digestate from the wastewater biosolids digester was used in this experiment as a control as it has a low TAN concentration compared to food waste digestate. It is also widely accepted, based on some very early studies, that sewage sludge digestion is dominated by acetoclastic methanogenesis and this contributes about 70% of the total methane production. This conclusion was first reached by Jeris and McCarty (1964) when studying cultures from

domestic sewage sludge and using ^{14}C labelled CO_2 (in the form of bicarbonate) as tracer. The conclusion was based on the assumption that all methane not originating from the reduction of labelled CO_2 was formed from acetic acid. Smith and Mah (1966) confirmed the findings of the previous study using [2-^{14}C] acetic acid and domestic sewage sludge samples: by calculating the turnover rate of labelled acetic acid, acetoclastic methanogenesis was estimated to account for 73% of methane produce by the sludge.

During stable food waste digestion the ammonia nitrogen concentration fluctuated in the range 5-6 g l^{-1}. It was postulated that under such high TAN, the methanogenic pathway shifts to being hydrogenotrophic due to the higher tolerance of hydrogenotrophic methanogens to ammonia. If this assumption is valid, a much higher quantity of C-14 labelled carbon dioxide molecules would be detected in the biogas produced compared to those from low nitrogen sewage sludge digestate where the acetoclastic route is thought to predominate.

The results from the C-14 acetate labelling experiment (summarised in Table 6.9) indicate a route to methane formation by the hydrogenotrophic methanogens and this strongly supports the hypothesis put forward.

For the sewage sludge samples, only 5.7% and 5.3% of the total ^{14}C in labelled acetate that took part in methanogenesis flowed into CO_2. The $^{14}CO_2$:$^{14}CH_4$ ratios were 0.067 and 0.056, respectively, which indicates the major methanogenic pathway was acetoclastic. In the food waste digestate samples tested, between 66-74% of the total ^{14}C in labelled acetate went into CO_2 with $^{14}CO_2$:$^{14}CH_4$ ratios in the range of 1.94 to 3.07, therefore strongly suggesting the dominant pathway in all food waste digesters tested was via syntrophic methanogenesis.

Table 6.9. Results from ^{14}C acetate labelling experiment

Sample	Subsample	Eff.* (%)	^{14}C (kBq)	Total ^{14}C recovered (kBq)	Total ^{14}C added (kBq)	$^{14}CO_2$:$^{14}CH_4$	Recovery Rate
Sewage sludge control-1	sludge	94.5	0.45				
	CO_2	94.02	0.57				
	CH_4	94.05	9.40	10.41	10	0.06	104%
Sewage sludge control-2	sludge	94.51	0.33				
	CO_2	93.88	0.48				
	CH_4	94.09	8.57	9.38	10	0.06	94%
F1	Digestate	32	8.69				
	CO_2	94.37	0.38				
	CH_4	94.42	0.13	9.19	10	3.05	92%
F1 Duplicate	Digestate	32.5	9.17				
	CO_2	94.6	0.39				
	CH_4	95.02	0.13	9.70	10	2.93	97%
F5+6	Digestate	46.2	0.26				
	CO_2	95.68	3.33				
	CH_4	94.31	1.34	4.93	5	2.48	99%
F5+6 Duplicate	Digestate	46.4	0.37				
	CO_2	94.39	3.13				
	CH_4	94.36	1.32	4.82	5	2.38	96%
F7+8	Digestate	45.2	0.83				
	CO_2	94.98	2.90				
	CH_4	95.27	1.15	4.88	5	2.52	98%
F7+8 Duplicate	Digestate	56.17	1.12				
	CO_2	94.38	3.10				
	CH_4	96.5	1.16	5.38	5	2.67	108%
F9	Digestate	47.56	1.14				
	CO_2	95.56	3.32				
	CH_4	95.48	1.28	5.74	5	2.60	115%
F9 Duplicate	Digestate	46.15	1.16				
	CO_2	95.49	3.19				
	CH_4	95.5	1.31	5.67	5	2.43	113%
F11+12	Digestate	83.13	4.90				
	CO_2	93.91	3.89				
	CH_4	93.90	2.00	10.79	10	1.94	108%
F11+12 duplicate	Digestate	83.71	4.74				
	CO_2	93.91	4.71				
	CH_4	93.77	2.03	11.48	10	2.32	115%

* Counting efficiency

The above results are in strong agreement with existing literature. Karakashev et al. (2006) carried out an extensive survey on methanogenesis in full-scale digesters fed on high ammonia

manure and low ammonia wastewater sewage sludge using a similar radioactive isotope method. Selected results ($^{14}CO_2$:$^{14}CH_4$ ratios) and the relevant operational parameters in those digesters are list in Table 6.10.

Table 6.10. Isotopic tracer results from manure and wastewater sludge digesters. (Karakashev et al. 2006)

ID	Digester feed type	Temp (^{o}C)	TAN (g l^{-1} ±SD)	$^{14}CO_2$:$^{14}CH_4$ (±SD)
M1	Manure	38	5.6±0.12	2.9±0.18
M2	Manure	37	4±0.1	2.75±0.11
M3	Manure	52.5	2.6±0.08	2.45±0.15
M4	Manure	37	4.5±0.01	2.2±0.14
M5	Manure	52	2.2±0.09	2.21±0.16
M6	Manure	37	4.4±0.11	1.9±0.18
S1	WW sludge	55	1.44±0.05	0.11±0.007
S2	WW sludge	37	1.00±0.04	0.08±0.003
S3	WW sludge	37	1.50±0.03	0.065±0.004
S4	WW sludge	37	0.50±0.001	0.04±0.002
S5	WW sludge	37	0.86±0.03	0.025±0.001

In other work at the University of Southampton (Banks et al., 2012), researchers using molecular biology methods (FISH and PCR) found that *Methanosaetaceae,* a known acetoclastic species, appeared exclusively as the dominant methanogen in the sewage sludge digestate sample. Using the same food waste digesters a sample taken on day 316 showed that the methanogenic groups in all the digesters (controls and TE supplemented) were members of the order *Methanoimicrobiales,* no acetoclastic methanogens could be observed in samples from any of the digesters. This phylogenetic study further supported the conclusion drawn from the C-14 tracer experiment. It therefore appears conclusive that, under a high ammonia concentration, food waste digestion adopts a syntrophic methanogenic pathway as the major route for methane production.

CHAPTER 7

Conclusions and Recommendations

7.1. General Conclusion

- The study elucidated trace element requirements for the anaerobic digestion of two distinctive but related waste streams: carbohydrate rich vegetable waste and high nitrogen source segregated food waste. Experimental data showed supplementation of trace elements, at a suitable concentration, is critical for maintaining operational stability and digestion efficiency in both cases.

- The metabolic pathways leading to methane production and their underpinning science were extensively reviewed and showed that enzyme structures involved in syntrophic methane production e.g. FDH, CODH etc were complex and had a dependency on trace elements

7.2. Vegetable Waste Digestion Trials

- Semi-continuous test on vegetable waste showed it could not be used as the sole substrate for anaerobic digestion without suitable trace elements supplementation. Digesters failed after approximately 3 HRT as a result of the build-up of volatile fatty acids and reduction in pH. These changes could not be compensated for in the long term by the use of chemical buffer solutions.

- The use of a trace element solution made to the Gonzalez-Gil et al. (2001) recipe allowed stable digestion at a low loading of 2 g VS l^{-1} day^{-1} but the recommended dose may not have been sufficient to satisfy trace element requirements when higher loadings were applied. The formulation was also shown to be deficient in tungsten and supplementation with this element was required even at low OLR.

- An improved TE recipe excluding EDTA and containing a higher concentration of elements allowed stable operation at OLR of 4 g VS L^{-1} day^{-1}. The use of yeast extract improved gas production by adding a further carbon source with a high BMP value, and also possibly by contributing additional cobalt and selenium.

- Use of card packaging and cattle slurry as co-substrates proved to be an effective means of restoring stable operating conditions.

- The research showed that anaerobic digestion was an ideal method for energy recovery from vegetable wastes with high water content. 68.6% of the measured calorific value could be recovered in the form of methane. Operation of the digestion process was feasible providing careful attention was paid to supplementation of the feedstock material with trace elements so as to maintain the correct concentration within the digester.

7.3. Food Waste Digestion Trials

- In non-TE supplemented digesters VFA accumulation indicated inhibition of either propionate oxidising bacteria or formate reducing hydrogenotrophic methanogens, resulting in loss of syntrophic interspecies electron transfer. In either case propionic acid will accumulate. The work clearly demonstrated that suitable trace elements supplementation could prevent this, and is required for interspecies electron transfer at high ammonia concentrations. Supplementation allowed stable operation at higher OLR with enhanced methanogenic efficiency.

- A radioisotope experiment conclusively proved the dominant metabolic pathway for methane formation in food waste digesters at high ammonia concentrations was via the syntrophic route. This confirmed work undertaken by others on identification of methanogenic groups which showed a loss of acetoclastic methanogens.

- A reliable gas chromatographic method was developed and validated for quantification of palmitic, stearic and oleic acid without the requirement for further sample methylation. During repetitive analytical runs, the relative standard deviations (RSD) of the results were satisfactory. Good LCFA recoveries were shown using a spike addition of LCFA to digestate sample. The simplicity of the sample preparation procedure reduces time for analysis therefore making the routine analysis of LCFA in digestate samples more realistic as a monitoring tool. Digestate samples from food waste digesters at different lipid loads and with and without trace element addition showed LCFA concentrations below values considered inhibitory in other studies, but concentrations of palmitic and stearic acid were lower in the TE supplemented digester in proportion to the lipid loading applied than they were in the unsupplemented control.

- Total ammonia nitrogen (TAN) decreased sharply in food waste operated at high loading rates. The reason for this was uncertain and requires further investigation.

7.4. Recommendations

- Digesters treating vegetable waste have a high hydrolysis rate which is intrinsic to this type of waste stream due to the material being rich in carbohydrates. The rate limitation appears to be preventing adequate methanogenic activity and this can be improved by applying TE to stimulate this and stabilise the carbon flow within the process. It is therefore recommended that to ensure stable operation of digesters at an OLR of 4 g VS L^{-1} day^{-1}a non-complexed trace element supplement is used.

- Co-digestion of vegetable waste with co-substrates which are complementary to achieve optimal C/N ratio is recommended, providing these co-substrates are readily available and do not introduce inhibitors to the system.

- In order to maintain a stable food waste digestion system the minimal required concentrations of Se and Co were estimated to be 0.16 and 0.22 mg kg^{-1} fresh matter feed material at moderate loading of 3 g VS $l^{-1}day^{-1}$; these concentrations may need to

be increased at higher loadings. Supplementation with other additional TE species was not regarded as necessary from the results of the study. Application of full TE supplementation containing other trace elements is not harmful, but not essential.

7.5. Future work

- Further work is still needed to be certain at which point the addition of Se unblocks the metabolic route from propionic acid through to methane.

- This further work could focus on testing the response of different enzyme systems in response to TE additions e.g. critical enzymes such as FDH and CODH

- There is scope for further use of molecular biology techniques (such as DNA sequencing and quantitative PCR) to monitor the methanogenic population quantitatively before and after TE supplementation.

- Radioisotope methods offer a rapid and semi-quantitative method of monitoring changes in the methanogenic pathway in food waste digesters. This technique could be used to follow the transition in metabolic route from acetoclastic to hydrogenotrophic in response to increasing ammonia concentrations.

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APPENDIX

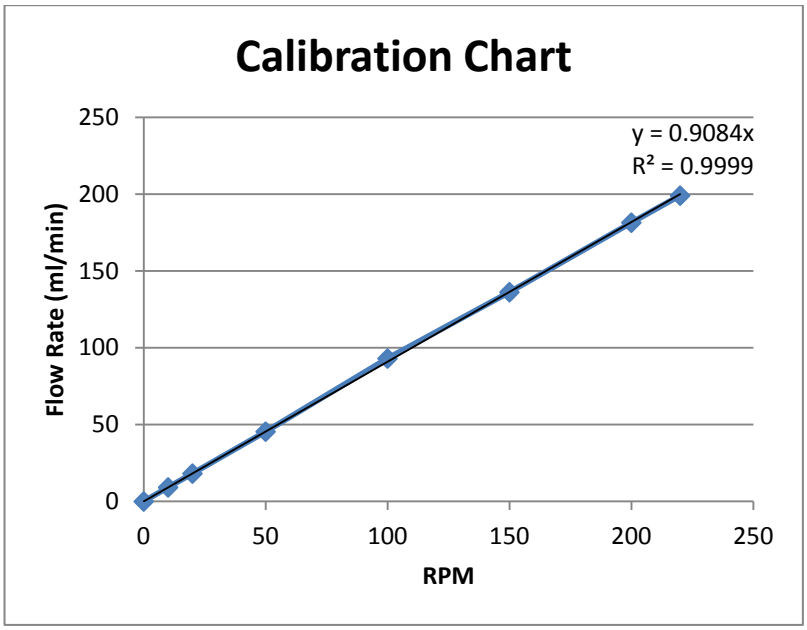
Appendix 1

Calibration data of a Watson Marlow 505 U peristaltic pump fitted with a 323 pump head and 3.2/1.6 mm (Bore/Wall) Marprene tubing.

Pump speed were set at 10, 20, 50, 100, 150, 200 and 220 RPM. Time and the water volume displaced from the measuring cylinder have been recorded. For each pump speed tested, 3 measurements were taken.

Pump speed (RPM)	Vol. (ml)	Time (Sec)	Flow Rate (ml/s)	Flow rate (ml/min)	Ave. from 3 measurement (ml/min)
10	31	203.98	0.152	9.1	
	21	136.22	0.154	9.2	
	28	182.63	0.153	9.2	9.19
20	39	129.32	0.302	18.1	
	31	102.48	0.302	18.1	
	29	95.11	0.305	18.3	18.18
50	48	64.04	0.750	45.0	
	55	72.86	0.755	45.3	
	67	87.17	0.769	46.1	45.46
100	69	44.83	1.539	92.3	
	67	42.91	1.561	93.7	
	74	47.71	1.551	93.1	93.03
150	75	33.05	2.269	136.2	
	78.5	34.66	2.265	135.9	
	78	34.26	2.277	136.6	136.22
200	69	22.48	3.069	184.2	
	71.5	23.85	2.998	179.9	
	64.5	21.46	3.006	180.3	181.46
220	79.6	23.72	3.356	201.3	
	85	25.61	3.319	199.1	
	79	24.1	3.278	196.7	199.06

Calibration chart was plotted below; the R^2 indicates very good linearity.



Appendix 2

Determination of pH

Principle of method:
pH is measured potentiometrically in the undiluted liquid sample or in the 1:2 (*V*/*V*) sample/water slurry for semi-solid or solid sample.

Apparatus:
1. pH meter with means for temperature compensation
2. Combination electrodes
3. Magnetic stirrer and Telfon-coated stirring bar
4. Plastic or glass containers, of sufficient capacity to accommodate the volume of the sample, deionized water and 10% air volume.

Reagents:
1. Buffer solutions: pH 4.0, 7.0 and 9.2.

Procedure:
1. Calibration of the pH-meter:
a. Calibrate the pH-meter as prescribed in the manufacturer's instruction;
b. Use at least two of the buffer solutions that bracket the expected pH of the samples and are approximately three pH units or more apart.

2. Liquid sample (when the aqueous phase constitutes at least 20% of the total volume of the sample):
a. Place the sample in a clean glass beaker using a sufficient volume to cover the sensing elements of the electrodes and to give adequate clearance for the magnetic stirring bar;
b. Stir the sample at a constant rate to provide homogeneity and suspension of solids;
c. Thoroughly rinse and gently wipe the electrodes prior to measuring pH of the samples;
d. Immerse the electrodes into the sample beaker;
e. Record sample pH to one decimal place after stabilisation is reached.

3. Semi-solid or solid sample:
a. Place a weight equivalent to 20 ml of the sample volume into a container;
b. Add 40 ml deionized water, secure the cap and mix for 1 h on the magnetic stirrer;
c. Stop stirring just before the measurement;
d. Immerse the electrodes into the settling suspension;
e. Record the pH when the meter has stabilized and report the result as pH (water 1:2).

References:
1. BS EN 13037:2000 Soil improvers and growing media – Determination of pH;
2. US EPA SW-846 9045D Soil and waste pH;
3. US EPA SW-846 9040C pH electrometric measurement.

Notes:
1. Samples should be analyzed as soon as possible;
2. If the waste is hygroscopic and absorbs all the deionized water, begin the experiment again using 20 ml of waste and 100 ml of deionized water;
3. Minimum stirring is required when measuring sample with high volatile components.

Appendix 3

Determination of Total and Volatile solids

Principle:
The test portion of sample is dried to constant mass in an over at 105 ± 5°C. The difference in mass before and after the drying process is used to calculate the total solids and the water content.

Then, the dried sample is heated in a muffle furnace at 550 ± 10°C. The difference in mass before and after the ignition process is used to calculate the content of VS and ash.

Apparatus:
1. Drying oven, capable of maintaining a temperature of 105 ± 5°C;
2. Electric muffle furnace, capable of maintaining a temperature of 550 ± 10°C;
3. Porcelain crucibles;
4. Dessiccator with active silica gel desiccant with indicator;
5. Analytical balance, with an accuracy of 0.1 mg.

Procedures:
1. Place the crucibles in the drying oven for a minimum of 30 minutes. Put them in the desiccator to ambient temperature. If the crucibles are brand new, place them in the muffle furnace at 550°C for 30 minutes to burn off any organic residue and then put in the desiccator to cool;
2. Weigh the empty crucible using a balance of accuracy of at least 0.1 mg. Record the weight (W_1);
3. Add the sample to the crucible to make up around 2/3 of the capacity of crucible. Weigh the loaded crucible and record the weight (W_2). At least triplicate analysis should be done for one sample;
4. Place the crucibles containing the sample in the drying oven until constant mass has been reached, typically overnight;
5. Cool the crucibles with dried samples in the desiccator and weigh. Record the weight (W_3);
6. Place the crucibles with dried sample in the muffle furnace for 2 hours at 550°C.
7. Cool the crucibles with ash in the desiccator and weigh. Record the weight (W_4);
8. Clean the crucibles by washing thoroughly in water. Rinse with deionized water and dry.

Calculation:

$$\%TS = \frac{W_3 - W_1}{W_2 - W_1} \times 100$$

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

and

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

References:
1. BS EN 12880:2000 Characterisation of sludges – Determination of dry residue and water content;
2. BS EN 12879:2000 Characterisation of sludges – Determination of the loss on ignition of dry mass;
3. BS EN 13040:2000 Soil improvers and growing media – Sample preparation for chemical and physical tests, determination of dry matter content, moisture content and laboratory compacted bulk density;
4. Soil improvers and growing media – Determination of organic matter content and ash.

Appendix 4

Determination of gas composition and volatile fatty acid by gas chromatography

Principle:
A gas chromatograph is an instrument for separating different components in a complex sample. It uses a flow-through narrow tube known as the column, through which different chemical constituents of a sample pass in a gas stream (carrier gas, mobile phase) at different rates depending on their various chemical and physical properties and their interaction with a specific column filling, called the stationary phase. As the chemicals exit the end of the column, they are detected and identified electronically.

Instrument analysis:

Test	Gas composition		Volatile fatty acid (VFA)	
GC model	GP-3800, Varian, USA		GC-2010, Shimadzu, Japan	
Standard	Standard biogas with 35% CO_2 and 65% CH_4		Mixture of VFAs in 10% of formic acid at concentrations of 50, 250, and 500 mg l^{-1} for each component	
Retention time (minutes)	CO_2	2.34	Acetic	6.33
	CH_4	3.27	Formic	6.92
			Propionic	7.32
			iso-Butyric	7.67
			n-Butyric	8.39
			iso-Valeric	8.86
			n-Valeric	9.64
			Hexanoic	10.81
			Heptanoic	11.94

Notes:
1. A gas sample should be measured as soon as it is taken;

2. For VFA measurement, the sample should be centrifuged at 13000rpm for 10 min. Then the supernatant is mixed with pure formic acid at the ratio of 9:1;

3. The concentration of each targeted component is positively proportional with the peak area it creates;

4. The detailed operation procedures are attached to the instruments.

Appendix 5

Elemental analysis for CHNSO

Principle:
The equipment used (elemental analyzer) operates for analysis of CHN, and also S, using a flash combustion in which a sample contained within a tin capsule is dropped into a combustion/reduction reactor held at 900°C. This short flash combustion is accomplished when the tin capsule is exposed to a gas flow temporarily enriched with ultra high purity oxygen. The resulting oxidation raises temperatures to higher than 1700°C. The encapsulated sample, depending on its composition, combusts generating one or more of these gases: N_xO_x, CO_2, H_2O, and SO_2 in the oxidation zone. Then N_xO_x is reduced to N_2 in the reduction zone. After passing the reactor, the gas mixture enters the gas chromatrographic column where the different components are time-separated and then measured by detectors.

Oxygen in solid sample is converted to carbon monoxide by pyrolysis at 1060°C in the presence of metallised carbon but with the absence of oxygen gas. The carbon monoxide is then separated from the other pyrolozates under steady state conditions, and measured as a function of thermal conductivity.

Instrument:
FlashEA 1112 Elemental Analyzer, Thermo Finnigan, Italy.

Application:
1. CHN analysis for solid sample;
2. S analysis for solid sample;
3. O analysis for solid sample;
4. CN analysis for liquid sample;
5. S analysis for liquid sample.

Analysis methods:
Shown in table A8.1. The detailed operational procedure is attached to the EA instrument.

Note:
EA analysis can be used to determine TOC of the solid sample:

$$TOC = A \times 10 - B \times (1 - \frac{VS}{100}) \times 10$$

where:
TOC = total organic carbon, mg/g;
A = carbon concentration in dried sample, %;
B = carbon concentration in ashed sample, %;
VS = VS,

Table A8.1. Elemental analyser – summary of methods and principle of technique used

		Analytical determination		
		CHN	S	O
Reactors	Configuration	*Oxidation zone:* Chromium oxide *Reduction zone:* Reduced copper *SO_2 removal:* Silvered cobaltous/cobaltic oxide	*Oxidation zone:* Copper oxide *Reduction zone:* Electrolytic copper	*Pyrolysis zone:* Nickel plated carbon
	Temperature (°C)	900	900	1060
Adsorption filters			*H_2O removal:* Magnesium perchlorate	*H_2O removal:* Magnesium perchlorate *Acid gas removal:* Soda lime
Gas chromatographic columns		Multiseparation column	Sulphur separation column	Oxygen separation column
Detector	Type	Thermal conductivity detector (TCD)	Flame photometric detector (FPD)	Thermal conductivity detector (TCD)
	Temperature (°C)	75	90	65
Standards		L-Aspartic acid; Atropine; Nicotinamide	Cystine; Methionine; Sulphanilamide	L-Aspartic acid; Atropine; Nicotinamide
Catalyst			Vanadium pentoxide	

Appendix 6

Determination of calorific value by bomb calorimetry

Theory:
Calorific value can be defined as the amount of energy released on burning by each unit of combustible mass. There are two types of calorific value (i.e., the gross calorific value and the net calorific value), and bomb calorimeter measures the gross calorific value.

The gross calorific value, also known as higher heating value (HHV), is the amount of energy released on burning by complete combustion of a mass unit of sample, at constant volume in an oxygen atmosphere, assuming that the final products of combustion consist of O_2, CO_2, SO_2, and N_2 in the gas phase together with water, that contained in the sample and that generated from the combined hydrogen, in liquid form.

The net calorific value, also known as lower heating value (LHV), is defined as the amount of heat released by combusting a specified quantity and returning the temperature of the combustion products to 150°C. LHV assumes the latent heat of vaporisation of water in the reaction products is not recovered. It is useful in comparing fuels where condensation of the combustion products is impractical, or heat at a temperature below 150°C cannot be put to use.

Both calorific values are related through the equation:

$$LHV = HHV_d - 2.442 \times (W + 9 \times H_d) \times 0.01$$

where,
LHV: the lower heating value of the sample, kJ/g;
HHV_d: the higher heating value of the dry sample, kJ/g;
W: the moisture percentage of the sample, %;
H_d: the hydrogen percentage of the dry sample, %;
2.442: the heat of vaporisation of water, kJ/g;
9: molecular weight ratio of water to hydrogen.

Principle:
A bomb calorimeter is a type of calorimeter used in measuring the heat of combustion in pure oxygen environment at high pressure. Electrical energy is used to light the sample. As the sample is burning, it will heat up the bomb vessel which is placed in the static polystyrene jacket. The temperature rise of the bomb vessel allows for calculating calorific content of the sample.

Apparatus:
1. CAL2k bomb calorimeter system: the filling station + bomb vessel + calorimeter + stainless steel crucible + cotton thread fuse;
2. Analytical balance with an accuracy of 1 mg.

Reagent:
1. Benzoic acid as standard, with a HHV of 26.454 kJ/g.

Operation procedure:
1. Place the stainless steel crucible onto its stand and attach a cotton thread fuse to the ignition wire and crucible;
2. Put the stand into the bomb vessel. Secure the lid;
3. Fill the bomb vessel with pure oxygen in the filling station until the pressure inside reaching 3 MPa;
4. Put the vessel into the static polystyrene jacket of the calorimeter. Secure the lid. Ignition will happen automatically when the temperature inside the jacket is stable;
5. Record the calorific value shown on the screen of calorimeter. A blank is made to account for the electrical energy input and the energy released in burning the fuse;
6. Input this blank value in the operation program at the data entry space 'baseline';
7. Open the lid of calorimeter. Take the bomb vessel out.
8. Release the pressure of the vessel by pressing the gas value with the special tool;
9. Open the lid of the vessel. Clean the vessel and the crucible;
10. Leave the vessel until it cools to room temperature;
11. Select 'calibration' program using the calorimeter screen;
12. Weigh around 1.0 g of benzoic acid with an accuracy of 0.1 mg in the crucible. Record the weight and input it to the operation program of the calorimeter;
13. Place the crucible onto its stand and connect the ignition wire and benzoic acid with a cotton thread fuse;
14. Repeat the steps 2-4;
15. Weigh around 1.0 g of sample with an accuracy of 0.1 mg. Record the weight and input it into the calorimeter;
16. Record the calorific value shown on the calorimeter screen;
17. Follow the steps of 7-10, and then start the next measurement.

Notes:
1. When measuring the calorific value of liquid samples, benzoic acid may be added as a spike to assist the ignition;
2. The solid sample should be pressed inside the crucible if this is not done it will tend to 'splash' when igniting.

Appendix 7

Determination of Kjeldahl Nitrogen

General discussion:
The Kjeldahl method is a means of determining the nitrogen content (in organic and ammonia form) of substances. This method may be broken down into three main steps:

1. Digestion – the decomposition of nitrogen in organic samples utilizing a concentrated acid solution. This is accomplished by boiling a homogeneous sample in concentrated sulphuric acid. The end result is an ammonium sulphate solution;
2. Distillation – adding excess base to the acid digestion mixture to convert NH_4^+ to NH_3, followed by boiling and condensation of the NH_3 gas in a receiving solution;
3. Titration – to quantify the amount of ammonia in the receiving solution.

Apparatus:
1. Tecator 1007 digestion system;
2. Kjeltec 1002 distilling unit;
3. Kjeldahl digestion tubes;
4. 250-ml Erlenmeyer flasks;
5. Analytical balance with an accuracy of 1 mg.

Reagents:
1. Sulphuric acid concentrated;
2. Digestion catalyst: Kjeltabs Cu 3.5;
3. Standard Ammonium Chloride Solution: dissolve 0.382 g anhydrous ammonium chloride (dried at 105ºC for at least 2 h) in Milli-Q water, and dilute to 100 ml using volumetric flask: 1.00 ml = 1.00 mg N = 1.22 mg NH_3. Stored in a stoppered glass bottle, this solution is stable for at least 1 month;
4. Mixed indicator solution: Dissolve 200 mg methyl red indicator in 100 ml 95% ethyl or isopropyl alcohol. Dissolve 100 mg methylene blue in 50 mL 95% ethyl or isopropyl alcohol. Combine solutions. Prepare monthly;
5. Indicating boric acid solution: Dissolve 20 g H_3BO_3 in water (heat if needed), add 10 ml mixed indicator solution, and dilute to 1 litre. Prepare monthly;
6. Standard sulphuric acid titrant, 0.10N or 0.25N: Dilute 2.72 or 6.80 ml concentrated sulphuric acid to 1000 ml with deionized water: 1.00 mL titrant = 14 × normality mg N. (For 0.1N, 1.00 ml = 1.4 mg N)

Safety:
The Kjeldahl method requires the digestion of the sample using strong acid at high temperatures. Careful handling of the solutions is mandatory for laboratory safety. For added protection, acid digestions should be performed in a fume hood with adequate ventilation. Eye protection should be worn at all times and care should be taken when handling hot digestion tubes.

Sample weight:
The actual weight of sample required is dependent on nitrogen content and homogeneity. When homogeneity of sample is not a controlling factor the sample weight can be selected relative to the nitrogen content. Using a titrant concentration of 0.25 N the analytical

sample should ideally contain 10-100 mg N. For selecting the size of sample a rough guide is given below:

Homogeneous solid samples	0.1-1.0 g
Non-homogeneous semi-solid samples	1.0-3.0 g or more
Liquid samples (depend on N content)	1.0-100 ml

Procedure:
1. Digestion
a. Switch on the digestion block and set the temperature to 420°C;
b. Weigh an appropriate amount of sample to an accuracy of 0.1 mg, or measure a certain amount of sample if sample is liquid, into a digestion tube. A blank should be run through all steps of the procedure to compensate for any contribution from the reagents used;
c. Add two Kjeltabs Cu 3.5;
d. Carefully add 12 ml of concentrated H_2SO_4 and gently shake to 'wet' the sample with acid. If sample contains high-fat or carbohydrate, then use 15 ml H_2SO_4 and 1-3 drops of octanol as anti-foaming agent;
e. Attached the exhaust system to the digestion tubes and secure it with PTFE tape;
f. Set the water aspirator to full effect;
g. Load the rack with exhaust into the preheated digestion block;
h. After about 5 minutes turn down the water aspirator until the acid fumes are just contained within the exhaust head;
i. Continue to digest until all samples are clear with blue / green solution. This will normally be after 60-120 minutes;
j. Remove the tubes with exhaust still in place from digestion block and put them in the stand to cool for 10-20 minutes;
k. Carefully add deionized water to the tubes to make up the volume to about 100 ml. For solid samples, this step should be done while the digestion mixture is still warm to avoid K_2SO_4 salting out.

2. Distillation
Starting up the distilling unit, which can be done when cooling the digestion mixture:
a. Make sure that a empty digestion tube and a receiver flask are placed in their proper positions in the distilling unit and that the safety window in pulled down. Check that the two valves at the rear of the unit are closed (handles parallel to the back);
b. Connect the tube labelled 'tap water' to the water tap, and put rest three tubes in the sink;
c. Switch on power, and open for steam by keeping the small black handle labelled 'steam' in the down position. (This valve should always be open when the unit is not in use);
d. Turn on the cold water tap to a flow of about 1.5litre/min for about half a minute and the water level should be visible through the top and then the bottom window at the left side of the distilling unit. Close the tap;
e. After a minute or so, open the water tap again for 10-15 seconds and watch the steam entering the digestion tube through the white Teflon tubing. After another minute open the water tap to a flow about 1.5litre/min and leave it open;
f. Let the distillation continue until about 150 ml of distillate has been collected. Move the platform with the receiver flask to its lower position. Then close the steam valve on the front panel;
g. Remove the digestion tube using heat protective gloves and the receiver flask.

Distilling the digestion mixture:

a. Place the digestion tube with diluted digestion mixture in its position in distilling unit and place the receiver flask with 25 ml indicating boric acid solution on the platform;
b. Close the safety window;
c. Move the platform for the receiver flask to its upper position so that the distillate outlet is submerged in the receiver solution;
d. Gently press the alkali handle half way down to dispense about 50 ml of 40% NaOH;
e. Open the steam valve. The boric acid receiver solution in the distillate flask will soon be green indicating the presence of ammonia;
f. When the indicating solution reaches the 150 ml mark of the flask, lower the platform for the receiver flask to its lower position. Close the steam valve and wait for a few more seconds to clean out the outlet tip;
g. Replace the digestion tube and the receiver flask with the next ones and continue in the same manner with all the samples. When removing a digestion tube, the teflon tube through which the steam enters the sample, should be placed in the metal clip. This makes it possible to replace a new digestion tube without touching the teflon tube;
h. It is better to titrate ammonia in distillate before distilling the next sample to allow the water to cool down in the distilling unit.

Closing down the distilling unit
a. Put an empty digestion tube and receiver flask into their position and close the water tap;
b. Remove the drain trough under the tube holder and the platform for the receiver flask and clean it with water. Wipe the unit clean from any spillage. Close the safety window;
c. Switch off power and open the valve in the middle position at the rear of the unit (handles vertical to the back) and drain out the water left in the distillation unit;
d. Leave the steam valve open to prolong the life of the tubing in the valve.

3. Titration
a. Titrate ammonia in distillate with standard 0.10 or 0.25 N of H_2SO_4 titrant until indicator turns to pale lavender.

Calculation:

a. Liquid samples: $mgN/L = \dfrac{(A-B)\times 14.0\times N\times 1000}{mL(sample)}$

b. Solid samples: $\%N = \dfrac{(A-B)\times 14.0\times N\times 100}{mg(dry\cdot wt\cdot sample)}$

where:
A = volume of H_2SO_4 titrated for sample, ml;
B = volume of H_2SO_4 titrated for blank, ml;
N = normality of standard sulphuric acid titrant.

Notes:
1. When the sample content of fats and / or carbohydrates is high, 1-3 drops of octanol, an antifoam agent, should be used to control the tendency for foaming;

2. Pure substances of known nitrogen content can be used as the calibration substances, for example acetanilide (C_8H_9NO), L-aspartic acid ($C_4H_7NO_4$), or amino acids of known composition;

3. Kjeldahl digestion does not always recover all forms of nitrogen in a sample. Nitrate and nitrate ions (which are unlikely to be present in digestion) in a sample must first be reduced prior to acid digestion for quantitative recovery. Salicylic acid followed by sodium thiosulfate has been used to pretreat the mixture to ensure complete reduction. For detailed procedure, please refer to BS EN 13654-1:2001 Soil improvers and growing media – Determination of nitrogen – Part 1: Modified Kjeldahl method.

Appendix 8

Extraction of Metal Elements in soils, sediments and sewage sludge and plants using Acid Digestion (SCA 1986)

Principle:
The objective of the method is to remove the organic matrix of the sample and leave the elements dissolved in the solution phase. The final elemental concentrations represent closely the total concentration of the element present in the sample.

A representative sample of up to 2.0 g dry matter is digested in 10 ml of a concentrated hydrochloric/nitric acid mix, first by leaving at room temperature for a minimum of 16 hours, followed by boiling for 2 hours in tall condensing tubes in a heating block.
After cooling, the contents of the tube are filtered and washed 5 times using 12.5% nitric acid and then made up to 50 ml in a volumetric flask volume. The diluted extract is then analysed for: extracted phosphorus (P) using a spectrophotometer method; and potassium (K) and heavy metals (Cd, Co, Cu, Fe, Ni, Se, W and Zn etc.) are using atomic absorption spectrometer (AAS) or ICP-OES.

Reagents:
1. *Concentrated nitric acid, HNO_3:* purified and certified for trace element analysis.
2. *Concentrated hydrochloric acid, HCl;*

Apparatus:
Before use, all digestion tubes should be cleaned in acid solution and rinsed with deionized water and dried in a clean environment;

1. Volumetric flasks, 50 ml capacity;
2. Funnels, suitable for 50 ml volumetric flask;
3. Whatman No.1 filter papers;
4. Analytical balance, with an accuracy of 1 mg.

Procedure:
1. Weigh accurately about 2 grams of the dried sample which has been finely ground, add this to the digestion tube.

2. Add 7.5 +/- 0.2 ml of hydrochloric acid (d_{20} 1:10) and 2.5 +/- 0.1 ml of analytical grade nitric acid (d_{20} 1:40).

3. Digest at room temperature for a minimum of 16 hours

4 Boil the sample for 2 hours

5. Cool the tubes and then filter the material through acid resistant cellulose paper and washed 5 times using 12.5% nitric acid. Make up the volume of the filtrate to 50 ml using 12.5% HNO_3 in a volumetric flask volume.

6. Two blanks are prepared by boiling and filtering the acid mix as if it were a sample.

Expression of results:
1. When calculated on a real ‘wet’ weight basis:

$$C_w = \frac{(C_d - C_b) \times 50}{m}$$

where,
C_w: Concentration of elements in sample, mg/kg;
C_d: Concentration of the corresponding element in the digestion supernatant, mg/l;
C_b: Concentration of the corresponding element in blank, mg/l;
50: The volume of final diluted solution, ml;
m: The mass of the sample, g.

2. To determine results on a dry weight basis, a separate determination of percent solids must be performed in parallel with the microwave digestion process:

$$C = \frac{C_w}{TS}$$

where,
C: Concentration of elements in sample on a dry weight basis, mg/kg;
TS: Total solids, %.

Reference:
Standing Committee of Analysts (1986), Methods for the determination of metals in soils, sediments and sewage sludge and plants by hydrochloric-nitric acid digestion, with a note on the determination of the insoluble metal contents, 1986. Methods for the Examination of waters and associated materials, HMSO, UK

Appendix 9

Acid Digestion of Aqueous Samples and Extracts for Total Metals for Analysis by FLAA or ICP Spectroscopy (EPA 3010A)

1.0 SCOPE AND APPLICATION

1.1 This digestion procedure is used for the preparation of aqueous samples, EP and mobility-procedure extracts, and wastes that contain suspended solids for analysis, by flame atomic absorption spectroscopy (FLAA) or inductively coupled argon plasma spectroscopy (ICP). The procedure is used to determine total metals.

1.2 Samples prepared by Method 3010 may be analyzed by FLAA or ICP for the following:

Aluminium	Magnesium
*Arsenic	Manganese
Barium	Molybdenum
Beryllium	Nickel
Cadmium	Potassium
Calcium	*Selenium
Chromium	Sodium
Cobalt	Thallium
Copper	Vanadium
Iron	Zinc
Lead	

* Analysis by ICP
NOTE: See Method 7760 for the digestion and FLAA analysis of Silver.

1.3 This digestion procedure is not suitable for samples which will be analyzed by graphite furnace atomic absorption spectroscopy because hydrochloric acid can cause interferences during furnace atomisation. Consult Method 3020A for samples requiring graphite furnace analysis.

2.0 SUMMARY OF METHOD

2.1 A mixture of nitric acid and the material to be analyzed is refluxed in a covered Griffin beaker. This step is repeated with additional portions of nitric acid until the digestate is light in colour or until its colour has stabilized. After the digestate has been brought to a low volume, it is refluxed with hydrochloric acid and brought up to volume. If sample should go to dryness, it must be discarded and the sample reprepared.

3.0 INTERFERENCES

3.1 Interferences are discussed in the referring analytical method.

4.0 APPARATUS AND MATERIALS

4.1 Griffin beakers - 150-ml or equivalent.
4.2 Watch glasses - Ribbed and plain or equivalent.
4.3 Qualitative filter paper or centrifugation equipment.
4.4 Graduated cylinder or equivalent - 100 ml.
4.5 Funnel or equivalent.
4.6 Hot plate or equivalent heating source - adjustable and capable of maintaining a temperature of 90-95^{o}C.

5.0 REAGENTS

5.1 Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

5.2 Reagent Water. Reagent water will be interference free. All references to water in the method refer to reagent water unless otherwise specified. Refer to Chapter One for a definition of reagent water.

5.3 Nitric acid (concentrated), HNO_3. Acid should be analyzed to determine levels of impurities. If method blank is < MDL, the acid can be used.

5.4 Hydrochloric acid (1:1), HCl. Prepared from water and hydrochloric acid. Hydrochloric acid should be analyzed to determine level of impurities. If method blank is < MDL, the acid can be used.

6.0 SAMPLE COLLECTION, PRESERVATION, AND HANDLING

6.1 All samples must have been collected using a sampling plan that addresses the considerations discussed in Chapter Nine of this manual.

6.2 All sample containers must be prewashed with detergents, acids, and water. Plastic and glass containers are both suitable.

6.3 Aqueous wastewaters must be acidified to a pH of < 2 with HNO_3.

7.0 PROCEDURE

7.1 Transfer a 100-mL representative aliquot of the well-mixed sample to a 150-mL Griffin beaker and add 3 mL of concentrated HNO_3. Cover the beaker with a ribbed watch glass or equivalent. Place the beaker on a hot plate or equivalent heating source and cautiously evaporate to a low volume (5 mL), making certain that the sample does not boil and that no portion of the bottom of the beaker is allowed to go dry. Cool the beaker and add another 3-mL portion of concentrated HNO_3. Cover the beaker with a nonribbed watch glass and return to the hot plate. Increase the temperature of the hot plate so that a gentle reflux action occurs.

NOTE: If a sample is allowed to go to dryness, low recoveries will result. Should this occur, discard the sample and reprepare.

7.2 Continue heating, adding additional acid as necessary, until the digestion is complete (generally indicated when the digestate is light in colour or does not change in appearance with continued refluxing). Again, uncover the beaker or use a ribbed watch glass, and evaporate to a low volume (3 mL), not allowing any portion of the bottom of the beaker to go dry. Cool the beaker. Add a small quantity of 1:1 HCl (10 mL/100 mL of final solution), cover the beaker, and reflux for an additional 15 minutes to dissolve any precipitate or residue resulting from evaporation.

7.3 Wash down the beaker walls and watch glass with water and, when necessary, filter or centrifuge the sample to remove silicates and other insoluble material that could clog the nebulizer. Filtration should be done only if there is concern that insoluble materials may clog the nebulizer. This additional step can cause sample contamination unless the filter and filtering apparatus are thoroughly cleaned. Rinse the filter and filter apparatus with dilute nitric acid and discard the rinsate. Filter the sample and adjust the final volume to 100 mL with reagent water and the final acid concentration to 10%. The sample is now ready for analysis.

8.0 QUALITY CONTROL

8.1 All quality control measures described in Chapter One should be followed.

8.2 For each analytical batch of samples processed, blanks should be carried throughout the entire sample-preparation and analytical process. These blanks will be useful in determining if samples are being contaminated. Refer to Chapter One for the proper protocol when analyzing blanks.

8.3 Replicate samples should be processed on a routine basis. A replicate sample is a sample brought through the whole sample preparation and analytical process. A replicate sample should be processed with each analytical batch or every 20 samples, whichever is greater. Refer to Chapter One for the proper protocol when analyzing replicates.

8.4 Spiked samples or standard reference materials should be employed to determine accuracy. A spiked sample should be included with each batch of samples processed and whenever a new sample matrix is being analyzed. Refer to Chapter One for the proper protocol when analyzing spikes.

8.5 The method of standard addition shall be used for the analysis of all EP extracts and delisting petitions (see Method 7000, Step 8.7). Although not required, use of the method of standard addition is recommended for any sample that is suspected of having interference.

9.0 METHOD PERFORMANCE

9.1 No data provided.

10.0 REFERENCES

1. Rohrbough, W.G.; et al. Reagent Chemicals, American Chemical Society Specifications, 7th ed.; American Chemical Society: Washington, DC, 1986.

2. 1985 Annual Book of ASTM Standards, Vol. 11.01; "Standard Specification for Reagent Water"; ASTM: Philadelphia, PA, 1985; D1193-77.

Appendix 10

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 non-carbohydrates 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% H_2SO_4, 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, $C_{10}H_{14}N_2Na_2O_8x2H_2O$) and 6.81g of Sodium Borate decahydrate, ($Na_2B_4O_7x10H_2O$), in a beaker and add some distilled water and heat until dissolved. Add 30g Sodium Lauryl Sulphate,

($C_{12}H_{25}OSO_3Na$), 10 ml of 2-ethoxyethanol, ($C_4H_{10}O_2$) and 4.56g Disodium Hydrogen phosphate, (Na_2HPO_4). 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 H_2SO_4 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 (W_1), tare and weigh around 1 g of ground sample to an accuracy of ±0.1 mg (W_2) into each capsule, secure lids. Place the capsules in the tray holder, 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 (CH_3COCH_3);

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 (W_3);

18. Place the capsules in pre-dried and pre-weighed (W_4) 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 (W_5);

22. Calculation:

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

where,
W_1 = Initial capsule weight, g;
W_2 = Sample weight, g;
W_3 = Weight of capsule + residue sample after extracting and drying, g;
W_4 = Weight of empty ashing crucible, g;
W_5 = 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:

$$C = \frac{blank \cdot capsule \cdot weight \cdot after \cdot extractions}{blank \cdot capsule \cdot weight \cdot at \cdot start}$$

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;

22. Calculation:

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

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

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 15-21;

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.