Integrating mineral wastes in the anaerobic digestion of OFMSW for improved recovery of renewable energy

Burhan Shamurad

bshamurad@yahoo.com

March 2020

A thesis submitted for the degree Doctor of Philosophy in the School of Engineering, Newcastle University, UK

Abstract

This thesis investigated the effects of mineral wastes (MW) on laboratory-scale anaerobic reactors treating organic wastes. Different MW resources were used, incineration bottom ash (IBA), fly ash (FA) and boiler ash (BA), taken from a municipal solid waste incineration (MSWI) plants, as well as a cement-based waste (CBW) from construction demolition wastes. The hypothesis was that these MW would provide trace elements (TEs) deficient in the organic fraction of municipal solid waste (OFMSW), and offer moderate alkalinity to prevent reactor acidification of mesophilic anaerobic digestion of the OFMSW. The control and operation of batch biomethane potential (BMP) reactors and continuous stirred tank reactors (CSTRs; single-stage and two-stage reactors), was studied under different feeding regimes, different organic loading rates and hydraulic/solid retention times, in order to determine potential benefits of mineral waste amendments and aqueous trace metal supplements on anaerobic digestion efficiency, methane productivity and process stability.

Co-digestion of different solid MW and organic wastes in single-stage CSTR using a liquid-recycled feeding method (LRFM) enhanced process stability (pH of 6.8 – 7.2), increased methane production by 25 - 45%, and yielded 450 – 520 mL CH_4/g VS (near to the theoretical maximum) compared to the control. Whereas draw-and-fill feeding method (DFFM) also enhanced digestibility but to a lesser degree. Pre-treatment of the OFMSW with the MW at 37^{o}C improved substrate hydrolysis, and enhanced the performance and stability of the DFFM digestion processes further to values similar to those of LRFM reactors. Amending two-stage CSTRs with aqueous MW extracts provided the reactors with the necessary trace elements deficient in the OFMSW, maintained alkalinity and pH, and hence enhanced hydrogen/methane production and processes stability of both acidogenic and methanogenic reactors.

Amendments of IBA, BA and CBW provided trace metals that supported anaerobic digestion processes without adverse effects; however, the metals released from FA provided much lower enhancement of the digestion processes, as some trace metal concentrations were within the toxic range for methanogenic processes.

To elucidate and compare the effect and importance of commercial TE supplementation and substrate co-digestion techniques in improving organic waste anaerobic digestion, especially for the single-stage reactors with high organic loading rates, different CSTR feed compositions were studied. Different feedstocks were investigated including synthetic organic waste (SOW), SOW supplemented with TE, SOW supplemented with wheat straw (WS) and

SOW supplemented with WS and TE. Results showed that high methane yields (450 - 550 mL/g VS), higher microbial numbers and process stability at higher OLRs, were achieved in all reactors having TE supplementation compared to the equivalent reactors without TE supplementation.

From analysis of molecular microbial data, the effect that different feeding methods, reaction times and WS co-digestion had on reactor performance was found to be associated directly with microbial community selection and stability. Different feeding regimes altered the microbial communities; *Methanoculleus* (hydrogenotrophic) and *Methanosaeta* (acetoclastic) were the most abundant methanogenic genera in the LRFM reactors, and the more metabolically versatile *Methanosarcina* genus dominated under DFFM. Interestingly, at 25% WS supplementation, the *Methanosarcina* were found to be acetoclastic (based on indicative coenzyme F_{420} measurements), but with no WS amendment with highest NH_3-N levels the F_{420} values indicated a predominantly hydrogenotrophic metabolism. These results suggest that, WS co-digestion reduced biological stress on the anaerobic community by reducing the net concentration of ammonia in the feedstock.

Acknowledgements

The time of producing this thesis of my PhD degree was the hardest and most challenging period of my life, but I have enjoyed the experience by achieving as much as information, skills and knowledge as possible.

To my colleagues, friends and the staff of the school of engineering at Newcastle university, you are supported me and provided me the happy environment to finish this study.

To my wife Laila and my children Rozh, Ramyar and Diwa you worked hard and tolerated the difficulties to give me love and a happy foundation to finish this degree.

Finally, to my supervisors Paul and Neil, your patience and support throughout will be remembered fondly.

"*Dei perfecta est*"

Better things will follow indeed!

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Chapter 1. Introduction

1.1. Motivation of the research

The last three decades have witnessed an unprecedented increase in the world population with a better life quality for individuals. According to the UN (2007), the world population will rise to 9.2 billion by 2050. This population growth and economic development correlate highly with the amount of municipal solid waste (MSW) generation. It is predicted that the global generation of MSW will reach 3 billion tonnes by the year 2025 (Karthikeyan and Visvanathan, 2013). In the European countries, an annual rise of about 2 - 3% in MSW production can be estimated (Salhofer *et al.*, 2008). In some developing countries, the organic fraction of this municipal solid waste (OFMSW) comprises ~ 80% (Figure 1-1) of that mixed waste (Aziz *et al.*, 2011).

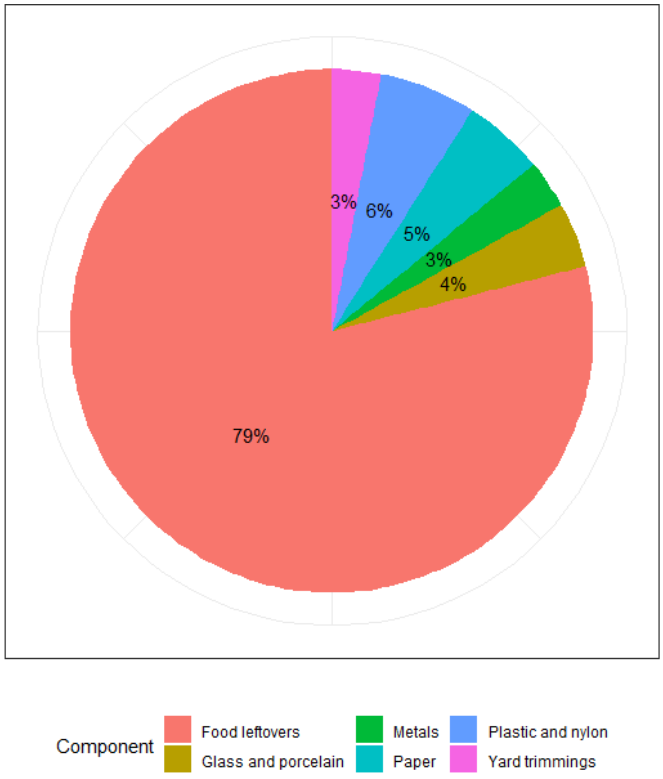


Figure 1-1. Percentage for different solid waste component (average weight) of Erbil city- Kurdistan Region of Iraq in 2009 (Aziz et al., 2011).

This study developed and investigated a feasible and an affordable treatment strategy that considered treatment scenarios for the high organic component of a municipal solid waste. The objective was to develop an integrated approach utilising other waste streams generated as a result of population growth and economic development, which ensures sustainable treatment of OFMSW, production of renewable energy.

Two categories of MSW were investigated in this study: the organic rich fraction i.e. OFMSW typically collected from households, restaurants and food markets and a solid waste stream from the construction industry known as construction demolition waste (CDW). The OFMSW stream can typically be sorted further into a fraction with good combustibility and low moisture content that has particle sizes of > 45 mm; this stream could be incinerated for energy recovery in municipal solid waste incineration (MSWI) plants, and another putrescible organic portion of raw OFMSW materials, which is relatively wet, and has particle sizes < 45mm; this fraction could be diverted for biological treatments such as anaerobic digestion (AD).

Mineral wastes (MW) produced from the incineration of organic wastes in MSWI plants and include incineration bottom (IBA), boiler (BA) and fly (FA) ashes are enriched with minerals and nutrients, and as such have a reasonable acid neutralising capacity. These properties make MW an interesting material to study in the context of the AD processes.

Furthermore, another MW, cement-based waste (CBW), the main component of CDW, represents a significant component of MSW (because of the increased uses of cement materials in construction due to urbanisation, especially in the developing countries). A large amount of Portland cement (2.4 Gt/year) is manufactured worldwide (Renforth *et al.*, 2011), and most of the produced cement enters the construction industry. Most of the CBW eventually produced will be disposed of either in areas outside of cities, or in landfill sites. However, the collected CBW could be utilised as another MW material in an integrated MSW treatment system that is proposed in this study, due to it being an alkaline/and trace element source that could improve biological treatment process in AD treatment facilities.

1.2. Thesis Overview

As AD is the key treatment step in the proposed integrated treatment process, this research focused on studying the AD process in order to identify the best methods for integrating MW from MSW into the AD of OFMSW. These methods included:

(1) Direct addition of different solid MW to anaerobic reactors digesting OFMSW (co-digestion of the organic OFMSW and mineral MW).
(2) Pre-treatment and co-digestion of the organic and MW.
(3) Using aqueous MW extracts for optimising two-stage AD of the OFMSW.

In addition, to provide a broader context for the assessment of mineral waste streams and their effect on AD, this project also comparatively investigated

(4) Regulation of macronutrients (C/N ratio), with and without trace element supplements, on AD reactor performance (methane production and yield) and process stability.

Regulation of C/N typically was achieved by OFMSW co-digestion with wheat straw (WS) and standard trace element solutions produced from stock metal solutions simulated the major minerals components found in the MW. Moreover, within the context of these four research themes, the main phases of the AD process routinely investigated for distribution and diversity of microorganisms.

1.3. Thesis Novelty

Many studies have been published in the literature regarding the AD of OFMSW, either in its raw state or when is has been pre-treated physically, chemically or thermally to make the carbon more bioavailable e.g. by hydrolysis. Most of the pre-treatment approaches are energy intensive, which ultimately diminishes the net energy recovery efficiency; one of the main goals of the AD processes. Moreover, most of the pre-treatment methods suggested in the literature might lead to rapid hydrolysis which causes problems for reactor stability via acidification. The novel aspect of this study was to investigate the feasibility of establishing an integrated treatment system with minimal energy input that utilised MW to stimulate bacterial hydrolysis minimising the need to do energy expensive pre-treatments, and increase microbial growth and activity in anaerobic digesters. It was proposed that this could be achieved through the AD of OFMSW with necessary nutrient supplementation, and better operating conditions (balanced alkalinity and pH levels in the reactor) using very low economic value, and widely available, waste materials as MW. Consequently, improvements to the digestion process were expected to result in higher organic loading rates, and higher solids removal and greater renewable energy (as methane) production efficiency.

Acidification of anaerobic reactors and pH drop exerts tremendous effects on the anaerobic processes (Chen *et al.*, 2008), which may lead to the inhibition of bacterial and archaeal communities, and consequently causes instability in the AD process, leading to lower biogas production, and reduced solids destruction efficiency. In order to stabilise pH at the optimal range (pH 6.5 - 7.5) for AD process (Liu *et al.*, 2008)), digesters have often been supplied with alkalinity from basic materials like NaOH, KOH, etc. Therefore, the second novel aspect in this study was to balance reactor pH at favourable ranges for methanogenic microorganisms by adding alkalinity in the form of low cost alkaline waste materials such as MW. Moreover, AD enhanced through alkali pre-treatment (alkaline hydrolysis) of the feedstock substrates, using the alkaline property of MW. This could provide better conversion

of lignocellulosic contents of OFMSW to biogas by disrupting the strong bonds between lignin and hemicellulose, resulting in improved solubilisation of the hemicellulose component of the feedstock material (Reilly *et al.*, 2015).

Certainly, MW need sustainable management, but currently there are no sustainable MSW management techniques for them. IBA usually contains 15% of materials unchanged by combustion like glass, soil minerals and metal. The other 85% of IBA is ash; which usually comprises less than 4% w/w organic matter with the remaining mass being enriched with mineral elements such as (Al, As, Ba, Ca, Cd, Cl, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Si, Ti, and Zn) (Banks and Lo, 2003). IBA also has moderate alkalinity with pH 10.5 - 12.2 (*Municipal solid waste incinerator residues*, 1997).

Banks and Lo (2003) reported that, according to their mass fractions, the metals found in the IBA can be classified as major (Ca, K, Na, Mg, Al, Fe), minor (Cd, Cu, Cr, Ni, Pb, Zn) and trace (As, Ba, Cl, Mn, P, S, Si, Ti) elements. However, although most of these elements are useful for optimising the biological process in AD, they are toxic to microorganisms above specific threshold concentrations (Demirel and Scherer, 2011; Karlsson *et al.*, 2012; Zhang and Jahng, 2012; Facchin *et al.*, 2013; Cai *et al.*, 2017). The relative toxicity of heavy metals to acidogenic and methanogenic consortiums are Cu > Zn > Cr > Cd > Ni > Pb and Cd > Cu > Cr > Zn > Pb > Ni, respectively (Lin, 1993b). However, Banks and Lo (2003) concluded that the heavy metal concentrations released by IBA of MSWI are well below inhibitory concentrations resulting in operational problems in the AD process. Moreover, previous studies have shown that accumulation of volatile fatty acids (VFA) are more likely to occur in the AD of food waste without addition of trace metal elements (Banks *et al.*, 2011). Therefore, the proposed integrated approach might be expected to provide the necessary trace elements required for enhancing the population growth of AD microorganisms and produce an optimum pH value that would result in a stable digestion process. In addition, the proposed integrated AD process is expected to produce a digestate with a minimum level of heavy metals suitable for composting as soil conditioner without dangerous toxicity effects on the food chain of humankind; however, this will be need to be validated by full characterisation of the digestate composition.

Similarly, the flux materials out of the construction and demolition industry is a considerable waste stream mainly composed of hydrated cement paste containing a high amount of calcium and other minerals (see Section 3.3.2). These characteristics make the cement-based waste

(CBW) from construction demolition waste (CDW) another mineral waste resource to be studied in the context of the reactor systems or digestion conditions studied in this thesis.

Finally, the suggested integration treatment process between MW and OFMSW has two main cost benefits, treatment of OFMSW, and producing renewable energy and a utilisable digestate. Moreover, the applied process would manage treatment of MW that might inflict environmental damages through leaching of these materials to the soils and waterways if disposed without proper treatment. Furthermore, the digestate of the integrated process might contain some metals that are the contaminants; therefore future studies need to address the quality of the digestate before its use as a compost or a fertiliser for land applications.

Chapter 2. Research gap

2.1. Aims

This research aimed to evaluate and determine the best methods of integrating MW as an economically feasible trace element and alkaline amendment for the anaerobic digestion of the organic fraction of municipal solid waste (Figure 2-1).

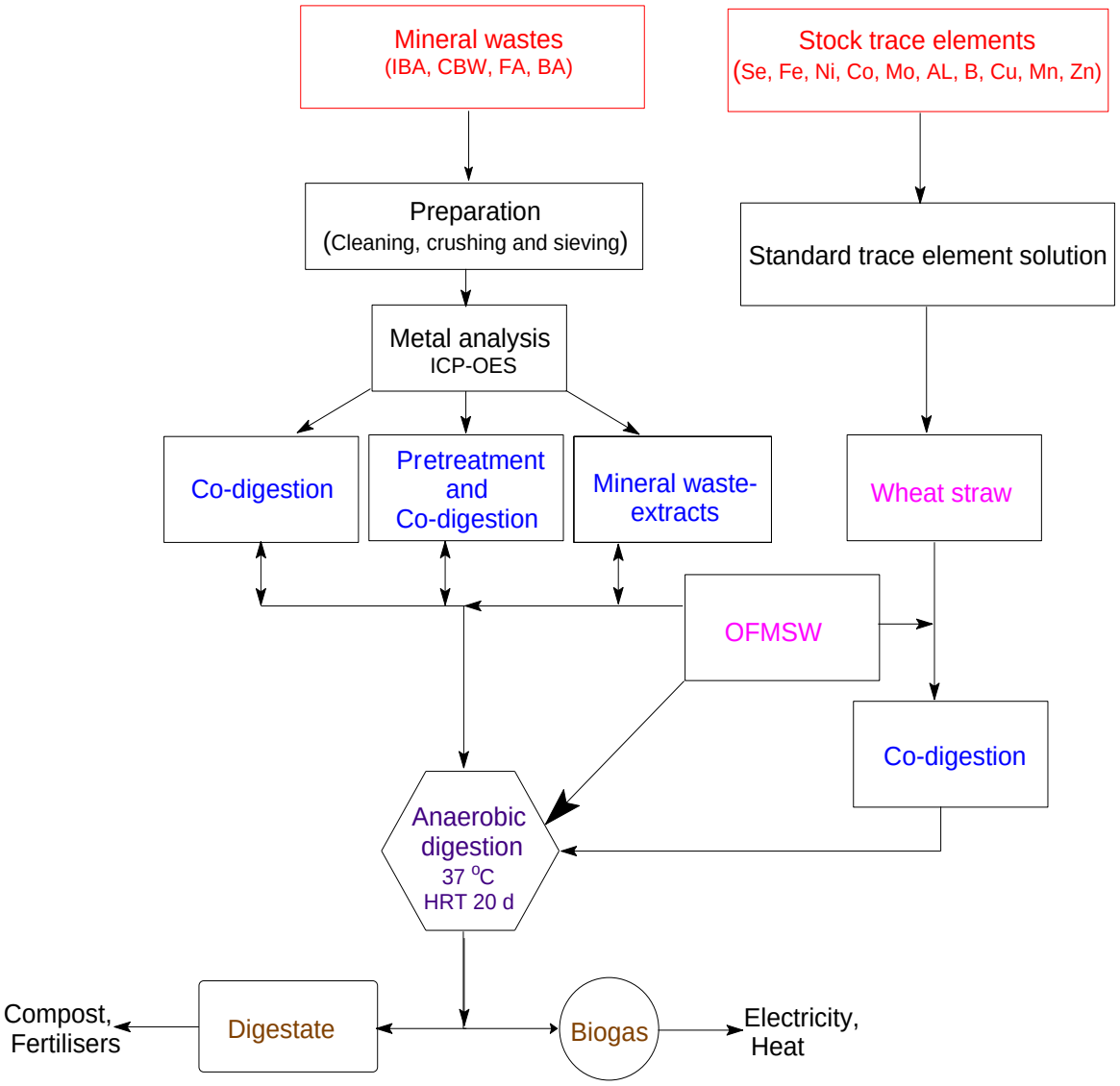


Figure 2-1. Identified research areas for the integration of mineral wastes and trace elements into the AD of OFMSW. ICP-OES: inductively coupled plasma - optical emission spectrometry, IBA: incineration bottom ash, CBW: cement-based waste, FA: fly ash and BA: boiler ash.

2.2. Objectives

- To investigate the elemental composition and chemical properties of MW and to measure the soluble components that would arise from them, for comparison with the typical range of the values cited in the literature.
- To investigate the biomethane potential (BMP) of OFMSW after co-digestion / pre-treatment with MW at a mesophilic temperature of 37ºC in batch reactors.
- To investigate the effects of anaerobic co-digestion of OFMSW and MW on CSTR reactor performance (methane production rate and yield), process stability and microbial community composition and dynamics at a mesophilic temperature of 37ºC.
- To investigate the effects of pre-treatment of OFMSW with MW at a mesophilic temperature of 37ºC on CSTR reactor performance (methane production rate and yield), process stability and microbial community composition and dynamics.
- To investigate the effect of mineral waste extracts (MW-extracts) on the performance of AD reactors treating OFMSW (methane production rate and yield), process stability and microbial community composition and dynamics at a mesophilic temperature of 37ºC.
- To assess the effect of wheat straw (WS) co-digestion with or without trace element concentration on the performance of AD reactors treating OFMSW (methane production rate and yield), process stability and microbial community composition and dynamics at a mesophilic temperature of 37ºC.
- To carry out microbial community analysis to identify and quantify the microbial communities in the digestate samples, and mechanistically understand in addition to empirically document, the individual and combined impacts of waste amendments on the AD process of OFMSW at a mesophilic temperature of 37ºC.

Chapter 3. Literature review

3.1. Anaerobic digestion processes

Anaerobic digestion (AD) is a series of linked oxygen-free biological processes producing biogas, primarily CH_4 and CO_2, by the degradation of organic matter catalysed by a large group of bacteria and archaea classified as guilds of hydrolysers, acidogens, acetogens and methanogens (Rittmann, 2001; Karthikeyan and Visvanathan, 2013). At standard pressure and temperature (STP) (0^oC and 1 bar), each gram of COD of organic matter is converted to 0.35 L of CH_4 (Rittmann, 2001).

The population growth of these microorganisms, and their microbial diversity and functions, are among the main indicators of a robust AD process. In the AD of OFMSW, the microbial communities involved in the hydrolysis and acidogenesis processes comprise fast growing microorganisms, therefore for the highly hydrolysable substrates (such as food waste) these two stages are considered non-rate limiting stages (Raposo *et al.*, 2012). In contrast, both the acetoclastic and hydrogenotropic methanogens, the two main archaeal guilds of the methanogenesis phase, are known for their low growth rates that normally makes this stage the rate-limiting stage in the AD process for OFMSW treatment (Rozzi and Remigi, 2004). Microbial growth in reactors systems is affected greatly by internal reactor parameters such as pH, alkalinity, oxidation-reduction potential (ORP), volatile fatty acids (VFA) concentration, temperature, physical mixing, presence of toxic materials, and availability of trace elements (Mao *et al.*, 2015). Most of these parameters are related to the characteristics of the feedstock substrate. Therefore, the feedstock characteristics play a major role in enhancement (high solids conversion rate and methane production) of the AD processes (Karthikeyan and Visvanathan, 2013).

The four stages of AD processes are hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 3-1). In the hydrolysis stage, hydrolytic bacteria convert long chain organic components of the substrate such as carbohydrates, proteins and fats to short and soluble monomers of glucose, amino acids and fatty acids, respectively. Three main hydrolytic enzymes mediate the hydrolysis stage, cellulase, protease and lipase ((Gerardi, 2003); Figure 3-2). Lignocellulosic substrates are difficult to degrade by hydrolytic bacteria, therefore, for lignocellulosic substrates the hydrolysis stage can be the most rate-limiting stage of the overall AD process (Li et al., 2018a). The stage following the hydrolysis stage is the acidogenesis stage, at this stage; products of the hydrolysis stage are converted to volatile fatty acids (VFA) such as acetic acid, butyric acid, valeric acid, and propionic acid, as well as

alcohols, CO_2 and H_2. Depending on the characteristics of the feeding substrate, some trace gases such as ammonia and hydrogen sulphide are also produced. The acetogenesis, is the process of acetate production from products of the hydrolysis and acidogenesis (such as propionate) stages (Zieminski and Frac, 2012).

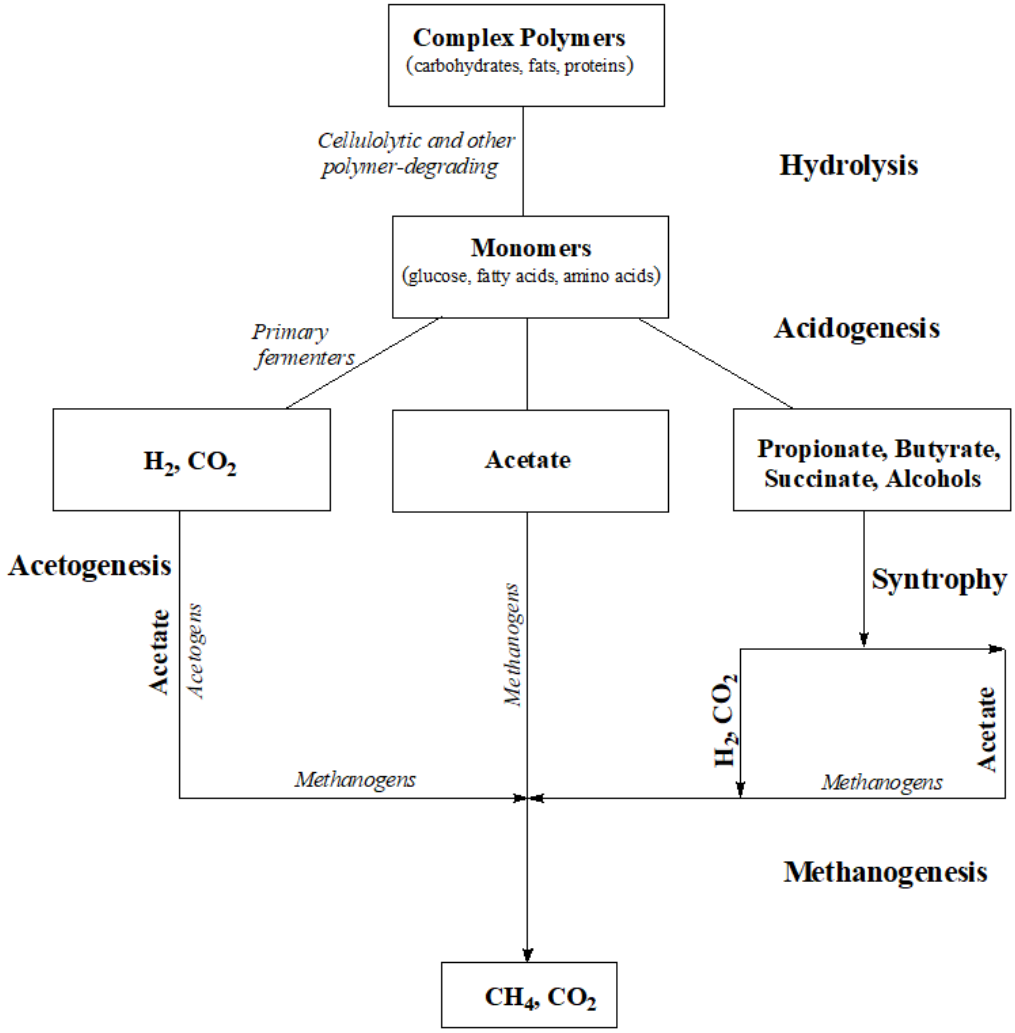


Figure 3-1. Biological processes for the anaerobic conversion of organic matter to methane (Madigan, 2015).

The final stage of the AD process is methanogenesis (for details see Section 3.1.4), which consists in the production of methane principally by two groups of methanogens: the acetoclastic and hydrogenotrophic methanogens. In addition, methylotrophic methanogens are the third group of methanogens which utilise methylated substrates (Madigan, 2015). Acetoclastic methanogens split acetate molecules to methane and CO_2, while hydrogenotrophic archaea use H_2 to reduce CO_2 to methane. About 70% of the methane which is produced in anaerobic digesters treating organic wastes is from acetate (Zieminski and Frac, 2012).

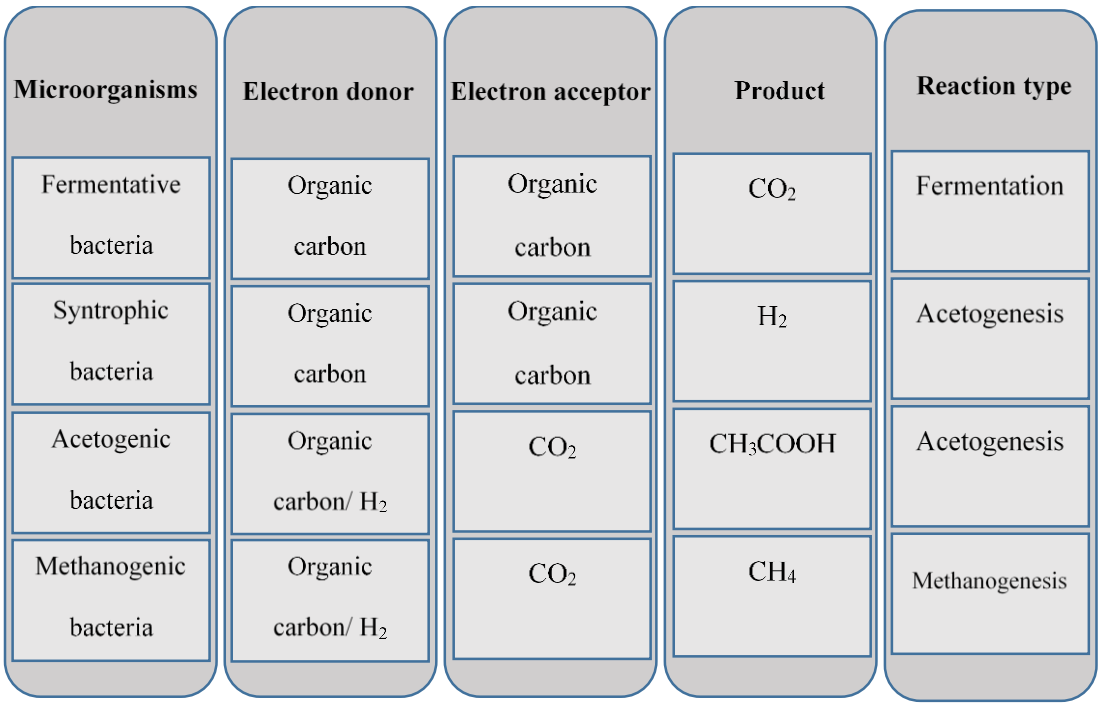


Figure 3-2. Cooperation of microorganisms to degrade organic matter (Zieminski and Frac, 2012).

During the fermentation processes, two different groups of microorganisms need a symbiosis to degrade organic matter and conserve energy; this form of metabolic processes is referred to as *syntrophy*. Most of the syntrophic reactions are secondary fermentations, the fermentation products produced by a group of anaerobes microorganisms are utilised by the next fermenter groups. The interspecies H_2 transfer is the common syntrophic reaction between the anaerobic species. For instance, fermentation of ethanol to methane and acetate is carried out by the ethanol-oxidising syntroph and methanogens. Oxidation of ethanol by an ethanol oxidiser is an endergonic reaction i.e. consumes energy, while consumption of H_2 by its hydrogenotrophic methanogen partner is an exergonic reaction i.e. releases energy (Eqs. 3-1, 3-2 and 3-3). The overall energy release from the two reactions is exergonic, i.e. both of the microorganisms share the free energy released from the two reactions (Madigan, 2015).

Ethanol Fermentation

$$2\ CH_3CH_2OH + 2\ H_2O \longrightarrow 4\ H_2 + 2\ CH_3COO + 2\ H^+ \qquad \sigma = +19.4\ KJ/reaction \qquad \textbf{(3-1)}$$

Methanogenesis

$$4\ H_2 + CO_2 \longrightarrow CH_4 + 2\ H_2O \qquad \sigma = -130.7\ KJ/reaction \qquad \textbf{(3-2)}$$

Coupled reaction:

$$2\ CH_3CH_2OH + CO_2 \longrightarrow CH_4 + 2\ CH_3COO + 2\ H^+ \qquad \sigma = -111.3\ KJ/reaction \qquad \textbf{(3-3)}$$

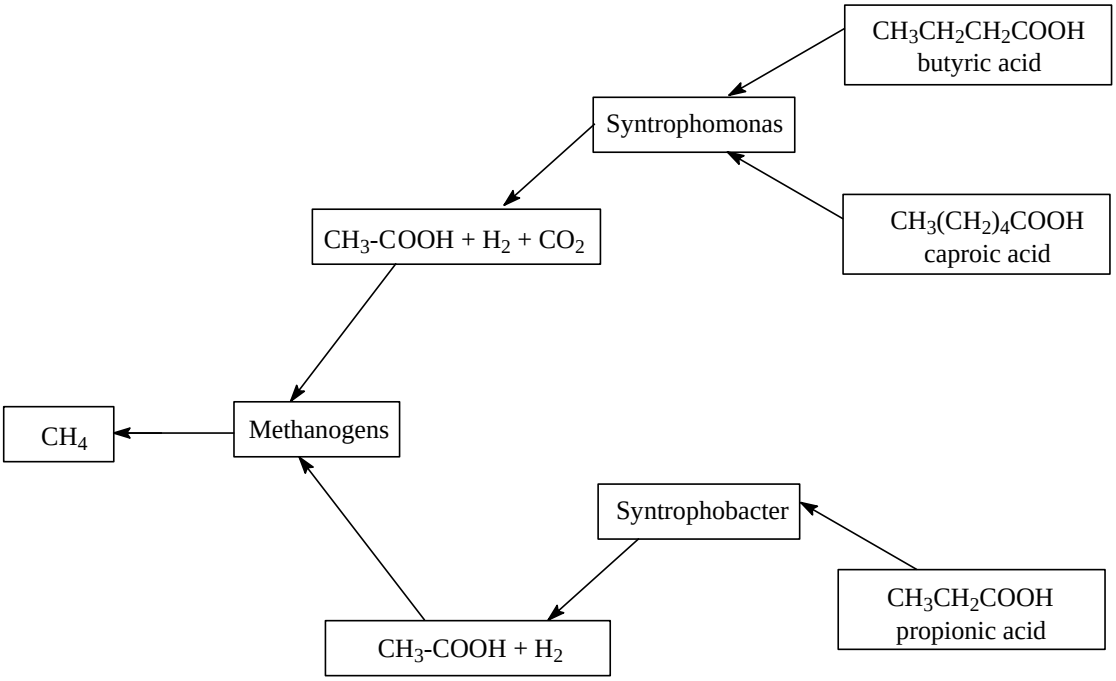


Figure 3-3. Syntrophic metabolism of fatty acids (Zieminski and Frac, 2012).

Another example of a syntrophic reaction is the oxidation of butyrate (Figure 3-3). In this reaction, oxidation of butyrate by the fatty-acid-oxidiser i.e. *Syntrophomonas* is an endergonic reaction, while consumption of the free H_2 from the reaction by their partner methanogens will release excess free energy which can be utilised by both of the microorganisms to drive the overall reaction of the fatty acids oxidation to methane (Madigan, 2015).

3.1.1. *Hydrolysis processes*

During the hydrolysis stage, polymeric material, mostly insoluble organic compounds such as carbohydrates, proteins, fats are hydrolysed to short and soluble molecules of mono-sugars, amino acids and fatty acids well as energy conservation in the form of ATP (Madigan, 2015). Different groups of bacteria carry out the hydrolysis process among them anaerobes from the *Streptococcus* and *Enterobacterium* genera (Zieminski and Frac, 2012), and some genera from the *Alphaproteobacteria* and *Firmicutes* phyla (Cirne *et al.*, 2007). According to (Toerien and Hattingh, 1969) proteolytic, denitrifying, lipolytic, and cellulolytic bacteria are common groups during the non-methanogenic stages (hydrolysis, acidogenesis and acetogenesis phases) of the AD processes. Bacteria hydrolyse organic matter using endoenzymes and exoenzymes. All bacterial groups produce endoenzymes while only a few

group of bacteria produce exoenzymes. Moreover, each bacterial group can usually produce a specific enzyme to degrade a specific substrate, but not to degrade all types of substrates (Gerardi, 2003). Bacteria release exoenzymes outside the cell to hydrolyse insoluble substrates attached to the exocellular slime, then the endoenzymes degrade the soluble wastes, which is entered the cell (Figure 3-4). The most abundant extracellular enzymes of the hydrolysis phase are amylases, proteases and lipases. Figure 3-5 shows the enzymes of the hydrolysis phase with their specific substrates and products (Gerardi, 2003). Different digestion parameters affect the rate of the hydrolysis process and include: size of particles; pH; enzyme production; and interaction between the enzymes and waste particles (diffusion and adsorption) (Gerardi, 2003).

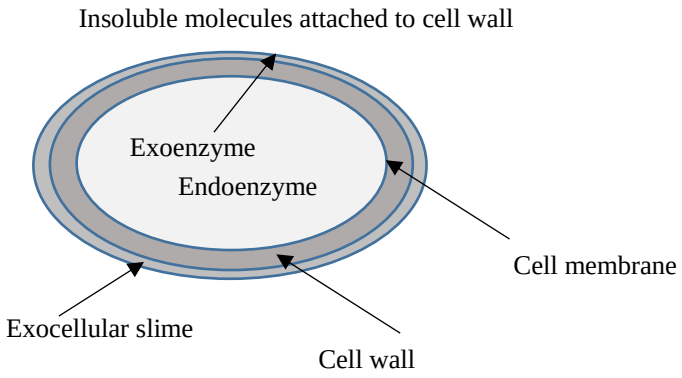


Figure 3-4. The process of enzymatic transfer through the bacterial cell wall (Gerardi, 2003).

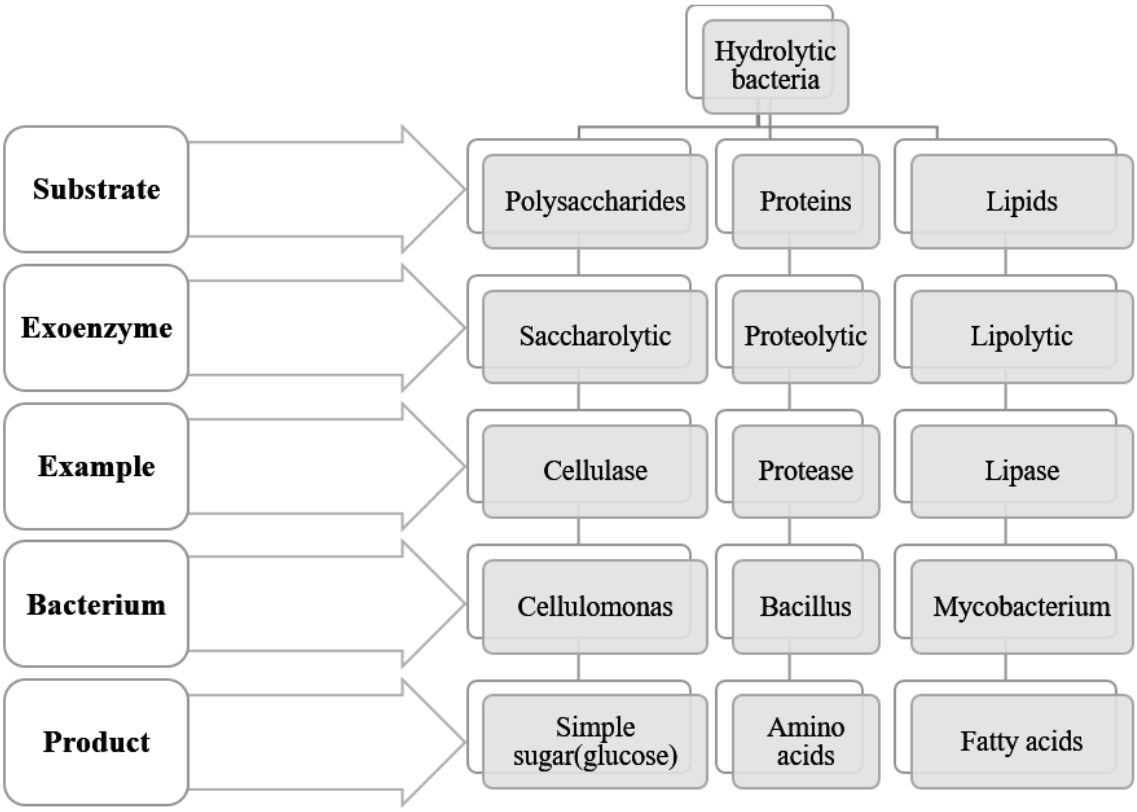


Figure 3-5. Exoenzymes of hydrolytic bacteria and their substrates (Gerardi, 2003)**.**

3.1.1.1. *Hydrolysis of carbohydrates*

Plants are the main source of carbohydrates, the general chemical structure of the carbohydrates is in the form of $C_n(H_2O)_m$. Plant materials mainly comprises cellulose (25% - 60%), hemicellulose (15% - 30%) and lignin (15% - 20%), as well as some small components such as tannins, soluble sugars and ash (Batstone, 2000a). Cellulose and hemicellulose are degradable under AD conditions, while lignin is difficult to degrade anaerobically (Batstone, 2000a).

Cellulose ($(C_6H_{12}O_6)n$) consists of long polymer fibrils of glucose molecules bonded together by strong β-1, 4 glucosidic chemical bonds. Cellulose is insoluble in water; therefore, microorganisms need to hydrolyse cellulose to soluble glucose monomers before degradation. Microorganisms such as *Cellulomonas* release extracellular enzymes such as the cellulase to break down the glucosidic bonds (Figure 3-6) and produce D-glucose molecules, then the microorganisms (usually the same microorganism) utilise the hydrolysed products to ferment to fatty acids (Batstone, 2000b; Gerardi, 2003). Batstone (2000b) reported that although the optimum pH of the cellulases ranges between pH 4 - 6, however, each hydrolysis step has its specific optimal pH value.

Cellulase enzymes act on three different positions of the cellulose chain: exoglucanase which acts on the non-reducing end of the cellulose chain to release cellobiose from cellulose, endoglucanase which randomly depolymerise internal units, and cellobiase which hydrolyses cellobiose to yield D-glucose units (Zehnder, 1988). Structurally, cellulose can be classified into crystalline and amorphous celluloses. The former contains regular and strong hydrogen bonds connecting cellulose fibrils which makes this type of cellulose less hydrolysable compared to the later which possess irregular and weak hydrogen bonds (Batstone, 2000a).

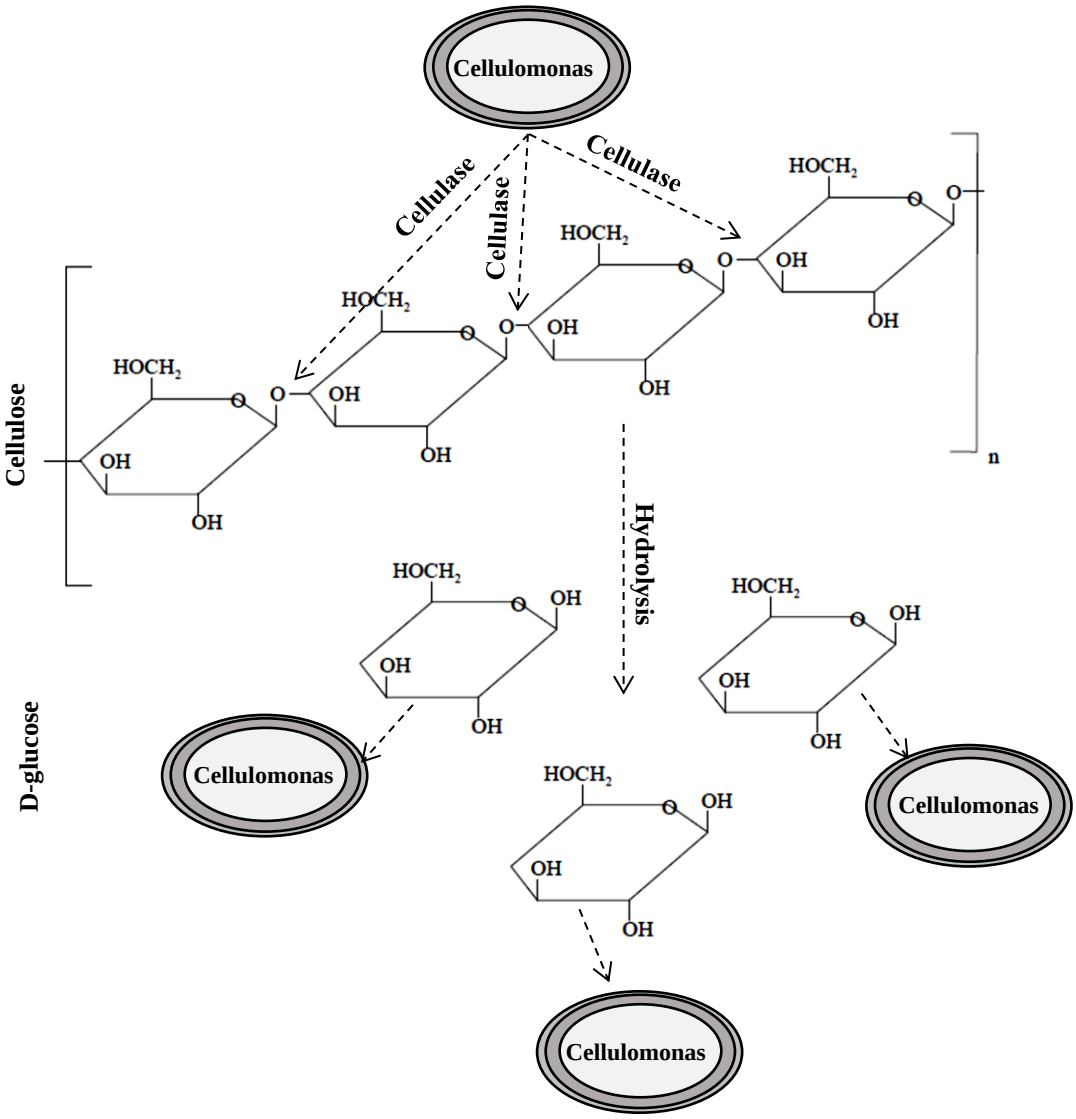


Figure 3-6. Hydrolysis of glucose to D-glucose by *Cellulomonas* (Gerardi, 2003)**.**

Hemicellulose is an amorphous structured component of polysaccharides in plants. Although hemicellulose similar to cellulose consists of linear polymers of D-glucose linked β-1, 4, however it is not considered cellulose (Zehnder, 1988). Hemicellulose is more hydrolysable than cellulose and is easily solubilised in weak alkali and acid conditions to produce products include L-arabinose, uronic acid, and hexoses such as D-glucose, D-mannose, and D-

galactose (Zehnder, 1988). Pectin, which comprises 12 - 30% of the carbohydrate in young plants, is sometimes considered a type of hemicellulose.

Lignin is the second abundant naturally occurring polymer after cellulose. It consists of dense, three dimensional, and randomly linked phenylpropane subunits, of aromatic polymers. The subunits are linked by variety of carbon-carbon as well as ether bonds. Lignin is less degradable by AD microorganisms, however, aerobic bacteria use ligninase, and H_2O_2 as an oxidant, to degrade lignin (Batstone, 2000a). From an AD perspective the problem with lignin is not just that it is difficult to degrade without oxygen and peroxidases but that the lignin physically protects the cellulose from exoenzymes, for this reason, the steam explosion is sometimes used for substrate pre-treatment (Theuretzbacher *et al.*, 2015).

3.1.1.2. *Hydrolysis of proteins*

Proteins are polymers of amino acids, the backbone structure of the proteins. Amino acids are joined by peptide bonds to form a polypeptide. Proteins consists of one or more polypeptides (Madigan, 2015). The structure of amino acids consists of two carbon atoms and one nitrogen atom arranged in three groups: an amino group ($-NH_2$), a carboxyl group (-COOH) and a side chain (R) connected to the central carbon atom (α-carbon) (Figure 3-7). The side chain (R) ranges from a simple hydrogen atom as in glycine, to aromatic rings as in phenylalanine, tyrosine, and tryptophan. The side chains of the amino acids (and ultimately the protein) governs the chemical properties such as acidic, basic, hydrophobic and hydrophilic properties of the amino acids (Madigan, 2015).

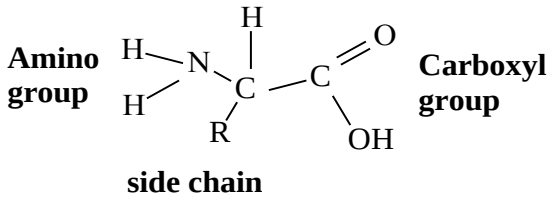


Figure 3-7. Common structure of the amino acids.

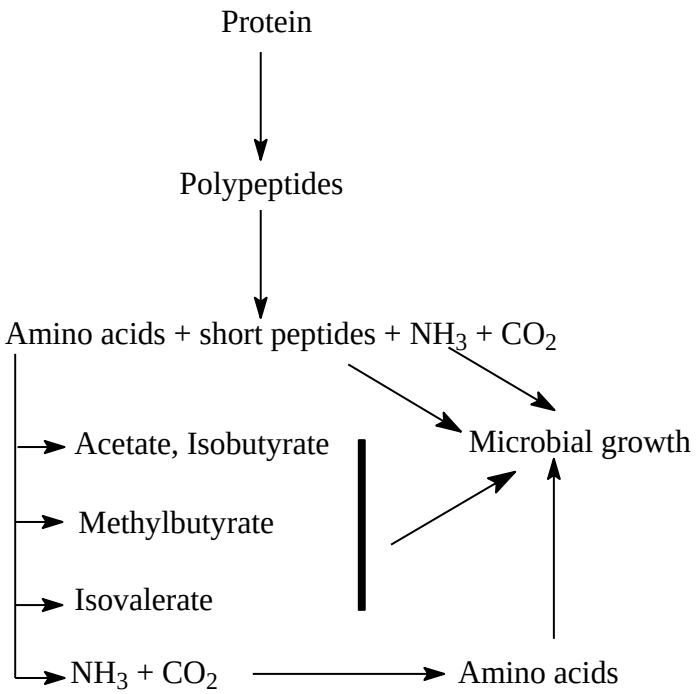


Figure 3-8. Degradation of protein and metabolism of nitrogen (Madigan, 2015).

Structurally, proteins are either fibrous or globular. The role of fibrous proteins is either connective (such as collagen) or protective (such as keratin), while the role of globular proteins is chemical functions, such as enzymes, hormones, and transport/storage proteins. Globular proteins are more hydrolysable than the fibrous proteins. There are three groups of proteases: serine-, metallo- and acid-proteases, the optimal pH of their chemical functions are pH (8 - 11), (6 - 8) and (4 - 6), respectively (Batstone, 2000a).

There are about 20 different amino acids (White, 2007). Hydrolysis of proteins releases the amino acids (Figure 3-8) as a substrate for microbial metabolism. Metabolism of the amino acids follow an oxidation-reduction reaction which converts the 20 amino acids to seven intermediates: pyruvate, acetyl-CoA, acetoacetyl-CoA, α-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate to enter the citric acid cycle (White, 2007). In this study, the organic feed substrate for the anaerobic reactors was a synthetic organic waste (SOW); it was rich of protein, which likely gave rise of a lot of the ammonia released during degradations.

3.1.1.3. *Hydrolysis of fats and phospholipids*

Lipids consist of long chains of glycerol and fatty acids connected by ester bonds in molecules called triglycerides. In the hydrolysis stage, the ester bonds between the glycerol and fatty acid chains are broken down by an extracellular enzyme i.e. the lipase, to produce glycerol and long chain fatty acids, which are metabolised by chemoorganotrophic

microorganisms such as acetogens (Madigan, 2015). Fatty acids are classified as saturated (without double bonds); monounsaturated (one double bond), polyunsaturated (two double bonds) and branched fatty acids ((White, 2007); Table 3-1).

Table 3-1. Structure of some long-chain fatty acids (White, 2007).

Fatty acids	Chemical structure	Number of carbon atoms
Palmitic	$CH_3(CH_2)_{14}COOH$	16
Stearic	$CH_3(CH_2)_{16}COOH$	18
Oleic	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	18
Linoleic	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	18
Lactobacillic	$CH_3(CH_2)_5CH(CH_2)CH(CH_2)_9COOH$	9
Tuberculostearic	$CH_3(CH_2)_7CH(CH_3)(CH_2)_8COOH$	19

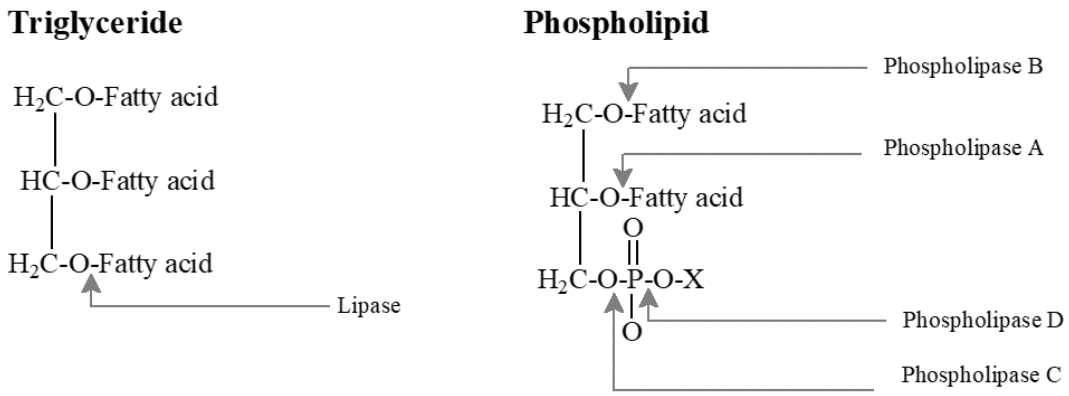


Figure 3-9. Activity of lipase and phospholipases on the fat and phospholipids respectively. The A, B, C and D designations denote the locations that phospholipase attacks the ester bonds (Madigan, 2015).

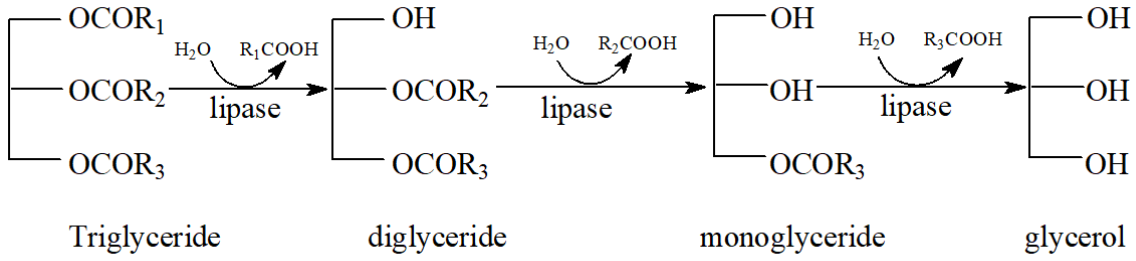


Figure 3-10. Lipase-catalysed hydrolysis of triglyceride.

Phospholipids consist of a glycerol molecule connected to phosphate by an ester bond. During the hydrolysis of phospholipids, phospholipase attacks the ester bonds at different locations

(Figure 3-9 and Figure 3-10). The phospholipase attack on the phospholipid at the A and B locations, cleaves the ester bonds between the glycerol and fatty acids. While the phospholipase attack on the C and D locations of the ester bonds produces free molecules of phospholipids and glycerol as a substrate for metabolism by microorganisms (Gerardi, 2003).

3.1.2. *Acidogenesis processes*

The products of acidogenesis stage are th883e necessary substrates for the metabolism of acetogens and methanogens. The metabolic processes of this stage are carried out by some groups of the hydrolytic bacteria that can remain active throughout this stage as well as the acidogenic bacteria (Manyi-Loh *et al.*, 2013). Facultative anaerobic bacteria, which can survive under both aerobic and anaerobic conditions, and the strict anaerobes, are the main groups of bacteria in this phase. Facultative species of this stage protect the strict anaerobic species by consuming trace O_2 molecules that might enter the AD digester through the feeding substrate. According to Anderson *et al.* (2003), the main fermentative genera and species of this phase are *Clostridium*, *Bacteroides*, *Ruminococcus*, *Butyribacterium*, *Propionibacterium*, *Eubacterium*, *Lactobacillus*, *Streptococcus*, *Pseudomonas*, *Desulfobacter*, *Micrococcus*, *Bacillus* and *Escherichia*. Typical cell concentration of the acidogenic species ranges between 10^6-10^8 cell/mL (Jiang, 2012). Some of the organic compounds are oxidised while some other compounds are reduced. Bacteria use the oxidation-reduction process to produce their required energy and produce soluble and simplest organic components. The main products of this stage are acetate, alcohol (ethanol), butyrate, lactate, mixed acid, mixed acid and butanediol, propionate and succinate, sulphide, CO_2 and methane (Gerardi, 2003). However, potential products of this stage are highly correlated to the feedstock characteristics, bacteria groups involved in the process and operational conditions of the AD digester (like pH and temperature). Some products of this stage, including formate and acetate, can be converted to methane by methanogens, while other products such as butyrate and propionate need to be converted to acetate before being used by methanogens (Gerardi, 2003).

3.1.3. *Acetogenesis processes*

The three main products of the acetogenesis stage are acetate, carbon dioxide and hydrogen. Quantitatively acetate is the most important precursor of the mesophilic AD and accounts for about 60 - 80% of the CH_4 produced by methanogens in the methanogenesis stage (Mackie and Bryant, 1981). There are two distinct groups of anaerobic species involved in the acetogenesis process, the first group known as the obligate hydrogen-producing acetogens

(OHPA). This group uses the products of the fermentation processes (hydrolysis and acidogenesis stages) including the fatty acid products (mainly propionate and butyrate), alcohols and other fatty acid components like valerate, isovalerate stearate, palmitate and etc. to produce acetate, CO_2 and H_2 via the β-oxidation pathway (Eqs. 3-4 and 3-5; (Anderson et al., 2003)). The second group known as homoacetogens, utilise hydrogen or another electron donor (alcohol) to produce acetate from the reduction of CO_2 (Eqs. 3-6 to 3-11), this metabolic pathway is also known as Wood-Ljungdahl pathway for acetate production (Jiang, 2012).

Acetogenesis via β-oxidation pathway (specific for fatty acids):

β-oxidation of propionate (Hanaki *et al.*, 1981)**:**

$$CH_3CH_2COO^- + 3\,H_2O \longrightarrow CH_3COO^- + HCO_3^- + 3H^+ + 2\,H_2 \qquad \sigma = +76.1\ kJ/mol \qquad \textbf{(3-4)}$$

β-oxidation of n-Butyrate (Hanaki *et al.*, 1981)**:**

$$CH_3(CH_2)_2COO^- + 2\,H_2O \longrightarrow 2\,CH_3COO^- + H^+ + 2\,H_2 \qquad \sigma = +48.1\ kJ/mol \qquad \textbf{(3-5)}$$

Acetogenesis via Wood-Ljungdahl pathway (specific for CO_2; (Jiang, 2012)**:**

$$2\,CO_2 + 4\,H_2 \longrightarrow CH_3COOH + 2\,H_2O \qquad \textbf{(3-6)}$$
$$4\,CO + 2\,H_2O \longrightarrow CH_3COOH + 2\,CO_2 \qquad \textbf{(3-7)}$$
$$4\,HCOOH \longrightarrow CH_3COOH + 2\,H_2O \qquad \textbf{(3-8)}$$
$$4\,CH_3OH + 2\,CO_2 \longrightarrow 3\,CH_3COOH + 2\,H_2O \qquad \textbf{(3-9)}$$
$$2\,CO + 2\,H_2 \longrightarrow CH_3COOH \qquad \textbf{(3-10)}$$
$$2\,HCOOH + 2\,H_2 \longrightarrow CH_3COOH + 2\,H_2O \qquad \textbf{(3-11)}$$

Continuous production of H_2 by OHPA species would potentially increase the H_2 concentration inside the anaerobic digester systems, which ultimately inhibits the growth of OHPA followed by the reactor failure and low methane production (Anderson *et al.*, 2003). However, the syntrophy of acetogenic bacteria with hydrogenotrophic methanogenic archaea (which need H_2 to reduce CO_2 in order to produce methane at the methanogenesis stage; Eq. 3-13) maintains the favoured H_2 partial pressure ($< 10^{-4}$ atm) for optimal syntrophic growth rates (Anderson *et al.*, 2003; Gerardi, 2003; Jiang, 2012).

The OPHA and methanogens relationship is considered a weak relationship, whereby, any substantial accumulation of fatty acids will inhibit methanogens, which in turn results in an increased H_2 partial pressure in the reactor and ultimately the failure of the reactor. However, Anderson *et al.* (2003) reported that most of the methanogenic processes are unlikely to result

in hydrogen pressure above 10^{-4} atm. The most recently isolated acetogenic species are *Syntrophomonas wolfei* and *Syntrophobacter wolinii*, each gram of mesophilic sludge contains about 4.5 x 10^{6} *Syntrophomonas wolfei* cells (Anderson *et al.*, 2003).

3.1.3.1. *Glycolysis and lactate production*

Glycolysis also known as *Embden-Meyerhof-Parnas pathway* is the most common metabolic pathway for fermentation of organic compounds by anaerobic bacteria to produce energy-rich compounds for microorganisms in form of ATP by substrate-level-phosphorylation process. Organic compounds such as carbohydrates, especially hexose sugars like glucose, acts as both an electron donor and an electron acceptor in the fermentation reactions. The products of the glycolytic process are two molecules of pyruvate, as well as energy conservation in the form of ATP (Figure 3-11).

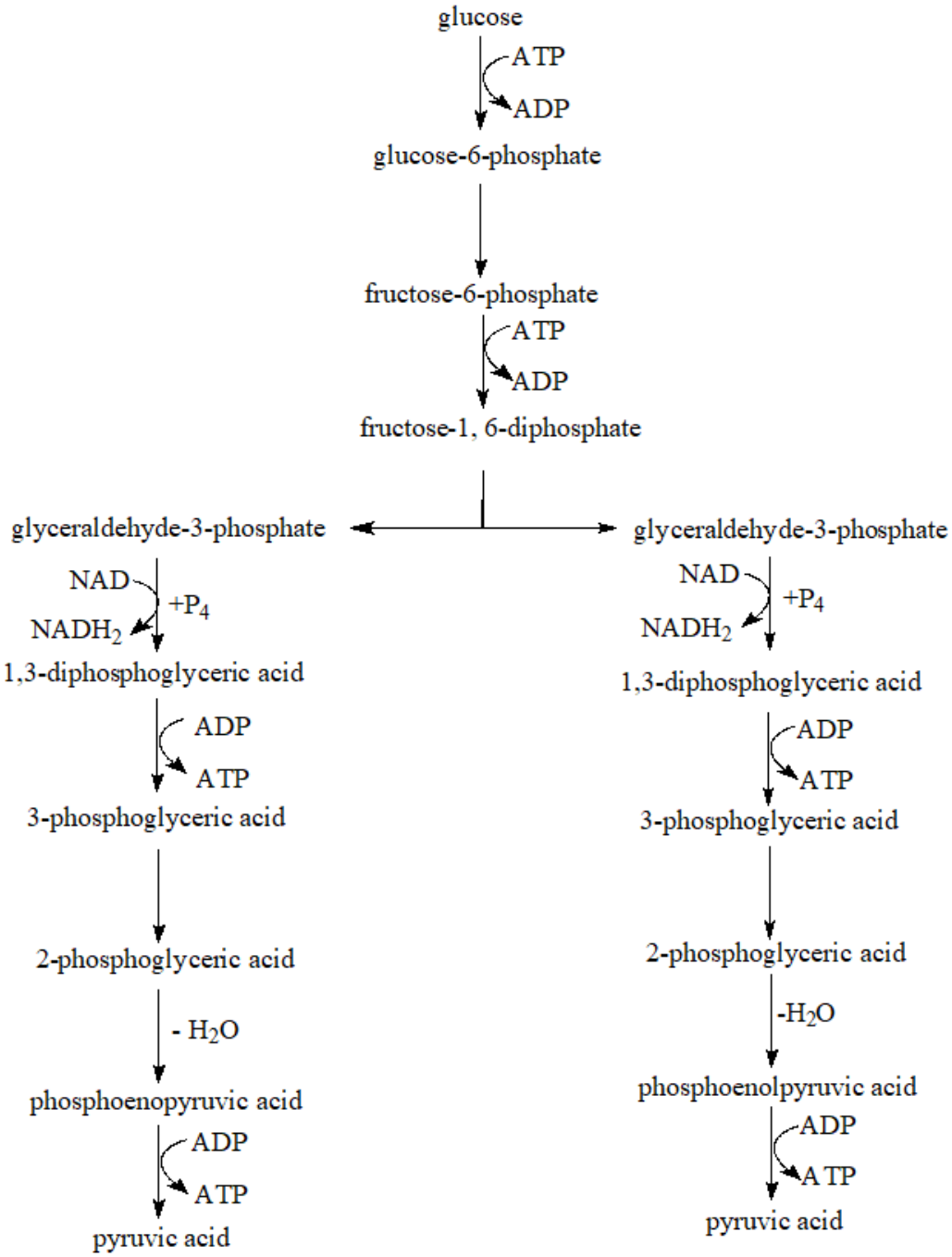


Figure 3-11. Degradation of glucose through the Embden-Meyerhof pathway (Madigan, 2015).

The formation of pyruvate is the terminal step for glycolysis. However, the cells need to achieve their redox balance; therefore, the fermentation reaction is necessary to continue producing further fermentation products such as lactate, butanol, butyrate, Ethanol, isopropanol, propionate and acetate. Although lactic acid is the main fermentation product of the glycolysis pathway, however after formation of pyruvic acid the fermentation reactions continue to produce wide array of fermentation products depending on the metabolic pathway adapted by the reductive bacteria to ferment pyruvic acids ((Madigan, 2015); Figure 3-12).

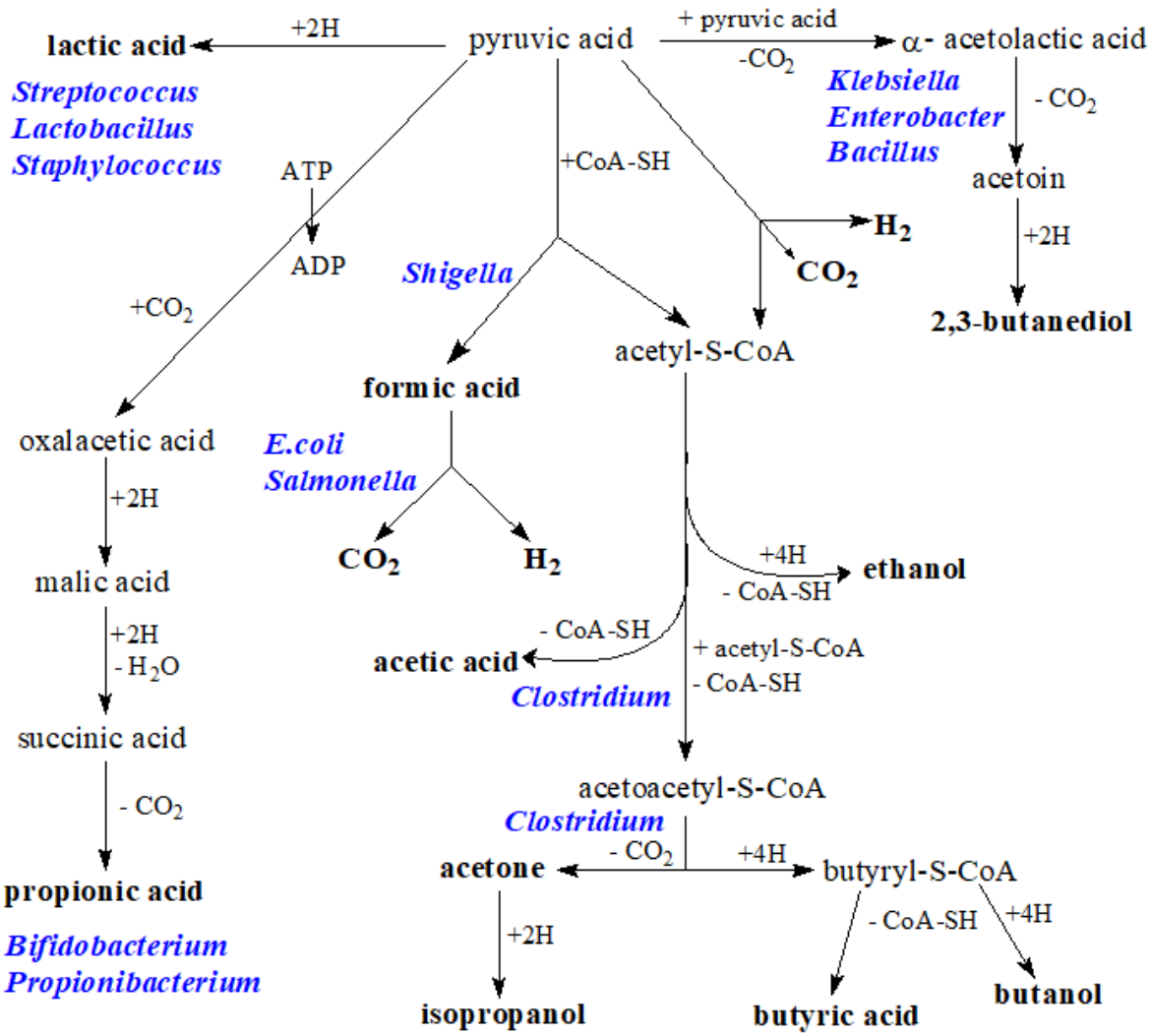


Figure 3-12. Fermentations of pyruvic acid through the Embden-Meyerhof pathway. Representative bacteria that utilize these pathways are shown in BLUE.

There are three biochemical reactions for lactate formation from carbohydrates (mainly glucose) as well as other sugars such as fructose, galactose, mannose, saccharose, lactose, maltose, and pentoses (Eqs. 3-12, 3-13 and 3-14):

$$\text{glucose} \longrightarrow 2\ \text{lactate} \qquad \textbf{(3-12)}$$

$$\text{glucose} \longrightarrow \text{lactate} + \text{ethanol} + CO_2 \qquad \textbf{(3-13)}$$

$$2\ \text{glucose} \longrightarrow 2\ \text{lactate} + 3\text{acetate} \qquad \textbf{(3-14)}$$

Two groups of bacteria are responsible of lactate formation, *Homofermentative* and *Heterofermentative* bacteria. The former contains an *aldolase* enzyme and therefore uses the glycolysis pathway while the later lacks *aldolase* and uses pentose phosphate pathway for lactate fermentation. The most common microbial genera for lactate fermentation are

Bifidobacterium, Lactobacillus, Leuconostoc, Pediococcus, Sporolactobacillus, and *Streptococcus.*

Homofermentative bacteria for each molecule of glucose can produce only one fermentation product which is two molecules of lactate (Eq. 3-15), while the hetrofermentative bacteria, in addition to lactate can produce other fermentation products, mainly ethanol and CO_2, (Eq. 3-16; (Madigan, 2015)).

Overall fermentation reaction of Homofermentative:

$$\text{glucose} + 2\ \text{ADP} + 2\ \text{Pi} \longrightarrow 2\ \text{lactate} + 2\ \text{ATP} \qquad \textbf{(3-15)}$$

Overall fermentation reaction of Heterofermentative:

$$\text{glucose} + \text{ADP} + \text{Pi} \longrightarrow \text{ethanol} + \text{lactate} + CO_2 + \text{ATP} \qquad \textbf{(3-16)}$$

The third pathway for lactate formation is the *Bifidum pathway*. The bacteria pattern of this fermentation pathway uses both the pentose phosphate pathway and the *homofermentative* pathway reactions. This pathway ferments two glucoses to two lactates, three acetates and five ATPs (Eq. 3-17; (Madigan, 2015).

Overall fermentation reaction of Bifidum pathway:

$$2\ \text{glucose} + 5\ \text{ADP} + \text{Pi} \longrightarrow 3\ \text{acetate} + 2\ \text{lactate} + 5\ \text{ATP} \qquad \textbf{(3-17)}$$

3.1.3.2. *Acetate formation (acetogenesis)*

As described in Section 3.1.3 most of bacteria produce acetate from H_2 and CO_2, or from the oxidation of other VFA. Acetogens can convert hexose molecules e.g. glucose to pyruvate through the glycolysis pathway, by-products of this fermentation reaction are two molecules of pyruvate and 4[H]. The Pyruvate molecules are then oxidised to two molecules of acetyl-CoA, 4[H] and two CO_2 molecules by using pyruvate: ferredoxin oxidoreductase enzyme (White, 2007; Schuchmann and Müller, 2016). Then from these two molecules of acetyl-CoA two molecules of acetate are produced (Figure 3-13).

The third molecule of acetate of this reaction is produced from the fixation of the two molecules of CO_2 and four [H] generated from the oxidation of two pyruvates to two acetates, and the four [H] generated during glycolysis via the Wood-Ljungdahl pathway (Figure 3-14). One of the CO_2 molecules is reduced to the methyl group and the other CO_2 is reduced to the carbonyl group of the third acetate molecule produced in this reaction (Figure 3-14).

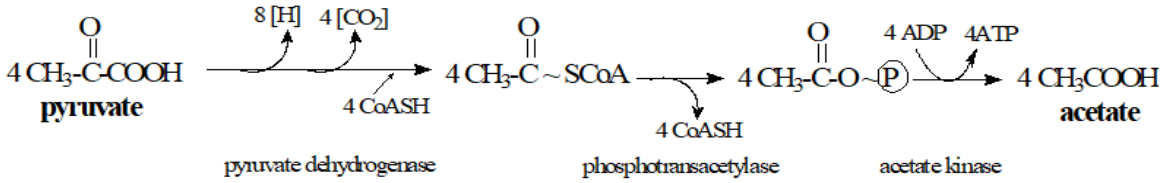


Figure 3-13. Acetogenesis from pyruvate by *Desulfotomaculum thermobenzoicum* (White, 2007) .

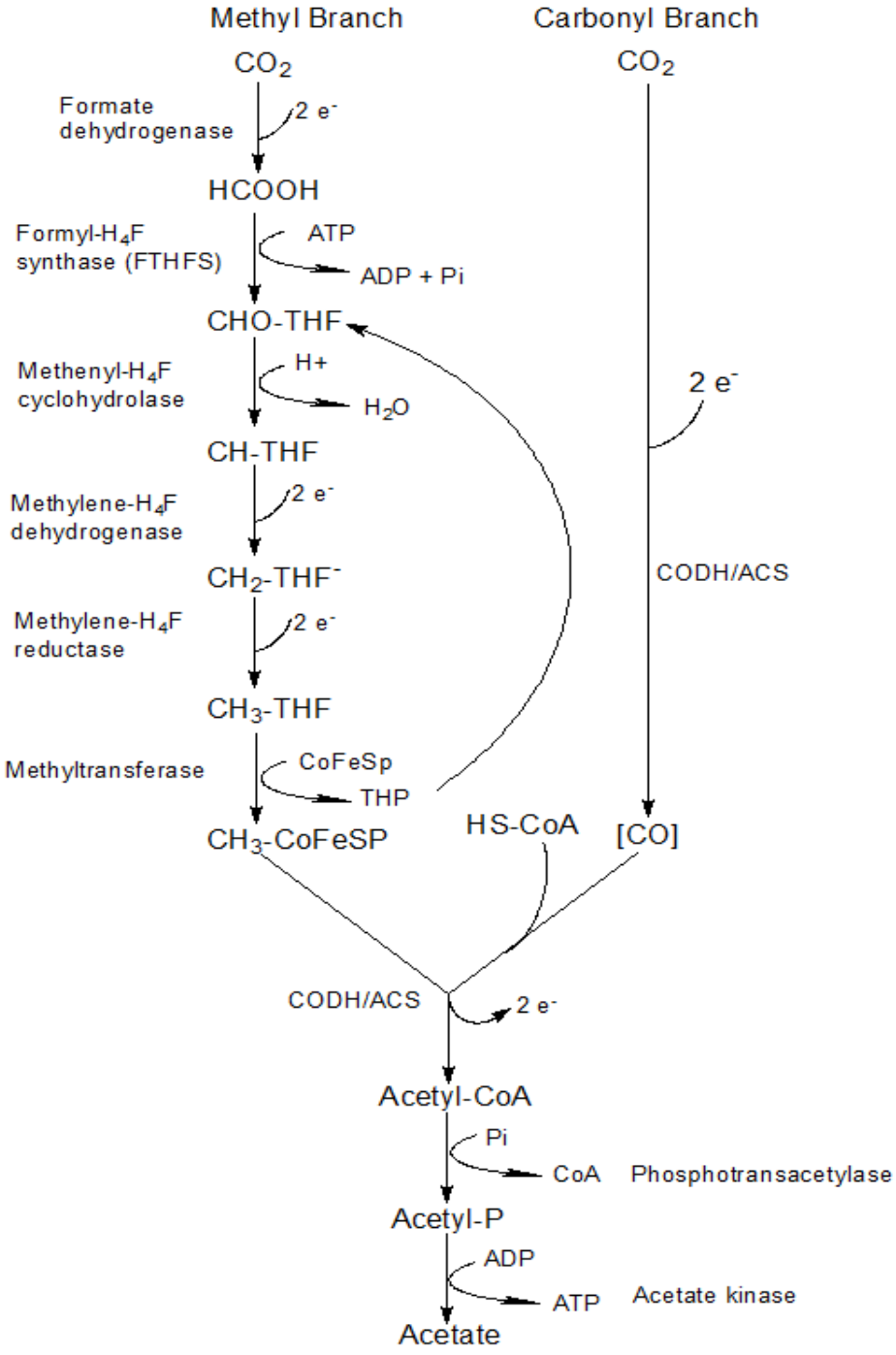


Figure 3-14. Acetogenesis from CO_2 following the Wood–Ljungdahl pathway (Schuchmann and Müller, 2016).

3.1.3.3. *Propionate formation*

Propionate is one of the main products of acidogenesis that must be converted to acetate for methane production by methanogens. Accumulation of propionate in the AD digester is an indicator of the start-up of the failure of the AD processes (Chen *et al.*, 2008).

Propionic bacteria (*Corynebacteria*, *Propionibacterium* and *Bifidobacterium*) can either produce propionate from glucose via pyruvate fermentation or from the lactate fermentation produced from glucose by glycolysis reactions (Eqs. 3-18 and 3-19). Succinate is an intermediate product of fermentation, in addition some amount of succinate can be found as an end product of propionate fermentation (Gerardi, 2003).

$$1.5\ \text{glucose} \longrightarrow 2\ \text{propionate} + \text{acetate} + CO_2 \quad \textbf{(3-18)}$$

$$3\ \text{lactate} \longrightarrow 2\ \text{propionate} + \text{acetate} + CO_2 \quad \textbf{(3-19)}$$

The acrylate pathway and the succinate-propionate pathway are two common pathways for fermentation of lactate to propionate. Each of the two pathways uses 3 molecules of lactate, produced from the fermentation of glucose via glycolysis. During the acrylate pathway one molecule of lactate is oxidised to pyruvate then to acetyl-CoA and CO_2. After that acetyl–CoA is converted to acetate and ATP via acetyl-P. This oxidation process yields 4[H] which must be utilised by an electron acceptor to balance the reaction. The second molecule of lactate acquires a CoA from propionyl-CoA to produce lactyl-CoA which is then dehydrated to produce the unsaturated molecule of acrylyl-CoA. Acrylyl-CoA is reduced to propionyl-CoA, using 2[H] of the 4[H] produced from the oxidation of the first lactate molecule. Similarly, the third molecule of lactate is oxidised to consume the last 2[H] molecules of the first lactate molecules, this balance the fermentation reaction. The transfer of CoA from propionyl-CoA to the second and third lactate molecules would produce two molecules of propionate. Ultimately, the overall reaction of three moles of lactate by acrylate pathway would produce one mole of acetate, two moles of propionate, one mole of CO_2 and one mole of ATP (Figure 3-15).

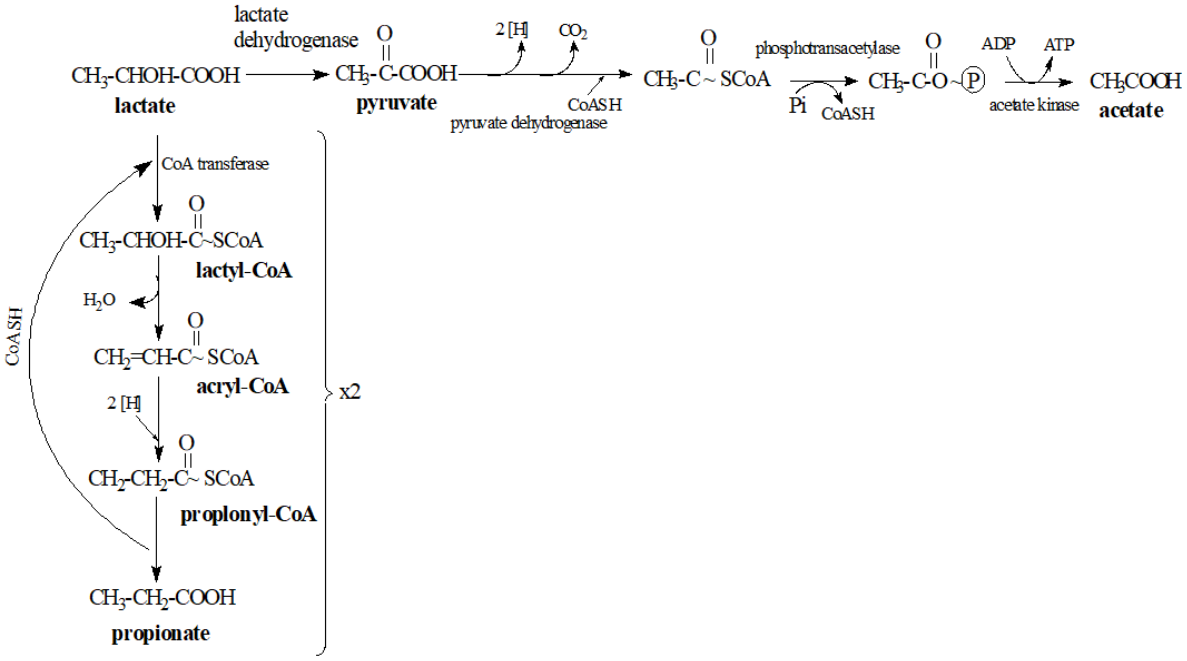


Figure 3-15. Propionate formation via acrylate pathway (White, 2007).

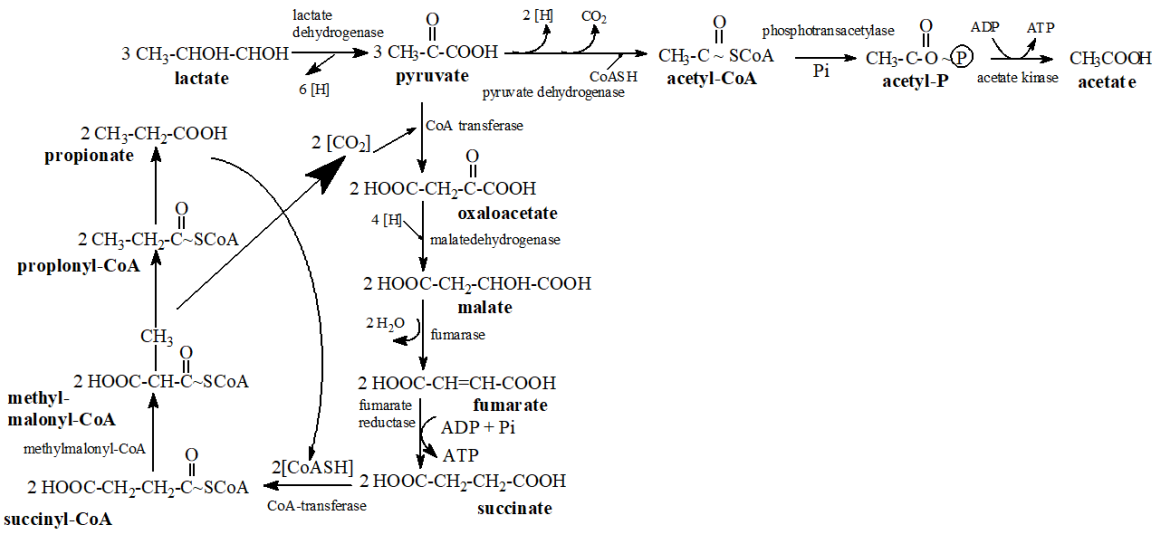


Figure 3-16. Propionate formation via succinate-propionate pathway (White, 2007).

One bacterium *Clostridium propionicum* uses this pathway to yield only one-third of an ATP per lactate, this low ATP production per reaction results in low growth yield for these organisms. Therefore, in order to produce more ATP per three molecules of lactate, many bacteria; among them *Propionibacterium*, use the succinate-propionate pathway to produce propionate as a fermentation product (Figure 3-16). *Propionibacterium* ferments three molecules of lactate, to produce a mixture of propionate, acetate, succinate and CO_2 (White, 2007).

3.1.3.4. *Butyrate formation*

A heterogeneous group of anaerobic bacteria classified under the genus *Clostridium,* including saccharolytic, proteolytic clostridia can ferment carbohydrates and proteins respectively. Other clostridia groups can ferment substrates like ethanol, acetate, and certain purines and amino acids. The butyrate formers are strict anaerobes in the *Clostridium* genera, and *Butyrivibrio* ferment carbohydrates (Eq. 3-20) to butyric acid, H_2, CO_2 and small amount of acetate (Gerardi, 2003).

$$\text{Hexose} \longrightarrow CH_3\text{-}CH_2\text{-}CH_2\text{-}COOH \qquad \textbf{(3-20)}$$

In the AD process, two molecules of acetyl-CoA condense to form acetoacetyl-CoA; which reduces to butyryl-CoA. After that, phosphotransacetylase converts butyryl-CoA to butyryl-P. Finally, kinase produces butyrate and ATP from butyryl-P. Conversion of one mole of glucose through the glycolytic pathway to butyrate produces three moles of ATP (two ATP from glycolytic pathway and one ATP from butyryl-P) and one mole of butyrate (White, 2007). With respect to impacts on AD, the accumulation of butyrate drops pH, the low pH conditions (pH < 4.5) push the cells especially *Clostridium acetobutylicum* to convert the butyrate molecules to butanol and acetone. Butanol is highly toxic to bacteria because it affects the functions of cellular membrane (Gerardi, 2003).

3.1.3.5. *Lipids and oxidation of fatty acids (β-oxidation)*

Lipids are a heterogeneous group of organic compounds soluble in nonpolar solvents and relatively insoluble in water. As described in Section 3.1.1.3 hydrolysis of lipids in AD produces long chain fatty acids. The role of fatty acids in the structure of the cells is related to the functions of the cell wall. In addition, many bacteria can use fatty acids as a source of energy production by oxidation through the β-oxidation pathway (Figure 3-17). For β-oxidation of fatty acids with even number of carbon atoms, the fatty acid is activated by coenzyme-A, then the oxidation reaction cleaves the carbon chain between the α and β carbon atoms of the fatty acid chain to produce acetyl-CoA. After that, acetyl-coA is oxidised to produce acetic acid, CO_2 and H_2. If a branched chain or a fatty acid with odd number carbon atoms is oxidised, the product of β-oxidation is either propionyl-CoA or branched-chain fatty acid-CoA and an acetyl-CoA molecule. Therefore, the final β-oxidation product of the odd carbon fatty acids is always propionic acid (White, 2007).

According to White (2007) acrylyl-CoA pathway (Figure 3-18) and methylcitrate pathway (Figure 3-19) are two possible pathways for the conversion of propionyl-CoA to acetyl-CoA.

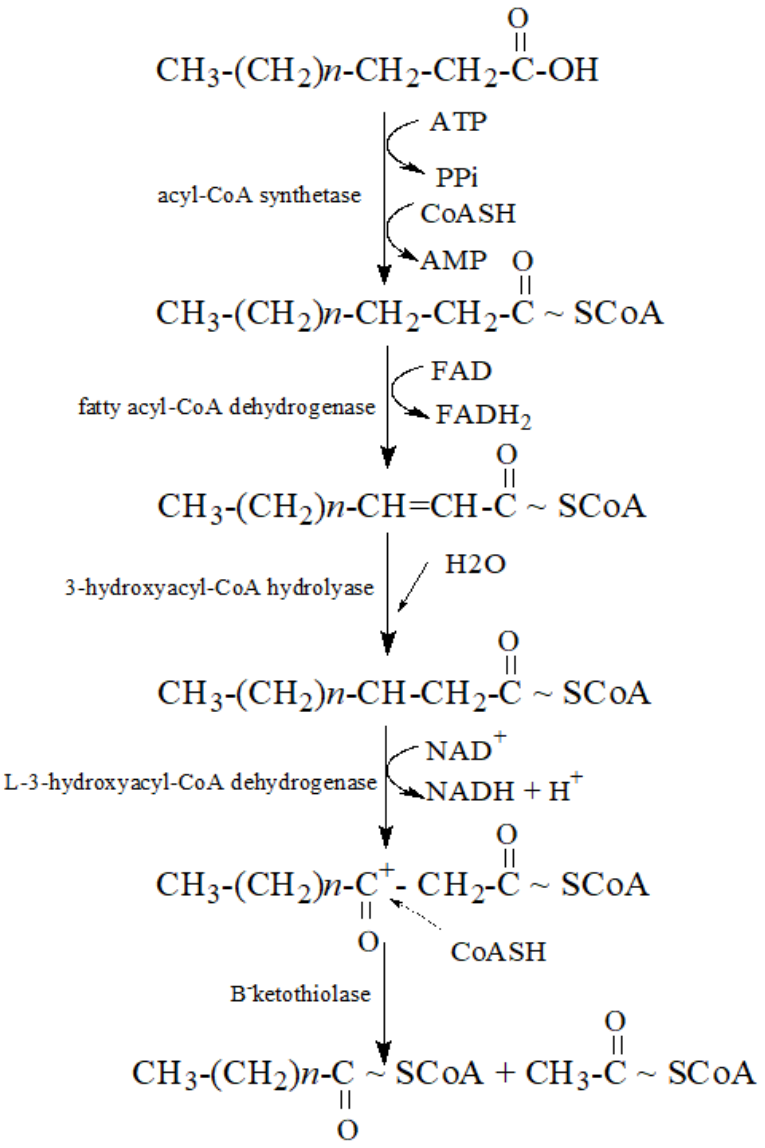


Figure 3-17. β-oxidation of fatty acids (White, 2007).

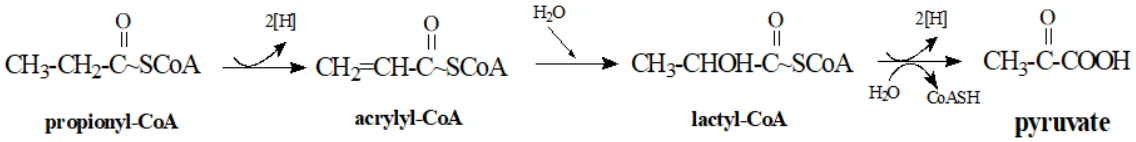


Figure 3-18. Oxidation of propionyl-CoA to pyruvate via acrylyl-CoA pathway (White, 2007).

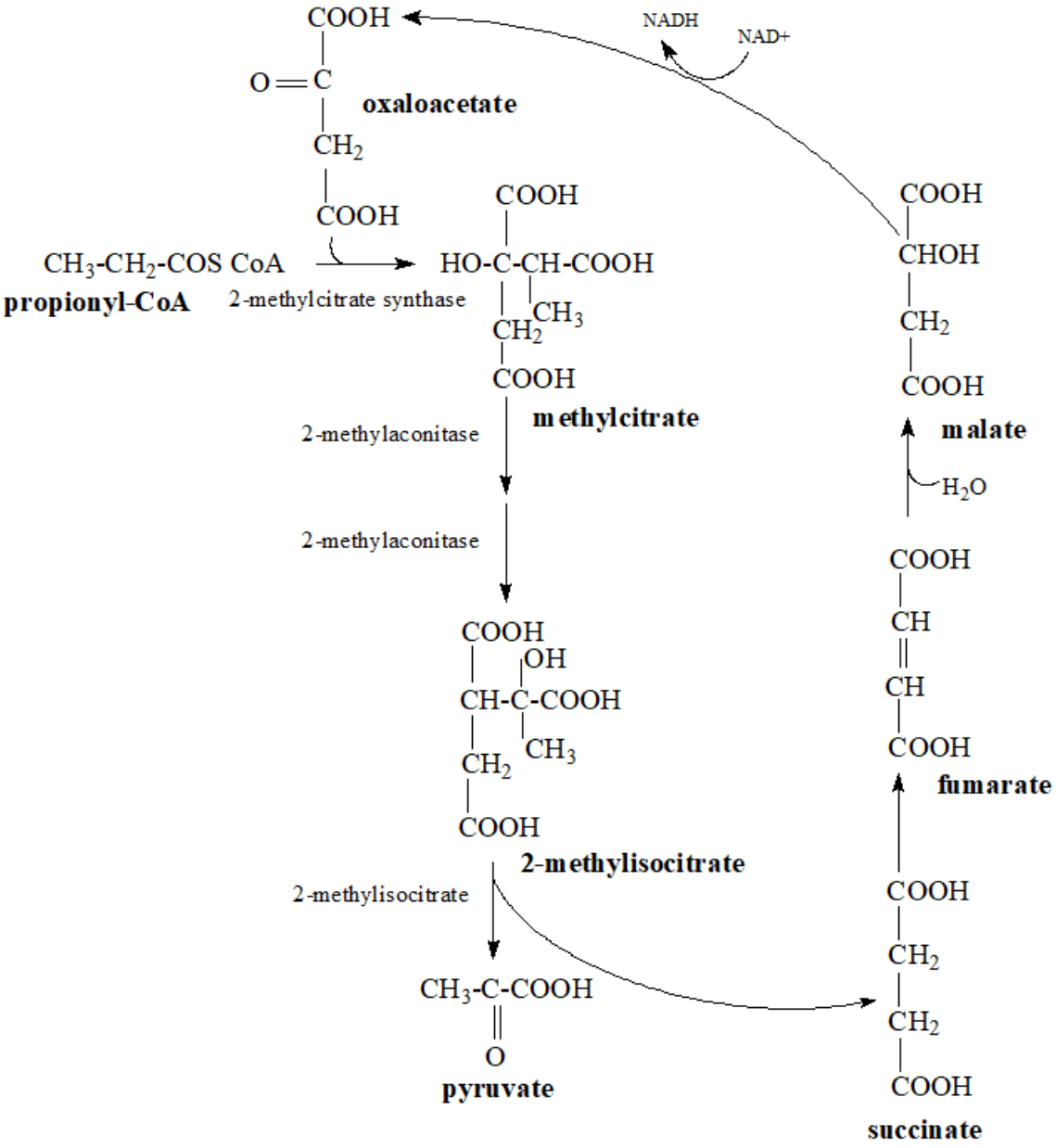


Figure 3-19. Oxidation of propionyl-CoA to pyruvate via the methylcitrate pathway (White, 2007).

3.1.4. *Methanogenesis*

The final stage of the AD processes is Methanogenesis. As described in Section 3.1 two groups of methanogenic archaea can produce methane from carbon dioxide (CO_2), hydrogen (H_2), formate (HCOOH), and carbon monoxide ((CO); Eqs. 3-21, 3-22 and 3-23).

$$CO_2 + 4\ H_2 \longrightarrow CH_4 + 2\ H_2O \quad \textbf{(3-21)}$$

$$2\ HCOOH \longrightarrow CH_4 + CO_2 \quad \textbf{(3-22)}$$

$$4\ CO + H_2O \longrightarrow CH_4 + 3CO_2 \quad \textbf{(3-23)}$$

Methylotrophic methanogens can produce methane from the substrates carrying methyl group rather than using CO_2, mong these substrates are acetate, methylamine and methanol (Eqs. 3-24, 3-25 and 3-26). Practically, acetate, and CO_2 and H_2 are the only industrially important substrates for methane production (Galagan *et al.*, 2002).

$$CH_3COOH \longrightarrow CH_4 + CO_2 \quad \textbf{(3-24)}$$

$$4\ (CH_3)_3\text{-}N + 6\ H_2O \longrightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_3 \quad \textbf{(3-25)}$$

$$3\ CH_3OH + 3\ H_2 \longrightarrow 3\ CH_4 + 3\ H_2O \quad \textbf{(3-26)}$$

Table 3-2. Some of the important reactions related to the acetogenesis and methanogenesis phases of AD (Kirsop and Wolfe, 1983).

	Substrates	Products	σ KJ/reaction
Acetigenic reactions	$Propionate^- + 3\ H_2O$	$Acetate^- + HCO_3^- + H^+ + 3H_2$	+ 76.1
	$Butyrate^- + 2\ H_2O$	$2\ Acetate^- + H^+ + 2\ H_2$	+ 48.1
	$Caproate^- + 4\ H_2O$	$3\ Acetate^- + 2\ H^+ + 4\ H_2$	+ 96.2
	$Lactate^- + 2\ H_2O$	$Acetate^- + HCO_3^- + H^+ + 2H_2$	- 4.2
	$Ethanol^- + H_2O$	$Acetate^- + H^+ + 2\ H_2$	+9.6
	$Lactate^- + SO_4^-$	$2\ Acetate^- + 2\ HCO_3^- + 2\ H^+$	-218.8
	$2\ CO_2 + 4\ H_2$	$Acetate^- + H^+ + 2\ H_2O$	-95.0
Methanogenic reactions	$4\ H_2 + H^+ + HCO_3^-$	$CH_4 + 3\ H_2O$	-135.6
	$Acetate^- + H_2O$	$CH_4 + HCO_3^-$	-31.0
	4 Methanol	$3\ CH_4 + HCO_3^- + H^+ + H_2O$	-315.1
	Methanol + H_2	$CH_4 + H_2O$	-112.5
	4 Methanol + Acetate	$4\ CH_4 + 2\ HCO_3^- + H^+$	- 471.4
	4 Methylamine + $3\ H_2O$	$3\ CH_4 + HCO_3^- + 4\ NH_4^+ + H^+$	- 225.4
	2 Dimethylamine + $3\ H_2O$	$3\ CH_4 + HCO_3^- + 2\ NH_4^+ + H^+$	-220.0
	4 Trimethylamine + $9\ H_2O$	$9\ CH_4 + 3\ HCO_3^- + 4\ NH_4^+ + 3\ H^+$	-159.8
	Formate + $H^+ + 3\ H_2$	$CH_4 + 2\ H_2O$	-134.3

In AD, a high organic loading rate or short retention time will lead to accumulation of fatty acids, which puts pressure on the methanogens and decreases their activity to degrade the products of acetogenesis stage; therefore, for an organic rich feedstock such as OFMSW, the methanogenesis stage is the rate-limiting stage.

Methanogens are classified under the archaea kingdom, the five phylogenetic orders of the methanogens are *Methanobacteriales, Methanopyrales, Methanococcales, Methanomicrobiales* and *Methanosarcinales* (Bapteste *et al.*, 2005). *Methanosarcina* is the most versatile genus of methanogens that can accomplish three pathways of methanogenesis using at least nine different methanogenic substrates including acetate, while the other orders

possess only single pathway for methanogenesis using no more than two methanogenic substrates (Galagan *et al.*, 2002).

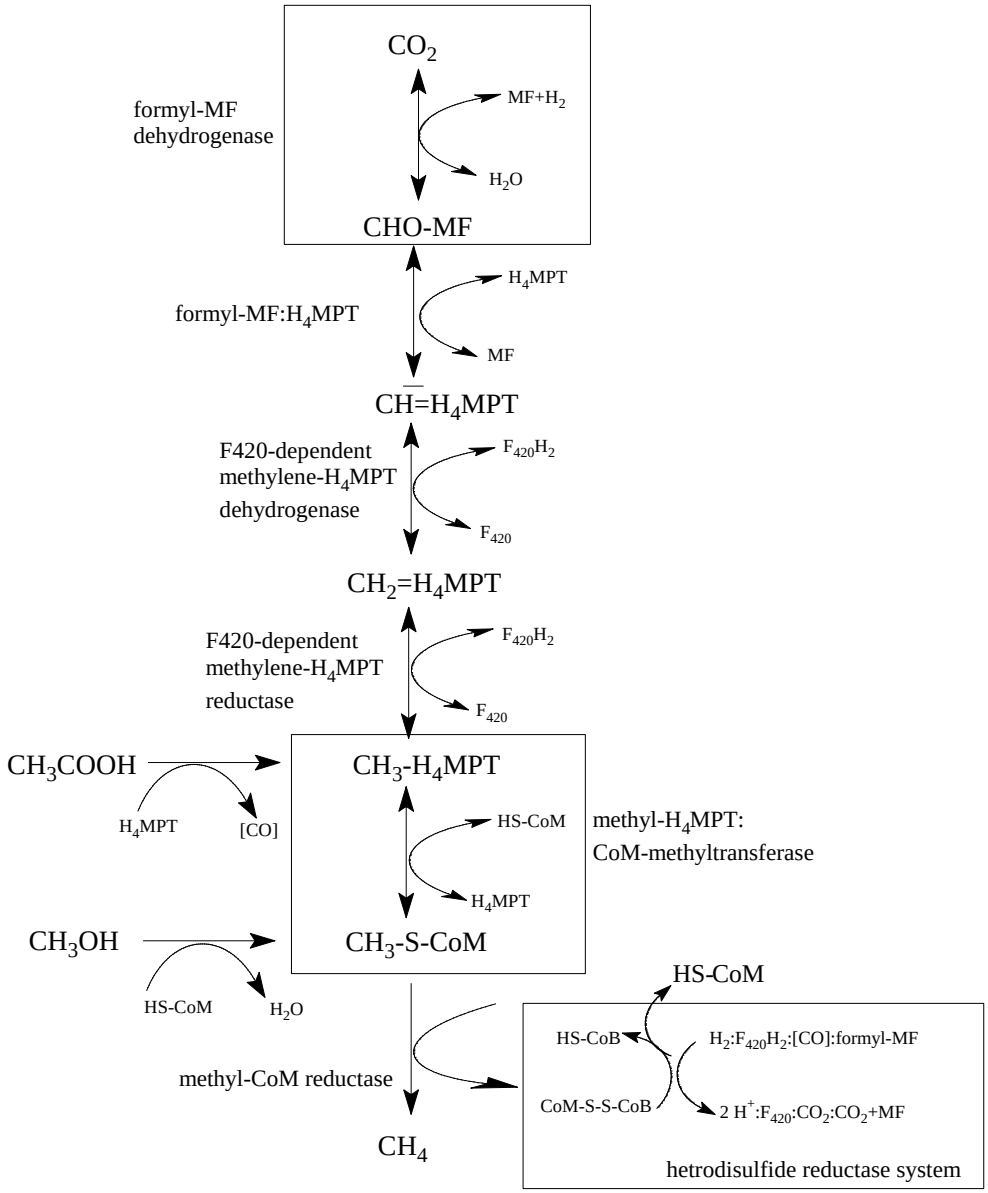


Figure 3-20. Hydrogenotrophic pathway of methanogenesis. The boxes show reactions which result in energy conservation (Schäfer et al., 1999).

Hydrogen is the electron donor for the reduction pathway of CO_2 to CH_4 (Figure 3-20). Firstly, methanofuran activates CO_2 to reduce to a formyl group. After that, the formyl functional group is transferred to co-enzyme containing methanopterin, which reduces it to methylene. Then the methyl functional group is transferred to co-enzyme M (CoM) to produce methyl-CoM. Finally, methyl-CoM is reduced by methyl reductase to methane with the presence of two coenzymes F430 and CoB. The two coenzymes CoM and CoB are regenerated by the reduction of CoM-S-S-CoB with H_2 (Madigan, 2015). Similarly, Figure

3-20 shows the acetoclastic disproportionation of acetate to methane and CO_2 by two species of methanogens *Methanosarcina* and *Methanosaeta*.

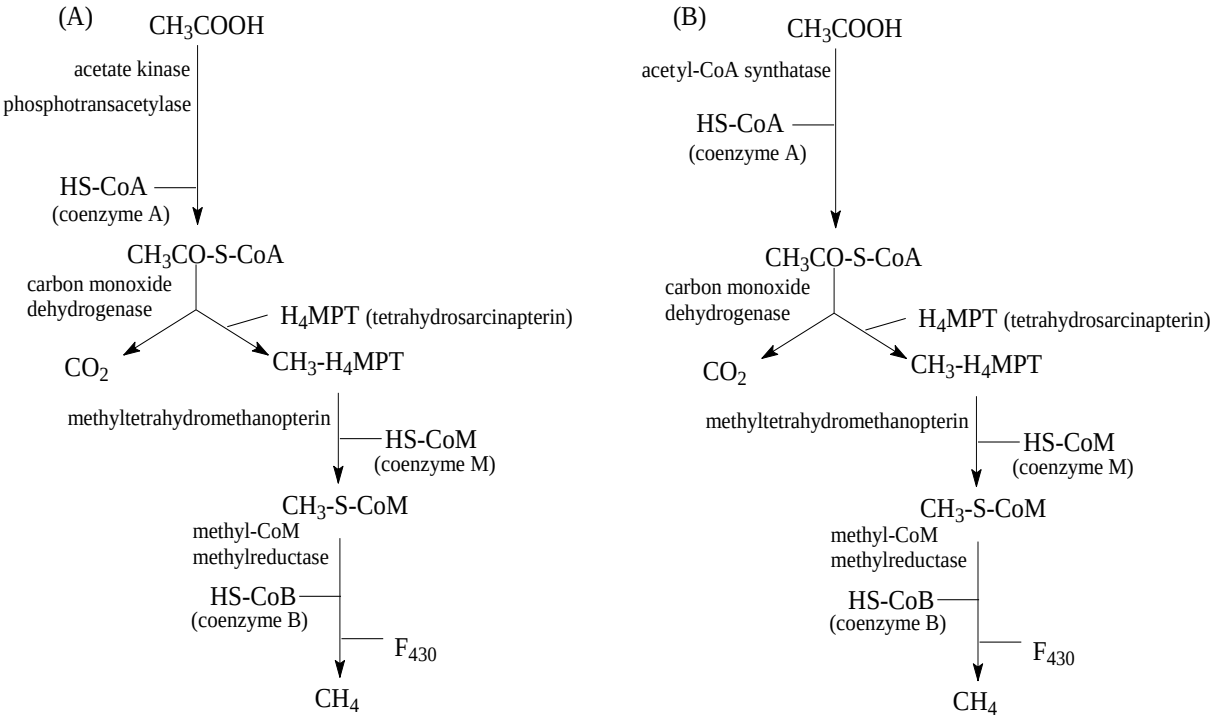


Figure 3-21. Acetoclastic pathway of methanogens (A) *Methanosarcina* and (B) *Methanosaeta* (Madigan, 2015).

3.2. Parameters of anaerobic digestion

3.2.1. *Operational parameters*

3.2.1.1. *Temperature*

Variations in temperature affects metabolic and synthetic activities of both hydrolytic/fermentative bacteria and methanogenic archaea, therefore the temperature of AD can significantly affect the substrate conversion, kinetics, process stability, digestate quality and consequently the methane yield (Sanchez *et al.*, 2001). The optimal temperature ranges for mesophilic AD is 35 - 37^{o}C, and that for thermophilic AD is from 55 - 60^{o}C, respectively (Rittmann, 2001). Although thermophilic AD results in higher biogas production rates, pathogen removal etc. than mesophilic digestion, the thermophilic process is more sensitive to environmental change, expensive to build and energy consuming to run (Kim *et al.*, 2002). Whereas, compared to the thermophilic reactors, mesophilic reactors are more susceptible to inhibitions due to ammonia accumulation, and less tolerant to increases in the organic loading rates and decreases in retention time (Gallert and Winter, 1997; Sanchez *et al.*, 2001; Kim *et*

al., 2006). However, in general, conventional anaerobic digestion is often performed at mesophilic temperature of 35 – 37ºC (Ahn and Forster, 2002; Kim *et al.*, 2006). OFMSW is a nitrogen rich substrate (from proteins in food waste) on fermentation forms high NH_3-N concentration inhibitory to methanogens. Ammonia toxicity increases as temperature increases due to the increase in free ammonia concentration (Kayhanian, 1999). Therefore, ammonia concentration above 0.7 g N/L limits the temperature tolerance in AD (Angelidaki and Ahring, 1994). Zeeman *et al.* (1985) suggested a 3 g N/ L as a critical threshold concentration in thermophilic AD at 50ºC. Angelidaki and Ahring (1994) obtained a stable conversion of VFA to biogas and better process stability at high ammonia loads by decreasing the digestion temperature below 55°C to the range of 37 - 40ºC. Moreover, thermophilic temperature at high OLR may lead to excessive accumulation of VFA in AD. Xu *et al.* (2014) reported intensive accumulation of VFA at thermophilic degradation of kitchen wastes at 55ºC with the increase in OLR, specifically the build-up in acetic acid concentration, which became inhibitory for methanogens at 1.5 – 2.5 g/L.

Furthermore, changes in temperature have effects on the syntrophic relationships between the acid producers (bacteria) and acid consumers (archaea) in AD. Montañés *et al.* (2015) studied the effect of temperature on the degradation of sewage sludge and sugar beet pulp lixiviation by *Eubacteria* and Archaea; they obtained higher volatile solids conversion to methane under mesophilic conditions than under thermophilic conditions.

Temperature increase enhances the release of metals in AD. Anjum *et al.* (2017) found that the release of metals such as Zn and Cu exceeded the toxic thresholds under thermophilic anaerobic conditions. In this regard, in this study, the anaerobic experiments were carried out at mesophilic temperature (37ºC); to ensure that the amount of trace elements and nutrients released from the feed substrate and MW; remain within the optimal thresholds for AD.

Among the changes, studies have also shown the variation in the diversity of microorganisms in anaerobic bioreactors with the increase in the digestion temperature. For instance, Levén *et al.* (2007) studied the effects of process temperature at 37°C (mesophilic) and 55°C (thermophilic) on the bacterial and archaeal community structure in bioreactors have been treating household organic waste for several years at constant conditions. They reported higher diversity of mesophilic communities compared to thermophilic communities. They also demonstrated that *Bacteroidetes* (34%) and *Chloroflexi* (27%) have dominated the mesophilic bacterial community, while *Thermotogae* phyla (61%) dominated the thermophilic bacterial community. The archaeon phylum *Euryarchaeota* (assigned to the genera *Methanospirillum*, *Methanoculleu*s, *Methanosarcina* and *Methanomethylovorans*) was the

most dominant archaeal phylum in both the mesophilic and thermophilic reactors. The archaeal genera *Methanospirillum* was representing 56% of the mesophilic clones, whereas the archaeal genus *Methanosarcina* was the most dominant sequence with more than 80% of the clones in the thermophilic reactors. For these reasons mentioned above and, as one of the aims of the proposed integrated digestion system in this study was to optimise a low-energy intensive digestion process, therefore, the author of this thesis preferred the mesophilic temperature at 37^{o}C for running reactors in the AD experiments.

3.2.1.2. *pH and buffering capacity*

Concentration of hydrogen ion (pH) in the reactor digestate is an important indicator of the performance and process stability of the anaerobic digester (Nayono, 2010). Ye *et al.* (2007) reported inhibition of fermentation at pH 4. Other studies suggested varied fermentation activity of acidogens from pH 4 to 8.5 (Yuan *et al.*, 2006; Appels *et al.*, 2008; Maspolim *et al.*, 2015). Acidogenic bacteria need a pH of around 5 for optimal enzymatic activity, while methane production by methanogenic archaea proceeds optimally at a pH of 6.8 - 7.6 (Nayono, 2010). The volatile fatty acids produced during the early stages of AD (acidogenesis and acetogenesis stages) consume most of the buffering capacity of the feeding substrate. Therefore, a stable AD process needs adequate methanogenic growth and activity to avoid excessive VFA accumulation, rapid pH drop and, ultimately the failure of the digester, which when it occurs needs a long restarting period in the range of weeks to months to recover full functionality (Rittmann, 2001). The imbalance in the enrichment between the hydrolytic/fermentative bacteria from one side and acetogenic and methanogenic microbes on the other side, will cause VFA accumulation and subsequent decrease in biogas production (Nguyen *et al.*, 2019a). Although digester failure usually associated with significant economic loss, however, only a few previous studies have investigated different recovery strategies (i.e. water dilution, bentonite addition, feeding cessation, inoculum addition, pH adjustment, trace elements supplement); (Nguyen *et al.*, 2019a).

Variations in the pH have important effects on the dominance of core microbial populations of AD reactors. Ye *et al.* (2007) demonstrated highest bacterial diversities at pH 7 to 8 compared to at pH 5 to 4, they also found that the pH 7 - 8 shifted metabolic pathways from alcohol to acid production, especially butyric acid. Whereas, both the alcohol and acid producers dominated the pH 5 - 6 conditions.

The AD of an organic waste such as OFMSW which is expected to produce a high concentration (> 200 mg/L) of total ammonia nitrogen (TAN), the variations in pH would

affect the growth of microorganisms as well as the composition of TAN (Chen *et al.*, 2008). The low pH and excessive acidity inhibits the digestion process which ultimately decreases the microbial growth (Viessman *et al.*, 1998). Whereas, the high pH (i.e. pH > 8) would results in higher concentration of free ammonia (FA) compared to the ionised ammonia ((NH_4^+)), and the FA has been suggested as a toxic agent for the anaerobic microorganisms (Kayhanian, 1999). Therefore, the pH close to 7 is recommended for the optimal activity of the anaerobic microorganisms (Nayono, 2010).

Lime ($Ca(OH)_2$), sodium bicarbonate (Na_2CO_3), potassium bicarbonate ($KHCO_3$), sodium hydroxide (NaOH) and ammonium bicarbonate (NH_4HCO_3) are the most common chemicals usually used to raise the pH of the anaerobic digesters (Hilkiah Igoni *et al.*, 2008). However, the excess use of lime will result in the precipitation of calcium carbonate, therefore other studies suggested the use of Na_2CO_3 and $KHCO_3$ as they provide bicarbonate alkalinity necessary for methanogens as well as being readily soluble and easily controlled chemicals having less adverse effects (Nayono, 2010). In the full-scale digesters, the high fluctuation in the pH due to the substrate composition and biological reactions make the addition of the exact amount of chemicals required to control the pH a difficult to apply process. For these reasons, other studies suggested making the pH in the AD reactors a self-stabilizing process as the best strategy to control the pH and prevent acidification (Nguyen *et al.*, 2019a). The presence of sufficient alkalinity (available at all times) in the feedstock substrate is the best strategy for maintaining a well-buffered digestion condition. Therefore, one of the hypothesis behind the use of MW in the current study was to control the pH by providing a readily and continuously available amount of alkalinity, which could maintain the pH and prevent the acidification.

3.2.1.3. *Solids retention time (SRT) and hydraulic retention time (HRT)*

Solid retention time is the period that biomass and substrate resides in the digester, while hydraulic retention time is the period that liquids take to pass through a digester completely. In AD, the SRT and HRT are either equal (1:1 e.g. continuous stirred tank reactors (CSTR)) or decoupled, depending on the requirements and designs of the digester systems. The shortest possible SRT is usually defined by the microbes with slowest growth-rate i.e. methanogens. Shorter retention time leads to washout of methanogens, whereas longer retention times require larger digester volumes and increased cost. Therefore the smallest practicable digester volume which prevents microbial washout usually indicates the digester volume used in practice (Rittmann, 2001).

In the AD of solid waste, characteristics of the substrate, as well as temperature, design and stages of the digester, specify the HRT required. For instance, during AD of a highly hydrolysable substrate like food waste, methanogenesis stage is the rate limiting step, therefore the shortest retention time possible is the period required by methanogenic community to provide a stable methanogenesis process. Dry (20 - 40% TS) AD needs a longer (by 3 days) HRT (typically 14 - 30 days) than wet (<5% TS) AD (Nayono, 2010). Usually, a stable methanogenic process requires a minimum HRT of 10 days, and the typical HRT for the AD of solid wastes ranges from 15 to 25 days (Rittmann, 2001). Anaerobic digestion of wastes under mesophilic conditions requires an average retention time of 15 - 30 days (Mao *et al.*, 2015). Kim *et al.* (2006) reported highest methane yields from thermophilic (thermophilic methanogens have faster growth rates) reactors (55°C) digesting food waste at an HRT of 12 days, while digestion stability decreased when HRT was decreased to 8 days. Moreover, longer retention time of 50 - 100 days was also reported for anaerobic digesters treating organic wastes rich in protein and fats; such as the poultry slaughter house waste (Salminen and Rintala, 2002).

3.2.1.4. *Mixing*

Mixing is an important operational parameter to distribute and transfer the substrate, biomass and chemicals, as well as to reduce sedimentation, flotation and foaming inside the anaerobic digester (Lindmark *et al.*, 2014) . Mechanical mixing, digestate recirculation and re-injection of the produced biogas are among the methods of digester mixing. The continuous stirred tank reactor (CSTR) is a very common full-scale digester design in Europe where the digester contents are usually homogenised by mechanical mixing using different types of propellers and agitators (Lindmark *et al.*, 2014). However, there are conflicting views on the exact design of mixing intensity for the CSTR systems. Hoffmann *et al.* (2008) reported no significant effects of mixing intensity on biogas production from CSTR systems treating animal manure at a wide range between 50-1500 rpm. Other studies found that mixing affects the balance between hydrolysis/fermentation and methanogenesis during startup, causing an accumulation of VFA and pH drop that inhibits the methanogens (Karim *et al.*, 2005). Moreover, Vavilin and Angelidaki (2005) studied effects of mixing intensity on the AD process of municipal household solid waste. They reported positive effects of low mixing intensity over high mixing intensity at high organic loading rates, while mixing intensity had no significant effects on the digestion processes at low organic loading rates. The same study also demonstrated the effects of mixing on the microbial community, they revealed that the low mixing intensity and successful digestion process were associated with the presence of

large irregular cocci of methanogens, which survived and spread in the digester. However, at high mixing intensity, mainly dead microbial cells were found, therefore low mixing intensity; at least at the startup period of the AD was recommended.

3.2.2. *Substrate characteristics*

3.2.2.1. *Particle size*

Reduction of particle sizes is one of the pre-treatment methods in AD. Grinding, comminution and blending are the common methods to reduce the particle size and increase the homogeneity of feedstock in AD. The particle size reduction of a substrate has two main effects:

1- To increase the amount of degradable components of the lignocellulosic substrate (high fibre content) available for degradation and ultimately increasing biogas production of the substrate.
2- To increase surface area of the substrate thus increasing the amount of food available for bacterial growth which will lead to rapid digestion of the substrate.

Sharma *et al.* (1988) found that decreasing the particle size of agricultural and forest residues to 0.088 and 0.40 mm increased biogas production effectively. Kim *et al.* (2000) reported the increase in the food waste utilisation rate constant from 0.0015 hr^{-1} to 0.0033 hr^{-1} with particle size reduction from 2.14 mm to 1.02 mm.

On the other hand, increasing surface area of a substrate through decreasing the particle size of the substrate will accelerate the hydrolysis and acidogenesis processes as well as VFA production. The imbalance between VFA production and consumption will lead to VFA accumulation and thus digester acidification and pH drop, resulting in inhibition of methanogens and decreasing the biogas production rate (Izumi *et al.*, 2010). Therefore, accelerating the microbial growth is also necessary to improve the methane yield in the anaerobic digestion processes.

3.2.2.2. *C/N ratio*

The nutrient level of a substrate is typically indicated by its C/N ratio. Biological degradation of nitrogenous matter such as proteins present in the feeding substrate of the AD reactor gives a low C/N ratio, the breakdown of which increases ammonium nitrogen and free ammonia concentrations which might lead to ammonia inhibition of methanogens (Wang *et al.*, 2014b). The optimal C/N ratio for maximum methane potentials are 25 and 30 at 35°C (mesophilic)

and 55°C (thermophilic), respectively (Wang *et al.*, 2014b; Mao *et al.*, 2015). A high C/N ratio prevents ammonia inhibition, however, it decreases the required nitrogen concentration necessary for microbial growth in the anaerobic digesters, resulting in low biogas production due to low utilisation of the carbon content of the feeding substrate (Mao *et al.*, 2015).

Co-digestion of different wastes is a common approach to balance the carbon and nitrogen of AD feedstocks. For instance, co-digestion of low C/N dairy/chicken manure with a high C/N wheat straw was found to enhance digestion performance of anaerobic digesters. Wu *et al.* (2010) found that co-digestion of swine manure with wheat straw at a combined C/N ratio of 20 increased daily biogas production by 6-fold and provided better digestion performance with stable pH and low concentrations of total NH_3-N and free NH_3. Yong *et al.* (2015) suggested 5:1 as an optimal mixing ratio of food wastes with wheat straw which increased methane yield by about 39.5% and 149.7% compared to that obtained from individual digestion of food waste and wheat straw, respectively.

3.2.2.3. *Ammonia*

AD of the OFMSW produces inorganic ammonia in the form of ammonium ion (NH_4^+) and free ammonia ((NH_3); Eqs. 3.27 to 3.30), both forms can directly or indirectly cause inhibition /and shifts in metabolic pathways of microbial communities (specifically methanogens) in anaerobic systems (Yenigün and Demirel, 2013).

The reactions of ammonia gas with water to produce ammonium and hydroxide ion depending on the hydrogen ion concentration, and the relation of free ammonia concentration to total ammonia concentration are given by the following equations:

$$NH_3 + H_2O \longrightarrow NH_4^+ + OH^- \quad \textbf{(3.27)}$$

$$CO_2 + HOH \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3 \quad \textbf{(3.28)}$$

$$H_2CO_3 + OH^- \rightleftharpoons HCO_3^- + HOH \quad \textbf{(3.29)}$$

$$NH_3 = \frac{TAN \times \frac{K_a}{[H]}}{\frac{K_a}{[H]} + 1} \quad \textbf{(3.30)}$$

Where, NH_3 = free ammonia concentration in mg/L, TA = total ammonia nitrogen in mg/L, K_a = temperature dependant dissociation concentration, [H] = hydrogen ion concentration (Kayhanian, 1999).

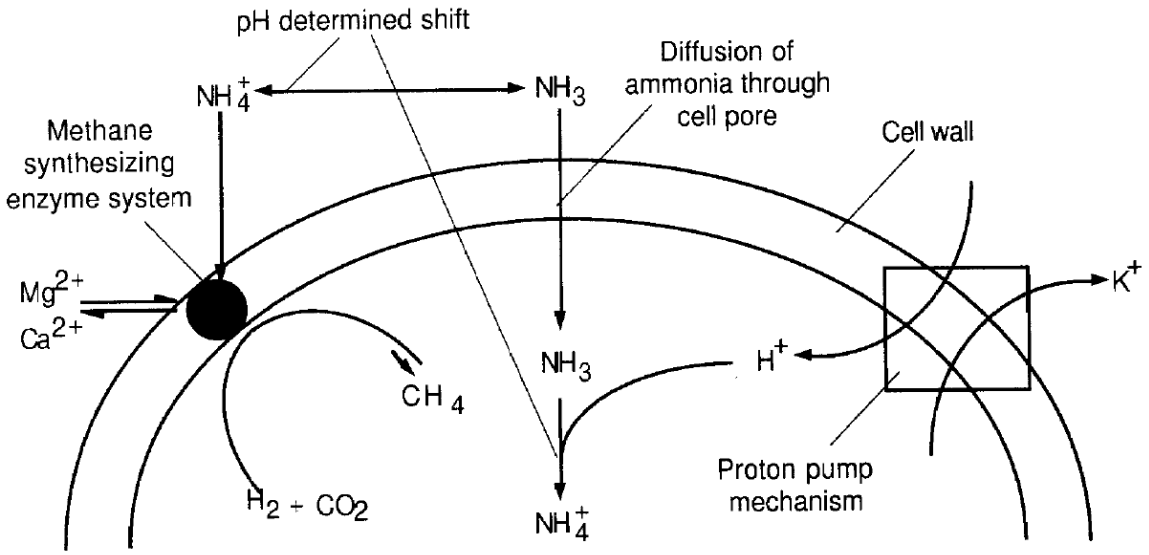


Figure 3-22. Proposed mechanisms of ammonia inhibition in methanogens (Kayhanian, 1999).

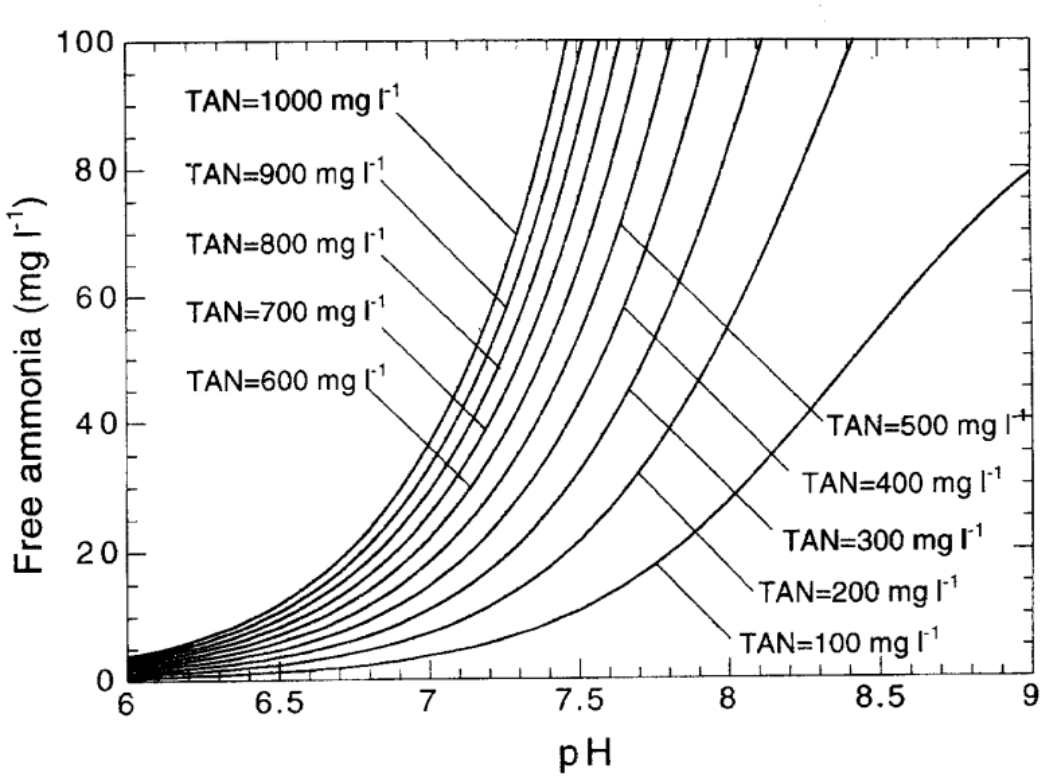


Figure 3-23. Variations in free ammonia concentration of a thermophilic digester at different total ammonia concentrations and varying pH (Kayhanian, 1999).

Only small amounts of ammonia are utilised for cell synthesis and higher levels of ammonia are inhibitory to the methanogens present in the anaerobic digesters. There are two proposed mechanisms for ammonia inhibition (Figure 3-22): (1) ammonia inhibits the synthesis of the enzymes utilised by methanogens in methanogenesis, and (2) the hydrophobic ammonia

molecules diffuse passively into the cell, causing proton imbalance and /or potassium deficiency. Ultimately, the both mechanisms of ammonia inhibition limit the efficiency (activity) of methanogens to degrade organic matter (Kayhanian, 1999).

The archaeal community, including both hydrogenotrophic and acetoclastic methanogens, are sensitive to ammonia inhibition (Kadam and Boone, 1996; Lay *et al.*, 1998; Kayhanian, 1999; Fujishima *et al.*, 2000; Schnurer and Nordberg, 2008; Tian *et al.*, 2018). However, Tian *et al.* (2018) found that hydrogenotrophic methanogens (such as *Methanosarcina*) were more tolerant to ammonia inhibition compared to acetoclastic methanogens (such as *Methanosaeta*). Moreover, these studies indicated that the AD process inhibition by ammonia is related to the characteristics and loading rate of the feeding substrate as well as the pH and temperature (mesophilic / thermophilic) of the digester. Increasing the digestion temperature increases the ammonia toxicity (Weiland, 2010). It can be concluded from equation 3.30 that at a given pH and TAN concentration, the free ammonia concentration for a digester at thermophilic temperature is six times higher than at a mesophilic temperature (Kayhanian, 1999). Angelidaki and Ahring (1994) reported positive effects of temperature reduction (below 55°C) on the biogas yield and process stability of thermophilic reactors with high ammonia concentrations.

In the literature, the reported inhibitory concentration of total ammonia for methanogens ranges between 1.5 g N/L to 4 g N/L (Rajagopal *et al.*, 2013; Yenigün and Demirel, 2013). Other studies reported archaeal tolerance of ammonia inhibition between 7.8 - 13 g N/L under acclimation conditions (Pechan *et al.*, 1987; Sung and Liu, 2003).

3.2.2.4. *Organic loading rate (OLR)*

Organic loading rate (OLR) is an important parameter in AD; it indicates the amount of volatile solids (VS) fed into the digester per day. In AD, to an extent, most of the VS is biodegradable and converted to biogas; therefore, the increase in OLR will increase the biogas production. However, excess increase in OLR (overloading) can disturb the equilibrium and productivity of the anaerobic digester. Extreme high OLR would lead to an imbalance of hydrolysis/acidogenesis activity and methanogenesis activity leading to accumulation of excessive VFA, which eventually causes the irreversible acidification of the anaerobic digester (Mao *et al.*, 2015). By arranging periods without organic loading (intermittent feeding), stable digestion process with high methane yields (364 - 489 mL/g VS) and VS reduction (83 - 91%) could be achieved from mesophilic single-stage wet (<5% TS) anaerobic digestion of food waste with maximum OLR of 10.5 g VS/L. d (Nagao *et al.*, 2012).

However, with continuous loading of the feeding substrate, the maximum possible stable loading rate for wet AD remained at 1- 4 g VS/L. d (Nagao *et al.*, 2012). Unlike in wet AD, semi-dry (10 - 20% TS) and dry (20 - 40% TS) AD provided stable digestion process without acidification at OLRs of 7 - 15 g VS/L. d. However, the methane yield (140 - 314 mL/ gVS) and VS reduction (31 - 48%) were lower (Vallini *et al.*, 1993; Dong *et al.*, 2010).

3.2.2.5. *Macro-nutrients and trace elements*

The growth and survival of microorganisms needs macronutrients and trace elements. Deficiency in macronutrients and trace elements causes significant effects on the AD process. Important macronutrients for biological processes include carbon, nitrogen, phosphorus, potassium, magnesium and sulphur. In addition, a large number of trace elements (TE) have been reported in the literature to be stimulatory for AD processes (Banks *et al.*, 2011; Takashima *et al.*, 2011; Facchin *et al.*, 2013; Westerholm *et al.*, 2015b).

The main element of microbial cell structure is carbon, which is usually supplied through the feeding substrate. Nitrogen is required during protein biosynthesis, and sulphur is necessary for amino acids. The energy carriers ATP and NADP needs phosphate for energy transfer in AD metabolic pathways. The optimal C: N: P ratio for methane yield enhancement is 200:5:1 (Weiland, 2010; Mao *et al.*, 2015).

Trace elements like iron, nickel, cobalt, selenium, molybdenum, and tungsten are important for the growth rate of microorganisms (Table 3-3). All methanogens need nickel for synthesis of cofactor F_{430} required in methanogenesis. Cobalt-containing corrinoid factor III, needs adequate cobalt for optimal cell growth. Addition of Ca and Mg salts were found to support methane production and prevent foaming. Tungsten enhances propionate degradation, Se and Co enhances processes stability of AD at high ammonia concentration such as digestion of food waste (Demirel and Scherer, 2011; Zhang and Jahng, 2012; Zhang *et al.*, 2012; Facchin *et al.*, 2013; Zhang *et al.*, 2015b; Cai *et al.*, 2017).

The necessary concentration of TE generally ranges from 0.05 - 0.06 mg/L, except iron which requires a concentration between 1 to 10 mg/L (Weiland, 2010). However, the bioavailability of TEs does not always associate with the measured concentration of the TE, the limited bioavailable concentration of TEs affects process stability and methane production significantly (Oleszkiewicz and Sharma, 1990). Finally, as suggested by Mao *et al.* (2015), "*Although supplementation of micronutrients and trace elements could be a simple way to achieve AD process stabilization and efficient biogas generation, the economic feasibility of trace elements should be dependent on their cost*". Therefore, finding economically feasible

sources of TEs able to support efficient AD is one of the key questions to answer in future research.

Table 3-3. Enzymes in anaerobic microorganisms and their related trace elements (Choong et al., 2016).

Enzymes or groups	Metals	Exist form	Affected microbial groups
Methyltransferase	Co	Corrinoid	Methanogenic archaea, Homoacetogenic bacteria
Methyl-CoM reductase	Ni	F430	Methanogenic archaea
Acetyl-CoA synthase	Ni		Methanogenic archaea
Hydrogenase	Ni		Methanogenic archaea
Formylmethanofuran dehydrogenase	W,(Se, Fe)	Tungstopterin	Methanogenic archaea
	Mo, (Se, Fe)	Molybdopterin	
Carbon monoxide dehydrogenase	Ni, Fe	Fe-Ni-S	Methanogenic archaea, Homoacetogenic bacteria, Sulfate-reducing bacteria
Hydrogenase	Fe	Fe-S	Methanogenic archaea, Homoacetogenic bacteria, Sulfate-reducing bacteria
	Fe, Ni, Se	Fe-S-Se	
Formate dehydrogenase	W, (Se, Fe)	Tungstopterin	Methanogenic archaea, Homoacetogenic bacteria, Sulfate-reducing bacteria
	Mo, (Se, Fe)	Molybdopterin	
Cabonic anhydrase	Zn		Methanogenic archaea, Homoacetogenic bacteria
Hydrogenase, formatedehydrogenase	Zn		Methanogenic archaea, Homoacetogenic bacteria
Nitrogenase	Mo		Methanogenic archaea
Superoxide dismutase	Cu, Zn		Methanogenic archaea
Cytochrome	Fe	Haem, Fe-S	Homoacetogenic bacteria, Sulfate-reducing bacteria
Ferredoxin	Fe	Fe-S	Methanogenic archaea, Homoacetogenic bacteria, Sulfate-reducing bacteria

3.2.2.6. *Co-digestion*

Co-substrate is a substrate produced by mixing two or more feedstock with varying physicochemical and microbial characteristics, and the AD of the co-substrate is known as co-digestion. Co-digestion is a method of increasing the digestion performance (methane production rate and yield and solids reduction) and process stability in AD. The positive effects of using co-substrates on AD include dilution of the toxicants, supplying missing nutrients (see Section 3.2.2.2 above) , and maintaining the required moisture percentage of the feeding substrate as well as the handling of the co-substrate would be easier (Mata-Alvarez *et al.*, 2000a; Salminen and Rintala, 2002). Moreover, changes in the microbial composition in the digester depend on the characteristics of the digester medium, such as VFA and ammonia concentrations (Sundberg *et al.*, 2013; Mata-Alvarez *et al.*, 2014). Therefore, mixing different substrates in a co-substrate means mixing microbial communities from these substrates in the digester medium, which is a feasible option to enhance the digestion performance of mono-

digestion by increasing the robustness of the microbial communities in the anaerobic digester to tolerate intermediate digestion products (Zhang *et al.*, 2011b; Sundberg *et al.*, 2013).

3.2.2.7. *Pre-treatment*

Pre-treatment is a method to optimise biological processes and improve the yields in AD. The applied pre-treatment method depends on the characteristics of the substrate. The most common pre-treatment methods in AD are mechanical, thermal, chemical and biological. These pre-treatment methods can be performed either singly or in combination. The main purpose of pre-treatment is to resolve the rate-limiting steps in AD. For complex substrates and low degradable substrates such as cellulosic wastes, the hydrolysis step is typically the rate-limiting linked to the presence of non-degradable components in the substrate (such as lignin of the cellulosic substrates), as well as formation of toxic by-products and accumulation of non-desirable VFA (Raposo *et al.*, 2011; Raposo *et al.*, 2012; Ariunbaatar *et al.*, 2014b). However, methanogenesis is the rate-limiting step for the easily and quickly degradable substrates such as food waste (Rozzi and Remigi, 2004). The key factors which govern the choice of the best pre-treatment method are the substrate characteristics, technology of the digester used, quality and subsequent utilisation of the digestate (Cesaro and Belgiorno, 2014). For OFMSW, all the potential pre-treatment methods can be applied (Cesaro and Belgiorno, 2014); however, the applicability and sustainability of such pre-treatment methods at full scale need to be considered (Ariunbaatar *et al.*, 2014a).

3.2.3. *Reactor configuration and operation*

Figure 3-24 shows classification of AD digesters according to the operational conditions and digester categories. Depending on the total solids content (TS %) of the feedstock substrate, anaerobic digesters are classified as being wet (< 10% TS), semi-dry (15 – 20% TS) and dry (22 - 40% TS) digestion. Furthermore, anaerobic digesters can be classified into single-stage, two-stage /and multi-stage digesters (Verma, 2002) according to the separation of the different biological processes within different reactor compartments or tanks. In single-stage reactors all the stages of AD occur in one digester, while in two-stage digesters the acidogenic and methogenic stages are performed in two different digesters (Verma, 2002) Usually the total solids content of OFMSW is higher than 15% and therefore can be processed through semi-dry or dry digester systems (Li *et al.*, 2011).

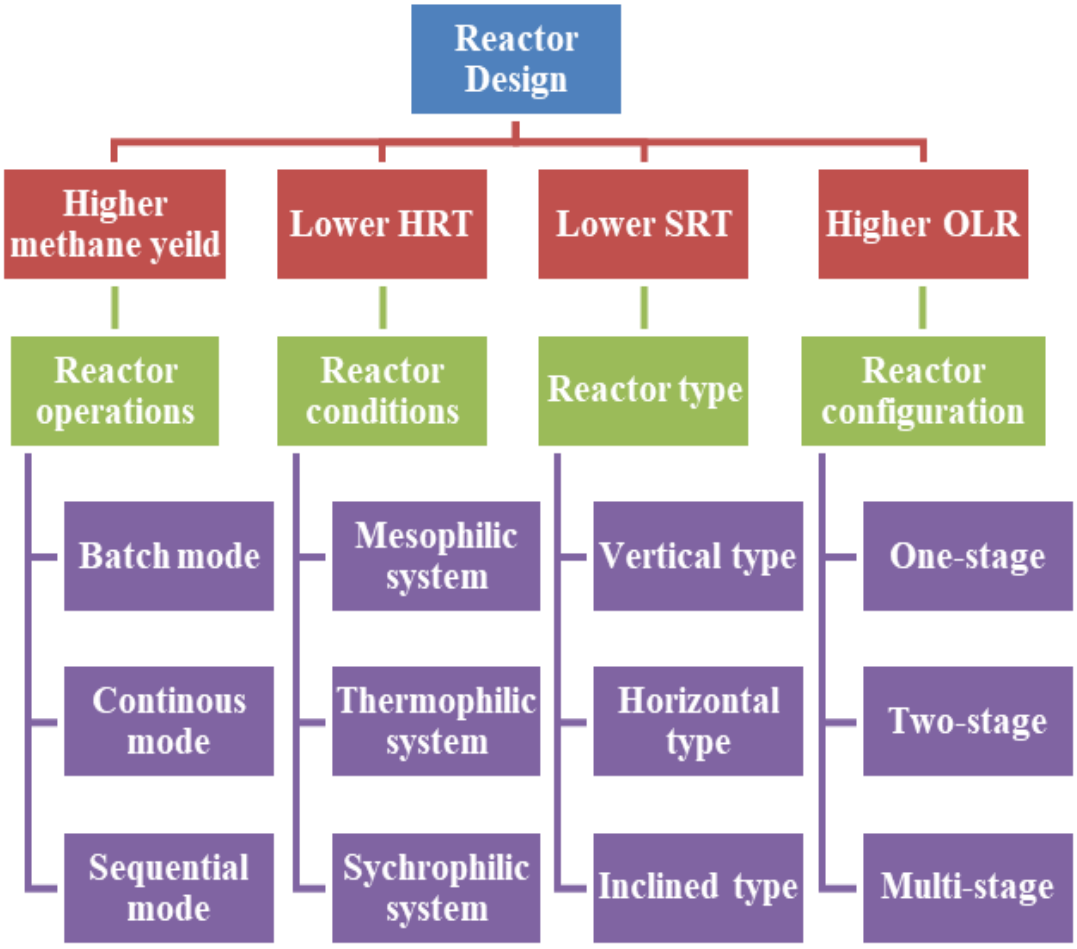


Figure 3-24. Classification of AD digester designs according to the operational condition and digester categories (Rapport *et al.*, 2008).

Depending on the feeding frequency, anaerobic digesters can be classified as batch or continuous systems. A batch digester is fed once until it totally degrades most of the solids in the substrate and then stops producing biogas, while continuous reactors need regular feeding as short intervals (typically minutes to hours). In continuous reactors, a fixed volume of the feeding substrate will replace an equal volume of digestate removed. Due to its relative simplicity, minimum maintenance requirements, low parasitic energy loss, and minimum capital cost, most of the digesters treating solid waste (i.e. MSW, food waste, and agricultural waste with 15 – 40% solids concentrations) for energy production in the EU are operated in single-stage and at mesophilic or thermophilic conditions (Li *et al.*, 2011).

The plug-flow digester is another single-stage anaerobic digester design. Inside the plug-flow digesters, the substrate is not completely mixed; it moves in plug-flow through the reactor from the feeding port to the digestate disposal port, while the continuous stirred tank reactor (CSTR) systems mix the digester content either continuously or periodically. The plug-flow

digesters require heavy process equipment to push dry and viscous substrates to move through the digester. This type of reactor is only useful for a substrate which can maintain at least 20% solids in the reactor, otherwise at lower solids content, the solids settle and produce a layer on the bottom of the reactor decreasing the active volume of the reactor (Li *et al.*, 2011).

In the CSTR systems to reduce the amount of water/or inoculum needed, the digestate can be recycled (either partially or totally) to the digester either directly or after mixing with the new feedstock substrate (Li *et al.*, 2011). The digester with a recycled digestate also supports the colonisation of bacteria in the digester and prevents the washout of microbial communities as well as preserves the useful macro-/micro-nutrients inside the digester (Li *et al.*, 2011). Therefore, in this thesis, the use of CSTR with a recycled method of digestate (mostly the liquid part of the digestate) was expected to accommodate the integration of MW in the AD of OFMSW.

3.3. Integrating mineral wastes into anaerobic digestion of organic waste

3.3.1. *Mineral wastes of municipal solid waste incineration (MSWI) plants*

3.3.1.1. *Introduction of mineral wastes*

Municipal solid waste (MSW) comprises different components coming from various waste streams such as household solid waste, industrial solid waste (non-hazardous), agricultural solid waste (garden waste) and local market solid waste (commercial waste). In term of combustibility, MSW can be divided into combustible and non-combustible components. There are two main advantages of incineration of MSW: it provides substantial decrease (~ 90%) in the waste volume requiring disposal in landfill, and produces energy; as heat or electricity (Cheng and Hu, 2010).

In many countries, incineration is the management method of choice for the combustible component of MSW. In England in 2011/2012, incineration accounted for 15.1% treatment of the total MSW produced, and the incineration-by-products (MW) was equated to ~ 4 million tonnes per annum (DEFRA, 2013). In Denmark, Germany, Sweden and France, incineration accounts for 48%, 36%, 55% and 42% by weight of total solid waste arising in these countries, respectively (Ecke and Åberg, 2006). In 2009, there were 449 operating incineration plants across 20 of the Western and Central European countries, with total throughput of 69.4 million tonnes of waste (DEFRA, 2013). In Japan, land for landfill is very scarce, therefore, incineration is indispensable for the management of MSW (74% of MSW is managed by incineration; (Ecke *et al.*, 2000)).

Generally, in an MSWI plant (Figure 3-25), management of MSW takes place in three sequential stages: incineration of the waste at 1000°C; energy recovery stage and air pollution control (APC) stage (Lam *et al.*, 2010). The incineration process releases heat energy for boiler systems and leaves an ash residue on the grate system. The generated heat from incineration process is used to produce super-heated and pressurised steam that in turn drives a turbine winch generates electricity transferred to the electrical network. The main by-products of an incineration plants can be categorised into gaseous and solid residues.

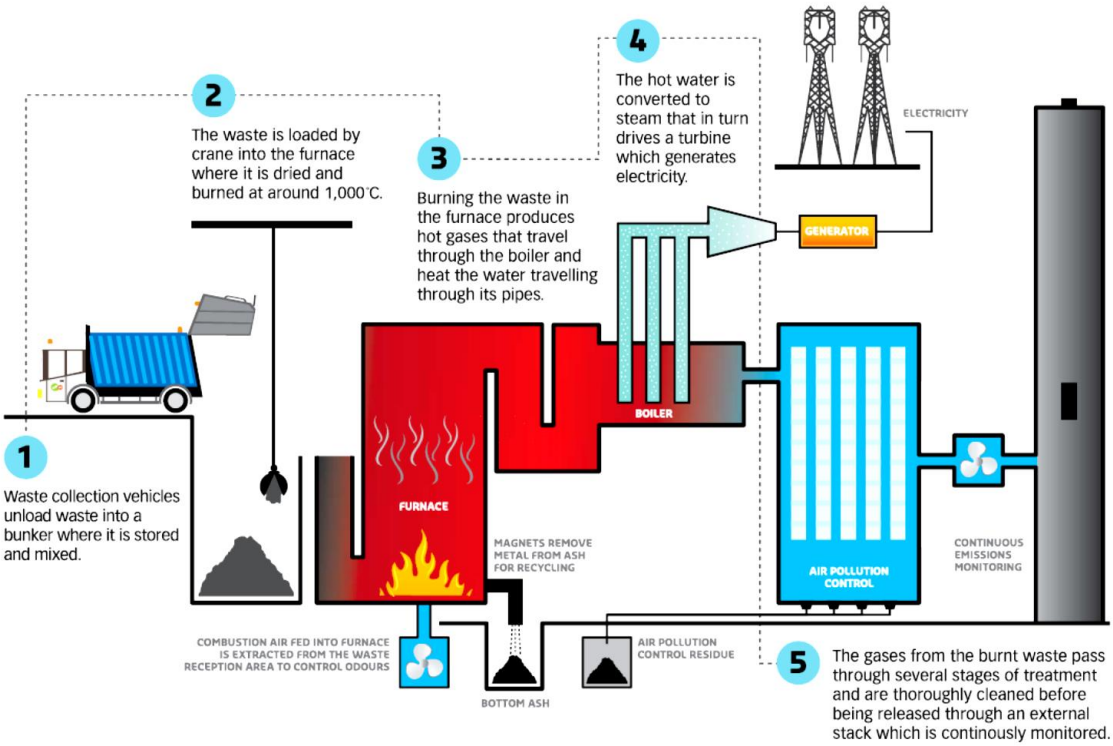


Figure 3-25. Schematic diagram of an incineration plant of MSW (SITA Company, Teesside, UK).

The incineration conditions and composition of the waste governs the composition and microstructure of the incineration by-products. After combustion, the solid residue of the incinerated waste is screened for recycling ferrous and non-ferrous metals. The solid material collected at the bottom of the incineration furnace is known as incineration bottom ash (IBA); it makes up to 85 - 90% of the MSWI solid residues. IBA is rich in trace elements and contains up to 61 - 94% of the heavy metals produced during incineration (Zhang *et al.*, 2008). The gaseous products from incineration plants are purified at the APC stage. The solid residuals from the APC stage contain particles from the incineration stage, lime used in spray absorbers and dust. The gas passes through a series of filters to be cleaned for specific standard limits before it is emitted to the ambient environment through high stacks. Collected

solid residues from the APC stage and heat exchange boiler are named as fly ash (FA) and boiler ash (BA), respectively.

3.3.1.2. *Applications of the mineral wastes from MSWI plant*

Sustainable use of mineral wastes (MW) from MSWI plants depends on a controlled contaminant release strategy (Hjelmar, 1996). Currently, the extension of existing MSWI landfill capacities and reduction of disposal costs has motivated many countries to assess the potential reuse of MW.

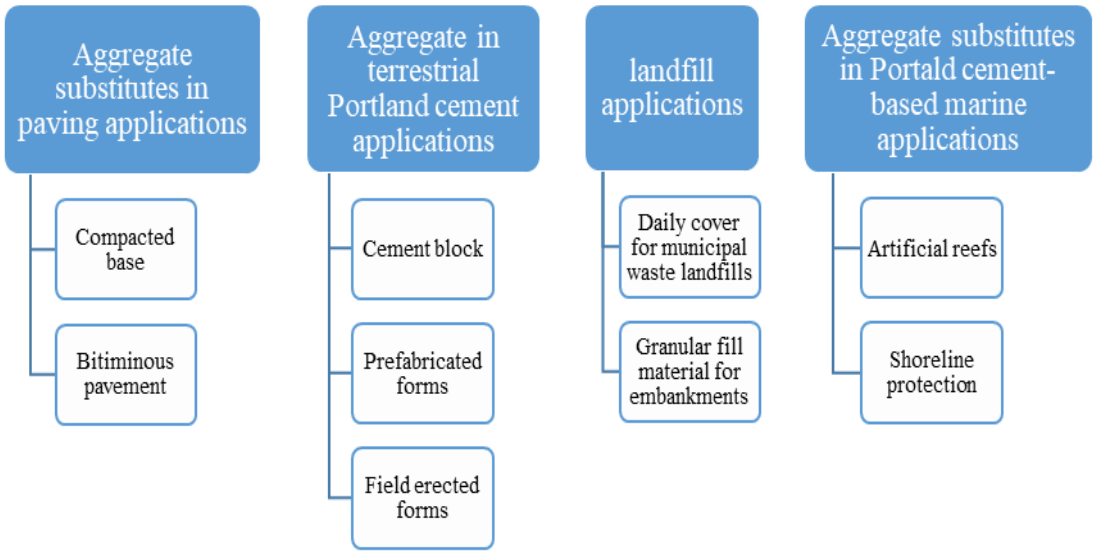


Figure 3-26. Primary applications of solid residues from MSWI plants (Chandler *et al.*, 1997).

In the Europe, incineration bottom ash is often utilised as substitute material for production of aggregate and other construction materials in roads, ramps, noise barriers etc. (Figure 3-26). However, emissions to the surroundings caused by leaching of heavy metals from MW exposed to the environment, apply some restrictions on the use of MW as building material in construction (Chandler *et al.*, 1997). In other countries such as the USA, MW are used with daily cover soil at landfill sites with MW: soil ratios between 1:4 and 1:5 (Rhew and Barlaz, 1995; Banks and Lo, 2003).

3.3.1.3. *The use of mineral wastes from MSWI plants in anaerobic digestion*

Mineral wastes from a MSWI plant contain many of the trace elements essential for AD metabolic pathways, and contains some other elements such as K, Ca, Na and Mg that may increase the buffering capacity of anaerobic digesters (see Section 4.2 of this thesis). To date, few studies have investigated the potential use of MW in AD. Banks and Lo (2003) assessed

the use of IBA to optimise mesophilic (37°C) AD of the degradable organic fraction of municipal solid waste. They demonstrated the increase in biogas production (two to four times higher than the control reactor without IBA amendment), alkalinity and pH, coupled with a low concentration of nutrients in the digestate of continuous reactors (CSTRs) amended with IBA. del Valle-Zermeño *et al.* (2015) investigated the use of IBA for upgrading biogas from CSTRs. They obtained a sorption capacity of 50 kg CO_2/t and 35 kg CO_2/t for dry fine IBA and weathered IBA, respectively. Zhang *et al.* (2015a) studied co-digestion of food waste with a fresh leachate obtained from a waste storage bunker of a MSWI plant. They obtained much better reactor performance and process stability in terms of high CH_4 yields (375.9 - 506.3 mL/gVS added), high VS reduction (66.9 - 81.7%), and stable pH (7.2 - 7.8) from the co-digestion compared to mono digestion of food waste. Consequently, published research has shown there to be potential benefits from incorporating MSWI plant wastes into AD processes treating OFSMW. For this reason, the current study investigated comprehensively the reuse of MW as a feedstock amendment for stabilising and optimising AD reactors treating OFMSW. The anaerobic digestion strategies applied during the current study for integrating MW into AD of OFMSW were expected to provide a mechanistic understanding of how these wastes enhance biogas production, as well as to provide information on the supply of trace elements and nutrients necessary for optimising the AD processes. Such an information could be used to improve the efficiency of the AD of OFSMW, and to improve disposal options for MW fractions of MSW. For instance, the combined waste streams (the ash and digestate) after AD can be reworked into normal ash re-use routes as described in Section 3.3.1.2.

3.3.2. *Mineral wastes from construction demolition waste (CDW)*

The past decade has shown high levels of growth of the construction industry worldwide (Renforth et al., 2011). One of the environmental impacts of the construction industry is that construction demolition waste (CDW). CDW represents 20 - 30% of the MSW produced each year (Gomes *et al.*, 2013). In 2012, about 821 million tons of CDW produced in the EU (Eurostat, 2014). As describe in Section 1.2, low amounts of these waste products are reused in the construction industry as aggregates for concrete production, while most of the CDW from the construction industry is either used as filling material or illegally dumped on empty open areas (Gomes *et al.*, 2013). Moreover, this waste stream of MSW comprises components can support buffering capacity of anaerobic digesters. Washbourne *et al.* (2015) reported that the CBW components of CDW could react with dissolved CO_2 to change alkalinity and precipitate carbon in the form of carbonate mineral as $CaCO_3$.

About 35.5 - 67% of CDW is cement-based waste (CBW) which contains a notional CaO content of about 10 - 20 % (Renforth et al., 2011), calcium silicate and hydroxide minerals. Under weathering conditions, CBW reacts with dissolved CO_2 to change the alkalinity and precipitate carbon in the form of carbonate minerals such as $CaCO_3$ (Washbourne *et al.*, 2015). There are three possible carbonation reactions for calcium silicate (Eq.3-31), hydrated cement mineral (Eq.3-32) and portlandite (a component of portlant cement) (Eq.3-33) (Washbourne *et al.*, 2015). The precipitation of carbonate minerals by calcium silicates scrubs CO_2 from biogas, this increases the methane concentration of the biogas produced from anaerobic reactors. Moreover, the CO_2 capture by the CaO content of the wastes diminishes CO_2 emission from AD systems. Furthermore, precipitation of $CaCO_3$ increases the alkalinity in reactors, which prevents inhibition of methanogens due to pH reduction. Therefore, the current study aimed to utilise CBW in AD of the OFMSW as an alkaline and a mineral resource for optimising the digestion processes.

$$CaSiO_3 + CO_2 + 2H_2O \longrightarrow CaCO_3 + H_4SiO_4 \quad \textbf{(3-31)}$$

$$Ca_9Si_6O_{18}(OH)_6.8H_2O + 9H_2CO_3 \longrightarrow 9CaCO_3 + 6H_4SiO_4 + 8H_2O \quad \textbf{(3-32)}$$

$$Ca(OH)_2 + H_2CO_3 \longrightarrow CaCO_3 + 2H_2O \quad \textbf{(3-33)}$$

3.4. Anaerobic co-digestion of the OFMSW with wheat straw

Wheat straw (WS) is a widely available (especially in the author's home country), non-competitive with food applications, easy to obtain, and low in cost agricultural by-product biomass obtained from agricultural wheat production (Maas *et al.*, 2008) and, therefore, it is of particular interest for co-digestion with OFMSW. About 60% of dry weight of WS is carbohydrates (Reilly *et al.*, 2015) in the form of cellulose, hemicellulose and lignin. WS is a good substrate for producing each of bioethanol, biohydrogen and biogas, however, the use of WS for biogas production is the most energy efficient process (Pohl *et al.*, 2013). Carbohydrates are the important source of energy for microorganisms, anaerobic bacteria can produce acetate and hydrogen from the fermentation of carbohydrates (Madigan, 2015). Methanogens metabolise the fermentation products for energy conservation and growth. The increase in the population of methanogens inside digester enhances the conversion of solids into bioenergy. Moreover, the un-degraded lignin component remained from WS after AD is a useful bulking agent for the later composting process of digestates from the anaerobic digesters. Furthermore, in the current study, it was hypothesised that co-digestion of WS with the OFMSW then production of acids at the acidogenesis stage will increase the disruption of

the strong bonds between lignin and hemicellulose in the WS, hence improves the solubilisation of hemicellulose (Reilly *et al.*, 2015). Solubilisation of hemicellulose in addition to cellulose will increase the amount of the fermented substrate from WS available for methanogens for biogas production.

Previous studies (Wang *et al.*, 2012) found that optimising the reactor feed (such as OFMSW) composition to a C/N ratio between 25 – 30 has improved the reactor performance by mitigating the risk of ammonia inhibition of methanogens. Whereas, a low level of nitrogen (i.e. high C/N ratio) in the feed substrate decreases the buffering capacity in the reactor which causes high VFA concentrations, pH reduction and hence inhibition of methanogens. Likewise, low C/N ratio of the feed substrate enhances the buffering capacity in reactors due to the hydrolysis of nitrogen to ammonia, however higher ammonia concentration might cause inhibition of methanogen (Yenigün and Demirel, 2013).

As described in Section 3.2.2.5 trace elements are crucial nutrients for the synthesis of metalloenzymes involved in the metabolic pathways of AD. In the literature, no previous studies have investigated the combined effect of C/N ratio and trace elements on AD. Therefore, the effects of these two factors singly/in combination; on AD of the OFMSW was studied in detail in the Chapter 9 of this thesis.

3.5. Conclusions

In the next few decades, it is likely dissemination of anaerobic biotechnology for renewable energy production will become an economically feasible alternative to fossil fuels. The success of AD for biogas production depends on minimising the operational and capital costs of this technology to produce a usable and an affordable biogas at low cost for the production of electricity, heat, and hydrogen or compressed methane as a sustainable fuel for transport vehicles. Nowadays, the theory behind the AD processes and the physicochemical parameters affecting these processes are well established. The key question for the future are about optimisation of the AD processes to increase the biogas production efficiency of the AD technology. The OFMSW is a nutrient rich substrate for AD; it is available for biogas production at almost every place in the world. However, in most cases OFMSW is deficient in some of the crucial trace elements necessary for the activation of the enzymes known to be crucial of the AD process. Nowadays, many types of commercial trace element amendments are available to anaerobic digesters. However, the economic feasibility and availability of these trace element sources are still limited to their use. Therefore, using low cost sources of trace elements, such as mineral wastes, which can release most of these trace elements, has

the potential to minimise the operational costs of AD. Moreover, the use of mineral wastes as a source of trace elements for AD plants is an attractive alternative to commercial trace element preparations not only from a cost perspective, but also because it offers a more sustainable method for the disposal of this MW material.

Chapter 4. Materials and Methods

Chapter contents

The general material and methods, which are applied in most of the chapter results (Chapter 5 to Chapter 9), are described in details in this chapter, whereas the specific material and methods are described in the related chapters. Moreover, to avoid the repetition, some of the results obtained from the physicochemical analysis of the inoculum, feedstock substrate and mineral wastes are presented in this chapter.

4.1. Reactor substrates

4.1.1. *Synthetic organic waste*

The substrate used for AD trials of the current research was a synthetic organic waste (SOW) to minimise the impacts of composition variability of the OFMSW on the reproducibility of results. The SOW was produced from different organic waste components to simulate typical composition of the OFMSW at the author's home country (Sulemani, Kurdistan region, Iraq). The substrate composed of 79% cooked food leftovers (such as rice 13.6%, meat 1.5%, beans 5.6%, fat 1.4% etc.), 20% of uncooked fruit and vegetable wastes (such as apple 1.3%, orange 1.7%, banana 2%, lemon 1.2% and pomegranate 1.4%, herbs ~ 6% etc.), and 1% of packing cardboard, simulating the OFMSW going to landfill (Table 4-1). The substrate components were blended together to particle sizes less than 5 mm using a food blender. The SOW total solids (TS) concentration was adjusted within the optimal TS value of 10 - 20% by wet weight (Forster-Carneiro *et al.*, 2008) using distilled water, about 30% dilution by volume of the blended organic waste was required. After adjustment, the substrate was stored at -20^{o}C until use.

During this research, in order to produce a SOW substrate for the AD trials with less variation possible in the physicochemical characteristics, two batches of the SOW were prepared. The first batch of the SOW substrate (Table 4-2) was used for the AD trials described in Chapter 5 and chapter 6, whereas the second SOW batch (Table 4-3) was used as the feedstock substrate for the AD trials described in Chapter 7, Chapter 8 and Chapter 9.

Table 4-1. Proportions among components used in the simulated OFMSW (SOW), the proportions were calculated as the wet weight of each component per total wet weight of the SOW substrate

Cooked food leftovers		**Uncooked fruit and vegetable wastes**		**Cardboard**	
Component	Proportion (%)	Component	Proportion (%)	Component	Proportion (%)
Rice	13.6	Watermelon	3.9	Cardboard	1
Bulgur	4.8	Muskmelon	2.5		
Bread	5.3	Orange	1.7		
Loaf	2.3	Banana	2		
Beans	5.6	Apple	1.3		
Eggplant	1.5	Lemon	1.2		
Dry apricot	1.6	Pomegranate peels	1.4		
FAT	1.4	Herbs, onions and etc.	6		
Tomatoes	5.6				
Squash	2.3				
Parsley	2.1				
Swiss chard	1.4				
Spinach	4.1				
Okra	3.1				
Sweet potato	5.4				
Garlic	1.2				
Yoghurt	1.9				
Cheese	1.5				
Chicken meat	2.2				
Red meat	1.2				
Desserts	1.9				
Spices and salts	0.7				
Onion	2.3				
Eggs	0.5				
Biscuits	1.2				
Tea (lees)	2.3				
Others	2				
Total	**79%**		**20%**		**1%**

Table 4-2. Characteristics of the feedstock substrate (SOW) used for the AD trials in Chapter 5 and Chapter 6

Parameters	Value*	Standard deviation
pH (1:2) **	4.27	±0.1
Total solids (%W/W)	18.6	±0.035
Volatile solids (%W/W)	17	±0.057
Volatile solids (%TS)	92	-
C (%)	46.47	±0.3
H (%)	6.76	±0.035
N (%)	2.21	±0.02
O (%)	37.52	±0.42
S (%)	0.16	±0.01
C/N	22.4	±0.05
Al***	45	±23
As	0.61	±0.3
B	4.2	±1.2
Ba	3.5	±0.9
Ca	4958	±245
Cd	0.02	±0.001
Co	0.03	±0.001
Cr	0.85	±0.23
Cu	4.3	±0.67
Fe	62.5	±.3.6
K	7523	±1220
Mg	657	±143
Mn	11.8	±2.1
Mo	0.48	±0.21
Na	323	±82
Ni	0.73	±0.17
Pb	0.28	±0.05
Se	0.85	±0.2
Si	69	±3.3
Ti	0.4	±0.1
V	1.1	±0.07
Zn	13.8	±2.5

* All values in this table represent mean value of three triplicate samples measured.
** One volume of each sample dissolved in two volume of distilled water mixed with magnetic stirrer for one hour then measured for pH.
*** All concentrations of trace elements are in mg/kg TS.
%W/W = percentage of the dry weight of total or volatile solids per wet weight of the feedstock substrate.

Table 4-3. Characteristics of the feedstock substrate (SOW) used for the AD trials in Chapter 7, Chapter 8 and Chapter 9

Parameters	Value*	Standard deviation
pH (1:2) **	4.3 2	±0.15
Total solids (%W/W)	12.8	±0.1
Volatile solids (%W/W)	11.3	±0.1
Volatile solids (%TS)	88	-
C (%)	44.8	±0.2
H (%)	NM	-
N (%)	3.3	±0.03
O (%)	NM	-
S (%)	0.3	±0.01
C/N	13.8	±0.06
Al***	340	±12
As	BD	-
B	11.3	±0.8
Ba	6.6	±0.7
Ca	2329	±205
Cd	0.05	±0.001
Co	0.2	±0.001
Cr	1.4	±0.18
Cu	12.4	±0.47
Fe	366	±.2.8
K	412	±53
Mg	142.5	±25
Mn	10.3	±1.1
Mo	0.9	±0.20
Na	167	±25
Ni	1.3	±0.15
Pb	8.3	±0.4
Se	BD	-
Si	85.8	±2.5
Ti	7.6	±0.18
V	1.8	±0.1
Zn	29.7	±1.8

* All values in this table represent mean value of three triplicate samples measured.
** One volume of each sample dissolved in two volume of distilled water mixed with magnetic stirrer for one hour then measured for pH.
*** All concentrations are in mg/kg TS.
%W/W = percentage of the dry weight of solids per wet weight of the feedstock substrate.
NM = not measured.
BD = below the detection limits.

4.1.2. *Inoculum*

The AD inoculum was obtained from a mesophilic (37ºC) AD plant treating cattle slurry and food waste or farm silage (Cockle Park Farm, Newcastle University, UK).

Table 4-4. Characteristics of the inoculum used to start up the AD systems

Parameter	Value*	Standard deviation
pH	8.2	± 0.31
TS (%W/W)**	1.28	± 0.005
VS (%W/W)**	0.65	± 0.06
VS (%TS)	51.7	-
TKN (mg/L)	2848	± 170.8
NH_3-N (mg/L)	2654	± 6.3
FAN (mg/L)	449	± 1
Total alkalinity (mg/L)	9792	± 157
Total VFA (mg/L)	3700	± 518
Total COD (mg/L)	8100	± 225
C (%)	40.9	± 3
N (%)	4.1	± 0.3
S (%)	0.15	± 0.04
C/N	10	-
Al ***	973	± 21
B	51	± 2.5
Ba	28	± 0.5
Ca	19465	± 183
Cd	0.29	± 0.1
Co	1.2	± 0.2
Cr	6	± 0.8
Cu	103	± 1.5
Fe	1818	± 9.8
K	6304	±19.0
Mg	2181	± 5.4
Mn	163	± 3.0
Mo	10	± 0.5
Na	637	±11.0
Ni	12.5	± 0.5
Pb	29.6	± 0.7
Si	304	± 3.0
Ti	38.8	± 0.4
V	8	± 0.1
Zn	389	± 2.7

*All values in this table represent mean value of three triplicate samples measured.
** %W/W = percentage of the dry weight of solids per wet weight of the inoculum.
*** All concentrations are total concentration of metals in µg per g TS.

The inoculum (seed) was passed through a 5 mm sieve to remove large particles of undigested organic matter then stored at 5°C until use. At the beginning of each study, the amount of inoculum required for starting up reactors was activated for 7 days according to the VDI method (VDI, 2006a) at 37°C, then acclimated to the feeding substrate and reactor environments for a period of 10 - 20 days. The activity of the inoculum (compared to the original seed) was checked by measuring the methane content of the biogas produced from the inoculum on day 20 of the acclimation period, and the inoculum was considered suitable for co-digestion experiments when the methane content of the biogas produced by the inoculum was > 50%.

4.1.3. *Mineral wastes*

Three of the MW (Table 4-5) were incineration bottom ash (IBA), fly ash (FA) and boiler ash (BA) obtained from a domestic Waste-to-Energy incineration power plant, Teesside, UK. In this plant, household food and garden wastes are dried and burned at ≈ 1000°C; steam turbines then convert the produced heat to energy. For the homogenisation, within a period of 3 week different samples of the MSWI solid residues (particle size of IBA was 14 - 40 mm) were collected from two production lines at the incineration plant. The fourth MW was from CDW produced from two CBW samples (with nominal particle sizes of 10 mm and 1 mm) collected from a CDW recycling site in Newcastle upon Tyne, UK. All the MW samples (IBA, FA, BA, and CBW) were dried overnight (104°C) and visible metals, glass and plastic materials removed. Prior to being used in the trials, all MW were ground by a mill (Vibratory Disc Mills, SIEBTECHNIK-TS, Germany), sieved to less than 212 µm (BS410 standard sieves 212 µm diameter) and stored at room temperature in airtight containers until use.

Table 4-5. Main characteristics of the mineral wastes*

Parameter	Incineration bottom ash (IBA)	Cement based waste (CBW)	Fly ash (FA)	Boiler ash (BA)
pH (1:2)**	10.37	11.07	10.3	11.84
Total solids(%WW)	99.15	97.1	97.1	99.5
Volatile solids (%WW)	2.86	2.44	2.9	1.2
Volatile solids (%TS)	2.88	3	3	1
C (%)	1.84	2.77	2.92	1.37
N (%)	0.04	0.02	0.02	0.01
S (%)	0.41	0.13	1.97	2.32
C/N	46	139	146	137

*All values in this table represent mean value with standard deviation (not shown) of three triplicate samples measured.
**20 g of each mineral waste was dissolved in 40 mL of distilled water and mixed with magnetic stirrer for one hour then measured for pH.

4.1.4. *Synthetic trace element solutions*

The synthetic (pure) TE solution used in this research (Chapter 9) was composed of multiple stock solutions (1000 mg/L) of each TE. The stock TE solutions were prepared with high purity element metals (Sigma Aldrich) in 2% HNO_3 solution. The acidified (pH ~ 5) stock solutions were stored at 5^oC until use. On start-up of experiments, reactors receiving TE supplementation were given appropriate volumes of these stock solutions to achieve the designed concentration of each TE in the reactor. The concentration of the TEs in the TE solution were chosen according recipes suggested by previous studies (Zhang *et al.*, 2012) as well as to simulate approximately the expected concentration of TEs released from mineral wastes of MSWI plant and construction demolition waste when supplemented to AD of OFMSW (Chapter 6).

Table 4-6. Concentration of standard trace elements used in this research (Chapter 9)

Component	Elements	Element concentration in individual stock solution of elements (mg/L)	Designated element concentration in reactor (mg/L)
$Na_2SeO{\cdot}5H_2O$	Se	1000 as Se	0.3
$FeCl_2{\cdot}4H_2O$	Fe	1000 as Fe	120
$NiCl_2{\cdot}6H_2O$	Ni	1000 as Ni	1.3
$CoCl_2{\cdot}6H_2O$	Co	1000 as Co	1
$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$	Mo	1000 as Mo	0.33
$AlCl_3{\cdot}6H_2O$	Al	1000 as Al	0.1
H_3BO_3	B	1000 as B	0.1
$CuCl_2{\cdot}2H_2O$	Cu	1000 as Cu	0.1
$MnCl_2{\cdot}4H_2O$	Mn	1000 as Mn	1
$ZnCl_2$	Zn	1000 as Zn	0.2

4.2. Metals analysis

Total concentration of metals in raw SOW, MW and dried reactor digestate samples, as well as soluble (dissolved) concentration of metals in reactor digestate samples were performed according to the EPA method 3010A (EPA, 1992) (see Section 4.2.1, 4.2.2 and 4.2.3).

4.2.1. *Total metals analysis*

For total metals concentration analysis, representative mass (2 g) of dried (at 50 - 70°C) and crushed samples were measured then transferred to long digestion glass tubes prior to acidic digestion with concentrated HNO_3 (2.5 mL/2 g TS) and HCl (7.5 mL/ 2 g TS) at room temperature for 16 hours, and then boiled on a heating block for another one hour at 100°C.

After cooling, the acid digested samples were filtered through acid resistant filter papers (Whatman ash-less filter papers) then diluted using 0.5 M HNO_3. The diluted samples were analysed for total metal concentrations using inductively coupled plasma optical emission spectroscopy (ICP- OES) (Vista MPX simultaneous ICP-OES, USA). Table 4-7 shows total concentration of metals in the mineral wastes (See Table 4-2 and Table 4-3 for total concentration of elements in the SOW).

Table 4-7. Total concentration of the acid-extracted (aqua regia) elements in the mineral wastes.

	Total metal concentration (mg/kg MW as TS)			
Elements	IBA	CBW	FA	BA
Al	28483 ± 1515	9653 ± 1320	15067 ± 518	35153 ± 2420
As	8 ± 0.5	3.7 ± 0.7	58 ± 0.8	49 ± 1.5
B	73.7 ± 3	90 ± 2.1	67 ± 4.3	128 ± 1.6
Ba	208.15 ± 49.1	122.32 ± 1.6	102.82 ± 48.3	70.61 ± 2.3
Ca	71199 ± 9745	136465 ± 2079	222346 ± 7198	197962 ± 1808
Cd	5.66 ± 1.1	0.51 ± 0.1	120.45 ± 2	51.42 ± 5.1
Co	40.39 ± 4	5.24 ± 0.4	10.78 ± 0.3	21.23 ± 0.2
Cr	97 ± 6.8	31.7 ± 1.7	41 ± 0.6	100 ± 3.9
Cu	2579 ± 102.4	17.4 ± 04	534.3 ± 40.7	283 ± 35
Fe	83655 ± 7521	19328 ± 491	6858 ± 111	12920.5 ± 374
K	3161 ± 96	1490 ± 79	38024 ± 725	21506 ± 18.3
Mg	6716 ± 684	10813 ± 661	5758 ± 492	13272 ± 652
Mn	1177 ± 118	348.6 ± 9.5	389 ± 2.9	1176 ± 104
Mo	6.73 ± 0.3	1.37 ± 0.01	13.85 ± 0.4	19.80 ± 0.7
Na	184 ± 3.2	22.6 ± 6.4	1553 ± 604	311 ± 55
Ni	114 ± 10	12.52 ± 0.6	85.6 ± 0.7	132.6 ± 4
Pb	1174 ± 120	15 ± 0.6	2109 ± 47	920 ± 54.5
Se	12.15 ± 1.6	5.40 ± 3.3	16.43 ± 0.6	24.68 ± 0.2
Si	70.2 ± 1.2	110.2 ± 38.7	180.2 ± 58	85.8 ± 2.9
Ti	1675.1 ± 147	687.6 ± 82	1594.7 ± 175	213 ± 20.6
V	212.8 ± 11	30.5 ± 1.1	472.4 ± 22.6	615.2 ± 34.8
Zn	3290 ± 102	51 ± 4.8	11114 ± 235	8699 ± 200

4.2.2. *Soluble metal analysis*

The concentration of water-leached (soluble) metals (Table 4-8) from MW under neutral pH ranges (6.4 - 7.5) of AD were measured according to the EPA method 3010A (EPA, 1992). This was carried out at the aqueous solutions obtained after adding 10 g of each MW in 500 mL distilled water. The MW-distilled water solutions were incubated in a reciprocating shaker

at 150 rpm for 100 h at 37°C. After that, the samples were filtered (Whatman ash-less filter papers), acidified (1-2 drops of concentrated HNO_3 per sample) and measured with ICP-OES (see Section 4.2.3 below).

Similarly, soluble (dissolved) metal concentrations in the inoculum (Table 4-4) and anaerobic digestate samples (Chapter 5, Chapter 6, Chapter 7 and Chapter 9) were measured from supernatant solutions discarded from digestate samples centrifuged for 30 min at 3392 g (Sigma centrifuge, UK). The samples were acidified with concentrated HNO_3 (1 - 2 drops per sample) then diluted with 0.5 M HNO_3 and stored at 5°C until analysed by ICP-OES (see Section 4.2.3 below).

Table 4-8. Soluble concentration of the water-extracted elements in the mineral wastes.

	Metal concentration (mg/L)			
Elements	IBA	CBW	FA	BA
Al	4874.13 ± 824	161.75 ± 15	10.00 ± 1.1	2.50 ± 0.24
As	0.92 ± 0.56	0.45 ± 0.29	0.30 ± 0.13	0.90 ± 013
B	14.11 ± 7.5	14.07 ± 7.6	21.00 ± 1.8	2.80 ± 0.4
Ba	5.98 ± 1.27	5.61 ± 0.68	22.50 ± 2.3	7.00 ± 0.7
Ca	6333 ± 1074	5586 ± 920	77538 ± 1220	52633 ± 1725
Cd	0.01 ± 0.001	0.01 ± 0.002	0.13 ± 0.04	0.13 ± 0.11
Co	0.06 ± 0.03	<0.01	0.07 ± 0.01	<0.01
Cr	2.31 ± 0.3	0.53 ± 0.19	2.00 ± 0.11	9.60 ± 1.5
Cu	4.11 ± 0.7	0.36 ± 0.13	0.73 ± 0.2	0.60 ± 0.2
Fe	16.44 ± 1.2	4.68 ± 0.13	1.25 ± 0.05	1.15 ± 0.21
K	757.4 ± 195	404 ± 87	10200 ± 1200	6745 ± 789
Mg	4.15 ± 0.65	1.90 ± 0.4	16 ± 1.8	42 ± 0.95
Mn	0.25 ± 0.07	0.06 ± 0.01	0.09 ± 0.003	0.04 ± 0.01
Mo	1.23 ± 0.1	0.28 ± 0.06	6.80 ± 0.87	8.60 ± 1.4
Na	112.3 ± 22	10.00 ± 2	727.50 ± 112	124.00 ± 21
Ni	0.94 ± 0.3	0.09 ± 0.01	0.08 ± 0.01	0.03 ± 0.001
Pb	0.55 ± 0.05	<0.25	2.55 ± 023	0.20 ± 0.03
Se	<0.02	<0.02	<0.02	<0.02
Si	3.000 ± .1	23.00 ± 1.2	36.00 ± 0.86	21.00 ± 2.17
Ti	0.10 ± 0.01	0.13 ± 0.02	0.370 ± 0.12	0.26 ± 0.07
V	0.84 ± 0.14	0.21 ± 0.03	0.98 ± 0.15	3.44 ± 0.23
Zn	2.01 ± 0.2	1.04 ± 0.16	5.35 ± 1.35	2.40 ± 0.2

4.2.3. *Elemental analysis by ICP-OES*

Total and soluble concentrations of the elements in samples prepared according to the Section 4.2.1 4.2.2 were quantified by ICP-OES (Vista MPX simultaneous ICP-OES, USA), which uses emission spectra of a sample to identify the elements and measure their concentrations. A main calibration standard solution (Standard-1; Table 4-9) was prepared from stock solutions (concentration of each element in its stock solution was1000 ppm) of the measured elements. Then another two calibration standard solutions were prepared by diluting the main calibration standard solution (dilution factors were 1/10 and 1/100) to construct multipoint standard curves covering the range of element concentrations anticipated in the samples. The 0.5 M HNO_3 solution used for diluting the acid digested samples (Section 4.2.1 4.2.2) was used as a matrix solution for preparing the calibration standard solutions.

Table 4-9. Concentration of elements in the standard solutions used for constructing calibration curves of the ICP-OES machine.

Designated element concentration in the standard solution (mg/L)			
Elements	Standard-1	Standard-2	Standard-3
Ca	150	15	1.5
Mg	15	1.5	0.15
Na	15	1.5	0.15
K	150	15	1.5
Fe	50	5	0.5
Mn	50	5	0.5
Al	150	15	1.5
Si	50	5	0.5
Cd	5	0.5	0.05
Cr	10	1	0.1
Co	1	0.1	0.01
Cu	10	1	0.1
Ni	5	0.5	0.05
Pb	25	2.5	0.25
Ti	50	5	0.5
V	3	0.3	0.03
As	2	0.2	0.02
B	2	0.2	0.02
Ba	50	5	0.5
Se	2	0.2	0.02
Mo	5	0.5	0.05
Zn	5	0.5	0.05

4.2.4. *Quality control*

In order to ensure the absence of sample contamination, blank and standard samples were prepared following the same sample-preparation and analytical processes. Three samples were processed, and the accuracy of the ICP-OES machine was determined by running the blank and standard samples (Table 4-9) after every 10 samples analysed.

4.2.5. *Alkalinity of mineral wastes*

The alkaline capacity of MW were determined using 1N HNO_3 according to the method described by Banks and Lo (Banks and Lo, 2003; Lo, 2005). The alkaline capacity tests were conducted in 250 mL laboratory glass beakers. For these tests, sixty samples of MW solutions (2.5 g of a MW+100 mL distilled water) were prepared, and each of the beakers were acidified with a volume (ranged between 0 - 20 mL) of 1N HNO_3 solution. The acidified MW solutions were then stirred for 1h using magnetic stirrers at room temperature (25^{o}C) prior measuring pH. Alkalinity at each pH value was calculated using Equation 4-1, then acid titration curves were produced (Figure 4-1) for each MW solution as a function between measured pH values and amounts of acid added; calculated as mM H^{+}/g MW. Partial alkalinity (PA) and total alkalinity (TA) at pH 7.5 and pH 4.4 respectively were also calculated using Equation 4-1 (Table).

$$Alk = V_{pH} \times N \times E_w / V_s \quad \textbf{(4-1)}$$

Where Alk = alkalinity of a solution (mg $CaCO_3$/L), V_{pH} = amount of acid amended (mL) to reach a pH value, N = normality of acid amended (eq/L), E_w = 50,000 (mg $CaCO_3$/eq), and V_s = solution volume (mL).

Table 4-10. Partial alkalinity (PA) and total alkalinity (TA) of the MW samples before using in the AD trials.

Mineral wastes	Partial alkalinity (PA) (meq/g $CaCO_3$)	Total alkalinity (TA) (meq/g $CaCO_3$)	PA/TA ratio	pH
Incineration bottom ash	1.2 ± 0.03	2.6 ± 0.04	43%	10.4 ± 0.5
Cement based waste	1.3 ± 0.1	6.7 ± 0.06	18%	10.3 ± 0.39
Fly ash	0.8 ± 0.1	5.3 ± 0.04	15%	11.8 ± 0.24
Boiler ash	2.6 ± 0.1	6.0 ± 0.2	43%	11.0 ± 0.7

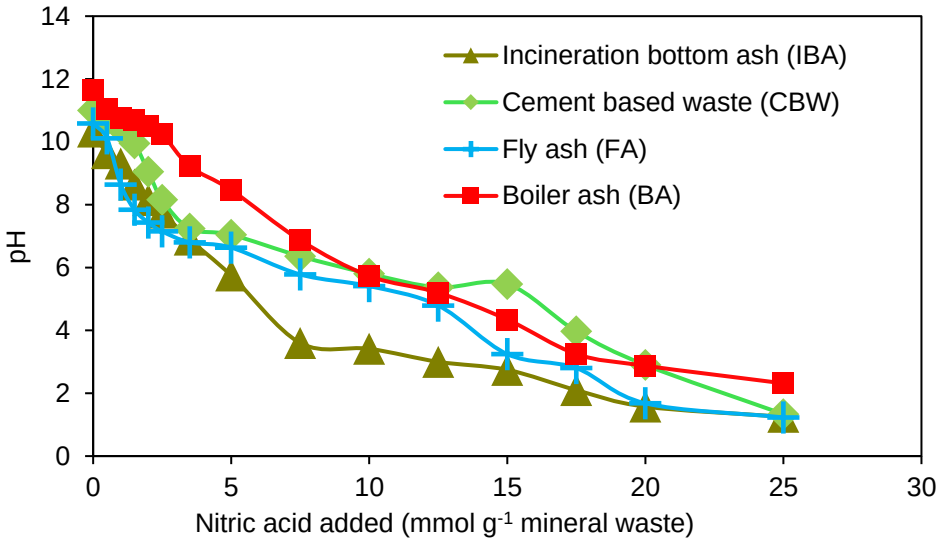


Figure 4-1. Titration curves of MW solutions (2.5 g MW/100mL distilled water) with nitric acid at room temperature 20^{o}C. The values are mean values of triplicate samples with standard deviation (not shown).

4.2.6. *Elemental composition analysis and theoretical methane yield*

The homogenised MW samples were dried at 50 - 70^{o}C then analysed for elemental compositions to determine the percentage of C, N, and S in these samples (Table 4-5). Elemental composition analysis was performed using an organic element analyser (Elementar Vario MAX CNS, Germany) according to the manufacturer's instructions and the standard method (SCA, 1986).

Another elemental composition analysis was carried out at an external lab (Elemental Microanalysis Ltd, UK) to measure the C, N, S, H and O elements of the SOW feedstock. The molecular formula of the substrate (SOW) was estimated via C, H, N, O and S microanalysis (Table 4-2 and Table 4-3).

4.2.7. *Estimation of theoretical methane potential*

Using the elemental composition (C, H, N, O, and S) analysis of the substrate ((SOW), Table 2), the estimated molecular formula of the substrate was $C_{3.87}H_{6.76}O_{2.35}N_{0.16}S_{0.005}$. Theoretical methane potential from the SOW based on VS conversion was calculated using the stoichiometric equation (Eq. 4-2) and Eq. 4-3A (Nielfa *et al.*, 2015). Similarly, theoretical methane potential of the substrate based on COD conversion was calculated from the stoichiometric oxidation equation of the substrate (Eq. 4-3B). The values are for the saturated gas under the standard temperature and pressure conditions (STP; 0^{o}C and 1 bar).

$$C_n H_a O_b N_c + \left(n - \frac{a}{4} - \frac{b}{2} + \frac{3c}{4}\right) H_2O \rightarrow \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - \frac{3c}{8}\right) CH_4 + \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4} + \frac{3c}{8}\right) CO_2 + cNH_3$$

(4-2)

A- Theoretical biomethane potential based on the compositional analysis (Buswell equation) at standard temperature and pressure (STP, 0°C and 1 bar).

$C_{3.87} H_{6.76} O_{2.35} N_{0.16}$ + 1.125 H_2O	⟶	2.1325 CH_4 + 1.7375 CO_2 + 0.16 NH_3	**(4-3A)**
93.04 + 20.25	⟶	34.12 + 76.45 + 2.72	
113.29	⟶	113.29	
1.12 g TS + 0.237 g H_2O	⟶	0.41 g CH_4 + 0.92 g CO_2 + 0.032 g NH_3	
1.03 g VS + 0.218 g H_2O	⟶	0.3773 g CH_4 + 0.846 g CO_2 + 0.0294 g NH_3	
		0.528 L CH_4 + 0.43 L CO_2 + 0.0387 L NH_3	
1 g VS + 0.211 g H_2O	⟶	0.5126 L CH_4 + 0.417 L CO_2 + 0.0375 L NH3	
		53% CH_4 + 43.11%CO_2 + 3.8% NH_3	
Theoretical methane potential ~	513 mL/ g VS		
Density of CH_4 =	0.77 g/L		
Density of CO_2 =	2.14 g/L		
Density of NH_3 =	0.83 g/L		

B- Theoretical biomethane potential based on the on COD conversion (at STP).

$C_{3.87} H_{6.76} O_{2.35} N_{0.16}$ + 4.195 O_2	⟶	3.8 CO_2 + 3.14 H_2O + 0.16 NH_3	**(4-3B)**
COD/VS	⟶	4.195 * 32/93.04	
		1.443 g COD/g VS of the substrate	
1 g COD is converted to 350 mL of CH_4 (Filer *et al.*, 2019))	⟶	1.443* 350	
Theoretical methane potential ~	504 mL/ g VS		

4.3. Reactor configuration

4.3.1. *Biomethane potential (BMP) reactors*

Biomethane potential (BMP) assays were conducted in in 0.5 litre glass bottles (Duran®, Germany) incubated at 37°C, in accordance with German standard method (VDI, 2006a). Prior to the incubation, in order to simulate anaerobic conditions, the BMP assays were purged with nitrogen gas (99.9%) for 5 minutes, then they were closed with rubber bungs with an opening for connection with gasbags (0.6 or 1 litre Tedlar gasbags, VWR) for biogas collection. All gasbags were disconnected daily, the volume of biogas and methane concentration (section 4.4.7 below) were measured then the gasbags reconnected to the BMP assays. The biogas and methane volumes were corrected for water-vapour content of saturated gas at standard temperature and pressure (STP, 0°C and 1bar) using Eq. 4-4. Mixing of BMP assays was performed manually by shaking the bottles two times per day for 1 - 2 minutes.

The BMP assays were ended after 30 days or when daily methane production was less than 1% of the accumulated methane (VDI, 2006a; Nielfa *et al.*, 2015).

Blank BMP assays with the inoculum only and inoculum plus MW (Chapter 5, Chapter 6 and Chapter 7) were prepared to correct (Eqs. 4-5) the rates from the interference of the methane formed from the organics of the inoculum and the effect of the methane generated from the MW when in contact the inoculum only. Moreover, the capacity and the performance of the inoculum was assessed in BMP assays (reference assay) contained inoculum and pure cellulose.

$$V_{STP} = P_{gas} / P_{STP} * (T_{STP} / T_{gas}) V_{gas} \quad \textbf{(4-4)}$$

Where, V_{STP} is the biogas volume adjusted to standard temperature and pressure, P_{gas} is the pressure of the measured biogas, T_{gas} is the temperature of the measured gas in Kelvin (K), T_{STP} is the standard temperature in K and V_{gas} is the measured gas volume from gasbags.

$$Y_{m\ Experimental} = (V_{substrate} - V_{blank} * (VS_{is} / VS_{ib})) / VS_s \quad \textbf{(4-5)}$$

Where, BMP is the biomethane potential of the substrate, $V_{substrate}$ is the accumulated methane volume from substrate, V_{blank} is the accumulated methane volume from the inoculum, VS is the mass of volatile solids of inoculum in substrate bottle, VS_{ib} is the mass of volatile solids of inoculum in blank bottle, VS_s is the mass of volatile solids of the substrate in substrate bottle.

4.3.1.1. *First-order and modified Gompertz models*

Mathematical models are mathematically derived equations have been applied for predicting methane production from the AD substrates without undertaking extensive and costly experiments (Kafle and Chen, 2016). Moreover, the mathematical models which have been developed from mechanistic studies, are considered a simple method to validate the results of empirical methods, and to enhance the design and optimisation of AD processes (Yu *et al.*, 2013; Ware and Power, 2017). In this thesis, methane production of the BMP assays was modelled by fitting the experimental methane production data with first-order (FO) (Eq.4-6) and Modified Gompertz (GM) (Eq. 4-7) models (Nielfa *et al.*, 2015) in MATLAB software (The MathWorks, 2015) with 95% confidence bounds. In addition to the kinetics of methane production rates (K), the kinetic parameters of microbial growth rates (μ) and lag phase time (λ) were determined from the first-order and Gompertz models, respectively. These parameters provided further insight into the results obtained from BMP assays; in particular, biodegradation patterns of the feed substrate (SOW) when co-digested with the MW.

$$Y = Ym[1 - exp(-\mu t)] \quad \textbf{(4-6)}$$

$$Y = Ym \times exp\left\{-exp\left[\frac{K \times e}{Ym}(\lambda - t) + 1\right]\right\} \quad \textbf{(4-7)}$$

Where Y is the cumulative methane production at time (t), Y_m is the maximum methane potential (mL CH_4/g VS) at an infinite digestion time (t), μ is the specific microorganisms growing rate (d^{-1}). K is the methane production rate (mL CH_4/g VS. d), "e" is a mathematical constant (2.718) and λ is the lag phase time constant (d).

Moreover, the accumulated methane volume achieved from the experimental BMP assays ($Y_{m\ Experimental}$) was then compared (D = difference between experimental and theoretical methane yield values (Eq. 4-8)) with the (1) maximum methane production ($Y_{m\ Theoretical}$) obtained by applying the two models at different operation times of the experiments, and (2) theoretical methane yield estimated from the elemental composition analysis (Nielfa *et al.*, 2015) as described in Section 4.2.4.

$$D\ (\%) = (Y_{m\ Experimental} - Y_{m\ Theoretical}) / Y_{m\ Experimental} * 100 \quad \textbf{(4-8)}$$

4.3.2. *Continuous stirred tank reactors (CSTRs)*

The continuous AD experiments were conducted in continuous stirred tank reactor (CSTR) systems with working volume of 5 L or 1 L. Each reactor was a borosilicate glass (Duran®, Germany) quick fit flask 100 mm diameter, with three ports for feeding, sampling and mixing. Mixing of CSTRs was by overhead stirrer motors rotating a 5 cm x 2 cm flat paddle mixer at 180 - 200 rpm fitted through a water seal port into the central reactor opening. The temperature of reactors was maintained at 37ºC ±1 automatically by a temperature and mixing controlled water bath; which maintained the temperature in reactors at 37ºC ±0.5. Biogas produced from each reactor was collected in 5 L or 10 L gasbags (Tedlar, VWR) and checked for methane volume and composition (Section 4.4.7) daily/every two days, emptied then reconnected to reactors. Daily volumetric methane production was calculated using Eq. 4-9.

Volumetric CH_4 production (L CH_4/L reactor/d) = V biogas x CH_4%/V reactor **(4-9)**

Usually, before starting the continuous AD experiments, each of the CSTR systems was thoroughly filled (5 L) with inoculum to get acclimated to the feedstock substrate (i.e. the SOW) and reactor environment for 20 days. During acclimation, the reactors were fed every 2 - 3 days with a SOW and distilled water mixture containing 1 g SOW volatile solids and the biogas produced by the CSTR systems were collected in gasbags and checked for methane

volume and composition (Section 4.4.7 below) to ensure the microorganisms were active. On day 20, a methane content of > 50% was observed in all the reactors, indicating that they were ready for operation in the continuous AD experiments.

4.4. Analytical methods

4.4.1. *pH*

pH was measured according to APHA standard method 4500-H (APHA, 2005) using a pH meter (Jenway- 3310, UK). Prior to use, the pH meter was calibrated with pH 4 and pH 7 standard solutions (VWR, UK). The pH of the feedstock and MW was measured by mixing (magnetic stirrer for one hour) one volume of each sample with two volumes of distilled water then measured for pH. While, pH of digestate samples was measured directly after they withdrawn from reactors.

4.4.2. *Total solids and volatile solids*

Total solids (TS) and volatile solids (VS) were measured in duplicate using crucibles (30 mL) according to APHA standard methods 2540D and 2500E, respectively (APHA, 2005). For solid samples (feedstock or MW) two-third of crucibles were filled with each sample, whereas for liquid samples (digestate) crucibles were filled with 20 mL of samples. Total solids (TS) were calculated as the mass of solids remaining after oven-drying (Gallenkamp, Hotbox Oven Size 2) samples overnight (105^{o}C) then volatile solids (VS) mass was calculated after oven-drying in the Muffle furnace (S. H. Scientific, Carbolite) at 550^{o}C for 30 minutes.

4.4.3. *Alkalinity and volatile fatty acids*

On weekly basis digestate samples from reactors were centrifuged (3392 x g, 30 min), after centrifugation the supernatant of samples were discarded and used for physicochemical analysis. Total alkalinity (total ALK) and total volatile fatty acids (total VFA) of digestate samples were measured by titration according to the Lossie and Pütz method (Lossie and Pütz, 2008). The titrant was 0.1 N H_2SO_4 and the sample volume (supernatant) was 20 mL. Total ALK referred to as TAC and intermediate alkalinity referred to as FOS; which equals to total VFA in samples, were calculated using Eqs. 4-10 and 4-11. The alkalinity ratio known as FOS/TAC ratio was calculated using the Eq. 4-12 to determine process stability in reactors, with values < 0.3 indicating a stable process (Lossie and Pütz, 2008).

$$TAC = H_2SO_4\text{-Volume added from start to pH 5 in mL} * 250 \quad \textbf{(4-10)}$$

$$FOS = (H_2SO_4\text{-Volume added from pH 5 to pH 4.4 in mL} * 1.66 - 0.15) * 500 \quad \textbf{(4-11)}$$

$$Alkalinity\ ratio = FOS / TAC \quad \textbf{(4-12)}$$

Concentration of individual VFA; including acetic acids, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid was measured by Ion Chromatography (ICS) (Dionex ICS-1000), which was calibrated with standard VFA solutions of 2 ppm to 500 ppm concentration. The samples used for the VFA analysis were prepared by mixing 0.4 mL of different filtered supernatant samples (0.22 µm polyethylene syringe filter; (VWR international, UK)) with 0.4 mL of 0.1N Octane sulfonic acid (OSA reagent; Thermoscientific, UK), and sonicated (50/60 Hz, Decon Ultrasonics Ltd,UK) for 40 minutes to drive off carbonate.

4.4.4. *Total Kjeldahl nitrogen and total ammonia nitrogen*

The supernatant samples (Section 4.4.3) obtained from digestate samples were used for total ammonia nitrogen and total Kjeldahl nitrogen (TKN) analyses. Total ammonia nitrogen was measured using Vapodest steam distillation unit (Gerhardt, Vapodest 30 S) according to APHA standard method 4500-NH_3 B and 4500-NH_3 C. Whereas for TKN analysis, the supernatant samples were acid digested with a Turbotherm digestion unit (Gerhardt, UK) followed by Vapodest steam distillation.

4.4.5. *Soluble chemical oxygen demand (sCOD)*

For the anaerobic experiments described in chapter 5 and chapter 6 of this thesis, sCOD concentration of the supernatant samples was measured according to APHA closed reflux titrimetric method 5220C (APHA 2005) in triplicate. While for the results demonstrated in Chapter 7, Chapter 8 and Chapter 9 concentration of sCOD was measured using COD kits (Merck, VWR) according to the manufacturer's instruction. The accuracy of analysis was checked by measuring blank samples following the same sample preparation and measurement methods.

4.4.6. *F_{420} analysis*

The relative florescence intensity (RFI) of coenzyme F_{420} in reactor digestate was determined according to the method described in (Dolfing and Mulder, 1985; Kida *et al.*, 2001). Digestate samples (10 mL) were autoclaved (120^{o}C, 30 min) followed by two times centrifugation (3392 x g, 30 min). Prior to the first centrifugation the autoclaved samples were mixed with

equal volume (10 mL) of 2-propanol. The supernatant obtained from centrifugation discarded, its pH adjusted to pH >13 with KOH, and the RFI of F_{420} determined by Spectro-fluorescence (Spectra Max M3) according to the manufacturer's instruction. The samples (200 µL per sample in triplicate) were loaded into the spectrometer machine in 96-well microplates, and the spectrometer was set up to shake the plates once before measuring the excitation at 425 nm and absorbance at 460 nm.

4.4.7. *Biogas measurement and analysis*

Biogas production from reactors was measured daily/every two days using a 500 mL graduated glass syringe (Trajan Jumbo Syringe, VWR, UK) from the volume of biogas collected in gasbags (Section 4.3.2). Methane and carbon dioxide compositions of biogas were measured by gas chromatography (GC) (Carlo Erba HRGC S160 GC with MFC 500 detector, Germany), the carrier gas of the GC was hydrogen (250 mL/min) with an oven temperature held isothermally at 35°C. Prior to analysis, the GC was calibrated with a standard gas of ~ 80.2% methane. Injection of gas (biogas/standard gas) was by a 100 µL lock tight gas syringe (SGE, Australia) in triplicate (50 µL gas per each injection). Measurements of methane percentage of biogas for each reactor were performed in triplicate. Volumes of biogas and methane were reported according to conditions of saturated gas under standard pressure and temperature (STP; 0^{o}C and 1bar) according Eq. 4-4 shown in Section 4.3.1.

4.5. Microbial analysis

4.5.1. *DNA extraction*

Total genomic DNA of biomass samples (obtained after centrifugation (5 min, 15.000 x g) of 1 mL of each digestate sample) were extracted according to the method described in (Griffiths et al., 2000). The absence / or presence of PCR inhibitors in the DNA was evaluated using a Nanodrop (Thermo Fisher, UK). The acceptable range between 1.8 and 2.2 was ensured for the DNA quality ratios of 260:280 and 230:260. For quality control, with each batch of DNA extraction, blank DNA samples were prepared following the same sample-preparation and DNA extraction methods. The blank samples were analysed with each batch of Real-time PCR and Illumina sequencing analyses.

4.5.2. *Real-time PCR (qPCR) analysis*

4.5.2.1. *qPCR standards*

The mcrA gene was targeted to measure the abundance of methanogens in the digestate

samples. *Methanosarcina barkeri* pure cultures were used to prepare the mcrA gene standard for qPCR analysis. The DNA extraction was carried out using an MP-bio 'for soil DNA' extraction kit (UK) based on the manufacturer's directions. The amplification of the mcrA gene was carried out using the mlas-F primer (Steinberg and Regan, 2009). The generated PCR products were employed for the mutation of *Escherichia coli* cells as per the manufacturer of the kit used (TA cloning kit; Invitrogen, UK). The clones that were found mutated (positives) were then incubated at 37°C using LB broth as growth medium (spiked with ampicillin). The generated plasmids were then extracted and cleaned (purification) using a purification kit for plasmids (ROCHE, UK). The yields were then quantified using Quant-It (Invitrogen, UK). Quantification enabled dilution (using PCR-grade distilled water) of the plasmid DNA from each clone to generate serial dilutions with known populations (ranged between 10^2 to 10^8 gene copies/µL). These populations were used for the qPCR standards.

The 16S rRNA gene was targeted for measuring bacteria abundance. To prepare the 16S rRNA standard for qPCR analysis, the complete 16S rRNA gene was amplified from *E. coli* using the PA/PH primers (pA and pH primers (Edwards et al., 1989); Table 4-11)). PCR reaction was conducted using Phusion Flash High-fidelity PCR master mix (ThermoFisher), using the following thermocycle program: (i) 10 sec denaturation (98°C), (ii) 35 cycles of 1 sec denaturation (98°C), (iii) 5 sec annealing (98°C), (iv) 15 sec elongation (72°C), and (v) 1 min elongation (72°C). The products were separated on 1.5% agarose gel electrophoresis containing SYBR® safe DNA gel stain (Sigma) and visualized using GelDoc (Biorad). The generated PCR products were then purified using the GenElute PCR clean-up kit (Sigma-Aldrich) as per the manufacturer's instructions. The TOPO pCR4 vector (Invitrogen) kit was used for the cloning of the purified products. The fresh cloned plasmids were re-purified with the PureYield Plasmid Miniprep System (Promega). The Quant-iT Picogreen dsDNA Assay kit (Invitrogen, Life Technologies, Inc.) with the SpectraMax® M3. The plasmid was used for the quantification of the DNA concentration. The absolute number of the gene copies of the genes used for the standards was calculated based on the plasmid size and insert length (3,973 and 1,515 bp respectively) assuming a mass of 660 Da/bp (molecule). The reference DNA solution contained 10^9 gene copies/µL.

4.5.2.2. *qPCR analysis*

For the quantification of the methanogenic and bacterial population in the bioreactor (digester) Real-time PCR analysis (qPCR) was used. The methanogenic population was quantified followed the protocol described above (Section 4.5.2.1) as well as the protocol

provided by Steinberg et al. (Steinberg and Regan, 2009). For the qPCR a CFX96 real–time PCR system (Biorad, UK) was used. The conditions set for the reaction (mcrA gene) included: (i) 3 min initial denaturation at 98°C, (ii) 39 cycles of denaturation at 95°C for 5 sec, (iii) annealing at 66°Cfor 10 sec, (iv) extension at 65°C for 5 sec with a 0.5°C increment, and (v) final extension step at 95°C for 0.5 min as per the manufacturer's protocol (BIORAD, UK for Ssofast Evergreen® Supermix). The reaction solution contained 1 µL sterile de-ionized water, 3 µL of sample DNA template, 0.5 µL each of the forward and reverse primers (the primers were diluted to concentration of 10 pmol/µL) and 5 µL of Ssofast EvaGreen Supermix solution (Biorad, UK). The analysis was carried out based on a 5-point calibration curve using the mcrA gene standards that were prepared followed the protocols described above (Section 4.5.2.1). For the dilutions filter sterile de-ionized water was used. All qPCR reactions were performed in triplicates; the reaction efficiency was estimated based on the curve generated by the standards. This was automatically assessed by the instrument's software.

Total bacteria was quantified using a SYBR green-based method assay (forward (1055F) and reverse (1392R) primers (Harms et al., 2003); (Table 4-11)). SYBR-green reactions were conducted using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad) as reagent. The reaction followed a thermocycle program with: (i) 2 min of initial denaturation (98°C), (ii) 40 cycles of 5 sec denaturation (98°C), and (iii) 5 sec annealing/extension (60°C). All assays were carried out in triplicates using a BioRad CFX C1000 System (BioRad, Hercules, CA USA). To avoid inhibition phenomena during the amplification the DNA samples were diluted to a working solution of 5 ng/µL. An internal control DNA was also employed in the SYBR-green reactions to assure there is no errors in the quantification process related to contamination. A standard curve with known copy numbers (10^3 and 10^8) was incorporated using plasmid clones of target sequences (Section 4.5.2.1). The reactions were all carried out in triplicates. For enumeration of the 16S rRNA gene via qPCR the following mixture was prepared: 3 µL template DNA, 5 µL Ssofast EvaGreen Supermix (Bio-Rad, UK), 0.5 µL of forward (1055F) and reverse (1392R) primers (Harms *et al.*, 2003) ; (Table 4-11), and 1 µL sterile de-ionized water (total volume was 10 µL).

Table 4-11. Primer design of the qPCR analysis targeting 16S rRNA gene

Target	Primer	Sequence (5' – 3')	Product size (bp)	Tm	Reference
16S rRNA	pA pH	AGAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCCGCA	1515	55	(Edwards *et al.*, 1989)
16S rRNA	1055F 1392R	ATGGCTGTOGTCAGCT ACGGGCGGTGTGTAC	337	60	(Harms *et al.*, 2003)

4.5.3. *Illumina sequencing of 16S rRNA gene*

The sequencing data was obtained from a 16S rRNA library (Illumina HiSeq (16S V4)) prepared by Earlham Institute (Norwich, UK) after sample quantification (Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Q33231)) and purification. The purity was inspected using the Drop Sense 96 (Perkin Elmer). Prior PCR amplification the DNA samples were diluted to reach a mass of 10 ng. The amplification (PCR) was carried out using 2 µL of the forward and reverse primers (each) (Kozich et al., 2013) at a concentration of 2.5 µM; 0.1 µL of Kapa 2G Robust polymerase (Kapa Bio systems KK5005), 0.5 µL 10 mM dNTPs were also added. Finally, Qiagen nuclease free water (Qiagen 129114) was added to make a volume of 25 µL. The amplification program had (i) 3 min of initial denaturation at 94°C, and 25 cycles of (ii) denaturation at 94°C for 45 sec, 55°C for 15 sec, and 72°C for 30 sec, then (iii) final extension for 3 min at 72°C, and (iv) holding for 3 min at 4°C. All amplified DNA samples were then purified using the Agencourt AMPure XP bead clean-up kit (Beckman Coulter A63882) using the manufacturer's protocol modified by two 80% EtOH washes and re-suspension of the samples in 25 µL of elution buffer (10 mM Tris). The generated libraries were quantified (Qubit dsDNA HS Assay Kit) and sized via PerkinElmer GX using a highly sensitive DNA chip (PerkinElmer CLS760672). Afterwards, all libraries were equimolar-pooled; the pool was quantified via qPCR using a Kapa Library Quantification Kit (Kapa Biosystems KK4828).

4.5.3.1. *Sequenced data processing*

Raw sequence data (FastQ) files obtained from Illumina HiSeq sequencing were de-multiplexed and quality filtered and reads were binned into amplicon sequence variant (ASV) using DADA2 (Callahan *et al.*, 2016) default parameters in the Quantitative Insights into Microbial Ecology (QIIME2) pipeline (Caporaso *et al.*, 2010). The taxonomical assignment was then accomplished using the SILVA119 database (Quast *et al.*, 2012). Canonical

Correspondence Analysis (CCA) was performed in R (R, 2013) using the "vegan" package (Oksanen *et al.*, 2010) to determine the correlations between the abundance of bacterial and archaeal communities and measured physicochemical parameters such as TAN, total VFA, soluble COD etc. In addition, representative sequences of selected, predominant, ASVs were compared (BLAST) to the NCBI nucleotide database to identify cultured and environmental closely related sequences. Representative 16S rRNA sequence fragments and close relatives were aligned using MUSCLE in MEGA7 to construct a neighbour-joining phylogenetic tree supported by bootstrap analysis (Kumar *et al.*, 2016). Evolutionary histories were inferred using the Neighbour-Joining method (Saitou and Nei, 1987). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2011) and the percentage of replicate trees in which the associated taxa clustered together was determined by bootstrap analysis of 1000 replicates (Westerlund and Edgerton, 2007).

For representative visualization of the ASV data and to highlight the differences in the structure of microbial community further analysis was carried out. Plots of principle component analysis (PCA) of bacterial and archaeal communities were generated using STAMP software (Parks *et al.*, 2014) and phyloseq R package (McMurdie and Holmes, 2013a).

The significant difference analysis between groups and within groups (treatment conditions) for archaeal and bacterial gene abundances was conducted using the Analysis of Similarity (ANOSIM) (Clarke, 1993) with Bray-Curtis indices. The package "vegan" in R platform (Oksanen *et al.*, 2010) was used for the dissimilarity matrix constructions and figure visualisations.

Alpha diversity (richness and Shannon index) were calculated then visualised using the global function in microbiome R package (Leo Lahti, 2017). In addition, beta diversity analysis, and Spearman correlation coefficients between reactor variables (*physico-chemical* parameters and reactor performances) and microbiome composition at family and genus levels were calculated and visualised using the microbiome R package (Leo Lahti, 2017).

4.6. Statistical analysis

The one-way analysis of variance (One-way ANOVA) in SPSS (IBM-SPSS, 2017) / R (R, 2013) were applied to determine whether there were any statistically significant differences between the means of the measured physicochemical parameters under different digestion conditions. The three assumptions of the ANOVA analysis were 1) no significant outliers in data; 2) the data of dependent variables were approximately normally distributed for each category of the independent variable; and 3) the homogeneity of variances. These three

assumptions were checked in SPSS before the ANOVA analysis. The one-way ANOVA analysis was followed up with a post hoc test (we assumed equal variance and used Tukey test with significance level of 0.05) to determine the statistically significant differences between different groups (for instance different digestion conditions or different feeding methods etc.).

Moreover, Pearson bivariate correlation analysis was also conducted in SPSS/ R to understand the correlations between 1) digestion conditions and measured physicochemical parameters and biological parameters such as the enumerations of bacteria and archaea or relative abundances of different bacteria and archaea species (see Chapter 6)), 2) different digestion conditions and measured parameters (such as methane yield, methane production rate, COD, F_{420}, VFA, concentrations of metals and etc.) and abundances of bacteria and archaea, fermentation and methanogenesis activities etc. (see Chapter 7)). The significant of correlations were measured at 0.05 and 0.01 levels (2-tailed). The assumptions made for the Pearson correlation analysis were 1) the two variables were continuous such as the operating time or changes in organic loading rates of reactors; 2) there was a linear relationship between the two variables (checked by creating a scatter plot for the two variables); 3) there was no significant outliers in the data if found removed; and 4) the data of variables was approximately normally distributed.

4.7. Microbial specific activity

The cell specific methanogenic and fermentation activities (Chapter 6, Chapter 7 and Chapter 9) in reactors was estimated from the daily methane production and total COD/or total VFA concentration (total COD/or total VFA concentration equals to the sum of the soluble COD (sCOD)/or VFA concentrations in the digestate plus the methane production expressed as COD/or VFA). The average number of the methanogenic and bacterial populations was estimated from the qPCR analysis. For total bacteria enumeration, the 16S RNA gene abundances were divided by 4 and for total methanogens the *mcr*A gene abundances were divided by 2 (Klappenbach *et al.*, 2001). The formulas (Eqs. 4-13 and 4-14) provided by Petropoulos *et al.* (2017) were employed for the estimation of the two specific activities:

$$\text{Cell specific methanogenesis} = \frac{Methane\ production\ (ml)}{Number\ of\ methanogen\ cells} \quad \textbf{(4-13)}$$

$$\text{Cell specific hydrolysis} = \frac{Total\ COD\ or\ TVFA\ concentration}{Number\ of\ bacteria\ cells} \quad \textbf{(4-14)}$$

4.8. Definitions of diversity and diversity indices

Measurement of diversity of species is a useful tools for characterisation of communities in reactors operated under different digestion conditions (Tuomisto, 2010; Li, 2013). As described in Section 4.6, the ASV data table, which obtained from the QIIME2 pipeline of the 16S rRNA sequenced data; was used for the alpha diversity and beta diversity analyses (Chapters 6 – 9).

Alpha diversity shows the richness and evenness of species within a habitat (Whittaker, 1977). In this thesis, richness indicates the number of species / evenness of species in digestate samples of reactors at specific time or HRT period or under different digestion conditions. While beta diversity compares the richness and evenness of species among different habitats (Whittaker, 1977), for example different reactor performances or different digestion conditions. The widely used diversity indices Cho1 (a nonparametric estimate of species richness), Shannon and Simpson (Chapters 6 – 9) were calculated for alpha diversity analyses. Both Cho1 and Shannon indices are known to confirm the richness component of diversity, while the Simpson index is confirming the evenness component of diversity (Nagendra, 2002). The Simpson index values range between 0 – 1, indicating whether the two communities are completely homogenous (Simpson index = 0) or completely heterogeneous (Simpson index = 1) (Simpson, 1949; Li, 2013).

Beta diversity shows the distance or dissimilarity between the digestate samples, this was estimated using Bray-Curtis and Unifrac methods and local contribution of beta diversity (LCBD) coefficients. Bray-Curtis is a non-phylogenic method, which depends on the abundance of species in the samples to produce the distance matrix of beta diversity, while the Unifrac method of beta diversity depends on the phlyogenic trees to generate a distance matrix of the species present in the samples (Li, 2013). The LCBD coefficients are comparative indicators of the ecological uniqueness of different samples in terms of community composition (Legendre and De Cáceres, 2013). For example, a digestate sample with higher LCBD value means it contains higher unique species than other digestate samples and vice versa. Moreover, the analysis of similarity (ANOSIM) was also performed to assess the statistical significance of differences of similarity within and among groups (reactors or digestion conditions) (McCord *et al.*, 2014).

Chapter 5. Predicting the effects of integrating mineral wastes in anaerobic digestion of OFMSW using first order and modified Gompertz model from BMP assays

Abstract

Previous studies found mineral wastes (MW) are promising resource for the macro-/micronutrients necessary for anaerobic digestion (AD) processes. The current study used both BMP assays and mathematical models to investigate the effects (buffering capacity, release of trace elements and kinetic parameters of methane production) of integrating MW into the AD of OFMSW at 37^{o}C. The Gompertz model results showed that most of the examined MW enhanced methane production rate from OFMSW (44 - 73% higher than control) without significant adverse effects on the hydrolysis and acetogenesis processes. The relative positive effects of MW on methane production rates were in the order: incineration bottom ash (IBA) > boiler ash (BA) > cement-based waste (CBW) > Fly ash (FA) > control. Results of the first order model showed ~ 37% increase in the specific growth rate of microorganisms (μ) in the MW- supplemented BMP compared to the control. The values obtained from the modified Gompertz model applied to the methane production rates showed no significant effect of co-digestion of OFMSW with MW on the length of the lag phase (λ) in BMP assays (average λ of 1.89 ± 0.07 days for IBA, CBW and BA amended assays compared to 1.5 ± 0.01 days for the control). Chemical analysis showed that the MW provided both alkalinity to increase BMP buffering capacity assays, and released several trace elements at concentrations within the optimal ranges for anaerobic bacteria.

5.1. Introduction

Mineral wastes (MW) have a reasonable acid neutralising capacity and they are enriched with minerals and nutrients with different concentrations (Section 4.2) either stimulatory or inhibitory to microorganisms; which carry out the AD processes (Section 3.3). The objective of the current chapter of this study was to evaluate the stability and productivity of co-digestion of organic and mineral wastes in batch BMP reactors to inform operators in optimising methane yields by integrating MW in their full-scale digesters. Experimental methane production data obtained from batch BMP assays fed with mineral and organic wastes were modelled with first order and modified Gompertz models then compared with 1) the theoretical values obtained from compositional analysis of the organic waste, 2) the

published values in the literature. Moreover, these results were then used to compare with data from CSTRs in later chapters.

5.2. Materials and methods

5.2.1. *Feedstock and seed inoculum*

The feedstock substrate used for the BMP assays was taken from the first batch of the SOW as described in Synthetic organic waste (Section 4.1.1). The SOW substrate was diluted (30% dilution) with distilled water to achieve a feedstock substrate with total solids (TS) and volatile solids (VS) values of 18.7% and 16.6%, respectively, and the characteristics of the feedstock substrate are shown in Table 4-2. The inoculum used for seeding the BMP assays was the mesophilic inoculum as described in (Section 4.1.2), and the characteristics of the inoculum are shown in Table 4-4. Prior to use, the inoculum was activated at 37^{o}C for 7 days (VDI, 2006a) then acclimated to the feed (SOW) by incubating for another 20 days (Section 4.1.2) at 37^{o}C ±1^{o}C with a single addition of one gram VS_{SOW} per litter of the inoculum. During the acclimation period, biogas produced from the acclimated inoculum was measured for methane content; on day 20, a methane content of > 50% was observed indicating that the inoculum was ready for the co-digestion experiments.

5.2.2. *Sources and preparation of MW samples*

The four MW (IBA, CBW, FA and BA) described in Section 4.1.3 were used for codigestion with the SOW substrate in the BMP assays. Preparation and characterisation of the MW are described in Section 4.1.3 and Section 4.2, respectively.

5.2.3. *Setup of BMP assays*

The co-digestion experiments were BMP assays performed (feedstock substrate was 1g VS of SOW + 1 g TS of MW) to evaluate the effect of MW addition on methane yield from the digestion of SOW. The BMP assays were prepared in triplicates as described in Section 4.1.3. The working volume of each BMP assay was 400 mL and the mass ratio of the VS in the feed (SOW) to that in the inoculum was 1:2. Gasbags of 0.6-litre (Tedlar, VWR) were used for biogas collection from the BMP assays.

5.2.4. *First-order and modified Gompertz models*

Methane production of the BMP assays was modelled by fitting the experimental methane production data with first-order (FO) and Modified Gompertz (GM) models as described in First-order and modified Gompertz models description (Section 4.3.1.1).

5.2.5. *Analytical methods*

General analytical procedures are as described in Section 4.2.5, Section 4.2.6 and Section 4.4.

5.3. Results and discussion

5.3.1. *Substrate and inoculum analyses*

The macro-parameters including total and volatile solids (TS and VS) showed that, the TS of the substrate (SOW) was 18.6% of wet weight, the VS accounted for 92% of TS (Table 4-2), similar to the typical food waste composition found in OFMSW (Dai *et al.*, 2013). The C/N ratio of the SOW (Table 4-2) was found to be 22.4 and therefore slightly below the optimal C/N ratio of 25 - 30 suggested for mesophilic AD (37^{o}C) (Wang *et al.*, 2014b). The molecular formula of the substrate (SOW) was estimated via C, H, N, O, S microanalysis (Table 4-2). The estimated molecular formula of the SOW was $C_{3.87}H_{6.76}O_{2.35}N_{0.16}S_{0.005}$, and the calculated theoretical methane yield ($Y_{m\ Theoretical}$; 4.2.6section 4.2.6) was 514 mL CH_4/g VS. This values was representative for this type of waste containing fractions of carbohydrates, proteins and lipids (Angelidaki and Sanders, 2004). The concentrations of Ni, Co, Mo, Se and Fe in the SOW (Table 4-2) were below the TE concentration required for the metabolism of bacteria /and archaea as per (Hinken *et al.*, 2008; Facchin *et al.*, 2013).

The NH_3-N concentration and the pH of the inoculum used were 2654 mg/L and 8.2, respectively (Table 4-4) associated with the presence of cattle slurry present in the inoculum (sourced from a digester, Cockle Park Farm, Newcastle University, UK). The inoculum showed lower levels of TS and VS (1.28 and 0.65% W/W respectively; Table 4-4) compared to the SOW (18.6 and 17.0% W/W respectively; Table 4-2). The inoculum contained a relatively high concentration of TE (Table 4-4). Therefore, the final mixture of the inoculum-substrate employed for the BMP provided with sufficient alkalinity (to neutralise pH) and macro and micronutrients for microbial growth. However, a previous study (Hinken *et al.*, 2008) suggested that the nutrients and TE concentrations necessary for anaerobic bacteria depends on the operating time of AD reactors, and the substrate composition and type.

The raw MW components were rich in TE and nutrients (Table 4-7); which could offset the nutrients deficient (Table 4-2) in the SOW. Table 5-1 shows the concentration of trace elements required by archaea (adapted from (Hinken *et al.*, 2008; Facchin *et al.*, 2013)) compared to the concentration of TE in the SOW and MW. The values shown in Table 5-1 indicate that the MW metals were always at higher concentrations than the TE requirements of archaea but the degree of exceedance differed for different MW. Clearly, the CBW, IBA and BA showed lower metal content compared to FA (the solid residues from air pollution control (APC)) process. Excess supplementation of TE and/or metals has been shown to limit methane production (Oleszkiewicz and Sharma, 1990). However, in anaerobic processes, it is the free form of these metals that are the most bioavailable (Banks and Lo, 2003). Moreover, the bioavailability of metals as nutrient or toxicants in the BMP assays will also be affected by other environmental conditions such as pH, redox potential, the kinetics of precipitation, and complexation (Oleszkiewicz and Sharma, 1990; Aquino and Stuckey, 2007).

Titration curves (Figure 4-1) of MW samples with 1N HNO_3 showed that the partial alkalinity (PA; Table 4-5) of mineral wastes was found to be in the order as BA>CBW>IBA>FA; with values of 2.56 ±0.09, 1.24 ±0.14, 1.12 ±0.03 and 0.8 ±0.12 meq $CaCO_3$/g MW, respectively. Therefore, it can be concluded that the MW additives (specifically IBA, CBW and BA) could provide digesters with moderate levels of alkalinity to maintain a favourable pH and support methanogen growth.

Table 5-1. Trace element requirement for archaea in comparison to measured trace elements in the substrate (SOW) and mineral (MW) wastes.

			TE in MW (mg/kg TS)			
Element	*TE in archaea (mg/kg TS)	TE in substrate (SOW) (mg/kg TS)	Incineration bottom ash (IBA)	Cement based waste (CBW)	Fly ash (FA)	Boiler ash (BA)
Ca	456	4958	71199	136465	222346	197962
Cu	1	4.36	2579	534	534	283
Ni	11	0.73	114	12.5	86	133
Co	9	0.03	40.4	5.2	10.8	21.2
Mo	7	0.48	6.7	1.4	14	20
Se	1.5	0.85	12.2	5.4	16.4	24.7
Mn	2	11.8	1177	349	389	1176
K	1140	7523	3161	1491	38024	21506
Fe	205	62.5	83655	19328	6858	12920
Mg	342	657	6716	10813	5758	13272
Zn	7	13.8	3290	51	11114	8699

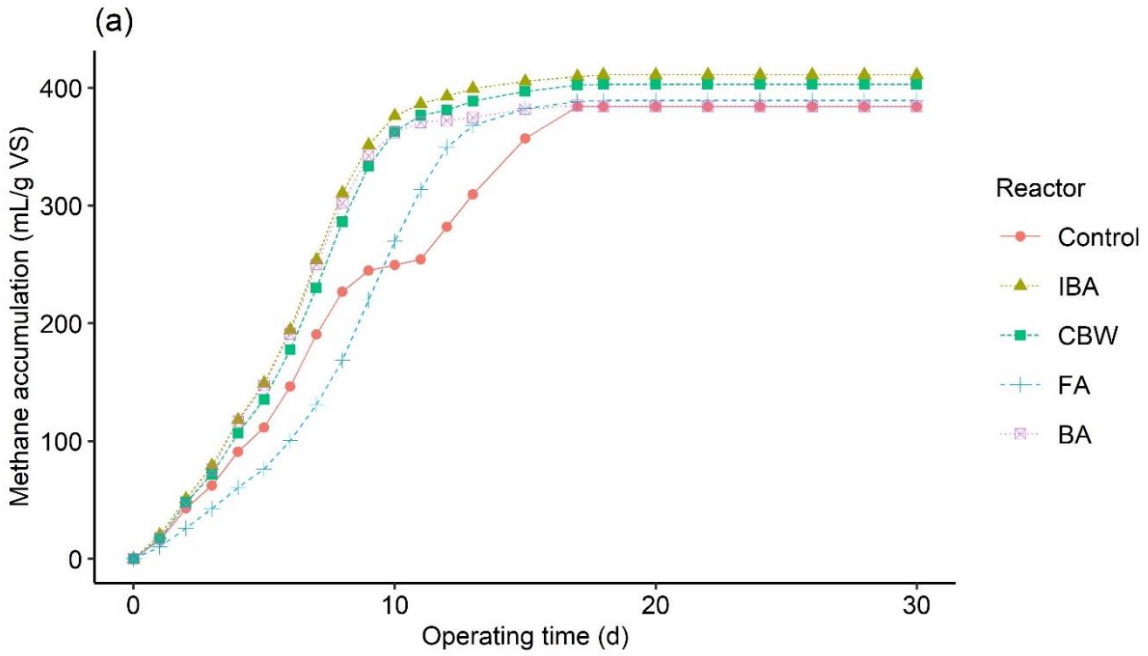
*(Hinken *et al.*, 2008; Facchin *et al.*, 2013).
TE = trace elements.

5.3.2. *Experimental methane production of BMP assays*

The main objective of the BMP assays was to determine a) the biomass activity and degradability of the substrate (SOW) in presence or absence of MW supplementation, and to b) ascertain the beneficial use of the MW as an alternative to nutrients that could offset potential deficiency from the SOW nullifying the likelihood for inhibition.

Figure 5-1 shows the cumulative methane yields (Y_m) for the SOW (OFMSW) from the BMP assays. By the end of the BMP tests (day 30), approximately similar Y_m values for the SOW (394.0 ±12.0 mL CH_4/g VS) were obtained from all the BMP experiments.

The Y_m obtained from the IBA, CBW, FA, BA and control BMP tests were 411.0 ± 5.2, 403.0 ± 5.6, 389.0 ± 4.8, 384.0 ± 3.3 and 384.0 ± 2.5 mL CH_4/g VS, respectively. These values represent 80, 78.5, 76, 75 and 75% of the theoretical methane yield (~ 514 mL CH_4/g VS) calculated from the elemental composition analysis method (Section 4.2.6) as described in (Angelidaki and Sanders, 2004; Nielfa *et al.*, 2015). The difference between the theoretical and experimental value is due the fact that the theoretical Y_m does not account for substrate biodegradability and the fraction of organic matter that can be used for synthesis of cellular material (Labatut *et al.*, 2011).



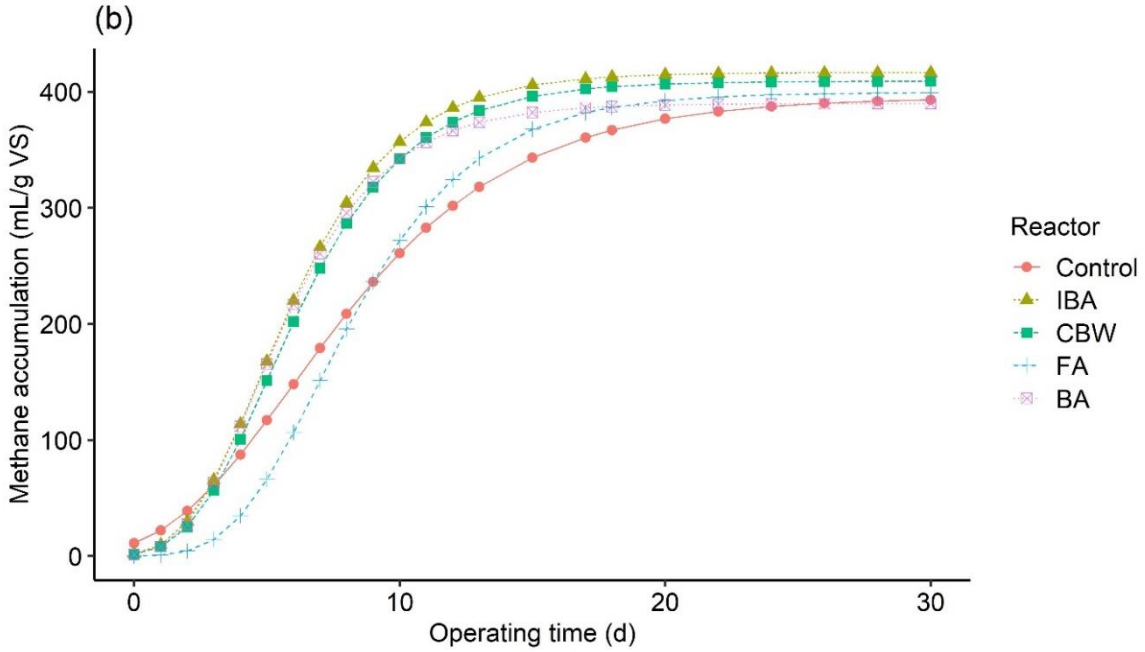


Figure 5-1. Cumulative methane production curves of control BMP assay (SOW only), mineral-amended (incineration bottom ash (IBA), cement-based waste (CBW), fly ash (FA) and boiler ash (BA)) BMP assays at mesophilic temperature 37°C (a) from experimental data and (b) from Gompertz model data The values are mean values of triplicate BMP assays with standard deviation (not shown).

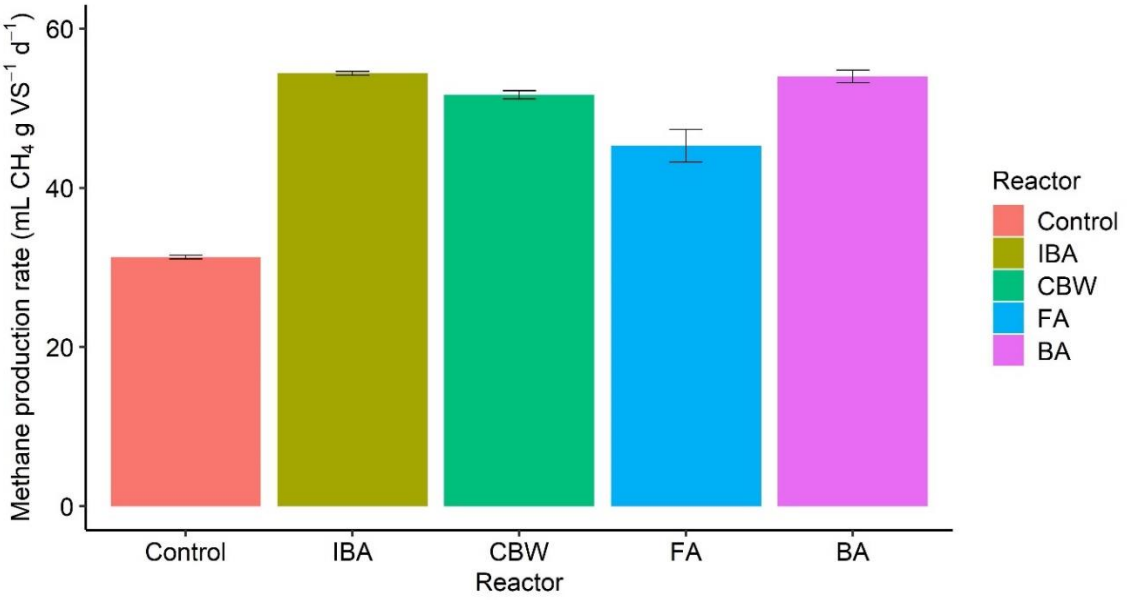


Figure 5-2. Bar-plot of maximum methane production rates (K) of the BMP assays calculated from Gompertz model. Control BMP assay fed with SOW only, and mineral-amended (Incineration bottom ash (IBA), cement-based waste (CBW), fly ash (FA) and boiler ash (BA)) BMP assays at mesophilic temperature 37°C. The values are mean values of triplicate BMP assays with error bars of standard deviation.

The maximum methane production rates (K) which were calculated from the Gompertz model applied to the data of BMP assays for each BMP condition (Figure 5-2) were different. The MW-amended BMP assays showed higher K values than the control. The IBA, CBW, FA and BA BMP conditions obtained the highest K values (45 - 54 mL CH_4/g VS. d), or 44 - 73% higher than the control (SOW only, ~ 31 mL CH_4/g VS. d).

These results show that MW can improve biogas production, and that the MW amended into the BMP assays contained beneficial levels of trace elements (Table 4-7 and Table 4-8) that were bioavailable. Microorganisms uses metalloenzymes (Formate dehydrogenase, Hydrogenase, Formyl-methanofuran dehydrogenase, Methyl-H4MPT: HS-CoM methyltransferase, Heterodisulfide reductase, and Methyl-CoM reductase) in the hydrogenotrophic methanogenesis pathway to reduce CO_2 to methane. The trace elements, known to be involved in these metalloenzymes, are Se, Mo, W, Fe, and Ni (Banks and Zhang, 2010c).

Hydrolysis of protein rich substrates increases ammonia, which may then inhibit methanogenesis (Kayhanian, 1999; Fujishima *et al.*, 2000). This issue is relevant to the BMP tests in the current study because the inoculum was rich in ammonia (Table 4-4). However, Selenium (Se) and Cobalt (Co) are known as the key TE elements essential for the biodegradation of organic matter under high ammonia concentrations (Banks and Zhang, 2010a; Banks *et al.*, 2012; Facchin *et al.*, 2013). These authors have shown that at organic loading rates below 3 g VS/L. d; the minimum concentrations of Se and Co of 0.16 and 0.22 mg/kg food waste (wet weight), respectively, are required to achieve the efficient VFA conversion under high ammonia concentrations. Although the relationship between ammonia inhibition and MW amendment could not be directly confirmed in this BMP based study, it was evident that the SOW methanogenesis as well as the metabolic capability of the methanogens (in the MSWI and CBW amended assays) were considerably higher compared to the control despite the potentially inhibitory levels of FAN (Table 5-4). This presumably results from the positive effects of the TE released by MW.

5.3.3. *Results of Gompertz and first order models*

The mathematical models (Section 4.3.1.1) which can be developed from mechanistic studies are considered an easy method to validate the results of empirical methods, and to enhance the design and optimisation of AD processes (Yu *et al.*, 2013). For this reason, at different time intervals (3, 7, 13 and 18 days), the cumulative methane production ($Y_{m\ Experimental}$) obtained from the BMP assays were modelled using the first-order (FO) and Gompertz (GM) models

(Section 4.3.1.1), the results are shown in (Table 5-2). It can be noted that among the four time intervals modelled, 'day 18' was the best day to fit both models (R^2 = 95 - 99%) with lowest D (percentage of difference between experimental values and modelled values) values of 10.2% ± 6.0 and 6.4% ± 3.4 for FO and GM, respectively. However, FA supplemented assays showed higher D values compared to the other MW amended assays on day 18 (D values were 19% and 11% for FO and GM, respectively; Table 5-2).

Table 5-3 shows that on day 18 the specific growth rate of microorganisms (μ) was ~ 0.22 d^{-1} in the IBA, CBW and BA amended BMP assays, about 57% higher than the control, while for the FA assay the μ value (0.15 d^{-1}) was approximately equal to that of the control (0.14 d^{-1}). Similarly, the value of the maximum methane production rates (K) of the MW-amended assays IBA, CBW and BA were ~ 43% higher than the control as shown in Table 5-3 and Figure 5-3. These results indicate the positive effect that MW have on methanogenesis, by increasing the specific growth rate of microorganisms (Table 5-3, Figure 5-3 and Figure 5-4). Future studies need to be conducted to determine whether the higher methanogenesis observed in the MW supplemented reactors was due to population growth only, or due to methanogenic activity (the capacity of methanogens to produce methane), or both.

Table 5-2. Parameters from modelling analysis using first order (FO) and Gompertz (GM) models.

Reactor & Time (d)		Y_m (mL/gVS added)		D (%)		R^2		Kinetic parameters		
		FO	GM	FO	GM	FO	GM	FO	GM	
								μ (d^{-1})	K (mL/g VS. d)	λ (d)
$Y_{m\ Experimental}$: Control		384	mL/gVS added							
	3	94	76.6	-76	-80	0.94	0.99	0.56	28.78	0.48
	7	998.0	428.3	160	12	0.95	0.99	0.08	34.2	1.56
	13	381.0	339.4	-1	-12	0.92	0.99	0.17	33.2	1.33
	18	439	418.6	14	9	0.95	0.94	0.14	36.8	1.5
$Y_{m\ Experimental}$: IBA		411	mL/gVS added							
	3	151.0	109.2	-63	-73	0.94	0.99	0.43	33.7	0.47
	7	1340.7	573.3	226	39	0.94	0.99	0.08	47	1.7
	13	497.7	453.6	21	10	0.95	0.99	0.18	45	1.74
	18	434.1	425.8	6	4	0.96	0.95	0.22	52.8	1.84
$Y_{m\ Experimental}$: CBW		403	mL/gVS added							
	3	114.8	90.9	-72	-77	0.93	0.99	0.52	32.95	0.53
	7	1184.4	513.5	194	27	0.94	0.99	0.08	42.4	1.68
	13	511.0	554.4	27	38	0.95	0.99	0.169	47.08	1.85
	18	429.3	420	7	4	0.96	0.94	0.21	49.91	1.98
$Y_{m\ Experimental}$: FA		389	mL/gVS added							
	3	97.8	64.4	-75	-83	0.94	0.99	0.36	18	0.53
	7	694.7	296.2	79	-24	0.94	0.99	0.08	24.1	1.69
	13	1346.5	713	246	83	0.92	0.99	0.058	41.6	3.73
	18	462.2	432.1	19	11	0.93	0.95	0.15	41.7	3.4
$Y_{m\ Experimental}$: BA		384	mL/gVS added							
	3	178.2	116.2	-54	-70	0.94	0.99	0.35	32.4	0.56
	7	1146.8	527	199	37	0.94	0.99	0.08	46	1.68
	13	453.1	420.6	18	10	0.94	0.99	0.2	49.7	1.74
	18	404.7	398.1	5	4	0.94	0.96	0.23	52.4	1.86

Table 5-3. Kinetic parameters of mean CH_4 production from BMP assays from modelling and biodegradability equations.

		Modified Gompertz model				First-order model		
BMP	Experimental methane yield	Maximum methane production rate	Lag time	Modelled methane yield	R^2	Specific microbial growth rate	R^2	Biodegradability
	$Y_{m\ Experimental}$	K	λ	Y_{GM}		μ		$Y_{m\ Experimental}$ / Y_{th}*
	(mL/g VS added)	(mL/g VS. d)	(d)	(mL/g VS added)		(d^{-1})		(%)
Control (SOW only)	384 ± 3.1	31.2	1.27	395	0.99	0.14	0.94	75
Incineration bottom ash (IBA)	411 ± 5.2	54.4	1.92	416	0.99	0.22	0.96	80
Cement based waste (CBW)	403 ± 5.6	51.7	2.1	409	0.99	0.21	0.96	78.5
Fly ash (FA)	389 ± 4.8	45.3	3.66	399	0.99	0.15	0.93	76
Boiler ash (BA)	384 ± 3.3	54.0	1.94	390	0.99	0.23	0.95	75

* Y_{th} = theoretical methane yield estimated from elemental composition analysis = 514 mL CH_4/g VS, as described in Section 4.2.6.

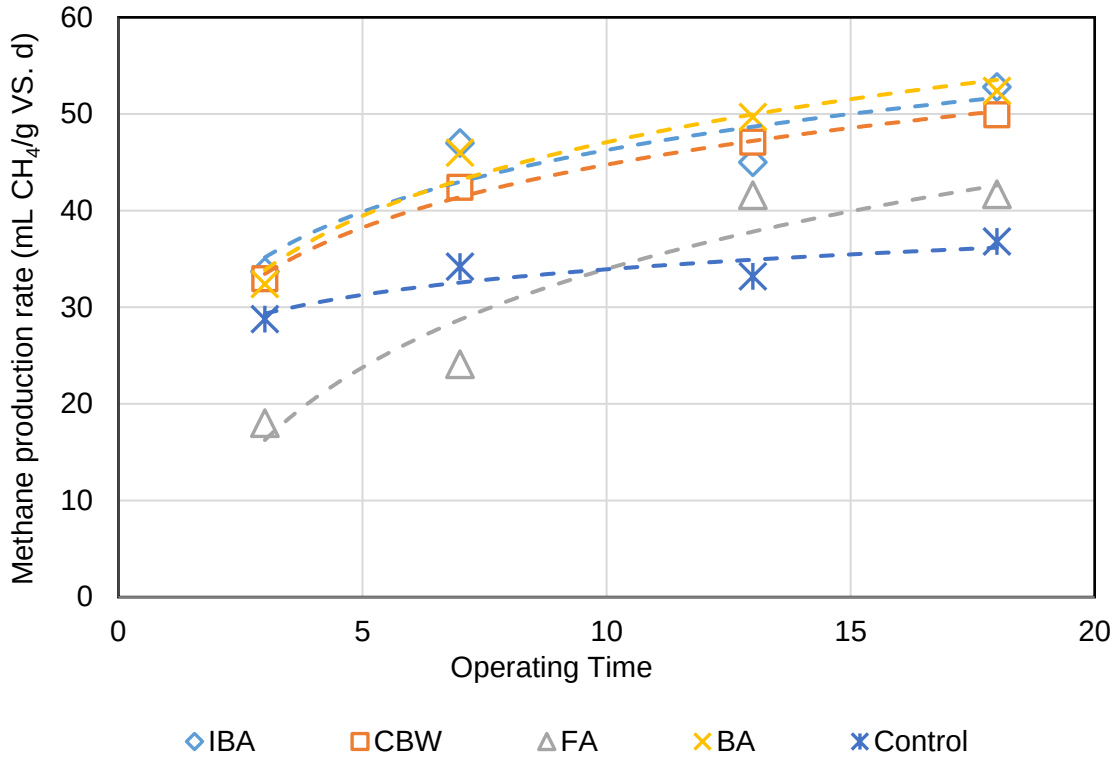


Figure 5-3. Variations in maximum methane production rates (K) calculated from Gompertz model in control and MW-amended BMP assays at 37°C.

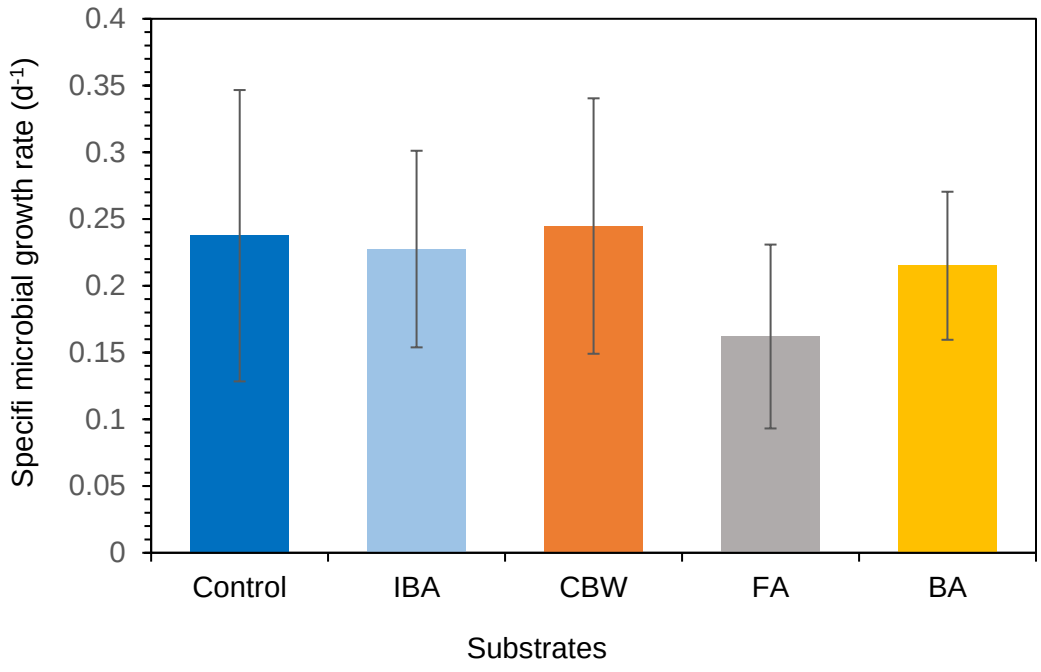


Figure 5-4. Variations in specific microbial growth rate (μ) calculated from 1st order model in control and MW-amended BMP assays at 37°C. Bars plots show mean value of (μ) calculated for each digestion condition in triplicates, and error bars are standard deviation of the mean values.

Table 5-4. Characteristics of the digestate from BMP assays co-digested synthetic OFMSW (SOW) with MW.

Parameter	Control	Incineration bottom ash	Cement-based waste	Fly ash	Boiler ash
pH	7.82 ± 0.01	8.05 ± 0.8	8.11 ± 0.12	8.02 ± 0.08	8.0 ± 0.1
Total soluble COD (mg/L)	4200 ± 250	3242 ± 51	3000 ± 250	3025 ± 225	2800 ± 50
Total Kjeldahl nitrogen (mg/L)	2217.6 ± 11	2226 ± 33	2206 ± 3	2243 ± 20	2215 ± 14
NH_3-N (mg/L)	1750 ± 23	1880 ± 37	1750 ± 28	1848 ± 22	1862 ± 18
Free NH_3 (mg/L)	187	339	362	311	344
NH_3-N/TKN (%)	79%	84%	79%	82%	84%
Total solids (g/L)	7.67 ± 0.3	9.2 ± 2.4	8.89 ± 1	10.67 ± 0.1	8.7 ± 1.3
Volatile solids (g/L)	2.67 ± 0.8	4.2 ± 0.7	3.17 ± 0.5	3.33 ± 1.3	4.3 ± 1
Volatile solids (%TS)	35%	48%	40%	33%	45%
FOS/TAC*	0.12	0.09	0.09	0.06	0.10
Total VFA (mg/L)**	1028 ± 89	751 ± 282	779 ± 249	530 ± 27	862 ± 223
Total alkalinity (mg/L)	8850 ± 135	8667 ± 125	8800 ± 100	8400 ± 118	8550 ± 156

*FOS/TAC = Total VFA / Total alkalinity.
** Calculated as acetate.
TKN = Total Kjeldahl nitrogen.

The lag time (λ) of the control reactor on day 18 was about 1.51 ± 0.1 day; while λ was 1.84 ± 0.2, 1.98 ± 0.3, 3.4 ± 0.3 and 1.86 ± 0.1 days for BMP assays amended with the IBA, CBW, and FA and BA, respectively (i.e. λ was ~ 22%, 31%, 125% and 19.2% longer, respectively, than the control reactor) (Table 5-3). Therefore, the lag times observed in the current study were only slightly increased for MW-amended reactors. Possibly, additional time was required by the microorganisms for acclimation in the altered environment of the MW reactors. After the lag phase, acclimation and bioavailability of beneficial trace elements took place, and the positive effects of the MW addition on the biogas production was evident in MW-amended reactors (Figure 5-1). In summary, it can be concluded that the preliminary lag phase that occurred in the MW-amended assays was indicative of a classic inhibition followed by adaptation; therefore, this inhibition would not be an issue in a continuous reactor system because microorganisms can get adaptation after a short period of reactor operation and this adaptation would be maintained in the CSTR. Moreover, in full scale digesters, the microorganisms adapted to MW can be more resistant to these toxicants, because the work of Lin (1993a) has shown that microorganisms in a seed sludge acclimated to heavy metals were more resistant to the toxicity of heavy metals than microorganisms in an un-acclimated seed sludge.

5.3.4. *Characteristics of BMP digestates: hydrolysis, acetogenesis and methanogenesis activity*

Table 5-4 shows the characteristics of digestate taken from BMP assays at the end of the experiment. It can be noted that total COD (tCOD) and total volatile fatty acids (tVFA) in the digestate of the control assay were 4200 ± 250 and 1028 ± 89 mg/L, respectively, expressed as acetate. These values were ~39 and ~ 41% higher than the mean total soluble COD and total VFA concentrations (3017 ± 189, 731 ± 142) of MW-amended assays. This suggests that although residual amounts of VFA substrates were available for methanogenesis in the control reactor, methanogens were unable to convert all the accumulated VFA to methane.

Table 5-5 shows that in the control BMP assay, the concentration of elements (required by archaea) was relatively lower than element concentrations in the MW amended assays. This could have contributed to lower methanogenic activity of the control.

Table 5-5. Concentration of elements in BMP assay digestates on the final day.

	Element concentration in digestates (mg/L)				
Element	Control	IBA	CBW	FA	BA
Ca	88 ± 0.2	187 ± 2.4	185 ± 2.4	524 ± 3	205 ± 3
Cu	0.08 ± 0.006	0.18 ± 0.001	0.081 ± 0.003	0.018 ± 0.006	0.016 ± 0.003
Ni	0.007 ± 0	0.04 ± 0.002	0.019 ± 0.002	0.048 ± 0	0.062 ± 0.001
Co	0.001 ± 0.001	0.025 ± 0.001	0.004 ± 0	0.003 ± 0	0.003 ± 0
Mo	0.002 ± 0	0.004 ± 0.003	0.004 ± 0.001	0.005 ± 0.003	0.011 ± 0.002
Se	ND	ND	ND	ND	ND
Mn	0.1 ± 0	0.9 ± 0.008	0.41 ± 0.003	0.154 ± 0	0.22 ± 0
K	467.0 ± 0.9	551.0 ± 0.4	489.0 ±1.0	1098 ± 10	766 ± 7
Fe	0.22 ± 0.001	8.7 ± 0.04	1.6 ± 0.006	0.641 ± 0.003	1.1 ± 0.007
Mg	22.7 ± 0.126	48.7 ± 0.14	32.2 ±0.15	81.284 ± 0.96	143 ± 3
Zn	0.3 ± 0.005	0.4 ± 0.01	0.12 ± 0.003	0.465 ± 0.022	0.26 ± 0.01

ND = not detected

Moreover, an imbalance in the syntrophic relationship between fermentation bacteria and methanogens can be hypothesised from the accumulation of fermentation products (VFA). Table 5-6 shows that the SOW hydrolysis and fermentation rates in the control assays were higher for the MW amended reactors (as estimated from the methane, expressed as COD equivalent, and the measured COD remaining in the digestate at the end of BMP tests). Whilst methanogenesis rates in the amended reactors were higher than the control. Furthermore, all the BMP assays maintained the mean alkalinity concentration and pH of 8653 ± 184 mg/L and 8.0 ± 0.1, respectively (Table 5-4) (i.e. there was no inhibition in the BMP assays due to

acidification). These results further support the previous observation (Section 5.3.2) that a larger microbial population (as can be noted from calculated μ values in Table 5-3) or higher methanogenic activity (as can be noted from calculated K values in Table 5-3) or both can be maintained presumably due to the TE released by the MW.

Table 5-6. Performance characteristics of anaerobic treatment of SOW in the control reactor and reactors supplemented with mineral wastes.

BMP condition	Methane yield	Total COD	Total VFA	Hydrolysis rate	Acetogenesis rate	Methanogenesis rate
	mL CH_4/g VS added	mg/L	(mg/L acetate)	mg COD/g VS. d	mg acetate/g VS. d	mL CH_4/g VS. d
Control	384	4200	1028	154	23	24
IBA	411	3242	751	137	17	40
CBD	403	3000	779	131	17	37
FA	389	3025	530	129	12	30
BA	384	2800	862	123	19	39

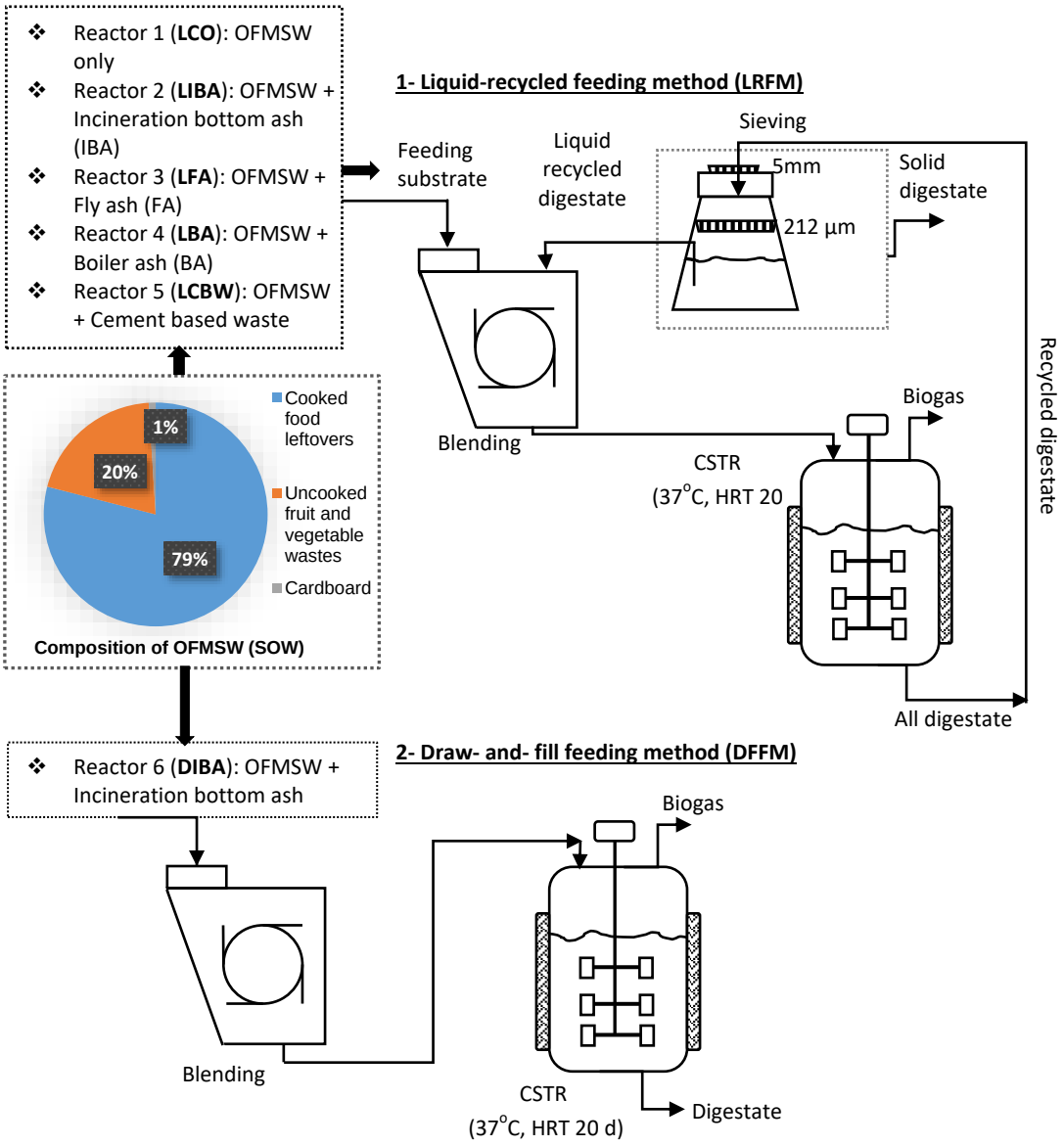
The soluble concentrations of measured elements (Table 5-5) in the digestate on the final day of BMP assays were found to be lower relative to the total concentrations of measured elements in the raw MW (Table 4-7) initially added. For instance, total Ni and Se concentrations in the IBA were 114.0 and 12.2 mg/kg TS respectively, while on final day of BMP assays, Ni concentration was 0.038 mg/L and Se concentration was below the limit of detection 0.01 mg/L. This suggests that only partial leaching of elements from MW occurred in the BMP assays and/ or soluble elements may have precipitated or adsorbed onto the anaerobic bacteria as has been observed by others (Facchin *et al.*, 2013).

5.4. Conclusions

Mineral waste (MW) originating from MSWI and CDW can be integrated into the digestion of OFMSW to promote biogas production and methane yield. MW can release soluble micronutrients (trace elements) essential for the growth and activity of methanogenic populations. The addition of MW enhanced methanogenesis of OFMSW despite potentially inhibitory levels of free ammonia. During the experiments, no leaching of heavy metals inhibitory to microorganisms was found from the MW materials originating from MSWI and CDW. In the presence of such amendments, the pH was also maintained at optimal levels (6.2 - 7.5) suitable for the anaerobic conversion of mixed organic waste streams to methane.

Chapter 6. Co-digestion of organic and mineral wastes for enhanced biogas production: Reactor performance and evolution of microbial community and function

Graphical abstract



Abstract

Mineral wastes (MW) from municipal solid waste incineration plants and construction demolition sites are rich in minerals, heavy metals and have acid neutralising capacity. This renders such MW a promising source of bulk and trace elements to enhance and stabilize biogas production in anaerobic processes. However, finding a MW with typical heavy metal concentrations, which promotes anaerobic digestion (AD) without adverse effects on the microbial community of the reactor is of major importance. To investigate the impact of several MW additives (1. incineration bottom ash; 2. fly ash; 3. boiler ash; 4. cement-based waste) as AD co-substrates, six 5 L single stage mesophilic, continuously stirred tank reactors (CSTR) were setup. Two different feeding regimes were employed including: a) a liquid-recycled feeding method (LRFM); b) a draw-and-fill feeding method (DFFM). Under the LRFM regime, 1 g MW/gram organic waste enhanced process stability (pH), increased methane production (25 - 45% increase), and yielded (450 – 520 mL CH_4/g VS); DFFM enhanced digestibility to a lesser degree. Illumina HiSeq 16S rRNA community sequencing of reactors showed that the microbial community compositions were unaffected by the presence of MW additives in comparison to unamended controls, but MW amendment accelerated bacterial growth (determined by qPCR). In contrast, different feeding regimes altered the microbial communities; *Methanoculleus* (hydrogenotrophic) and *Methanosaeta* (acetoclastic) were the most abundant methanogenic genera in the LRFM reactors, and the more metabolically versatile *Methanosarcina* genus dominated under DFFM.

6.1. Introduction

The potential reuse of mineral wastes such as incineration ash can effectively lower disposal costs of MSW and provide valuable materials to countries where natural resources are either expensive and/or unavailable (Liu *et al.*, 2015a). The analysis and BMP assays conducted in Chapter 5 of this thesis showed that the MW are rich in nutrients (TE), have acid neutralising capacity and have associated heavy metals. The presence of nutrients and pH buffering capacity are properties that render such MW a resource that may have a promising impact on AD in CSTR systems. However, in AD, the heavy metals present in such MW might be stimulatory, inhibitory or toxic depending upon their concentration (Hickey *et al.*, 1989; Lin, 1993b; Mudhoo and Kumar, 2013; Franke-Whittle *et al.*, 2014). Microorganisms can utilise metals at certain trace concentrations for activation and/or function of enzymes and co-enzymes (Zandvoort *et al.*, 2006; Abdel-Shafy and Mansour, 2014). However, metal concentration above certain thresholds may cause inhibition co-depending on other

physicochemical characteristics in AD digesters (VS/TS, humic substances, pH, VFA, alkalinity and ammonia (Dong *et al.*, 2013)). The key parameter controlling the potential toxicity of metals is the concentration of the solids (VS or TS) (Hickey *et al.*, 1989; Mudhoo and Kumar, 2013). Moreover, (Gu and Wong, 2004) identified impacts of VFA concentration (acetic and propionic acid) on metal solubilisation during bioleaching of sewage sludge. The authors of this study found that the presence of 10.8 mM acetic acid and 9.88 mM propionic acid delayed solubilisation of Cu and Cr by 6 and 7 days respectively compared to a one-day lag period in the control with low organic acid concentration.

The aim of the experiments conducted in this chapter was to investigate the impact of integrating the MW in AD of the OFMSW (i.e. the SOW, Table 4-2) using CSTR systems. The hypothesis was that the release of alkalinity, necessary macronutrients (Ca, Na, K and Mg) and trace metals (Fe, Zn, Mn, B, Co, Ni, Cu, Mo, Se, Al, W and V) would benefit the process and promote digestibility as assessed by biogas production and stability with additional insights into mechanistic effects of additives provided by microbial community abundances and dynamics.

In this chapter, the co-digestion of the MW (Section 4.1.3) and SOW substrate (Section 4.1.1) was conducted in CSTR systems (Section 4.3.2) using a liquid-recycled feeding method (LRFM) based on the hypothesis that recycling the liquor part of digestate could affect AD through retaining metals considered either stimulatory or inhibitory (toxic) to the digestion processes (Gu and Wong, 2004; Mudhoo and Kumar, 2013). In addition, for the purpose of comparison, one of the reactors was fed using the conventional draw-and-fill feeding method (DFFM). With this feeding regime, the substrate was IBA co-digested with the SOW substrate.

6.2. Materials and methods

6.2.1. *Inoculum, substrate and mineral wastes*

The preparation and characteristics of the inoculum, feedstock substrate (SOW) and MW are described in Section 4.1.2, Table 4-4, Section 4.1.1, Table 4-2 and Section 4.1.3, Table 4-7, respectively.

6.2.2. *Reactors*

Six anaerobic, lab-scale continuous stirred tank reactors (CSTR, Section 4.3.2) were setup. The working volume of each of the six reactors was 5 L. The reactors identified as LFA,

LCO, DIBA, LCBW, LBA and LIBA, respectively, reflecting their respective feeds (Table 6-1) operated at mesophilic temperature (37^{o}C) with HRT/SRT of 20 days (Viswanath *et al.*, 1992; El-Mashad and Zhang, 2010). Initially, each of the six reactors was thoroughly filled (5 L) with inoculum to get acclimated to the substrate and environment for 20 days (Section 4.3.2). The codigestion experiments (CSTR systems) were run for 75 days to ensure pseudo-steady state conditions had been achieved (> 3 HRT, (Dai *et al.*, 2013)) with two organic loading rates (OLRs): 0.5 g VS/L. d for days 0 - 40 and 1 g VS/L. d for days 41 - 75 successively with or without additions of MW to give $MW_{(TS)}/SOW_{(VS)}$ at a mass ratio of 1:1.

6.2.3. *Feeding*

The current study of the co-digestion of mineral and organic wastes was conducted under a reactor-feeding method named as 'liquid-recycled feeding method' or LRFM. This is based on the hypothesis that recycling the liquid fraction of the digestate could reduce losses of metals that are considered either stimulatory or inhibitory (toxic) to the digestion processes (Gu and Wong, 2004; Mudhoo and Kumar, 2013) by returning them to the reactor in each feeding cycle. For comparison, the two feeding methods, conventional draw-and-fill feeding method (DFFM) and the LRFM were used in the reactor fed with SOW feed and supplemented with IBA to determine the effect of feeding regime on MW supplementation in AD of organic waste (SOW).

Table 6-1. Experimental design of the CSTR systems

Reactor ID	Feeding method	Mineral waste added
LFA	LRFM*	Fly ash (FA)
LCO	LRFM	Control reactor (no MW added)
DIBA	DFFM**	Incineration bottom ash (IBA)
LCBW	LRFM	Cement-based waste (CBW)
LBA	LRFM	Boiler ash (BA)
LIBA	LRFM	Incineration bottom ash (IBA)

*LRFM = liquid-recycled feeding method.
**DFFM = draw-and-fill feeding method.

The liquid-recycled feeding method (LRFM) was applied for the LFA, LCO, LCBW, LBA and LIBA reactors ((Table 6-1), the first letter of the reactor name refers to the feeding method used whereas the following letters refers to individual MW additions). For this feeding method, instead of using distilled water for preparing the required volume of the daily feed (a 250 mL mixture), the liquid fraction of the discharged digestate from each reactor (sieved with a 212 µm sieve) was used (the solid fraction of the digestate was discarded). The

draw-and-fill feeding method (DFFM) was applied for the DIBA reactor whereby the volume was maintained at 5 L by withdrawing digestate and feeding equal volumes of feed prepared with DW instead of the liquid fraction of the digestate.

6.2.4. *Analytical methods*

Five-litre gasbags (Tedlar, VWR) were used for biogas collection from the reactors. Each day, the gasbags were disconnected, biogas volume measured, emptied with samples of biogas from each analysed for methane and carbon dioxide composition by gas chromatography then the gasbags were reconnected to the reactors (Section 4.4.7). Contamination of the reactor headspace with lab air was prevented during the feeding and sampling processes because the sampling port extended below the liquid surface level of the reactor.

Digestate pH was measured daily (Section 4.4.1). Samples of digestate were also taken on a weekly basis and centrifuged (3392 g, 30 min) and the supernatant was used for determining: chemical oxygen demand (COD, Section 4.4.5), ammonia-nitrogen (NH_3-N) and total Kjeldahl nitrogen (TKN) (Section 4.4.4), total volatile fatty acids (total VFA) and total alkalinity (total ALK) (Section 4.4.3). Total solids (TS) and volatile solids (VS) of reactor digestates were calculated as described in Section 4.4.2.

Metal analysis for the raw SOW and MW, and digestate samples on day 75 were performed as described in Metals analysis (Section 4.2).

Statistical analysis (Section 4.6) was conducted between various physiochemical parameters including the concentrations of metal elements in the digestate together with the reactor performances on day 75.

6.2.5. *Molecular analysis*

Microbial community analyses were performed for a sample of inoculum on day 0 (before the acclimation period) and in digestate samples collected on day 20 and day 75. Genomic DNA was extracted (Section 4.5.1), then real time quantitative PCR (qPCR) (Section 4.5.2) and 16S rRNA gene sequencing analyses (Section 4.5.3) were performed.

6.2.5.1. *Sequenced data processing and statistical analysis*

Raw sequencing data (FastQ files) obtained from the Illumina sequencing platform were de-multiplexed and quality filtered using dada2 (Callahan *et al.*, 2016) within the QIIME2 analysis pipeline (Caporaso *et al.*, 2010) as described in Section 4.5.3.1. Further analysis was conducted on these data to generate figures and check microbial diversity using the phyloseq

(McMurdie and Holmes, 2013a) and STAMP v2 (Parks *et al.*, 2014) software packages. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7.0 (Kumar *et al.*, 2016) (Section 4.5.3.1). The cell specific methanogenic and fermentation activities on day 75 was estimated from the daily methane production and total COD concentration, and the bacterial and archaeal gene copies (Section 4.7).

6.3. Results and discussion

6.3.1. *Performance characteristics of the AD reactors*

The experimental BMP value of the SOW was 480 ± 50 mL CH_4/g VS. This methane yield value was close to the calculated (theoretical) value (514 mL CH_4/g VS) estimated from the elemental composition analysis according to the method described in Section (4.2.6), and was within the typical range of BMP values reported for food wastes ((435 - 489 mL CH_4/g VS); (Zhu *et al.*, 2008; Banks *et al.*, 2011; Nielfa *et al.*, 2015)). This outcome indicates that the substrate used in this study was suitable for the digestion studies conducted.

Table 6-2 and Figure 6-1 show the performance profiles and physicochemical parameters of the six reactors operated in this study. On day 20, in the six reactors, the average methane yield was 499 ± 38 mL CH_4/g VS, with close to equal values of pH (6.9 ± 0.16) and NH_3-N (533 ± 49 mg/L), indicating similar and stable reactor conditions. According to (Koster and Lettinga, 1984; Ward *et al.*, 2008; Franke-Whittle *et al.*, 2014) AD occurs optimally at pH values of 6.8 - 7.2 and total ammonia nitrogen concentrations below 1700 mg/L. Presumably, the inoculum used in the CSTR set-ups contained enough alkalinity (to balance pH) and nutrients (Table 4-4) for the digestion processes to be stable until day 20, therefore between days 0 - 20 all the reactors showed approximately similar digestion conditions. Thereafter, and with continuous daily feeding and gradual dilution of the set-up inoculum and substrate inside the reactors, the parameters inside the reactors represented the conditions induced by the daily feeds which now diverged (SOW or SOW and MW).

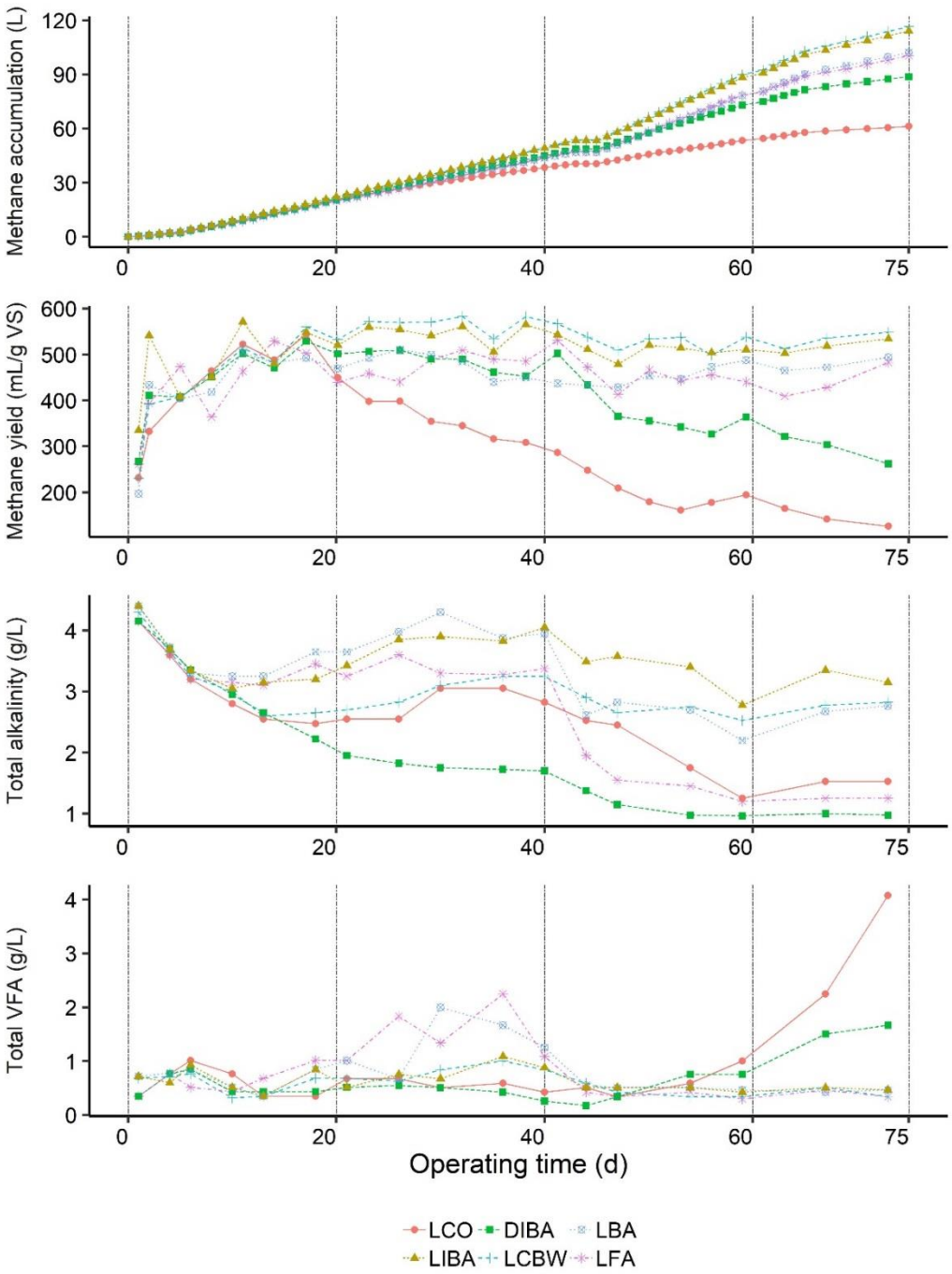


Figure 6-1. Profiles of methane accumulation, methane yield, total alkalinity and total volatile fatty acids during the single-stage co-digestion of synthetic organic waste and mineral wastes from MSWI plants and cement-based waste (IBA=incineration bottom ash, FA=fly ash, BA=boiler ash, CBW=cement-based waste) in comparison to mineral free control. L and D indicate the reactors feeding method liquid-recycled feeding method and draw-and-fill feeding method respectively. The values for total alkalinity and total VFA are mean values of triplicate samples with standard deviations (not shown).

Table 6-2. Summary of reactor parameters on day 20 and day 75*.

	Reactors	Methane Yield [1]	Hydrolysis activity [2] **	Methanogenesis activity [3]	Mixed liquor soluble COD [4] ***	pH	Mixed liquor NH_3-N [5]
Day 20	LCO	484	-	-	2831 ± 516	6.99 ± 0.1	538 ± 59
	LFA	476	-	-	4502 ± 1284	6.7 ± 0.1	521 ± 23
	DIBA	476	-	-	4014 ± 687	6.7 ± 0.1	447 ± 50
	LCBW	558	-	-	4194 ± 788	6.9 ± 0.1	539 ± 36
	LBA	464	-	-	4647 ± 534	6.9 ± 0.1	559 ± 36
	LIBA	536		-	5272 ± 758	7.1 ± 0.05	595 ± 38
Day 75	LCO	219	0.068	0.015	3619 ± 1020	5.8 ± 0.3	378 ± 14
	LFA	454	0.081	0.049	4250 ± 742	6.4 ± 0.03	410 ± 8
	DIBA	286	0.106	0.363	3630 ± 1270	5.7 ± 0.2	33 ± 27
	LCBW	536	0.033	0.016	2625 ± 625	6.9 ± 0.03	399 ± 11
	LBA	480	0.087	0.017	3940 ± 860	6.8 ± 0.02	402 ± 14
	LIBA	522	0.028	0.014	2850 ± 450	7.0 ± 0.01	477 ± 1

*All values are mean values for triplicate samples with standard deviation.
** Methanogenesis and hydrolysis activities were calculated for the whole operation time of the reactors i.e. 75 days.
***Errors show standard deviation of triplicate measurements from the same reactor.
Units are [1] (mL/gVS added), [2] (pgram COD/cell. d), [3](pmol CH_4/cell. d), [4] (mg/L) and [5] (mg/L).

In the LRFM, the contribution of the MW in the LBA, LCBW and LIBA reactors toward the alkalinity balance was detectable; these reactors showed 1000 - 1500 mg/L more alkalinity than that in the control reactor, however, in the DIBA reactor (fed with DFFM) the buffering capacity of the IBA was limited with a noticeable decrease in the alkalinity. A similar decrease in the alkalinity was observed in the control (LCO) reactor, specifically from day 40 and onwards when organic loading rates were increased from 0.5 g VS/L. d to 1.0 g VS/L. d. The alkalinity in the LCO and DIBA reactors declined from an average concentrations of 2500 mg/L on day 40 to about 1000 - 15000 mg/L by day 75 (Figure 6-1), resulting in a pH drop from 6.9 ± 0.1 on day 40 to ~ pH 5.8 on day 75. Among the reactors amended with the MW and fed with LRFM only the LFA reactor showed a lower alkalinity (~ 1250 mg/L) with a pH (6.4) on day 75.

Under the LRFM feeding regimen, co-digestion of the SOW and mineral wastes (IBA, FA, BA and CBW) resulted in higher methane yields and stable digestion process compared with the control (LCO) (Figure 6-1). The highest daily methane production was from LCBW,

LIBA, LBA and LFA reactors with 528, 513, 468 and 446 mL/L/d respectively. Daily methane production in the DIBA reactor (operated with DFFM) was 376 mL/L/d, which was about 30% lower than the mean daily methane production of the reactors operated with LRFM. Correspondingly, on day 75, the accumulated methane volume produced by the LFA, LCBW, LBA and LIBA reactors were 27, 45, 28 and 44% higher than the LCO (control) reactor, respectively. For the DFFM, the accumulated methane volume of the DIBA reactor (amended with IBA) was 24% higher than the control but about 25% lower than that of the LCBW and LIBA reactors.

6.3.2. *The influence of reactor amendments and feeding regimens on microbial abundances*

The overall performance of the reactors i.e. higher stable biogas production and stable pH and VFA levels with MW supplements compared to when MW is absent suggests that these materials primarily promote the growth and survival of the microbes present in the reactors increasing the volumetric rate of hydrolysis and fermentation. This growth leads to the increased formation of intermediate substrates for methanogens but equivalent increase in consumption of these products by the methanogens (Hude Moreshwar and Yadav Ganapati, 2014) (following a Monod and Michaelis-Menten approach respectively). To test this hypothesis, microbial abundances were determined in these reactor systems and cell specific activities calculated.

6.3.2.1. *Methanogenic populations*

The *mcr*A gene abundances representing methanogenic populations in the control and MW amended reactors showed an increase in numbers between days 0 to day 75. On day 75, the methanogen abundances in the control and MW amended reactors were 80 (4.44×10^9 genes/mL) and 90 - 118 - fold (4.94×10^9 - 6.50×10^9 genes/mL) higher than the inoculum (5.52×10^7 genes/mL) on day 0 respectively (Figure 6-2). In the LFA and DIBA reactors, the methanogen abundances were lower than in the control by day 75. Methanogenic populations in the DIBA reactor by day 75 (1.08×10^8 genes/mL) had only increased two-fold compared to the inoculum was the lowest population size observed among all the reactors (Figure 6-2).

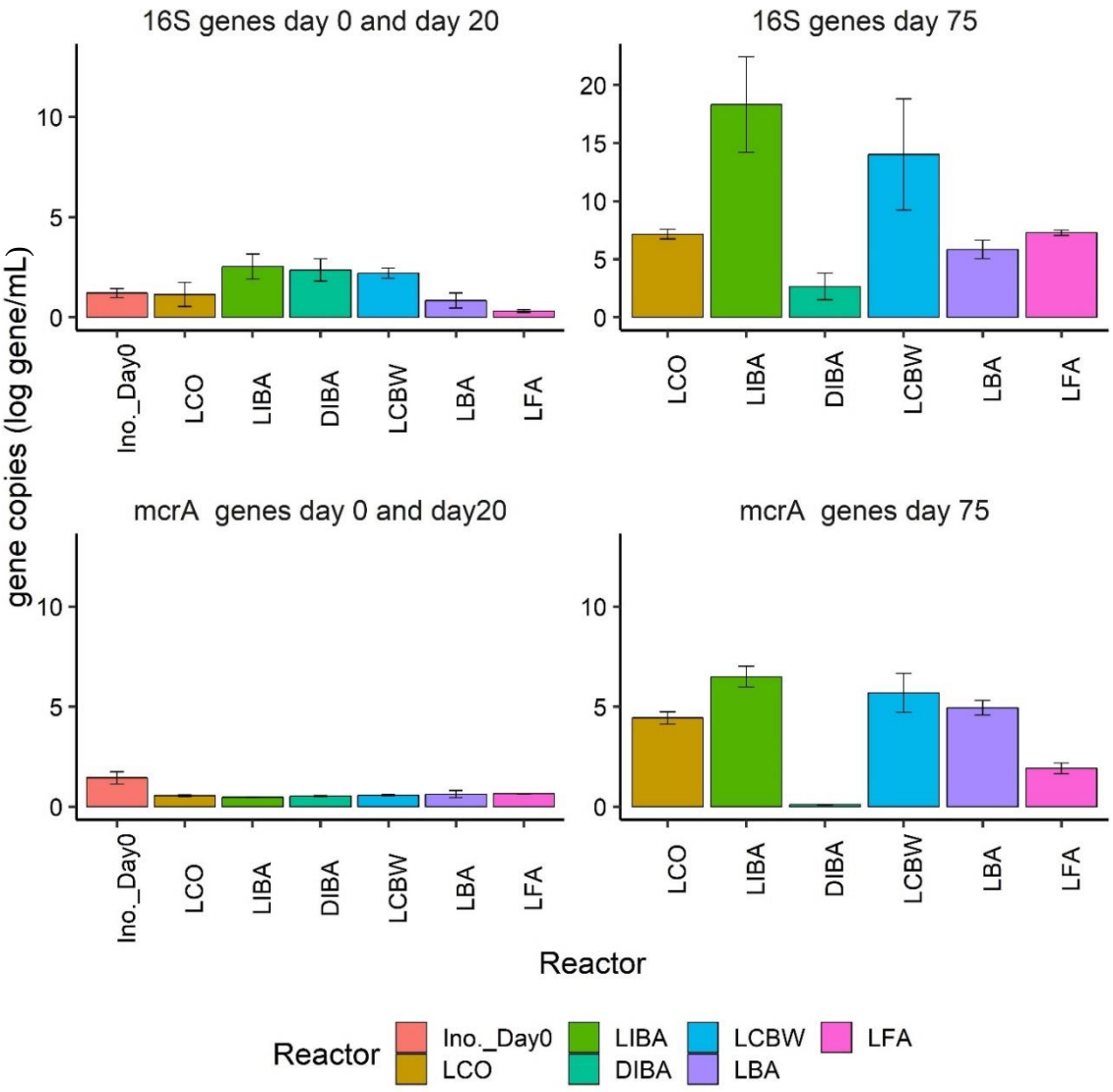


Figure 6-2. Microbial gene abundances for bacteria and methanogens in the inoculum on day 0 and digestates on day 20 and 75 calculated from qPCR analyses. Error bars represent standard deviations of microbial gene abundances calculated for triplicate samples from qPCR analyses.

6.3.2.2. *Bacterial populations*

On day 75, the 16S gene abundances representing bacterial populations in the LCBW and LIBA reactors were 11- and 14-fold (1.4×10^{12} and 1.8×10^{12} genes/mL) higher respectively than that in the inoculum (1.2×10^{11} genes/mL) on day 0. While in the LCO (7.2×10^{11} genes/mL), LBA (5.8×10^{11} genes/mL) and LFA (7.3×10^{11} genes/mL) reactors the 16S gene abundances were 4 - 5-fold higher than that in the inoculum on day 0. The lowest 16S gene abundance increase was in the DIBA reactor (2.7×10^{11} genes/mL) which was only one-fold higher that than in the inoculum on day 0 (Figure 6-2). There was no notable difference in the bacterial population between the LFA reactor (7.3×10^{11} genes/mL) and the control (LCO)

reactor on day 75; however, bacterial gene abundances in the LCBW and LIBA reactors were about 2.0 - 2.6-fold higher respectively than the control.

The cell specific hydrolysis and methanogenesis activities for the reactors were estimated (Section 4.7) from measured COD, biogas production and the relative abundances of the bacteria and methanogens from the qPCR analysis on day 75 (Table 6-2). In the LRFM reactors, the LFA reactor showed the highest cell specific methanogenic and hydrolytic activities at 0.049 pmol CH_4/cell. d and 0.081 pgram COD/cell. d, respectively. That is to say in this reactor, which actually sustained the lowest LRFM with MW biogas production, the growth/maintenance of individual cells apparently required higher rates of substrate turnover especially with respect to the methanogen population. The control reactor (LCO) had a moderately high cell specific hydrolysis activity of 0.069 pgram COD/cell. d and relatively similar methanogenic activity (0.015 pmol CH_4/cell. d) compared to reactors amended with MW amended reactors operated with LRFM (except for LFA). The LIBA reactor had cell specific hydrolytic and methanogenic activities of 0.028 pgram COD/cell. d and 0.024 pmol CH_4/cell. d, respectively. Whilst, the DIBA reactor (which had the same MW added as the LIBA reactor) showed the highest cell specific hydrolytic and methanogenic activities (0.106 pgram COD/cell. d and 0.363 pmol CH_4/cell. d, respectively).

In the AD, hydrolysis and the primary and secondary fermentation process are mainly linked to bacteria (Liebetrau *et al.*, 2017). However, in methanogenic syntrophic partnerships it is well understood that the activity of the fermentative bacteria can be limited by the inhibition (failure) of the methanogens, because such inhibition results in the accumulation of both sCOD and total VFA (as summation of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate) in the reactor which will eventually stop fermentation (Berlanga Herranz, 2008). It is also well understood that the success of such syntrophic partnerships is principally controlled by thermodynamic trade-offs between the partners and the efficient transfer of substrate intermediates between them whereby the energy yield for each participant in the partnership is maximal (Hamilton *et al.*, 2015). The accumulation of VFA as a control of bacterial hydrolysis and fermentation was certainly evident in the control reactor (LCO) on day 75 (~ 4 g/L) (Figure 6-1). Suggesting that the methanogen population was limiting VFA conversion to methane, while in the reactors amended with the MW (LFA, LCBW, LBA and LIBA) and operated with LRFM the VFA concentration on day 75 remained less than 0.5 g/L. Presumably, the MW in particular stimulated methanogenesis (as is obvious in LFA), which in turn stimulated bacterial growth (as it is obvious in LBA and LCBW) whereby the removal

of the fermentation products increased the energy yield for fermentation (Berlanga Herranz, 2008).

Optimising reactor performance by additions of MW may allow not only the reduction of the applied HRT, but also a capital and maintenance cost minimization as a direct consequence. Since biodegradation is an intrinsic property linked to biomass growth kinetics, an increase of the cell concentration within a reactor, combined with an increase in the activity of each cell gives process intensification, and the possibility of using smaller reactors (Akay *et al.*, 2005). Importantly, the readily available source of the MW supplements, combined with its minimal processing requirement, makes it a promising material for AD optimization in regions where commercial trace element additive solutions are either expensive and/or unavailable. Moreover, MW supplementation may have benefits in other sectors, for example where hydrolysis/fermentation is carried out at low temperatures and metabolic reaction rates are consequently reduced (Petropoulos *et al.*, 2017). Furthermore, the use of MW from MSWI plants in AD decrease the amount of MW need to be sent to landfills as a daily cover material (Banks and Lo, 2003). The use of MW as a daily cover material of landfill will enhance biological degradation of wastes and increase the landfill capacity to receive an increasing quantity of the daily wastes. Indeed, the landfills with a daily cover of mineral wastes could themselves be converted to an AD bioreactor for biogas production (the leachate produced from the landfill can be recycled again to the landfill like the LRFM applied in this study) giving an economic value to the MW.

6.3.3. *The contributions of metals from MW to AD digestates and correlation of physiochemical parameters with reactor performances*

Additions of trace elements like Ni, Co, Fe, Mn, Zn Mo etc. either singly or, in combination, to anaerobic reactors are known to be sometimes necessary for the activity of the enzymes improving methanogenesis (Oleszkiewicz and Sharma, 1990; Feng *et al.*, 2010; Pobeheim *et al.*, 2010; Takashima *et al.*, 2011; Zhang *et al.*, 2011a; Zhang *et al.*, 2012; Facchin *et al.*, 2013; Westerholm *et al.*, 2015a; Westerholm *et al.*, 2016; Wu *et al.*, 2016).

Accordingly, the positive effects of MW amendments on the microbial populations involved in the reactors described above may be due to the increased supply of such required nutrients in addition to the provision of alkalinity to keep pH values within optimum range. Certainly, the analysis of metals in the MW revealed that the concentration of metals (major elements, minor elements and trace elements, (Table 4-7) in the MW was many orders of magnitude higher than that of the SOW substrate (Table 4-2). This high concentration of metals was

reflected in the compositions of the digestate solids (Figure 6-3), whereby the control reactor (unamended with MW) had lower levels of most metals in comparison to the MW-amended reactors. Furthermore, this variation in the concentration of the minor elements and trace elements was clearly observed in the LRFM reactors, which showed increased concentrations of B, Ba, Cd, Co, Cr, Mn, Mo, Ni and Pb (Figure 6-3).

Pearson correlation analysis (Table 6-3) between various physiochemical parameters including the concentrations of metal elements in the digestate together with the reactor performances on day 75 were studied. For instance, in reactors that were fed by the LRFM regimen and amended with the MW (LFA, LBA, LCBW and LIBA) which showed higher and stable biogas production compared to the control, significant correlations (Pearson correlation = 0.945, $p < 0.05$) was found between methane yields and dissolved Mn concentration (Table 6-3). The data from the DIBA reactor fed using such a different feeding regimen were not included in this correlation analysis and the possible inhibitory impact of Mn in this reactor is discussed in the next section.

In addition, an apparent positive correlation (albeit not significant; $p > 0.05$) between the methane yield and single element concentrations like Ni, Mo, Zn, Mg, Co, B and Ba with Pearson correlations of 0.49, 0.33, 0.26, 0.315, 0.4, 0.33 and 0.24 were also detected (Table 6-3). On one hand, significant correlations (Pearson correlations of 0.923 and 0.964, $p < 0.05$) between Co concentrations and both VFA and NH_3-N concentrations were observed. Furthermore, significant correlations (Pearson correlation = 0.967, $p < 0.05$) between NH_3-N concentrations and VFA concentrations, and a positive correlations (Pearson correlation = 0.67 and 0.64, $p > 0.05$) between NH_3-N and alkalinity and NH_3-N and pH in the reactors operated on the LRFM were also detected (Table 6-3). These results refer to the dual positive effects of the MW on the microbial activity and alkalinity in the AD reactors. Specifically, some trace elements like Fe, Cu, Zn, Mn, Co, Ni etc. are previously reported to have important roles in the synthesis of coenzymes involved in the metabolic pathways of methanogenesis (Jiang *et al.*; Pobeheim *et al.*, 2010; Demirel and Scherer, 2011; Ünal *et al.*, 2012; Zhang *et al.*, 2015b; Westerholm *et al.*, 2016; Cai *et al.*, 2018).

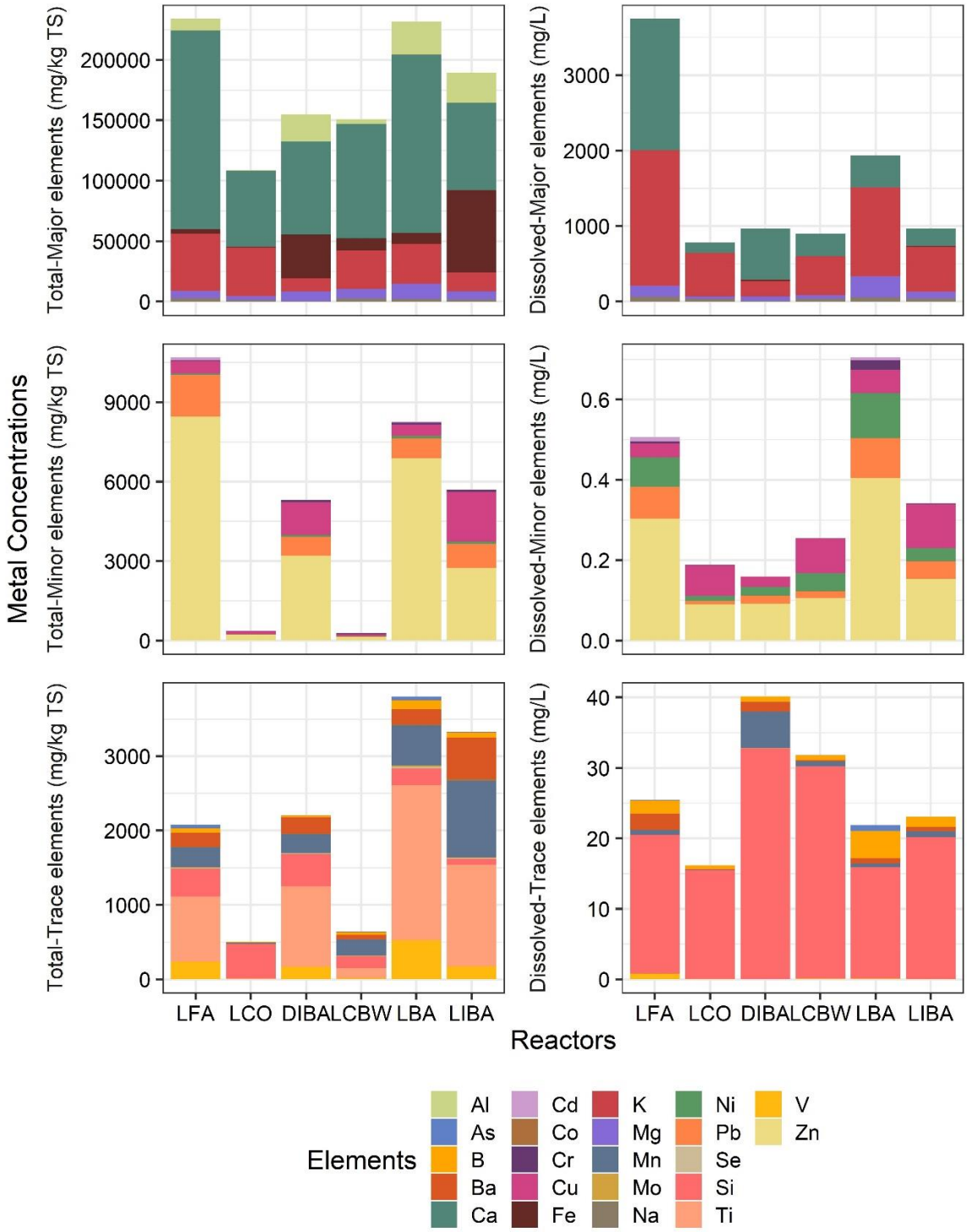


Figure 6-3. Total and dissolved metal concentrations in the reactor digestates on day 75. 'Total' is total metal concentrations, 'Dissolved' is dissolved (soluble) metal concentrations.

Table 6-3. Correlation analysis of physiochemical parameters in digestates on day 75.

Parameters	Methane yield	pH	Alkalinity	total VFA	NH_3-N
pH	.405				
Alkalinity	.670	.944*			
total VFA	.022	.447	.416		
NH_3-N	.187	.647	.632	.967**	
Al	.198	.324	.390	-.116	.042
As	.161	.214	.268	-.378	-.217
B	.331	.108	.263	-.238	-.103
Ba	.246	-.741	-.480	-.284	-.384
Ca	.148	-.844	-.623	-.459	-.578
Cd	.169	-.726	-.494	-.512	-.571
Co	.400	.603	.664	.923*	.964**
Cr	.122	.153	.219	-.302	-.165
Cu	-.079	.708	.517	.655	.695
Fe	.539	.644	.745	.850	.924*
K	-.008	-.833	-.645	-.541	-.637
Mg	.315	.033	.193	-.324	-.197
Mn	.945*	.399	.633	.193	.314
Mo	.330	.340	.422	-.343	-.149
Na	-.239	-.865	-.746	-.536	-.653
Ni	.488	-.013	.182	-.580	-.429
Pb	.418	-.178	.064	-.279	-.204
Si	.299	.363	.305	-.110	-.038
Ti	-.064	.127	.132	-.293	-.177
V	.009	-.910*	-.730	-.462	-.610
Zn	.261	-.206	-.021	-.430	-.350
Ni+Co+Mn	.969**	.403	.650	.160	.293
Ni+Co	.679	.221	.453	-.261	-.085

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
Positive and negative correlations with significant are highlighted in dark green and dark red respectively.
Lower and lowest positive and negative correlations are highlighted in lighter and lightest green and red respectively.

6.3.4. *Assessment of inhibitory and toxicity effects of mineral wastes during anaerobic digestion*

Biogas production is an obvious key indicator of the performance and stability of an AD process (Masebinu *et al.*, 2018). Accordingly, the high biogas yields from the SOW in reactors amended with MW, specifically the reactors fed with LRFM which were expected to contain higher concentrations of metals (Table 4-7); suggests that there was no obvious inhibitory/toxicity effects from the metals released by the MW on the microbial activities and

hence the biogas production in the reactors (Figure 6-1) or the relative growth of both bacterial and methanogenic populations in relation to the unamended controls (Figure 6-2). Dissolved concentration of Cd, Cr, Cu, Ni, Pb, and Zn in the digestates after 75 days of rector operations are shown (Figure 6-3). These concentrations were well below the inhibitory thresholds for AD processes (Jiang *et al.*; Hickey *et al.*, 1987; Hickey *et al.*, 1989; Oleszkiewicz and Sharma, 1990; Lin, 1992; Lin, 1993a; Lin, 1993b; Banks and Lo, 2003; Chen *et al.*, 2008; Banks and Zhang, 2010b; Banks *et al.*, 2011). The inhibition of AD is expected when the total weight (meq) of the heavy metals Zn, Ni, Pb, Cd and Cu per kg of dry solids in the digesting sludge is ≥ 400 meq/kg (Facchin *et al.*, 2013; Mudhoo and Kumar, 2013; Abdel-Shafy and Mansour, 2014). However, in all the reactors of current study lower magnitudes were detected (1.2, 0.6, 0.6, 0.8, 1.7 and 0.85 meq/kg for LFA, LCO, DIBA, LCBW, LBA, and LIBA respectively). Moreover, the results of this study were in line with other research (Lo *et al.*, 2009) which reported that heavy metals released from co-disposal of fly ash with MSW exerted no instability and toxicity effects on the digestion processes.

Focusing specifically on manganese, although Cai *et al.* (2018) observed a 48.9% increase in the methane yield from rice straw at a Mn concentration of 1.0 mg/L, they found that acetic acid was accumulated when excessive Mn concentrations were added. This study suggested Mn concentrations were at half-maximal inhibitory concentration (IC50) at 773.9 mg/L. It should be noted here that the dissolved concentration of Mn (5.2 mg/L) was found to be considerably higher in the poorer performing DIBA reactor compared to the LRFM reactors (0.66 ± 0.12 mg/L) and the control reactor (0.07 mg/L) which might suggest inhibition by this metal. On balance, a Mn induced reason for the poor performance of the DIBA reactor seems unlikely as levels observed were considerably closer to the stimulatory rather than inhibitory levels determined by (Cai *et al.*, 2018).

An alternative indicator of possible negative effects of the mineral wastes is an assessment of their impacts on microbial diversity. This assessment assumes that the toxicity of metals may reduce diversity and select for specific communities tolerant to the imposed conditions. Such effects have been observed in numerous studies of microbial communities (Huang *et al.*, 2003; Nettmann *et al.*, 2008; Nelson *et al.*, 2011; Ünal *et al.*, 2012; Xia *et al.*, 2012; Koch *et al.*, 2013; Wang *et al.*, 2014a; Westerholm *et al.*, 2015a). In a general sense, the dominant bacterial and archaeal communities in all the reactor communities, regardless of time or treatment were consistent with those that might be expected to proliferate in anaerobic digesters treating food waste for methane production. Evidence for this is provided in the phylogenetic trees shown in Figure 6-6 and Figure 6-7 which include close relatives randomly

selected from BLAST searches of the Genbank database and in particular their source environments which are dominated by conventional anaerobic digester studies without reports of toxic stress.

Furthermore, although the diversity of the microbial communities clearly decreased in the reactors as might be expected from toxicity (Figure 6-4 and Figure 6-5), it was actually operation time and feeding mechanism (i.e. LRFM or DFFM) rather the presence or absence of MW which were the factors that controlled the dynamics and compositions of the microbial communities. This operational driver for community change was also clear from a principal component analysis of sequence libraries (Figure 6-4 and Figure 6-5) where the community compositions (both bacterial and archaeal) in all the reactors was principally influenced by time of operation and not specific amendments. In the inoculum and at 20 days all the communities were dominated by taxa assignable to the candidatus genus *Cloacamonas* (*Cloacimonadaceae*); genus *Thermovirga* (*Synergistaceae*); family *Syntrophomonadaceae*; family *Rikenellaceae;* and (data not shown) family *Bacteroidetes vadinHA17* (see below for a discussion of specific taxa functions). However, by 75 days all the LRFM reactors including the control were dominated by taxa assignable to the *Cloacimonadaceae* W5 group; some *Synergistaceae* (not so closely related to the genus *Thermovirga*) and; the genus *Proteiniphilum (Dysgonomonadaceae*). Likewise, in all the LRFM reactors including the control, the archaea underwent substantial changes with a shift from the domination of the genera *Methanosphaera*, Candidatus *Methanoplasma* and *Methanobrevibacter* by 20 days to a general increase in the proportion of archaea and domination of the genera *Methanoculleus* and *Methanosaeta* at day 75. In contrast, by day 75 the DIBA reactor (which was also amended with IBA similar to LIBA but was fed with DFFM) was dominated by bacterial taxa assignable to the family *Dysgonomonadaceae* (but unrelated to the genus *Proteiniphilum*) and to a taxa related to the genus *Georgenia* (*Bogoriellaceae*). The archaeal taxa were dominated by the genus *Methanosarcina* with only a minor presence of *Methanosaeta* and only moderate increase in *Methanoculleus* (Figure 6-6 and Figure 6-7).

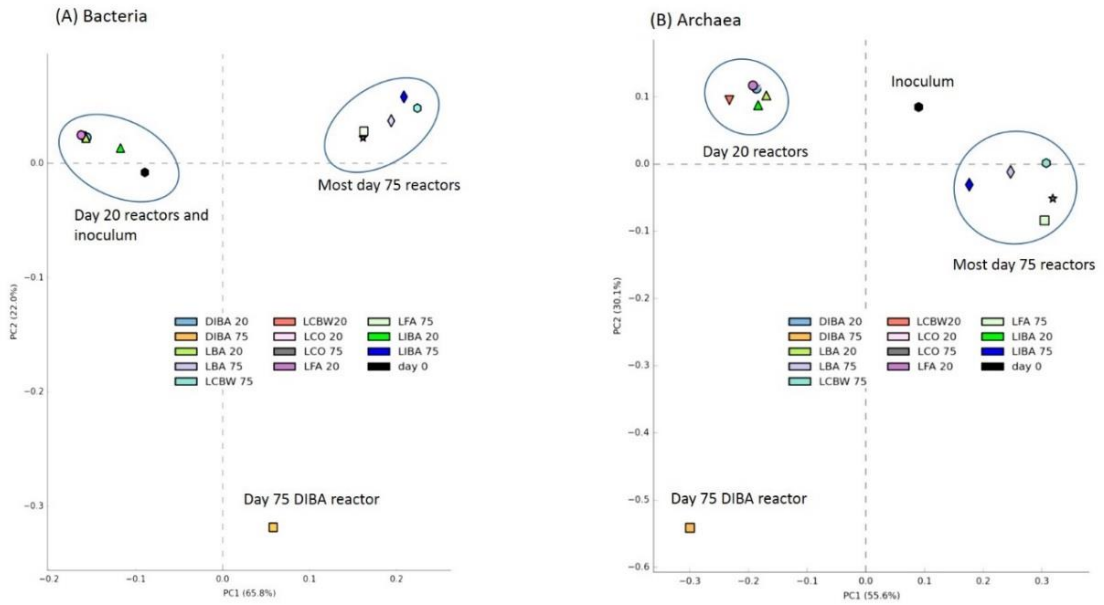


Figure 6-4. Principle component analysis of bacterial (A) and archaeal (B) communities of digestate samples collected from anaerobic reactors on days 0, 20 and 75.

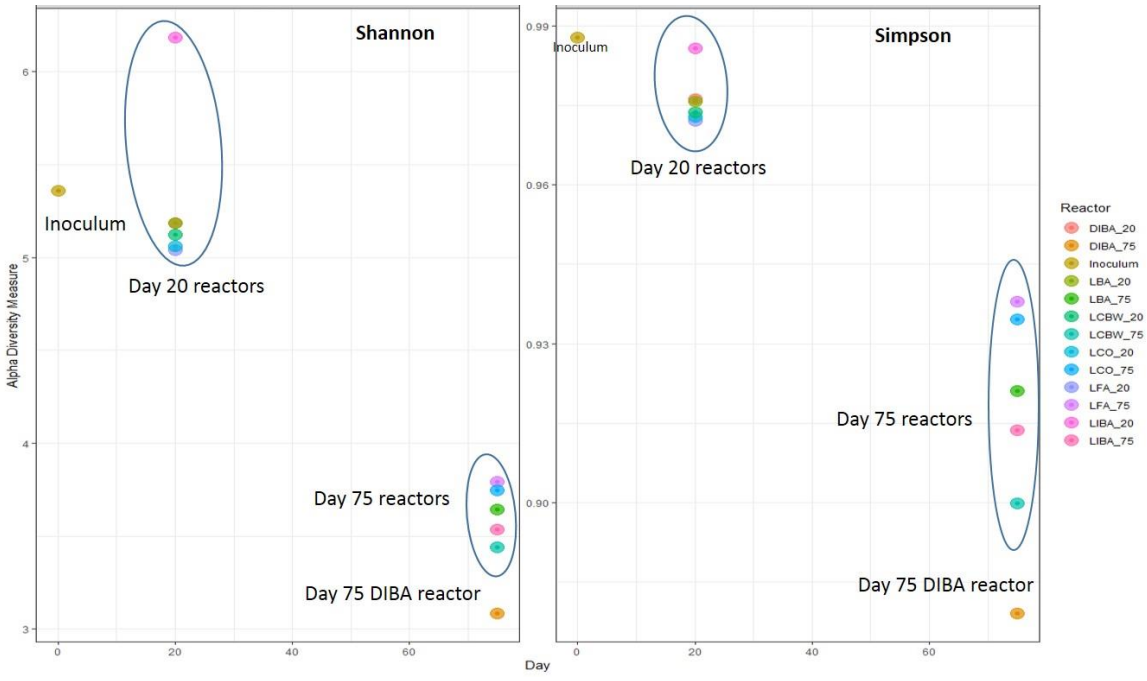


Figure 6-5. Alpha diversity metrics of microbial communities of digestate samples collected from different anaerobic reactors on days 0, 20 and 75

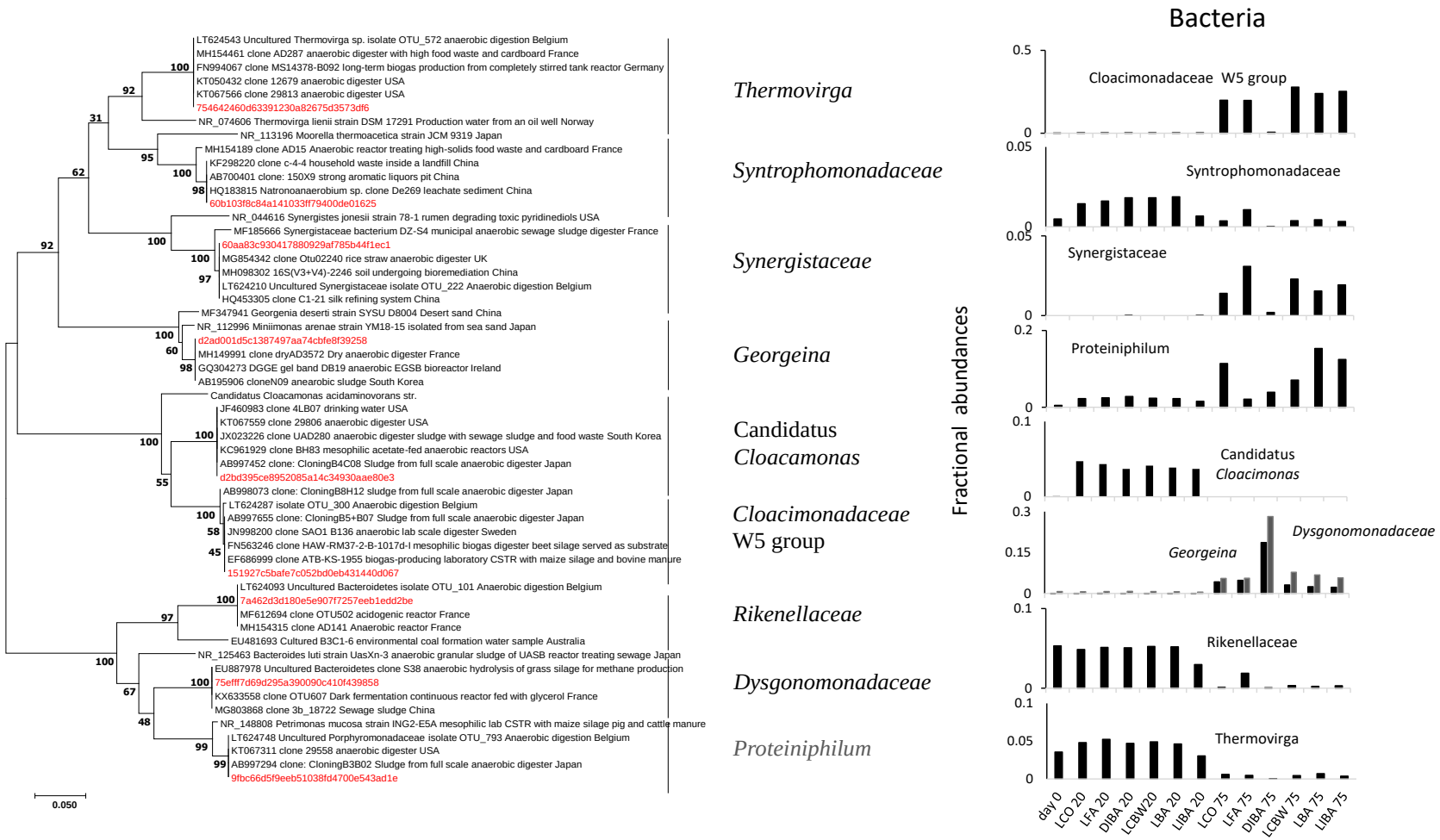


Figure 6-6. Phylogenetic distance tree (Neighbour-Joining) of key AD reactor bacterial taxa and close relatives (left) and plots of the fractional abundances of these taxa in individual reactor sequence libraries (right). The tree is based on comparative analysis of selected partial 16S rRNA sequences recovered from the anaerobic reactors at day 20 and 75 and indicated by individual codes assigned during pipeline analysis. The percentage of replicate trees in which the associated taxa clustered together in bootstrap analysis (1000 replicates) are shown next to the branches. The analysis involved 252 nucleotide positions.

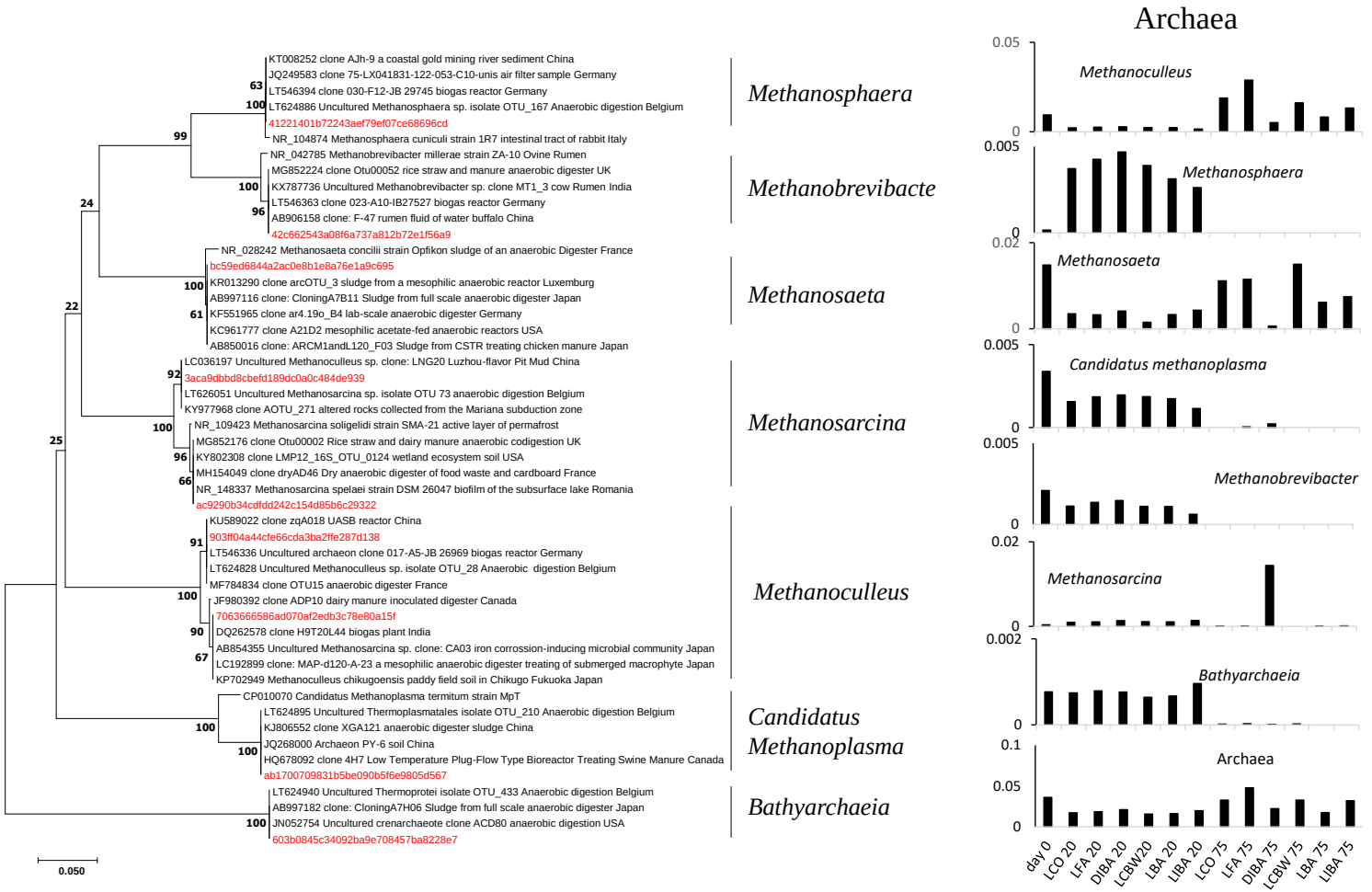


Figure 6-7. Phylogenetic distance tree (Neighbour-Joining) of key AD reactor archaeal taxa and close relatives (left) and, plots of the fractional abundances of these taxa in individual reactor sequence libraries (right). The tree is based on comparative analysis of selected partial 16S rRNA sequences recovered from the anaerobic reactors at day 20 and 75 and indicated by individual codes assigned during pipeline analysis. The percentage of replicate trees in which the associated taxa clustered together in bootstrap analysis (1000 replicates) are shown next to the branches. The analysis involved 252 nucleotide positions.

6.3.5. *Inferred functions, syntrophic relationships and community selection pressures under different operating conditions*

The recent study by Lee *et al.* (2018) has pointed out that taxa such as *Rikenellaceae, Proteiniphilum, Candidatus Cloacimonas, Cloacimonadaceae W5, Bacteroidetes vadinHA17* which were enriched in anaerobic digesters treating food wastewater or sewage sludge is 'known (or suspected) to be' anaerobic mesophilic acetogens. In the case of *Candidatus Cloacimonas* this genus has been implicated in syntrophic partnerships and hydrogen generation from the fermentation of carbohydrates and proteins (Pelletier *et al.*, 2008). Accordingly, the transient (20 days) or ultimate (75 days) enrichment of these groups in the LRFM reactors, coincident with the transient or ultimate enrichment of hydrogenotrophic (*Methanoculleus*) and acetoclastic (*Methanosaeta*) methanogens is entirely consistent with biogas production from the SOW. What is less clear is the reason for the succession between the 20 and 75-day communities with, for instance, the transient dominance of putative methanol reducing and hydrogen oxidising methanogens (*Methanosphaera*, Candidatus *Methanoplasma*) indicating that at 20 days of reactor operation methanol was a major intermediate product of mixed fermentation. Bio-methanol has been observed during the anaerobic co-digestion of animal and agriculture wastes (Anitha *et al.*, 2015). Furthermore, in this study methanol was an early stage product. It has been suggested (Chandra *et al.*, 2012) that products such as methanol are formed in the early phases of continuous or semi-continuous anaerobic digestion because the build-up of acidic products of hydrolysis.

By 75 day, the relative dominance of *Methanoculleus* methanogens over *Methanosaeta* suggested the dominance of hydrogenotrophic over acetoclastic methanogenesis indicating the likely occurrence of syntrophic acetate oxidation in the LRFM reactors. *Methanoculleus* spp. have certainly been found in mesophilic syntrophic acetate oxidising digesters (Schnürer *et al.*, 1999; Franke-Whittle *et al.*, 2014; Westerholm *et al.*, 2016), predominating over other hydrogenotrophic methanogens at extreme environmental conditions (i.e. high salt, ammonia and VFA concentrations). In contrast, however, the growth of *Methanosaeta* is known to be sensitive to changes of operational conditions such as VFA and NH_3-N concentrations (Demirel and Scherer, 2008; Franke-Whittle *et al.*, 2014). That being said a significant positive correlations was found (not shown) between NH_3-N concentrations and both *Methanosaeta* and *Methnoculleous* at day 75 and, furthermore, NH_3-N concentrations in the LRFM reactors were below likely inhibitory levels (Westerholm *et al.*, 2015a) especially after their substantial decline from the levels measured at 20 days. It can be concluded that the

sufficient concentration of NH_3-N in the LRFM reactors supported the growth of microorganisms (Kayhanian, 1999) rather than exerting inhibitory effects.

In this study and as discussed above, the control reactor (LCO) had an approximately similar microbial community composition and dynamics to that of the MW amended reactors operated on the same feeding mechanism (LRFM) (Figure 6-6 and Figure 6-7). However, methane production in the LCO reactor decreased gradually and total VFA concentration increased rapidly from 340 mg/L on day 46 to about 4073 mg/L on day 73 (Figure 6-1) this led to pH drop and a drastic decrease in methane yield. Based on the high degree of similarity in community composition, the low methane production efficiency of the LCO reactor was probably related to two main reasons. Firstly, a lower relative population growth of acetoclastic methanogens (especially *Methanosaeta*; Figure 6-2 and Figure 6-7) in this reactor compared to the MW amended reactors (specifically LCBW, LBA and LIBA). Secondly, low trace element concentrations in the LCO reactor affected methanogenic activity in this reactor especially when the OLR was increased to 1 g VS/L. d. The deficiency of the required trace elements in the SOW substrate caused an alteration in methanogenic pathways and a decline in digestion performance (Westerholm *et al.*, 2015a), since this decrease in methanogenesis was not observed in the other reactors that were operated on LRFM and amended with the MW (i.e. LFA, LCBW, LBA, and LIBA). In contrast, in the DIBA reactor a low NH_3-N concentration was existed, therefore presumably the growth of *Methanosarcina* (which is known for its high growth rates and dominance when high levels of VFA present (Franke-Whittle *et al.*, 2014)) was limited due to the lack of enough N nutrient needed for the population growth.

The dominance by day 75 of very different bacterial and archaeal taxa in the DIBA reactor fed by DFFM feeding regimen was likely dictated by the prevailing conditions within this reactor. With respect to the bacterial sequences enriched, a taxa closely related to the genus *Georgenia* (family *Bogoriellaceae*) is notable as isolates of this genus range from aerobic, microaerophilic to facultative anaerobic metabolisms (Ward and Bora, 2009) and this genus does not appear to be a commonly associated with anaerobic digestion. However, a close relative has identified as a dominant component of the granular sludge of a low temperature glucose fed anaerobic digester (O'Reilly *et al.*, 2010) in a reactor where the dominant methanogen was the putative hyrdrogenotroph *Methanocorpusculum*. However, in contrast, in the present study this substantial enrichment of the *Georgenia* taxon along with a taxa from the family *Dysgonomonadaceae* in the DIBA reactor was associated with enrichment of the methanogenic genus *Methanosarcina* which is also known to be metabolically more versatile

and robust with shorter doubling times and tolerance to environmental stress such as low pH (Calli *et al.*, 2005; Conklin *et al.*, 2006; Thauer *et al.*, 2008). Several previous studies have linked *Methanosarcinaceae*-related populations to high residual acetate concentrations often associated with poor COD removal (Hulshoff Pol *et al.*, 2004). However, high COD, VFA and low pH was actually a property of the LCO reactor which sustained a similar microbial community to all other reactors which included the presence of *Methanosaeta* typically considered less tolerant to such stresses. The most obvious distinguishing feature of the DIBA reactor in comparison to all the other reactors was the relatively low NH_3-N concentrations which is of interest because it is another selection factor for *Methanosarcina* , since previous studies have reported the predominance of *Methanosarcina* at high ammonia concentrations (Calli *et al.*, 2005; Tian *et al.*, 2018).

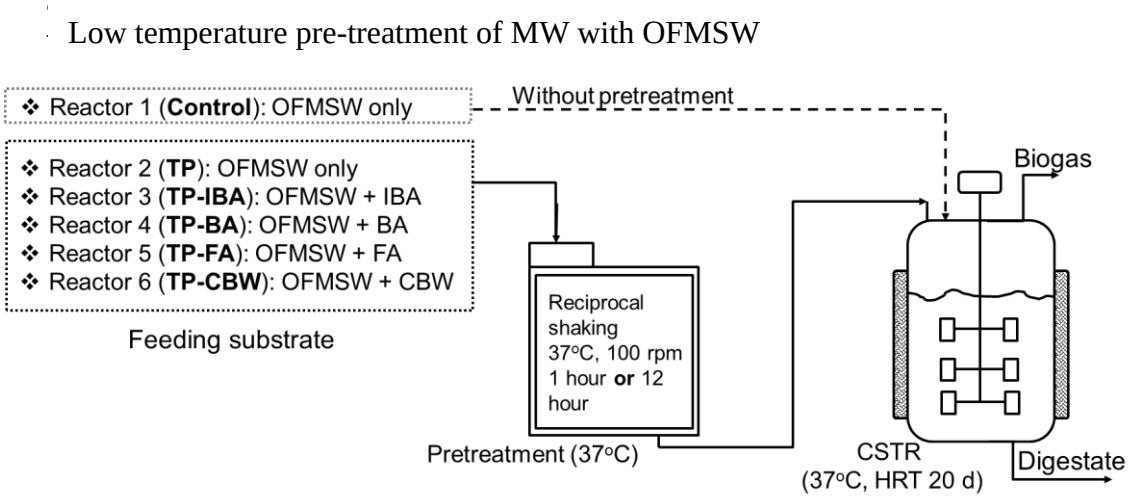
6.4. Conclusions

1- The MW from MSWI plants and CDW can be utilised as trace element supplements for optimising (high biogas production and stable digestion process) the AD of organic materials.
2- The metals released from the MW enhanced the buffering capacity and metabolic/catabolic activities in the AD reactors without inhibitory/toxicity effects.
3- The LRFM feeding method can be considered as a proper feeding method for anaerobic co-digestion of OFMSW with MW from MSWI plants and CDW.

Feeding methods and time were the key factors affecting microbial diversity in AD reactors supplemented with or without the MW.

Chapter 7. Low temperature pre-treatment of organic feedstocks with selected mineral wastes sustains anaerobic digestion stability through trace metal release rather than enhanced hydrolysis

Graphical abstract



Abstract

Anaerobic co-digestion of mineral (MW) and organic wastes can improve the performance of mesophilic (37^{o}C) anaerobic digestion (AD) of organic wastes via a liquid-recycling feeding method (LRFM). However, a limited improvement was achieved with a conventional draw-and-fill feeding method (DFFM) due to low retention / and concentration of trace elements / and heavy metals released from MW. In order to overcome the low concentrations of metals and alkalinity released from MW in reactors amended with these MW with a DFFM as described in chapter 6, the current chapter investigated pre-treatment of the organic waste with MW at mesophilic temperature ((37^{o}C); TP-MW) before their co-digestion at 37C. The use of low temperature (37^{o}C) for pre-treatment was to avoid excessive use of energy for the pre-treatment and avoid high release of heavy metals which might inhibit the digestion processes. Digestion experiments were carried out using biomethane potential (BMP) and continuous (CSTR) trials. BMP of substrates prepared with TP-MW (Organic waste$_{(VS)}$/MW$_{(TS)}$ mass ratio of 1:2.5 and 110 hour of pre-treatment) showed limited inhibition effects on biogas production rate and methane yield of organic waste compared to a control BMP fed with a substrate prepared by pre-treatment (TP) only (without MW additives). The biodegradation efficiency of BMPs fed with TP-MW substrates was close to ~ 90% of that obtained by thermochemical pre-treatment using 0.6% NaOH (TP-Alk). The continuous AD reactors (CSTR) fed with substrates prepared with TP-MW showed stable digestion process with higher methane production compared to TP and control reactors. Methanogenesis/fermentation activity (determined by qPCR) in CSTRs fed with TP-MW substrates was (3 - 4)-fold higher than that in the control and TP reactors. Illumina HiSeq 16S rRNA analysis showed that concentration of trace elements in the CSTRs fed with the TP-MW substrate was the key factor shaped the final diversity of microbial populations in these reactors. In the CSTRs fed with TP-MW substrate microbial community structure either shifted to a mixed community of acetoclastic (*Methanosaeta*) and hydrogenotrophic (*Methanobacterium*) methanogens, or totally shifted to hydrogenotrophic methanogens dominated by *Methanosarcina*, *Methanobacterium* and *Methanocuelleous*. While the acetoclastic methanogens (*Methanosaeta*) dominated the control and TP reactors. There was no evidence of enhanced hydrolysis of organic waste due to pre-treatment with the selected MW.

7.1. Introduction

Anaerobic digestion (AD) of organic wastes generates biogas with a high methane content, carbon dioxide, ammonia and trace gases (Hilkiah Igoni *et al.*, 2008). AD processes consist of three main stages: hydrolysis, fermentation and methanogenesis (Nguyen *et al.*, 2019b). In hydrolysis, long chain organic materials are converted to short chain monomers, for instance, proteins are hydrolysed to amino acids, and sugars and carbohydrates are hydrolysed to glucose (Gavala *et al.*, 2003). Acetogens use hydrolysis products to produce organic acids (VFA), CO_2 and H_2, and these intermediates are utilized by methanogens for biogas production (Mata-Alvarez *et al.*, 2000b). Hydrolysis is often the rate-limiting step for complex organic wastes (Fdez.-Güelfo *et al.*, 2011; Ariunbaatar *et al.*, 2014a), therefore pre-treatment are often considered as a mean to increase solubilisation of organic matter to simple monomers to improve the bioconversion (fermentation and methanogenesis) steps. Whilst methanogenesis is the rate-limiting step for easily degradable organic waste (Ariunbaatar *et al.*, 2014a), energy exchange relationships between fermentative and methanogenic communities (Hamilton *et al.*, 2015) through syntrophic relationships is considered a key factor for stable performance and productive AD processes (Schnurer and Nordberg, 2008; Amani *et al.*, 2011; Li *et al.*, 2012; Westerholm *et al.*, 2016). Sufficient growth and activity of specific microbial populations is required for successful and accelerated syntrophic interactions between the cells participating at the fermentation and the methanogenesis stages in AD (Zhang *et al.*, 2019).

Changes in AD temperature (psychrophilic, mesophilic and thermophilic) and organic composition of the feed substrate (total and volatile solids) can affect the biodegradation efficiency, microbial growth and diversity significantly (Yi *et al.*, 2014; Gaby *et al.*, 2017; Petropoulos *et al.*, 2017). Additionally, sufficient nutrients, such as trace elements within specific concentrations considered crucial for balanced metabolic pathways towards methane production (Demirel and Scherer, 2011; Takashima *et al.*, 2011; Facchin *et al.*, 2013; Cai *et al.*, 2017).

Positive effects of commercially available trace elements such as Se, Co, Ni, Mo, Zn, Cu, Mn, Mg etc. on AD have been studied widely in the literature (Demirel and Scherer, 2011; Zhang *et al.*, 2011a; Banks *et al.*, 2012; Facchin *et al.*, 2013; Zhang *et al.*, 2015b). Mineral wastes (MW) from municipal solid waste incineration (MSWI) plants and construction demolition waste (CDW) from recycling sites are also rich in trace and heavy metals, and have moderate alkaline content. This renders MW a potentially attractive TE resource for AD reactors, and a

possible alternative to commercial TE solutions (Chapter 5 and Chapter 6). The results obtained from experiments in Chapter 6 showed that direct amendments of MW from MSWI and CDW produced positive effects on AD of organic waste using a liquid-recycled-feeding method (LRFM), whereas limited effects were observed when conventional draw-and-fill feeding method (DFFM) was adopted.

The objective of the current study was to determine a method for increasing the concentration of trace elements released from MW in order to improve the performance and productivity of anaerobic reactors fed with organic wastes using the conventional DFFM. To the best of authors' knowledge so far, no previous research has been conducted using MW to pre-treat organic wastes for the enhancement of AD fed with OFMSW. The current study incorporated MW as additives during pre-treatment (TP) of organic wastes at 37^{o} (named as TP-MW) to produce a substrate for BMP assays and subsequent continuous experiments. The hypothesis was that the TP-MW would enhance the hydrolysis of organic waste, whilst simultaneously increase the concentration of TEs released from MW, and improve digestion efficiency and methane yields. As described in chapter 6, physicochemical characteristics in digesters such as the concentration of the solids (VS or TS), humic substances, ammonia pH, alkalinity and VFA are key parameters controlling the bioavailability and potential toxicity of metals is the AD systems (Hickey et al., 1989; Gu and Wong, 2004; Dong et al., 2013; Mudhoo and Kumar, 2013). Moreover, the ability to generate increased concentrations of TEs from TP-MW could help offset the potentially negative effects of hydrolysis i.e. more rapid accumulation of VFA , and compensate gradual decreases in the concentration of TEs seen in full-scale digesters due to regular substrate feeding and digestate discharge (Zhang *et al.*, 2019).

Three MW were used from a MSWI plant, namely, incineration bottom ash (TP-IBA), fly ash (TP-FA) and boiler ash (TP-BA), and a fourth MW, cement-based waste (TP-CBW), was obtained from CDW. Preliminary batch anaerobic assays (BMP) were conducted on a substrate comprising the simulated organic waste (SOW) and SOW incorporated into the TP-MW during preparation as indicated above. The potential hydrolysis and inhibition effects (sCOD concentration and CH_4 production) of BMP assays with TP-MW were evaluated by comparing outcomes with BMP assays fed with the same organic substrate (SOW) but pre-treated (at 37^{o}C) with alkali pre-treatment (TP-Alk) using a 0.6% NaOH solution. Moreover, in order to account for any possible loss in nutrients of the feedstock substrate during pre-treatment with or without MW, control BMP assays were run which fed with the same

feedstock substrate which was either frozen at -20°C then thawed (FrTh) before digestion, or pre-treated at 37°C without MW (TP).

Depending on the results obtained from the BMP assays, expected adequate pre-treatment time (12 hours and 1 hour) with TP-MW of organic waste (SOW) was applied to produce substrates for CSTRs fed with the DFFM method. Microbial analyses using qPCR and Illumina HiSeq analyses were conducted to determine the alterations in microbial population activity and composition in the CSTRs due to TP-MW method of the tested organic and MW.

7.2. Methodology

7.2.1. *Organic waste and inoculum*

The substrate used for this study was the SOW, its characteristics are shown Table 4-3. The final TS and VS concentrations of the SOW were within optimal ranges for AD (10 - 20%; (Forster-Carneiro *et al.*, 2008)) of 18.2% and 16.6%; respectively. The inoculum was a digestate from a full-scale mesophilic reactor digested cattle slurry and farm silage (Cockle Park Farm, Newcastle University, UK) (Table 4-4). The inoculum was reactivated for two weeks at 37°C before starting the BMP and CSTR experiments.

7.2.2. *Mineral wastes*

The four MW (IBA, CBW, FA and BA) were used for the experiments in this chapter. The preparation and characteristics of the Mineral wastes are described in (Section 4.1.3, Table 4 7), respectively.

7.2.3. *Experimental design of BMP assays*

7.2.3.1. *Substrate pre-treatment for BMP assays*

Pre-treatment assays focused on soluble COD (sCOD) and pH levels. Seven pre-treated samples (in triplicate) of SOW were prepared. Pre-treatment of the substrate (SOW) was carried out for 110 hours using the following methods 1) freezing SOW at -20°C then thawing (FrTh); 2) pre-treatment of SOW at 37°C (TP); 3) pretreatment of SOW with MW at 37°C (TP-MW). For (TP-MW) four MW were used individually as additives for pre-treatment of SOW; they named as TP-IBA, TP-CBW, TP-FA and TP-BA reflecting the name of the MW used; and 4) alkali pre-treatment (using 0.6% NaOH) of SOW at 37°C (TP-Alk). The pre-treatment assays (except FrTh) were conducted in 250 mL open-capped plastic containers with 100 mL working volume incubated at 37°C. The pre-treatment processes included

mixing the samples in reciprocating speed of 150 rpm. The control and TP assays comprised 1g by VS of the SOW made up 100 mL with distilled water. The $SOW_{(VS)}$ to $MW_{(TS)}$ mass ratio of each of the TP-MW assays was 1:2.5 made up 100 mL with distilled water. The TP-Alk of 1 g by VS of the SOW was conducted according to the method described by Lin *et al.* (2009). Sampling during the pre-treatment period took place at three time intervals (after 0 hour, 0.5 hour and 110 hour) where, the pH and sCOD of the seven TP assays were measured. The developments in alkalinity due to the MW and their effects on the hydrolysis of organic matter (the SOW) was evaluated by comparing the pH and sCOD obtained from the TP-MW assays with the pH and sCOD values obtained from the control, FrTh, TP, and TP-Alk assays. Whereas, effects of the pre-treatment method on the bioavailability and toxicity of metals released were assessed by digesting the whole pre-treated feedstock samples (i.e. the whole solid and liquid products) in BMP batch reactors.

7.2.3.2. *BMP assays*

BMP assays for the pre-treated and control feedstock samples (Section 7.2.3.1) were carried out in 500 mL glass bottles (Duran bottles, VWR) as described in Section 4.3.1. The working volume of BMP assays was 400 mL (200 mL inoculum, 100 mL pre-treated or control SOW sample (see Section 7.2.3.1) and 100 mL distilled water) and the headspace was 190 mL. The inoculum to substrate (SOW) mass ratio of each BMP assay was 2:1 on VS basis.

7.2.4. *Experimental design of anaerobic CSTR experiments*

7.2.4.1. *Substrate pre-treatment for CSTRs*

Due to limited resources of current study, only six CSTR systems were operated at the continuous experiments. The pre-treatment methods were the TP and TP-MW (TP-IBA, TP-CBW, TP-FA and TP-BA) methods, and the whole substrate of TP-MW (i.e. the whole solid and liquid products of the SOW and MW) was added to reactors. The SOW feedstock used (Table 4-3) for the control reactor and pre-treatment methods (TP and TP-MW) was stored in a cold room at 5^{o}C during CSTR experiments, on daily basis, required amount of this feedstock was separated for pre-treatment (to feed TP and TP-MW reactors) and feeding the control reactor. Then the rest of the substrate was returned to the cold room. Depending on the results obtained from the BMP assays, lower $SOW_{(VS)}/MW_{(TS)}$ mass ratio and shorter substrate pre-treatment time were chosen for the TP and TP-MW for the CSTRs operation. The $SOW_{(VS)}/MW_{(TS)}$ mass ratio decreased to 1:1 and the pre-treatment time decreased to 12 hours (overnight) for day 1- 60 (HRT-1, HRT-2 and HRT-3) then to one hour for day 61- 80 (HRT-

4). Pre-treatment of the substrates before digestion was carried out using an orbital incubator (Stuart S1500, UK) with a heater power of 250 W. The estimated energy required for pre-treatment (assuming an energy consumption of 0.25 KWh and a loading capacity of 3 kg of the substrate i.e. 1.5 kg of MW plus 1.5 kg of SOW) of the substrate for 1, 12 and 110 hours was 0.06, 0.72 and 6 MJ/kg of the organic waste (SOW), respectively.

7.2.4.2. *Reactors*

The continuous experiments were conducted in six 5 L working volume CSTR systems operated at mesophilic temperature (37^{o}C) (Section 4.3.2). Four of the reactors fed with a substrate pre-treated with the TP-MW method (i.e. the SOW was mixed with one of the MW then pre-treated at 37^{o}C), and the reactors were identified as TP-IBA, TP-CBW, TP-FA and TP-BA reactors reflecting the name of the MW (IBA, CBW, FA and BA; respectively) used in pre-treatment of the SOW at 37^{o}C. The other two reactors were a control reactor (named as Control) which was fed with SOW without pre-treatment and MW additives, and a second reactor which was named as TP fed with a substrate (SOW) pre-treated at 37^{o}C but without MW additives. The reactors were operated for 80 days with HRT of 20 days. Choice of the 20 days HRT was based on the results from the previous study (Chapter 6) which studied anaerobic co-digestion of the same substrate (SOW) and MW and found that this HRT is optimal. The organic loading rates (OLRs) were 1 g VS/L. d for day 1- 40 and 2 g VS/L. d for day 41- 80 successively. The feeding method was the draw-and–fill feeding method (DFFM). For this feeding method, working volume of each reactor was maintained at 5 L by withdrawing 250 mL/d of digestate and feeding with equal volume of feed. The biogas was collected in 5L (for day 1- 40) or 10 L (for day 21-80) gasbags (Tedlar, VWR).

7.2.5. *Analyses and analytical methods*

Methods for measuring physicochemical parameters, as well as metal and microbial analyses are described in Materials and Methods (Chapter 4). Biogas and pH analysis were conducted daily. Digestate sample were analysed for TS, VS, sCOD, total ALK, total VFA and individual VFA concentrations every 10 days. Moreover, after each HRT (20 days), digestate samples of the CSTR systems were collected for the soluble metal concentrations and microbial analyses.

7.2.6. *Statistical Analysis*

Statistical analysis was conducted as described in Section 4.6.

7.2.7. *Calculations of fermentation and methanogenesis activities*

In the current study, a novel parameter named as methanogenesis to fermentation ratio (M/F ratio) was defined to derive the processes stability or failure in continuous reactors (Eqs. 7.1, 7.2 and 7.3). Moreover, the cell specific fermentation (CSF) and cell specific methanogenesis (CSM) activities during each HRT for each reactor were calculated (Eqs. 7.4 and 7.5).

$$F\ (g\ COD) = total\ VFA_{in\ reactor}\ (g\ COD) + total\ VFA_{to\ CH4}\ (g\ COD) \quad \textbf{(7.1)}$$

$$M = total\ VFA_{to\ CH4}\ (g\ COD) = VMP\ (mL)\ /350\ (mL/\ g\ COD) \quad \textbf{(7.2)}$$

$$M/F\ ratio = M/F \quad \textbf{(7.3)}$$

$$CSF\ activity\ (pg\ COD\ Cell^{-1}\ d^{-1}) = (F/bacteria\ cell\ numbers) \times 10^{12} \quad \textbf{(7.4)}$$

$$CSM\ activity\ (pg\ COD\ Cell^{-1}\ d^{-1}) = (M/methanogen\ cell\ numbers) \times 10^{12} \quad \textbf{(7.5)}$$

Where F is the total fermentation by the reactor, total $VFA_{\text{in reactor}}$ are the total mass of VFA in the reactor, total $VFA_{\text{to CH4}}$ are the total mass of VFA converted to methane, M is the total methanogenesis, and VMP is the volume of methane produced.

7.3. Results and discussion

7.3.1. *Results of BMP assays*

7.3.1.1. *Influence of pre-treatment on pH and sCOD concentration*

The influence of pre-treatment (FrTh, TP, TP-MW and TP-Alk) on pH and sCOD concentration of SOW was determined and compared with the same values obtained from the control and TP (Figure 7-1). At startup (0 hour), TP-MW increased the pH of SOW to 10 - 11 compared to pH = 12 and pH = 6.5 in the TP-Alk and control assays respectively. Indicating that the MW could provide an alkaline condition and increase alkalinity associated with high concentration of some metals such as Ca as calcium oxide which can form $Ca(OH)_2$ in aqueous solution (Yin *et al.*, 2018). With the progress in pre-treatment time, the pH decreased in all pre-treatment assays, with high decrease in the control assays (pH <= 3), moderate decrease (pH 6.5 - 9) in the TP-MW assays and low decrease (pH ~12) in the TP-Alk assays. As can be seen from Figure 7-1 a, after 110 hours of pre-treatment, TP-BA showed the highest pH value of ~ 8.5 among the four MW.

Effects of TP-MW on sCOD concentration were different (Figure 7-1 b), after 110 hours of pre-treatment, among the TP-MW assays, the highest sCOD concentration was in the TP-

CBW and TP-FA assays (5 - 6 g COD/L) while sCOD concentration in the TP-IBA and TP-BA assays were close to the sCOD of the control and FrTh assays. An apparent effect of TP-MW on the sCOD concentration was observed. Specifically, the sCOD concentration in the TP-CBW and TP-FA assays were about 80 - 90% of the sCOD concentration in the TP-Alk assay. This is considered a promising result for a readily available substrate as MW for the pre-treatment of organic wastes applications at a pre-treatment temperature of 37ºC.

The use of MW for solid waste pre-treatment can ensure environmental sustainability through promoted bioconversion and reduce the investment for pre-treatment giving value to the MW (which tended to be disposed to landfills). The reason behind the lower sCOD concentration at the TP-IBA and TP-BA batch BMP assays compared to sCOD concentration in the TP-CBW and TP-FA batch BMP assays was not clear; possibly, the abiotic / biotic reactions in the TP-IBA and TP-BA assays resulted in some losses of biodegradable material/nutrients in these assays. Moreover, it was found that dissolution kinetics of ashes differs from that of alkali/alkaline chemicals (e.g. $Ca(OH)_2$) (Yin *et al.*, 2018). In the current study, the pre-treatment effect of the liquid NaOH solution was very instant (0.5 hours) whereas solid phase MW gave a slower change in sCOD and could not sustain the higher pH during this hydrolysis. The slow alkaline effect of the MW on the hydrolysis can be considered useful for conditions where the high hydrolysis rate (such as OFMSW) might be a rate limiting which cause rapid accumulation of VFA and acidification followed by the inhibition of methanogenesis (Guerrero *et al.*, 1999; Munk *et al.*, 2010; Lerm *et al.*, 2012).

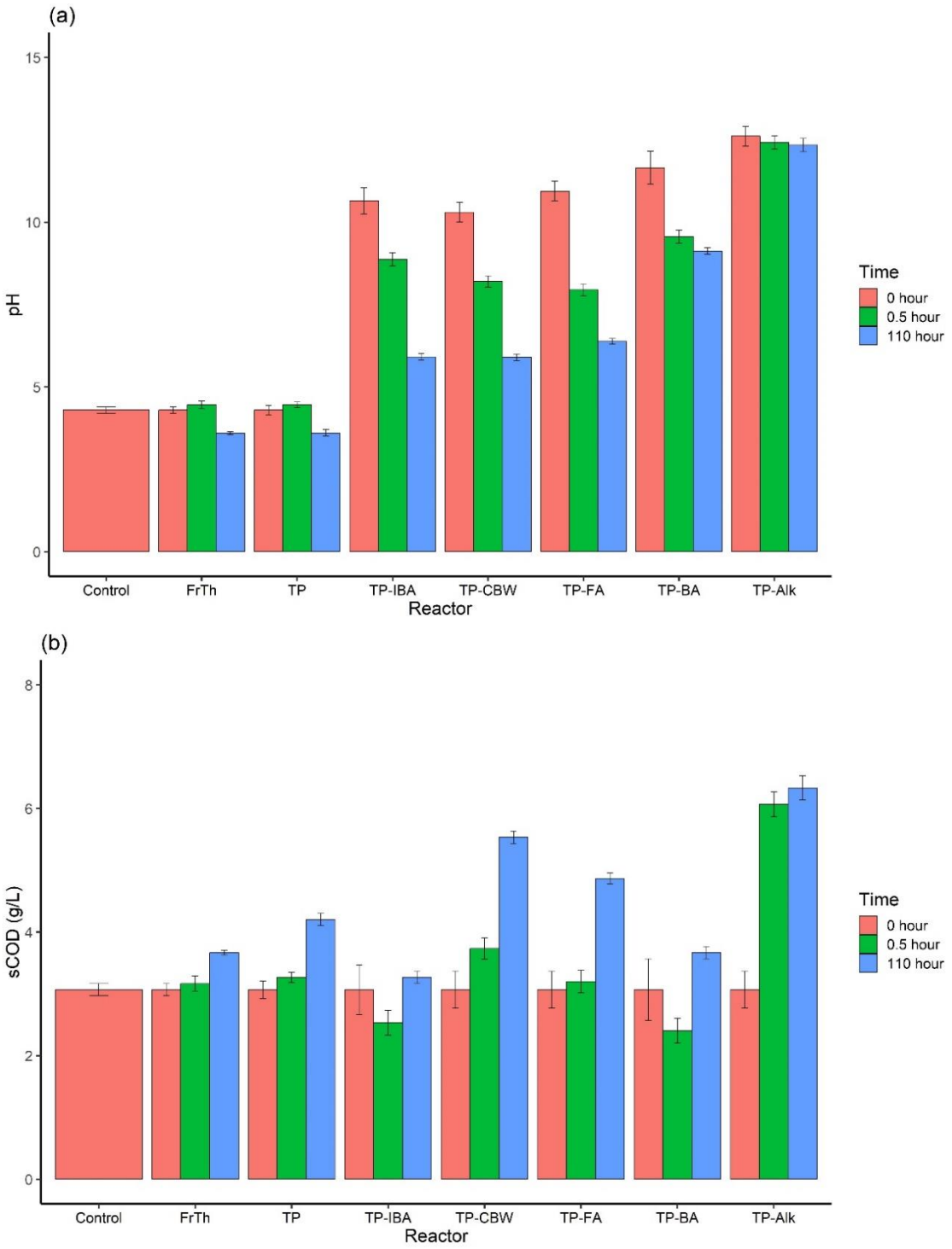


Figure 7-1. Variations in pH and $_{S}$COD concentration in pre-treatment assays of organic waste. TP-Alk = pre-treatment with 0.6 % NaOH solution. Control = raw organic waste prior to start the experiments (i.e. without pre-treatment and mineral waste addition). TP = organic waste pre-treated at 37^{o}C but without MW addition. TP-IBA, TP-CBW, TP-FA and TP-BA refer to the MW used in the pre-treatment (TP-MW) assays. The values are mean values of triplicate measurements with standard error.

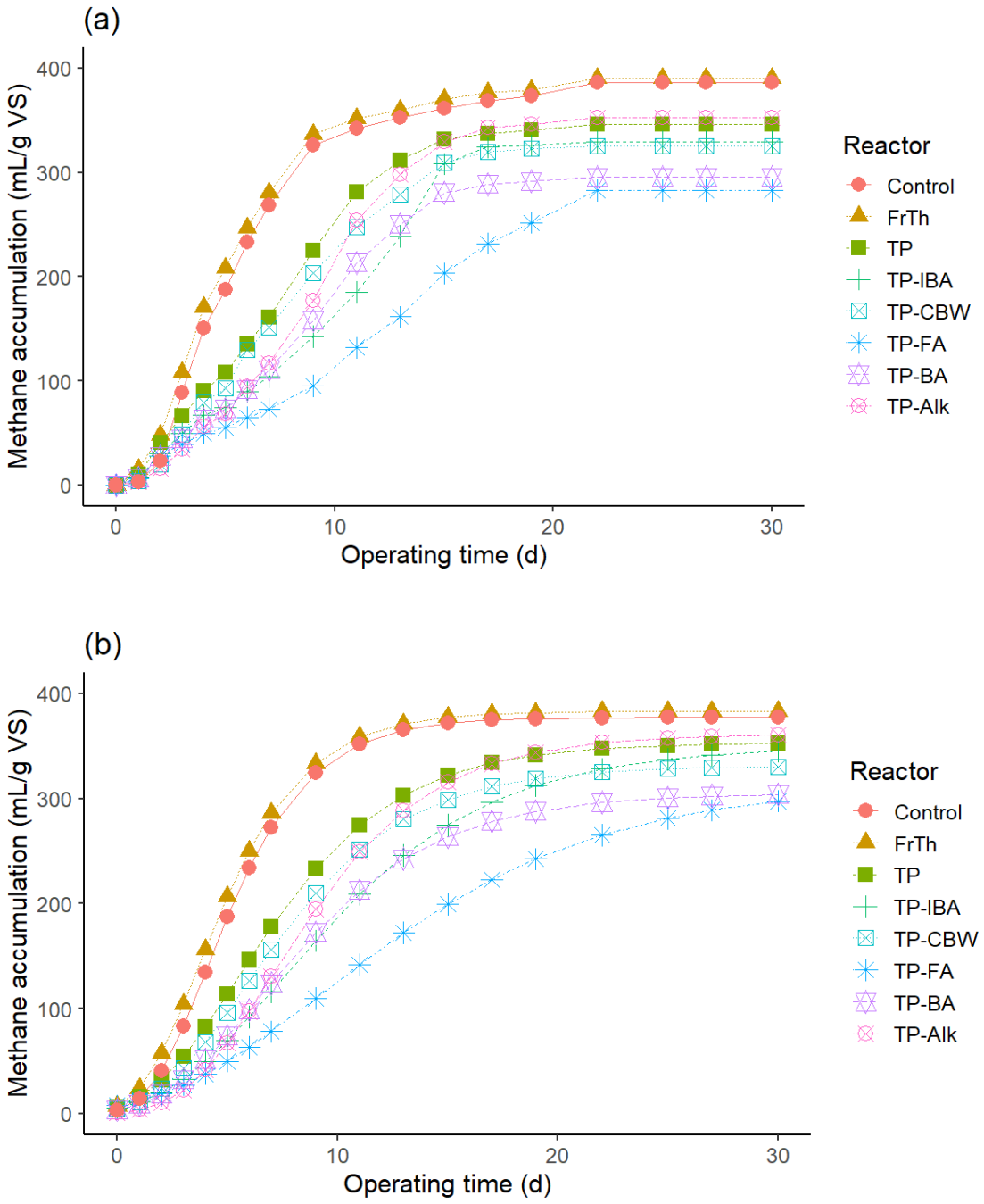
7.3.1.2. *Influence of pre-treatment on methane production rate and yield*

The BMP assays (BMPs) were conducted to assess the influence of TP-MW on biodegradation of the SOW and to investigate for any possibility of methanogenic inhibition. Previous studies (Banks and Lo, 2003) and Chapter 5 in this thesis demonstrated the possibility of extraction of water-soluble trace and heavy metals from MW within 96 - 110 hours at 20 - 37^{o}C. Therefore to investigate any potential biological inhibition due to TP-MW the longest pre-treatment time (110 hour) and relatively high $MW_{(g\ TS)}$ / $SOW_{(g\ VS)}$ mass ratio 2.5:1 (Yin *et al.*, 2018) were chosen for the TP-MW for the BMP assays.

It can be seen from Figure 7-2 that pre-treatment (TP, TP-MW, TP-Alk) of the SOW at 37^{o}C decreased the maximum methane production rate (K) compared to the control and FrTh pre-treatment. Mean K value of the BMPs digested substrates with pre-treatment (TP, TP-MW and TP-Alk) was 27 ± 6.4 mL/g VS/d, this K was about 96% lower than the K of the control and FrTh assays. Consequently, accumulated methane production (Y_{exp}) of the control and FrTh assays (Figure 7-2 a and b) were higher (388 ± 3 mL/g VS) compared to the other BMPs (322 ± 28 mL/g VS). This difference was more likely due to losses of some nutrients during the pre-treatment (as discussed in Section 7.3.1) and inhibition of methanogenesis in these reactors which can be noted from the lag phase time (λ) values. The lag phase time was one-fold (2.1 ± 0.2 d) and two-fold (3.1 ± 0.1 d) higher in the TP-MW and TP-Alk reactors compared to the control, FrTh and TP reactors (1.3 ± 0.26 d) Table 7-1. A study conducted by Yin *et al.* (2018) found that the IBA dosage of 1.5 g IBA /g TS of activated sludge resulted in the greatest sludge hydrolysis and VFA production but inhibited methanogenesis. This same study found that 0.9 g IBA/ g dry activated sludge was the optimal dosage increased the methane production by 26.6%, shortened the lag phase time by 32.4% and increased the maximum methane production rate by 36.0%. Moreover, in the current study, the high lag phase time (3.1 ± 0.1 d) in TP-Alk assays was likely to be related to the high pH (pH > 12; Figure 7-1 a) at the beginning of the experiments (lag phase time) then it decreased to 8.1 in the reactor digestate on final day of the experiments (Table 7-2).

The BMP of TP-FA showed lowest K and Y_{exp} values (16.2 mL/g VS. d and 282 mL/g VS respectively), whilst the mean K and Y_{exp} values for the TP-IBA, TP-CBW and TP-BA assays were 26.8 ± 3.2 mL/g VS. d and 316 ± 19 mL/g VS, respectively. These values were about 83% and 91% of the K and Y_{exp} values obtained from the TP assay, which fed with pre-treated SOW but without MW addition. The slight decrease in the K and Y_{exp} of the BMPs with TP-MW compared to the TP (Figure 7-2 and Table 7-1) was likely to be related to a slight inhibition (microbial adaptation time required) of methanogens to the digestion

environments produced by MW (as discussed above). Therefore, it was expected that this effect would be negligible/ or disappear in continuous AD (CSTR) experiments.



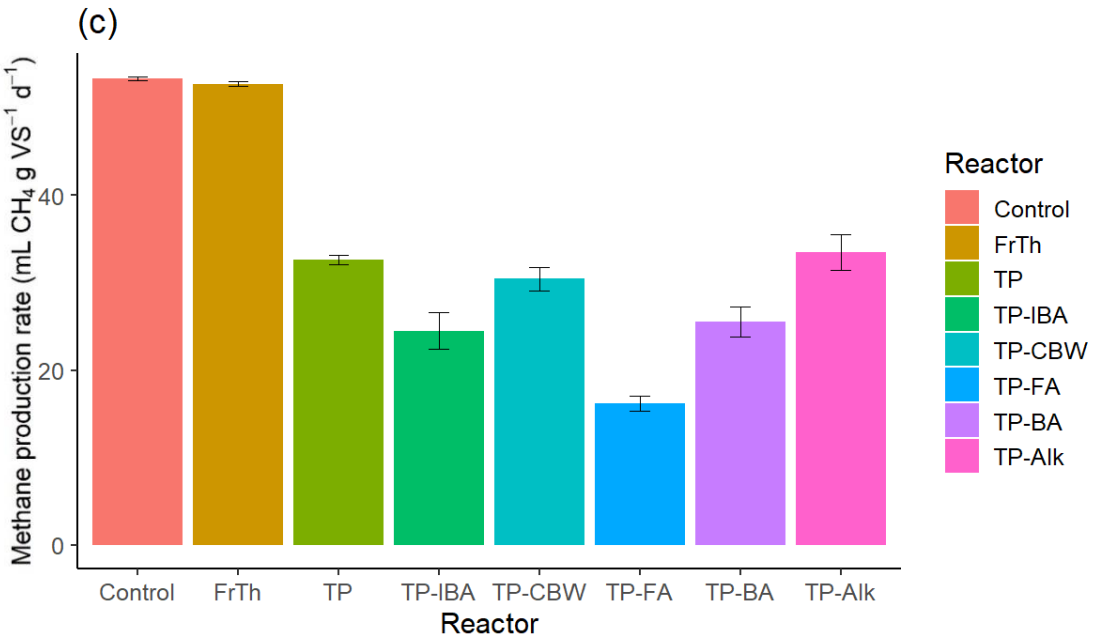


Figure 7-2. Performance of mesophilic BMP assays of organic waste pre-treated with mineral wastes. (a) and (b) methane accumulation obtained from experimental data and Gompertz model respectively, and (c) maximum methane production rate (K) calculated from Gompertz model. TP-Alk= pre-treatment with 0.6 % NaOH solution. Control = raw organic waste prior to experiments (i.e. without pre-treatment and mineral waste addition). TP = organic waste pre-treated at 37°C but without MW. TP-IBA, TP-CBW, TP-FA and TP-BA refer to the MW used in the TP-MW pre-treatment assays. The values are mean values of triplicate measurements with standard error.

Table 7-1 Parameters of BMP assays from modelling of methane accumulation

		Modified Gompertz model				First-order model		
BMP	Y_{exp}	Lag time (λ)	Y_{GM}	K	R^2	μ	R^2	Biodegradability[1]
	(mL/g VS_{in})	(d)	(mL/g VS_{in})	(mL/g VS/d)		(1/d)		(%)
Control	386	1.47	378	53.3	0.996	0.18	0.96	75%
FrTh	390	1.04	383	52.7	0.997	0.19	0.96	76%
TP	346	1.5	353	32.2	0.984	0.087	0.99	67%
TP-IBA	329	2.27	350	24.5	0.983	0.05	0.99	64%
TP-CBW	325	1.8	331	30.5	0.998	0.09	0.96	63%
TP-FA	283	2.2	315	16.2	0.990	0.04	0.98	55%
TP-BA	295	2.14	306	25.5	0.993	0.06	0.99	57%
TP-Alk	352	3.1	362	33.5	0.995	0.06	0.96	68%

[1] Biodegradability calculated as a ratio of experimental methane yield (Y_{exp}) to theoretical methane yield of SOW (514 mL/g VS; as described in Section 4.2.6.

K= maximum methane production rate calculated from Gompertz model.

Y_{GM} accumulated methane yield calculated from Gompertz model.

μ = coefficient of microbial growth rate calculated from first order model.

R-squared (R^2) is the goodness-of-fit of models.

In term of alkaline pre-treatment effects from the MW and NaOH solution in this study, it can be seen from Table 7-1 that the K and Y_{exp} values obtained from the TP-Alk assays were very close (K = 33.5 mL/g VS. d and Y_{exp} = 352 mL/g VS) to those values obtained from the TP-IBA, TP-CBW and TP-BA assays which had the average K and Y_{exp} values of 26.8 ± 3.2 mL/g VS. d and 316 ± 19 mL/g VS, respectively. This renders MW a promising substitute for a wide range of chemicals (for instance NaOH) used in the alkali thermochemical pre-treatment of organic wastes, specifically in places where these MW are already available and need to be managed or useful for integration in AD before their disposal in landfills or other management routes of MW as described in Section 3.3.1.2.

7.3.2. *Effect of the MW pre-treatment on biodegradation in CSTR*

7.3.2.1. *Effect of the MW pre-treatment on methane yield and performance*

The continuous (CSTR) experiments to investigate the effect of pre-treatment of the substrate (SOW) with mineral wastes (collectively named as TP-MW) at 37^{o}C lasted for 80 days (four HRT of 20 days) (Figure 7-3). During the steady state (day 11 - 20) period of HRT-1 the methane yields of the six reactors were all similar (527 ± 20 mL/ g VS) and these results suggest that there was no chemical alteration (hydrolysis) of the organic feed as a result of interaction with the mineral wastes during pre-treatment. However, after day 20, methane yield profiles diverged. For the TP-FA reactor (pre-treatment with FA), methane yields decreased sharply between day 20 and 40 until this reactor stopped producing biogas on day 40 (see discussion of putative toxicity effects of the FA treatment below) and the continuous feeding of the reactor was stopped. In contrast, although, the two control reactors (i.e. without any pre-treatment or without any MW amendment to the pre-treatment) showed a notable but gradual decrease in methane yield after day 20, a sharp decline in the methane did not occur until day 60 when OLR in these reactors was increased to 2 g VS/L. d (Figure 7-3, Table 7-2). Intriguingly a better performance (before day 60) of the TP-only reactor compared to the control highlighted the positive effect of even this mild pre-treatment on the AD of the SOW (Figure 7-3). Suggesting that the organic compounds of the feedstock such as proteins and carbohydrates were solubilized or exposed to some extent during the pre-treatment perhaps through the physical agitation and slightly elevated temperature.

All the other CSTR reactors were still producing high levels of methane at the end of the 80 days operation, however, the TP-BA and TP-CBW reactors (pre-treatment conditions with BA or CBW) showed highly fluctuating methane yields from day 40 onwards which only stabilized (to the range of methane yields in the TP-IBA) when the pre-treatment time was

decreased to one hour between day 60 and 80. Decreasing pre-treatment times from 12 hours (day 1 - 60) to one hour (day 60 - 80) had no obvious effect on methane yields in the TP-IBA (with pre-treatment and IBA amendment). Regardless, this reactor sustained the most consistent yields of methane during the experimental period and so clearly benefitted without any negative impacts but from the addition of MW regardless of pre-treatment times.

From an energetic point of view, the amount of energy which is needed for pre-treating the substrates (MJ/kg of substrate Section 7.2.4.1) for the TP-MW reactors was very low (below 0.3% and 3.4% for one hour and 12 hours pre-treatment times, respectively (Table A-7-2.)) in comparison to the output energy as methane from these reactors (assuming 40 MJ/M^3 as the calorific value of methane (Table A-7-2.)). This output energy estimation was excluded the TP-FA reactor, as this reactor failed as biogas-producing reactor after day 40 (Figure 7-3). During the steady state of HRT-4 (days 60 – 80 and before the control and TP reactors stopped producing biogas), the estimated output energy obtained from the TP-IBA, TP-CBW and TP-BA rectors were 23% (± 9%) and 60% (± 12%) higher than that obtained from the control and TP reactors, respectively. In full-scale digester conditions, increasing the temperature of the pre-treated substrate to 37ºC would substantially decrease the amount of energy required for heating the substrate in the methanogenic digester (i.e. 37ºC) and therefore, would compensate most of the energy which was required for pre-treatment. Moreover, shorter pre-treatment times (less than one hour) should be assessed in future studies.

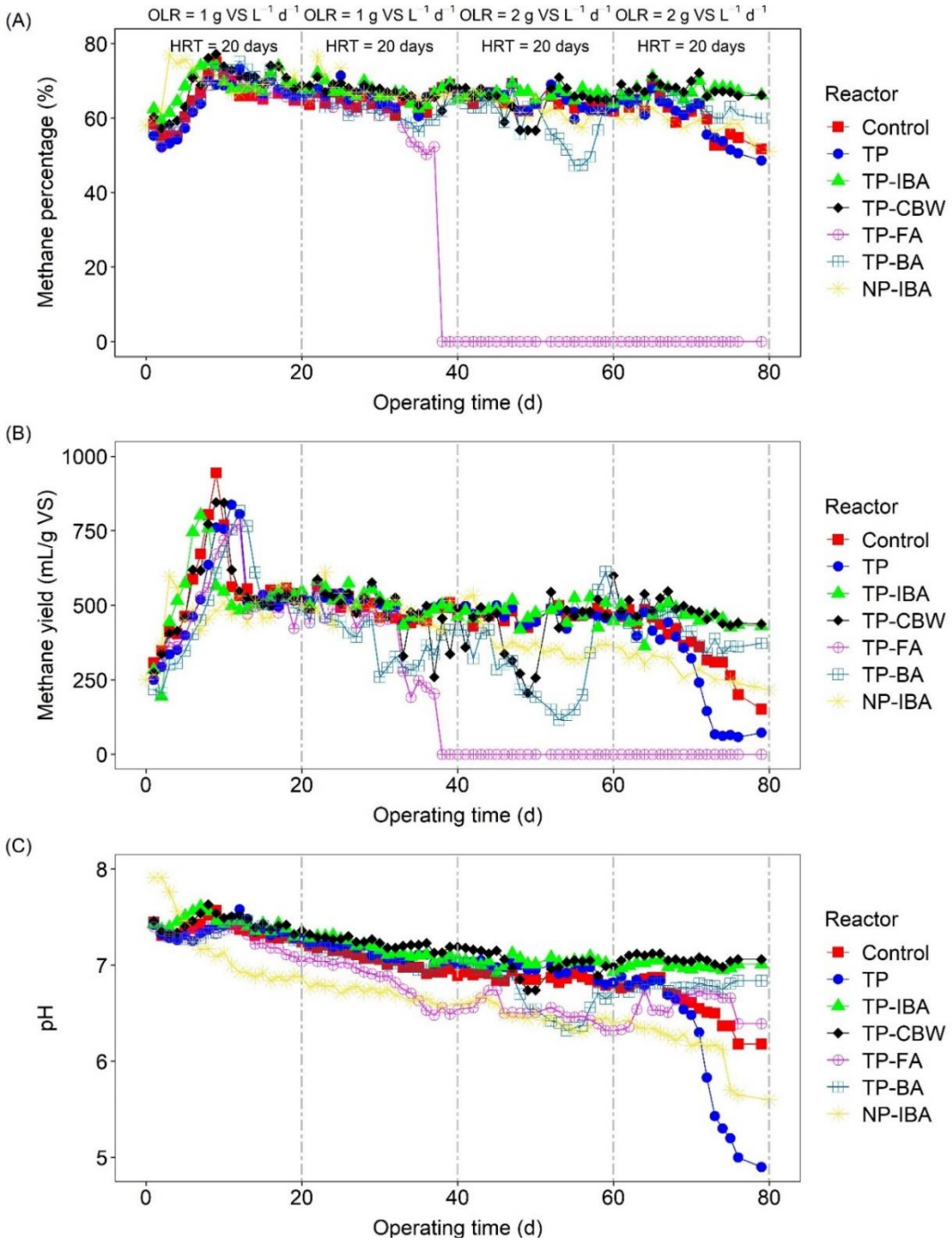


Figure 7-3. Profile performance of single-stage mesophilic AD (CSTR systems) of organic waste pre-treated with mineral wastes at 37°C (TP-IBA, TP-CBW, TP-FA and TP-BA) compared to 1) a control reactor (Control) without mineral waste amendment and without pre-treatment, 2) a control reactor (TP) without mineral waste amendment but with pre-treatment, and 3) a reactor (NP-IBA) from a previous study (DIBA reactor in Chapter 6) amended with IBA but without pre-treatment. The OLR for NP-IBA reactor was 0.5 and 1 g VS L^{-1} d^{-1} for days 0 – 40 and 40 – 80, respectively. These OLR values shown are for the Control, TP, TP-IBA, TP-CBW, TP-FA and TP-BA reactors in the current study. The vertical lines show HRT periods.

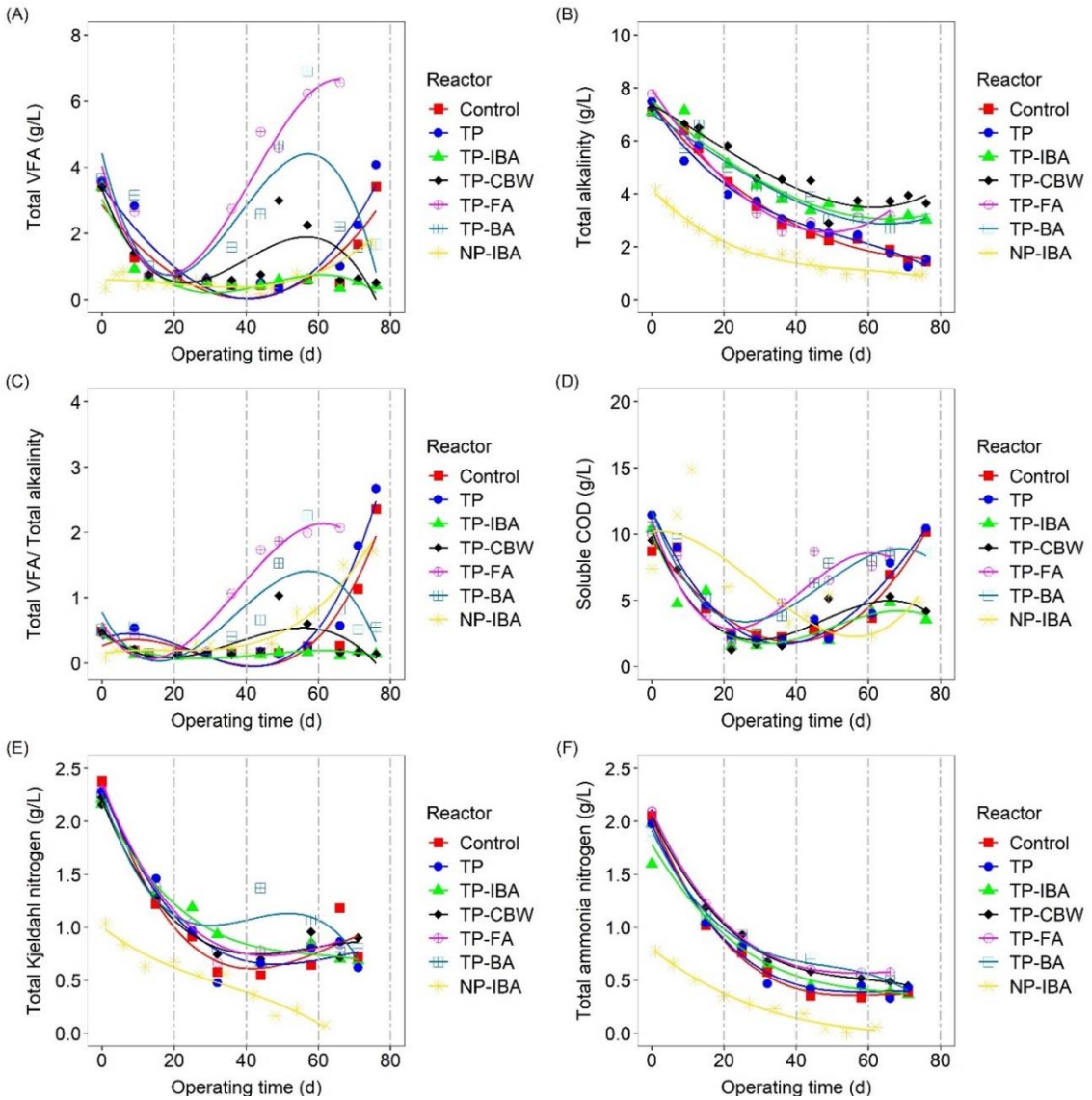


Figure 7-4. Variation in measured parameters of single-stage mesophilic AD (CSTR systems) of organic waste. The description of legends is similar to that described in Figure 7.2 above. The values are mean value of triplicate samples with standard deviation not shown.

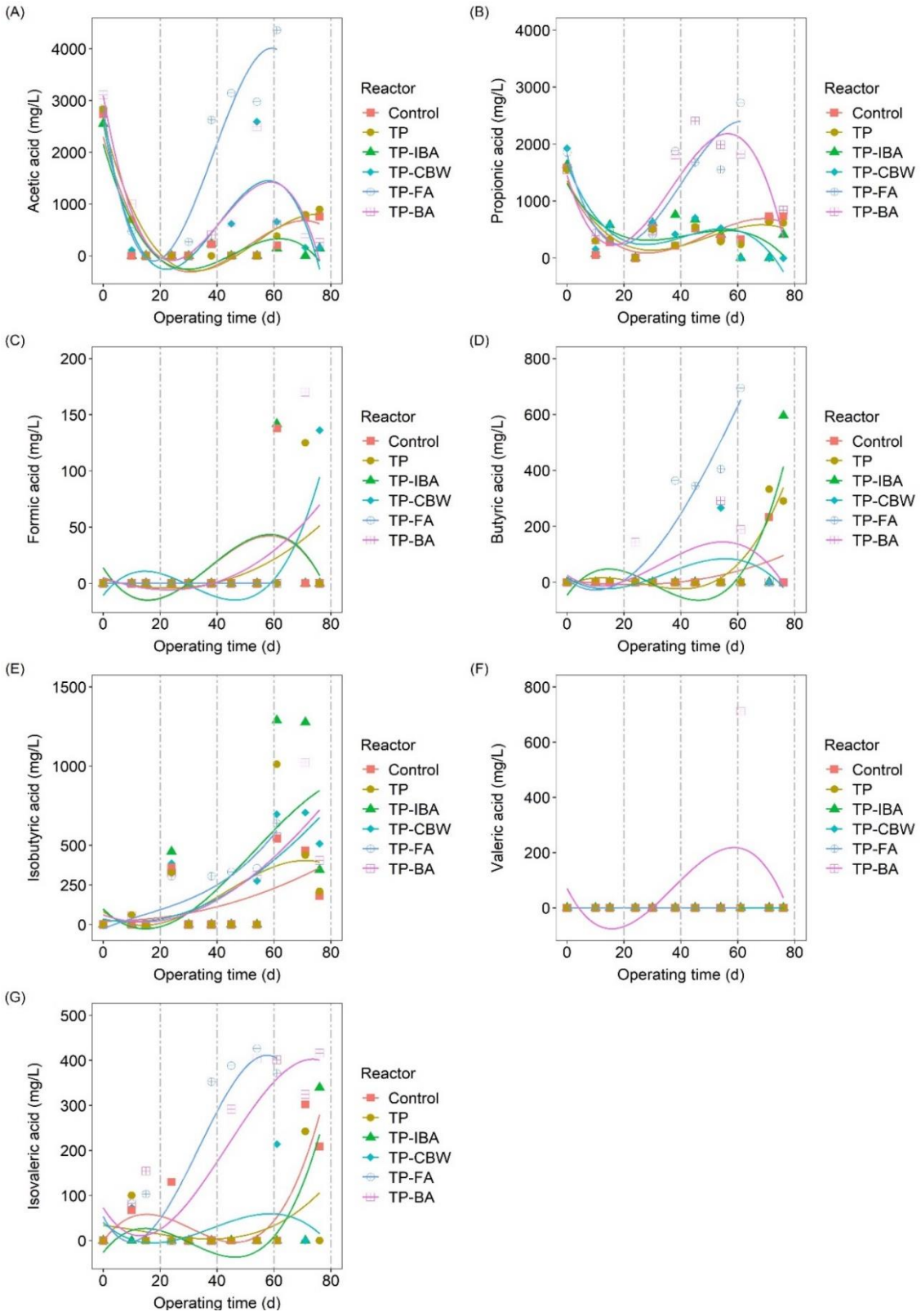


Figure 7-5. Variations in VFA concentrations in digestate samples of single-stage mesophilic AD (CSTR systems) of organic waste pre-treated with/without mineral wastes. The labels are as described in previous figures.

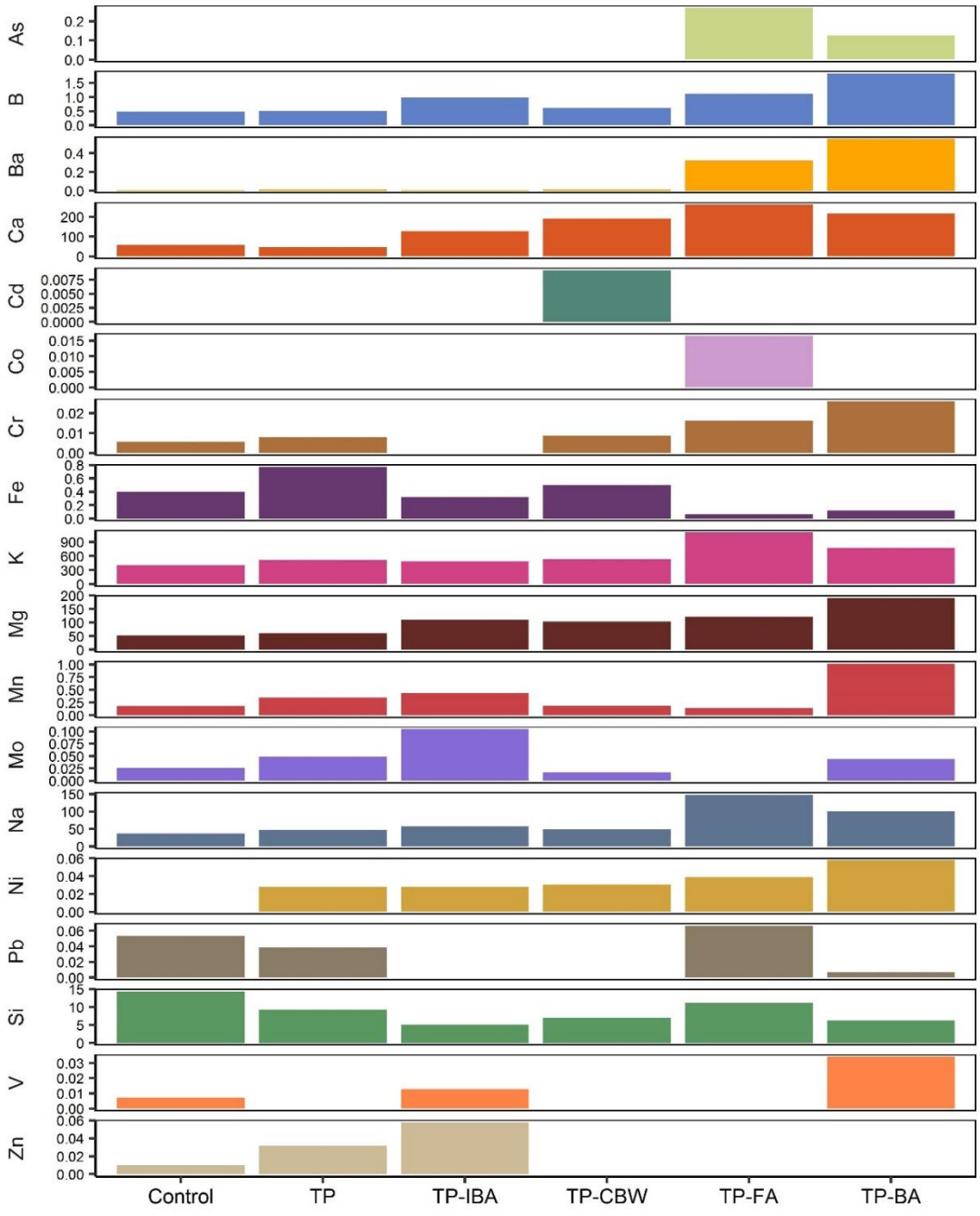


Figure 7-6. Soluble concentration of metals (mg/L) in CSTRs on day 40. The concentration of elements that were not detected during metal analyses are denoted as blank in the figure. The concentration of metals on the other days were measured (not shown here) and used for the correlation analysis with the microbial taxa relative abundances (see the last figure in this chapter).

As the TP-IBA reactor was the best biogas-producing reactor, therefore, the effect of IBA pre-treatment on the digestion performance and process stability of this reactor was assessed by comparing with that of the DIBA reactor in Chapter 6. The DIBA reactor was also operated with the DFFM but the IBA waste was added to this reactor (DIBA reactor in Chapter 6 here it labelled as NP-IBA reactor in Figure 7-3 and Figure 7-4) directly without pre-treatment. It

can be seen from Figure 7-3 that , although the OLR of the NP-IBA reactor was lower (0.5 and 1 g VS/L. d for days 0 - 40 and 40 – 80, respectively; (Chapter 6)) compared to that of the TP-IBA reactor (1 and 2 g VS/L. d for days 0 - 40 and 40 – 80, respectively) however, the NP-IBA reactor showed a lower performance and process stability (low methane yield and VFA accumulation) compared to the TP-IBA reactor (Figure 7-3). This low performance of the NP-IBA reactor (Chapter 6) was found to be related to the inhibition of methanogens caused by VFA accumulation which led to a decrease in alkalinity (Figure 7-4) and drop in pH (Xu *et al.*, 2014). However, the performance of the TP-IBA reactor was stable until the end of experiments as discussed below.

In current study, the reason for differences in process stability is apparent on examination of the similarities and differences in measured parameters in the CSTR digestates (Figure 7-4, Figure 7-5 and Figure 7-6). For instance, for the first 40 days there was a universal decline in buffering capacity (measured as total alkalinity, total ALK) in all reactors reflecting the high alkalinity (9.8 g/L $CaCO_3$) of the start-up inoculum (Table 4-4) and its progressive dilution during CSTR operation. However, after 20 days, the reactors diverged and alkalinity was variably controlled by three factors i.e. further dilution of the inoculum, a greater proportional contribution of alkalinity from the TP-MW treatments and the accumulation of alkalinity consuming VFA. In the case of the control and TP-only reactors, alkalinity change progressed by inoculum dilution, however, after 60 days alkalinity consuming VFA concentrations increased dramatically coincident with an increase in the OLR (Xu *et al.*, 2014) (Figure 7-4 and Figure 7-5). The fall in alkalinity below 2 g/L resulted in a pH drop below the optimal values (pH 6.8 - 7.2; (Franke-Whittle *et al.*, 2014)) required for sustaining methane production. In contrast, the limited methane yields of the TP-FA reactor after 20 days (ultimately leading to reactor failure) as well as the fluctuating performances of the TP-BA and, to a lesser extent, the TP-CBW reactors (from day 30 onwards) cannot be ascribed to alkalinity loss through dilution or VFA based consumption. For these reactors, as for the best performing reactor TP-IBA, alkalinity remained stably above 2 g/L. Neither can ammonia inhibition be invoked to explain instability since total ammonium nitrogen (TAN) concentrations in all the reactors were very similar regardless of performance and were below the inhibition limits thought to apply for AD (2 - 2.5 g TAN/L; (Lay *et al.*, 1998; Latif *et al.*, 2017). These results suggest that the poor stability (low biogas production and associated accumulations of VFA) of the TP-FA reactor and the fluctuating performance of the TP-BA and TP-CBW reactors were most likely related to inhibition caused by metals leached from the respective MW (Figure 7-6). This suggestion is supported by soluble metal concentrations

in the digestate samples measured on day 40 (Figure 7-6), the point with the highest concentrations of most of the metals found in the failed TP-FA reactor compared to the concentrations in the other MW amended reactors. For instance, As, Ba and Cr had high concentrations in the TP-FA and TP-BA reactors, the presence of these heavy metals in the anaerobic digester above certain thresholds (depending on the characteristics of the substrate in the digester) has been found to decrease the efficiency of the anaerobic digestion processes (Ahring *et al.*, 1995; Sierra-Alvarez *et al.*, 2004; Abdel-Shafy and Mansour, 2014). In support of this metal inhibition hypothesis it was interesting to note that when pre-treatment times were decreased to 1 hour (day 60 - 80) a notable increase in methane yields and process stability was observed in the TP-BA and TP-CBW reactors (Figure 7-3 and Table 7-2).

It is worth mentioning that the TP-IBA reactor which showed consistently the highest methane yield and process stability contained high concentrations (dissolved) of Zn, Mo (~ 0.06 mg/L and 0.1 mg/L respectively) and moderate concentration of other elements such as V ~ 0.01 mg/L, Ni ~ 0.02 mg/L, Na ~25 mg/L, Mn ~ 0.25 mg/L, Mg ~ 100 mg/L, Fe ~ 0.25 mg/L) (Figure 7-6). These elements are known as important trace elements in AD. Uemura (2010) demonstrated a restoration in methane yield of mesophilic AD of organic waste after the addition of Ni, Co and Fe. Zhang *et al.* (2012) reported the decline in the performance of semi-continuous single stage mesophilic reactors of food waste due to the decrease in concentration of trace elements such as Co, Mo, Ni and Fe. In the current study, using the concentration of TEs in the TP-IBA reactor as an indicator for higher methane production from the TP-IBA reactor compared to the control and TP reactors, it could be concluded that the eventual the failure of the control and TP reactors was more likely related to the deficiency /and low concertation of the metals Zn, V, Mo, Ni, Mn and Mg (Figure 7-6).

Table 7-2. Summary performance data for mesophilic continuous reactors without or with mineral waste pre-treatment at 37^{o}C

		Control	TP	TP-IBA	TP-CBW	TP-FA	TP-BA
Day 0 - 20	OLR (g VS/L. d)	1	1	1	1	1	1
	Pre-treatment time (h)	12	12	12	12	12	12
	HRT (d)	20	20	20	20	20	20
	Yield (mL CH_4/g VS $_{added}$)	553 ± 151	526 ± 164	529 ± 138	545 ± 147	506 ± 141	503 ± 173
	pH	7.4 ± 0.09	7.4 ± 0.09	7.4 ± 0.08	7.4 ± 0.08	7.3 ± 0.1	7.3 ± 0.07
	Methane percentage (%)	65 ± 5	64 ±6	68 ±4	68 ± 5	64 ±5	66 ± 6
Day 21 - 40	OLR (g VS/L. d)	1	1	1	1	1	1
	Pre-treatment time (h)	12	12	12	12	12	12
	HRT(d)	20	20	20	20	20	20
	Yield (mL CH_4/g VS $_{added}$)	492 ± 34	503 ± 35	508 ± 39	481 ± 81	408 ± 183	391 ± 76
	pH	7.1 ± 0.1	7 .1 ± 0.08	7.2 ± 0.1	7.2 ± 0.06	6.8 ± 0.2	7.1 ± 0.09
	Methane percentage (%)	64 ± 2	66 ± 2	66 ± 2	67 ± 2	51 ± 22	62 ± 2
Day 41 - 60	OLR (g VS/L. d)	2	2	2	2	2	2
	Pre-treatment time (h)	12	12	12	12	12	12
	HRT (d)	20	20	20	20	20	20
	Yield (mL CH_4/g VS $_{added}$)	466 ± 20	467 ± 22	462 ± 51	428 ± 103	174 ±30	308 ± 146
	pH	6.9 ± 0.04	7.0 ± 0.07	7.0 ± 0.05	7.0 ± 0.13	6.5 ± 0.1	6.7 ± 0.24
	Methane percentage (%)	64 ± 1	64 ± 2	66 ± 1	64 ± 4	N/A	59 ± 6
Day 61 - 80	OLR (g VS/L. d)	2	2	2	2	2	2
	Pre-treatment time (h)	1	1	1	1	1	1
	HRT (d)	20	20	20	20	20	20
	Yield (mL CH_4/g VS $_{added}$)	368 ± 97	284 ± 167	457 ± 33	487 ± 44	145±25	420 ± 58
	pH	6.6 ± 0.2	6.4 ± 0.5	7.0 ± 0.03	7.1 ± 0.04	6.6 ± 0.1	6.8 ± 0.04
	Methane percentage (%)	59 ± 5	59 ± 5	67 ± 1	67 ± 1	N/A	63 ± 2

HRT =hydraulic retention time.
OLR = organic loading rate.
Control = SOW without pre-treatment and without MW, TP = SOW + pre-treatment only, TP-IBA = SOW + pre-treatment+ incineration bottom ash, TP-CBW = SOW + pre-treatment+ cement-based waste, TP-FA = SOW+ pre-treatment + fly ash, TP-BA = SOW + pre-treatment+ boiler ash.
N/A = not available i.e. the reactor has stopped producing biogas.

7.3.2.2. *Effects of mineral waste pre-treatment on microbial growth and activity*

The abundance of mcrA and 16S rRNA genes determined by qPCR analysis (Figure 7-7) were used to estimate the numbers of archaeal and bacterial cells in the reactors and a number of general features are revealed by examining these abundance trends with time and in relation to reactor operation. For instance, in most of the reactors the abundances of both bacteria and archaea increased with time consistent with growth during the start-up phase and in response to an increase in the OLR. However, in all the reactors there was a consistent and considerable numerical dominance of bacteria over the methanogenic archaea. These bacterial and archaeal numbers were then used in combination with measured VFA and methane values for calculation of total fermentation (F), total methanogenesis (M), cell specific fermentation activity (CSF) and cell specific methanogenesis activity (CSM) activities in the CSTRs (Figure 7-8 and Table 7-3).

One-way ANOVA analysis conducted on the overall data (i.e., gene abundances from qPCR analysis) obtained throughout the current study showed that there was no significant difference ($p > 0.05$) in the abundance of either bacteria or archaea between the six reactors. However, bacterial abundance in the TP-MW reactors (TP-IBA, TP-CBW, TP-FA and TP-BA) was significantly ($p = 0.07$) different from that in the control and TP reactors (Figure 7-7). Interrelationships between acetogenic bacteria and methanogenic archaea in AD to degrade VFA is considered an essential factor for the stability of the digestion process (Amani *et al.*, 2011; Xu *et al.*, 2014). The differences in archaeal abundance between TP-MW reactors and the control and TP- only reactors were not significant ($p > 0.05$) ((Figure 7-7). On day 80 (at the end of the experiments), the archaeal abundance in the TP-only reactor was the highest ($> 5 \times 10^8$ gene sequences/mL), while in the other reactors which had pre-treatment with MW (TP-IBA, TP-CBW, TP-FA and TP-BA reactors) archaeal gene abundances were ~ 2.5×10^8 gene. These results suggested that variations in methane yield between reactors were most likely related to consortia directly related to the final steps in methanogenesis either due to the inhibition of methanogens and/or due to low specific methanogenic activity, i.e. the reactors had equal or very similar numbers of cells but with different methane production activities.

Table 7-3 and Figure 7-8 show that in current study the variation in the methanogenesis to fermentation ratio (M/F) was consistent with the performance (methane yield and process stability) of the CSTRs. For instance, during the stable performance period of the control and TP-only reactors (day 1 - 60), the M/F increased from 0.53 ± 0.02 from day 1 to 20 (HRT-1) to 0.83 ± 0.04 from day 21 to 40 (HRT-2) and then remained constant from day 41 to 60

(HRT-3; 0.87 ± 0.04). While, the M/F of the control and TP reactors decreased to 0.41 ± 0.01when methanogenesis started to decline between day 61 - 80 (Figure 7-3, Table 7-2 and Figure 7-8). A subsequent decrease in the cell specific methanogenic activity compared to fermentation activity (M/F =0.41 ± 0.01) in the control and TP reactors between day 61 - 80 (Figure 7-3, Table 7-2 and Figure 7-8) reflected the accumulation of VFA (most obvious after day 60) and the pH drop observed. As discussed in Section 7.3.2.1, these changes in reactor performance after day 60 were most likely due to the deficiency in TEs, however, on the basis of theses accompanying low cell specific methanogenic activities in the control and TP reactors after 60 days, it can be concluded that this metal deficiency principally affected the methanogenic archaea, and the relative abundance of archaeal communities were positive correlated with the concentration of measured metal elements in reactors (Figure 7-12). During HRT-2 (day 21 - 40) the TP-FA reactor showed the lowest M/F observed among all CSTRs (Figure 7-8); which is consistent with the likely failure of this reactor due to an accumulation of VFA (Figure 7-4 and Figure 7-5). In this reactor the low cell specific methanogenic activity in the TP-FA reactor is more likely related to the inhibition of methanogens due to high metal concentrations rather than deficiency as seen in the controls (as discussed in Section 7.3.2.1).

In contrasts the M/F activity of the TP-IBA and TP-CBW reactors remained higher compared to other reactors (Figure 7-8 and Table 7-3) supporting a conclusion that the successful performance of these two reactors was due to sufficient methanogenesis activity sustained by sufficient metal supply resulting in a balanced syntrophic relationship between acetogens and methanogens. As discussed in Section 7.3.2.1 the TP-IBA reactor contained most of TEs (Figure 7-6) important for AD (Zhang *et al.*, 2012) and it is supposed that these TEs were bioavailable in the moderate concentrations suitable for microbial metabolism.

Interestingly, two novel findings could be revealed from the calculations of fermentation and methanogenesis activities in this study. Firstly, an M/F of > 0.4 was necessary to avoid the instability (as it was in the TP-CBW and TP-BA reactors) or failure (as it was in the TP-FA reactor) of the continuous reactors of the OFMSW. Secondly, the optimum M/F for anaerobic digestion of the OFMSW ranged from 0.6 to 0.8, with this optimal M/F the methane production was maximum and the digestion process was stable (as in the TP-IBA) (Figure 7-8 and Table 7-3).

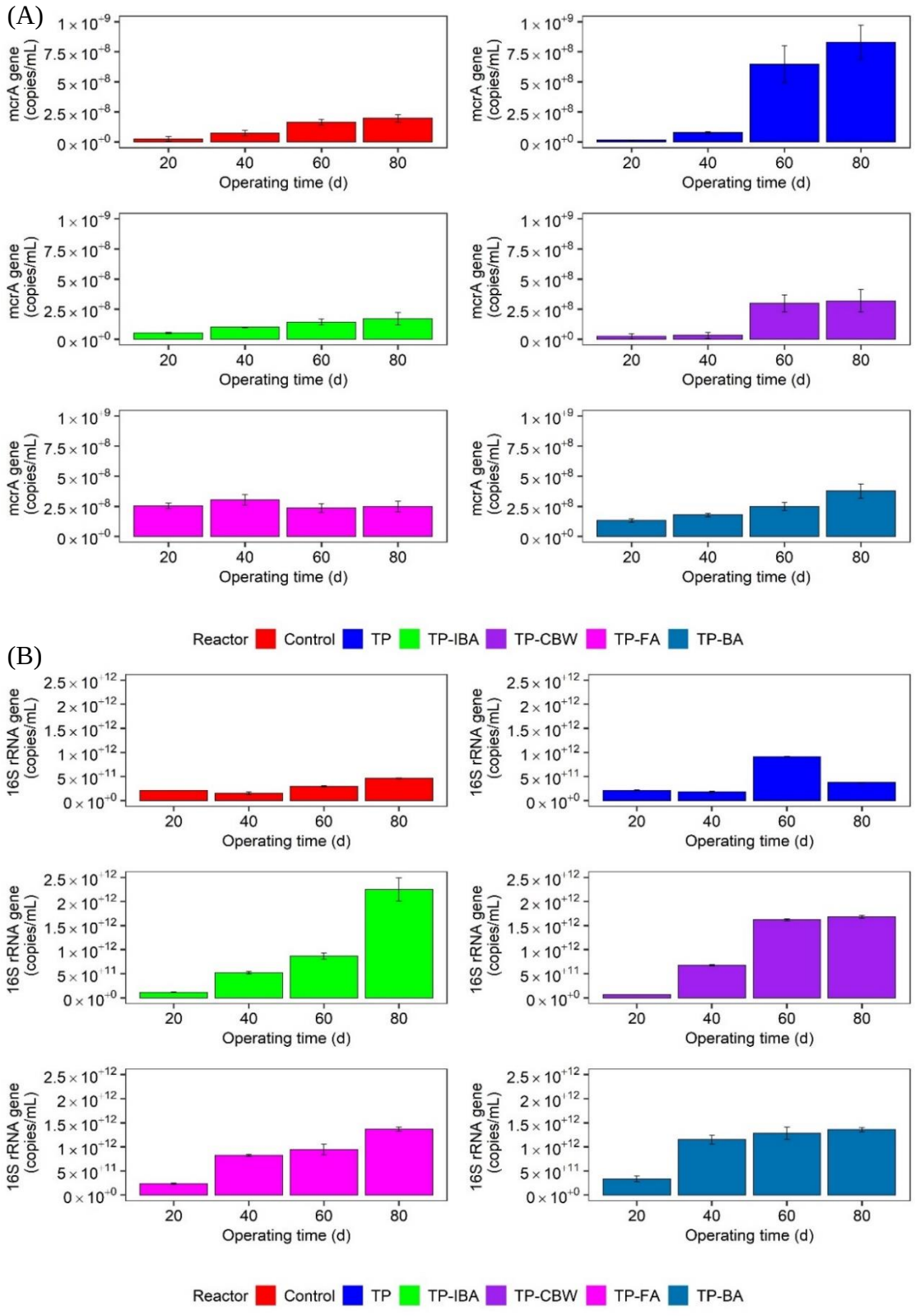


Figure 7-7. Variations in microbial community population ((A) mcrA = archaea, (B) 16S rRNA = bacteria) of single-stage mesophilic AD (CSTR systems) of organic waste pretreated with/without mineral wastes at 37°C.

Table 7-3. Microbial population growth and activity in the CSTRs during four HRT of 20 days, the values are mean values (in triplicate) with standard deviation (not shown)

	Reactors	Average total VFA (mg/L)	Total fermentation (g COD)	Bacteria cells (Cell/ mL)	Cell specific fermentation activity (pg COD/Cell. d)	Archaea cells (Cell/mL)	Cell specific methanogenesis activity (pg COD/Cell. d)	*M/F	**B/A
HRT-1	Control	1523	15.5	4.97×10^{10}	0.063	1.73×10^{7}	91	0.51	2864
	TP	1896	21	4.09×10^{10}	0.102	9.99×10^{6}	230	0.55	4095
	TP-IBA	1378	17.8	2.86×10^{10}	0.125	2.09×10^{7}	105	0.61	1371
	TP-CBW	1585	19.1	3.62×10^{10}	0.106	2.21×10^{7}	101	0.59	1634
	TP-FA	1913	20.6	5.21×10^{10}	0.079	7.37×10^{7}	30	0.54	706
	TP-BA	2145	21.3	8.49×10^{10}	0.050	4.98×10^{7}	43	0.50	1704
HRT-2	Control	451	12	3.75×10^{10}	0.064	3.68×10^{7}	53	0.81	1020
	TP	506	17.5	4.45×10^{10}	0.079	3.87×10^{7}	77	0.86	1149
	TP-IBA	478	17.3	1.30×10^{11}	0.027	4.96×10^{7}	60	0.86	2628
	TP-CBW	672	17.2	1.68×10^{11}	0.020	1.60×10^{7}	173	0.80	10522
	TP-FA	2802	23.5	2.06×10^{11}	0.023	1.52×10^{8}	13	0.40	1360
	TP-BA	1585	20	2.87×10^{11}	0.014	8.96×10^{7}	27	0.60	3201
HRT-3	Control	478	15.2	7.50×10^{10}	0.040	8.24×10^{7}	31	0.84	910
	TP	644	23.2	2.27×10^{11}	0.020	3.24×10^{8}	12	0.86	703
	TP-IBA	506	21.7	2.17×10^{11}	0.020	7.13×10^{7}	54	0.88	3038
	TP-CBW	1945	27.8	4.05×10^{11}	0.014	1.49×10^{8}	24	0.65	2718
	TP-FA	5790	32.5	2.36×10^{11}	0.027	1.18×10^{8}	6	0.11	2001
	TP-BA	4587	36.8	3.19×10^{11}	0.023	1.25×10^{8}	22	0.38	2556
HRT-4	Control	2540	21.6	1.16×10^{11}	0.037	9.84×10^{7}	18	0.41	1178
	TP	3162	26.9	9.38×10^{10}	0.057	4.15×10^{8}	5	0.41	226
	TP-IBA	485	18.9	5.63×10^{11}	0.007	8.54×10^{7}	39	0.87	6585
	TP-CBW	568	20	4.19×10^{11}	0.010	1.59×10^{8}	21	0.86	2631
	TP-FA	2415	15.7	3.44×10^{11}	0.009	1.24×10^{8}	6	0.23	2769
	TP-BA	1627	24	3.40×10^{11}	0.014	1.89×10^{8}	17	0.66	1801

* Methanogenesis to fermentation ratio.
**Total bacteria to total archaea cells ratio.

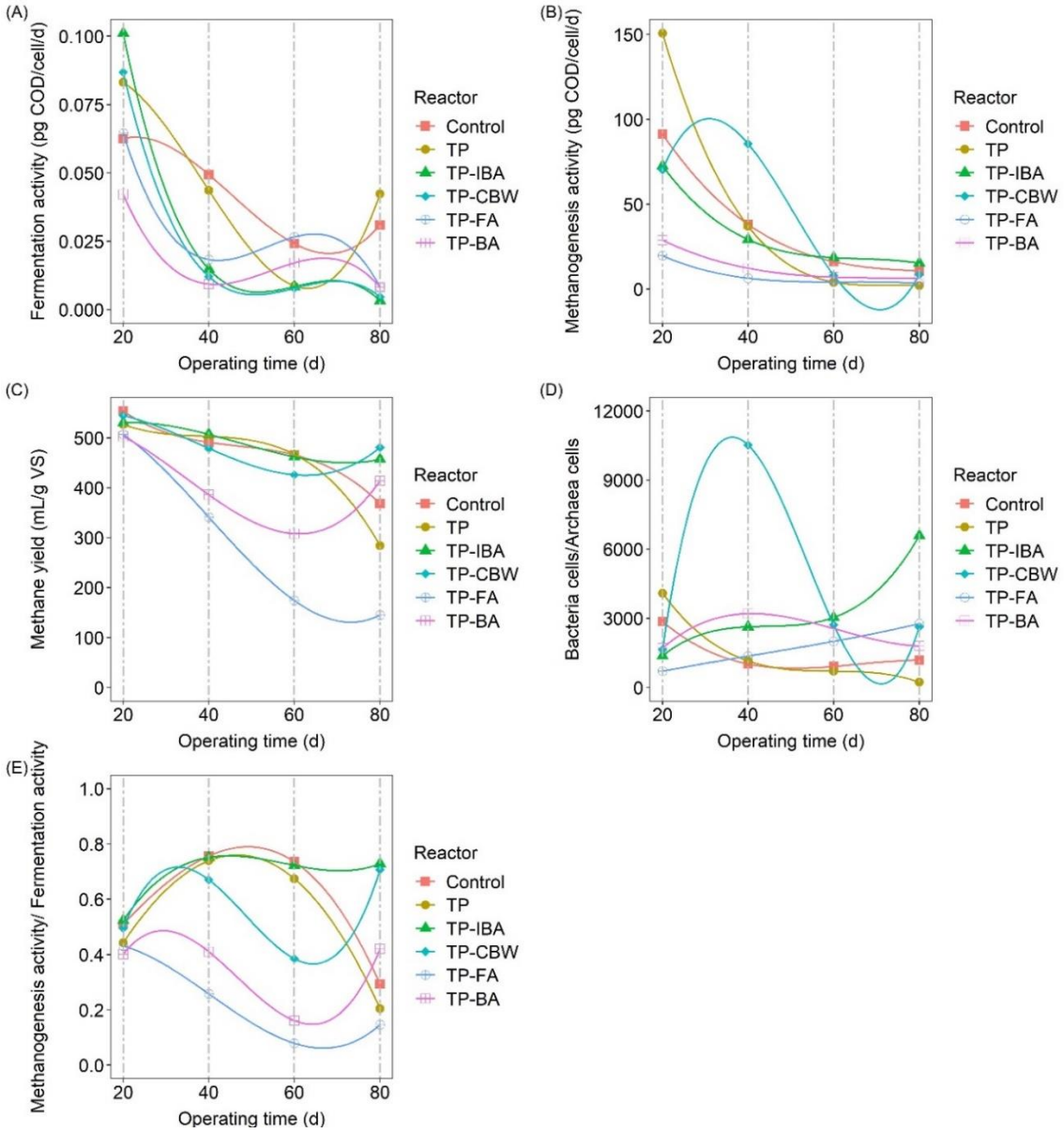


Figure 7-8. Variations in microbial growth (counts) and performance of single-stage mesophilic CSTRs of organic waste pre-treated with/without mineral wastes at 37^{o}C. The values are mean values with standard deviation not shown.

7.3.3. *Developments in microbial community compositions and function due to the pre-treatment of organic and mineral waste mixtures*

In AD, the availability of substrates, nutrients and catabolic inhibitory products drives the diversity of the microbial populations. For instance, the presence of inhibitors such as accumulations of VFA, ammonium and toxic metals etc. has been found to shift the archaeal methanogenic community from acetoclastic to hydrogenotrophic taxa (Banks *et al.*, 2012; Williams *et al.*, 2013; Town *et al.*, 2014; Westerholm *et al.*, 2015b). Williams *et al.* (2013) noted a 69% decrease in *Methanosaeta* due to the accumulation of VFA in a full-scale anaerobic digester fed with food waste; the same study also observed that the addition of trace

elements and alkalinity stimulated the growth of acetogens which supported propionate degradation. In the current study, the average number of reads in individual 16S rRNA sequence libraries after quality filtering was 4,927,452 ranging from 161,113 sequences in the smallest library to 277,982 sequences in the largest. However, individual libraries were rarefied for comparative analysis. An ASV table which showed the number of features per the digestate samples was produced, it comprised 540 taxa (> 96% of sequences) belonging to the bacterial domain and 19 taxa (about 3.5 - 4% of sequences) belonging to archaeal domain. The effects of TP-MW on microbial community composition and dynamics are shown in Figure 7-9, Figure 7-10 and Figure 7-11. In general, the overall diversity in reactors decreased with time and increases in organic loading rates (OLR). Moreover, the concentration of metal elements was correlated with the abundance of archaeal communities (Figure 7-12), and these metals were the key factor shaped the archaeal community composition in reactors.

7.3.3.1. *Bacterial diversity*

The bacterial community of the reactors at the genus level showed a high number of unassigned bacterial genera, therefore, differences in bacterial diversity between the CSTRs are broadly discussed at family level with reference to lower taxonomic assignments where appropriate and possible. The most frequently observed bacterial family in all digestate samples was the *Dysgnonomonadaceae* (≤ 30% of bacterial sequences), followed by *Cloacimonadaceae*, *Synergistaceae* and *Ruminococcaceae* with each family contributing ~ 5 - 10% of the sequences in the libraries (Figure 7-9). The presence of these bacterial families is not surprising given that they are common constituents of AD communities including those we have previously documented degrading the same synthetic organic waste (Chapter 6). These organisms are known to work collaboratively to degrade organic matter to precursors for methanogenesis through several metabolic activities including hydrolysis, fermentation, acetogenesis and even syntrophic hydrogen-production (Guo *et al.*, 2014). To be more specific and illustrative bacterial communities belonging to phylum *Bacteroidetes* i.e. the *Dysgnonomonadaceae* are known to be responsible for degradation of proteins or complex sugar polymers and in particular the *Dysgnonomonadaceae* sequences identified were mostly dominated by sequences related to the genera *Proteiniphilum* and *Fermentimonas* which have been isolated from anaerobic digesters and are considered strictly anaerobic proteolytic or sacchrolytic bacteria generating acetic acid and NH_3 (Chen and Dong, 2005; Hahnke *et al.*, 2016). Interestingly, previous studies (Westerholm and Schnürer, 2019) have even indicated that some pre-treatments such as ultrasonic, microwave, and electrokinetic methods have increaseed the relative abundance of the *Cloacimonadaceae ,Synergistaceae* and

Ruminococcaceae families (which all belong to the phylum *Firmicutes*) while decreasing the relative abundance of *Proteobacteria*. In the current study, sequences affiliated with the *Proteobacteria* only made a minor contribution to the sequence libraries and, indeed, particularly were even a minor component of the control reactors. Despite these generic bacterial components in all the reactors a multi-dimensional scaling (MDS) Unifrac distance analysis (Figure 7-11) clearly identified a time based progression and divergence of the bacterial communities, most clearly observable in the separation of the best performing (TP-IBA and TP-CBW) stable reactors from the failed reactor TP-FA. This separation is largely explained by the progressive enrichment of the families *Kosmotogaceae* and *Anaerolineaceae* in TP-IBA and TP-CBW reactors and to a lesser extent in the other ultimately stable reactor TP-BA. Interestingly, and of relevance to the discussion below about the dynamics of the methanogenic archaea in the different CSTRs, the *Kosmotogaceae* sequences were all from the genus *Mesotoga* which have been previously implicated in syntrophic acetate oxidation to hydrogen in a methanogenic bioreactor degrading terephthalate (Nobu *et al.*, 2015). It has subsequently been speculated that syntrophic acetate oxidation by this organism only occurs however under low acetate concentrations (Nesbo *et al.*, 2018). Of related interest, the *anaerolineacea* sequence type similarly enriched (Figure 7-9) was found to be most closely related (100% sequence homology) to *anaerolineacea* species identified in other methanogenic AD systems e.g. a methanogenic full scale bioreactor treating food waste (Bengelsdorf *et al.*, 2013). Furthermore, an identical sequence type was found to be present in a methanogenic CSTR reactor fed only with acetate and supplemented with Ni and Co but run at a low dilution rate (Shigematsu *et al.*, 2003). In this reactor it was concluded, based on a slight dominance of bacterial over archaeal sequences in the clone libraries and the selection for hydrogenotrophic methanogens that syntrophic acetate oxidation was favoured (Shigematsu *et al.*, 2003).

7.3.3.2. *Archaeal diversity*

In AD, the biodegradation of organic matter to volatile fatty acids is an important step followed by degradation to form acetate, H_2 and CO_2, a process collectively known as acidogenesis. Then there is the potential for syntrophic acetate oxidation (to hydrogen and CO_2) or acetogenesis (CO_2 reduction to acetate in the presence of a reductant i.e. H_2 or formate). Acidogenesis, syntrophic acetate oxidation, and acetogenesis are largely carried out by the bacterial taxa as described in the previous section. In the final AD step principally but not exclusively, acetoclastic methanogens, mainly *Methanosaeta* and, or, *Methanosarcina*, utilise the acetate to produce CH_4 and CO_2 (Fukuzaki *et al.*, 1990) and hydrogenotrophic

methanogens e.g. *Methanobacterium* or *Methanoculleus* typically produce CH_4 from H_2 and CO_2. Critically, a failure to convert acetate or hydrogen to methane regardless of the pathway leads to a build-up in VFA and reactor instability (Xu *et al.*, 2014).

Some environmental factors may contribute to changes in the relative contribution of different methanogenic pathways and the organisms that mediate. For instance, the growth of the obligatory acetoclastic *Methanosaeta* is known to be sensitive to VFA and NH_3-N concentrations (Demirel and Scherer, 2008; Franke-Whittle *et al.*, 2014). In contrast, Zhang *et al.* (2019) has reported for an anaerobic digester that a deficiency of essential TEs such as Fe, Co, Mo, and Ni in food waste limited the methanogenesis ultimately leads to the build-up of propionate which then causes further inhibition of methanogens. The same study of Zhang *et al.* (2019) showed that TE addition eliminated this methanogenesis imbalance by stimulating the growth of *Methanosarcina* which can utilise acetate to produce methane, reduce carbon dioxide with hydrogen and use methyl compounds (not formate) to produce methane (Zhang *et al.*, 2019).

These contrasting impacts on the selection of archaeal methanogens are of interest and relevance to the current study, because the archaeal communities in the six reactors receiving different substrates developed into distinct methanogenic diversity compositions. The methanogenic diversity compositions were ranging from dominantly acetoclastic through to dominantly hydrogenotrophic methanogenesis as illustrated in the stacked bar charts provided in Figure 7-9 and in the multi-dimensional scaling (MDS) Unifrac distance analysis shown in Figure 7-11. For instance, in the control and TP reactor libraries (Figure 7-9) throughout the experimental period there was a dominance of the obligatory acetoclastic *Methanosaeta* (~ 40 - 60% of archaeal sequences), to a lesser extent the obligatory hydrogenotrophic genera *Methanobacterium* and *Methanoculleus* (<10 - 25%), and members of the methylotrophic methanogen family *Methanomethylophilaceae* and the genus *Methanoplasma* (<10 - 25%). The occurrence of the methylotrophic methanogens suggests that methanol was a major catabolic product of fermentation in these reactors especially in control reactor from day 60 onwards. This is interesting because it has been suggested (Chandra *et al.*, 2012) that products such as methanol are formed in continuous or semi-continuous anaerobic digestion because of the build-up of acidic products of hydrolysis. Given the stable performance of the controls during the first three HRT periods before 60 days it can be concluded that these methanogen community compositions were capable of consuming the acetate, methanol and hydrogen products of fermentation in these reactors, however, with an increase in OLR after 60 days this was not the case. One obvious explanation for this restriction was that the deficiency of

TE in the substrate limited the growth and metabolism of the hydrogenotrophic methanogens as previously observed by Zhang *et al.* (2019) which lead to a considerable build up in VFA in both control reactors.

In contrast to the controls, the methanogenic diversity in the most stable TP-IBA reactor was equally dominated by obligatory acetoclastic and hydrogenotrophic methanogens with only a minor presence of *Methanomethylophilaceae*. Between days 40 and 80, about 75% of archaeal population in the TP-IBA reactor divided fairly equally between *Methanosaeta* and *Methanobacterium.* However, it is interesting to note that during this time there was a gradual increase in the proportion of hydrogenotrophic methanogens coincident with the enrichment of the putative bacterial syntrophic acetate oxidizers (*Kosmotogaceae* and *Anaerolineaceae*) highlighted above and as discussed above putatively driven by the required supply of trace metals. In addition, there was an enrichment of a sequence related to the recently proposed *candidatus* genus *Methanofastidiosum* (Nobu *et al.*, 2015). This organism is considered nutritionally fastidious, as the name suggests whereby methanogenesis is achieved through methylated thiol reduction linked to hydrogen oxidation. As such, these organisms likely compete with hydrogenotrophic methanogenesis and, due to favourable thermodynamics, may maintain and thrive under the low H_2 partial pressures which favours efficient syntrophic acetate oxidation and fermentation (Nobu *et al.*, 2015). The greater presence of this methanogen was in the TP-IBA reactor; which sustained the highest most stable biogas production throughout the 80 days experiment, therefore, may be a useful barometer of digester health. Likewise, for the consistent but balanced presence of *Methanosaeta* since it has been widely recognised that this genus is sensitive to stress i.e. low pH and high VFA, ammonia and heavy metals ((Franke-Whittle *et al.*, 2014) and Chapter 6 of this thesis). In contrast, the archaeal diversity in the, TP-FA, TP-BA, and to a lesser extent the TP-CBW reactors progressed to very different community compositions reflecting the more negative impacts of the specific MW pre-treatments. On day 80, about 80 - 95 % of archaeal diversity in the TP-CBW, TP-FA and TP-BA reactors was dominated by *Methanosarcina*, *Methanobacterium*, *Methanomethylophilaceae*, *Methanocuellous* and *Methanoplasma* with hardly any of the more stress sensitive *Methanosaeta*. Of particular note, was the metabolically flexible *Methanosarcina* which represented, by the end of the experiment, almost 70% of archaeal composition in these reactors. *Methanosarcina* is known to resist VFA inhibition (Vavilin *et al.*, 2008) and found to survive at harsh environmental conditions e.g. extreme pH, high ammonium, salt (Na^+), and acetate concentrations (De Vrieze *et al.*, 2012). However, an added burden (Mudhoo and Kumar, 2013) in these reactors and

documented in Figure 7-6 was the presence of higher levels of putatively toxic elements released during pre-treatments and in particular from the BA and FA wastes. On balance, it appears from both the process stability and archaeal community dynamics that there was a gradient in the magnitude of the negative impacts on these reactors in the order TP-FA>TP-BA>TP-CBW. In the TP-FA reactor which had failed as a biogas-producing reactor prior to day 60, it was noticeable that in addition to the dominance of the stress tolerant *Methanosarcina* there was a decline in sequences from the *Methanobacterium* but retention of *Methanoculleus. Methanoculleus spp.* have certainly been found in mesophilic digesters (Schnürer et al., 1999; Franke-Whittle et al., 2014; Westerholm et al., 2016), predominating over other hydrogenotrophic methanogens at extreme environmental conditions (i.e. high salt, ammonia and VFA concentrations). With respect to the TP-CBW and TP-BA reactors which did not fail, they both retained *Methanobacterium*. Moreover, similar to the TP-IBA reactor, the TP-CBW reactor also retained *Methanofastidiosum* on day 80 (Figure 7-9), as discussed above suggesting efficient syntrophic acetate oxidation and fermentation, which supported the stability of digestion process in this reactor. Furthermore, the presence of metals as well as the level of their concentrations was the key drive for shaping ecological functions of methanogens in the reactors of the current study. Metal concentrations in the digestate of reactors throughout 80 days of operation time (one digestate sample at the end of each HRT) were measured. Some significant correlations ($p < 0.05$) were detected between concentration of some metal elements and relative abundance of archaeal communities from sequencing data (Figure 7-6 and Figure 7-12).

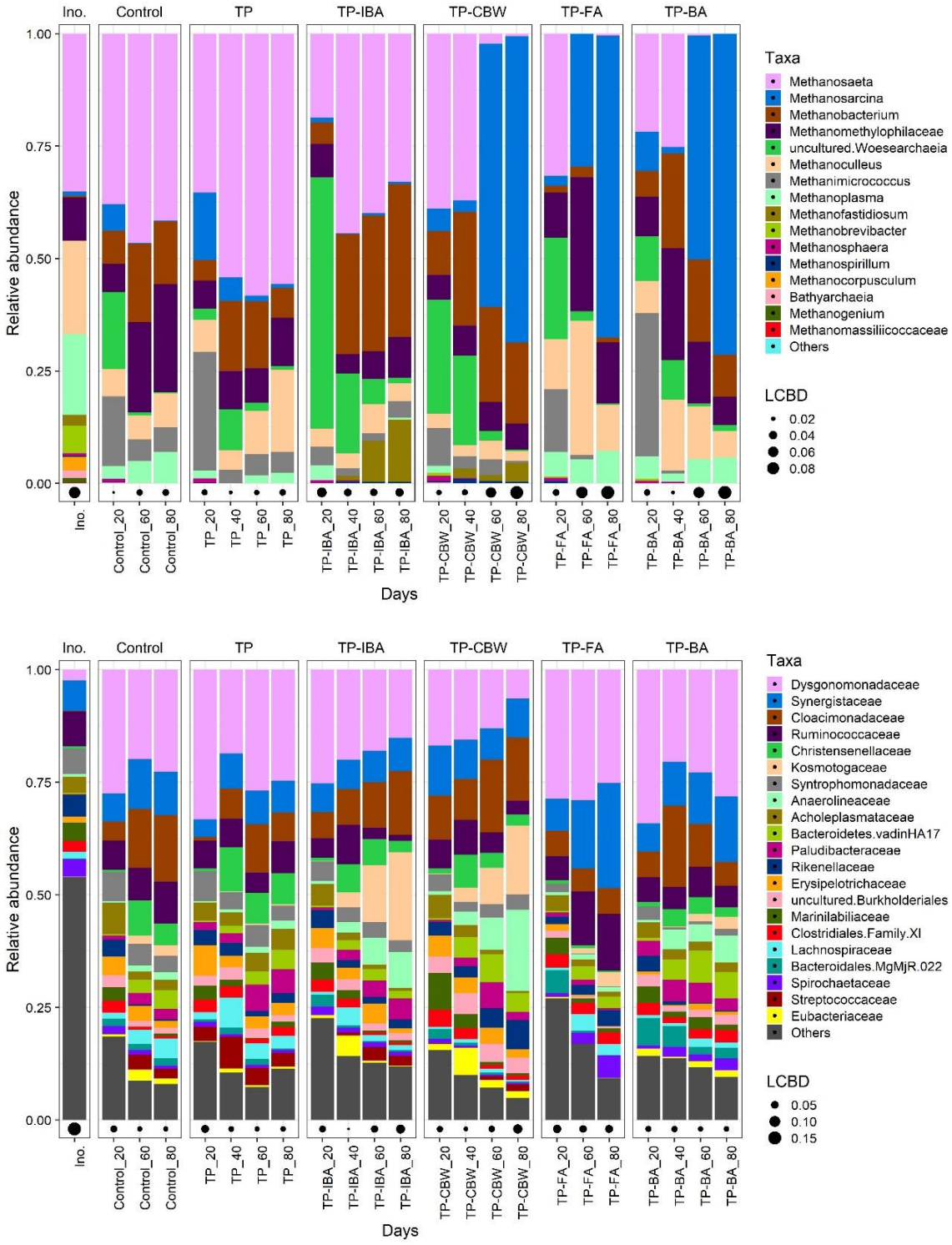


Figure 7-9. Taxonomic composition of archaeal genera (all genus taxa) and bacterial families (top 20 families taxa) in the CSTRs. LCBD = local contribution of beta diversity.

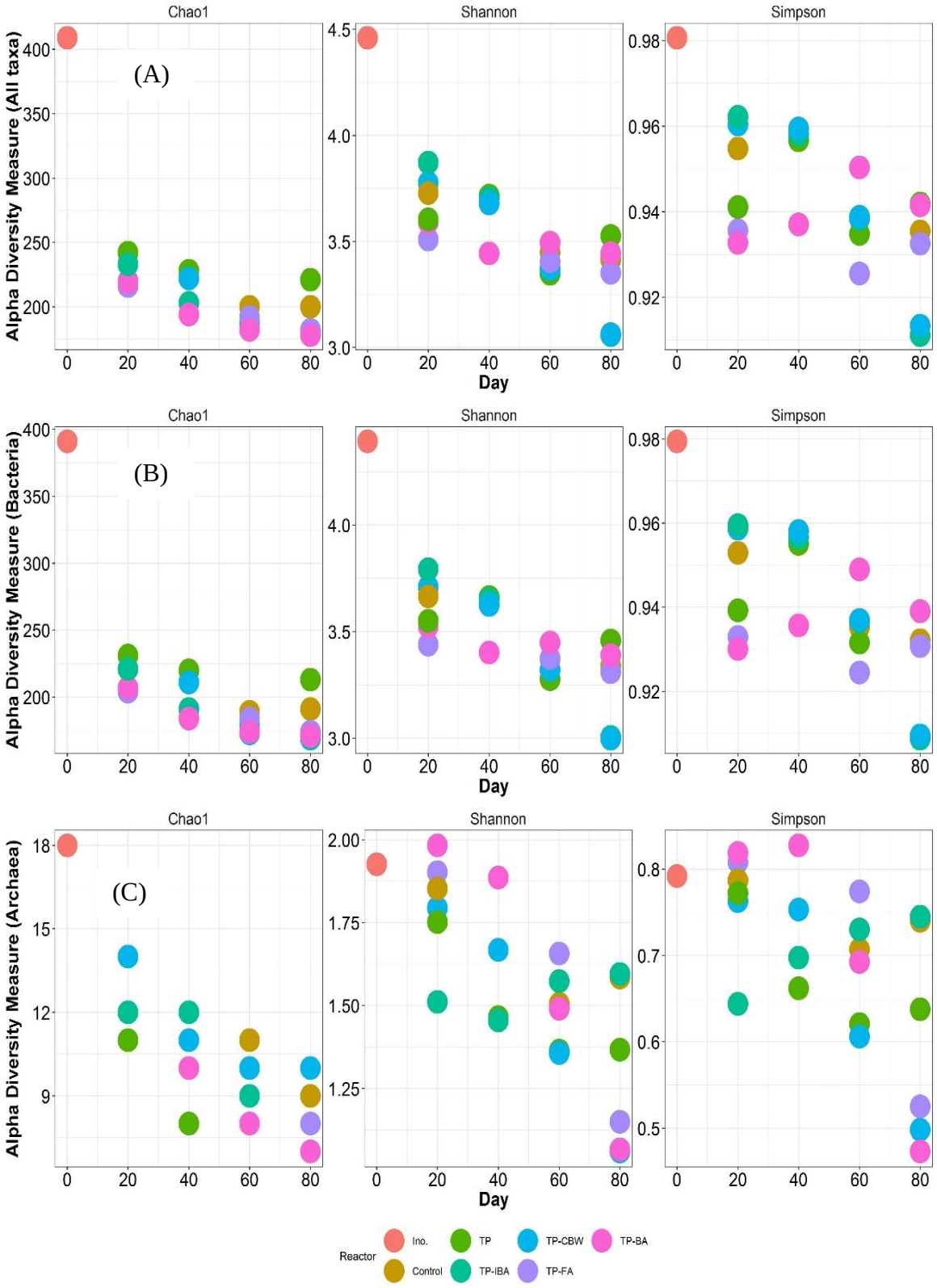


Figure 7-10. Alpha diversity for (A) All taxa (B) Bacterial taxa and (C) Archaeal taxa in CSTRs. The values are from the sequenced data of digestate samples were collected on the days shown on the x-axis.

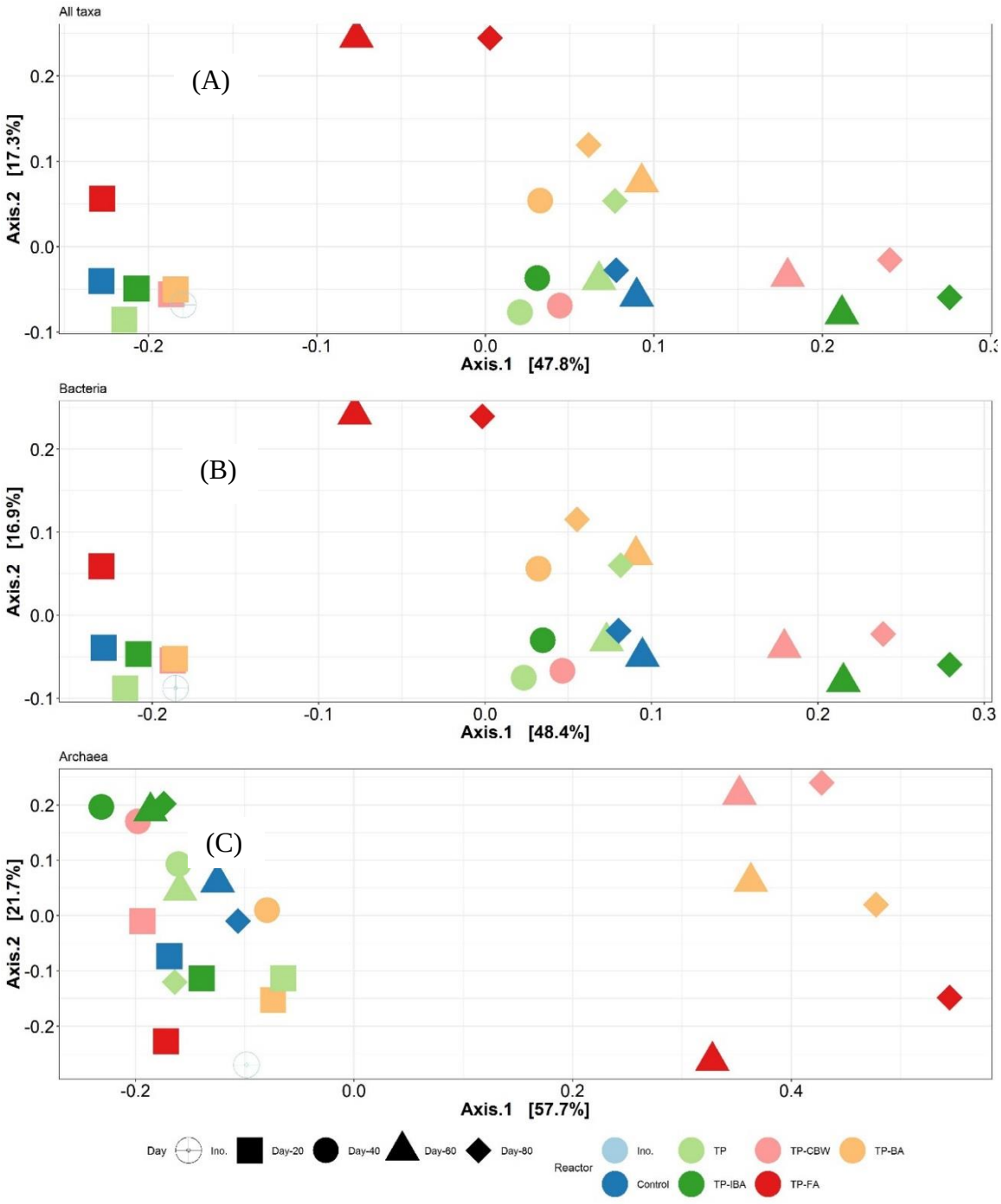


Figure 7-11. Multidimensional scaling analysis (MDS) on Unifrac distance for (A) All taxa, (B) Bacterial taxa (C) Archaeal taxa from 16S rRNA sequencing data of the startup inoculum (Ino.) and digestate samples from the CSTRs on days 20, 40, 60 and 80.

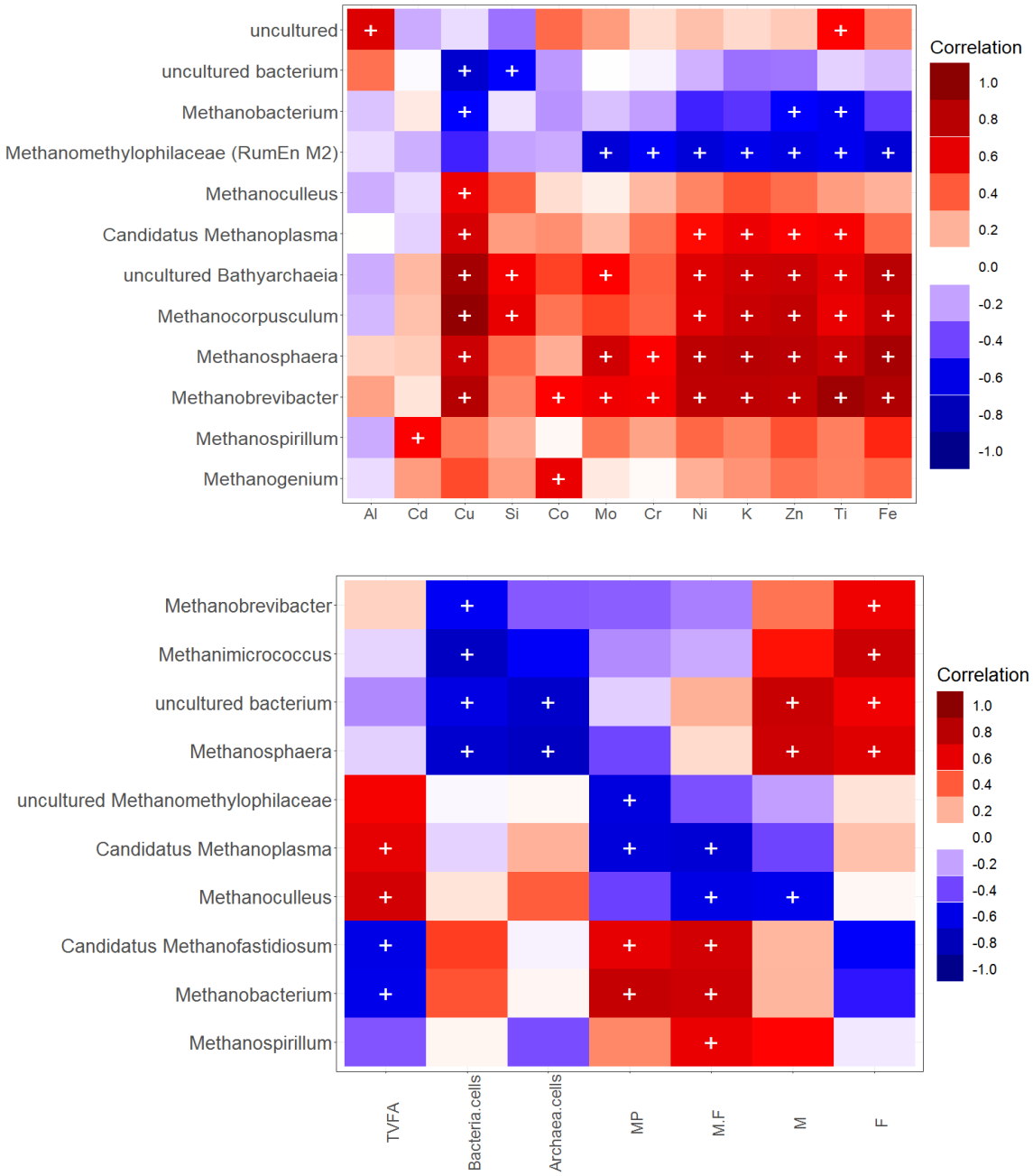


Figure 7-12. Pearson correlations between the archaeal community relative abundance and parameters measured in CSTRs. MP = methane production, M = total methanogenesis activity, F = total fermentation activity and M.F = methanogenesis to fermentation ratio (M/F). Bacteria and archaea cell numbers were estimated from their abundances from the qPCR analysis. The metal concentrations are the soluble metal concentrations in the digestate of reactors throughout this study.

7.4. Consideration for full-scale application of MW

Results of the microbial diversity suggested that the stable biodegradation performance of the TP-IBA reactor was most likely related to the presence of both acetoclastic and hydrogenotrophic pathways in this reactor. While in the reactors with lower methanogenic performance compared to TP-IBA reactor the metabolic pathways were mostly dominated by

one of the main metabolic pathways acetoclastic or hydrogenotrophic. The metabolic pathway in the control and TP reactors was mainly acetoclastic with low concentrations of metals present, but shifted to hydrogenotrophic in TP-MW reactors with high metal and VFA concentrations present (TP-CBW, TP-FA and TP-BA). Therefore, it is suggested that in full-scale digesters, to achieve a stable AD process, the environmental and operational parameters need to be adjusted to assure the presence of both acetoclastic and hydrogenotrophic microbial communities.

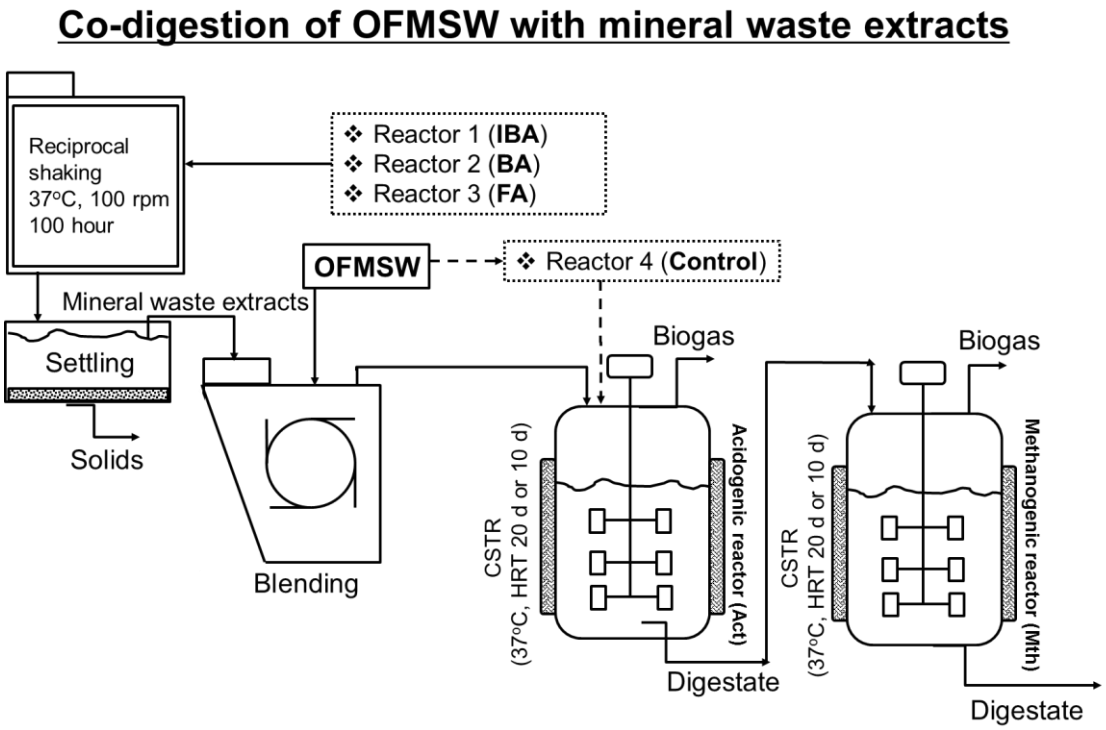
In current study, although a decrease in the pre-treatment time from 12 hours to one hour during only 20 days (between days 60 to 80) was not adequate to affect the microbial diversity, it revealed its positive effect on methanogenesis for all TP-MW reactors with draw-and-fill feeding method (DFFM). Therefore, in full scale digesters with TP-MW the startup period with a longer pre-treatment time (12 hours) followed by a shorter pre-treatment time (one hour) is recommended to avoid plausible overloading accumulation of metals released from MW. The integration method of MW in the AD of OFMSW in this study could resolve the limited effect (low methane production and yield) of MW when used with DFFM.

7.5. Conclusions

Thermal pre-treatment of organic waste with mineral wastes as TEs supplements at mesophilic temperature compensates for the trace element's deficiency of the OFMSW. Starting-up CSTRs with TP-MW found to be sufficient for adequate provision of the required TEs for robust AD. Among the four MW used in the current study (IBA, CBW, FA and BA) IBA was the most beneficial for AD, CBW was the second best followed by BA; FA was found the least suitable for pre-treatment of OFMSW. The microbial ecology and function of methanogenic populations developed at different TE compositions and concentrations showed that at low TE concentrations acetoclastic methanogenesis is the predominant pathway to methane. At moderate TE-concentration, a balance between acetoclastic and hydrogenotrophic metabolic pathways was observed whilst at high TE-concentration the metabolic pathway shifted towards to hydrogenotrophic pathway. No significant differences in methane production performance and process stability were observed between the TP and control reactors with and without pre-treatment at 37^{o}C. The results which obtained from the TP-MW reactors suggested that the stability of digestion processes in theses reactors were mainly related to the release of trace elements useful for methanogenesis rather than enhanced hydrolysis due to pre-treatment with these MW amendments.

Chapter 8. Use of dissolved extracts from municipal solid waste incineration ash in two-stage anaerobic reactors treating the organic fraction of municipal solid waste

Graphical abstract



Abstract

Previous experiments in Chapter 6 and Chapter 7 of this thesis showed positive effects of amending MW of MSWI plants in anaerobic digestion of the OFMSW, when they were amended directly to CSTR systems or they were used in the pre-treatment of the feedstock substrate before co-digestion in reactors. In this chapter, we investigated the effects of mineral waste extracts (MWE) on laboratory-scale two-stage anaerobic digesters treating synthetic organic waste. MWE was prepared as aqueous extracts from different ash samples, incineration bottom ash (IBA), fly ash (FA) and boiler ash (BA), taken from a municipal solid waste incineration plant. At 20 days hydraulic retention time, all three MWE stimulated hydrogen production in their respective acidogenic reactor by around 35% (c.f. control acidogenic reactor), whilst no difference was seen in the methane productivity of the linked methanogenic reactors (average 527 ± 45 mL CH_4/g VS, including control methanogenic reactor). Following a step reduction in hydraulic retention time from 20 to 10 days and doubling of organic loading rate from 2.5 g to 5 g VS/L. d, no significant change was seen in hydrogen production ($p > 0.05$) in acidogenic reactor amended with MWE from IBA and BA, or the control acidogenic reactor, however, acidogenic reactor receiving MWE from FA had 45% lower hydrogen productivity. The step change in hydraulic retention time and organic loading rates led to the failure of most methanogenic reactors (≤ 100 mL CH_4/g VS), however, the methanogenic reactor receiving feed containing MWE from IBA showed stable performance without signs of failure, and had higher volumetric methane productivity, albeit at lower methane yields (370 ± 20 mL CH_4/g VS). 16S rRNA analysis using the Illumina sequencing platform revealed acidogenesis by *Lactobacillaceae* in the acidogenic reactor and syntrophic acetate oxidation by *Synergistaceae* linked to enrichment of the *candidatus* genus *Methanofastidiosum*, in the stable methanogenic reactor receiving MWE from IBA.

8.1. Introduction

Capital costs of anaerobic digestion can be minimised through improvements in reactor performance such as high and continuous organic loading rate and maximum methane production if they lead to a decrease in reactor volume (short hydraulic retention time; HRT) and better process energy balance (Cecchi *et al.*, 1991; Ward *et al.*, 2008). Previous studies have applied different methods for increasing the efficiency of single-stage anaerobic reactors (Lo *et al.*, 2009; Banks *et al.*, 2012; Serna-Maza *et al.*, 2015). However, low organic loading rates (OLR; 1– 4 g VS/L. d) are still one of the limitations of single-stage anaerobic reactors in wet AD (< 5% TS) (Nagao *et al.*, 2012). For semi-dry (10 – 20% TS) and dry (20 – 40%

TS) anaerobic digestion despite higher OLRs of 7 – 15 g VS/L. d it is low levels of VS reduction (31– 48%) and methane yields (140 - 314 mL/g VS) which are the main limitations (Dong *et al.*, 2010; Nagao *et al.*, 2012).

In simpler single stage AD reactors, volatile fatty acid (VFA) produced from the fermentation stage accumulates quickly therefore methanogens need a longer HRT to convert the accumulated VFA to biogas (Aslanzadeh *et al.*, 2014). Moreover, methanogenic archaea are more sensitive to VFA accumulation and a consequent pH drop than hydrolytic/acidogenic bacteria, which might usually lead to irreversible acidification, which causes inhibition of methanogens and the failure of anaerobic digesters. For this reason, in full-scale digesters, some none-feeding periods (intermittent feeding) are arranged to allow methanogens to consume the accumulated VFA before the irreversible acidification of digesters occurs (Nagao *et al.*, 2012).

In contrast to the acidification problems encountered for single stage digestion during AD of easily hydrolysable feedstocks like food waste with two-stage anaerobic reactors , separation of the acidogenic stage from the methogenic stage is reported to improve the stability of AD (Ward *et al.*, 2008). Two-stage anaerobic reactors provide separate favourable environments (pH and nutrients) for acidogenic bacteria and methanogenic archaea (Aslanzadeh *et al.*, 2014). Further advantages of separating the acidogenic stage from methanogenic stage include the easy selection and enrichment of different bacteria/archaea in each stage, increasing process stability, and the possibility of operation at a higher OLR with shorter HRT (Jung *et al.*, 2000; Demirer and Chen, 2005; Wang *et al.*, 2014a). Due to the possibility of AD at shorter HRT in two-stage reactors compared to the single-stage reactors, a smaller rector volume is required. FOR instance, Aslanzadeh *et al.* (2014) obtained 65% decrease in the reactor volume for digesting the OFMSW when they used two-stage reactors instead of single-stage reactors.

Previous studies have investigated the optimization of two-phase anaerobic processes for hydrogen and methane production. Reject water as well as sludge recirculation are common methods which have been applied for optimizing the acidogenic stage of two-stage anaerobic reactors. For instance, Cavinato *et al.* (2011) and Kobayashi *et al.* (2012) obtained a better optimization of two-stage pilot scale reactors by reject water and sludge recirculation to buffer the system and keep the pH around 5.5 optimal for the acidogenic reactor, this method, consequently increased the hydrogen production rate and methane yield.

Previous experiments in Chapter 6 and Chapter 7 of this thesis showed positive effects of amending MW of MSWI plants in anaerobic digestion of the OFMSW. The amendment of MW in AD was through their addition directly with the feeding substrate to reactors and through their use in the pretreatment of the feedstock substrate before AD. The current study in this chapter, investigated the use of water-extracted minerals from MW (MWEs) to optimize the digestion processes of two-stage AD reactors of OFMSW. For this, the water MWE obtained from MW (Section 4.1.3) of MSWI plant (incineration bottom ash (IBA), fly ash (FA) and boiler ash (BA)) were used to prepare feeding substrates of acidogenic reactors, digestates obtained from these acidogenic reactors were then used as the feeding substrates for subsequent methanogenic reactors. The hypothesis was that the moderate alkalinity provided by the MW (Chapters 4-6) would support the buffering capacity in the acidogenic reactors to maintain optimal pH (4.5 – 6) required for acidogenic bacteria (Yu *et al.*, 2002; Cavinato *et al.*, 2011). Subsequently, the dissolved metals provided in the MWE are still present in the digestate of acidogenic reactor and the principle benefit of the dissolved elements will be carried over and be of most benefit in the methanogenic reactor and will increase the methane production rate and process stability of the methanogenic reactors.

The novel aspect of this study was to investigate the feasibility of using very low economic value, and widely available, waste materials such as MW to stimulate bacterial hydrolysis and increase microbial growth and activity in anaerobic digesters, with the ultimate aim of increasing the OLR whilst maintaining the methane production efficiency of anaerobic reactors. The hypothesis was that the moderate alkalinity provided by the MW would support the buffering capacity in the acidogenic reactors to maintain optimal pH (4.5 – 6) required for acidogenic bacteria (Yu *et al.*, 2002; Cavinato *et al.*, 2011). Subsequently, the dissolved metals provided in the MWE and still present in the acidogenic digestate and carried over to the methanogenic reactor will sustain methane production and process stability.

8.2. Materials and methods

8.2.1. *Substrate and inoculum*

The feedstock substrate was the synthetic organic waste (SOW) described previously (Section 4.1.1), and the feedstock characteristics are shown in Table 4-3. The inoculum was obtained from a mesophilic (37^{o}C) digester (Table 4-4). Before starting the continuous experiments (CSTR) and in order to kill the maximum possible number of methanogen species, the inoculum used for the acidogenic reactors (Act) was boiled at 100^{o}C (Rogers, 1986; Valdez-Vazquez *et al.*, 2005) for one hour, left to cool down to room temperature, and then had its pH

adjusted to pH 6 ± 0.5 using 1N HCl solution. The methanogenic (Mth) reactors were filled with 1 L of the inoculum (Table 4-4) without boiling. Before staring the experiments, the inoculum in the reactors (acidogenic and thermophilic reactors) were reactivated and acclimated to the digestion environments and the feedstock substrate (SOW) for 20 days as described in Section 4.1.2.

8.2.2. *Preparation of the feeding substrate*

The MW used in this chapter were only the incineration ash (i.e. IBA, FA, and BA) however, the CBW (i.e. the MW which was from CDW) was not used in this chapter due to the limited resources available for this study, and it was difficult to operate and manually feed more than 8 continuous reactors (CSTRs) in a single experimental. The same orbital incubator, which was used pre-treating the substrates in Chapter 7 (Section 7.2.4.1), it was also used for metals extraction in this chapter. The metals were extracted from the MW by reciprocal shaking (100 rpm, 100 hours and 37^{o}C) of 20 g of each MW in 500 mL of distilled water, left to settle for one hour then the supernatant discarded, hereafter it named as mineral waste extracts (MWEs). From each run of the metals extraction, about 3 L of MWEs were produced (which was enough for preparing 300 g VS of the SOW). The feeding substrates of the MW amended Act reactors (referred to hereafter as the Act_IBA, Act_FA and Act_BA reactors) were prepared by using these MWEs and it referred hereafter as the SOW-MWE feeding substrate. Distilled water was used for preparing the feeding substrate of the control Act reactor (referred hereafter as the Act_control reactor), and the control substrate was referred to as SOW-DW feeding substrate. Each batch of the feeding substrate for each reactor was composed of 50 g VS of the SOW made up of 500 mL with MWE /or DW to give a mixture of a feeding substrate with solid contents of 5 g VS/50 mL. The substrates were mixed thoroughly then stored at 5^{o}C until use.

8.2.3. *Reactor setup and operation*

The continuous AD experiments were carried in four sets of one-litre CSTRs (Section 4.3.2). Each CSTR set consisted of a one Act reactor and a one Mth reactor and each reactor was fed every two days. After reactivation and acclimation of the inoculum inside reactors (Section 4.3.2), the CSTR were operated for 90 days in three stages. During the first stage (day 1 - 40), the OLR of CSTR was of 2.5 g VS/L. d with an HRT of 20 days, requiring the draining of 50 mL/d of digestate from the Act reactors and replacing with an equal volume of the feedstock substrate (SOW-MWE/ or SOW-DW); Section 8.2.2). Simultaneously, 50 mL/d of digestate was drained from each Mth reactor then replaced (fed) with the digestate (50 mL/d) which

was drawn from the Act reactors. The same feeding method was applied for the second stage (day 40 – 60), during this stage, the organic load was maintained at 2.5 g VS/L. d; however, the HRT was decreased to 10 days.

Table 8-1. Feeding design of CSTR systems

Day	OLR	HRT	Feed (mL)	Act_ Control	Act_ IBA	Act_ FA	Act_ BA	Mth_ Control	Mth_ IBA	Mth_ FA	Mth_ BA
Day 1-40	2.5 g VS/L. d	20 d	DW	_	_	_	_	_	_	_	_
			MWE	_	_	_	_	_	_	_	_
			SOW-MWE	_	50	50	50				
			SOW-DW	50							
			Digestate-rem	50	50	50	50	50	50	50	50
			Digestate-add	_	_	_	_	50	50	50	50
Day 41-60	2.5 g VS/L. d	10 d	DW	50	_	_	_	_	_	_	_
			MWE	_	50	50	50	_	_	_	_
			SOW-MWE	_	50	50	50				
			SOW-DW	50	_	_	_				
			Digestate-rem	100	100	100	100	100	100	100	100
			Digestate-add	_	_	_	_	100	100	100	100
Day 61-90	5 g VS/L. d	10 d	DW	_	_	_	_	_	_	_	_
			MWE	_	_	_	_	_	_	_	_
			SOW-MWE	_	100	100	100	_	_	_	_
			SOW-DW	100							
			Digestate-rem	100	100	100	100	100	100	100	100
			Digestate-add	_	_	_	_	100	100	100	100

DW =distilled water.
MWE= mineral waste extracts.
SOW-MWE = feed prepared with mineral waste extracts.
SOW-DW = feed prepared with distilled water.
Digestate-rem = digestate removed.
Digestate-add = digestate added.

During this second stage, 100 mL/d of digestate was drawn from the Act reactors and then fed to the Mth reactors after draining 100 mL of the Mth digestate, the Act reactor was fed with 50 ml of the SOW-MWE/ or SOW-DW substrate and 50 ml of either MWE (for the MW amended Act reactors) or DW (for the control reactor). For the operation period between days 60 - 90, the HRT was maintained at 10 days while the OLR was increased to 5 g VS/L. d. During this stage, 100 mL of digestate from the Act reactors was drained and replaced with 100 mL of the feeding substrate. The 100 mL of digestate, which was drained from the Act reactors was fed to the Mth reactors after draining 100 mL of the Mth digestate (Table 8-1).

8.2.4. *Analytical methods*

Analytical methods were performed as described previously (Section 4.4). The biogas produced from the Act and Mth reactors was collected in 5 L gasbags (Tedlar, VWR), the Act gasbags were disconnected once per HRT, while the Mth gasbags were disconnected every two days to measure the biogas volumes and compositions (methane and hydrogen contents) by a gas chromatograph (GC)(Section 4.4.7). On day 90 for all experiments, samples of digestates were collected from the Act and Mth reactors to be used for the microbial analysis as described in Microbial analysis (Section 4.5).

8.3. Results and discussion

8.3.1. *Performance of acidogenic reactors*

The pH of the start-up inoculum of the Act reactors was initially pH 6 ± 0.3 but decreased to pH 5 ± 0.3 within 10 days remained approximately constant up to day 25 (Figure 8.1), then the pH decreased gradually to pH 4 ± 0.5 on day 60. Indicating that the alkalinity in the Act reactors was not sufficient to buffer the acids (VFA) produced (VFA not measured in the Act reactors but could be inferred from the sCOD and drop in pH). However, when the OLR was increased to 5 g/L i.e. after day 60, the pH recovered (pH 4.5 ± 0.5) in the Act reactors. In two-stage anaerobic reactors ammonia is a major contributor to the alkalinity (Qin *et al.*, 2018), therefore this increase in the pH of the Act reactors was likely linked to the NH_3-N released by the hydrolysis of the feedstock substrate (SOW). The low C/N ratio (13.8; Table 4-2) of the organic substrate (SOW) was principally due to the high levels of protein present which is degraded to ammonia during AD (Kayhanian, 1994).

The performance summary of the Act and Mth reactors are shown in Table 8-2 and figures Figure 8.1 to Figure 8-5. Hydrolysis of the SOW and production of sCOD were not significantly different ($p > 0.05$) in the Act reactors (Figure 8-5), and the concentration of soluble COD (sCOD) in the Act reactors was very similar and maintained at 23 ± 6 g/L. Higher accumulation of hydrogen compared to methane in acidogenic reactors is a sign of rapid fermentation of the feeding substrate (Parawira et al., 2008). Up to day 60 (HRT of 20 days and OLR of 2.5 gVS/L. d) the accumulation of hydrogen and methane in the gasbag of the control reactor was similar to that in the reactors amended with MWE, except the Act_BA rector which gave up to 50% higher hydrogen and methane accumulation (Table 8-2). Indicating that that fermentation in the Act_BA was higher than other reactors. The H_2% and CH_4% of biogas for the Act_control reactor were generally similar to that of the Act reactors with MWE, on day 60, the H_2% of the Act_control was 25 ± 8% whereas the average H_2% of

the Act reactors amended with MWE was 24 ± 3%. An obvious increase in the H_2% of biogas was observed in the Act_IBA and Act_BA reactors Table 8-2. The lowest H_2% was in the Act_FA reactor, the minerals released by the FA were inhibitory/ or were less stimulatory to the acidogenic bacteria (Figure 8-4). This was evident from the metal analysis data shown in (Table 4-8) which shows higher concentrations of dissolved metals such B, Na, Pb, Zn etc. in the FA extracts compared to the IBA and BA extracts.

8.3.2. *Performance of methanogenic reactors*

The pH of the Mth reactors were also not significantly different (pH 7.1 ± 0.2; $p > 0.05$) with reactor operation at an HRT of 20 days (days 1- 40) or 10 days (days 40 - 60) and an OLR of 2.5 g VS/L. d. However, increasing the OLR to 5 g VS/L. d at the HRT of 10 days (days 60-90), led to the pH drop linked to accumulation of VFA which consumed most of the alkalinities available in all the Mth reactors except in the Mth_IBA reactor (Figure 8.1 and Figure 8-5). It worth noting that the CH_4% of biogas on day 60 (OLR = 2.5 g VS/L. d and HRT of 10 days) remained ~ 70% in all the Mth reactors, this was comparably higher than the CH_4% values obtained from the CSTRs in the previous chapters (60 - 65% of CH_4) of this thesis. Indicating, beneficial effects of using two-stage reactors over one-stage reactors in the AD of OFMSW. Two-stage AD allow the selection and enrichment of different microorganisms in each stage due to different operational parameters such as pH in each stage (De La Rubia *et al.*, 2009). Moreover, degradation of the protein component of the substrate to ammonia in the acidogenic stage may act as metabolic buffer preventing sudden pH drop in the methanogenic stage (Solera *et al.*, 2002; De La Rubia *et al.*, 2009).

Despite a decrease in the HRT to 10 days (2.5 gVS/L. d) from day 40, the methanogenic reactors showed a gradual increase in methane production up to day 50, then methane production stabilised between days 50 to 60. During the pseudo steady state period (day 50-60) similar CH_4% (~ 70%) and methane yields were obtained from both the methanogenic reactors amended with MWE (527 ± 45 mL CH_4/g VS) and the control reactor (523 ± 47 mL CH_4/g VS), and differences in methane yield between the Mth reactors never exceeded 10% (Figure 8-3). However, increasing OLR to 5 g VS/L. d at HRT to 10 days after day 60, led to a rapid drop of pH in the Mth_control, Mth_FA and Mth_BA reactors, consequently the methanogenesis process deteriorated in these reactors due to acidification and hence inhibition of methanogenic activity. This acidification and likely inhibition of methanogenic activity lead to the failure of these reactors (Figure 8.1, Table 8-2, Figure 8-3, Figure 8-4 and Figure 8-5).

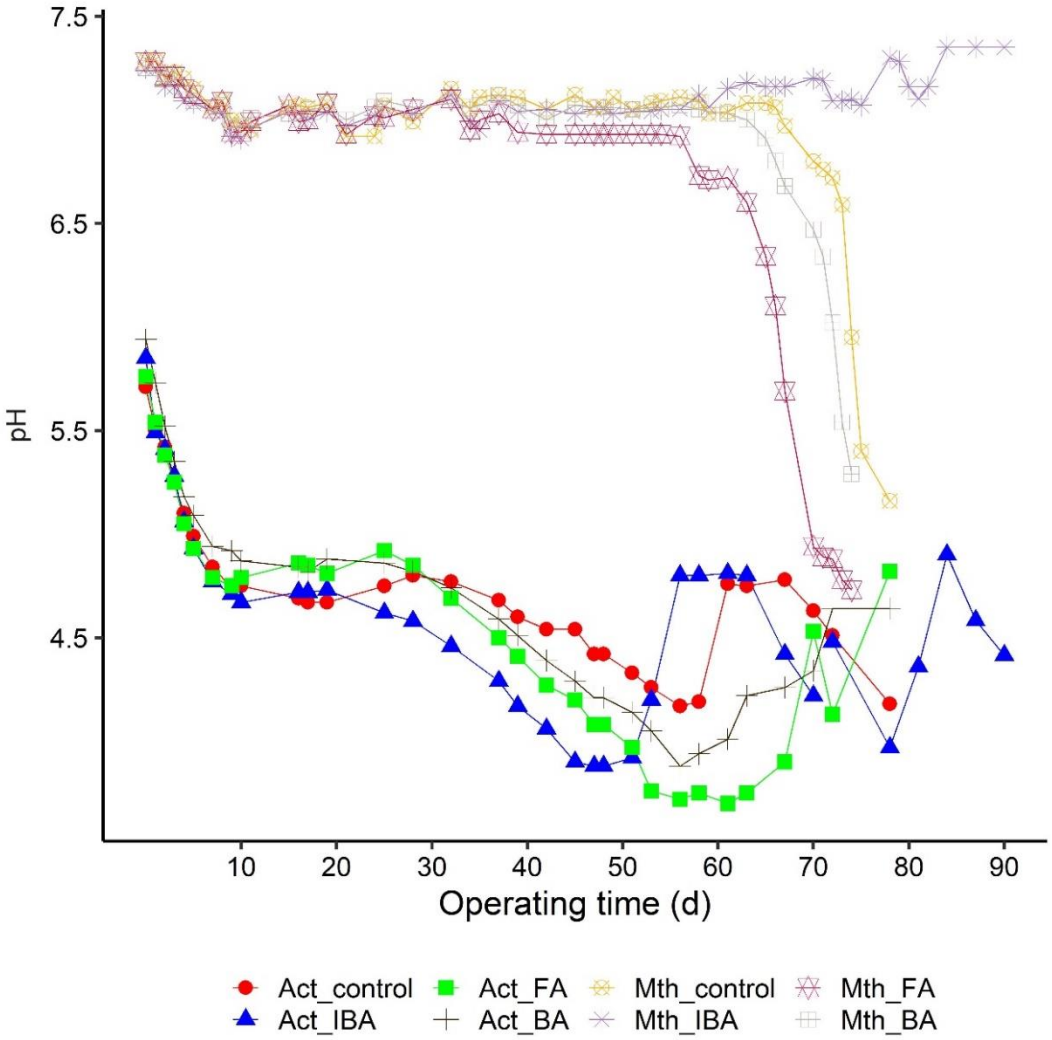


Figure 8-1. Profile of pH in the acidogenic and methanogenic reactors.

Table 8-2. Profile performance of two-stage anaerobic reactors.

Time	Day 60				Day 90			
Methanogenic reactors	Mth_ control	Mth_ IBA	Mth_ FA	Mth_ BA	Mth_ control	Mth_ IBA	Mth_ FA	Mth_ BA
OLR (g VS/L. d)	2.5	2.5	2.5	2.5	5	5	5	5
HRT (d)	10	10	10	10	10	10	10	10
pH	7.04	7.15	6.72	7.03	5.16	7.35	4.7	5.29
sCOD (mg/L)	1515	1305	1545	1245	5805	345	17415	9255
Total VFA (mg/L)	589	465	1170	589	4490	838	6565	4324
Total ALK (mg/L)	2575	3000	2250	2975	625	4250	300	400
MPR (L/L. d)	1201	1319	1053	1249	40	2300	30	170
Y (mL/g VS)	423	572	482	526	4	370	5.2	90
CH_4% (%)	67.5	70	64	64	17	70	22	40
Total CH_4 (L)	46.5	48	44	43	64	91	50	56
RFI	5118	4422	1281	3699	2445	8161	1293	1875
Acidogenic reactors	Act_ control	Act_ IBA	Act_ FA	Act_ BA	Act_ control	Act_ IBA	Act_ FA	Act_ BA
pH	4.76	4.81	3.7	4.0	4.5	4.5	4.4	4.6
H_2% (%)	23	33	26	36	23	32.6	13	36
CH_4% (%)	5	5	25.6	5	5	5.1	13.5	5.1
Total H_2 (mL)	1529	1175	1349	2216	3284	2897	1695	3175
sCOD (mg/L)	20940	21810	24210	20250	23490	26520	28170	27420
RFI	684	1117	801	806	724	1168	1446	955

sCOD = soluble COD.
total VFA = total volatile fatty acids.
total ALK = total alkalinity.
MPR =methane production rate.
Y= methane yield.
RFI= relative fluorescence intensity of F_{420}.
H_2% of biogas collected in gasbags attached to Act reactors.

Interestingly, among all the Mth reactors, the Mth_IBA reactor showed a stable methanogenesis process despite of doubling the OLR (5 g VS/L. d) and decreasing HRT to half (10 days). After day 60, digestion of the Mth_ IBA reactor gave up to a three-folds higher methane yield (370 ± 20 mL CH_4/g VS) than the control (≤ 100 mL CH_4/g VS) (Figure 8-3). Inhibitory and toxic effects of heavy metals on the acid-phase of two-phase anaerobic digestion processes have been investigated in the literature (Demirel and Yenigün, 2002). However, in the current study, the successful methanogenic activity of the Mth_IBA reactor was likely associated with the mineral composition of this MW which were mostly within the stimulating ranges for AD (Chapter 6). Moreover, in general, the acidogenic and methanogenic processes of both of the Act_FA and Mth_FA reactors were lower than the

other MW amended reactors. This behaviour of the FA amended reactors in the current chapter of this study was similar to that observed from FA in the co-digestion and pre-treatment chapters in this thesis (chapter 5, 6 and 7). Indicating that, the FA as a MW was a detrimental MW for integrating in the AD of OFMSW due to the high concentration of metals leached from FA and appeared toxic to anaerobic microorganisms. For instance, the dissolved concentrations of Cr, Mg and V (9.6, 42.0 and 3.44 mg/kg TS respectively) in the FA extract was higher compared to their concentrations in the IBA and BA extracts and appeared toxic to anaerobic microorganisms (Chapters 5, 6 and 7).

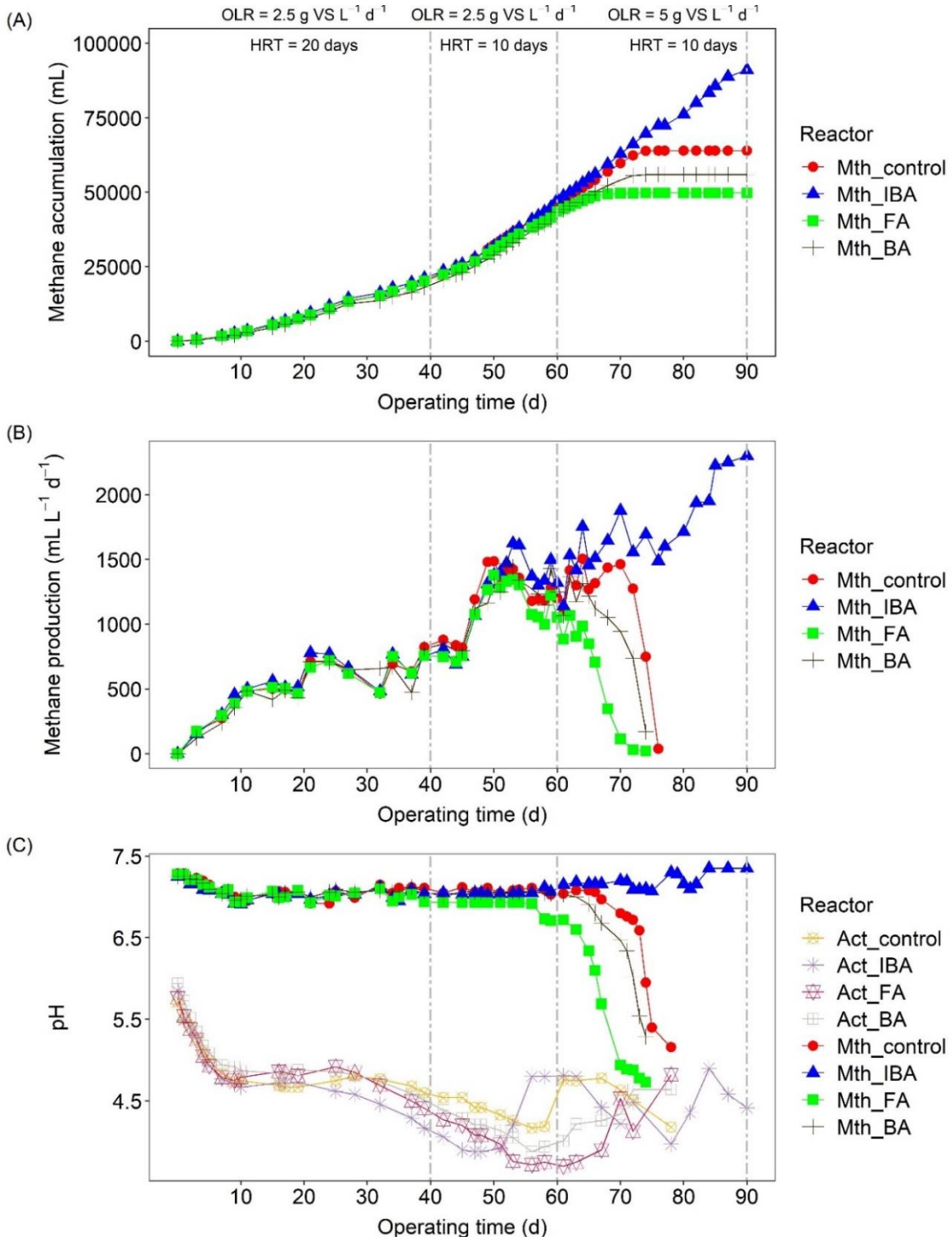


Figure 8-2. Profile of methane accumulation (throughout 80 days of the operation time) and methane production rate from methanogenic reactors in parallel to the variations in the pH of the acidogenic and methanogenic reactors.

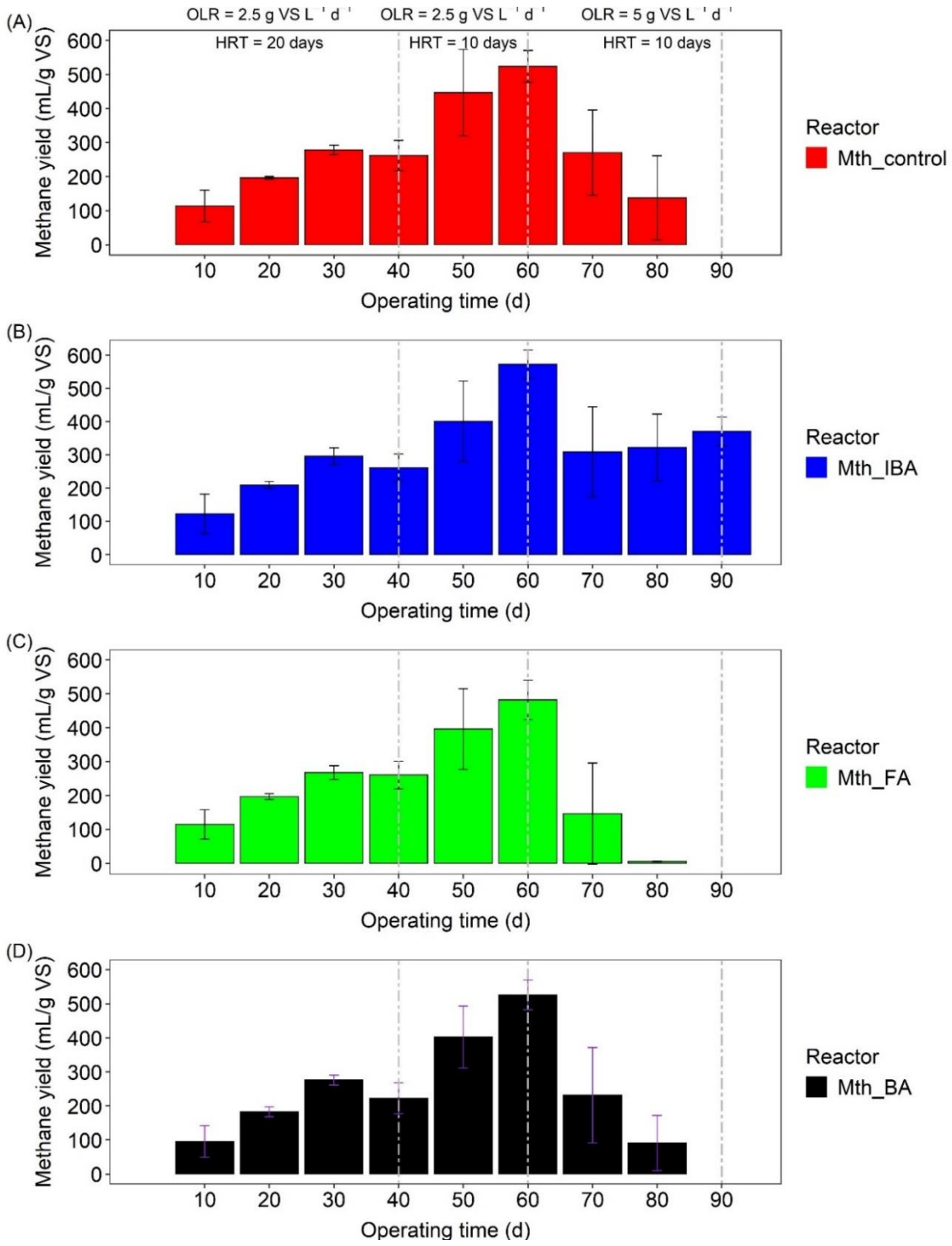


Figure 8-3. Variations in the methane yield from methanogenic reactors. The values are average of 10 days over time intervals and the error bars are showing the standard deviation of the average values.

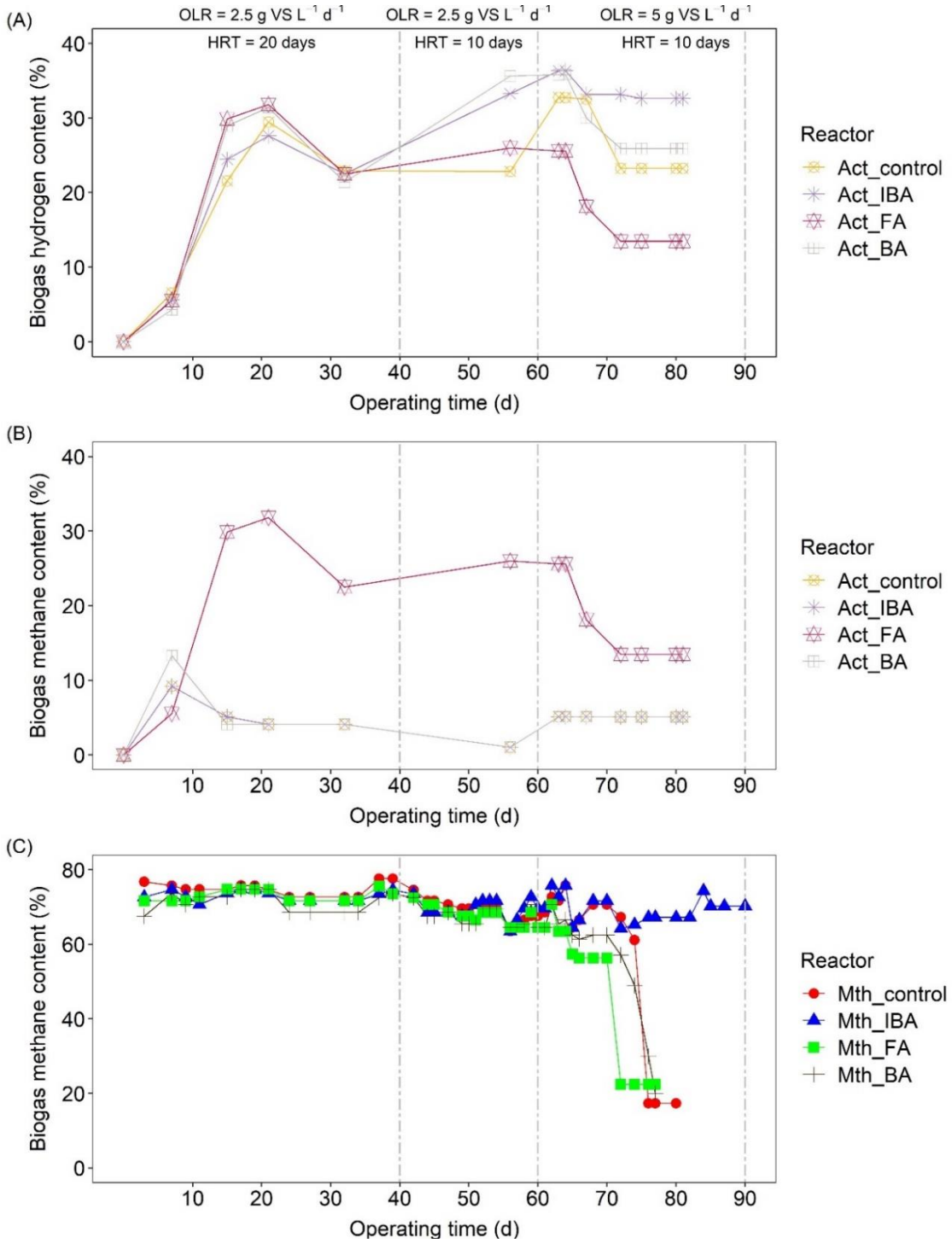


Figure 8-4. Variations in the hydrogen and methane percentage of biogas (by volume) from gasbags were connected to the acidogenic and methanogenic reactors.

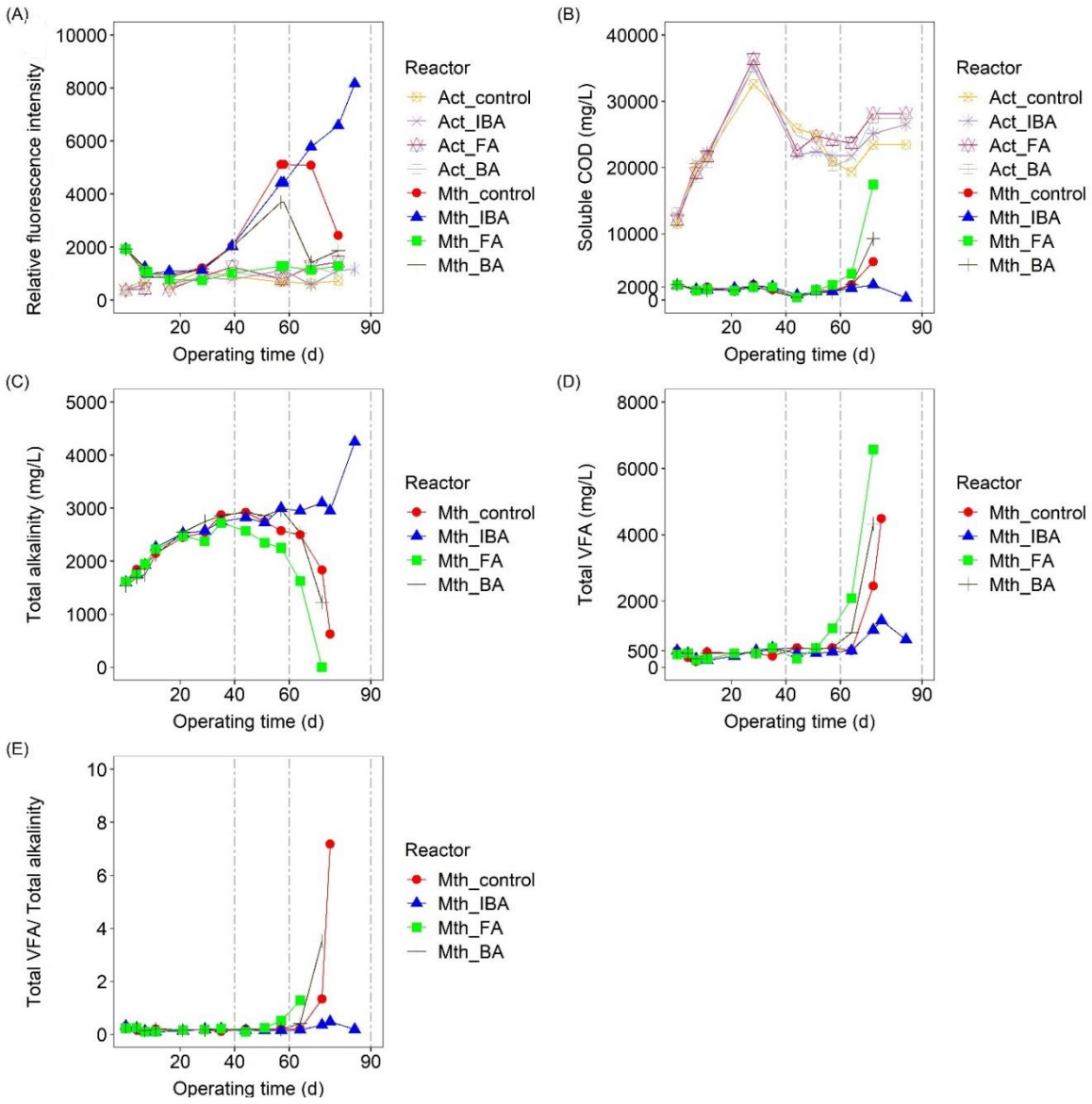


Figure 8-5. Physicochemical parameters from digestate of acidogenic and methanogenic rectors.

8.3.3. *Microbial population characterisation*

Microbial analysis was conducted on digestate samples collected from the four Act and four Mth reactors on day 90. The total number of reads in 16S rRNA sequence libraries after quality filtering was 639,199 ranging from 43,432 sequences in the smallest library to 109,999 sequences in the largest. However, individual libraries were rarefied for comparative analysis. Pipeline analysis of the 16S rRNA amplicon sequences from these samples identified a total of 1347 taxa, ~ 97.8 % (1318 taxa) of which were bacterial and ~ 2.2% (29 taxa) of which were archaeal. Bacterial proportion represented ~ 99.95% of the reads in the Act reactors, while in the Mth reactors the proportion of bacteria reads: archaea reads were 95% : 5%, 80% : 20%, 96% : 4% and 95% : 5%; in the Mth_control, and Mth_IBA, Mth_FA and Mth_BA reactors respectively. Indicating higher relative proportion of archaea reads in

the Mth_IBA reactor compared to the other Mth reactors. This high proportion of archaea in the Mth_IBA reactor coincided with the high relative florescence intensity (RFI) of co-enzyme F_{420} in this reactor (Table 8-2). For instance on day 90, the average RFI in the Mth_control, Mth_FA and Mth_BA was 1871 ± 576 nm compared to 8161 nm in the Mth_IBA reactor (i.e. RFI in the Mth_IBA reactor was about 3.36 folds higher than the average RFI in the other reactors).

Figure 8-6 shows the alpha diversity measures of Chao1, Shannon, Simpson for each reactor. For both bacteria and archaea, the richness in the Mth reactors was higher than that in the Act reactors. Interestingly, the Mth_IBA reactor, which showed higher performance among all the methanogenic reactors, had lowest bacterial and archaeal richness compared to the other reactors. The possible reason for this is that the Mth_IBA reactor was the only reactor which was performing by day 90. Such a behaviour was similar to that observed in the other chapters of this thesis (Chapter 6, Chapter 7 and Chapter 9) where the rectors with high process stability and methane productivity showed gradual decrease in alpha diversity with time. The high alpha diversity in other reactors (Mth_control, Mth_FA and Mth_BA) which showed a decline in performance after day 60 and failed on day 90 this might have increased alpha diversity in these reactors as there was no strong selection pressure.

In AD, such a decrease in the alpha diversity is usually associated with a decrease in relative abundance of archaeal community rather than the bacterial community. Yi *et al.* (2014) studied the performance of anaerobic reactors digesting food waste based on differences in total solids in the reactor feed; they demonstrated that an increase in reactor performance was associated with increases in the relative abundance of archaeal community in sequence libraries. For instance, the richness of *Methanosarcina* related sequences showed an increase with the increases in the total solid contents. However, the changes in the relative abundance of bacterial taxa were not consistent and showed a fluctuating trend in response to increases or decreases in the performance of these reactors.

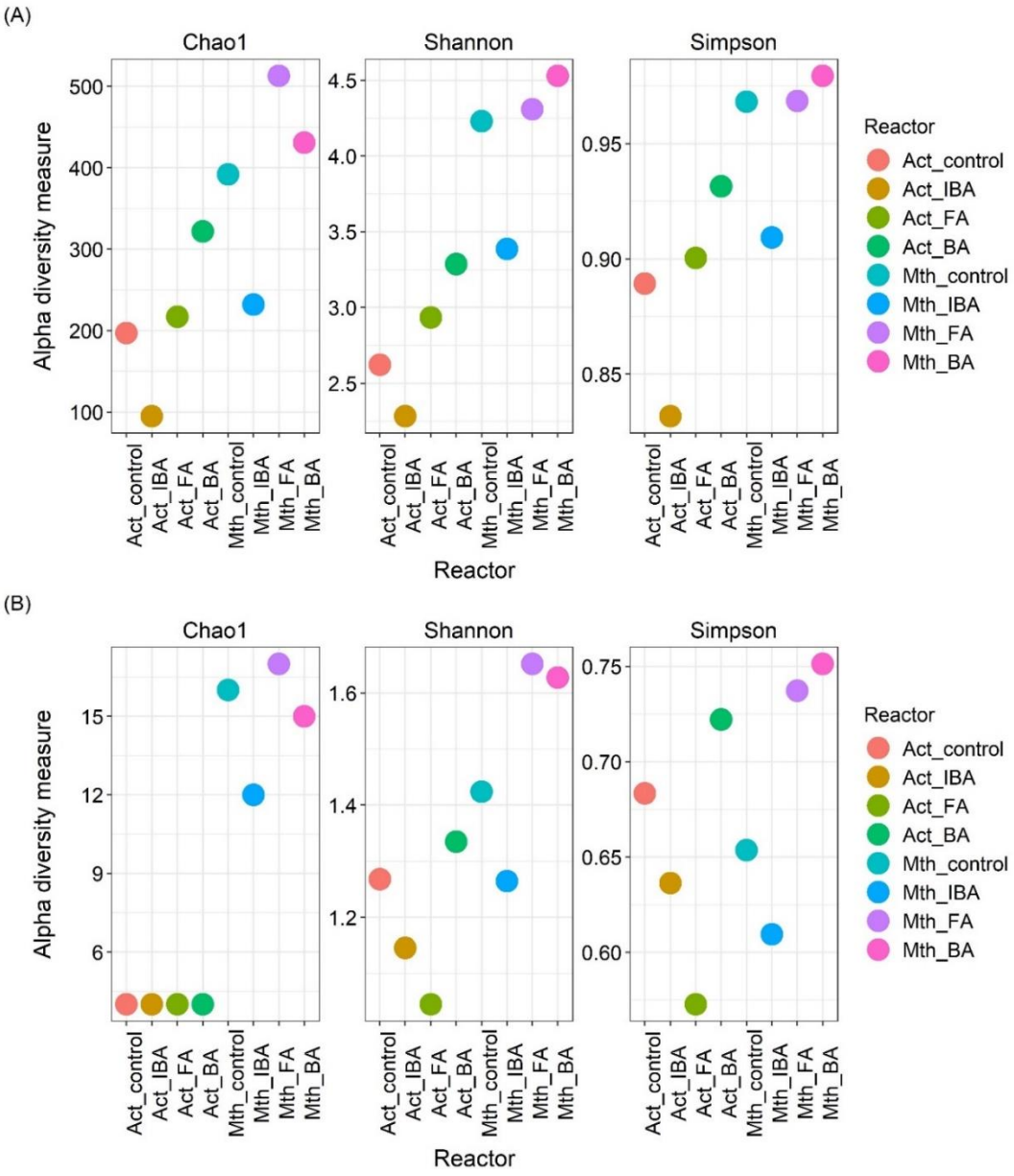


Figure 8-6. Alpha diversity of microbial community bacteria (A) and archaea (B) in relation with reactor stages and feeding substrate (Act = acidogenic reactor, Mth = methanogenic reactor, IBA = incineration bottom ash, FA = fly ash and BA = boiler ash) in eight AD reactors digested a synthetic organic waste as mono substrate or prepared with a mineral waste extracts.

8.3.3.1. *Composition of bacterial community*

Based on the operational data presented in Figure 8-1 and Table 8-2, namely, elevated sCOD concentrations at a low pH, it can be predicted that the Act reactor bacterial sequence libraries are dominated by taxa with a principally hydrolytic and acidogenic function. On this basis, the three major bacterial families observed (Figure 8-7), namely, the *Ruminococcaceae*, *Clostridiaceae* and *Lactobacillaceae* can be confidently linked to this role. Collaboratively, the bacterial family *Ruminococcaceae* are known fermenters and their role in AD (including the genera *Fastidiosipila* and *Ercela* present) is widely documented and linked to the

cellulolytic digestion of plant fibres (Ziganshin *et al.*, 2013), specifically, the degradation of hemicellulose (van Gelder *et al.*, 2014; Pandit *et al.*, 2016; Cai *et al.*, 2018). With respect to the high abundance of *Lactobacillaceae*, their presence suggests that the reactors were able to efficiently convert lactic acid produced by these bacteria to acetate in subsequent AD steps. It is postulated (Detman *et al.*, 2018) that lactate oxidation to acetate as a substrate for methanogens is the most energetically attractive process in comparison to butyrate, propionate, or ethanol oxidation.

Obviously, the bacterial taxa selected in the Act reactors are then physically transferred to the linked Mth reactors and to varying extents can be observed in these reactors (Figure 8-7). However, it can also be seen from Figure 8-7 that the bacterial communities in these reactors were not simply a reflection of those carried over from the Act reactors. Therefore, the roles of these subsequently selected bacterial families are potentially attributable to the next steps in the AD process, namely, acetogenesis and syntrophic acetate oxidation. Such an insight into the functions of different bacterial taxa is an interesting outcome of studying two-stage reactor systems rather than single stage ones. For instance, the bacterial family *Synergistaceae* was the most abundant (~ 30%) bacterial family in the Mth_IBA reactor but was largely absent in the preceding Act reactor. This family is a common AD constituent (Riviere *et al.*, 2009b) and has been associated with acidogenesis, the degradation of amino acids (Riviere *et al.*, 2009b), and carbohydrates (Godon *et al.*, 2005). However, in the context of this study, Westerholm and Schnürer (2019) have previously suggested that the *Synergistaceae* contain bacteria capable of performing a syntrophic metabolism in association with hydrogenotrophic methanogens. Moreover, Ito *et al.* (2011) identified a novel syntrophic acetate-oxidizing bacterium candidate belonging to *Synergistes* group 4 in an anaerobic digester of sludge. The bacterial family *Spirochaetaceae* was also dominant in some of the Mth reactors; their role in anaerobic digesters is known to be linked to the acetogenesis from the reduction of CO_2 by H_2 (Leadbetter *et al.*, 1999). On the basis of the putatively different functions of the *Synergistaceae* and the *Spirochaetaceae* it is interesting to note that the Mth_IBA reactor had the lowest dominance of the *Spirochaetaceae* and the highest dominance of the family *Synergistaceae* (Figure 8-7) which is consistent with the likely dominance of syntrophic acetate oxidizers as evidenced by the greater dominance of hydrogenotrophic methanogens in this reactor (see below).

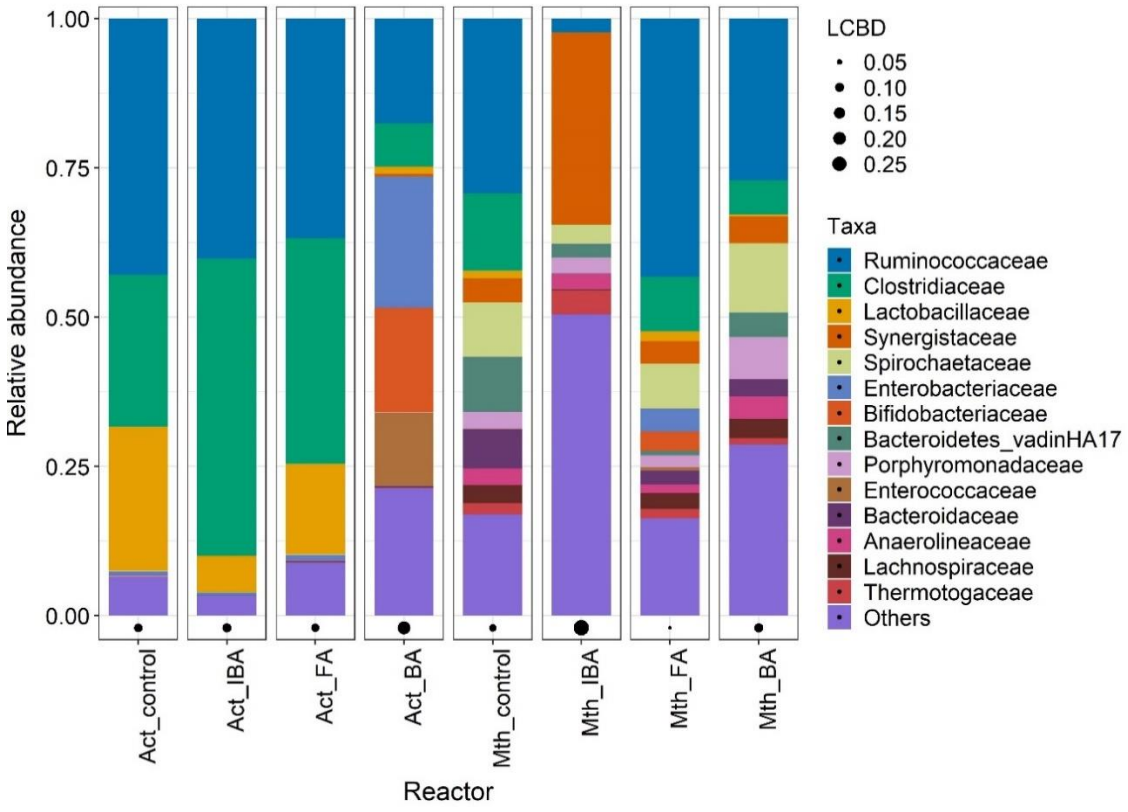


Figure 8-7. Relative abundance of the most dominant 15 bacterial families identified in reactors on day 90. LCBD is local contribution of beta diversity in the digestate samples. The Act_BA and Mth_IBA samples were with high LCBD values which means that these two reactors had higher unique species compared to the other reactors. There was a clear enrichment of *Lactobacillaceae* in the Act reactors. The Mth_IBA reactor had higher relative abundance of *Synergistaceae* compared to the other Mth reactors.

8.3.3.2. *Composition of archaeal community*

It can be seen from Figure 8-8 that about 90% of the archaeal sequences in the Mth reactors were dominated by hydrogenotrophic (*Methanospirillum* and *Methanobacterium*), acetoclastic (*Methanosaeta*) methanogens and the metabolically more flexible methanogens (i.e. *Methanosarcina*, which use either acetate or H_2/CO_2 to produce methane). In addition to those well described methanogens which are common constituents of anaerobic digestion systems, there was an enrichment of a sequence in the Mth-IBA reactor related to the recently proposed as the *Candidatus* genus *Methanofastidiosum* (Nobu *et al.*, 2015). This organism considered nutritionally fastidious whereby methanogenesis is achieved through methylated thiol reduction linked to hydrogen oxidation likely competes with hydrogenotrophic methanogens and may maintain and thrive under lower H_2 partial pressures favouring efficient syntrophic acetate oxidation and fermentation (Nobu *et al.*, 2015).

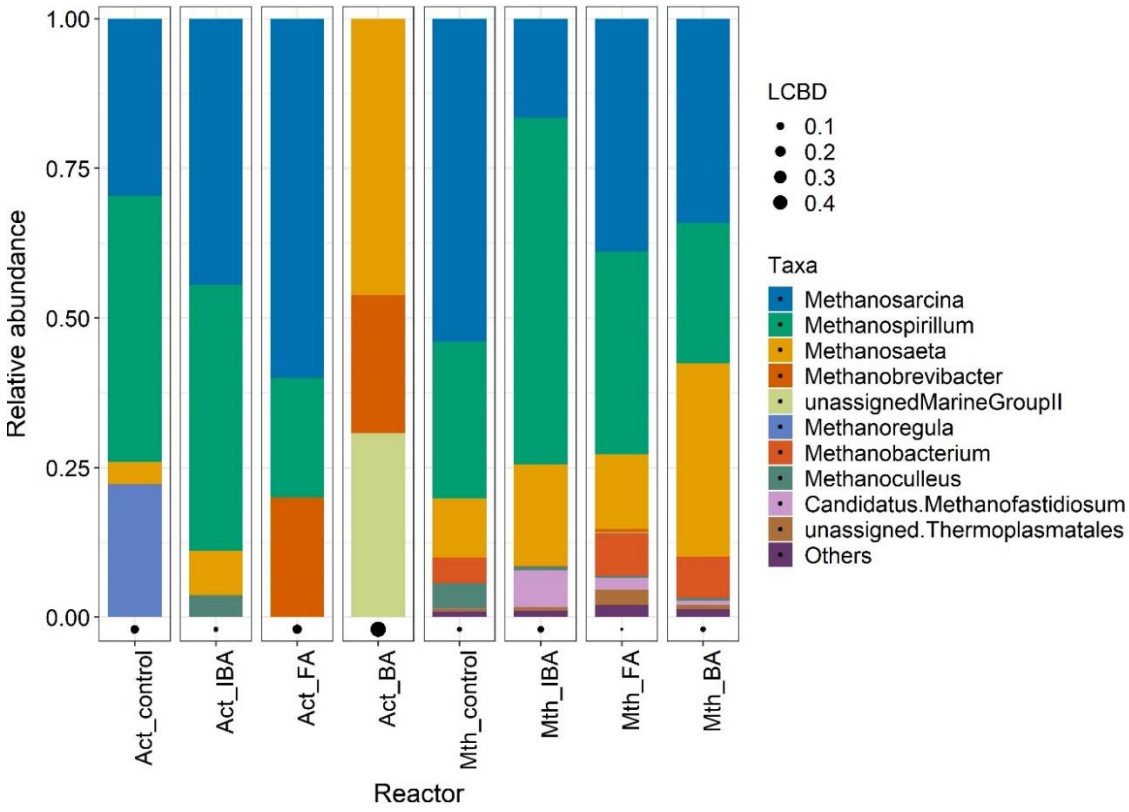


Figure 8-8 Relative abundance of highest 10 archaeal genera in the CSTRs on day 90. LCBD is local contribution of beta diversity in the digestate samples as described in Figure 8.7. There was an enrichment of the *Candidatus* genus *Methanofastidiosum* in the Mth-IBA reactor which showed stable digestion processes.

The dominance of the hydrogenotrophic methanogens *Methanospirillum* and especially *Methanofastidiosum* in the Mth_IBA reactor, which showed greater process stability and higher methane production at an OLR of 5 g VS/L. d with an HRT of 10 days, is of particular interest. This hydrogenotrophic dominance and likely maintenance of low hydrogen partial pressures is coincident and consistent with the presence and dominance of the *Synergistaceae* bacteria as putative syntrophic acetate oxidizers as discussed above. While the parameters measured in this study were not sufficient to conclude clearly, why the Mth_IBA reactor maintained a stable and efficient digestion process compared to the Mth_control, Mth_FA and Mth_BA reactors which ultimately failed under a OLR and short HRT it seems likely that the formation of stable syntrophic bacterial/archaeal partnerships supported by beneficial concentrations of trace elements available from the IBA (Table 4-8) played an important role. While, inefficient conversion of VFA to acetate and, or, oxidation of acetate to CO_2 and H_2 (either linked to high concentrations of trace and heavy metals released from the FA and BA MW or their absence in the control) resulted in the accumulation of VFA, pH drop, and consequent inhibition of methanogenesis in the other reactors.

8.4. Practical application and future work

The biogas produced from the Act and Mth reactors can be used separately as renewable energy or can be mixed together to obtain a hydrogen-methane blend known as biohythane fuel, which usually comprise 10% H_2, 30% CO_2 and 60% of CH_4 v/v (Cavinato *et al.*, 2011). From energetic point of view, as can be seen from Table 8-3, with 100 h metals extraction time, the net energy output from the Mth_IBA reactor was negative with the incubator, which was used for metals extractions in this chapter. However, both the 1h and 12 h metals extraction times gave positive net energy output from the Mth_IBA rector. For this reason, future works should investigate the shortest metals extraction time required for each MW to provide sufficient amount of metals for achieving stable digestion processes of Mth_reactors. Moreover, the effect of using mineral wastes (in solid phase) in the acidogenic reactors should be assessed instead of using the liquid MW extracts to enhance the hydrolysis and increase the alkalinity and concentration of trace elements necessary for the next stage methanogenic reactors.

Table 8-3. Estimated net energy output from Mth_IBA reactor under different metals extraction times. The values between parentheses show the energy required for the specific pre-treatment time.

			Net energy output (MJ/kg of SOW)		
Day	Methane production (m^3/kg)	Total energy output as methane (MJ/kg)	1 h pre-treatment (0.3 MJ/kg)	12 h pre-treatment (3.6 MJ/kg)	100 h pre-treatment (30 MJ/kg)
60	0.527	21.08	20.78	17.48	-8.92
90	0.46	18.4	18.1	14.8	-11.6

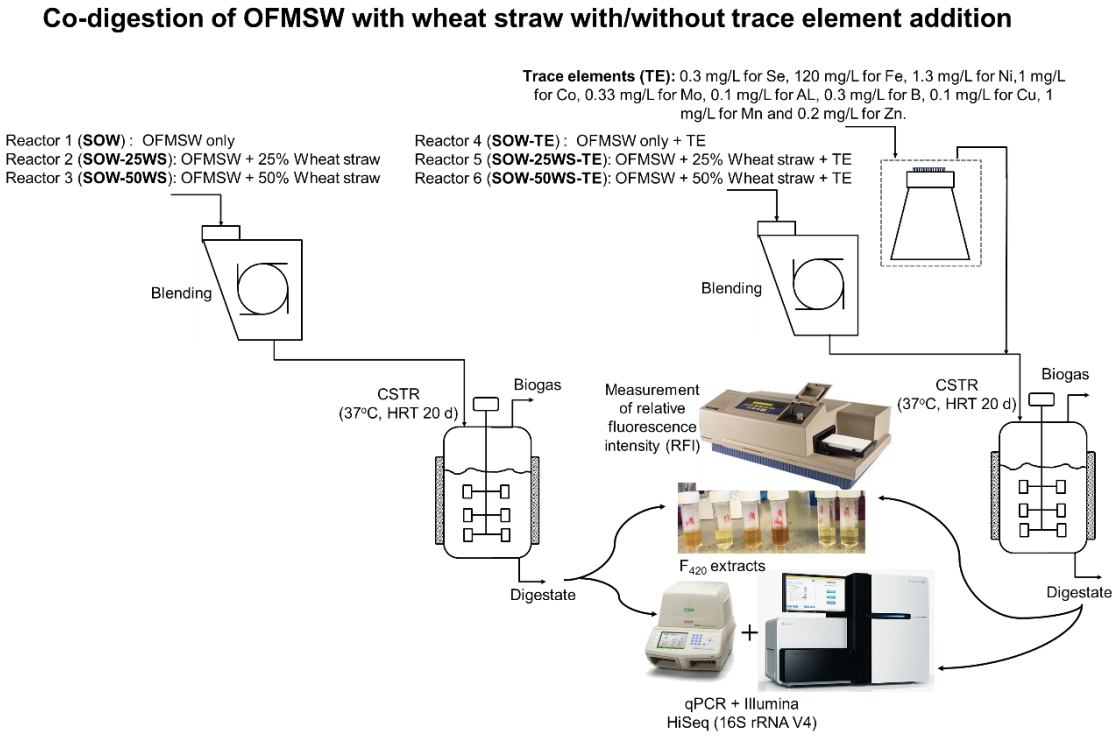
8.5. Conclusions

This chapter investigated the effects of MW extracts on the AD of the OFMSW. In contrast to MWE from FA, the MWE from IBA and BA clearly enhanced hydrogen production in the acidogenic reactors. The MWE from IBA was also found to enhance greatly the performance of the methanogenic reactor stage, providing nutritionally important trace elements deficient in the OFMSW feedstock, and also provided additional levels of alkalinity that helped regulate pH, support methane productivity, and provide process stability. Moreover, following a sudden step decrease in HRT (from 20 to 10 days), which reduced biogas production in most reactors, the MR-IBA showed no signs of failure i.e. it maintained key syntrophic partnerships in the microbial community, allowing higher volumetric methane productivity, albeit at a reduced specific methane yield.

The study in this chapter has demonstrated that MW extracts can act as a highly effective nutritional supplement that supports the growth of important members of the microbial community in AD systems, and can be used to substitute standard trace element nutrient solutions during the AD of OFMW. Furthermore, the low economic value and widespread availability of MW means the preparation and use of MW extract amendments offers an affordable approach to integrating mineral wastes into organic waste disposal through anaerobic digestion.

Chapter 9. Enhancing reactor stability and biogas production for food waste AD: trace element addition has more impact than wheat straw co-digestion

Graphical abstract



Abstract

Trace element (TE) supplementation and substrate co-digestion are techniques to improve organic waste anaerobic digestion (AD), especially for single-stage reactors with high organic loading rates (OLR). To compare these techniques, different AD reactor feed compositions were studied in parallel, including synthetic organic waste (SOW); SOW supplemented with TE; SOW supplemented with wheat straw (WS); SOW supplemented with WS and TE. Feeds were digested in 20 days HRT mesophilic continuously stirred tank reactors (CSTRs) with successively greater organic loads (1, 2 and 4 g VS/L. d). High methane yields (450 - 550 mL/g VS), microbial numbers and process stability at higher OLRs were only maintained with TE supplementation, regardless of co-digestion. The principal effect of WS co-digestion was on microbial community selection and stability. Although bacterial communities were all similar at day 40, communities in stable reactors with TE subsequently diverged, most notably with selection of *Cloacimonadaceaea* in the absence of WS. WS co-digestion had more effect, however, on the more sensitive methanogenic archaea. 50%WS co-digestion + TE selected for a stable community comprising the stress intolerant acetoclastic *Methanosaeta*, however, TE amended reactors with less (25%) or no WS comprised the metabolically more flexible *Methansarcina* selected as a result of ammonia stress. Interestingly, at 25% WS the *Methanosarcina* were acetoclastic (based on indicative coenzyme F_{420} measurements); with no WS and highest ammonia levels they were hydrogenotrophic. These results imply TE amendment was hierarchically more important than co-digestion but co-digestion was beneficial in reducing biological stress linked to lower ammonia.

9.1. Introduction

Anaerobic digestion has been widely used for the management of the organic fraction of municipal solid waste (OFMSW) whilst recovering energy by methane production (Liebetrau *et al.*, 2017). The advantages of single-stage anaerobic reactors over multi-stage anaerobic reactors due to low installation and operation costs are well recognised, but, multi-stage reactors enhance biogas production by separating the acidogenic and methanogenic phases (Hernández and Edyvean, 2011; Schievano *et al.*, 2012). As a consequence, although in the EU about 90% of full scale anaerobic plants are single-stage systems (Zhang *et al.*, 2011a) there are concerns related to their failure (Rincón *et al.*, 2008) due to low acid buffering capacities at high organic loading rates (OLR) specifically when using a highly hydrolysable substrates like OFMSW. Regardless of reactor design, trace element (TE) deficiency is

considered a common cause for the failure of both single and multi-stage reactor systems (Chen *et al.*, 2008; Gu *et al.*, 2014). These operational problems require new strategies to be developed which can improve reactor performance, such as optimising reactor feed composition for long-term and high OLR applications.

Each year, worldwide, CO_2 fixation by photosynthesis is responsible for the production of ~10^{11} tons of dry plant matter comprised of cellulose (> 50%) (Leschine, 1995). For instance, wheat straw (WS) is a lignocellulosic plant material in which celluloses and hemicelluloses comprise 50 - 70% of the composition with 10 - 20% as lignin (Xi *et al.*, 2014). Therefore, at least 50% of WS can theoretically be degraded anaerobically for biogas production despite lignin's resistance to AD (Xi *et al.*, 2014). Cellulose contains glucose monomers but is nitrogen deficient which places limits on degradation because the nutrient requiring microbial biomass yield from glucose by methanogenesis can be >20% (Kalyuzhnyi, 1997). Therefore, co-digestion of organic wastes (with high nitrogen content) with WS (high carbon content) can be a favourable strategy for optimising feeding composition by balancing C and N. Importantly, two readily available low cost substrates (OFMSW and WS) contain complementary C and N contents making them ideal co-substrates for AD and renewable energy production (Romero-Güiza *et al.*, 2016). The main benefits of co-digestion of OFMSW with WS are:

a) lignin in wheat straw, and presumably the cellulose it occludes, is slowly degraded (Noike *et al.*, 1985), so the risk of rapid hydrolysis and acidification in AD reactors is reduced for mixtures of OFMSW and WS;

b) the non-degradable components of WS (lignin) can work as a biofilm carrier in the reactor (Pohl *et al.*, 2013) decreasing the risk of biomass washout;

c) improved biodegradation efficiency arises from communities growing on mixed wastes (Wang *et al.*, 2012). Theoretically, two or more distinct microbial communities, i.e. microbes that grow on OFMSW and those that grow on WS, are expected to utilize substrates more efficiently than individual communities (Wang *et al.*, 2012; Sierocinski *et al.*, 2017).

d) a balanced C:N ratio i.e. between 20 to 30, is achieved by mixing N-rich (e.g. OFMSW) and N-deficient (e.g. WS) feedstocks, preventing the onset of ammonia inhibition (Yao et al., 2018).

Another method to optimize the nutrient balance of OFMSW for AD feedstocks is via supplementation of trace elements (TEs). The deficiency of TEs like Se, Fe, Ni, Co, Mo, AL,

B etc. in food waste (food waste represents the largest component fraction of OFMSW) has been reported (Banks et al., 2012, Zhang et al., 2015). The absence/low concentration of TEs is often considered the main reason for limited performance of anaerobic digesters (Zhang et al., 2015, Demirel and Scherer, 2011). The positive effect of TE supplementation during the mono-digestion of OFMSW and its crucial role in the synthesis of enzymes and co-enzymes (like F_{420}) necessary for methanogenesis, are widely reported in the literature (Dolfing and Mulder, 1985; Takashima and Speece, 1989; Pobeheim *et al.*, 2010; Banks *et al.*, 2011; Takashima *et al.*, 2011; Ünal *et al.*, 2012; Facchin *et al.*, 2013; Westerholm *et al.*, 2015a).

Recent studies have identified the risk of failure for AD reactors fed with wheat straw and organic waste co-substrates due to the accumulation of volatile fatty acids and pH drop (Cai *et al.*, 2017). However, in addition Liu *et al.* (2015b) has shown a 22 - 56% higher solids bioconversion of Corn-Stover (a lignocellulosic material) by adding 1.0, 0.4, and 0.4 mg/L. d of Fe, Co, and Ni, respectively. The current study explicitly assumed that:

a) Co-digestion of WS with OFMSW enhances the hydrolysis and fermentation of the cellulose and hemicellulose fractions of WS.

b) The release of carbon from WS (glucose) increases the substrates required for microbial growth in the mixed liquor.

c) TE supplementation enhances enzymatic activity to promote methanogenesis, and that the combined effect of TE supplementation and WS/OFMSW co-digestion results in a balanced fermentation-methanogenesis synergy and thus increased methane production and the long-term stability of AD.

The aim of the study in current chapter was for to directly compare the relative individual and combined effects of TE supplementation and WS/OFMSW co-digestion on process stability (as measured by pH and alkalinity status), performance (biogas production) and specific methanogenic activity (relative fluorescence intensity of coenzyme F_{420} in digestate). The novelty and strength of the current study was the simultaneous comparison of six mesophilic reactors fed with a synthetic organic waste as a mono substrate (only OFMSW), or as a co-substrate with WS (25% and 50% WS), with and without TE addition. In all these reactors, 16S rRNA gene abundances were measured and microbial community compositions analysed for correlation with the measured physicochemical parameters.

9.2. Materials and methods

9.2.1. *Inoculum and feedstock materials*

The feedstock of reactors was the synthetic organic waste (SOW) substrate described in Synthetic organic waste (Section 4.1.1) and its characteristics are shown in (Table 4-3). The total (TS) and volatile (VS) solids concentrations of the SOW feed were adjusted to 12.8% and 11.3% respectively by adding distilled water (~ 35% dilution of the organic waste was required).

The wheat straw (WS) was collected from Cockle Park Farm, Newcastle University. The WS dried at 50 - 60^{o}C, ground with a dry food grinder, sieved to pass a 1mm sieve, and stored in airtight bags at room temperature until use. The inoculum for reactor start-up was obtained from a mesophilic (37^{o}C) digester treating cattle slurry and farm silage (Cockle Park Farm, Newcastle University, UK) as described in Inoculum (Section 4.1.2) and physicochemical characteristics of the inoculum of reactors are shown in Table 4-4 . Table 9-1 shows the physicochemical properties of the SOW, WS and inoculum used in the current study.

Table 9-1. Physicochemical characteristics of the wheat straw used in the feedstock substrate of CSTR.

Parameters [1]	Wheat Straw
pH (1:2)	6.3 [2]
TS (%W/W)	92.2
VS (%W/W)	81.4
VS (%TS)	88.0
C (%)	45.5
N (%)	0.56
S (%)	0.0
C/N	81.3
Al	153.80
B	7.00
Ba	38.0
Ca	2255.0
Cd	0.08
Co	0.22
Cr	4.90
Cu	4.40
Fe	443.0
K	1532.0
Mg	498.0
Mn	24.50
Mo	0.24
Na	60.60
Ni	2.60
Pb	2.40
Si	51.40
Ti	6.10
V	0.84
Zn	20.40

[1] All metal concentrations are total concentration of metals in µg per g TS, and all values in this table represent mean value of triplicate samples measured with standard deviation not shown.
[2] One volume of wheat straw was added to 1.5 volume of distilled water mixed with magnetic stirrer for one hour then measured for pH.

9.2.2. *Trace elements solution*

The composition of TE solution was prepared according to the recipe reported by (Zhang *et al.*, 2012) as described in Synthetic trace element solutions (Section 4.1.4). The reactor concentration of the TEs (see Table 4-6) was chosen to simulate the approximate concentrations of measured TEs released from the mineral wastes such as incineration ash and

construction demolition waste that were recently supplemented to the AD of OFMSW (Chapter 4 and Chapter 6). On experimental start-up, reactors receiving TE supplements were given appropriate volumes of these stock solutions to achieve the designed concentration of each TE in the reactor (Table 4-6). Although reactors were fed daily with organic feedstocks, the TE concentration was maintained every 5 days by adding an appropriate amount of each stock solution to match the volume of feed added, and digestate removed, over that period. The reactors receiving TEs received the same amount of the TE solution on each addition.

9.2.3. *Reactor start-up and operation*

9.2.3.1. *Biomethane potential tests*

Biomethane potential (BMP) tests for each of the SOW and the WS substrates were carried out separately with the same inoculum used for the continuous trials (CSTRs) to find the individual potential methane yields from the substrates. The potential methane yields of the SOW and WS combinations (25% or 50% WS/SOW co-digestion) was then inferred mathematically. The BMP tests conducted according to a standard method (VDI, 2006b) as described in Biomethane potential (BMP) reactors (Section 4.3.1). The inoculum to substrate (SOW or WS) volatile solid (VS) mass ratio was 2:1.

9.2.3.2. *Continuous digestion trials*

Six continuously stirred anaerobic reactors (CSTRs) of 1L working volume were employed for continuous digestion trials. The CSTRs were operated at a mesophilic temperature (37^{o}C) over a period of 100 days (five HRTs of 20 days). An HRT of 20 days was chosen based on results of previous studies (Chapter 6) which studied the AD of a similar substrate. The startup of CSTR and inoculum was similar to that described in Continuous stirred tank reactors (CSTRs) of Section 4.3.2. The CSTR were fed with three successive OLRs 1, 2 and 4 g VS/L. d. The six reactors were designated: SOW (fed with SOW only (C/N = 13.6)); SOW-TE (fed with SOW and supplemented with TEs (C/N = 13.6)); SOW-25WS (fed with 75% SOW and 25%WS (C/N = 30.5)); SOW-25WS-TE (fed with 75% SOW, 25%WS and TEs (C/N = 30.5)); SOW-50WS (fed with 50% SOW and 50%WS (C/N = 47.5)) and; SOW-50WS-TE (fed with 50% SOW, 50%WS and TEs (C/N = 47.5)).

9.2.4. *Analytical methods*

Biogas produced from each reactor was collected in a five-litre gasbag (Tedlar, VWR). Each day gasbags were disconnected, biogas volume measured, emptied with samples of biogas

from each reactor analysed for biogas composition (CH_4 and CO_2) by gas chromatography (GC). All analytical methods were similar to those described in Chapter 4.

9.2.5. *Microbial community analysis*

Microbial community analyses were performed for the inoculum (before the acclimation period) and digestates; samples for the later were collected from the reactors on days 20, 40, 50, 60, 80 and 100. DNA extraction, Real time quantitative PCR (qPCR) and 16S rRNA gene sequencing analyses are described in Microbial analysis (Section 4.5).

9.2.6. *Statistical analysis*

Coefficients of Pearson's bivariate correlations for measured reactor parameters and performances (replicate values per HRT per reactor were obtained for methane yields, methane production, COD, F_{420}, and VFA etc.) were conducted in SPSS (version 23.0). One-way ANOVA analysis was conducted in R (R, 2013) to compare the abundance of bacteria and archaea (from qPCR and Illumina analyses) between reactors (SOW, SOW-TE, SOW-25WS, SOW-25WS-TE, SOW-50WS, SOW-50WS-TE) . All figures were produced in R (R, 2013). Figures of microbial composition (Beta diversity) and Local Contributions of Beta Diversity ((LCBD); is a comparative indicator of the degree of the uniqueness of digestate samples in terms of community composition)) were produced using MicrobiomSeq package in R (Ssekagiri *et al.*, 2017). Alpha diversity (the variation in species composition among the reactors) indices (Chao1, Shannon and Simpson) were calculated then visualised using phyloseq package (McMurdie and Holmes, 2013b) in R.

9.2.7. *Determination of the relative fluorescence intensity of F_{420}*

The relative florescence intensity (RFI) of coenzyme F_{420} in digestates was determined according to F_{420} analysis (Section 4.4.6). For each reactor three separate samples of digestate at different periods of HRT were analysed (~ 15 samples per rector during 100 days).

9.3. Results and discussion

9.3.1. *The relative effects of trace element supplementation and co-digestion on the performance characteristics of CSTRs*

Three distinct periods of CSTR performance were observed during the current study (Figure 9-1). During the first period (day 1- 20) all reactors operated satisfactorily. During the second period (day 21- 60) all reactors without TE showed progressively declining performance and

failure (between day 41- 60), while TE supplemented reactors continued with stable methane yields. During the third period (day 60 - 100) TE supplemented reactors showed stable methane production and yields, even when the OLR was increased to 4 g VS/L. d between day 80 - 100. An overall conclusion that can be drawn from these results is that under an equivalent set of AD operating conditions, TE supplementation was effective as a single AD enhancement strategy, while co-digestion with wheat straw was not.

A more forensic analysis of the reactor characteristics mechanistically explains the positive impact of TE addition. Between day 1 to 20 (OLR of 1 g VS/L. d) the reactors showed similar performances in terms of biogas production, methane yield, pH and F_{420} content. During this period, the average methane yields were 80 - 100% of those achieved in BMP tests (Figure 9-2). However, between day 20 and 40 (OLR = 1 g VS/L. d) the methane production performance of the reactors started to diverge, with volatile fatty acids (VFA) gradually accumulating in the SOW and SOW-50WS reactors (Figure 9-3). For instance, acetate and propionate showed sharp increases in these control reactors which did not receive TE; the varied timing of these increases coincided with reactor declines in methane production (Figure 9-1). This VFA accumulation (~ 1700 mg/L on day 40) consumed most of the alkalinity in the SOW and SOW-50WS reactors and pH decreased from 7.2 ± 0.1 on day 30 to less than 5 ± 0.2 on day 43 (Figure 9-1), resulting in inhibition of methane production and failure of SOW and SOW-50WS reactors by day 43 (Figure 9-1). The SOW-25WS reactor, which also operated without TEs, showed a better performance up to day 60. However, when the OLR was increased to 2 g VS/L. d on day 60, methane production from this reactor also declined rapidly and the VFA concentration increased to 2360 mg/L, with a pH drop to ~ 5.8 (Figure 9-3 and Figure 9-1).

In contrast to the control reactors no decline in methane production was observed in the reactors supplemented with TEs (SOW-TE, SOW-25WS-TE and SOW-50WS-TE), and, furthermore, methane production rates increased consistent with increases in OLRs (Figure 9-1). Methane yields obtained from SOW-TE, SOW-25WS-TE and SOW-50WS-TE were close to methane yields obtained in the BMP trials (Figure 9-2). Throughout the 100 days of reactor operation, these three reactors showed stable digestion with methane yields of 450 - 550 mL/g VS consistent with optimal methane yield values reported for OFMSW and/food waste (Angelidaki and Ellegaard, 2003; Angelidaki et al., 2006; Banks et al., 2011; Fdez.-Güelfo et al., 2011; Elbeshbishy et al., 2012). Moreover, concentrations of the coenzyme F_{420}, which is an essential cofactor only found in methanogens in anaerobic environments (Xu *et al.*, 2014; Madigan, 2015) and thus considered a reliable measure of methanogenic

populations and activities (Reuter et al., 1986; Greening et al., 2016), increased commensurately with increases in methane production rates (Figure 9-1) in all the TE supplemented reactors. Statistical analysis showed that F_{420} fluorescence correlated very well with methanogenic activity (Table 9-2), confirming its suitability as parameter to gauge methanogenic activity (Dolfing & Mulder 1985).

The satisfactory performance of all reactors during the start-up implied that sufficient levels of nutrients (micronutrients such as Fe, Co, Ni and Mo and macronutrients such as Na, K, Ca and Mg) and alkalinity were likely present in the start-up inoculum (Table 9-1). The subsequent decrease in the performance of SOW, SOW-25WS and SOW-50WS (all without TE addition) on day 20 and onwards was obvious was therefore presumably related to the dilution of the TEs in these reactors due to the daily discharge of digestate and feed addition.

Focusing specifically on alkalinity, its origins and relationship to TE, Figure 9-3 shows that despite increasing the OLR to 2 g VS/L. d (day 40 - 60) and 4 g VS/L. d (day 80 – 100), alkalinity tended to increases in TE amended reactors (SOW-TE, SOW-25WS-TE and SOW-50WS-TE). However, the highest alkalinity and NH_3-N concentrations were in the SOW-TE amended reactor (3 - 5 g/L and 1.25 g/L respectively on day 100), while lower alkalinity and corresponding lower NH_3-N concentrations were observed in the SOW-25WS-TE and SOW-50WS-TE reactors. This pattern suggests that increases in alkalinity in TE supplemented reactors was most likely related to microbially enhanced NH_3-N release from the SOW substrate, and that this enhanced release is linked to bacterial growth and activity. Certainly, the total number of bacteria (estimated from 16S rRNA gene abundances; Figure 9-4) in reactors with TE supplementation increased with increases in the OLR and there was a highly significant ($p < 0.01$) strong ($R = - 0.78$) negative correlation between reactor NH_3-N concentrations and the C/N ratio of the substrate feed. However, it is worth noting here that despite observed differences in NH_3-N, with higher levels associated with decreasing inputs of WS, these levels never exceeded the 2 g/L considered inhibitory for AD (Chen *et al.*, 2016). Non-inhibitory levels of NH_3-N were supported by the observed maximal biogas production in all these TE amended reactors, however, differences in NH_3-N may have affected archaeal community selection, stability and dominant methanogenic pathways (see below).

Alkalinity and NH_3-N and their relationship to process stability and TE supplementation are however only two of a number of possible impacts of TE. For instance, in AD to ensure a stable digestion processes, most of the VFA produced by acidogenic and acetogenic bacteria at the fermentation stage need to be consumed by acetogenic bacteria in syntrophy with

methanogenic archaea (Zheng *et al.*, 2015). Previous studies have highlighted the importance of TE for VFA degradation (Jiang *et al.*; Karlsson *et al.*, 2012) and considered TE deficiency as the main cause of failure (Chen *et al.*, 2008; Gu *et al.*, 2014). In the current study, the total number of bacteria (estimated from 16S rRNA gene copies; Figure 9-4) in reactors with TE supplementation demonstrably increased with increases in the OLR, however, intriguingly increases in the total number of methanogenic archaea occurred essentially only between day 0 - 40. Thereafter archaeal numbers in the TE reactors remained approximately constant which suggests that later increases in methane production with higher OLRs were actually linked to higher archaeal metabolic activities of individual cells sustained by TE rather than as a result of TE supported growth. Corroborative evidence of different individual archaeal cell activities is provided by the initial low OLR feeding regimen. Here no accumulation of VFA occurred in the TE supplemented reactors but did in the TE free reactors (Figure 9-3), despite an equal number of archaeal methanogen cells and, indeed, similar RFI values.

Table 9-2. Pearson correlation analysis of measured parameters and reactor performances for the data obtained from the six CSTRs at 37°C.

	HRT	OLR	C/N	Y	MP	F_{420}	16S rRNA	mcrA	pH	total VFA	COD	CH_4%	TE	TAN
Y	-.089	.102	-.569**	1										
MP	.698**	.867**	-.189	.438*	1									
F_{420}	.509*	.587**	-.312	.573**	.761**	1								
16S rRNA	.550**	.732**	.308	.102	.670**	.386	1							
mcrA	.678**	.738**	.250	.102	.650**	.214	.759**	1						
pH	-.474*	-.259	-.102	.588**	.167	.145	.146	.030	1					
total VFA	-.347	-.280	-.098	-.345	-.450*	-.353	-.478*	-.570**	-.405	1				
COD	-.196	-.039	.066	-.241	-.144	-.299	-.324	-.248	-.318	.750**	1			
CH_4%	-.174	-.057	-.097	.567**	.339	.221	.200	.214	.726**	-.625**	-.370	1		
TE	0.000	0.000	0.000	.616**	.485*	.431	.471*	.531*	.399	-.800**	-.494*	.524*	1	
TAN	-.147	.076	-.777**	.687**	.312	.488*	-.155	-.247	.310	.188	.125	.150	.068	1
TS	.466*	.710**	.396	.013	.619**	.215	.896**	.728**	.041	-.301	-.015	.213	.362	-.194
VS	.596**	.751**	.411	-.089	.619**	.198	.879**	.773**	-.093	-.345	-.060	.167	.339	-.308

*Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). Positive and negative correlations with significant are highlighted in dark-green and dark-red respectively. Lower and lowest positive and negative correlations are highlighted in lighter and lightest green and red respectively. MP = methane production rates, Y = Methane yield and TE = trace elements.

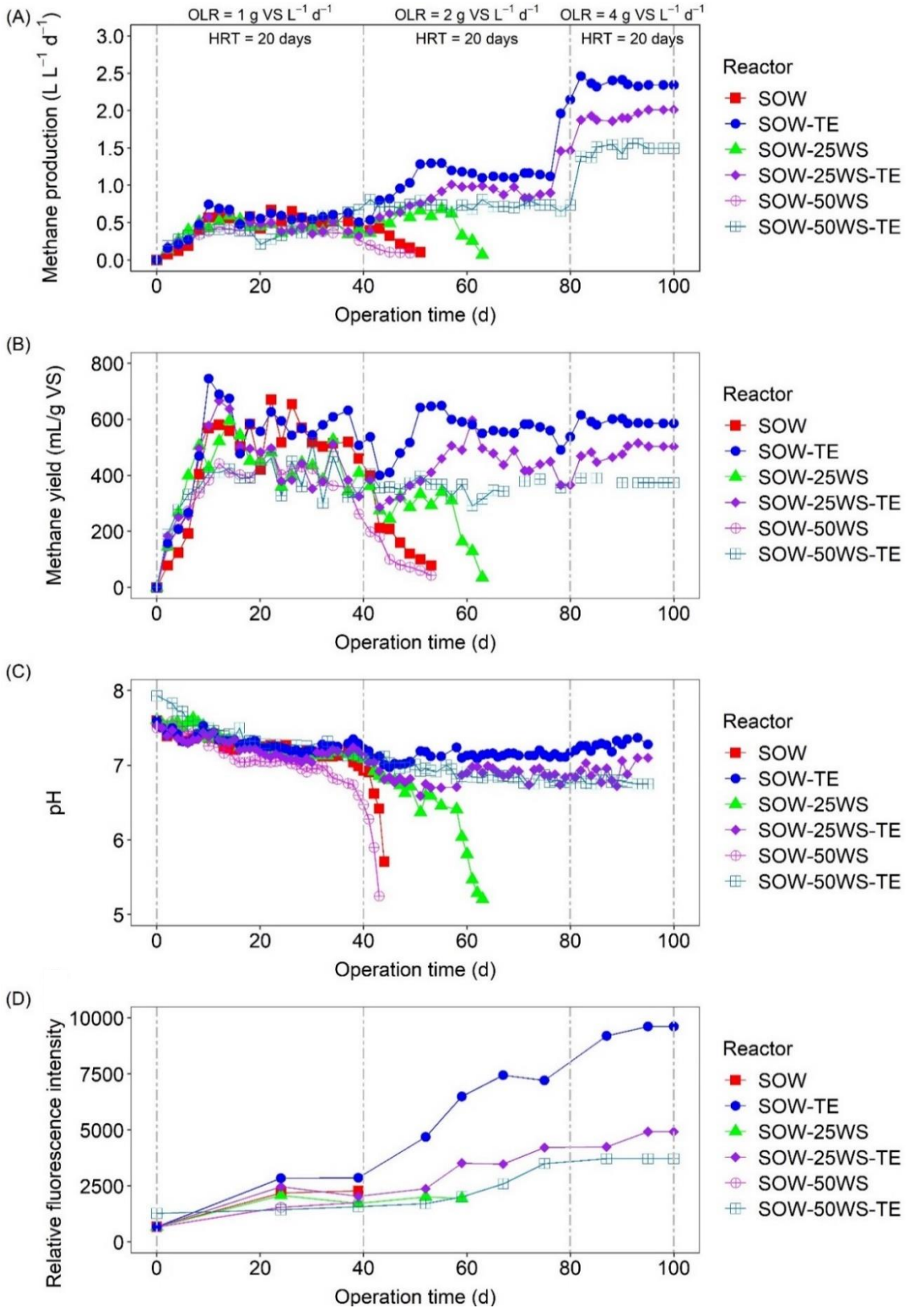


Figure 9-1. Performance profile of six CSTR systems at mesophilic 37°C and three organic loading rates (OLR) of 1, 2 and 4 gVS/L. d. (A) daily methane production, (B) methane yield, (C) pH, (D) relative florescence intensity of F_{420} in digestates. The values for pH and relative fluorescence intensity are mean values of triplicate samples with standard deviations (not shown).

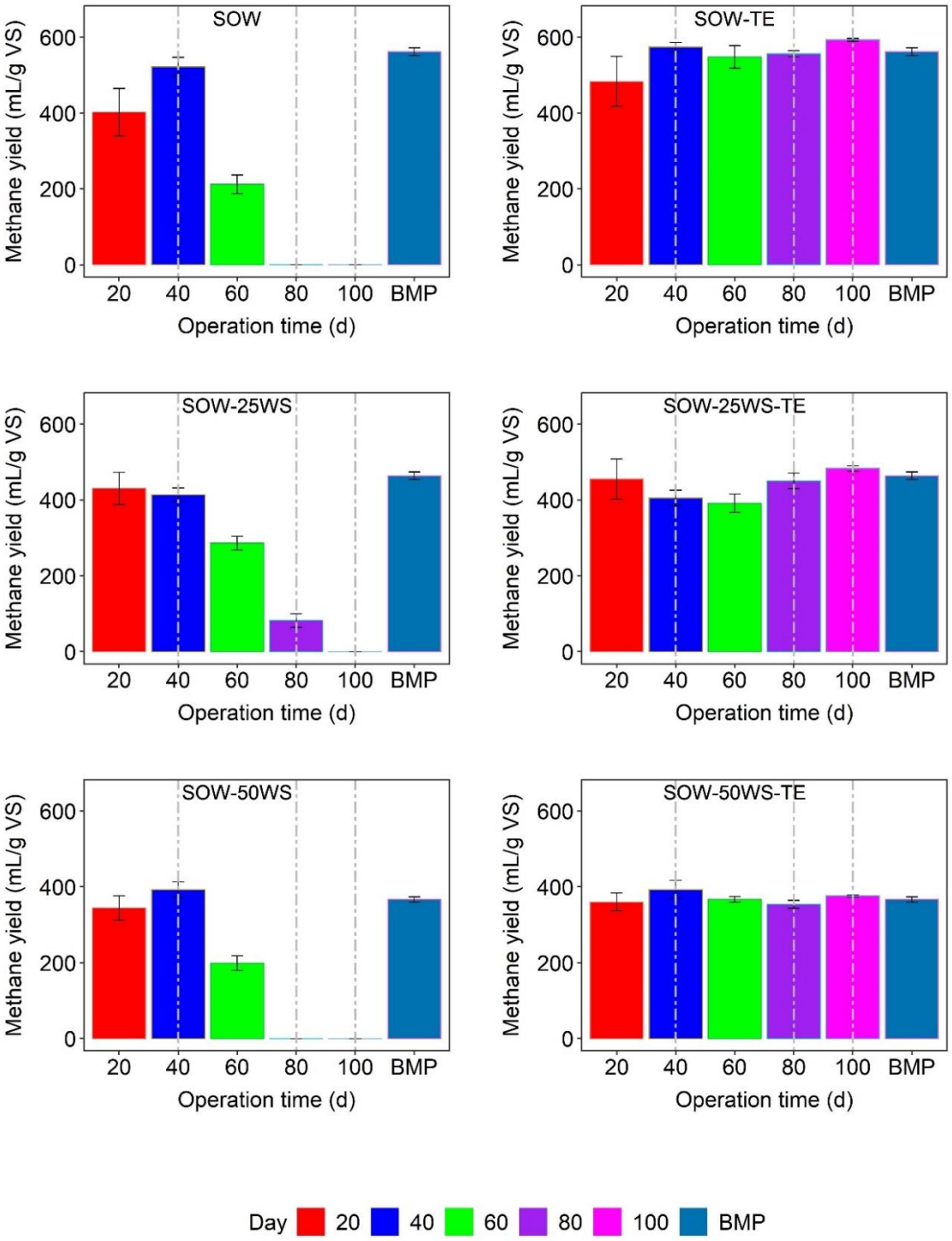


Figure 9-2. Measured methane yields from the 6 CSTR systems in comparison with theoretical methane yields calculated from the individual biomethane potential (BMP) tests carried out separately on the individual SOW and WS substrates. The BMP x-axes therefore refer to BMP values (mL CH_4/ g VS) obtained from the batch reactor BMP tests, whereas the bars on days 20, 40, 60, 80 and 100 show mean biomethane potential values per 20 day periods of CSTRs operation. The error bars represent standard deviations of the mean values.

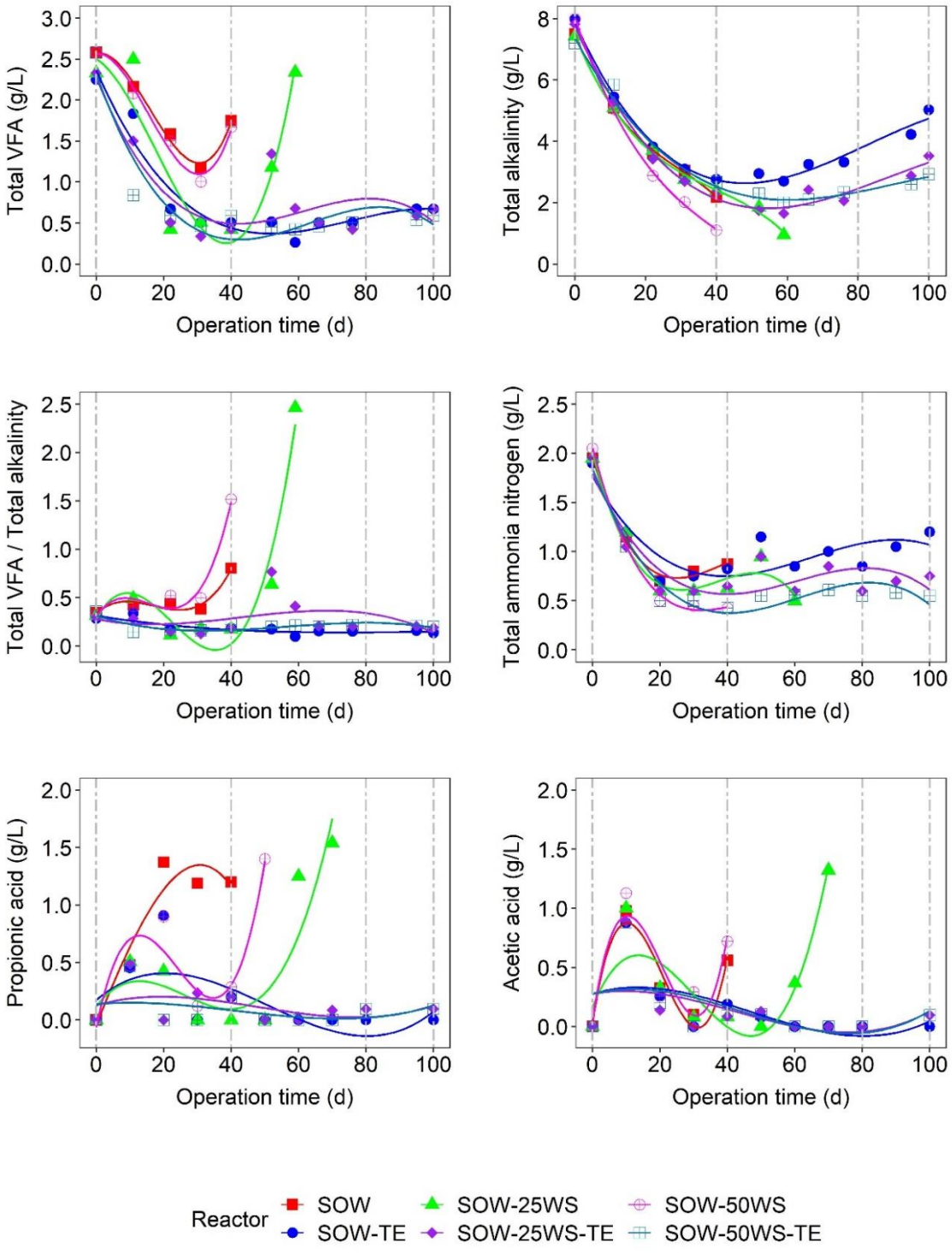


Figure 9-3. Variations in total VFA, total alkalinity, total ammonia nitrogen, acetate and propionate with time in six CSTR systems fed with six different substrate compositions at mesophilic temperature 37^{o}C. The values are mean values of duplicate digestate samples with standard deviations (not shown).

9.3.2. *Microbial community diversity analysis based on 16S rRNA sequence libraries*

The average number of reads in individual 16S rRNA sequence libraries after quality filtering was 7,074,589 ranging from 96,867 sequences in the smallest library to 265,689 sequences in the largest. However, individual libraries were rarefied for comparative analysis. Sequences have been deposited in the Mendeley Data repository and can be accessed at http://dx.doi.org/10.17632/6wnfvkz6gb.1. Pipeline analysis of sequence libraries identified a total of 609 bacterial taxa (accounting for ~ 96% of sequence reads) and 24 archaeal taxa (~ 4% of sequences reads) which is broadly consistent with the numerical dominance of bacteria over methanogenic archaea identified by qPCR assays. A high number of bacterial sequences were not taxonomically assignable below family level and therefore taxonomic based bacterial composition analysis (as opposed to ASV clustering metrics) are presented in this manuscript at the family level with finer taxonomic resolutions indicated where appropriate. In contrast, archaeal sequences were all assignable to the genus level.

A comprehensive sampling strategy was adopted in this study to assess impacts on microbial communities as a function of time and generic reactor operation as well as individual reactor conditions. For instance, diversity represented by Chao1 richness (Chao1), Shannon-Weaver (Shannon), and Simpson diversity indices showed a marked and progressive reduction for both bacterial and archaeal domains as a function of time irrespective of reactor feed (Figure 9-5). This general diversity decline was in all cases clearly the result of the selection, after startup, of a small number of taxa commonly observed in anaerobic digesters (see below). This selective enrichment is entirely consistent with other AD studies which have investigated start-up (e.g. (Alcántara-Hernández *et al.*, 2017)) and long term reactor operation (e.g. (Jia *et al.*, 2016)). The specific bacterial and archaeal compositional changes observed and the insights drawn from them are described and discussed in the following two sections.

9.3.2.1. *Bacterial community composition and dynamics*

Sequence types related to seven different families variably dominated the bacterial communities of the reactors: *Ruminococcaceae*, *Spirochaetaceae*, *Synergistaceae*, *Cloacimonadaceae*, *Dysgonomonadaceae, Rikenellaceae* and *Kosmotogaceae* (Figure 9-6 and Figure 9-7). These bacterial families are, as mentioned above, common constituents of anaerobic digesters and their selection is consistent with the complexity of the food waste based reactor feed as indicated by the environmental source and function of the cultured and uncultured close relatives (Figure 9-8). For instance, the family *Ruminococcaceae* are known fermenters. Despite their eponymous title, their role in AD (including the genera

Fastidiosipila and *Ercela* present in the reactors, see Figure 9-7 and Figure 9-8) is widely documented and linked to the cellulolytic digestion of plant fibres (Ziganshin et al., 2013), specifically, the degradation of hemicellulose (van Gelder *et al.*, 2014; Pandit *et al.*, 2016; Cai *et al.*, 2018). Likewise, the *Spirochaetaceae* are ubiquitous in anaerobic digestion systems where they participate in methanogenic-syntrophic partnerships e.g. taxa related to the genera *Treponema* (Poirier *et al.*, 2016) and *Spirochaetes* (Lee *et al.*, 2018) both of which were present in the reactors. This family is sometimes associated with lignocellulose hydrolysis (Pandit *et al.*, 2016). *Synergistaceae* as common AD constituents (Riviere *et al.* (2009a) are associated with acidogenesis, the degradation of amino acids (Riviere *et al.*, 2009a), and carbohydrates (Godon *et al.*, 2005). Likewise, *Cloacimonadaceae* are also 'known (or suspected) to be' anaerobic mesophilic acetogens. Constituent genera such as *Candidatus Cloacimonas* (present in the reactors) are known to be involved in syntrophic partnerships via hydrogen generation from the fermentation of carbohydrates and proteins in mineral rich anaerobic reactors (Riviere *et al.*, 2009a). Moreover, *Cloacimonadaceae* are known as homoacetogens (Lee *et al.*, 2018). *Dysgonomonadaceae* (*Fermentimonas*) and *Rikenellaceae* (DMER64) (see Figure 9-7) have both been previously documented in food waste AD digesters (Lee *et al.*, 2018). *Dysgonomonadaceae* are known as a degraders of various polysaccharides (Murakami *et al.*, 2018) and can be associated with the enrichment of the methanogenic genus *Methanosarcina* which is known to be a metabolically more versatile and robust archaeal genus in AD (see below). The co-enrichment of *Dysgonomonadaceae* and *Methanosarcina* was supported in this study based on a significant ($p = 0.013$, albeit weak ($R= 0.427$) correlation of these two groups in the reactors. Finally, the family *Kosmotogaceae* (specifically, the genus *Mesotoga*, see Figure 9-8 and Figure 9-9) has been implicated in the oxidation of sugars including cellobiose and xylose produced from the hydrolysis of cellulosic biomass during the anaerobic digestion of cow-grass (Lee *et al.*, 2018).

At day 40, the dominance of these different bacterial groups in the three reactors with or without TE addition were remarkably similar to each other, but clearly different to the original inoculum, suggesting their selective enrichment during AD without any influence from variations in TE supply or WS co-digestion. Some of these enriched taxa were consistently present and retained in all the reactors for the duration i.e. the *Dysgonomonadaceae*, however, the abundance and long term stability of others were tentatively attributable to differences in WS%. For instance, at 40 days the dominance of *Spirochaetaceae* apparently increased with the presence and increasing amount of WS. In contrast, the relative abundance of the enriched *Cloacimonadaceae* was comparatively lower. Furthermore, by days 80 and 100 the SOW-TE

and SOW-25WS-TE reactors were dominated by ~ 55% *Cloacimonadaceae* supporting the selection of this family at lower WS%. Conversely, the *Synergistaceae* were still a dominant (~ 40%) component of the bacterial communities in the SOW-50WS-TE reactor along with the families *Rikenellaceae* and *Kosmotogaceae* (Figure 9-6, Figure 9-7, Figure 9-8 and Figure 9-9).

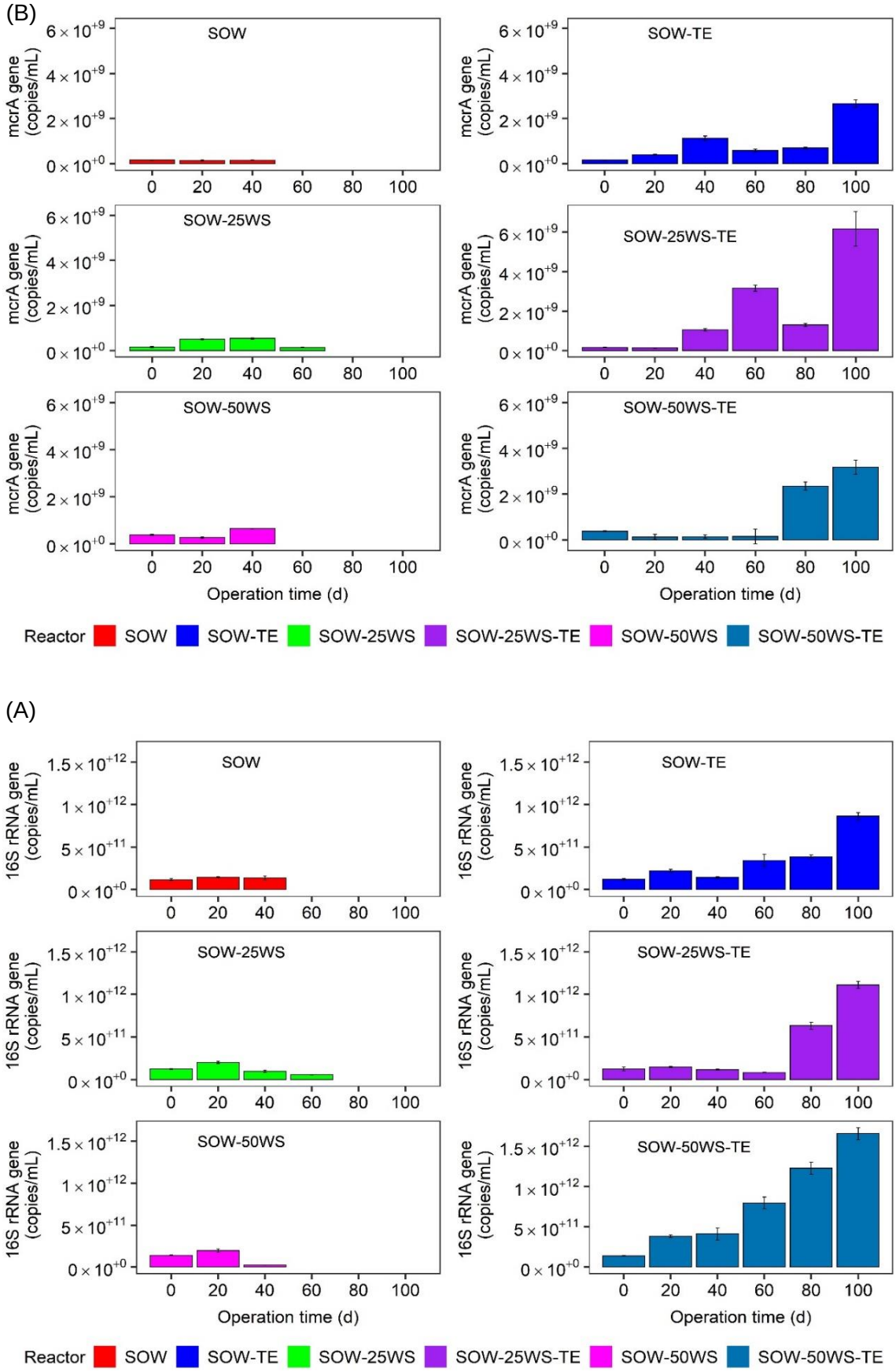


Figure 9-4. Variations in archaeal mcrA gene (A) and bacterial 16s RNA (B) abundances derived copies from qPCR analysis. Days after SOW, SOW-25WS and SOW-50WS reactor failure were not analysed.

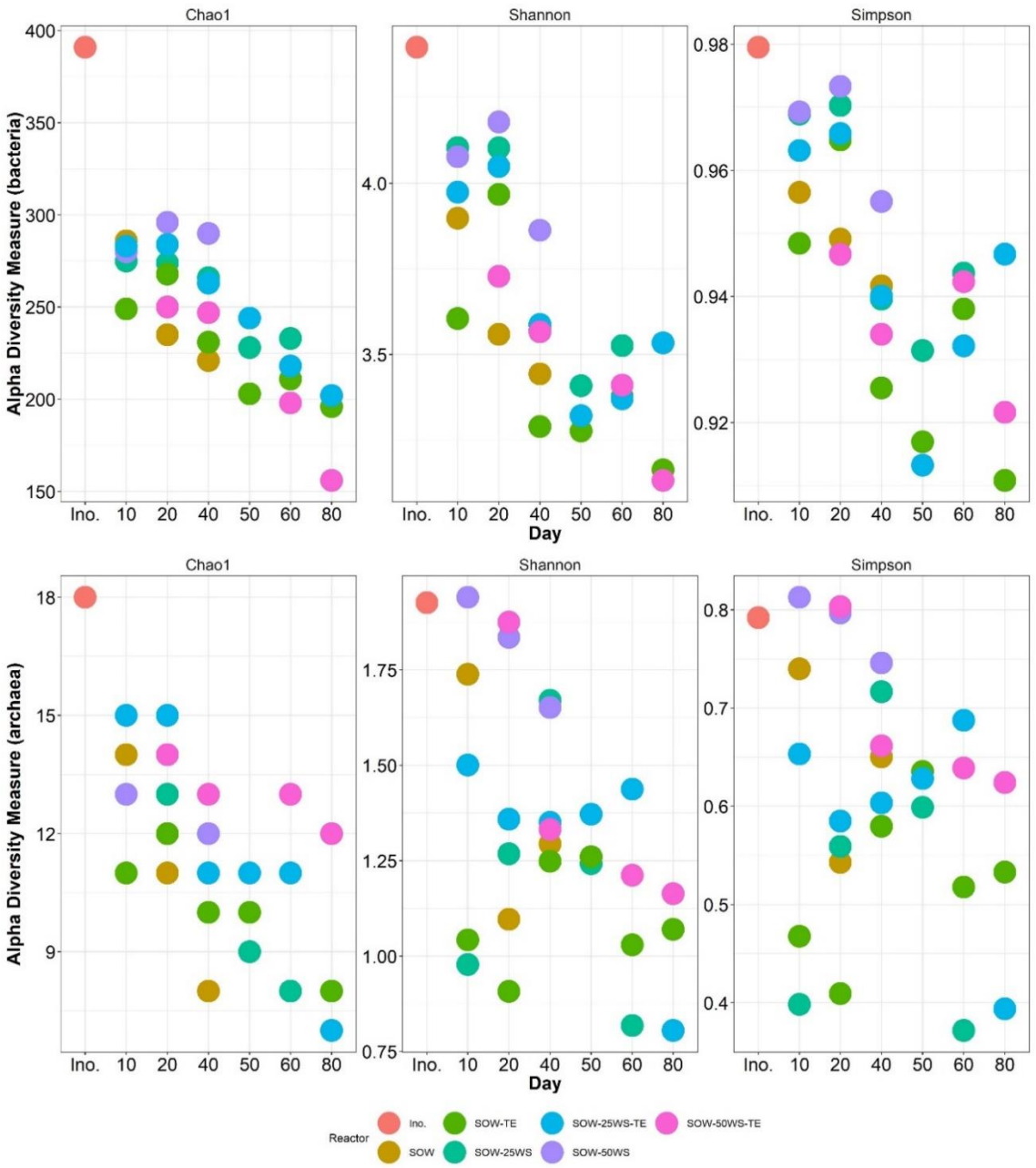


Figure 9-5. Alpha diversity of microbial community (both bacteria and archaea) in relation with feed composition and time (the numbers on the x-axis denote days and Ino. = inoculum) in six AD reactors digested a synthetic organic waste as mono substrate or co-digested with wheat straw with/without trace element supplementations.

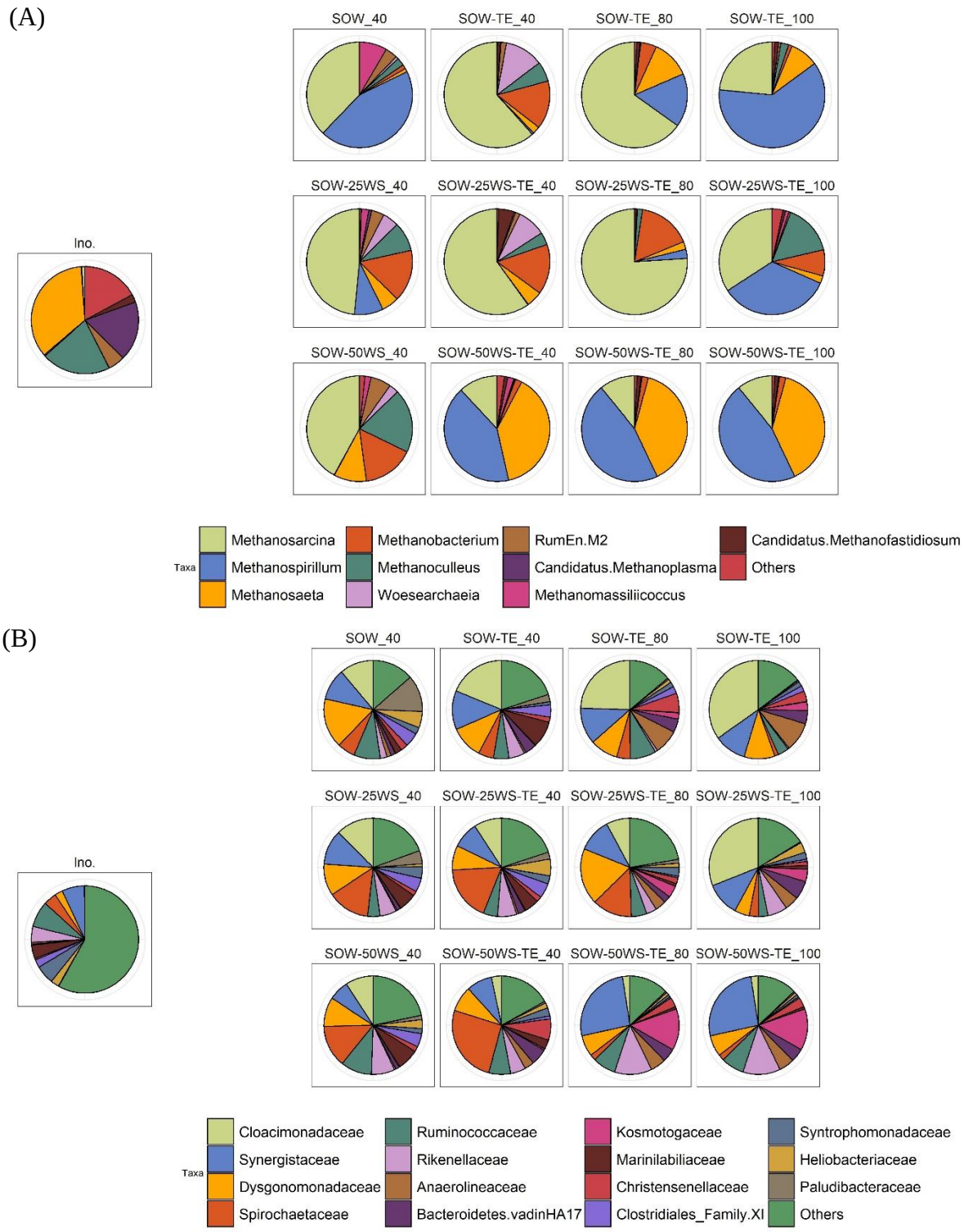


Figure 9-6. Beta diversity of microbial community taxa in relation with feed composition and time on day 0 (Ino. = inoculum), day 40, day 80 and day 100 in six AD reactors which digested a synthetic organic waste as mono substrate or co-digested with wheat straw with/without trace element supplementations. The acetoclastic methanogen Methanosaeta was the most abundant genus in the reactor with trace elements added and 50% wheat straw codigestion, while in the reactor without wheat straw codigestion and 25% wheat straw co-digestion the hydrogenotrophic methanogens were the most abundant archaeal genera. ((A) bacteria represented by the15 most abundant families and (B) archaea represented by the 13 most abundant genera). Families and genera with lower abundances are combined in “Others”.

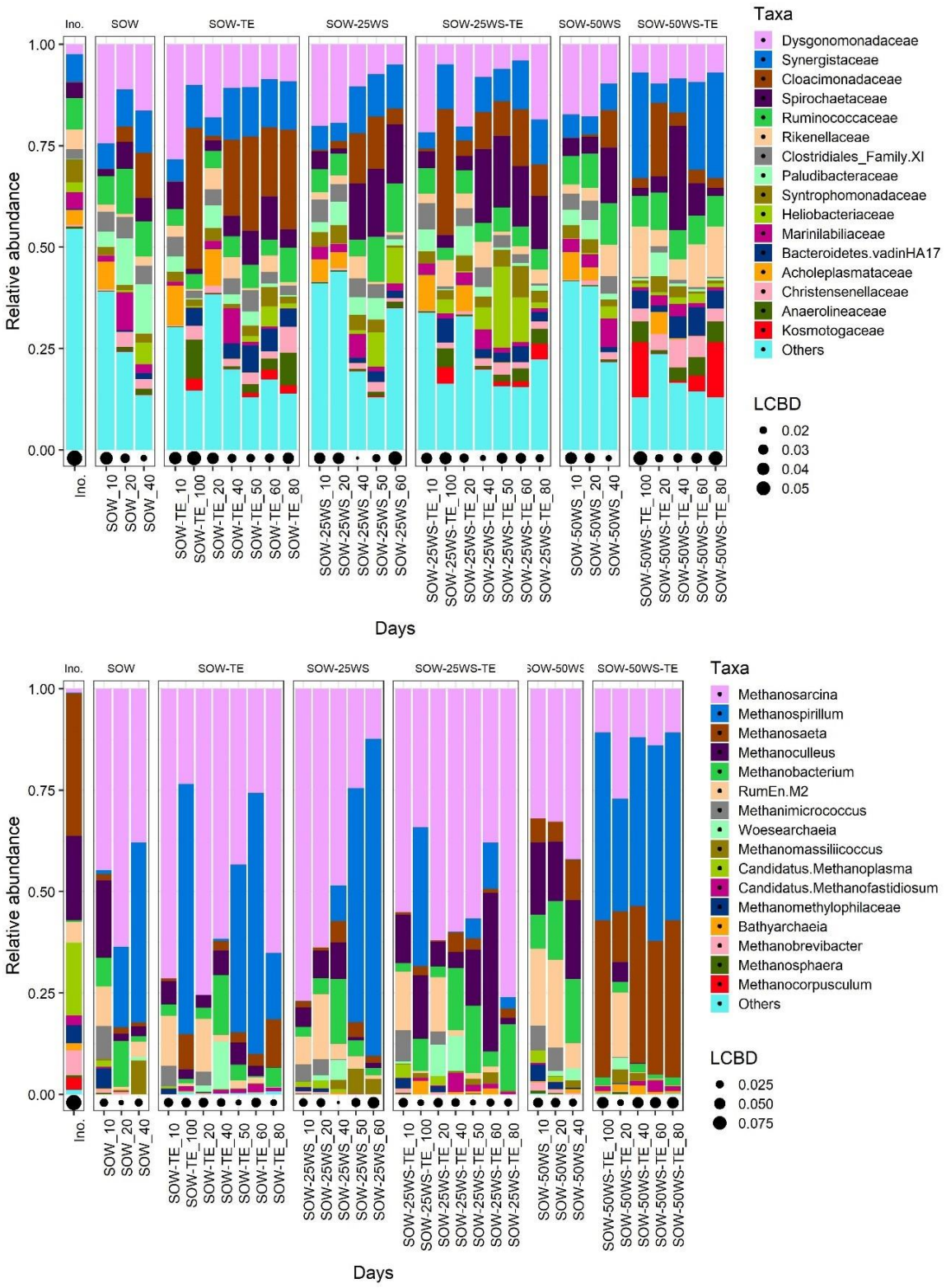


Figure 9-7. Beta diversity of microbial community taxa in relation with feed composition and time (the numbers with x axis texts denotes days and Ino. = inoculum) in six AD reactors digested a synthetic organic waste as mono substrate or co-digested with wheat straw with/without trace element supplementations (bacteria highest 16 families and archaea highest 13 genera). The bacterial families and archaea genera below these limits and with lower abundances are combined in “other”.

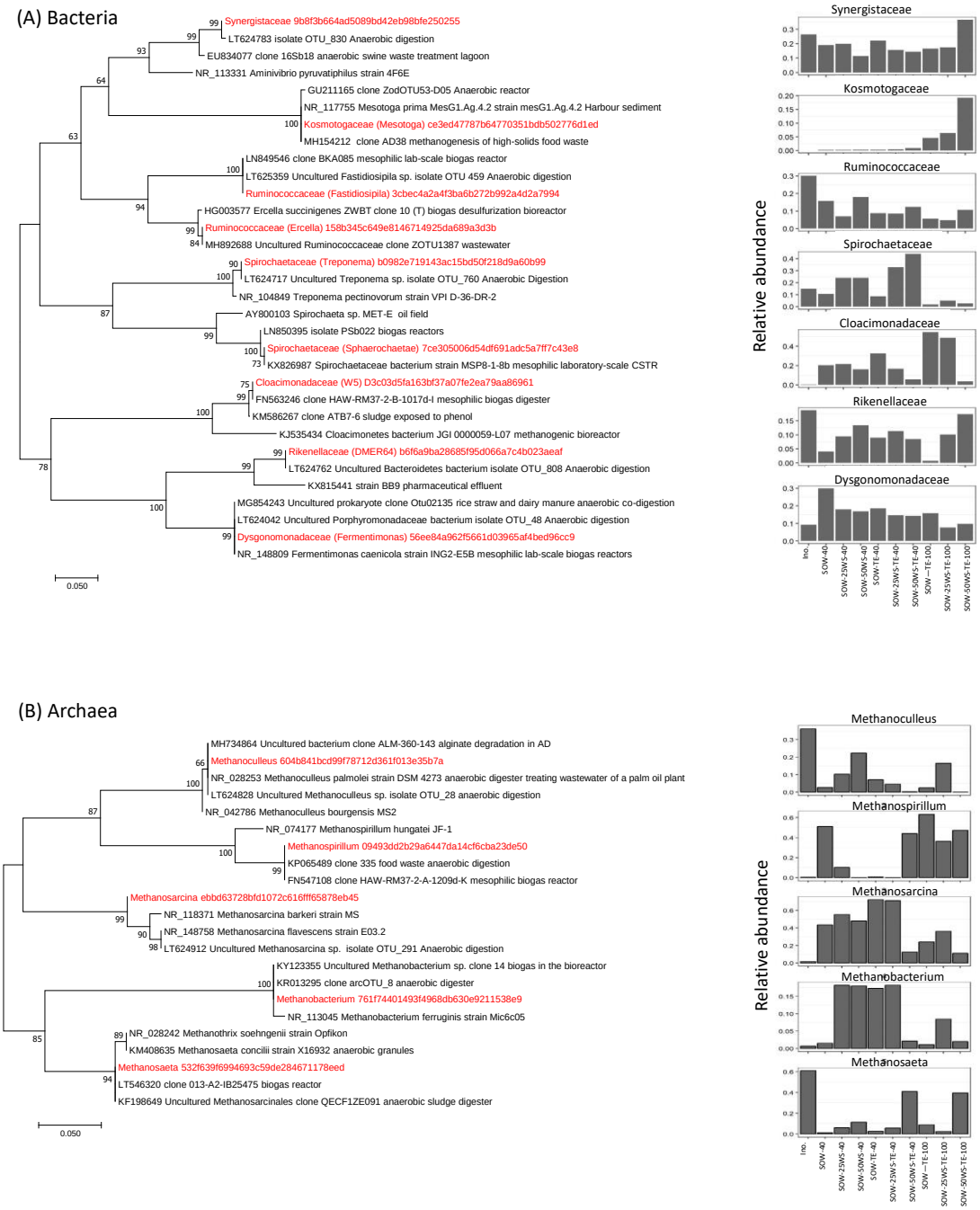


Figure 9-8. Phylogenetic distance tree (Neighbour-Joining) of key AD reactor bacterial / and archaeal taxa and close relatives (left) and, plots of the fractional abundances of these taxa in individual reactor sequence libraries (right). The tree is based on comparative analysis of selected partial 16S rRNA sequences recovered from the anaerobic reactors at day 0, 40 and 100 and indicated by individual codes assigned during pipeline analysis. The percentage of replicate trees in which the associated taxa clustered together in bootstrap analysis (1000 replicates) are shown next to the branches. The analysis involved 252 nucleotide positions.

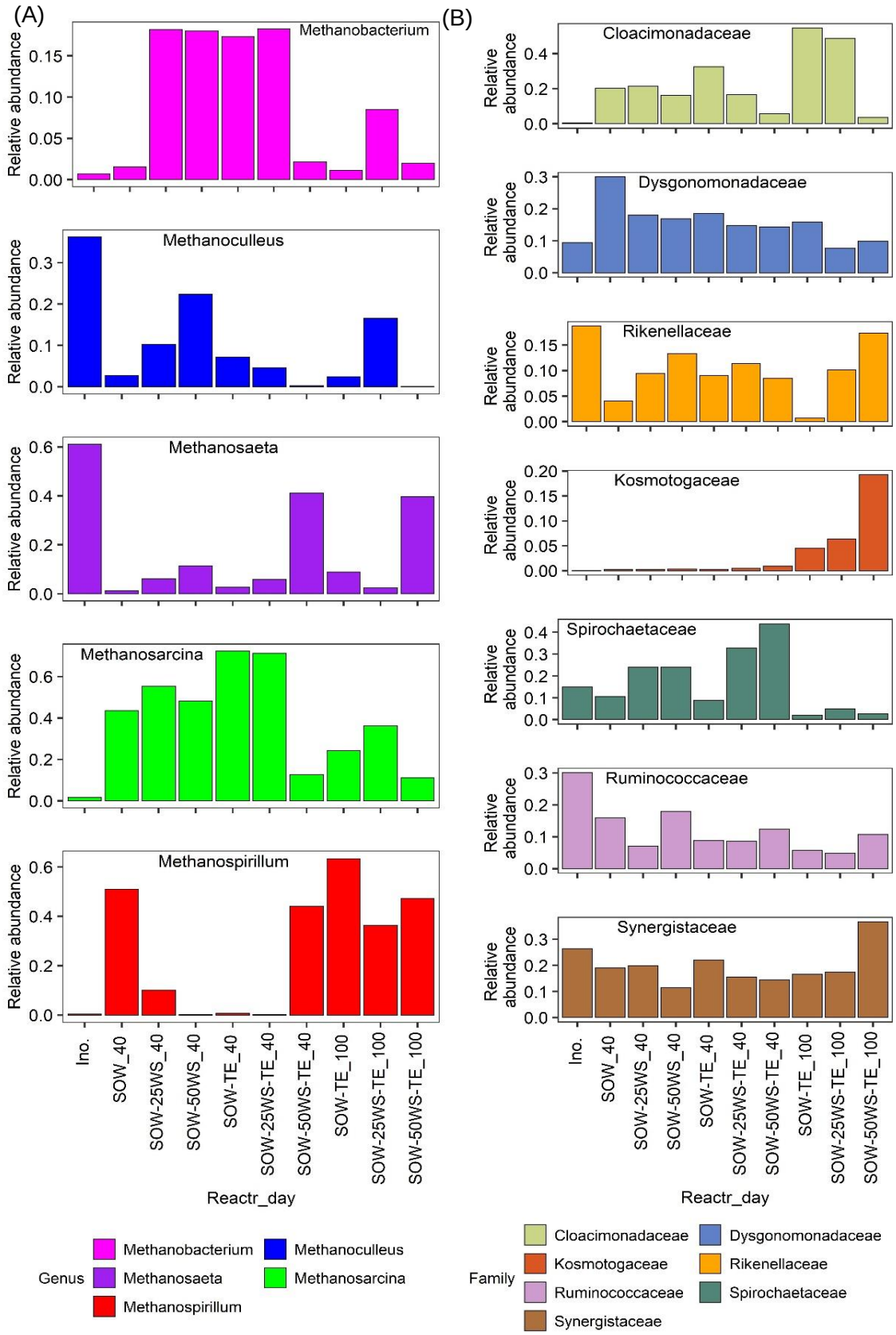


Figure 9-9. Relative abundance of key AD reactor archaeal (A) and bacterial (B) taxa in individual reactor sequence libraries. Substrate compositions changed the archaeal and bacterial compositions and their abundances with time. The figures are based on 16S rRNA sequences recovered from the anaerobic reactors at day 0 (Ino.), 40 and 100. The x-axes show reactors and sampling days. Ino. = the inoculum which was used to start up the 6 reactors.

9.3.2.2. *Archaeal community composition and dynamics*

In AD, microbial communities develop in response to the availability of nutrients including trace metals (Wintsche *et al.*, 2016; Li *et al.*, 2018; Koo *et al.*, 2019; Mao *et al.*, 2019) and the presence of inhibitors such as high concentrations of VFA (Karlsson *et al.*, 2012), heavy metals (Chapter 6) and ammonia (Principi *et al.*, 2009). The methanogenic archaea are often considered particularly sensitive to such parameters (Demirel, 2014) which is supported in the current study, whereby, in comparison to the bacteria, more indicative and interpretable changes in community composition occurred (Figure 9-6). Archaeal communities of the inoculum and the six reactors sampled at different time points were mainly dominated by sequence types related to just five archaeal genera: *Methanosarcina*, *Methanospirillum*, *Methanosaeta, Methanobacterium* and *Methanoculleus* consistent with the core archaeal microbiome of anaerobic digestion (Riviere *et al.*, 2009a). In the start-up inoculum the archaeal acetoclastic methanogenic genus *Methanosaeta* dominated (~ 40% of sequences) along with, to a lesser extent, the hydrogenotrophic *Methanoculleus*, *Methanobacterium* and *Methanoplasma* but by day 40, the six reactors had changed.

A key feature of this community shift was the universal enrichment of the metabolically more flexible methanogen *Methanosarcina*. However, while *Methanosarcina* was dominantly maintained in the 0 and 25%WS + TE amended reactors with successively higher loading rates; the *Methanosaeta* recovered and remained remarkably stable in the reactor with 50% WS + TE. These results suggest, given that *Methanosaeta* had a significantly positive ($p < 0.05$) correlation with the C/N ratios in the reactor feeds, that co-digestion with WS% was responsible for *Methanosaeta* stability and retention. Such selection with respect to feeding regimen has been reported previously. For instance, Conklin et al. (2006) found that infrequent (daily) and hence overload feeding of a mesophilic AD reactor increased the dominance of *Methanosarcina* while hourly feeding enriched for *Methanosaeta*. These authors concluded that selection for *Methanosarcin*a resulted from their ability to cope with periodic increases in acetate. However, high concentrations of acetate and VFA in general were apparently not important in the current study as these were all maintained at similarly low levels in the three sturdy TE reactors. As described above, one consistent measured difference between the more stable *Methanosaeta* dominated 50%WS + TE reactor and the other two reactors was the level and variation in NH_3-N concentrations (Figure 9-3) and their likely impact on community or pathway selection. Of particular relevance is the recent study of Chen *et al.* (2016) on the effects of ammonia on the semi-continuous anaerobic digestion of food waste which found that added ammonia inhibited methanogenesis but minimally affected

bacterial hydrolysis and acidification. Critically, in this study levels of NH_3-N at 2 g/L gradually led to reactor failure but also shifted the dominance of *Methanosaeta* and acetoclastic methanogenesis towards a dominance of hydrogenotrophic methanogens (*Methanobacterium* and *Methanospirillum*). This selection for hydrogenotrophic methanogenesis at higher ammonia concentrations in mesophilic reactors is well documented (e.g. (Schnurer and Nordberg, 2008)). In the current study, none of the three long term reactors had ammonia levels as high as those imposed by Chen *et al.* (2016) and all three maintained maximal reactor performance as a result of TE addition. However, there was a clear dominance of the facultatively hydrogenotrophic *Methanosarcina* and the obligately hydrogenotrophic *Methanospirillum* in the SOW only + TE and 25WS% + TE reactors in which NH_3-N levels were typically higher suggesting a community selection effect even at these NH_3-N levels. *Methanospirillum* (Ferry *et al.*, 1974) are commonly found in anaerobic bioreactors treating organic wastes (e.g. (Padmasiri *et al.*, 2007; Krakat *et al.*, 2010)).

9.3.3. *The metabolic flexibility of Methanosarcina (inferred from RFI measurements) as a function of WS co-digestion*

In the more stable 50% WS + TE amended reactor the overall contribution of putatively acetoclastic (*Methanosaeta*) and hydrogenotrophic (*Methanospirillum*) methanogens to biogas production was in balance for the period of reactor operation at successively higher loading rates. This balance cannot, however, be assumed in the other reactors given the common dominance of *Methanosarcina* which, in addition to a documented greater tolerance for environmental parameters such as high VFA, pH or ammonia (De Vrieze *et al.*, 2012), can be both acetoclastic, hydrogenotrophic and even methylotrophic. To help resolve potential differences in methanogenic activities in the reactors we examined in more detail variations in coenzyme F_{420} levels based on fluorescence at 420 nm (RFI) which is typically considered indicative of overall methanogenic activity (Figure 9-10). A critical ecophysiological characteristic of coenzyme F_{420} in methanogens is that its level is higher in hydrogenotrophic than in acetoclastic methanogens (Dolfing and Mulder, 1985; Reynolds and Colleran, 1987; Poorter, 2005) because acetoclastic methanogenesis is mediated by different enzymes (Greening *et al.*, 2016). In this context, we analysed the individual relationship between fluorescence (RFI) and methane production rates in each of the three long term stable (TE amended) reactors (Figure 9-10). Intriguingly, slopes for the 25%WS +TE and 50%WS + TE reactors were essentially the same and higher than found for the TE only reactor, indicating that hydrogenotrophic methanogenesis was dominant in the latter (more F_{420} was needed for the same rate of methanogenesis) while acetoclastic methanogenesis was more important in

the two co-digesting reactors. This data independently supports the conclusion drawn from community analysis through the dominance of *Methanosaeta*, that acetoclastic methanogens made up a significant part of the methanogenic community in the 50%WS + TE reactor. Interestingly, however, these observations also allow us to speculate that in the 25%WS + TE reactor, the *Methanosarcina*, functioned as acetoclastic methanogens. In contrast, when *Methanosarcina* dominated in the TE only reactor they likely functioned as hydrogenotrophs, a shift in activity which is indicative of the subtle effects that WS co-digestion had on these AD reactors.

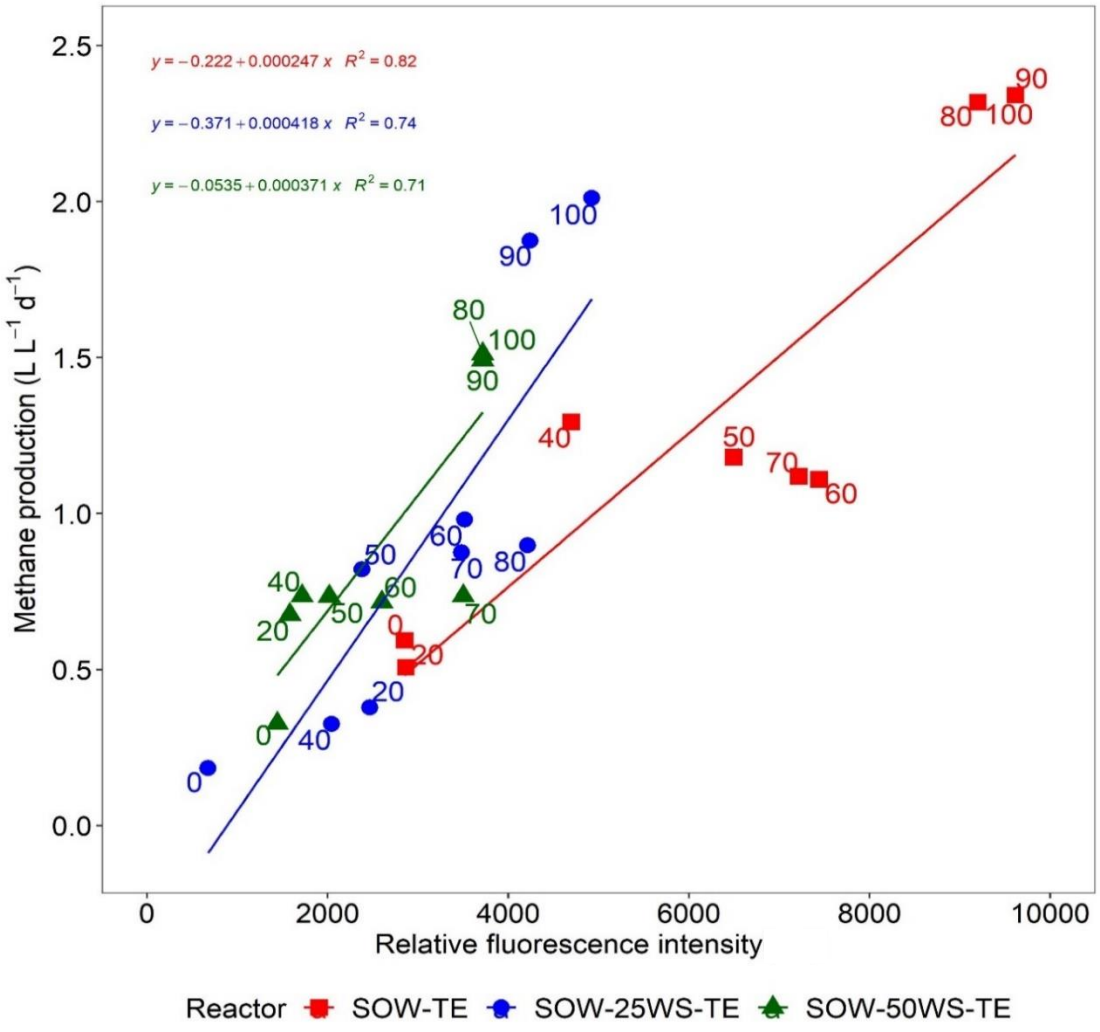


Figure 9-10. Relationship of co-enzyme F_{420} levels in reactor digestates with methane production in reactors with trace elements added. The labels (numbers) next to the scatter points are the collection days of samples from reactors. The reactors without co-digestion and low wheat straw co-digestion (25%WS) which had highest hydrogenotrophic methanogen abundances showed highest relative florescence intensity of co-enzyme F_{420} compared to the reactor with 50% wheat straw co-digestion which had a highest acetoclastic methanogen abundance.

9.4. Conclusions

Co-digestion and trace element supplementation are strategies known to affect reactor performance of easily digestible organic material such as the organic fraction of municipal solid waste. In the present study, involving a parallel, long term operation of reactors with and without trace element supplementation and addition of wheat straw as co-substrate we have been able establish the relative merit of these two strategies, and evaluate their link to community composition and operational stability.

Interestingly, and significantly, trace element addition was an absolute requirement for the long-term operational stability of the reactors, both with and without addition of wheat straw as co-substrate. Without trace element addition reactors failed after about two months: pH dropped and methane production stopped, indicating that trace element limitation first and foremost affected the methane producers. In the reactors that had been supplemented with trace elements as well as wheat straw loading rates of up to at least 4 kg volatile solids /m^3.day were consistently digested to methane for well over three months. The microbial population in these reactors was affected by the ratio between OFMSW and wheat straw. More wheat straw not only increased methane production but also resulted in a more diverse community and a shift in the physiology of the Methanosarcina population from hydrogenotrophic to acetoclastic methanogenesis. The findings of this study are valuable for the development of anaerobic digestion as a sound technology for the stabilisation of the organic fraction of municipal solid waste. For this technology to be viable, it needs to be robust and cheap. We show that this technology is robust as long as trace elements are provided, and that the process can be run cheaply in that the whole digestion process can be run in one single reactor with energy recovery from biogas as an additional “plus”. The current intense discussions about global warming emphasizes the need for production of renewable energy; biogas from the organic fraction of municipal solid waste fits in this paradigm.

Chapter 10. Conclusions and recommended future work

10.1. Conclusions

This thesis studied the effects of integrating four MW (IBA, CBW, FA and BA) from MSWI plants, and a CDW into the AD of OFMSW to enhance process stability and increase the methane productivity of anaerobic digesters. Amendment of MW at 1 g MW/g VS provided the optimum AD performance, producing biogas with 60 - 70% methane volume percentages in the produced biogas and increasing the total methane production by 25 - 45%. Analysis of VFA and COD, as well as kinetics analysis, showed that MW amendments had substantial effects on hydrolysis and acidogenesis without inhibitory effects on methanogenesis.

The feasibility of using such MW as additives to promote the AD of OFMSW was investigated by determining the physicochemical characteristics of the MW, the inoculum for reactor startup, and the synthetic organic waste (SOW) feedstock, after which AD experiments were performed using both batch reactors for biomethane potential (BMP) experiments, and CSTR systems for continuous experiments.

The BMP experiments (Chapter 5 and Chapter 7) showed that MW could be integrated into the AD of OFMSW to provide moderate alkalinity, maintain pH at optimal levels (6.2 - 7.5), enhance the hydrolysis of organic matter, and consequently promote biogas production and methane yield. Moreover, the BMP experiments showed that despite the presence of relatively high concentrations of trace and heavy metals in the raw MW, under mesophilic AD conditions, the leaching of heavy metals from most of the MW was very low and not inhibitory to microorganisms. Three of the four MW used in this study (IBA, CBW and BA), had positive effects on the methane production kinetics and the specific growth rates of microorganisms. However, FA had only a minor effect on the kinetics of methane production and microbial growth rate, which was linked to the presence of high concentrations of heavy metals in FA compared to the other three MW (i.e. IBA, CBW and BA).

Three CSTR experiments were performed that used co-digestion of the OFMSW and mineral wastes using both liquid-recycled (LRFM) and draw-and-fill (DFFM) feeding methods (Chapter 6); pre-treatment of the OFMSW and mineral wastes at 37^{o}C then AD at mesophilic temperature (37^{o}C) (Chapter 7); and integration of MW-extracts into two-stage mesophilic AD of the OFMSW (Chapter 8), to assess how the reactor performance (methane production rate and yield, and process stability) and microbial ecology and function changed under different integration methods for combining the MW and OFMSW. All three CSTR studies

showed that the metals released from MW enhanced buffering capacity and microbial activities (metabolic/catabolic activities) in the AD reactors without producing inhibitory/toxicity effects. Moreover, LRFM was found to be the best feeding method for anaerobic co-digestion of OFMSW with MW. Results in Chapter 7 showed that pre-treatment of the OFMSW with MW at 37^{o}C can enhance the limited positive effects of MW under DFFM. However, similar to the results obtained from the BMP experiments, any benefits observed with FA under all the CSTR treatment conditions were very limited compared to IBA, CBW and BA mineral wastes linked to high concentrations of some of the metals leached from the FA waste. The results obtained from integrating MW-extracts into a two-stage CSTR experiments showed that the shock decrease in HRT from 20 days to 10 days affected methanogenic performance in the second stage (methanogenic) reactors, however, the methanogenic reactor which was amended with IBA mineral extracts, tolerated the high OLR and short HRT without any signs of failure, albeit with a lower methane yield at the10 day HRT. The good performance of reactors amended with IBA was a general feature in this thesis linked to the metals present in this MW and the bioavailability of metals (within optimum levels for AD) under mesophilic conditions. All three studies showed that both feeding method and operation time had significant effects on the microbial diversity in the CSTR amended with or without the MW. Moreover, at low TE concentrations, metabolic pathways were dominated by acetoclastic methanogenesis represented by *Methanosaeta;* at moderate TE-concentration, a balance between acetoclastic (*Methanosaeta*) and hydrogenotrophic (*Methanobacterium* and *Methanospirillum*) metabolic pathways was observed, whilst at high TE concentration the metabolic pathway shifted towards the hydrogenotrophic pathway with a predominance of *Methanosarcina* /and *Methanoculleus*.

The fourth CSTR experiment was conducted with amendments of a pure (standard) TE solution and wheat straw as a co-digestion feedstock with the OFMSW. The results indicated that high reactor performance and microbial numbers, and good process stability at higher OLRs could only be maintained with TE supplementation, regardless of co-digestion with wheat straw. However, co-digestion was beneficial in reducing biological stress linked to lower ammonia; this condition was clearly observed from the dominance of both acetoclastic (represented by *Methanosaeta*) and hydrogenotrophic methanogens (such as *Methanospirillum*) in the co-digestion reactors. Moreover, in this experiment the F_{420} levels in the digestate of reactors showed that *Methanosarcina* was metabolically the more versatile archaeal methanogen since it could follow both an acetoclastic pathway with 25% wheat straw, and a hydrogenotrophic pathway with no WS and highly elevated NH_3 levels.

In summary, the broad conclusion from this thesis is that the integration of MW into the AD of OFMSW has two main cost benefits of commercial significance, better treatment of the OFMSW, and greater production renewable energy. These resulted from an improved performance of the microbial communities in the digesters caused by several factors, the main one being the release of TE from MW that improved the growth and activity of the methanogenic community. However, it was also shown that the different MW integration methods applied in this study could have further positive effects on methanogen community composition and stability, and hence enhance further the productivity and process stability of anaerobic reactors fed with the OFMSW.

10.2. Future work

Mineral wastes are readily available resources rich in trace and heavy metals that can be recovered for integration in AD systems. Further studies are possible and necessary to identify practically applicable technologies for integrating MW in full-scale AD systems. Most importantly, identifying novel systems for preparing MW, extracting minerals and nutrients, as well as developing technologies for amending the MW in digesters are essential. The effective sorting of the MSW at the source is the best way to achieve a high-quality feedstock for the AD of OFMSW according to the integration systems proposed in this thesis, thereby minimising the risk of excessive concentrations of contaminants in the digestate so that it could be used for land application as fertiliser or soil conditioner. Another method for obtaining optimal quality of the digestate requires other studies to be undertaken for controlling the quality and composition of the minerals which can be extracted from MW before their amendment into the AD reactor systems.

The experiments in the current study were only conducted under mesophilic conditions, so it would be important for future studies to be conducted under thermophilic conditions and at varied HRT/SRT and mixing conditions to elucidate the changes in the concentration and composition of the minerals that can be released from or recovered from MW as the stability and especially the solubility of the metals from the wastes are likely to be very different. Depending on these results mathematical models could be developed to predict the relationship between the composition and concentration of minerals leached from MW under different feedstock characteristics (such as organic contents, pH, particles size and etc.) and reactor operation parameters (such as temperature, pH, mixing, reactor-stages and etc.) to ensure optimal reactor performance and process stability. Moreover, the effects of MW amendments on the CO_2 sequestration in anaerobic digesters could be studied as this must be

affected by the alkalinity provided by MW. Furthermore, improved molecular biological analysis techniques could be conducted to monitor the methanogenic populations more quantitatively under different MW amendments and reactor operation parameters.

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Appendix

Table A-7-1. Measured parameters of digestate from anaerobic BMP assays

Characteristics of digestate								
BMP	pH	sCOD	TS	VS	VS/TS	FOS/TAC	Total VFA	Total alkalinity
		(g/L)	(g/L)	(g/L)	(%)		(g/L) as acetate	(g $CaCO_3$/L)
Control	7.5	2.9	10.1	7.3	72%	0.12	0.75	6.3
FrTh	7.7	3.1	10.0	7.0	70%	0.11	0.68	6.2
TP	7.8	2.7	10.5	7.3	69%	0.13	0.85	6.3
TP-IBA	7.8	1.8	13.3	7.3	55%	0.11	0.76	6.7
TP-CBW	7.9	2.4	13.7	7.0	51%	0.14	1.0	7.3
TP-FA	7.6	2.6	14.3	7.0	49%	0.14	0.85	6.3
TP-BA	7.8	2.4	14.5	7.3	50%	0.11	0.68	6.3
TP-Alk	8.1	3.4	12.0	7.3	60%	0.13	1.0	7.8

Table A-7-2. Estimated energy consumption for pre-treatment conditions.

Pre-treatment time	**Incubator Energy (kwh)**	**Incubator capacity (Kg)**	**Energy consumption (MJ/kg**				
one hour pre-treatment	0.025	3	0.06				
12 hours pre-treatment	0.3	3	0.72				
110 hours pre-treatment	2.75	3	6				
	Methane Yield (M^3/kg)						
HRT	**Pre-treatment time**	**Control**	**TP**	**TP-IBA**	**TP-CBW**	**TP-FA**	**TP-BA**
1	12	0.53	0.53	0.53	0.54	0.51	0.5
2	12	0.49	0.50	0.51	0.48	0.41	0.4
3	1	0.46	0.47	0.46	0.43	0.17	0.3
4	1	0.37	0.28	0.46	0.49	0.15	0.4
	Total energy of OFMSW as methane (MJ/kg) (*assume CV CH_4 = 40 MJ/M^3)						
HRT	**Pre-treatment time**	**Control**	**TP**	**TP-IBA**	**TP-CBW**	**TP-FA**	**TP-BA**
1	12	21.3	21.0	21.2	21.8	20.2	20.1
2	12	19.7	20.1	20.3	19.2	16.3	15.6
3	1	18.6	18.7	18.5	17.1	7.0	12.3
4	1	14.7	11.4	18.3	19.5	5.8	16.8
	Net (MJ/kg) (*assume CV CH_4 = 40 MJ/M^3)						
HRT	**Pre-treatment time**	**Control**	**TP**	**TP-IBA**	**TP-CBW**	**TP-FA**	**TP-BA**
1	12	21.3	20.3	20.4	21.1	19.5	19.4
2	12	19.7	19.4	19.6	18.5	15.6	14.9
3	1	18.6	18.6	18.4	17.1	6.9	12.3
4	1	14.7	11.3	18.2	19.4	5.7	16.7
		% control		122%	130%	37%	112%
		%TP		163%	174%	49%	149%

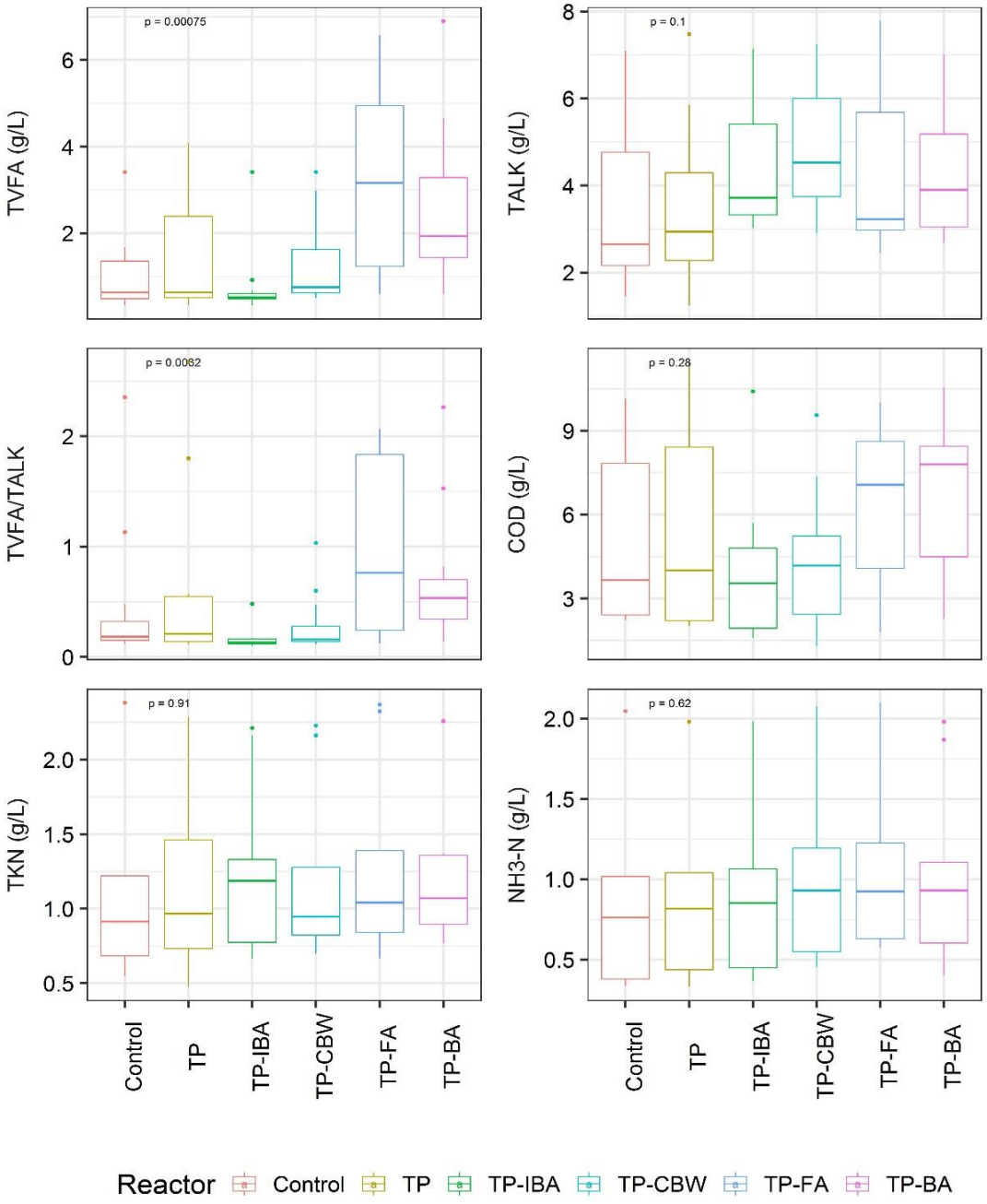

Figure A-7.1. Box-plot showing different parameters measured in CSTRs throughout 80 days (four measurements for each reactor i.e. one measurement per each HRT). P-values show one-way ANOVA analysis for the significance difference in the mean value of parameters between reactors.

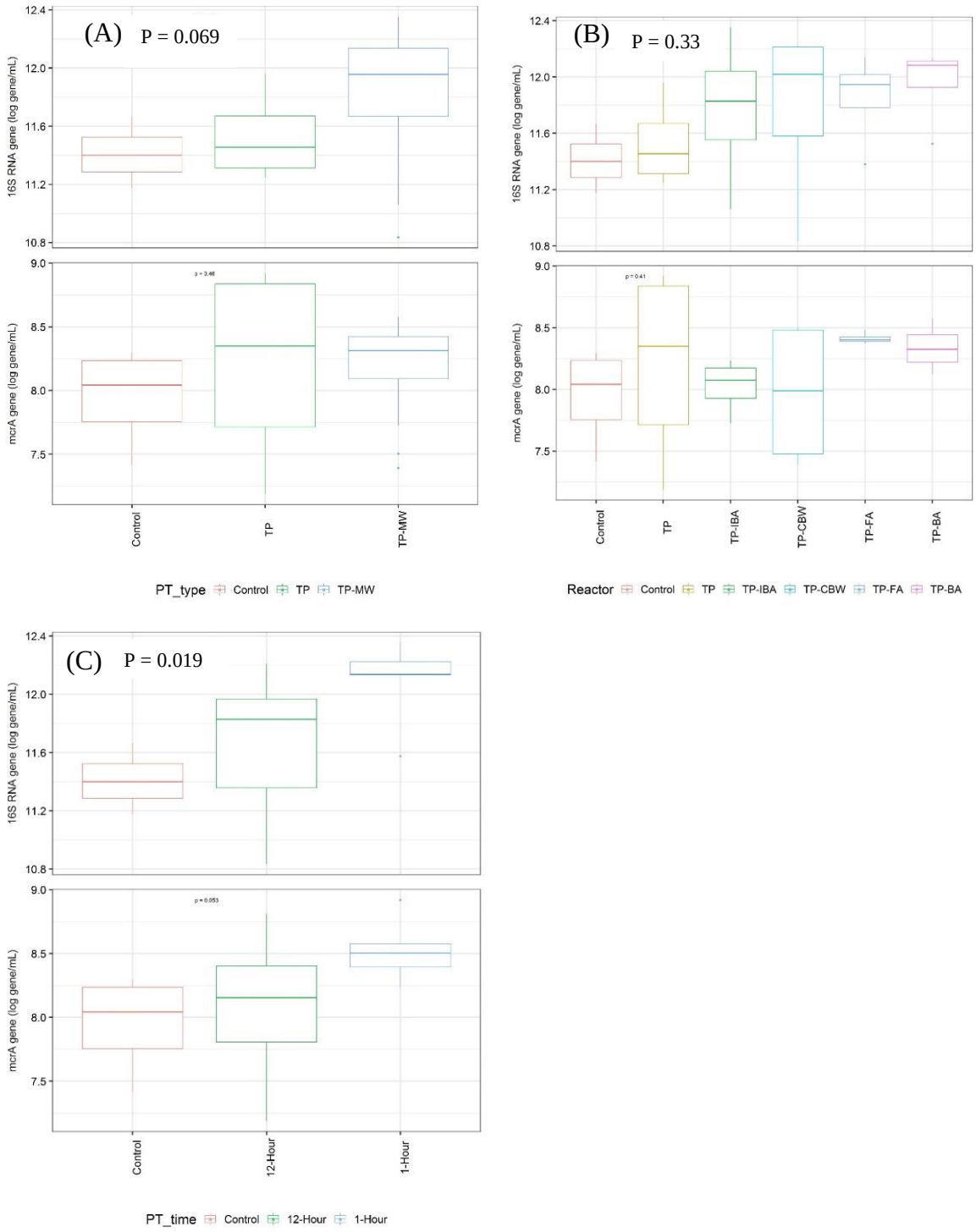

Figure A-7.2. Box-plot and p-value of the difference in means using one-way ANOVA of the abundance of bacteria and archaea from qPCR in CSTRs throughout this study (A) between reactors; (B) between control, TP and TP-MW reactors and (C) between reactors with different pre-treatment times of the feedstock. PT_type = pre-treatment type of the feedstock (SOW) i.e. "Control" without pre-treatment. "TP" only pre-treatment without MW addition and "TP-MW" pre-treatment using MW addition. PT_time = the pre-treatment time i.e. "Control" without pre-treatment, "12-Hour" = pre-treatment for 12 hours and "1-Hour" = pre-treatment for one hour.

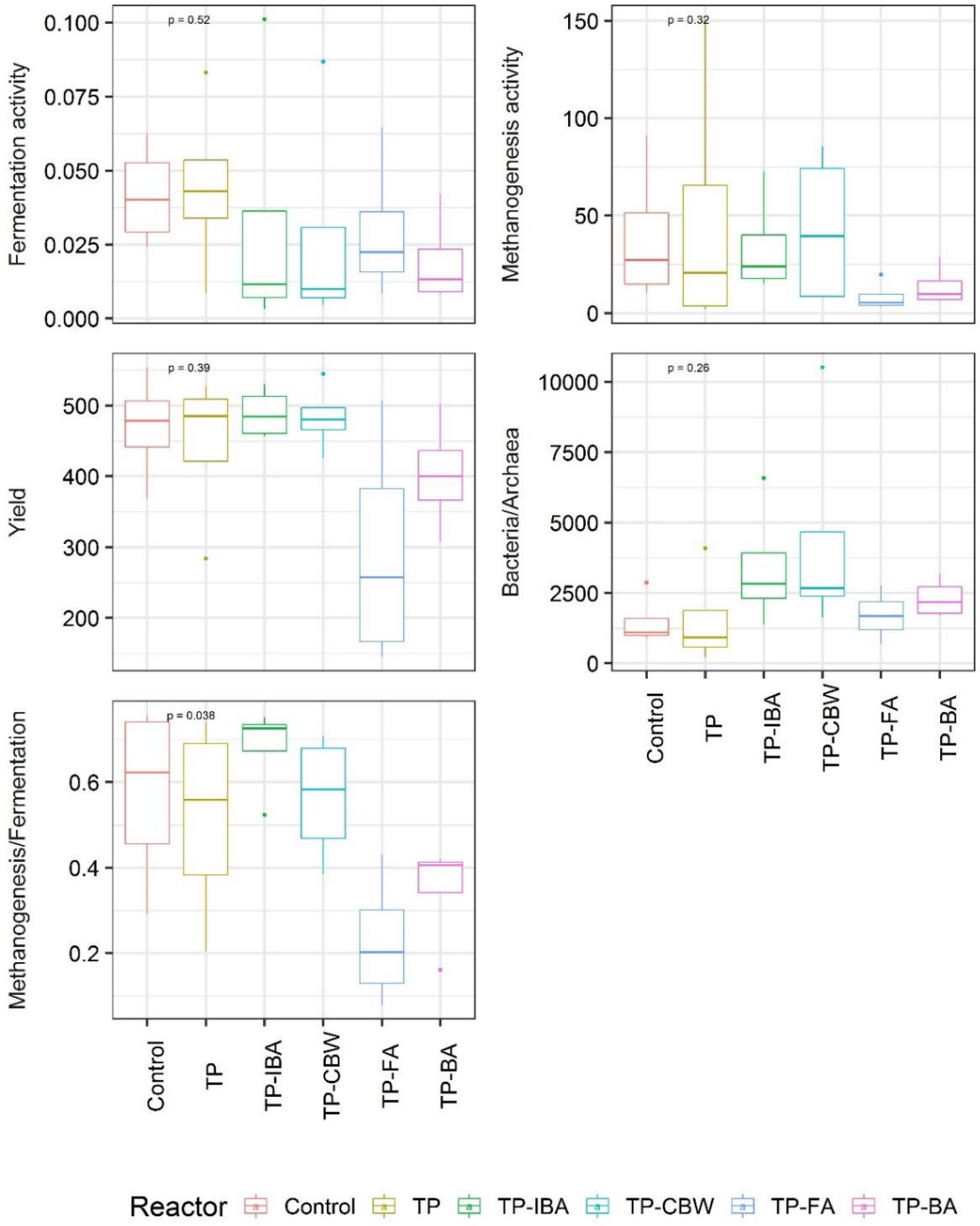

Figure A-7.3. Box-plot of mean values and p-values using one-way ANOVA for variations in the microbial growth and activity in CSTRs. The values are for the whole period throughout this study.

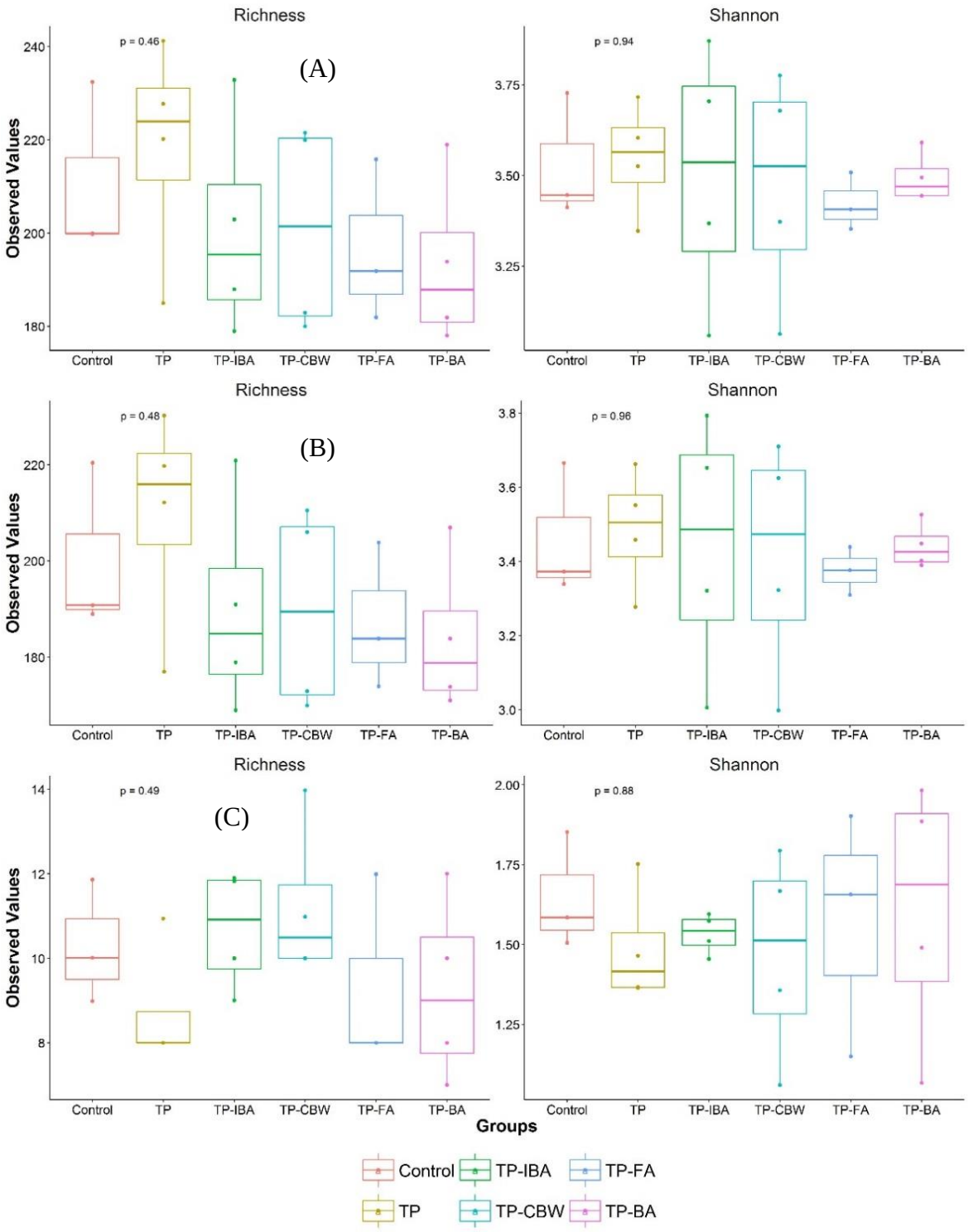

Figure A-7.4. Box-plot for mean alpha diversity (four digestate samples from each reactor i.e. one sample per each HRT) values Richness and Shannon and p-values for significant difference in the values between reactors measured using one- way ANOVA (A) All taxa (B) Bacterial taxa (C) Archaeal taxa in CSTRs throughout 80 days.

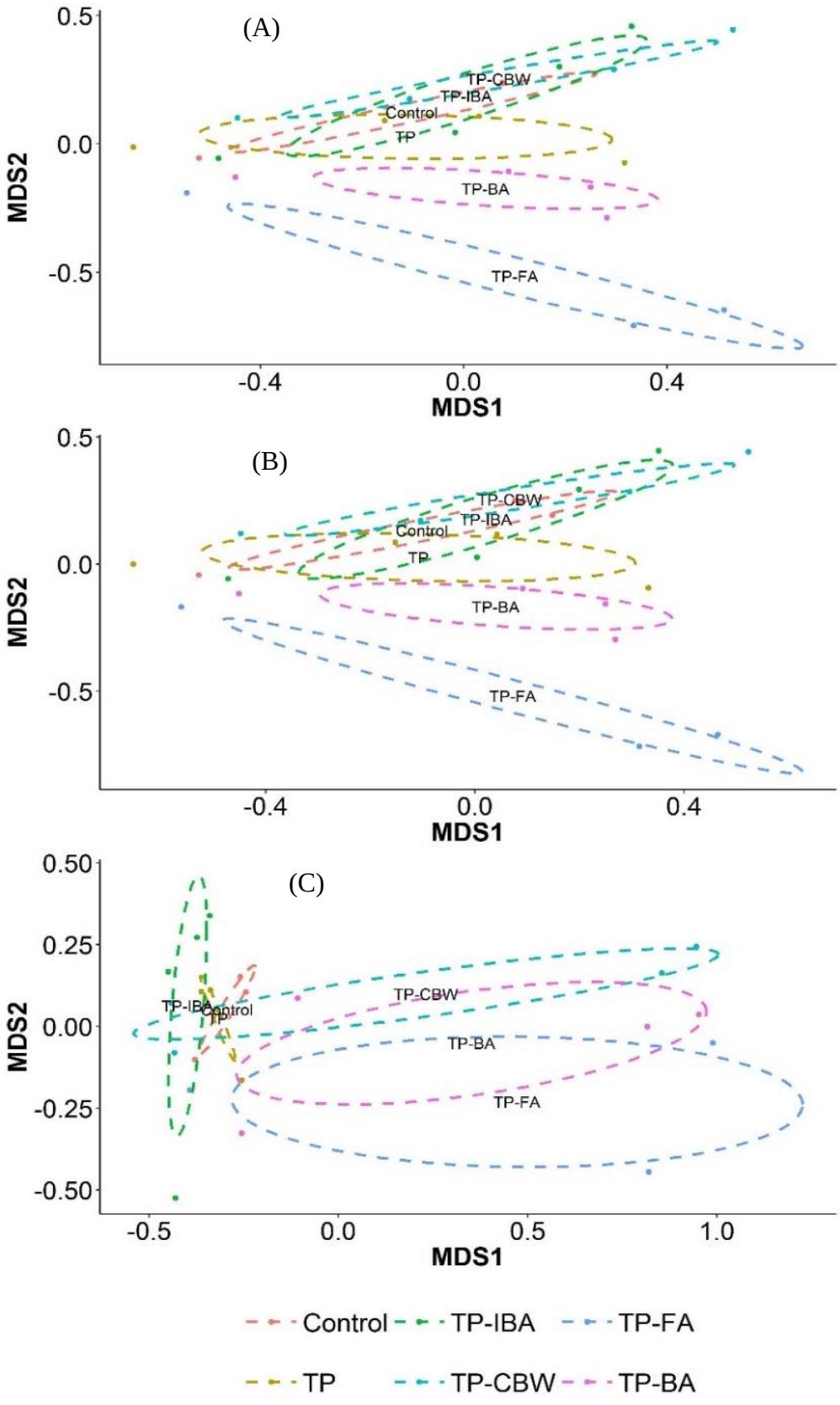

Figure A-7.5. Separation of microbial community using MDS in (A) All taxa; (B) Bacterial taxa and (C) Archaeal taxa abundances from 16S rRNA sequencing data of digestate samples from CSTRs according to their substrates (reactor names) with/without pre-treatments (TP) and with/without MW amendments (IBA, CBW, FA and BA). Calculated from four digestate samples per reactor collected at four HRTs.

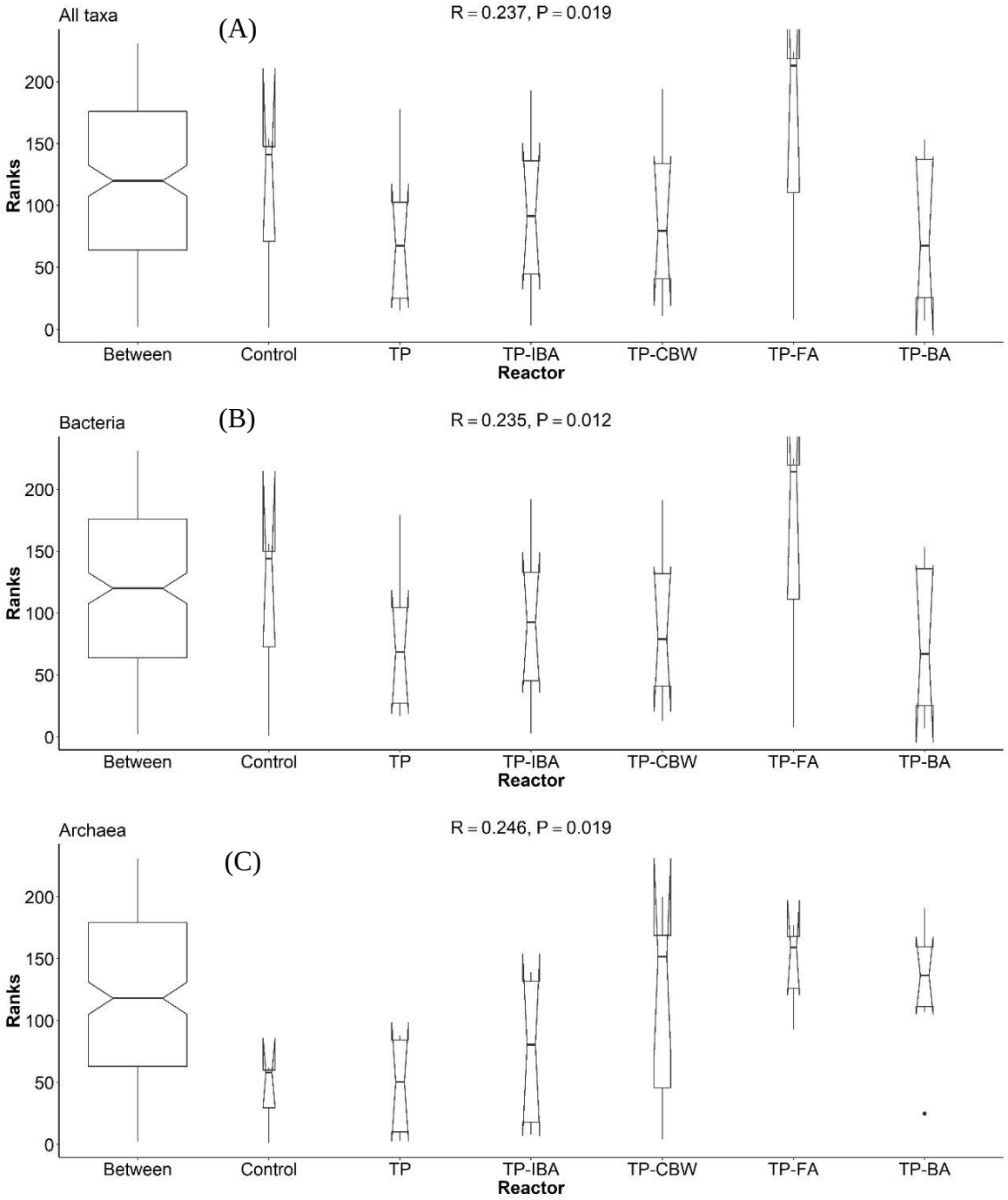

Figure A-7.6. Analysis of similarity ANOSIM for (A) All taxa; (B) Bacterial taxa and (C) Archaeal taxa abundances in CSTRs. Calculated from four digestate samples per reactor one sample of digestate per each HRT.