Microbial Community Dynamics and Operational Performance in Rice Straw Anaerobic Digestion

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Abstract

Waste rice straw (RS) is generated in massive quantities around the world and is often burned or left to rot in the fields creating environmental and health issues. Work has shown anaerobic digestion (AD) of RS is feasible, but is primarily associated with expensive and/or hazardous pretreatment methods. Therefore, this study sought to determine if RS AD was possible without pretreatment and under what conditions.

Biomethane potential (BMP) batch tests (~200 days) found methane yields were similar at organic loading rates (OLR) of 1 to 3 g VS/L; 425 µm and 1.0 mm RS particle sizes were superior to 30 mm and 70 mm; and addition of dairy manure reduced yields. Nigerian RS methane yields were higher than Chinese, Indian, and Philippine RS, with BMP yields increasing with lower lignocellulose. Using the BMP data, 2.5 L continuously-stirred reactors were operated (~500 days) at five feeding frequencies (FF) and two different OLRs. Less frequent feeding at low OLR was more effective probably because of the relative recalcitrance of RS; a FF well-suited to acyclic harvesting at RS in rural settings. However, less frequently fed units (1/14 and 1/21) at higher OLRs both failed (soured) due to apparent organic acid accumulation owing to overloading. 16S rDNA amplicon sequencing showed 'healthy' reactors were dominated by *Methanogens* and *Bacteroidetes* and 'souring' of reactors led to dominance by *Firmicutes*. Overall, increasing the OLR had a much greater impact on AD microbial communities and infrequent feeding did not have a negative impact at low OLR. The substrate, not the feeding regime, dictated the community. In this case the traditional markers of reactor stability e.g. pH, did not suggest impending reactor failure. However, microbial population shifts, such as increases in fermenters and decreases in methanogens, reacted earliest and could be used as an early warning of forthcoming system failure. Further, four RS:DM ratios were tested (to assess different C:N ratios) in the reactors. 100 % RS had the highest methane yield with increasing DM reducing yields. Sequencing data indicated microbial community richness increased with the increasing DM addition. Predominant OTUs in the RS only unit were *Bacteroidetes*/*Firmicutes* and the 30:70 RS:DM unit was *Proteobacteria*. DM reactors had lower abundances of cellulosic hydrolysing bacteria such as, *Christensenellaceae* and *Bacteroidetes*. Data suggest DM-amended reactors were underfed. As such, the benefit of co-digestion would be decreased VFA production and VS reduction, which might cope with higher OLR, enabling a greater throughput of RS. Overall, results show that RS AD without major pretreatment is feasible, especially at lower OLRs with less frequent feedings, although co-digestion with manure could allow higher OLR operations, although this needs to be proven.

Acknowledgements

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To Denise, "*You are my everything. My life without you would be a lonely, alcoholic abyss. Undoubtedly without your excellent communications skills I would be a friendless loser. I owe you everything but in particular 4 years of part time hours (where you can be a smug arsehole as much as you like), a billion pounds in printer credits, a holiday where emails are not checked and a night out where statistical analysis is not mentioned and some nice earrings/a watch.*" Denise, 2017.

Seriously…you've probably earned a handshake.

Statement of publications and declaration

The following contributions to this thesis and publications of the authors and others is acknowledged.

Chapter 4 is based on a publication, with some edits and further details added.

- Zealand, A.M., Roskilly, A.P. and Graham, D.W. (2017) 'Effect of feeding frequency and organic loading rate on biomethane production in the anaerobic digestion of rice straw', *Applied Energy*.

16S DNA amplicon data used in Chapters 5 and 6 has also been deposited online at the NCBI GenBank under accession numbers MG808422 - MG811525 and MG852175 - MG855654, respectively.

Due reference is given to literature and any research collaborations where appropriate and I hereby certify that the work presented in this thesis is my original research work. No part of this thesis has been submitted previously for a degree at this or any university.

Andrew Zealand

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Abbreviations

AD	Anaerobic Digestion
ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
BMP	Bio(chemical) Methane Potential
CHP	Combined Heat and Power
CSTR	Continually Stirred Tank Reactor
DGGE	Denaturing Gradient Gel Electrophoresis
DistLM	Distance based Linear Model
DM	Dairy Manure
FF	Feeding Frequency
GHG	Greenhouse Gas
HRT	Hydraulic Retention Time
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
I:S	Inoculum:subsrate
MDS	Multi-Dimensional Scaling
NGS	Next Generation Sequencing
PCA and PCO	Principal Components and Coordinates Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Analysis of Variance
PS	Particle Size
OLR	Organic Loading Rate
OTU	Operational Taxonomic Unit(s)
RS	Rice Straw
RS AD	Rice Straw Anaerobic Digestion
STP	Standard Temperature and Pressure
TDT	Technical Digestion Time
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VS	Volatile Solids
VSR	Volatile Solids Reduction

Chapter 1 Introduction

World energy demand is forecast to increase by 48 % by 2040 and, although this demand will be shared worldwide, increased industrial growth of Asia, particularly China and India, will account for more than half this increase (U.S. Energy Information Administration [USEID], 2016). Further, worldwide greenhouse gas (GHG) emissions from agriculture, forestry and fisheries have nearly doubled since the 1960s and could further increase by 30 % if no actions are taken (Food and Agriculture Organization of the United Nations [FAO], 2014). Coupled with increasing demand on the energy sector, the world's climate continues to change with warm and more extreme weather, including increased serious air pollution events (Zhang *et al.*, 2006; United Nations News Centre, 2015). Given many areas across Asia already suffer from poor air quality (Figure 1.1) and associated negative health impacts, such as stroke, lung disease and chronic pulmonary problems, all efforts should be made to develop new energy sources as well as reducing GHG emissions.

Figure 1.1: Smog in Beijing, China.

Over the recent decades there has been a significant economic expansion in China, driven mostly by fossil fuels, with consequentially increasing GHG releases. The combination of increased population, air pollution and climate change has led China

to overtake the US as the greatest carbon dioxide emitter (in 2007) as reported by Kan (2011). Further, many countries, including China, have been slow to recognize the twin issues of energy demand and climate change despite attempts to limit their combined effect (e.g. the Kyoto Protocol). The Chinese government knows about the links between energy, climate change and health, but has been slow to react with declaring war on air pollution (BBC, 2016). However, China has recently targeted reducing emissions through, for example, the Chinese Certified Emission Reduction (CCER) scheme, with the resulting reduced coal use, GHG releases are expected to decline (Green and Stern, 2015).

Of worldwide GHGs, it is thought that methane (CH_4) will account for 20 % of future emissions (Intergovernmental Panel on Climate Change [IPCC], 2002). Increased CH_4 emissions are of extra concern as NASA (2007) found that the effects of individual methane compounds on world warming may be double what was previously thought. Rohde and Muller (2015) found that air pollution comes from a wide range of sources and effects areas other than cities and low lying basins, although the worst affected area in China is that spanning Shanghai to north of Beijing. They also calculated that the air pollution in China has contributed to 1.6 million, or 17 % of deaths per year. Kim *et al.* (2017) also found the relationship between particulate matter smaller than 10 μm (PM_{10}) and mortality rates varies across seasons and locations in China, Japan and South Korea though it tends to be worse in winter. The exposure to soot and smoke causes respiratory issues amongst farmers and local people (Blanca-Ferrer, 2013). Therefore, renewable energy options that reduce GHGs and improve health outcomes are needed, including bioenergy production from agricultural waste streams, such as waste rice straw (RS).

Approximately 620 Mt of rice was produced worldwide in 2009 (Figure 1.2), equating to around 840 Mt of RS waste, although production levels are increasing (Mussoline *et al.*, 2013a). RS is a waste product (crop residue) that remains after the harvesting of the rice grains (seeds) that is collected shortly after the main harvest (Lim et al., 2012). Worldwide, RS production is dominated by Asia, which produces > 90 % (Zhao *et al.*, 2010; Lim *et al.*, 2012). Across two-three growth cycles per year China and India share approximately 51 % of world production (Gadde et al., 2009), with China producing higher yields than India (Mohanty *et al.*, 2013).

Figure 1.2: Rice terraces from above by Gao (2015)

RS is a fibrous, lignocellulosic biomass with high volatile solids and low bulking density (Rice Knowledge Bank [RKB], 2009b; Mussoline *et al.*, 2013a), represents around 62 % of total crop residues in China and is the third largest crop residue in the world behind maize and wheat(Mussoline *et al.*, 2013b). RS tends to be produced in large quantities, but in irregular cycles related to seasonal harvests, which has always posed a problem for using RS as a bioresource. As such, RS is usually left in the fields and-or burnt (Figure 1.3), resulting in 13400 t of CH_4 and 800 t of nitrous oxide (N_2O) per year. In fact, Li *et al.* (2002) estimated rice cultivation accounts for up to 5.1 Mt of CH_4 a year, approximately 10 % of world's emissions. Incorporation of RS into the soil can improve crop yield, but is difficult due the relatively short time between harvest and re-seed (RKB, 2009a). As RS decomposes in naturally anaerobic field conditions, it releases CH_4 that makes up 10-15 % of world CH_4 emissions (IPCC, 2002; Mussoline *et al.*, 2013a; International Rice Research Institute [IRRI], 2014b).

Figure 1.3: Farmer burning rice straw in Vietnam (Eng, 2015)

The global quantities of RS and other agricultural crop wastes makes them a huge source of potential energy. For example, China produces approximately 200 Mt of rice each year with each tonne producing ~ 1.35 t of RS (Lim *et al.*, 2012). Current practices make RS a large source of air pollution, that exacerbates respiratory and smog issues, as well as an underutilised potential energy source (IPCC, 2002; IRRI, 2014a). Therefore, utilising RS as a renewable energy source could provide numerous benefits including reduced air pollution, improved environmental quality, and a decrease in the health issues associated with current RS management.

Anaerobic digestion (AD) is a process in which the microbial community breaks down organic matter in the absence of oxygen, creating digestate and biogas. AD can utilise a range of substrates to generate bioenergy, including food waste, energy crops, wastewater sludge, and slurry. However, lignocellulosic material, such as RS, is not a preferred substrate as it is considered recalcitrant to AD and offers lower biomethane yields compared to other crops (Mussoline *et al.*, 2013a). As the use of traditional household fuels such as wood, produce high amounts of residential and environmental pollution as particulate matter and deforestation, the need for biogas is increasing. Local biogas production could reduce this pollution and offer an

alternative for lighting, heating, and-or electricity generation (Krishania *et al.*, 2013a). The process has the potential to remove the associated pollution and generate useable energy whilst also removing the problematic waste stream of rice straw (Figure 1.4).

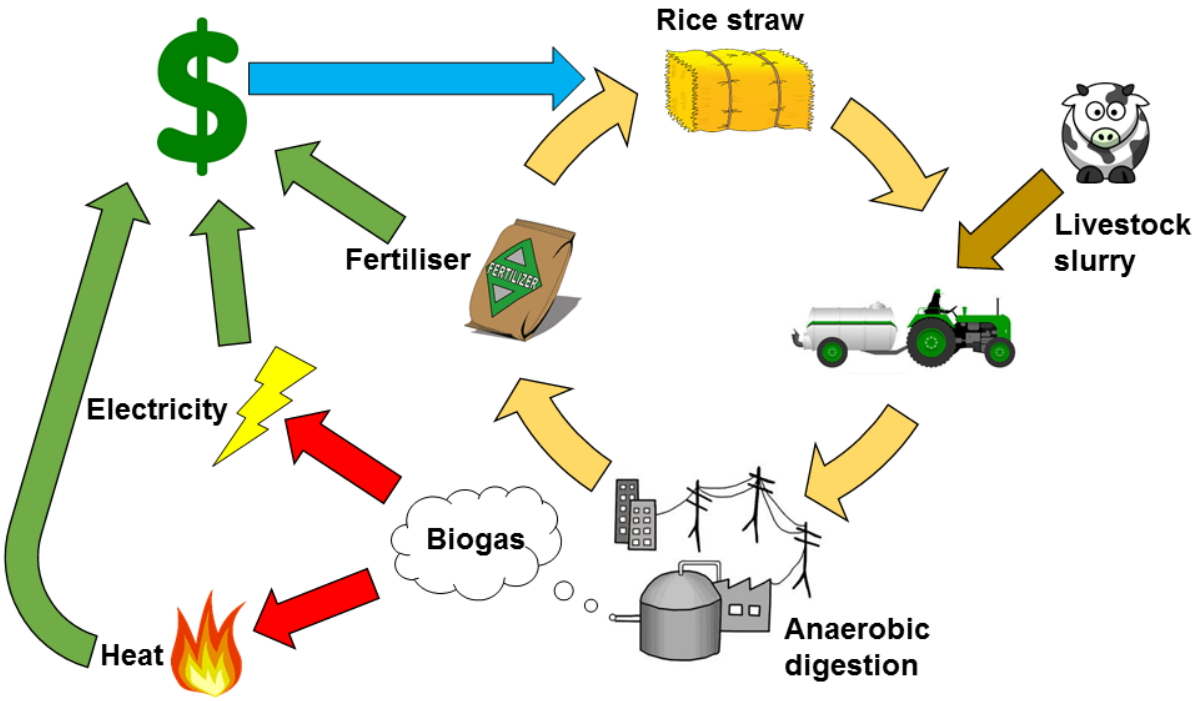

Figure 1.4: Possible rice straw anaerobic digestion life cycle to heat and power.

As a result, the energy value of agricultural waste streams has received increased interest, such as the prospect of harnessing energy from RS (Phutela *et al.*, 2012; CGIAR, 2014). In fact, China is pushing for complete use of RS as a fuel in the near future (Zhiqiang *et al.*, 2011). However, the recalcitrance under anaerobic conditions of most straws, due to high lignocellulose content, results in lower CH_4 biogas yield compared with other waste biomass; e.g. RS has only 193-240 L/kg TS compared with oilseed rape cake with 300-350 L/kg TS (Dinuccio *et al.*, 2010; Lei *et al.*, 2010; Mussoline *et al.*, 2013a). This aspect of RS means theoretical yields are often much higher than experimental or field values Mussoline *et al.* (2013b.

Many believe that RS pretreatment by biological, chemical, or a combination of methods can improve biogas yields (Taherzadeh and Karimi, 2008; Fernandes *et al.*, 2009; Agbor *et al.*, 2011), and some state that lignocellulosic materials must have pretreatment to ensure degradation (Labatut *et al.*, 2011; Brown *et al.*, 2012). Pretreatment can conditionally help. For example, Chen *et al.* (2014) found that

extrusion pretreatment improved CH_4 yield by 32 % compared with milling pretreatment, Bauer *et al.* (2009) increased CH_4 yields 20 % by steam explosion and Zhao *et al.* (2010) increased CH_4 yield by 35 % using mild acid pretreatment. However, Angelidaki and Ahring (2000) also found that combining chemical pretreatment and milling did not increase yields and Gu *et al.* (2014) found inoculum source was a more important factor to yield. Therefore, although pretreatment is sometimes effective, it comes at a cost (monetary, technical, and-or energy), often making full-scale operations impractical or operationally incompatible with actual farming practices (Ariunbaatar *et al.*, 2014; Ferreira *et al.*, 2014; Croce *et al.*, 2016).

As global energy demand increases, concerns about security, environmental impact, and fluctuating oil prices support the expanded use of renewable energy sources and cleaner technologies (USEID, 2016). Thus, avoiding pretreatment, despite possibly lower yields, has major advantages.

1.1 Aims and objectives

The aim of this study was therefore to assess under what conditions RS AD was achievable without the need for expensive and-or hazardous pretreatment steps. This was met through the following objectives:

1. Evaluate the impact of inoculum:substrate ratio (organic loading rate, OLR), particle size, C:N ratio (through co-digestion), and the geographic origins of RS on AD performance using batch digestion tests.
2. Identify optimal feeding frequencies and OLRs for RS AD at the semi-continuous-fed reactor level, including the potential for identified conditions for scale-up.
3. Determine the effect of FF/OLR on AD microbial communities, particularly focussing on how FF and low versus high OLRs alter methanogenic guilds.
4. Evaluate the effect of dairy manure (DM) co-digestion on RS AD performance and differences in microbial communities between reactors with and without DM addition.

Chapter 2 Literature Review

2.1 Anaerobic Digestion (AD)

Anaerobic digestion converts organic carbon into carbon dioxide (CO_2), methane (CH_4), and minor amounts (< 1 %) of other gases, and reduces substrate volume, providing other potentially recoverable products such as organic acids (Angelidaki and Sanders, 2004; Bajpai, 2017). Anaerobic Digestion (AD) systems can utilise a wide variety of substrates, including household wastes, food, sewage and crop residues, separately or in combination (Lim *et al.*, 2012), but AD always depends on the complex biological relationships of bacteria and archaea (Cabezas *et al.*, 2015). In order for these microorganisms to reduce organic materials into CO_2 and CH_4 (majority of gases) AD can be divided into four separate processes shown by Figure 2.1 - hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Appels *et al.*, 2008; Cabezas *et al.*, 2015).

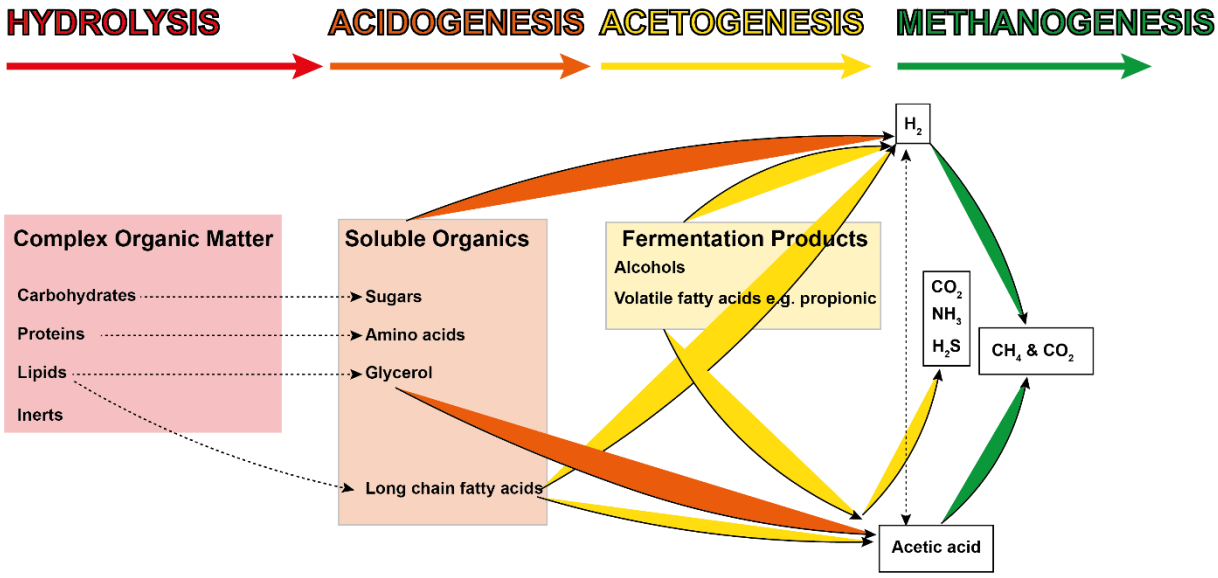

Figure 2.1: Anaerobic pathway based on Panico *et al.* (2014), Cabezas *et al.* (2015) and Bajpai (2017)

Microorganisms involved in the AD process fall into general functions: fermenting bacteria (fermenters), secondary fermenters or syntrophs (and acetogens), and methanogens (two types). Each group degrades certain products into new forms that are then used by the next group, and so on. First, fermenters degrade substrate constituents to organic acids and hydrogen (H_2) which is then used by syntrophs and

converted to acetate and H_2, before the methanogens further convert these products to CH_4.This synergistic relationship is essential, particularly in the latter stages for the methanogenic group that produces the biogas (Jessica *et al.*, 2012; Fayyaz *et al.*, 2014).

2.1.1 *Hydrolysis*

Hydrolysis is the first AD step in which bacteria release enzymes, such as proteases, to break down complex organics. Generally, only ~ 50 % of the organics are degraded, due to the lack of specific enzymes, with leftovers remaining in the digestate (Fayyaz *et al.*, 2014). Successful hydrolysis depends on enzymatic production, pH, and particle size, and is often considered rate limiting, as it is the slowest step, particularly when digesting lignocellulosic materials (Angelidaki and Sanders, 2004). Fermenting bacteria, such as *Firmicutes, Bacteroidetes, and Spirochaetes*, first disintegrate the organic components of the substrate into carbohydrates, proteins, lipids, and inert material, ready for hydrolysis. These bacteria excrete intracellular and extracellular enzymes, e.g. cellulase and xylanase, to hydrolyse these organic compounds. In this stage, the presence of lignin can be inhibitory to AD as it can release phenolic compounds and furan derivatives (Wei, 2016). These are then more easily utilised by other bacteria with any acetate and hydrogen produced in this stage directly used by the methanogens. The conversion of complex polymers to glucose and hydrogen can be seen in Eq. (2.1) (Bajpai, 2017).

$$C_6H_{10}O_4 + 2H_2O \rightarrow C_6H_{12}O_6 + H_2 \quad (2.1)$$

2.1.2 *Acidogenesis and Acetogenesis*

Fermentative bacteria, such as the *Firmicutes* phylum, in the acidogenesis phase break down the hydrolysis products into volatile fatty acids (VFAs) such as acetic, propionic, and butyric, as well as ammonia (NH_3), CO_2, and hydrogen sulphide (H_2S), and some other products. Acidogenesis can be a two-way pathway from which methanogens may directly utilise any hydrogen produced. The majority of products, such as higher volatile fatty acids, must be converted through acetogenesis before they can be utilised by the methanogens (Fayyaz *et al.*, 2014). The roles of bacteria early in the process overlap and can be generalist until the latter stages, however,

secondary fermenters and syntrophs include *Firmicutes, Proteobacteria,* and *Syntrophomonas*. These further convert monosaccharides and amino acids (among others) through different pathways. For example, amino acids are converted by the Strickland reaction or through uncoupled oxidation if hydrogen levels are low. Whereas, the Entner-Doudoroff (ED) or Emben-Mayerhof-Parnas (EMP) pathways ferment monosaccharides to different acids, including acetate and propionate Angelidaki *et al.* (2011). Eq. 2.2 and Eq 2.3 show the conversion of glucose to ethanol and acetic acid respectively (Bajpai, 2017).

$$C_6H_{12}O_6 \leftrightarrow 2C_2H_6O + 2CO_2 \quad (2.2)$$

$$C_6H_{12}O_6 + 2H_2O \leftrightarrow 3CH_3COOH \quad (2.3)$$

Acetogenic bacteria in the acetogenesis phase digest the organic fatty acid products of acidogenesis to acetic acid (mainly), CO_2, and H_2, by syntrophic bacteria such as *Syntrophomonas*. This is achieved through anaerobic oxidation of acidogenesis products mostly by *Firmicutes* but also *Proteobacteria* and *Actinobacteria* (Stams and Plugge, 2009). Eq. 2.4 shows the conversion of ethanol to acetate and hydrogen (Banks *et al.*, 2012)

$$C_2H_6O + 2H_2O \leftrightarrow CH_3COO^- + 3H_2 + H^+ \quad (2.4)$$

Acetogenesis can be done by hydrogen-utilising or hydrogen-producing acetogens. The first group reduce CO_2 in order to synthesize acetate whereas the second group oxidise organic acids, e.g. propionate, and alcohols such as ethanol Angelidaki *et al.* (2011). The symbiotic part of the AD process appears as the acetogenic bacteria produce hydrogen, which is toxic to them, this is reduced by the methanogens, which in turn enables the acetogens to release more hydrogen, and so on (Stams and Plugge, 2009). Acetate is a key indicator of AD efficiency as ~ 70 % of methane is produced by acetate reduction (Fayyaz *et al.*, 2014; Bajpai, 2017).

2.1.3 *Methanogenesis*

Methanogenesis is the final stage in producing CH_4, CO_2, and water from the products of the previous stages. Methanogenic archaea mainly utilise either acetic acid (acetoclastic) as well as some other acids, or, hydrogen and CO_2 as electron

donor/acceptor (hydrogenotophic). Eq. 2.5 and Eq. 2.6 show acetic acid and hydrogen methanogenesis pathways respectively (Stronach *et al.*, 1986; Banks *et al.*, 2012).

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (2.5)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (2.6)$$

The number of microorganisms able to produce methane from acetic acid is relatively small, although the majority of AD methane is produced using this pathway. As approximately 70 % of methanogenesis is acetoclastic, at mesophilic temperatures, and the relatively low numbers of methanogens this stage is often rate limiting (if the rest of the process has been optimised) (Speece, 1983). Obligate anaerobes, methanogens come from the *Euryarchaeota* phylum and include dominating genera such as *Methanobrevibacter, Methanoseata* (which tends to be most abundant)*, and Methanosarcina.* That *Methanosaeta* tends to dominate is likely due to its versatility, i.e. it can produce methane via acetic acid but also from CO_2 by direct inter-species electron transfer (DIET). This is the process by which free electrons may flow between cells without bound to reduced molecules such as hydrogen (Stams and Plugge, 2009; Dube and Guiot, 2015).

Efficient AD is a balance between the outputs of these organisms and their interactions where small changes can provide enough system shock to inhibit the biogas production and yields.

2.2 Molecular analysis

The AD process is used to treat a wide variety of wastes, including wastewater, petrochemical wastes, and agricultural residues. Methods for achieving biogas production are generally well known, but the complexity of the microbial community is less well understood. Studying these complex systems has always required molecular tools to answer specific questions. Over recent years the number of tools available has increased, improved in quality, and reduced in cost. Choosing the analysis method depends on what is being asked, often based on: Who is there? How many of each group are there? What do they do? How do they change over

time? Choosing the correct tool is essential, for example next generation sequencing (NGS) for ‘who’, quantitative PCR or fluorescent *in-situ* hybridisation (FISH) for ‘how many’, meta-omics for ‘function’, and (DGGE) for ‘changes’ (Cabezas *et al.*, 2015).

NGS is a popular method among AD researchers as it comes with a number of benefits, essentially, it is quicker, cheaper, provides more data than older techniques, and indirectly identifies uncultivable strains, which are very common in AD processes. Within NGS there are four main options, Roche 454 pyrosequencing, Illumina, Ion Torrent, and Sanger sequencing. Roche 454 pyrosequencing gives long read lengths providing more accurate annotation, which is why this is often used for environmental samples. Illumina and Sanger offer high coverage, but at a shorter read length, whilst Ion Torrent has long reads, but lower coverage than 454. Of these, Illumina sequencing has become the most widely used for identifying microbial communities (‘who’) in environmental research (Aydin, 2016).

Illumina uses sequencing by synthesis. This approach utilises DNA (fragmented by physical, enzymatic, or chemical methods), which then have adapters ligated to each end by annealing. These fragments are then randomly attached to a flow cell that has a surface of oligonucleotides complementary to the adaptors. The initial fragments are converted to their sequence (via a PCR step that is repeated several times to cluster the copies and amplify the signal), with both forward and reverse strands bound to the flow cell. Sequencing primers are attached to the free ends of the bound fragments and one base is labelled with an allocated fluorescent colour. The clusters (of identical DNA sequences) are then read as one base while a scanning camera reads the entire flow cell as in Figure 2.2 (Tufts University, 2014; Goodwin *et al.*, 2016; Eurofins Genomics, 2017).

If a paired end method is used then both fragments are sequenced where the template strand is used to build a bridge to the second, and can then be synthesised and used for the second read (Aydin, 2016). This has enabled the identification of many microorganisms that was not possible with previous technologies (Ahmed *et al.*, 2017). However, due to shorter read lengths of 200 - 400 base pairs (bp)it is not possible to fulfil exact phylogenetic characterisation (Bruneel *et al.*, 2017; Dai *et al.*, 2017). Tan *et al.* (2015) reported that Illumina provided higher sequence sensitivity at lower dilutions due to high throughput compared to Roche 454.

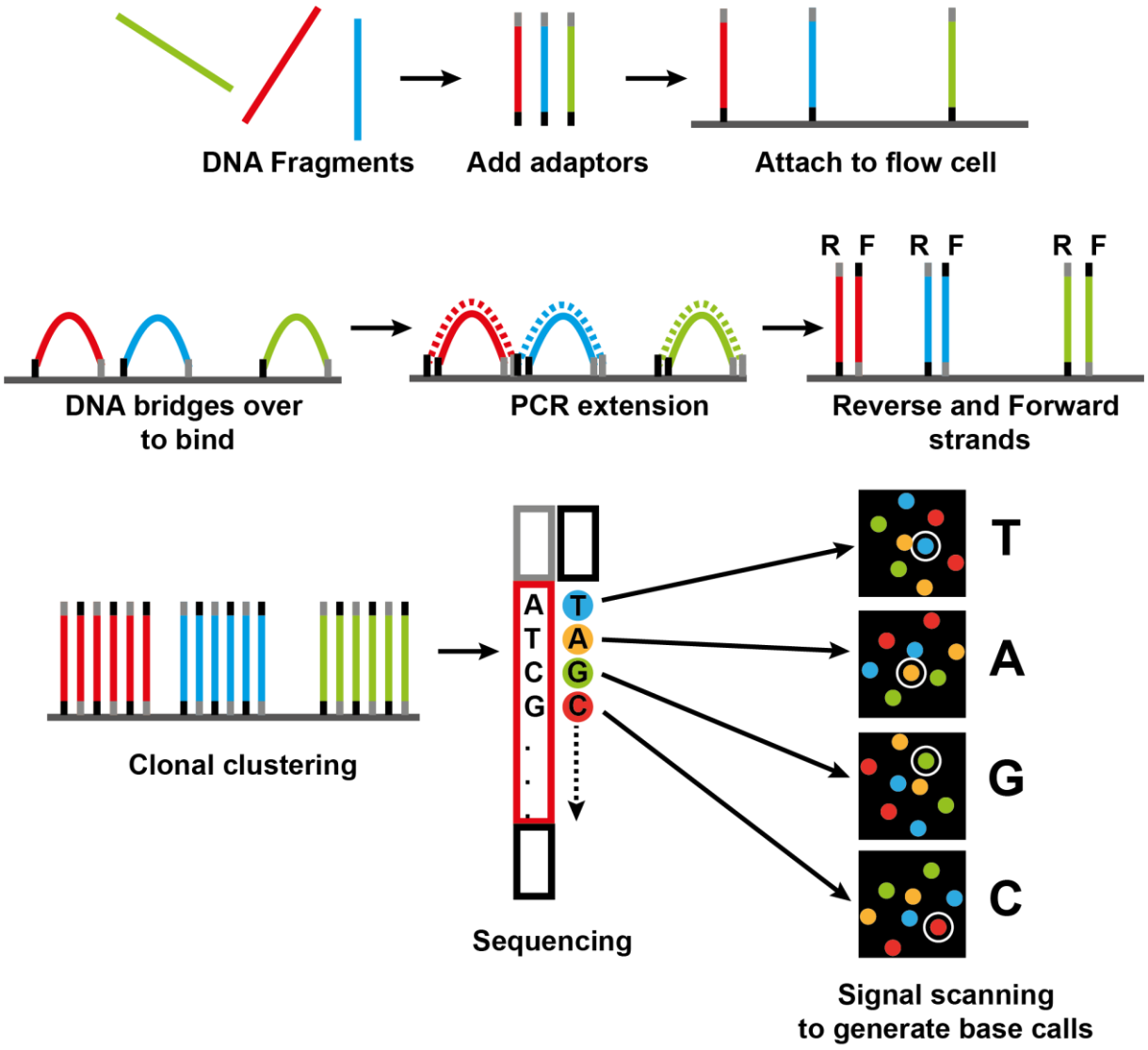

Figure 2.2: Bridge PCR amplification and Illumina sequencing technique adapted from Tufts University (2014), Goodwin *et al.* (2016) and Anonymous (2017)

As Illumina platform costs decrease and coverage increases (generating millions of partial 16S rDNA amplicon reads) its use in microbial ecology has increased (Lee *et al.*, 2017). In AD systems this has led to the identification of core bacterial phyla, including *Firmicutes* and *Bacteroidetes* (De Vrieze *et al.*, 2015),with *Methanosarcina* and *Methanosaeta* predominating in mesophilic reactors (Campanaro *et al.*, 2016; Fontana *et al.*, 2016; Dai *et al.*, 2017). A number of researchers have utilised Illumina sequencing in AD studies, for example, De Vrieze *et al.* (2015) found > 85 % of the AD microbiome was Firmicutes, Bacteroidetes, and Proteobacteria, Kuroda *et al.* (2016) used it to identify the microbial diversity of different UASB granules, and Mei *et al.* (2017) used it to identify a core AD microbiome at a global scale.

The sequences provided by these techniques are gathered into operational taxonomic units (OTUs), which are an arbitrary method for defining sequences that share > 97 % DNA identity similarity (Cabezas *et al.*, 2015). Once determined, there are a number of OTU analyses that can be used. Samples can be grouped based on dissimilarity (as a dendogram or tree) to determine community differences due to operational changes. Further analysis of dissimilarity includes, Principal Component Analysis (PCA), Principal Coordinate Analysis (PCO), Multi-Dimensional Scaling (MDS), and Canonical Correspondence Analysis (CCA). Each enables the researcher to plot taxonomic abundance with any environmental data, such as pH and VFAs, to visualise potential relationships. Determining statistical differences between samples or defining relationships can be done using for example, analysis of similarities (ANOSIM) and distance-based linear matrix (DistLM), among others. Further tests into species abundance include richness - the number of different OTUs, evenness - the equitability of the sample, and Shannon's index - calculated using abundances and the number of different OTUs (Ramette, 2007).

2.3 Rice Straw Anaerobic Digestion (RS AD)

Biofuel crops can be anaerobically digested to recover their stored energy. Traditionally rice straw (RS) has not been used because, although it is abundant and has high carbon, digesting complex lignocellulose structures is extremely difficult (Sanderson, 2011; Xu *et al.*, 2012; Mussoline *et al.*, 2013a). However, in a time of growing population, water use, and food scarcity, using land for biofuels might increase food prices and affect biodiversity as nutrients are removed from the land (FAO, 2002; United Nations Department of Economic and Social Affairs [UNDESA], 2014).

Developments in emissions targets and the energy value of waste products have increased the interest in harnessing energy from RS (Phutela et al., 2012; Consultative Group on International Agricultural Research [CGIAR], 2014). RS has not been widely used despite its massive potential for bioenergy because it is considered recalcitrant to AD (Xu *et al.*, 2012; Mussoline *et al.*, 2013a). However, attitudes are changing. If RS AD can be made economically feasible, it would address many problems, including air pollution and associated health effects, whilst also reducing a voluminous waste stream. It could provide a renewable source of

methane-rich gas that could be coupled with combined heat and power (CHP) systems.

China is the world leader in rice production with ~195 million tonnes per year at an average of 6.5 tonnes of rice hectare (Mohanty *et al.*, 2013). The huge volume of RS produced by this industry, and its disposal, causes a range of problems including environmental and health issues due to open field burning and the resultant smog (Zhiqiang *et al.*, 2011). Energy from RS is advantageous compared with other bioenergy crops as it does not divert land use from crop production, does not overly affect soil quality, removes a large GHG source, and high production and harvest rates ensure a consistent fuel supply (Blanca-Ferrer, 2013; Mussoline *et al.*, 2013a). Modification of the crop, standards, and practices to improve anaerobic digestibility, profit and reduce GHG emissions are ongoing (IRRI, 2014b). However, RS AD results in a lower CH_4 potential than other agricultural and bioenergy biomass, e.g., RS 46 - 195 L CH_4/kg VS (Dinuccio *et al.*, 2010; Mussoline *et al.*, 2013b), compared with sunflower oil cake, 213 mL CH_4/g VS (De la Rubia *et al.*, 2011), and, 388 mL CH_4/g VS from food waste with cattle manure (Zhang *et al.*, 2013).

If RS can be collected and stored in sufficient volume, and AD can produce useful biogas yields, what can be done with it? See Figures 2.3 and 2.4. A combined heat and power (CHP) system is a catchall for a number of technologies that provide heat and electricity from one fuel or energy source. Usually close to the point of use encompassing almost any fuel but natural gas currently dominates. As heat is more costly to transport than electricity, a CHP system would enable the heat to be used locally (not just for the AD system) whilst the electricity could be sold or used elsewhere. CHPs can reach up to 90 % fuel conversion efficiency and could reduce CO_2 emissions from biofuel generation by as much as 10 % by 2030 whilst providing real savings now by reducing the reliance on more expensive power generation (International Energy Agency [IEA], 2008). Lim *et al.* (2012) found that a number of countries, including China, have the rice resources to generate heat and electricity that could be utilised by local farms or mills with any excess potentially transported further in the grid. As an added benefit, once the rice straw is digested the sludge could also be used as an effective fertiliser adding nutrients, N, P, and trace elements (FAO, 1992). However, there are range of operational options and cellular characteristics to consider.

Figure 2.3: Rice straw collection from Berto and Nidoy (2016)

2.3.1 *Biodegradability*

Lignocellulose is a biomass complex of lignin (5 - 25 %), cellulose (35 - 45 %), and hemicellulose (25 - 40 %) (Wei, 2016). Cellulose is the most common organic compound in the world but lignin, which among other things provides plants their elasticity and stability, is the second. Lignin is a polyphenyl aromatic compound (with ester bonds) that is the glue that binds cellulose and hemicellulose to form the primary and secondary cell wall to protect the plant from microbial decomposition and provide elasticity. It is this 'toughness' that causes difficulties in anaerobic digestion systems as there is a negative correlation between lignin content and biogas yield. However, even if this structure is broken down within the process or with pretreatment, as an aromatic compound, it can release phenolic compounds and actually inhibit AD (Wei, 2016). Cellulose is the main framework of the cell (the wall) consisting of glucose chains linked by strong hydrogen bonds into crystalline microfibrils. Although this can make cellulose difficult to degrade it can also be (partially) amorphous and therefore more easily digestible. The cellulose can then be further embedded into lignin, hemicellulose, and pectin which can fill the spaces

between the microfibrils (Van Dyk and Pletschke, 2012). This can be degraded by cellulose-producing microbes that often produce an extra-cellular enzyme and a multienzyme cellulosomes (expressed on the surface of anaerobes). However, this process is relatively unknown and it is clear that aerobes and anaerobes operate in different ways (Wei, 2016).

There are a number of types of hemicellulose, making it more varied than cellulose, such as xylan (most common), glucuronoxylan and mannan. Within a plant the xylan creates a layer over the hydrogen bonds of the cellulose and covalently links with the outer lignin to protect the plant (Van Dyk and Pletschke, 2012). It contains a variety of different sugar monomers, such as glucose, that is relatively easy to hydrolyse in comparison with cellulose though it requires a large number of enzymes such as endo-xylanase and endo-mannanase. As one enzyme depolymerises the other removes the substituents. Once hydrolysed, the monomeric sugars and acetic acid can be utilised by other bacteria and archaea to produce biogas as previously described in Section 2.1 (Wei, 2016).

This means that although cellulose and hemicellulose are relatively easy to degrade microbiologically they are protected by the lignin. When it comes to AD, lignocellulosic biomass is not indigestible if it has enough carbohydrates (cellulose and hemicellulose), carbon, nutrients, etc., but it may provide lower biogas yields than material with less lignin (Lübken *et al.*, 2010). Lignocellulose composition varies between species of the same biomass, batches, seasons, harvest, and the methods of analysis. That lignocellulose is recalcitrant to AD is not simply the presence of lignin but also the amount of crystallinity, polymerisation (of polysaccharides such as pectin), ferulate cross-linking within the lignin, surface area and moisture content (Van Dyk and Pletschke, 2012). How these three components combine is shown in Figure 2.4.

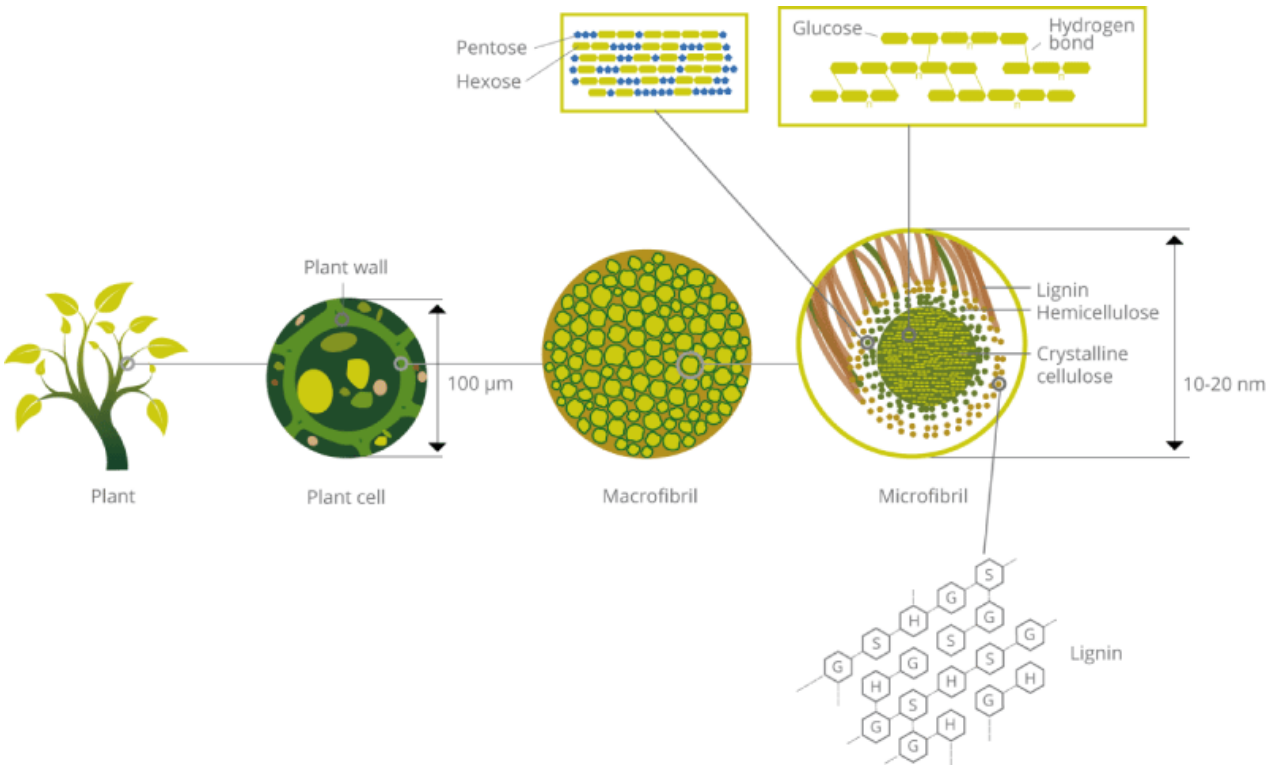

Figure 2.4 Structure of lignocellulose (Streffer, 2014)

It has therefore often been reported that RS requires pretreatment; i.e., mechanical, chemical or biological, or supplementation such as trace metal addition, to digest anaerobically and-or produce sufficient yields (Yan et al., 2015). RS pretreatment by biological, chemical, or a combination of methods aim to degrade the lignin and improve biogas yields (Taherzadeh and Karimi, 2008; Fernandes *et al.*, 2009; Agbor *et al.*, 2011). For example, Chen *et al.* (2014) found that extrusion pretreatment improved CH_4 yield by 32 % compared with milling, Bauer *et al.* (2009) increased CH_4 yields 20 % by steam explosion and Zhao *et al.* (2010) increased CH_4 yield by 35 % using mild acid pretreatment. However, Angelidaki and Ahring (2000) found that combining base chemical pretreatment and milling did not further increase yields and Gu *et al.* (2014) showed that inoculum source can be a significant factor. Some studies even suggest that lignocellulosic materials must have pretreatment to ensure sufficient degradation (Labatut *et al.*, 2011; Brown *et al.*, 2012). This means that operational decisions can be key factors in the success of RS AD.

Figure 2.5: Inventive ways of reuse of rice straw from Miller (2015)

2.4 AD Operational considerations

2.4.1 *Reactor configuration and inhibition*

Reactor design depends on waste type, solids content, composition, cost, and location among many others. There are a number of designs, however, they fall generally into, dry or wet digestion, batch or (semi) continuous, and one-stage or two-stage, with different combinations and options available to improve biogas yields (Krishania *et al.*, 2013a).

Wet digestion has total solids content of 6 - 10 % whilst dry digestion is 10 - 40 % (Monnet, 2003; Yadvika *et al.*, 2004; Montero *et al.*, 2008; Krishania *et al.*, 2013a). The decision of wet or dry is determined by the waste substrate as this influences the AD system performance (e.g. pH) and, therefore, the efficacy of the microbial community. Wet digestion also tends to be the cheapest as pumps, pipes etc. required for pumping slurries is cheaper than the dry system equivalents, however, larger reactor volumes and dewatering may negate this benefit (Monnet, 2003).

Batch systems are fed once with the chosen substrate and progress through all stages of the AD process, often without mixing, as a 'dry' digestion (30 - 40 % TS) (Monnet, 2003). Batch systems are used at all scales, including for the biochemical methane potential (BMP) test that is used to provide insight into potential CH_4 yield from a substrate and biodegradability of organic substrates, usually across a wide variety of conditions (Strömberg *et al.*, 2014). There are a number of methods so comparing data is sometimes difficult, but the VDI Standard: 4630 (2006) method has been used by many, including Membere *et al.* (2015) and Steinmetz *et al.* (2016). The BMP test provides three distinct phases, which all inform the assay, including: 1. Lag-phase, i.e. how long the microbial community needs to break the substrate structure and start methanogenesis; 2. Production phase, i.e., the period between lag-phase and technical digestion time when methanogenesis is at maximum rate; and 3. Technical digestion time (TDT_{80}), i.e., the time it takes to reach 80 % of the ultimate yield (Palmowski and Muller, 2000); the 'maximum methane' yield (i.e., when daily production is at its lowest, < 10 % of ultimate) and the 'ultimate methane yield', the CH_4 yield a particular substrate could produce given infinite time, can be derived for BMP data. Compared with continuously-fed AD systems, BMP tests require a fraction of the facilities, cost and energy, and so enables a wider variety of test conditions (Owen *et al.*, 1979). The major benefit of BMP testing is for optimisation studies.

Continually-fed reactors require careful balancing of system inputs and outputs to ensure maximum degradation of the feedstock and steady biogas production (Figure 2.5). Regular substrate feeding of a continuous system reduces the impact of shock loading the microbial community and in turn maintains the sensitive methanogen population (Appels *et al.*, 2008). Semi-continuous reactors are intermittently fed and whose use is usually determined by feedstock and location, e.g. intermittent crop growth. Whichever design is chosen the draw and fill feeding method (removing digestate before feeding new substrate) is most used as this provides improved pathogen kill over other methods (Farrell *et al.*, 1988; Appels *et al.*, 2008).

One-stage digesters are the oldest, most well-known, simplest systems in which, the whole AD process occurs from hydrolysis through methanogenesis. Two-stage digesters split the process, often with a hydrolysis reactor that feeds into a secondary digester (Nallathambi Gunaseelan, 1997). Due to build costs and limited biogas gains this approach is less common than it was in the past (Appels *et al.*, 2008).

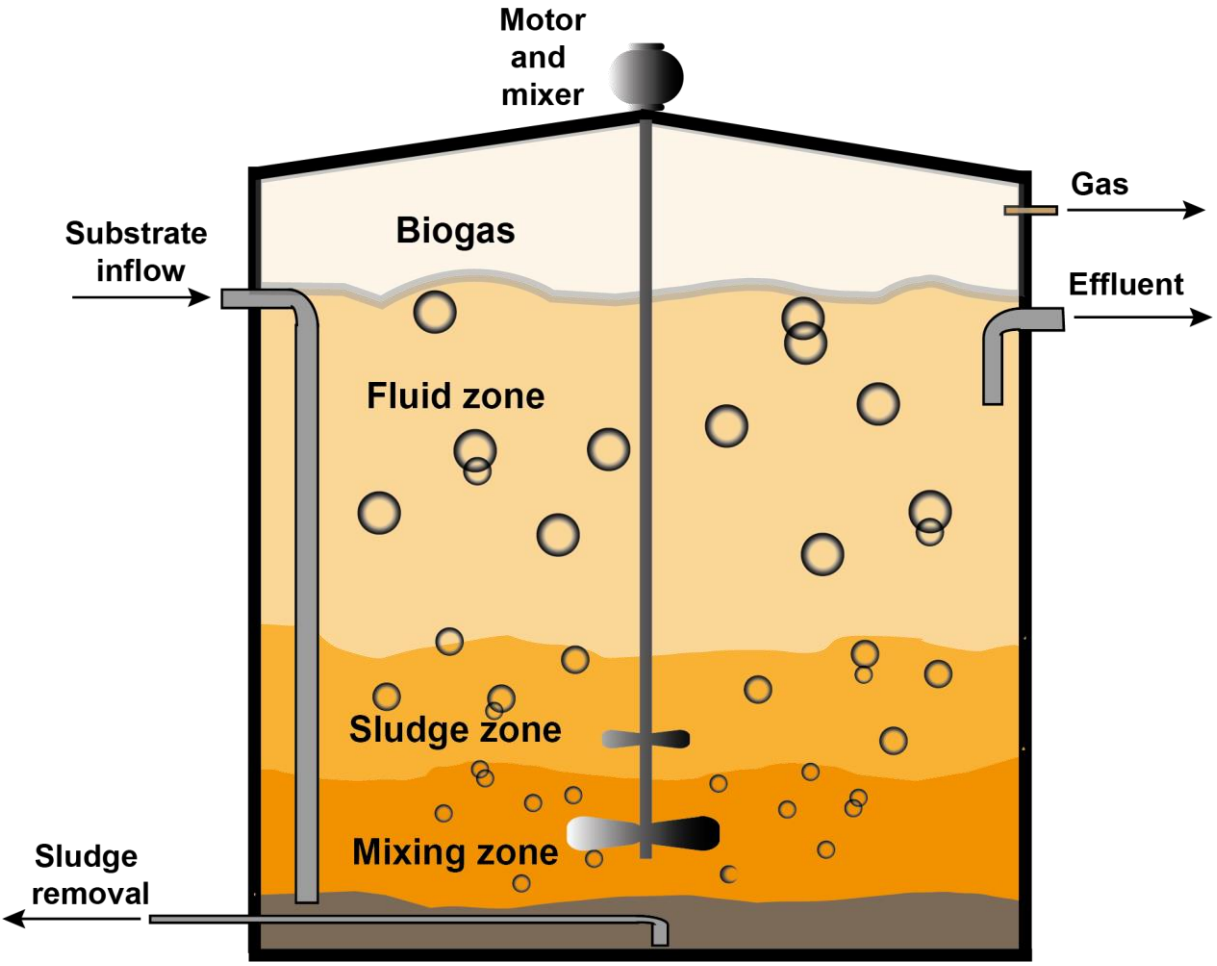

Figure 2.6: Typical stirred anaerobic digester diagram influenced by Narra *et al.* (2016) and Aquafix (2017)

Toxicity or inhibition of an AD system is extremely varied and can occur in a number of ways, depending on the individual system. This can be due to many reasons including, salt and calcium toxicity, though ammonia inhibition is frequently studied as a main inhibitor in manure co-digestion (Chen *et al.*, 2008; Mussoline *et al.*, 2013a). Ammonia inhibition can limit an AD system in a number of ways, for example, inhibiting specific enzyme reactions or adjusting intracellular pH. It mostly affects the sensitive methanogen population whilst the acidogenic bacteria may be relatively unaffected (Chen *et al.*, 2008). Rajagopal *et al.* (2013) noted that free ammonia was the largest issue as high membrane permeability inhibits the methanogenic cells. However, it is possible to acclimate AD flora to high ammonia by adding carbon rich substrates and controlling pH and temperature (Chen *et al.*, 2008). Depending on the substrate and other operational inputs, heavy metals can also cause inhibition as they do not biodegrade and so, can accumulate to potentially toxic levels. Many of

these heavy metals are essential to optimal AD performance, such as cobalt, copper, zinc, and nickel, with acidogens again, more resistant than methanogens (Kong *et al.*, 1994; Chen *et al.*, 2008).

2.4.2 *pH, temperature, and mixing*

AD has two optimal pH ranges, which is when two-stage reactors can be useful. Acidogenesis produces acids that can reduce the pH to as low as 5.5 (as previously described), which not only inhibits the bacteria at this stage but pH below 6.4 can be toxic to the methanogens (Appels *et al.*, 2008). Therefore, an optimal pH range for AD is 6.4 - 7.2 (Monnet, 2003), but can range from 4.0 - 8.5 (Appels *et al.*, 2008). Low pH, due to the accumulation of VFAs and higher pH, due to ammonia accumulation, can both result in failure or 'souring' of the reactor as methanogens become inhibited (Krishania *et al.*, 2013a); i.e. the bacteria produce more VFAs than the archaea can use to produce methane and the system acidifies (Franke-Whittle *et al.*, 2014).

There are two main AD temperature ranges, mesophilic (between 20 - 40 °C and usually ~ 35 °C), and thermophilic (between 50 - 65 °C and usually 55 °C). Temperature is determined on a case by case basis taking into consideration multiple factors including, substrate, location, and digester type. Higher temperature offers the option of sterilisation without additional steps such as pasteurisation, though this requirement depends on the end-use of the digestate. Mesophilic digestion is less efficient, as regards to OLR, hydraulic retention time (HRT), and often, gas production. However, mesophilic AD requires lower heat input and has more diverse microbial communities that are more resilient to changes in environmental variables such as pH (Monnet, 2003; Moset *et al.*, 2015). Higher temperature AD (when substrates are co-digested with animal wastes) can cause the additional problem of inhibitory levels of ammonia. As the temperature is increased, the pH rises and ammonia is ionized, increasing methanogen toxicity (Appels *et al.*, 2008; Labatut *et al.*, 2014). There has also been a range of studies into psychrophilic AD (often < 20 °C) but these have so far proved unsuccessful in matching mesophilic or thermophilic methane yields. However, a full life cycle assessment has not been performed so it may be that this approach offers a new method in the future.

Mixing comes naturally in a digester as gas bubbles and thermal currents agitate the digestate but, as this is often insufficient for complete mixing, additional mixing is required. This is mostly done using internal shaft mixers (Appels *et al.*, 2008). The main aims of mixing a reactor are to reduce foam/scum on the top and sludge settling, maintain maximum contact of microorganisms and substrate, enable uniform fluid and concentration gradients, further enhancing the microorganisms' ability to obtain nutrients. All of which aim to improve digestion and biogas yields (Yadvika *et al.*, 2004; Appels *et al.*, 2008). If co-digesting different wastes, these should be mixed before entering the digester to ensure quick homogeneity. Rapid mixing can be worse than no mixing as it can disturb the microbial community and reduce degradation and fatty acid destruction (Monnet, 2003; Krishania *et al.*, 2013a).

2.4.3 *Organic loading rate (OLR)*

The OLR reflects the true biological capacity of AD; i.e., the ability of the system to degrade the feedstock. Feeding too little or too much can be equally inhibitory, with reduced biogas and pH, and high VFAs particular indicators. OLR can either be calculated as chemical oxygen demand (g COD/L) or using volatile solids (g VS/L) and is often linked with HRT to a particular feedstock. Once combusted, volatile solids are considered the organic matter of a substrate (Monnet, 2003). A high VS feed is desirable but the recalcitrance of RS makes it difficult to run a high organic loading rate (OLR). The potential increase of volatile fatty acids (VFA) and VS accumulation at high OLRs can inhibit the process. Experiments in RS AD have therefore been run at a range of OLRs but most have used a range of pretreatments and-or co-digestion, for example: RS and pig manure at 3 - 12 kg VS/m^3/d (Li *et al.*, 2015b), RS and paper mill sludge (Mussoline *et al.*, 2013b), wheat straw and pig manure at 1 - 4 kg VS/m^3/d (Babaee *et al.*, 2013), and a range of biomass-manure digestions at 0.85 - 2.25 kg VS/m^3/d (Menardo *et al.*, 2011).

2.4.4 *Hydraulic and solids retention time (HRT/SRT)*

HRT is the mean time the digestate remains in the digester and SRT is the time the solids remain. HRT and SRT can be the same, when there is no sludge recirculation, or distinctly different, for example, in an upflow anaerobic sludge blanket (UASB), and can range from 5 to greater than 100 days depending on the system (Ndegwa *et al.*, 2008; Krishania *et al.*, 2013a). When sludge is recirculated the SRT has its own

calculation as microorganisms are added to the system at a different rate. The HRT/SRT is extremely important as if it is too short incomplete digestion occurs with increased VFAs and methanogen washout whilst too long and the system may accumulate less biodegradable VS (Appels *et al.*, 2008).

Difficult feedstocks tend to require longer a HRT to provide the microbial community adequate digestion time and can also play a significant role in shaping that community (Dareioti and Kornaros, 2014). Some methods enable shorter HRTs, for example co-digestion of crops and manure has been shown to reduce HRT compared to sole crop digestion (Nges and Björnsson, 2012). Shi *et al.* (2017) found a 60 day HRT outperformed 40 and 20 days as regards VS and methane content. However, there is still limited literature on the effect of HRT on agricultural wastes, specifically RS AD.

2.4.5 *Feeding frequency (FF)*

Continuous feeding provides a stable AD output of biogas whilst semi-continuous or batch feeding provide more varied effects as the AD process is flooded with early AD intermediaries before they can be utilised by the methanogens (Lv *et al.*, 2014). Continuous feeding may not be the most efficient option for agricultural biomass as the microbial community requires time to hydrolyse the feedstock. Less frequent feeding, but not batch, may therefore lead to a more efficient system. For example, Piao *et al.* (2016) found no difference in the acetoclastic community between daily and bi-daily fed reactors but it did change the dominance of methanogens - *Methanosaeta* increased with less frequent feeding. Whilst Manser *et al.* (2015) reported weekly feeding gave higher methane yields and improved faecal bacteria destruction but Golkowska *et al.* (2012) found an increase in biodegradation rate with increased FF. There have been few studies into the effect of infrequent feeding regimes i.e. extended starvation periods and bouts of plenty with RS AD studies often choosing to use batch or continuous feeding regimes. The large input of RS to the waste cycle each harvest means conventional AD feeding frequency options (little and often) would struggle to cope without a large storage capability. Studies often focus on a narrow time margin between feeds such as Bombardiere *et al.* (2007) at 1-12 feeds/day with manure, or focus on products other than methane such as biopolymers (Albuquerque *et al.*, 2011) or hydrogen production (Valdez-Vazquez *et al.*, 2005).

2.4.6 *Carbon:nitrogen (C:N) ratio and co-digestion*

The microbial community utilises carbon approximately 25 - 30 times more than nitrogen (Yadvika *et al.*, 2004) and it has been shown that rebalancing the C:N to 25:1 improves biogas yields (Lei *et al.*, 2010). In low nitrogen systems methanogens struggle to produce optimal biogas levels, whereas in a low C:N system, excess nitrogen causes ammonia accumulation, pH increase, and inhibition (Monnet, 2003). RS has a natural C:N ratio of up to 80:1 (Mussoline *et al.*, 2013a). Low C:N can lead to ammonia inhibition, whilst high C:N can result in incomplete digestion, though feedstocks can be considerably higher or lower (Monnet, 2003; Ward *et al.*, 2008). There are a number of methods that introduce nitrogen into RS AD in order to improve the C:N, such as co-digestion with activated sludge (Abudi *et al.*, 2016), or AD sludge (Xu *et al.*, 2013), but co-digestion with animal wastes is most favoured.

Co-digestion with manure is seen as a cost-effective method of improving the C:N ratio in AD whilst also using another large waste stream, eventually producing a rich fertiliser (Li *et al.*, 2014a; Li *et al.*, 2015a). RS co-digestion with manure, of various animals, has been shown by many researchers to improve the C:N ratio and subsequently the methane yield of RS AD (Table 2.1). Additionally, manure co-digestion can add moisture and valued microbes into the system, improving syntrophic relationships and growth (El-Mashad and Zhang, 2010; Silvestre *et al.*, 2013a). Co-digestion can also add essential nutrients such as phosphorus. AD has a favoured N:P ratio of 7:1, whilst RS is ~ 16:1 (Mussoline *et al.*, 2013a). Experiments that have supplemented P, such as Acharya (1935), Hussain *et al.* (2008), and Lei *et al.* (2010) found it had minimal or no effect.

Table 2.1: Co-digestion used in the anaerobic digestion of rice straw or similar

Feed substrate	Range or ratio	Highest Gas yield	Reference
RS:DM	1:9, 3:7, **5:5**[a], :7:3, 9:1	~ 175 mL biogas /g VS	Li *et al.* (2014a)
RS:DM	15:1, 20:1, **25:1**, 30:1, 35:1 (C:N)	272 mL CH_4/g VS	Wang *et al.* (2014b)
RS:Cow manure	0:1, 1:2, **1:1**, 2:1, 1:0	196 L CH_4/kg VS	Li *et al.* (2015a)
RS:Pig manure	0:1, **1:2**, 1:1, 2:1, 1:0	268 L CH_4/kg VS	Li *et al.* (2015a)
RS:Buffalo dung	1:9, 2:8, **3:7**, 4:6, 5:5, 6:4	184 L CH_4/kg VS	Sahito and Mahar (2014)
RS:Pig wastewater	5:10		
RS:Paper mill sludge	**5:10**	340 L CH_4/kg VS	Mussoline *et al.* (2013b)
RS:Pig WW:PM sludge	5:5:5		
RS:Cow manure	1:99, 2:98, **5:95**	0.8 m^3 biogas /m^3	Silvestre *et al.* (2013a)
RS:Kitchen waste:Pig manure	1:2:0, **1:1.6:0.4**, 1:1.2:0.8, 1:0.8:1, 1:0.4:1.6, 1:0:2	384 L CH_4/kg VS	Ye *et al.* (2013)
RS:Pig dung	50:50	390 L/kg[b]	Successful case studies International Rice Research Institute [IRRI] (2016)
RS:Cow dung	75:25	320 L/kg	
RS:Animal manure	75:25	300 - 400 L/kg	
RS:Cow dung	95:5	350 - 400 L/kg	

Note: [a] Bold shows highest performing condition.
[b] It was unclear if this reference was reporting biogas or methane

2.4.7 *Substrate pretreatment*

A pretreatment is any method designed to improve the AD process, usually in terms of gas yield, that occurs before the substrate enters the system. The efficacy of pretreatments is highly varied with researchers using different methods and different substrates. Lignocellulosic pretreatment has been a popular target (Ariunbaatar *et al.*, 2014). Generally, pretreatments come in the form of physical, chemical, biological, or a mixture of methods (Mussoline *et al.*, 2013a).

Physical: Before thinking of gas yields, many researchers engage a physical pretreatment, such as milling or freeze-thaw, to reduce the RS (lignocellulosic biomass) to an apt experimental size (Table 2.2). A reduction in size, through milling for example (Figure 2.6), is designed to improve hydrolysis by enabling greater microbial - substrate contact and improved microbial access via destruction of the cell walls. Although a reduction in size can potentially give faster methane production and higher yields the energy required for size reduction can often outweigh the potential benefits (Menon and Rao, 2012). It remains an essential tool in improving AD slurry flow and reducing blockages.

Figure 2.7: Small scale rice straw chopping from Feedipedia (2017)

Table 2.2: Particle sizes used in the anaerobic digestion of rice straw or similar

Feed substrate	Particle size range[a]	Highest gas yield	Reference
Rice straw	3.0 – 5.0 mm (mean)	0.29 m^3 CH^4/kg	Lei *et al.* (2010)
Rice straw	1.0, **30**[b], **50** mm	239 - 245 mL CH_4/g VS/d	Ferreira *et al.* (2014)
Rice straw	5, 20, **50** mm	203 L CH_4/kg VS	Menardo *et al.* (2012)
Rice straw	**0.88**, **0.4**, 1, 6, 30 mm	365 - 367 L CH_4/kg VS	Sharma *et al.* (1988)
Rice straw (and buffalo dung)	1.0, **2.0**, 4.0, 6.0, 8.0, 10 mm	184 mL CH_4/g VS	Sahito and Mahar (2014)
Wheat Straw	0.1, **0.7**, 1.4 mm	115 mL CH_4/g VS	Motte *et al.* (2013)
	0.1, 0.67, **1.45** mm	192 mL CH_4/g VS	Motte *et al.* (2014)
Grass silage	<10 mm and **>30** mm	342 L CH_4/kg VS	Wall *et al.* (2015)
Sisal fibre waste	**2.0** – 100 mm	0.22 m^3 CH_4/g VS	Mshandete *et al.* (2006)
Water hyacinth	16, 64, **127** mm	0.18 L CH_4/g VS	Moorhead and Nordstedt (1993)
Ensiled sorghum forage	0.25, 0.5, 1.0, **2.0** mm	298 mL CH_4/g VS	Sambusiti *et al.* (2013)
Corn stover	0.075 - 0.25, **0.25 -1.0**, 1.0 - 5.0, 5.0- 20 mm	249 mL CH_4/g VS	Xiao *et al.* (2013)

Note: [a] Particle sizes have been normalised to mm.
[b] Bold shows highest performing condition

Heating: Heating or freezing samples can also disrupt the lignocellulose and provide improved degradation (Ward *et al.*, 2008). Chang *et al.* (2011) found that enzyme digestibility improved from 48 - 84 % after freezing pretreatment. When samples are

heated, rapidly depressurised, and cooled the water in the cells ‘explodes’ resulting in hydrolysis and further cell destruction (Menon and Rao, 2012).

Chemical: Chemical treatments involve pretreating RS with alkalis, acids, or a mixture of the two. Alkali pretreatment uses bases, often lime ($Ca(OH)_2$), ammonium hydroxide (NH_4OH), or particularly, sodium hydroxide (NaOH) to remove the lignin of a substrate and increase enzyme accessibility to the hemicelluloses and cellulose (Ward *et al.*, 2008; Menon and Rao, 2012; Obata *et al.*, 2015). It has also been noted by Krishania *et al.* (2013a) that pretreating AD sludge can improve VS reduction (VSR). An additional benefit of alkaline pretreatment is that it can be performed at ambient temperatures with samples soaked for a prescribed amount of time (Menon and Rao, 2012), and is less hazardous than other pretreatments. Alkali pretreatment produced improved methane yields (of up to ~ 90 %), methane content, and VS reduction for Wang *et al.* (2015), Shen *et al.* (2014), and Romero-Güiza *et al.* (2017). Abudi *et al.* (2016) showed pretreatment with NaOH gave a higher reduction in lignin than hydrogen peroxide (H_2O_2), 22 and 7 %, respectively, and Chandra *et al.* (2012) used NaOH to increase methane by 124 %. The addition of urea (4 % concentration) to RS optimised its biodegradability for Luo *et al.* (2013), whilst Cann *et al.* (1994) favoured a mixture of chlorite and acetic acid, and Angelidaki and Ahring (2000) increased methane by combining NaOH and NH_4OH. Conversely, acid pretreatment uses dilute and concentrated acids, sulphuric acid (H_2SO_4) most commonly, to break the lignocellulose and hydrolyse hemicellulose (Liu *et al.*, 2012; Menon and Rao, 2012; Hude and Yadav, 2014). Zhao *et al.* (2010) used nitric acid (HNO_3, 0.75 mol/L) to remove almost 35 % of lignin and increase RS methane yield by the same percentage, whereas Park *et al.* (2015) used it to improve VFA production from RS AD. Testing the effect of H_2O_2 and ammonia, Song *et al.* (2012) showed that 3 - 4 % (w/w) H_2O_2 provided higher biogas yields of 320 - 328 mL/g VS. Teghammar *et al.* (2012) also found that pretreatment with the organic solvent NMMO increased RS methane yield to 203 CH_4/g VS, a 79 % increase on the untreated.

Biological: Biological pretreatments have a number of advantages over chemical pretreatments, for example, more environmentally friendly, no fermentation inhibition, no effluent, and no toxic compound release. However, it can take far longer, and more space (Menon and Rao, 2012; Sindhu *et al.*, 2016). A biological treatment is often one that utilises fungi or bacteria to degrade the biomass to make it more amenable to the AD process. A reason biological pretreatments can be less attractive

is that lignin degrading organisms often solubilise hemicellulose and cellulose as well (Menon and Rao, 2012). The main area of focus in biological pretreatment has been fungi, though there are others including researching the potential of termite enzymes and bacteria. Mustafa *et al.* (2016) showed fungal treatment using *Pleurotus ostreatus* and *Trichoderma reesei* decreased lignin by > 30 % and led to a methane yield increase of 120 %. Deng *et al.* (2015) had similar results with *Trichoderma reesei* whilst Ghosh and Bhattacharyya (1999) used *Phanerochaete chrysosporium* and *Polyporus ostreiformis* to improve RS methane production by 46 and 32 %, respectively. Whilst Phutela *et al.* (2011), Phutela *et al.* (2012), and Phutela and Sahni (2013) reported improved RS digestibility, decreases in lignin, and biogas increases when using *Pleurotus florida,* and, *T. reesei* and *Coriolus versicolor*. A soft-rot fungus in the *Fusarium solani* complex that contributes to wood degradation was found by Geib *et al.* (2008) in the lignin degrading long-horned beetle (*Anoplophora glabripennis*). The ability of termites to degrade lignocellulose provides them an important role in the carbon cycle and makes them an attractive research proposition (Ni and Tokuda, 2013; Brune, 2014; Lima *et al.*, 2014). Mathew *et al.* (2013) identified the functions of *Bacillus* and *Clostridium* in the termite *Odontotermes formosanus*, providing further information for future biological treatments. Pretreating wastes with specific bacteria strains has also been shown to be successful in improving methane yields. Zhang *et al.* (2016) treated RS with rumen fluid, Niu *et al.* (2012) composted corn straw with a cellulose degrading strain, Zhong *et al.* (2011) dosed corn straw with a microbial agent, and Martin and Martin (1998) pretreated paper with *Fibrobacter succinogenes*. Each method saw the bacteria pretreated substrate produce higher methane yields and shorter digestion times.

There are clearly a number of pretreatment options for RS, and other lignocellulosic AD, but a leading method is unclear, which is perhaps why a number of researchers have combined treatments. For example, Chandra *et al.* (2012) found 3 % NaOH for five days followed by 200 °C for 10 minutes improved methane production by 222 % compared with untreated RS. Using dilute H_2SO_4 at high temperature and pressure to hydrolyse RS Karimi *et al.* (2006) depolymerised hemicellulose by 80 %. Mustafa *et al.* (2017) combined fungal and RS milling, Liu *et al.* (2011) NaOH and H_2O_2 on corn straw, Wang *et al.* (2014a) combined alkali on corn stalk, Arisutha *et al.* (2016) mixed thermal and H_2SO_4, and Ma *et al.* (2010) used *Echinodontium taxodii* and H_2SO_4.

2.5 AD Supplementation

2.5.1 *Metals*

An alternative to pretreatment, or perhaps in addition, is supplementation. AD systems can be supplemented to aid the process and improve the results, with the addition of certain metals particularly useful (Ward *et al.*, 2008). Optimal biogas production requires the efficient growth and survival of microorganisms with elements such as Fe, Cu, and Ni, identified as essential to this process (Krishania *et al.*, 2013a). Co-digestion of RS and food waste supplemented with Co, Ni, and a combination of both, increased the kinetic rate for Zhang *et al.* (2017), with sole Ni supplementation significantly increasing VS reduction. Narra *et al.* (2016) also used micronutrients to increase RS AD biogas by 37 % and Cai *et al.* (2017) found Mn addition provided the highest methane yield and Fe, Mo, Se, and Mn all reduced VFA. Within their experiment, *Methanosaeta* increased by up to 12 % compared to the control. Gustavsson *et al.* (2011) found that Co, Ni, and Fe stabilised high OLR wheat stillage AD, whilst Hinken *et al.* (2008) used the same metals to increase maize silage biogas yield by 35 %. This was similar to Pobeheim *et al.* (2010), whilst Jarvis *et al.* (1997) increased OLR above the control with Co addition. The majority of metal supplemented AD has been researched using food waste as a substrate by researchers such as Feng *et al.* (2010), Banks *et al.* (2012), Facchina *et al.* (2013) and Zhang *et al.* (2015b).

2.5.2 *Biosupplementation*

Biosupplementation, bioaugmentation, and biostimulation all aim to achieve the same result as the metal dosing but in a more environmentally friendly way by dosing with microorganisms. For example, a proprietary cellulolytic culture was shown Martin-Ryals *et al.* (2015) to increase methane production by > 15 % and acetic acid by > 30 %. As a cellulosic bacteria, many of the researchers have focussed on supplementation with phylum *Clostridia*. Bioaugmentation using *Clostridium stercorarium* and *Bacteroides cellulosolvens* was shown by Hu *et al.* (2016) to increase the degradation of lignin, cellulose, and hemicellulose, and consequently increased methane production by almost 250 %. Ozbayram *et al.* (2016) supplemented BMP tests with enriched sheep rumen cultures (4 % addition) that increased the abundances of *Lachnospiraceae* and *Ruminococcaceae* to enhance

methane production. Wheat straw inoculated with the cellulose degrading bacteria *Clostridium cellulolyticum* enhanced methane yields of 8 - 13 % for Peng *et al.* (2014) but they could not characterise the effect of the supplement on the microbial community. Tsapekos *et al.* (2017) bioaugemented with *Clostridium thermocellum* and found wheat straw methane yield increased by 35 %, however, in continuous reactors the increase was only by 7.5 %. It was also seen in the same study that *Melioribacter roseus* did not improve AD performance. Interestingly, neither strain was over-represented in the digester microbiome. Using different bacteria to supplement wheat straw Zhang *et al.* (2015a) reported an increase in methane yield of 19 - 23 % and cellulose and hemicellulose removal rates of 12 and 5 %, after 10 % inoculation with *Acetobacteroides hydrogenigenes*. Nielsen *et al.* (2007) also showed a methane increase of 10 - 24 % from cow manure when inoculated with *Caldicellusiruptor*, which is also *Clostridia* and *Dictyoglomus*, a thermophile of a different phylum.

2.6 Conclusion

AD is therefore a promising technology in the management of waste RS with the opportunity to recover energy as methane. However, as a biological system, RS AD is not an optimised process and is also subject to shock changes such as, volatile fatty acid (VFA) accumulation or pH decreases, which can cause system failure producing little or no methane. As much of the worldwide RS is produced by small-medium rural farms a sustainable AD process needs to be accessible at this level. Many countries have enough RS resources to generate heat and electricity at the farm or mill-level, and even export surplus power to the grid. Unfortunately, the acyclic production of RS and its reputed poor digestibility still has influenced many against RS AD.

There has been many studies on AD but relatively few on RS AD, specifically focussing on RS AD without pretreatment but not one method has been provided as a generally accepted, best solution. The mixture of methods and research goals of others means there are gaps to fill, including, for example, the impact RS collected from different countries has on AD, as well as differences in OLR and FF. From these it is also unknown as to how these operational changes would affect the microbial communities of the AD process and whether their reactions could be used as a predictor of RS AD failure. That is what this thesis sought to achieve.

Chapter 3 Anaerobic Biochemical Methane Potential (BMP) Preliminary testing - P addition, OLR, Particle size, Co-digestion, and effect of RS origin country

3.1 Introduction

The Biochemical Methane Potential (BMP) test is widely used to quantify rates and yields of biogas production as well as substrate degradation. Although other methods are sometimes used (Angelidaki *et al.*, 2009; Contreras *et al.*, 2012), BMP tests are quick, easy, cheap, and require less space than continuous reactor experiments (Feng *et al.*, 2013; Koch and Drewes, 2014).The common VDI Standard: 4630 (2006) method was designed to provide a unified method for researchers, although it is not perfect. For example, it presumes an inoculum:substrate (I:S) ratio of 2:1, which is not always optimum and requires optimisation based on the substrate (Akunna *et al.*, 2007; Chen *et al.*, 2014). Regardless, BMP tests are a good starting point for any anaerobic digestion (AD) study.

Rice straw (RS) has not regularly been used in anaerobic digestion due to its recalcitrant lignocellulose structure and naturally high C:N ratio (Mussoline *et al.*, 2013a). This structure requires additional considerations, for example feeding too little or too much can be equally inhibitory (loading rate), whilst a reduction in particle size can improve microbial - substrate contact by destroying the cell walls. Within this context, conditions needed to be developed for the rice straw (RS) BMP tests herein. For example, I:S can be considered as organic loading rate (OLR) and previous work showed OLRs of 1 to 2 g VS/L were typical for RS (Pohl *et al.*, 2013), but higher OLRs might be beneficial for reducing reactor size. Testing the effect of particle size is not new, for example, Sharma *et al.* (1988) tested RS particle sizes of 0.09 mm – 30 mm (RS) and Chen *et al.* (2014) RS up to 50 mm, however, researchers have often avoided larger particle sizes and RS as the sole substrate for the reasons given in the previous chapter. Whether achieved by milling, hand cutting, threshing or other techniques, reducing the size of RS requires energy. Generally, the smaller the particle size, the more energy is required for processing, thus reducing the potential benefits of higher methane yields. Phosphorus is of particular importance to AD but

as a limited resource its addition to AD is not feasible, but it can be added via manure co-digestion. C:N is of particular importance with the ideal C:N range is given as 25 - 30:1 (Wang *et al.*, 2014b), whilst the addition of P has been shown to stimulate and improve AD. This has led to researchers co-digesting RS with manure (as an N and P source) such as Wang *et al.* (2012) and Silvestre *et al.* (2013b) who used DM and RS. Co-digesting RS with manure aims to decrease the C:N ratio of RS, which is naturally high and exceeds the targeted 25:1. Anaerobic co-digestion has been shown to enhance methane production as well as reducing odours and pathogens in the manure, improve the fertiliser quality of the sludge, whilst also providing a renewable fuel (Holm-Nielsen *et al.*, 2009; Marañón *et al.*, 2012).

The majority of rice straw anaerobic digestion ('RS AD') research has been done in China (the largest rice producer), so RS from China was used in the majority of tests. There is some research on other south-east Asian and European RS, with Nigeria also producing a significant amount of RS. However, the literature does not directly compare AD performance of RS from different countries, especially how RS chemistry from different regions might impact AD performance.

The aims of this Chapter were to determine the effects of five factors using the BMP method:

1. Inoculum:substrate (I:S) ratio (OLR) - 'I:S-test'
2. RS particle size - 'PS-test'
3. C:N adjustment (via dairy manure co-digestion) - 'Codi-test'
4. P addition - 'P-test'
5. The geographic origins of the RS - 'GO-test'
6. Assess the reproducibility of the VDI Standard: 4630 (2006) BMP method

3.2 Materials and Methods

3.2.1 *Rice straw and composition analyses*

RS was provided by Xiamen University (China); Institute of Engineering and Technology, Delhi (IET, India); the International Rice Research Institute (IRRI, the Philippines); and a Newcastle University colleague (Nigeria), although limited information was available on the variety, harvest, or storage conditions of straws

tested. RS from China was used for all particle size tests as RS production there is most plentiful. Anaerobic sludge inoculum was stock from within Newcastle University Environmental Engineering department that had previously been acclimated to RS.

Total solids (TS), volatile solids (VS), moisture content (MC), ash content (AC), total C, N, H, S were analysed using American Public Health Association standard methods (APHA, 1998), whilst lignin was analysed by Sciantec Analytical. Trace element concentrations were determined using ICP-MS after nitric acid digestion (Environmental Protection Agency [EPA], 1996). Calorific value was calculated by adding 1 g of dry sample to a crucible and igniting under 100 % oxygen using a bomb calorimeter (Parr 6100, Parr, USA) with benzoic acid tablets used to calibrate. Composition analyses are presented in Table 3.1 and elemental analyses in Table 3.2.

3.2.2 *BMP test method*

The VDI Standard: 4630 (2006) method requires seeding inoculum to be stored for at least a week in the test apparatus to 'degas' i.e., sufficiently reduce its own biogas production. The method per 500 mL bottle is, briefly, 300 mL of AD inoculum sludge, 100 mL distilled water, and RS added in a 3:1 inoculum:substrate (I:S) ratio (≥ 2:1 is suggested and preliminary tests shown later suggested 3:1 would be best) capped with a bung with 1.0 L gas bag attached. Blanks, containing only inoculum and distilled water, were also prepared. All test conditions, including controls, were prepared in triplicate and performed under mesophilic temperature conditions (37 °C) in an incubator shaker (Innova 4300) at 100 rpm and operated until daily methane production was ≤ 1 % of cumulative methane yield (minus mean blank yield) and substrate gas yields were ≥ 80 % of total biogas. The daily and cumulative data were then subjected to Gompertz modelling and production/biodegradation calculations.

Table 3.1: Characteristics of the rice straw, dairy manure, and anaerobic digester inoculum

Parameter (%DW [a])	Rice Straw Country of Origin					
	China	India	Philippines	Nigeria	Dairy Manure	Inoculum
Total Solids	93.5 ± 0.1[b]	96.4 ± 0.1	95.9 ± 0.2	95.6 ± 0.0	11.1 ± 0.3	2.4 ± 0.0
Volatile Solids	87.5 ± 0.2	86.9 ± 0.1	78.3 ± 0.2	82.6 ± 0.1	84.5 ± 0.3	76.6 ± 0.3
Moisture Content	6.50 ± 0.1	3.57 ± 0.1	4.47 ± 0.2	4.36 ± 0.0	89.7 ± 0.3	97.6 ± 0.0
Ash Content	12.5 ± 0.2	12.9 ± 0.1	21.5 ± 0.2	17.4 ± 0.1	25.7 ± 0.7	23.3 ± 0.3
C	39.0 ± 0.4	38.6 ± 0.3	34.5 ± 0.3	31.9 ± 0.3	40.9 ± 0.4	54.6 ± 0.1
N	0.86 ± 0.1	0.81 ± 0.1	0.79 ± 0.1	0.66 ± 0.1	4.09 ± 0.0	4.72 ± 0.1
C:N (ratio)	44.6	44.7	44.2	48.7	10.0	11.6
Calorific Content (MJ/kg)	14.7 ± 0.7	15.3 ± 0.2	12.2 ± 0.4	15.1± 0.4	No data	No data

Notes [a] is an abbreviation of ‘dry weight’ at STP and is for all samples but C:N ratio and Calorific content
[b] Standard error (n = 3)

Table 3.2: Elemental composition of the rice straw from the four countries

Element (mg/kg)	Rice Straw Country of Origin			
	China	**India**	**Philippines**	**Nigeria**
Aluminium (Al)	216 ± 1.3 [a]	27.7 ± 3.3	14.7 ± 0.2	27.3 ± 0.0
Arsenic (As)	1.2 ± 0.4	0.5 ± 0.5	0.9 ± 0.5	0.7 ± 0.5
Barium (Ba)	3.0 ± 0.1	1.3 ± 0.5	3.3 ± 0.0	2.8 ± 0.0
Calcium (Ca)	1552 ± 3.2	762 ± 5.6	539 ± 2.2	962 ± 2.5
Cobalt (Co)	0.5 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Chromium (Cr)	0.7 ± 0.0	2.6 ± 0.2	1.6 ± 0.0	1.7 ± 0.0
Copper (Cu)	1.7 ± 0.1	4.4 ± 0.5	2.2 ± 0.0	2.0 ± 0.0
Iron (Fe)	238 ± 1.0	34.8 ± 0.5	24.4 ± 0.1	39.2 ± 0.2
Potassium (K)	3322 ± 1.0	5397 ± 19	6290 ± 25	4878 ± 52
Magnesium (Mg)	364 ± 10	944 ± 44	232 ± 6.4	118 ± 5.0
Manganese (Mn)	294 ± 0.9	33.4 ± 0.2	163 ± 45	65.4 ± 0.2
Sodium (Na)	14.6 ± 1.3	16.1 ± 1.6	28.2 ± 2.3	3.7 ± 0.4
Nickel (Ni)	0.5 ± 0.0	0.4 ± 0.1	0.6 ± 0.0	0.8 ± 0.0
Silica (Si)	80.6 ± 0.4	21.8 ± 0.9	85.0 ± 0.2	89.2 ± 0.1
Titanium (Ti)	13.5 ± 0.0	3.7 ± 0.1	1.3 ± 0.0	2.4 ± 0.0
Zinc (Zn)	14.3 ± 0.2	7.7 ± 0.6	5.7 ± 0.1	7.6 ± 0.2

Notes: [a] Standard error (n=3)
[b] Phosphorus was below detection limits (< 0.1 mg/kg) throughout

3.2.3 *Biogas analysis*

Daily biogas volume was determined using a 100 µL gas tight syringe (SGE, Australia). Biogas samples were collected at the same time of day and analysed immediately when possible. To quantify methane content (% CH_4) the sample was injected into a Carlo Erber HRGC 5160 GC-FID fitted with a HP-PLOT Q column at 35 °C with hydrogen as the carrier gas and Atlas software. A seven-point calibration was performed before and after each analysis session by injecting a standard that spanned the range of expected methane concentrations (80 % CH_4, Scientific Technical Gases, UK). All injections were made in triplicate and the standard calibration required a minimum R^2 of 0.99. The volume of biogas was collected over time in 1.0 L Supel-Inert Multi-Layer Foil bags (Sigma Aldrich) before daily extraction using a 1 L gas tight syringe (SGE). Biogas and methane analysis was normalised to specific production (mL/g VS/d), corrected for moisture, standard temperature and pressure (STP), and headspace, using VDI Standard: 4630 (2006) (Appendix A).

3.2.4 *Gompertz modelling, production rate and biodegradation*

The modified Gompertz equation (Eq. 3.1) assumes methane production corresponds to microbial activity within a BMP system and has been used by many researchers, for example, Abudi *et al.* (2016). The Gompertz equation was fitted to the observed cumulative BMP data to provide a fitted curve, lag-phase, maximum daily methane production, and ultimate methane yield.

$$\mathrm{M_t} = \mathrm{M_{ult}} \exp\left(-\exp\left(\frac{\mathrm{D_{max}}}{\mathrm{M_{ult}}}\ (\mathrm{LP} - \mathrm{t})\right) + 1\right) \qquad (3.1)$$

Where, M_t is the cumulative specific methane yield (mL CH_4 /g VS) at any time (t), M_{ult} is the ultimate methane yield (mL_{ult} CH_4/g VS) produced in the duration of the BMP test, D_{max} is the maximum methane produced per day (mL CH_4/g VS/d), LP is the lag phase (days), and t is the duration of the BMP test.

The production constant (k_h-value) for a first order hydrolysis model, as shown by Angelidaki *et al.* (2009), used cumulative methane curve data between lag-phase and TDT_{80} (Chen *et al.*, 2014) (Eq. 3.2).

$$\frac{\mathrm{dS}}{\mathrm{dt}} = -k_h \mathrm{S} \tag{3.2}$$

Where, S is the available substrate, t is time and k_h the first order hydrolysis constant (day^{-1}). The relationship between the biodegradable substrate and the cumulative methane (Eq. 3.3) produced a linear slope, providing the value of k_h, which is the maximum production rate at a particular methane volume and is specific to the tested substrate.

$$\ln \frac{\mathrm{M_{ult}} - \mathrm{M}}{\mathrm{M_{ult}}} = -k_h \mathrm{t} \tag{3.3}$$

Where, M_{ult} is the ultimate methane yield (mL_{ult} CH_4/g VS) at the end of the BMP test, M is the methane yield (mL CH_4/g VS/d) at any point t is time and k_h the first order hydrolysis constant. The x-axis intercept provided production rates for each sample, a steeper slope relates to a faster microbial rate of methane production.

Biodegradability of the rice straw was calculated using the theoretical methane yields obtained using methods from Buswell and Mueller (1952) and the experimental methane yields as shown in Eq. 3.4 (Angelidaki and Sanders, 2004).

$$\text{Biodegradability}\ (\%) = \frac{\mathrm{M_{ult}}}{\mathrm{M_{theo}}} \times 100 \tag{3.4}$$

Where, M_{ult} is the ultimate methane yield (mL_{ult} CH_4/g VS) at the end of the BMP test, M_{theo} is the theoretical methane yield (mL_{Theo} CH_4/g VS/d).

3.2.5 *Theoretical methane yield*

Using the atomic composition and the elemental constituents of RS the theoretical methane potential yield (mL_{Theo} CH_4/g VS) was calculated with Eq. 3.5 (Buswell and Mueller, 1952), which was then used to determine the theoretical yields of each RS

as Eq. 3.6 (assuming total stoichiometric conversion) (Li *et al.*, 2013; Membere *et al.*, 2015).

$$C_nH_aO_b + \left(n - \frac{a}{4} - \frac{b}{2} + \frac{3c}{4}\right) H_2O$$

$$\rightarrow \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - \frac{3c}{8}\right) CH_4 + \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4} + \frac{3c}{8}\right) CO_2 \quad (3.5)$$

$$mL_{Theo}\ CH_4/g\ VS = \frac{22.4 \times 1000 \times \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - \frac{3c}{8}\right)}{12n + a + 16b + 14c} \quad (3.6)$$

3.2.6 *Statistical analysis*

Analysis of variance (ANOVA) with Tukey comparison was used to compare mean methane content, and 'maximum' methane yield (90-100 % of cumulative yield), with significance defined as 95 % confidence i.e. $p \leq 0.05$. Outliers were removed. As PS1 and GO-Chi were essentially same condition (from different BMP test runs) the two sample *t*-test was used to compare data and assess method reproducibility. All statistical analyses and Figure plots were conducted using, Microsoft Excel and Minitab 17 (Leadtools Technologies Inc, version 17.1.0, 2014). MatLab 2016a (The MathWorks Inc.) was used to fit the Gompertz model for lag-phase, daily and ultimate methane yields. PRIMER 7 (PRIMER-E, Plymouth, UK) was used to produce Principal Coordinates Analysis (PCO) and DistLM (distance-based linear modelling) of the log-transformed, normalised, RS composition data which is shown in full in Appendix A.

3.2.7 *BMP Conditions*

Five factors were assessed in a series of BMP tests using background information summarized in Chapter 2 as a guide. The I:S (OLR) test used a particle size of 1.0 mm (for practicality) and assessed ratios of 5:1, 4:1, 3:1, 2:1, and 1:1 (1.0 g VS/L, 1.5 g VS/L, 2.0 g VS/L, 3.0 g VS/L, and 6.0 g VS/L). Particle size means of 425 µm, 1.0 mm, 30 mm, and 70 mm, given the prefix 'PS'. Mean particle sizes were achieved using the hammer mill at Cockle Park farm (1.0 mm), followed by a 425 µm sieve, or scissors and a tape measure. C:N ratio (60:1, 50:1, 30:1, 25:1, and 15:1) was varied through adjusting the RS:DM ratios (100:0, 96:4, 80:20, 75:25, 40:60, and 0:100) and named based on the percentage of RS e.g. 96:4 is 'RS96'. P addition with a fixed C:N ratio, using hydrogen phosphate, HPO_4^{-2} (C:N:P = 60:1:0, 60:1:0.1, 60:1:1,

60:1:2.5, and 60:1:5). All ‘geographic origin’ samples were milled to a mean size of 1.0 mm with no additions at an I:S of 3:1 and have sample codes that reflect the geographic origin ‘GO-Chi’, ‘GO-Ind’, ‘GO-Phi’, and ‘GO-Nig’.

3.3 Results and Discussion

The maximum daily and ultimate methane yields of the experimental data showed that the BMP data falls within the 95 % confidence limits of the Gompertz predicted data, and, the R-Sq values were all ≥ 0.993 which indicates that the BMP test data was strong and the test operated for an appropriate time frame. One BMP bottle of RS96, RS75, RS40, DM (Codi-test), and GO-Chi, GO-Phi and GO-Nig (GO-test), was removed due to significant differences between replicate bottles in cumulative methane yields. All other bottles were analysed. Ultimate methane yields for each condition are summarised in Figure 3.1.

The IS-test, Figure 3.1a, showed no difference in ultimate yields between 5:1 to 2:1 (179 - 159 mL_{ult} CH_4/g VS) however, 1:1 (62.9 mL CH_4/g VS) yielded significantly lower levels of CH_4 ($p = <0.001$). The VDI Standard: 4630 (2006) suggests I:S of ≥ 2:1 and although there was no significant difference, 3:1 was higher than 2:1 (176 vs. 159 mL_{ult} CH_4/g VS), so that was used for the following tests. The PS-test (Figure 3.1b), showed PS425 and PS1 were similar and had higher yields than PS30 and PS70. The 1.0 mm cut was used for all subsequent tests, as the joint highest performer and most practicable size, which may be important when extending results to larger scales e.g., commercial use. To assess the impact of N addition in the Codi-test, DM was added to make different C:N ratios. In contrast to Yan *et al.* (2015) who suggested 30:1 was optimal, Figure 3.1c shows that 60:1, which had no DM addition, had the highest specific CH_4 yield and yields progressively declined with increasing DM addition. Only RS96 (50:1, C:N) yielded a statistically similar amount of CH_4 to no DM addition (RS100, $p \geq 0.05$). Similarly, adding P did not significantly enhance specific CH_4 yields (Figure 3.1d) as also found by Lei *et al.* (2010). Given a goal of this thesis was to keep operations simple, DM and P supplements were not used in the continuously fed reactor experiments of Chapter 4 and 6. The GO-test provided significant differences between source countries where the Nigerian RS yielded the highest methane, whilst Indian RS was the poorest performing (Figure 3.1e).

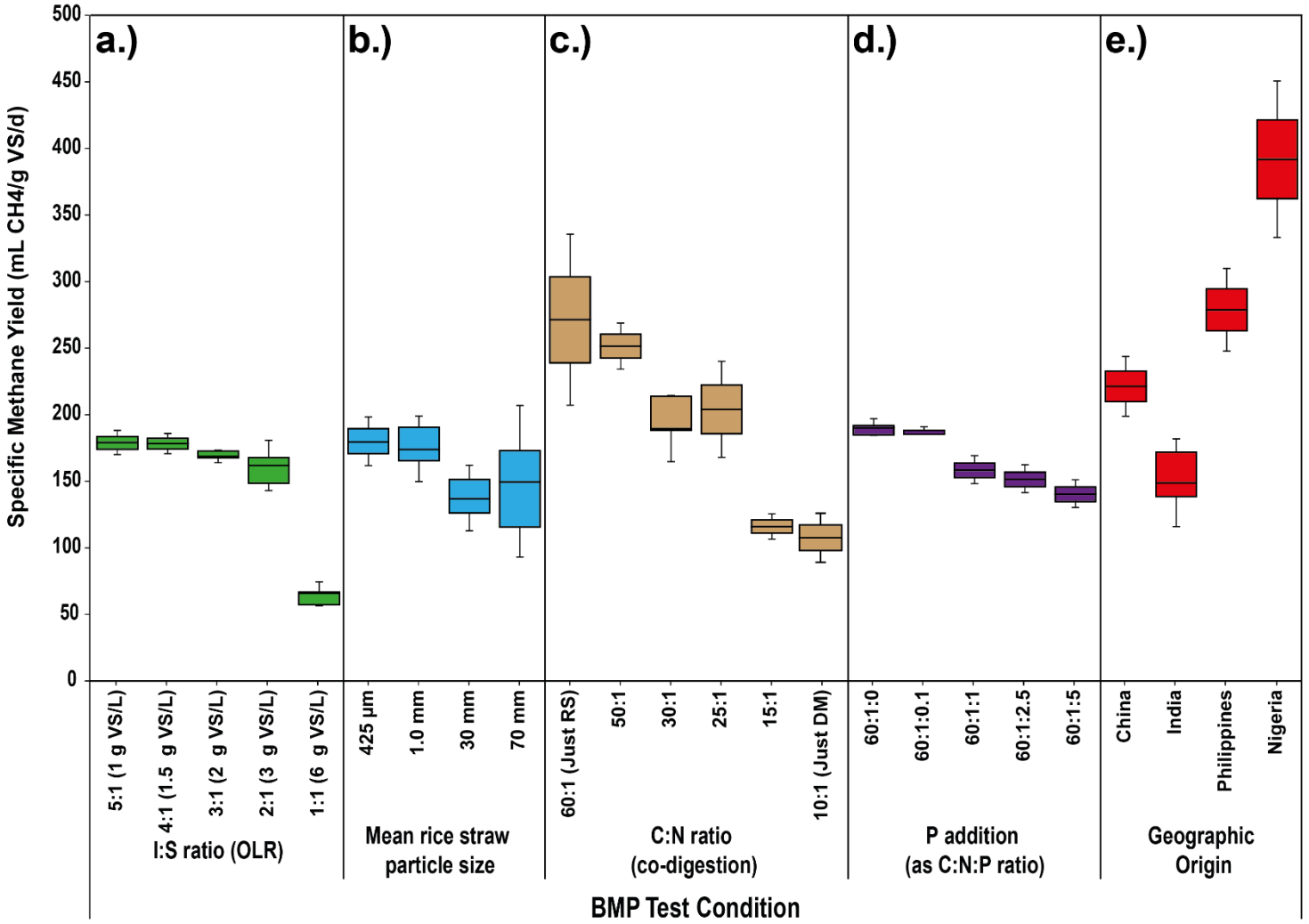

Figure 3.1: Ultimate methane yield (mL_{ult} CH_4/g VS) for, **a.)** Inoculum:substrate ratio (OLR), **b.)** Particle size, **c.)** C:N ratio (dairy manure co-digestion), **d.)** P addition, and **e.)** Geographic origin at 1.0 mm. Standard error bars (standard error, n = 3 for all tests except, n = 2 for, GO-Chi, GO-Phi, and GO-Nig.

To validate the above tests, experimental methane yields in Figure 3.1 were compared with expected theoretical levels based on the elemental make-up of the RS substrate, calculated using previous methods (Buswell and Mueller, 1952; Yoon *et al.*, 2014; Hidalgo and Martín-Marroquín, 2015). Shown later in Section 3.3.3. The calculated theoretical yield from Chinese RS was 412 mL_{Theo} CH_4/g VS, which suggests experimental yields were approximately 45 - 55 % of the maximum and typical of previous experimental studies; e.g. Nielfa *et al.* (2015) (40 - 50 %). The theoretical yield of Indian RS was similar, but Philippine RS was higher (75 %) and Nigerian RS (108 %) exceeded the theoretical which shows theoretical assumptions can provide errors when compared with experimental data (Achinas and Euverink, 2016).

3.3.1 *Particle size (PS-test)*

The lag phase for the PS-test (and the Codi-test) was very short (< 2.5 days), which indicated that the inoculum was highly active and acclimated to RS.

The time each PS sample took to reach its TDT_{80} decreased with particle size i.e. PS425 took 12 days, followed by PS1 (13 days), and, PS70 and PS30, which both took 14 days (Figure 3.2a). Matching the TDT_{80} with the feeding regime can offer the option of reducing the digester volume (Palmowski and Muller, 2000). This was supported by the methane production rates, which were consistently higher with smaller particle sizes; i.e., PS425 and PS1 had k_h-values of 0.15 and 0.14 d^{-1}, whilst PS30 and PS70 were lower at 0.11 and 0.12 d^{-1}, respectively (Table 3.3 and Figure 3.2b). These k_h-values were comparable to Contreras *et al.* (2012) (0.08 d^{-1}).

Similar trends are also apparent in the cumulative methane production data shown in Figure 3.3 where PS425 and PS1 display steeper slopes relative to larger particle sizes, indicating that smaller particles tend to enhance methane production rates in the BMP tests. Ultimate methane yields continued this trend as the PS425 and PS1 conditions were statistically similar (PS425 was highest) and had significantly higher yields than PS30 and PS70 ($p = <0.001$). However, it is suspected slightly higher methane yields with 425 µm particles do not justify the energy required for such size reduction, and it is not likely to be practical at small to medium rural-scale sites, as suggested by Sahito and Mahar (2014). Differences were observed in the mean methane content of the conditions, PS1 (50.4 %) outperformed PS425 (44.8 %),

which was unexpected, but not statistically significant. Methane content of PS1 was significantly higher than both PS30 and PS70 (43.1 & 39.8 %) p = <0.001, but PS425 was not significantly different to PS30 or PS70.

Table 3.3: Mean performance data for different rice straw particle sizes

Parameter	Particle Size			
	≤ 425 µm	**1.0 mm**	**30 mm**	**70 mm**
Mean CH_4 content (%)	44.8 ± 1.9 [a]	**50.4 ± 1.8** [b]	43.1 ± 1.6	39.8 ± 2.4
Ultimate methane yield (mL_{ult} CH_4/g VS)	**180 ± 2.0**	177 ± 6.0	139 ± 7.5	140 ± 9.0
Biodegradation (%)	**43.7**	43	33.7	34
Production rate (d^{-1})	**0.15**	0.14	0.11	0.12
Technical Digestion Time (TDT_{80}) (days)	12	13	15	14

Note: [a]Standard error was calculated: For mean methane content n = 63, for Ultimate methane n = 3.
[b]Bold indicates highest performing condition for each parameter

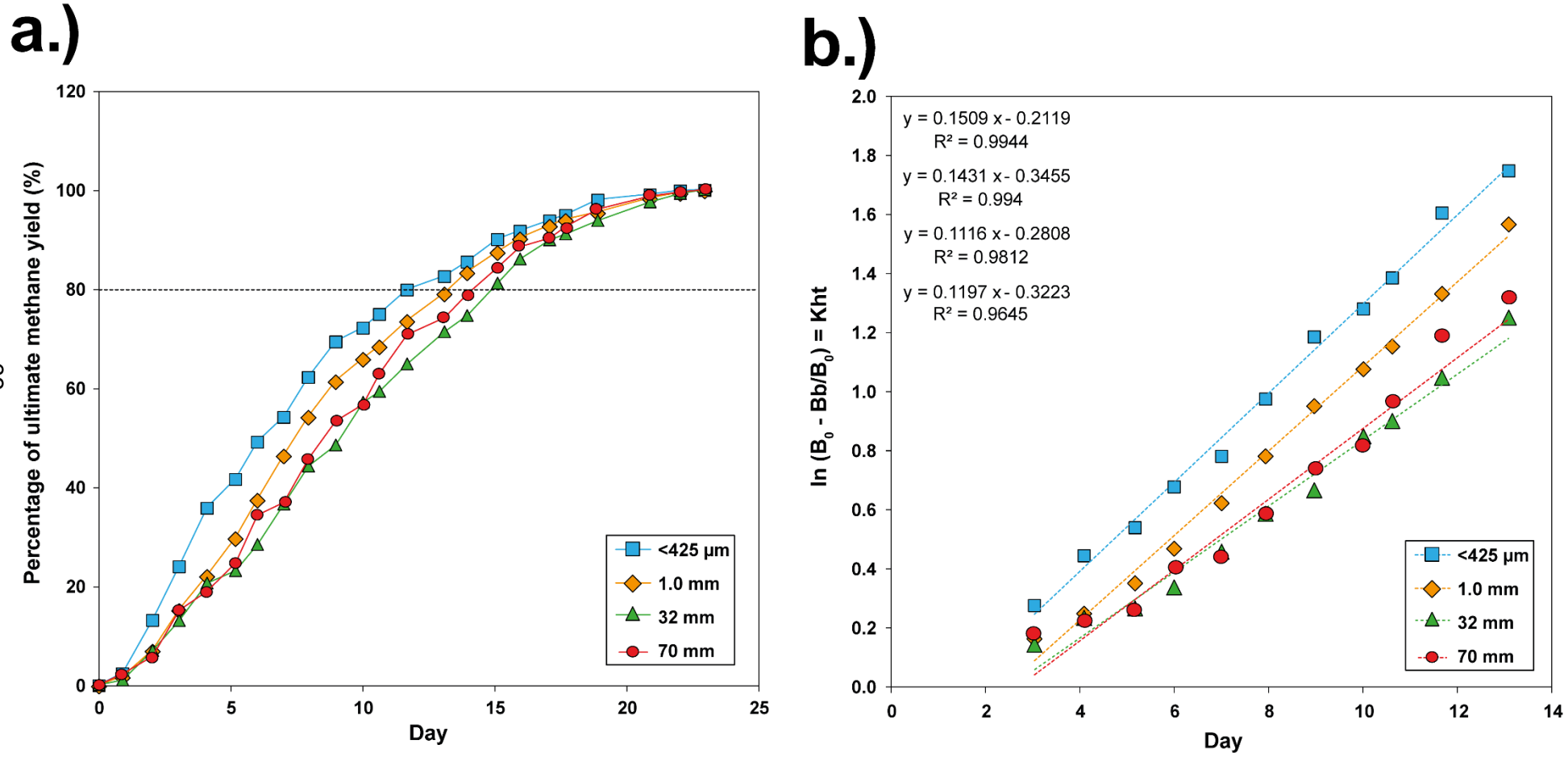

Figure 3.2: **a.)** Time-course data showing technical digestion time of 80 % (indicated by the dotted line) for particle size test and **b.)** Hydrolysis rate constant (k_h) with linear trend line and slope equation. Equation order (top to bottom) is analogous to legend order.

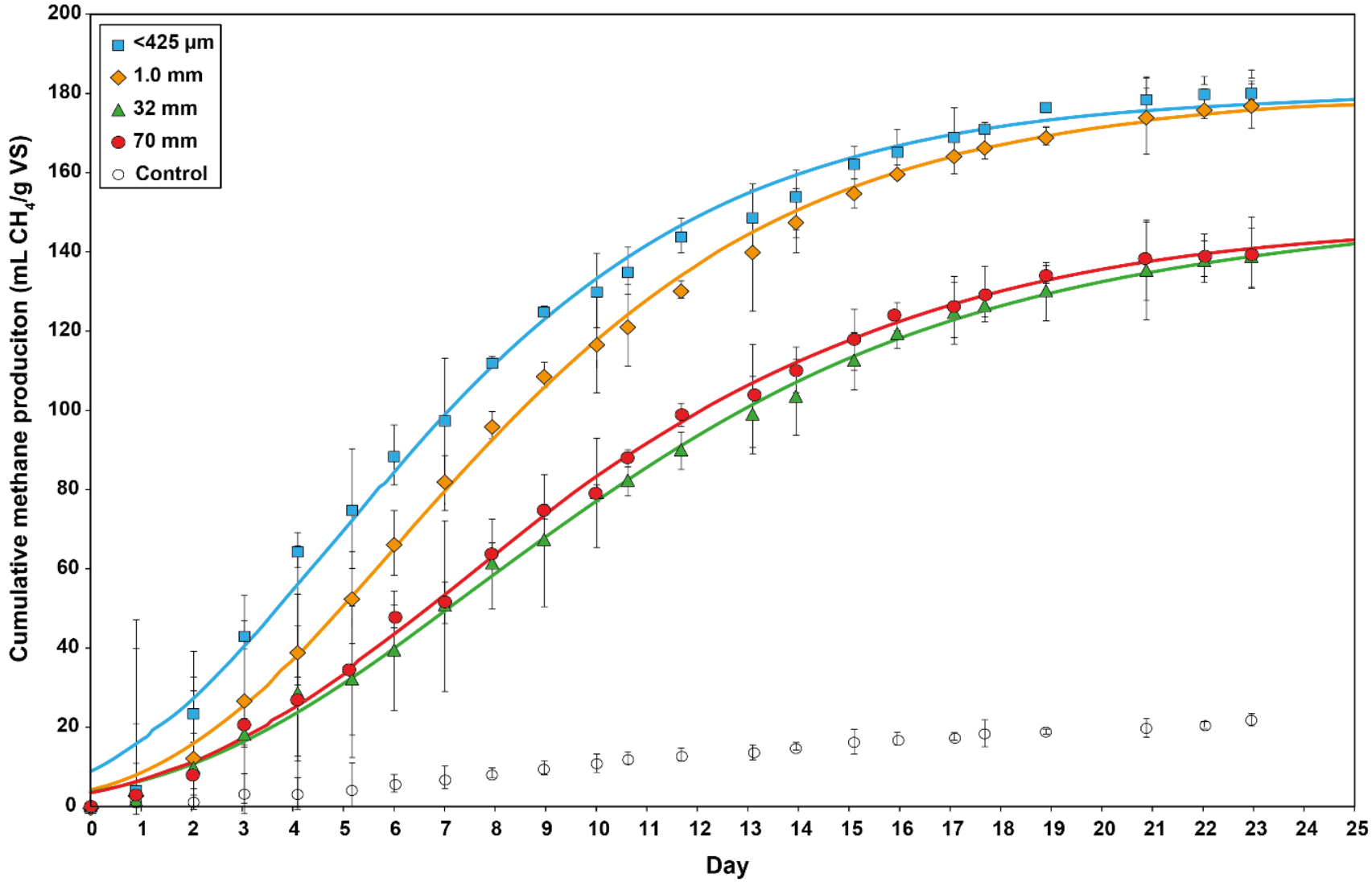

Figure 3.3: Time-course cumulative methane yields (mL CH_4/g VS) for particle size test. Figure includes cumulative methane fitted line given by the Gompertz equation. Standard error bars (n = 3 per day)

The biodegradation (%) was calculated using the ultimate methane yield of each PS condition, the theoretical methane yield of Chinese RS, Eq. 3.6 (shown in Table 3.3). Results were similar to those of Li *et al.* (2013) who reported RS theoretical and experimental methane yields of 460 mL_{Theo} CH_4/g VS and 281 mL CH_4/g VS; however, they also observed higher biodegradation rates (~ 62 %). Following the ultimate methane yield trends, PS425 and PS1 biodegradation rates were 44 and 43 % with PS32 and PS70 the being lower (both 34 %). This was expected as decreasing particle size increases surface area, which provides greater potential microbial access to RS for degradation. A number of researchers have found that milling improved substrate methane potential including, Chen *et al.* (2014) whilst Bruni *et al.* (2010) found that reducing size to 2 mm increased yield by 10 %. However, De la Rubia *et al.* (2011) showed below 1.4 mm and 1.0 mm, respectively, size made no difference. Zhang and Zhang (1999) showed ground, chopped (both 25 mm), and whole RS had similar biogas yields and methane contents i.e., 0.4, 0.38 and 0.38 L/g VS, and, 49.4, 49.4 and 50 % CH_4. However, the results reported here are similar those reported by Krishania *et al.* (2013b) (0.191 m^3 CH_4/kg VS, unground wheat straw) and show PS is significant.

3.3.2 *C:N ratio (codi-test)*

Differences in the methane production rates of each co-digestion condition were seen in the relatively gentler TDT_{80} slopes of the RS40 and DM conditions (Figure 3.4a), and confirmed by results shown in Table 3.4. This was supported by Figure 3.4b, which indicate methane production rate was highest at RS100 (0.23 d^{-1}) and decreased with increasing DM down to RS40 (0.17 d^{-1}) and DM only (0.18 d^{-1}). This is also reflected in cumulative methane curve data (Figure 3.5) with RS40 and DM having the gentlest slopes. RS100 and RS96 (C:N, 60:1 and 50:1) produced the highest methane yields (259 & 240 mL_{ult} CH_4/g VS), highest CH_4 content (both 46 %), and at the highest rates (0.23 and 0.22 d^{-1}) with both RS40 and DM being significantly lower ($p = <0.001$).

This contradicts work by Li *et al.* (2015a) who found that a 1:1 RS:cow manure ratio produced the highest biogas production. Further, Sahito and Mahar (2014) found 43 % RS mixed with buffalo dung as effective, whilst International Rice Research Institute [IRRI] (2016) found co-digestion with a range of manures was generally beneficial. Silvestre *et al.* (2013b) found that small additions of RS to manure

digestion significantly increased biogas production. However, Callaghan *et al.* (2002) found that increasing chicken manure levels resulted in AD performance deterioration, and Li *et al.* (2015b) and Li *et al.* (2015a) found that increased loading of a RS with pig manure and RS with cow manure co-digestion resulted in inhibition.

Table 3.4: Mean performance data for different rice straw:dairy manure ratios

Parameter	RS:DM (C:N)					
	RS100 (60:1)	**RS96 (50:1)**	**RS80 (30:1)**	**RS75 (25:1)**	**RS40 (15:1)**	**DM (10:1)**
Mean CH_4 content (%)	**46.2** [a] **± 2.5** [b]	45.9 ± 2.9	43.2 ± 2.1	40.1 ± 2.7	30.9 ± 2.2	34.7 ± 2.4
Ultimate methane yield (mL_{ult} CH_4/g VS)	**259 ± 8.9**	240 ± 4.4	186 ± 3.9	186 ± 7.0	108 ± 33	98.0 ± 3.0
Biodegradation (%)	**63.3**	58.7	45.5	45.5	26.4	24.0
Production rate (d^{-1})	**0.23**	0.22	0.19	0.18	0.17	0.18
Technical Digestion Time (TDT_{80}) - days	9	9	9	10	10	9

Note: [a] Bold indicates highest performing condition for each parameter
[b] Standard error was calculated: For mean daily methane content n = 60 (40 for RS96, RS75, RS40, and DM), for ultimate methane n = 3 (2 for RS96, RS75, RS40, and DM).

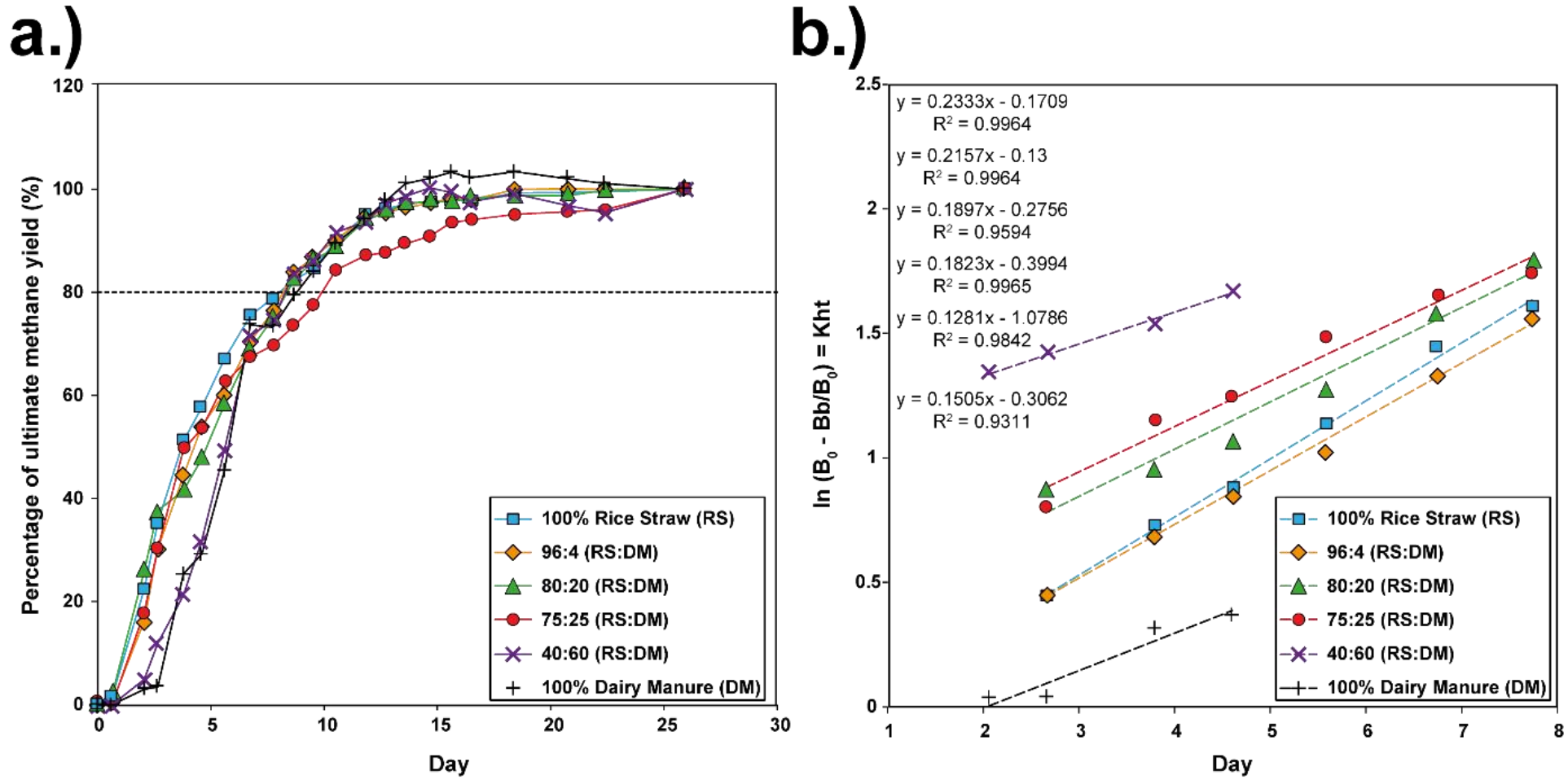

Figure 3.4: a.) Time-course data showing technical digestion time of 80 % (indicated by the dotted line) for co-digestion test, and **b.)** Hydrolysis rate constant (k_h) with linear trend line and slope equation. Equation order (top to bottom) is analogous to legend order.

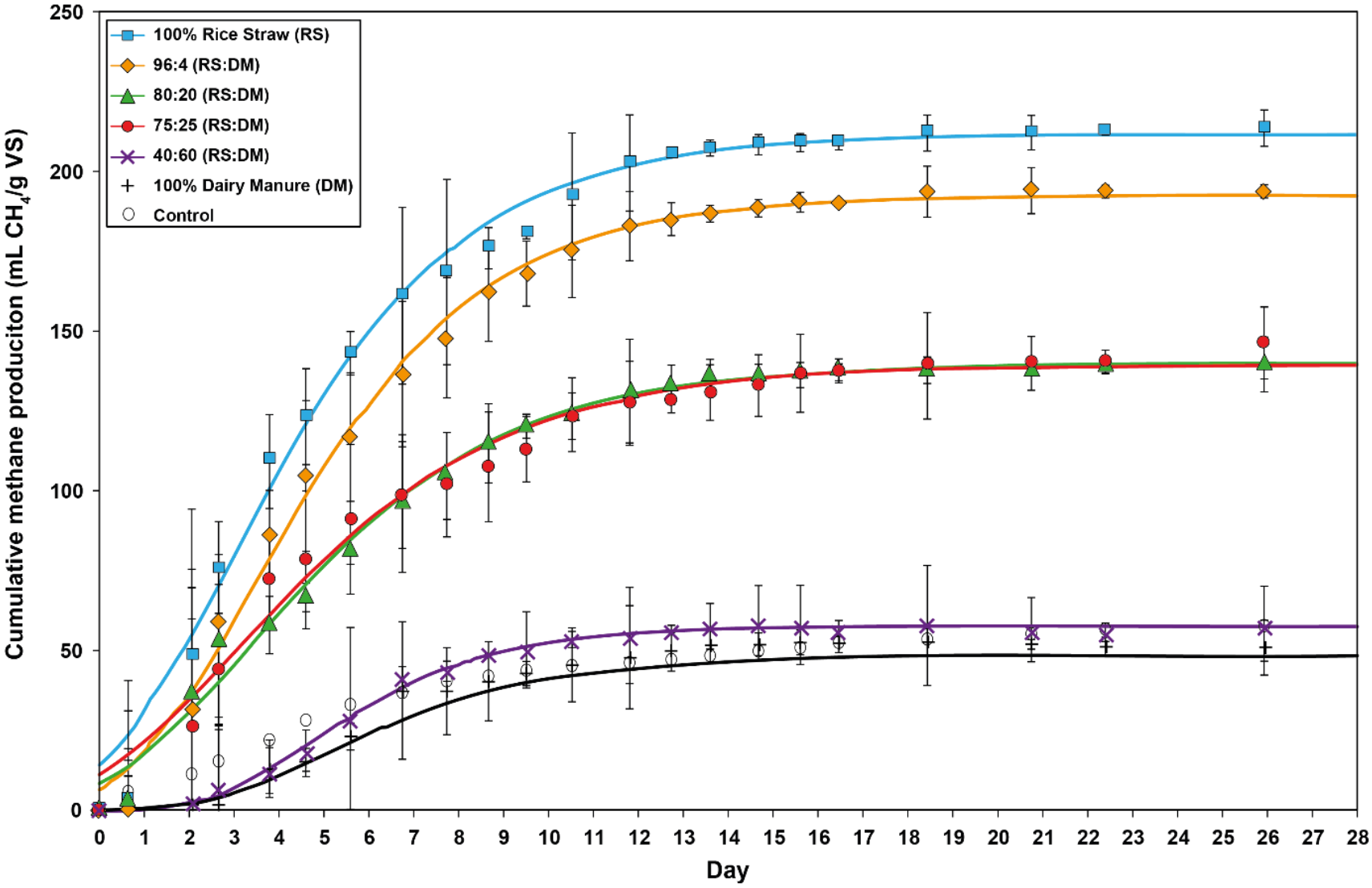

Figure 3.5: Time-course cumulative methane yields (mL CH_4/g VS) for co-digestion test. Figure includes cumulative methane fitted line given by the Gompertz equation. Standard error (n = 3 per day except, n = 2 per day for RS96, RS75, RS40, & DM).

A high C:N ratio is seen as a limiting effect in AD and the ideal is 25 - 30:1 (Hills, 1979; Khalid *et al.*, 2011; Dioha *et al.*, 2013). The C:N of RS is high (60:1) so co-digestion should reduce this, improve performance, and provide essential elements that may not naturally occur in the substrate. However, Wang *et al.* (2014c) found a lower C:N can increase ammonia inhibition and that a higher C:N can be beneficial to the AD process, and Hussain *et al.* (2008) noted that unamended substrates may perform better as essential nutrients are released during cell decay.

In this case, BMP bottles with wholly RS had better biogas performance than those with increasing levels of DM due to the type and biodegradability of the substrates as shown by the biodegradation results in Table 3.4. Though all bottles were given the same level of VS the bottles with higher levels of DM were receiving higher levels of recalcitrant lignin. Dairy herds, like many other ruminants are fed on grasses or other lignocellulosic material. By the time this is manure the most easily digestible forms of carbon, e.g. cellulose, have been digested and the less biodegradable constituents such as lignin have been concentrated as previously reviewed in Section 2.3.1. This difference in biodegradability was likely the main determinant of biogas production and yields in this experiment.

Differences in biogas results also may have been significantly impacted by the BMP test inoculum source. Gu *et al.* (2014) found that using DM as an inoculum starter in RS AD had higher cellulase activity and produced higher methane yields than five others, including anaerobic granular sludge. As the inoculum used in this experiment was acclimated to RS but originated from wastewater AD treatment it may have had lasting inhibitory effects.

3.3.3 *Geographic origins (GO-test)*

The GO-test lag-phase was longer than the previous experiments, ranging from 3.5 - 6.8 days with GO-Chi the shortest and GO-Nig the longest. TDT_{80} showed minimal differences between samples though overall, it was slightly lower than the PS-test with the GO-Nig samples were the slowest (17 days) to reach TDT_{80} (GO-Phi, GO-Chi, and GO-Ind took 15 - 16 days) (Table 3.5 and Figure 3.6a). The K_h-values were contrary to this; i.e., GO-Nig (0.17 d^{-1}) was higher than GO-Phi (0.14 d^{-1}), which was higher than GO-Ind (0.12 d^{-1}) and GO-Chi (0.11 d^{-1}), and were similar to Li *et al.*

(2013) (0.15 d^{-1}). These values are shown by the steep slope of GO-Nig and gentle slope of GO-Chi in Figures 3.6b and 3.7.

Table 3.5: Mean performance data for RS of different geographic origins

Parameter	Rice Straw Country of Origin			
	China	**India**	**Philippines**	**Nigeria**
Mean CH_4 content (%)	42.1 ± 2.2 [a]	39.5 ± 2.1	46.7 ± 2.8	**54.1 ± 2.8** [b]
Ultimate methane yield (mL_{ult} CH_4/g VS)	221 ± 4.6	153 ± 4.9	275 ± 5.1	**388 ± 2.5**
Biodegradation (%)	53.6	37.1	**74.6**	*108.2*[c]
Production rate (d^{-1})	0.11	0.12	0.14	**0.17**
Technical Digestion Time (TDT_{80}) - days	16	16	15	17
Theoretical methane yield (mL_{Theo} CH_4/g VS)	409	412	368	360
Neutral Detergent Fibre (%DW)	65.1	64.6	60.9	54.6
Crude Fibre (%DW)	32.8	33.2	30.7	26.2
Cellulose (%DW)	33.5	34.0	31.4	26.8
Hemicellulose (%DW) (%DW)	27.5	27.0	26.4	24.9
Acid Detergent Lignin (%DW)	4.0	3.6	3.2	2.8
Acid Detergent Fibre (%DW)	37.6	37.6	34.6	29.7

Note: [a]Standard error was calculated: For mean daily methane content n = 44 (66 for GO-IND), for ultimate methane n = 3 (n = 2 for GO-CHI, GO-PHI, GO-NIG
[b]Bold indicates highest performing condition for each parameter
[c]Italicised indicates that the experimental methane yield exceeded the theoretical

The lag-phase and methane production rate are important factors for full-scale operations as the faster RS AD can reach stable production the sooner the AD unit becomes viable. However, the use of kinetic values to predict the operations of a continuously fed AD system should only be used as an approximate as they tend to be a slight overestimation (Strömberg *et al.*, 2014).

Differences in methane content between GO-samples were clear; i.e., GO-Nig was significantly higher than GO-Chi and GO-Ind (54 % versus 42 & 40 %, p = <0.001), but not significantly higher than GO-Phi (47 %). Differences in ultimate methane yields between each substrate were also significant; i.e., GO-Nig was highest (388 mL_{ult} CH_4/g VS), then GO-Phi (275 mL_{ult} CH_4/g VS), GO-Chi (221 mL_{ult} CH_4/g VS), and GO-Ind (153 mL_{ult} CH_4/g VS) at p = <0.001.

Comparing ultimate methane yields with theoretical yields provided biodegradation rates (BD) (Table 3.5) and showed both GO-Chi and GO-Ind had BD rates similar to rates reported by Nielfa *et al.* (2015), but GO-Phi samples outperformed these CO (75 %), and GO-Nig gave over 100 % BD. This was because the ultimate methane yield of GO-Nig outperformed its theoretical yield (388 mL_{ult} CH_4/g VS to 360 mL_{Theo} CH_4/g VS).

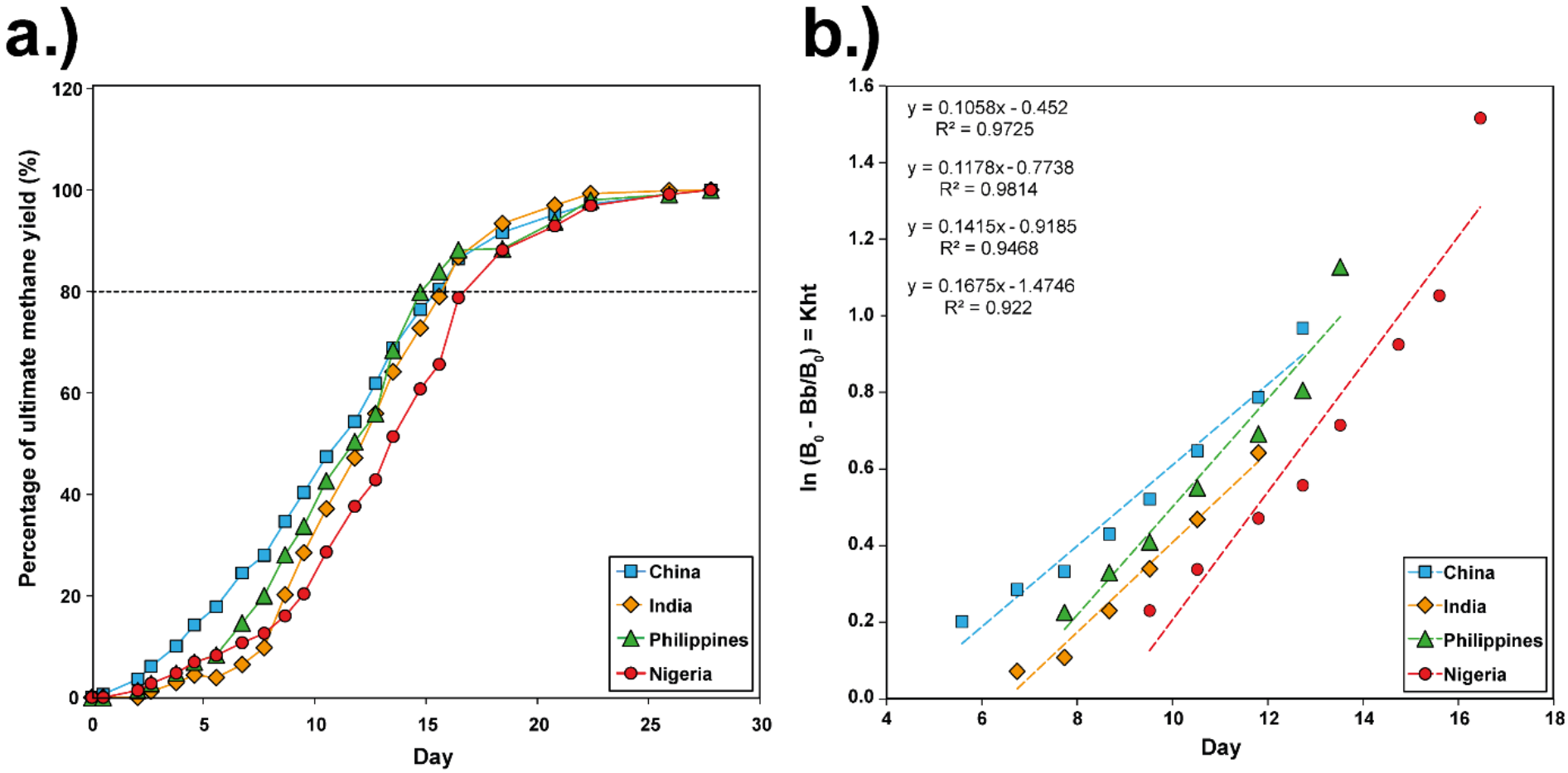

Figure 3.6: **a.)** Time-course data showing technical digestion time of 80% (indicated by the dotted line) for geographic origins test, and **b.)** Hydrolysis rate constant (k_h) with linear trend line and slope equation for all RS countries. Equation order (top to bottom) is analogous to legend order.

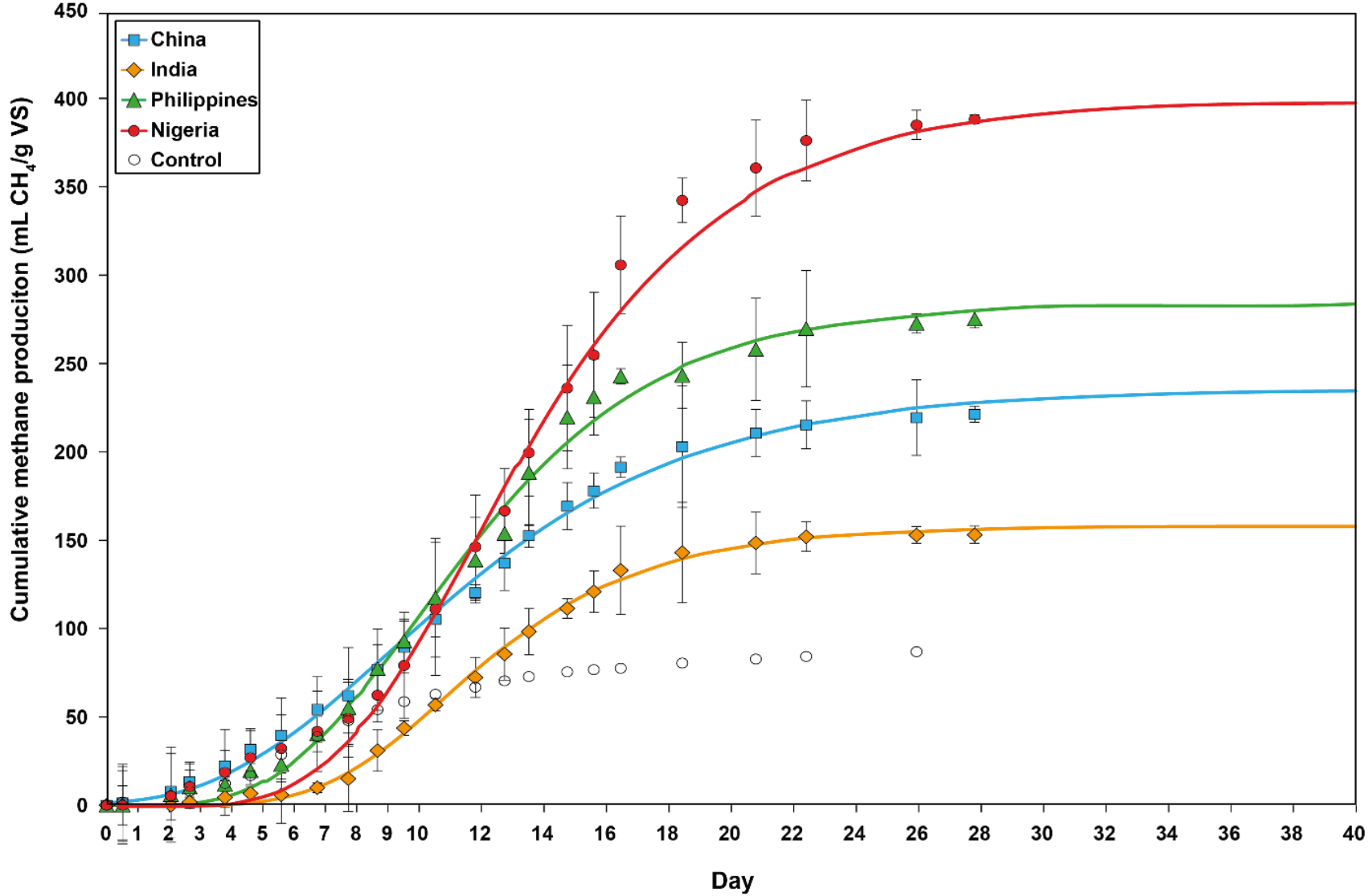

Figure 3.7: Time-course cumulative methane yields (mL CH_4/g VS) for GO-test. Figure includes cumulative methane fitted line given by the Gompertz equation. Standard error bars (n = 3 per day for GO-Ind, n = 2 per day for others)

To determine if RS composition (Table 3.1 and 3.2) was a significant factor during AD performance, PCO clustering was used and showed clear groups between the RS of different countries (Figure 3.8a & b). DistLM was used to determine any statistical significance between parameters and geographic origin, including individual and step-wise variable sequential significance. Full results can be found in Appendix A. GO-Nig and GO-Phi had the highest gas production and biodegradation and clustered relatively closely whilst GO-Chi and GO-Ind were plotted separately based on their differences.

Decreasing VS content, hemicellulose, acid detergent lignin (ADL), and calorific content correlated with improved performance as they point away from the higher performing GO-Nig and GO-Phi (Figure 3.8a). Lignin is insoluble in water, has great chemical stability and acts as the glue that binds the potentially biodegradable cellulose and hemicellulose (Watkins *et al.*, 2015). Typically, lignin constitutes around 10 - 25 % of lignocellulosic biomass, cellulose (2 - 50 %), and hemicellulose (19 - 35 %) (Watkins *et al.*, 2015; Mustafa *et al.*, 2016). Methane content and ultimate methane yields increased as acid detergent lignin (ADL) decreased, e.g., GO-Chi (221 mL CH_4/g VS and 42 % CH_4 at 4.0 ADL % DW) versus GO-Nig (388 mL CH_4/g VS and 54 % CH_4 at 2.8 ADL % DW). There was a similar trend with crude fibre, cellulose, and hemicellulose. As cellulose hydrolysis is the rate limiting step, due to slow degradation and-or recalcitrance, higher levels of lignin and cellulose have been found to yield less methane than substrates with lower lignin (den Camp *et al.*, 1988). That methane yields increased with decreases in these characteristics supports this.

Figure 3.8b shows that RS composition may also affect biogas yields. Increasing Ni correlated with the higher performing GO-Phi and GO-Nig. The benefits of Ni has been reported by a number of researchers including, Gonzalez-Gil *et al.* (2003); Demirel and Scherer (2011) and Pobeheim *et al.* (2010). The lower amounts of Ni in GO-Chi and GO-Ind may account for their difference in performance here compared with GO-Nig and GO-Phi. Of all the individually significant elements, Ni (p = 0.041) and Mg (p = 0.014) correlated with biomethane performance. GO-Ind yielded the lowest biomethane and had the lowest Ni (0.4 mg Ni/kg) and highest Mg (944 mg Mg/kg), whilst GO-Nig had the highest biomethane yield with the highest Ni (0.8 mg Ni/kg) and lowest Mg (118 mg Mg/kg). Chen *et al.* (2008) noted that in the right

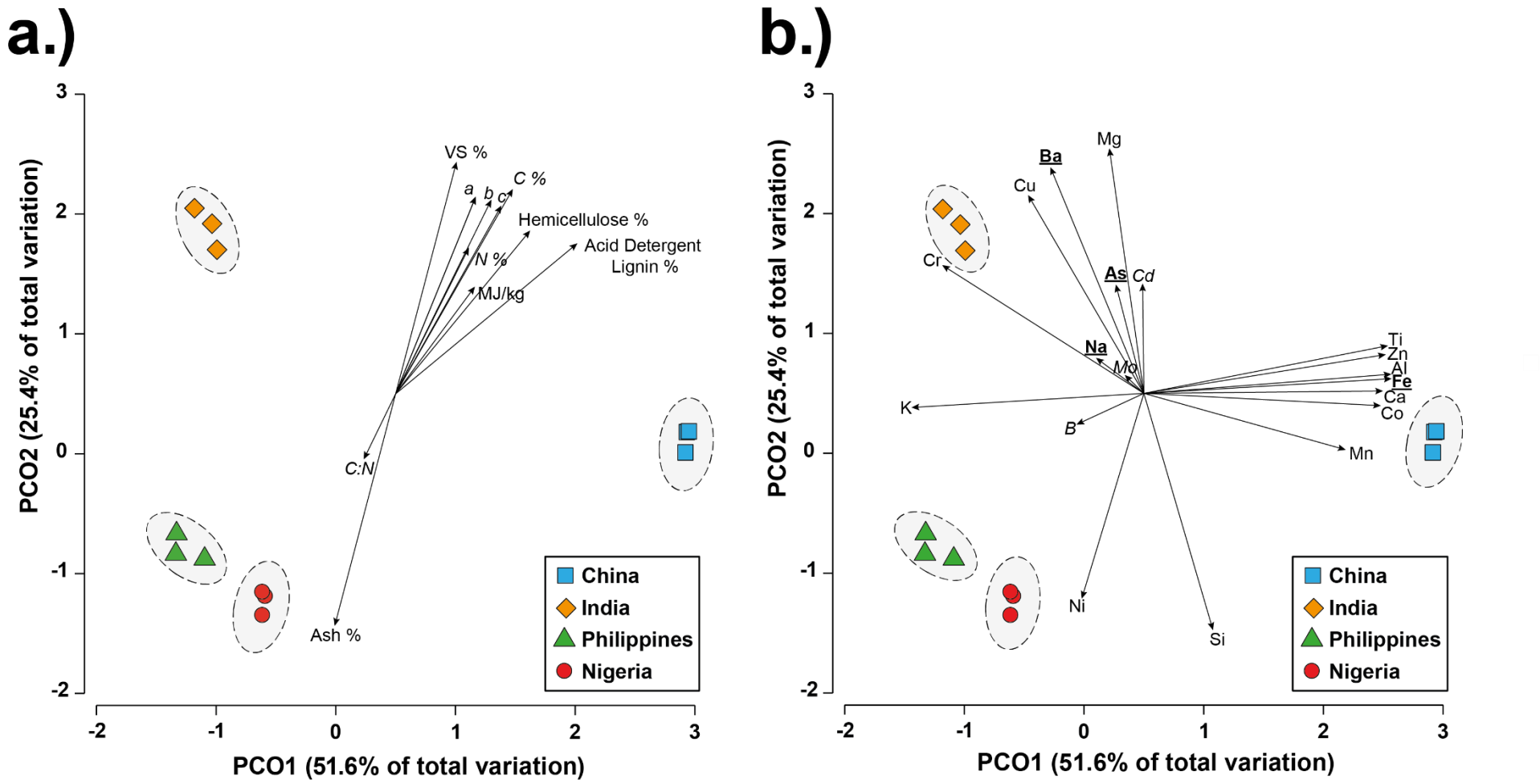

Figure 3.8: Principal coordinates Analysis (PCO) plots of GO-test conditions: **a.)** Composition analysis, *a* = Crude Fibre and Cellulose (%), *b* = Acid Detergent Fibre (% ADF), and *c* = Neutral detergent Fibre (% NDF); and, **b.)** Elemental analysis. Arrows show significant individual influence, underline/bold indicates significant combined group influence, and italics indicates non-significant variables (given by DistLM and multiple regression analysis);

amounts Mg can stimulate growth but above ~ 400 mg/L, such as GO-Chi and GO-Ind, it can be inhibitory. GO-Chi and GO-Ind also differed from each other, with elemental differences possibly responsible for the difference in performance of GO-Chi and GO-Ind. Increased Fe, Co, and Zn (as in GO-Chi) can provide improved performance, (Kong *et al.*, 1994; Demirel and Scherer, 2011). Whilst, excess Cr and Cu, as in GO-Ind, can cause AD inhibition (Yenigün *et al.*, 1996; Abdel-Shafy and Mansour, 2014). Fermoso *et al.* (2009) reported that an excess of metals can inhibit enzymatic processes and-or compete with the substrate.

Although cellulose, hemicellulose, and lignin were likely the deciding factor on AD performance between these straws, the combination of trace elements could have provided a significant impact, as shown by Feng *et al.* (2010); Gustavsson *et al.* (2011); Moestedt *et al.* (2016) and Banks *et al.* (2012). DistLM step-wise analysis showed iron (Fe), barium (Ba), sodium (Na), and arsenic (As), significantly correlated (all $p = 0.001$) with improved biomethane yields (cumulative R^2 of 0.98). Increasing Fe, Ba, Na, and As, correlated with the poorest RS AD performance (GO-Ind and GO-Chi), whilst a reduction of these correlated with improved performance (GO-Phi and GO-Nig). Multiple regression analysis of the significant elements (As, Ba, Fe, and Na), gave a *p*-value of < 0.001 and an R^2 of 86.7 for the RS AD ultimate methane yield model Equation 3.7. However, due to the small sample size (n = 3 per element) and being untested against other RS elemental analysis in the literature due to a lack of available data, this model and *p*-value should be considered carefully.

$$\text{Ultimate mL } CH_4/\text{gVS} = 195.9 - 34.72Ba + 8.87Fe - 0.0350Fe^2 \qquad (3.7)$$

At the time of writing, there is no literature comparing the effect of RS geographic origin (composition) on anaerobic digestion performance. Most AD research on RS has been carried out in China, but there is some yield data from other countries, including India (Fotidis *et al.*, 2016), 364 mL CH_4/g VS; the Philippines and Vietnam (Nguyen *et al.* (2016), 225 - 325 L CH_4/kg organic dry matter; Nigeria, (Okeh *et al.* (2014), 77 - 382 mL biogas/d; Cuba (Contreras *et al.* (2012), 0.226 m^3 CH_4/kg VS; and Italy, (Dinuccio *et al.* (2010), 195 L/kg VS. RS grown in different countries will have different growing conditions, farming techniques, harvesting, storage etc. that will all affect composition and therefore AD performance. It may also be that different varieties were provided, *Oryza sativa* (typical of Asia) or *O. glaberrima* (Nigeria) for example, but unfortunately not all suppliers could confirm. It is likely that differences

in the ‘same’ feedstock due to natural and nurtured changes, such as lignin and external factors such as pesticide residues (Chen *et al.*, 2008), could alter the microbial communities in the digesters, and affect biomethane potential. The results show that lignin content plays a defining role in biogas production and yields and that any elemental supplementation should be substrate specific for maximum effect, as suggested by Lebuhn *et al.* (2008).

3.3.4 *Method reproducibility*

The maximum daily and ultimate methane yields of the experimental data shows that the BMP data falls within the 95% confidence limits of the Gompertz predicted data, and, the R-Sq values were all ≥ 0.993 indicates that the BMP test was operated for an appropriate time frame.

PS1 and GO-Chi were compared to assess VDI Standard: 4630 (2006) reproducibility in this case. There were differences between PS1 and GO-Chi in Gompertz lag phase (1.5 & 3.5 days), experimental methane content (50.4 & 42.1 %), p = 0.005, TDT_{80} (13 & 16 days), maximum methane yields (168 & 214 mL CH_4/g VS) p = <0.001, and ultimate yields 177 & 221 mL_{ult} CH_4/g VS. Differences in daily methane production and max daily methane yield were not significant. This indicates that reproducibility of the method is affected by the seed inoculum as that was the main difference between PS and GO-tests. Though there were differences here, perhaps the VDI Standard: 4630 (2006) is the unified method needed to compare research findings.

3.4 Conclusion

RS AD was limited to less than 3 g VS/L OLR (I:S, 2:1), whilst decreasing RS particle size increased methane production rate and maximum yield, 425 µm and 1.0 mm particles were best. Although the reproducibility of the VDI Standard: 4630 (2006) was not exceptionally high, it was a sufficiently reliable method.

Decreasing DM addition to balance C:N, and P addition did not improve methane production. Though all bottles were given the same level of VS, the bottles with higher levels of DM received higher levels of recalcitrant lignin as dairy herds, like many other ruminants are fed on grasses or other lignocellulosic material. This process digests the most easily digestible forms of carbon, e.g. cellulose, and the

less biodegradable constituents such as lignin have been concentrated. It was this difference in biodegradability was likely the main determinant of biogas production and yields in this experiment.

GO-Nig provided the highest yield but the poorest rate (388 mL $_{ult}$ CH_4/g VS and 1.4 d^{-1}), GO-Chi the fastest rate (0.14 d^{-1}), and GO-Ind the lowest yield (153 mL $_{ult}$ CH_4/g VS). This indicates that the ‘same’ feedstock behaves differently depending on natural and nurtured changes and more universal testing is required. The greatest impact here was due to the differences in lignin. Lignin is insoluble in water, has great chemical stability and acts as the glue that binds the potentially biodegradable cellulose and hemicellulose. Therefore, higher levels of lignin and cellulose yielded less methane than RS with lower lignin.

Chapter 4 Effect of Feeding Frequency and Organic Loading Rate on Biomethane Production in the Anaerobic Digestion of Rice Straw

4.1 Introduction

The current ‘go to’ method to improve RS AD is to use some form of pretreatment and-or co-digestion, which in some cases has improved biogas yields. However, these options come with costs (monetary, technical, or energy), often being impractical at full scale or unworkable within the context of rice farming practice (Ariunbaatar *et al.*, 2014; Ferreira *et al.*, 2014; Croce *et al.*, 2016). Avoiding pretreatment and supplements, despite potentially lower yields, has advantages. For example, if it were possible to show that RS AD systems can operate effectively under irregular feeding conditions, AD coupled with CHP becomes more attractive, although suitable RS organic loading rates (OLRs) must be defined; i.e., feeding frequency (FF) and OLR must be co-optimised. Would it be optimum to operate with higher, but more intermittent loads for short periods or lower loads spread out over a longer time, and at what OLR? Balancing FF and OLR must be assessed in tandem, although few studies have examined infrequent and-or extreme feeding regimes, such as glut-starve versus steady and regular-fed systems. Most previous work has focused on a narrow time-margins between feeds or tested ranges, such as Bombardiere *et al.* (2007) who examined 1-12 feeds/day for chicken litter waste; Golkowska *et al.* (2012) who tested batch vs semi-batch vs continuous feeding frequencies; Piao *et al.* (2016) twice daily, once daily and bi-daily; and Manser *et al.* (2015) that compared bi-daily vs weekly feeding regimes. An AD system that focussed on infrequent feeding regimes could provide a suitable outlet for the periodic harvest and RS availability. As the use of biomass derived gases become more economically viable they will be increasingly important as a method of utilising waste streams to provide useable energy and play an important role in the reduction of GHGs (International Energy Agency [IEA], 2008). Limiting the frequency with which an AD system is fed would allow the process to synchronise with the acyclic nature of RS production whilst also reducing the level of operational worker input.

To our knowledge, no studies have assessed a wide range of glut-starve feeding regimes on the biogas productivity and yields in RS AD. Therefore, Biomethane Potential (BMP) assays were first performed in Chapter 3 to determine conditions for long-term reactor experiments. Five different feed-starvation regimes then were assessed in lab-scale AD units to quantify the co-influence of FF and OLR on CH_4 yields and process stability. Two different OLRs were used, creating a two by five matrix of AD operating conditions. Biomethane yields, volatile solids (VS) reduction, and VFA production were monitored to identify optimum feed/OLR options to inform and guide prospective large-scale commercial applications.

4.2 Materials and methods

4.2.1 *Substrate and inoculum*

Chinese RS was ground and homogenized to a 425 µm mean size and characterised using methods described in Chapter 3. Inoculum was RS acclimated and both are summarised in Table 4.1.

Table 4.1: Characteristics of the rice straw feed and the anaerobic digester inoculum

Parameter	Unit	Rice Straw	Anaerobic Inoculum
Total Solids	% DW [a]	96.1 ± 0.1[b]	2.4 ± 0.0
Volatile Solids	% DW	87.3 ± 0.2	76.6 ± 0.3
Moisture Content	% DW	3.76 ± 0.1	97.6 ± 0.0
Ash Content	% DW	12.3 ± 0.2	23.3 ± 0.3
Fixed Solids	% DW	11.3 ± 2.1	6.18 ± 0.4
C	% DW	39.0 ± 0.4	54.6
N	% DW	0.86 ± 0.1	4.72
C:N	Ratio	45.3	11.6
Calorific Content	MJ/Kg	15.4 ± 0.1	- [c]

Notes: [a] DW is an abbreviation of dry weight – the weight of sample at standard temperature and pressure
[b] Standard error (n=3)
[c] No data for inoculum calorific content

4.2.2 *AD reactor conditions and operations*

Five 2.5-L reactors with control towers were used as the AD units, with working volumes of 2.0 L. Each glass, airtight-sealed reactor consisted of a heating jacket set to 37 °C, a biogas sampling bag, and a paddle stirrer (Figure 4.1). Overall, the reactors were operated for 252 days of which 112 days were used for sludge acclimation to RS feed. During acclimation, the anaerobic sludge inoculum was operated in draw-fill mode (digester sludge removal prior to feed addition) with a 50-day hydraulic retention time (HRT) and an OLR of 1.0 g VS/L/d (chosen based on BMP assays) fed once every seven days. After two HRTs, pH and VFA levels had become stable with time, and the formal experiment was commenced (defined as Time 0). Operationally, OLR1 (defined as 'Low') was where 280 mL of reactor volume was removed per week and 14 g VS/week was provided in 280 mL distilled water (as 425 µm RS). For OLR2 (defined as 'High'), 28 g VS/wk was provided to the reactors with the same water volume removed as in OLR1.

The first part of the experiment assessed the effect of FF on performance by varying the frequency at which the reactors were fed, including: five feeds every seven days (5/7); three every seven days (3/7); one every seven days (1/7); one every fourteen days (1/14); and one day every twenty-one days (1/21). The reactors were operated at OLR1 for 56 days at a mean RS feed rate of 1 g VS /L/d; i.e., some reactors received RS frequently in small amounts, whereas others received less frequent, larger doses. After 56 days, OLR was increased in all reactors to 2 g VS/L/d, which were operated for 84 more days using the same FFs.

Example feed sequences are as follows. For the 5/7 reactor at OLR1, 56 mL of reactor volume was removed per feed and then 56 mL of distilled water, containing 2.8 g VS of RS was provided. This was done five times per week. In contrast, the 1/21 unit had 840 mL removed (per feed) after which 47.7 g RS and 840 mL distilled water were added, but this was only done once every three weeks. The same mean mass of RS was added in both cases, but 5/7 received 15 small feeds in three weeks, whereas 1/21 received one large feed over the same time. Similar withdrawal-feed schedules were used for other FF and OLR reactors, as appropriate.

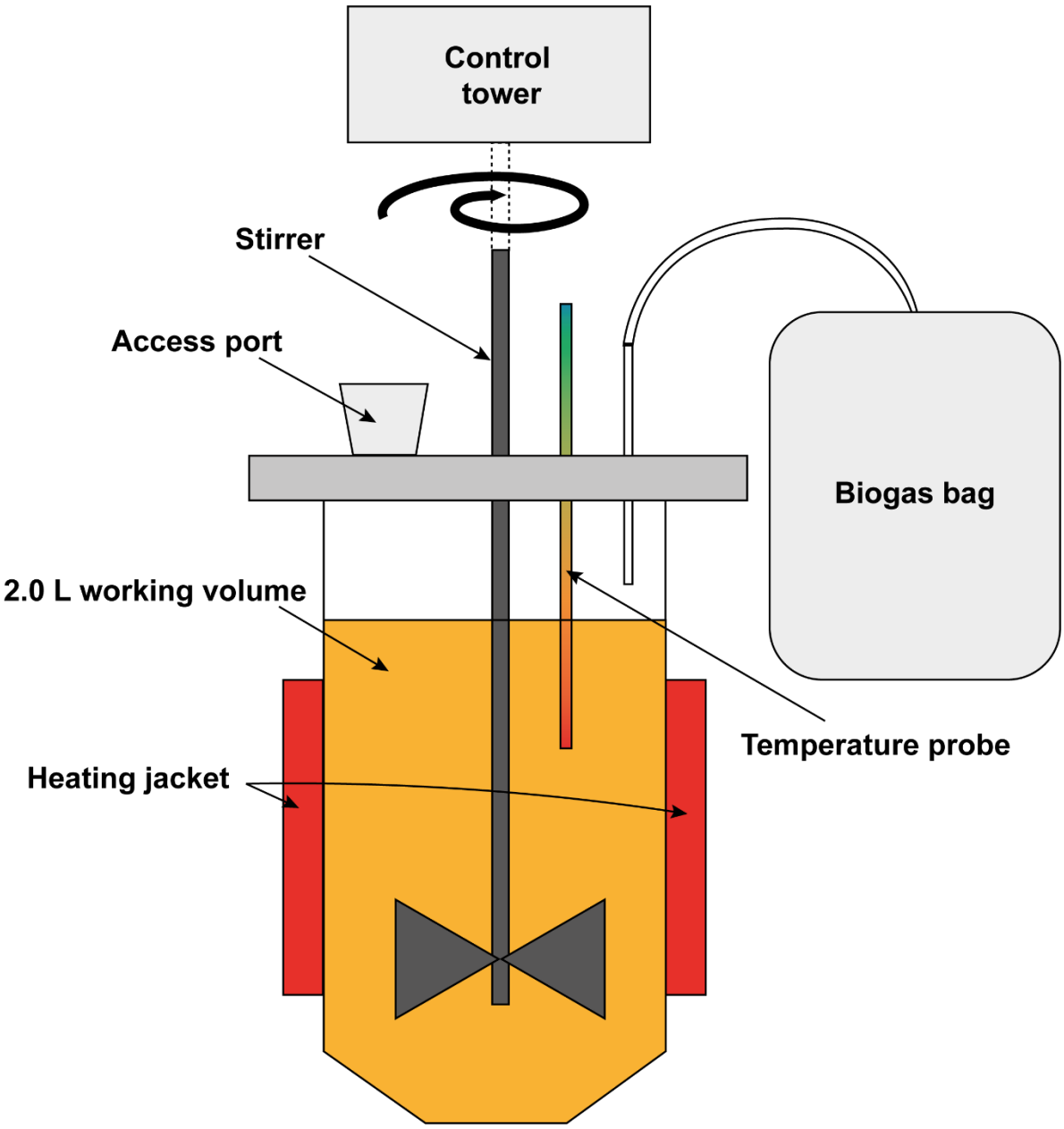

Figure 4.1: Schematic of the anaerobic digesters used for rice straw digestion.

4.2.3 *CH_4 production and other routine analyses*

Gas samples were collected from the biogas-bag using gas tight syringes (SGE and Samco), and total/volatile solids were measured as in Chapter 3.2. A Hach HD40q probe was used to measure pH three times per week (Monday, Wednesday, and Friday) and was unadjusted throughout.

VFA analysis only was performed in the long-term experiment, typically three or four days per week, depending on the ambient stability of the reactors. Analysis consisted of filtering the sample through a 0.2µm PES syringe filter before mixing 1:1 with 0.1M

Octane Sulphonic Acid before sonicating for 40 minutes. Samples were then analysed using the Ion Chromatography Dionex Aquion system equipped with an AS-AP auto sampler with Chameleon 7 Software.

4.2.4 *Data analysis and statistics*

Statistical analysis of sample data was performed using analysis of variance (ANOVA) with the Tukey comparison and-or *t*-tests. Comparisons of mean performance were contrasted among FFs and between OLRs. Significance was defined as 95 % confidence in differences (i.e., $p < 0.05$). All statistical analyses were conducted using Minitab 17 (Version 17.1.0)

4.3 Results

4.3.1 *Effect of feeding frequency on reactor performance*

Mean biogas yields, specific, and volumetric methane yields, and VS reductions (% VSR) for OLR1 (low loading, 1 g VS/L/d) and OLR2 (high loading, 2 g VS/L/d) are summarised in Table 4.2 and Figure 4.2, which are drawn from time-course data typical of Figure 4.3a-h.

At OLR1, mean biogas yields ranged from 295 ± 9.9 to 317 ± 8.8 mL/g VS/d across the five FF conditions, which did not significantly differ implying FF did not impact overall biogas production when VS loadings were low. However, specific CH_4 yields (mL CH_4/g VS/d) differed among reactors with the most infrequently fed reactor, 1/21, having significantly higher mean specific yields than the most frequently fed reactor, 5/7 (i.e., 148 ± 6.3 vs 112 ± 4.6 CH_4/g VS/d, respectively; $p = 0.001$). Significant differences between these two FFs also were seen in biogas quality; i.e. 5/7 had a mean CH_4 content of 40.2 % ± 1.3 in contrast to 49.3 % ± 1.4 for 1/21. Biogas yields, specific CH_4 yields and biogas quality varied among the middle three FFs, but not significantly, although 1/7 tended to have slightly higher yields than 3/7 and 1/14. In contrast to gas results, 5/7 had the highest % VS reduction (44.1 % ± 1.8) and 1/21 had the lowest (38.0 % ± 3.2) (Table 4.2), although differences were not significant.

Table 4.2: Overall mean performance data for reactors with different feeding regimes and organic loading rates

Feed Frequency	**5/7** [a]		**3/7**		**1/7**		**1/14**		**1/21**	
Organic loading rate (g VS/L/d)	**1.0**	**2.0**	**1.0**	**2.0**	**1.0**	**2.0**	**1.0**	**2.0**	**1.0**	**2.0**
Biogas	301	239	299	215	**317** [c]	249	295	139	303	42.0
(mL/g VS/d)	± 8.4 [b]	± 5.1	± 6.8	± 4.6	**± 8.8**	± 5.8	± 9.9	± 10.0	± 11.5	± 7.8
% CH_4	40.2	52.1	42.4	52.2	46.7	**55.4**	45.4	38.7	49.3	21.7
	± 1.3	± 1.4	± 1.2	± 1.7	± 1.7	**± 1.7**	± 1.4	± 2.4	± 1.4	± 1.4
Specific CH_4	112	125	127	112	146	138	134	63.4 [d]	**148**	7.7 [d]
(mL CH_4/g VS/d)	± 4.6	± 4.4	± 4.5	± 4.4	± 6.0	± 5.3	± 6.0	± 5.6	**± 6.3**	± 0.7
Volumetric CH_4	112	251	127	224	146	**276**	134	127	148	15.4
(mL CH_4/L/d)	± 4.6	± 8.7	± 4.5	± 8.7	± 6.0	**± 10.6**	± 6.0	± 11.1	± 6.3	± 1.3
g VS/L	25.9	38.3	25.7	37.0	25.4	41.1	26.9	43.8	27.1	54.1
	± 0.5	± 1.7	± 0.6	± 1.8	± 0.8	± 2.0	± 1.0	± 2.6	± 1.2	± 3.4
% VS	**44.1**	41.6	42.5	42.8	42.5	40.5	39.4	31.7	38.0	41.3
Reduction	**± 1.8**	± 2.3	± 1.9	± 1.9	± 1.8	± 2.2	± 2.6	± 1.6	± 3.2	± 3.0
Total VFA	147	432	135	495	252	383	354	1730	1250	3470
(ppm)	± 29.4	± 109	± 18.2	± 163	± 43.7	± 57.8	± 77.2	± 336	± 312	± 355
pH	6.8	6.7	6.8	6.7	6.8	6.7	6.7	6.3	6.6	5.7
	± 0.02	± 0.01	± 0.02	± 0.01	± 0.01	± 0.01	± 0.01	± 0.06	± 0.02	± 0.04

Note: [a] The feeding frequency of each reactor e.g. 5/7 = feed five days out of seven. All had the same net load of 1 g VS/L/d then 2 g VS/L/d.
[b] Standard error (For OLR 1.0g VS/L/d n = 76 for biogas and methane, n = 12 for VS and total VFA, n = 30 for pH, and, n = 3 - 12 for individual VFAs.
[c] Bold indicates highest performing condition for biogas, % CH_4, specific and volumetric methane yields, and % VS reduction.

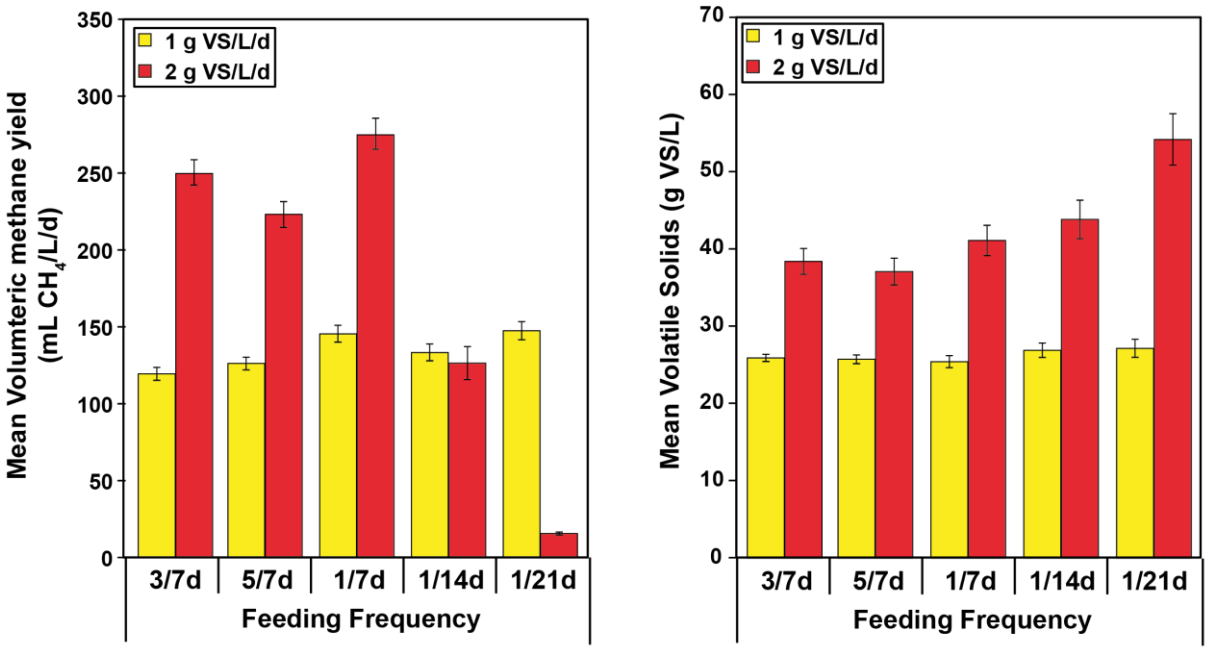

Figure 4.2: a.) Mean volumetric methane yield per day for all feeding frequencies and organic loading rates. Standard error bars (n = 56 at OLR 1 g VS/L/d and n = 84 at OLR 2 g VS/L/d). **b.)** Mean volatile solids for each feeding frequency condition for the whole of each OLR. Standard error (n=9 for VS at OLR 1 g VS/L/d and n=12 for VS OLR = 2.0g VS/L/d).

At OLR2, mean biogas volumes ranged from 42.0 ± 7.8 to 249 ± 5.8 mL/g VS/d; however, both 1/14 and 1/21 failed, which explains the wide range (Figures 4.2g and h). Of the surviving reactors, specific CH_4 yields were significantly different between 3/7 and 1/7 (i.e., 112 ± 4.4 vs 138 ± 5.3 mL CH_4/g VS/d, respectively), and there were no significant differences observed in biogas quality (i.e., % CH_4 content).

4.3.2 *Effect of loading rate on reactor performance*

Inter-OLR comparison, i.e. 5/7 at OLR1 versus 5/7 at OLR2 etc., showed specific biogas and CH_4 yields were always higher at OLR1. However, significant differences were only seen in inter-OLR specific CH_4 yields for 1/14 and 1/21 (p = 0.001), although these were biased by the fact that both 1/14 and 1/21 failed at OLR2. At OLR1, the highest specific CH_4 yield 148 ± 6.3 mL CH_4/g VS/d observed at 1/21, whereas at OLR2, 1/7 was highest at 138 ± 5.8 mL CH_4/g VS/d; however, these were not significantly different from each other. Although biogas and specific yields were always higher at the lower OLR, the reactor with the highest CH_4 content in biogas

was 1/7 at OLR2 (55.4 ± 1.7 % CH_4), significantly higher than 1/21 at OLR1 (49.3 ± 1.4 % CH_4; p = 0.006).

In contrast to specific biogas and CH_4 yields (where differences were not significant), volumetric biogas (as mL/L/d) and CH_4 (as mL CH_4/L/d) yields were significantly higher at OLR2 (p = 0.001). Volumetric CH_4 production at OLR2 ranged from 224 ± 8.7 (3/7) to 276 ± 10.6 (1/7) mL CH_4/L/d compared with 112 ± 4.6 (5/7) to 146 ± 6.0 (1/21) mL CH_4/L/d at OLR1 (Figure 4.2a). In summary, greater CH_4 volumes were produced at OLR2 when the reactor did not fail (i.e., was not overloaded), but more stable operations and higher specific CH_4 yields were seen at OLR1.

4.3.3 *Other indicators of reactor performance*

Across all FFs at OLR1, no significant differences were observed in the pH (range 6.6 to 6.8), VS removal (range ~38 to 44 %), or in VS accumulation (~25.4 to 27.1 g VS/L), although 1/14 had significantly lower VS removal than 5/7 (p < 0.05). At OLR2, 1/14 and 1/21 were significantly different for various parameters: i.e., pH (6.3 ± 0.06 and 5.7 ± 0.03, respectively) and were significantly lower than 1/7, 3/7 and 5/7 (all pH 6.7 ± 0.01). VS % removal in 1/14 was significantly lower than the other FFs at OLR2 (32 % compared with 40.3 to 44.1 %, p = 0.006) and VS accumulation was always greater at OLR2 relative to OLR1 (i.e., 37.0 to 54.1 g VS/L vs 25.4 to 27.1 g VS/L, respectively), which may have practical implications for actual RS AD operations.

Time-course data (Figures 4.3) shows that when the OLR was doubled at day 56, declines in performance in 1/14 and 1/21 were almost immediately apparent. For 1/14, Figure 4.3a - c & g show VS and VFA became more variable and pH dropped rapidly after feeding, ultimately leading to reactor failure on day 112. Whereas, for 1/21, failure occurred almost immediately after the loading change (Figure 4.3a - c & h). In both cases, mean VFA levels were significantly higher than other OLR2 reactors; i.e., 1730 ± 336 and 3470 ± 355 ppm for 1/14 and 1/21, respectively (Figure 4.3c). Mean VFA levels were significantly higher in 1/21 at OLR2 compared with the other FF units (p = 0.015); ~1250 ppm versus < 353 ppm in the other reactors.

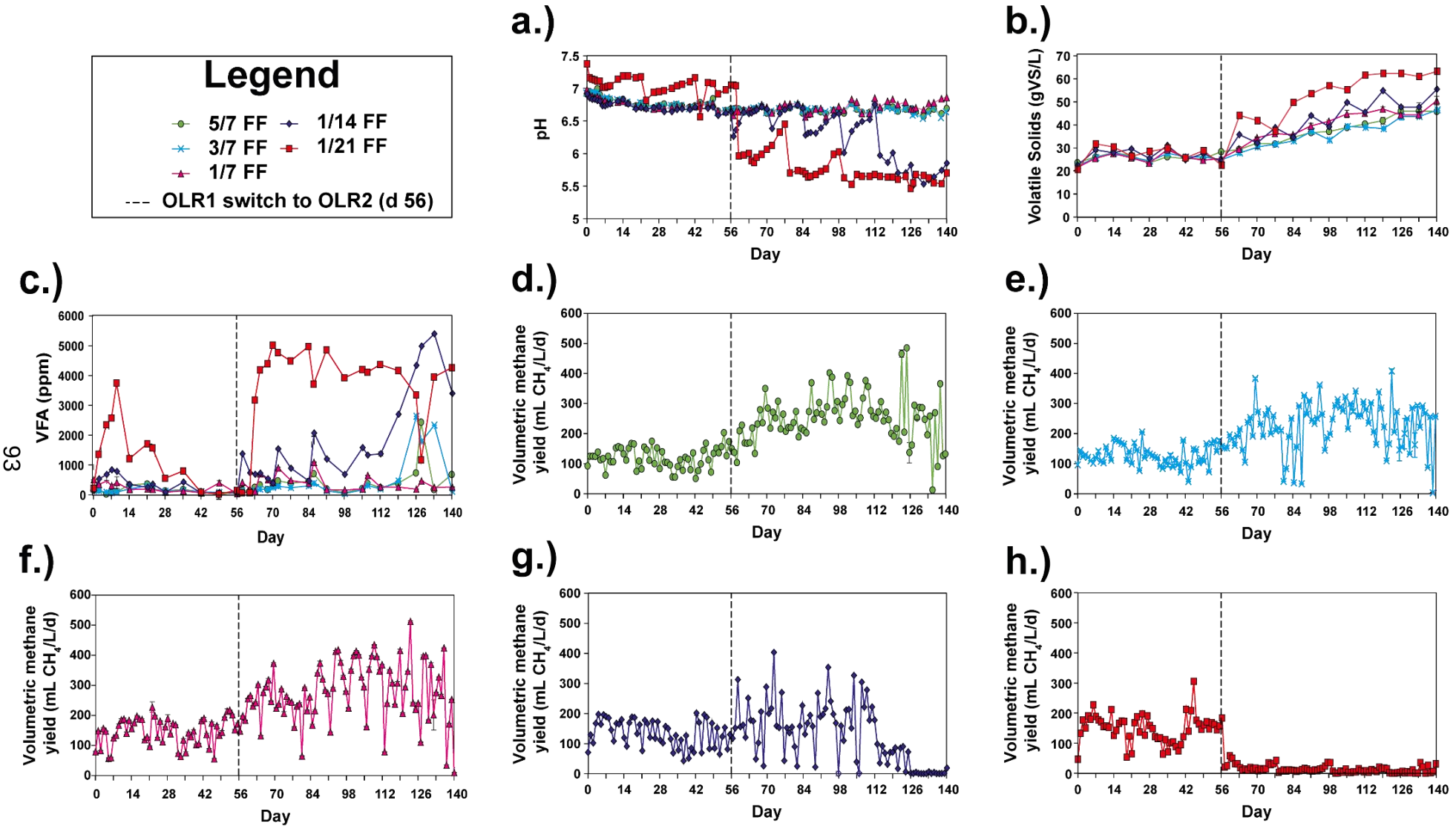

Figure 4.3: Time-course performance data for reactor operations post acclimation, **a.)** pH, **b.)** VS, **c.)** Total VFA, and volumetric methane yields for each feeding frequency i.e. **d.)** 5/7d, **e.)** 3/7d, **f.)** 1/7d, **g.)** 1/14d, **h.)** 1/21d

4.4 Discussion

Methane yields observed in long-term experiments performed here (see Table 4.2) were higher than Gu *et al.* (2014) who combined RS and granular sludge (~125 mL CH_4/g VS), Lianhua *et al.* (2010) (120 mL CH_4/g VS), and Mussoline *et al.* (2013b) (46 mL CH_4/g VS). However, yields were lower than batch experiments by Lei *et al.* (2010) (240 mL CH_4/g VS) and the large scale digesters of Mussoline *et al.* (2014) (181 mL CH_4/g VS). Therefore, yields are roughly comparable to previous work with variation among studies due to differences in feeding regimes, pH-balancing, scale, and-or pretreatment. However, results are promising in a practical sense because we show RS AD can operate without major pretreatment and with less frequent feeding, especially at lower OLRs.

4.4.1 *Biomethane yields and operating options*

Rice farms typically have two to three harvests each year, which produce massive amounts of RS over short periods. Given this operating reality, it is surprising few studies have been performed on how irregular RS production patterns influence AD; a bioprocess that usually requires a stable and regular feedstock. Bombardiere *et al.* (2007) did assess the influence of 1 to 12 RS feeds per day and found more stable operations at less frequent feeding rates. However, their feed frequencies were very short compared with seasonal cycles in rice fields, which work here was designed to assess.

Overall, data show infrequent feeding at low OLRs can provide comparatively higher specific levels of biogas and CH_4 at 1/21 day feed rate (148 mL CH_4/g VS/d), which was higher than the more frequently-fed reactors (Figure 4.2). This is promising for RS AD field applications. However, infrequent feeding at higher OLRs can overload AD systems (Figures 4.3a - h), causing reactor failure. Whereas, higher OLRs with more frequent feeding can produce larger biogas volumes. Therefore, two clear operating options exist for RS AD; less frequent feeding at low OLRs or more frequent feeding at higher OLRs. The preferred option will depend on the space available for RS storage prior to use as well as the quality of the biogas for direct combustion and associated costs, although other considerations exist.

First, the low OLR produced higher specific biogas volumes, whereas the higher OLR produced more volumetric biogas of higher quality in reactors 5/7, 3/7 and 1/7.

Doubling the substrate load should, logically, result in increased gross biogas and-or CH_4 yield as seen by Bezerra *et al.* (2011), assuming the AD units are not overloaded, such as 1/14 and 1/21. Although, increasing OLR does not always increase specific biogas yield as seen here and by Babaee and Shayegan (2011). Differences in inter-OLR specific yields in this study were not significant, probably due to the general recalcitrance of RS and greater VFA production during infrequent feeding at OLR2. Nevertheless, inter and intra-OLR volumetric biogas and CH_4 yields were significant i.e., OLR2 1/7 (276.1 mL CH_4/L) yielded almost 20 % more CH_4 than OLR2 5/7 (224.1 mL CH_4/L at p = 0.001), and almost double that of OLR1 1/21 (148 mL CH_4/L at p = 0.001).

Second, significantly greater VS accumulation was apparent at the higher OLR. Reactor VS reflects the organic fraction of the substrate that is not degraded by the system (Babaee and Shayegan, 2011), and suggest reactors at OLR2 may be receiving more 'substrate' than can actually be degraded. Finally, the more infrequently fed reactors tended to have greater VFA accumulation, especially at OLR2; i.e., 1/21 produced significantly more than all other conditions followed by 1/14. Such acidification can be irreversible and cause a massive drop in CH_4 as seen by Neves *et al.* (2004), or it can be reversible, as indicated by the VFA peaks in 1/7 (Figure 4.3c), and CH_4 yield can recover as seen by Kawai *et al.* (2014). However, elevated VFA levels at OLR2 with 1/14 and 1/21 very probably explain failure, which is important to future RS AD practical applications.

As background, rapidly growing, pH-insensitive, acidogenic bacteria tend to overproduce VFAs that the slow growing acetogenic bacteria cannot oxidise (Wang *et al.*, 1999). The high VFA values, and large fluctuations in pH, indicate an imbalance between the acid producing bacteria and the CH_4-producing archaea. Excess acid production in AD systems is a common reason for systems to fail or sour as reported by Tait *et al.* (2009) and Franke-Whittle *et al.* (2014), or at least negatively affect specific yields (Raposo *et al.*, 2006; Wang *et al.*, 2009; Ye *et al.*, 2013). There are a number of opinions as to which acids are the best causes/indicators of failure; e.g. Wang *et al.* (2009) and Zhang *et al.* (2012) had low biogas production at 900 and 1000 mg/L of propionic acid, whilst Lianhua *et al.* (2010) and Xu *et al.* (2014) suggested acetic acid was more influential. Both the 1/14 and 1/21 produced average VFA levels of over 1000 ppm, mostly acetic and propionic, with 1/21 having an equal volume of butyric acid. This indicates that the

microbial community had reached substrate saturation point and could not progress through complete methanogenesis. However, this might be avoided in prospective applications by identifying microbial 'tipping points' through growth rate analysis and removing a proportion of solids before the system became unproductive.

4.4.2 *Energy implications*

The potential for RS AD was assessed to provide sufficient biogas and electrical power for a rural community where the average household requires 4 kWh/d (World Energy Council [WEC], 2016). As background, the average rice farm in Asia is one hectare, producing approximately 7.5 tonnes of RS per hectare per year (Bouman, 2014; Wiggins and Keats, 2014). Therefore, if one scaled-up the feed rate from 1.0 g VS/L/d to 1.0 t VS/1000 m^3/d, one would require 50 hectares to produce 1.0 tonne of RS per day.

Using data from Table 4.1 and that of Munder *et al.* (2012) and RKB (2016), the average energy content of RS is 15.5 MJ/kg. Converting MJ/kg into kWh at a 3.6:1 ratio provided by Cuéllar and Webber (2008), means that 1 tonne of RS has the potential energy of 4300 kWh. Therefore, using 1 tonne RS per day in an AD unit of 1000 m^3 volume, and CH_4 yields from the OLR1 (and also from Mussoline *et al.* (2013a) and Wu *et al.* (2016)), RS AD-CHP could potentially generate between 400-500 kWh/d (assuming 1 m^3 CH_4 equates to an energy content of 36 MJ and an electrical generating efficiency of 35 % for the CHP system). However, 800 to 1000 kWh/d electricity could be produced by RS AD-CHP at OLR2, assuming low FFs. If this energy were wholly recovered from the RS AD process, the energy yields are similar to average values reported by Mussoline *et al.* (2014) (i.e., 1100 kW/d), and could provide electrical power to 1000 rural households. Conversely, smaller versions of this theoretical system, such as 100 m^3 capacity, may be suitable stepping stones in scaling up the system. This size falls within the range of most small-scale digesters in China, where there are over 30 million AD plants sized 1-150 m^3 (Rajendran *et al.*, 2012). If the potential energy within RS could be released through AD then 100 rural homes would benefit from our method.

Feasibility depends on the costs and impact of RS storage, RS production frequency, the economics of the electricity generation, and the usefulness of heat produced from the CHP system. In a full-scale system, some electrical power and heat would be

used on-site to maintain the digester, as well as providing additional electrical power and heat for local community use. For example, heat can be used locally for crop drying whilst the electricity could be sold or used elsewhere. CHP systems can reach up to 90 % fuel conversion efficiency and could reduce CO_2 emissions from biofuel generation by as much as 10 % by 2030 whilst providing real savings now by reducing the reliance on more expensive power generation (International Energy Agency [IEA], 2008).

As an added benefit, using anaerobic digestate as a fertiliser has been shown by Nguyen and Fricke (2015) to be an effective N, P, and trace metal supplement for soils (FAO, 1992). This 'fertiliser' is organic and aids local farmers in reducing their variable costs, simultaneously mitigating other environmental impacts and increasing self-sufficiency and financial security (Sawatdeenarunat *et al.*, 2016). This was shown as feasible by Luo *et al.* (2016), who reported small-scale digesters (operated by trained farmers) can produce usable biogas for a local community with digestate being used to improve rice yields by ~15 %.

Finally, as the use of waste biomass-derived gases become more economically viable, they will become increasingly important source of useable energy and play an important role in the reduction of GHGs (International Energy Agency [IEA], 2008). AD is not a new process, but the way in which it is harnessed may prove important for remediating these global issues and reaching these energy goals. RS AD will not produce as much gas as other agricultural wastes (per biomass), but due to its massive abundance, it could provide local, national and international benefit if used optimally. However, the scale up of AD is not linear and, as such, any data extrapolated to a larger scale would first require modelling and pilot scale testing.

4.5 Conclusions

RS is abundant and has high carbon content, but its potential as a renewable energy source has been underutilised due to its perceived poor biodegradability and infrequent production cycles. Long-term, CSTR-scale AD experiments were performed to assess the impact of FF and OLR on specific CH_4 yields and biogas volumes. Highest specific CH_4 yields were seen in least frequently fed AD unit at a lower OLR (i.e., 1/21 at 1 g VS/L/d). In contrast, highest volumetric yields were observed with moderately frequent feeding at a higher OLR (i.e., 1/7 at 2 g VS/L/d).

Although both operating options have benefits, low loading with less frequent feeding is probably better in tune with acyclic waste RS production cycles and may be a better option than current practices. In fact, with sufficient storage, infrequently-fed RS AD with CHP has the potential to generate large quantities of renewable heat and electrical power via a simple process, providing other benefits, such as reduced air pollution, limited pretreatment and no co-digestion, and improved environmental quality.

Chapter 5 Effect of Feeding Frequency, Organic Loading Rate and Reactor Failure on Microbial Communities during Rice Straw Anaerobic Digestion

5.1 Introduction

It was shown in the previous Chapter that less frequent rice straw (RS) feeding can produce higher methane yields compared with more frequent feeding when organic loading rates (OLR) are lower. However, at higher OLRs, methane yields reversed versus feeding frequency (FF) patterns, with lower FFs leading to reactor failure, and mid-range FFs performing comparatively well at both lower and higher OLRs. Failed reactors displayed low biogas/methane yields and pH, high VFA and volatile solids (VS) accumulation, but if such operating parameters are related to changes in the microbial communities is not known.

Overall, microbial communities in RS AD are poorly understood, especially under different operating conditions and in the presence of 'shock loads' (Mei *et al.*, 2016b). Some work has been done on RS AD microbial communities, such as Nakakihara *et al.* (2014); Chen *et al.* (2016) and Yan *et al.* (2015), but the range of conditions has been limited and more basic data are needed. Specifically, few studies have assessed the influence of glut versus starve feeding regimes on AD microbial communities, particularly associated with RS. Integral explanations for AD failure also are unknown as it is a biological process built on a web of interlinked interactions at the microbial scale.

Chapter 4 showed VFA and VS accumulation, as a product of infrequent mass loading at extreme feeding frequencies, correlates strongly with reactor failure, indicating that the syntrophic degradation of these acids is a critical step. To determine the effect of these feeding regimes on the bacterial and archaeal communities, 16S rDNA amplicons were analysed. This was used to assess changes in microorganism abundance, in conjunction with physio-chemical data, to identify predominant taxa and potential ecological roles. Identifying predominant microorganisms that are key to system stability and shock recovery could provide

further research into biomarkers, biostimulation, and-or bioaugmentation (Lebuhn *et al.*, 2014; Liu *et al.*, 2017).

To determine the effect of FF and OLR bacterial and archaeal communities, 16S rRNA genes were characterised using Illumina MiSeq to assess how microbial community structure varied in bench reactors with different physio-chemical operating conditions, especially identifying predominant taxa and their potential ecological roles in RS AD. Communities leading to reactor failure at elevated OLRs are particularly highlighted with the goal of identifying microbial shifts that might be useful to foretell failure in RS AD.

5.2 Materials and methods

5.2.1 *Experiment background*

Samples were collected from five continuously stirred tank AD reactors (CSTRs) previously described in Chapter 4. Briefly, five reactors ran at different FFs (i.e., 5 days in 7, 3 days in 7, 1 day in 7, 1 day in 14, and, 1 day in 21) and were labelled '5/7', '3/7', '1/7', '1/14', and, '1/21', respectively. Reactors were maintained for 252 days, including a 112-day period of acclimation followed by 140 days of actual monitoring. Monitoring at an OLR of 1 g VS/L/d was performed for 84 days (OLR1), and for 56 days at an OLR of 2 g VS/L/d (OLR2).

Highest specific methane yields were observed in the 1/21 FF reactor at OLR1 (148 mL CH_4/g VS/d) whilst highest volumetric yields were seen in 1/7 operated at OLR2 (276 mL CH_4/L/d). At OLR2, the 1/14 and 1/21 FF reactors failed in association with high VFA accumulation and decreasing pH.

5.2.2 *Sample collection and preparation*

One original inoculum sample and four samples at each OLR for the five reactors were collected on days 0, 17, 36, & 56 at OLR1, and days 64, 92 130, & 140 at OLR 2, which resulted in 39 samples across the experiment. Due to a rapid decline in performance (souring) only days 64 and 140 were analysed at OLR2 for the 1/21 day reactor. Samples were always collected in triplicate and stored at - 20 °C before further analyses. For each sample, genomic DNA was extracted following the instructions of the FastDNA SPIN Kit for Soil (MP Biomedicals, Carlsbad, CA, USA).

Extracted DNA was quantified using a Qubit 2.0 Fluorometer (Invitrogen) to ensure quantity for analysis and suitability for subsequent sequencing.

5.2.3 *Sequencing analysis*

Analysis of the extracted DNA (concentration of between 1 and 10 ng/µL and a volume of 10-20 µL) was undertaken by LGC Genomics GmbH in Berlin, Germany and briefly consisted of PCR amplification using universal forward primer (U341F) CCTAYGGGRBGCASCAG and universal reverse primer (U806R) GGACTACNNGGGTATCTAAT targeting the V3-V4 16S DNA region (Klindworth *et al.*, 2013). The published protocol consisted of 10 cycles of touchdown PCR (annealing 61 °C - 55 °C, decreasing by 0.6 °C per cycle), followed by 26 standard PCR cycles at an annealing temperature of 55 °C. Quality control (agarose gel check), library preparation including tagging, equimolar mixing and clean-up was completed. 16S rDNA amplicon sequencing was then performed on Illumina MiSeq V3 (2 x 300 bp).

Bioinformatics analysis was undertaken and consisted of inline barcode demultiplexing, adaptor clipping, and amplicon pre-processing using Mothur (Schloss *et al.*, 2009): pair joining, filtering, alignment against Silva (128) 16S, subsampling 5,000 - 25,000 reads per sample, denoising and chimera removal. OTU picking used Mothur: clustering aligned sequences at 97 % identity. Further details can be found in Appendix B. Additional OTU analysis was undertaken to confirm and complement the work of LGC Genomics, including assignment of taxonomy on the Greengenes database (version 13_8). Predominant OTUs were defined as having ≥ 0.5 % abundance in any sample. Phylogenetic analysis of predominant OTUs was performed with the ARB programme (Ludwig *et al.*, 2004), using the neighbour-joining and parsimony methods with 1,000 bootstrap replication (McDonald *et al.*, 2012; Kuroda *et al.*, 2016).

5.2.4 *Statistical analysis*

Alpha diversity and beta diversity based on weighted UniFrac distances were calculated in QIIME. PRIMER 7 (PRIMER-E, Plymouth, UK) was used for principal component analysis (PCA), and metric-multidimensional scaling (MDS), permutational analysis of variance (PERMANOVA) with 1,000 permutations, analysis of similarities (ANOSIM), RELATE, BEST and DistLM (distance-based linear model)

of the weighted UniFrac distances (even sampling at 12,069 reads) using Bray-Curtis after square-root transformation, and physiochemical data (Ling *et al.*, 2016a; Mei *et al.*, 2016b). Observed OTUs, Chao1, and, Simpson's and Shannon's Indexes were plotted and compared using ANOVA (analysis of variance) with Tukey comparison in Minitab 17 (Version 17.1.0). Group significant differences were compared using STAMP v2.1.3 and the two sample *t*-test. Significance was always defined as 95 % confidence in differences (i.e., $p < 0.05$).

5.3 Results and Discussion

5.3.1 *Impact of FF and OLR on beta-diversity and physio-chemical parameters*

The clustering analysis based on Bray-Curtis distance indicated that samples did not cluster based on FF (Figure 5.1a) or OLR (Figure 5.1b) but grouped into 'healthy' and unhealthy or 'sour' reactor points (3/7 d130 as an outlier). In Figure 5.1b, OLR1 samples were all 'healthy' whilst OLR2 was split between 'healthy' and 'sour', denoted as 'OLR2-H' and 'OLR2-S', respectively. The influence of physiochemical variables on microbial community was represented by direction and length of corresponding arrows. VFA and VS build up related to OLR2-S samples whilst all other OLR1 and OLR2-H samples were related to increased biogas production and pH value. This infers that physiochemical differences between reactor conditions were strongest when reactors soured due to OLR rather than FF.

Clustering analysis based on weighted UniFrac distances (Figure 5.1c) grouped the different OLR conditions into three. OLR1 samples related to higher pH and biogas production, OLR2-S reactors were correlated to higher VFA and VS, and OLR2-H reactors fell between them. The higher levels of individual and total VFAs for OLR1, OLR2-H and, OLR2-S in Figure 5.1d support the observations in Figures 5.1a-c that increasing VFAs and decreasing pH is associated with OLR2-S. In addition, all day-64 samples did not conform to these groups, i.e., OLR2-H day-64 samples grouped with OLR1 and 1/21 day-64 samples grouped with OLR2-H samples. In progressing from OLR1 to OLR2 there were a number of stages in the microbial community i.e., from OLR1 came transition (OLR2-T: day 64 for all FF), healthy (OLR2-H, day 92 - 130 for 5/7, 3/7, and 1/7, as well as day 92 at 1/14) and, sour (OLR2-S, day 130 and 140 for 1/14 with day 140 of 1/21).

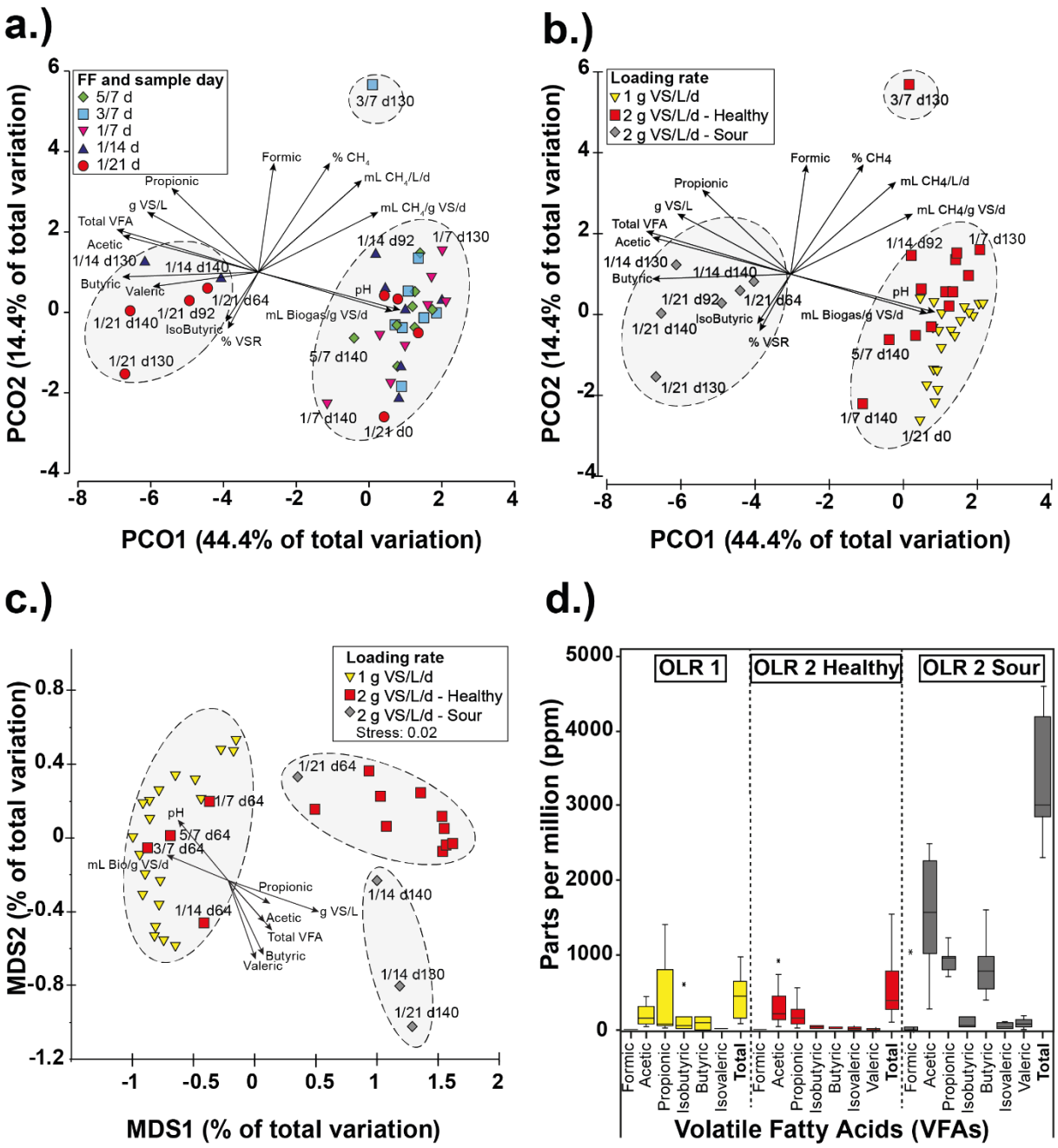

Figure 5.1*:* Analyses of beta diversity showing variation of microbial community structure and the influence of physiochemical data. **a.)** and **b.)** are same figures based on PCA of Bray-Curtis distance, but coloured differently by FF or OLR. **c.)** MDS of weighted UniFrac distance and has two 1/21d samples fewer than a/b. **d.)** boxplot of individual and total VFAs for OLR1, OLR2-H and OLR2-S (there was no valeric acid in OLR1). Physiochemical data overlaid arrows and dashed elliptical shapes indicate sample groupings).

To statistically examine the effects of FF and OLR on RS AD beta-diversity and to determine any correlations with the physiochemical data, RELATE, BEST, DistLM,

ANOSIM, and PERMANOVA were calculated (Table 5.1). Influence of physiochemical data on the beta-diversity was reflected by a significant (0.1 %) 0.40 correlation with VS and pH (RELATE), followed by butyric acid, which BEST related to the microbial community structure (BEST, R = 0.77; DistLM, *p* = 0.001). ANOSIM and PERMANOVA analysis showed that FF was not significant, whereas OLR was shown as significant at 0.02% and *p* = 0.001, respectively.

Table 5.1: Test statistics of beta diversity and physiochemical variables and operational factors

Method [a]: RELATE		
Variable	**Significance (%)**	**Rho.**
Physiochemical data	**0.1 [b]**	0.403
Method: BEST		
Variable	**Physiochemical Correlation (R)**	
g VS/L	0.772	
+ pH	0.768	
Method: DistLM		
Variable	**p-value**	**Cumulative variance explained (%)**
g VS/L	**0.001**	63.9
+ pH	**0.001**	72.7
+ Butyric acid	**0.001**	73.8
Method: ANOSIM		
Factor	**Global R**	**Significance level (%)**
Feeding Frequency	- 0.055	75.5
Organic Loading Rate	0.532	**0.02**
Method: PERMANOVA		
Factor	**p-value**	**Sq.root of estimates of component of variation**
Feeding Frequency	0.823	- 2.08
Organic Loading Rate	**0.001**	11.0
FF x OLR	0.959	- 3.58

Notes: [a] RELATE, giving correlation of comparisons (Rho); BEST, trend correlation; DistLM, distance based linear model; ANOSIM, analysis of similarities; PERMANOVA, permutational multivariate analysis of variance.
[b]Bold indicates statistically significant results

5.3.2 *Impact of OLR and reactor souring on alpha-diversity*

A comparison of α-diversity indices was undertaken to determine differences in community richness and evenness, and it was found that there were no differences among FFs within each OLR, which was consistent with Colwell and Coddington (1994); Hughes *et al.* (2001) and Lemos *et al.* (2011). However, there were significant differences between observed OTUs and Chao1 estimations for OLR1 and those of OLR2-H and OLR2-S (p = <0.001). This was not the case between OLR1 and OLR2-T (p = 0.635 & p = 0.323), indicating OLR2-H and OLR2-S were less rich than at OLR1 (Figure 5.2a). Such differences suggest the observed OTUs outweighed the number of single OTUs among conditions.

A Simpson's index score was calculated and shown in Figure 5.2b to compare evenness. OLR1 had greater diversity than OLR2-H and OLR2-S (p = <0.001), as did OLR2-T (p < 0.001). Within OLR1, there also were differences with 1/21 FF being significantly less diverse than 3/7 (p = 0.013) or 1/7 d (p = 0.024). It had been hypothesized that the less frequently fed reactors would have a lower diversity as infrequent loading would select for K-strategist community. For example, there may be acetic acid tolerant species within *Methanosarcina*, as this can be a scavenging genus capable of withstanding environments more hostile to others, including *Methanosaeta* (Conklin *et al.*, 2006). It has been shown that *Methanosarcina* can dominate Archaea at once per day feeding and when hydrogenotrophic methanogenesis is required (Conklin *et al.*, 2006). It is also apparent that OLR2-H and OLR2-S had lower OTU richness and evenness, probably resulting from higher loadings, VFA levels, a pH decrease, and comparatively poor reactor performance. Richness and evenness has been seen by Wittebolle *et al.* (2009) as essential for a 'happy' AD system with evenness providing a greater buffer in high stress situations, such as an increased organic loading. It is possible with more time, all reactors at 2.0 g VS/L/d would sour. In this case, the traditional markers of reactor stability e.g. pH, did not react quickly enough, whereas, the effect of FF and community changes such as richness and unevenness would play more significant roles. Population shifts such as increases in fermenters and decreases in methanogens could therefore be used as an early warning of forthcoming system instability.

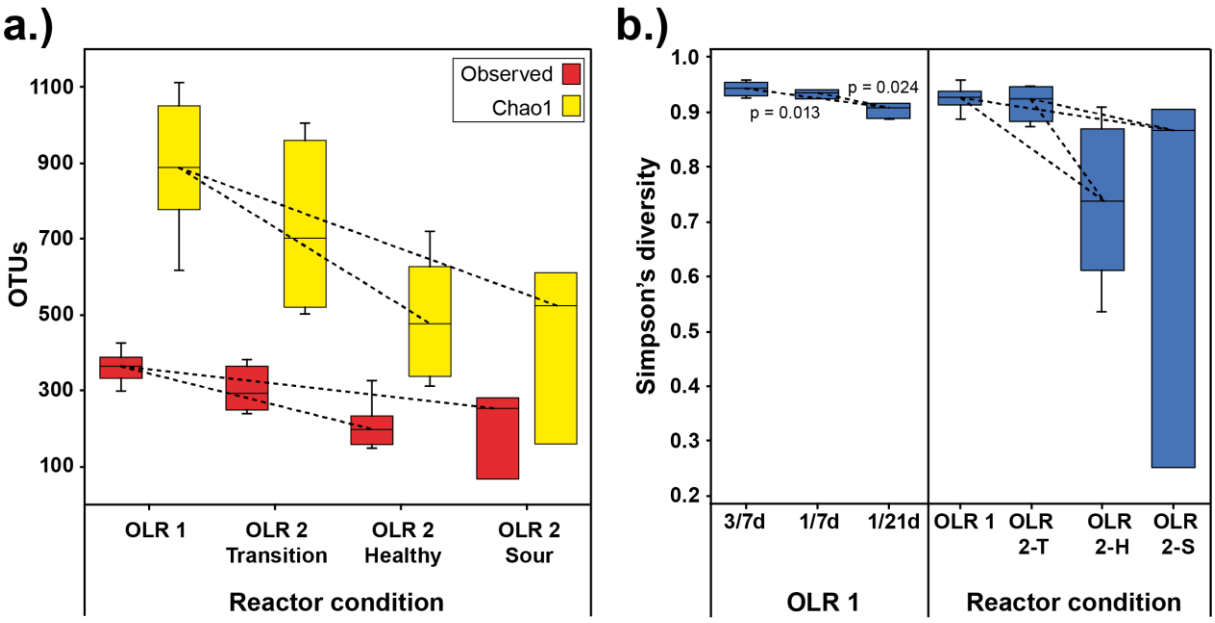

Figure 5.2: Boxplots between, **a.)** Observed OTUs (lower) and Chao1 (upper boxes), and, **b.)** Simpson's index scores at OLR1 FFs that showed significant differences (left) and the mean scores of OLR1, OLR2-H and, OLR2-S. Dashed lines and *p*-value indicate 2-sample t-test statistical significance between linked samples.

5.3.3 *Predominant OTUs*

Predominant OTUs (≥ 0.5 % relative abundance, 60 OTUs) are shown as Figure 5.3, Figure 5.4 (to illustrate the differences between operating conditions), and Figure 5.5 to assess whether any perceived changes among conditions were statistically significant. The 60 OTUs are summarised in Appendix B as a phylogenetic tree and OTU table to the genus level where possible.

All samples at OLR1, regardless of FF, were similar in community composition and mostly consisted of *Bacteroidetes* and *Firmicutes,* previously noted in straw digestion by Heeg *et al.* (2014)*,* then *Actinobacteria, and Euryarchaeota*, which has been previously noted in mesophilic reactors by Heeg *et al.* (2014). Day 64 samples (transition, 'OLR2-T') had similar predominant OTUs, after which OLR2-H became dominated by *Bacteroidetes, Actinobacteria* and *Firmicutes,* whereas OLR2-S became dominated by *Firmicutes*.

Overall, the number of predominant OTUs in OLR1 (30) declined slightly into OLR2-T to 25 OTUs; however, the largest drop was to OLR2-H and OLR2-S, 17 and 18 OTUs, respectively. Phyla in OLR1 and OLR2-T were similar with a relatively even

spread of OTUs between *Bacteroidetes, Actinobacteria, Firmicutes*, and, *Euryarchaeota*, with abundances not exceeding ~ 17 %. That the number of predominant OTUs in OLR2-H and OLR2-S was lower than OLR1 and OLR2-T infers that differences in OTU presence and abundance at OLR2-H and OLR-S was needed for optimal reactor performance. Relative abundances of individual OTUs in OLR2-H and OLR2-S increased with highs of 44 % and 52 %, respectively, whilst the number of OTUs almost halved. Missing taxa were generally from *Bacteroidetes* and *Actinobacteria* with an increase in *Firmicutes*, particularly in OLR2-S.

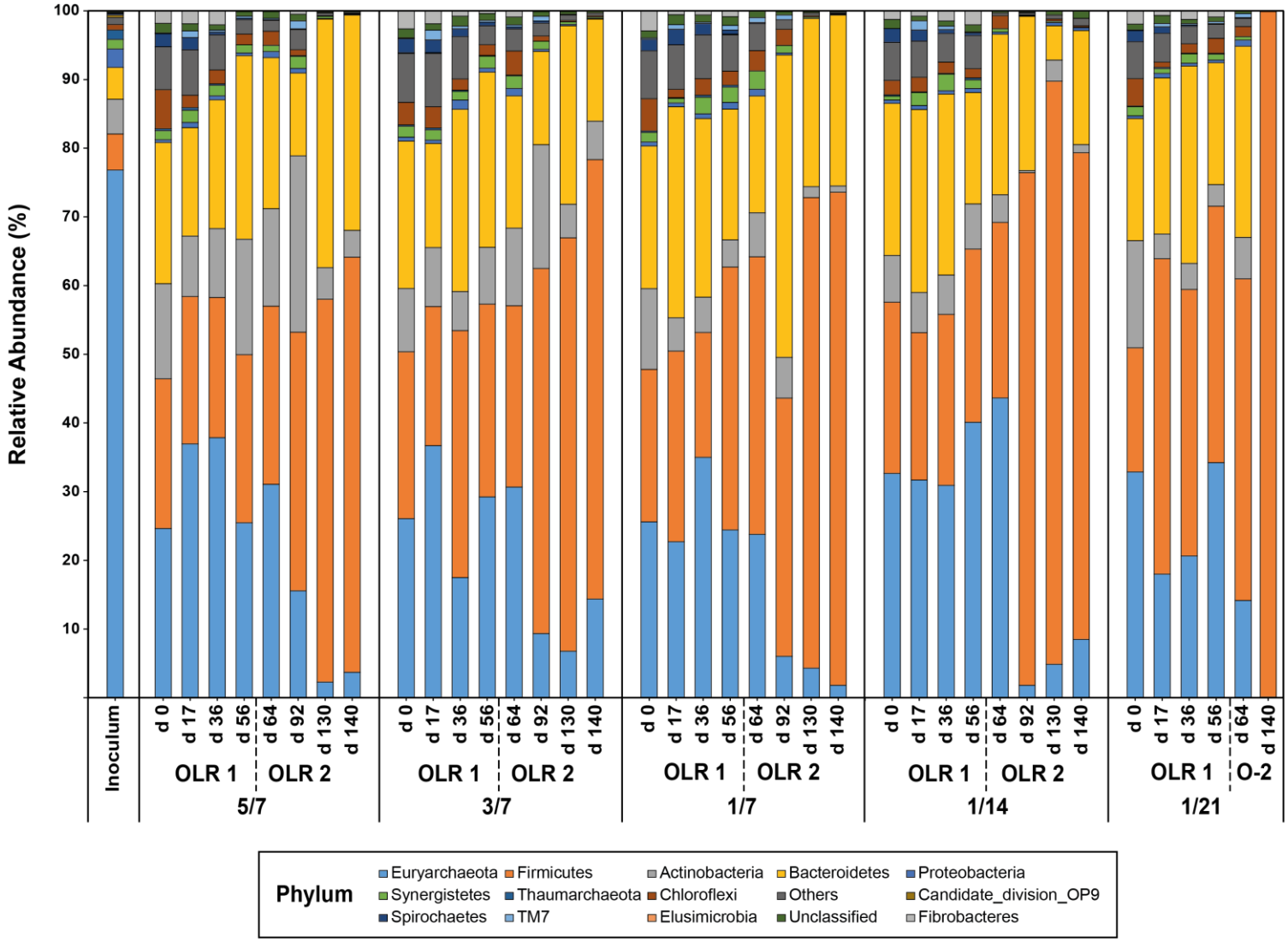

Figure 5.3*:* Microbial composition at phylum level. Each section represents initial inoculum, FF (5/7, 3/7, 1/7, 1/14, and 1/21) across time with each split into OLR1 and OLR2.

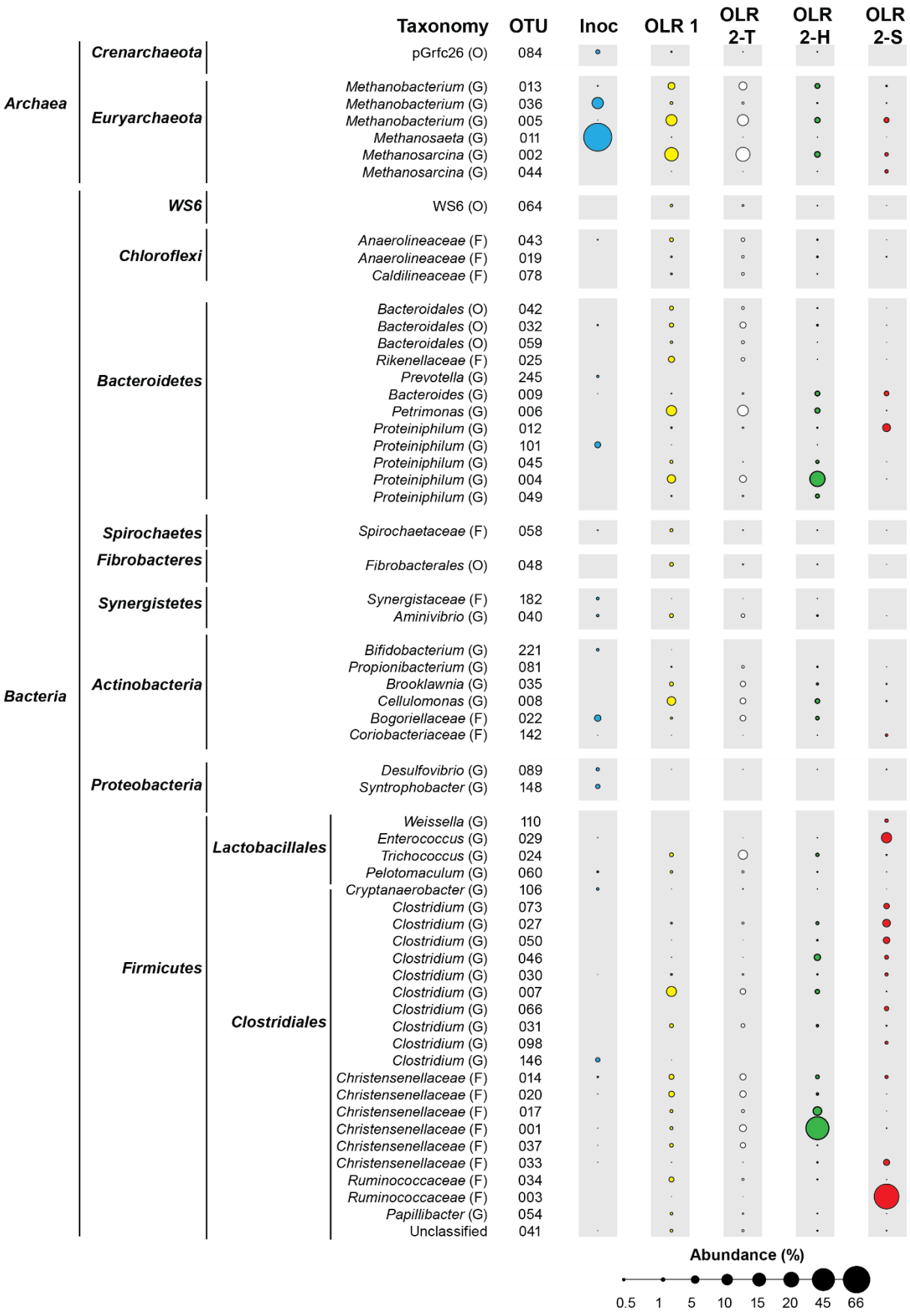

Figure 5.4*:* Predominant OTUs (≥ 0.5 % abundance) grouped based on ARB phylogenetic tree construction to genus where possible for OLR1, OLR2-T, OLR2-H, and, OLR2-S. Area of bubbles represents relative abundance. 'Inoc' = inoculum

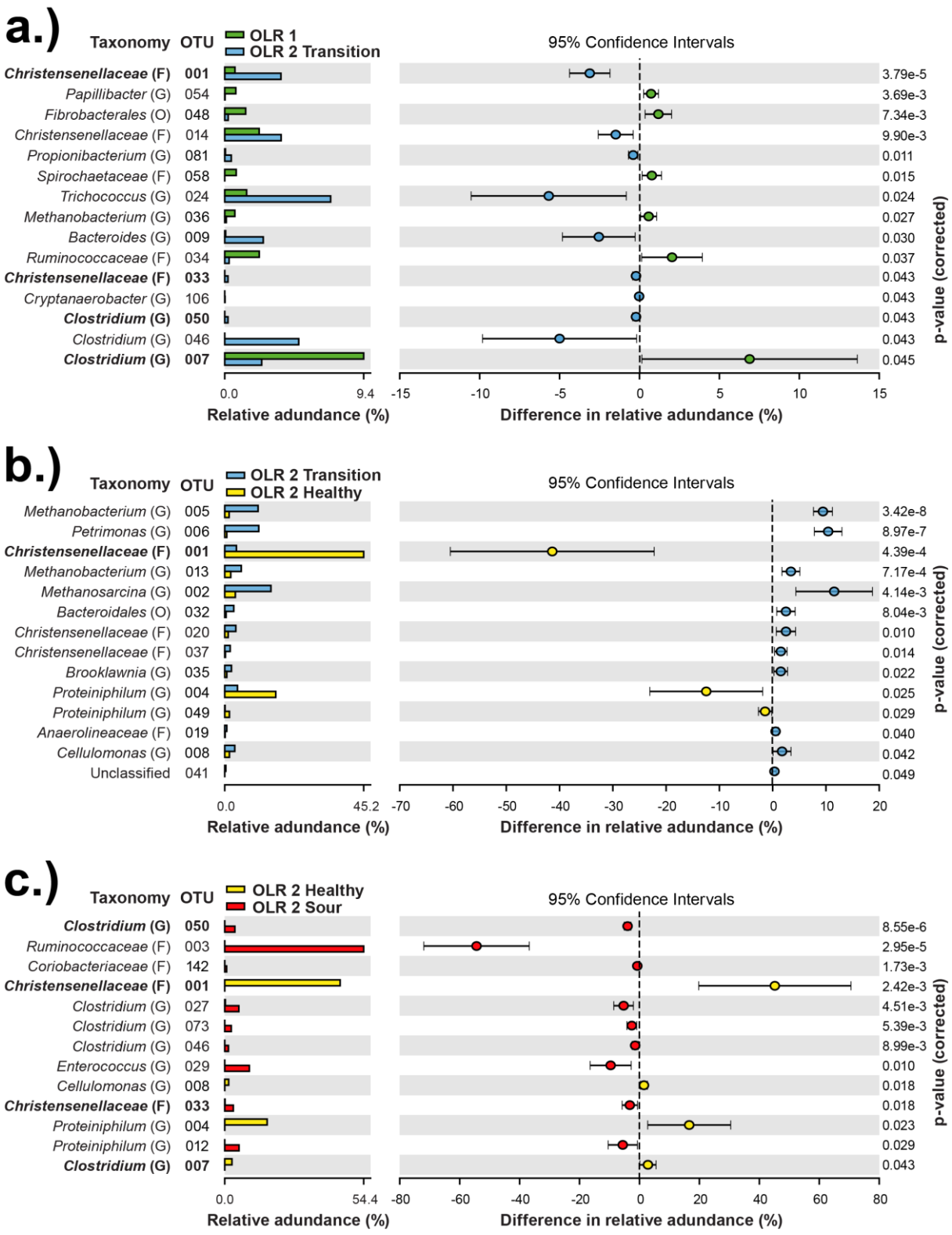

Figure 5.5: Extended error bar plot showing predominant OTUs that have significantly different abundances between organic loading conditions, **a.)** OLR1 and OLR2-T, **b.)** OLR2-T and OLR2-H, and, **c.)** OLR2-H and OLR2-S. Only OTUs with ≥ 0.5 % abundance are shown and bold type indicates OTUs that appear in more than one panel.

Although overall OTU presence and relative abundance decreased, methanogens were present under all operating conditions. *Methanobacterium* remained steady through OLR1 (4.0 and 10.4 %) and OLR2-T (5.2 and 10.3 %) before dramatically reducing in apparent abundance in OLR2-H (1.8 and 2.2 % at p = 0.0017) and OLR2-S (0.1 and 1.1 %). *Methanosarcina* followed the same general pattern, decreasing significantly over time from 15.2 and 16.6 % to 2.6 % (p = 0.004) and 1.0 %. There may be acetic acid tolerant species within *Methanosarcina*, as this can be a scavenging genus capable of withstanding environments more hostile to others, including *Methanoseata* (Conklin *et al.*, 2006). It has been shown that *Methanosarcina* can dominate Archaea at once per day feeding and when hydrogenotrophic methanogenesis is required (Conklin *et al.*, 2006). That *Methanobacterium* was highly abundant among the shared OTUs was unexpected as this was previously observed under thermophilic operation, potentially due to an increase in H_2 partial pressure (Goberna *et al.* (2010); Sun *et al.* (2015)).

The lack of obligate syntrophic bacteria, such as *Syntrophobacter* was unexpected, as Mei *et al.* (2017) has shown these are integral to 'optimal' AD. However, strict *Firmicutes* anaerobes such as *Bacilli* and *Clostridia* include facultative syntrophs, such as *Syntrophomonas* (Morris *et al.*, 2013), *Clostridium ultunense* (Li *et al.*, 2015c), *Ruminococcus albus* (Stams and Plugge, 2009), and *Desulfovibrio* (Saia *et al.*, 2016). As theorised in our original study, and also noted by Tait *et al.* (2009) and Franke-Whittle *et al.* (2014), the accumulation of VFAs, propionate, butyrate, and particularly, acetate, under OLR2 conditions imply acid production by syntrophs outweighed the conversion rates by methanogens, eventually overwhelming the system.

There were multiple changes in OTU abundances between OLR stages with a number being statistically significant. *Rikenellaceae* (OTU 025) decreased from OLR1 to OLR2-T and *Bacteroides* (OTU 009) increased from OLR2-T to OLR2-S, but not significantly (3.0 – 1.0 % and 0.2 - 1.8 %). *Rikenellaceae* are mostly found in faecal samples (Graf, 2014), therefore the decline here is not surprising given the original inoculum would likely have contained faecal matter. Their decrease indicates that they are being selected against, as the sludge moved from faecal to RS substrate. As an order, *Bacteroidales* are very common, hydrolysing bacteria in anaerobic digesters fermenting carbohydrates (Ju *et al.*, 2017). *Bacteroides cellusolvens* growth is supported by cellulose and cellobiose (Lin *et al.*, 1994), whilst

Bacteroides xylanolyticus uses cellobiose but not hemicellulose or cellulose (Scholten-Koerselman *et al.*, 1986). The *Bacteroidetes* phylum can dominate cellulose and hemicellulose degradation (van der Lelie *et al.*, 2012; St-Pierre and Wright, 2014), as found by Ziganshina *et al.* (2015) in the AD of dried grains, and by Sun *et al.* (2015) when digesting RS. Within this, *Petrimonas* (OTU 006) abundance decreased significantly from OLR2-T (9.5 %) to OLR2-H (2.2 %, $p = 0.03$), and then again in OLR2-S (0.0 %, $p = 0.00$). Whilst, OTU 004 and OTU 049, associated with *Proteiniphilum*, increased significantly from OLR2-T to OLR2-H, 3.9 - 19.6 % ($p = 0.02$) to 0.2 - 1.2 % ($p = 0.03$), respectively, before rapidly decreasing in OLR2-S (0.0 %). These fermenters almost wholly produce acetate and H_2, however, *Petrimonas* does not use cellulose (Grabowski *et al.*, 2005), and *Proteiniphilum* does not utilise cellobiose or cellulose (Chen and Dong, 2005), so it is logical they declined with a highly cellulosic substrate.

Spirochaetaceae (OTU 058) and *Fibrobacterales* (OTU 048) had low abundance, but declined significantly from OLR1 to OLR2-T (0.8 - 0.1 % to 1.2 - 0.2 % at $p = 0.015$ and 0.007). *Spirochaetaceae* are facultative anaerobes with few cultivated species (Paster, 2015), whilst *Fibrobacterales* utilise carbohydrates and are capable of growth on cellulose, but they are understudied (Spain *et al.*, 2015). The *Synergistes* phylum has previously been noted as lignocellulosic-degrading systems and can increase with increasing OLR (Leite *et al.*, 2016). Here, *Synergistaceae* (OTU 182) was present in the inoculum (0.7 %), but declined to 0.0 % during the acclimation phase (pre-OLR1). Whereas, *Aminivibrio* (OTU 040) was stable at 1.1 - 1.2 % through to OLR2-T before decreasing significantly to 0.2 and 0.0 % ($p = 0.069$) through OLR2-H to OLR2-S. The *Aminivibrio pyruvatiphilus* species prefers a pH of 6.4 - 8.4 (Honda *et al.*, 2013) and as there was a glut of acid at high loading, particularly OLR2-S, it could have caused its decline.

Firmicutes contain syntrophic bacteria that can degrade a range of fatty acids to produce H_2, which is further degraded by the methanogens (Rivière *et al.*, 2009) as well as by hydrolysers and fermenters that can utilise straw (Qiao *et al.*, 2013). *Pelotomaculum* (OTU 060) and *Papillibacter* (OTU 054) both declined from 0.5 and 0.7 % in OLR1 to 0.4 % in OLR2-T to 0.0 and 0.1 % by OLR2-S, which was expected for *Papillibacter* as it is not known to grow well on carbohydrates, but only on aromatics. *Pelotomaculum* has previously been shown as a syntrophic degrader in conjunction with *Methanosaeta* (Kuroda *et al.*, 2016), the abundance of which also

declined across conditions. As a cellulolytic bacteria, *Cellulomonas* (OTU 008) increased during acclimation to OLR1 (to 6.1 %), but significantly decreased to 2.8 % (p = 0.042) when the load doubled to OLR2, it stabilised at 1.7 % in OLR2-H, before declining to 0.1 % in OLR2-S (p = 0.018). That *Cellulomonas* declined here was unexpected given the number of cellulolytic and ligninolytic species in this genus that are associated with rice pant degradation (Akasaka *et al.*, 2003; Ventorino *et al.*, 2015).

There were ten OTUs associated with *Clostridium*. Among them, OTU 007 and 031 decreased significantly from OLR1 to OLR2-T (8.4 - 2.6 %, p = 0.045) before dropping again into OLR2-S (p = 0.043). It is possible that these OTUs represent species that utilise cellobiose but not cellulose, such as *Clostridium grantii* (Mountfort *et al.*, 1994), or species that cannot use either; e.g. *Clostridium paradoxum* (Li *et al.*, 1993). The remaining eight *Clostridium* OTUs all increased in OLR2-S and are discussed shortly. OTUs 014, 020 and 037 of the *Christensenellaceae* family showed significant increase from OLR1 (2.1, 2.7, and 1.1 %) to OLR2-T (3.2, 3.5, and 2.2 %) before decreasing in OLR2-H (1.1, 0.4, and 0.0 %). However, OTU 001 and 017 increased from 0.7 and 3.9 % in OLR2-T to 6.1 and 43.7 % in OLR2-H respectively, (p = <0.001 and 0.079) before an equally dramatic decrease to 0.0 % in OLR2-S (p = 0.002 and 0.134). There is currently only one described species of *Christensenellaceae*, *Christensenella minuta*, which was found in the human gut (Morotomi *et al.*, 2012; Rosa *et al.*, 2017), so it logically declined in RS AD.

There were two OTUs assigned to the *Ruminococcaceae* family, of which OTU 034 decreased from OLR1 (2.1 %) to OLR2-T (0.4 %, p = 0.034) then to 0.0 % in OLR2-S, whilst OTU 003 (discussed later) significantly increased from 0.0 % throughout to 52 % in OLR2-S (p = <0.001). This was unexpected as this family ferments cellulose to produce hydrogen (Tian *et al.*, 2014).

5.3.4 *Thriving OTUs*

There were also some OTUs that either only appeared in OLR2-S or thrived under this condition. These were associated with *Proteiniphilum*, *Clostridium*, *Christensenellaceae*, *Coriobacteriaceae*, *Weissella*, *Ruminococcaceae*, and *Enterococcus*.

Contrary to declines discussed earlier, *Proteiniphilum* (OTU 012) and *Christensenellaceae* (OTU 033) significantly increased from OLR2-H into OLR2-S from 0.1 % to 5.1 and 3.1 %, respectively, (p = 0.029 and 0.018). *Proteiniphilum* are fermenting bacteria capable of producing acetic and propionic acid, which fits with the increase in acids in this latter condition (Whitman *et al.*, 2015), but they do not use cellulose. *Clostridium* (OTU 050, 073, and, 027) increased significantly from OLR2-H (0.0, 0.7 and, 0.1 %) into OLR2-S (2.5, 4.9, and 3.6 % at p = ≤ 0.005), whilst OTUs 030, 098, 031 and 066 showed small increases of ≤ 1.6 %. It has been found by Zhao *et al.* (2012) that many of the *Clostridia* class ferment butyrate to produce acetate. Evidence of this acid production can be seen in the increase of acetic and butyric acids in OLR2-S, which were more than double the levels seen in OLR2-H.

The *Clostridiales* order, which includes *Christensenellaceae,* has been shown to degrade cellulose by Fontes and Gilbert (2010); Zverlov *et al.* (2010) and Ziganshina *et al.* (2015), which can explain their increase in conjunction with higher RS loading. The anaerobic cellulolytic ability of the microbial community is generally through *Clostridia* though it contains few species that can directly degrade cellulose. However, it has been found that some *Clostridium* species (e.g. *Clostridium clariflavum*, *C. thermosuccinogenes*, and *Clostridium thermocellum*) can use cellulose and-or cellobiose to produce acetate and formate (Li *et al.*, 2011; Lebuhn *et al.*, 2014; Lü *et al.*, 2014), with cellobiose fermentation occurring more rapidly than cellulose processing (Weimer and Zeikus, 1977). *Clostridium cellulovorans* is a specialist cellulose-degrader that can be limited by the amount of lignin in a substrate through lack of ligninolytic activity (Valdez-Vazquez *et al.*, 2015). However, contrary to OLR2-S, Lebuhn *et al.* (2014) showed that low pH and high VFA can constrain their growth.

The facultative anaerobes *Coriobacteriaceae* (family), *Weissella* (genus), which both increased into OLR2-S from 0 to 0.6 % and 1.0 %, *Ruminococcaceae* (family) and, *Enterococcus* (genus), significantly increased from 0.0 % to 52 % and 8.9 %. *Ruminococcaceae* are known for breaking down carbohydrates in the intestinal system, but this family also contains a large amount of acetogenic species that degrade cellulosic products, such as *Acetivibrio cellulolyticus* (Dassa *et al.*, 2012). The genus *Ruminococcus* has previously been noted to hydrolyse cellulose in the rumen (Sun *et al.*, 2015), whilst the species *Ruminococcus flavefacians* and *Ruminococcus champanellensis* can utilise cellobiose as well as cellulose (Sun *et al.*,

2013), which could explain the huge increase in the OLR2-S samples. In these samples, where high loading was coupled with failure, there was likely to have been the greatest level of substrate available for this family. *Enterococcus* has a number of species that could thrive at high organic loading, cellulose environments (Valdez-Vazquez *et al.*, 2015). *Enterococcus faecium* was found to increase fermentation rate and acid production from lignocellulosic substrate (Pang *et al.*, 2014) whilst *Enterococcus saccharolyticus* was found during silage fermentation that degrade cellobiose and decrease pH (Kuikui *et al.*, 2014), and, *Enterococcus saccharolyticus* and *Enterococcus gallinarum* were found producing H_2 in a microbial consortium composting cellobiose (Adav *et al.*, 2009).

The, sometimes, extreme increase in relative abundance of some *Clostridium*, *Ruminococcaceae*, *Enterococcus*, and, *Weissella* here, suggests that they thrived on the increase in cellulosic substrate, the RS, whilst other *Bacteroidetes*, *Actinobacteria*, and *Synergistetes* decreased. Vartoukian *et al.* (2007) wrote that *Synergistetes* are adaptable to high acid situations, though generally at low abundances, so their decrease into OLR2-S may be due to preference for lactic acid rather than acetate (Delbès *et al.*, 2001). Increasing *Firmicutes* and decreasing *Bacteroidetes* in the OLR2-S samples indicate that, although these are both cellulose utilising bacteria, *Firmicutes* outcompetes and may cope better with more extreme conditions.

5.3.5 *Shared-core OTUs*

Identifying the predominant OTUs that were shared between loading conditions is shown as a network in Figure 5.6 and enabled us to characterise those that provided an important function within the reactors, as did Rui *et al.* (2015a); Ling *et al.* (2016a); St-Pierre and Wright (2014) and Mei *et al.* (2016a). For example, OTU 002 (*Methanosarcina*), 005 (*Methanobacterium*), 014 (*Christensenellaceae*), and 022 (*Bogoriellaceae*) were found in all samples, and thus were likely to play an important role in the process compared to others only found in healthy reactors.

OTUs that were shared with OLR2-S were mostly *Firmicutes* (3), with two *Euryarchaeota* and with only one *Bacteroidetes*, whereas those shared between healthy loading conditions were more even, *Firmicutes* (6), *Euryarchaeota* (5), *Bacteroidetes* (4), *Actinobacteria* (2), and *Synergistetes* (1). A similar core

composition was found by Nelson *et al.* (2011), and shared OTUs were relatively low in number, but contained many of the high abundance OTUs (Ling *et al.*, 2016b); e.g., OTU 001.

The microbial community dynamics, and the predominant OTUs were slow to react to loading change and were not significantly affected by FF, however, an increase in fermentation acidic products at OLR2 resulted in the ‘souring’ of the high FF reactors. Described as OLR2-T and OLR2-H it is possible that with more time, all reactors at 2 g VS/L/d would sour and the effect of FF, richness and unevenness would play more significant roles (Wittebolle *et al.*, 2009).

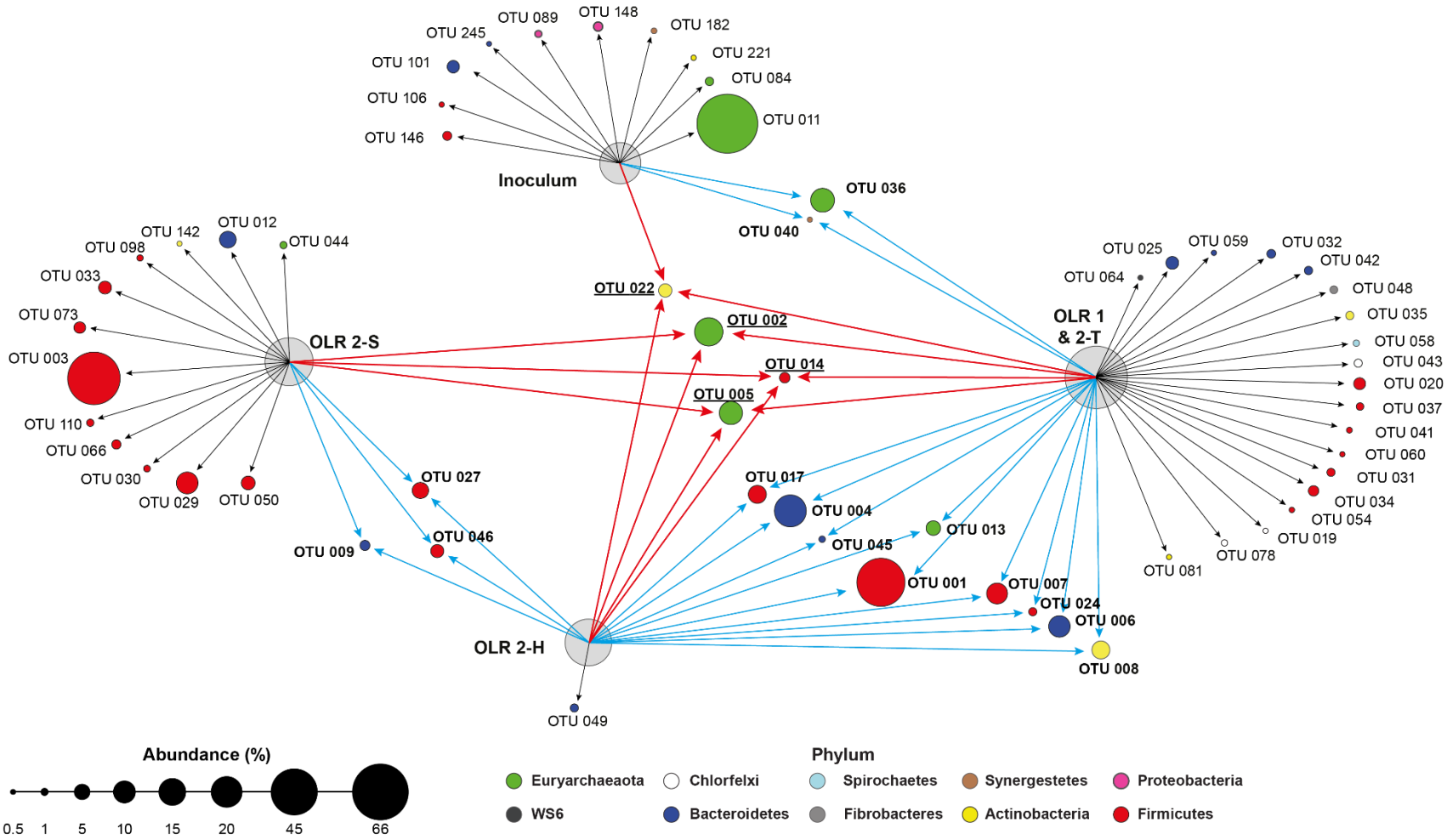

Figure 5.6: Shared predominant OTUs (only ≥ 0.5 % abundance) based on sample appearances i.e. in OLR1, OLR2-T, OLR2-H, and/or, OLR2-S. Area of bubbles represents relative abundance and in the case of shared OTUs, closeness to an OLR 'hub' indicates higher abundance e.g. OTU 001 is nearer to OLR2-H than OLR1. Bold indicates OTUs shared by two conditions and bold/underlined indicates OTUs shared by three conditions

5.4 Conclusion

Microbial community dynamics and predominant OTUs were slow to react associated with changes in OLR and were not significantly affected by FF. However, an increase in fermentation acidic products at OLR2 resulted in the 'souring' of the higher OLR FF reactors, denoted as OLR2-T and OLR2-H.

There were clear community differences between low and high loading, as well as transitional and 'sour' (failed) reactors. When OLR was increased, the microbial community appeared to become more reliant on fermenters, such as *Clostridia* and *Christensenellaceae*. This community shift was also seen as reactors transitioned from 'healthy' to 'sour' whilst others, such as *Methanosarcina* decreased. It is possible that with more time, all reactors at 2 g VS/L/d would sour. In this case the traditional markers of reactor stability e.g. pH, would not react quickly enough. Whereas, community changes, such as richness and unevenness, and population shifts, such as increases in fermenters and decreases in methanogens reacted early and could therefore be used as an early warning of forthcoming system instability.

As 16S taxonomic assignments are not based on full sequences they should be interpreted carefully, though this is an excellent window to determine further, deeper sequencing analysis. The data here also suggests that by specific bacterial group augmentation, such as adding populations shared by the healthy reactors, could process higher levels of acid, although this needs to be proven. Alternately, optimising operating procedures to better control acid production, could provide additional benefit to RS AD.

Overall, this experiment shows that OLR rather than FF most strongly impacts RS AD microbial community composition and diversity. However, this work has broader implications to operating any AD unit with a less degradable substrate. We show that infrequent feeding does not negatively impact that core microbial community as long as OLR is moderately low, which explains why higher specific CH_4 yields are seen in low FF units; i.e., the substrate (in this case RS) dictates the community, not the feeding regime. The idea of intentionally feeding a biological treatment unit less frequently goes against traditional views, but if the substrate is less degradable, infrequent feeding can improve performance. We visualise this effect as like providing a slow-release drug to a patient where release rate is designed to match

biological need for the drug. Therefore, analogously, feeding RS or any less degradable substrate to an AD unit might also benefit from infrequent feeding. This now needs to be examined with other “less degradable” substrates to verify that low FF may be the better strategy for any AD application like rice straw.

Chapter 6 Effect on the Microbial Community of Co-digesting Rice Straw Co-digestion with Dairy Manure

6.1 Introduction

In the previous chapters, a range of conditions that impact RS AD were evaluated, including feeding frequency (FF) and organic loading rate (OLR). However, the lignocellulosic structure of RS makes it recalcitrant to digestion and the production rates/methane yields were sometimes quite low (Chapters 3 - 5). There are a number of potential strategies to improve RS AD, for example, RS pretreatment or supplementation using mechanical, biological, or chemical means, as well as alternate reactor configurations (Ward *et al.*, 2008; Fernandes *et al.*, 2009; Mussoline *et al.*, 2013a). However, no options have been defined as the 'best' solution (Sun *et al.*, 2015).

As the overall goal of this project was to determine if RS AD was feasible without expensive and-or technically difficult methods, co-digestion to adjust C:N ratios was the next experiment. Although co-digestion was assessed in BMP tests reported in Chapter 3, the results were contrary to much of the literature, which suggests that the addition of manure to balance C:N ratio would improve gas yields (Li *et al.*, 2014a; Mussoline *et al.*, 2014; Li *et al.*, 2015a). However, many co-digestion studies, including Estevez *et al.* (2012) and Xavier *et al.* (2015) did not compare RS as a sole substrate alongside co-digestion units so it is difficult to evaluate results here against the literature.

Therefore, this Chapter investigated the effects of co-digestion of RS with dairy manure (DM) to assess the value of altered C:N ratios. Four reactors similar to Chapter 4 were fed different ratios of RS:DM to determine if the BMP results of Chapter 3 would be replicated, or if the continuously-fed systems behaved differently. The reactors were monitored for the same physio-chemical conditions and microbial communities were compared using 16S rDNA amplicon sequences to assess the effect of DM additions on AD performance and also the abundance and predominance of eubacterial and methanogenic organisms. Note that a 50 day HRT in Chapter 4 was not feasible here, therefore a lower HRT of 25 days was assessed.

(Yadvika *et al.*, 2004) reported long HRTs require larger digesters, and cost, whilst very short HRTs may cause bacterial washout. HRTs as low as 15 - 30 days have been shown to work for agricultural residue AD by Jarvis *et al.* (1997); Alkaya *et al.* (2010) and Babaee *et al.* (2013). Although decreases in methane yield were reported, the potential of smaller reactors was considered beneficial enough to test a 25 day HRT in the DM study. Data were compared with that from Chapter 4.

6.2 Materials and methods

6.2.1 *Experimental conditions and analyses*

Four 2.5 L CSTRs were set-up with 2.0 L working volume using inoculum, RS milled to 1.0 mm, and dairy manure (DM) previously characterised and described in Chapters 3 and 4.The reactors operated with a HRT of 25 days for 150 days, of which 70 days were used to acclimate to the addition of manure by which time, pH and VFAs had become stable. At the end of acclimation, time was defined as 'Time 0' for reporting purposes (full acclimation data is provided in Appendix C).

Operationally, a feeding frequency (FF) of one in seven days and an OLR of 1 g VS/L/d was used as determined from Chapter 4 data. This constituted of removing 560 mL of reactor volume before adding 14 g VS (as 1.0 mm RS) with 560 mL of distilled water each week. The experiment assessed the effect of DM as an N supplement on RS AD by varying the amount of RS:DM fed to each reactor whilst maintaining the same OLR (Table 6.1).

Table 6.1: Rice straw and dairy manure feeding ratios for each reactor

Reactor	**1**	**2**	**3**	**4**
Rice straw (% of VS)	100	90	70	30
Dairy Manure (% of VS)	0	10	30	70
C:N	60:1	40:1	24:1	13:1
Reactor code	RS100	RS90	RS70	RS30

One DM and one reactor sample were collected at each HRT for the four reactors; at days 0 (70), 37 (107), & 75 (145) (after acclimation), resulting in 15 samples stored at

- 20 °C before sampling, DNA extraction, sequencing, and bioinformatics similar to Chapter 5.

All routine analyses; i.e., biogas, methane content, VFA, pH, solids, and composition, were performed as described in Chapter 3 whilst the sequencing analyses were performed as per Chapter 5. Total Ammonia Nitrogen (NH_3-N) analysis was achieved using a Vapodest 30S steam distillation unit according to the APHA standard method (APHA, 1998).

Statistical analysis of the physiochemical data was performed as described in Chapter 4 whilst sequencing data analysis was performed as per Chapter 5. Additionally, the heatmap of beta-diversity abundance analysis was performed using PRIMER 7 (PRIMER-E, Plymouth, UK).

6.3 Results and Discussion

6.3.1 *Effect of dairy manure on reactor performance*

Stable bioreactor operations were confirmed before any samples were collected for microbial community characterisation. Stability was defined as when no statistically significant differences (using ANOVA) in biogas yields were apparent when comparing sequential HRTs of operating time. Biogas production “stability” was achieved after the third HRT, after which specific gas yields, CH_4 content, VS, pH, and total VFA data were tallied and summarised (see Table 6.2). Time-course data of pH, VS, methane yields, and total VFA are shown as Figure 6.1a-d. Ammonia readings were zero throughout as the relatively low pH deterred free ammonia.

Mean biogas yields ranged from ~115 to 222 mL/g VS/d whilst methane yields ranged from ~48 to 112 mL CH_4/g VS/d. For both parameters, increasing the ratio of RS to DM increased biogas yield significantly ($p = <0.001$), indicating DM addition had a significant, negative impact on gas yields. Relative percent methane content (% CH_4) was more similar; i.e., RS100 and RS90 ~51 % and 48 %, respectively, which were significantly higher than RS30 and RS70 (41 % and 43 %), respectively.

Comparing RS100 methane yields at 25 d HRT (i.e., 112 mL CH_4 /g VS/d) to those reported in Chapter 4 at the same FF and OLR (148 mL CH_4 /g VS/d at 50 d HRT), indicate the lower HRT resulted in significantly lower methane yields ($p = <0.001$). Increasing HRT was also seen to increase methane yields by Shi *et al.* (2017) and

Nges and Björnsson (2012). However, a shortened HRT may still be of benefit in the building of a reactor as it could have a smaller volume, reducing the costs, due to quicker throughput (Yadvika *et al.*, 2004).

Table 6.2: Overall Mean Performance Data for Reactors with Different Feeding Regimes and Organic Loading Rates

Reactor	RS100	RS90	RS70	RS30
Biogas yield (mL/g VS/d)	**222 [a] ± 5.2 [b]**	193 ± 5.2	156 ± 4.3	115 ± 4.7
Methane content (% CH_4)	**50.9 ± 1.7**	48.2 ± 1.5	43.2 ± 1.8	40.8 ± 1.6
Methane yield (mL CH_4/g VS/d)	**112 ± 4.6**	94.2 ± 4.3	69.9 ± 3.5	47.5 ± 2.7
Volatile Solids (g VS/L)	13.9 ± 1.0	11.9 ± 0.6	10.6 ± 0.7	**4.7 ± 0.2**
% VS Reduction	26.3 ± 2.5	22.8 ± 2.5	24.7 ± 3.2	**37.5 ± 3.2**
pH	6.1 ± 0.01	6.1 ± 0.01	6.0 ± 0.01	6.3 ± 0.01
Total VFA (ppm)	506 ± 69	420 ± 28	401 ± 44	**97 ± 31**
Formic acid (ppm)	11.6 ± 9.7	15.3 ± 12	16.8 ± 15	18.0 ± 10
Acetic acid (ppm)	145 ± 32	118 ± 15	116 ± 21	106 ± 37
Propionic acid (ppm)	319 ± 50	259 ± 20	256 ± 32	**36.9 ± 12**
Isobutyric acid (ppm)	51.9 ± 21	57.0 ± 26	64.5 ± 32	64.2 ± 33

Notes: [a] Bold indicates the highest performing condition for that parameter
[b] Standard error (For OLR 1.0g VS/L/d n = 76 for biogas and methane, n = 12 for VS and total VFA, n = 30 for pH, and, n = 3 - 12 for individual VFAs.

In contrast to biogas results, RS30 had the highest %VS reduction (38 %), which was significantly greater than the others (range from 23 to 26 %) at $p = 0.003$. RS30 also had the lowest amount of VS (4.7 g VS/L), significantly lower than the others (between 11 and 13 g VS/L) at p = <0.001. Differences in pH also were significant with RS30 having the highest (6.3), although all four reactors were always > 6.0. Contrary to pH data, difference in total VFA levels were wider; i.e., RS30 had 97 ppm, which was significantly lower than the other three reactors (range from 401 to

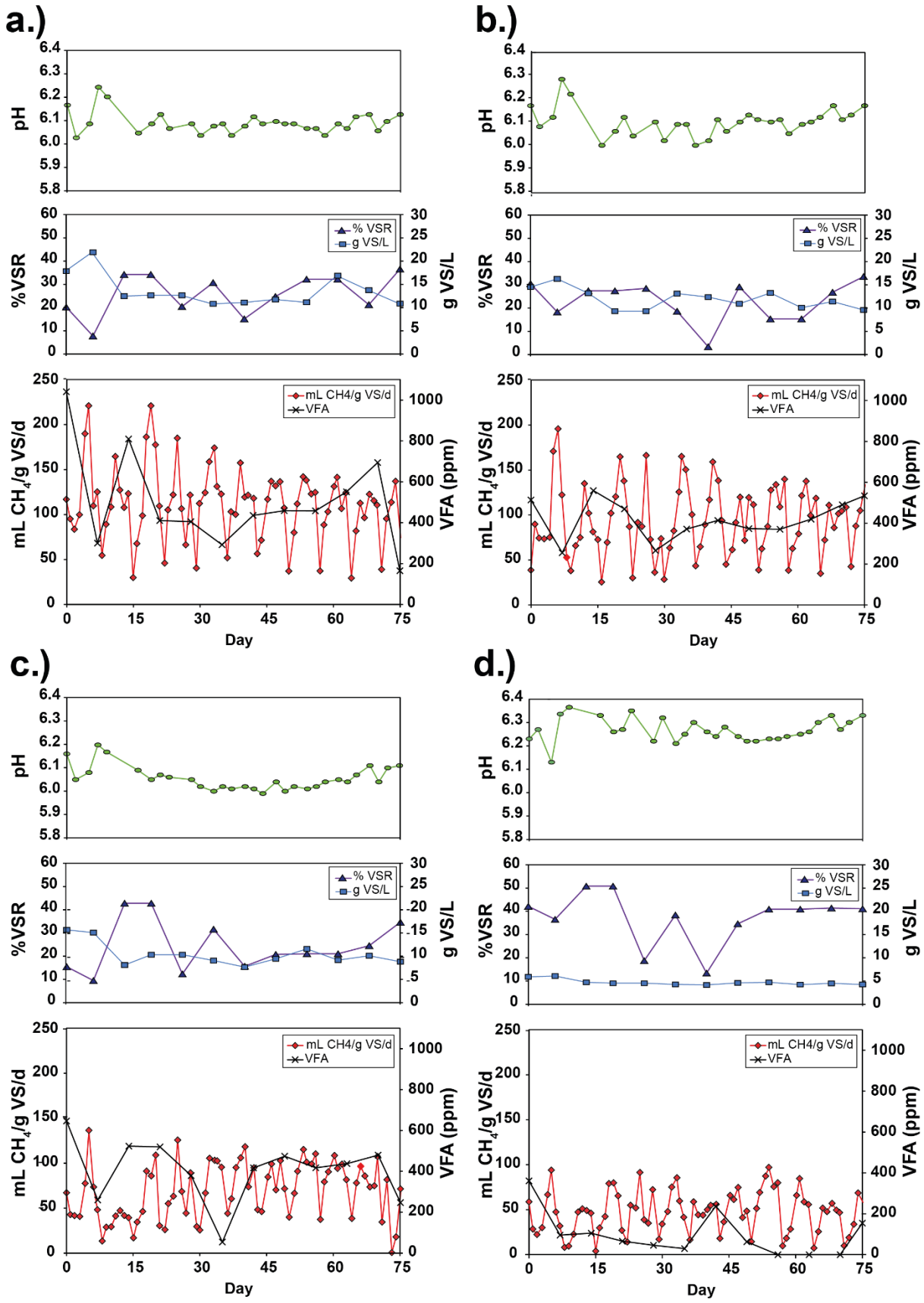

Figure 6.1: Time-course performance data - pH, % VSR with g VS/L, and methane yield with VFA concentration, for: **a.)** RS100, **b.)** RS90, **c.)** RS70, **d.)** RS30

506 ppm) at p = 0.003. Only formic, acetic, isobutyric, and propionic acid had enough data points for statistical testing, but of these, propionic showed significant differences with RS30 (37 ppm), which was significantly lower than the other conditions (range 246 to 319 ppm) at p = 0.003. Time course data (Figure 6.1a-d) show that measured parameters were generally consistent with time, particularly pH and specific methane yield.

Results from this experiment were somewhat unexpected as co-digestion with manure is a popular and often used method to add nutrients to AD processes (Marañón *et al.*, 2012). Based on RS stoichiometry, a limiting factor in RS AD should be N because C:N ratios are often too high (Wang *et al.*, 2014b). However, in BMP tests (Chapter 3) and in the CSTR experiment here, the addition of manure was detrimental to the performance of the reactors. Similar results were seen by Callaghan *et al.* (2002) and Dechrugsa *et al.* (2013) who found increasing levels of manure also had a detrimental effect on biogas yields. However, many co-digestion studies, such as Estevez *et al.* (2012), Wang *et al.* (2014c), Sahito and Mahar (2014), Li *et al.* (2014a), Xavier *et al.* (2015), and Jiménez *et al.* (2016), report that co-digestion with manure improves methane yields, but none of these previous studies did side-by-side comparisons with RS as a sole substrate, which make previous conclusions tenuous.

That these reactors were fed different ratios of RSDM, though at equal amounts of VS (1g VS/L/d), may be the reason for the differences in biogas yields seen between RS100 to RS30. Each ratio of RSDM provided a different quality, or type, of solids, i.e., although they all received 1 g VS/L/d, the VS fed as DM was less accessible at a microbial level than the RS solids. Dairy cattle are often fed on grasses, or similar materials, that are high in lignocellulose as is RS. Cellulose and hemicellulose are relatively easy to degrade but are protected from microbial attack as they are bound by the lignin (second most common organic compound. Therefore, the difference between RS and DM VS content is that the easily degradable material in DM has already been degraded within the ruminant system and the AD recalcitrant lignin has been concentrated (Triolo *et al.*, 2011). Lignin data was not available for the DM in this experiment but Hills (1979); Hills and Roberts (1981); Labatut *et al.* (2011); Triolo *et al.* (2011) and Li *et al.* (2013) found that DM has higher lignin content (11.9, 13.8, 14.0, 17.4 and 17.4 %) than in RS as found by Hills and Roberts (1981); Lee (1997); He *et al.* (2009); Phutela *et al.* (2011); Li *et al.* (2013) and here (4.9, 7.4, 8.2, 9.9,

10.8, 2.8 - 4 %). This means that although the VS content may be equal, the enhanced biodegradability of the substrate fed to RS100 compared with RS30 differs and, in this case, outweighs any positive effects of C:N balancing.

In experiments here, increasing %DM reduced biogas production, although higher DM did balance pH, increase VS removal, and reduce VS and VFA accumulation. Therefore, a major benefit of co-digestion is a more stable system (Babaee *et al.*, 2013), reflected by non-methane operating parameters. The addition of manure not only balances the C:N ratio to the predicted 'ideal' zone, but also provides additional nutrients that improve the AD process (Li *et al.*, 2014a). These additions have been shown by Li *et al.* (2015a) and Cornell *et al.* (2012) to facilitate higher RS loading (both 6 kg VS/m^3/d) of an AD system than was seen in Chapter 4 or used here. In a higher loading situation it is feasible that the lower biodegradability of the DM will be outweighed by the possible benefits of a co-digestion system to deal with VFAs and VS. However, the addition of N-rich manure also can lead to inhibitory increases in ammonia levels (Estevez *et al.*, 2012), which was not seen here (likely due to the low loading rate). 16S rDNA amplicon sequencing is discussed in the next section to better understand the differences in microbial communities between each of the reactor conditions.

6.3.2 *Beta-diversity and physio-chemical parameters*

Samples collected from the four reactors clustered into HRT-based (Figure 6.2a) and RS:DM-based groupings (Figure 6.2b) based on Bray-Curtis distance, with the RS:DM ratio having the greater effect according to x-axis variation (> 80 %). The influence of individual variables on each sample point was represented by the direction and length of corresponding arrows. As RS decreased from RS100 to RS30, sample clusters migrated from left to right (Figure 6.2b), moving further from higher biogas and VFA production, but lower pH and VS removal.

Weighted Unifrac distances (Figure 6.2c) grouped the samples based on HRT (ellipses) and RS:DM ratio (shown by coloured lines) with RS30 grouping separately. Over the three HRTs the samples migrate upwards from higher acetic, formic, and propionic acids to greater methane content and isobutryic acid, although the axis explains only 13.9 % of the variance. Higher levels of VFAs (see Figure 6.2d) confirm

the inference that samples with higher percent RS have higher biogas yields and also higher VFA levels whilst decreasing RS leads to higher pH and VS removal.

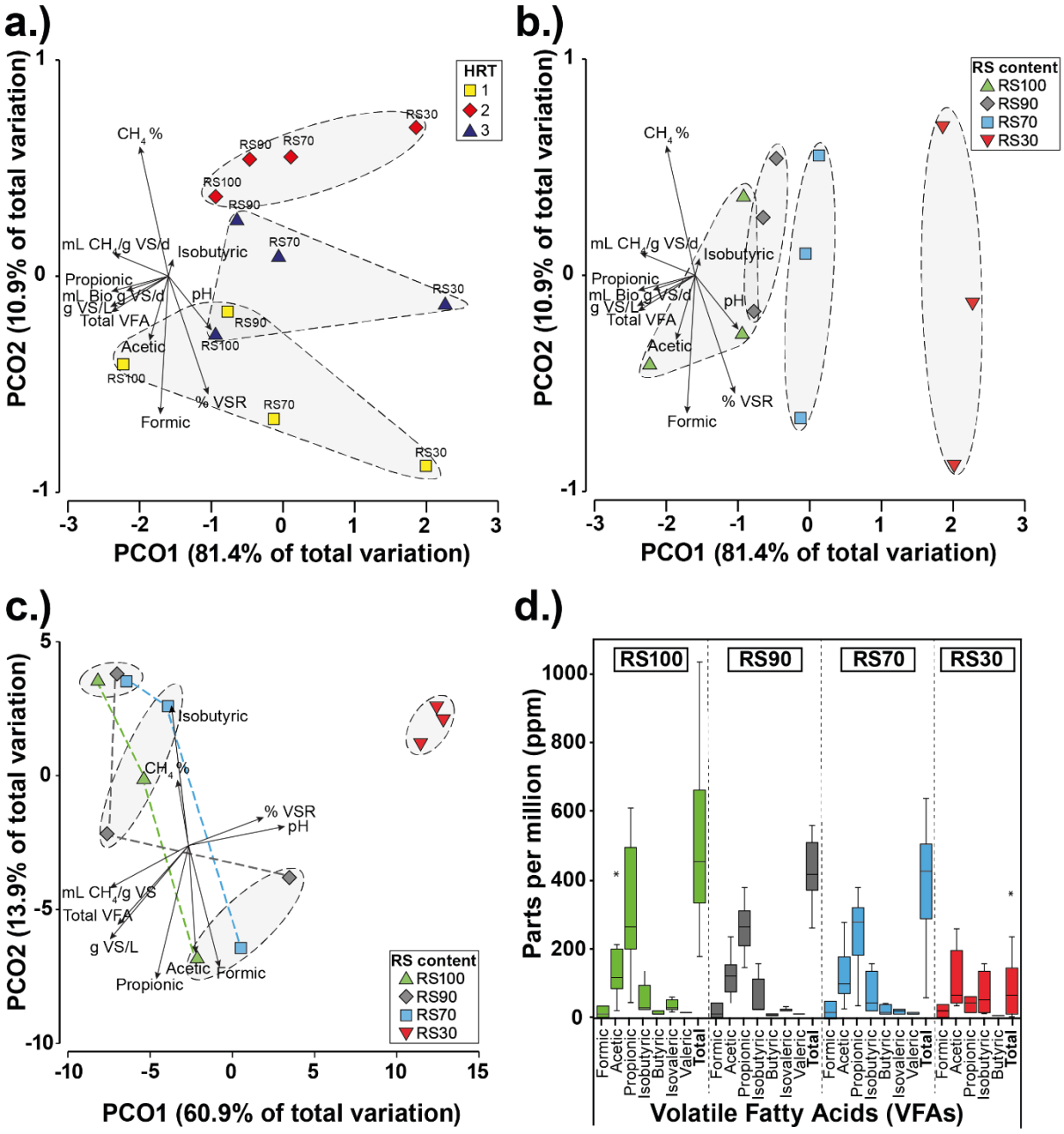

Figure 6.2*:* Analyses of beta diversity showing variation of microbial community structure and the influence of physiochemical data as a PCO with groupings of **a.)** HRT, and **b.)** RS:DM, **c.)** PCO of weighted UniFrac distance. **d.)** boxplot of individual and total VFAs for RS100, RS90, RS70, and RS30 (there was no isovaleric or valeric acid in RS30). Physiochemical data overlaid arrows and dashed elliptical shapes and-or coloured lines indicate sample RS:DM or HRT groupings.

To determine whether the groupings and correlations seen in Figure 6.2 were statistically significant, RELATE, BEST, DistLM, ANOSIM, and PERMANOVA were performed on the data and reported in Table 6.3 and Appendix C.

The influence of physiochemical variables on the beta-diversity was reflected by a significant (0.2 %) overall correlation (Rho = 0.68, RELATE) whilst pH was found to have the greatest influence with a correlation of 0.73 (BEST). DistLM analysis was used to show a number of variables were significant in this correlation, seen in Table 6.3, including VS accumulation (p = 0.004). When undertaking sequential analysis, pH was the best descriptor (p = 0.002). ANOSIM and PERMANOVA showed that RS:DM was a significant factor influencing beta-diversity (2.2 % and p = 0.018).

6.3.3 *Impact of OLR and reactor souring on alpha-diversity*

Alpha-diversity comparisons (Hughes *et al.*, 2001; Lemos *et al.*, 2011) were used to determine differences in microbial community richness and evenness, using a combination of observed and Chao1 OTU numbers, and Simpson's and Shannon's indices (Figure 6.3). Observed OTUs and Chao1 estimations showed DM addition generally resulted in significantly higher observed OTUs. For example, observed OTUs in RS30 were higher than all other conditions, but only significantly higher than RS100 (p = 0.041) Chao1 estimations were less ambiguous as DM was significantly higher than all other conditions (p = <0.001), showing that it contained a higher amount of OTUs sequenced just once.

Simpson's and Shannon's scores (Figure 6.3b and c) showed no statistical differences among reactors, although Shannon's Index, which considers both richness and evenness, indicated RS30 and DM had higher scores (5.6 & 5.5) than RS100, 90, and 70 (4.8, 5.1, and 4.9, respectively). Alpha diversity scores of 0.90 - 0.95 (Simpson's Index) and 4.8 - 5.6 (Shannon's Index) were similar to Zhao *et al.* (2012) (3.5 - 5.5 for Shannon's) and Sun *et al.* (2015) (> 0.93 for Simpson's). These indicate that as the level of DM decreased in the sample so did the richness and is supported by trends shown on the beta-diversity abundance heatmap (Figure 6.3d).

Table 6.3: Test statistics of beta diversity and physiochemical variables and operational factors

Method [a]: RELATE		
Variable	**Significance (%)**	**Rho**
Physiochemical data	**0.2 [b]**	0.677
Method: BEST		
Variable	**Physiochemical Correlation (R)**	
pH	0.725	
Method: DistLM		
Variable	**p-value**	**Cumulative variance explained (%)**
mL Biogas/g VS/d	**0.012**	-
CH_4 %	**0.039**	-
mL CH_4/g VS/d	**0.006**	-
g VS/L	**0.004**	-
% VSR	**0.021**	-
pH	**0.003**	-
Total VFA	**0.005**	-
Sequential		
+ pH	**0.002**	49.4 %
Method: ANOSIM		
Condition	**Factor**	**Global R**	**Significance level (%)**
Physiochemical	RS:DM	1.0	**0.3 %**
Beta-diversity	RS:DM	0.81	**2.2 %**
Method: PERMANOVA		
Condition	**Factor**	**p-value**	**Sq.root of estimates of component of variation**
Physiochemical	RS:DM	**0.002**	1.49
Beta-diversity	RS:DM	**0.018**	2.62

Notes: [a]Tests - RELATE, giving correlation of comparisons (Rho); BEST, trend correlation; DistLM, distance based linear model; ANOSIM, analysis of similarities; PERMANOVA, permutational multivariate analysis of variance.
[b]Bold indicates statistically significant results

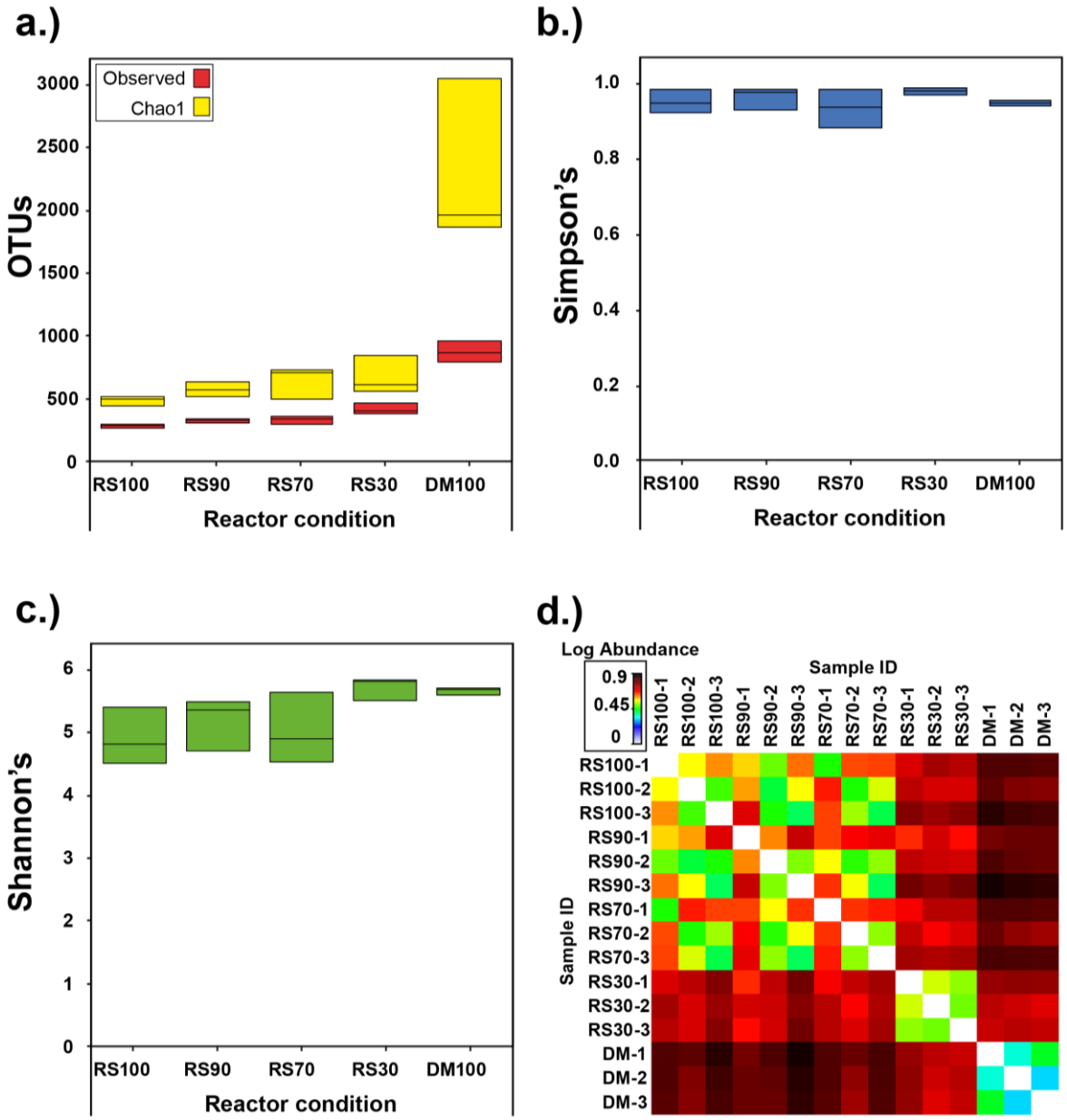

Figure 6.3: Boxplots between, **a.)** Observed OTUs (lower) and Chao1 (upper boxes). **b.)** Simpson's index scores. **c.)** Shannon's index scores. **d.)** Heatmap of beta diversity abundance

6.3.4 *Predominant OTUs*

As HRT was not a significant factor in determining microbial diversity (shown in the previous section), the three samples from each stage were combined and discussed as one; i.e., all three samples for each reactor were combined.

Predominant OTUs (≥ 0.5 % relative abundance, 76 OTUs) are shown to the phylum level in Figure 6.4 and to genus where possible in Figure 6.5 to illustrate apparent

differences in microbial communities among operating conditions, and Figure 6.6 shows statistically significant differences. The 76 predominant OTUs are presented in Appendix C as a phylogenetic tree and genus OTU table.

RS100, 90, and 70 were all similar in phyla and were dominated by *Firmicutes* and *Bacteroidetes*, with the remaining 30 - 40 % made up of *Proteobacteria*, *Spirochaetes*, *Euryarchaeota*, and *Chloroflexi*. RS30 differed in that *Bacteroidetes* was less dominant with higher levels of *Proteobacteria* and *Spirochaetes*. *Bacteroidetes* is a dominant gut bacteria so it was expected to increase in RS30 (Wexler, 2007). However, Li *et al.* (2015c) found *Proteobacteria* can thrive in low VFA conditions (such as RS30) and *Spirochaetes* have the ability to ferment plant polymers and have been found in large numbers in bovine rumen fluid (Paster and Canale-Parola, 1982). In contrast, the DM samples (raw) were > 65 % *Firmicutes* and had almost no *Spirochaetes*.

Overall, the number of predominant OTUs in each sample increased with increased DM from RS100 and RS90 (32 & 31) through RS70 (35) to RS30 (38). DM samples had a higher number of “rare” OTUs as their predominant OTU was the lowest (26). High microbial diversity was not essential to have high biomethane production, i.e., RS100 had the highest biomethane and fewest predominant OTUs.

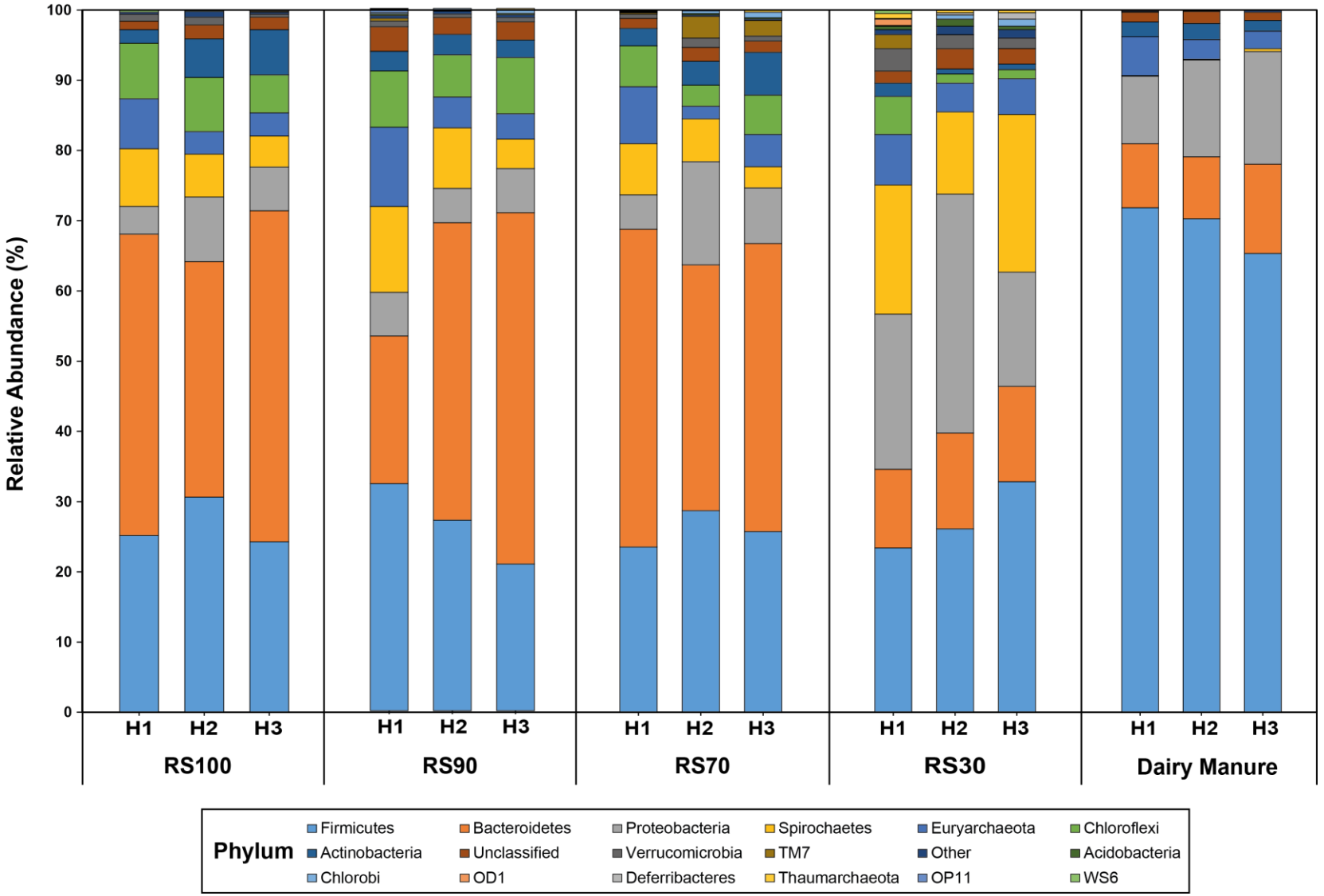

Figure 6.4: Shared predominant OTU table to phylum level (only ≥ 0.5 % abundance) based on sample appearances

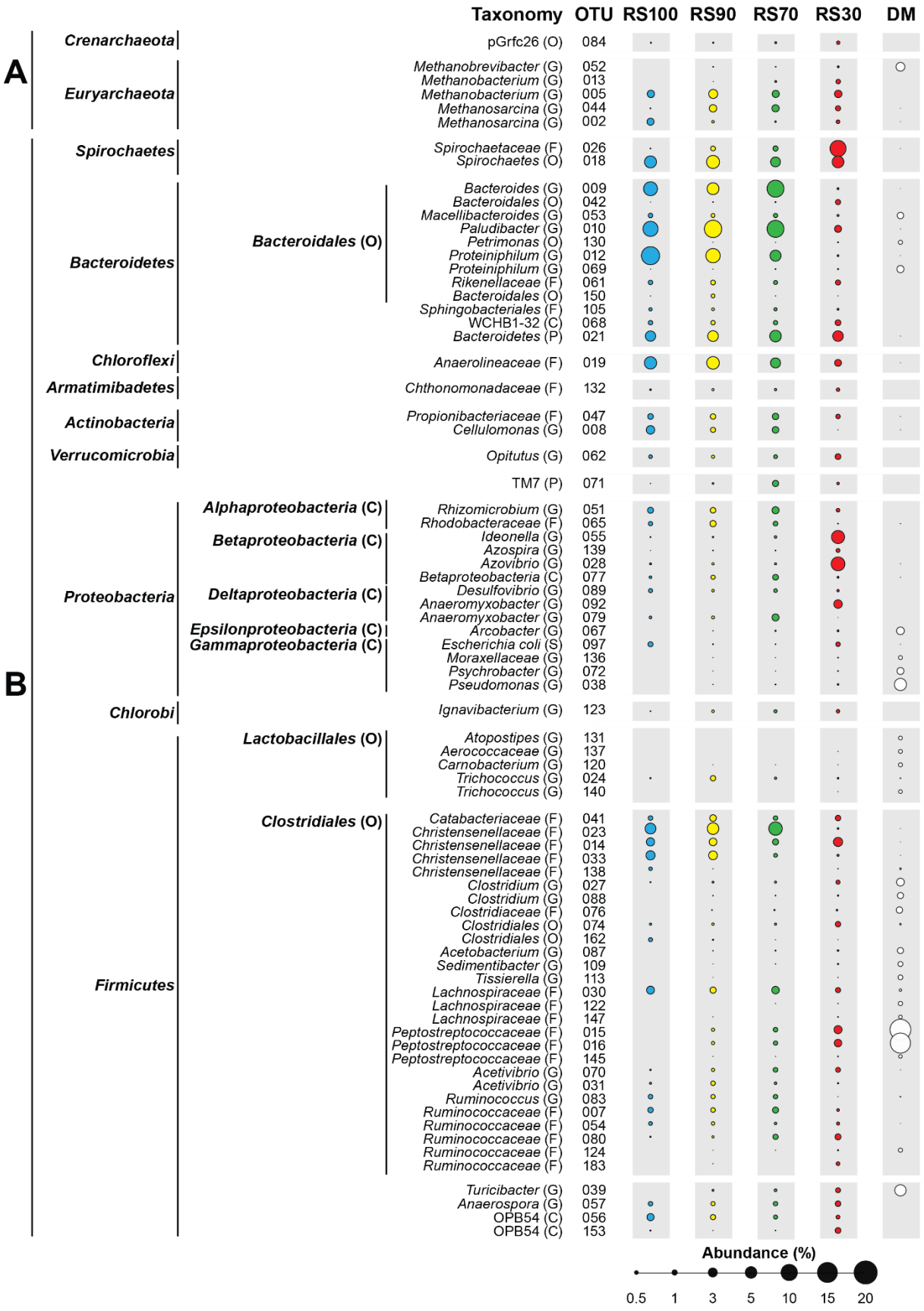

Figure 6.5: Predominant OTUs (≥ 0.5 % abundance) to genus level where possible for RS100, RS90, RS70, RS30, and DM. A = Archaea, B = Bacteria. Area of bubbles represents relative abundance.

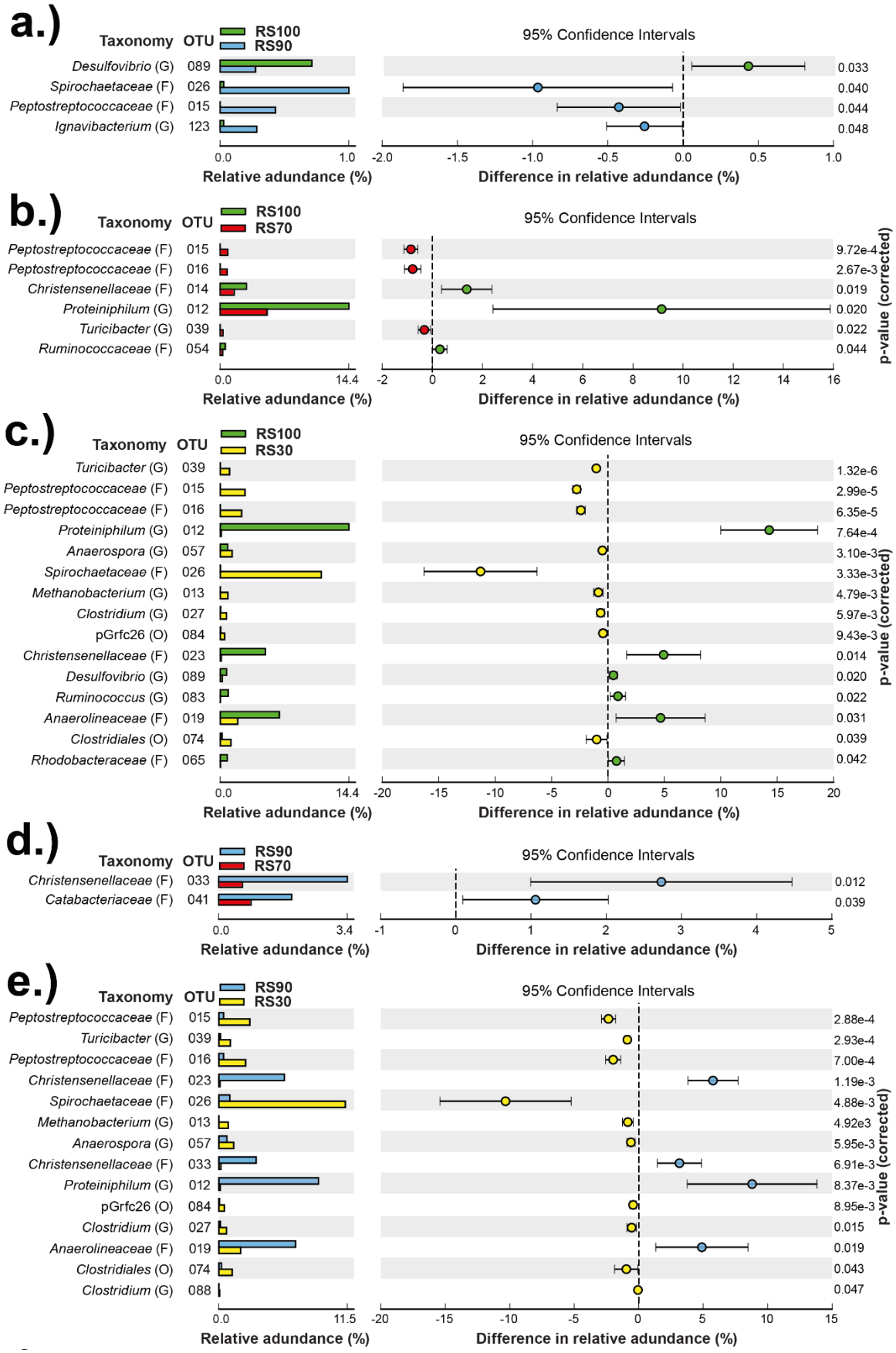

Figure 6.6: Extended error bar plot of significant differences between predominant OTU, **a.)** RS100 vs RS90, **b.)** RS100 vs RS70, **c.)** RS100 vs RS30, **d.)** RS90 vs RS70, **e.)** RS90 vs RS30, and **f.)** *continued over page*

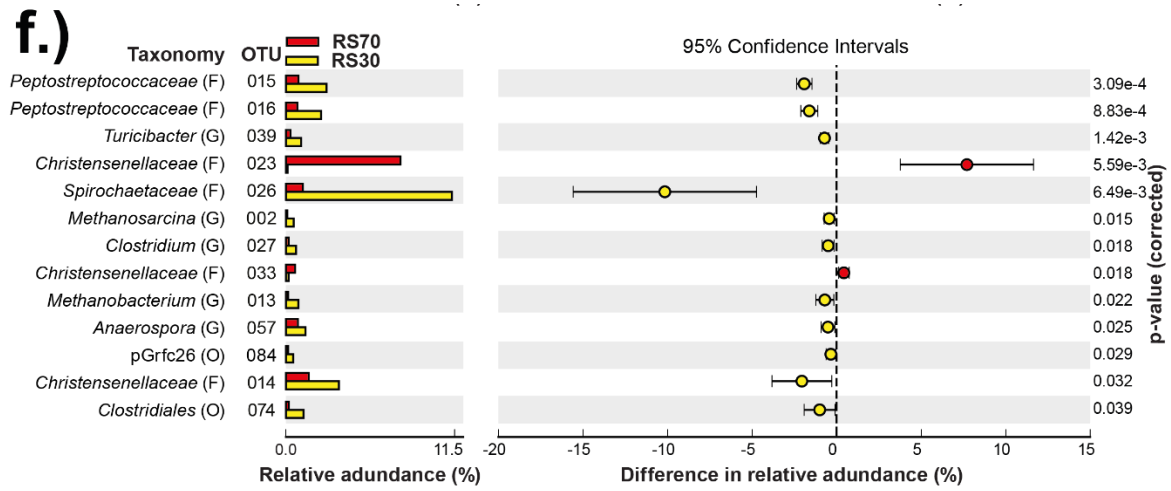

Figure 6.6: f.) RS70 vs RS30

Methanogens were present in all reactors, typically between 4.5 and 6.2 % of the relative abundance within each reactor. The highest abundance was seen in RS90 and the lowest in RS100. *Methanobacterium* and *Methanosarcina* were the main methanogens present. Their major OTUs were 005 and 044/002 with abundances ranging from 2.2 to 3.5 % and 1.1 to 2.4 %, respectively. However, these methanogens were below detection (i.e., 0.0 %) in the raw DM samples, whereas *Methanobrevibacter* (OTU 052) was below 0.2 % in all reactors but had a much higher abundance (~3.4 %) in the DM samples. *Methanosarcina* was also dominant in RS100, possibly due to its, versatility, acid tolerance, and high growth rate (Conklin *et al.*, 2006; Yi *et al.*, 2014; Leite *et al.*, 2015). Where *Methanosarcina* was less dominant (in all but RS100) may be due to its acetoclastic preferences (FitzGerald *et al.*, 2015), i.e., as acetic acid decreased with RS so did *Methanosarcina* (Fontana *et al.*, 2016). Leite *et al.* (2015) also found that *Methanosarcina* preferred mono digestion rather than co-digestion environments.

As in Chapter 5, no well-known syntrophic bacteria were in relatively high abundance. This may be because high hydrogen production during RS AD, as suggested by Kim *et al.* (2012) reduces habitable zone for syntrophs that require low hydrogen pressure (Stams and Plugge, 2009). This was different to other studies on sewage sludge digestion (Mei *et al.*, 2017), although Liu *et al.* (2017) found that highly active fermenting bacteria also could produce inhibiting levels of VFAs and hydrogen. Facultative syntrophic bacteria, such as *Ruminococcus albus*, can grow syntrophically with hydrogenotrophic methanogens (Stams and Plugge, 2009).

However, the increase in acetic and propionic acid with increased RS indicates that the syntrophic breakdown of acetate and propionate was overwhelmed (Amani *et al.*, 2011; Banks *et al.*, 2012). Deeper studies are needed to analyse the obligate syntroph community in RS AD and further our knowledge of the microbial community, which could potentially further improve the efficiency of RS AD.

There were a number of significant community changes across the reactors with some bacteria thriving under higher RS conditions. *Bacteroidetes* was most dominant when RS was high, and *Proteiniphilum* (OTU 012) also was significantly more abundant in RS100 and RS90 (14.4 % and 8.9 %) than in RS30 (0.1 % at p = < 0.001 and 0.008). Although its abundance in RS70 was 5.3 %, this was not significantly different to RS30. As background, *Proteiniphilum* do not use cellulose (Chen and Dong, 2005), so their abundance in high RS reactors was unexpected. However, they often produce acetic and propionic acid (Whitman *et al.*, 2015), which marries with higher VFA levels in RS100 and RS90. Among *Chloroflexi*, *Anaerolineaceae* (OTU 019) displayed a similar pattern, being more abundant under higher RS conditions. Their abundance in RS100 and RS90 was similar (6.6 and 6.9 %), lower in RS70 (4.4 %), but significantly lower in RS30 (2.0 % at p = 0.031 and 0.019). Xia *et al.* (2016) found the cellulolytic capacity of *Anaerolineaceae* was not likely to be its main attribute, but there are very few isolates and genome sequences, and its ecological role is uncertain. Of the four *Christensenellaceae*, which can degrade cellulose (Fontes and Gilbert, 2010), OTU 033 was significantly higher in RS90 (3.4 %) than in RS30 (p = 0.007). *Ruminococcus* (OTU 083) had lower abundance, but was significantly higher (p = 0.022) in RS100 (0.9 %) than in RS30 (0.0 %). This genus is noted as a cellulose hydrolyser and some species use cellobiose (Sun *et al.*, 2015).

A number of bacteria were more dominant under the RS30 condition. For example, *Spirochaetaceae* (OTU 026) was higher in RS90 than RS100 (1.0 % vs 0.0 % at p = 0.040) and was very different from RS70 (1.2 %) to RS30 (11.3 %). However, there are few cultivated species of this facultative anaerobe with which to compare data (Paster, 2015). The *Firmicutes* phylum showed the greatest abundance increase in RS30 compared with RS100. *Clostridium* (OTU 027) and *Clostridiales* (OTU 074) were of low abundance with only one reactor breaching the 0.5 % predominant condition. However, the abundances of *Clostridium* (OTU 027) and *Clostridiales*

(OTU 074) in RS30 (0.7 % and 1.2 %) were significantly higher than in any other reactor (all $p \leq 0.05$).

The fact that these were low in abundance in the higher RS reactors was unexpected, as it is contrary to analysis in Chapter 5, and a number of the *Clostridiales* Order have been shown to ferment cellulose (Fontes and Gilbert, 2010; Zverlov *et al.*, 2010; Ziganshina *et al.*, 2015). However, although cellulolytic ability of the microbial community is assumed to be primarily via *Clostridia*, there are few species that can directly degrade cellulose, for example *Clostridium paradoxum* (Li *et al.*, 1993).

Peptostreptococcaceae (family) (OTU 015 & 016) were both < 1.0 % in RS100, 90, and 70, but were significantly higher in RS30 at 2.8 and 2.4 % (all $p = < 0.001$). Li *et al.* (2014b) found *Peptostreptococcaceae* contains a number of genera isolated from manure and Mao *et al.* (2012) noted it negatively correlates with VFAs, which may be why it was limited in the RS dominant reactors. Similarly, *Turicibacter* (OTU 039) abundance was < 0.5 % in RS100, 90, and 70, but 1 % in RS30 ($p = < 0.001$) and, *Anaerospora* (OTU 057) abundance was < 0.8 % in RS100, 90, and 70, but 1.3 % in RS30 ($p = <0.05$). *Turicibacter* has previously been shown as a core population and a taxa key in hydrolysis (Li *et al.*, 2015c; Rui *et al.*, 2015b) and has been found in cattle faeces Liu *et al.* (2016), whilst Cersosimo *et al.* (2015) found *Anaerospora* in impala rumen, explaining their preference for RS30. However, the *Firmicutes* phylum is extremely complex and partial rDNA amplicon sequences should be interpreted with caution (Lü *et al.*, 2014).

There were a number of ‘Goldilocks’ bacteria, favouring neither RS100 nor the DM heavy RS30, but something in the middle. *Rhodobacteraceae*, which is typical of slurry (FitzGerald *et al.*, 2015), was highest in RS90 (1.6 %) and lowest in RS30 (0.0 %; significantly different at $p =0.042$) with 0.8 and 1.0 % in RS100 and RS70. *Christensenellaceae* (OTU 023) was 5.0 and 5.9 % in RS100 and RS90, but was highest in RS70 (7.8 %) and lowest in RS30 (0.1 %, $p = 0.006$). There is currently only one described species of *Christensenellaceae*, *Christensenella minuta*, which was found to favour gut environments (Morotomi *et al.*, 2012; Rosa *et al.*, 2017).

There were OTUs that only appeared, or thrived, in the DM samples, which were selected against in RS90, RS70, and RS30. For example, *Clostridiaceae* (OTU 076,

1.8 %), *Clostridium* (OTU 027 & 088, 2.6 & 1.7 %), and *Peptostreptococcaceae* (OTU 015 and 016, 19.0 and 17.7 %) all play important roles in the rumen (Sun *et al.*, 2015), were not seen in the reactors. Wang *et al.* (2016) found *Proteiniphilum* (OTU 069, 2.2 %) hydrolyses proteins in manure and Liu *et al.* (2016) found *Turicibacter* (OTU 039, 5.5 %) dominated faecal samples. *Pseudomonas* (OTU 038) was part of a successful bioaugmentation experiment by Duran *et al.* (2006) and although it did not survive well in the RS reactors (< 0.1 %), it was 6.5 % in DM. It is typically an "aerobic" organism (Miller *et al.*, 2016) and it may be beneficial as a niche bio-supplement. *Escherichia coli* (OTU 097) was also found in RS100 and RS30 (1.1 and 0.8 %) whilst *Arcobacter* (OTU 067), which has been associated with enteritis and diarrhoea, was found in the DM samples (2.6 %).

6.4 Conclusion

RS100 (25 d HRT) methane yields (112 mL CH_4 /g VS/d) were lower than those reported in Chapter 4 under the same FF and OLR (148 mL CH_4 /g VS/d at 50 d HRT), which suggests a higher HRT results in greater specific biogas production. In the DM addition experiments, highest specific methane yields were seen in the unit without DM addition; RS100 had 112 mL CH_4/g VS/L/d, whereas lowest yields were observed in the unit with the highest level of DM (RS30; 48 mL CH_4/g VS/L/d). In contrast, as DM increased, both VS and VFA accumulation decreased, and VS removal increased. These benefits of co-digestion may offer the option of higher OLRs in practical applications.

Increasing DM content in the feed resulted in greater microbial richness compared with reactors with higher levels of RS. Evenness was similar among all RS:DM ratios, although the predominant OTUs differed among reactors. RS only reactors were dominated by *Firmicutes* and *Bacteroidetes*, whereas the highest DM reactor, RS30, had higher levels of *Proteobacteria* and *Spirochaetes*. Methanogen abundances were similar among the reactors, therefore lower abundances of cellulosic hydrolysing bacteria such as, *Christensenellaceae* and *Bacteroidetes*, best explain lower methane production levels when higher DM was in the feed, implying carbon short-circuiting was prevalent in those reactors. Overall, the main benefit of co-digestion with RS and DM appears to be decreased VFA production and higher rates of VS removal, which suggest co-digestion systems may be able to operate at higher OLRs, increasing RS throughput

Chapter 7 Conclusions and Recommendations

7.1 Conclusions

The impact burning millions of tonnes of waste rice straw has on local communities and the environment is a substantial problem and an alternative method is needed. The aim of this study was to assess under what conditions rice straw anaerobic digestion (RS AD) was achievable without the need for expensive and-or hazardous pretreatments. Four main sets of experiments (Chapters 3 - 6) were performed to achieve the objectives set at the start:

1. Evaluate the impact of inoculum:substrate ratio (organic loading rate, OLR), particle size, C:N ratio (through co-digestion), and the geographic origins of RS on AD performance using batch digestion tests.
2. Identify optimal feeding frequencies and OLRs for RS AD at the semi-continuous-fed reactor level, including the potential for identified conditions for scale-up.
3. Determine the effect of FF/OLR on AD microbial communities, particularly focussing on how FF and low versus high OLRs alter methanogenic guilds.
4. Evaluate the effect of dairy manure (DM) co-digestion on RS AD performance any differences in microbial communities between reactors with and without DM addition.

Using batch tests in Chapter 3 it was shown that RS AD was limited to an OLR of ≤ 3 g VS/L and that additions of N and P, including dairy manure (DM), had a negative impact on biomethane yields. In this case, the reduction in performance with DM addition was likely due to the less biodegradable nature of the DM VS content. Ruminants digest the most easily digestible forms of carbon in their lignocellulosic feed, e.g. cellulose, and the less biodegradable constituents such as lignin have been concentrated. It was this difference in biodegradability was likely the main determinant of biogas production and yields in this experiment. Whereas, decreasing particle size proved the opposite, for example at the extremes tested, 425 µm versus 70 mm yields were 180 and 140 mL $_{ult}$ CH_4/g VS. By far the most interesting result in this chapter came from the geographic origins test. RS from Nigeria had the highest methane yield (388 mL $_{ult}$ CH_4/g VS) followed by Philippine RS (275 mL $_{ult}$ CH_4/g VS)

and Chinese RS (211 mL $_{ult}$ CH_4/g VS). This trend was the same for methane production rate, with Nigerian RS highest (0.17 d^{-1}), except that Philippine (0.11 d^{-1}) and Chinese RS (0.14 d^{-1}) reversed positions. Indian RS lagged in both (153 mL $_{ult}$ CH_4/g VS and 0.12 d^{-1}). The greatest impact to methane yield/production was due to the differences in lignin which has great chemical stability and acts as the glue that binds the potentially biodegradable cellulose and hemicellulose. Therefore, higher levels of lignin and cellulose yielded less methane than RS with lower lignin. It was possible to develop in Chapter 3 an equation to model the methane yield one may expect from RS based on its compositional analysis but this is so far untested due to lack of external data. Further, the batch method used was not wholly reproducible, though it is seemingly the best of its kind.

Long-term CSTR experiments in Chapter 4 suggested that frequent feeding (5/7 and 3/7d FF) performs relatively well at low and high loading. However, the highest specific methane yields were from the least frequently fed reactor, one in twenty-one days, i.e., 148 mL CH_4/g VS/d (at 1 g VS/L/d), and the highest volumetric yields were seen at a moderately frequent feeding (1/7d) at the higher loading, i.e., 276 mL CH_4/g VS/d and 2 g VS/L/d. Infrequent feeding led to VFA and VS accumulation and pH drops that caused two reactors to quickly fail once at the higher loading rate (1/14 and 1/21d FF). Both operating options have benefits for an acyclic waste stream, but low loading and less frequent feeding is probably better as it may provide a more holistic option with current practices. If there were sufficient storage then infrequently fed RS AD with CHP could generate large quantities of renewable heat and electrical power as well as providing other local, and global benefits, such as reduced air pollution and improved environmental quality.

The microbial dynamics of the FF/OLR (Chapter 4) provided unexpected results in Chapter 5. Even though the physiochemical variables were significantly affected by less frequent feeding, i.e., VFA accumulation, the microbial community was not significantly affected. However, there were clear community differences between low and high loading, as well as transitional and ‘sour’ (failed) reactors. The balance of predominant OTUs in low loading reactors was notably higher than in high loading reactors, regardless of whether they were healthy or sour. The mean number of OTUs at the low loading (OLR1) was 30 OTUs with none exceeding ~ 17 % abundance, compared to healthy and sour reactors at OLR2, which had 17 and 18 OTUs with highs of 44 and 52 % relative abundance. Low loading reactors were

dominated by *Bacteroidetes* and *Firmicutes,* then *Actinobacteria, and Euryarchaeota*, whereas high loading (healthy) reactors had comparatively limited *Euryarchaeota*, *Bacteroidetes,* and Actinobacteria, as Firmicutes thrived. When reactors failed they were wholly dominated by *Firmicutes.* Overall, the reduction in evenness and the abundance of fermenters, such as *Clostridia* and *Christensenellaceae*, increased with loading and eventual failure, whilst others, such as *Methanosarcina* decreased. Overall, the work here shows that OLR rather than FF most strongly impacts RS AD performance as well as microbial community composition and diversity. Traditional markers of reactor stability e.g. pH, would not react quickly enough, whereas, the effect of FF and community changes such as richness and unevenness would play more significant roles. Population shifts such as increases in fermenters combined with decreases in methanogens could therefore be used as an early warning of forthcoming system instability.

Although the addition of manure in Chapter 3 (to balance C:N ratios) had not been a success, insofar as the batch tests with DM performed more poorly than the RS bottles, co-digestion was evaluated at the CSTR scale in Chapter 6. This provided similar biomethane results, with RS100 yielding 112 mL CH_4/g VS/L/d whilst the reactor with the highest ratio of DM comparatively underperformed (DM30, 48 mL CH_4/g VS/L/d). This difference was likely due to the type of VS content of the DM, which, though the same as RS, was less accessible at a microbial level and thus provided lower methane yields as found in Chapter 3. Interestingly, reactors with DM had lower VS and VFA accumulation, and higher VS reduction and pH, than RS reactors. This experiment also enabled the comparison of 25 to 50 day HRTs (Chapter 4 versus Chapter 6), which showed 50d HRT methane yields to be significantly higher. RS100 (25 d HRT) methane yields (112 mL CH_4 /g VS/d) were lower than those reported in Chapter 4 under the same FF and OLR (148 mL CH_4 /g VS/d at 50 d HRT), which suggests a higher HRT results in greater specific biogas production.

Microbial richness increased with increased DM content compared with reactors that were predominantly RS though microbial evenness was similar among all conditions. Overall, the number of predominant OTUs in each reactor increased with DM, RS100 (32 predominant OTUs), RS90 (31), RS70 (35), and RS30 (38), indicating that high microbial diversity was not essential to have high biomethane production. DM samples had the highest number of “rare” OTUs, i.e., those < 0.5 % abundance. RS

reactors were dominated by *Firmicutes* and *Bacteroidetes* whilst RS30 had higher levels of *Proteobacteria* and *Spirochaetes*. Lower abundances of cellulosic hydrolysing bacteria such as, *Christensenellaceae* and *Bacteroidetes*, as well as lower methane production suggests that the AD process was short-circuiting as methanogens were similarly abundant regardless of DM addition. The main benefit of co-digestion, in this instance, would appear to be that the decreased VFA production and VS reduction indicates that these could cope with a higher OLR enabling greater throughput of RS.

Each year hundreds of millions of tonnes of rice are produced worldwide, on average this leaves a massive volume of rice straw as agricultural residue, 1-2 t per tonne of rice. The straw is often burned or left to rot in the fields adding to the already drastic issue of localised smog, particularly in China. This work has broader implications to operating any AD unit with a less degradable substrate. It shows, among others, that infrequent feeding does not negatively impact core microbial communities (though it is OLR dependent) and offers a new potential bio-predictor of reactor instability. Overall, RS AD without pretreatment is achievable and could provide a worthwhile volume of biomethane at a larger scale with pretreatment(s) or supplementation used to further enhance the process. The experiments and ideas shown here now need to be further examined and expanded on, including with other “less degradable” substrates, to verify the findings and produce a better strategy for any AD application like rice straw.

7.2 Recommendations for future work

Overall, due to its composition and recalcitrant structure, the biomethane potential of RS AD will always be limited without treatment or supplementation. With this in mind, there are a number of directions I would take this work in the future:

7.2.1 *The impact of geography and farming*

It has become apparent that researchers often consider that rice straw behaves the same under digestion regardless of source i.e., rice straw from India is the same as that from China or Nigeria. However, Chapter 3 showed this was not the case, meaning a ‘catch-all’ response is not appropriate. Comparing the AD performance of RS from different countries in this way has not been done before. I suggest expanding on Chapter 3 by assessing compositional analysis of RS from a wide

range of countries and batch testing to determine theoretical and ultimate biogas yields. This should be supported by continuous reactors to collect a greater array of data, as well as microbial sampling. Additionally, I would suggest including more thorough background information and, if possible, soil samples. From this, the microbial community should be sequenced to better understand the effect of AD operation, environment, and-or RS composition on AD performance. This work would show that RS AD performance is dependent on a range of factors and traditional AD methods are not appropriate in every case. Using these data a more expansive model could be developed to further evaluate RS AD based on compositional and environmental factors and provide more realistic theoretical yields.

7.2.2 *Co-digestion and metal supplementation*

The co-digestion experiments in Chapter 3 and 6 did not produce methane yields necessarily expected, partly due to a lack of consensus in methods used in the literature, to the lack of biodegradable VS in manure, and the reactors being underfed. AD digestate that has been mixed with animal manures requires further treatment and-or is subject to strict re-use regulations due to the pathogenic potential. Pathogens were not evident (in significant abundances) in the data from Chapter 6. Increasing loading rate, whilst following the same RS:DM experimental methods, would provide further insight into any pathogenic accumulation in the digestate.

In some small experiments not provided here it was suggested that metal or ash additions could increase the biomethane potential of RS by providing essential elements inherently missing in RS such as Co. The ash used was produced in a way to mimic pyrolysis and was alkali. By utilising this product it could lead to linked pyrolysis and RS AD processing plants to achieve the highest methane yields possible using a simple supplementation.

7.2.3 *Holistic pretreatments*

One option discussed early in this project was the option of using enzymes as a pretreatment. Specifically, white-rot fungus (that is known to degrade lignin) though there are few studies that combine white-rot and RS or that provide a method that could work at a large scale. To overcome this, a twist on the rotating bioreactor design could maximise biofilm growth and enzymatic production, which could then be

used as an RS pretreatment. In the same vain, using the microbial data here (preferably boosted with functional analyses), the biosupplementation of bacteria is an exciting possibility that could increase RS AD yields as with Liu *et al.* (2017). Based on the 16S data from Chapter 5 and 6, as well as the literature, I would suggest pretreatment with a fermenter from *Clostridiales* before the RS is fed into a digester. However, taxonomic assignments are not based on full sequences they should be interpreted carefully (Lü *et al.*, 2014), though this is an excellent window to determine further, deeper sequencing analysis.

7.3 Summary

Further microbial work into the functions of the RS AD community would provide invaluable knowledge into each of these options as well as suggesting which bacteria would be best for biosupplementation or similar pretreatments. Each of these future directions needs the added research depth of scaling up to pilot/farm scale, to confirm or refute the lab-scale outcomes and determine whether small-medium farms could actually benefit from this technology.

Although I would change some aspects of this project if I completed it again and the RS research community is unfocussed regards a common goal, by virtue of being too focussed, I am confident that AD is an appropriate method to deal with this waste stream. By combining a global approach with composition analysis at the laboratory scale and metagenomics I would suggest an overall solution to RS AD, regardless of variety or location, is possible. This would enable local operations to tailor their AD to their RS, decreasing the need for potentially expensive additional treatments and improve biomethane yields.

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Appendix A

List of Equations:

List of Tables:

Equations:

$$\text{Day corrected to 24h } = \frac{\text{H}}{24} + \text{d}^{-1} \quad \text{(A.1)}$$

$$\text{mL Biogas/g VS or mL } CH_4\text{/g VS} = \frac{\left(\frac{\text{Bio mL}}{\text{H}} * 24\right)}{\text{gVS R d}} \quad \text{(A.2)}$$

Where: H is the number of hours between gas measurements and d^{-1} is the day corr. from the previous day.

$$VhPA = 6.112 * exp^{\left(\frac{17.62*T}{243.12+T}\right)} \quad \text{(A.3)}$$

Where: VhPA is vapor pressure, T=temperature in °C

$$mlDbiogas.gVS = \frac{mlBiogas * \left(\frac{(LhPA-VhPA)*K}{SLhPA*IK}\right)}{gVS} \quad \text{(A.4)}$$

Where: Dbiogas is ml of dry biogas, LhPA is GC-FID analysis day air pressure, VhPA as Goff Gratch, K is 273.15 Kelvin, SLhPA is normal sea level air pressure, IK is K plus incubator temperature.

$$\%CH_4\ Corr. = \%CH_4 + (\%CH_4 - Pd\%CH_4) * \left(\frac{pdmlB}{pdmlB+H}\right) \quad \text{(A.5)}$$

Where: $PdCH_4$ is the corrected CH_4 from the previous day, PdmlB is the ml wet biogas from the previous day, and H is headspace in the bottle.

Table A.1: Gompertz modelled data for different rice straw particle sizes

Gompertz Fitted data[a]	**425 µm**	**1.0 mm**	**30 mm**	**70 mm**
Lag-phase (days)	0.45 (-0.06 - 0.95)	1.49 (1.20 - 1.78)	1.77 (1.44 - 2.11)	1.74 (1.41 - 2.07)
Maximum daily production rate (mL CH_4/g VS/d)	15.2 (13.9 - 16.6)	14.4 (13.7 - 15.1)	9.45 (8.99 - 9.91)	10.1 (9.63 - 10.7)
Ultimate methane yield (mL CH_4/g VS)	180 (175 - 186)	180 (176 - 184)	149 (145 - 154)	148 (144 - 152)
R-Sq.	0.994	0.998	0.998	0.998

Note: [a] Data from the Gompertz equation showing how well the experimental data fits the expected curve. (Figures in brackets indicate confidence intervals of > 95%)

Table A.2: Gompertz modelled data for co-digestion

Gompertz Fitted data[a]	RS100	RS96	RS80	RS75	RS40	DM100
Lag-phase (days)	0.46 (-0.04 - 0.48)	0.64 (0.29 - 0.99)	0.16 (-0.33 - 0.66)	-0.19 (0.94 - 0.55)	2.43 (2.17 - 2.70)	2.42 (2.1 - 2.8)
Maximum daily production rate (mL CH_4/g VS/d)	26.7 (23.9 - 29.5)	24.9 (22.7 - 27.1)	15.9 (14.1 - 17.7)	15.2 (12.9 - 17.5)	9.4 (8.6 - 10.2)	6.7 (5.9 - 7.4)
Ultimate methane yield (mL CH_4/g VS)	211 (206 - 215)	192 (189 - 196)	140 (136 - 143)	139 (134 - 144)	57.7 (56.7 - 58.7)	48.3 (47.2 - 49.4)
R-Sq.	0.992	0.995	0.991	0.982	0.996	0.995

Note: [a] Data from the Gompertz equation showing how well the experimental data fits the expected curve. (Figures in brackets indicate confidence intervals of > 95%)

Table A.3: Gompertz modelled data for different geographic origins

Gompertz Fitted data[a]	China	India	Philippines	Nigeria
Lag-phase (days)	5.1 (4.6 - 5.6)	5.7 (5.2 - 6.2)	5.1 (4.5 - 5.7)	4.9 (4.1 - 5.9)
Maximum daily production rate (mL CH_4/g VS/d)	15.1 (13.9 - 16.2)	13.6 (12.3 - 14.9)	17.7 (16.0 - 19.5)	19.5 (17.1 - 21.8)
Ultimate methane yield (mL CH_4/g VS)	234 (223 - 245)	155 (148 - 161)	216 (207 - 226)	362 (324 - 401)
R-Sq.	0.995	0.994	0.994	0.986

Note: [a] Data from the Gompertz equation showing how well the experimental data fits the expected curve. (Figures in brackets indicate confidence intervals of > 95%)

Table A.4: BMP statistics

Method: PERMANOVA		
Factor	***p-value***	***Sq.root of estimates of component of variation***
Country of origin	0.001	6.48
Method: DistLM		
Variable	***p-value***	***Cumulative variance explained (%)***
MARGINAL TESTS		
MJ/kg	0.078	-
VS (%)	0.031	-
Ash (%)	0.027	-
C (%)	0.009	-
N (%)	0.176	-
C:N	0.825	-
Neutral Detergent Fibre (%)	0.009	-
Crude Fibre (%)	0.014	-
Cellulose (%)	0.024	-
Hemicellulose (%)	0.009	-
Acid Detergent Lignin (%)	0.002	-
Acid Detergent Fibre (%)	0.012	-
Al (mg/kg)	0.002	-
As (mg/kg)	0.241	-

B (mg/kg)	0.206	-
Ba (mg/kg)	0.007	-
Ca (mg/kg)	0.001	-
Cd (mg/kg)	0.637	-
Co (mg/kg)	0.005	-
Cr (mg/kg)	0.001	-
Cu (mg/kg)	0.01	-
Fe (mg/kg)	0.001	-
K (mg/kg)	0.001	-
Mg (mg/kg)	0.014	-
Mn (mg/kg)	0.001	-
Mo (mg/kg)	0.475	-
Na (mg/kg)	0.164	-
Ni (mg/kg)	0.041	-
Si (mg/kg)	0.008	-
Ti (mg/kg)	0.003	-
Zn (mg/kg)	0.001	-
SEQUENTIAL TESTS		
+ Fe	0.001	51.3
+ Mg	0.001	76.5
+ Na	0.001	90.3
+ As	0.001	98.1

Appendix B

List of Figures:

NGS additional:

The PCRs included about 1-10 ng of DNA extract (total volume 1µl), 15 pmol of each forward primer and reverse primer (in 20 µL volume of 1 x MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline) and 2 µl of BioStabII PCR Enhancer (Sigma). For each sample, the forward and reverse primers had the same 10-nt barcode sequence. PCRs were carried out for 30 cycles using the following parameters: 2 min 96°C pre-denaturation; 96°C for 15 s, 50°C for 30 s, 70°C for 90 s. DNA concentration of amplicons of interest was determined by gel electrophoresis. About 20 ng amplicon DNA of each sample were pooled for up to 48 samples carrying different barcodes. If needed PCRs showing low yields were further amplified for 5 cycles. The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other small mispriming products, followed by an additional purification on MinElute columns (Qiagen). About 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN). Illumina libraries were pooled and size selected by preparative gel electrophoresis. Sequencing was done on an Illumina MiSeq using V3 Chemistry (Illumina).

A total of 910,226 quality filtered samples were obtained from 39 samples with the number of 16S rDNA sequences ranging from 12,069 to 58,577 (mean, 23,339). After removing the chimera sequences the operational taxonomic units (OTUs) were picked at more than 97 % similarity representing 9,720 OTUs. The alpha and beta diversity of the microbial community were then assessed.

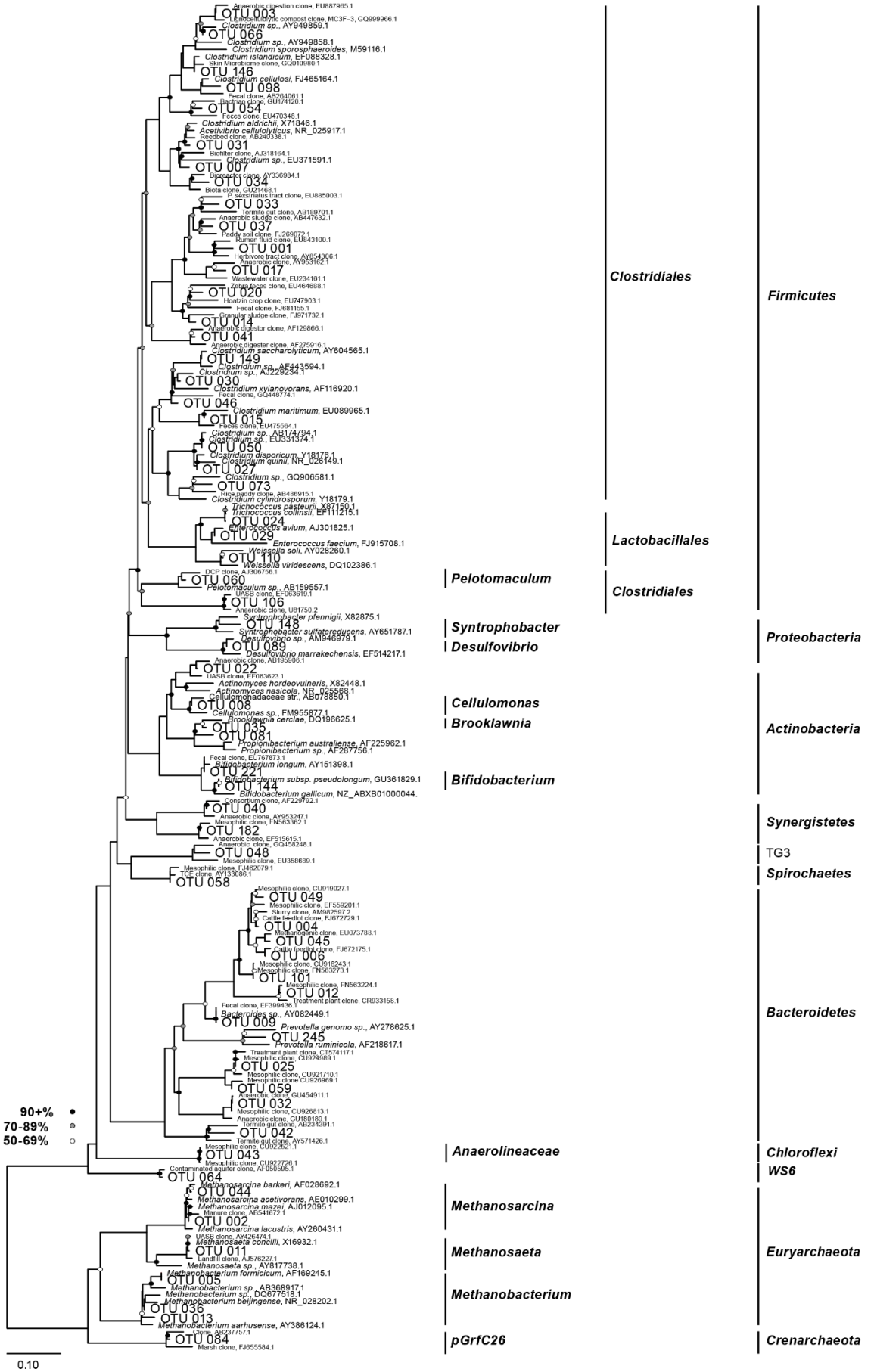

Figure B.1: Phylogenetic tree of shared predominant OTUs (only ≥ 0.5 % abundance)

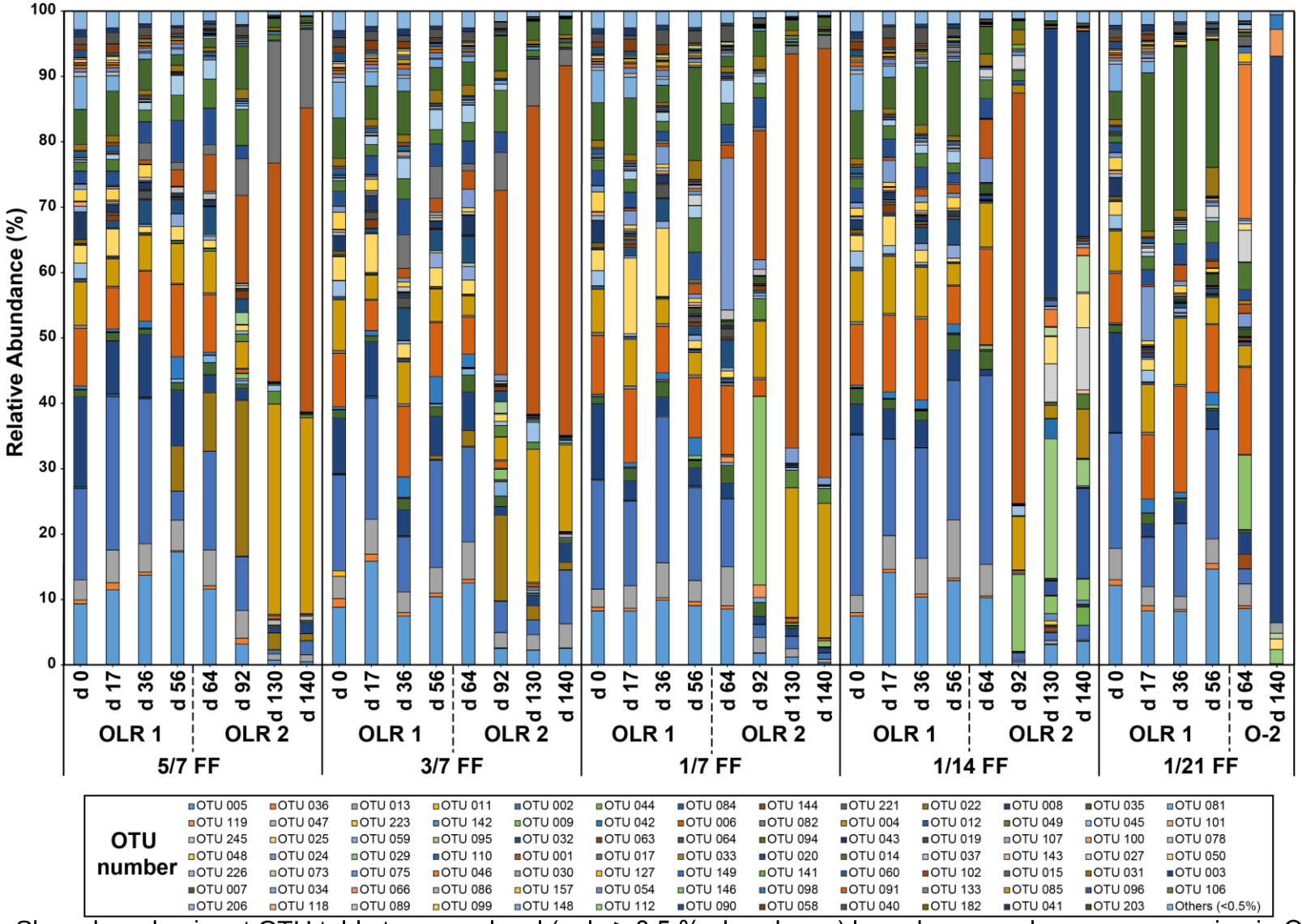

Figure B.2: Shared predominant OTU table to genus level (only ≥ 0.5 % abundance) based on sample appearances i.e. in OLR1, OLR2-T, OLR2-H, and/or, OLR2-S.

Appendix C

List of Tables:

List of Figures:

NGS additional:

A total of 419,747 quality filtered samples were obtained from 15 samples with the number of 16S rDNA sequences ranging from 10,864 to 48,127 (mean, 27,983). After removing the chimera sequences the operational taxonomic units (OTUs) were picked at more than 97 % similarity representing 9,720 OTUs. The alpha and beta diversity of the microbial community were then assessed.

Table C.1: Test statistics of beta diversity and physiochemical variables and operational factors

Method [a]: RELATE		
Variable	**Significance (%)**	**Rho**
Physiochemical data	**0.2 [b]**	0.677
Method: BEST		
Variable	**Physiochemical Correlation (R)**	
pH	0.725	
CH_4 %, Total VFAs, Formic and Isobutyric acid	0.724	
Method: DistLM		
Variable	**p-value**	**Cumulative variance explained (%)**
mL Biogas/g VS/d	**0.012**	-
CH_4 %	**0.039**	-
mL CH_4/g VS/d	**0.006**	-
g VS/L	**0.004**	-
% VSR	**0.021**	-
pH	**0.003**	-
Total VFA	**0.005**	-
Sequential		
+ pH	**0.002**	49.4 %

Method: ANOSIM			
Condition	**Factor**	**Global R**	**Significance level (%)**
Physiochemical	RS:DM	1.0	**0.3 %**
	HRT	0.75	**4.7 %**
Beta-diversity	RS:DM	0.81	**2.2 %**
	HRT	0.67	7.3 %

Method: PERMANOVA			
Condition	**Factor**	**p-value**	**Sq.root of estimates of component of variation**
Physiochemical	RS:DM	**0.002**	1.49
	HRT	**0.004**	0.57
Beta-diversity	RS:DM	**0.018**	2.62
	HRT	0.276	7.26

Notes: [a]Tests - RELATE, giving correlation of comparisons (Rho); BEST, trend correlation; DistLM, distance based linear model; ANOSIM, analysis of similarities; PERMANOVA, permutational multivariate analysis of variance.

[b]Bold indicates statistically significant results

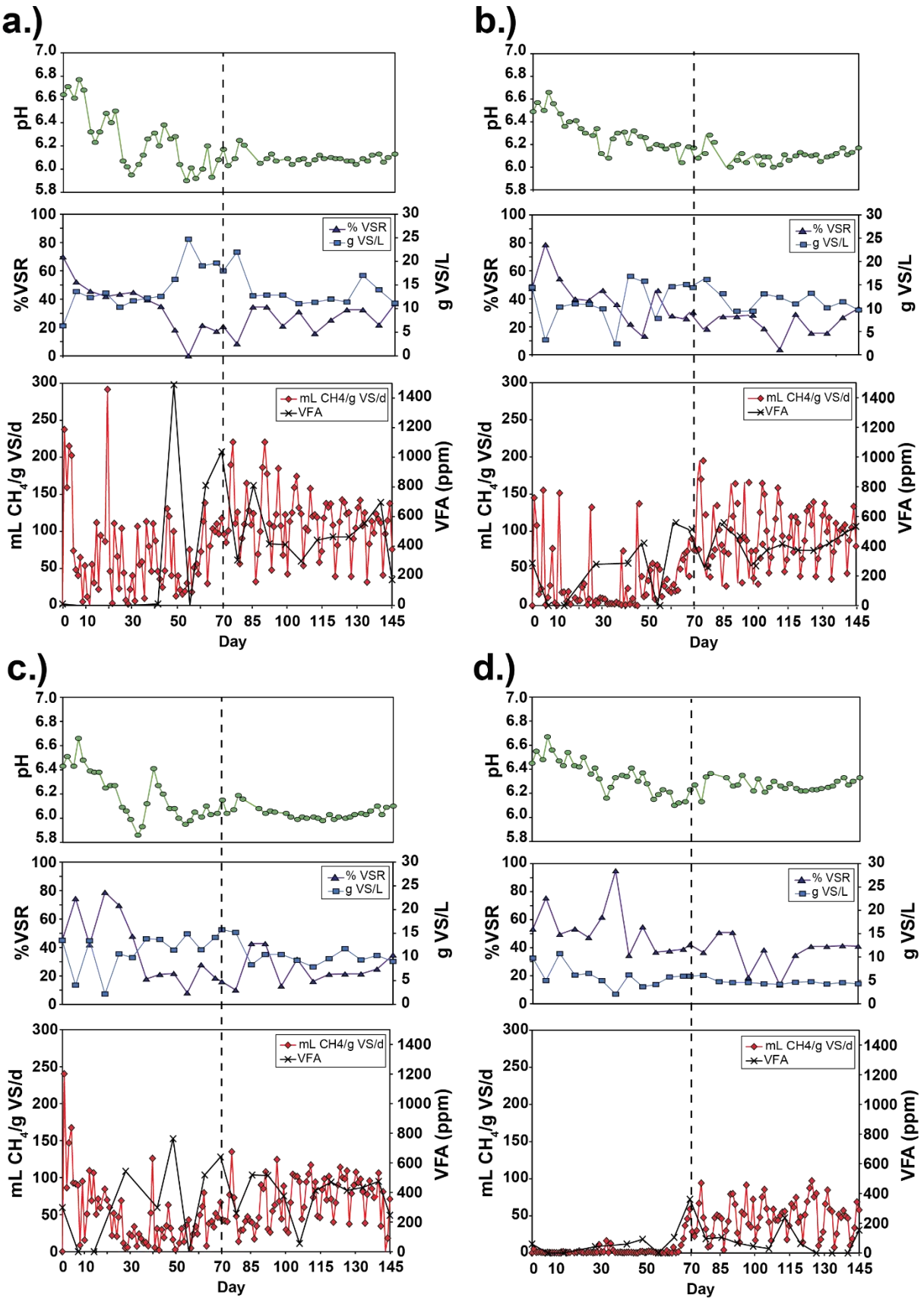

Figure C.1: Time-course performance data including acclimation (experiment 'Time 0' shown by dashed line)- pH, % VSR with g VS/L, and methane yield with VFA concentration, for: **a.)** RS100, **b.)** RS90, **c.)** RS70, **d.)** RS30f

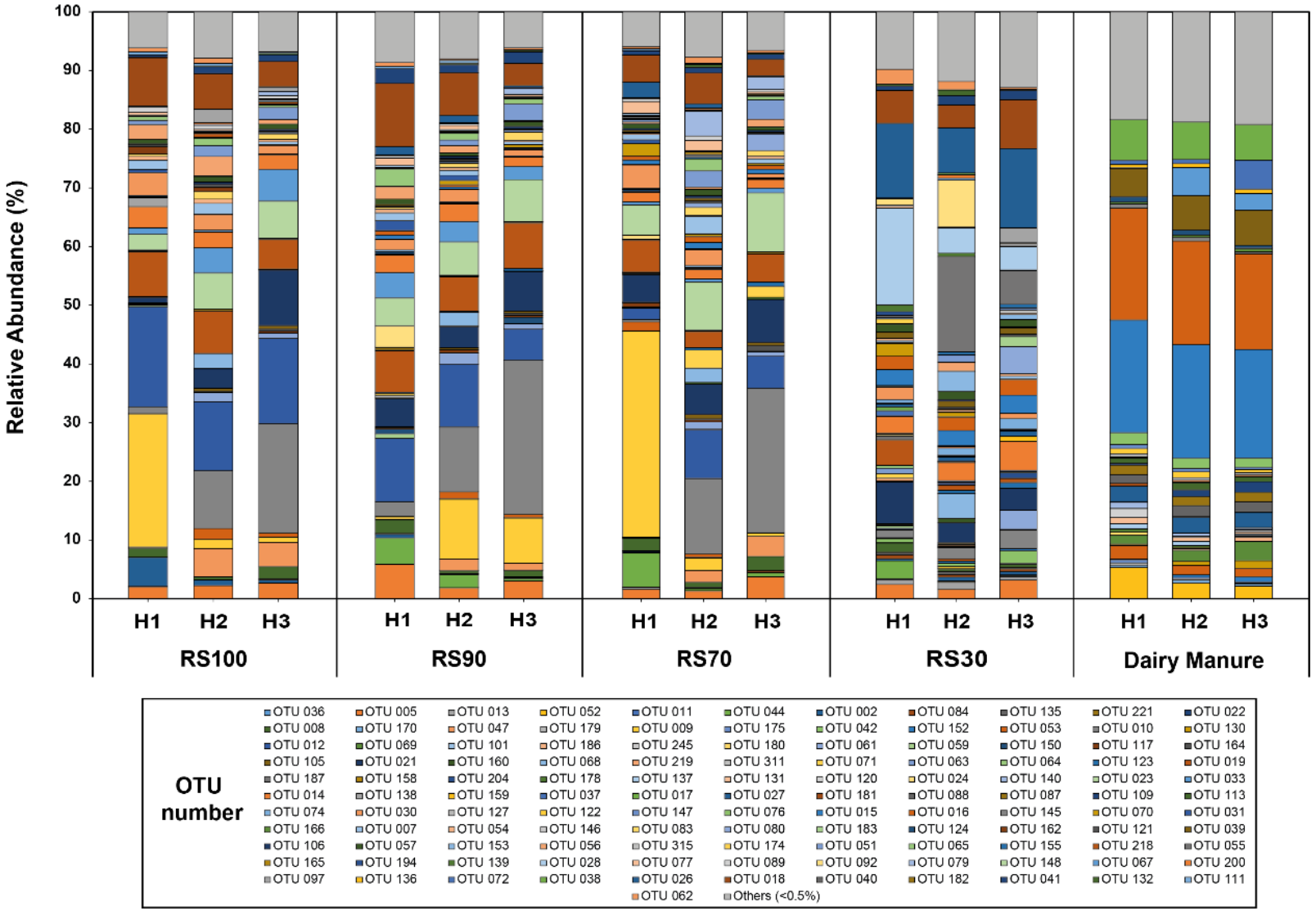

Figure C.2: Shared predominant OTU table to genus level (only ≥ 0.5 % abundance) based on sample appearances

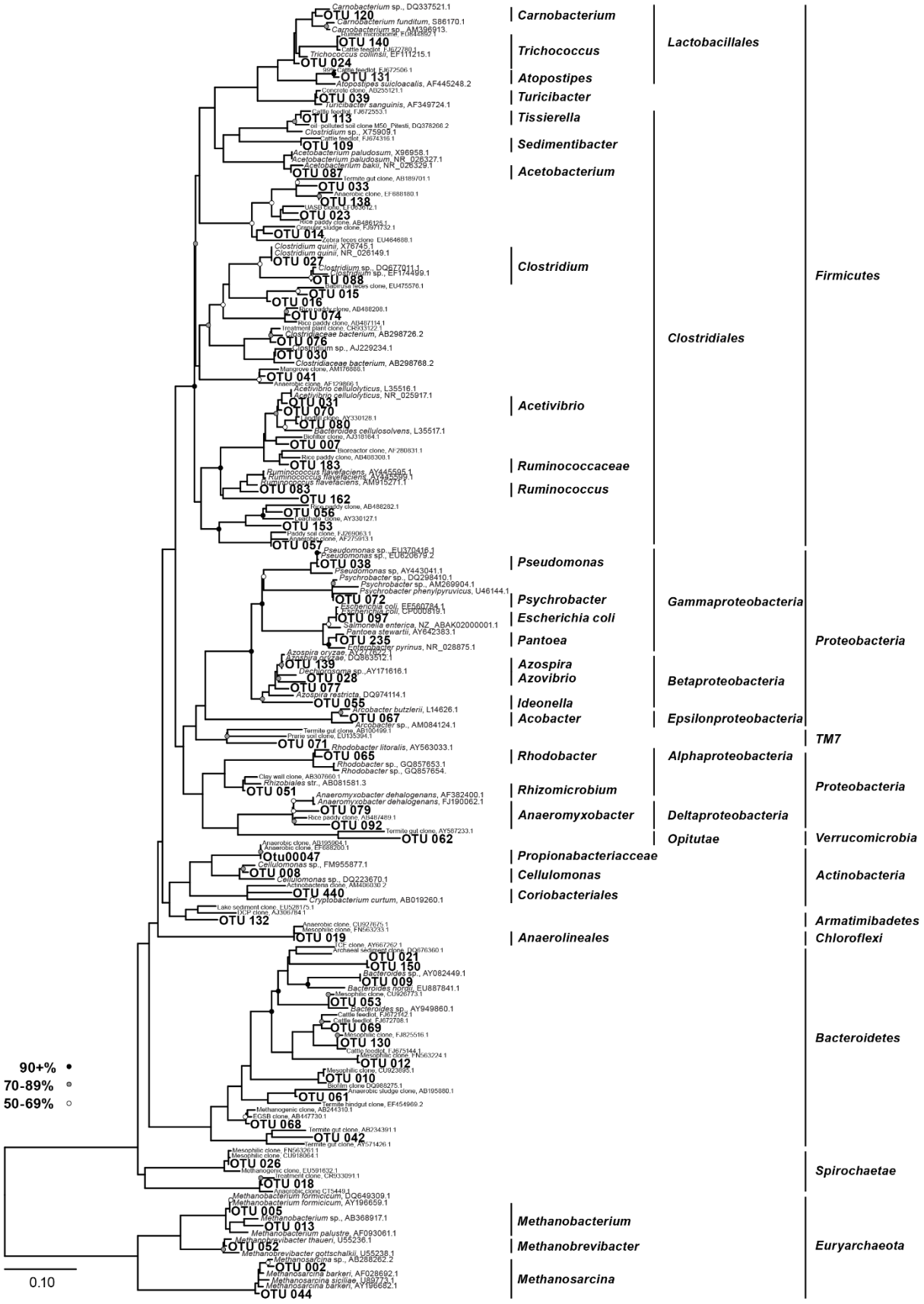

Figure C.3: Phylogenetic tree of shared predominant OTUs (only ≥ 0.5 % abundance)

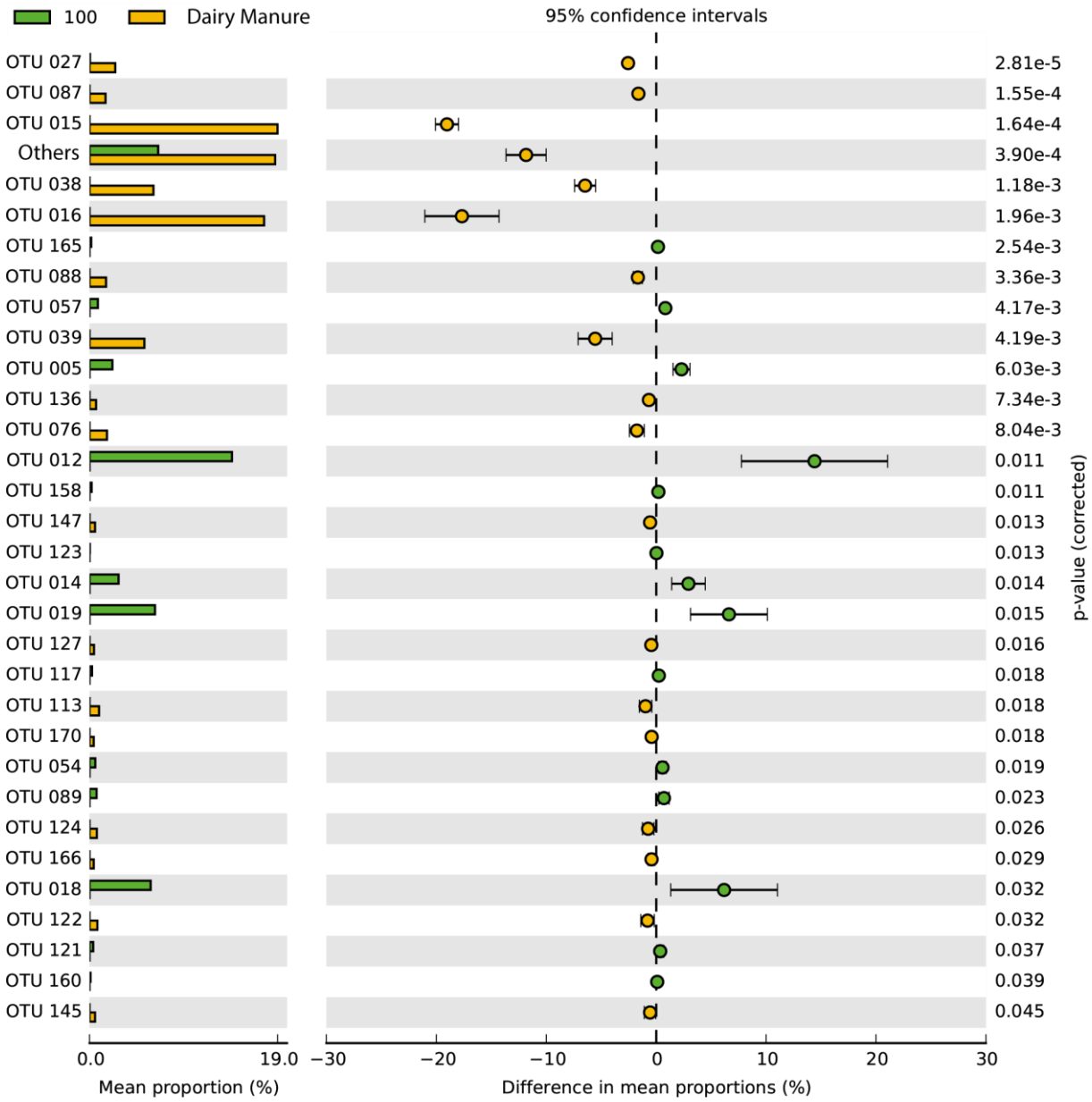

Figure C.4: Extended error bar plot of significant differences between predominant OTU of RS100 vs DM

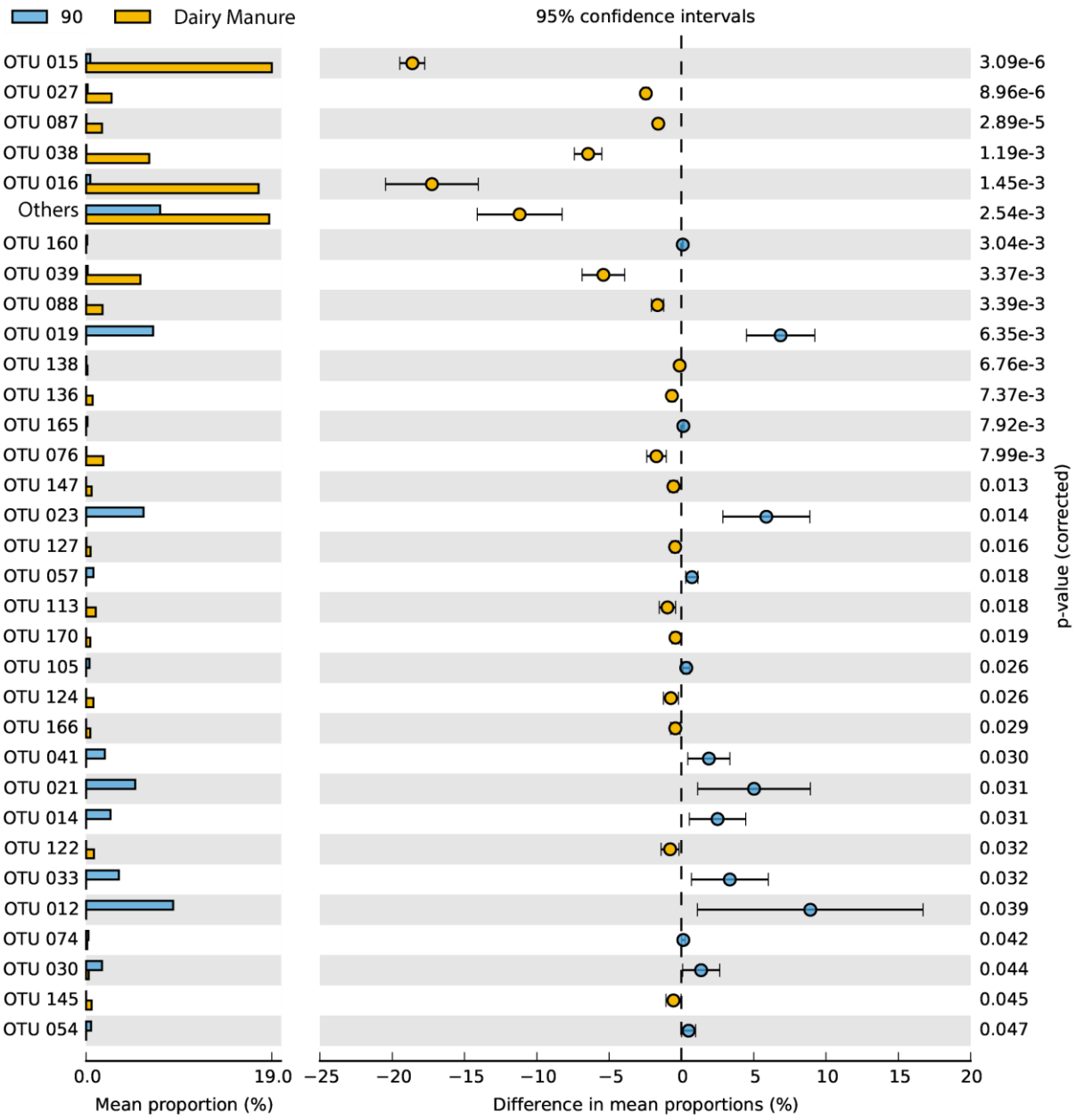

Figure C.5: Extended error bar plot of significant differences between predominant OTU of RS90 vs DM

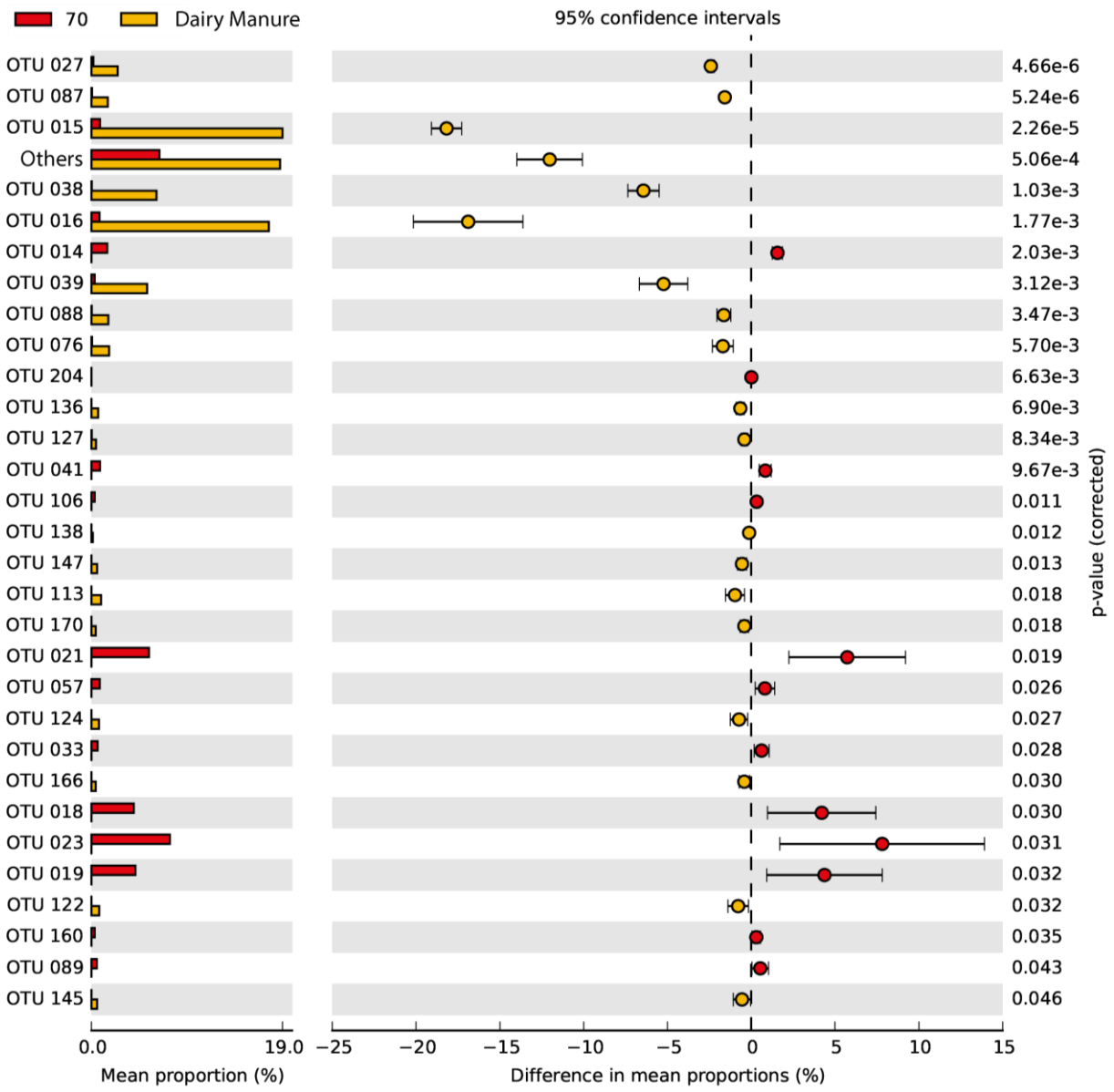

Figure C.6: Extended error bar plot of significant differences between predominant OTU of RS70 vs DM

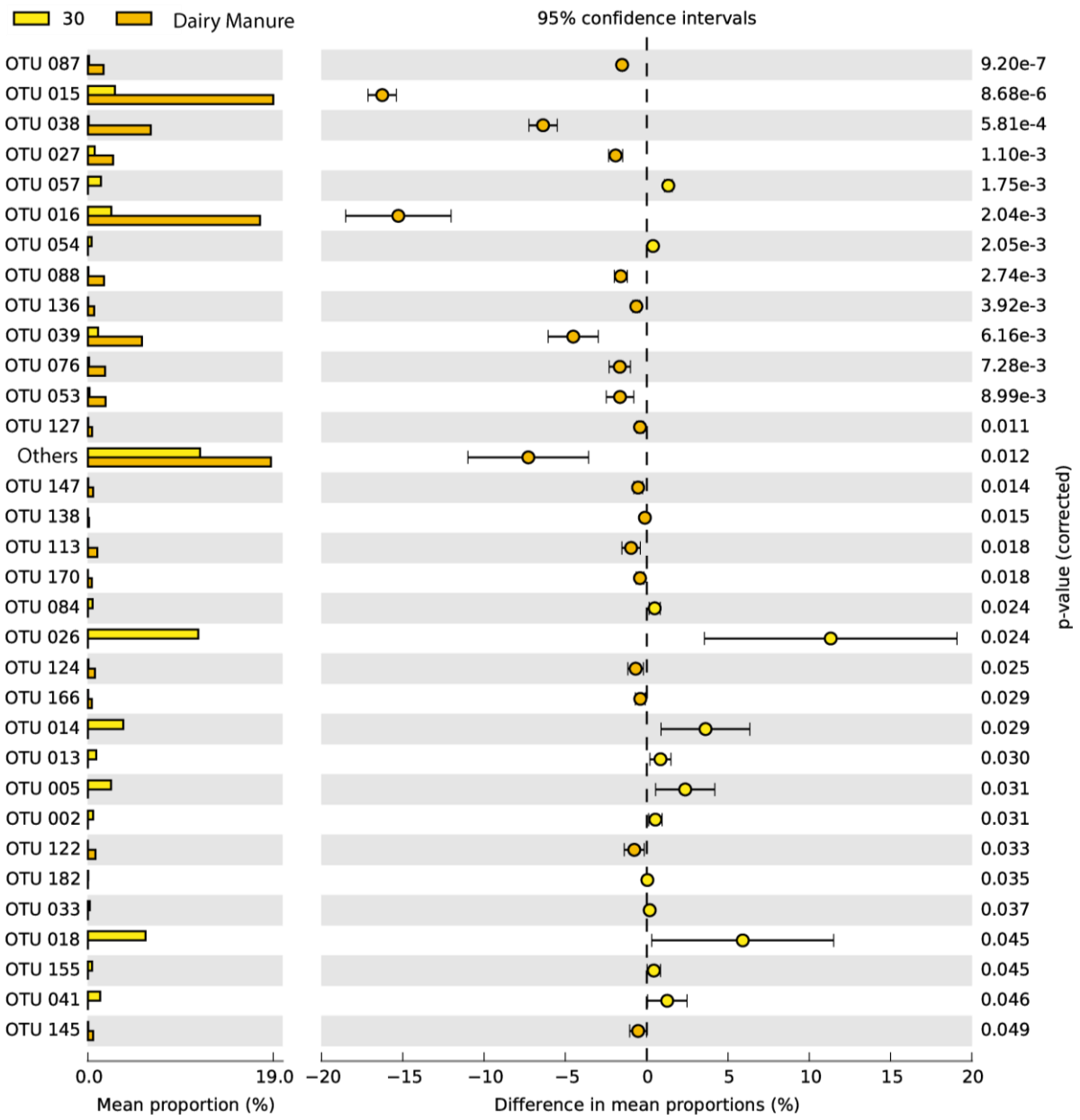

Figure C.7: Extended error bar plot of significant differences between predominant OTU of RS30 vs DM

The End