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**PURE CULTURE, CO-CULTURE AND
WHOLE ECOSYSTEM INVESTIGATIONS
OF SINGLE ANAEROBES, PARTNERSHIPS
AND MICROBIAL COMMUNITIES IN
ANAEROBIC DIGESTION**



A thesis submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy

by

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2017

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Table of contents

Acknowledgements.....	viii
Scientific Communications.....	x
Abstract.....	x
List of abbreviations and chemicals	xii
Chapter 1 - General Introduction	2
1.1 Short introduction to thesis.....	2
1.2 Motivations, aims and scope of the thesis	3
1.3 Organisation of thesis	4
1.4 References	5
Chapter 2 - Literature Review.....	7
2.1 Microbes and Anaerobic Environments	7
2.2 Anaerobic Digestion.....	8
2.2.1 Trophic community dynamics and energetics within AD	11
2.2.1.1 Hydrolysers	12
2.2.1.2 Acidogens / Fermenters	13
2.2.1.3 Acetogens.....	13
2.2.1.3.1 <i>Acetobacterium woodii</i>	15
2.2.1.4 Methanogens	17
2.2.1.4.1 <i>Methanosarcina barkeri</i>	19
2.2.1.4.2 <i>Methanococcus maripaludis</i>	23
2.2.1.4.3 Methanogenic Inhibition	25
2.2.1.5 Syntrophy	26
2.2.1.5.1 <i>Acetobacterium woodii</i> and <i>Methanosarcina barkeri</i>	28
2.2.1.6 Competition.....	28
2.2.1.6.1 <i>Methanosarcina barkeri</i> and <i>Methanococcus maripaludis</i>	29
2.3 Wastewater Treatment Systems	29
2.3.1 Sequencing batch reactors	32
2.3.2 Anaerobic granular sludge biofilms.....	33
2.3.3 Microbial community structure and function	37
2.3.4 Physiological analysis of AD systems	38
2.4 Development of microbial ecology	39
2.4.1 Plate count anomaly.....	39

2.4.2 Techniques used in molecular microbial ecology.....	40
2.4.2.1 Cloning.....	41
2.4.2.2 DGGE/TGGE.....	41
2.4.2.3 T-RFLP	43
2.4.2.4 FISH	44
2.4.2.5 SIP	47
2.4.3 High-throughput / Next generation sequencing (NGS) approaches	47
2.4.3.1 Sanger sequencing to NGS	48
2.4.3.2 Illumina sequencing platform	50
2.4.3.2.1 Active community insights.....	51
2.4.4 Sequencing data and statistical analyses.....	52
2.4.4.1 Sequencing data analysis.....	52
2.4.4.2 Alpha diversity	53
2.4.4.3 Beta diversity	53
2.4.5 Quantitative microbial ecology.....	55
2.4.6 The ‘Omics Era.....	58
2.5 Central concepts of the thesis	62
2.5.1 Microbial interplay in the acetogenic-methanogenic realm	62
2.5.2 The individual anaerobic sludge granule – a whole ecosystem.....	62
2.6 References	64

Chapter 3 - An investigation into microbial dynamics and interplay within and between model anaerobic digestion acetogenic and methanogenic players 100

Abstract..... 100

3.1 Introduction	101
3.2 Materials and Methods	108
3.2.1 Growth conditions and experimental set-up for anaerobic pure and co-cultures.....	108
3.2.2 Spectrophotometry.....	113
3.2.3 VFA and methane profiling	113
3.2.4 DNA extractions from cultures.....	114
3.2.5 Quantitative polymerase chain reaction analyses	114
3.2.6 Statistical analysis.....	116
3.3 Results	116

3.3.1 An examination of the adaptive flexibility within the metabolically versatile <i>Methanosarcina barkeri</i> (MB temperature study)	116
3.3.2 The growth response of methanogenic archaea co-culture to competitive growth (MB-MM co-culture experiment).....	119
3.3.3 Synergistic growth effects of a methanogenic archaea-homoacetogen co-culture (MB-AW co-culture study)	122
3.3.3.1 <i>M. barkeri</i> and <i>A. woodii</i> metabolic and growth profiling on H ₂ -CO ₂ at 35°C.....	122
3.3.3.2 Growth effects of co-culturing <i>M. barkeri</i> and <i>A. woodii</i> fed with H ₂ -CO ₂ at 15°C	125
3.3.3.3 Growth impacts of the <i>M. barkeri</i> and <i>A. woodii</i> co-culture grown on acetate at 35°C on pure cultures	127
3.3.3.4 <i>M. barkeri</i> and <i>A. woodii</i> growth and chemical profiles grown on acetate at 15°C.....	130
3.4 Discussion	132
3.4.1 Temperature impacts on the metabolism and growth of the flexible methanogen <i>Methanosarcina barkeri</i> (MB temperature study)	132
3.4.2 Competitive methanogenic study demonstrates preferential growth on co-substrates towards <i>Methanococcus maripaludis</i> (MB-MM co-culture experiment)	134
3.4.3 Characterisation of synergy between <i>Methanosarcina barkeri</i> and <i>Acetobacterium woodii</i> under various environmental conditions (MB-AW co-culture study)	135
3.4.3.1 <i>M. barkeri</i> and <i>A. woodii</i> growth on H ₂ -CO ₂	136
3.4.3.2 <i>M. barkeri</i> and <i>A. woodii</i> incubation on acetate.....	138
3.5 Conclusions	140
3.6 References	143
Chapter 4 - Physical, physiological and phylogenetic characterisation of individual, and size-resolved, anaerobic sludge granules	149
Abstract.....	149
4.1 Introduction	150
4.2 Materials and Methods	152
4.2.1 Biomass sources, particle size distribution and physical characterisation of granules.....	152
4.2.2 Specific Methanogenic Activity (SMA) assay	154
4.2.3 DNA/RNA co-extraction from biomass	155

4.2.4 Illumina MiSeq Analysis	155
4.2.5 Statistical Analysis.....	157
4.3 Results	157
4.3.1 Biomass characterisation of the three sludges	157
4.3.2 Physical profiles of size-resolved anaerobic granular sludges on a whole-sludge and single-granule level	158
4.3.3 SMA determination for anaerobic granular sludge from different sources according to specific size distributions.....	161
4.3.4 Phylogenetic characteristics of individual granules from various granular sources of specific sizes.....	164
4.4 Discussion	175
4.5 Conclusions	181
4.6 References	183
Chapter 5 - Physiological characterisation of size-resolved anaerobic granules and phylogenetic investigation of replication between individual granules.....	188
Abstract.....	188
5.1 Introduction	189
5.2 Materials and Methods	191
5.2.1 Source of biomass and granular sludge characteristics	191
5.2.2 Specific Methanogenic Activity (SMA) and Methanogenic Activity (MA) Assays	191
5.2.3 Sugar and volatile fatty acid profiling during the MA assay.....	191
5.2.4 Characterisation of individual granules	193
5.2.5 DNA/RNA co-extraction and cDNA synthesis	193
5.2.6 Illumina MiSeq Analysis	193
5.2.7 Statistical Analysis.....	194
5.3 Results	194
5.3.1 Effect of granular size on methanogenic activity	194
5.3.2 Sugar monitoring among granular size fractions during the MA assay	197
5.3.3 VFA dynamics in three granular size fractions during the MA assay	198
5.3.4 Physical characterisation of large-size individual granules.....	201
5.3.5 Microbial community structure of large-size single granules	201

5.3.6 Comparison between the potential and active community diversities within individual granules	205
5.4 Discussion	209
5.4.1 Impact of size on MA and VFA metabolism.....	209
5.4.2 Physical parameters of one hundred individual anaerobic granules...	211
5.4.3 Active community composition of sixteen individual granules	212
5.4.4 Potential versus active granular microbial community structures	213
5.5 Conclusions	215
5.6 References	216
Chapter 6 - High Throughput Characterisation of Single Whole Ecosystems: Anaerobic Granules in micro-Sequencing Batch Reactors.....	221
Abstract.....	221
6.1 Introduction	222
6.2 Materials and Methods	226
6.2.1 Anaerobic medium and stock solutions	226
6.2.2 Micro Sequencing Batch Reactors (μ SBRs).....	227
6.2.3 VFA analysis.....	229
6.2.4 Soluble sugar profiles	229
6.2.5 DNA/RNA co-extraction and cDNA synthesis from biomass	229
6.2.6 High-throughput cDNA sequencing and processing	230
6.2.7 Statistical Methods.....	230
6.3 Results	230
6.3.1 The metabolic impact of VFA-fed single granules incubated under various environmental parameters	230
6.3.2 The fate of VFAs within single granules incubated under acetate-feeding	234
6.3.3 Fermentative profiling of individual granules under cellulose- and glucose-feeding.....	235
6.3.4 16S rRNA bacterial and archaeal community composition.....	238
6.3.4.1 The effect of pH on VFA-metabolised single whole granules.....	240
6.3.4.2 Effect of substrate on the active 16S rRNA community structure of single granules.....	242
6.3.4.3 Impact of temperature on active 16S rRNA community structure of individual granules	245

6.3.4.4 Effect of cobalt-deprivation on active 16S rRNA community structure of a single granule	248
6.3.4.4.1 VFA-fed cobalt-deprived granules	248
6.3.4.4.2 Acetate-fed cobalt-deprived granules	251
6.3.4.5 Effects of methanogenic inhibitor on active 16S rRNA community structure of single granules	253
6.4 Discussion	265
6.4.1 Physiological and phylogenetic pH impacts on VFA-fed communities	266
6.4.2 Differences and similarities in the active community structure of single granules fed with a panel of substrates	267
6.4.3 The effects of low temperature on individual granules	270
6.4.4 Impact of cobalt deprivation on individual granular community metabolism and structure	271
6.4.5 The inhibitory effect of BES on the VFA and community structure profiles of individual granules	273
6.5 Conclusions	274
6.6 References	277

Chapter 7 - Final Conclusions, Perspectives and Future

Recommendations	286
7.1 Concluding Discussion	286
7.1.1 Detailed characterisation of anaerobic pure cultures and the influences of co-cultures	286
7.1.2 Differences between granule sizes and types	288
7.1.3 Single granules as whole eco-systems	288
7.1.4 High-throughput characterisation for granular ecological studies and modelling	289
7.1.5 Novel and main findings from the thesis	289
7.2 Future directions	290
7.2.1 What the findings of the thesis mean for the AD field of research	290
7.2.2 Alternative lines of investigation	291
7.2.3 Opportunities following this work	292
7.3 Final comments and some outlooks on the future of anaerobic microbial ecology	294
7.4 References	296

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Scientific Communications

Presentations

Oral

1. International Symposium on Microbial Ecology (ISME), Montreal 2016
“High-throughput characterisation of whole ecosystems: anaerobic granules in micro-sequencing batch reactors”.

Poster

1. Society of Microbiology (SGM), NUI Galway 2015
“An insight into the comparative kinetic behaviour of methanogenic and acetogenic microbial strains in co-culture systems”.

2. Martin Ryan Institute Research Day, NUI Galway 2015
“An insight into the behaviour of acetate and methane producers in co-culture systems”.

Papers in Preparation:

1. Examination of growth and chemical profiles of the acetogenic methanogen *Methanosarcina barkeri* with the homoacetogen *Acetobacterium woodii* and with the hydrogenotrophic methanogen *Methanococcus maripaludis*.
2. Physical, physiological and phylogenetic traits of size-resolved anaerobic sludge granules from different wastewater treatment plants.
3. High-throughput physiological and phylogenetic characterisation of individual anaerobic granules as whole ecosystems under environmental stresses.

Abstract

Anaerobic digestion (AD) is a multi-stage process whereby biodegradable material is broken down by complex microbial consortia into renewable CH₄ in the absence of oxygen during waste and wastewater treatment. Anaerobic granules are spherical microbial biofilms which form in digesters such as upflow anaerobic sludge blanket (UASB) and expanded granular sludge blanket (EGSB) systems. There is a fundamental knowledge gap in the microbial dynamics and metabolic interactions among microorganisms within AD bioreactors.

This thesis addressed some growth and chemical aspects resulting from microbial interplay between three model pure cultures found in AD digesters under various temperature and substrate conditions. The methanogen *Methanosarcina barkeri* is a metabolically diverse organism, independent of temperature, which was demonstrated to grow synergistically with the homoacetogen *Acetobacterium woodii*. The competitive interaction between *Methanosarcina barkeri* and the hydrogenotrophic methanogen *Methanococcus maripaludis* is novel within the literature. *Methanococcus maripaludis* out-competed *Methanosarcina barkeri* for H₂-CO₂ but both pure cultures were able to grow together.

Physical, physiological and phylogenetic properties of anaerobic granules were significantly different at three designated size distributions. Physiological and phylogenetic characteristics of anaerobic granules from three sources were significantly different. Individual anaerobic granules had significantly similar physical characteristics and active community structures based on 16S rRNA Illumina sequencing. Single anaerobic granules were described as “whole ecosystems” because they had a statistically similar active microbial profile.

Micro Sequencing Batch Reactors were employed to analyse how individual anaerobic granules/“whole ecosystems” respond to various environmental stresses. Results from VFA profiling and sequencing indicated that single granules demonstrated replicated shifts in metabolic and community structure patterns.

In conclusion, a combination of both pure cultures and high-throughput sequencing studies of mixed microbial communities show potential as one tool to underpin the understanding of complex systems such as anaerobic digestion.

List of abbreviations and chemicals

AD	Anaerobic Digestion
<i>A. woodii</i>	<i>Acetobacterium woodii</i>
BES	2-Bromoethanesulfonate
CH ₄	Methane
CO ₂	Carbon Dioxide
CO	Carbon Monoxide
CSTR	Continuously Stirred Tank Reactor
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
EGSB	Expanded Granular Sludge Bed
FB	Fluidised Bed
FISH	Fluorescent <i>in situ</i> Hybridisation
GC	Gas Chromatography
H ₂	Hydrogen
HRT	Hydraulic Retention Time
<i>M. barkeri</i>	<i>Methanosarcina barkeri</i>
<i>M. maripaludis</i>	<i>Methanococcus maripaludis</i>
MB-AW	<i>Methanosarcina barkeri</i> - <i>Acetobacterium woodii</i>
MB-MM	<i>Methanosarcina barkeri</i> - <i>Methanococcus maripaludis</i>
μSBR	micro-Sequencing Batch Reactor
NGS	Next Generation/High-Throughput Sequencing
qPCR	quantitative Polymerase Chain Reaction

rRNA	ribosomal Ribonucleic Acid
SIP	Stable Isotope Probing
SMA	Specific Methanogenic Activity
SRT	Sludge Retention Time
TGGE	Temperature Gradient Gel Electrophoresis
TPAD	Temperature Phase Anaerobic Digestion
TRFLP	Terminal Restriction Fragment Length Polymorphism
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acid

Chapter 1

Chapter 1 - General Introduction

1.1 Short introduction to thesis

Microorganisms are important to the environment and are arguably vital to human life due to the vast range of roles they play in biogeochemical cycling, decomposition, manufacture of pharmaceuticals, food production, water treatment and gut immunity and digestion. The global environment contains many natural variations, such as disparities in temperature, pH, and substrate availability. A difference in the water, sunlight, metals, carbon sources and, particularly, oxygen present can have an effect on a microorganism's survival rates. Aerobic and anaerobic systems contain vastly different microbial communities due to the different capabilities of the trillions of microbes in existence around Planet Earth. It is estimated that there are between 9.2×10^{29} and 31.7×10^{29} microbes in the global sub-seafloor sediments alone (Kallmeyer et al., 2012). Anaerobic communities prevail in habitats ranging from rumen to gastrointestinal tracts and from geothermal sources to sediments. Anaerobic microbes have a broad metabolic potential to survive in many types of environments, including under extreme conditions.

Anaerobic digestion (AD) is a multi-stage series of interactions between different types of microorganisms resulting in the breakdown of complex organic materials into CH_4 in the absence of oxygen. CH_4 is one of the most significant greenhouse gases, having contributed to approximately 20% of post-industrial global warming (Knittel and Boetius, 2009). AD plays an important role in waste and wastewater treatment. Although CH_4 has the potential to increase global warming, biogas can be used as a renewable energy source if this energy is harnessed. Although there have been many advancements in the fields of AD microbiology and the ecology surrounding the microorganisms, it remains a challenge for researchers to understand this complex system fully. There is a knowledge gap regarding the microbiology that underpins the AD process, especially the specific synergies and competitions between the individual species involved. Both pure culture and whole-community insights may help to provide essential insight into the AD process. This work can then be combined with process data to optimise reactor performance and maximise CH_4 production for the use of this renewable source of energy.

1.2 Motivations, aims and scope of the thesis

The overall objective of this thesis was to investigate the microbial interplay among individual key microorganisms responsible for methanogenesis on a pure culture level and on a co-culture level, as well as to gain an insight into the anaerobic granular biofilms within AD systems in order to investigate their response to environmental stresses at a single granule level.

The aim of the first part of this work was to investigate and follow the growth and temporal chemical profiles of key anaerobic pure cultures and co-cultures involved in waste and wastewater treatment at different temperatures and substrates. The three model AD organisms studied were the methanogen *Methanosarcina barkeri*, the homoacetogen *Acetobacterium woodii* and the hydrogenotrophic methanogen *Methanococcus maripaludis*. These were chosen because they are commonly found in anaerobic digesters and their genomes have previously been sequenced. The objectives were to investigate the microbial interplay between the synergistic *M. barkeri* and *A. woodii* and the competitive *M. barkeri* and *M. maripaludis* as well as to conduct a temperature profile study of the metabolically-flexible *M. barkeri* in order to examine whether the flexibility is dependent on temperature. These experiments gave an indication of fitness and this information can be applied to running these digesters e.g. by providing more of the preferred or most desirable species to a digester in order to create a higher yield of methane.

As well as examining key individual microorganisms that are found within AD systems, anaerobic granules were also investigated. Individual anaerobic granules provide key information about the community structure that forms spontaneously within an anaerobic digester. The second part of this thesis (Chapter 4) aimed to focus on the study of these granules, based on three specific sizes and from three separate sources of granular sludge, in order to determine whether there are differences or similarities between granular size and granular sludge type from physical, physiological and phylogenetic perspectives.

Subsequently, physical characteristics and the active community structures of individual granules were analysed to determine whether single granules were similar to each other (Chapter 5). The last part of this thesis (Chapter 6) aimed to

examine the physiological and phylogenetic response of the active microbial community structure of individual granules to various environmental stresses.

Collectively, the benefits of this series of studies are in advancing the understanding of the microbial community that underpins the AD process by the elucidation of the anaerobes' models and the investigation of anaerobic granules at a single granule level. Henceforth, these types of studies and potential data could be used as model systems when predicting how changes in operational parameters can affect the anaerobic microbial communities within bioreactors.

1.3 Organisation of thesis

The purpose of this introductory chapter is to introduce and engage the reader with the motivations for this research. Chapter 2 is a literature review which details the microbiology associated with AD in waste and wastewater treatment technologies and the microbial techniques used to study anaerobic pure cultures and co-cultures and the anaerobic granular microbial community systems. The literature review is followed by four experimental chapters. Chapter 3 presents growth and chemical information regarding the microbial interplay of model microorganisms involved in AD based on growth rates, substrate uptake and product accumulation.

Chapter 4 presents work on physico-chemical and ecological insights into various types of anaerobic granular sludge from different sources and at different sizes.

Chapter 5 sets out the investigation into the fermentative and methanogenic activities of different size fractions from one sludge type and analysed the active community structure of 16 individual granules. Chapter 6 presents the examination of the active whole-community response of individual granules to various environmental stresses. Finally, Chapter 7 summarises this research and the general conclusions drawn from the entire pure culture, co-culture and mixed-culture community investigations. Future work on these topics is also recommended. The references for each piece of work are listed at the end of the relevant chapter.

1.4 References

- KALLMEYER, J., POCKALNY, R., ADHIKARI, R. R., SMITH, D. C. & D'HONDT, S. 2012. Global distribution of microbial abundance and biomass in subseafloor sediment. *Proceedings of the National Academy of Sciences*, 109, 16213-16216.
- KNITTEL, K. & BOETIUS, A. 2009. Anaerobic oxidation of methane: Progress with an unknown process. *Annual Review of Microbiology*, 63, 311-334.

Chapter 2

Chapter 2 - Literature Review

2.1 Microbes and Anaerobic Environments

Through the many years of microbial ecological research, it has become abundantly clear that bacteria and archaea play a crucial role in global systems processes. Their vast capabilities to survive in virtually every part of the earth and the adaptations they undergo in order to do so demonstrate their importance in terms of their role in biogeochemical cycling processes (Prosser et al., 2007). Calculations have suggested that microbial carbon and nitrogen could be approximately ten times greater than the carbon and nitrogen that is stored within plants (Whitman et al., 1998). A thorough understanding the ecology of microorganisms is vital to several different contemporary anthropogenic interests, from the mitigation of climate change to sustaining the natural ecosystems and industrial processes such as pharmaceutical production and wastewater treatment (Prosser et al., 2007). It is the microbial processes that have driven and will drive these important developments. The more that is understood about the communities and the factors which influence them, the more efficiently they can be optimised.

There are countless areas throughout the globe where oxygen fails to reach. These anaerobic communities include soil, lakes, marine sediments, guts of animals and landfill sites (Figure 2.1). Anaerobic microorganisms are ubiquitous among these types of environments.

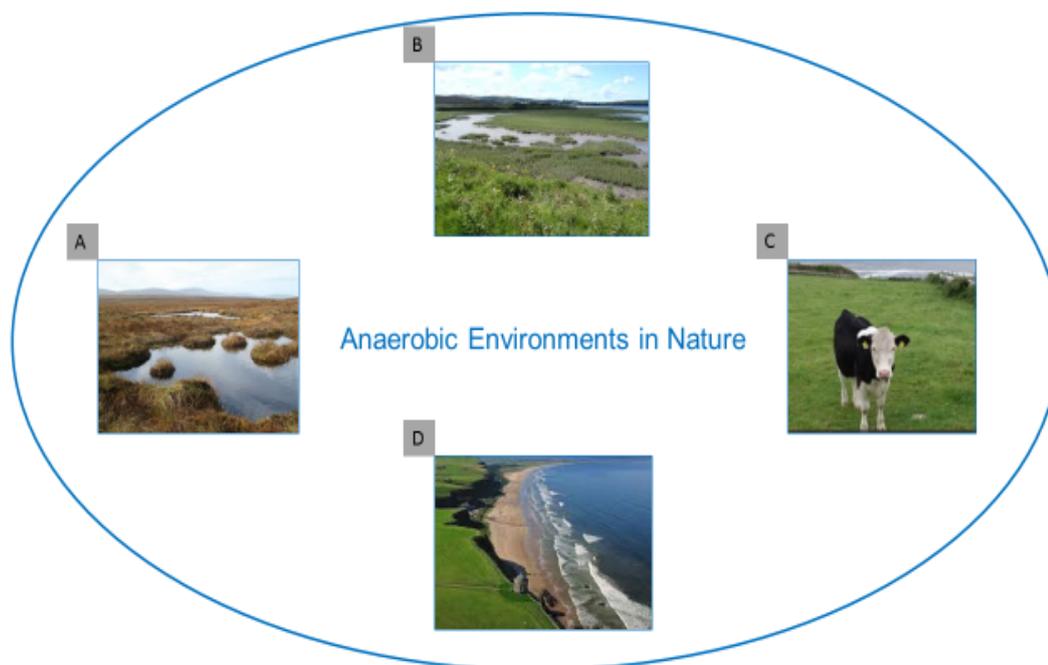


Figure 2.1: Examples of anaerobic environments: (A) bogs, (B) marshes, (C) intestinal tract of animals and (D) ocean sediments.

2.2 Anaerobic Digestion

Global CO₂ emissions have become a major concern in terms of their impact on climate change and global warming. It has been estimated that flue gas exhausts from power plants have been between 3-15% CO₂ in majority N₂ (Aaron and Tsouris, 2005). Despite commitments and strategies put into writing, sequestration has become less popular due to several societal factors. As a result, the focus of remedial efforts reverts to attempting to convert these large CO₂ emissions to useful products such as renewable fuels. Anaerobic digestion (AD) is one such process that can produce a renewable fuel (biogas) due to acetogenesis and methanogenesis.

AD is a process by which consortia of microbial communities break down organic materials into simpler molecules, and ultimately result in the production of CH₄ and CO₂. There are several advantages of AD to aerobic digestion. These include the use of digestate from AD as a valuable fertiliser because of its better nitrogen availability and short-term fertilisation effect, the reduction of pathogenic survival and the production of biogas as a renewable alternative to fossil fuels for heating, power generation and fuel for vehicles (Weiland, 2010). Biogas was termed one of the most efficient and environmentally valuable bioenergy production technologies

(Fehrenbach, 2008). The disadvantages associated with AD are that there is a long start-up time associated with anaerobic digesters and that the biomass that is produced by the reactors is itself a type of waste. The capture and conversion of CO₂ from industrial plant emissions would also require some removal of oxygen prior to treatment of this flue gas because it generally contains some residual oxygen (Goyal et al., 2016). Its main environmental aim is to reduce pollution and to produce CH₄ to be used as a biofuel.

Currently the majority of full-scale treatment systems operate above 18°C for optimal microbial activity. Interest in developing low temperature AD (LtAD) has increased over the past couple of decades due to the reduced treatment costs associated with wastes streams at sub-ambient temperatures, resulting in little or no heating requirements. This increases the attractiveness of AD as a waste and wastewater treatment technology (Lettinga et al., 2001, Connaughton et al., 2006, McHugh et al., 2006, McKeown et al., 2009b).

Microorganisms have been found in many cold environments that have been studied, from mountains to the deep sea, and these psychrophilic ecosystems are involved in the global nutrient cycles. The integrity of the microbial community is compromised under low temperatures, and effects on cellular structure and various cellular functionalities, such as decreases in diffusion, transcription, translation and cell division, have been observed (D'Amico et al., 2006). There have been studies that reported anaerobic degradation of organic matter being observed at temperatures as low as 2°C (Nozhevnikova et al., 1997).

Other studies have been conducted using bioreactors operated at low temperatures and these have proven to be successful as a potential alternative to mesophilic AD, with acclimatisation being followed by enhanced methanogenic activity (Rebac et al., 1999, Lettinga et al., 2001, Collins et al., 2003, McHugh et al., 2003b, McHugh et al., 2004, Collins et al., 2006, Connaughton et al., 2006, O'Reilly et al., 2009). The design and development of the granular sludge-based EGSB-style bioreactor (more detailed discussion in section 2.3) proved to be a milestone for LtAD research (Kato et al., 1999).

Its primary disadvantage is that growth rates are lower as degradation takes longer to achieve in AD at low temperatures with much longer lag phases corresponding to decreasing temperatures. Homoacetogenesis is considered to be one of the most important steps in low temperature ecosystems such as in tundra soil (Kotsyurbenko et al., 1996). A dominance of acetoclastic methanogens have been observed in engineered LtAD reactors (Akila and Chandra, 2007), which is consistent with the finding that it is acetogens in particular that appear to benefit at low temperatures due to energy gain from the syntrophic volatile fatty acid (VFA) degradation limitation and elevated autotrophic acetogenesis (Nozhevnikova et al., 2000, Kotsyurbenko, 2005).

The acetoclastic methanogen *Methanosarcina* (section 2.2.1.4.1) was demonstrated to be associated with a psychrophilic homoacetogenic bacteria in cold climates, and acetogens could potentially have an increased role at lower temperatures through a syntrophic interaction (Kotsyurbenko et al., 1993). Despite the findings that acetogens thrive at low temperatures, other studies have reported that under low H₂ partial pressures, hydrogenotrophic methanogens may be capable of out-competing acetogens due to their higher affinity for H₂ leading to CH₄ production through this pathway as demonstrated in engineered systems (Syutsubo et al., 2008, Enright et al., 2009, McKeown et al., 2009a, McKeown et al., 2009b, O'Reilly et al., 2009, Madden et al., 2010).

Although mesophilic and thermophilic methanogenesis in AD bioreactors are comparatively well documented, psychrophilic methanogenesis occurs in most aquatic and terrestrial environments, with up to approximately 75% of the Earth's biosphere being maintained at cold temperatures. Psychrophilic archaea are found in these permanently cold environments (Madigan, 2000, Schleper et al., 2005, Cavicchioli, 2006). Due to this, there is a growing interest in LtAD as a viable technology with financial and sustainability advantages. In order to progress in this field, additional investigations into biochemical pathways and microbial interactions are required (Kotsyurbenko et al., 2004, Metje and Frenzel, 2007).

2.2.1 Trophic community dynamics and energetics within AD

AD comprises the degradation of complex organic materials in the absence of oxygen by means of a multi-stage pathway (Figure 2.2). Various groups of microorganisms are required for the breakdown of organic compounds.

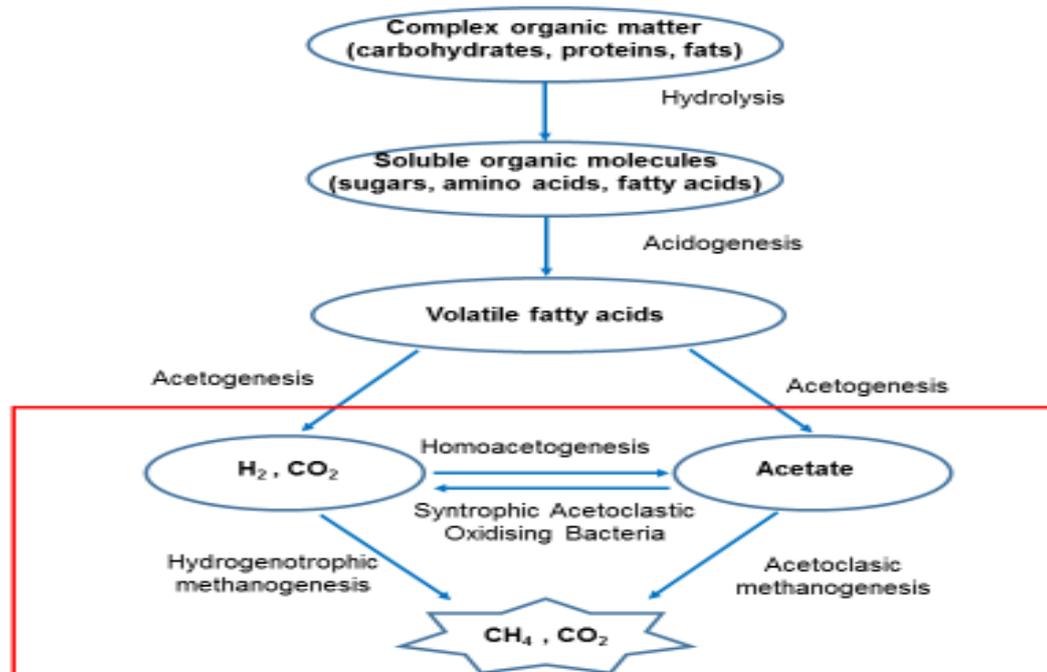


Figure 2.2: The stages and microorganism trophic groups of AD. The red box highlights the trophic groups to which the pure cultures studied for this work belong (Chapter 3). Adapted from (Chen et al., 2014).

Since their discovery, archaea have garnered much attention for their ability to grow in difficult and extreme environments and their important role in industrial processes (Schiraldi et al., 2002, Stetter, 2006, Sharma et al., 2012). Anaerobic microorganisms have a tremendous ability to survive under energetic limitations by evolving strategic mechanisms which have enabled archaea to conserve even small amounts of energy (Mayer and Müller, 2014). Gibbs free energy is one way of measuring thermodynamic potentials. It calculates either the maximum or reversible work that occurs within a thermodynamic system at both constant pressure and temperature. ΔG^0 is favourable for reactions that are less than zero and are unfavourable for reactions zero or greater. The calculation is as follows:

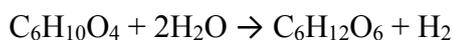
ΔG (change in Gibbs free energy) = ΔH (change in enthalpy) – T (standard temperature) * ΔS (change in entropy)

The standard change in enthalpy refers to the total heat evolved due to the reaction at both constant pressure and temperature in the absence of work and is often the same as the change in enthalpy provided that the solutions are equal (Alberty, 1969). Entropy is a measure of how dispersed a systems energy is. Gas phase entropies are larger than for liquids or solids phases of the same substance because a gas can expand throughout a space easier and faster than a liquid for example. It can be calculated from thermodynamic tables.

Redox potentials measure the potential balance between electron acceptors and donors in chemical species that are subsequently reduced and oxidised, respectively. Environments with a low redox potential and short electron transport chains are ubiquitous throughout nature, particularly in the deeper zones on earth due to oxygen depletion and hydrogen sulphide formation when organic matter is anaerobically degraded (Worm et al., 2014). Methanogens are dependent on low redox environments to maintain their growth. Mass balances are crucial for thermodynamics processes to ensure mass conservation along the AD pathway. The major players in this process are:

2.2.1.1 Hydrolyzers

Hydrolyzers are organisms that are present in the initial stage of AD: hydrolysis. They degrade complex organic polymeric substrates (carbohydrates, lipids and proteins) into either soluble monomeric or dimeric substrates (monosaccharides, long-chain fatty acids plus glycerol, and amino acids) (Sevier and Kaiser, 2002, Kim et al., 2003). Examples of common hydrolyzers include bacteria such as *Streptococcus* and *Enterobacterium*. Hydrolysis can limit the rate of the overall AD process involving particulate matter such as adipic acid:



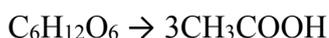
(Batstone et al., 2002) and thus can suffer from low efficiency (Appels et al., 2008).

2.2.1.2 Acidogens / Fermenters

Acidogens/fermenters initiate the second stage in the AD process: acidogenesis, whereby there is continued fermentation of the monomeric products and further degradation into various short-chain fatty acids and alcohols. The various end-products consist typically of propanoic acid, butyric acid, acetate, formate, methanol, H₂ and CO₂ (Fang and Liu, 2002). The H₂ partial pressure impacts on the products in this phase so that, if it was too high, it would decrease the amount of reduced compounds (Gerardi, 2003).

The acid phase bacteria that are facultative anaerobes can consume any oxygen that has been accidentally introduced into the reactor and can subsequently create some favourable conditions for the growth of obligatory anaerobes such as *Clostridium*, *Bacillus* and *Pseudomonas* (Shah et al., 2014). Fermentation in anaerobic processes usually results in intermediates production from complex substrates to create organic acids and solvents for example (Temudo et al., 2007).

Several types of reactions can occur during acidogenesis such as:



2.2.1.3 Acetogens

Acidogenic products are further oxidised to H₂-CO₂ or acetate in processes referred to as acetogenesis, and are completed by either obligate H₂ producing acetogens (OHPAs) (Schink et al., 1992, Stams, 1994) or homoacetogens. Acetogenesis was discovered in 1932 (Fischer, 1932). CO₂ is ubiquitous within nature, especially in anaerobic habitats, due to its importance for chemoorganotrophic metabolism. Acetogens are one of the groups of strictly anaerobic prokaryotes that use CO₂ as an electron acceptor. Acetogenic bacteria are important members of the AD pathway as they are capable of utilising various other common compounds found within the anaerobic food web such as alcohols, sugars, organic and amino acids or Cl compounds to form acetate. Acetogens utilise the Wood-Ljungdahl or the

reductive acetyl-CoA pathway for acetyl CoA synthesis and for the conservation of their energy, i.e. to fix carbon and to synthesise ATP.

Beginning in the 1930s, microorganisms were found to convert H₂ and CO₂ into acetic acid. Their general reaction which they carry out is:



Organisms such as *Acetobacterium woodii* (*A. woodii*) (section 2.1.1.3.1) and *Clostridium acetivum* can grow either by fermentation of sugars (chemoorganotrophically) (reaction 2) or chemolithotrophically and autotrophically by CO₂ reduction to acetate with H₂ as the electron donor:



(Kalogo, 2001)

Acetogens can stoichiometrically convert one mole of glucose into three moles of acetate. Homoacetogens grow autotrophically using H₂ and CO as electron donors and CO₂ as an electron acceptor. Other pathways can metabolise many different electron donors, acceptors and carbon sources (Ragsdale and Pierce, 2008). CO is another sole energy source for some acetogens, resulting in acetate and CO₂ as the fermentation products (Lorowitz and Bryant, 1984, Martin et al., 1985, Zeikus et al., 1985):



Almost all acetogenic bacteria that produce acetate are gram-positive. Bacteria and many of these are from the genera *Clostridium* and *Acetobacterium*. The generated acetate is either used directly by acetoclastic methanogens (*Methanosarcina* spp. and *Methanosaeta* spp.) or by syntrophic acetate oxidising bacteria (SAOBs) and hydrogenic methanogens. This was first observed in a thermophilic reactor (Zinder and Koch, 1984) and subsequently at a low temperature in anaerobic sludges with high ammonia content (Schnürer et al., 1996).

Acetogenesis is of interest industrially because it can synthesise acetic acid from biomass-derived sugars readily with the possibility of recovering additional valuable products like vitamin B₁₂. However, it also has technical and economic

problems when it comes to sourcing inexpensive substrates and developing an economically competitive fermentation process (Wang, 1978, Ng et al., 1981, Klibanov, 1983, Zeikus, 1983, Wang and Wang, 1984).

Over the years, acetogens have become more studied within the field of AD. The original rationale was that methanogens were more important because they are thermodynamically more favourable but this has evolved to the understanding that acetogens are a highly diverse group of microorganisms regarding their metabolic range. This was demonstrated by their ability to utilise chemolithoautotrophic substrates to their heterotrophy capabilities of converting sugars to acetate to their oxidation of methoxylated aromatic compounds (Xu et al., 2009). From 3% to 11% of total bacteria in anaerobic sludge have been reported previously to be H₂-utilising acetogens (Wang et al., 2007). Homoacetogenic bacteria have been observed in environments spanning a wide pH range of 4-8 (Drake, 2006).

2.2.1.3.1 *Acetobacterium woodii*

A. woodii is a fastidious homoacetogenic bacteria which is strictly anaerobic, gram-positive, oval and motile due to one or two sub-terminal flagella at the end of short rods measuring 1 by 2 µm. Colonies are circular and grow to 1 mm in diameter. It is part of the *Eubacteriaceae* family and is named in honour of Harland G. Wood (Balch et al., 1977).

The acetogenic bacterial strain studied in this thesis (Chapter 3) was *A. woodii* WB1 (DSM 1030) and was isolated from black sediment of a marine estuary called Oyster Pond Inlet in Woods Hole, Massachusetts (Balch et al., 1977). It is a rod-shaped bacterium that metabolises via the Wood-Ljungdahl pathway (Figure 2.3).

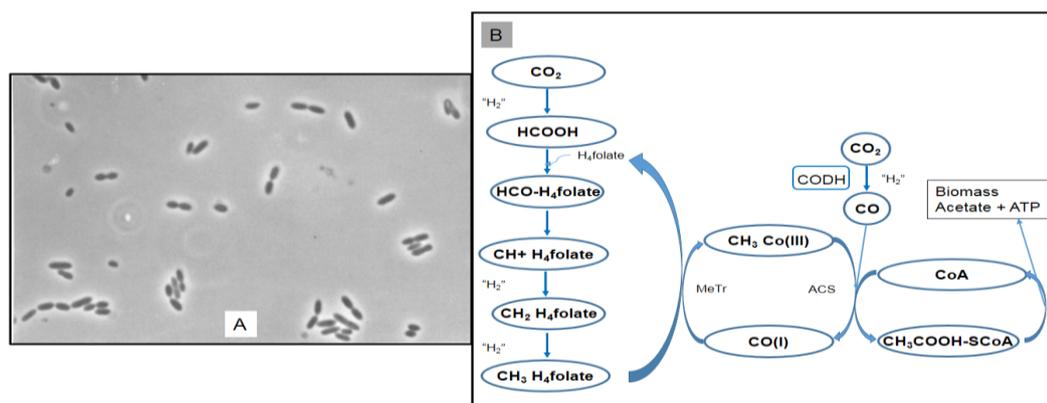


Figure 2.3: (A) A phase contrast photomicrograph illustrating *A. woodii* WB1 cells (taken from Balch, 1977) and (B) the Wood-Ljungdahl pathway from H_2 - CO_2 (adapted from Ragsdale and Pierce, 2008).

Its substrates include H_2 - CO_2 and organics such as formate, fructose, glucose, glycerate, lactate and methoxylated aromatic compounds. Fructose fermentation occurs by means of a homoacetic fermentation as reported previously (section 2.2.1.3, reaction (2)):



Every mol of fructose is fermented to 2 mol of acetate in glycolysis and the phosphoroclastic reaction of pyruvate generating 4 mol of ATP (Godley et al., 1990).



During autotrophic growth, *A. woodii* uses the exergonic oxidation of H_2 and 2 mol of CO_2 and is then reduced to 1 mol of acetic acid for ATP synthesis (Bertsch and Muller, 2015). Heterotrophic growth involves the fermentation of 1 mol of fructose to 3 mol of acetate (Balch et al., 1977). Acetate has been reported to be the only VFA detected during *A. woodii* growth studies (Diekert et al., 1986).

Sodium has been demonstrated to stimulate faster growth of *A. woodii* at specific concentrations and influences the amount of acetate that is produced when grown on fructose or on H_2 - CO_2 . There is no such sodium dependence on acetate formation present when grown on methanol plus H_2 - CO_2 (Heise et al., 1989). There has been conflicting evidence on whether *A. woodii* can grow on CO as the sole energy

course (Genthner and Bryant, 1987, Bertsch and Muller, 2015). Optimal parameters for *A. woodii* include pH 5-8.2 at mesophilic temperatures.

Applications for acetogens, and *A. woodii* in particular, now include the bacterial development as a production platform for bio-commodities from syngas, a combination of CO, H₂ and CO₂. Acetogens are involved in bio-commodities production strategies because they can convert each of these molecules to the starter molecule acetyl CoA for various chemicals (Köpke et al., 2010, Daniell et al., 2012, Bengelsdorf et al., 2013).

Furthermore, the *A. woodii* end-product acetic acid represents an important building block within the petrochemical industry where polymers such as vinyl acetate and cellulose acetate are produced and the major greenhouse gas CO₂ is consumed. 10 million tons of acetic acid are produced annually (Diekert and Wohlfarth, 1994, Muller, 2004, Schiel-Bengelsdorf and Durre, 2012, Kantzow and Weuster-Botz, 2016). Additionally, low aqueous gas solubility in the fermentation of H₂ gas results in low bioreactor productivities. *A. woodii* can be applied to stirred-tank bioreactors with continuous increased H₂ partial pressure in order to overcome this mass transfer limitation by increasing H₂ solubility (Kantzow and Weuster-Botz, 2016).

2.2.1.4 Methanogens

Methanogens are a group of strictly anaerobic *Archaea* that biologically produce CH₄. The ecological strategies of methanogens are crucial to understanding many global processes as well as many anaerobic environments, but they are also relevant to human health conditions such as periodontal diseases and colon cancer, where they have been found to be present (Conway de Macario and Macario, 2009, Jarrell et al., 2011). Methanogens exhibit enormous diversity in various aspects such as their ultrastructure and morphology and their wall and membrane chemistry, as well as their nucleic acid homology (Barker, 1956, Zeikus, 1977, Balch et al., 1979, Taylor, 1982, Vogels and Visser, 1983, Zeikus, 1983, Daniels et al., 1984). Membrane lipids of methanogens are made of ether-linked isoprenoid units and their walls are composed of a variety of polysaccharides, polypeptides or of a mixture of N-acetyltalosaminuronic acid, N-acetylglucosamine, and polypeptides. They do not contain peptidoglycan (Jones et al., 1977, Kandler and König, 1978,

Makula and Singer, 1978, König and Kandler, 1979, Tornabene and Langworthy, 1979).

They have been studied in depth for over fifty years due to their importance in the carbon cycling of compounds by the degradation of low carbon simple molecules and due to their involvement in the global carbon flux. Accordingly, methanogens are used in the agricultural, industrial and toxic wastes processing industries (Zinder, 1993, Deppenmeier, 2002). Methanogenesis catalyses the terminal stage of the AD process and maintains a tremendously low partial pressure of H₂ in the range of 10 to 1000 ppm due to syntrophic associations between H₂ producing and consuming microorganisms in well-running anaerobic digesters (Harper and Pohland, 1986, De Corte et al., 1988).

Microorganisms including methanogens produce approximately 80% of atmospheric CH₄, a significant greenhouse gas (Le Mer and Roger, 2001). They use anaerobic respiration metabolism whereby CO₂ is reduced by H₂ to form CH₄, and acetate can also be used as a substrate to initiate methanogenesis by acetate decarboxylation (Ferry, 1999). Fermentation of acetate accounts for approximately 70% of the global CH₄ formed from biologically produced CH₄ (Lovley and Klug, 1982). These two subdivisions of methanogenesis are referred to hydrogenotrophic (reaction 1) and acetoclastic methanogenesis (reaction 2) respectively.



There are two genera of archaea that can utilise acetate: *Methanosaeta* and *Methanosarcina*. It has been reported that some species within the *Methanosarcinaceae* family are capable of both acetate and H₂-CO₂ utilization (Boone et al., 1993). Contrastingly to mesophilic and thermophilic temperatures, there has been research into psychrophilic methanogenic metabolism within various environments (section 2.2). *M. barkeri*, for example, has been studied at a low temperature and is discussed in the next section.

There are six known methanogenic orders, five of which are termed “hydrogenotrophic”. Hydrogenotrophic methanogens mainly utilise H₂-CO₂, but rumen methanogens can also utilise formate, sodium formate in particular:



They lack a cytochrome-containing electron transport chain, and electron bifurcation is the proposed mechanism to conserve energy from CO₂ (Thauer et al., 2008, Costa et al., 2010, Kaster et al., 2011, Lie et al., 2012). The maintenance of low partial pressures of H₂ keeps the fermentative pathways energetically favourable. The *Methanococcaceae* family was originally discovered to have fragile cells, which occur singly or in pairs and have an optimal growth range from 32°C to 40°C (Barker, 1936, Kluyver and van Niel, 1936, Balch et al., 1979). There are four species in this family that have been categorised as mesophilic: *Methanococcus maripaludis* (*M. maripaludis*) (described further below in section 2.2.1.4.2.), *M. vannielii*, *M. voltae* and *M. aeolicus* (Keswani et al., 1996). *M. maripaludis* displays an advantage over the other species at low temperatures as it is better able to maintain a low partial pressure of H₂ during wastewater treatment (Goyal et al., 2016).

2.2.1.4.1 *Methanosarcina barkeri*

Methanosarcina barkeri (*M. barkeri*) is termed an acetoclastic methanogen and a member of the *Methanosarcinaceae* family but it is capable of consuming H₂-CO₂. It was named after H. A. Barker who made conclusive studies on this species and on other methanogenic species. The cells are generally non-motile mesophiles or thermophiles and have a thick cell wall of approximately 500 nm which is comprised of the acid heteropolysaccharide. *M. barkeri* cells are clustered together in clumps which vary in size from several to several hundred micrometres and are gram-positive irregular cocci (Figure 2.4). They are coccoid bodies of cells ranging from 1.5 to 2.0 µm in diameter. Without a microscope they have a grainy appearance with active gas formation present (Balch et al., 1979, Bryant and Boone, 1987).

The strain studied in this thesis (Chapter 3) was *M. barkeri* MS (DSM 800), which was isolated in an axenic culture, lost and re-isolated from a butyrate enrichment derived from an anaerobic sewage sludge digester (Schnellen, 1947, Bryant and Boone, 1987). Other strains isolated and studied at the time included strains 227 (also taken from a sewage sludge digester), strain W (which was a gas-vacuolated

strain) and strain DM (ATCC 29894) (which was isolated from an anaerobic digester supplemented with bovine waste) (Mah et al., 1977, Mah et al., 1978, Mountfort and Asher, 1979). *M. barkeri* can be found in a wide range of very different environments such as marine habitats, Antarctic lakes, submarine hydrothermal vents, freshwater soil and sediments, landfills, rice paddies, sewage digesters and as symbionts in rumen, termites, protozoa, human large intestines, and in the gastrointestinal tracts of various animals (Zinder, 1993, Sowers, 2009).

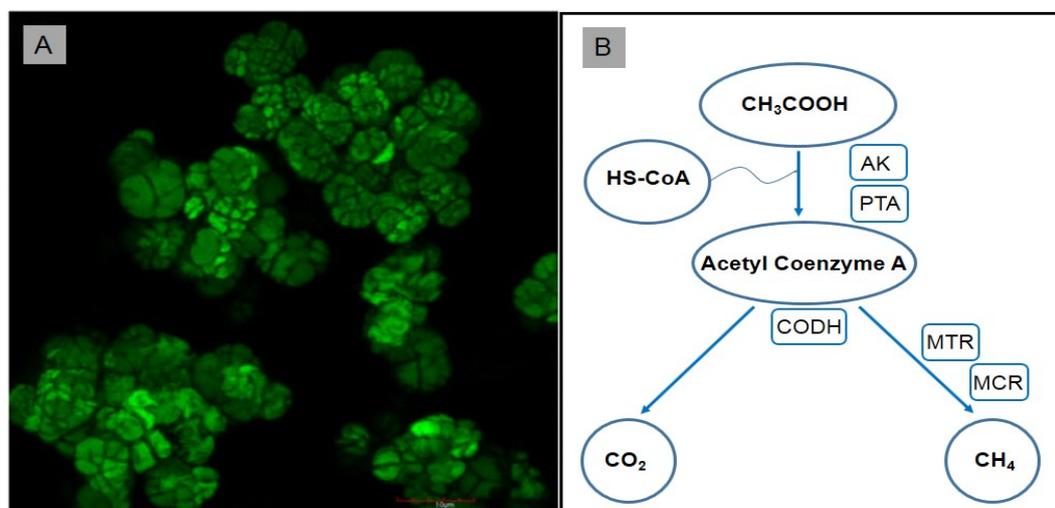
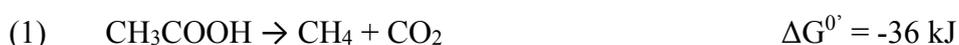
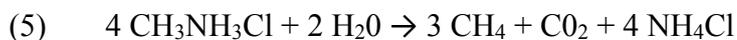
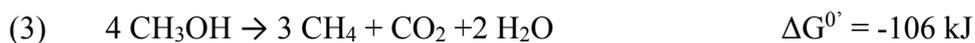


Figure 2.4: (A) A micrograph image of *M. barkeri* CM1 cells using a laser confocal scanning inverted microscope (Lambie et al., 2015) and (B) the methanogenic pathway from acetate. Adapted from (Qiao et al., 2014). Abbreviations: AK, acetate kinase; PTA, phosphotransacetylase; CODH, carbon monoxide dehydrogenase/ acetyl-CoA synthase complex; MTR, methyltetrahydromethanopterin: CoM methyltransferase; MCR, methyl-CoM methylreductase; HS-CoA, coenzyme A.

As well as coveting the largest environmental distribution, *M. barkeri* is known as the most metabolically diverse methanogen due to its ability to grow both chemoautotrophically and chemoorganotrophically (Stadtman, 1967, Zeikus, 1977, Weimer and Zeikus, 1978b). Along with acetate consumption and the oxidation of H₂/ reduction of CO₂ substrate processes, *M. barkeri* can metabolise methanol, CO and methylamine as sole electron donors. CH₄ is formed by the following reactions:





CH₄ formation and growth are faster in H₂-CO₂- or in methanol-containing medium than in a medium containing acetate (Weimer and Zeikus, 1978b, Balch et al., 1979, Hippe et al., 1979, Müller et al., 1986, Bryant and Boone, 1987, Sancho Navarro et al., 2016). It has been suggested that acetate-adaptation growth has a lag period of approximately three weeks before growth is observed (Winter and Wolfe, 1979). H₂ was considered to be a requirement for methanogenic growth until *M. barkeri* was adapted to grow on acetate in the absence of H₂ (Mah et al., 1978).

It was also observed that the acetate-fed *M. barkeri* strain MS was capable of producing H₂ as well as CH₄, and it was suggested that the amount and rate of both products formation was directly correlated to and dependent on acetate concentration (Krzycki et al., 1987). Hydrogenase is the key enzyme for the metabolism of H₂ and can distribute between the particulate and soluble fractions of a cell (Fauque et al., 1984). It was also reported that H₂ was a potent inhibitor of methanogenesis from acetate in some *M. barkeri* strains (Smith and Mah, 1978, Ferguson and Mah, 1983, Eikmanns and Thauer, 1984) but that H₂ was not an inhibitor of CH₄ or of CO₂ production from acetate's methyl group and it did not inhibit methanogenesis for the strain *M. barkeri* MS.

It was also speculated that the clumping structural morphology could be advantageous for *M. barkeri* due to the possibility of any H₂ lost during consumption being regained by an adjacent cell for subsequent use. Cells that contain hydrogenase in either the membrane or the cytosol would then be able to consume any H₂ that has been produced by their neighbouring cells in the physical cluster (Krzycki et al., 1987, Kemner and Zeikus, 1994).

In relation to multiple substrates (acetate and methanol), a previous study has reported that *M. barkeri* can exhibit a diauxic response when there is an acetate and methanol mixed substrate feed. In that experiment methanol grows first (Smith and Mah, 1978). The increased unavailability of acetate over a partner substrate was

also demonstrated in another mixotrophic study of methylation where the methyl group of methanol was activated easily whereas the methyl group of acetate was not available (Shapiro and Wolfe, 1980). Acetate is known to be a key intermediate within the metabolism of *M. barkeri* and is able to contribute as much as 60% of the cells carbon formed during heterotrophic growth and during autotrophic growth on H₂-CO₂ (Weimer and Zeikus, 1978b, Weimer and Zeikus, 1979). Although it is a key intermediate, acetate's utilisation rates were substantially lower for single substrate growth while cell weights of cells produce per mole of substrate were generally similar for acetate with methanol. Several other studies observed simultaneous consumption of acetate and methanol (Hutten et al., 1980, Scherer and Sahm, 1981, Krzycki et al., 1982).

The bioenergetics of *M. barkeri*, and in particular the acetate conversion pathway, did not begin to unravel until the late 1980s (Peinemann et al., 1988), when it was demonstrated that acetoclastic methanogenesis was accompanied by ATP generation from AK and PTA (Figure 2.4). A sodium motive force is required for certain reactions involved in CH₄ formation from methanol, CO₂ plus H₂ to acetate (Peinemann et al., 1988). From the ability to utilise a wider range of substrates, to adaptation within a wider range of environments and to forming complex multicellular structures in comparison to most other methanogens, *M. barkeri* has proven to be an extremely versatile archaeon. It also has a relatively large genome size of 4.8 Mb, which perhaps accounts for its adaptable nature (Maeder et al., 2006).

M. barkeri has been partnered with other species in various co-cultures to further analyse its metabolic and genetic reactions with a competitor or a syntroph (Winter and Wolfe, 1979, Winter and Wolfe, 1980, Rocheleau et al., 1999, Plugge et al., 2010, Qi et al., 2014). In general, *Methanosarcina* species have proven to be an exceptional model organism for genetic studies of methanogenesis. (Guss et al., 2005, Welander and Metcalf, 2005, Welander and Metcalf, 2008).

Its optimal growth conditions are at pH 5.5 to 7.5 and at mesophilic temperatures. Expanding on the versatility of *M. barkeri*, it has been reported that it is capable of growing at low temperatures by their presence in Lt bioreactors during periods of reactor instability brought about through operational parameter changes such as

temperature (McKeown et al., 2009a) and hydraulic retention times (HRTs) (O'Reilly et al., 2010). A separate study looked into the growth and functional aspects of *M. barkeri* when it was subjected to a heat shock (37°C down to 15°C) and found that methanol-based methanogenesis was found to be significant at 15°C and methylamine methanogenesis was suggested to be successful, conferring a survival advantage for *M. barkeri* at low temperatures (Gunnigle et al., 2013).

2.2.1.4.2 *Methanococcus maripaludis*

Methanococcus maripaludis grows autotrophically on H₂-CO₂ in a defined medium. It was discovered in marine sediments, particularly in a salt marsh in South Carolina. It is Gram negative, non-spore-forming and weakly motile. It has been found to couple motility through flagella and pili to H₂ concentration sensing resulting in the phenomenon of hydrogentaxis. Hydrogentaxis could act as a competitive strategy for *M. maripaludis* to outcompete other microorganisms for H₂ gas as a substrate (Briley et al., 2013). The specific role of its pili is still unknown (Ng et al., 2011). Its pleomorphic coccoid-rod cells (Figure 2.5 A) average 1.2 by 1.6 µm and a 0.9-1.3 µm diameter with electron micrographs illustrating a regular array of protein subunits on the outer surfaces. The pale yellow colonies are both translucent and circular with a smooth surface and an entire edge (Jones et al., 1977, Balch et al., 1979, Jones et al., 1983a).

The particular strain studied in this work (Chapter 3) was *M. maripaludis* JJ (DSM 2067) and was isolated from salt marsh sediment in Pawley's Island, S.C. Observations in plating efficiencies suggested that even low levels of O₂ during preparation and incubation seems to inhibit both growth and colony formation of plated cells that were well-separated (Jones et al., 1983b).

Substrates for *M. maripaludis* have thus far been confined to formate or a mixture of H₂ and CO₂ (Figure 2.5 B) described as reactions 1 and 2 below. CO₂ is reduced to CH₄ by a modified Wood-Ljungdahl pathway which is referred to as the Wolfe cycle (Escalante-Semerena et al., 1984). Unlike other methanogens that require more complex substrates and intermediates, *M. maripaludis* grows on a simple carbon source such as CO₂ and N₂ as the sole nitrogen source (Zellner and Winter, 1987). Sodium, selenium and magnesium are important nutrients required for

growth (Jones et al., 1983a). If H₂ was a renewable source of energy for *M. maripaludis*, it would be capable of capturing and converting CO₂ emissions, the rise of which comprises a global problem, into the beneficial biofuel (Goyal et al., 2016). Even though it can assimilate other carbon substrates like acetate and pyruvate, both of these substrates are not relevant physiologically for CH₄ production (Shieh and Whitman, 1987, Yang et al., 1992).



M. maripaludis is an ideal methanogen for genetic studies because of its short generation time and because its nutrition has been characterised (Jones et al., 1983b). More than one hundred experimental studies have examined specific parts of *M. maripaludis*'s genetics of CO₂ and N₂ and its biochemistry (Goyal et al., 2016). Its genome of 1722 protein-coding genes in a 1.66 Mb single circular chromosome has been sequenced along with a number of studies of global regulation such as responses to nutrient limitations and growth rates within archaea as well as metabolic flux (Haydock et al., 2004, Hendrickson et al., 2004).

M. maripaludis is polyploid and it can contain from 30 to 55 genomes per cell. This is dependent on which growth phase it is at. Remarkably, the results of a recent study indicated a similarity of essential gene numbers between this methanogenic archaeon of approximately 526 genes and bacteria that range from 271-642 genes (Sarmiento et al., 2013). A reconstructed genome-scale metabolic model has also been generated from a combination of genomic, biochemical and physiological data resulting in 605 metabolites and 570 reactions across 52 distinct pathways (Goyal et al., 2014). The only other similar model that was found in the literature thus far is for the central metabolism of the co-culture *M. maripaludis* and *D. vulgaris*, which looked at their syntrophic association as well as investigating CH₄ production from lactate (Stolyar et al., 2007). Its optimal growth conditions are at pH 6.8 to 7.2 and at 35°C to 39°C (Jones et al., 1983a, Haydock et al., 2004).

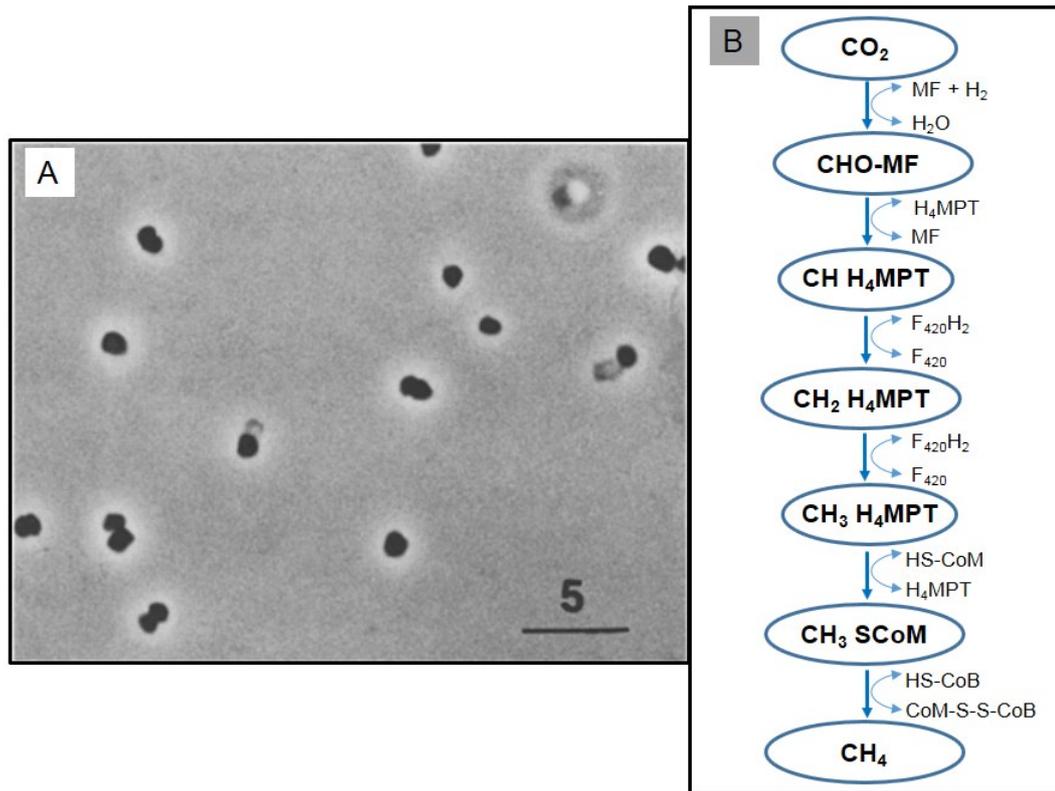


Figure 2.5: (A) Image of *M. marispludis* JJ of cells viewed by phase contrast microscopy (taken from Jones, 1983) and (B) general methanogenic pathway from H₂-CO₂. Adapted from (Schäfer et al., 1999) and (Lambie et al., 2015). Abbreviations: CHO-MF, formyl methanofuran; H₄MPT, tetrahydromethanopterin; CoM, coenzyme M.

2.2.1.4.3 Methanogenic Inhibition

Several inhibitors have been used in AD studies to test the influences and impacts of various trophic groups. In order to study the effects of methanogenesis and the AD pathway in more detail, a potent inhibitor called 2-bromoethanesulfonate (BES) is commonly used. It is the coenzyme M (2-mercaptoethanesulfonate) analogue which inhibits the process of reducing methyl-coenzyme M to CH₄ in some cell extracts (Gunsalus et al., 1978, Zinder and Koch, 1984). In particular, 1 nmol/ml of BES is capable of inhibiting methanogenesis by *Methanosarcina* cells (Smith and Mah, 1981). Several ecological studies have used BES as a methanogenic inhibitor at concentrations of approximately between 0.1 and 1 μmol/ml (Healy et al., 1980, Zehnder and Brock, 1980, Oremland et al., 1982, Bouwer and McCarty, 1983). Although a significant amount of inhibition or complete inhibition has been achieved in these short studies (Zinder and Koch, 1984), it should not be

definitively concluded that adaptation to or degradation of BES may occur in long term experimental studies (Smith and Mah, 1981, Bouwer and McCarty, 1983).

Antibiotics vary in their effectiveness against methanogenic microorganisms, especially in accordance with their concentrations, as with most compounds. For *M. maripaludis*, D-cycloserine (1 mg/ml) and chloramphenicol (0.1 mg/l) completely inhibited growth. Comparatively, penicillin G (2 mg/ml), vancomycin (0.1 and 0.5 mg/ml) and tetracycline (10 µg/ml) all had no effect on growth (Jones et al., 1983a).

2.2.1.5 Syntrophy

Synergy and competition are the cornerstones to how all organisms survive, thrive or disappear within a community, a niche, an ecosystem or an environment. The interactions of specific species can result in their survival in a specific environment in which they otherwise would be unable to survive (Stolyar et al., 2007). Syntrophy is a key metabolic process within anaerobic catabolism which involves two different organisms cooperating in the consumption of a substrate. They require each other to complete this degradation, and they achieve energy conservation in the process. This is also a form of mutualism where archaeal and bacterial interactions with each other ensure the anaerobic oxidation of CH₄ (Boetius et al., 2000). Interspecies H₂ transfer is the heart of these syntrophic reactions where one species consumes H₂ and the partner produces H₂. Although it is difficult to eke out a living on a severely marginal energy economy, they can somehow survive and understanding their strategy still poses a significant challenge to scientists today.

It is known that syntrophs degrade highly reduced fermentation products and that they issue an important product for anaerobic H₂ consumers as H₂ released during acetogenesis may have an inhibitory effect on the microbial community (Shah et al., 2014). This proves that a bottleneck would be triggered where electron acceptors aside from CO₂ are doing the limiting if syntrophs did not exist.

Early acetogenic and methanogenic microorganism studies have demonstrated a parallel course presumably due to both groups residing in similar environments and

utilising similar substrates (Fischer, 1932, Wieringa, 1936, Schnell, 1947, Barker, 1949, Zeikus et al., 1985).

As described previously, fermentation to short-chain fatty acids, alcohols, H₂ and CO₂, results in H₂ and acetate being consumed by methanogenesis but most of the carbon remains and cannot be catabolized directly. Syntrophs are secondary fermenters and require two organisms; one being a H₂-consuming partner organism. Syntrophy is the interactions of acetogens with methanogens and is vital for AD performance (Schink and Stams, 2006). These syntrophs degrade fatty acids, coupled with growth of and distances between acetogens. Hydrogen-scavenging microorganisms have a bearing on specific growth rates (Batstone, 2006). Thermodynamic equilibrium is required and chemical energies are shared amongst the syntrophic organisms.

Syntrophic acetate oxidation requires acetotrophic bacteria (SAOBs) to convert acetate to H₂ and CO₂. This conversion is followed by hydrogenotrophic methanogenesis (Schnürer et al., 1999, Schnürer and Nordberg, 2008). Thus far, most of the SAOBs have been classified as acetotrophs such as *Thermacetogenium phaeum*, *Clostridium ultunense*, *Syntrophaceticus schinkii*, and *Tepidanaerobacter acetatoxydans* (Lee and Zinder, 1988, Schnürer et al., 1997, Hattori et al., 2005, Westerholm et al., 2011).

Biogas production relies on a symbiotic relationship between syntrophic bacteria such as *Syntrophobacter*, *Synthrophospora* and *Syntrophomas* with methanogens (Shah et al., 2014). Syntrophic bacteria convert acid-phase products into acetate and/or H₂ which can be subsequently used by the methanogens. Recent studies have been investigating the possibility of nanowires/pili initiating direct electron transfer between the syntrophs. Acetogenesis cannot progress in the absence of methanogenesis (Song, 2003, Summers et al., 2010, Morita et al., 2011, Rotaru et al., 2014).

According to the literature, most syntrophic interactions have been demonstrated to have optimum temperatures in the range of 25 to 45°C and optimum pH levels in the range of 6.3 to 8.5 (Stams, 1994, Schink, 2000, Schink, 2002, O'Flaherty et al., 2006). It has been reported that syntrophic activity reduced slightly after BES exposure indicating that both methanogenesis and syntrophy can be affected by the

inhibitor's presence (Webster et al., 2016) demonstrating a delicate balance among syntrophs.

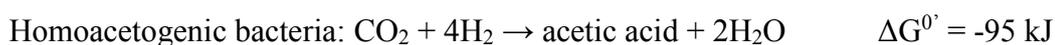
2.2.1.5.1 *Acetobacterium woodii* and *Methanosarcina barkeri*

Syntrophy between the homoacetogen *A. woodii* and *M. barkeri* was previously examined in studies where acetate-adapted *M. barkeri* was co-cultured with fructose-fed or glucose-fed *A. woodii*. The acetate produced by *A. woodii* was then consumed by *M. barkeri*. This pairing led to the complete conversion of the carbohydrate to CO₂ and CH₄. It was observed that *A. woodii* formed 3 mol of acetate per mol of fructose followed by *M. barkeri* utilising acetate to form CH₄ and CO₂ in this mixed culture (Winter and Wolfe, 1979). Adaptation to acetate-adapted *M. barkeri* cells took approximately 3 weeks for three early sets of studies (Mah et al., 1978, Winter and Wolfe, 1979, Hutten et al., 1980).

In a continuous co-culture of H₂-grown (i.e. not acetate-adapted) *M. barkeri* and *A. woodii*, it was reported that there was a doubling time of 6h at 33°C when grown on fructose both *A. woodii* individually and when grown together. It was suggested that autotrophically-grown methanogens develop a syntrophic association with *A. woodii* as opposed to acetate-pregrown *M. barkeri* fermenting the acetate produced by *A. woodii* (Winter and Wolfe, 1980).

2.2.1.6 Competition

It has been suggested that methanogens can out-compete homoacetogens for H₂-CO₂, in particular due to the thermodynamic favourability of methanogenic archaea.



This advantage is presumably the reason that methanogens can utilise H₂ which is approximately tenfold lower in its concentrations than homoacetogenic bacteria (Lovley, 1985, Cord-Ruwisch et al., 1988, Conrad and Wetter, 1990, Peters et al., 1998). Even though it is the methanogens who should energetically prevail over

homoacetogens, H₂-consuming homoacetogens do persist in environments alongside methanogens. It was speculated that this may be due to non-homogeneous distribution of these physiotypes at different sites present in the same environment. This suggestion was made in the context of a study of the hindgut of a termite in which coexistence of both homoacetogens and methanogens resulted in the absence of a direct H₂ competition or of another mutual resource (Leadbetter and Breznak, 1996, Ebert and Brune, 1997, Peters et al., 1998).

2.2.1.6.1 *Methanosarcina barkeri* and *Methanococcus maripaludis*

There has been no study found in the literature that is directly focused on the co-culture of *M. barkeri* and *M. maripaludis*.

2.3 Wastewater Treatment Systems

Aerobic wastewater technology is the core system used in activated sludge treatment plants. The aerobic digestion process is faster than AD but requires higher energy inputs. Biomass production is low for anaerobic processes and high for aerobic processes (Gasparikova et al., 2005). However anaerobic technology has enabled the exploitation of naturally-occurring microbial consortia for production of biogas from renewable organic wastes (Murto, 2003).

The septic tank is a simple common form of domestic sewage treatment where solids settle out and an anaerobic community develops at the bottom of the tank. This community enables the decomposition and treatment of applied waste. In parallel, abiotic and biotic processes within a piped drainfield aid effluent purification in the tank and subsequent dispersion into groundwater (Withers et al., 2014). Beyond the septic tank system, there are many different reactor configurations that have been developed and studied in detail for full-scale AD treatment. Microorganisms can adhere to solid material in reactors such as trickle filters or fluidized beds. In the event of microorganisms having poor adhesion properties, they can be artificially immobilised in polymers such as alginate (Cassidy et al., 1996). This allows for high conversion rates of organic and inorganic compounds in small reactors (Stams and Oude Elferink, 1997).

Anaerobic moving bed biofilm reactors (AMBBRs) are able to retain high concentrations of biomass for applications such as domestic wastewater treatment (Kim et al., 2015). Consequently, they are used for high-strength wastewaters as they are capable of providing higher rates than other digesters (Ødegaard et al., 1994, McQuarrie and Boltz, 2011). Their main advantage is the ability to handle high loading rates at a low HRT, leading to a decrease in reactor volume and therefore, a smaller footprint (di Biase et al., 2015). Within these AMBBRs, floating carrier media, usually polyethylene, encourage microorganism surface growth and biofilm development (Ødegaard, 1999, Jähren et al., 2002, di Biase et al., 2015).

The Upflow Anaerobic Sludge Blanket (UASB) system began as the preferred high rate granular sludge-based reactor design (Lettinga et al., 1980, Guiot et al., 1992, Kato et al., 1997, Frankin, 2001). High reactor volumes are retained in a UASB due to aggregation of clumps of bacterial cells into a suspended solids granular structure which can settle within the bioreactor (MacLeod et al., 1990). The gases produced due to microbial interactions helped to fluidise the granules for enhanced wastewater contact (Rittmann and McCarty, 2001). UASBs dominate the industrial marketplace in terms of full-scale granular sludge-based bioreactors and represent a typical reactor which is common for work with AD.

Expanded Granular Sludge Bed (EGSB) reactors and Internal Circulation[®] (IC) systems are also advantageous due to their increased organic loading and mixing capacity, which result in a preferable smaller bioreactor. This was achieved through effluent recirculation and high hydraulic loads creating turbulence and high mixing intensities, and thus eliminating dead zones within the bioreactor (de Man et al., 1988, van der Last and Lettinga, 1992). It has been suggested that these benefits will lead to the replacement of UASBs in the future (Frankin, 2001), but UASBs are still widely used (Abbasi and Abbasi, 2012).

Other bioreactors involved in AD technology include the anaerobic filter (AF), the continuously stirred tank reactor (CSTR), the anaerobic lagoon (AL), the hybrid sludge bed-anaerobic filter (HYB), the fluidised bed (FB) and temperature phase AD (TPAD) reactors. A specific type of HYB bioreactor has been developed from the EGSB design by placing an anaerobic filter (AF) section at the top of the bioreactor, enabling active biomass which would otherwise have been lost from the

granular sludge bed to be trapped within the reactor and retained on the inert support matrix as fixed biofilm growth (McHugh et al., 2004, Collins et al., 2005).

Further to this, anaerobic membrane bioreactors (AnMBRs) also retain biomass successfully within domestic wastewater treatment systems as well as anaerobic baffled reactors, both of which are operated at low temperatures (Feng et al., 2008, Smith et al., 2013). Both of these approaches ensure that immobilised biomass is effectively and efficiently retained, with the result that methanogenic community enrichment is promoted through low decay rates and is successful at low temperatures (Lettinga et al., 2001). Additionally, it has been demonstrated that a fixed-film filter section is a beneficial modification to the EGSB (Collins et al., 2006).

CSTRs are applied in low-rate suspended biomass systems where microbial studies have been conducted. This implies that dynamic shifts may occur in the absence of measurable variations in their performance (Fernández et al., 1999, Fernandez et al., 2000, Zumstein et al., 2000, Wang et al., 2010, Wang et al., 2011). The inverted FB reactors were described as promising due to their use of floatable particles containing a lower specific density than the liquid with the result that the particles were fluidised downwards (García-Bernet et al., 1998, Garcia-Calderon et al., 1998).

TPAD reactors involve the sequential use of a thermophilic digester followed by a mesophilic digester (Kaiser et al., 1995, Schmit and Ellis, 2001, Sung and Santha, 2003). The four stages of AD are separated according to digester type. Firstly, feedstock is dispensed into the thermophilic digester where hydrolysis and acidogenesis take place so that the often rate-limiting hydrolytic phase can be accelerated by applying elevated temperatures. The process then proceeds into the mesophilic digester to promote acetogenesis followed by methanogenesis with the use of different pH, HRT and loading rate according to optimal conditions for these stages (Lv et al., 2010). Its digestion efficiencies were not consistent among different feedstocks in different case studies indicating certain limitations. TPAD is still in its infancy and its potential for large-scale use remains open to question (Demirel and Yenigün, 2002, Bolzonella et al., 2007). More studies need to be conducted on additional feedstocks to provide sufficient data for commercial-scale design and operation (Lv et al., 2010).

2.3.1 Sequencing batch reactors

Sequencing batch reactors (SBRs) treat wastewater using activated sludge. They evolved from the CSTR system whereby the HRT was uncoupled from the sludge retention time (SRT) for biomass retention. The SRT refers to the average time activated-sludge solids are in a system or bioreactor. One such mode of cycle involves pumping in fresh medium over a certain period of time while the reactor is continuously stirring. After this filling period both the influent supply and stirrer cease for a short period of time to allow the aggregates to settle within the bioreactor. This is followed by the near complete removal of spent medium along with suspended material. New fresh medium is pumped in once again and this pattern repeats until the experiment ends. Anaerobic gas / N₂ flushing also continuously occurs (Figure 2.6).

The advantages of SBRs include their efficient biomass retention, the homogeneity of feed chemicals throughout the reactor, the products and seed biomass that they generate, their long-term operation stability of over a year and their steadiness under substrate-limiting conditions. The original goal of Strous et al. was to develop a bioreactor technology that can enrich for a large amount of slow-growing microorganisms that were unattainable and uncultivated for AD research (Strous et al., 1998). An adapted version of this technology was applied to a batch-type experiment involving 48-well plates where individual anaerobic granules were fed with a specific type of medium simulating a certain environmental condition or stress. Spent medium was replaced with fresh medium every 48 hours. These experimental plates were called micro SBRs (μ SBRs) (Chapter 6).

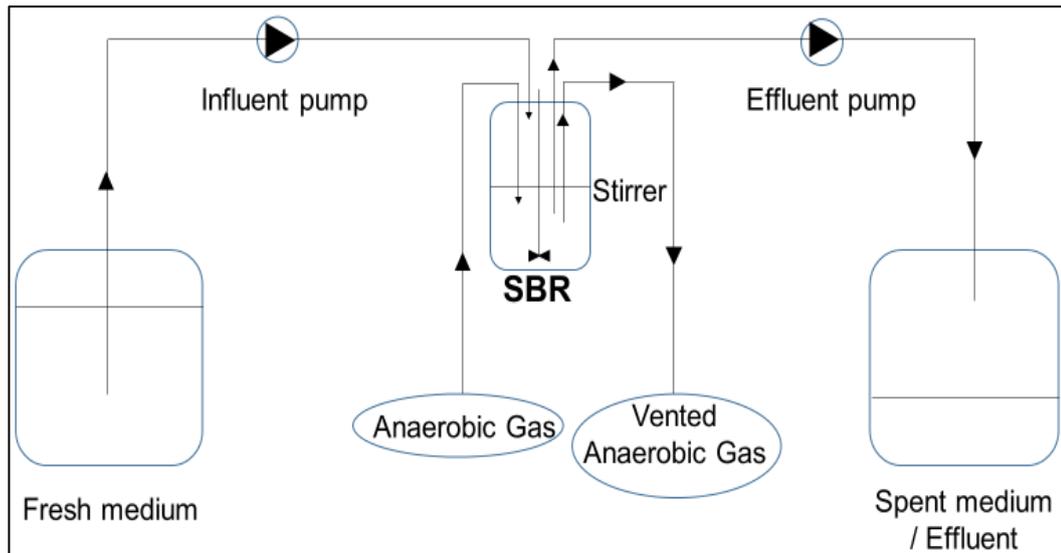


Figure 2.6: Experimental set-up of an SBR (adapted from Strous et al., 1998).

2.3.2 Anaerobic granular sludge biofilms

As described in 2.3 the success of anaerobic bioreactors and especially the UASB, is attributable to the formation and maintenance of anaerobic sludge granules in which an array of microorganisms from each trophic group along the AD pathway can survive as a niche community. Anaerobic sludge granules are spontaneously-occurring biofilms of mixed communities of species from the various AD trophic groups. Granules are self-immobilised and retained within anaerobic digesters. They dispense with the need for costly support materials and facilitate the inter-species transfer of various substrates to ensure the complete degradation of organic constituents from the wastewater present in the bioreactor (Grotenhuis et al., 1991, O'Flaherty et al., 1997, Stams and Plugge, 2009).

Anaerobic granular sludge was first discovered in 1976 at a sugar factory in Breda in the Netherlands and superior results and efficiencies were observed in this pilot plant than in previous laboratory studies in the University of Wageningen (Lettinga, 1977). Granulation of sludge is anomalous and it occurs in upflow anaerobic sludge bioreactors, thereby allowing for much higher loading rates than previous conventional activated sludge systems. There have been a variety of theories given to explain granulation. They can be categorised into physical, microbial and thermodynamic groups.

Physical explanations for the occurrence of granulation include the presence of inorganic nuclei, the wash-out of suspended biomass under high selection pressure conditions and the readily-acidifiable chemical oxygen demand which enhances microbial extra-cellular polymer formation (Hulshoff Pol et al., 1983, Alibhai and Forster, 1986, Hulshoff Pol, 1989, Vanderhaegen et al., 1992, Thaveesri et al., 1995b). Another group examined the growth of colonised suspended solids from the influent and put forward the suggestion that the increase in granule size is solely attributable to growth. The same group theorised that granular size distribution resulted from small particles being washed into the reactor or by attrition within the reactor. Additionally, it was found that there was a wider distribution of the size of granules when there were fewer suspended solids within the influent (Pereboom, 1994). Extra-cellular polymeric substances were found on the anaerobic granules and it was possible to predict granular strength and loss by observing the flotation of gas bubbles (de Beer et al., 1996, O'Flaherty et al., 1997).

Microbial theories are often integrated with the physical theories mentioned above. The Cape Town Hypothesis proposed that the autotrophic hydrogenotroph *Methanobacterium* strain AZ grown under high H₂ pressures is crucial for granulation (Sam-Soon et al., 1987) but that it is unable to produce the essential amino acid cysteine, limiting cell synthesis. The “Spaghetti theory” stated that granules were formed in UASB reactors treating acidified wastewaters due to acetate solutions or VFA mixtures with an abundance of *Methanothrix* bacteria/*Methanosaeta* (Wiegant, 1987). One group observed that a layered structure was present in glucose-fed granules, for example (Lens et al., 1993). This suggests direct agglomeration of microbes. It was observed that small aggregates of *Methanosaeta* were present at the core, due to turbulence generated by gas production (Hulshoff Pol et al., 2004). Similarly, another group detected this type of nucleus formation in a fermented alcohol stillage-fed UASB (Chen and Lun, 1993).

Microscopic and activity studies have demonstrated that granulation begins by the layering of acidogenic bacteria over filamentous *Methanothrix*, forming microflocs and the establishment of bridges, reaffirming the importance of *Methanothrix* in granulation (Dubourgier et al., 1987). This demonstrates that a layer of syntrophic acetogenic and hydrogenotrophic bacteria surrounds this aggregated *Methanothrix*

core and that a combination of acidogenic, sulfate-reducing, and hydrogenotrophic archaea formed the outermost layer, presumably, in part, because they are the least strict anaerobes in the AD process, which suggests that mature granules have a variety of other bacteria on their surfaces (Figure 2.7).

The products of the outer layers serve as substrates to the inner layers and hydrogenotrophic microorganisms could consume free H_2 or H_2 produced by the acetogens, which implies that strategic positions are determined by the microorganisms rather than by a random aggregation of suspended bacteria (MacLeod et al., 1990, Guiot et al., 1992, Chen and Lun, 1993, Fang, 2000).

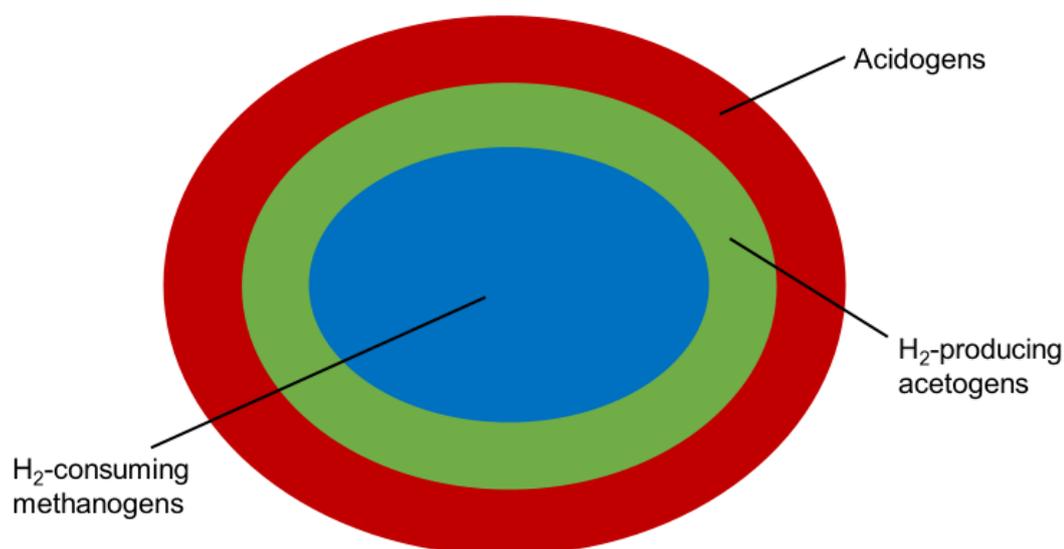


Figure 2.7: Illustration of the physical structure of an anaerobic granule, showing different layers of trophic groups: a central core of methanogens is fed by the substrates of the surrounding hydrogen-producing acetogens. The outermost layer of acidogens breaks down complex organic molecules producing acids for acetogenic consumption. Adapted from (Agapakis et al., 2012).

Strong evidence has suggested that *Methanosaeta concilii* in particular is a key species involved in the granulation process (Hulshoff Pol et al., 2004). Subsequently, other substrates utilised by the granules were also studied such as sucrose, brewery and potato wastes, wheat-starch and papermill wastewaters (Fang et al., 1994, Quarmby and Forster, 1995, Jianrong et al., 1997). Conversely, this layered structure was not observed in other types of wastewater such as that used

to treat propionate, ethanol, glutamate, sugar refinery wastewaters, and methanol waste (Bhatti et al., 1993, Fang et al., 1994).

The thermodynamic group of theories suggest that energy is involved in the adhesion of the granules due to interactions of a physio-chemical nature between cell walls and, in some cases, inert surfaces. Key features such as hydrophobicity, electrophoretic mobility and proton translocating membrane activities add weight to these granulation research and hypotheses (Hulshoff Pol et al., 2004). A four-step proposal has been created to describe granule and biofilm formation: cell transportation to either an inert object or to other cells, reversible physio-chemical adsorption to the other cells, irreversible adhesion to these cells by polymers or appendages, and cell growth of the granules/biofilm (Costerton et al., 1987, Verrier, 1988, Gantzer, 1989, van Loosdrecht and Zehnder, 1990, Schmidt and Ahring, 1996).

There is also a surface tension model, which was developed to relate surface dynamics to bacterial adhesion within UASB reactor communities (Thaveesri et al., 1995a). Acidogens are generally hydrophilic whereas acetogens and methanogens are usually hydrophobic, and it was found that high surface tensions lead to hydrophobic bacteria aggregation and low surface tensions resulted in hydrophilic bacteria aggregation. Having acidogens as solid-phase emulsifiers around methanogens allows more stability within a reactor, which reduces gas bubble adhesion or subsequent wash-out (Daffonchio et al., 1995, Thaveesri et al., 1995b).

Granular sludge is harmed in certain circumstances, including where there is a high concentration of poorly-flocculating suspended particles (Lettinga et al., 1980) and in the presence of clay and other inorganics (Lettinga et al., 1980). Many inert solids that have a very large surface area have also been shown to harm granular sludge due to the concentrated growth limitations leading to a slowdown of granulation (Hulshoff Pol et al., 1988). In summary, *Methanosaeta* is believed to play a vital role in granulation with some suggesting that *Methanosarcina* aids cell clumping for granule generation. Bacterial adhesion is thought to be the initial step, followed by the retention of sludge agglomerates, and growth conditions appear to be more important than growth itself. *Methanosaeta* conditions are considered optimal (Hulshoff Pol et al., 2004).

2.3.3 Microbial community structure and function

As well as bioreactor operation studies utilising the different configurations outlined in section 2.3 above, the fundamentals of the underlying microbiology can be used to develop strategies to improve efficiencies within the AD bioreactors. Many studies have been conducted to investigate the structure and function of microbial communities in various reactor types and under various conditions to improve reactor operation by maintaining the healthiest and most efficient environment for microbes to grow and perform AD.

One full scale study examined the microbial community of thirty-two digesters over six years. The authors found that only 300 operational taxonomic units (OTUs) were present in 80% of the total reads across all bioreactors and that some of the abundant OTUs could not be classified, thus making it difficult to infer their function and role within the reactor. The archaea community comprised of *Methanosaeata* and *Methanolinea* for the mesophilic reactors and *Methanobacter* and *Methanosarcina* for the thermophilic plants. For bacteria, the dominant phyla were composed of *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Chloroflexi* (Kirkegaard et al., 2017). Similar amplicon studies have previously reported that there appears to be a common set of abundant microbes in anaerobic digesters that operate similarly (Riviere et al., 2009, Werner et al., 2011, Sundberg et al., 2013b, De Vrieze et al., 2015).

Environmental changes such as changes to temperature, substrate and ammonia concentration appear to shape the community structure but it must be noted that anaerobic digesters probably have additional deoxyribonucleic acid (DNA) present that originated from the influent seed biomass, which would likely have an effect on the microbial dynamics (Saunders et al., 2016). Experiments have been conducted to avoid these false conclusions, including by removing or binding extracellular DNA prior to lysing the cells (Nocker et al., 2006, Wagner et al., 2008, Lee et al., 2015) and by monitoring influent composition (Lee et al., 2015, Mei et al., 2016, Saunders et al., 2016, Seib et al., 2016). The curated MiDAS database taxonomy protocols have been standardised for wastewater treatment plant sludges, but it is presently deficient for AD (Albertsen et al., 2015, McIlroy et al., 2015). This database promises to become a good foundation for studies of anaerobic digester system ecology (Kirkegaard et al., 2017). It is worth mentioning that key

species could be absent from these studies due to PCR biases, and in order to bypass this issue primer-free alternatives are required (Eloe-Fadrosh et al., 2016, Karst et al., 2016). With regard to lab-scale bioreactors, the presence of acetoclastic *Methanosaeta* species appear to be necessary for strong formation and maintenance of granular sludge, which UASBs require to operate successfully (McHugh et al., 2005).

2.3.4 Physiological analysis of AD systems

Various methodologies based on anaerobic microbial activity have provided important information in relation to community metabolism under various environmental conditions and changes, including physiological assays. One such assay is the specific methanogenic activity (SMA) assay (Colleran et al., 1992), which ascertains an activity level for a particular substrate along the methanogenic pathway and determines the methanogenic route for a particular biomass type either through CO₂ reduction or acetate decarboxylation (Willkie, 1987).

The assay consists of monitoring the biogas pressure of sealed vials containing biomass and either a soluble (acetate, butyrate, ethanol or, propionate) or a gaseous (H₂-CO₂) substrate supplied on an individual basis to the biomass in order to assess the activity of the trophic group followed by the determination of the SMA as ml CH₄ g VSS⁻¹ day⁻¹ (Chapters 4 and 5).

Gas chromatography (GC) was performed to measure the CH₄ contained within the sealed vials. A gas chromatograph fitted with a thermal conductivity detector (TCD) ensures that compounds are detected by means of the difference between the thermal conductivity properties of the carrier gas and the target sample. GC is advantageous over other instrumentation methods (e.g. non-dispersive infrared, non-dispersive ultraviolet and chemiluminescence) because it makes it possible to rapidly and simultaneously separate and analyse a complex mixture of volatile organic compounds, it works for a wide range of chemicals and it is capable of measuring at both micro and macro-sample scale (Hilborn and Monkman, 1975, Budiman et al., 2015).

The soluble substrates acetate, butyrate and propionate are measurable using a gas chromatograph fitted with a flame ioniser detector (FID) which is capable of ionising organic compounds by burning them in the H₂ air flame (Budiman et al., 2015). FID is more used in gas chromatography than any other signal detection type and is sometimes called a “carbon counting” system because hydrocarbons induce the same number of ionisation responses as carbon atoms (Holm, 1999, Amirav, 2001).

One particular study compared the two types of gas chromatography methods (GC-TCD and GC-FID) for their determination of C₃H₈ (propane) in N₂ matrix and the authors concluded that the GC-FID method was able to offer higher sensitivity and had a wider linearity range than the GC-TCD method. Generally speaking, however, both detectors do have adequate sensitivity, linearity ranges and are very stable. This may differ to various extents based on the compounds being analysed (McNair, 1998, Grob, 2004, Budiman et al., 2015).

Monitoring sugars involved examining the fermentative stage of the AD pathway and their levels and rates of consumption required a chemical assay. One such technique available is the Dubois method. The Dubois method is a colorimetric straightforward quantitative approach. It utilises the knowledge that phenol mixed with sulphuric acid can determine ketoses, aldoses and their methyl derivatives on a sub-micro scale, and this technique is applicable to all carbohydrates with a free or a potential-reducing group. Therefore, when a sugar-containing solution is added to the phenol-sulphuric acid mixture its optical density is measured and concentration is determined with reference to a standard curve (Dubois et al., 1951). This method works for simple sugars, oligosaccharides, polysaccharides, and their respective derivatives (DuBois et al., 1956).

2.4 Development of microbial ecology

2.4.1 Plate count anomaly

In the seventeenth century Antonij van Leeuwenhoek made observations of “little animals” in water and subsequently built the first microscope that was capable of verifying this observation (Dobell, 1932). Microbial ecology evolved from culture-

dependent microbiology whereby only microorganisms that can be grown under laboratory conditions were studied in independent culture studies. Although staining and microscopy methods have proven crucial in bacterial classification through work achieved by scientists such as Robert Koch, who discovered the relationship between diseases and causative microbes (Ben-David and Davidson, 2014), this culture-dependent work only represented approximately one per cent of the microorganisms that actually exist (Staley and Konopka, 1985, Amann et al., 1995, Alain and Querellou, 2009, Lewis et al., 2010).

Isolates of microorganisms proved difficult to culture for several reasons, including the interdependency of microorganisms and the lack of knowledge in relation to the specific living conditions for these species (Muyzer and Smalla, 1998). These culture-dependent approaches require complex media, consisting of specific ingredients such as certain vitamins, micronutrients, cofactors etc. In order to investigate the true diversity within an environmental sample, other non-culture-dependent methods are required.

2.4.2 Techniques used in molecular microbial ecology

This led to a “renaissance” within the scientific biological community known as the development of molecular microbial ecology. This area of research was able to bypass the past shortcomings of culture-dependent microbiology. A vast number of additional species were thus identified, as well as information regarding their evolutionary histories, community metabolism, phenotypes and genotypes, using techniques such as cloning, denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal-restriction fragment length polymorphism (T-RFLP), fluorescent *in situ* hybridisation (FISH), stable isotope probing (SIP), high-throughput/next generation sequencing (NGS) and quantitative polymerase chain reaction (qPCR) technologies (Leclerc et al., 2004, Sousa et al., 2007, Malin and Illmer, 2008). Many of these techniques rely on targeting the conserved 16S ribosomal ribonucleic acid (rRNA) gene among different species of bacteria and archaea and thus is a useful target for complex environmental samples (Woese, 1987).

2.4.2.1 Cloning

Clone libraries have been extensively employed for microbial ecology community analysis. Molecular cloning has aided in the research of individual genes within living organisms (Arnheim and Erlich, 1992). Initially, DNA is extracted from samples and polymerase chain reactions (PCRs) amplified the 16S rRNA phylogenetic marker gene in many microbial diversity studies related to wastewater treatment systems and other environmental systems (Bond et al., 1995, Jackson et al., 2001, Freitag and Prosser, 2003, Sanz and Kochling, 2007, Riviere et al., 2009, Leigh et al., 2010, Cardinali-Rezende et al., 2013, Shah, 2014).

The PCR method was developed over thirty years ago, at Cetus Corporation where substantial amounts of a specific fragment of DNA was amplified and produced from a complex DNA sample in a straightforward reaction. PCR involves the synthesisation of a targeted DNA fragment using a heat-resistant DNA polymerase (*Taq* isolated from *Thermus aquaticus*) and two oligonucleotide primers and involves the following steps: denaturation to form a single strand, annealing of the first (forward) primer with the single strand, and extension of the forward primer to include the second reverse primer on the other single strand. These steps are sequentially repeated and amplified over a number of cycles until a satisfactory product DNA band is generated (Saiki et al., 1985, Mullis and Faloona, 1987, Saiki et al., 1988, Arnheim and Erlich, 1992). Subsequently, clean-up and insert preparation are carried out, the insert is then ligated into a plasmid vector, competent *Escherichia coli* cells are transformed and screened. Finally, the samples are sequenced for clone identification. The limitations of clone library analyses include missing rare taxa (Kröber et al., 2009, Nelson et al., 2011) and the substantial time required to generate all of the possible clone libraries in a complex ecosystem.

2.4.2.2 DGGE/TGGE

Both DGGE or TGGE and T-RFLP are used for screening and community pattern recognition purposes (Theron and Cloete, 2000). DGGE and TGGE are types of methods which are also commonly used for mutational analysis and for microbial diversity studies. DGGE separates similar-sized genes based on their denaturing

ability whereas TGGE separates nucleic acids based on temperature dependant changes in structure. Each has been used extensively in the study of community complexity. Originally DGGE of PCR-amplified 16S rDNA fragments was implemented in the community analysis of microbes present in a microbial mat and within bacterial biofilm samples (Muyzer et al., 1993). Subsequently, a study was carried out to identify the resultant bands by DNA sequencing directly from the bands that were excised from the gradient gel (Muyzer and de Waal, 1994).

In both DGGE (Fischer and Lerman, 1979, Fischer and Lerman, 1983, Myers et al., 1987) and TGGE (Rosenbaum and Riesner, 1987, Riesner, 1991), same-length fragments of DNA with distinguishing sequences are separated and these fragments of DNA are differentiated based on their melting behaviour (Janse et al., 2004). Fragments proceed down a gradient gel and once the fragment with the lowest melting temperature reaches its melting temperature at a specific location, a transition from a helical to a partially melted molecule occurs and its migration ceases (Muyzer and Smalla, 1998). As previously mentioned, 16S ribosomal RNA (rRNA) genes are widely used as a diversity marker within community studies. The 16S rRNA gene sequences possess the capability of identifying bacteria due to their hypervariable regions which give a species-specific sequence. This allows the recognition of non-cultured bacteria (Clarridge, 2004).

DGGE fingerprinting is based on the principle that 16S rRNA gene amplicons are able to separate in a linear gradient due to differential denaturing characteristics of the PCR product generating characteristic band patterns of complex bacterial communities. It has become a valuable means of microbial ecology analysis. The use of a polyacrylamide gel with an increasing chemical denaturant gradient allows DNA molecules to pass by electrophoresis at different rates depending upon the % GC of the particular molecule as well as the precise base arrangement making up the sequence. In order to accurately complete this technique, PCR primers, one of which contains a 35-40 nucleotide GC clamp, are used. This ensures that the GC clamp remains double stranded, and thus retards and stops at its formation point. Visual analysis alone is not sufficient to produce a clear quantitative analysis of whether two bands represent the same species or not (Muyzer and Smalla, 1998, Sekiguchi et al., 2002, Huber and Peduzzi, 2004). TGGE is also used in a similar

capacity. The difference is that there is a temperature gradient across the gel and the species will melt at different points according to their sequence.

Even though many studies have utilised the 16S rRNA biomarker, PCR products attained from functional genes have also been used. One particular study designed PCR primers to target and amplify the [NiFe] hydrogenase gene from *Desulfovibrio* microorganisms (Wawer and Muyzer, 1995). Simpler communities such as co-cultures have also been studied using DGGE of rDNA fragments. Authors reported that, by sequencing and applying more selective environmental conditions, they isolated a *Desulfovibrio* and an *Arcobacter* strain in pure culture (Teske et al., 1996).

The DGGE and TGGE methods have several advantages, including their high sensitivity, high detection rate and absence of radioactivity, as well as the fact that sequencing can be performed on the PCR fragments post-technique. Disadvantages and limitations lie in the primer cost, coarse taxonomic resolution, qualitative or semi-qualitative data and specialised equipment, as well as the fact that there is frequently a lack of reproducibility of results both between and within laboratories (Crosby and Criddle, 2003, Talbot et al., 2008). The small fragments of up to 500bp limits the amount of phylogenetic sequence information that can be inferred (Myers et al., 1985). Moreover, the attempt to excise individual bands may be compromised due to co-migration of DNA fragments, leading to problems in sequencing (Muyzer and Smalla, 1998).

2.4.2.3 T-RFLP

T-RFLP (Liu et al., 1997) relies on size polymorphism measurements of terminal restriction fragments from a PCR product. It emerged as another fingerprinting method after techniques such as DGGE and TGGE described above. It has some clear advantages, including high taxonomic resolution, good reproducibility between and within laboratories, ability to directly correlate the sequence database with the reference fragments or sample and immediate gel analysis. It has a significant cost advantage over clone libraries (section 2.4.2.1). Combining these techniques by processing large sample numbers with T-RFLP followed by sample selection for the generation of key species identification using the clone library

method utilises both techniques to their full and most efficient potential (Widmer et al., 2006, Lindahl et al., 2007). Thus, this method is combined with sequence databases in order to identify which microorganisms correspond to the different TRFs (Dickie and FitzJohn, 2007, Roberts et al., 2012).

However, T-RFLP has some disadvantages and limitations in that there are both shared T-RFLP profiles among two different species and multiple T-RFLP profiles within a specific species. Generating absolute abundances rather than relative abundances presents a challenge, as do the requirements for multivariate statistical analyses, cluster analysis for group identification and either principal components analysis or multi-dimensional scaling to investigate continuous variation (Marsh, 1999, Blackwood et al., 2003, Egert and Friedrich, 2003, Grant and Ogilvie, 2003, Lueders and Friedrich, 2003, Rees et al., 2004, Hartmann et al., 2005, Dickie and FitzJohn, 2007, Talbot et al., 2008).

In summary, the method includes an initial PCR of environmental DNA extracts with fluorescently-labelled primers, followed by digestion using a restriction enzyme of the terminally labelled amplicons from the PCR amplification, and lastly the resulting terminal restriction fragments (TRFs) size measurements are determined using an electrophoresis platform (Liu et al., 1997). Fluorescent peak profiles are then referred to as “community fingerprints” in environmental samples for diversity and composition analyses where the fluoresce intensities can determine the relative abundance of a fragment within a sample (Cotton et al., 2014).

2.4.2.4 FISH

Since 1990, fluorescence in situ hybridisation (FISH) has become a very successful method because it is able to simply and rapidly quantify methanogens (Stabnikova et al., 2006, Weiland, 2010) in their natural environment in a culture-independent way (Amann et al., 1990a, Amann et al., 1990b, Dedysh et al., 2001). It is very useful for many different types of microbiology. It can identify, visualise, enumerate and localise microbial cells. It aids the understanding of extremely complex microbial ecosystems as it can determine both spatiotemporal dynamics and physiological properties of methanogens within their natural environment conditions.

This method can overcome the challenges faced in traditional cultivation-dependent methods when studying methanogens, such as their requirement for obligate anaerobiosis and the nature of their slow growth rates (Kumar et al., 2011). There are both advantages and downfalls associated with using this technique. Originally, *in situ* hybridisation was developed independently by two research groups (John et al., 1969, Pardue and Gall, 1969). FISH detects sequences of nucleic acid using a probe which is fluorescently labelled and it hybridises specifically to a target sequence which is complementary to it.

The process begins with fixation of sampled cells followed by sample preparation, hybridisation, unbound probe wash-off, mounting, visualisation and, finally, the documentation of the results. 16S rRNA is the most commonly used target molecule. There can be pitfalls, such as the autofluorescence of the microorganisms themselves, a lack of specificity from the oligonucleotide probe and its binding, insufficient probe penetration, a low rRNA content due to low physiological activity and photobleaching due to the fading of fluorochromes resulting in destruction of them over time.

Probes are usually approximately 15 to 30 nucleotides in length and are covalently labelled with a fluorescence dye at the 5' end. This method is being improved upon continuously. Group-specific probes for taxonomies such as the Bacteria, Archaea and Eukarya domains are still proving valid and popular. They ensure an initial rapid assessment of a group which is the overall dominant one (Moter and Göbel, 2000, Amann et al., 2001, Amann and Fuchs, 2008). Additionally, due to the hundreds of thousands of rRNA sequences that are now known, new oligonucleotide probes can be designed for community and community dynamics analyses (Amann and Fuchs, 2008).

FISH investigation has provided a better analysis for microbial diversity within wastewater treatment environments. Many bacteria and archaea have been examined and numerous insights into their genetics have been gained through this technique. Methanogens are differentiated from other microorganisms due to a few unique features such as pseudomurein, phospholipid etherlipid and CH₄ production (Balch et al., 1979, Woese et al., 1990, Leahy et al., 2010, Sirohi et al., 2010)

It has been reported recently that the combination of micro-sensors with FISH has enabled the simultaneous analysis of bacterial communities and metabolic activities. This suggests that anaerobes are in anaerobic micro-niches within an anaerobic environment. This combination can demonstrate community structure change and biofilm growth over time. The main advantages of FISH are that it is easy to perform, inexpensive and rapid. However, FISH is not properly automated as of yet. Automation would increase analysis throughput at a much higher rate as compared with what is possible at present. The tediousness of manually counting cells through a microscope is a major drawback (Schramm et al., 1999, Moter and Göbel, 2000)

Micro-autoradiography is a powerful method for tracing radio-labelled compounds within a sample using particular bacteria down to the resolution of a single cell. It enables eco-physiological features to be distinguished, such as the production of bacterial biomass. However it is regarded as a challenging technique (Alonso, 2012). If specifically labelled substrates are accessible, microautoradiography coupled with FISH (MAR-FISH) is possibly the best way to discover the physiological activity of bacteria in conjunction with their environment. There is another approach, that of combining FISH with 5-cyano-2, 3-tolyl-tetrazolium chloride staining but this method misses out on the discovery of numerous active cells. The main characteristic of MAR-FISH which has enticed increased study is that it can be applied to identify specific members of a range of phylogenetic groups which are active. It enables direct analysis of *in vivo* substrate uptake under various conditions.

As well as MAR-FISH, the Raman microscopy and nano-scale secondary-ion mass spectrometry (NanoSIMS) techniques are also used. In brief, Raman microscopy involves using Raman spectra to detect labelled substrate incorporation. NanoSIMS enables the imaging of multi-isotopes (Lee et al., 1999, Nielsen et al., 2003, Wagner et al., 2003, Amann and Fuchs, 2008). Increased sensitivity has paved the way for more multiplexing options to identify microbes in their own environments (Wagner and Haider, 2012). The FISH method has been constantly improving for almost 25 years. It has great potential for analysis of complex communities present within the AD process. FISH has also been a beneficial technique for the study of anaerobic

granular ultrastructure (Saiki et al., 2002) and methanogenic identification (Rocheleau et al., 1999).

2.4.2.5 SIP

Stable-isotope probing (SIP) of nucleic acids has become a robust and suitable tool capable of linking microorganism identity with activity in many various types of environments (Dumont and Murrell, 2005) under culture-independent means. Firstly, a stable isotope such as ^{13}C (Radajewski et al., 2003, Dumont and Murrell, 2005, Neufeld et al., 2007, Uhlík et al., 2009, Chen and Murrell, 2010, Madsen, 2010), ^{15}N (Buckley et al., 2007a, Buckley et al., 2007b, Roh et al., 2009, Bell et al., 2011) or, less often, ^{18}O and ^2H (Aanderud and Lennon, 2011, Woods et al., 2011), is incorporated into specific substrates in the system which is to be analysed. DNA or RNA is then extracted from the sample and an ultra-centrifuge separates out the labelled and unlabelled fractions of the sample into different densities. The microorganisms which assimilated the labelled substrate into their cells by phylogenetic biomarkers such as their phospholipid-derived fatty acids (Boschker et al., 1998), DNA (Radajewski et al., 2000) or rRNA (Manefield et al., 2002) are screened using molecular techniques.

Phylogeny can subsequently be linked to function. These types of short-term label application experiments are capable of determining which specific microorganisms are responsible for a particular substrate degradation in a steady-state environment (Hori et al., 2007). Moreover, this provides information on microorganisms that cause environmental changes (Neufeld et al., 2007) as well as associated genes that are crucial to the potential of microbial community functions and roles. Metagenomic integration has elevated researchers' insights into both the potential processes and applications within the microbial community (Uhlík et al., 2013).

2.4.3 High-throughput / Next generation sequencing (NGS) approaches

Sequencing technologies play a significant role in the field of microbial ecology. Their origins date back thirty years to the discovery and identification of the ancient

prokaryotic 5S, 16S and 18S rRNA marker gene sequences (Woese, 1987, Head et al., 1998, Suau et al., 1999, Eckburg et al., 2005, DeSantis et al., 2006). The conserved regions of the 16S gene are of paramount importance as they are widely used today as higher taxa identifiers and as consensus markers for microbial assessments using each technique outlined in section 2.4.2. The variable regions are capable of identifying closely-related species (Rudi et al., 2007).

These analyses remain the standard for culture independent microbiological techniques and their accuracy is highly dependent on primer choice (Klindworth et al., 2013). The widely-used 16S/18S rDNA databases including SILVA (Pruesse et al., 2007), greengenes (DeSantis et al., 2006) and RDP II (Cole et al., 2007), come from PCR amplification assays. The authors of one particular study re-evaluated their own results using the Global Ocean Sampling (GOS) database, which consists of 6.3 billion base pairs (bp) of Sanger sequence reads (Rusch et al., 2007) with additional samples taken from the Atlantic and Indian Oceans (Yooseph et al., 2010). The authors reported that even the commonly-used primers exhibit big differences in coverage of the phylum spectrum and they recommended just 10 primers as broad range primers. This is out of 175 primers and 512 primer pairs examined (Klindworth et al., 2013).

The Earth Microbiome Project (<http://www.earthmicrobiome.org/>) is a collaborative effort to comparatively analyse and seek out universal primers for genes akin to the 16S rRNA gene in terms of conserved and common genes. From this work, certain universal sets of primers have been established (Caporaso et al., 2012) but bias is introduced because of the information that is restricted to the sequence between primers (Zhou et al., 2015).

2.4.3.1 Sanger sequencing to NGS

The use of ribosomal RNA genes along with Sanger sequencing propelled the field of microbial ecology and microorganism identification and classification to where it is today. Sanger sequencing (Sanger et al., 1977) is a technique whereby dye-labelled normal deoxynucleotides (dNTPS) are mixed with dideoxy-modified dNTPs followed by a PCR reaction. During elongation, some of the strands take up a dideoxy-dNTP and terminate the process. The strands subsequently separate out on a gel where the terminal base label is determined for each strand by laser

excitation and emission results (Goodwin et al., 2016). It is currently capable of attaining up to 96 sequences per run with approximately a 650 bp in length. That may be enough for phylogenetic marker analysis but NGS platform technologies can generate millions of DNA molecule sequences in parallel of various lengths and yields (Escobar-Zepeda et al., 2015).

Many innovations originating in the above instrumentation from Sanger sequencing paved the way for the Human Genome Project (Mardis, 2013), and ultimately this stimulated the development and commercialisation of NGS (van Dijk et al., 2014). It was only in the mid-2000s that the cost of human genome sequencing began to decline with the release of the Human Genome Project. This led to ideas for understanding genome complexities (Wetterstrand, 2016), including the ability to use read lengths the size of some full genomes (Goodwin et al., 2016). Although NGS technologies can result in a large amount of data, the error rates are higher and the read lengths tend to be shorter than had previously been the case when using traditional Sanger sequencing.

The pioneering technology for the development of NGS was the 454-sequencing platform or “pyrosequencing”. This method involved a cycle of single nucleotide additions whereby the pyrophosphate (PPi) becomes luminescent when freed from the DNA polymerisation reaction. A micro-well plate containing millions of wells with different DNA fragments emits light that is read by a machine and interpreted into nucleotide sequences along with base quality values. Its primary bias is the long homopolymeric regions generating artificial insertions and deletions, now rendering it obsolete (Margulies et al., 2005, Escobar-Zepeda et al., 2015).

Amplicon software has been designed and developed for both Sanger and 454 ribosomal pyrotags, including Mothur (Schloss et al., 2009), Quime (Caporaso et al., 2010), MEGAN (Huson and Weber, 2013) and CARMA (Krause et al., 2008). In recent years, the metagenomics software considers short sequences similar to Illumina reads or else much longer PacBio reads (Escobar-Zepeda et al., 2015).

454 sequencing technology is analogous to the Ion Torrent platform in that a similar yield and fragment length is produced within the middle stage of the sequencing run. It was the first NGS technology that did not utilise optical sensing, and instead it relied on semi-conductor technology (Rothberg et al., 2011). It is hailed as the

smallest potentiometer in existence as it can detect H₂ potential changes with the release of a proton once a new nucleotide joins the reaction occurring in each micro-well (Rothberg et al., 2011). Its maximum yield is approximately 500 million reads of up to 400 bp fragment lengths (Glenn, 2014). Ion Torrent sequencing carries the added financial benefit of costing only one tenth as much as 454 sequencing (Whiteley et al., 2012).

2.4.3.2 Illumina sequencing platform

Illumina technology has fast become one of the most popular sequencing choices because of its high yield and low cost, which facilitates multiple lines of research. The principle of Illumina technology is the synthesis of DNA fragments, with fluorescently-labelled nucleotides undergoing reversible-termination sequencing. It involves flow cell technology whereby DNA fragments are attached and distributed followed by the addition of a labelled nucleotide. A laser then excites this fluorescent molecule in order to relay a signal to the machine. Subsequently the fluorophore is detached and the next nucleotide is incorporated as before in an amplification manner known as “bridge amplification”. The DNA molecules are capable of being sequenced from one or both ends creating single or pair-end sequencing of up to 300 bp read lengths (Bennett, 2004, Metzker, 2010). Because it accounts for the most outputs among this second generation sequencing technology, it is also capable of multiplexing hundreds of samples (Glenn, 2014). Illumina technology, and particularly 16S rRNA profiling from DNA samples, has been applied to a wide variety of environments, including AD systems.

One particular concern in this age of sequencing is whether the biological results and final conclusions from one platform are consistent with other platforms. One study has reported consistent biological conclusions across sequenced amplicon regions for Illumina HiSeq2000 and MiSeq systems (Caporaso et al., 2012). Further studies would be beneficial to test this question of sequencing consistency to ensure the validity of scientific results and conclusions that have been drawn thus far and into the future.

Third generation sequencing technologies are currently in ongoing development, as attempts are made to reduce or eliminate the issues caused by the trade-offs of either

read length, yield or cost. Presently there are two technologies which are giving cause for optimism. PacBio RS from Pacific Bioscience (Fichot and Norman, 2013) and the Oxford Nanopore (Kasianowicz et al., 1996) are single molecule, real-time technologies that have reduced the bias caused by amplification and the short fragment length issue, as well as improving the time and cost problems. The disadvantage to these technologies lies in the error rates that are generated in comparison with the other platforms, but this can be alleviated provided that the sequencing is deep enough. Both of these sequencing generations are more time-efficient due to the absence of DNA cloning vectors of bacterial hosts requirements, which both simplifies the library preparation and also reduces DNA contamination that can be picked up from foreign organisms that are not part of the study (Escobar-Zepeda et al., 2015).

From this section and the previous section (summarised in Table 2.1), it is clear that NGS has evolved to a level in fundamental scientific research whereby it is also capable of translational research across many biological fields to enable the better understanding of environments, health and disease (van Dijk et al., 2014).

Table 2.1 Specifications of NGS technologies adapted from several publications (Buermans and den Dunnen, 2014, van Dijk et al., 2014, Escobar-Zepeda et al., 2015, Goodwin et al., 2016).

Platform	Read length (bp)	Reads	Runtime	Error rate (%)
454/Pyrosequencing	1200	200000	20 h	1
Ion Torrent	200-400	4 million	4 h	~1
Illumina MiSeq	2 x 300	25 million	65 h	~0.1
Illumina HiSeq	2 x 100	3 billion	12 days	~0.1

2.4.3.2.1 Active community insights

Active communities generally contain more 16S rRNA than starved or dead cells. RNA is less stable than DNA because of its shorter lifetime (Novitsky, 1986), which demonstrates that it is representative of the active section within a sample (Kahlisch et al., 2012). This has led to more in-depth analysis of the microbes that are actively involved in AD (Ito et al., 2012, Zakrzewski et al., 2012) and can be expanded to

microbial community analysis under different environmental conditions/stresses (Chapter 6). Generally speaking, the use of rRNA can be more representative than DNA and these are the reasons why the cDNA of granular communities was examined and investigated in Chapters 5 and 6.

However, similar to all techniques, RNA-based methods have their limitations. It has been suggested that RNA work undergoes identical biases to DNA work (Norris et al., 2002a, Griffiths et al., 2003a, Griffiths et al., 2003b, Hoshino and Matsumoto, 2007). RNA-Sequencing / whole transcriptome shotgun sequencing identifies the presence and quantities of RNA within biological samples by using NGS. This method enables the monitoring of a changing transcriptome over time as a gene expression investigation (Morin et al., 2008, Chu and Corey, 2012, Wang et al., 2009b). There have been many published opinions promoting either DNA or RNA work but this thesis aimed to investigate community structures and compare the active with the potential communities (Chapter 5).

2.4.4 Sequencing data and statistical analyses

2.4.4.1 Sequencing data analysis

Despite the substantial advantages in the novel microbial community discoveries that NGS technologies furnish scientists with, they also present great computational challenges. These very powerful second and third generation sequencing platforms generate large datasets which demand higher and more complex levels of bioinformatics as well as vast amounts of storage and computational power and speed within computers (Logares et al., 2012). For example, a single run with the Illumina HiSeq2500 is capable of generating as much as 600 Gigabases of data (Scholz et al., 2012) requiring approximately 0.6 Terabytes of disk space (Glenn, 2011, Logares et al., 2012). High-end servers and UNIX operative system knowledge and training are also required (Escobar-Zepeda et al., 2015).

Although increased computer space requirements may surpass sequencing quotas, pre-processed data are capable of successful analysis on personal computers (Logares et al., 2012). Subsequently, statistical analysis is used on most personal computers using R software for example (Team, 2016). Multivariate statistical

analysis has become increasingly important for microbial community studies regarding the environment (Mouser et al., 2005).

2.4.4.2 Alpha diversity

Almost sixty years ago species diversity analysis was divided into three categories: alpha (α), beta (β) and gamma (γ) diversity (Whittaker, 1960). Both α and γ diversity are designated as inventory diversity (Jurasinski et al., 2009) and have the same characteristics (Zhang et al., 2014). Both of the following formulae have been used as a definition of diversity: $H\alpha + H\beta = H\gamma$ or $H\alpha \times H\beta = H\gamma$ (Jost, 2007). 16S rRNA gene microbial ecology studies generally focus on just α and β diversity, relating community organisation between different samples (Lemos et al., 2011). α diversity within a community is described based on species richness, species evenness (abundances) or by indices which combine both variables (Lozupone and Knight, 2008). Types of α analysis have been widely applied, such as rarefaction curves (qualitative) as well as Simpson (Simpson, 1949) and Shannon (Shannon and Weaver, 1949) diversity indices (quantitative) (Chapter 6). This allows a good estimation of phylogenetic relationships among single microorganisms to be extrapolated and generates a clearer picture of α diversity (Lozupone and Knight, 2008).

2.4.4.3 Beta diversity

β diversity represents differences of species between communities, and β research has increased in recent years (Anderson et al., 2011, Sfenthourakis and Panitsa, 2012, de Juan et al., 2013). Patterns of distribution have been placed under ecological scrutiny and ideas as to how to conserve diversity under environmental changes have become a topic of interest (Melo et al., 2009, Wang et al., 2009a, Meynard et al., 2011). β diversity investigations study differences in two or more communities and allow researchers to infer how they change over time (Lozupone and Knight, 2008).

Divergence-based measures are more reliable than species-based measures because the latter relies on a poorly-understood species concept but is capable of evaluating

whether similar environments contain the same species (Noguez et al., 2005, Lozupone and Knight, 2008). Divergence-based measures have often been applied to β diversity to determine whether two communities or two samples are significantly different, including the Phylogenetic test (P test). The P test determines significance between two communities which are randomly distributed over a phylogenetic tree (Martin, 2002) and is therefore subject to errors imposed from a single tree topology (Lozupone and Knight, 2008).

Multiple statistically-equivalent phylogenetic trees have bypassed this issue by using bootstrapping or Bayesian phylogenetic methods to create a more powerful and robust test (Jones and Martin, 2006). Varying β diversity measures can be required depending on either significant differences between communities or distances between pairs of communities, which can require the use of multivariate statistical techniques such as ordinations or clustering (Lozupone and Knight, 2008). β diversity analyses often involve producing Canonical Correspondence Analysis (CCA) plots, bar plots, cluster trees (Chapter 6) and PERMANOVA statistical analysis.

Different views have been expressed as to which type of diversity should be prioritised. Some recommend α diversity and some β diversity but others believe that both work together (Jost, 2007, Chiarucci et al., 2010, Meynard et al., 2011, Zhang et al., 2014). α diversity tends to be essential in homogenous community environments with strong-diffusion species and β diversity is more crucial in heterogeneous-driven communities that contain weak-diffusion species (Crist and Veech, 2006, Chiarucci et al., 2010). It has been suggested that environments can be compared adequately between samples according to microbial diversity even with a relatively low sequencing effort (Lemos et al., 2011). There has been a widespread development in the area of species diversity measurements and analysis and this has been crucial to the understanding of ecological properties within microbial communities (Koleff et al., 2003, Magurran, 2004). There is no single answer that can determine all hypotheses and questions surrounding microbial diversity so a multi-viewing approach is necessary to build up a whole picture of an environmental community (Lozupone and Knight, 2008).

2.4.5 Quantitative microbial ecology

In sections 2.4.2, 2.4.3 and 2.4.4, the techniques described were generally qualitative or semi-quantitative. The 16S rRNA gene is universally conserved with a reasonably constant evolutionary rate and at least one copy of it is contained within the genome (Gray et al., 1999, Acinas et al., 2004). The gene is suitable for bioinformatics analysis because approximately 1,600 bp make up the gene also allowing for phylogenetic discrimination of prokaryotes (Van de Peer et al., 1996, Prosser, 2002). Once species have been identified using a sequencing method, the number of species can be subsequently quantified using primers applicable to specific microorganisms or groups of microorganisms from various environmental samples.

General and functional primers are both used as quantification tools to discern the abundance of a particular class, order, family etc. or functional parameter within an environmental sample. Primer choice is crucial to ensuring the highest coverage of species and taxa possible. Otherwise there will likely be underestimation or even complete neglect of certain species (von Wintzingerode et al., 1997, Kanagawa, 2003). Even though there is no “perfect” primer pair, especially for Archaea, further research into primer design and validation is required (Klindworth et al., 2013). Many experiments have failed due, in part, to improper selection or design of primer-probe sets (Lim et al., 2011).

Even though qualitative analysis of microbial diversity is informative, quantitative data provides more applicable data for species development and growth, which can lead to more insight into bioreactor processing (Yu et al., 2006). For example, this absolute quantification is required in order to evaluate the biokinetics and growth of specific microorganisms (Ahn et al., 2004) (Chapter 3).

Real-time quantitative polymerase chain reaction (qPCR) is another cultivation-independent method. This uses certain fluorescent reporter molecules to monitor and quantify amplification products after each PCR cycle. This method removes the necessity of gel electrophoresis for product detection and eliminates the requirement to use either Southern blotting or sequencing techniques to identify each amplicon sample. Its simplicity, sensitivity and high throughput capacity means that qPCR is ideal for the detection and comparison of nucleic acid levels

(Bustin et al., 2005). This method can detect even a few copies per reaction (Khot and Fredricks, 2009) and its linear quantification ability can exceed eight orders of magnitude (Song et al., 2010).

It has become increasingly commonplace in environmental microbial ecology to detect and target either specific species or groups of species such as the genera *Nitrosomonas* and *Nitrospira* for example (Lim et al., 2008) and this is among the best and most robust methods for gene amplification across many biological disciplines (Suzuki et al., 2000, Takai and Horikoshi, 2000, Klein, 2002, Schena et al., 2004, Hori et al., 2006). When growing pure cultures, growth yields and doubling times can be monitored by using quantitative data from the qPCR methodology. qPCR technology can be confidently applied to questions surrounding strains or sequences which have previously been analysed and described. Otherwise qPCR data needs to be linked with other molecular tools such as fingerprinting, clone libraries or FISH as described in section 2.4.2 to enhance information gathered on community structure complexities (Lim et al., 2011). The level of sensitivity can go down as far as the gene expression within a single cell. Other methods have proved to be less flexible than qPCR in the past. These include Northern blot and *in situ* hybridisation for example (Freeman et al., 1999).

qPCR achieves its aim of quantification by incorporating several steps. The first part involves extracting DNA from the sample(s) and this is followed by the development of quantification assays. An assay employs DNA binding dye such as the fluorescent SYBR green or a more specific assay involving a quencher and a fluorescent report (Goel et al., 2005) such as TaqMan. The TaqMan assay involves two primers and one fluorescent dual-labelled probe which has both reporter and quencher dyes. There are two biochemical reactions that initiate the assay: (a) 5' → 3' exonuclease activity of *Taq* polymerase and (b) the fluorescence resonance energy transfer which occurs between the quencher and reporter (Giulietti et al., 2001). The SYBR Green assay is less specific as it does not require a probe. Next, a standard curve is generated for either assay. Possible contamination and control of inhibitors needs to be addressed so that any suspect results are removed from the analysis. The final stage is data analysis. A distinctive advantage of qPCR is that it can be used to quantify bacteria which are essentially impossible to culture (Wéry et al., 2008).

Real-time PCR studies have used many different primers and probes for methanogen quantification (McSweeney et al., 1993, Odenyo et al., 1994, Krause et al., 1999, Tajima et al., 2001, Nadkarni et al., 2002, Ouwerkerk et al., 2002, Sawayama et al., 2004). The first report on the development of seven primer-probe sets was published for methanogens found in engineered systems including the Archaea domain, order-specific primers for *Methanosarcinales*, *Methanococcales*, *Methanobacteriales*, *Methanomicrobiales* and family-specific primers targeting *Methanosarcinaceae* and *Methanosaetaceae* (Yu et al., 2005). This method has been employed for many anaerobic digester community structure studies. For example, *Methanoculleus* sp. was found to be dominant in a thermophilic anaerobic digester using qPCR (Hori et al., 2006). The group-specific primers listed above have been implemented in methanogen quantification within anaerobic digesters.

A SYBR green I qPCR assay capable of quantifying total gene numbers of the methyl coenzyme M reductase-subunit was created by a group along with TaqMan probes targeting nine different methanogenic phylogenetic groups both from anaerobic digesters and acidic peat samples. Species from within *Methanosaetaceae*, *Methanosarcina*, *Methanobacteriaceae* and *Methanocorpusculaceae* families and from the Fen cluster were detected (Steinberg and Regan, 2009).

Since this technique is so sensitive to any DNA contamination, it can seriously undermine the resulting data. Detecting pathogens using traditional end-point PCR had problems, which the qPCR methodology was able to overcome. Negative controls are generally used with this technique to make sure that any fluorescence signal which was seen was an indication of PCR amplification of the template DNA rather than other components of the PCR mixture. A study reported that the TaqMan probe was used in conjunction with qPCR to detect pathogens throughout their different stages of wastewater treatment. This group did suggest that pure genomic DNA from wastewater was extremely difficult to find and the qPCR experiments were time-consuming with regard to set-up. However, qPCR is faster and more specific than culture-based methods. This demonstrates a big potential for qPCR to be used specifically for detecting pathogens within wastewater (Shannon et al., 2007).

qPCR experiments do have limitations due to both technical and biological inconsistencies and therefore, there is a significant chance of error being introduced into these experiments. An accurate design is crucial. One study suggested that the most successful way of increasing the validity of qPCR trials is to use more independent replicates of a biological nature which have been randomly picked from every subpopulation (Kitchen et al., 2010). More recent work has begun to connect the population dynamics of methanogenic groups with the various functions and processes which occur in a large-scale anaerobic treatment facility (Song et al., 2010). The suitability of qPCR as a technique for complex community analysis has yet to be determined (Kumar et al., 2011).

In summary, microbial ecology has progressed tremendously over the past number of decades. It can be concluded that molecular methods in ecology need to be selected on the basis of determining and detecting meaningful ecological insights. With the vast array of techniques at researchers' disposal, deciding the best way to answer questions while trying to incorporate as much precision as possible in relation to variables such as species abundances, frequencies and identifications is always a difficult task (Dickie and FitzJohn, 2007). Wherever and whenever possible, it is usually most desirable to use multiple techniques to attain the best possible assessment of a microorganism, a co-culture or a microbial community (Bougoure and Cairney, 2005, Allmér et al., 2006).

2.4.6 The 'Omics Era

In parallel with the sequencing revolution in microbial ecology (Section 2.4.3), researchers have been able delve deeper into understanding environments at a "whole community" level. This entails linking collective microbial communities to specific metabolic pathways at metagenomic (the study of all genes in a microbial system at the DNA level), metatranscriptomic (the study of gene expression in a microbial community at the RNA level), metaproteomic (the study of proteins within a community at the protein level) and metabolomic (the profile of metabolites present and their fluxes) levels (Figure 2.8). Together this allows for the culture independent analysis of entire specific ecosystems (Handelsman et al., 1998).

Many genomes of previously uncultured microorganisms that are important within wastewater treatment bioreactors have been discovered such as *Candidatus Accumulibacter phosphatis* (a polyphosphate-accumulater), *Candidatus Nitrospira defluvii* (a nitrite oxidiser) and *Candidatus Kuenenia stuttgartiensis* (an anammox bacterium), for example. The complete genomes for eleven methanogenic archaea which have been isolated from anaerobic sludge are now available. These include four genomes from Methanosarcinales, three from Methanomicrobiales and four genomes from Methanobacteriales. It has been suggested that these types of metagenomic studies, integrated with the other forms of ‘omics studies listed previously and resulting data generation, provide a deeper understanding of community interactions and responses to environmental changes within ecosystems (Rodríguez et al., 2015).

Metatranscriptomics analyses are required to establish a functional profile of the microbial sample. One AD study implementing metatranscriptomics analysis reported expression of enzyme-encoding transcripts for substrate hydrolysis, acidogenesis and the formation of acetate as well as a large number of archaea (Zakrzewski et al., 2012). Metaproteomics investigates key enzymes within important pathways and links these with both genomes and transcriptomic profiles of microbial communities. However, this method is unable to define which proteins are in their active form. One such study implemented proteomics to find the link between protein function and such key metabolic pathways within an anaerobic EGSB reactor treating synthetic glucose-fed wastewater (Abram et al., 2011).

Metabolomics examines which by-products are produced by the microbial community and released into the environment. Metabolomics in AD studies is challenging due to the sheer number of possible metabolites that can be present or formed during different operational conditions. To alleviate the workload presented by this technique, only specific metabolites are monitored to focus on their underlying mechanisms (de Kok et al., 2013, Vanwonterghem et al., 2014). Each method enables the discovery of important information separately (Aguiar-Pulido et al., 2016).

Together, meta-omics provide a more comprehensive outlook on the community being studied. For example, it allows for the discovery of new microbes and new

pathways relevant to wastewater treatment and AD with a potential to optimise bioreactors based on these fundamental microorganism meta-omics approach studies. It also has the potential for targeted biomarker development to be used as a predictive tool for bioreactor performance (Rodríguez et al., 2015). Thus, it is becoming increasingly important to increase the knowledge and insights of structure-function microbial interactions in order to better understand AD processes (Daims et al., 2006).

Overall, this combination of meta-omics allows for the understanding of intricate and complex systems of communities, like in AD, which have previously been regarded as ‘black boxes’. Integration of meta-omics data into engineering applications remain a continuing challenge for researchers. To date, each of the methods mentioned have been applied to wastewater treatment biotechnologies except for community- or meta-metabolomics. These methods have primarily focused on fundamental biological information but have the potential to progress onto a systems biology view of optimising operational procedures, for example by optimising resource recovery (Sales and Lee, 2015). Modern systems biology collates comprehensive datasets and allows for molecular-level analyses on whole systems performances from high-throughput sequencing technologies (Kitano, 2002).

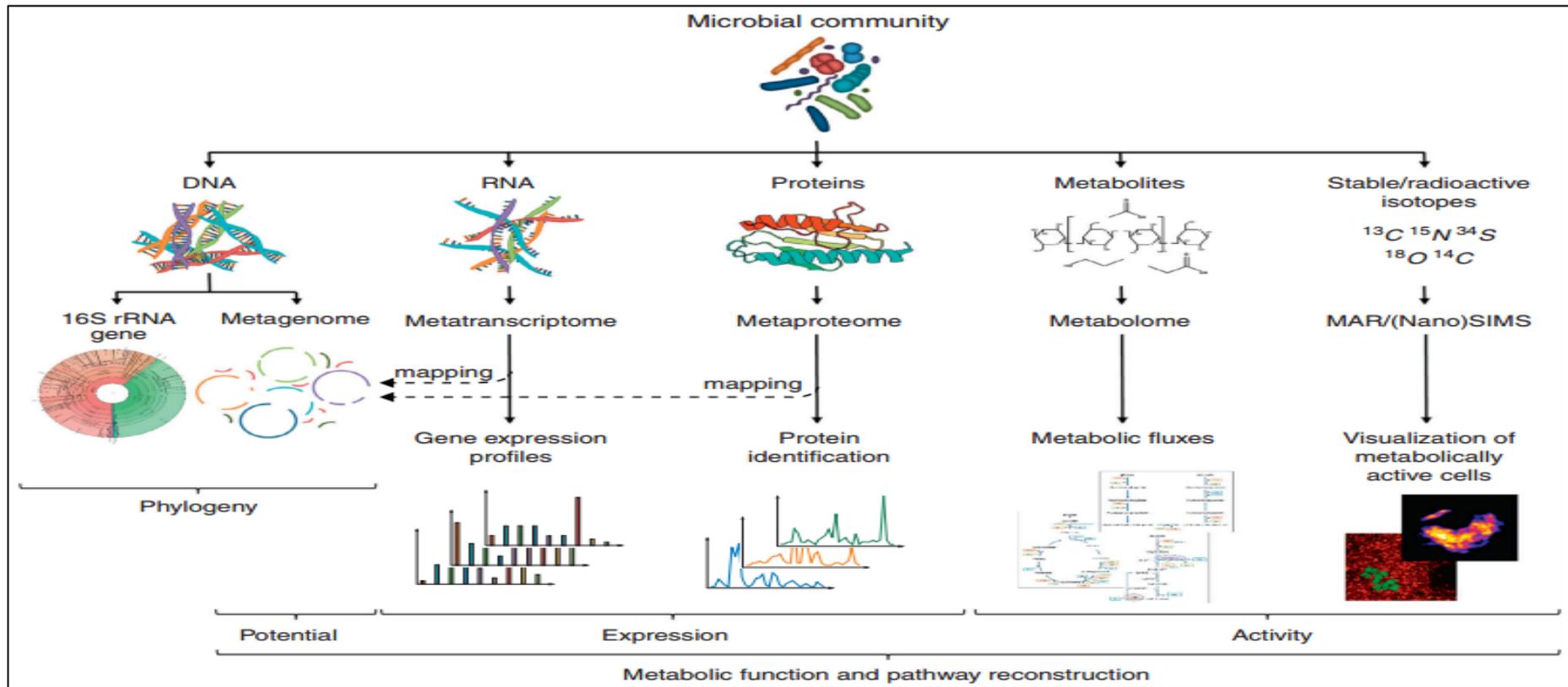


Figure 2.8: A summary of the main methods involved in determining both community phylogeny and functionalities. Taken from (Vanwonterghem et al., 2014).

2.5 Central concepts of the thesis

2.5.1 Microbial interplay in the acetogenic-methanogenic realm

The basic metabolism of AD is fairly well known but there is still a lot to understand and discover about the microbes involved in AD (Weiland, 2010). Anaerobic digesters have been referred to as “black boxes” because the role of many of the microorganisms involved in AD is still poorly understood. Understanding their pathways and characteristics will aid in counteracting reactor failure and making AD more efficient (Malin and Illmer, 2008). Chapter 3 aims to analyse the growth and metabolic interplay that exists within a select few key microorganisms within the acetogenic and methanogenics stages of AD.

2.5.2 The individual anaerobic sludge granule – a whole ecosystem

It is widely speculated that systems biology is the way forward in microbial ecology in terms of obtaining as close to “the big picture” of naturally-occurring ecosystems as is possible. Debates have occurred which have discussed the merits of looking at DNA or cDNA or proteins or metabolites for the best insight into the workings of a microbial system. Now, with systems biology, it is generally accepted that all of these components are needed to obtain the most conclusive and accurate findings and observations about particular microbial communities. The interactions of the microbial community within the environment have been dubbed the “environmental microbial interactome”; this refers to the great opportunity that fully investigating the complexity of microbial worlds in a variety of environments presents to scientists (Larsen et al., 2012).

Studies of single cell microbiology which took particular interest in labelling experiments occurred in the mid-2000s. Single-cell imaging has been widely used for cell growth, cell structure, cell division and biofilm studies. It is the potential of ‘omics and whole ecosystem technologies that are just beginning to be realised. The ‘omics era has developed technologies from metagenomics to metatranscriptomics to metaproteomics to metabolomics. They each give a comparative analysis of different types of signals according to which genes, proteins or metabolites are

switched on within cells. There are advantages of using ‘omics as tools for generating a plethora of data.

As described previously in section 2.1, anaerobic environments include soil, lakes, etc. but these types of communities present difficulties in capturing a true representative sample due to the size of the field or lake, for example. Sampling from a particular site will not guarantee that each and every species from all of the trophic groups will be present.

With regard to anaerobic sludge granules, it is speculated that each individual sample contains microorganisms from the different trophic groups in the AD process. This implies that each granule physically contains a whole entire community or ecosystem within a spherical granule of approximately 1-3 mm in diameter. Previous studies have reported the granular ecology and microbial structure (MacLeod et al., 1990, Guiot et al., 1992, Chen and Lun, 1993, Fang, 2000) of these anaerobic granular communities. However, few have tested an individual granule as a “whole ecosystem” and linked physical characteristics with high-throughput phylogenetic testing of what happens to the granular community structure under a variety of different synthetic environmental stresses. Several experiments, detailed in Chapters 4, 5 and 6, investigate the individual granule in terms of its wastewater source, granular size fraction, granular potential community structure and granular active community composition.

2.6 References

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Chapter 3

Chapter 3 - An investigation into microbial dynamics and interplay within and between model anaerobic digestion acetogenic and methanogenic players

Abstract

Anaerobic Digestion (AD) comprises the microbiological breakdown of complex organic materials in the absence of oxygen to produce biogas, which can be used as a renewable fuel. However, there is a knowledge gap regarding the microbiology that underpins the AD process, especially the specific synergies and competitions, between the individual species involved.

Growth and substrate consumption profiles of pure cultures, and co-cultures, of three model AD organisms, *Methanosarcina barkeri*, *Acetobacterium woodii* and *Methanococcus maripaludis*, were investigated to determine their interactions under H₂-CO₂ and acetate feeding at both moderate and low temperatures. A common medium was also used for two of the strains for this study. Temporal CH₄ and volatile fatty acid (VFA) concentrations in assays were determined for all combinations at each growth phase. Results showed that the substrates and temperatures strongly influenced the growth rates of each strain and co-culture. *M. barkeri* grew fastest in H₂-CO₂ at 37°C (doubling time (dt): 12.44 hours) compared to under acetate (dt: 34.34 hours) or at 15°C (dt: 28.25 hours). *A. woodii* grew fastest in H₂-CO₂ at 35°C (dt: 17.13 hours) compared to at 15°C in H₂-CO₂ (dt: 27.90 hours) and *M. maripaludis* grew at an optimum of dt = 32.30 hours in the CP medium on its preferred substrate H₂-CO₂ as the main energy source, at 37°C. Co-cultures of *M. barkeri* partnered with *A. woodii* and *M. barkeri* partnered with *M. maripaludis* demonstrated the impact of competitions on their pure culture counterparts regarding growth and metabolisms.

Future studies will include analyses of individual strains and mixed-species consortia by genomics and transcriptomics, and metagenomics and metatranscriptomics, respectively.

3.1 Introduction

Anaerobic environments are ubiquitous throughout the planet and scientists have been working to understand how these communities operate, i.e. how microorganisms can survive in these extreme atmospheres deprived of oxygen. Many uses have been found for these microorganisms which have been isolated in various anaerobic environments. The microorganisms involved undergo AD. Different trophic groups of organisms are implemented at each step: hydrolysers for hydrolysis, acidogens for acidogenesis, acetogens for acetogenesis and methanogens for methanogenesis.

Although there have been many studies which examined anaerobic degradation by naturally-occurring complex mixed cultures, there have been relatively few carried out on the metabolism and growth profiles differences between pure cultures and co-cultures with a combination of defined temperatures, substrates and organisms. The final two steps of the AD process are crucial to the formation of CH₄, which can be used as a biofuel. Homoacetogenesis is the process whereby microorganisms consume H₂-CO₂ and produce acetate.



The homoacetogen *Acetobacterium woodii* (*A. woodii*) can readily utilise an important substrate to methanogenesis, H₂-CO₂, it has a relatively fast doubling time of several hours in comparison to other acetogens with doubling times in the region of days, and its fundamental biochemistry has been widely studied since its discovery (Balch et al., 1977, Fuchs, 1986, Ljungdahl, 1986, Wood et al., 1986, Heise et al., 1989, Van Lier et al., 2008).

The methanogen, *Methanosarcina barkeri* (*M. barkeri*), generally consumes acetate and forms CH₄ and it has been reported to be an important microorganism due to the extensive range of anaerobic environments which it inhabits. It is considered the most metabolically-diverse methanogen, making it an attractive pure culture model (Stadtman, 1967, Zeikus, 1977, Weimer and Zeikus, 1978b).



Studies have reported faster growth on H₂-CO₂ than on acetate (Ferguson and Mah, 1983). Prolonged acetate adaptation is required for *M. barkeri* to use acetate as an energy source (Weimer and Zeikus, 1978a, Winter and Wolfe, 1979). Its optimal growth temperature is 37°C (Kunow et al., 1996) and few studies have examined it incubated at sub-optimal temperatures (Westermann et al., 1989, Gunnigle et al., 2013).

The hydrogenotrophic methanogen, *Methanococcus maripaludis* (*M. maripaludis*), produces CH₄ by consuming H₂-CO₂. It is also a good methanogenic model which has been utilised for many genetic and biochemical studies due to its fast doubling time and its previously characterised nutrition (Jones et al., 1983b, Goyal et al., 2016). Therefore, the key substrates involved in the terminal two stages of AD are acetate and H₂-CO₂ (Figure 3.1). Homoacetogens and methanogens are known to compete for available H₂ in anaerobic environments (Lovley and Klug, 1983). H₂ is a rapidly turned-over intermediate within AD of organic matter, resulting in occurrences of H₂ at extremely low partial pressure of just a few pascals (Westermann, 1994, Conrad, 1996, Conrad, 1999).

In summary, these three microorganisms were chosen because they had their entire genomes previously sequenced (Hendrickson et al., 2004, Maeder et al., 2006, Poehlein et al., 2012) and they are each important species that have been found in AD bioreactors.

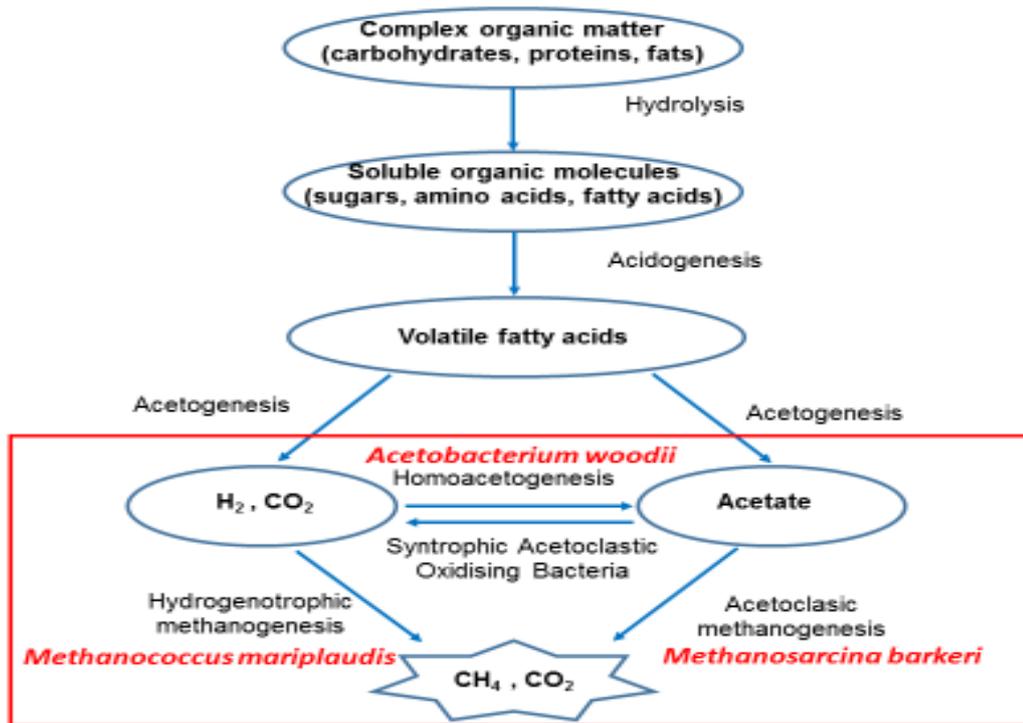


Figure 3.1: AD pathway highlighting the final two stages of acetogenesis and methanogenesis in the box with the three anaerobic pure cultures studied in this work placed in their trophic group accordingly – the homoacetogen *Acetobacterium woodii*, the hydrogenic methanogen *Methanococcus mariplaudis* and the methanogen *Methanosarcina barkeri*.

Along with substrate investigations and studies, temperature impacts on anaerobic microorganisms have become very important in AD research. Anaerobic digesters usually operate at mesophilic temperatures because the microbial communities function optimally at these temperatures. In the recent past research has become heavily involved in advancing the use of low-temperature wastewater treatment to optimise the AD process through biofilm growth and operational parameters (Collins et al., 2003, Collins et al., 2005, Connaughton et al., 2006, McKeown et al., 2009a, Madden et al., 2014). The advantages of low temperature AD (LtAD) over mesophilic temperatures include lower energy and financial costs. Most pure culture data has been obtained under optimal growth conditions but natural ecosystems are subject to temperature deviations, especially seasonally. By harnessing information from microorganisms at lower temperatures, metabolic models can then predict which biofilm communities are most efficient and metabolically flexible, without the additional costs associated with mesophilic AD.

Work from Westermann *et al.* has studied kinetic responses of *Methanosarcina barkeri* strain 227 to temperature modulations (Westermann *et al.*, 1989).

LtAD has gained acceptance as a successful method of both treating wastewater and producing CH₄ as a biofuel without the need for expensive fuel. Average temperatures within a bioreactor can be around 15°C and lower during the winter months. It has also been demonstrated that acetogenesis may have an increased importance at the process level in cold habitats and can compete with methanogens for H₂-supplying substrates (Nozhevnikova *et al.*, 1992, Kotsyurbenko *et al.*, 1993, Parshina *et al.*, 1993). In fact, homoacetogenesis has been observed to completely out-compete hydrogenotrophic methanogens in psychrophilic environments (Conrad *et al.*, 1989, Kotsyurbenko *et al.*, 1996, Schulz and Conrad, 1996) but, at the same time, there have also been studies that show hydrogenotrophic methanogens playing an important role in LtAD (McHugh *et al.*, 2004, Syutsubo *et al.*, 2008, McKeown *et al.*, 2009b), possibly due to their greater affinity for H₂ than the homoacetogens (Kotsyurbenko, 2005). It has been widely commented that chemical and biological reactions perform slower under low temperatures in comparison to their higher, moderate temperature (25 - 45°C) counterparts but that decay rates are also thereby lower (Pennington, 1908).

Although many aspects of anaerobic, acetogenic and methanogenic metabolisms are now understood, there are still knowledge gaps and open questions regarding the biochemistry, the bioenergetics and the way they interact with other species in their environment. Axenic cultures have proven crucial to answering the basic and fundamental questions about what a single organism is capable of through genomics and transcriptomic studies. Pure cultures are examined in order to avoid the uncontrollable variables present within natural communities.

Progressing from pure cultures, synthetic co-cultures have built upon the basic knowledge with a view to understanding important competitive and syntrophic relationships. To better understand the metabolic capabilities, axenic pure cultures were set up with single and paired substrates and compared with a synthetic culture of two combined pure cultures. A combination of growth and chemical data were evaluated to gain insight into the synergistic/competitive steps taken when pure cultures are synthetically paired up.

The aims of this work were to unravel some of the physiological and chemical interplay amongst and between specific key anaerobic microorganisms present in the final two stages of AD: acetogenesis and methanogenesis. This motivation stems from the fundamental work of investigating key representative methanogens and an important homoacetogen which are prevalent within the AD system. These are noted microorganisms from main trophic groups and focus on an important stress point of acetogenesis within the AD pathway. This work asked some fundamental questions on specific aspects of pure cultures and co-cultures which could then integrate the chemical and growth data into the holistic schematics that are already present. The pure culture studies were divided into three main pieces of work.

1. ***M. barkeri* (MB) temperature study:** It is well known that *M. barkeri* can grow chemolithoautotrophically on H₂ with CO₂ or heterotrophically on acetate, the primary substrates required for methanogenesis within many anaerobic ecosystems (Stadtman, 1967, Mah et al., 1977, Zeikus, 1977, Mah et al., 1978, Weimer and Zeikus, 1978b, Zinder, 1993). In this study, the effect of temperature was investigated by taking measurements at 25°C, 30°C and 37°C on acetate and H₂-CO₂.

The primary hypothesis was that *M. barkeri* is a flexible organism that has a preference for a substrate independent of temperature.

The central question was what methanogenic pathway does *M. barkeri* take when grown on an acetate and H₂-CO₂ medium at a variety of temperatures in terms of growth rates and qPCR abundances subsisting at specific phases. Additionally, it was asked whether its growth rates decrease in correlation with decreasing temperatures (Westermann et al., 1989)?

The objective in this study was to use chemical analyses to demonstrate how acetate consumption, CH₄ production and growth rates are generally highest at 37°C and lowest at 25°C in the case of *M. barkeri*.

2. ***Methanosarcina barkeri* – *Methanococcus maripaludis* (MB-MM) co-culture experiment:** It has been reported that *M. barkeri* and, separately, *M. maripaludis* can utilise H₂-CO₂ at moderate temperatures (Jones et al., 1983a). As previously mentioned, *M. barkeri* can also use acetate. However, there have been fewer studies on the effect of acetate on *M. maripaludis*

(Shieh and Whitman, 1987, Yang et al., 1992). MB-MM co-culture has not been previously studied according to the literature.

The hypothesis for this piece of work was that *M. maripaludis* will out-compete *M. barkeri* under H₂-CO₂ when in co-culture and acetate will not be consumed because *M. barkeri* is not acetate-adapted.

The key questions were which microorganism will out-compete the other in the co-culture, and which methanogen will utilize the most H₂-CO₂ and grow faster than the other.

The objectives were to demonstrate that growth rates, and hence cell concentrations, will increase in the competitive co-culture in similar proportions to the respective pure cultures but that *M. maripaludis* will out-compete *M. barkeri* due to its faster growth rate. This experiment will analyse the impact of two H₂-utilising methanogens on each other but also in comparison to each microorganism as a pure culture.

3. ***M. barkeri* – *A. woodii* (MB-AW) co-culture study:** As in study 2, it has been previously reported that *M. barkeri* and *A. woodii* can also utilise H₂ with CO₂ (Balch et al., 1977). Of the two, only *M. barkeri* has been demonstrated to utilise acetate according to the literature. The pure and co-cultures were separated into studies based on different conditions.

(a) 35°C H₂-CO₂: The hypothesis for this set-up was *M. barkeri* and *A. woodii* grow synergistically as a pair grown under H₂-CO₂.

The questions included which microorganism utilises the most H₂-CO₂ when in pure culture and comparatively when in a co-culture system. It was also asked whether both microorganisms can survive synergistically and, if so, what their metabolic growth development would be in terms of growth and qPCR abundance patterns in both individual pure cultures and in the co-culture?

Additional to growth profile analyses, the objective was to follow the chemical profiles through the pure culture and co-culture growth phases so that several aspects of their growth could provide a big picture of how these organisms grow alone and respond with a partner.

(b) 15°C H₂-CO₂: similar hypothesis, questions and research objectives to (a) except that there is the additional feature of investigating how a low temperature further impacts on these species.

(c) 35°C acetate: The hypothesis was that *M. barkeri* out-competes *A. woodii* in an acetate-rich environment when in a dual culture because *A. woodii* is not a reversible acetogen.

The research questions associated with acetate as the sole substrate included what the differences were between growth and chemical profiles of acetate-adapted *M. barkeri* in comparison to non-acetate-adapted *M. barkeri* and whether *A. woodii* will grow on acetate as the sole substrate either alone or paired with *M. barkeri*? Furthermore, it was asked what impact, if any, there would be on each pure culture's growth and metabolic profiles when they are partnered?

The research aims were to analyse these aspects of growth in real time at lag, log, stationary and death phases of each (co-) culture: OD readings, qPCR cell numbers, VFA concentrations and, acetate rates.

(d) 15°C acetate: similar research questions and aims to part (c), except with the replacement of moderate temperature with low temperature stresses on each culture. The additional objective was to determine if/how they survive in a psychrophilic acetate-limited environment.

For each of the three experiments in this study, either or both acetate and H₂-CO₂ were used as substrates. Previous studies have demonstrated that acetate accounts for up to two thirds of the CH₄ produced within anaerobic digesters (Weimer and Zeikus, 1979, Zinder, 1993). The co-culture studies (2 and 3) aimed to decipher the impact or influence of the co-cultures on the growth and substrate utilisation in the individual pure cultures.

In summary, the novelties of this work include:

- examining whether the substrate preference of *M. barkeri* is independent of temperature.
- investigating the competition of a member of the *Methanosarcinaceae* family with a member of the *Methanococcaceae* family – *M. barkeri* and *M. maripaludis* have not been studied previously according to the literature.

- identifying the growth impacts of the synergistic pairing of the acetate-adapted *M. barkeri* with the homoacetogen *A. woodii* – this co-culture has not been studied at low temperature.

3.2 Materials and Methods

3.2.1 Growth conditions and experimental set-up for anaerobic pure and co-cultures

Methanosarcina barkeri (Schnellen, 1947) strain MS (DSM 800), *Acetobacterium woodii* (Balch et al., 1977) strain WB1 (DSM 1030) and *Methanococcus maripaludis* (Jones et al., 1983a) strain JJ (DSM 2067) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and grown under strictly anaerobic conditions. All strains were grown in serum bottles fitted with blue butyl stoppers (Bellco) and aluminium crimp seals with 25 ml of medium per tube. All media were dispensed under N₂ and CO₂; the pH was approximately 7.0 (with filter sterilised 5 M HCl).

For the specific basal medium, DSMZ recommended medium 2771, several temperatures were tested for *M. barkeri* growth: 4°C, 15°C, 20°C, 25°C, 30°C and 37°C. It was found that, from the temperatures tested, the methanogen could grow sufficiently at 25°C, 30°C and 37°C in this specific minimal medium. The basal medium for the *M. barkeri* temperature study consisted of (all measurements are expressed in grams per litre of distilled water): KCl, 0.34, MgCl₂·6H₂O, 4.00, MgSO₄·7H₂O, 3.45, NH₄Cl, 0.25, K₂HPO₄, 0.14, NaCl, 18.00, Trace elements (nitrilotriacetic acid, 1.50, adjusted pH to 6.5 with KOH, MgSO₄·7H₂O, 3.00, MnSO₄·H₂O, 0.50, NaCl, 1.00, FeSO₄·7H₂O, 0.10, CoSO₄·7H₂O, 0.18, CaCl₂·2H₂O, 0.10, ZnSO₄·7H₂O, 0.18, CuSO₄·5H₂O, 0.01, KAl(SO₄)₂·12H₂O, 0.02, H₃BO₃, 0.01, Na₂MoO₄·2H₂O, 0.01, NiCl₂·6H₂O, 0.03, Na₂SeO₃·5H₂O, 0.0003, Na₂WO₄·2H₂O, 0.0004 and distilled H₂O, 1000.00 ml.), 10.00 ml, Fe(NH₄)₂(SO₄)₂·6H₂O, 0.002, Na-acetate, 1.00, Yeast extract (Oxoid), 2.00, Trypticase peptone (BD BBL), 2.00, Resazurin, 0.001, distilled water, 1000.00 ml. After autoclaving, NaHCO₃, 5.00 g, CaCl₂·2 H₂O, 0.14, vitamin stock solution

(Biotin, 2.00 mg, Folic acid, 2.00 mg, Pyridoxine-HCl, 10.00 mg, Thiamine-HCl. 2H₂O, 5.00 mg, Riboflavin, 5.00 mg, Nicotinic acid, 5.00 mg, D-Ca-pantothenate, 5.00 mg, Vitamin B12, 0.10 mg, p-Aminobenzoic acid, 5.00 mg, Lipoic acid, 5.00 mg, Distilled H₂O 1000.00 ml) 10.00 ml, L-Cysteine-HCl.H₂O, 0.50, and Na₂S.9H₂O, 0.50 solutions were added to the vials. H₂-fed cultures were pressurised to two atmospheres of 80% H₂: 20% CO₂ gas mixture. It was a pure culture with both acetate and H₂-CO₂ as substrates.

The basal medium for *M. maripaludis* and *M. barkeri* grown individually and as a co-culture was as described above for the *M. barkeri* temperature study. It was also a pure culture with both acetate and H₂-CO₂ as co-substrates.

The basal medium for *A. woodii* and *M. barkeri* was grown individually and as a co-culture contained (all measurements are expressed in grams per litre of distilled H₂O): NH₄Cl, 0.30, KH₂PO₄, 0.40, Na₂HPO₄.2H₂O, 0.53, MgCl₂.6H₂O, 0.10, NaCl, 0.30, Resazurin, 0.0005, trace element stock solution (HCl, 1.80, H₃BO₃, 0.0618, MnCl₂, .06125, FeCl₂, 0.9435, CoCl₂, 0.0645, NiCl₂, 0.01286, ZnCl₂, 0.0677, NaOH, 0.4, Na₂SeO₃, 0.0173, Na₂WO₄, 0.0294, Na₂MoO₄, 0.0205, all mixed in distilled H₂O, 1000 ml), 1 ml and distilled water, 1000 ml. After autoclaving, vitamin stock solution (Biotin, 0.02, Cyanocobalamin, 0.002, Pyridoxine-HCl, 0.5, Thiamine-HCl, 0.2, Riboflavin, 0.1, Nicotinic acid, 0.2, D-Ca-pantothenate, 0.1, Vitamin B12, 0.10 mg, p-Aminobenzoic acid, 0.1 and distilled H₂O, 1000 ml), 1 ml, CaCl₂, 1.10, L-Cysteine-HCl.H₂O, 0.50, NaHCO₃, 4.00 and Na₂S.9H₂O, 0.25 stock solutions were added. This medium recipe was kindly shared by the lab of Caroline Plugge in the University of Wageningen and will henceforth be referred to as “CP medium”. Unitrophic cultures were prepared with either acetate or H₂-CO₂. Anaerobic aqueous stock solution of acetate (prepared under N₂) was filter-sterilised by syringe injection to the medium. H₂-fed cultures were pressurised to two atmospheres of 80% H₂: 20% CO₂ gas mixture.

All DSMZ cultures were inoculated into their respective basal medium (Figure 3.2) within an anaerobic chamber (Coy) containing N₂ as the background gas and H₂ or Argon as the anaerobic gas. Throughout cultivation, pure culture and co-culture samples were taken for microscopic observation of purity using a light microscope (Nikon-Eclipse 600).

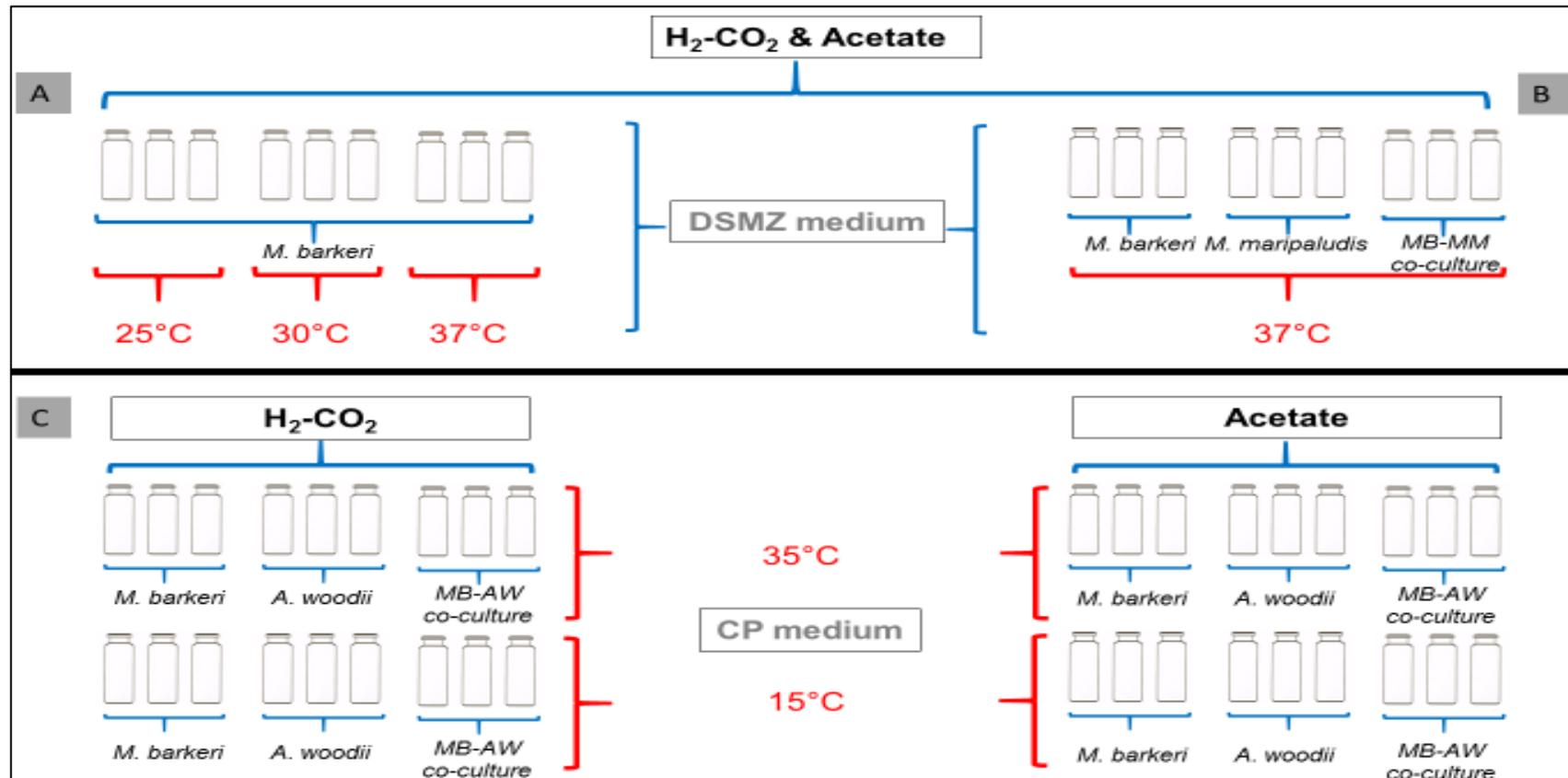


Figure 3.2: Experimental set-up of anaerobic pure and co-cultures. (A) *Methanosarcina barkeri* temperature study, (B) *Methanosarcina barkeri*-*Methanococcus maripaludis* co-culture study and (C) *Methanosarcina barkeri*-*Acetobacterium woodii* co-culture study. Abbreviations MB-MM: *Methanosarcina barkeri*-*Methanococcus maripaludis*, MB-AW: *Methanosarcina barkeri*-*Acetobacterium woodii*.

All cultures were grown and sub-cultured for at least 3 generations prior to inoculation for these studies of cultures at logarithmic growth. For co-cultures *M. barkeri* and *M. maripaludis*, as well as for *M. barkeri* and *A. woodii*, in all conditions tested, 5% of each pure culture at the same OD during log phase was inoculated into anaerobic hungate tubes containing fresh medium.

For the two final culture experiments in this pure culture study (*M. barkeri* and *A. woodii* pure cultures and co-cultures grown on acetate at both 35°C and 15°C separately), *M. barkeri* was acetate-adapted for over 3 months prior to commencement of experiments.

The growth curves of all cultures were determined in triplicate and subsequently four time points were chosen to represent the four main phases of growth: lag, log, stationary and death. Sacrificial vials were set up to take several measurements at these four phases of growth as described above in the methods section: CH₄ concentration from the headspace within the vial, duplicate liquid samples for both DNA extractions (10 ml samples) and VFA analysis (2 ml samples) (Figure 3.3).

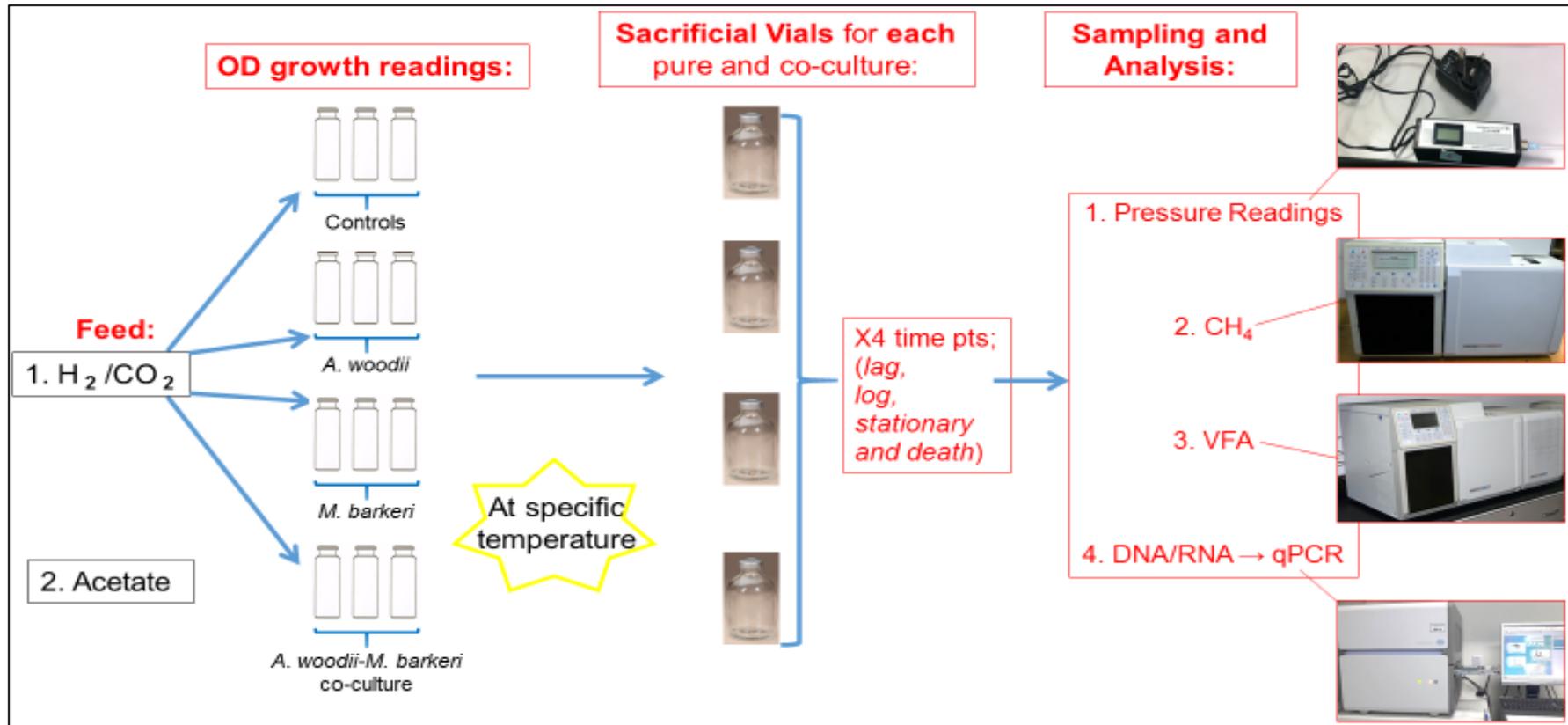


Figure 3.3: Flow diagram illustrating the growth and chemical analyses performed for each experiment in the study. The example given is for experiment (iii), the *M. barkeri* – *A. woodii* (MB-AW) co-culture study described previously in the Introduction (section 3.1). Growth curves are generated from triplicate cultures of *A. woodii*, *M. barkeri* and MB-AW co-culture. Four time points were chosen to represent lag, log, stationary and death phases based on previously-measured growth curves, and sacrificial culture vials were set up for analyses at specific times. At each designated time, sacrificial samples were taken and used to measure pressure and the concentrations of CH_4 and VFA, and for nucleic acid extraction followed by qPCR analysis.

3.2.2 Spectrophotometry

Acetate-fed culture tubes were incubated in a horizontal static position and hydrogen-fed cultures were incubated horizontally, continuously shaking at 150 rpm (Gyrotory water bath shaker, Model G76, New Brunswick Scientific, N.J.) in order to promote hydrogen substrate diffusion of the gas mixture into the pure culture or co-culture medium. Tubes were vigorously shaken prior to optical density measurements. Growth was measured as the optical density at 600 nm (OD_{600}) (Hach Odyssey DR/2500 scanning spectrophotometer); the optical path width (i.e., the culture tube's diameter) was 22.5 cm. Uninoculated medium served as the reference negative control for growth measurements. The results were representative of triplicate experiments. Doubling times and growth curves were calculated using the least square fitting exponential method (Weisstein, 2016) where each data point is weighted equally.

3.2.3 VFA and methane profiling

Samples were taken from each individual and co-culture at the four phases of growth (lag, log, stationary and death) for chemical and gaseous profiling. VFAs were measured in the Varian Saturn 2000 gas chromatographer/mass spectrometer, with a Combi PAL auto sampler (Varian Inc., Walnut Creek, CA). A Varian Capillary column, CP-WAX 58 (FFAP) CB (25 m length x 0.32 mm i.d. x 0.2 μ m film thickness, carried out separation of acids. 1 ml was injected at 250°C at a flow rate of 1 ml/min. Helium was supplied as the carrier gas. The method used was 60°C (0.1 min) to 110°C for 0.2 min at a rate of 30°C/min followed by 110°C to 200°C (2 min) at a rate of 10°C/min. An FID was used and VFAs were identified via assigning chromatographic retention times and spectra to the relevant compounds (acetic acid, propionic acid and butyric acid). Standard VFA calibration curves were used for comparison of relative VFA concentrations in culture medium and they were expressed as mg l⁻¹. The internal standard used was 2-Ethylbutyric acid.

CH₄ concentrations were analysed using the Varian gas chromatograph (Varian 3800, Agilent Technologies) connected to a hydrogen generator (Whatman). A standard curve was generated using different percentages of pure CH₄. Sample vials

were shaken and gas from the headspace was removed for CH₄ calculations using a sterile needle and syringe.

3.2.4 DNA extractions from cultures

All culture samples (in 10 ml volumes) were collected in falcon tubes and cells were harvested via centrifugation (Eppendorf – Centrifuge 5810) for 5 min at 4,000 rpm at 4°C, followed by decantation of the supernatant liquid. Duplicate samples were snap frozen for storage at -80°C. Total genomic DNA was extracted from the 10 ml culture samples obtained at each growth phase. Each sample pellet was resuspended in lysis buffer containing a chaotropic agent and detergent. DNA extraction was performed with the Maxwell® 16 automated system (Promega). Nucleic acids bound to magnetised silica particles. Purified DNA was eluted with 200 µl of elution buffer. DNA sample concentrations were measured on the Qubit fluorometer (Invitrogen) and samples were stored at -20°C for further analysis.

3.2.5 Quantitative polymerase chain reaction analyses

Real-time PCR (qPCR) analysis was performed using a LightCycler 480 instrument (Roche, Mannheim, Germany) and using a universal primer and probe set to target the 16S rRNA gene within *A. woodii* and two methanogenic order-specific primer and probe sets. *Methanosarcinales* (MSC) primers were used to target *M. barkeri* and *Methanococcales* (MCC) primers were used to target *M. maripaludis* as previously described (Yu et al., 2005). The LightCycler 480 Probe Master kit (Roche Diagnostics) was used to prepare 20 µl reaction mixtures containing 2 µl of PCR-grade water, 1 µl of each primer (with a final concentration of 10 mM), 1 µl of *TaqMan* probe (with a final concentration of 10 mM), 10 µl of 2x LightCycler® 480 Probes Master, and 5 µl of template DNA. The thermal cycling program used for each of the primer and probe sets used comprised of several steps, including pre-denaturation at 94°C for 10 min, 45 cycles of denaturation at 95°C for 10 s and simultaneous annealing and extension at 60°C for 30 s. All samples were analysed in duplicate.

Standard curves were constructed (Yu et al., 2006) using representative strains: 16S rRNA gene (*Thiobacillus denitrificans* strain ME16 16S ribosomal RNA gene, partial sequence: 99% ACCESSION EU546130) (AATTTTGGACAATGGGGGCAACCCTGATCCAGCCATTCCGCGTGAGT GAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCTGGAACGAAACGGT ACGCGCTAACATCGTGTGCTAATGACGGTACCGGCAGAAGAAGCACC GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTT AATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGATTGTTAAGCA AGACGTGAAAGCCCCGGGCTTAACCTGGGAATGGCGTTTTGAACTGGT AGTCTAGAGTGCGTCAGAGGGGGGTGGAATTCCACGTGTAGCAGTGA AATGCGTAGAGATGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTG GGATGACACTGACGCTCATGTACGAAAGCGTGGGTAGCAAACAGGAT TAGATACCCTGGTAGTCA), MSC (*Methanosarcina acetivorans* C2A DSM 2834) and MCC (*Methanococcus voltae* PS; DSM 1537).

Using each standard listed here, a 10-fold dilution series from 10^9 to 10^1 copies / μl was set up and analysis was conducted by qPCR with its analogous primer/probe set. Standard curves were generated using the data obtained from qPCR in conjunction with serial dilution copy concentrations. Data was normalised according to the number of copies of 16S rRNA genes in each pure culture genome. *A. woodii* cells contained five gene copies, *M. barkeri* had three gene copies and *M. maripaludis* cells had two gene copies in their cells. Results were expressed as cell abundance per ng DNA based on 16S rRNA gene copies. qPCR growth rates were calculated based on the slope of the line created using the qPCR data points at each growth phase.

Standard curves were created by performing a PCR using the primers 338F and 805R for the 16S bacterial set. PCR reactions were carried out on a GenDX Kyratec SuperCycler Trinity (Medical Supply Company). This PCR assay master mix consisted of 15 μl of PCR-grade, nuclease-free water, 0.5 μl of each primer (with a final concentration of 10 mM), 0.125 μl of *Taq* polymerase (Fisher), 2.5 μl of buffer (10X *Taq* buffer B), 5 μl of MgCl_2 (with a final concentration of 25 mM), 0.5 μl of dNTPs (with a final concentration of 10 mM of each) and 1 μl of template DNA. The thermal cycling program used comprised of several steps: pre-denaturation at 95°C for 1 min, 30 cycles of denaturation at 95°C for 15 s, annealing at 57°C for

15 s and extension at 72°C for 10 s followed by the final elongation at 72°C for 10 min.

The standards were cloned into pCRTM4 - TOPO vector (Promega, Mannheim, Germany) via chemical transformation of competent *E. coli* (One Shot® TOP10) using the TOPO TA Cloning kit (Invitrogen, Paisley, UK). 100 µl of each transformation was spread onto pre-warmed selective plates with a S.O.C. medium. A selection of positive colonies was picked for PCR subsequently using the reverse primer 805R and M13F primer to confirm that the insert was the correct size, as visualised on an agarose gel. The clones were regrown in LB and kanamycin overnight at 37°C and sent for Sanger sequencing (Source BioScience, Co. Waterford).

3.2.6 Statistical analysis

Statistical Package for Social Sciences (SPSS) software (IBM SPSS Statistics for Windows, version 23, IBM Corp., Armonk, N.Y., USA) was used to compute analysis of variance (ANOVA), Kruskal Wallis H, Mann Whitney or T tests of significance between pure culture and co-culture growth and qPCR rates analyses. All statistical results were analysed based on the level of significance, where $p < 0.05$.

3.3 Results

3.3.1 An examination of the adaptive flexibility within the metabolically versatile *Methanosarcina barkeri* (MB temperature study)

The doubling times, growth rates (Table 3.1) and growth curves (Figure 3.4 (A-C)) demonstrated that *M. barkeri* grew fastest at 37°C and slowest at 25°C. Statistical tests revealed that each of the doubling times for *M. barkeri* grown at all three temperatures were significantly different from each other ($p = 0.000$). Similarly, the qPCR results demonstrated that highest cell abundance per ng of DNA based on 16S rRNA gene copies of the *Methanosarcinales* family were found at 37°C and

the lowest numbers in the triplicate cultures grown were found at 25°C at each time point (Figure 3.4 (A-C)). For the pure cultures at both 25°C and 30°C, there was a two-fold increase in the cell abundance per ng of DNA, from 10^3 to 10^5 , whereas a three-fold increase, from 10^3 to 10^6 , was found in cultures at 37°C. Growth rates based on the slope of the cell abundance qPCR data profiles were calculated. 37°C cultures had a qPCR growth rate of 0.294 hr^{-1} , 30°C cultures had a growth rate of 0.246 hr^{-1} and the qPCR growth rate for 25°C cultures was 0.193 hr^{-1} .

Table 3.1: Doubling times (hours) and growth rates of *M. barkeri*

	Doubling times (h)	Growth rates (h⁻¹)
<i>M. barkeri</i> 37°C	12.44	0.0557
<i>M. barkeri</i> 30°C	19.75	0.0351
<i>M. barkeri</i> 25°C	22.66	0.0306

All values are means \pm SD ($n=3$)

The chemical and metabolic profiles (Figure 3.4 (D-F)) of *M. barkeri* were examined at a range of temperatures. The highest consumption of acetic acid occurred at 37°C (from 973 mg/l to 709 mg/l) and the lowest occurred at 25°C (from 979 mg/l to 759 mg/l). There was a smaller decrease in final acetate consumption from 30°C to 25°C (9 mg/l) than from 37°C to 30°C (35 mg/l). The acetic acid profiles reported a 27% consumption at 37°C, a 23.5% consumption at 30°C and a 22.5% consumption at 25°C.

Statistical tests showed that acetic acid profiles between the three temperatures were statistically similar ($p = 0.498$) and the acetic acid concentrations were statistically different between the growth phases ($p = 0.000$). By contrast, propionic acid profiles between the three temperatures were statistically different ($p = 0.000$) and they were statistically similar between the different growth phases ($p = 0.153$). Like the propionic acid statistical tests, butyric acid profiles between the three temperatures were statistically different ($p = 0.000$) and the butyric acid concentrations were statistically similar ($p = 0.767$) between the growth phases.

The amount of CH₄ gas (Figure 3.4 (D-F)) produced by *M. barkeri* correlated with an increase in temperature. The highest amount of CH₄ produced was 992 μmol at 37°C, 610 μmol at 30°C and 443 μmol at 25°C.

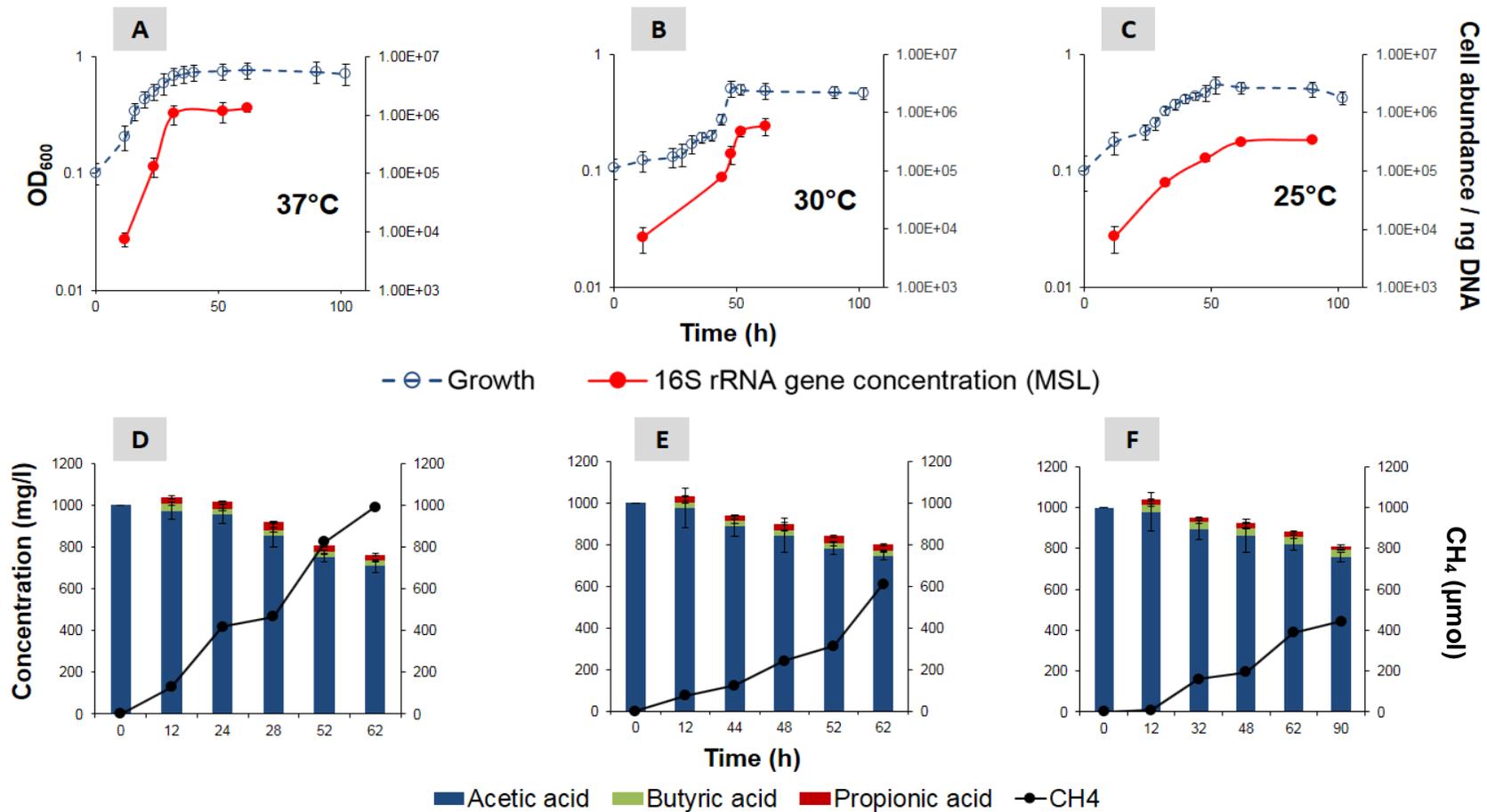


Figure 3.4: Growth profiles were generated via optical density measurements taken at 600 nm (log scale) and qPCR data showing 16S rRNA cell abundances / ng of DNA were determined for *M. barkeri* cultures grown on acetate and H₂-CO₂ at (A) 37°C, at (B) 30°C and at (C) 25°C. VFA (stacked bars) and CH₄ (lines) profiles are illustrated for cultures grown on acetate and H₂-CO₂ at (D) 37°C, at (E) 30°C and at (F) 25°C. Error bars represent the standard deviation from the mean; some error bars are smaller than the symbols. Time 0 represents the theoretical concentration of acetate at the beginning of the study (D-F).

3.3.2 The growth response of methanogenic archaea co-culture to competitive growth (MB-MM co-culture experiment)

To further investigate the dynamics of the flexible methanogen, *M. barkeri*, it was grown at its optimal temperature of 37°C fed with H₂-CO₂, and compared to *M. maripaludis* as individual pure cultures and with *M. maripaludis* as a co-culture.

From each of the doubling times, growth rates (Table 3.2) and growth curves (Figure 3.5 (A-C)), it was observed that *M. maripaludis* had a faster doubling time of 5.38 hours than *M. barkeri*, whose doubling time was 12.44 hours. The doubling times for *M. maripaludis* and the MB-MM co-culture were statistically similar to each other ($p = 0.837$) However, there were statistically significant differences between the doubling times for *M. maripaludis* and *M. barkeri* ($p = 0.000$) as well as between *M. barkeri* and the MB-MM co-culture ($p = 0.000$).

Results from qPCR assays reported that there was a 1-fold increase in cell abundance per ng of DNA based on 16S rRNA gene copies for *M. maripaludis* (*Methanococcales* family) (10^4 to 10^8) over *M. barkeri* (*Methanosarcinales* family) (10^3 to 10^6) pure cultures. The co-culture reported a higher difference in cell abundance for *M. maripaludis* (10^3 to 10^8) and a lower difference for *M. barkeri* (10^3 to 10^5) (Figure 3.5 (A-C)). qPCR growth rates from the slope of the lines reported 0.294 hr⁻¹ for *M. barkeri* in pure culture and 0.295 hr⁻¹ when in co-culture. *M. maripaludis* exhibited a qPCR 16S rRNA cell abundance increase rate of 0.823 hr⁻¹ in pure culture and 0.79 hr⁻¹ in co-culture.

Table 3.2: Doubling times (hours) and growth rates for *M. barkeri*, *M. maripaludis* and MB-MM co-culture.

	Doubling times (h)	Growth rates (h ⁻¹)
<i>M. barkeri</i>	12.44	0.0557
<i>M. maripaludis</i>	5.38	0.1289
MB-MM	5.49	0.1262

All values are means \pm SD ($n=3$)

Subsequently, the metabolic and gaseous profiles were investigated at each growth phase. Acetate was partially consumed by *M. barkeri* as the concentration was reduced to 709 mg/l. There was almost no consumption by *M. maripaludis* and there was less acetate consumption in the co-culture than in the *M. barkeri* pure culture (the concentration decreased to 880.5 mg/l at the same time point) (Figure 3.5 (D-F)).

Statistical tests were carried out on acetic, propionic and butyric acid profiles of each culture type. The acetic acid profiles were significantly different between the three cultures ($p = 0.038$). However, the acetic acid profiles of *M. maripaludis* compared only with the *MB-MM* co-culture reported statistical similarity ($p = 0.848$). P-values based on growth phase indicated that all acetic acid profiles were significantly different ($p = 0.001$). Butyric acid profiles were significantly different ($p = 0.019$) between the three cultures and statistically similar between the growth phases ($p = 0.404$). The propionic acid profiles were statistically different between the three cultures ($p = 0.000$). Statistical tests for propionic acid profiles presented statistical similarity among all of the phases ($p = 0.754$).

CH₄ profiles demonstrated that both pure cultures produced similar cumulative amounts of CH₄ by their death phases: 992 μmol for *M. barkeri* and 999 μmol for *M. maripaludis*. Although qPCR assays resulted in a higher yield in the triplicate co-cultures for the *Methanococcales* family and a lower yield for the *Methanosarcinales* family, there was a similar yield of CH₄ in the co-culture (996 μmol) when compared to their respective pure cultures.

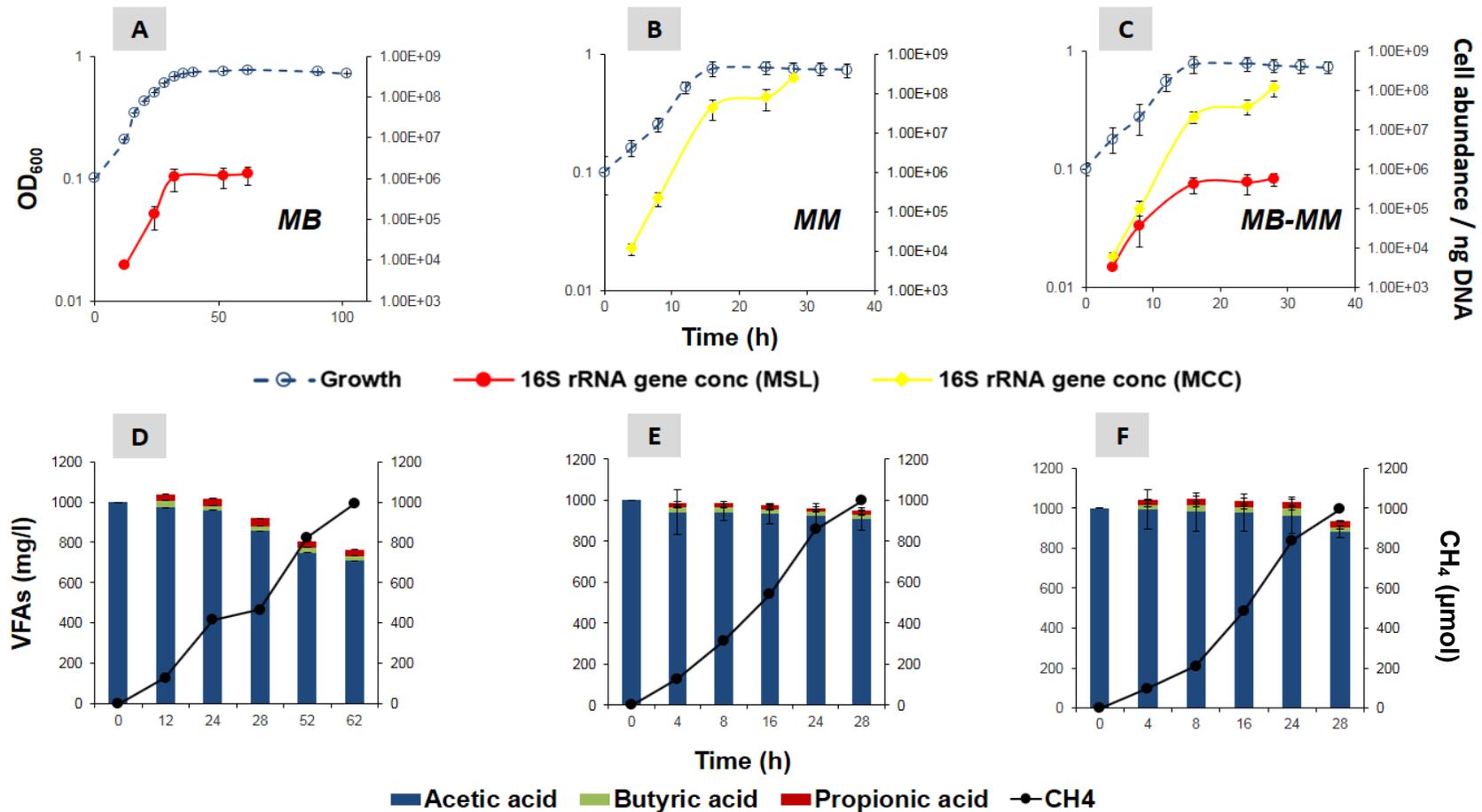


Figure 3.5: Growth profiles were generated via OD measurements taken at 600 nm and qPCR data showing cell abundance / ng of DNA based on 16S rRNA gene copies were determined for (A) *M. barkeri* pure cultures, (B) *M. maripaludis* pure cultures and (C) *MB-MM* co-cultures. VFA (stacked bars) and CH₄ (lines) profiles are illustrated for (D) *M. barkeri* cultures, (E) *M. maripaludis* cultures and (F) *MB-MM* co-cultures. A combination of acetate and H₂-CO₂ was utilised for all cultures. Error bars represent the standard deviation from the mean; some error bars are smaller than the symbols. Time 0 represents the theoretical concentration of acetate at the beginning of the study (D-F).

3.3.3 Synergistic growth effects of a methanogenic archaea-homoacetogen co-culture (MB-AW co-culture study)

M. barkeri and *A. woodii* were grown both individually and as a co-culture in a variety of conditions:

Table 3.3: Doubling times (hours) of *A. woodii*, *M. barkeri* and MB-AW co-culture. Results were an average of triplicate cultures.

	H ₂ -CO ₂		Acetate	
	35°C	15°C	35°C	15°C
<i>A. woodii</i>	17.13	27.9	115.64	155.69
<i>M. barkeri</i>	18.92	28.25	34.34	50.46
MB-AW co-culture	18.87	28.22	36.93	55.34

All values are means ±SD (n=3)

Table 3.4: Growth rates (hours) of *A. woodii*, *M. barkeri* and MB-AW co-culture. Results were an average of triplicate cultures.

	H ₂ -CO ₂		Acetate	
	35°C	15°C	35°C	15°C
<i>A. woodii</i>	0.0405	0.0248	0.006	0.0137
<i>M. barkeri</i>	0.0357	0.0245	0.0202	0.0045
<i>A. woodii</i> - <i>M. barkeri</i>	0.0367	0.0246	0.0188	0.0125

All values are means ±SD (n=3)

3.3.3.1 *M. barkeri* and *A. woodii* metabolic and growth profiling on H₂-CO₂ at 35°C

The growth results (Tables 3.3 and 3.4) for the condition H₂-CO₂-fed at 35°C indicated that *A. woodii* grows the fastest as a pure culture, with a doubling time of 17.13 hours. When the cultures were paired together, the doubling time of 18.87 hours was slower than *A. woodii* alone but slightly faster than *M. barkeri* alone (18.92 hours). Statistical tests showed that the doubling times between the pure cultures and co-cultures bore no statistically significant difference (p = 0.139). qPCR results showed similar increases in growth numbers for both pure cultures

and the co-culture. They both increased from 10^3 to 10^5 across both the pure cultures and the co-culture. The co-culture was inoculated with 50% of the inoculum that each pure culture was inoculated with. The co-culture cell abundances per ng of DNA based on respective 16S gene copy numbers of the individual microorganisms corresponded to approximately 50% of the pure culture 16S rRNA gene numbers. qPCR gene copy rates were calculated for both pure cultures and the co-culture. For *M. barkeri* in pure culture, a rate of 0.17 hr^{-1} was reported based on the slope of the line of the qPCR data, compared to a rate of 0.27 hr^{-1} in co-culture. Based on 16S rRNA gene copy numbers and cell abundances, a rate of 0.3 hr^{-1} was measured for *A. woodii* cells in pure culture, which decreased to 0.27 hr^{-1} when in co-culture.

The metabolic and gaseous profiles (Figure 3.6 (D-F)) of the three cultures showed that acetate was produced by *A. woodii* to a maximum of 449 mg/l from a theoretical starting yield of 0 mg/l. There was no acetate included in the basal medium, but there was a small quantity of acetate measured at the beginning of the *M. barkeri* pure culture (38.5 mg/l). This small amount of acetate detected depleted over the course of *M. barkeri* growth, and approximately 32.5 mg/l of acetate was remaining at the final sampling time point (hour 96). For the co-culture, there was a slight increase in acetate production due to *A. woodii* growth. Similarly, *A. woodii*'s cumulative acetic acid increased by 55.2% compared to it in pure culture. Based on acetic acid profiles, statistical tests on triplicate technical replicates confirmed that there were statistically significant differences between the three culture types ($p = 0.000$) and no significant differences between the growth phases ($p = 0.248$). Propionic acid and butyric acid were not detected on the VFA-GC.

CH_4 accumulation was also measured for the three cultures in parallel with the acetate profile. There was no CH_4 detected in the *A. woodii* cultures at each sacrificial sampling time point. There were 902 μmol of accumulated CH_4 measured in *M. barkeri* pure cultures and 532 μmol of accumulated CH_4 measured in the co-culture, which was 59% of that measured in the pure cultures.

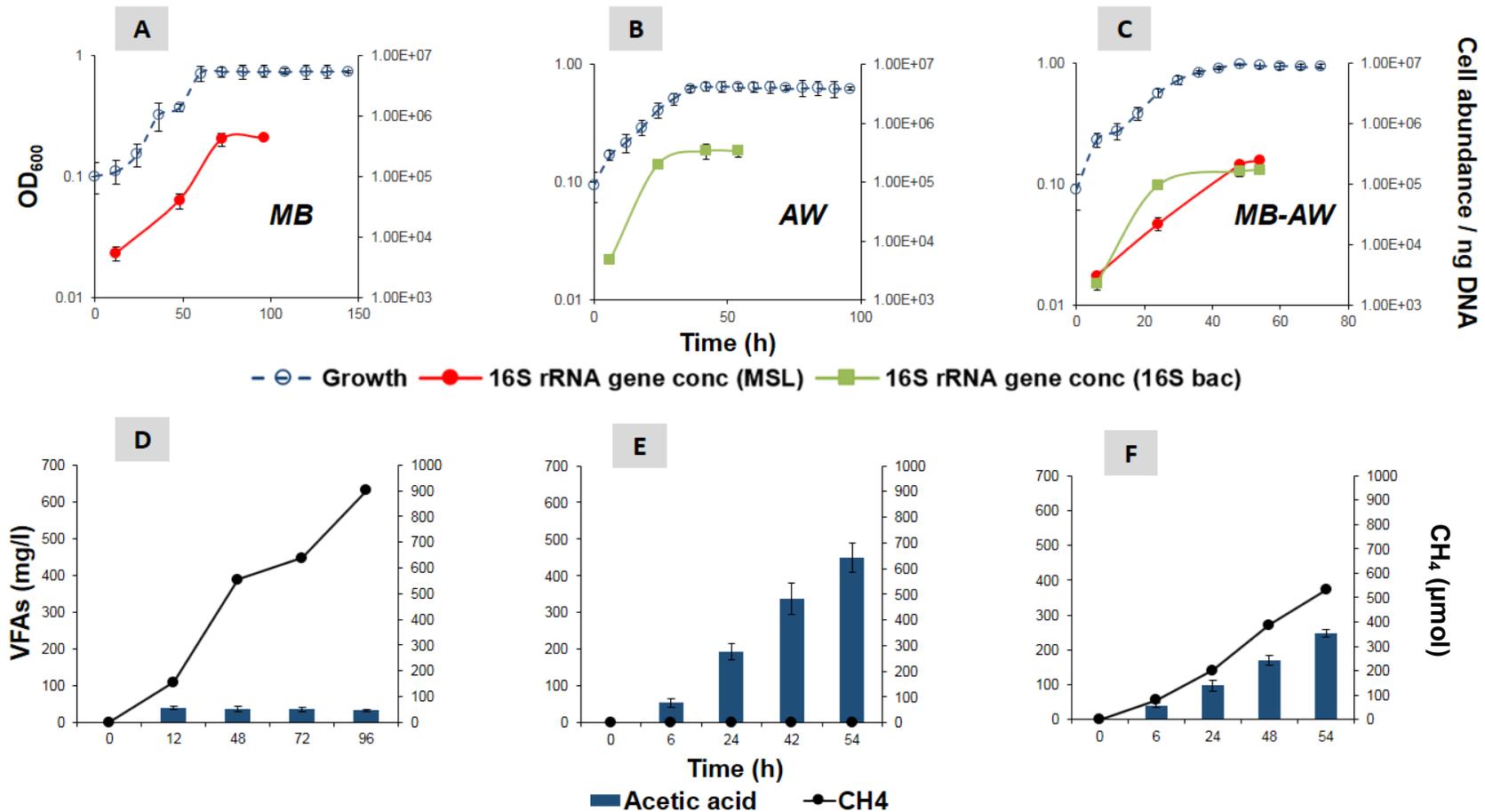


Figure 3.6: Optical density measurements and qPCR data for (A) *M. barkeri* pure cultures, (B) *A. woodii* pure cultures, and (C) the *MB-AW* co-cultures. VFA (stacked bars) and CH₄ (lines) profiles are illustrated for (D) *M. barkeri* pure cultures, (E) *A. woodii* pure cultures, and (F) *MB-AW* co-cultures. All cultures were grown on H₂-CO₂ at 35°C. Error bars represent the standard deviation from the mean; some error bars are smaller than the symbols. Time 0 represents the theoretical concentration of acetate at the beginning of the study (D-F).

3.3.3.2 Growth effects of co-culturing *M. barkeri* and *A. woodii* fed with H₂-CO₂ at 15°C

The growth results for the pure cultures and the co-culture grown on H₂-CO₂ at 15°C indicated that, similar to the results above at 35°C, *A. woodii* had a marginally faster doubling time of 27.9 hours than *M. barkeri*, which had a doubling time of 28.25 hours, and the co-culture had a doubling time in between both pure cultures of 28.22 hours (Table 3.3). Statistical tests showed that the doubling times for *M. barkeri* and *A. woodii* pure cultures showed no statistical significance between each other ($p = 0.993$). Cell abundances per ng of DNA based on 16S rRNA gene copies (Figure 3.7 (A-C)) were within one log of each other across all sampling time points for *A. woodii* (10^3 - 10^4). *M. barkeri*'s *Methanosarcinales* normalised gene copy numbers increased from 10^3 to 10^5 within the pure culture but the co-culture only had a one log difference by the end of its growth. The cell abundances per ng of DNA of both microorganisms in the co-culture were approximately 50% of their respective pure cultures. qPCR rates based on the slope of the lines of the qPCR data were revealed to be 0.08 hr^{-1} in the *A. woodii* pure culture and a higher rate of 0.17 hr^{-1} in the co-culture. *M. barkeri*'s qPCR rates were 0.13 hr^{-1} in pure culture and 0.167 hr^{-1} in co-culture.

An examination of the acetate profiles (Figure 3.7 (D-F)), showed that *A. woodii* accumulated up to 293 mg/l from lag phase to death phase. *M. barkeri* consumed a small amount of acetate (with measurements of 28 mg/l decreased to 18 mg/l). The co-culture accumulated about 57% of the acetate concentration by the end of the *A. woodii* pure culture growth. Statistical tests were performed using the acetic acid data. There were no significant differences in acetic acid profiles between the cultures ($p = 0.344$) but there were significant differences between the growth phases ($p = 0.000$). Propionic acid and butyric acid were not detected on the VFA-GC.

Gaseous profiles (Figure 3.7 (D-F)) showed an absence of CH₄ production by *A. woodii* as in the previous study. There was a similar increasing CH₄ pattern for both the pure *M. barkeri* and the co-culture, with a total CH₄ accumulation of 332 μmol and 129 μmol , respectively. The co-culture production equates to 39% of the pure culture production.

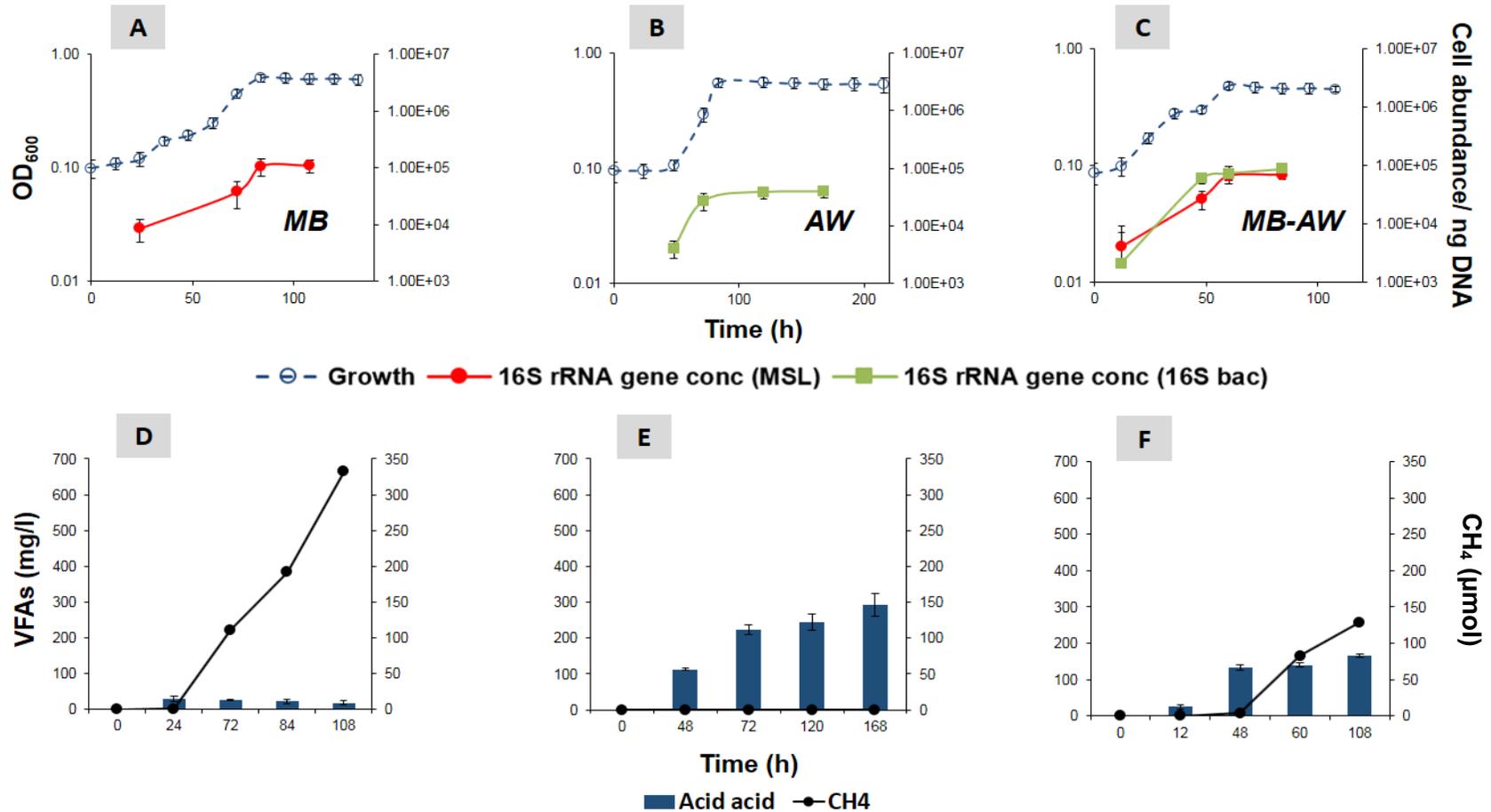


Figure 3.7: Optical density measurements and qPCR 16S rRNA gene copy numbers for (A) *M. barkeri* pure cultures, (B) *A. woodii* pure cultures, and (C) the *MB-AW* co-cultures. VFA (stacked bars) and CH₄ (lines) profiles are illustrated for (D) *M. barkeri* pure cultures, (E) *A. woodii* pure cultures, and (F) *MB-AW* co-cultures. All cultures were grown on H_2-CO_2 at $15^\circ C$. Error bars represent the standard deviation from the mean; some error bars are smaller than the symbols. Time 0 represents the theoretical concentration of acetate at the beginning of the study (D-F).

3.3.3.3 Growth impacts of the *M. barkeri* and *A. woodii* co-culture grown on acetate at 35°C on pure cultures

The growth results for both pure cultures and co-culture while acetate-feeding at 35°C (Tables 3.3 and 3.4) indicated that only *M. barkeri* can grow substantially on acetate. Very little growth was observed for *A. woodii* after several attempts and the shortest doubling time calculated was 115.64 hours. Acetate-adapted *M. barkeri* had a doubling time of 34.34 hours as a pure culture and had a doubling time of 36.93 hours when it was paired with *A. woodii*. Statistical tests confirmed that *M. barkeri* and *MB-AW* co-culture are statistically similar to each other ($p = 0.867$) while *A. woodii* is significantly different from both *M. barkeri* and *MB-AW* co-culture ($p = 0.000$ and $p = 0.000$, respectively).

The results from qPCR assays (Figure 3.8 (A-C)) revealed a similar profile for both *M. barkeri* alone and when it was paired with *A. woodii*, except that the cell abundances per ng of DNA based on 16S rRNA gene copies numbers were approximately halved when inoculated into co-culture in comparison with pure culture (59% for *M. barkeri* and 47% for *A. woodii* in co-culture compared to their respective pure cultures). While qPCR assay numbers for *A. woodii* remained within a one log range of 10^3 , there was an increase of two orders of magnitude in cell abundances of the *Methanosarcinales* family, from 10^3 to 10^5 , in both pure cultures and co-culture, which were approximately the same as the growth increase of *M. barkeri*. qPCR rates from the increase in cell abundance per ng of DNA for *M. barkeri* were 0.154 hr^{-1} and 0.124 hr^{-1} for the pure culture and co-culture, respectively. *A. woodii* had qPCR growth rates of 0.05 hr^{-1} and 0.06 hr^{-1} in pure and co-culture, respectively.

Acetate was consumed (Figure 3.8 (D-F)) under both *M. barkeri* in pure culture and in co-culture –an initial approximate concentration of 5000 mg/l decreased to 2275 mg/l for *M. barkeri* pure culture and to 2371 mg/l for the co-culture. *M. barkeri* alone consumed slightly more acetate than in the *MB-AW* co-culture. The acetic acid profiles between *A. woodii* and *M. barkeri* as well as between *A. woodii* and the *MB-AW* co-culture were statistically different ($p = 0.000$ in both cases). The acetic acid profiles between *M. barkeri* and the co-culture were statistically similar ($p = 0.243$). The acetic acid concentrations among all of the growth phases were

statistically different ($p = 0.000$). Propionic acid and butyric acid were not detected on the VFA-GC.

Both types of *M. barkeri* culture showed an accumulation of acetic acid (up to 205 mg/l for *M. barkeri* and 186 mg/l for the co-culture, which equates to 91% of what was produced in the pure culture), correlating with the measured decreasing acetate concentrations in Figure 3.8 (C-D)). There was no CH₄ detected in the *A. woodii* pure culture.

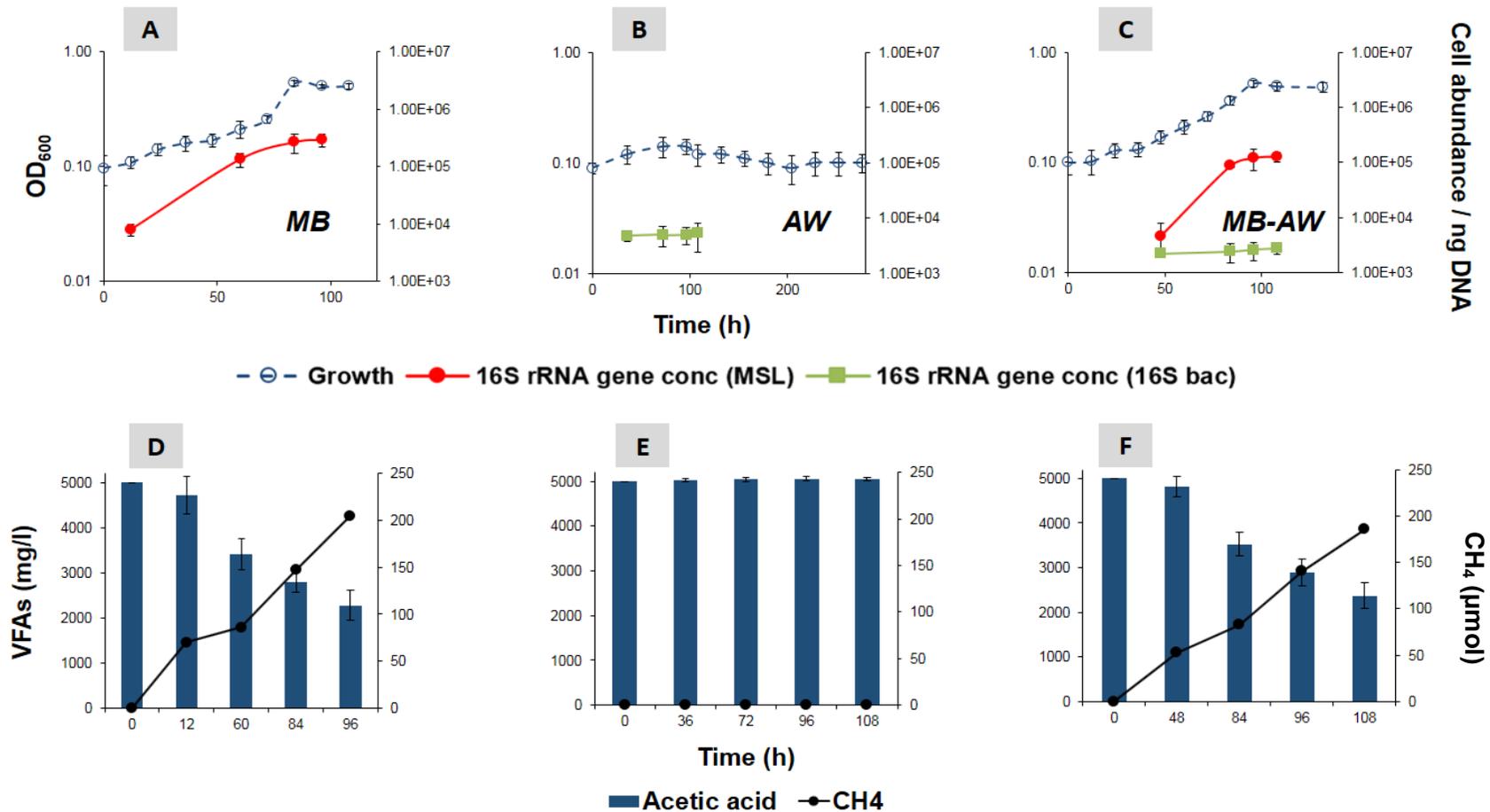


Figure 3.8: Optical density measurements and qPCR data gathered for (A) *M. barkeri* pure cultures, (B) *A. woodii* pure cultures and (C) the *MB-AW* co-cultures. VFA (stacked bars) and CH₄ (lines) profiles are illustrated for (D) *M. barkeri* pure cultures, (E) *A. woodii* pure cultures and (F) *MB-AW* co-cultures. All cultures were grown on acetate at 35°C. Error bars represent the standard deviation from the mean; some error bars are smaller than the symbols. Time 0 represents the theoretical concentration of acetate at the beginning of the study (D-F).

3.3.3.4 *M. barkeri* and *A. woodii* growth and chemical profiles grown on acetate at 15°C

Growth results for the above cultures incubated with acetate at 15°C indicated that *A. woodii* cannot grow on acetate, as expected and as reported at 35°C in the previous experiment. All doubling times (Table 3.3) were slower than acetate-fed cultures at 35°C. *M. barkeri* alone had a doubling time of 50.46 hours and *M. barkeri* paired with *A. woodii* gave a doubling time of 55.34 hours. Statistical tests were completed using the triplicate doubling times of each culture and indicated no statistical significance between *M. barkeri* and the co-culture ($p = 0.888$). Contrastingly, *A. woodii* cultures were significantly different to both *M. barkeri* ($p = 0.000$) and the *MB-AW* co-culture ($p = 0.000$).

The qPCR results revealed an increase in *Methanosarcinales* numbers of two orders of magnitude over the course of growth in both *M. barkeri* pure cultures, from 10^3 to 10^5 , and one order of magnitude when partnered with *A. woodii*, from 10^3 to 10^4 (Figure 3.9 (A-C)). *A. woodii*'s cell abundances per ng of DNA based on its 16S rRNA gene copies remained at approximately 10^3 . qPCR growth rates were 0.1 hr^{-1} and 0.094 hr^{-1} for *M. barkeri* pure culture and co-culture respectively. They were 0.03 hr^{-1} and 0.036 hr^{-1} for *A. woodii* pure culture and co-culture respectively.

The metabolite and CH₄ profiles (Figure 3.9 (D-F)) were studied and it was found that acetate was consumed by *M. barkeri* to concentrations of 3182 mg/l in the pure cultures and 3264 mg/l in the co-culture samples. Acetic acid profiles were significantly similar between *M. barkeri* and the *MB-AW* co-culture ($p = 0.59$) but were significantly different between *A. woodii* and *M. barkeri* and between *A. woodii* and the co-culture ($p = 0.000$ in both cases). There were significant differences in the acetic acid profiles between the growth phases ($p = 0.000$). Propionic acid and butyric acid were not detected on the VFA-GC.

As the *M. barkeri* and co-culture consumed their substrate by approximately 35%, up to 62.5 μmol CH₄ accumulated in the pure culture, and up to 59.4 μmol accumulated in the *MB-AW* culture.

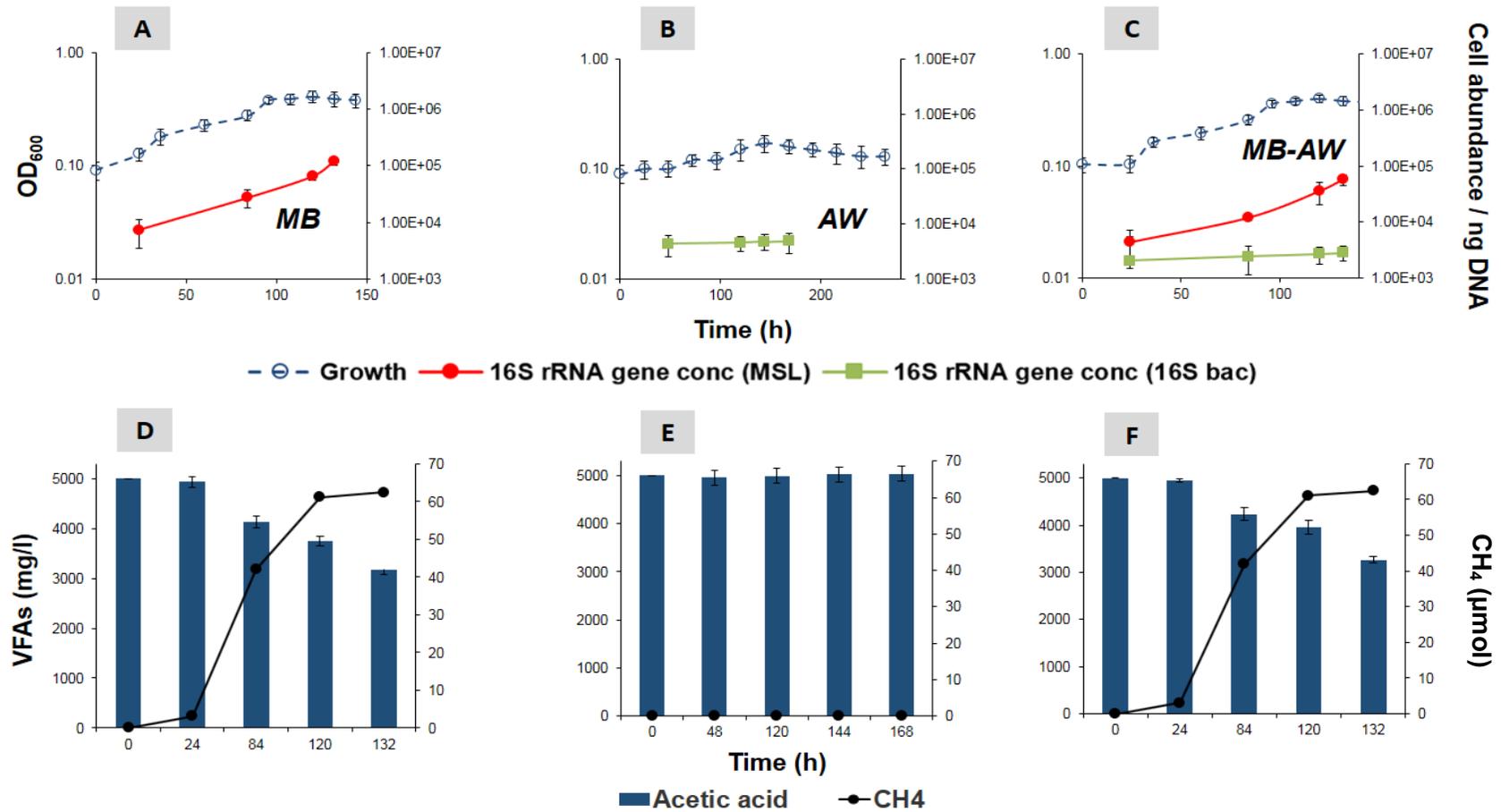


Figure 3.9: Optical density measurements and qPCR cell abundances / ng DNA based on 16S rRNA genes gathered for (A) *M. barkeri* pure cultures, (B) *A. woodii* pure cultures and (C) the *MB-AW* co-cultures. VFA (stacked bars) and CH₄ (lines) profiles are illustrated for (D) *M. barkeri* cultures, (E) *A. woodii* pure cultures and (F) *MB-AW* co-cultures. All cultures were grown on acetate at 15°C. Error bars represent the standard deviation from the mean; some error bars are smaller than the symbols. Time 0 represents the theoretical concentration of acetate at the beginning of the study (D-F).

3.4 Discussion

The results and analyses from these studies provide a broader foundation for understanding some important metabolisms and chemical and growth impacts that occur both within and between key anaerobic microorganisms in the acetogenic and methanogenic stages of the AD process under different environmental conditions. Recent tools for next generation sequencing and ‘omics, along with their integration with systems biology models, will be able to deepen the understanding of key AD microorganisms at the molecular level.

3.4.1 Temperature impacts on the metabolism and growth of the flexible methanogen *Methanosarcina barkeri* (MB temperature study)

Focusing on the growth results from OD measurements and qPCR assays, the fastest growth and highest cell numbers occurred during *M. barkeri* incubation at 37°C and decreased as the temperature decreased. This result was expected since lower temperature has been shown to slow down or halt growth (Pennington, 1908).

As a methanogen that is capable of consuming acetate, acetate had the highest consumption at 37°C, whereas at 25°C there was more acetate remaining, implying that it will either take the cells longer to consume the acetate because this strain is not acetate-adapted (Krzycki et al., 1982, Gokhale et al., 1993) or that there are few viable cells in the cultures. Since this strain of *M. barkeri* purchased from the DSMZ was not acetate-adapted and since previous studies have reported a higher affinity for H₂-CO₂ over acetate, it appears that it is quite difficult for the culture to consume the acetate. This culture has been described as a metabolic mutant devoid of CO dehydrogenase activity (Krzycki et al., 1982). This experiment is consistent with another study, which proposed that *M. barkeri* catabolises both acetate and H₂-CO₂ mixotrophically (Weimer and Zeikus, 1978b). Acetic acid results (section 3.3.1) suggest that there appears to be less of a difference in cumulative acetate metabolism between 30°C and 25°C than there is between 37°C and 30°C. There were some small measurable amounts of butyric and propionic acid. The quantification limits for liquid samples were found to be between 0.002 and 0.016 µg/L on the VFA-GC (Saadati et al., 2013). There may have been residual propionic and butyric acid within the DSMZ-recommended medium (used in the *M. barkeri*

temperature study and in the *M. barkeri* – *M. maripaludis* study) or it could have been below the limit of detection of the VFA-GC.

From the CH₄ production concentrations, the results indicate that the highest CH₄ accumulation occurs at 37°C (992 µmol), but there is a smaller observable difference in CH₄ concentrations between 25°C (443 µmol) and 30°C (609 µmol) at their death phases. This was likely due to the higher conversion of acetate to CH₄, as evidenced by the highest concentration decreases of acetate at 37°C. Since there was a difference in the CH₄ accumulations and similar levels in the VFA concentrations, this would imply that the CH₄ accumulated due to different rates of H₂-CO₂ consumption.

The fact that *M. barkeri* grown at 25°C and 30°C based on growth rates and chemical profiles were statistically similar could be relevant for decisions on what temperature is best to run bioreactors, which would have to take into account the process performance against the financial costs involved. Both the acetate and the CH₄ results also indicate that the VFA and CH₄ profiles are more closely correlated is between the lower temperatures than as between the higher temperatures. It is well known that mesophilic bioreactors at 37°C perform very well, but lower temperatures are proving economically viable and successful (Lettinga et al., 2001, Collins et al., 2003, McHugh et al., 2003b). If results showed statistically similar data at 25°C and 30°C in pilot scale reactors, this could create potential financial savings by reducing the temperature without losing many operational benefits. Overall, these results imply that as temperatures decrease the effects of temperature lessen, i.e. there are greater differences in VFA consumption and CH₄ production between higher temperatures than between lower temperatures. It has been demonstrated that the metabolic flexibility of *M. barkeri* is independent of temperature as it can grow at each temperature.

3.4.2 Competitive methanogenic study demonstrates preferential growth on co-substrates towards *Methanococcus maripaludis* (MB-MM co-culture experiment)

By growing these “competitive” methanogens together on a combination of H₂-CO₂ and acetate, their growth and chemical interactions were monitored at each phase of their growth as both pure cultures and co-culture. This specific co-culture has not been found previously in the literature. It has been reported in the literature that *Methanosarcinaceae* are important participants in anaerobic digesters. They have been found in several AD bioreactor studies (Kang et al., 2011, Kirkegaard et al., 2017). *Methanococcales* are less apparent in bioreactor studies but they have been found in granular sludge treating brewery wastewater (Liu et al., 2002).

The doubling time of *M. maripaludis* of 5.38 hours is less than that of the best that has been reported – 2.3 hours at 38°C (Jones et al., 1983a), but this could be due to the disparities in temperature differences studied and the different medium that was used. The fastest doubling of *M. barkeri* reported was 5 hours on a methanol-acetate (Zinder and Mah, 1979) and the doubling time of 12.44 hours reported in this work was presumably due to medium, substrate and temperature variations as well as it being a different strain.

The qPCR assays validated that the co-culture contained 50% of the starting inoculum of each pure culture. Overall, the results suggest that *M. maripaludis* can grow as well as in the pure culture, if not better, because it maintained approximately half of its yield at each growth phase (Figure 3.5 (B and C)). It had an accumulation of cell abundances per ng DNA based on 16S rRNA gene copy numbers that was one order of magnitude higher than when *M. maripaludis* was in the pure culture. Contrastingly, *M. barkeri* maintained a yield which was consistently lower than 50% of 16S rRNA genes than had been quantified when it was in pure culture. This implies that *M. maripaludis* was the “winner” of the competition. *M. barkeri* was also able to grow, but not to the same extent.

The growth and chemical results indicate that there was partial acetate consumption in both the *M. barkeri* and in the MB-MM cultures. This is probably because *M. barkeri* can consume both acetate and H₂, whereas *M. maripaludis* uses H₂ as its primary substrate and it thus less choice for growth and metabolism. Also, it can be

presumed that *M. barkeri* did not benefit from its substrate flexibility because it had a significantly lower doubling time than *M. maripaludis* had. There was incomplete consumption of acetate by *M. barkeri*, perhaps because it was not an acetate-adapted strain and it therefore had a much higher affinity for the H₂-CO₂. There was less consumption in the co-culture (27% in pure culture and 11% in co-culture). From the qPCR assay results and qPCR rates, it was noted that there was a higher growth in *M. maripaludis* and a lower growth in *M. barkeri*. This observation indicates that a decrease in *M. barkeri* could account for the lower overall acetate consumption within the co-culture.

For CH₄ production, the pure cultures' and co-culture's profiles were very similar. Both pure cultures could utilise the substrates (both H₂-CO₂ and some acetate in the case of *M. barkeri* and solely H₂-CO₂ for *M. maripaludis*) to produce CH₄. The co-culture was able to reach the same level of CH₄ accumulation because it had roughly half of each of the pure culture cells. There were higher than half of cell numbers of *M. maripaludis* present in the co-culture inoculum and there were less than half the number of *M. barkeri* cells as there were in their pure cultures and they both produced approximately 1000 µmol when in co-culture.

When partnered, they were **both** able to grow according to the qPCR and methane data and it appeared that, because *M. maripaludis* had the faster doubling time, it followed that it utilised a higher proportion of the H₂-CO₂ to give a similar doubling time to the co-culture as was measured for *M. maripaludis* in pure culture. Overall, both cultures were able to grow similarly as a pure culture and when in co-culture.

3.4.3 Characterisation of synergy between *Methanosarcina barkeri* and *Acetobacterium woodii* under various environmental conditions (MB-AW co-culture study)

Paired substrate investigations were not repeated in these studies because it has previously been reported that *A. woodii*/homoacetogenic bacteria are able to grow on paired substrates utilizing H₂/CO₂ simultaneously with an organic substrate (Braun and Gottschalk, 1981, Breznak and Blum, 1991, Peters et al., 1998).

3.4.3.1 *M. barkeri* and *A. woodii* growth on H_2 - CO_2

The doubling time of *M. barkeri* of approximately 19 hours was similar to a previous study where the strain 227 exhibited doubling times of between 12 and 24 hours when grown in a H_2 - CO_2 complex medium (Ferguson and Mah, 1983). The doubling time of *A. woodii* pure culture in this set of experiments was 17.13 hours. The optimal and fastest doubling time reported for *A. woodii* was 6 hours (Balch et al., 1977) but the conditions in this study diverged from those in Balch's study in some respects. For example, the incubation temperature was 35°C in this study compared to 30°C in Balch's study and this study had a minimal CP medium for growth of both cultures, unlike Balch's study.

The qPCR assays highlighted the similarity in both species' growth profiles. In all pure cultures and co-cultures, the cell abundances per ng of DNA of both *A. woodii* and *M. barkeri* increased in the range of approximately 10^3 to 10^5 (Figure 3.6 (A-C)), and the numbers in the co-culture were approximately half of pure cultures' starting numbers. For *A. woodii*, qPCR assays revealed that 46% of the original pure culture was detected in the co-culture and for *M. barkeri* 56% of the original pure culture was present at inoculation. By the final phase, 49% of the *A. woodii* and 54% of the *M. barkeri* pure culture counterparts was detected, which indicates that both microorganisms followed similar growth patterns in both pure and co-culture.

The absence of acetate in the basal medium accounts for its continuous accumulation from a starting point of approximately 0 mg/l in the *A. woodii* pure culture and the stationary acetate profile for the *M. barkeri* pure culture. In the co-culture, the acetate profile increases due to the utilisation by *A. woodii* of H_2 - CO_2 , i.e. acetate production. The acetate production in the co-culture was 45% less than the amount produced in the *A. woodii* pure culture. This was likely due to the fact that the *A. woodii* starting inoculum was half of that in the pure culture and that both cultures are able to survive and grow in this pure culture. Subsequently, there would presumably have been a decrease in acetate towards the end of (biphasic) growth due to *M. barkeri* consuming the acetate produced by the homoacetogen in a syntrophic manner but this strain of *M. barkeri* was not acetate-adapted, unlike in previous co-culture studies (Winter and Wolfe, 1979, Winter and Wolfe, 1980).

There was no detection of either butyric or propionic acid in the CP medium compared to the DSMZ-recommended medium as used in the previous two studies. This indicated that there may have been some VFA contamination in the DSMZ medium that was prepared such as from glassware or other equipment that was used.

CH₄ was only produced by *M. barkeri* and none was detected in the *A. woodii* pure culture. The co-culture contained 59% as much CH₄ (531 μmol) as the *M. barkeri* pure culture did (902 μmol). This difference is accounted for by the fact that the inoculum concentration present in the co-culture was approximately half of that present as was in the *M. barkeri* pure culture growth. Some utilisation by *A. woodii* of H₂-CO₂ did not produce any CH₄ and therefore the cumulative CH₄ could not reach 100% of what was produced by the pure culture:



In summary, the co-culture appears to exhibit similar growth and chemical profiles to the individual pure culture counterparts, and a synergistic partnership is demonstrated between *M. barkeri* and *A. woodii*.

Similar growth and chemical profile patterns were observed for both pure cultures and co-culture as at 35°C, except that the profiles were lower in all cases. The doubling times were significantly lower between the cultures at the higher and lower temperatures ($p = 0.047$). Psychrophilic growth of *M. barkeri* has previously been reported (Westermann et al., 1989, Gunnigle et al., 2013) but low temperature growth studies on *A. woodii* have not been sourced in the literature. It has been reported that homoacetogens have been capable of outcompeting methanogens at low temperatures (Conrad et al., 1989, Kotsyurbenko et al., 1996, Schulz and Conrad, 1996) and the results in this study demonstrate a faster doubling time for *A. woodii* than for *M. barkeri* (Table 3.4).

Homoacetogens are more metabolically diverse than hydrogenotrophic methanogens. Although acetogenesis is favourable at low temperatures and although it was observed that homoacetogenic bacteria outcompeted methanogens for H₂ (Conrad et al., 1989, Kotsyurbenko et al., 1993, Nozhevnikova et al., 1994,

Kotsyurbenko et al., 2001), *M. barkeri* is also as metabolically diverse like *A. woodii*. The similar growth rates and cell abundance per ng of DNA profiles and patterns detected (Figure 3.7 (A-C)) suggest the conclusion that *M. barkeri* has a similar level of potential diversity to *A. woodii*.

Acetate levels (Figure 3.7 (D-F)) were higher for *A. woodii* and remained stationary for *M. barkeri* at 15°C as predicted. The co-culture showed a smaller increase in acetate concentrations, which is likely due to the fact that the co-culture contained half as much of the starting inoculum as the pure cultures did, but there was 57% as much acetate production as there was with the pure culture.

CH₄ levels followed similar patterns as at 35°C except that they occurred at a lower level, most likely due to slower growth. There was no CH₄ accumulation for the homoacetogen, as anticipated, and the CH₄ production in the co-culture was 69% of what was produced in the *M. barkeri* pure culture, once again due to the cell volume of the former being half that of the latter and due to competition from, and the growth of, *A. woodii*.

3.4.3.2 *M. barkeri* and *A. woodii* incubation on acetate

The growth and doubling times (Tables 3.3 and 3.4) were similar for both *M. barkeri* and the co-culture because *A. woodii* did not show significant observable growth according to OD measurements and qPCR results (Figure 3.8 (A-C)). The *M. barkeri* growth rate of 0.02 hr⁻¹ was higher than a previous study which determined that an *M. barkeri* MS strain had a growth rate of 0.014 hr⁻¹ (Krzycki et al., 1982). The growth rate of *M. barkeri* in pure culture fed with acetate was lower than its counterpart fed with H₂-CO₂. This was consistent with their stoichiometries: the free energy change (ΔG°) when acetate was introduced into the pure culture was -36 kJ/mol compared to -131 kJ/mol when H₂-CO₂ was present.

Acetate levels were lower in the acetate-adapted *M. barkeri* pure culture as the acetate at 35°C was consumed, and levels were almost stationary for the *A. woodii* pure culture. For the co-culture, acetate levels were lower due to the flexible methanogen, *M. barkeri*. This was because *A. woodii* did not appear to have any observable growth and, therefore, acetate could not be produced. However, there

was a small difference in the overall profile in that acetate concentrations were marginally higher in the co-culture than in *M. barkeri* pure culture. This was perhaps due to minimal growth of some *A. woodii* cells, which was evident by way of a small increase in qPCR 16S rRNA bacterial gene copy numbers for the pure culture. The resulting acetate concentrations were not as low as at the death phase of *M. barkeri* pure culture growth, possibly due to some minimal levels of *A. woodii* growth (which causes acetate production) arising out of *M. barkeri*'s production of CH₄ and some CO₂.

CH₄ levels were similar in the *M. barkeri* pure culture as in the co-culture, except that the final concentrations were slightly lower in the co-culture, perhaps again due to some minor growth of *A. woodii* due to lower production of methane in the *A. woodii* compared to the *M. barkeri* pure culture. This potential growth could be because of residual CO₂ production from *M. barkeri* as previously mentioned. Thus, any marginal *A. woodii* growth would not be able to produce CH₄, resulting in lower overall CH₄ levels.

Similar growth and substrate profiles were detected as when the pure cultures and co-culture were incubated at 35°C except that all profiles were at lower levels due to the slower growth of microorganisms at low temperatures, as mentioned previously. Once again *A. woodii* had no observable growth, so *M. barkeri* and the co-culture had almost identical profiles.

Just as with the counterpart cultures grown at 35°C, acetate was consumed in both the *M. barkeri* and the co-culture but a higher acetate concentration was once more found at the death phase of the co-culture growth, implying possible minimal growth by *A. woodii* which accumulated a small amount of acetate and thus accounting for a slightly higher acetate level. This pattern also occurred with respect to the CH₄ profile. (Figure 3.9).

3.5 Conclusions

From this study, it has been demonstrated that pure culture work can add valuable knowledge and insights into how key players in the AD pathway specifically interact on a fundamental scale. Unquestionably, community-based studies are important to get a full picture of how the whole system works, but it is only from pure culture and synthetic co-culture studies that a clear picture can be attained as to how species adapt and interact with each other in an undisturbed way in particular circumstances, such as at a specific temperature or with a key substrate. By peeling away the layers of trophic groups, experimental insights can help to optimise the way in which microorganisms handle specific environmental stresses.

For the *M. barkeri* temperature study, a decrease in temperature resulted in decreased growth, as expected. CH₄ production decreased by 38.5% when temperature was reduced from 35°C to 30°C, but it only decreased by 27.3% when the temperature was reduced from 30°C to 25°C. This implied that there are more substantial growth and substrate changes at differences of higher temperatures. Similar findings were demonstrated in the acetate consumption profile analysis (sections 3.3.1 and 3.4.1). Additional statistically robust proof for this preliminary conclusion will be obtained if similar studies are conducted at further temperature levels.

For the *M. barkeri*-*M. maripaludis* experiment, both species were able to compete for H₂-CO₂. This co-culture impacted on the respective pure cultures' data by causing the growth and substrate profiles of *M. barkeri* to decrease slightly compared to the increase of *M. maripaludis* profiles. *M. maripaludis* marginally out-competed *M. barkeri* for growth. As already demonstrated, *Methanococcaceae* is a very important species within anaerobic bioreactors, and with its capability of fast growth this data suggests that lower numbers are capable of surviving and thriving in hydrogen-rich anaerobic environments. This experiment represented the first time this co-culture has been analysed according to the literature.

For the *M. barkeri*-*A. woodii* H₂-CO₂ study, a direct competition was observed for the substrate as evident from the CH₄ co-culture profile (Figures 3.6 (F) and 3.7 (F)), but overall, they were both able to grow similarly to their individual pure cultures. qPCR results reflect this observation where the cell abundances per ng of

DNA in co-culture were half of those in respective pure cultures at each time point and the co-cultures contained 50% of each starting inoculum when they were in pure culture. Previous studies have demonstrated a biphasic type of substrate utilisation in the co-culture, with acetate initially accumulating due to *A. woodii* growth with acetate subsequently decreasing due to acetate-adapted *M. barkeri* feeding on it after H₂-CO₂ had been depleted (Winter and Wolfe, 1979, Winter and Wolfe, 1980).

When acetate was the substrate, acetate-adapted *M. barkeri* consumed it and it was hypothesised that *A. woodii* would not grow. From the qPCR and metabolic data, it appeared that there was some marginal amount of *A. woodii* growth where there were increased acetate concentrations and qPCR cell abundances per ng DNA based on the 16S rRNA gene copy numbers. Error bars indicate that there may not have been any growth because they extend wider than the basal level starting points for the qPCR data (Figure 3.9 (B)) and the acetic acid data (Figure 3.9 (E)).

Both cultures are capable of growth at low temperatures under H₂-CO₂-feeding and acetate-adapted *M. barkeri* could grow under acetate-feeding. A deeper understanding of pure cultures at low temperatures will help to advance the wider LtAD field and to understand how microorganisms adapt and survive at low temperatures within bioreactors. A study which investigates these cultures at a gradient of low temperatures could give accurate information as to the differences in metabolic changes over those temperatures, which would aid and link in with the current understanding of bioreactor performance.

Overall, temperature did influence each of the competitive and/or synergistic relationships within the three studies. In all cases the higher temperature resulted in more growth and methane production.

Advancing from these experiments, an important subsequent step in understanding the pure cultures and co-cultures examined in this study would be to implement a suite of 'omics techniques to provide additional data, for example, investigating *A. woodii* growth under the sole acetate substrate. Under the various combinations of temperature, substrate and pure culture applied here, samples can be used further from each growth phase to look at specific genes that are responsible as the drivers under specific environmental changes using genomics, for example. Pure culture

studies will help to allow more detailed metabolic modelling of synthetic communities within an AD bioreactor environment.

An additional future direction of this pure culture work would be to build on these synthetic co-cultures with a tri-culture community. Synthetic biology serves as a powerful tool in order to create predictive models of microbial communities but pure culture work significantly add to these databases detailing how microorganisms interact with each other.

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Chapter 4

Chapter 4 - Physical, physiological and phylogenetic characterisation of individual, and size-resolved, anaerobic sludge granules

Abstract

Anaerobic digestion (AD) involves complex organic compound degradation in the absence of oxygen to produce biogas, a renewable fuel. In some configurations of engineered AD systems, the process is underpinned by anaerobic granules, which are self-immobilised, spherical biofilms. Anaerobic granules are naturally-occurring spherical biofilms that comprise of microbes from each trophic group along the AD pathway: hydrolysers, acidogens, acetogens and methanogens. Anaerobic granules have been widely studied, but rarely at the level of individual granules.

This research investigated size distributions, physical characteristics, specific methanogenic activities (SMAs) and community structures across three granule size fractions from three upflow anaerobic sludge blanket (UASB) bioreactors treating different types of wastewater. Based on size distribution, total solids, volatile solids and SMAs against acetate and H_2 - CO_2 , the results indicated significant differences between each size fraction but not between sludge types, except with volatile solids. Smaller granules had higher hydrogenotrophic activity and large granules had higher acetoclastic activity.

Five individual granules were taken from three size fractions of three different granular sludge bioreactors. These forty-five individual granules were analysed and found to have replicated community structures for each set of five granules. 16S rRNA MiSeq analyses based on the DNA community structure of single individual granules showed significant differences in the community structure for the three granule size fractions in two out of the three anaerobic sludges. There were significant differences studied between the three sources of anaerobic sludge based on phylogenetic data. These results indicate possible heterogeneity among different granule sizes from different sources at the level of a single granule.

4.1 Introduction

Anaerobic digestion (AD) is a multi-stage process whereby several trophic groups of microorganisms sequentially break down organic molecules into simpler molecules. The groups of microbes involved in AD include hydrolyzers, acidogens, acetogens and methanogens. Anaerobic granules are biofilms that spontaneously occur within digesters and are composed of these complex microbial consortia. They are exclusive to anaerobic bioreactors and particularly to the bioreactor configurations; the Upflow Anaerobic Sludge Blanket (UASB) and the Expanded Granular Sludge Bed (EGSB) systems that have been developed over the past few decades (Lettinga et al., 1980, de Man et al., 1988). It is estimated that 80% of global anaerobic wastewater treatment systems are based on UASB technologies (Abbasi and Abbasi, 2012).

Anaerobic granular sludge has been studied extensively in respect of granulation formation (Hulshoff Pol, 1989, Thaveesri et al., 1995a, Schmidt and Ahring, 1996), granular ultrastructure (MacLeod et al., 1990, Quarmby and Forster, 1995, Fang, 2000), community structure (Ito et al., 2012, Mei et al., 2016, Saunders et al., 2016, Kirkegaard et al., 2017), community function (Wang et al., 2010, Abram et al., 2011, Siggins et al., 2012) and its effects on reactor performance (Bhatti et al., 1993, McHugh et al., 2004, Lee et al., 2008). Despite the numerous studies and successes as a bioenergy technology, AD still has mostly undefined microbial communities with relatively poorly-understood dynamics (Werner et al., 2011).

There are various theories related to anaerobic sludge granulation and the development of granules including physical, microbial and thermodynamic theories, which have each been previously reviewed (Hulshoff Pol, 1989, Thaveesri et al., 1995a). Among the ecological microbial theories, the general hypothesis is that methanogens cluster together initially and then acetogens and syntrophic bacteria grow around the filamentous methanogens, creating a spherical biofilm core. Subsequently, fermenters, acidogens and hydrogenotrophs adhere as a syntrophic relationship occurs and they form an exterior layer surrounding the biofilm. Due to hydrogenotroph extracellular polymer excretion, it remains as an intact spherical biofilm as the individual trophic groups, and therefore the granule,

continues to grow (MacLeod et al., 1990, Vanderhaegen et al., 1992, Ahn, 2000, Fang, 2000).

Linking with these theories, the purpose of this experimental work was to examine the DNA community structure of five single individual granules from three sources of biomass source and at three granular size distributions to correlate species with granule size and source. Further to this, investigations were carried out to determine whether granules had significantly different physical and physiological characteristics from each other based on size and, separately, based on sludge type.

In this study, granular biomass was sourced from three UASB bioreactors in diverse companies treating various different types of wastewater: dairy wastewater, starch-rich wastewater and high-sugars wastewater. For each anaerobic sludge sample granules were separated into three different size fractions.

The primary hypotheses were that:

1. physical characteristics of granules at three different sizes are significantly different to each other;
2. physical characteristics of the three granular sludges (due to the variety of and differences between the types of wastewater being treated) are significantly different to each other;
3. there are significantly different methanogenic activity potentials between the different sizes and between the three types of anaerobic granular sludge;
4. based on single granules at a specific size, the community structures of differently-sized granules are significantly different (based on a granulation theory which suggested that different microbes attach as the biofilm grows);
5. however, the microbial community structure of granules from the same size fraction are similar;
6. based on single granules from different reactors, the phylogenetic characterisation of granules from different sources are significantly different from each other.

4.2 Materials and Methods

4.2.1 Biomass sources, particle size distribution and physical characterisation of granules

Granular sludge was sourced from three different anaerobic wastewater bioreactors. The first sludge, “Biomass A”, was obtained from Avecom (“A”) in Belgium where the company was treating wastewater from a sweetener factory at 35°C. “Biomass L” was from Lurgan (“L”), Northern Ireland. It came from a UASB bioreactor treating starch-rich wastewater at 37°C. The average granule diameter was 1.45 mm (Figure 4.1). The third sludge, “Biomass C”, was a mesophilic, anaerobic granular biomass obtained from a lab-scale UASB reactor treating a synthetic volatile fatty acid (VFA) wastewater at 37°C. The reactor was operated by Robert Dillon. Samples of Biomass C were taken from the UASB reactors after 112 days of operation and from a combination of reactor sampling ports. Previously, Biomass C was in a full-scale (1500 m³), internal circulation (IC) bioreactor at Carbery (“C”) Milk Products (Ballineen, Co. Cork, Ireland) and was used to treat ethanol production wastewater but the VFA stream was fed into the lab-scale reactor which was sampled for this work.

For the remainder of the thesis, the sludges will be referred to as follows:

1. Avecom (high sugar-fed granules): “A”
2. Lurgan: (high starch-fed granules): “B”
3. Carbery and lab-scale reactor (VFA-fed granules): “C”

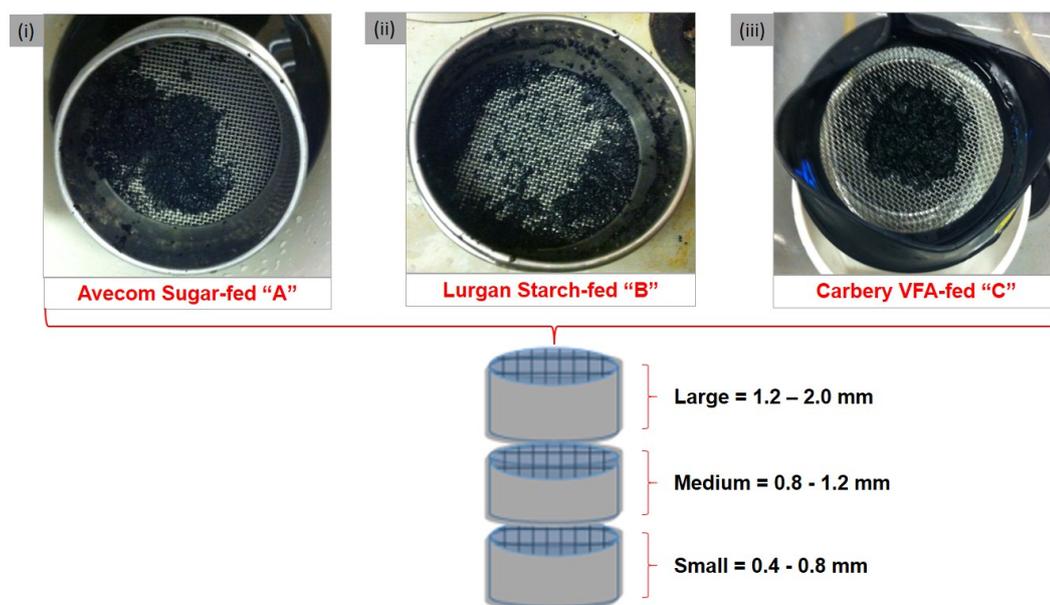


Figure 4.1: Separation of anaerobic sludge source and size. Granular sludge which treated (A) sugar-fed wastewater at Avecom, (B) starch-rich wastewater at Lurgan plant and (C) VFA-fed wastewater. Each sludge was sieved to separate granules into three different size fractions: large, medium and small.

Aliquots of 1 L of each sludge type were passed through a series of sieves of different pore diameters. Size fractions were classified as “large”, “medium” and “small”. Large granules were between 1.2 and 2.0 mm in diameter, medium granules were between 0.8 mm and 1.2 mm, and small granules were between 0.4 and 0.8 mm in diameter (Figure 4.1). The wet weight of each granular size (“small”, “medium” and “large”) from the three granular sludge sources was weighed to determine the relative contribution of each size fraction in the three bioreactors (Figures 4.2 and 4.3, section 4.3.1).

Following these measurements, ten individual granules from each size distribution of the three sludges were weighed individually on an analytical balance (minimum calibration setting, 0.000 g). Based on the averages of small-, medium- and large-sized granules, the size distribution based on a single granule was calculated and presented for each source (Figure 4.3, section 4.3.1).

Total solids (TS) and volatile solids (VS) were measured in accordance with standard methods (APHA, 1998). VS represent the organic matter in a sample. Briefly, TS was measured by the amount of solids remaining after oven-drying

samples overnight (at 105°C). Volatile solids are determined by the decrease in mass of granules after the oven-dried residue are incinerated at 550°C for two hours (Franke-Whittle et al., 2014).

4.2.2 Specific Methanogenic Activity (SMA) assay

The methanogenic activity of the granular samples at the three size fractions which were taken from the three sludge sources were each measured by the specific methanogenic activity (SMA) assay method. This test uses the pressure transducer technique as previously described (Colleran et al., 1992, Coates et al., 1996, Siggins et al., 2012, Bialek et al., 2013). The levels of acetate (30 mM) and H₂-CO₂ (80:20, v/v) present determined acetoclastic and hydrogenotrophic methanogenesis, respectively. Specific methanogenic activity was measured in ml CH₄ g VSS⁻¹ day⁻¹ (Colleran et al., 1992).

SMA tests are batch assays where the rate of methane production over time is measured. Direct methanogenic substrates included acetate (30 mM) and H₂-CO₂ (2 atm). Butyrate (15 mM) and propionate (30 mM) were higher carbon intermediates. Biomass samples were transferred to sterile hypovials to a final concentration of 2-5 g VSS l⁻¹ in an anaerobic activity test medium. Controls were set up either without substrate (in parallel with soluble substrate vials) or with an N₂-CO₂ pressurised headspace (in parallel with H₂-CO₂-fed vials).

The biogas presence was monitored by measuring the pressure of sealed vials containing biomass and either a soluble (acetate, butyrate, propionate) or a gaseous (H₂-CO₂) substrate supplied to the biomass on an individual basis. A gas chromatograph (Varian) was used to measure the CH₄ contained inside the sealed vials as described in Chapter 3 (section 3.2.3). This method assessed the maximum potential rates of methane generation against intermediates of the AD process followed by the determination of SMA levels in ml CH₄ g VSS⁻¹ day⁻¹.

4.2.3 DNA/RNA co-extraction from biomass

Co-extraction was performed using a previously-described protocol (Griffiths et al., 2000). Individual granules were transferred to separate 2 ml screw-cap tubes, which were pre-filled with 0.5 g sterile glass beads of 1.0 mm diameter and 0.5 g sterile glass beads of 0.15-0.2 mm diameter (Sigma). Each sample was mixed with 500 μ l 5% w/v cetyl trimethylammonium bromide (CTAB) extraction buffer and 500 μ l of phenol-chloroform-isoamyl alcohol (25:24:1; pH 8) in respective 2 ml tubes for sample-lysing by bead-beating for 10 min at 3,000 rpm using the Fine PCR Vortex (Medical Supply Company). For each sample, the phases were separated by centrifuging at 12,500 rpm for 5 min at 4°C and the clear aqueous supernatant was transferred into a sterile, Phase Lock Gel tube (MaXtract™ High Density – Qiagen). An equal volume of chloroform isoamyl alcohol (24:1) was transferred to the same tube, which was centrifuged again at 12,500 rpm for 5 min at 4°C to separate the nucleic acid phases. For each sample, the supernatant was transferred into a sterile, RNase-free tube, along with 0.1 vol of 3 M sodium acetate (pH 5.2) and 1 vol of ice-cold isopropanol. The tubes were subsequently centrifuged at 12,500 rpm for 30 min at 4°C and the supernatant was discarded. The nucleic acids were washed by using 1 ml 70% (v/v) ice-cold ethanol. The samples were centrifuged at 12,500 rpm for 5 min at 4°C and the supernatant was discarded. The final pelleted nucleic acids were air-dried on ice and were resuspended in 50 μ l DEPC treated water. The DNA quality of the samples was assessed using a nanodrop (Thermo Scientific) and agarose gel electrophoresis. The nucleic acids were stored at -80°C.

4.2.4 Illumina MiSeq Analysis

DNA samples of quintuplicate single granules from each size fraction from each of the three sludge sources (Figure 4.2) were sent to The Foundation for the Promotion of Health and Biomedical Research of Valencia Region, FISABIO (Valencia, Spain) for amplification of the 16S rRNA gene sequences using the universal bacterial/archaeal forward primer 515f and the reverse primer 806r on an Illumina MiSeq platform. The samples were processed *in vitro* to generate a library of short inserts. MiSeq produced between 15 and 28 million clusters passing filters per run.

A modified version of the MiSeq SOP pipeline in mothur (http://www.mothur.org/wiki/MiSeq_SOP) processed the raw sequences.

In summary, each paired raw sequence was combined, ambiguous base cells were removed (maxlength=300, minlength=200) (Weigel and Erwin, 2016), duplicate sequences were removed, sequences were aligned to the SILVA database (SILVA, V4, Release 119) (Quast et al., 2013), over-hangs at both ends were removed, sequences were de-noised by pre-clustering, chimeras were removed, sequences were clustered into operations taxonomic units (OTUs) and all remaining sequences were binned into phylotypes for analyses. Sequences were organised into groups corresponding to the level of family.

Both alpha and beta diversities were examined for each size fraction from each of the three sludge sources. Cluster analysis, dendrograms and canonical correspondence analysis (CCA) plots were constructed using RStudio. Quintuplet samples for the three size fractions of each of the three sludge types were used in the Illumina MiSeq analysis pipeline. However, one sample from the VFA-fed small-sized group of quintuplet granules had to be excluded as its sequence number was comparatively lower than each of the other granules.

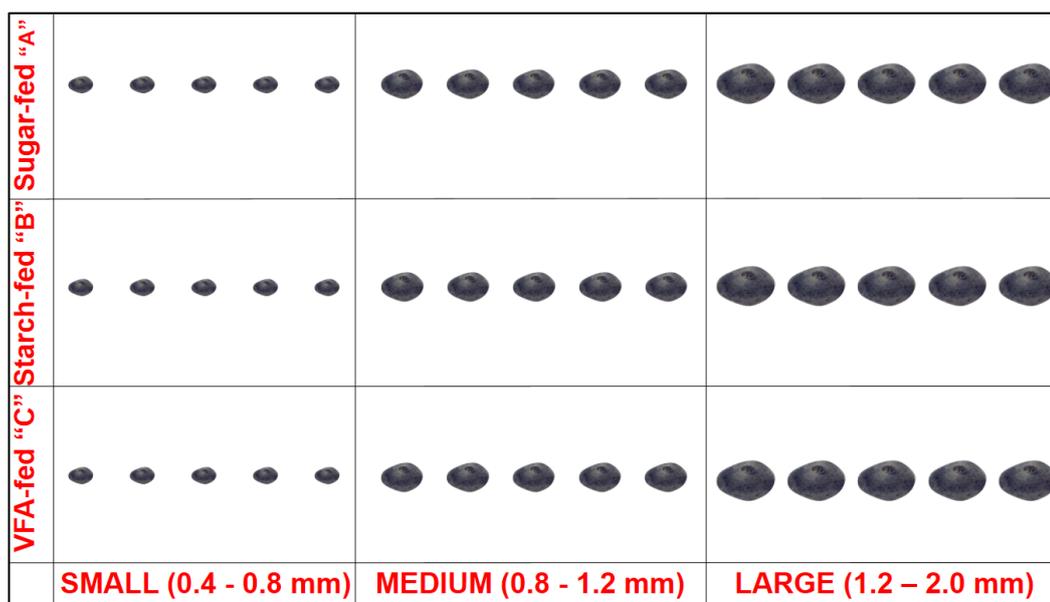


Figure 4.2: Set-up of individual granules for Illumina MiSeq analysis according to specific size fraction: five “small-”, five “medium-” and five “large-” sized granules from each of the three granular sources (sugar-fed “A”, starch-fed “B” and VFA-fed “C”).

4.2.5 Statistical Analysis

SPSS 23.0 version statistical software package for Windows was used to determine each of the statistical conclusions for the hypotheses outlined previously (section 4.1). The statistical tests employed included Kolmogorov-Smirnov tests to examine normal distributions. Two-way analysis of variance (ANOVA) was carried out to determine significant differences between granular sizes and between granule source based on SMA activity data. Significance was determined by a p-value of less than 0.05

For Illumina MiSeq analysis, statistics were performed on cleaned-up sequence data using the R statistical framework version 3.3.2 (Team, 2016) and version 2.4–1 of the Vegan package. The samples were subsampled (rarefaction analysis) to an even depth of 41,000 reads per sample to alleviate the effect of sample size bias on the microbial community composition.

Specifically, statistical analyses included taxonomic alpha-beta diversity analysis by Pielou's evenness, by Simpson and Shannon indices, by multivariate ANOVA based on similarities (Adonis tests) and by CCA to test the relationships between bacterial and archaeal community structures of single granules from each size fraction from the three different sludge sources. Rarefaction curves were also calculated using the Vegan package. Taxonomic beta-diversity matrices of Euclidean distances were calculated. Significance tests were computed based on the sequential sums of squares from permutations of the raw data. It partitioned the sums of squares of a multivariate data set. All analyses were performed using mothur and RStudio.

4.3 Results

4.3.1 Biomass characterisation of the three sludges

Each biomass source was initially characterised using a mixed granule sample for the three types of biomass: "A" was sugar-fed sludge, "B" was starch-fed sludge

and “C” was VFA-fed sludge. At the outset of the work, physical appearance, TS contents and VS contents were measured for the three sludges (Table 4.1).

Table 4.1: Anaerobic sludge physical traits based on a mixed sample from the three bioreactors.

Traits	“A” sugar-fed	“B” starch-fed	“C” VFA-fed
Colour and Morphology	Spherical and black	Spherical and brown	Spherical and brown
Total Solids (% m/v)	5.77	3.30	5.99
Volatile Solids (% of TS)	91.15	89.32	90.84

4.3.2 Physical profiles of size-resolved anaerobic granular sludges on a whole-sludge and single-granule level

Based on the wet weight of three size fractions, each of the three sludge types yielded different amounts of sludge (Figure 4.3(i)). The smallest granule fraction (0.4-0.8 mm) accounted for 10%, 11% and 4% of A (sugar-fed), B (starch-fed) and C (VFA-fed) sludge samples, respectively. The medium-sized granules (0.8-1.2 mm) accounted for 35.5%, 37% and 28.5% of A (sugar-fed), B (starch-fed) and C (VFA-fed) sludge samples, respectively. The largest granule fraction (1.2-2.0 mm) accounted for 54%, 51.5% and 67.5% of A (sugar-fed), B (starch-fed) and C (VFA-fed) granular samples, respectively.

Using two-way ANOVA, significant differences were measured between granular sizes ($p = 0.000$) but not between the sludge source ($p = 0.287$). Therefore, ‘small’ granules had a similar percentage composition in each source sample, as did ‘medium’ and ‘large’ granules.

Using the average values of ten ‘small’, ten ‘medium’ and ten ‘large’ granules from each source, compositions were determined for each sludge based on individual granules (Figure 4.3 (ii)). There was a difference in these distributions compared to the previous compositions. It was apparent that the ‘small’ granules now accounted for the largest proportion of each sludge type and the large granules accounted for the smallest proportion. This result was the inverse of the data based on size distributions. The only consistent observation was that medium-sized granules accounted for approximately one third of the granular distribution.

The smallest granule fraction (0.4 – 0.8 mm) accounted for 49.8%, 37.9% and 38.2% of A (sugar-fed), B (starch-fed) and C (VFA-fed) sludge samples, respectively. The medium-sized granules (0.8 – 1.2 mm) accounted for 37.4%, 38.6% and 40.3% of A (sugar-fed), B (starch-fed) and C (VFA-fed) granular samples, respectively. The largest granule fraction (1.2 – 2.0 mm) accounted for 12.8%, 23.5% and 21.5% of A (sugar-fed), B (starch-fed) and C (VFA-fed) sludge samples, respectively.

Statistical analysis reported the same findings for this size distribution study based on single granules as it did for the previous size distribution test based on bulk sludge samples. There were statistically significant differences between granular size ($p=0.000$) and no statistically significant difference for each of the three sizes between the granule sources ($p=0.997$).

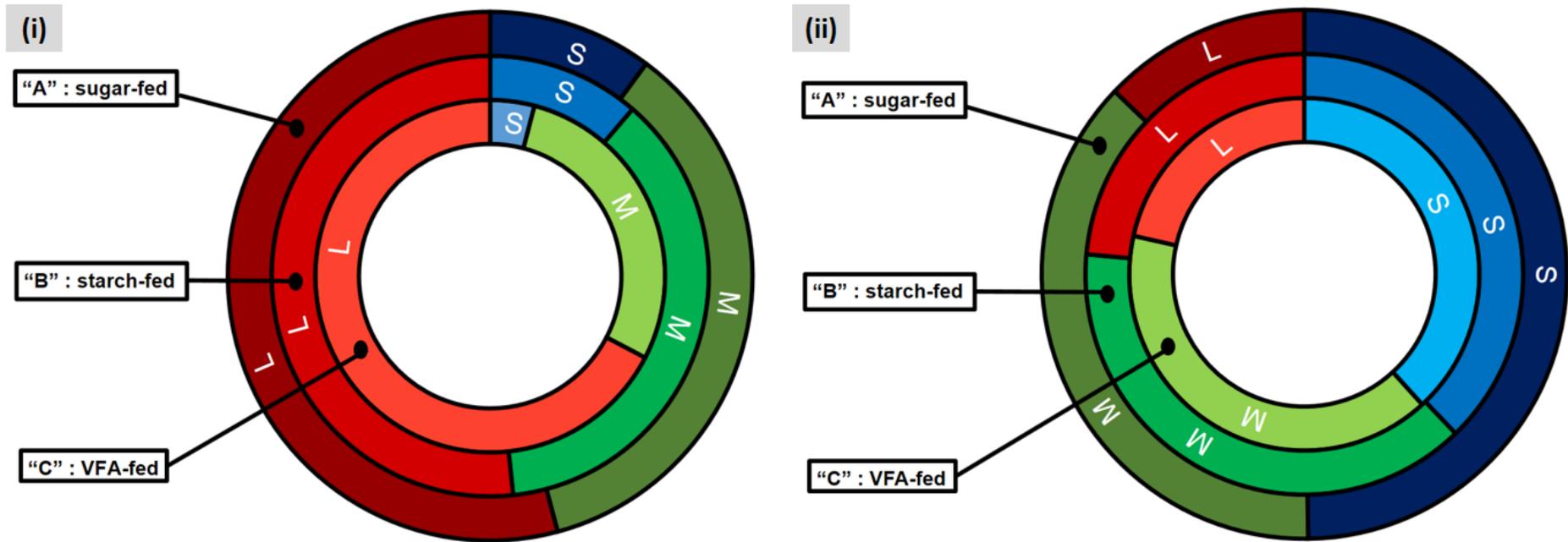


Figure 4.3: Contribution of granules of three size distributions (S=small [shades of blue], M=medium [shades of green] and L=large [shades of red]) taken from three different source (sugar-fed, starch-fed and VFA-fed) sludge samples. (i) Particle size distributions among the three sources and (ii) distributions of average wet weights of individual granules for each source and size fraction.

TS and VS contents were determined (Table 4.2) for each granular size fraction from the three sludge sources. Taking all sizes and sources into account, TS percentages ranged from 2.31% m/v to 7.29% m/v and the VS concentrations extended from 83.49% to 92.24% of the TS in each sample. The small-sized fractions for each sludge type had the lowest TS and VS measurements except for the TS of “C”. Medium-sized granules always contained the highest organic content.

The TS contents were statistically analysed and the results showed no significant difference between each of the sludge types ($p = 0.054$) but there was a significant difference reported between granular sizes ($p = 0.036$). The overall statistical analysis of VS percentages reported a statistically significant difference between the three sources of sludge ($p = 0.013$) and between granular sizes ($p = 0.05$).

Table 4.2: TS and VS concentrations for each size and source of anaerobic granular sludge.

	Size	“A” (%)	“B” (%)	“C” (%)
TS	Small	4.14	2.31	5.33
	Medium	6.29	3.36	4.99
	Large	7.29	5.13	6.18
VS	Small	88.93	83.91	83.49
	Medium	92.23	89.71	85.24
	Large	89.67	89.50	84.96

TS (% [m/v])

VS (% [m/v] of TS)

4.3.3 SMA determination for anaerobic granular sludge from different sources according to specific size distributions

SMA results showed that, at each size fraction and with each sludge type, there was higher activity against hydrogen than against acetate, except for large granules from the VFA-fed sludge (“C”). The mixed- and large-sized granules had the most similar profiles against both H_2 - CO_2 and acetate. Sugar-fed sludge (“A”) had the highest hydrogenic activity for small granules. Starch-fed granules (“B”) had the highest activity against H_2 - CO_2 at all sizes except for the small-sized granules. For activity against acetate, small- and medium-sized granules from the VFA-fed

reactor (“C”) had the highest activity and sugar-fed anaerobic sludge (“A”) had the highest activity for mixed and large-sized granules.

Based on the SMAs against H_2 - CO_2 , two-way ANOVA tests showed that there was no significant difference between A (sugar-fed reactor) and B (starch-fed reactor) sludges ($p=0.168$) but there was a statistically significant difference measured between A and C (VFA-fed reactor) sludges ($p=0.000$) as well as between B and C sludges ($p=0.000$).

It was found that there was not a statistically significant difference between large- and medium-sized granules ($p=0.344$). However, statistically significant differences between large- and small-sized granules ($p=0.001$), as well as medium- and small-sized granules ($p=0.016$), were found. Observations on the differences between the three size fractions with a mixture of granule sizes were also statistically represented (Table 4.3).

Acetate assays also used ANOVA tests to examine statistical differences between granular size and type. Acetate activities for A and B sludge types were not significantly different ($p=0.380$) but acetate activities between B and C ($p=0.001$) as well as between A and C sludges ($p=0.000$), were significantly different to each other.

The results showed that the acetate activities between large- and medium-sized granules ($p=0.000$), as well as large- and small-sized granules ($p=0.012$) differed to a statistically significant degree. Medium- and small-sized granules were not significantly different to each other ($p=0.242$).

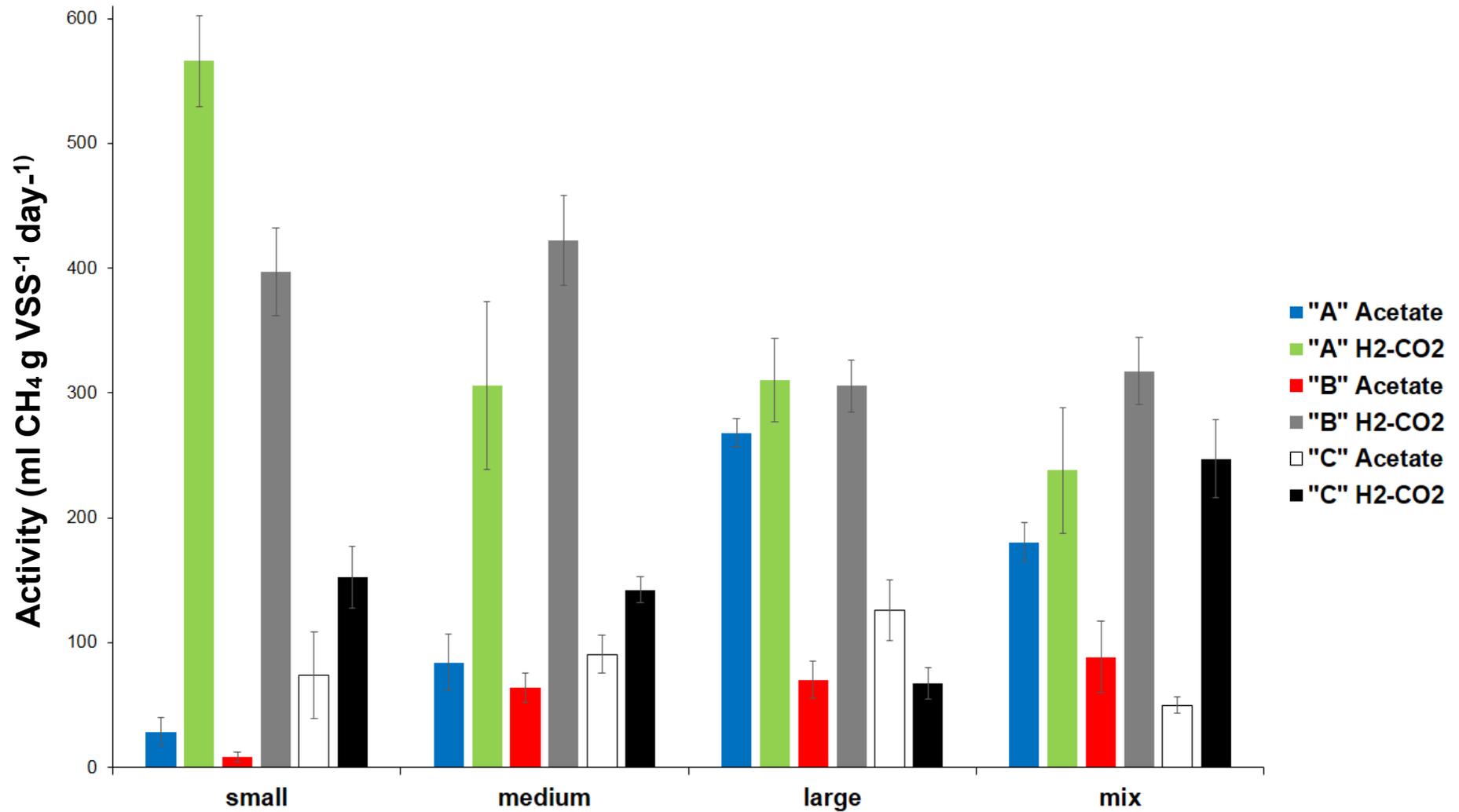


Figure 4.4: Specific methanogenic activity assays were performed on each size (small, medium, large and mixed granules) of each granular sludge sample (sugar-fed ("A"), starch-fed ("B") and VFA-fed ("C")) against substrates acetate and H₂-CO₂ (80:20). Error bars were based on triplicate assays.

Table 4.3: List of statistical significance values for specific methanogenic activity assays between the three sludge sizes and types. The level of significance was set at $p = <0.05$. Dark grey represents the tests where both substrates were significantly different. The paler grey represents the tests where one substrate showed statistical significance. Unshaded indicates that there was no significance.

	H₂-CO₂ Assays	Acetate Assays
<i>Size</i>		
Small + Medium	0.016	0.242
Small + Large	0.001	0.012
Small + Mixed	0.005	0.994
Medium + Mixed	0.854	0.000
Large + Mixed	0.948	0.001
Large + Medium	0.344	0.000
<i>Reactor Type</i>		
sugar-fed (A) + starch-fed (B)	0.168	0.380
sugar-fed (A) + VFA-fed (C)	0.000	0.000
starch-fed (B) + VFA-fed (C)	0.000	0.001

4.3.4 Phylogenetic characteristics of individual granules from various granular sources of specific sizes

Initial alpha diversity results showed by rarefaction curves that there was almost complete saturation of community diversity in many of the samples, demonstrating adequate sequencing depth for sample coverage (Figure 4.5). Pielou's evenness was also calculated (Figure 4.6 (i - iii)). Evenness is lowest in B (starch-fed) single granules and highest in C (VFA-fed) granules. Statistical tests showed that evenness values between each granule size were similar to a statistically significant degree ($p = 0.105$ to 0.78) but the evenness values for each sludge type were statistically different from one another ($p = 0.00$ to 0.01).

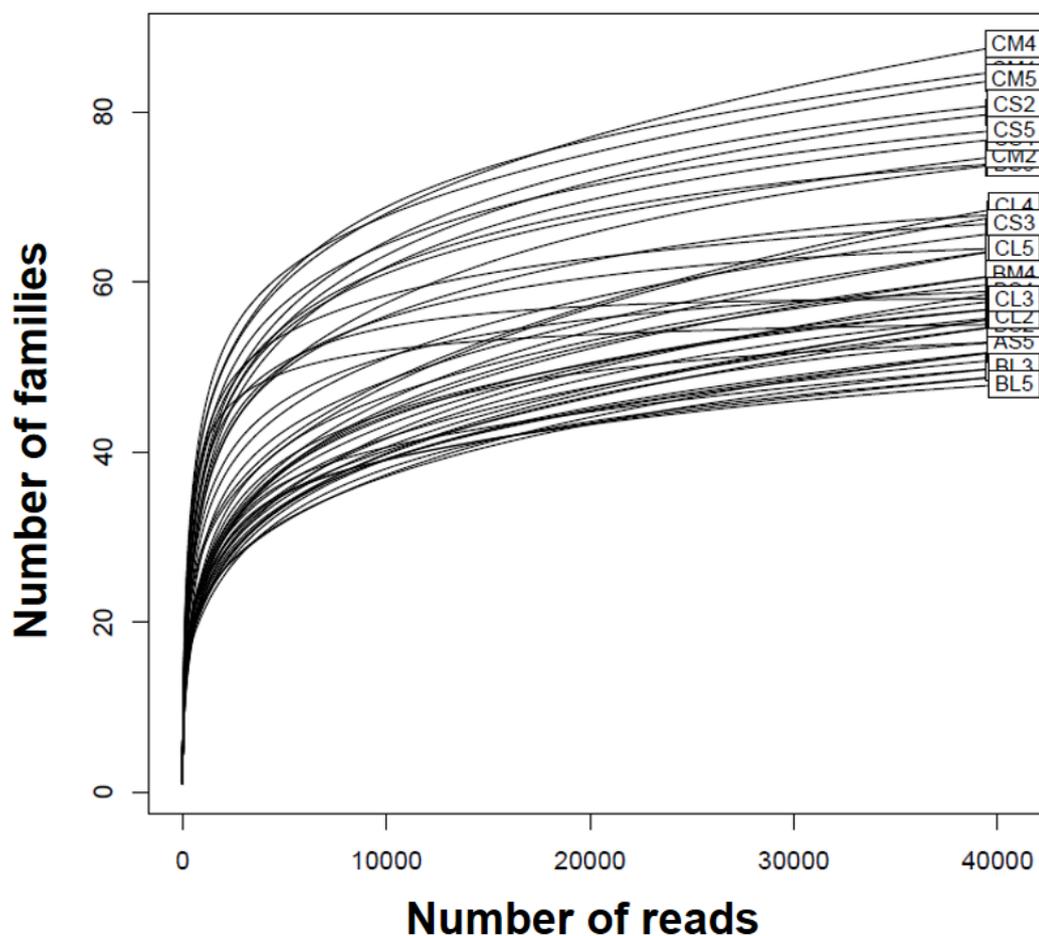


Figure 4.5: Rarefaction curves of all samples. AS = sugar-fed, small, AM = sugar-fed, medium, AL = sugar-fed, large, BS = starch-fed, small-sized granules, BM = starch-fed, medium, BL = starch-fed, large, CS = VFA-fed, small granules, CM = VFA-fed, medium granules and CL = VFA-fed, large granules.

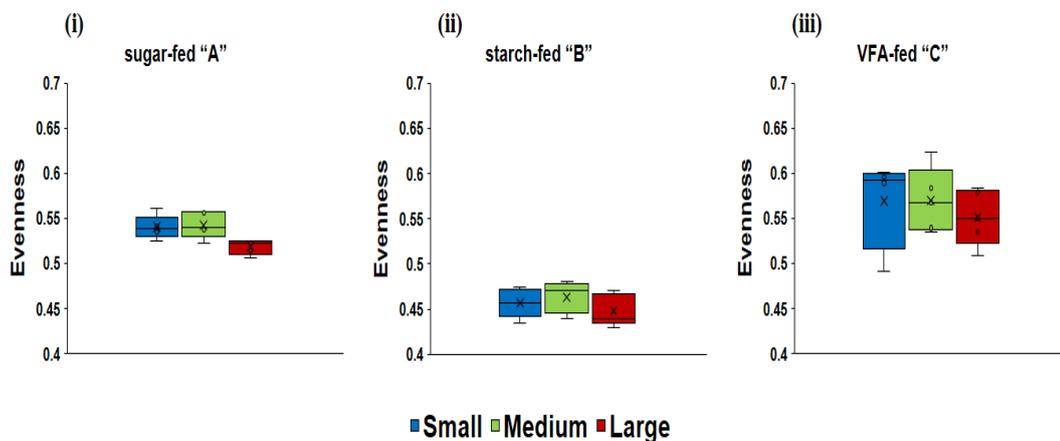


Figure 4.6: Pielou's evenness results for (i) sugar-fed "A", (ii) starch-fed "B" and (iii) VFA-fed "C" sludges at the three size distributions (small-blue, medium-green and large-red). The line in each box represents the median.

Simpson and Shannon diversity indices were also computed for each granule size and sludge type. The Simpson index (which gives more weight to common or dominant taxa) illustrated high diversity in all samples from the VFA- and sugar-fed sludges, but displayed noticeably lower diversity in starch-fed granules (Figure 4.7 (i-iii)).

The Shannon index also showed that the starch-fed sludge granules had the lowest diversity in comparison to the other two sludge types (starch-fed = 1.66 to 1.9, compared to sugar-fed = 1.91 to 2.12 and VFA-fed = 1.96 to 2.52 (Figure 4.7 (iv-vi))).

Two-way ANOVA statistical tests were carried out on the data for the Simpson and Shannon indices. The results were consistent with the evenness values. The Simpson diversities were statistically similar for the three granule sizes ($p = 0.923$) and statistically different between the three sludge types ($p = 0.000$). The Shannon diversities were statistically similar for the granule size ($p = 0.254$) and statistically different for the sludge type ($p = 0.000$). Their overall maximum diversity values were 0.865 for the Simpson index and 2.520 for the Shannon index.

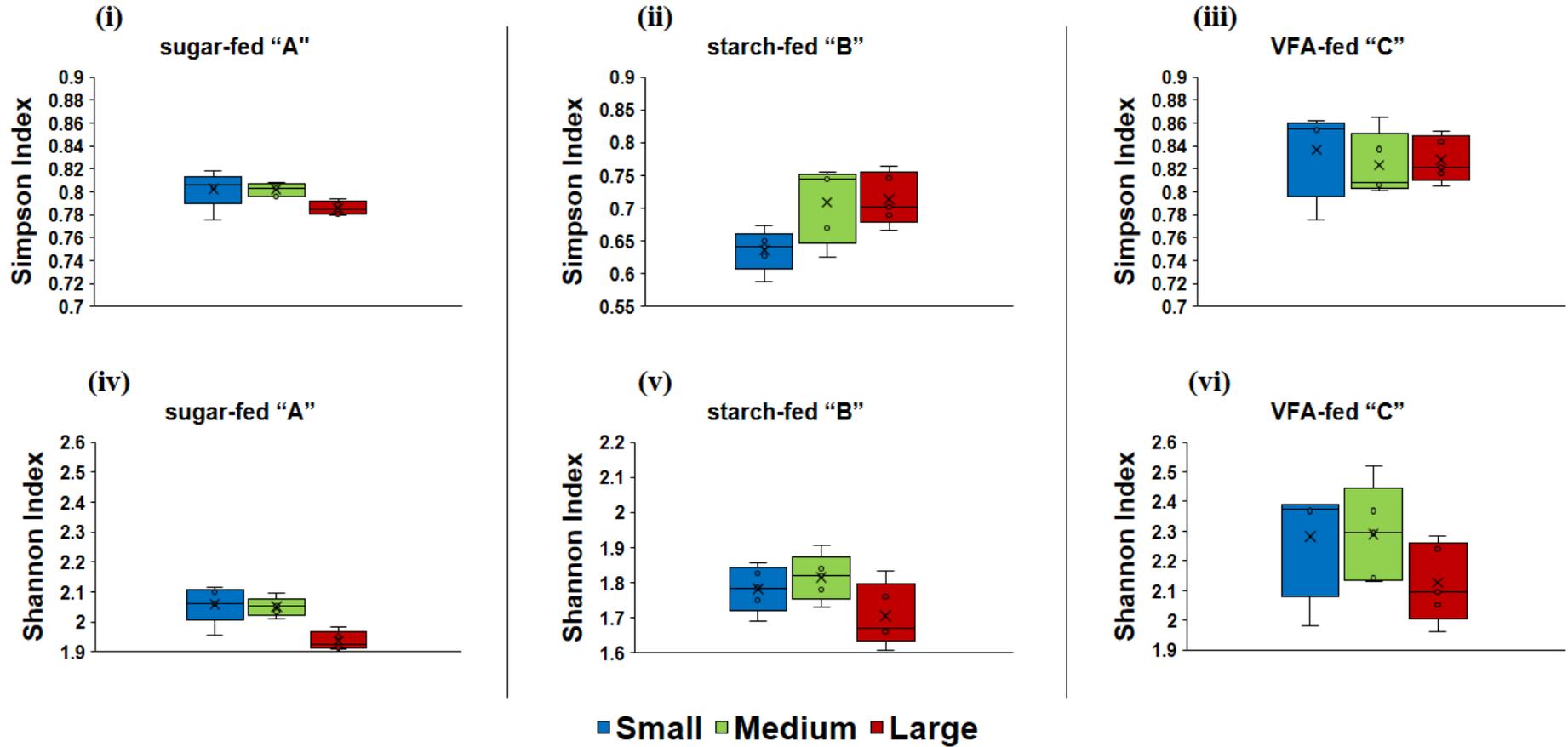


Figure 4.7: Simpson and Shannon indices results for each sample (sugar- (i and iv), starch- (ii and v) and VFA-fed (iii and vi) granular sludge) each at three size distributions (small-blue, medium-green and large-red). The line in each box represents the median.

Focusing on beta diversity among the community structure of the single granule samples, a barplot (Figure 4.8) and a bacterial/archaeal heatmap (Figure 4.9) were generated to illustrate the dominant communities in the various granule types. Sugar-fed (“A”) large granules (shortened to AL in Figures 4.8 and 4.9) had *Methanosaetaceae* (27.4-33.5%), *Anaerolineaceae* (13.6-17.0%), *Methanomicrobiales_incertae_sedis* (4.3-6.3%), *Synergistaceae* (3.3-3.8%) and *Syntrophobacteraceae* (1.7-2.4%) as dominant families, while 27.7-31.6% comprised of unclassified families.

Sugar-fed medium-sized granules (AM) had *Methanosaetaceae* (21.7-33.2%), *Anaerolineaceae* (9.7-16.8%), *Methanomicrobiales_incertae_sedis* (4.7-5.6%), *Synergistaceae* (3.4-4.1%) and *Syntrophobacteraceae* (2.9-4.1%) as well as *Methanobacteriaceae* (2.9-5.8%) and *Syntrophomonadacea* (1.2-2.3%), while 27.4-34.3% comprised of unclassified families. *Methanosaetaceae* (19.7-37.1%), *Anaerolineaceae* (10.6-17.1%), *Methanomicrobiales_incertae_sedis* (3.6-5.5%), *Synergistaceae* (3.8-4.9%), *Methanobacteriaceae* (2.3-5.4%) and *Syntrophobacteraceae* (2.7-3.7%) as well as *Methanosarcinaceae* (1.3-1.7%), as well as unclassified families (25.9-34.7%), were present in sugar-fed small granules (AS).

For the starch-fed (“B”) anaerobic granules, *Anaerolineaceae* (8.8-10.2%), *Methanobacteriaceae* (14.7-18.6%), *Methanomicrobiales_incertae_sedis* (3.6-6.3%), *Methanosaetaceae* (8.4-15.8%), *Syntrophobacteraceae* (2.2-7.2%) and unclassified families (41.2-54.0%) were dominant for its large-sized granules (BL). The same families were dominant for medium-sized granules (BM): *Anaerolineaceae* (6.3-10.4%), *Methanobacteriaceae* (4.6-17.5%), *Methanomicrobiales_incertae_sedis* (2.7-7.1%), *Methanosaetaceae* (6.1-14.4%), *Syntrophobacteraceae* (4.9-8.1%) and unclassified families (42.3-59.7%). Small granules also contained (BS) the same families of *Anaerolineaceae* (5.7-8.6%), *Methanobacteriaceae* (3.0-8.2%), *Methanomicrobiales_incertae_sedis* (2.4-8.4%), *Methanosaetaceae* (3.1-7.7%), *Syntrophobacteraceae* (1.5-7.4%) and unclassified families (55.0-63.2%).

VFA-fed (“C”) large-sized granules (CL) microbial communities revealed the following dominant families were *Anaerolineaceae* (4.9-6.3%),

Methanobacteriaceae (18.7-28.9%), *Methanosaetaceae* (14.1-25.5%), *Propionibacteriaceae* (1.2-8.3%), *Synergistaceae* (1.3-5.8%), *Syntrophaceae* (20.1-26.1%) and unclassified families (55.0-63.2%). The medium-sized granules (CM) had a similar dominant community structure of *Anaerolineaceae* (1.0-1.4%), *Methanobacteriaceae* (23.5-36.8%), *Methanosaetaceae* (6.6-22.8%), *Propionibacteriaceae* (3.9-11.1%), *Synergistaceae* (0.5-2.5%) and unclassified families (15.4-26.0%) with the addition of *Comamonadaceae* (1.4-4.7%) and *Desulfovibrionaceae* (1.6-10.2%). The small granules (CS) included the same families in their DNA community as the medium-sized granules: *Anaerolineaceae* (1.0-5.4%), *Methanobacteriaceae* (14.7-37.6%), *Methanosaetaceae* (12.2-22.0%), *Propionibacteriaceae* (2.6-12.9%), *Synergistaceae* (2.6-3.5%) and unclassified families (16.7-25.8%) with the addition of *Comamonadaceae* (1.4-14.8%) and *Desulfovibrionaceae* (0.4-4.8%).

The percentages in brackets for each family referred to results obtained amongst five replicates for each condition, except for small-sized VFA-fed granules, of which there were four single granules. The heatmap also indicated that sludge sources clustered together and that there was some clustering of granules according to size. The common families which had more than 10% relative abundance across most granules were *Methanobacteriaceae*, *Methanosaetaceae*, *Anaerolineaceae* and unclassified families. A few small and medium-sized granules from the VFA-fed reactor also reported that *Propionibacteriaceae* and *Comamonadaceae* were higher than 10% relative abundance (Figure 4.9).

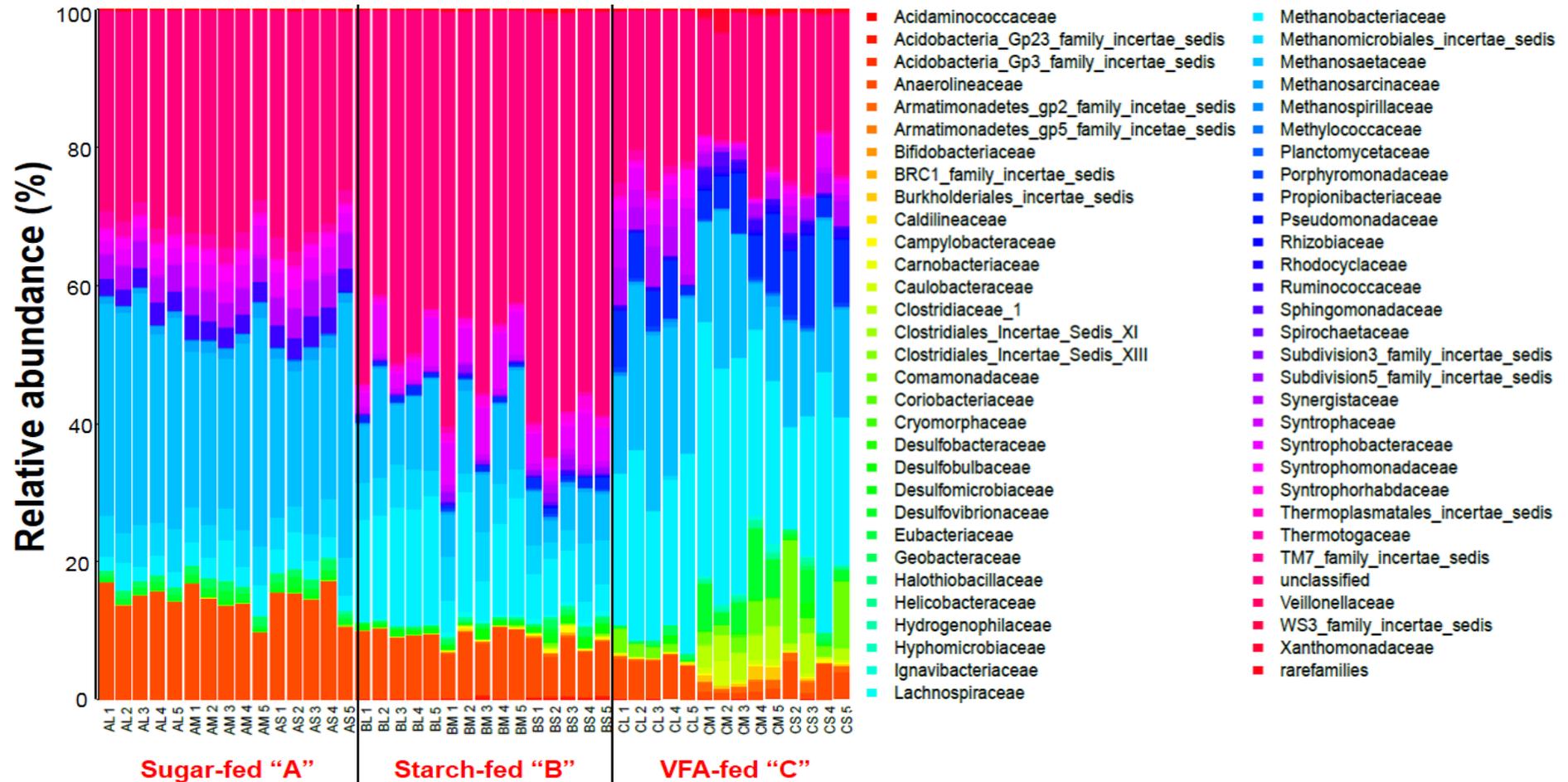


Figure 4.8: Stacked barplots depicting the percentage relative abundances of the 16S DNA community structure of single granules from different sludge sources and sizes. DNA community structure for individual granules from sugar- (“A”), starch- (“B”) and VFA-fed (“C”) bioreactors at granule sizes: small (S), medium (M) and large (L).

The cluster dendrogram (Figure 4.10) represents hierarchical clustering with the percentage approximately unbiased (AU) p-values of each cluster and bootstrap probability (BP) values, which showed that granules were clustered into three groups representing VFA-, sugar- and starch-fed granular sludges.

For sugar-fed sludge granules, the medium- and small-sized granules were more similar to each other than to the large granules. Large-sized VFA-fed granules were also clustered together but the small- and medium-sized granules were further away from each other on the dendrogram than the sugar-fed small- and medium-sized granules. For the starch-fed granules, some medium-sized granules clustered together with large-sized granules, while other medium-sized granules clustered with the small-sized granules on a separate branch. Based on AU p-values, VFA-fed granules were grouped altogether as 100% similar, as were sugar- and starch-fed granules. Sugar- and starch-fed samples were calculated to be 94% similar to each other.

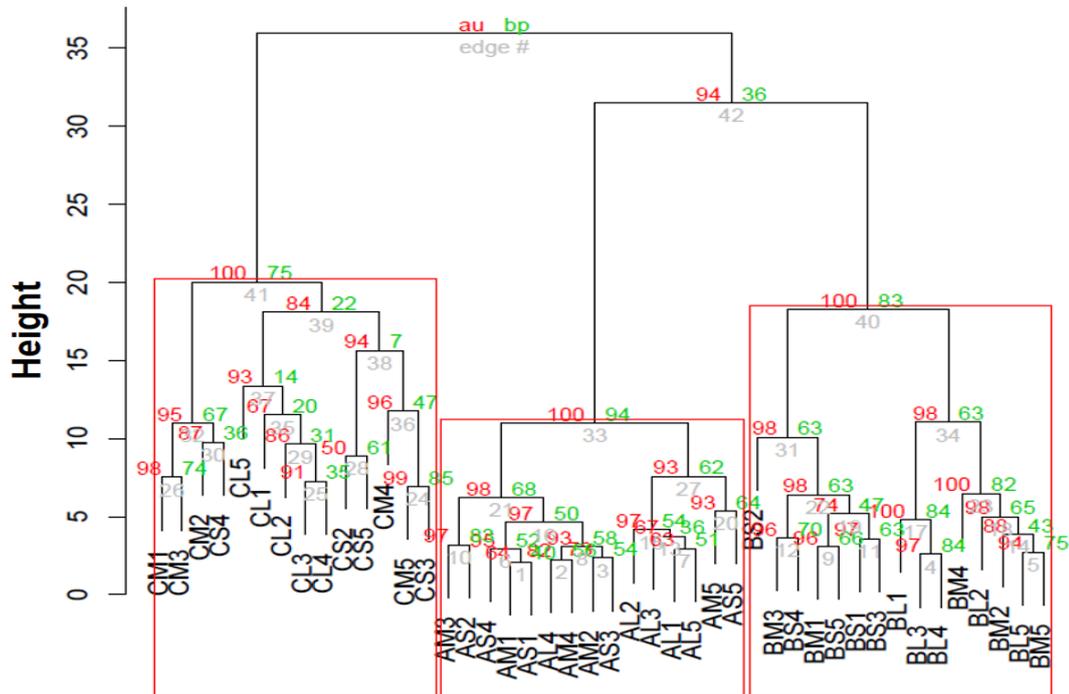


Figure 4.10: Hierarchical clustering with multiscale bootstrap resampling. The dendrogram shows separation of VFA-, sugar- and starch-fed sludges from each other based on Euclidean distances and their corresponding p-values. Labelling was as follows: sugar-fed (A), starch-fed (B) and VFA-fed (C) at granule sizes: small (S), medium (M) and large (L). Values at branches are AU p-values (left), BP values (right), and cluster labels (bottom). Clusters with $AU \geq 95$ are indicated by the rectangles.

The CCA plot displayed the observable relatedness of both granular size (size) and granular type (location) between five replicates of each of the three sizes and types of single granules (Figure 4.11A). Each granular source was distinctly separated from the others, as were the three sizes for VFA-fed and, to a lesser degree, starch-fed granules. All sugar-fed granules were clustered together tightly (Figure 4.11B).

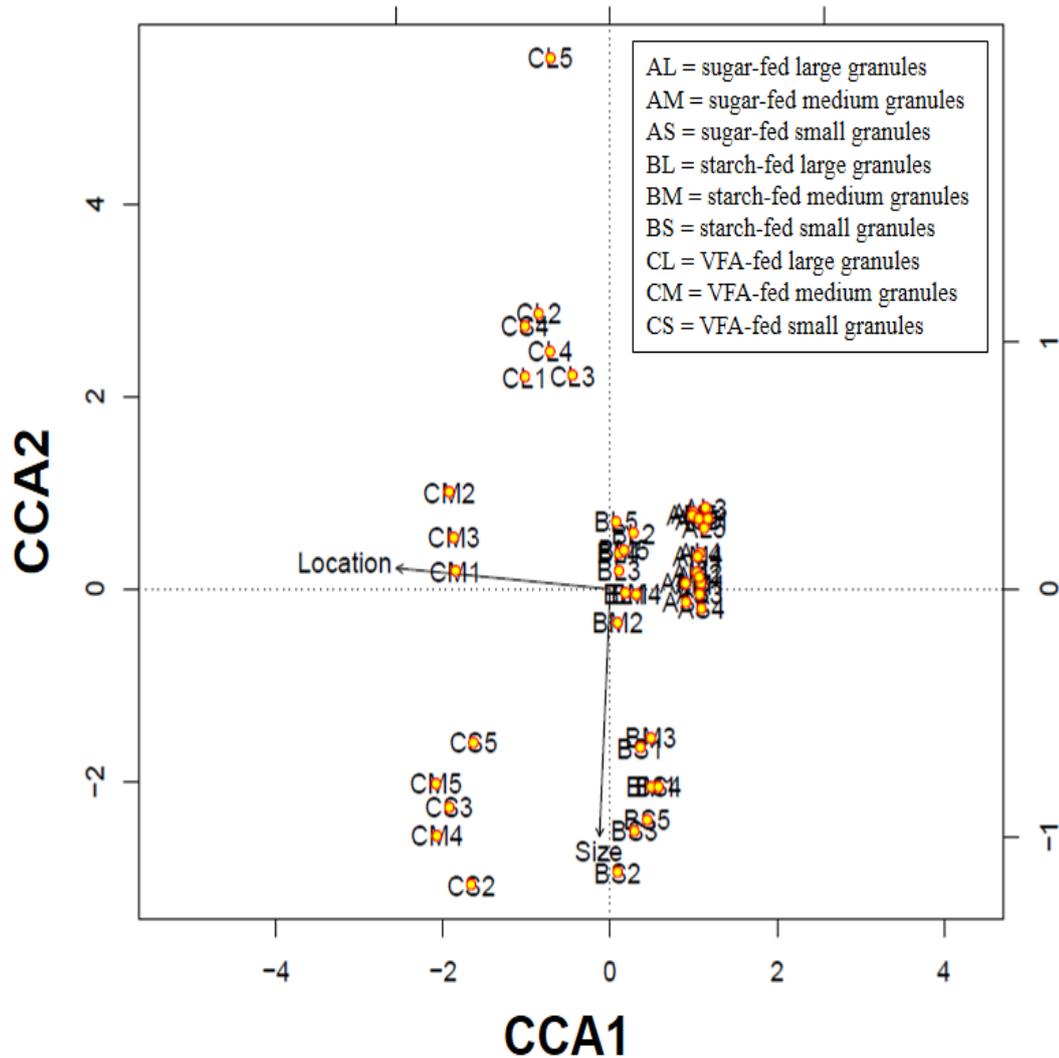


Figure 4.11A: Correspondence analysis (CA) based on size and granule source location. Single granules were separated based on 16S rRNA community structure for sugar-fed, starch-fed and VFA-fed granules at three size fractions. Samples were abbreviated and described in the box in the upper right hand corner of the plot.

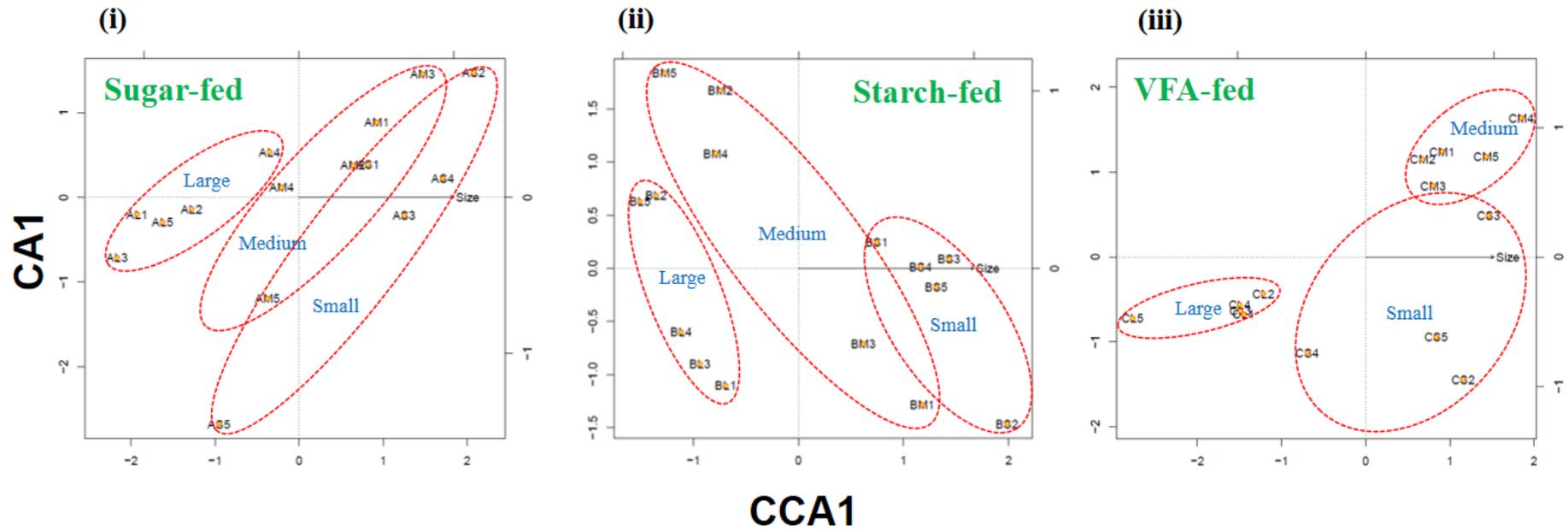


Figure 4.11B: Correspondence analysis (CA) based on granule size for each of the three granular sludges. Granules were separated based on 16S rRNA community structure for (i) sugar-fed granules, (ii) starch-fed granules and (iii) VFA-fed granules. Red dashed circles represent the separation of granules according to size distribution.

The final statistical analysis conducted on these samples was a multivariate ANOVA analyses based on dissimilarities tests (Adonis). The different sizes of individual granules were statistically similar ($p = 0.283$) and the different types of anaerobic sludge were statistically different ($p = 0.005$). These results were according to analyses based on Euclidean distances taking all samples into account. Dividing the samples into separate sets based on sludge type, it was found that only the sugar-fed sludge granules showed no statistically significant difference between the different sizes ($p = 0.214$). For starch-fed and VFA-fed granules, there was a statistically significant difference ($p = 0.015$ and $p = 0.025$, respectively) (Table 4.4 in Conclusions) between the three sizes (Figure 4.11B).

4.4 Discussion

The results suggest that there is a correlation as to the size distribution of granules from the three sludges; there are significant similarities in the proportion of small, medium and large granules. This could indicate that a similar percentage of each granular size fraction may be found because they are sludge-based bioreactors (UASBs in this study) and that this could be true at all time points, considering that the biomass came from reactors that were running for different periods of time. This may be due to similar rates of growth in the “life cycle” of granules corresponding with the three different relative size distributions. Perhaps there would be differences in relative granular size distributions across a larger number of UASBs. A wider UASB study is required in the future.

The results indicate that there is an inverse correlation between the size distribution of granules compared to the average weight of single granules at each size fraction for each of the three sludge sources. Large-sized granules based on their average weight of an individual granule comprised of the smallest proportion based on wet weight and the small-sized granules comprised the largest proportion. This is presumably because the weights of individual small-sized granule are much smaller than the weights of individual large granules. Small granules weigh up to 15 times less than large granules based on individual granule wet weight so it was expected that there would be a higher relative contribution of individual small granules and a lower relative contribution of individual large granules (Figure 4.3). The only

result which was consistent with the size distributions in the first part of the study was that medium-sized granules accounted for approximately one third of the biomass in each bioreactor. This type of size and single granular distribution study has not been found in the literature.

TS and VS results were similar to the granular size contribution results. Statistical differences were reported for the TS and VS concentrations between the three sizes. This could be due to the different amounts of inorganics and organics inside the granules. Small granules had the lowest TS and VS contents, presumably due to having the least amount of microbes. Large granules had the highest TS concentrations but medium granules had the highest VS concentrations. This implied that large granules may have more void spaces inside of the granules and the medium granules may have a higher number of microorganisms per gram of granule.

No statistically significant differences for the granular size contributions (Figure 4.3) and TS results (Table 4.2) were found between the three sources. However, VS concentrations showed significant differences among the three sludge sources. This could be due to different microbial communities emerging and adapting in the different types of wastewater being treated. Generally, VS has been reported to be in the region of 88.9% in wheat straw (Motte et al., 2013), from 90 to 336 mg l⁻¹ for domestic wastewater (Ruiz et al., 1998). Volatile suspended solids have been reported in municipal UASB bioreactors to range from 23 up to 62.3 g l⁻¹ over 150 days of operation (Rizvi et al., 2015) and 76% volatile solids was also reported for municipal waste (Amin and Vriens, 2014). These are considered typical for successfully running UASBs in some of the literature.

SMA profiles revealed higher hydrogenotrophic activity than acetoclastic activity across all granular sizes and types except in large granules from the VFA-fed bioreactor ("C"). Further statistical investigations showed that there was a significant difference between the small- and large-sized granules for both methanogenic activities against acetate and against H₂-CO₂ (Table 4.3). From various granulation theories, microbial models suggest that H₂-consuming organisms were found on the interior of granules and the acetogens and acidogens grow around them in larger granules. This suggests that the smaller granules are

prone to higher levels of hydrogenotrophic methanogenesis. This could also account for the significant difference reported between the small- and large-sized granules because the larger granules may have even more microorganisms that are not present in the small granules than the medium granules do (MacLeod et al., 1990, Hulshoff Pol et al., 1983).

Overall there was more hydrogenotrophic activity in the small and medium granules and visibly more acetoclastic activity in the large granules (Figure 4.4). Hydrogenotrophic activity was up to 48 times higher than acetoclastic activity for the small-sized granules and only up to 6.6 times higher than the medium-sized granules. This was the general pattern found across the three different types of sludge. This may be consistent with the microbial granulation theory where granulation initiates with methanogens like *Methanosaeta concilli*/*Methanotherix soehngeni* forming the granular core and then acetogens, followed by the acidogens and fermenters, forming layers around the hydrogenotrophs becoming more acetogenic in activity in larger granules (Hulshoff Pol, 1989, Thaveesri et al., 1995a).

The SMAs for sugar- (“A”) and starch-fed (“B”) granular sludges were not significantly different to each other when tested against both acetate and H₂-CO₂. This is as expected because “A” and “B” are both sugar-treated bioreactors. VFA-fed (“C”) sludge was significantly different to both sugar-fed and starch-fed sludges when tested against both acetate and H₂-CO₂. This may be because of their differences in feed. Starch is a complex sugar so it would be reasonable to expect that the starch- and sugar-fed sludges would have more similar activities than each would have in comparison to a VFA-fed sludge. However, this is a preliminary finding and further studies involving triplicate reactors of each sludge type need to be studied in order to make more statistically robust conclusions. Moreover, the studies on granules of the three different size fractions do indicate significant differences in each of the physical, physiological and phylogenetic methodologies. These size-related studies are supported by statistical replication between the three granule size fractions.

16S rRNA amplicon Illumina MiSeq analyses was conducted on forty-five single individual granules (Figure 4.2). Rarefaction curves illustrated that all samples

reached close to full saturation, indicating that almost a full picture of potential DNA diversity was achieved.

The highest evenness was present in the VFA-fed granules. This indicated that these granules may be the most capable of responding to new and fluctuating parameters because higher species evenness implies greater functional stability and robustness (Wittebolle et al., 2009). Statistical tests based on evenness, Simpson and Shannon metrics reported that there were significant differences between individual sludge sources, similar to the activity results, presumably due to the differences in community structure between the three bioreactors. However, evenness, Simpson and Shannon tests did not show differences between granular sizes whereas there were differences in the activity results (Figure 4.4) possibly due to variances in the community development of the SMA assay.

Differences of community structure between different types of sludge, such as bioreactors treating various wastewaters from synthetic to agricultural to food processing to petro-chemical effluents, have previously been reported and are expected (Leclerc et al., 2004, Li et al., 2010, Bialek et al., 2012). This contrasts with some of the above-mentioned physical results (size distribution and TS concentrations) and the SMA results of the starch-fed sludge compared to the sugar-fed sludge. These studies reported that sludge type was not significantly different. However, the sludge types were significantly different for VS concentrations and for the phylogenetic study. The barplot and heatmap analyses support the preliminary conclusion that there are observable differences in the DNA community structures between the three sludge types (Figure 4.8). Although there are differences between the three sources, the barplot does show that “A” and “B” have more similar profiles across the most relative abundant microorganisms. Hence, the results imply that a combination of chemical, physiological and ecological data are required when investigating single granules, for example for predictive microbial modelling.

There were some differences in families across the three granular sizes during the sequencing study. For example, there are two additional species in both small and medium VFA-fed granules, *Comamonadaceae* and *Desulfovibrionaceae*, and not in large granules. Additionally, sugar-fed samples found *Methanobacteriaceae* and

Syntrophomonadacea present in small- and medium-sized granules and *Methanosarcinaceae* was reported in small-sized granules. These results indicate that specific families are present in small, medium and large granules. Active community analyses can provide more of an insight (Chapters 5 and 6).

The heatmap did show better clustering between “A” and “C” than “A” and “B”. This was unexpected as “A” and “B” are both sugar-fed bioreactors (Figure 4.9). However, in the hierarchical clustering dendrogram (Figure 4.10) the expected results of “A” and “B” clustering better was observed. Perhaps it is because of the different statistical testing that generates both sets of data – ANOVA for heatmaps and Euclidean distance analysis for the cluster dendrogram. Overall, the three sources are statistically different from each other.

Moreover, clustering analyses (Figure 4.10) illustrates some differences in granule size on separate branches, implying that there is some species differentiation. The reason sugar- and starch-fed samples were more similar to each other in the cluster dendrogram could be that they were fed with starch and sugary wastewaters, which have similar hydrolytic and fermentative degradation pathways, whereas the substrate mixture in VFA-fed anaerobic sludge was methanogenic in nature. CCA illustrated that samples from the three anaerobic sludge biomass sources were located in unique specific areas of the plot. The quintuplet granule community structures were close together (Figure 4.11A). Granules taken from the VFA- and starch-fed sludges also separated according to granule size (Figure 4.11B (B and C)) but sugar-fed sludge samples were close together in the plot (Figure 4.11B (A)).

Adonis analysis was conducted on all samples together and the results were consistent with the above α diversity conclusions that there were significant differences between the types of anaerobic sludge but no significant difference was measured between granular sizes. The samples were subsequently separated and the same results were reported for sugar-fed granules, but the statistics revealed significant differences across the three sizes in both starch- and VFA-fed granules. This is consistent with the results extrapolated from the CCA analysis and the SMA data. These results imply that the microbial communities are heterogenous between differently-sized granules, as hypothesised at the outset but more single-granule studies using a larger panel of different granular sludges are necessary.

As granules, comprised of microbial communities, grow, they increase in size and, once they come under stress or die, they can either shrink or break apart without any appendages to hold them together (Figure 4.12). Small-sized granules could be this type of granule until they start growing again and become larger-sized granules. This indicates that individual granules could contain whole niches in their spherical biofilm and undergo a form of ‘life cycle’ during their growth and death phases.

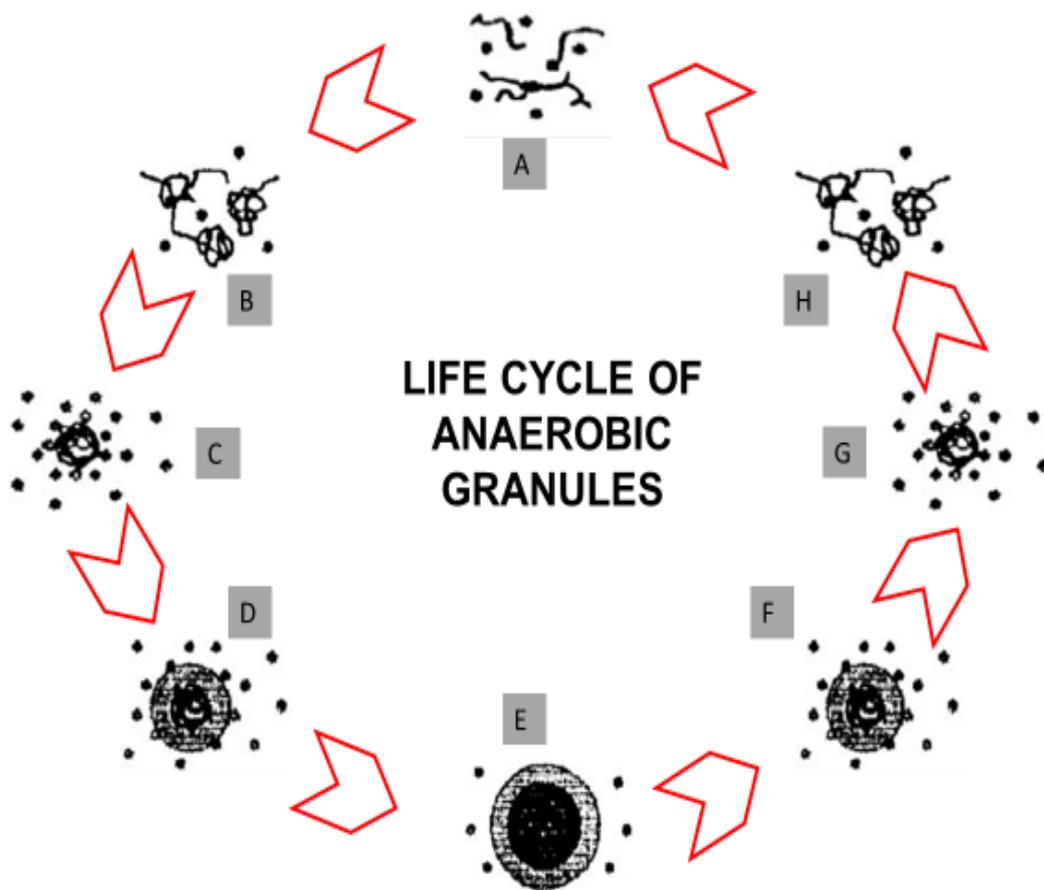


Figure 4.12: Proposed life cycle of single anaerobic granules; (A) filamentous methanogenic growth, (B) bridging of filaments, (C) formation of a core with syntrophic acetogens surrounding it, (D) growth of small granule with syntrophic hydrogenotrophs and acidogens around its core, (E) growth of large granule composed of multilayer groups of microbes, (F) breakdown/shrinkage of granule, (G) possible disintegration and breaking off of hydrogenotrophs and acidogens and (H) breakdown to smaller pieces of granules. Adapted from (Ahn, 2000) and proposed breakdown/disintegration of granule into “smaller granules” (van Lier et al., 1990, Grotenhuis, 1992, Subramanyam, 2013).

4.5 Conclusions

One of the preliminary findings of this work was that there may be generally consistent proportions of each granule size in the UASB bioreactors studied in that they followed a similar pattern. However a larger number of granular-based bioreactors are required to be sampled, such as EGSBs, EGSBs with Internal Circulation® (IC) systems, anaerobic filter (AF) reactors and anaerobic membrane (AnMBR) reactors, (de Man et al., 1988, van der Last and Lettinga, 1992, McHugh et al., 2004, Collins et al., 2005, Feng et al., 2008, Smith et al., 2013), to draw more statistically significant conclusions regarding granular size proportions across anaerobic bioreactors. In general, granules of three different sizes and from three sources found that their physical attributes (size distribution, TS and VS) were significantly different between the three sizes and were statistically similar in terms of size distribution, except when VS concentrations were measured (Table 4.4).

SMA assays indicated a methanogenic preference for H₂-CO₂ at smaller granular sizes and a higher acetoclastic activity profile across the three sludges in larger-sized granules. The total methanogenic activity (against H₂-CO₂ and acetate) was approximately similar across the various size fractions, but hydrogenotrophic activity was noticeably higher for smaller granules and the acetoclastic activity increased as the size of the granules tested increased (Figure 4.4). There was a consistent preference for H₂-CO₂.

Overall, physical (size distributions, TS and VS concentrations) and phylogenetic tests (SMAs) indicated that there were significant differences between granule sizes. Statistical analyses also demonstrated that there were significant differences among the active microbial communities of the three size fractions for VFA-fed and starch-fed sludges, but not for the sugar-fed single granules. VS content, SMAs and active community structure data (alpha and beta diversity tests) also reported significant differences between sludge types. Because definitive conclusions cannot be drawn by solely looking at physical or physiological granular sludge data or community-based datasets for individual granules, the results from this study (Table 4.4) suggest that both chemical and community composition need to be examined to obtain a thorough analysis of granular samples taken from bioreactors.

Table 4.4 Summary of statistical significant comparisons between sludge types and granular sizes across the whole study. Level of significance was set at $p < 0.05$. Size distribution refers to the relative proportions of size fractions in the wet weight study (Figure 4.3 (i)).

Type	Size/Feed	Size Distribution	TS/ VS	SMA- H ₂ -CO ₂	SMA- acetate	DNA community structure	
'A'	small medium large	}	0.000	0.036/ 0.013	0.017	0.003	0.214
'B'	small medium large						
'C'	small medium large						
'A'	sugar	}	0.997	0.054/ 0.050	0.000	0.000	0.005
'B'	starch						
'C'	VFA						

Further analyses into the active community structure of single individual granules is required such as comprehensive 'omics studies to pinpoint exact differences in granules at different sizes or stages of a granule's "life cycle".

4.6 References

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Chapter 5

Chapter 5 - Physiological characterisation of size-resolved anaerobic granules and phylogenetic investigation of replication between individual granules

Abstract

Anaerobic granules are biofilms that are discovered within certain configurations of anaerobic bioreactors. They are small spherical biofilms comprising many different microbes and hence have been referred to as “micro-ecosystems” within a bioreactor’s “macro-ecosystem”.

Previous work (Chapter 4) reported specific methanogenic activity (SMA) assays using large (1.4 - 2.0 mm), medium (0.8 mm - 1.4 mm) and small (0.4 – 0.8 mm) granules against key substrates. The complete SMA work found that large granules were the most active overall. The activity of large granules against acetate, H₂-CO₂, propionate, butyrate and a volatile fatty acids (VFA) mixture was measured as 125.84, 67.3, 197.84, 34.23 and 254.42 ml CH₄/g VSS/day, respectively. VFA concentrations were fully consumed after 48 hours. Total and volatile solids of one hundred individual large granules, as well as cDNA sequencing targeting 16S rRNA from sixteen large granules, indicated that granules can be considered as distinct, replicated, “whole ecosystems”.

DNA extracted from individual granules was compared against cDNA community structures of a specific size fraction and from the same sludge source. Results from 16S rRNA MiSeq analysis indicated that there were significant differences between the potential (DNA) and active (cDNA) community structures. *Methanosaetaceae* was the most abundant family common to both and is regarded as a key player in granular activity and metabolism.

5.1 Introduction

Previous studies have proven that anaerobic granules can contain microorganisms from each trophic group along the anaerobic digestion (AD) pathway: hydrolyzers, acidogens, acetogens and methanogens. This indicates that each granule may be regarded as a whole niche community.

AD is ubiquitous in nature and there is a variety of anaerobic ecosystems, from soils to lakes and animal rumen. Sampling these types of physically vast communities is challenging because it is difficult to conclusively say whether any one sample is truly representative, since most samples usually have varying community structures to some degree. Granules comprise a whole community of microbes from each stage of AD, implying that granules can be thought of as whole ecosystems within a type of “meta-ecosystem”.

Building on the results in Chapter 4, one of the three sludges was chosen for more in-depth analysis into phylogenetics based on granular size, followed by a detailed investigation into whether single individual granules of a specific size from a specific biomass source contain the same active physical traits and community structure.

Anaerobic granular sludge of three different sizes from the VFA-fed reactor granular sludge (‘C’) investigated in Chapter 4 was subjected to more detailed physiological examination. This specific anaerobic granular sludge was initially selected because previous DNA community structure analysis reported significant differences in three granular sizes (Chapter 4 section 4.3.4). Activity tests (Colleran et al., 1992) against a wider panel of methanogenic substrates were employed to determine whether anaerobic granular size was an influential factor in relation to methanogenic activity. Methanogenesis is paramount for biogas production in AD.

In parallel with the SMAs, general methanogenic assays were set up with a VFA mixture feed along with additional sacrificial samples for monitoring fermentation of sugars, such as cellulose and glucose, and to profile VFA concentrations throughout the AD process. Cellulose is the most abundant polysaccharide within organic waste (Khan and Trottier, 1978) and breaks down into cellobiose and then

glucose. The methanogenic assays were also employed as a prerequisite test for determining the length of time it takes for granules to utilise each of the substrates.

Subsequently, total solids (TS) and volatile solids (VS) of single individual granules were determined to investigate the range and distribution of these results amongst 100 batches of 10 single granules. This will help to understand whether individual granules have the same physical characteristics using these specific assays.

The cDNA of sixteen individual granules of one specific size fraction were sequenced using the Illumina MiSeq platform in order to further the physical investigations of single granules and physiological studies of three granule size fractions. RNA-targeted analyses were expected to be more responsive than DNA because metabolically-active cells usually contain more 16S rRNA than quiescent cells (Nomura et al., 1984, Poulsen et al., 1993, Aviv et al., 1996), and several studies have chosen RNA-based approaches to more accurately represent population dynamics at specific sampling points (Frias-Lopez et al., 2008) in AD microbial communities (Akuzawa et al., 2011, Ito et al., 2012, Zakrzewski et al., 2012). It is noteworthy that there have been suggestions that RNA-based and DNA-based approaches are both subject to the same biases (Norris et al., 2002b, Griffiths et al., 2003a, Griffiths et al., 2003b).

Lastly, if the active communities are concluded to be replicates, five random cDNA samples would be directly compared to the five DNA granule counterparts from the VFA-fed anaerobic sludge at the “large” (1.2 – 2.0 mm) fraction size.

The hypotheses for this study were that

1. methanogenic activity against an extended panel of substrates from Chapter 4 is significantly different between three granular size fractions;
2. the physical characteristics of 100 single granules are replicated; and
3. single individual anaerobic granules are entire “whole ecosystems” based on their active community structure pattern
4. there are significant differences between the DNA and cDNA microbial community structures of individual granules.

The overall aim was to ascertain whether the active microbial community was replicated across individual anaerobic granules and, if so, to compare the active and total community structure.

5.2 Materials and Methods

5.2.1 Source of biomass and granular sludge characteristics

As described previously in Chapter 4 (section 4.2.1), anaerobic granular biomass was obtained from a lab-scale upflow anaerobic sludge blanket (UASB) using a VFA-fed synthetic wastewater for 112 days. The reactor was operated by Robert Dillon.

5.2.2 Specific Methanogenic Activity (SMA) and Methanogenic Activity (MA) Assays

This assay was performed as described previously in Chapter 4 (section 4.2.2) but with additional substrates, propionate and butyrate. SMA assays against acetate, H_2 - CO_2 (both shown previously in Chapter 4), propionate and butyrate were performed for small- (0.4 – 0.8 mm), medium- (0.8 mm – 1.2 mm) and large-sized (1.2 mm – 2.0 mm) granules as well as a sample of granular sludge containing a random ratio of small, medium and large-sized granules (termed “mix”).

In parallel, a more general MA assay was set up using a VFA mixture as the substrate for each of the three granule sizes. The VFA mixture was identical to the feed used in the lab-scale UASB reactor (section 5.2.1). The VFA recipe consisted of acetic acid (15.6 mM), propionic acid (8.9 mM), butyric acid (6.3 mM) and ethanol (10.4 mM). Both SMAs and MAs were measured in $ml\ CH_4\ g\ VSS^{-1}\ day^{-1}$.

5.2.3 Sugar and volatile fatty acid profiling during the MA assay

Sugar concentration was measured at seven time intervals (0, 6, 12, 24, 36, 48 and 60 hours) over the duration of the MA assay against the VFA mixture (as described

in 5.2.2 above) in order to examine what happens to sugars during the methanogenic assay throughout the 60 hour assay as they are involved in fermentation. At each time interval, technical triplicates were measured from the sacrificial vials for each granular size. The degradation rates were measured via the slopes of the lines and results were measured in mg/l/hr.

During the MA assay of small-, medium- and large-sized granules sacrificial samples were taken periodically for sugar monitoring and VFA profiling. The rate of sugar degradation was measured to analyse consumption of the sugars present in the granules from the lab-scale reactor. Before measuring both sugar and VFA concentrations, the granules were briefly sonicated to release any residual or “trapped” chemicals inside the biofilm that were being consumed or produced by the innermost species community (Salsabil et al., 2009, Gunnigle et al., 2013). Granules were resuspended with 1% phosphate-buffered saline and were disrupted by sonication (Soniprep 150 Ultrasonic disintegrator, MSE, UK) at 50% amplitude for two pulse sessions of 30 s on ice. Intervals of 30 s were applied to prevent thermal damage of cellular proteins.

Soluble sugars were monitored according to the Dubois method (Dubois et al., 1951). In brief, each sample was diluted to 200 mg/l. A glucose calibration curve was generated in parallel. The range of glucose concentrations measured were 200 mg/l, 100 mg/l, 50 mg/l, 25 mg/l, 10 mg/l and a distilled H₂O “blank”. 5% v/v phenol was added to the standards and the samples. Each tube was vortexed and incubated at 4°C for 15 min. Subsequently, H₂SO₄ was pipetted into each tube, which was vortexed and incubated at 100°C for 5 min. The tubes were cooled to room temperature over 30 min, and all samples were transferred to a 96-well plate. Absorbance readings were taken at 490 nm using a spectrophotometer (LT-5000MS ELISA Reader coupled with Manta PC software).

In parallel with sugar monitoring, samples were taken at the same seven time intervals for VFA profiling. Three technical VFA samples were determined as described previously (Chapter 3, section 3.2.3).

5.2.4 Characterisation of individual granules

TS and VS were measured for one hundred batches of ten individual granules from the large size fraction as one single individual granule was below the level of detection of the analytical balance. Calculations were performed using Standard Methods (APHA, 1998) and described in Chapter 4.

5.2.5 DNA/RNA co-extraction and cDNA synthesis

The DNA/RNA co-extraction method was described previously in Chapter 4 (section 4.2.4). cDNA was generated from the frozen nucleic acids through several steps. RNA was purified via DNase treatment and removal according to the manufacturer's instructions on the TurboDNase free kit (AMBION- Invitrogen by Thermo Fisher Scientific). DNA removal was verified by 16S rRNA PCR, using a range of sample RNA dilutions. The PCR assay was performed as previously described in Chapter 3 (section 3.2.5) using the primer pair 338f and 805r. The final step was cDNA synthesis. The master mix used to accomplish cDNA synthesis included MgCl₂ (50 mM), Random Primer Mix (60 µM) (BioLabs) and dNTPs (10 mM). M-MuLV reverse transcriptase (BioLabs) was the enzyme used to catalyse the synthesis. cDNA sample concentrations were determined using the Qubit fluorometer (Invitrogen), as per the manufacturer's instructions.

5.2.6 Illumina MiSeq Analysis

Illumina 16S MiSeq sequencing was carried out on sixteen single granules from the large size fraction to determine if they had replicated active communities within the established UASB lab-scale reactor (section 5.2.1). cDNA samples of sixteen individual granules were sent for 16S rRNA amplicon Illumina MiSeq sequencing. The sequences were returned from the Research and Testing Laboratory (Lubbock, Texas), where the 16S rRNA gene sequences were amplified using the universal bacterial/archaeal forward primer 515f and the reverse primer 806r on the Illumina MiSeq platform. Mothur analysis was conducted as described previously in Chapter 4 (section 4.2.4). Analysis similar to the MiSeq analysis described previously in Chapter 4 (section 4.3.3) was carried out.

Total and active community structures between granules from the large size fraction of the same anaerobic sludge source were compared. A computer randomiser program (random.org) was used to pick out five unbiased samples from the sixteen granules whose cDNA was sequenced. The samples that were chosen by the computer were seed granules: cDNA_12, cDNA_5, cDNA_10, cDNA_2 and cDNA_14.

Alpha and beta analysis was performed on the DNA samples from chapter 4: CL (VFA-fed; large) 1, CL 2, CL 3, CL 4 and CL 5. They were renamed DNA_1, DNA_2, DNA_3, DNA_4 and DNA_5 and their community structures were compared to VFA-fed large cDNA samples: cDNA_12, cDNA_5, cDNA_10, cDNA_2 and cDNA_14.

5.2.7 Statistical Analysis

Statistical tests and data visualisation were performed using SPSS software and the Vegan package (version 2.4-1) platform within R Studio (version 3.3.2) (Team, 2016) as described previously in Chapter 4 (section 4.2.5). Analysis of variance (ANOVA) significance analysis and Kruskal Wallis tests determined whether differences were present between granular sizes in SMAs and between individual granules in the various phylogenetic studies. Alpha and beta diversities were examined as well as multivariate ANOVA based on dissimilarities (Adonis) analysis. Statistical significance was determined to occur where there was a p-value of less than 0.05. The single granule active community structures were subsampled to an even depth of 32,000 reads per sample.

5.3 Results

5.3.1 Effect of granular size on methanogenic activity

Both SMA and MA assays were carried out for granules at three size fractions against acetate, H₂-CO₂, propionate, butyrate and a VFA mixture in triplicate. The results demonstrated that the general MA assay had the highest activity across each

size distribution for the “C” VFA-fed anaerobic granules (Figure 5.1). Small- and medium-sized granules exhibited the same pattern for each substrate: The highest activity was against the VFA mixture, followed by H₂-CO₂, then propionate, followed by acetate and, lastly, butyrate. There was no distinguishable pattern for the large granules and the mixture of granules had quite low activity for propionate in comparison to the separated granules. VFA activity was the highest across the different size fractions in comparison to the other substrates.

Two-way ANOVA statistical analyses based on substrate type reported that activity against acetate was significantly different to H₂-CO₂ ($p = 0.003$), butyrate ($p = 0.000$) and VFA mixture ($p = 0.000$), but that there was no statistically significant difference between acetate and propionate ($p = 0.137$). Activity against H₂-CO₂ was statistically different to all substrates except propionate ($p = 0.553$). Activity against butyrate activity was statistically different to each other substrate ($p = 0.000$). Activity against propionate was statistically different to both butyrate and the VFA mixture to a statistically significant degree ($p = 0.000$ in both tests). The VFA mixture activity was statistically different from each substrate ($p = 0.000$). The only statistical similarities based on SMAs were between propionate and acetate and between propionate and H₂-CO₂ (Figure 5.2).

By examining the SMA results based on size, two-way ANOVA tests results showed that large granules were statistically different to both small ($p = 0.047$) and mixed ($p = 0.001$) granules. Medium granules were statistically similar to all granule sizes ($p = 0.052$ to 0.807). Small granules were statistically different from large granules ($p = 0.077$ as shown before). Mixed granules were statistically different from large granules ($p = 0.001$). In summary, there was a gradient pattern where large granules were statistically similar to medium granules ($p = 0.0395$) and medium were similar to small granules ($p = 0.807$). Both small- and medium-sized granules were statistically similar to mixed granules ($p = 0.305$ and 0.052 , respectively) (Figure 5.2).

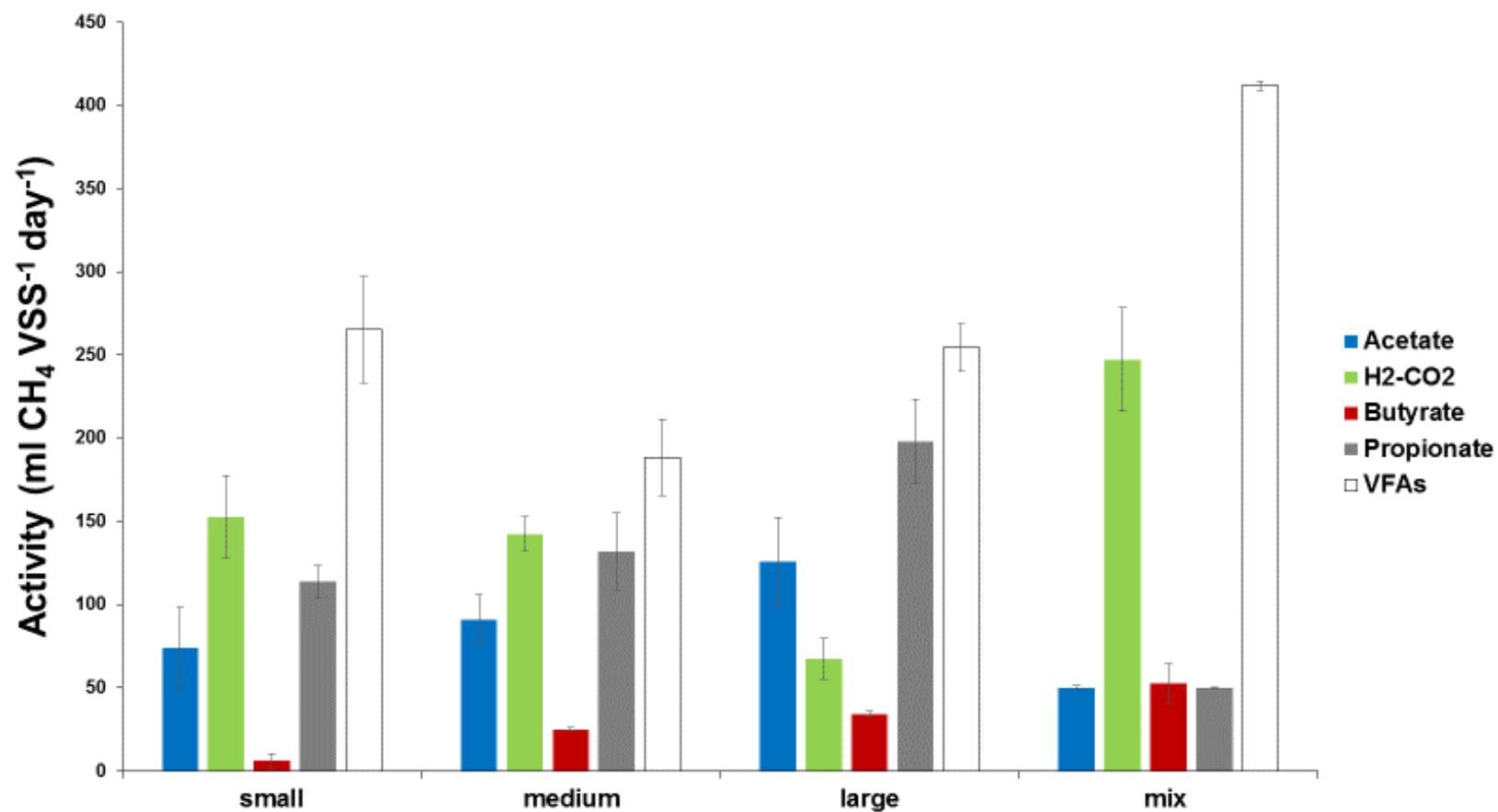


Figure 5.1: Methanogenic activities among three different size fractions. SMA assays against acetate, H₂-CO₂, butyrate and propionate and an MA assay against a mixture of VFAs were grouped together according to small granules, medium granules, large granules and a mix of granules. Error bars were based on triplicate assays.

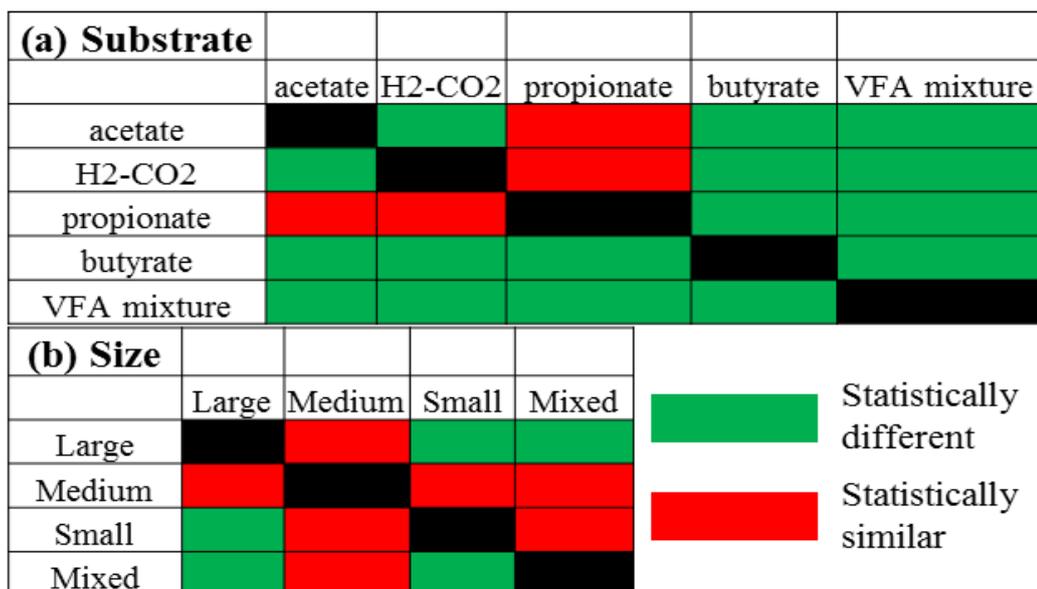


Figure 5.2: Heatmaps showing statistical significances between (a) methanogenic substrates and (b) granular sizes based on activity assays. Green boxes represent statistical differences and red boxes represent statistical similarities. Black boxes represent no statistical test.

5.3.2 Sugar monitoring among granular size fractions during the MA assay

Sugar degradation in granules resulted in the near-complete consumption of sugars by hour 48 of monitoring (Figure 5.3). The results showed that sugars were consumed the fastest in large-sized granules at $1.98 \text{ mg l}^{-1} \text{ h}^{-1}$, compared to the medium-sized granules, which had a fermentative depletion rate of $1.86 \text{ mg}^{-1} \text{ l}^{-1} \text{ h}$. Small-sized granules had the lowest consumption rate, $1.77 \text{ mg}^{-1} \text{ l}^{-1} \text{ h}$.

A one-way ANOVA test was carried out for the triplicate sugar depletion rates for the three granular sizes. The results showed that there was no statistical difference in sugar consumption rates between any of the sizes ($p = 0.257$).

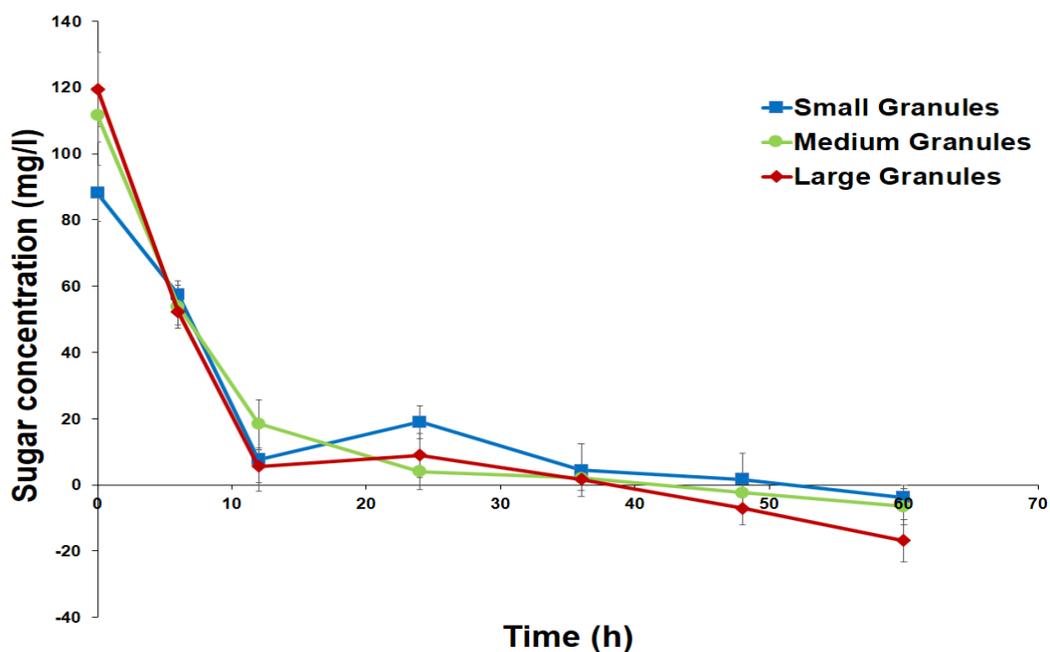


Figure 5.3: Average glucose concentrations (mg/l) at seven time points over 60 hours of the methanogenic assay. Each line refers to granule size: blue squares represent small granules, green circles represent medium granules and red triangles represent large sized. Error bars were based on triplicate sampling.

5.3.3 VFA dynamics in three granular size fractions during the MA assay

The VFAs under examination were acetic acid, propionic acid and butyric acid. The results showed that acetic acid and propionic acid had been completely consumed by the 48th hour of the MA assays (Figure 5.4 (A to D)). Butyric acid has also been fully consumed by the mixed and large granules by the 48th hour in both cases, but was still present in small and medium granules.

Before time point 24 hours, there was a similar pattern for each of the three granule sizes and the mixed granules. VFAs accumulated between time points 6 hours and 12 hours. From 12 hours until 60 hours, when the methanogenic assay was completed, there was a steady decline of all VFAs.

Statistical tests showed that acetic acid concentration profiles were significantly similar between the three granular sizes and the mixed granules ($p = 0.088$) and were significantly different between the six time points ($p = 0.000$).

The propionic acid profiles of the mixed, small, medium and large granules reported significant similarities ($p = 0.757$) between the granular sizes. There were significant differences between all time-points ($p = 0.000$).

For butyric acid profiles, significant differences were observed between the mixed granules and the three sizes of granules ($p = 0.028$) as well as between the six time points ($p = 0.000$), where significance was set at $p\text{-value} < 0.05$.

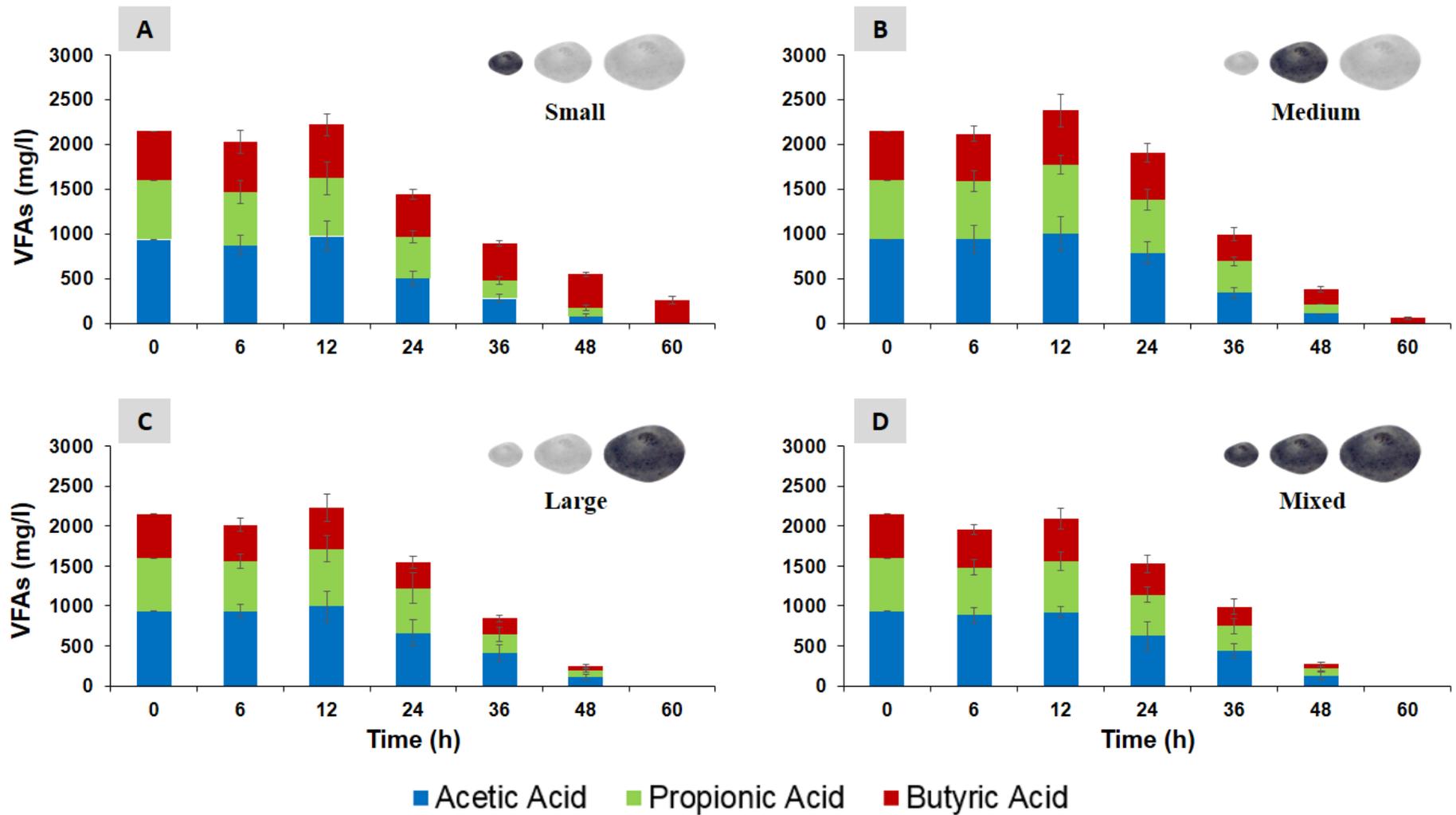


Figure 5.4: VFA profiles of (A) small, (B) medium, (C) large and (D) mixed granules at seven time intervals over 48 hours during the MA assay. Error bars were based on technical triplicate sampling.

5.3.4 Physical characterisation of large-size individual granules

One hundred batches of ten large-sized granules were collected and physical analysis was performed (section 5.2.4). The range of TS concentrations was 16 to 105 mg/l. Over three quarters of the results were between 30 and 70 mg/l. VS concentrations of single granules had a minimum of 3 mg/l and a maximum of 61 mg/l with the majority (over 80%) being between 21 and 46 mg/l. The range of VS contents was 8.1% to 94.29% of the TS concentrations. 87 of the 100 granules had VS values of above 50% and 73 granules were above 60%.

Average TS concentrations, VS concentrations and VS percentages were 50 mg/l, 32 mg/l (Figure 5.5) and 63.08% respectively. The organics (VS) result was approximately 63.07% of the TS concentrations found within single individual granules. Standard deviation values, as depicted by the error bars for TS and VS values, were 20.17 and 11.99 respectively (Figure 5.5).

Both the TS concentrations and the VS concentrations were statistically similar between the averages of one hundred batches of ten single granules (TS p-value = 0.457 and VS p-value = 0.635).

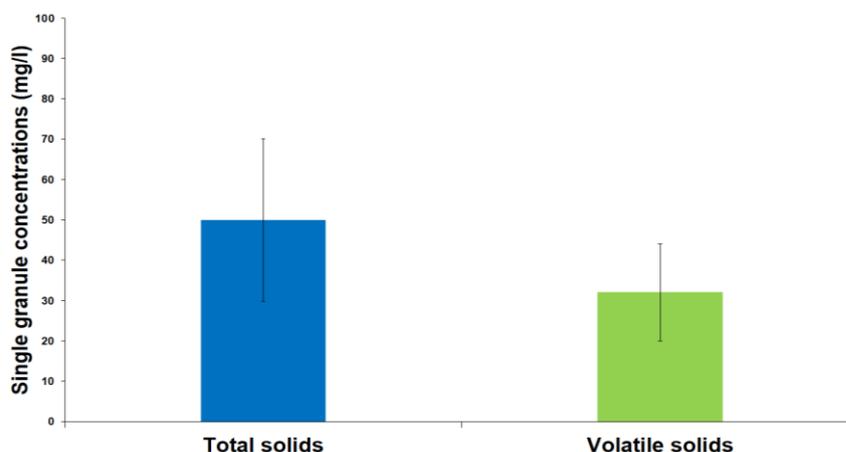


Figure 5.5: Average TS and VS concentrations in one hundred batches of ten single granules. Error bars were based on standard deviation of the one hundred tests.

5.3.5 Microbial community structure of large-size single granules

Rarefaction curves for the sixteen individual granules highlighted adequate saturation of the community diversity and the presence of between thirty and at least sixty-five families amongst thirty-two thousand sequences (Figure 5.6).

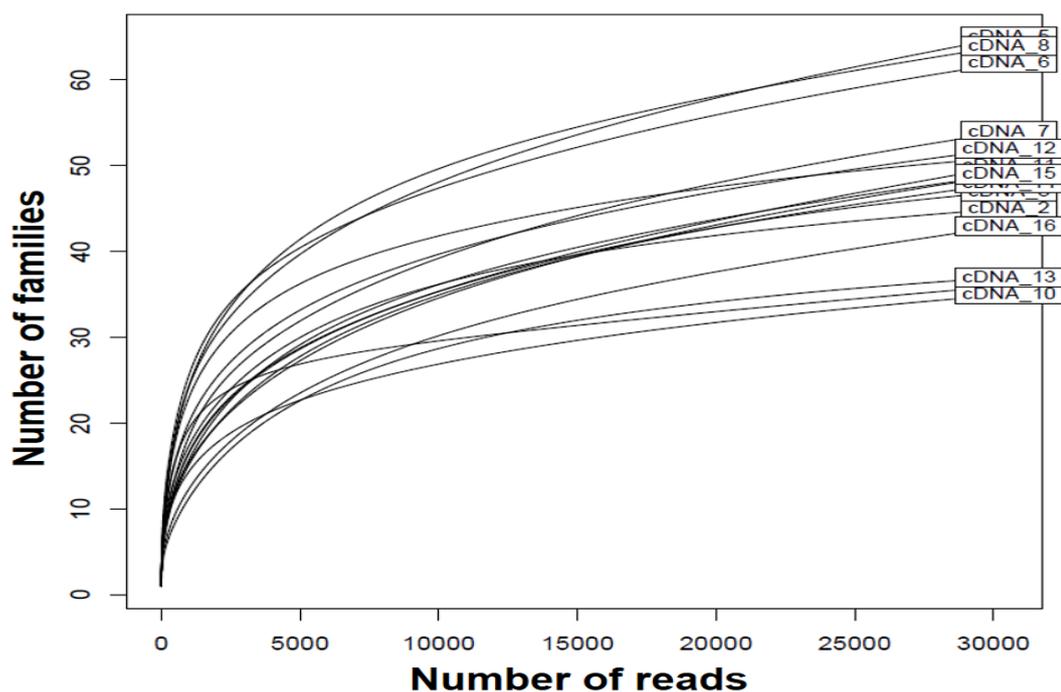


Figure 5.6: Rarefaction curves of all sixteen individual single granules taken from the lab-scale (seed) UASB reactor as described in section 5.2.1 (cDNA_1 to cDNA_16 granules).

Evenness for the sixteen granules were calculated and the standard deviation for the evenness data was 0.075 (Figure 5.7). The evenness results for the active community (0.33 to 0.43) were lower than those shown for the total community in Chapter 4 (Figure 4.6 (C-large granules)), i.e. between 0.52 and 0.58. The evenness between sixteen granules are statistically similar to each other ($p = 0.728$).

The other alpha diversity tests included both Simpson and Shannon indices tests (Figure 5.7). They illustrated high diversity among the sixteen samples. The maximum Simpson diversity was 0.746 and the standard deviation was 0.116. The maximum Shannon diversity was 1.723 and the standard deviation was 0.26. Statistical tests showed statistical similarities between the sixteen granules for both the Simpson index ($p = 0.686$) and for the Shannon index ($p = 0.803$).

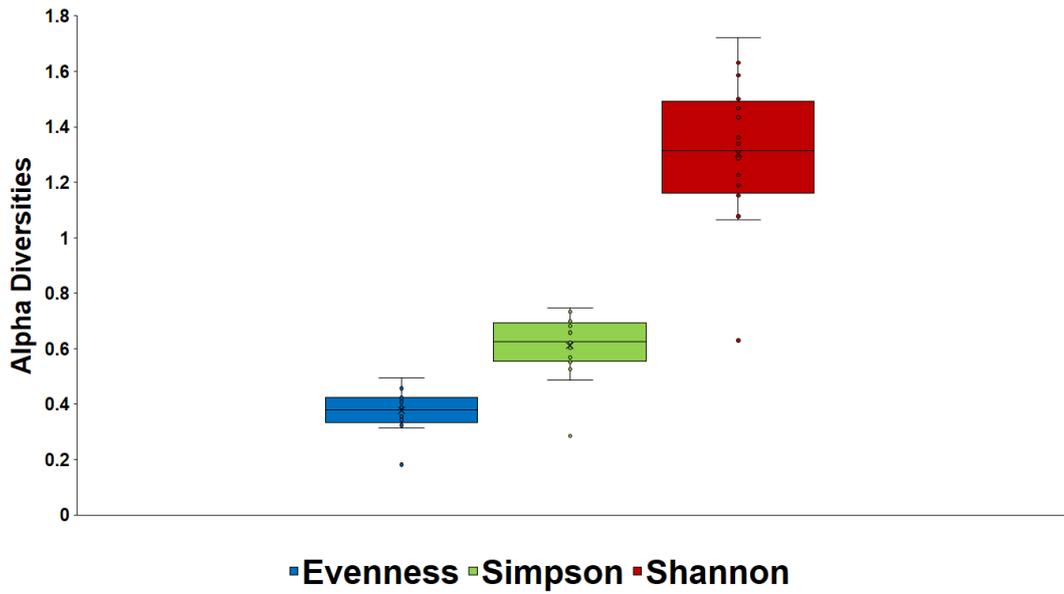


Figure 5.7: Alpha diversity calculations for Evenness (blue), Simpson (green) and Shannon (red) indices of the sixteen individual single granules from the large-size fraction. The small circles refer to outliers among the granules

A bar plot illustrating the cDNA community of sixteen granules was generated, and it was observed that the dominant families in each of the sixteen granules were *Anaerolineaceae* (0.4 - 4.6%), *Methanobacteriaceae* (10.9 - 38.0%), *Methanosaetaceae* (21.9 - 83.8%), *Syntrophaceae* (1.3 - 14.2%) and unclassified families (2.6 - 24.0) (Figure 5.8).

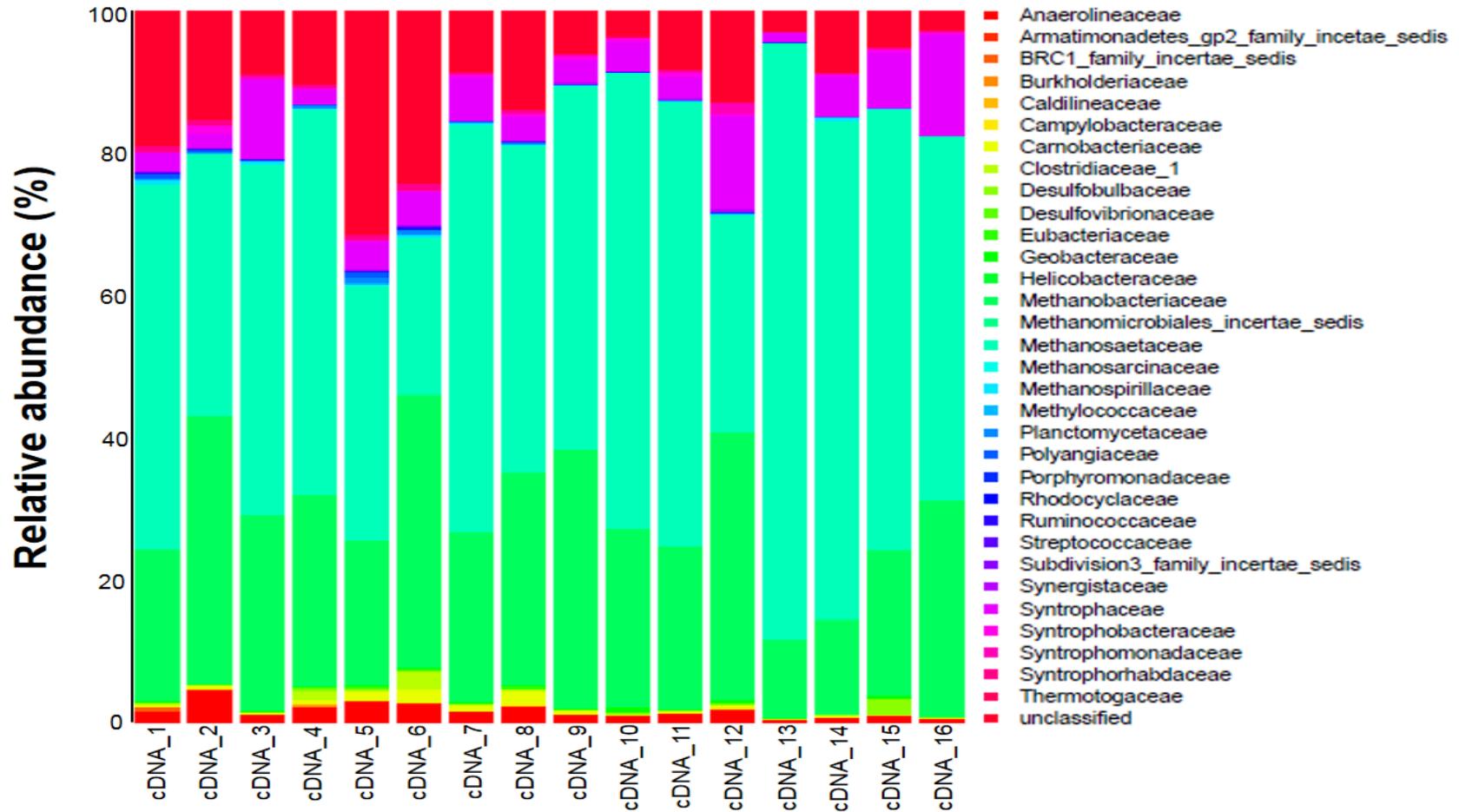


Figure 5.8: Barplot of sixteen individual single granules. cDNA community structure of sixteen granules that were sequenced from the 16S Illumina MiSeq sequencing platform.

When the Adonis test was applied, it was shown that there was no significant difference between the 16S cDNA structures of the sixteen communities ($p = 0.075$) based on Euclidean distances.

5.3.6 Comparison between the potential and active community diversities within individual granules

The results in the previous section indicated that the single individual granules had a statistically similar community amongst the sixteen individual granules. Similar analyses were performed on the five DNA-extracted samples from the VFA-fed “C” large-sized specific granules analysed in Chapter 4 (section 4.2.4). The DNA samples were compared directly with five random samples from their cDNA counterparts referred to earlier in this chapter to compare the total with the active communities in single granules.

Alpha diversity methods were employed to compare evenness and Simpson and Shannon diversity indices for the ten samples chosen, 5 DNA samples and 5 cDNA samples (Figure 5.9). The maximum evenness was 0.63. The maximum Simpson diversity was 0.85 and the maximum Shannon diversity was 2.3. The standard deviations for evenness, Simpson and Shannon values were 0.115, 0.13 and 0.454, respectively. One-way ANOVA tests showed that there were statistical differences between the DNA and cDNA evenness values ($p = 0.001$), Simpson values ($p = 0.007$) and the Shannon values ($p = 0.001$). The DNA samples were higher in each case.

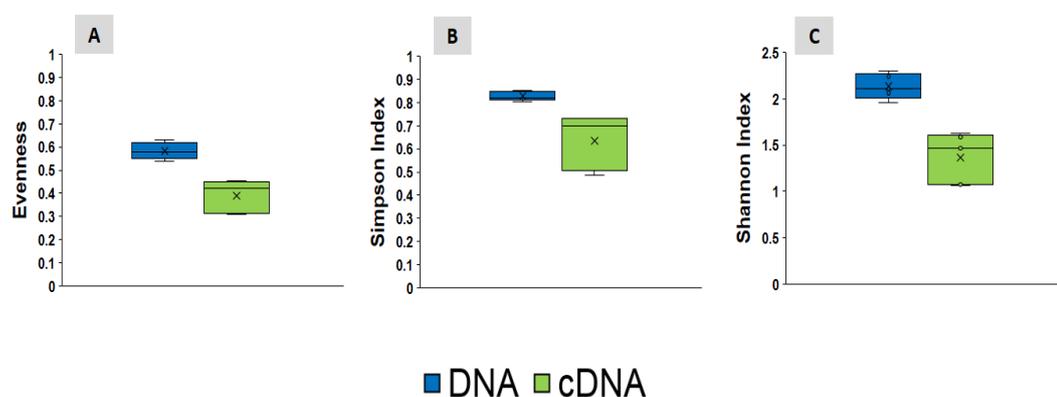


Figure 5.9: Evenness (A), Simpson (B) and Shannon (C) indices for DNA (blue) and cDNA (green) samples.

When studied together, the DNA and cDNA community structures both had similar families present based on relative abundances. The primary differences within the generated bar plot were in the relative abundances of dominant families in the DNA versus in the cDNA of single granules (Table 5.1).

Table 5.1: Families percentages in DNA vs cDNA of individual granules. Shaded boxes refer to families which were higher for each nucleic acid.

Families	DNA (%)	cDNA (%)
<i>Anaerolineaceae</i>	4.00-6.00	0.70-4.00
<i>Methanobacteriaceae</i>	18.00-28.00	13.00-37.00
<i>Methanosaetaceae</i>	13.00-25.00	30.00-70.00
<i>Propionibacteriaceae</i>	1.00-8.00	0.00
<i>Synergistaceae</i>	1.00-6.00	0.00-0.04
<i>Syntrophaceae</i>	2.00-10.00	2.00-13.00
<i>Syntrophobacteraceae</i>	1.50-3.00	0.10-1.00
<i>Syntrophomonadaceae</i>	1.00-4.00	0.01-1.00
Unclassified	20.00-25.00	3.00-15.00

When both DNA and cDNA microbial communities were examined as a whole, it transpired that there were more families present in the DNA samples than in the cDNA samples (e.g. *Propionibacteriaceae*) and there was a much higher percentage of unclassified families within the DNA community. Most of the dominant families were also noticeably more abundant in the DNA profiles (shaded boxes in Table 5.1). For the cDNA community profile the most dominant family was *Methanosaetaceae*.

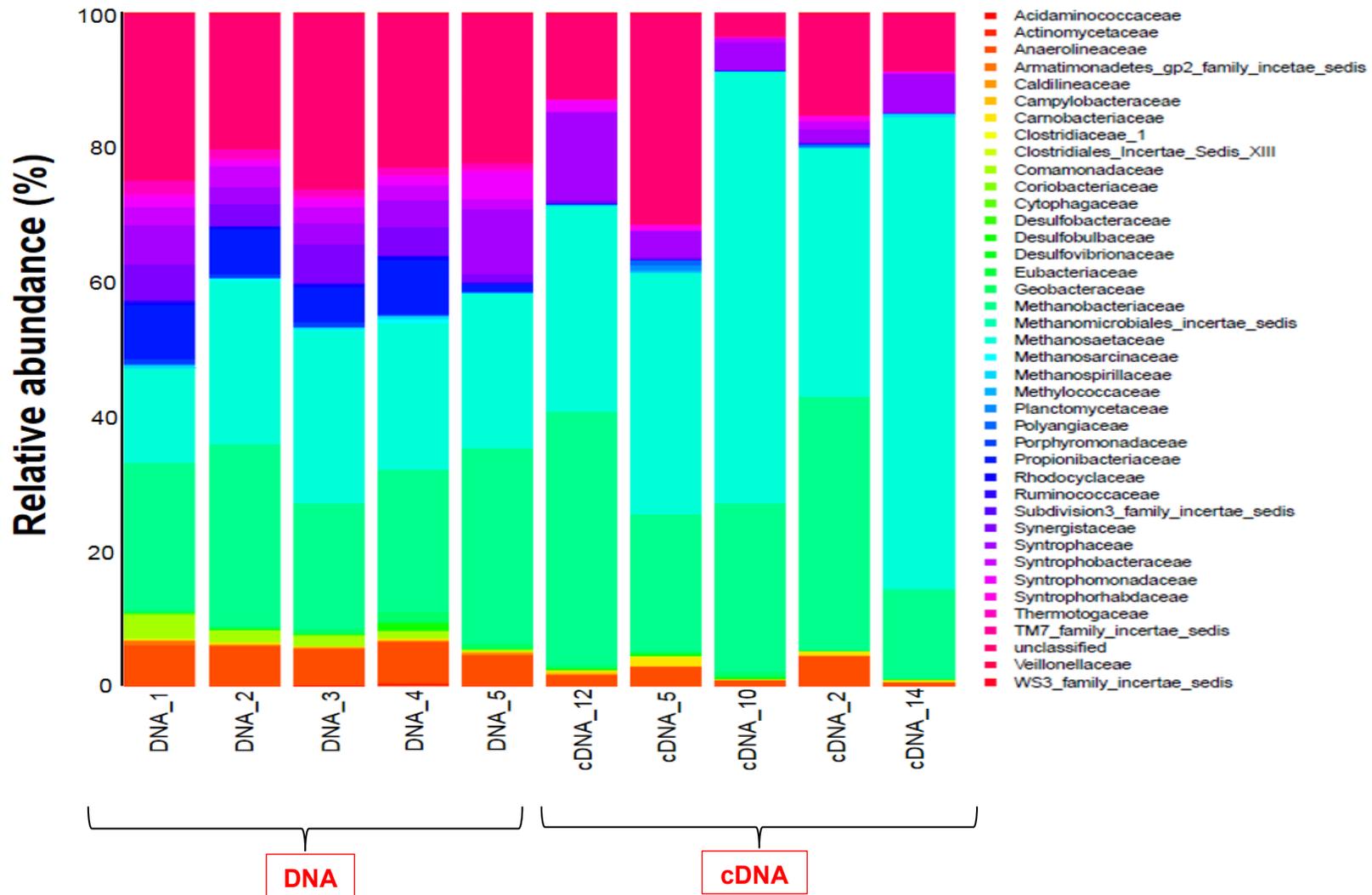


Figure 5.10: 16S community structure composition for both DNA and cDNA granule samples. The most abundant families from five granules based on their DNA structure (DNA_1 to DNA_5) and five granules based on their cDNA structure (cDNA_2, 5, 10, 12 and 14).

Both the cluster dendrogram (Figure 5.11) and the canonical correspondence analysis (CCA) (Figure 5.12) plots illustrated observable differences between the DNA and cDNA communities. The cluster dendrogram showed that the DNA samples (DNA_1 to DNA_5) were in a separate branch from all of the cDNA samples except for sample cDNA_5. The correspondence analysis showed that all five DNA samples were clustered tightly and 90% similar to each other. Each of the five cDNA samples was separated from the DNA samples on the opposite side of the plot. They were split and clustered into 3 samples on the left and 2 samples on the right main branch of the dendrogram showing 98% and 92% similarity to each other, respectively (Figure 5.11).

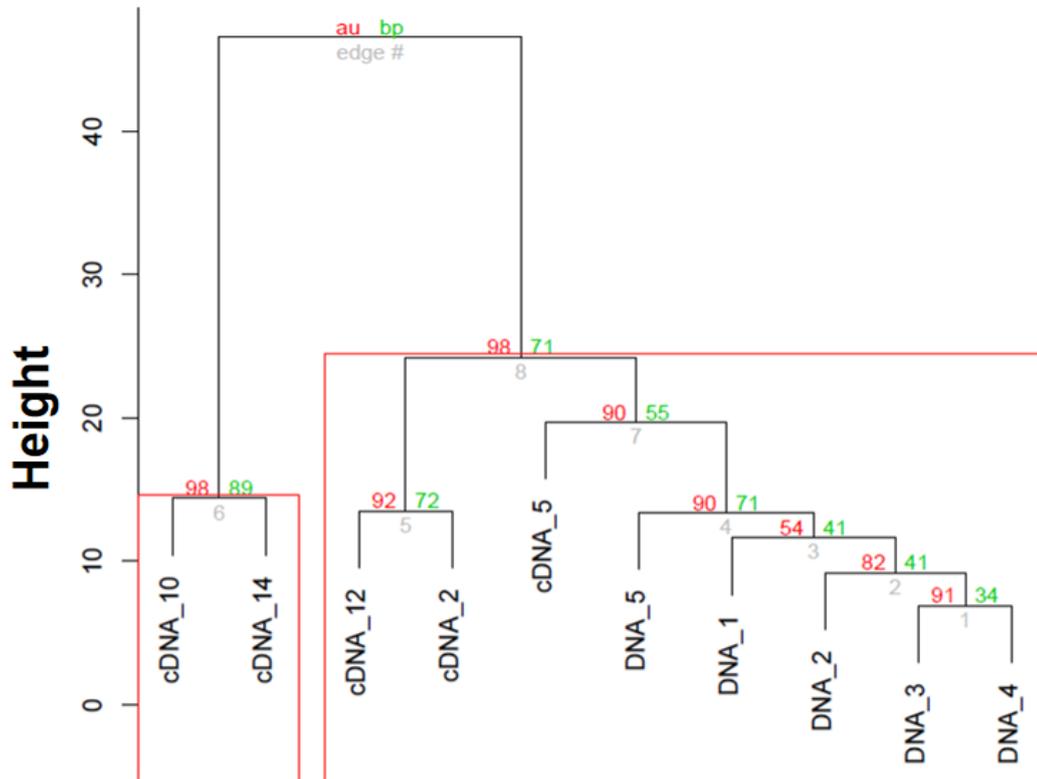


Figure 5.11: Cluster dendrogram of DNA and cDNA single granule samples based on Euclidean distances.

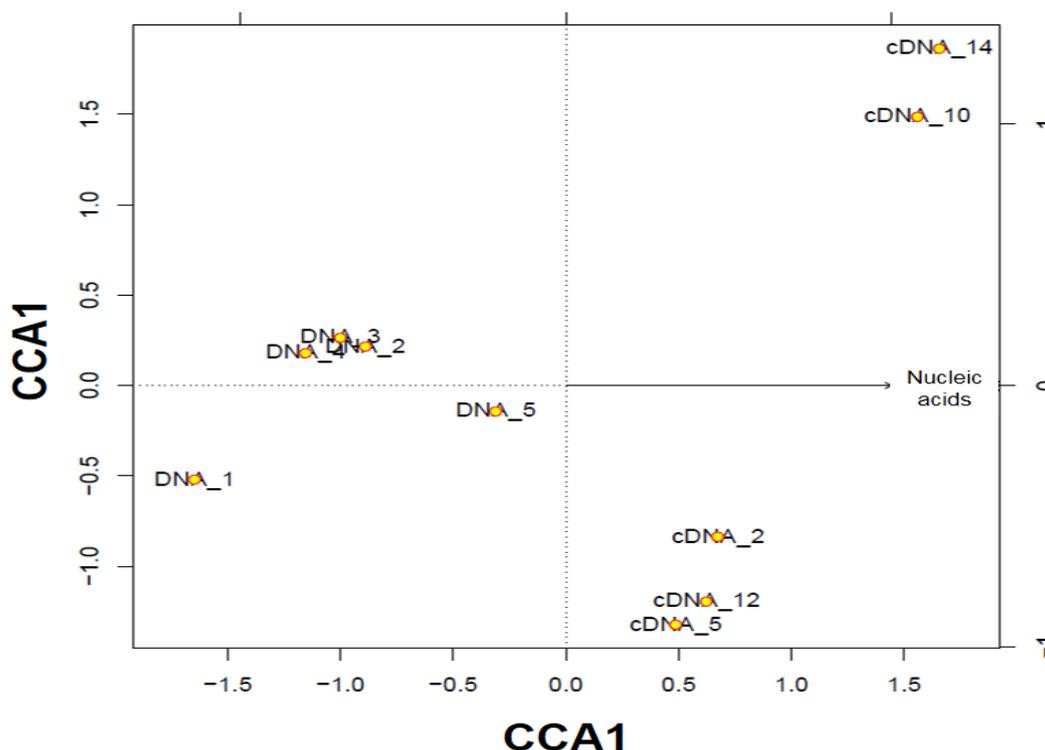


Figure 5.12: CCA ordination biplot of DNA (DNA_1 to DNA_5) and cDNA (cDNA_2, 5, 10, 12 and 14) individual granule samples (yellow symbols). Black arrow depicts “Nucleic acids” direction.

Adonis statistical testing reported a statistically significant difference between the microbial communities based on DNA in comparison to the communities based on cDNA. There was a statistically significant difference ($p = 0.011$) between the two types of communities based on Euclidean distances. The significance cut-off was $p < 0.05$.

5.4 Discussion

5.4.1 Impact of size on MA and VFA metabolism

SMA and MA assays generally indicated significant differences between substrate types. Neither butyrate nor propionate can be used directly by methanogens. First, they must be converted to acetate or H_2 - CO_2 for subsequent methanogenesis, and this process is mediated via bacterial syntrophic acetogens and methanogenic archaea (Schink and Stams, 2006). Perhaps the reason why propionate and H_2 - CO_2 ,

as well as propionate and acetate, were significantly similar was that propionate is a well-established precursor of a large fraction of methane from anaerobic digesters (Kaspar and Wuhrmann, 1978, Boone, 1982, Boone, 1984, Qiao et al., 2015).

Several studies have previously reported that propionate and acetate concentrations influence anaerobic oxidation of propionate (Nanba et al., 1983, Lin et al., 1986, Gorris et al., 1989, Fukuzaki et al., 1990, Mawson et al., 1991) and this correlation was evident in the SMAs (Figure 5.1) whereby propionate activity increased in correspondence with increased acetate activity. These SMA results highlighted an important degradation pathway through propionate rather than butyrate (Figure 5.1) but they were not significantly different in activity levels in the mixture of granular sizes. Acetoclastic activity dominated in the large VFA-fed granules that were chosen for further analysis. One unexpected result from the SMA study was that the H_2 - CO_2 and VFA mixture activities were much higher in the granular mix. It could be due to higher affinity for the substrates across the mixture of granules but this panel of substrates should be re-tested in the future. Perhaps having a mixture of granular sizes encourages the most activity.

Overall, it can be seen in this study that acetate activity increased as granular size increased. In contrast, as granular size increased, hydrogenotrophic activity decreased. Similar to acetate, as granular size increased, propionic acid and butyric acid activity increased. All activities can be compared to each other statistically.

In parallel with the VFA-fed MA assay, both sugar and VFA profiles were analysed. The results illustrated that sugars such as the macromolecule cellulose, which was present in the lab-scale UASB synthetic feed at 0.211 g/l, were still residually present in the anaerobic granules being assayed. Presumably cellulose was degraded by species in the granules which were capable of hydrolysis and fermentation as it was reduced from approximately 100 mg/l to complete consumption which occurred by hour 60 in each granular size distribution. It has been demonstrated previously that both bacterial and archaeal communities such as *Bacteroidetes* and *Firmicutes* play a crucial role in cellulose degradation (Azman et al., 2017).

Anaerobic cellulose degraders fall into four distinct families: *Syntrophomonadaceae*, *Clostridiaceae*, *Eubacteriaceae*, and *Lachnospiraceae*.

Most originate in the *Clostridiaceae* family (Schwarz, 2001). The latter three were all present in each granule size, indicating that these may have been the species that consumed the sugars in all granule sizes (Figure 5.3). It is therefore conceivable that the statistical similarity in the fermentation rate occurred because of similar residual sugar concentrations in the small-, medium- and large-sized granules.

VFA monitoring in AD can be considered as low throughput metabolomics whereby these regulatory compounds lead to metabolic activities at the fundamental process level. Although there was a decrease in the residual sugars present, there was no observable VFA increase indicating that VFA consumption occurred faster than any VFA production via sugar breakdown. VFA profiles correlated with the final methanogenic activity results, which reported higher activity against acetate and propionate (Figure 5.4) than against butyrate. The profiles illustrate complete consumption of acetic acid and propionic acid by hour 60 for each size fraction. Butyric acid remained in the small and medium granules by hour 60. This could be because they had the lowest SMA yields and, therefore, would have a substrate preference for propionate, acetate and H₂-CO₂.

These results further indicate the constant degradation of VFAs, regardless of granular size. This may be due to VFA degraders being at the core of anaerobic granules so they would be present in small, medium and large-sized granules. Microbial granulation theories have suggested that acetoclasts which degrade acetic acid are at the core of anaerobic granules (McHugh et al., 2003) As previously commented, propionate and acetate have been reported to be consumed easily in anaerobic digester studies (Kaspar and Wuhrmann, 1978, Boone, 1982, Boone, 1984). Both a general VFA mixture and specific methanogenic substrates were fully consumed by 48 hours in the large granules, that were chosen for further analysis.

5.4.2 Physical parameters of one hundred individual anaerobic granules

The results from the TS and VS tests demonstrated that there were wide ranges in solids contents. The VS average result of 63.07% for an individual “large” granule was much lower than the original VS of large-sized granules of 84.96%, which was

determined for 10 ml of large-sized granule sample taken from the VFA-fed anaerobic sludge in Chapter 4 (Table 4.1), but a standard deviation of 24.24 was measured for the VS of the one hundred batches of ten individual granules which would take this difference into account. As mentioned previously, organics quantities of up to approximately 90% have been reported in various types of anaerobic digesters (Motte et al., 2013, Amin and Vriens, 2014).

The error bars for the data (Figure 5.5) demonstrated that ten single granules were difficult to weigh accurately, especially after ignition to 550°C, and hence there were substantial standard deviations in the results. However, these deviations are reduced once some outlier data is removed. For TS, the standard deviation was almost halved when the outliers were removed (decreased from 20.17 to 11.47). When the outliers were removed for VS, the standard deviation decreased from 11.99 to 7.75. These low standard deviations among one hundred individual granules implied TS and VS similarity inside of single granules. Statistical analysis for the TS and VS concentrations confirmed that the one hundred single granules were not significantly different from each other ($p = 0.457$ and 0.635 , respectively). This indicated that individual granules have similar and perhaps replicated physical compositions.

5.4.3 Active community composition of sixteen individual granules

High yields of RNA were extracted from individual granules. 16S rRNA Illumina MiSeq analyses provided a rare insight into the active community structure of individual granules. A previous study investigated single granule community structures at a DNA level (Kuroda et al., 2016) but this work represents the activity of anaerobic granular community structures at the RNA level in parallel to single granules total community structures. Statistical tests on the alpha diversity results indicated similarity between the sixteen granules in terms of evenness, Simpson and Shannon indices. Rarefaction curves indicated that species diversity was close to saturation and was therefore representative of the microbial community that was present.

The archaeal portion of the community was dominated by the *Methanobacteriaceae* and *Methanosaetaceae* (Figure 5.8). *Methanobacteriaceae* is a hydrogenotrophic methanogen and *Methanosaetaceae* is part of the acetoclastic methanogenic group of microbes. Previous studies have suggested that both hydrogenotrophs and acetoclastic methanogens are required to maintain process stability (Leclerc et al., 2001, McHugh et al., 2003a). The current study revealed that there were no significant differences between the active community structures of sixteen granules. In combination, the sequencing results and the physical characterisation results in section 5.3.4 imply that individual granules within a bioreactor may be approximate replicates of each other. This holds great potential for high throughput characterisation of anaerobic granules under the plethora of conditions and variables that can occur within AD bioreactors and in the natural environments to monitor a community's response (Chapter 6).

Beyond Illumina MiSeq amplicon sequencing lies more integrated next generation sequencing. This includes methods such as metagenomics, which focuses on microbial community functional metabolic capabilities (Xu, 2006, Su et al., 2012, Shakya et al., 2013), metatranscriptomics, which examines their gene expression and has rarely been studied in the context of AD environments (Yu and Zhang, 2012, Zakrzewski et al., 2012, Bremges et al., 2015), metaproteomics (Wilmes et al., 2008, Abram et al., 2011) and metabolomics, which analyses metabolites within systems and are still in their infancy (Han et al., 2008, Sasaki et al., 2014). These types of studies have already been implemented in AD systems (Handelsman et al., 1998), but this involved sampling valuable biomass and impacts on these engineered systems by sampling disturbances. Reducing this disturbance would be extremely beneficial, especially for smaller lab- and pilot-scale bioreactors. Smaller amounts of biomass to be sampled would enable more temporally-resolved sampling.

5.4.4 Potential versus active granular microbial community structures

Since the cDNA of the sixteen granules was observed to replicate each of the others in terms of its microbial community, five cDNA samples were randomly picked for

direct comparison to five DNA samples (analysed in Chapter 4) to investigate how different they were from each other.

Alpha diversity tests demonstrated higher diversity among the total community structures compared to the active community structures. Evenness may have been higher in the DNA microbial community compared to the cDNA community because it may be more robust and more capable of adapting to new conditions and parameters (Wittebolle et al., 2009). This was further illustrated and confirmed by beta diversity analysis. The bar plot highlighted that a higher number of families were present in the DNA community structure of five single granules. However, the two most abundant families were in the cDNA community. Perhaps this could be because there were a larger number of lower abundant families in the DNA community structure. Interestingly the most abundant families in the DNA community were the same in the cDNA community. This showed that the abundant families of the potential community were a good indicator for which species would be relevant in the active community.

Clustering analysis and correspondence analysis were employed to visualise each sample and how they relate to one another. It was evident that the cDNA microbial communities formed a separate branch to the DNA samples. However, one cDNA sample was 90% similar to one DNA community sample. This could presumably have occurred due to their most abundant species being identical (Figure 5.10).

Adonis analysis highlighted significant differences between the datasets generated from the total community (DNA) of five granules and the active community (cDNA) of five granules from a specific size distribution and sludge type. This illustrates different DNA and RNA profiles from physically similar granules. These differences between DNA and RNA microbial communities have been reported in the AD field on numerous occasions via various methodologies (Hoshino and Matsumoto, 2007, De Vrieze et al., 2016, Mei et al., 2016). Future studies should be extended to include granules based on other size fractions.

In general, these results contrast with the only other previous study found in the literature on single granules. This study investigated individual granules at the DNA level and the authors found that there was heterogeneity within granules. They did however find that there were similarities in granular species composition at both

nucleic acid levels, such as for *Methanosaeta* for example. Also, they suggested that several microbial consortiums coexist in different granules within a single bioreactor. Additionally, both types of methanogens were established and dominate within the granules (Kuroda et al., 2016). This work also observed both types of methanogens.

5.5 Conclusions

The main conclusions were fivefold;

1. Methanogenic activities were significantly different among anaerobic granules at three size fractions. Therefore, granular size may be a strong influencer of AD activity. Large granules were chosen for further studies because they had the highest overall SMA results, after taking all substrate (acetate, butyrate, propionate and H₂-CO₂) activity yields into account.
2. Single individual granules from the size fraction (1.2 – 2.0 mm diameter) and from the source studied in this work have statistically similar physiochemical properties in terms of TS and VS concentrations.
3. 16S rRNA active community structures of sixteen individual granules from the size and source chosen in this study demonstrated microbial replication. *Methanobacteriaceae*, *Methanosaetaceae* and *Syntrophaceae* were the most abundant classified families across the sixteen samples.
4. There were significant differences between the microbial structures based on DNA and cDNA of large-sized VFA-fed granules. *Methanobacteriaceae*, *Methanosaetaceae* and *Syntrophaceae* species were the most abundant for DNA and cDNA communities, but many of the families that were less abundant in the potential (DNA) granular community were absent in the cDNA microbial structure. *Methanosaetaceae* was the most abundant family within the active community profile, which indicates its importance in granular metabolism and functioning.
5. If granule communities were replicated in individual anaerobic granules, smaller samples would be required, which would be very advantageous for lab- and pilot-scale reactors where biomass is valuable and can't be replaced during trials.

5.6 References

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Chapter 6

Chapter 6 - High Throughput Characterisation of Single Whole Ecosystems: Anaerobic Granules in micro-Sequencing Batch Reactors

“Everything is everywhere; the environment selects” (Bass-Backing, 1934).

Abstract

Anaerobic granules are characteristic of wastewater treatment bioreactors such as upflow anaerobic sludge blanket (UASB) reactors. Granules are naturally-occurring spherical biofilms in bioreactors composed of many microorganisms involved in the anaerobic digestion process: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Optimal bioreactor performance depends on the thermodynamics and the crucial metabolic interactions of fermenters, syntrophs and methanogens. Many of these processes remain poorly understood and the specific species involved in granular growth and development are rarely studied at the level of a single granule.

Previous data supported the idea that granules could be considered as distinct, replicated, whole ecosystems. On this basis, and by using novel, ‘micro-sequencing batch reactors’ (μ SBRs), a series of high-throughput experiments was set up to investigate the single, whole-ecosystem microbiology of granules under a number of distinct environmental conditions.

Individual granules in μ SBRs were fed every 48 hours over 42 days. Granules were subjected to various, distinct conditions of pH (4, 7 and 10), carbon source (a VFA mixture, acetate, cellulose and glucose), trace elements availability (e.g. cobalt-deprivation) and temperature (37°C and 23°C). For each condition, assays were set up with and without the methanogenic inhibitor, 2-bromoethanesulfonate (BES), to also investigate non-methanogenic pathways. RNA was extracted from individual granules at the conclusion of μ SBR trials and cDNA was sequenced.

An analysis of the volatile fatty acids (VFA) present in BES-treated granules indicated that there was homoacetogenic activity. The core findings were that community structure succession shifted in response to environmental conditions and that species patterns shifted in the same way within triplicate granules.

6.1 Introduction

Microorganisms are globally ubiquitous within highly diverse and complex community structures (Flint et al., 2007, Johnson et al., 2015). They underpin the earth's biogeochemical cycles, and thus examining and understanding microbial systems can pave the way for numerous applied advances in the areas of human health, biotechnology industries and environmental issues (Widder et al., 2016). Despite the large number of studies conducted in the field of AD, knowledge of microbial consortia is limited because of the deficiency of structural and functional data (Goswami et al., 2016) as well as the limitations from the continuous use of DNA-based technologies (Koch et al., 2014).

From the natural environment, soil aggregates have been examined and termed as “independent evolutionary incubators” whereby each one has its own microbial community. The authors described the stages which aggregates undergo: formation, stabilisation and disintegration followed by the release of the microbial communities into the bulk soil macro-community leading to a resulting increased genetic diversity (Rillig et al., 2017).

Sampling and investigations of microbial communities within individual granules has been less studied (Kuroda et al., 2016), especially at RNA level. The physical structure of an individual granule has been determined by using techniques such as FISH analysis (Rocheleau et al., 1999, Saiki et al., 2002). Each single anaerobic sludge granule comprises of a micro-community of the trophic groups involved in the AD process and this is what makes them interesting; this is a unique microbial niche evolutionary setting. Due to their physical separation and encapsulation of an isolated community every environmental trajectory of each specific granule can be investigated. Each individual granule can therefore be considered as a whole community, or a whole ecosystem in itself. The objective of this work was to test this ‘whole ecosystem’ concept in relation to the response of a whole community to various environmental conditions.

One drawback to anaerobic microorganisms and methanogens in particular, is that they are very sensitive to different and sudden changes in environmental conditions (Lau and Fang, 1997, Shah et al., 2014). It has been reported that an increase in salt

concentration or organic matter concentration, or the introduction of a toxin has resulted in system failure within a bioreactor (Chen et al., 2008, Ma et al., 2009).

It has been demonstrated in the literature that pH has a very strong effect on hydrogen production (Zoetemeyer et al., 1982, Fang and Liu, 2002, Horiuchi et al., 2002, Temudo et al., 2007). It has been found that low pHs tend to inhibit methanogenic activity. Acetoclastic methanogens are inhibited at a pH of less than 6, whereas hydrogenotrophic methanogens are not (Batstone et al., 2002). Previously it has been shown that acetogenic metabolic interactions in particular are influenced by the environmental parameters of pH and temperature, and that it is strain-specific which acetogens survive within these complex habitats of anaerobic digesters (Kotsyurbenko et al., 2001). Homoacetogenic bacteria in particular can survive in environments covering a wide pH range (Drake, 2006).

By contrast, methanogens cannot survive under large pH fluctuations (Chen et al., 2008). Fermentation reactors have been operated at psychrophilic temperatures (Levin et al., 2004). Granular sludge ultrastructure studies have demonstrated that both it and the bacterial community structure were dependent on temperature and on the substrate that was present in the bioreactors (Schmidt and Ahring, 1995, Banik et al., 1997). From these types of studies, it has been demonstrated that pH management as well as heat treatments may not be effective for H₂-consuming autotrophic inhibition (Dinamarca et al., 2011).

Temperature plays an important role in microbial ecophysiology, influencing growth and substrate utilisation rates; for example, growth rates decrease with decreasing temperature (Henze and Harremoës, 1983, Lettinga et al., 1999). However, it has been suggested that methanogens and acidogens obtain higher yields at lower temperatures (Lin et al., 1987, van Lier et al., 1997). LtAD as a whole process has been examined in detail in various studies but more fundamental research is required to elucidate clearly the role that microbes play at lower temperatures (Rebac et al., 1999, Lettinga et al., 2001, Collins et al., 2003, McHugh et al., 2003b, McHugh et al., 2004, McHugh et al., 2006). Within the AD pathway, it has been found that hydrolysis is the rate-limiting step, and under low temperatures this stage proceeds even slower than in mesophilic environments (Pavlostathis and Giraldo-Gomez, 1991, Fey and Conrad, 2003). There have been

studies suggesting that hydrogenotrophic methanogenesis is the main pathway to methane production even in a predominantly acetoclastic methanogenic community (Hughes, 2011).

Various trace elements are required for microbial growth, including tungsten, selenium, molybdenum, nickel, iron and cobalt. Cobalt is essential as it increases the Co-containing corrinoid factor III, which is necessary for growth to be optimal within cells. Between 0.05 and 0.06 mg/l has been determined as the requirement of each trace element except for iron, of which there must be approximately 1 to 10 mg/l (Bischoff, 2009). Cobalt is an important nutrient in both anaerobic digesters and anaerobic granules, and is among the most studied and sought after elements in terms of digester stability and higher biogas production (Choong et al., 2016)

Utilising substrates is paramount to microbial growth, metabolism, interaction with other microorganisms and survival. Acetate is a well-known and studied substrate for acetoclastic methanogenesis and syntrophic acetate-oxidising bacteria. VFAs are involved in AD fermentation. Cellulose and glucose are also widely studied AD substrates. Cellulose is known to be the most abundant organic polymer on earth and is synthesised primarily by plants with almost half of the global dry plant material comprising of cellulose (Bailey and Ollis, 1986). Because of its environmental abundance, cellulose is considered to be a large biomass component in various wastes and many studies have analysed it in relation to anaerobic digestion (Shang, 2000, O'Sullivan et al., 2005, Song et al., 2005). Glucose degradation in relation to anaerobic digestion has been widely studied as it is a key natural organic compound (Mosey, 1983, Oi et al., 1984, Kalyuzhnyi and Davlyatshina, 1997, Wu et al., 2008, Subramanyam and Mishra, 2013, Osman and Ahmed, 2015, Shahperi et al., 2016).

BES is a methanogenic inhibitor (Figure 6.1) so, if methanogenic pathways are blocked, substrates such as acetate can be utilised by sulfate-reducing bacteria instead of methanogenic archaea. Sulfonates are usually described as the electron acceptors for sulfate- and sulfite-reducing bacteria, but BES reduction by microbial communities has also been reported (Ye et al., 1999). The role that BES plays in AD is still unclear, although some have suggested that it is the adsorption to sludge particles or microbial uptake or its own possible degradation (Zinder et al., 1984).

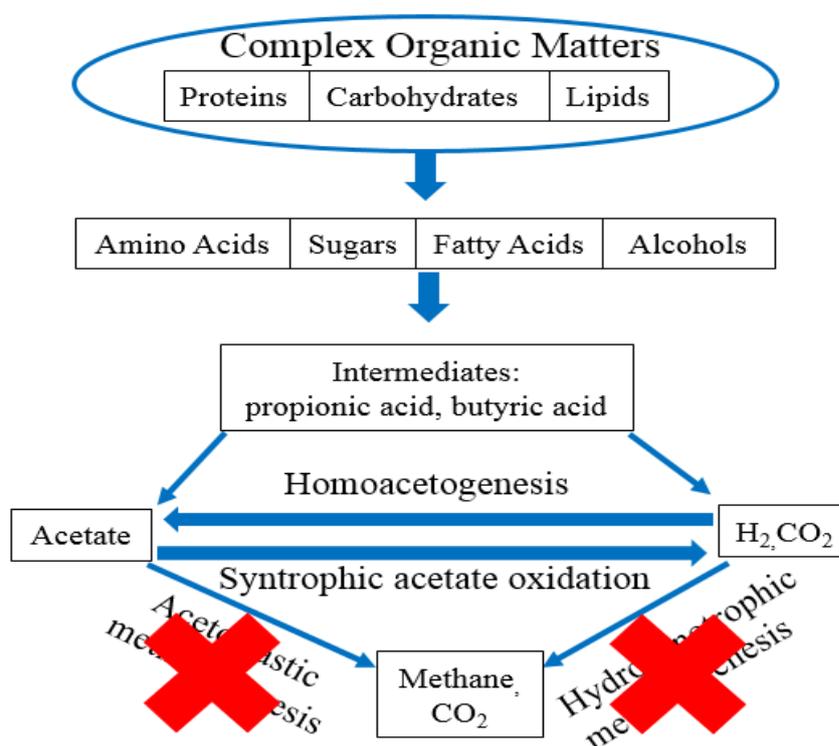


Figure 6.1. AD pathway with methanogenic inhibitor BES blocking the final stage of methanogenesis.

Regarding the microbial consortia studied in high rate, retained biomass systems, it has been observed that *Methanosaeta* may dominate within acetoclastic methanogenesis of healthy systems, whereas *Methanosarcina* dominance was found during unstable periods throughout some studies (Demirel and Scherer, 2008, Rincón et al., 2008, Blume et al., 2010).

In this study, single individual granules were fed with different kinds of media to create synthetic environmental changes (section 5.2.1) from the conditions that prevailed when they were originally sampled (Chapter 4, section 4.2.1 VFA-fed “C” granules). The environmental factors investigated were pH, substrate, metal deprivation, temperature and the methanogenic inhibitor BES. Each of these stresses, aside from BES, naturally occurs both in the environment and within anaerobic digesters. Many studies have been conducted to investigate the impact of these parameters on the bioreactor’s microbial community and some of these studies have been described here.

In summary, the main aim of this work was to identify the active microbial communities under different conditions and to determine whether active community structure replication is present at a single granule level when an environmental stress occurs on a high-throughput scale.

The primary hypotheses are:

1. Metabolic dynamics and active community structures are replicated among individual granules of a specific size and type under the same conditions.
2. Metabolic dynamics and active community structures among individual granules shift under an environmental stress.
3. Individual granules are “whole ecosystems” with a complex community structure that can be investigated in a high-throughput platform.

6.2 Materials and Methods

6.2.1 Anaerobic medium and stock solutions

The anaerobic basal medium for each individual granule consisted of (in grams per litre of distilled water): KH_2PO_4 , 0.27, K_2HPO_4 , 0.27, trace metal solution (H_3BO_3 , 0.05 g, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 g, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.03 g, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.5 g, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.01 g, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 g, Na_2SeO_3 , 0.05 g, ZnCl_2 , 0.05 g, in 1000ml of distilled water), 1 ml, trace element solution (NH_4Cl , 132.5 g, $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$, 25 g, CaCl_2 , 14.15 g, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 5 g, in 1000ml of distilled water), 4 ml and L-cysteine- $\text{HCl} \cdot \text{H}_2\text{O}$, 0.622. After boiling and sparging the medium with N_2 gas, 3.05 g of NaHCO_3 was added to the medium.

Sterile stock solutions of a VFA mixture (acetic acid, 15 mM, propionic acid, 9 mM and butyric acid, 6 mM), acetate (30 mM), cellulose (0.211 g/l or 6.17 mM), glucose (0.2346 g/l or 1.3 mM) and BES (50 mM) were stored at 4°C for the duration of the trial. A trace metal solution (as described above but without $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) was made up for cobalt-deprived granules. Granules that were not cobalt-deprived had a cobalt concentration of 2.11 μM . pHs were altered with 5 M HCl to lower the

solutions to pH 4 and with 8 M NaOH to raise the solutions to pH 10 using a pH meter for reference (HI-207 Bench pH Meter, Hanna, UK).

6.2.2 Micro Sequencing Batch Reactors (μ SBRs)

Granules were sampled from the large-size fraction (1.2 – 2.0 mm diameter) of the VFA-fed granular sludge (“C”) sample described in Chapters 4 and 5 (sections 4.2.1 and 5.2.1). Single granules were transferred into individual wells on 48-well plates and 1 ml of anaerobic basal medium and corresponding substrate were pipetted into each well inside an anaerobic chamber (Coy). Plates were either stored inside an anaerobic box (BD GasPak EZ) with an anaerobic gas-generating sachet (Oxoid AnaeroGenTM) (Figure 6.2) in a hot room (37°C) or kept in the anaerobic chamber (for granules incubated at 23°C) (Table 6.1) for the 42-day trial. Each plate was wrapped with parafilm (Parafilm M Wrapping Film, Fisher Scientific) to secure the lids and to prevent moisture entering the plates.



Figure 6.2. Anaerobic box for μ SBR plates incubated at 37°C.

PLATE A

	Well a	Well b	Well c	Well d	Well e	Well f	Well g	Well h
Well 1	pH 7, VFAs	pH 7, VFAs	pH 7, VFAs	pH 7, VFAs	pH 7, VFAs + BES			
Well 2	pH 7, VFAs	pH 7, VFAs	pH 7, VFAs	pH 7, VFAs	pH 7, VFAs + BES			
Well 3	pH 4, VFAs	pH 4, VFAs	pH 4, VFAs	pH 4, VFAs	pH 4, VFAs + BES			
Well 4	pH 4, VFAs	pH 4, VFAs	pH 4, VFAs	pH 4, VFAs	pH 4, VFAs + BES			
Well 5	pH 10, VFAs + BES							
Well 6	pH 10, VFAs + BES							

PLATE B

	Well a	Well b	Well c	Well d	Well e	Well f	Well g	Well h
Well 1	pH 7, acetate	pH 7, acetate	pH 7, acetate	pH 7, acetate	pH 7, acetate + BES			
Well 2	pH 7, acetate	pH 7, acetate	pH 7, acetate	pH 7, acetate	pH 7, acetate + BES			
Well 3	pH 7, cellulose + BES							
Well 4	pH 7, cellulose + BES							
Well 5	pH 7, glucose	pH 7, glucose	pH 7, glucose	pH 7, glucose	pH 7, glucose + BES			
Well 6	pH 7, glucose	pH 7, glucose	pH 7, glucose	pH 7, glucose	pH 7, glucose + BES			

37°C

PLATE C

	Well a	Well b	Well c	Well d	Well e	Well f	Well g	Well h
Well 1	pH 7, VFAs, - Co	pH 7, VFAs, - Co + BES						
Well 2	pH 7, VFAs, - Co	pH 7, VFAs, - Co + BES						
Well 3	pH 7, acetate - Co + BES							
Well 4	pH 7, acetate - Co + BES							

PLATE D

	Well a	Well b	Well c	Well d	Well e	Well f	Well g	Well h
Well 1	pH 7, VFAs + BES							
Well 2	pH 7, VFAs + BES							

23°C

Table 6.1: μ SBR environmental conditions. Each environmental condition was represented by eight granules in individual wells. Plate A contained granules fed at different pHs, Plate B examined granules fed with different carbon sources, Plate C granules were deprived of cobalt (- Co), Plate D granules were incubated at 23°C. Plates A-C were incubated at 37°C. BES was added for each condition to eight counterpart granules on the right half of each plate.

6.2.3 VFA analysis

VFAs within single granule environments under different conditions were monitored at various time points throughout the time span of one μ SBR's batch time of 48 hours through targeted metabolomics profiling. This was carried out on eight biological replicates from each different condition via the VFA-GC used and described previously in Chapter 3 (section 3.2.3). Four days before the end of the 42-day μ SBR trial, eight biological samples (i.e. individual granules) of the media were taken at various time points during the forty-eight-hour batch period. At hours 0, 4, 8, 12, 20, 24, 28, 32, 36, 40, 42 and 48, triplicate samples were taken and the respective medium was replaced.

Analyses performed on the granules were separated into the following categories: VFA-fed granules, acetate-fed granules and sugar-fed granules.

6.2.4 Soluble sugar profiles

Soluble sugars were monitored as previously described in Chapter 5 (section 5.2.3). The samples profiled were the single granules fed with cellulose (with and without BES) and with glucose (with and without BES) in the μ SBRs. Samples were taken from eight biological replicates and were analysed at the time points 0, 4, 8, 12, 24, 36 and 48 hours during the trial. Average fermentative depletion rates were measured based on the slope of each line.

6.2.5 DNA/RNA co-extraction and cDNA synthesis from biomass

Genomic DNA and RNA were extracted from each individual granule (3 granules per condition i.e. $3 \times 18 = 54$) at the end of the μ SBR trial (Day 42). DNA/RNA co-extraction was described previously in Chapter 4 (section 4.2.3) and cDNA synthesis methodology applied for this NextGen sequencing study was set out in Chapter 5 (section 5.2.5).

6.2.6 High-throughput cDNA sequencing and processing

Triplicate cDNA samples of single granules subjected to each separate condition during the μ SBR trial were sent to the Research and Testing Laboratory (Lubbock, Texas) for amplification of the 16S rRNA gene sequences using the universal bacterial/archaeal forward primer 515f and reverse primer 806r on an Illumina MiSeq platform. A modified version of the MiSeq SOP pipeline in mothur (http://www.mothur.org/wiki/MiSeq_SOP) processed the raw sequences and was described previously in Chapter 4 (section 4.2.4).

Analyses performed on the granules were separated into the following categories: pH, substrate, temperature, cobalt-deprivation and BES effects.

6.2.7 Statistical Methods

Statistical analyses and data visualisation were performed on cleaned-up sequence data using the R and VEGAN software and packages as described previously in Chapter 4 (section 4.2.5). Diversity indices were measured, multivariate ANOVA tests based on similarities were performed and Euclidean distances were calculated. All samples were subsampled to an even depth of 32,000 reads per sample. The level of statistical significance was $p < 0.05$.

6.3 Results

6.3.1 The metabolic impact of VFA-fed single granules incubated under various environmental parameters

pH: The VFA profiles of individual granules under different environmental conditions were analysed. For granules incubated at three different pH (Figure 6.3 (A-C)), there was a widespread consumption of each VFA across the three pH. The highest consumption was at pH 7 where the three VFAs were almost completely consumed and the lowest was at pH 10 where there was approximately 750 mg/l of acetic acid present after the 48-hour batch time. There was a statistically significant difference between both the acetic acid profiles ($p = 0.000$) and the butyric acid

profiles ($p = 0.003$) of the three pH, whereas there was no statistical difference between the propionic acid profiles of pH 7, pH 4 and pH 10 ($p = 0.071$).

pH and BES: Granules that were fed with the addition of the methanogenic inhibitor BES also had decreasing profiles at each pH but there was an observable higher level of acetic acid for each pH in comparison to the granules fed without BES. Butyric and propionic acids followed the same patterns in each granule both with and without BES. There were statistically significant differences reported between the acetic acid ($p = 0.000$), butyric acid ($p = 0.002$) and propionic acid ($p = 0.044$) profiles of the BES-fed granules at the three pH. BES-fed granules were statistically similar to non-BES-fed granules for pH 7 ($p = 0.482$), pH 4 ($p = 0.716$) and pH 10 ($p = 0.802$) across all three VFA profiles.

Temperature: The VFA profiles of granules incubated at 23°C were compared to those of granules at 37°C but under the same conditions (pH 7) (Figure 6.3 (A) (37°C) and Figure 6.3 (D) (23°C)). Although each of the VFAs steadily declined at both temperatures, the primary difference was that granules incubated at 23°C consumed VFAs more slowly. The final VFA concentrations were less reduced at 23°C. 91% of acetic acid had been consumed at 37°C whereas 63% had been consumed at 23°C. For propionic acid, 4% of the original concentration remained at 37°C and 14% remained at 23°C. Butyric acid declined by 98% of its original concentration at 37°C and there was a decrease of 90% at 23°C. Statistical tests measuring the difference between the acetic acid profiles at the two temperatures reported a statistical similarity ($p = 0.057$). Butyric ($p = 0.545$) and propionic ($p = 0.572$) acid profiles were also statistically similar between the two temperatures. BES-fed granules were statistically similar to non-BES-fed granules for granules incubated at 23°C ($p = 0.148$) across all three VFA profiles.

Temperature and BES: With the addition of BES, a similar pattern of consumption emerged: granules at 23°C consumed each VFA slower than at 37°C. However, acetic acid did not decrease to the same level as it did in granules where BES was absent. The acetic acid profiles were statistically different for the two temperatures ($p = 0.001$) but there were no statistically significant differences between the two

temperatures for their butyric ($p = 0.612$) and propionic ($p = 0.072$) acid profiles for the BES-fed granules.

Cobalt-deprivation: VFA-fed granules which were cobalt-deprived consumed acetic acid more slowly than granules which contained cobalt (Figure 6.3 (A) compared to Figure 6.3 (E), respectively). Acetic acid was reduced to 86 mg/l in granules containing cobalt but acetic acid was reduced to 189.5 mg/l in cobalt-deprived granules. The differences between of acetic acid profiles were statistically different ($p = 0.043$) whereas butyric ($p = 0.79$) and propionic ($p = 0.531$) acid profiles with the cobalt-fed and the cobalt-deprived granules were all statistically similar.

Cobalt-deprivation and BES: Granules containing cobalt and BES reduced acetic acid to 290.6 mg/l. The cobalt-deprived granules with BES had a steady decrease of acetic acid, to 296 mg/l. Butyric and propionic acid were almost completely consumed both for granules which did and did not contain cobalt. Statistical tests showed that acetic ($p = 0.678$), butyric ($p = 0.746$) and propionic ($p = 0.393$) acid profiles were all significantly similar between the granules BES-fed with cobalt and the BES-fed cobalt-deprived granules. Cobalt-deprived granules were statistically similar to the BES-fed, cobalt-deprived granules ($p = 0.407$) taking all of the VFA concentration profiles into account. There were however, differences between the cobalt-fed and cobalt-deprived granules at the last two time points in each case.

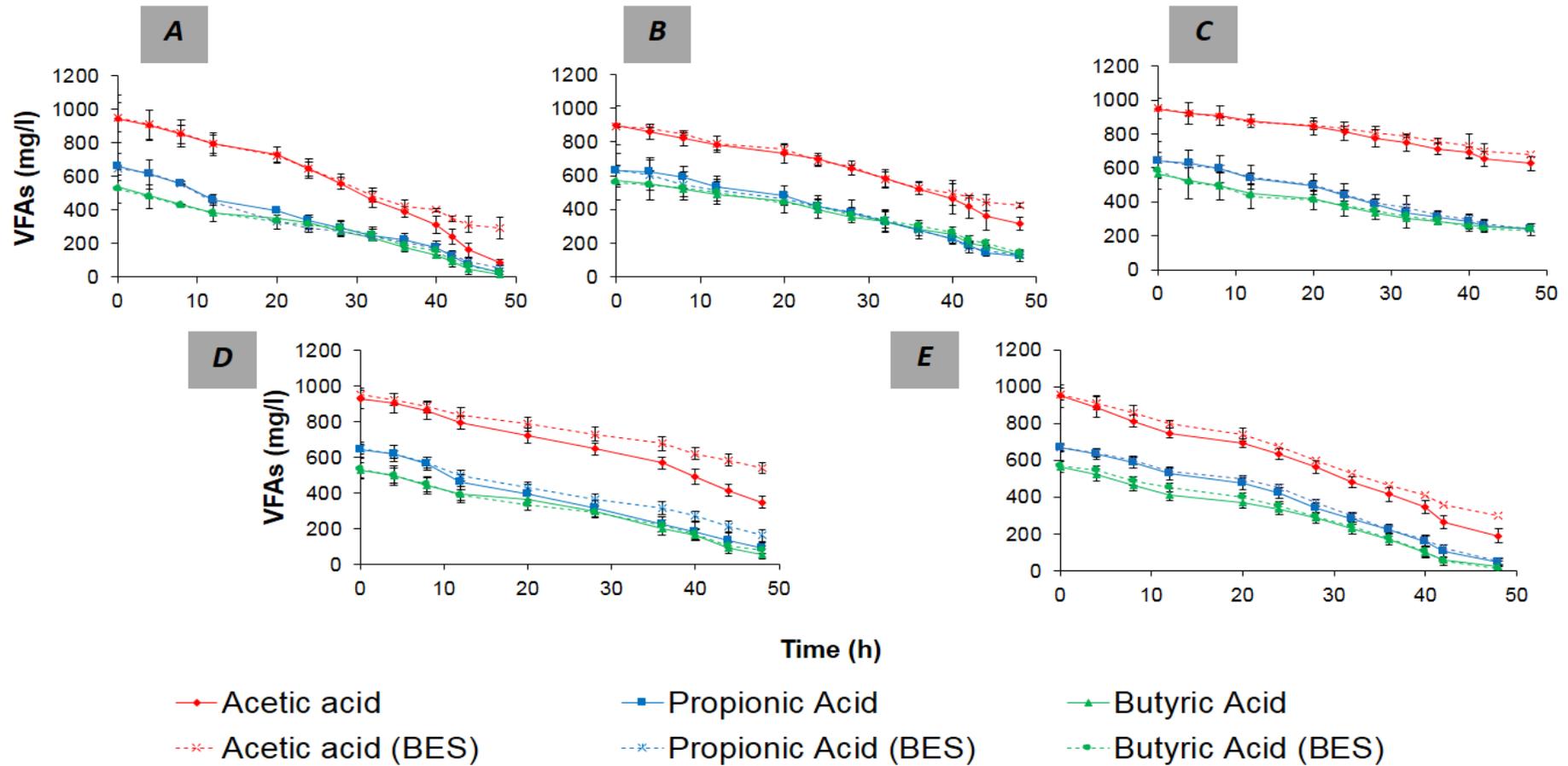


Figure 6.3: Effect of different pHs on VFA production and consumption. Metabolic profiles of granules fed with VFAs at (A) pH 7, 37°C, (B) pH 4, 37°C, (C) pH 10, 37°C, (D) pH 7 at 23°C and (E) pH 7, 37°C, cobalt-deprived. Concentrations of acetic acid (red markings), butyric acid (green markings) and propionic acid (blue markings) are with the methanogenic inhibitor BES (broken lines) and without BES (continuous lines).

6.3.2 The fate of VFAs within single granules incubated under acetate-feeding

Acetate-fed granules consumed most of the acetic acid by the end of each 48-hour batch (Figure 6.4 (A)). Acetic acid profiles were compared between granules fed with acetate and those fed with VFAs in section 6.3.1. There was a statistically significant difference between them ($p = 0.008$).

The acetic acid profiles of acetate-fed granules were not statistically different to acetate-fed granules with BES ($p = 0.058$). Butyric acid and propionic acid were not added to the basal medium and there was no increase or decrease for acetate-fed and acetate-fed cobalt-deprived granules, either with or without BES.

Acetate-fed cobalt-deprived granules (Figure 6.4 (B)) consumed 56% less acetic acid than granules containing cobalt. Final acetic acid, propionic acid and butyric acid concentrations were 124 mg/l, 40 mg/l and 56 mg/l for acetate fed granules, respectively whereas final VFA concentrations for acetate fed and cobalt-deprived granules were 283 mg/l (acetic acid), 60 mg/l (propionic acid) and 70 mg/l (butyric acid). Acetic profiles of granules fed with cobalt were statistically similar than those fed without cobalt ($p = 0.058$).

Acetic acid consumption for granules deprived of cobalt with BES was lower (where the concentration only decreased to 940 mg/l) than granules without BES (where it was consumed to a concentration of 283 mg/l). Approximately 50% of residual propionic acid and butyric acid was consumed in cobalt-deprived acetate-fed granules with BES, which was a little less than for acetate-fed granules with cobalt and BES, which decreased by approximately 60%. Although the results appear similar in the graph, acetic profiles of cobalt-deprived granules with BES were statistically different than the cobalt-deprived granules without BES ($p = 0.005$).

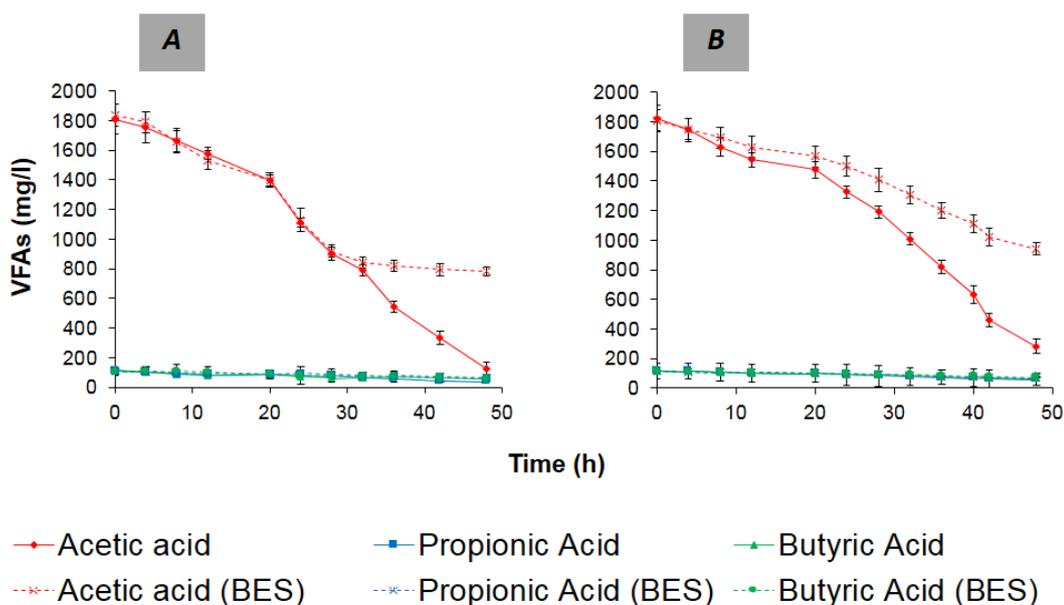


Figure 6.4: Effect of cobalt and BES on acetate-fed granules. VFA profiles of granules fed at 37°C at pH 7 with (A) acetate and (B) acetate and cobalt-deprived. Concentrations of acetic acid (red markings), butyric acid (green markings) and propionic acid (blue markings), are with the methanogenic inhibitor BES (broken lines) and without BES (continuous lines).

6.3.3 Fermentative profiling of individual granules under cellulose- and glucose-feeding

Both sugar and VFA profiles were monitored throughout the μ SBR trial. It was found that both cellulose and glucose were depleted. However, they were not fully consumed (Figure 6.5). Based on the slope of the sugar depletion lines for cellulose and glucose, the average consumption rates were as follows; glucose-fed granules with and without BES had the highest depletion rate of $4.13 \text{ mg sugar granule}^{-1} \text{ h}^{-1}$ and $4.22 \text{ mg sugar granule}^{-1} \text{ h}^{-1}$, respectively, while the consumption rate for cellulose was $3.5 \text{ mg sugar granule}^{-1} \text{ h}^{-1}$ and the rate for cellulose-fed granules with BES was $3.23 \text{ mg sugar granule}^{-1} \text{ h}^{-1}$.

Statistical tests were performed on triplicate fermentative depletion rates. Statistical results reported that there were no significant differences between the fermentative consumption rates within granules which were BES-fed and those which were not BES-fed ($p = 0.899$). There was a significant difference between the consumption rates of cellulose-fed and the glucose-fed granules ($p = 0.002$).

The VFA assay profiles were much lower (the VFA concentration scale was 0-300 mg/l) for granules fed with cellulose and glucose than for the granules fed with either acetate or the VFA mixture. The highest accumulation of acetic acid was 287 mg/l during glucose-fed granules' 48-hour batch incubations and 202 mg/l of acetic acid for cellulose-fed granules. There were significant differences between the acetic acid profiles ($p = 0.004$), the butyric acid profiles ($p = 0.000$) and the propionic acid profiles ($p = 0.001$) of cellulose- and glucose-fed granules. For BES-fed granules there were significant differences between the acetic acid profiles ($p = 0.006$), the butyric acid profiles ($p = 0.001$) and the propionic acid profiles ($p = 0.000$) of cellulose- and glucose-fed granules.

There were no significant differences between the acetic acid profiles of cellulose-fed granules with and without BES ($p = 0.592$) across the VFA profiles. The same was true of glucose-fed granules with and without BES across all VFA profiles. There was no statistical difference between them ($p = 0.705$). Granules that also contained BES in their media for each substrate had a similar VFA profile. VFAs accumulated as the sugar concentrations were depleted (Figure 6.5) in all granules fed with either cellulose or glucose. As sugars decreased, VFAs were produced.

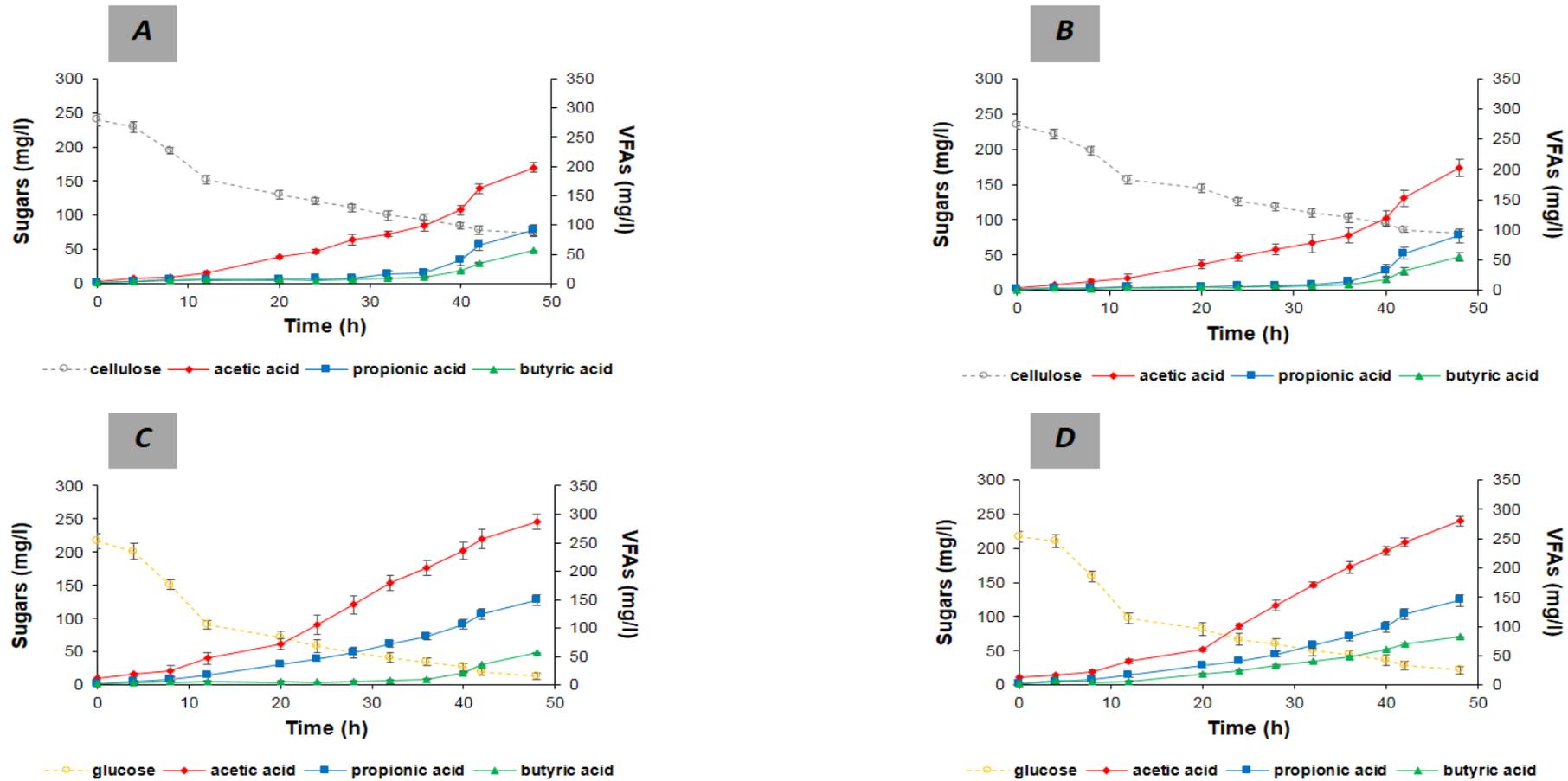


Figure 6.5: Sugar and VFA profiles for (A) cellulose-fed granules, (B) cellulose-BES-fed granules, (C) glucose-fed granules and (D) glucose-BES-fed granules. Dashed lines represent sugar consumption. Continuous lines represent VFA accumulation: acetic acid (red), propionic acid (blue) and butyric acid (green).

6.3.4 16S rRNA bacterial and archaeal community composition

Sequencing results for triplicate cDNA samples for each environmental condition (pH 7, pH 4, pH 10, acetate, cellulose, glucose, cobalt deprivation in VFA-fed medium, cobalt deprivation in acetate-fed medium, 23°C and, separately, each of these supplemented with BES) were analysed. Rarefaction curves (operational taxonomic unit (OTU) numbers versus sampled sequence numbers) were generated according to each set of conditions (Figure 6.6 (A-F)) and the resulting images suggested that there was close to complete saturation of species diversity across the samples.

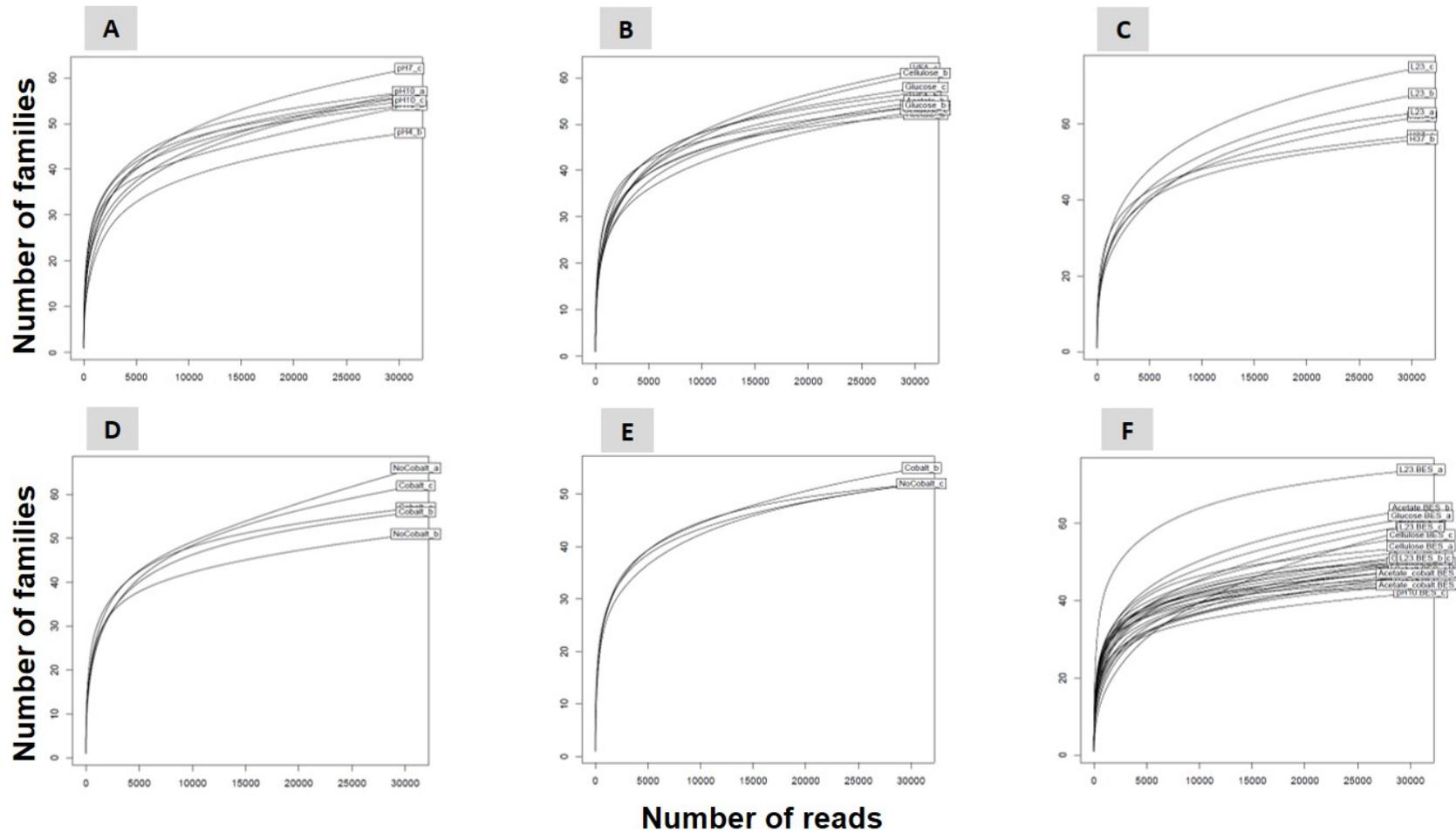


Figure 6.6: Species richness among individual granules exposed to various environmental pressures. Rarefaction curves with a cut-off of 32,000 sequences representing (A) pHs 7 (“control” granules), 4 and 10, (B) substrates acetate, cellulose, VFAs and glucose, (C) temperatures 37°C (labelled H37) and 23°C (labelled L23), cobalt deprivation for both (D) acetate- and (E) VFA-fed granules (labelled “Nocobalt”) alongside control acetate and VFA-fed controls (labelled “Cobalt”) respectively and each different environmental condition.

6.3.4.1 The effect of pH on VFA-metabolised single whole granules

The standard alpha diversity tests are species evenness, Simpson and Shannon indices (Figure 6.7). The standard deviations across the three pH were 0.1 for evenness, 0.15 for Simpson diversity and 0.4 for Shannon diversity. The maximum Simpson diversity was 0.86 and the maximum Shannon diversity was 2.27. There were statistically significant differences based on evenness, Simpson and Shannon diversity between granules at pH 4 and pH 7 ($p = 0.033$, 0.021 and 0.031 , respectively). Also, there were significant differences between granules at pH 10 and pH 4 ($p = 0.029$, 0.041 and 0.044 , respectively) based on evenness, Simpson and Shannon diversities. However, there were no significant differences between granules at pH 10 and pH 7 ($p = 0.336$, 0.168 and 0.322 , respectively).

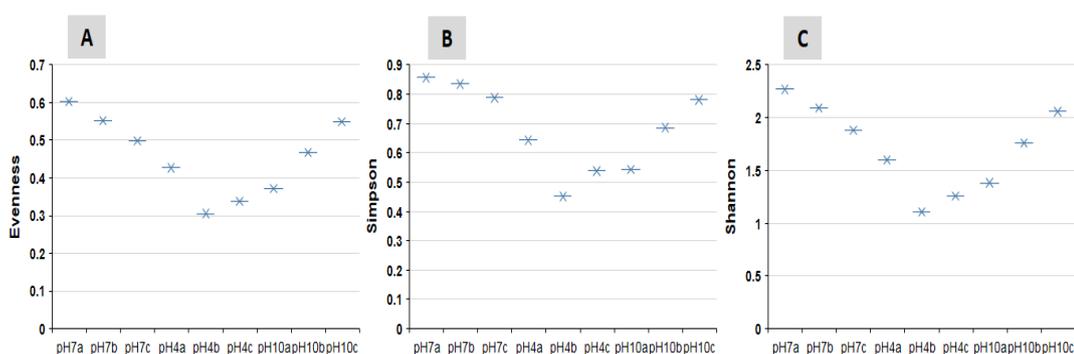


Figure 6.7: Alpha diversity calculations. Measurements of (A) Evenness, (B) Simpson diversity and (C) Shannon diversity metrics for granules incubated with media at pH 7, pH 4 and pH 10.

The most dominant families for the pH 7 incubated (control) granules were unclassified bacteria (8.8-14.6%), *Desulfuromonadaceae* (10.1-22.3%), *Methanobacteriaceae* (21.6-28.8%), *Methanosaetaceae* (22.1-30.4%) and *Rhodocyclaceae* (5.5-8.2%). For pH 4-incubated granules, the dominant families were *Campylobacteraceae* (56.3-72.9%) and *Methanobacteriaceae* (9.5-17.5%). For granules with media at pH 10, the dominant families were unclassified bacteria (8.3-15.3%), unclassified *Bacteroidetes* (4.4-10.8%) and *Methanobacteriaceae* (40.9-66.0%) (Figure 6.8).

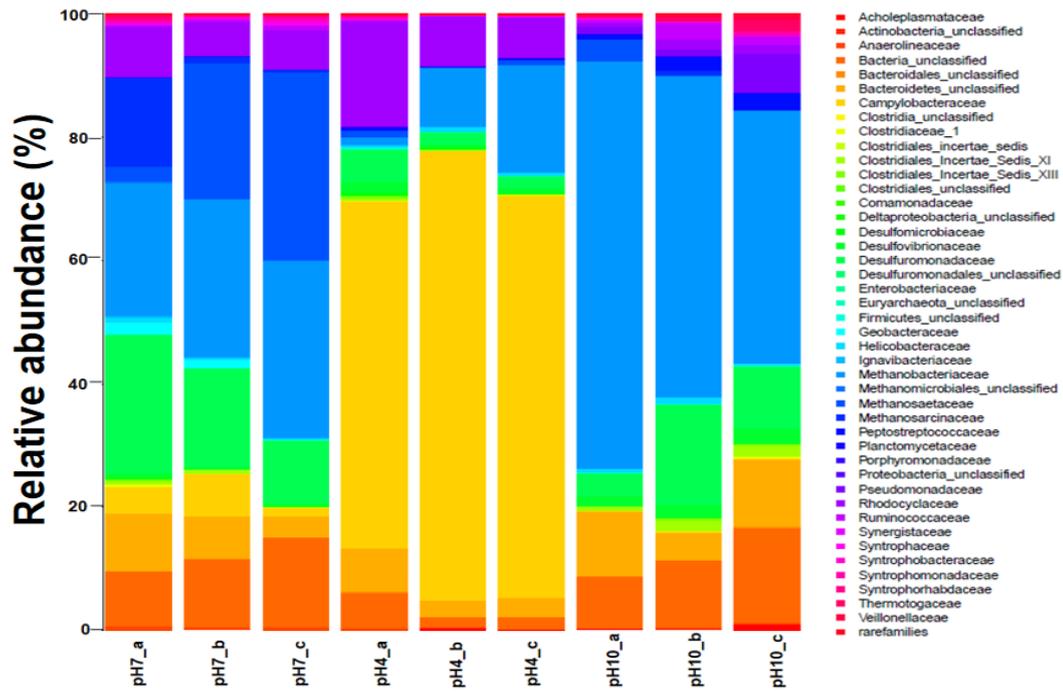


Figure 6.8: Barplot of the dominant families according to relative abundances from 16S rRNA Illumina sequencing of granules that were incubated in media at pH 7, pH 4 and pH 10.

Clustering occurred for each pH. pH 7, pH 4 and pH 10 caused similarities of 91%, 100% and 98% among each triplicate granule, respectively (Figure 6.9). This was further illustrated on a CCA plot (Figure 6.10), where the three pH were separated distinctly.

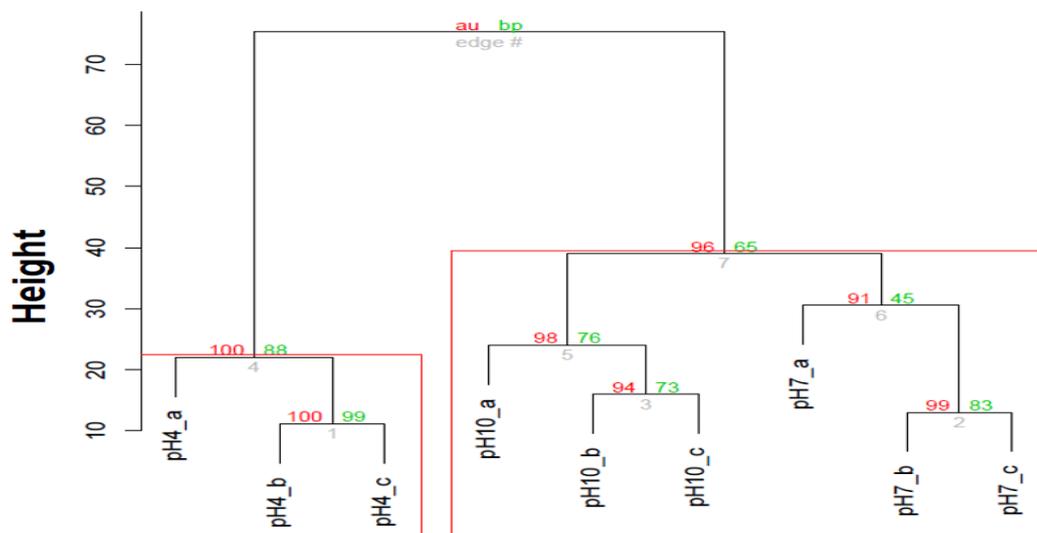


Figure 6.9: Hierarchical cluster dendrogram of the microbial community of single granules incubated at pH 7, pH 4 and pH 10, sequenced in triplicate from μ SBR samples.

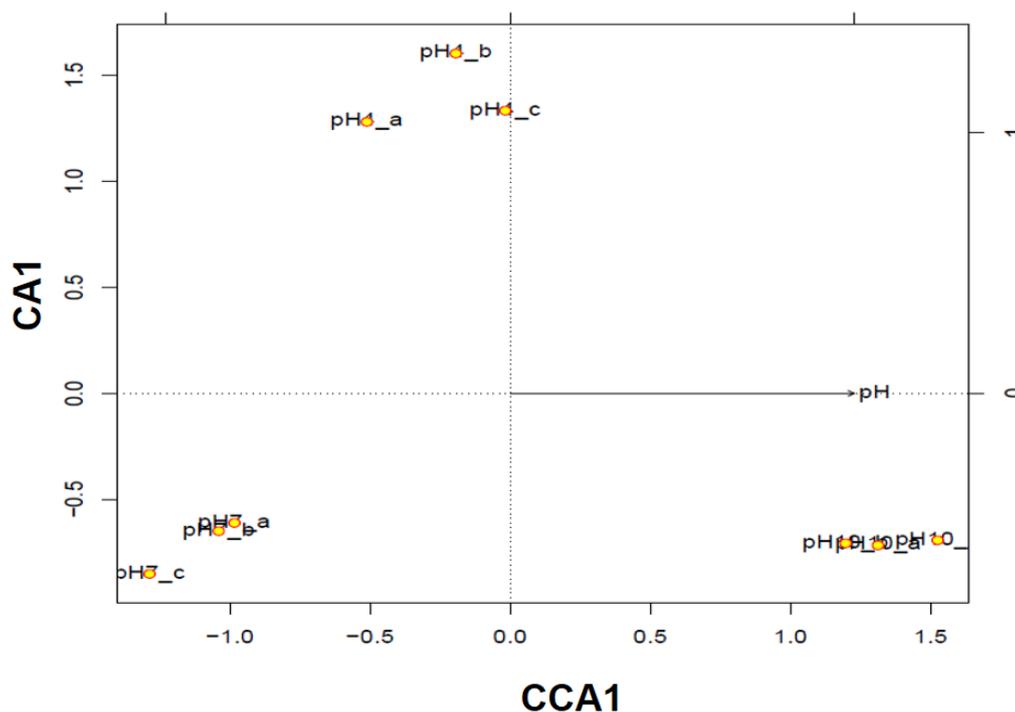


Figure 6.10: Canonical correspondence analysis of triplicate granules incubated with media at pH 7, pH 4 and pH 10 clustering according to pH.

Statistical analysis was carried out on these pH variables via the Adonis test and the results indicated that there was a statistically significant difference among the samples at a given pH when tested against z ($p=0.0199$) and Euclidean distances ($p=0.00497$). The level of statistical significance was $p < 0.05$. Granules incubated at each of pH 7, pH 4 and pH 10 were statistically different from one another.

6.3.4.2 Effect of substrate on the active 16S rRNA community structure of single granules

Granules fed with the four different substrates were initially analysed based on their alpha diversities - Evenness, Simpson and Shannon metrics (Figure 6.11). The maximum Simpson diversity across all four substrates was 0.86 and the standard deviation was 0.03. The maximum Shannon diversity was 2.27 and the standard deviation was 0.12. The standard deviation of evenness was 0.03. Statistical tests reported no significant differences between the four substrates based on evenness ($p = 0.766$), Simpson diversity ($p = 0.643$) and Shannon diversity ($p = 0.744$) data.

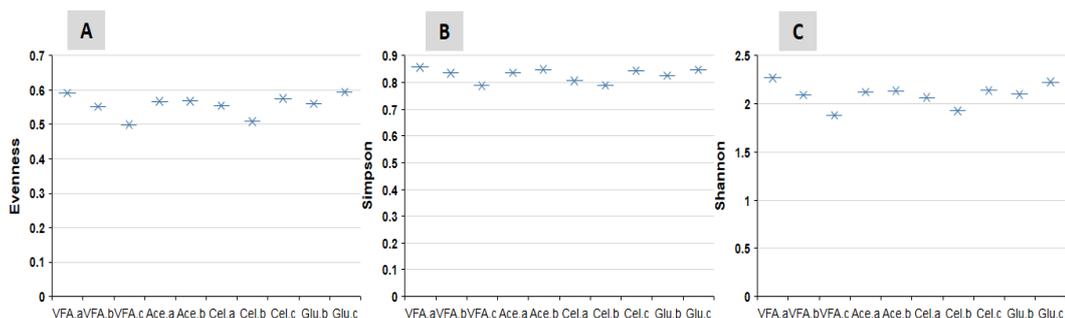


Figure 6.11: Evenness (A), Simpson (B) and Shannon (C) results for single granules fed with the following substrates: VFAs, acetate, cellulose and glucose. Some triplicate samples are missing because all samples were subsampled to an even depth of 32,000 reads per sample.

The most dominant families for the VFA-fed (control) granules (Figure 6.12 (“pH7”)) were unclassified bacteria (8.8-14.6%), *Desulfuromonadaceae* (10.1-22.3%), *Methanobacteriaceae* (21.6-28.8%), *Methanosaetaceae* (22.1-30.4%) and *Rhodocyclaceae* (5.5-8.2%).

The most dominant families for the acetate-fed granules were unclassified bacteria (15.6-23.9%), unclassified *Bacteroidetes* (10.5-17.6%), *Desulfovibrionaceae* (8.6-20.0%), and *Methanobacteriaceae* (12.4-20.0%).

The most dominant families for the cellulose-fed granules were unclassified bacteria (13.1-18.1%), *Methanobacteriaceae* (24.4-36.1%) and *Methanosaetaceae* (7.4-14.3%). Unclassified bacteria (17.0-19.3%), unclassified *Bacteroidetes* (18.7-23.0%), *Methanobacteriaceae* (13.2-23.0%) and *Methanosaetaceae* (21.45-21.48%), were dominant in glucose-fed granules, as illustrated in the barplot (Figure 6.12).

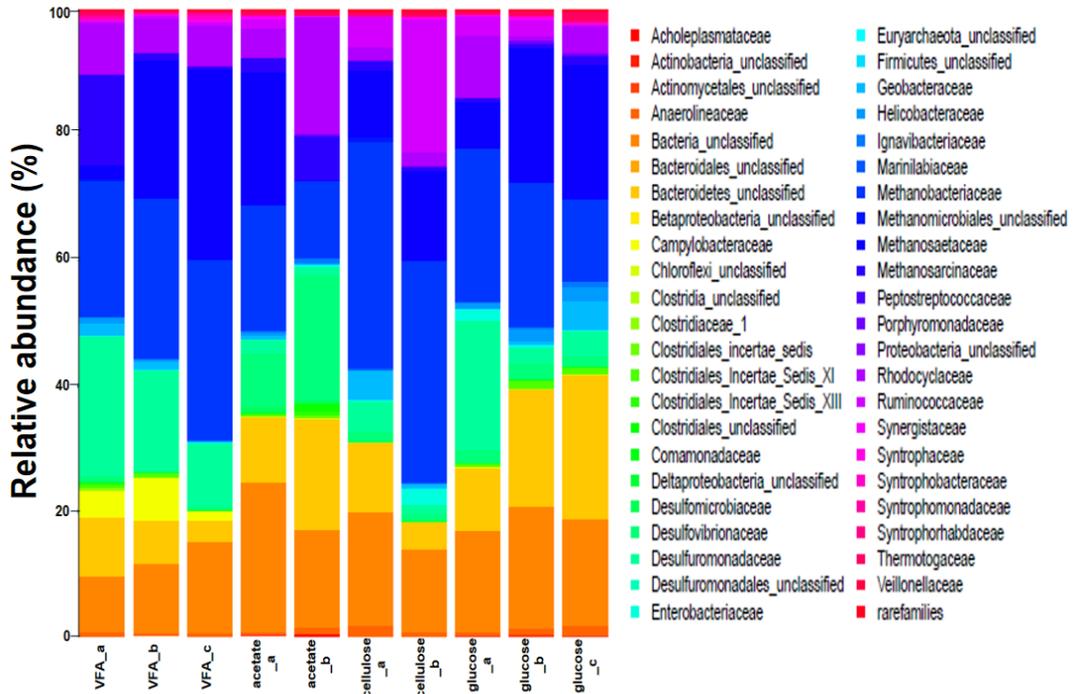


Figure 6.12: Barplot of dominant families present in individual granules fed with a VFA mix, acetate, cellulose and glucose. Some triplicate samples are missing because all samples were subsampled to an even depth of 32,000 reads per sample.

Separation occurred for each of the triplicate granules for the different substrates illustrated on a CCA plot (Figure 6.13). VFA-fed granules were the furthest apart from the other feeds in the CCA plot. Cellulose, glucose and acetate were closer together except for the acetate_b acetate-fed granule sample, which distinctly separated from the rest of the samples.

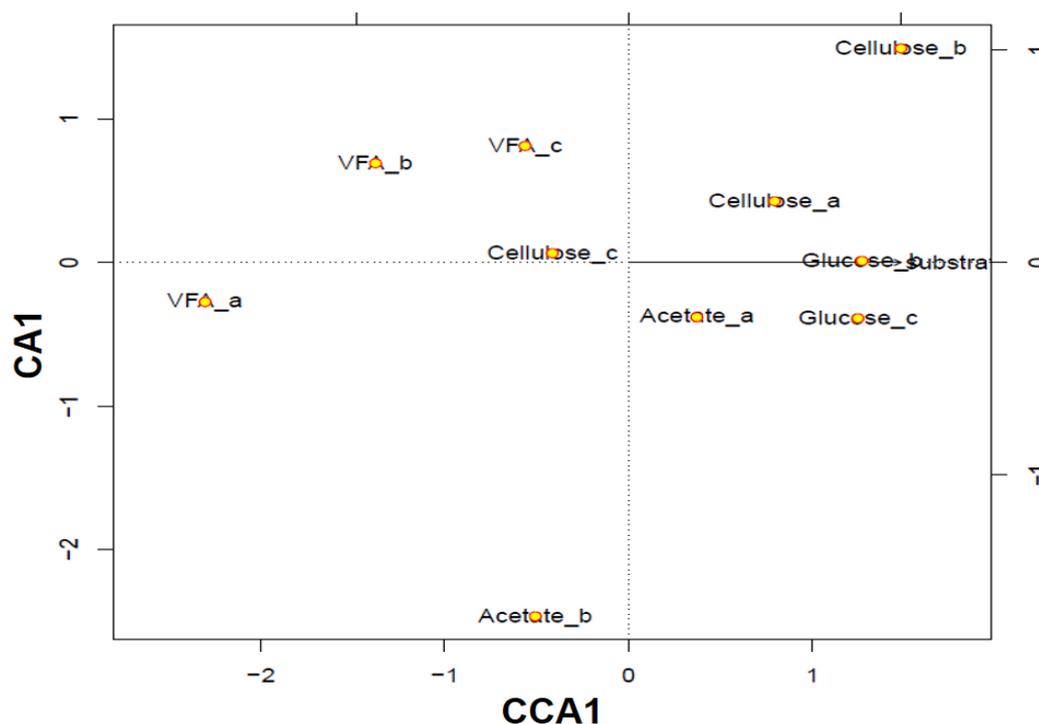


Figure 6.13: CCA ordination biplot of single granules fed with different substrates; VFA mix, acetate, cellulose and glucose (circle symbols) and environmental parameter: substrate (black arrow). Some triplicate samples are missing because all samples were subsampled to an even depth of 32,000 reads per sample.

Statistical analysis based on the Adonis test reported that there was a statistically significant difference among the substrate samples when tested against z ($p = 0.015$) and Euclidean distances ($p=0.04$). The level of statistical significance was $p < 0.05$. Granules fed with VFA mix, acetate, cellulose and glucose were each statistically different from one another.

6.3.4.3 Impact of temperature on active 16S rRNA community structure of individual granules

The alpha diversity tests reported that the maximum Simpson diversity across both temperatures was 0.86 and the standard deviation was 0.13. The maximum Shannon diversity was 2.27 and the standard deviation was 0.36. The standard deviation of evenness was 0.11 (Figure 6.14). Based on temperature, there were significant differences in the evenness ($p = 0.035$) and Shannon diversity ($p = 0.045$) of the

individual granules but there were no statistical differences in the Simpson diversity ($p = 0.075$) data across the two temperatures.

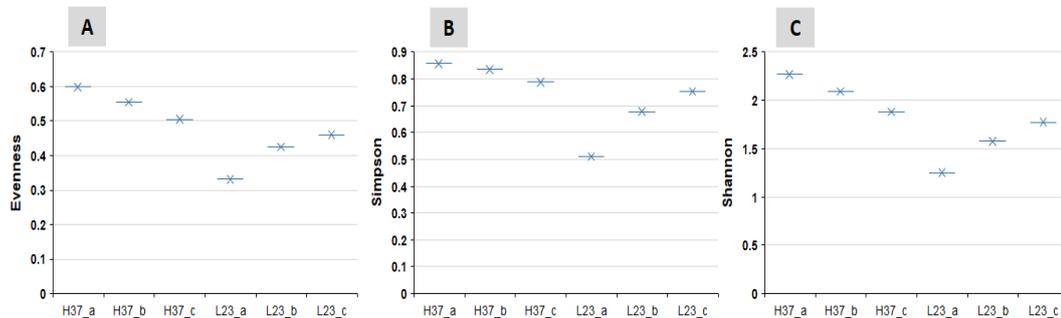


Figure 6.14: (A) Evenness, (B) Simpson and (C) Shannon diversities for each of the individual granules incubated at two different temperatures.

As described earlier, the most dominant families for the 37°C-incubated (control) (Figure 6.8 (“pH7”)) granules were unclassified bacteria (8.8-14.6%), *Desulphuromonadaceae* (10.1-22.3%), *Methanobacteriaceae* (21.6-28.8%), *Methanosaetaceae* (22.1-30.4%) and *Rhodocyclaceae* (5.5-8.2%). For the 23°C-incubated granules, the dominant families were *Alcaligenaceae* (3.9-39.1%), *Campylobacteraceae* (36.0-68.0%) and *Methanosaetaceae* (3.6-18.8%), as illustrated in the barplot (Figure 6.15).

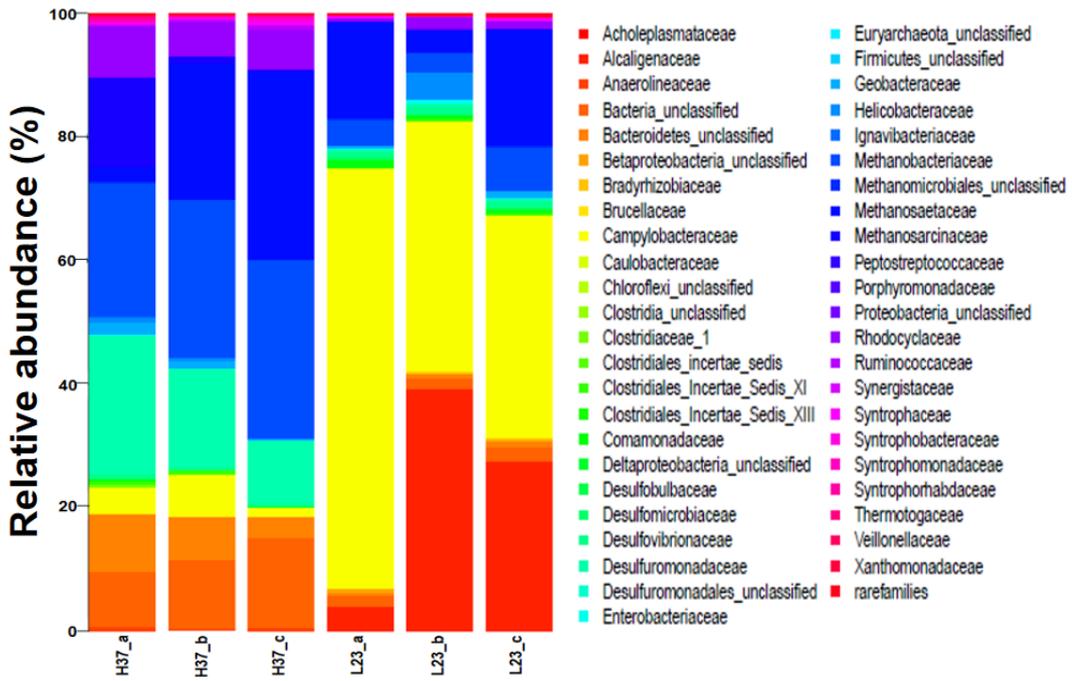


Figure 6.15: Barplot for each of the single granules incubated at 37°C (H37) and at 23°C (L23) based on 16S Illumina sequencing.

Clustering was illustrated for the different temperatures (Figure 6.16) and illustrated on a CCA plot (Figure 6.17). The cluster dendrogram had a definitive split, where the active community structures of granules incubated at 37°C were present at one side of the plot with 96% similarity to each other and the granules incubated at 23°C were on the other side of the plot with 92% similarity to each other.

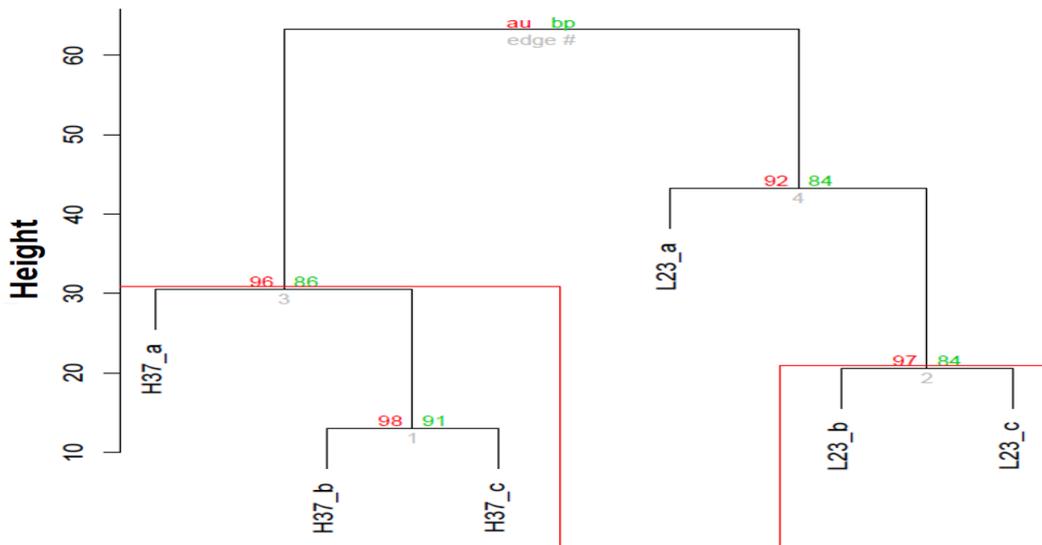


Figure 6.16: Cluster dendrograms of triplicate granules at 37°C (H37) and at 23°C (L23).

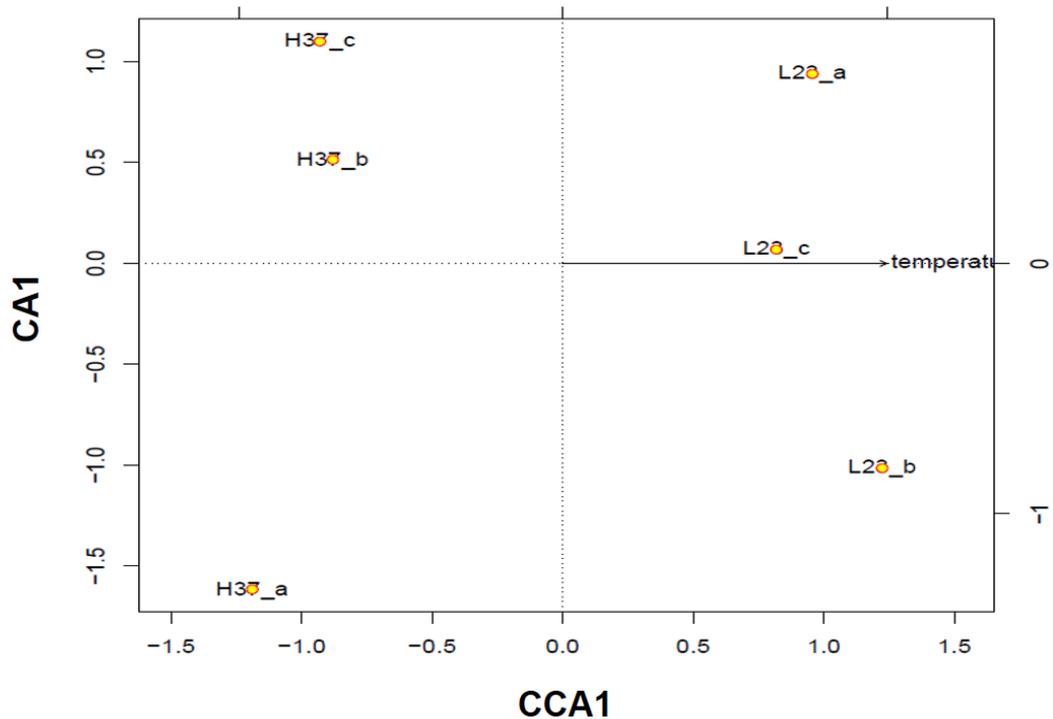


Figure 6.17: CCA ordination biplot of temperature-differentiated granules (37°C and 23°C) (circle symbols) and environmental parameter: temperature (black arrow).

Adonis statistical analysis was carried out for the different temperature samples, and the results specified that there was no statistical difference among the granular community structures at the two temperatures when tested against z ($p = 0.095$) and Euclidean distances ($p = 0.1$). The level of statistical significance was $p < 0.05$. Granules incubated at 37°C and 23°C were not statistically different from each other regarding their active 16S rRNA community structures.

6.3.4.4 Effect of cobalt-deprivation on active 16S rRNA community structure of a single granule

Granules fed with the VFA mix and granules fed with acetate were deprived of the macro nutrient cobalt. Differences were observed within their 16S rRNA community structures.

6.3.4.4.1 VFA-fed cobalt-deprived granules

The alpha diversity tests comprised of evenness and Simpson and Shannon indices (Figure 6.18). The maximum Simpson diversity across cobalt-fed and cobalt-deprived granules was 0.86 and the standard deviation was 0.03. The maximum

Shannon diversity was 2.27 and the standard deviation was 0.1. The standard deviation of evenness was 0.04. There were no statistically significant differences between the VFA-cobalt-fed and the VFA-fed cobalt-deprived granules based on evenness ($p = 0.76$), Simpson diversity ($p = 0.586$) and Shannon diversity ($p = 0.735$).

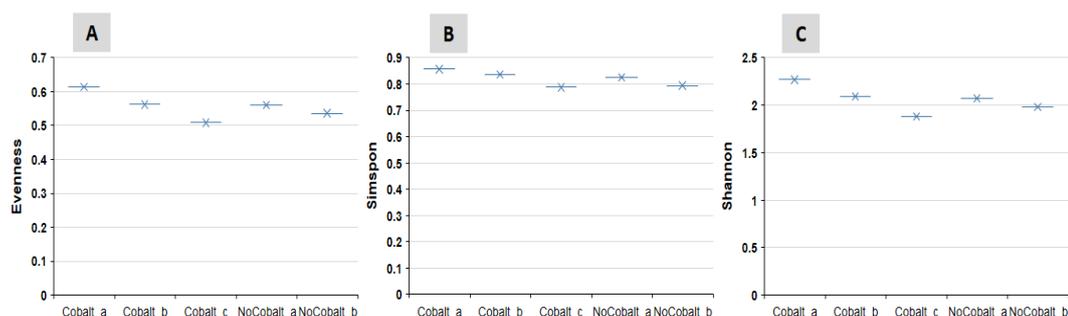


Figure 6.18: Standard alpha diversity boxplots of triplicate results of (A) evenness, (B) Simpson and (C) Shannon diversities. NoCobalt_c is missing because all samples were subsampled to an even depth of 32,000 reads per sample and it was below the threshold.

For the VFA- and cobalt-fed granules (Figure 6.19 (“cobalt”)) the dominant families were unclassified bacteria (8.8-14.6%), *Desulfuromonadaceae* (10.1-22.3%), *Methanobacteriaceae* (21.6-28.8%), *Methanosaetaceae* (22.1-30.4%) and *Rhodocyclaceae* (5.5-8.2%). It was reported that the dominant families for VFA-fed cobalt-deprived (“no_cobalt”) granules were unclassified *Bacteroidetes* (4.7-10.9%), *Desulfuromonadaceae* (20.5-24.0%), *Methanobacteriaceae* (15.7-35.0%) and *Methanosaetaceae* (9.2-29.0%) (Figure 6.19).

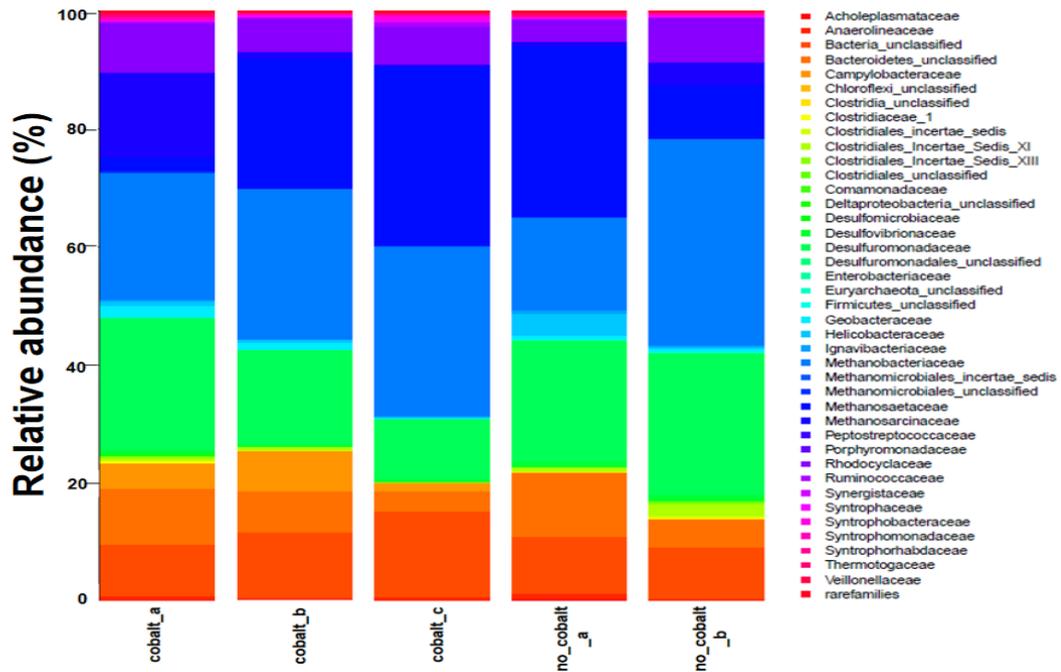


Figure 6.19: Barplot of the relative abundances of dominant families present within granules VFA-fed with (cobalt) and without cobalt (no_cobalt). NoCobalt_c is missing because all samples were subsampled to an even depth of 32,000 reads per sample and it was below the threshold.

Clustering was present for individual VFA-fed granules with and, separately, those without cobalt (Figure 6.20). Cobalt-deprived granules were separated from cobalt-fed granules within the CCA plot.

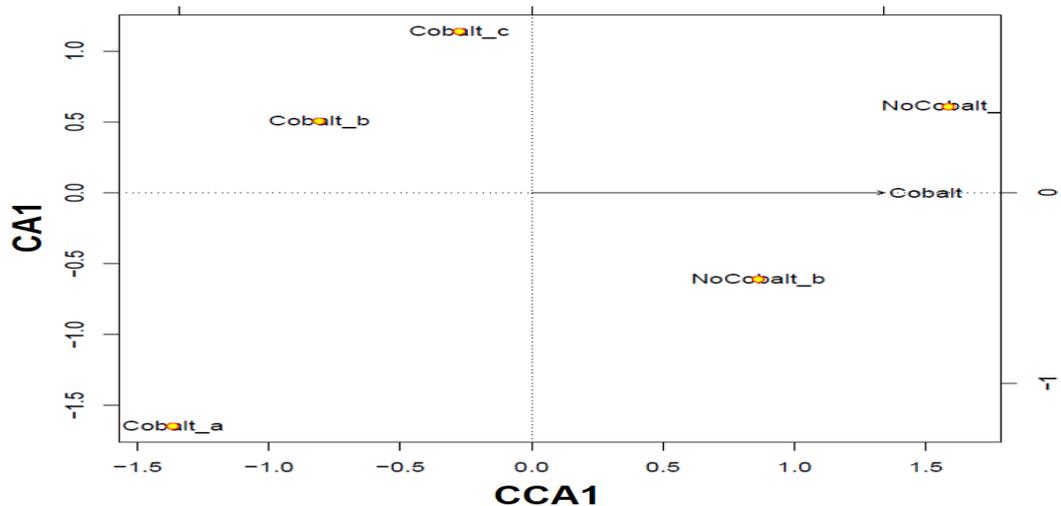


Figure 6.20: CCA ordination biplot of cobalt-fed and cobalt-deprived granules (circle symbols) and environmental parameter: cobalt deprivation (black arrow). NoCobalt_c is missing because all samples were subsampled to an even depth of 32,000 reads per sample and it was below the threshold.

The Adonis test resulted in no statistical differences among the cobalt dependent samples when tested against z ($p = 0.1$) and Euclidean distances ($p = 0.9$). The level of statistical significance was $p < 0.05$. Granules incubated with and without cobalt were not statistically different from each other in terms of their active community structure.

6.3.4.4.2 Acetate-fed cobalt-deprived granules

The alpha diversity metrics comprised of evenness, Simpson and Shannon indices (Figure 6.21). The maximum Simpson diversity across cobalt-fed and cobalt-deprived granules was 0.85 and the standard deviation was 0.07. The maximum Shannon diversity was 2.14 and the standard deviation was 0.23. The standard deviation of evenness was 0.06. There were no statistically significant differences between the acetate-cobalt-fed and the acetate-fed cobalt-deprived granules based on evenness ($p = 0.283$), Simpson diversity ($p = 0.121$) and Shannon diversity ($p = 0.121$).

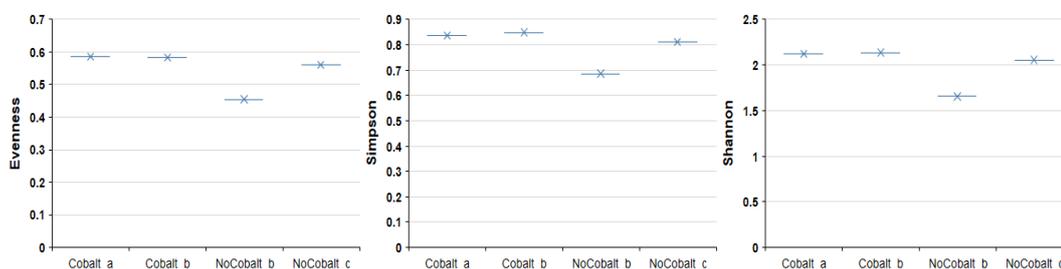


Figure 6.21: Boxplots depicting the alpha diversity tests of (A) evenness, (B) Simpson and (C) Shannon indices among individual granules. Cobalt_c and NoCobalt_a are missing because all samples were subsampled to an even depth of 32,000 reads per sample and they was below the threshold.

As illustrated earlier (Figure 6.12 (“acetate”)), the most dominant families for the acetate fed (control) granules were unclassified bacteria (15.6-23.9%), unclassified Bacteroidetes (10.5-17.6%), *Desulfovibrionaceae* (8.6-20.0%) and *Methanobacteriaceae* (12.4-20.0%). For the acetate-fed cobalt-deprived granules, the dominant families were unclassified bacteria (23.4-50.3%), *Campylobacteraceae* (9.3-11.1%), *Methanobacteriaceae* (21.8-30.9%) and *Rhodocyclaceae* (4.6-14.3%) (Figure 6.22).

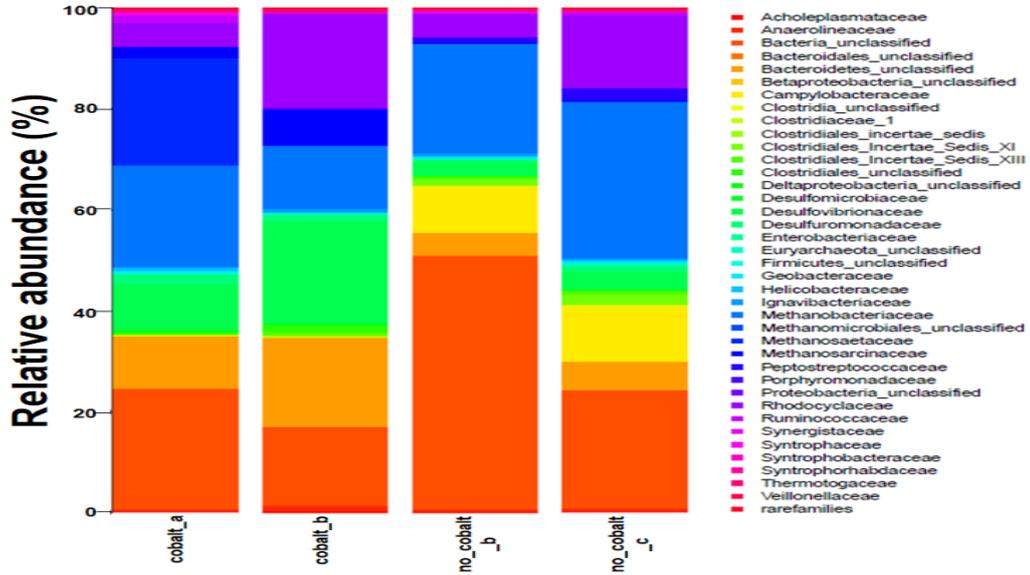


Figure 6.22: Barplot illustrating dominant families found from 16S rRNA amplicon Illumina sequencing of acetate-fed granules with and without cobalt. Cobalt_c and Nocobalt_a are missing because all samples were subsampled to an even depth of 32,000 reads per sample and they were below the threshold.

Some separation was visible between the two granule types on a CCA plot (Figure 6.23). They were not separated on the hierarchical dendrogram, but the cobalt-deprived granules were observably separated when correspondence analysis was performed.

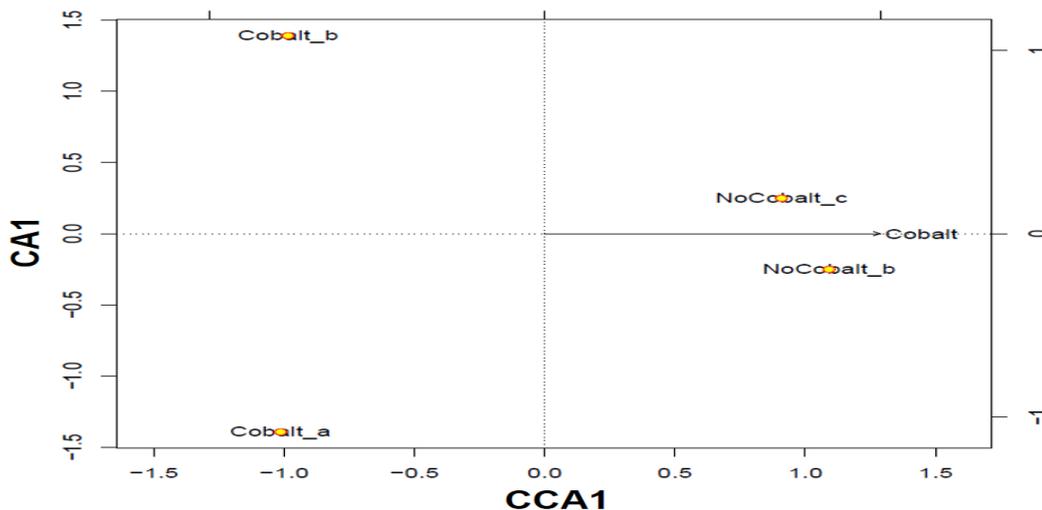


Figure 6.23: CCA plot illustrating the relatedness of active communities of anaerobic sludge granules that are fed acetate and cobalt (“Cobalt”) compared to individual granules that are acetate-fed but deprived of cobalt (“NoCobalt”). Cobalt_c and Nocobalt_a are missing because all samples were subsampled to an even depth of 32,000 reads per sample and they were below the threshold.

Adonis results indicated an absence of any statistically significant differences between the cobalt-dependent samples when tested against z ($p=0.67$) and Euclidean distances ($p=0.33$). Granules incubated with and without cobalt were not statistically different from each other.

6.3.4.5 Effects of methanogenic inhibitor on active 16S rRNA community structure of single granules

The evenness and Simpson and Shannon indices were illustrated in Figures 6.24-6.26. For all granules, both with and without BES, and at pHs of 7, 4 and 10, the maximum Simpson diversities were 0.86, 0.84 and 0.84 respectively and the standard deviations were 0.03, 0.16 and 0.08 respectively. The maximum Shannon diversities were 2.31, 2.24 and 2.24 respectively and the standard deviations were 0.18, 0.47 and 0.25 respectively. The standard deviations of evenness were 0.04, 0.13 and 0.07 respectively (Figure 6.24). Granules at pH 7 also contained the VFA mixture substrate and were incubated at a temperature of 37°C. There were no statistically significant differences and similarities between granules with and without BES granules based on evenness, Simpson diversity and Shannon diversity (Table 6.2)

Table 6.2: List of statistical significance results for all of the individual granules based on evenness, Simpson and Shannon indices. The level of significance was set at $p < 0.05$.

Condition	Evenness	Simpson	Shannon
pH 7 ± BES	0.677	0.93	0.825
pH 4 ± BES	0.268	0.234	0.273
pH 10 ± BES	0.763	0.96	0.67
acetate ± BES	0.876	0.423	0.925
cellulose ± BES	0.471	0.286	0.396
glucose ± BES	0.198	0.283	0.236
23°C ± BES	0.048	0.046	0.048
VFA-fed cobalt-deprived ± BES	0.1	0.223	0.164
acetate-fed cobalt-deprived ± BES	0.777	0.473	0.729

Across each of the granules fed with substrates acetate, cellulose and glucose, the maximum Simpson diversities were 0.85, 0.84 and 0.85 respectively and the standard deviations were 0.01, 0.03 and 0.01 respectively. The maximum Shannon diversities were 2.25, 2.14 and 2.29 respectively and the standard deviations were 0.07, 0.1 and 0.08 respectively. The standard deviations of evenness were 0.02, 0.03 and 0.02 respectively (Figure 6.25). There were no statistically significant differences between granules with and without BES granules based on both diversities (Table 6.2)

For granules at 23°C, VFA-fed cobalt-deprived granules and acetate-fed cobalt-deprived granules, the maximum Simpson diversities across granules with and without BES were 0.9, 0.88 and 0.82 respectively and the standard deviations were 0.28, 0.03 and 0.05 respectively. The maximum Shannon diversities for these environmental parameters were 2.72, 2.4 and 2.06 respectively and the standard deviations were 0.78, 0.17 and 0.15 respectively. The standard deviations of evenness were 0.18, 0.06 and 0.04 (Figure 6.26). The granules incubated at 23°C

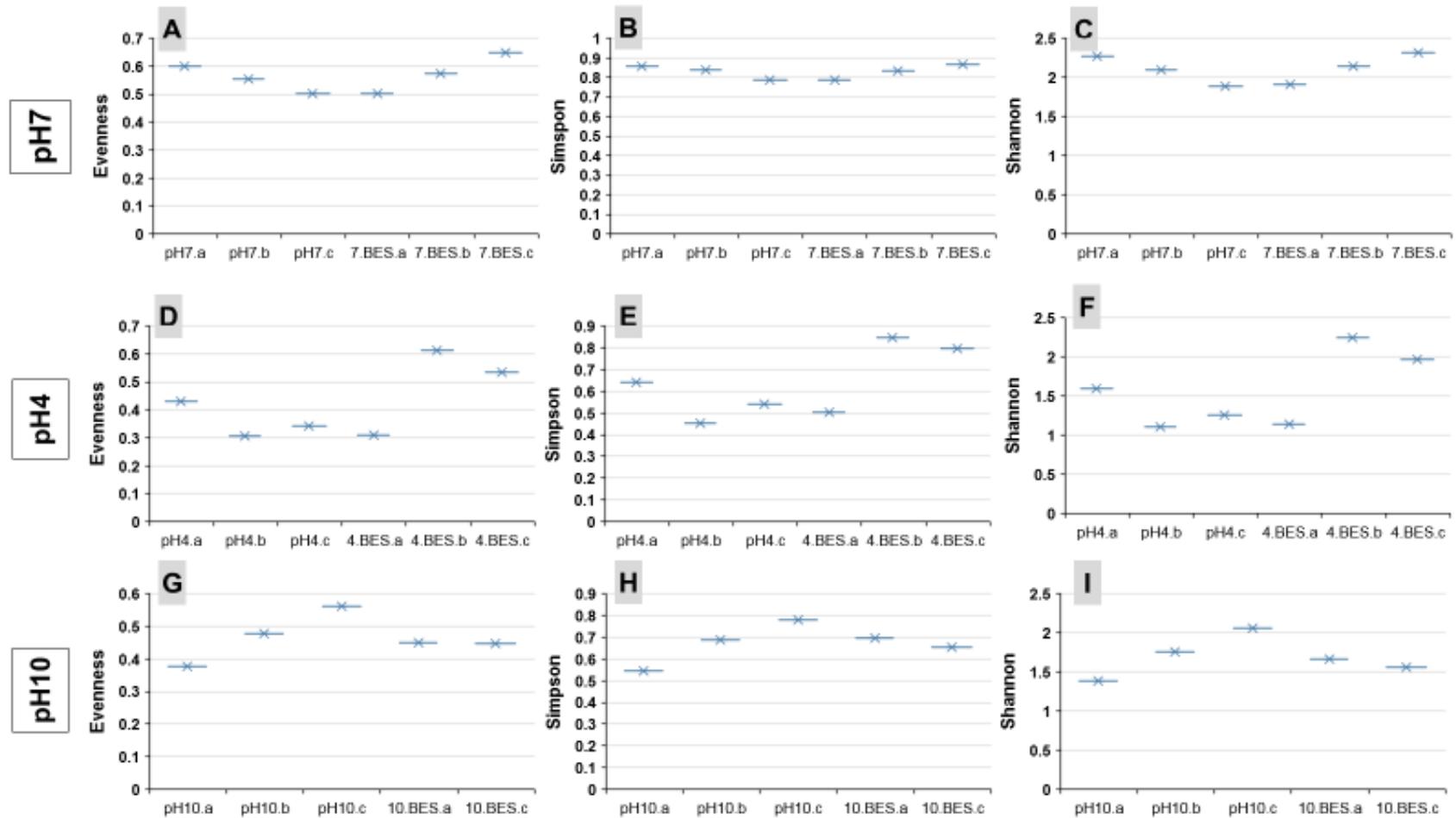


Figure 6.24: The alpha diversity tests of evenness and Simpson and Shannon indices as boxplots for each granule and set up to compare granules with and without BES under each pH: (A-C) pH 7, (D-F) pH 4 and (G-I) pH 10. 10.BES.b is missing because all samples were subsampled to an even depth of 32,000 reads per sample and it was below the threshold.

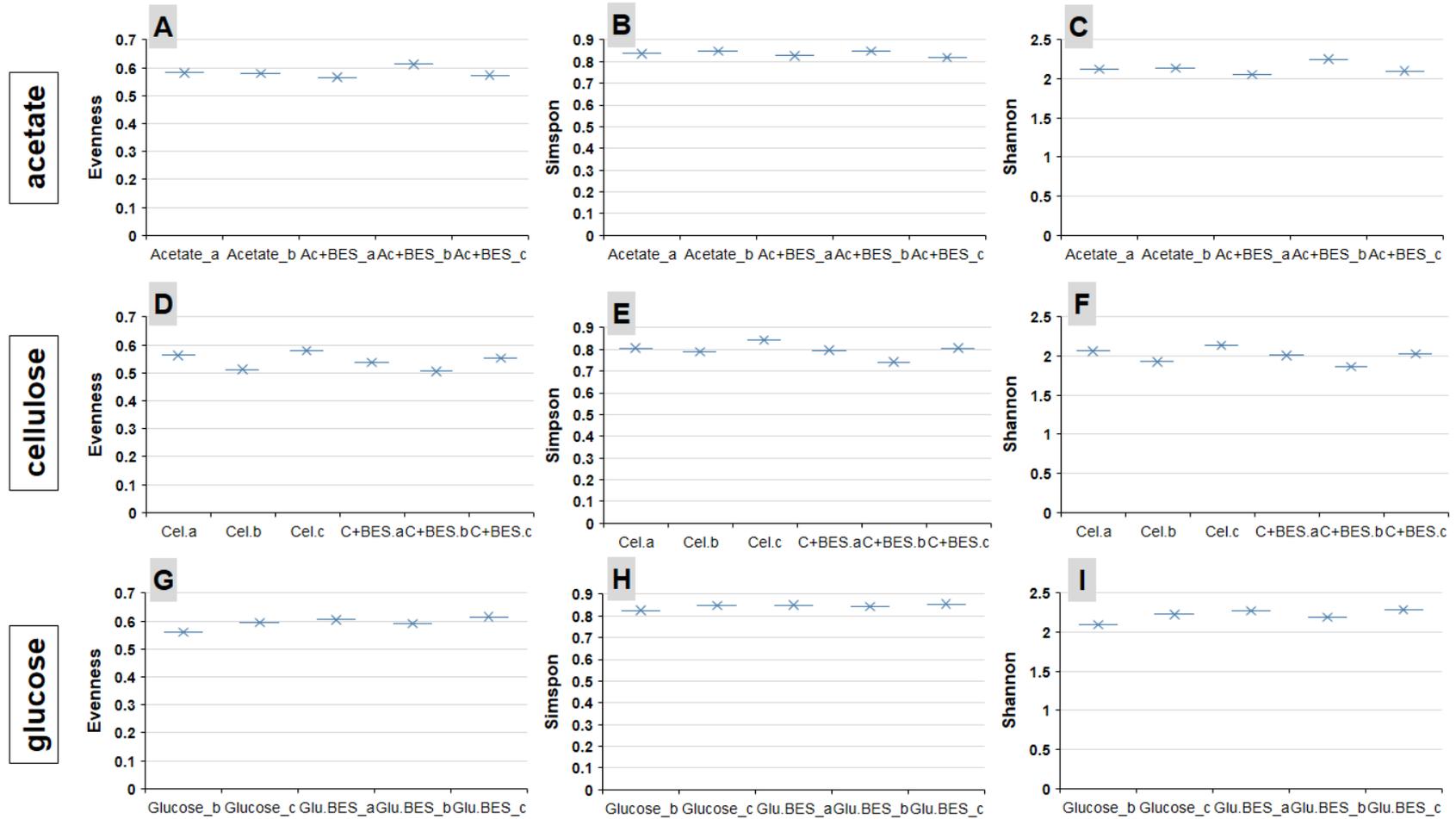


Figure 6.25: The alpha diversity tests of evenness, Simpson and Shannon indices for (A-C) acetate, (D-F) glucose and (G-I) cellulose. Some triplicate samples are missing because all samples were subsampled to an even depth of 32,000 reads per sample.

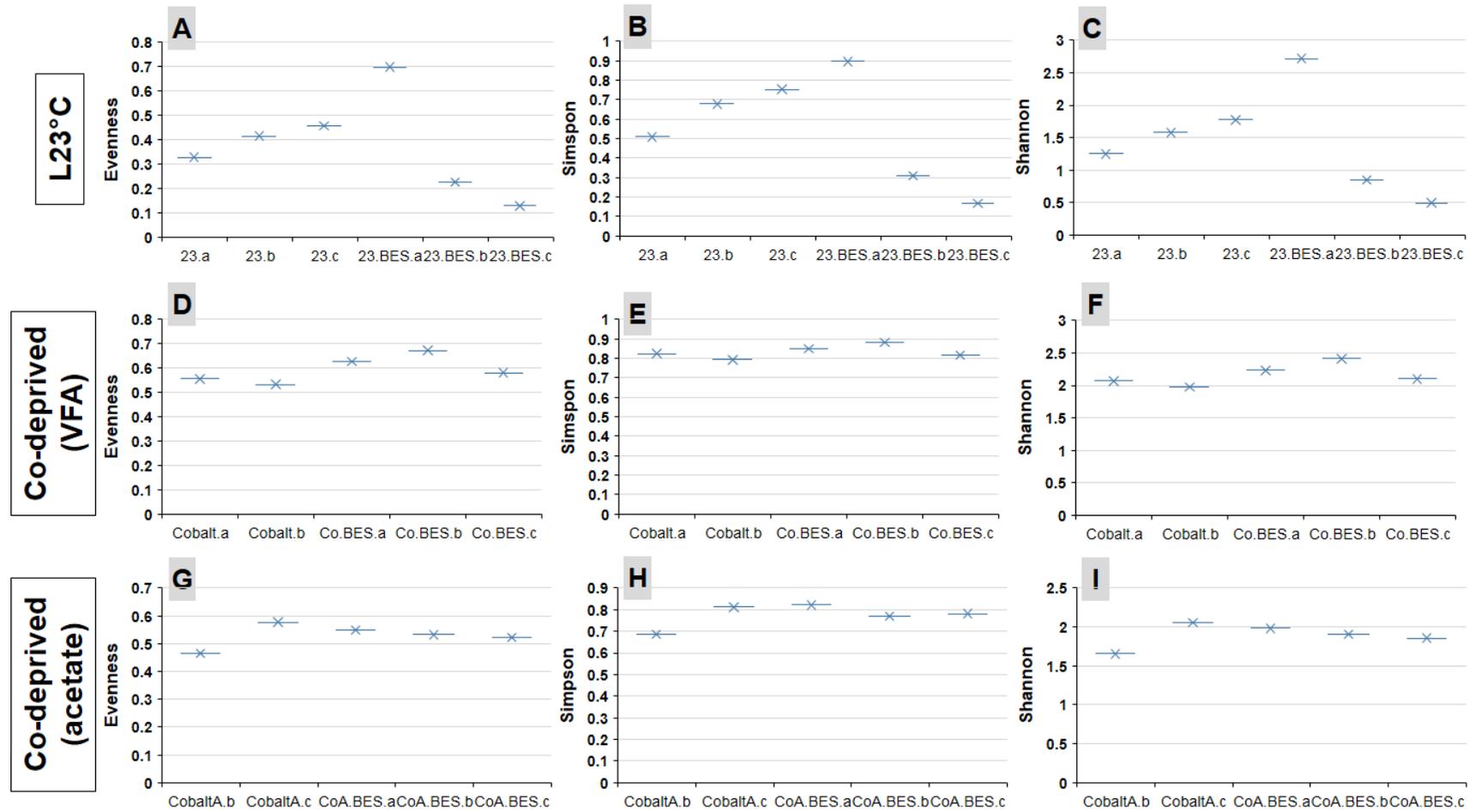


Figure 6.26: The alpha diversity tests of evenness, Simpson and Shannon indices for (A-C) 23°C, (D-F) VFA-fed cobalt-deprived and (G-I) acetate-fed cobalt-deprived granules. Some triplicate samples are missing because all samples were subsampled to an even depth of 32,000 reads per sample.

Using the information presented in the barplots (Figures 6.27-6.29), a summary of the two most dominant families was created (Table 6.3) for each environmental condition and compared against the same condition but with the addition of BES. The table excluded unclassified families. (9.3-11.1%). Overall, there was a BES effect on families, except for 23°C, and a lower effect for Co-deprived granules.

Table 6.3: The two most dominant families present in granules under the following environmental parameters: pH7, pH4, pH10, acetate-fed, cellulose-fed, glucose-fed, VFA-fed cobalt-deprived, acetate-fed cobalt-deprived. Each condition was compared to its counterpart containing BES.

Condition	Families	Condition	Families
pH 7	1. <i>Methanosaetaceae</i> 2. <i>Methanobacteriaceae</i>	pH 7+BES	1. <i>Desulphuromonadaceae</i> 2. <i>Desulfovibrionaceae</i>
pH 4	1. <i>Campylobacteriaceae</i> 2. <i>Methanobacteriaceae</i>	pH 4+BES	1. <i>Desulphuromonadaceae</i> 2. <i>Desulfovibrionaceae</i>
pH 10	1. <i>Methanobacteriaceae</i>	pH 10+BES	1. <i>Desulphuromonadaceae</i>
acetate	1. <i>Alcaligenaceae</i> 2. <i>Campylobacteriaceae</i>	acetate+BES	1. <i>Desulfovibrionaceae</i> 2. <i>Rhodocyclaceae</i>
cellulose	1. <i>Methanosaetaceae</i> 2. <i>Methanobacteriaceae</i>	cellulose+BES	1. <i>Desulfovibrionaceae</i> 2. <i>Rhodocyclaceae</i>
glucose	1. <i>Methanosaetaceae</i> 2. <i>Methanobacteriaceae</i>	glucose+BES	1. <i>Desulphuromonadaceae</i> 2. <i>Desulfovibrionaceae</i>
23°C	1. <i>Alcaligenaceae</i> 2. <i>Campylobacteriaceae</i>	23°C+BES	1. <i>Alcaligenaceae</i> 2. <i>Campylobacteriaceae</i>
VFA-fed, Co-deprived	1. <i>Desulphuromonadaceae</i> 2. <i>Methanobacteriaceae</i>	VFA-fed, Co-deprived +BES	1. <i>Desulphuromonadaceae</i> 2. <i>Desulfovibrionaceae</i>
acetate-fed, Co-deprived	1. <i>Campylobacteriaceae</i> 2. <i>Methanobacteriaceae</i>	acetate-fed, Co-deprived +BES	1. <i>Campylobacteriaceae</i> 2. <i>Pseudomonadaceae</i>

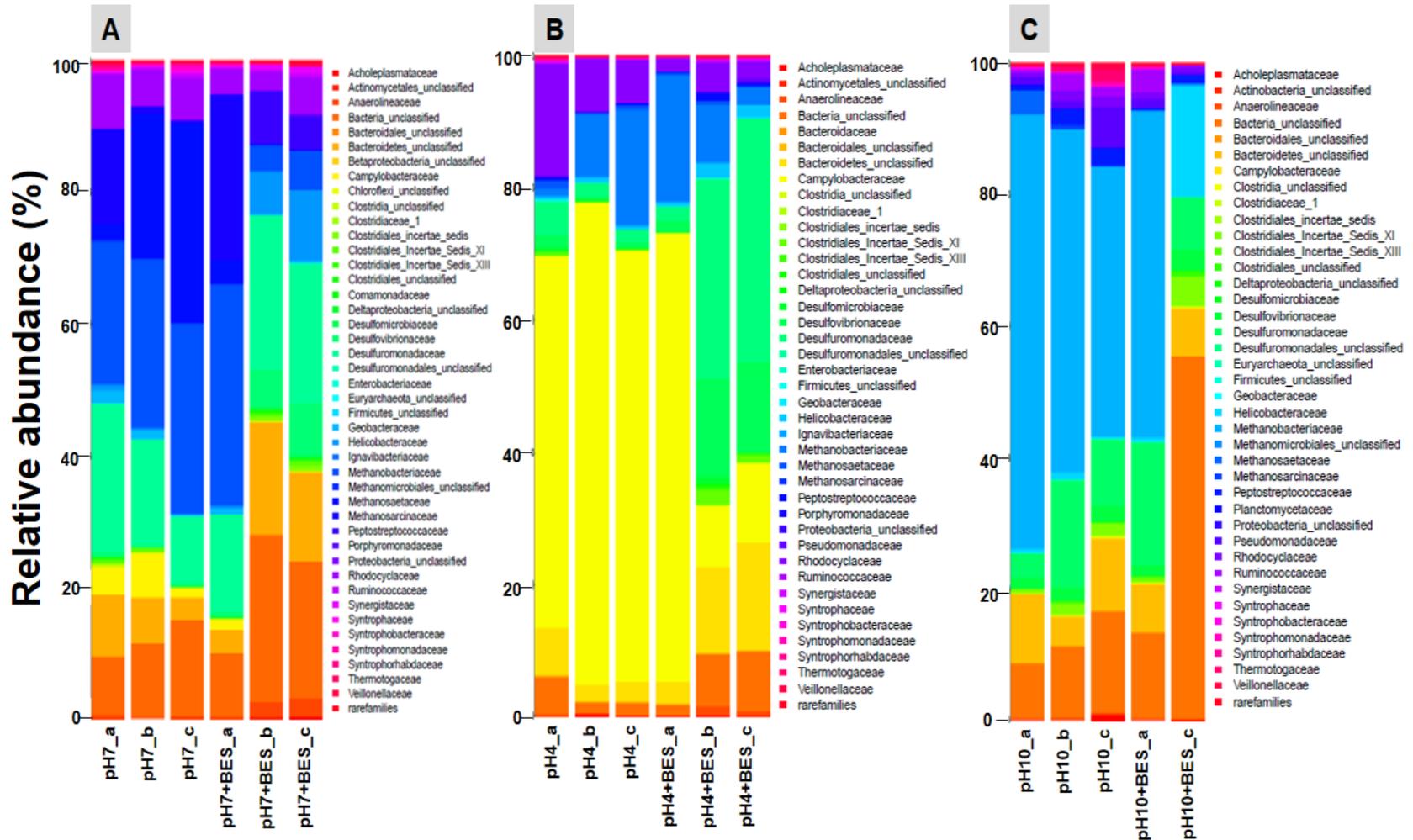


Figure 6.27: Barplot of each of the substrates VFA-fed, 37°C (A) pH 7, (B) pH 4 and (C) pH 10 with its counterpart inhibited condition i.e. addition of the methanogenic inhibitor BES on the right of each plot. 10.BES.b is missing because all samples were subsampled to an even depth of 32,000 reads per sample and it was below the threshold.

Clustering was observed in granules with and without BES for the different conditions studied (Figure 6.30) and illustrated on a CCA plot (Figure 6.31). There was some separation of granules fed with BES from granules whose media did not contain BES (which was most pronounced for granules at the following substrates and conditions; cellulose, glucose, VFA-fed cobalt-deprived and acetate-fed cobalt-deprived). BES separation was also observed in some CCA plots, such as those for granules at the following conditions: pH 10, acetate, cellulose, glucose, 23°C, VFA-fed cobalt-deprived and acetate-fed cobalt-deprived. Overall, there was no effect of BES on clustering of the granular active community structures at any condition.

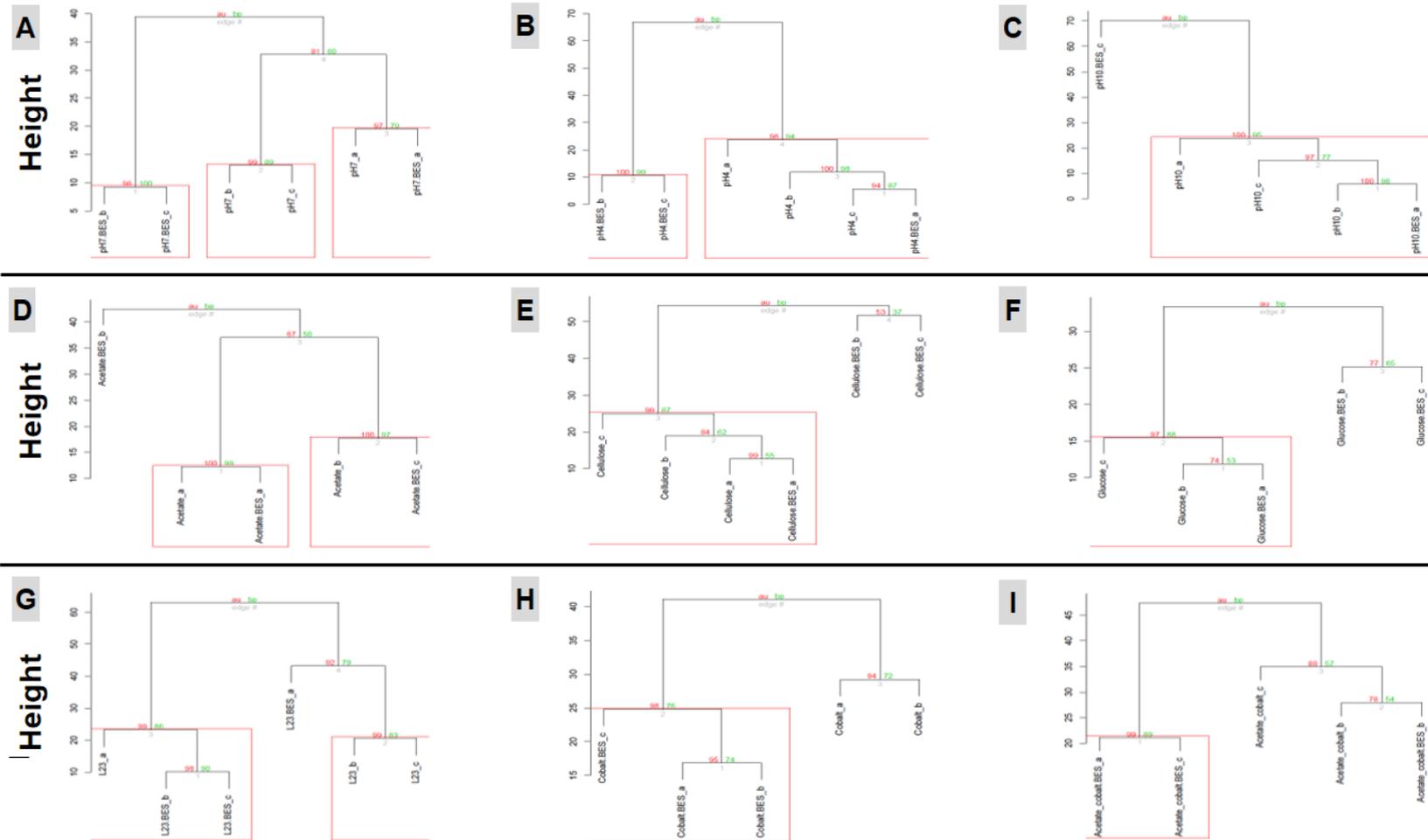


Figure 6.30: Cluster dendrograms for each condition with and without the BES inhibitor; (A) 37°C, VFA- and cobalt-fed, pH7 (control granules), (B) 37°C, VFA and cobalt-fed, pH4, (C) 37°C, VFA and cobalt-fed, pH10, (D) 37°C, acetate and cobalt-fed, pH7 (E) 37°C, cellulose- and cobalt-fed, pH7, (F) 37°C, glucose- and cobalt-fed, pH7, (G) 23°C, VFA- and cobalt-fed, pH7, (H) 37°C, VFA-fed, cobalt-deprived, pH7 and (I) 37°C, VFA-fed, cobalt-deprived, pH7. Some triplicate samples are missing because all samples were subsampled to an even depth of 32,000 reads per sample.

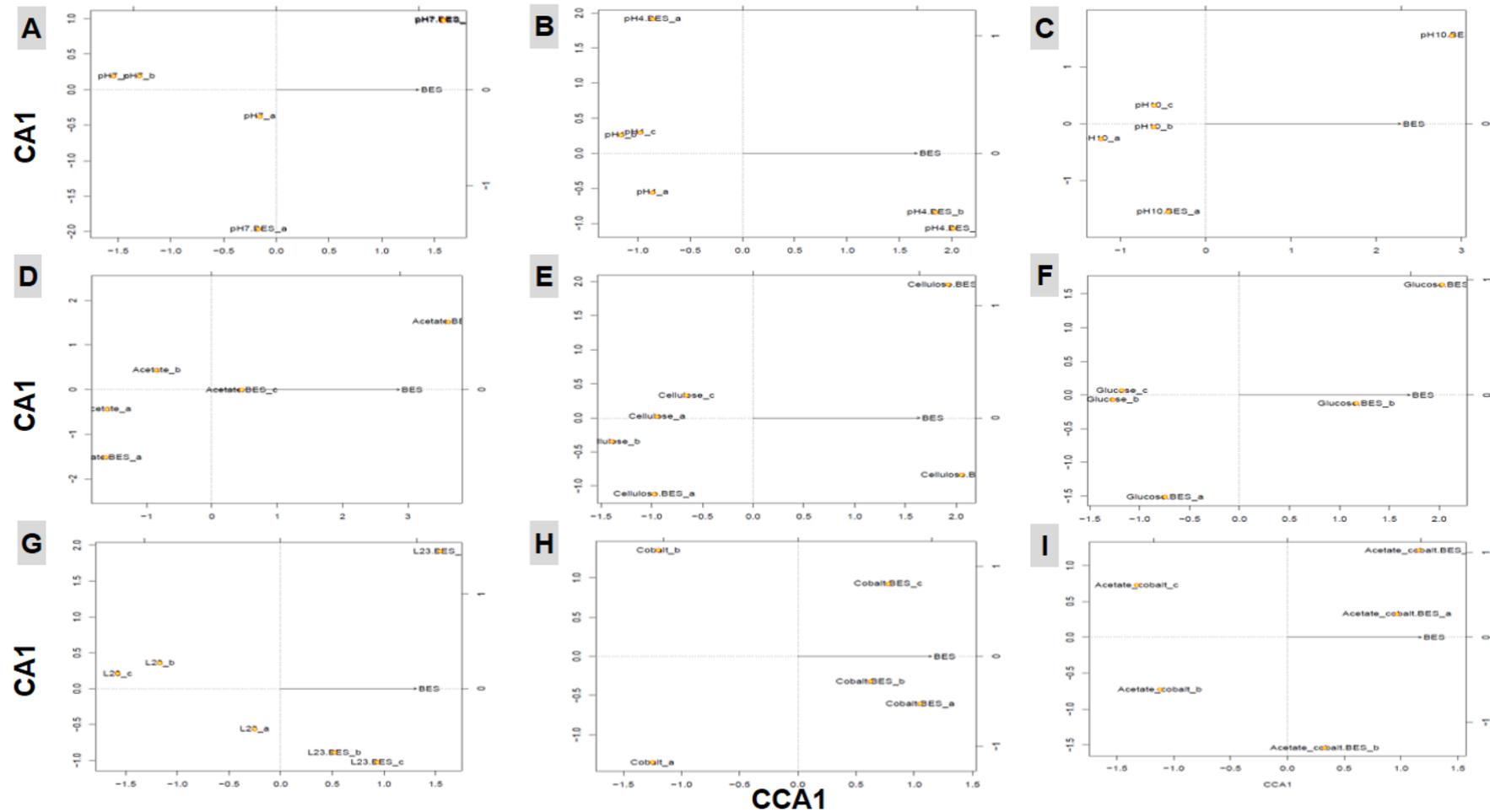


Figure 6.31: CCA ordination biplots for each condition with and without the BES inhibitor; (A) 37°C, VFA- and cobalt-fed, pH 7 (control granules), (B) 37°C, VFA and cobalt-fed, pH 4, (C) 37°C, VFA and cobalt-fed, pH 10, (D) 37°C, acetate and cobalt-fed, pH 7 (E) 37°C, cellulose- and cobalt-fed, pH 7, (F) 37°C, glucose- and cobalt-fed, pH 7, (G) 23°C, VFA- and cobalt-fed, pH 7, (H) 37°C, VFA-fed, cobalt-deprived, pH 7 and (I) 37°C, VFA-fed, cobalt-deprived, pH 7. Some triplicate samples are missing because all samples were subsampled to an even depth of 32,000 reads per sample.

Statistical analysis was carried out on each environmental condition both with and without BES via the Adonis test (Table 6.4). There were no significant differences in the single granular active community structures between those granules with and without BES for each condition.

Table 6.4: List of statistical significance results based on z and Euclidean distances. Each condition refers to the multivariate significance tests between individual granules with and without BES at a specific condition.

	z	Euclidean
pH 7	<i>0.18</i>	<i>0.3</i>
pH 4	<i>0.27</i>	<i>0.3</i>
pH 10	<i>0.2</i>	<i>0.3</i>
Acetate	<i>0.3</i>	<i>0.09</i>
Cellulose	<i>0.1</i>	<i>0.4</i>
Glucose	<i>0.2</i>	<i>0.3</i>
23°C	<i>0.5</i>	<i>0.7</i>
VFA-fed cobalt-deprived	<i>0.1</i>	<i>0.1</i>
Acetate-fed cobalt-deprived	<i>0.1</i>	<i>0.2</i>

p-value of significance is $p < 0.05$.

6.4 Discussion

This series of environmental impacts from specific stresses has presented a myriad of data from which some preliminary conclusions can be drawn (Rillig et al., 2017). Many results from both the VFA-sugar profiling and the study of active microbial community structures under various environmental stresses, were noteworthy. The primary result was that single granules had similar metabolic and phylogenetic profiles under specific environmental conditions.

6.4.1 Physiological and phylogenetic pH impacts on VFA-fed communities

The VFA profiles (Figure 6.3-6.5) and the main findings of general VFA consumption by anaerobic granules in this work concurred with a previous study focused on granular VFA profiles under different pHs. A VFA study on individual anaerobic granules was not found in the literature. Overall, the highest acetic acid consumption occurred at pH 7, followed by pH 4, and this is presumably due to the optimal pHs of many studied anaerobic bacteria and archaea being in the range of pH 6 to pH 7.5 (Capri and Marais, 1975, Liu et al., 2008). The lowest VFA concentrations across the 48-hour batch time occurred at pH 10, indicating that it is not a preferable pH level within the AD process. Previous studies have investigated certain species of extremophiles, the type of organism that can survive in harsh environments, and found that they can withstand very alkaline environments such as microalga *Spirulina* (Nolla-Ardèvol et al., 2015). However, for the majority of archaea and bacteria, the optimal pH levels are close to neutral.

The bacterial and archaeal cDNA consortia identified several abundant families that were involved in active metabolism. *Methanosaetaceae* dominated at pH 7 in VFA-fed single granules. *Methanosaeta concilli* is a key microorganism in the granulation process of anaerobic systems (Hulshoff Pol et al., 2004). *Methanobacteriaceae* were common at each pH. A previous study found *Methanobacteriaceae* to be the most abundant hydrogenotrophic methanogen (Griffin et al., 1998). The main difference in microorganism presence was found in granules incubated with a medium at pH 4. *Campylobacteriaceae*, a type of Proteobacteria which has not been previously reported to be found in mesophilic anaerobic sludge granules in the AD molecular ecology literature, was present in granules incubated at pH 4. Numerous studies have looked at the effect of pH differences in granular-based bioreactors with various substrates (Zoetemeyer et al., 1982, Horiuchi et al., 2002) and it has been suggested that pH may be an effective strategy for the control and maintenance of a stable microbial community (Zhang et al., 2016).

This study demonstrated the possibility of investigating the adaptation of granules' responses to pH stresses at a single-granule level. The MiSeq analyses (Figure 6.10)

illustrated that individual granules shift their active communities in a replicated pattern. The Adonis test indicated significant differences between the three pH studied.

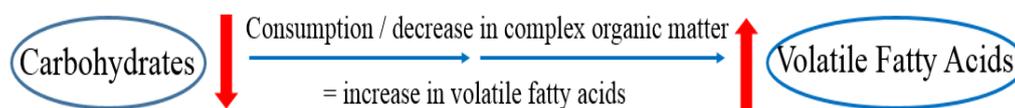
Previous studies have suggested that acetoclasts are inhibited at pHs of less than 6, whereas hydrogenotrophs may survive at that level (Batstone et al., 2002). Acetogens have a large pH gradient range of between pH 5 and pH 8, but their optimal level is circumneutral (Balch et al., 1977, Slonczewski and Foster, 2011) and there is less methanogenesis at very high pH levels. The optimal pH level is usually either close to neutral or slightly below (Ferry, 1993). *Methanobacterium* cells have an optimal growth at pH 6.9 to 7.2 and *Methanococcales* grow optimally at pH 6.7 to 7.4 (Balch et al., 1979).

The barplots (Figure 6.8) illustrated a dominance of *Methanosaetacea* and *Methanobacteriaceae* at pH 7, *Campylobacteriaceae* and *Methanobacteriaceae* at pH 4 and, surprisingly, *Methanobacteriaceae* were also dominant in the active community structure at pH 10. *Methanobacteriaceae* are presumably in granules at each pH because it has a wide range of metabolic potential in both methanogenesis and nitrogen fixation. Although it has a lower optimal pH, *Methanobacteriaceae* was detected in ultrabasic springs at pH 12 and was found to be dominant in the 16S rRNA sample sequencing in some previous studies. However, no *Methanobacteriaceae* sequences were found during metagenomic sequencing (Quéméneur et al., 2015, Brazelton et al., 2017).

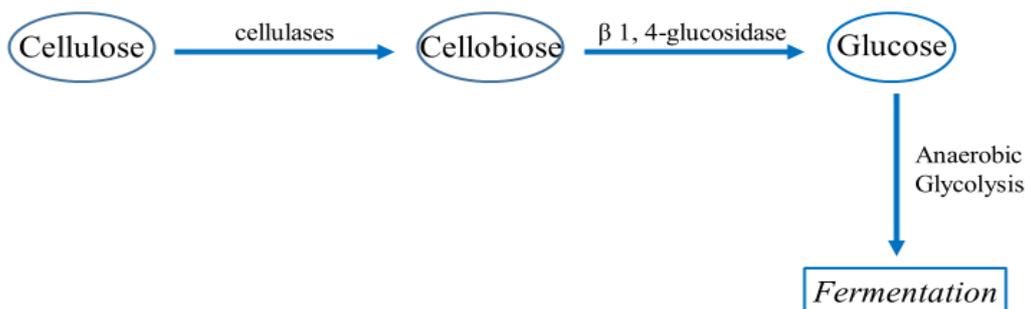
6.4.2 Differences and similarities in the active community structure of single granules fed with a panel of substrates

The sugar-VFA assay demonstrated similar concentrations of sugar consumption and VFA accumulation among eight individual granules based on the standard deviation error bars (Figure 6.5) and, therefore, that granular metabolism may be replicated. The results reported that granules fed with either cellulose or glucose had statistically different consumption rates. Moreover, BES did not have an impact on the fermentative consumption rates. This was as expected; BES should not have any effect on the fermentative activity as it is a methanogenic inhibitor further on

in the AD process. There was an overall decrease of sugars, which coincided with the increase in VFAs (Figure 6.5). This is consistent with the AD pathway thermodynamic process:



The main observable difference was that there was a faster degradation of glucose and hence a faster and higher increase of VFAs (Figure 6.5 (C and D)) than occurred with cellulose-fed granules (Figure 6.5 (A and B)). Glucose may have had a faster overall consumption rate because it is a simpler sugar than cellulose in that it has a shorter degradation pathway (Noike et al., 1985). Cellulose is a linear polymer made of glucose subunits linked by β -1, 4 bonds. Cellulose degradation occurs as follows (Sylvia et al., 2005):



Glucose utilisation has previously been reported in a UASB study where glucose concentrations of between 100 and 1000 mg l⁻¹ were applied as a substrate (Subramanyam and Mishra, 2007).

The sugar-fed granules – both those fed with cellulose and with glucose - had a lower VFA profile (Figure 6.5), with a maximum concentration of 350 mg/l for VFA production. By comparison, the VFA-fed (Figure 6.3) and acetate-fed (Figure 6.4) granules had a VFA concentration of up to 2000 mg/l. The lower VFA production in the cellulose- and glucose-fed granules may be explained by the smaller quantities of fermentative microbes within the granules. MiSeq active community analysis reported mainly acetogenic bacteria and archaea in all of the granules, indicating that methanogenesis was the main active process present.

These microbial populations have vital metabolic capabilities for AD systems and are important in global biogeochemical cycling (Offre et al., 2013, Shah et al., 2014).

The microbial consortia in all granules containing either VFA or carbon substrates had a large proportion of families that had a relative abundance of < 1.5% (Figure 6.12). *Methanobacteriaceae* was the common abundant family for each substrate and at each pH (section 6.4.1). Additionally, it was reported that one of the most abundant families for acetate- and glucose-fed granules was *Bacteroidetes*, whereas cellulose-fed and glucose-fed granules' active communities included a large proportion of *Methanosaetaceae*. *Bacteroidetes* is one of the four major phyla of bacterial domains (Nelson et al., 2011, Sundberg et al., 2013a, Carballa et al., 2011) and has been reported to be dominant within anaerobic digesters at mesophilic temperatures (Lee et al., 2012) where it can utilise a broad range of substrates (Tracy et al., 2012, Merlino et al., 2013), such as acetate and glucose as in this study.

It has previously been suggested that *Bacteroidetes* are responsible for polysaccharide breakdown and fermentative processes (Vanwonterghem et al., 2014), and a combined metagenomic-metaproteomic study of an anaerobic digester which degraded plant carbohydrates revealed that *Bacteroidetes* expressed a large number of sugar transporters (Hanreich et al., 2013). *Methanosaetaceae* is an acetate-utilising family of anaerobes which is inclined to decrease upon increases of VFA and the other acetoclastic family, *Methanosarcinaceae* (Padmasiri et al., 2007, Blume et al., 2010, Chelliapan et al., 2011). *Methanosaetaceae* has been reported in sewage sludge digesters with low amounts of VFAs, which accounts for its abundance in cellulose and glucose-fed granular communities over granules fed with acetate and the VFA mixture (Karakashev et al., 2005). Statistical analysis confirmed significant differences between the granules fed with different substrates, as expected, indicating a definite shift or adaptation of active communities from the original VFA-fed "control" granules.

6.4.3 The effects of low temperature on individual granules

23°C was a sub-mesophilic temperature for the single anaerobic granules in that the results reported a comparatively slower VFA consumption profile for granules incubated at this temperature (Figure 6.3 (D)) than for granules incubated at 37°C (Figure 6.3 (A)). These results demonstrated less acidogenic and acetogenic activity. This was as expected because studies of low and psychrophilic temperatures have previously reported decreases in microbial growth (Pennington, 1908, D'Amico et al., 2006) and, therefore, demonstrated slower metabolisms and substrate consumption and accumulation such as in this study.

16S rRNA MiSeq analysis revealed that active microbial consortia within granules at incubated 37°C were different to those within granules incubated at 23°C. This work highlighted an active 16S community shift to 23°C with such families such as *Alcaligenaceae* (3.9-39.1%), *Campylobacteriaceae* (36.0-68.0%) and *Methanosaetaceae* (15.5-18.8%) being abundantly present. The *Alcaligenaceae* family has not been reported within biogas reactor community analyses often at mesophilic or psychrophilic temperatures (Kratat et al., 2011, Xafenias and Mapelli, 2014, Campanaro et al., 2016). *Campylobacteriaceae* are generally not found in anaerobic digesters but they were reported in a study whereby they were dominant in a psychrophilic seeding community from farm effluent (Goux et al., 2016). *Methanosaetaceae* were previously found to be highly abundant in low temperature AD systems highlighting their importance at sub-mesophilic conditions (Bialek et al., 2013, Gunnigle et al., 2015) even with environmental stresses such as sulfate addition (Madden et al., 2014).

Cluster and CCA plots illustrated and verified these differences by their physically separating of triplicate granules according to temperature (Figures 6.16 and 6.17). Although Adonis tests did not show significant differences between the two temperatures, it is clear from the previous analyses that there were community separations. The Adonis result contrasted with all of the previous phylogenetic data (alpha diversity tests, barplots, cluster dendrogram and the CCA plots shown previously). This inconsistency indicates that the statistical test may not be robust enough for sequencing analysis and that the other sequencing analysis demonstrated that there were differences between the granular communities at the higher and

lower temperatures. Also, comparing to the pure culture studies in Chapter 3, it was seen that there were significant differences in growth between high and low temperatures. Further studies of these granules at additional lower temperatures would be beneficial as they would provide a specific insight into low temperature adaptation at a single-granule level. Ideally, such further studies would be coupled with 'omics studies to link structure with function and metabolisms.

6.4.4 Impact of cobalt deprivation on individual granular community metabolism and structure

Lower VFA consumption concentrations were observed for both VFA- and acetate-fed granules that were deprived of cobalt (Figure 6.3 (E) and Figure 6.4 (B), respectively) than for their counterpart cobalt-fed granules (Figure 6.3 (A) and Figure 6.4 (A), respectively). A previous study looked at cobalt in grass-clover silage AD and found higher rates of methane and acetate degradation (Jarvis et al., 1997). In comparison, a UASB that was methanol-fed and cobalt-deprived had low methanogenic activity and it was suggested that it had shifted from a methanogenic to a methylotrophic pathway (Zandvoort et al., 2002). The authors used a dosing strategy whereby the final high dosage of 0.84 μM had a significant impact upon the increase of methanogenic activity. Cobalt is important for the growth of acetogens but it is difficult to determine whether they require trace metals because they can efficiently use other trace metals within media (Ljungdahl, 1986). This μSBR experiment used 2.1 μM of cobalt. Perhaps there is a greater number of acetoclastic methanogens utilising acetate compared with acetogens producing it as evident in Figure 6.4. This equated to 0.5 mg/l of cobalt in the media; previous studies have determined that the stimulatory concentration of cobalt is between 0.03 – 19 mg/l with an inhibitory concentration of 35 – 950 mg/l (Kida et al., 2001, Gikas, 2007, Fermoso et al., 2009, Ma et al., 2009, Worm et al., 2009, Romero-Güiza et al., 2016).

The reported decrease in VFA metabolism in this study is possibly due to a decrease in the active community, further strengthening the case for the importance of cobalt to granular growth. A recent study suggested that the addition of cobalt alone increases acetogenesis rather than methanogenesis (Moestedt et al., 2016). There

was a decrease in the VFA profile of acetate-fed Co-deprived granules (Figure 6.4 B). Moreover, additional micro- and macro-nutrient deprivation would pinpoint the precise effect and impact they have on granules on an individual level and what changes they make to the active granular microbial communities.

The main distinguishing features of granular active communities which were cobalt-deprived included the presence of *Bacteroidetes* for VFA-fed cobalt-deprived granules and the addition of both *Campylobacteriaceae* and *Rhodocyclaceae* for acetate-fed cobalt-deprived granules. The abundance of *Bacteroidetes* could be due to it being a metabolically diverse family and it may have grown more in these granules in order to help them to survive with the absence of cobalt.

As previously mentioned, *Campylobacteriaceae* have rarely been found in anaerobic digesters but there have been reports of them (*Arcobacter*) in anaerobic systems such as an abundance found in continuous upflow reactor samples incubated in an anaerobic chamber (Saia et al., 2016). In this study, it was found in particularly harsh conditions such as at pH 4 and at 23°C. Perhaps it survived was because it is ubiquitous throughout nature and can be found in many different environments from the gut to anaerobic environments to some aerobic environments making it a very versatile family of microorganisms.

In one particular study, *Rhodocyclaceae*-related species were selectively enriched and were considered to be involved in acetogenesis (Yang et al., 2015), while another study connected them to the anaerobic benzene degradation process (van der Zaan et al., 2012). Final statistical analysis after CCA plotting (Figures 6.20 and 6.23) reported no significant difference between the triplicate cobalt-deprived granules and the non-cobalt-deprived granules, but earlier preliminary metabolic analysis did demonstrate observable differences in VFA profiles. There were also microbe abundance discrepancies in the 16S rRNA data, so further analysis or longer experimentation with a higher number of single granule replicates would be required to delve deeper into nutrient/metal deprivation and their precise roles in granular structure and function through 'omics studies.

6.4.5 The inhibitory effect of BES on the VFA and community structure profiles of individual granules

For each single granule studied, VFA profiles showed that the application of BES to media generally resulted in higher yields of VFAs at the end of each 48-hour batch (Figures 6.3, 6.4 and 6.5 (B) and (D)). This indicated that homoacetogenic activity might have been stimulated (Harriott and Frazer, 1997) with either a decrease or cessation of syntrophic acetate oxidation or methanogenic activity, or with both. One possible reason why the VFAs stopped being consumed at the end of the experiment could be that SAOBs could not consume as much acetate as when methanogens were not inhibited and could consume more acetate when they worked together. A related study examined antibiotics and BES in relation to the fate of VFA during the AD process via batch experiments. The authors found that BES demonstrated complete inhibition of VFAs during specific degradation pathways and inhibited both methanogenic routes (Lins et al., 2015).

Unexpectedly, there was no significant difference in the evenness, Simpson and Shannon indices between granules with and without BES. This could be because there appeared to be one outlier sample in each of the BES triplicate samples that was more similar to the non-BES-fed granules. This may have skewed the statistical results.

A further study has also demonstrated that BES is capable of enhancing the sulfate-reducing bacterial community within anaerobic paddy soil (Hu et al., 2012). A comparison between granules fed with BES and granules where BES was absent resulted in an abundance of two specific families, *Desulfuromonadaceae* and *Desulfovibrionaceae* (Table 6.3), across the conditions where granules were BES-fed. Possibly, when the methanogenic community was inhibited by BES, an alternative pathway was switched on - the sulfate pathway. It is noteworthy that the same top two families were present for granules incubated at 23°C. This could be due to insufficient time being allowed for granular sulfate-reducing active species to adapt and shift.

Desulfuromonadaceae and *Desulfovibrionaceae* are both strictly anaerobic sulfate-reducing families, although *Desulfuromonadaceae* has some tolerance for oxygen. Members of both families are all either mesophilic or slightly psychrophilic.

Desulfuromonadaceae family members are ubiquitous in the anaerobic environment, particularly in marine and freshwater sediments (Greene, 2014). *Desulfovibrionaceae* species have been found in biofilms and sewage sludge, where they incompletely oxidise organic substrates to acetate (Kuever and Rabenstein, 2005).

Individual cluster (Figure 6.30) and CCA (Figure 6.31) plots did not show a clear separation of BES-fed granules from their non-BES counterparts, but there did appear to be shifts away from the VFA-fed 37°C, pH “control” granules in the active community structure when BES is present. However, these shifts were not conclusively replicated. Given more time and/or an increase in BES concentration within the media, the active granular structure may be able to adapt in a more replicated manner with complete methanogenic inhibition. Further high-throughput studies are required.

6.5 Conclusions

In summary, the primary conclusions that can be drawn from this μ SBR study are that both metabolic (VFA profiles) and phylogenetic characteristics shift in a similar way when individual granules are subjected to environmental stresses or influences. This is a unique study which demonstrates both the chemical and the molecular traits of the active microbial community at the level of a single granule. The highest level of VFA consumption was observed at pH 7 and the lowest level at pH 10. Granules at pH 4 are the most different in terms of phylogenetic profile. *Methanobacteriaceae* was the common at the 3 pH values

Substrates were found to determine the active microbial structures. Acetate degraders include *Methanosaetaceae*, which have a much higher relative abundance in the pH 7 granules (22-30%) than in the pH 4 (0.1-1.1%) and pH 10 (0.1-3.56%) granules. Cellulose- and glucose-fed granules consumed their respective sugar feeds and accumulated more acetic, propionic and butyric acid than the VFA- and acetate-fed granules did due to increased acidogenesis or fermentation, whereas the latter two substrates were involved in acetogenesis.

BES-fed granules demonstrated higher VFA profiles because methanogenesis is decreased as shown by the active community MiSeq analysis barplots (Figures 6.8, 6.12, 6.15, 6.19, 6.22, 6.27-6.29). Illumina sequencing indicated that the presence of various microbial families, specifically *Desulfuromonadaceae* and *Desulfovibrionaceae*, were important for continued growth despite the inhibitor within the environment. Without methanogens to consume acetate, it accumulates via homoacetogenesis. Acetate could be consumed by syntrophic acetate oxidising bacteria but, if they were present, they did not consume as much acetate as methanogens could in the granules without BES. They were not found in the 16S rRNA analysis as an abundant microorganism. Both lower temperature and cobalt deprivation reported consistently lower VFA concentrations, indicating slower growth in these granules.

It is clear that the active communities based on 16S rRNA exhibited significant shifts within microbial populations of individual granules under most environmental stresses that were examined. The common species to all pHs was *Methanobacteriaceae*, which demonstrated its metabolic versatility. Granules can adapt to environmental shocks in a similar way, forming a similar species profile, which is significantly different to a control environment of VFA-fed granules at pH 7 incubated at 37°C.

An important result bias was indicated during the barplots analysis was observed where one BES sample had a very similar community profile to the three granule samples without BES. Perhaps this sample could be excluded for comparative analysis but was left in for this study to get a full picture with all of the samples.

This experimental set-up presents a great potential for high-throughput investigation of individual granules representing environmental whole ecosystems and could also represent a way to investigate whole sludge samples. From the VFA study, it demonstrated similar metabolisms among eight replicates under a specific environmental stress. Countless tests could be conducted on this type of unique anaerobic niche community. Results could then be expanded upon where this type of experiment could answer a multitude of important ecological questions regarding microbial community strategies towards environmental adaptation, climate change, chemicals influence, etc.

However, it was observed during this trial that the individual granules did break apart under most of the environmental stresses, except for the VFA-fed granules at pH 7, at both 37°C and 23°C, as well as the acetate-fed granules. Within anaerobic digesters, there are physical hydrodynamic stresses and shears that allow the anaerobic granules to maintain their structural integrity during the pumping in of effluent. This phenomenon is absent in this μ SBR system. On the other hand, the granules were confined and remained in their respective wells, so the entire communities were possible to analyse at the end of the trial.

Future work should integrate experimental data from high-throughput studies such as those demonstrated in this work with mathematical model-building to link composition with microbial function (Widder et al., 2016). Additional similar high-throughput studies along with 'omics work will add tremendous value and statistical robustness to predictive models. An additional beneficial aspect to this study would be to include real time CH₄ and H₂ analysis in order to analyse in more detail how environmental stresses affect these parameters which are important in climate change.

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Chapter 7

Chapter 7 - Final Conclusions, Perspectives and Future Recommendations

7.1 Concluding Discussion

Anaerobic Digestion (AD) has been studied for many decades and many advances have been made in the area of wastewater treatment. However, despite this, there is still a relatively poor understanding of the true complexity of the biological mechanisms and the microbial interactions that underpin the AD pathway. The focus of this thesis was to examine anaerobic microorganisms at an individual level and at a community level. Fundamental growth analyses, including growth rates, volatile fatty acid (VFA) profiles and CH₄ profiles, were conducted on key microorganisms in AD systems as pure cultures and as synthetic co-cultures. In the context of wastewater treatment, understanding these microbial dynamics is paramount to optimising bioreactor performance.

Following this study, an entire community of anoxic microorganisms were investigated in the form of anaerobic granules. Individual granules were considered as whole eco-systems. These individuals were shown to be replicated microbial biofilms which formed naturally inside granular sludge bioreactors. Single granules were subjected to various environmental stresses in μ SBRs in order to survey the active community structure response of granules to these stresses. Overall, this thesis has shown in detail, the growth and chemical impacts that specific co-cultures have on anaerobic pure cultures and that anaerobic granules are complex niche communities which are versatile and capable of shifting their active communities when under environmental pressures.

7.1.1 Detailed characterisation of anaerobic pure cultures and the influences of co-cultures

The work in Chapter 3 gave additional insights into three specific model microorganisms found in the AD system and their interactions when placed in a co-culture set-up. *M. barkeri* is known to be a metabolically-diverse species. An analysis of the growth, VFA and CH₄ production data showed that temperature

plays a key role in *M. barkeri*'s ability to utilise substrates and produce CH₄. The highest level of substrate consumption and CH₄ accumulation occurred at 37°C. There was a 38.5% reduction of CH₄ production at 30°C but there was a lower reduction of 27.3% at 25°C. This indicated that low temperatures may still produce a substantial amount of CH₄ while significantly reducing the energy costs associated with heating anaerobic digesters.

The *M. barkeri* and *M. maripaludis* co-culture has not previously been studied in the literature. They are both important methanogens to AD and are capable of utilising H₂-CO₂. Hence, a competitive interaction between them was hypothesised. Growth and chemical data analyses indicated that both *M. barkeri* and *M. maripaludis* were able to grow. The growth rate for the co-culture was higher than the *M. barkeri* pure culture growth rate but the co-culture growth rate was significantly similar to the *M. maripaludis* pure culture rate.

The flexible methanogen *M. barkeri* and the homoacetogen *A. woodii* are capable of synergy and syntrophy when in co-culture (Winter and Wolfe, 1979, Winter and Wolfe, 1980). H₂-CO₂ can be utilised by both, so this study looked at their relationship in co-culture when they were both competing for the same substrate. The growth and chemical results showed that the two microorganisms were both able to grow at similar growth rates and, based on their acetic acid profiles produce similar results in pure cultures and co-cultures. They grew synergistically where they were both able to utilise H₂-CO₂ as the sole substrate. Acetate can only be consumed by *M. barkeri*, so *M. barkeri* out-competed *A. woodii*, which was not able to grow with acetate as the sole substrate.

Both *M. barkeri*'s and *A. woodii*'s growth and substrate utilisation profiles were decreased at low temperature with H₂-CO₂ as their substrate. *M. barkeri*'s growth and chemical profiles also decreased under acetate-feeding. Together, this data gives a detailed understanding of the microbial interplay that occurs between some model AD microorganisms.

7.1.2 Differences between granule sizes and types

In Chapter 4 anaerobic granular sludge from three different sources and separated into three different sizes were characterised based on physical, physiological and phylogenetic methods. The physical approaches consisted of relative size contributions, total solids (TS) and volatile solids (VS) concentrations. All three demonstrated significant differences between the three granule sizes. Relative size contributions and TS concentrations did not demonstrate any significant differences among the three sludge types but VS concentrations did demonstrate significant differences between sludge types.

The physiological specific methanogenic activity (SMA) assays determined significant differences between sludge types against H_2 - CO_2 and acetate activities, except that no significant differences were reported between sludges from reactors treating starch and sugar wastewaters reported. There were also significant differences between most of the granule sizes except for large- and medium-sized granules when tested against H_2 - CO_2 .

The physiological and phylogenetic results contradicted the physical tests, whereby there were significant differences between the sludge types. Based on the active community structure of individual granules, it can be reported that there were significant differences between the sizes in two of the three sludges.

Overall, this work demonstrated that there were significant differences between granular sizes when considering physical, physiological and phylogenetic data.

7.1.3 Single granules as whole eco-systems

The focus of Chapter 5 was on granule characterisation of one specific sludge type of one specific size. The TS and VS concentrations of one hundred individual granules were revealed to be statistically similar to each other. It was also reported that there were no significant differences between the active community structure of sixteen single granules and that the overall bacterial and archaeal diversity between the sixteen granules was highly conserved. Based on these results, it was concluded that individual granules have similar physical and phylogenetic relationships between each other.

The potential and active communities of sixteen single granules of one specific anaerobic granular sludge type and size were found to consist of the same highly-abundant families but an analysis of the DNA demonstrated that the community had at least twice as many unclassified families than were revealed by an analysis of a single granule's cDNA structure. Moreover, what was found in this study was that the most relative abundant families in the DNA community structure were the same as for the cDNA structure.

7.1.4 High-throughput characterisation for granular ecological studies and modelling

Chapter 6 detailed the physiological and phylogenetic characteristics of single anaerobic granules in order to investigate the ecological response of whole ecosystems to various environmental pressures. Overall, the results demonstrated that eight replicated granules under a specific change of conditions undergo similar VFA (and sugar) consumption-depletion profiles. Similarly, the active community structure of triplicate individual granules under a specific condition shift in a similar way when exposed to an environmental stress. This demonstrated that environmental variables were drivers of microbial community change. Some of the active community structures were significantly different (for example, those of granules which were subjected to different pHs and different substrates) and some did not show significant differences (such as cobalt-deprived granules and BES-fed granules).

7.1.5 Novel and main findings from the thesis

1. *M. barkeri* - Metabolic flexibility is independent of temperature.
2. *M. barkeri* and *M. maripaludis* - Both cultures can grow synergistically under H₂-CO₂ at 37°C but *M. maripaludis* consumes over half of the substrate.
3. *M. barkeri* and *A. woodii* - Both can grow synergistically under H₂-CO₂ at 35°C and at 15°C whereas;
4. *M. barkeri* outcompetes *A. woodii* for acetate at 35°C and at 15°C.
5. Anaerobic sludge granules of different sizes are significantly different in terms of physical, physiological and phylogenetic characteristics.

6. Anaerobic granules from three different wastewater bioreactors were significantly different in terms of phylogenetics but had similar physical and physiological traits.
7. Individual granules from one size fraction and one source have similar metabolic and active community structure profiles.
8. The active community structure of individual granules shifted in a somewhat replicated manner when environmental stresses were applied.

7.2 Future directions

Both pure culture work and whole microbial community work are important to the future of the field of microbial ecology. Studying both provides a two-pronged approach to studying the effects and impacts of influences both within the microbial community, such as the syntrophic and competitive dynamics of different microorganisms and from outside environmental cues and stresses.

7.2.1 What the findings of the thesis mean for the AD field of research

From the work presented in this thesis several hypotheses have been tested and questions answered, while new hypotheses have also been formulated from the results. The hypotheses relating to how pure cultures interact with each other in terms of growth and chemical data were answered through the various methodologies described in Chapter 3. However, answers to many more probing questions may be extracted from the results surrounding the genetics of how these specific pure cultures interact with each other. For the anaerobic granule work in Chapter 4, the results and data that were generated answered many of the questions posed such as whether there are there significant differences between anaerobic granular sludges from different sources and between anaerobic granules of different sizes. Physical, physiological and phylogenetic characteristics were analysed and conclusions were drawn based on three specific types and sizes of anaerobic granules. More robust conclusions could be determined by conducting similar investigations into additional granular types and sizes.

Environmental sampling involves certain issues and challenges. A common difficulty that is encountered when sampling from an environment is determining where and how much to sample in order to get a true representation of the microbial community present. Single anaerobic granules were observed to demonstrate statistically similar active community structures following 16S rRNA sequencing analysis, as set out in Chapter 5. This result implied that individual granules could be replicated whole ecosystems and thus presented a unique opportunity to analyse a whole community on a high throughput basis. Unquestionably, more granules at different sizes and from different reactors are required to be sequenced in order to draw a more definitive conclusion on whether granules are replicated communities.

The method of utilising a simple μ SBR set-up and subjecting single granules in individual wells to a plethora of environmental stresses enabled unique characterisation of whole communities. This type of whole ecosystem community level analyses can enable valuable high-throughput datasets for microbial predictive modelling.

As demonstrated throughout this thesis there have been several molecular techniques utilised but sometimes contradictory results are found. This is a challenging issue in biology. What is the best technique to answer questions of a biological sample? Is it classical pure culture kinetics, microscopy and cloning? What is best to extract from a sample - DNA, RNA, proteins, metabolites? Is it quantitative PCRs followed by sequencing? What type of sequencing? These decisions are ongoing and constant and have to be based on the hypotheses proposed followed by considered planning.

7.2.2 Alternative lines of investigation

There were many ways in which these various growth experiments on the pure cultures and co-cultures could potentially have been tested. Some alternative methodologies, such as cell counting or flow cytometry (Moldavan, 1934) could have been used to determine growth rates and cell numbers instead of OD measurements and qPCR assays respectively. For the anaerobic granular sludge work, the composition of the sludges (categorised into “small”, “medium” and

“large” granules) could have been determined by VS content. There are also alternative ways to monitor sugar profiles, such as high-performance liquid chromatography (Horvath et al., 1967, Snyder, 1967).

The pure culture and co-culture experiments could potentially have been supplemented by taking measurements at an increased number of conditions in order to gain more detailed fundamental information on the microorganisms. Substrates such as fructose for *A. woodii* and methanol for *M. barkeri*, higher temperatures and other pure cultures that are also important in the AD pathway such as *Syntrophaceticus schinkii*, *Geobacter sulfurreducens*, *Clostridium ultunense*, *Methanocorpusculum labreanum* and *Methanosaeta concilii* could have been studied as co-cultures and tri-cultures. The information gained from these pure cultures and synthetic cultures would be beneficial in the field of synthetic biology. H₂ and CO₂ gas analyses would also have been beneficial for both the pure culture experiments (Chapter 3) and the μ SBR study (Chapter 6).

7.2.3 Opportunities following this work

Along with the alternative work listed in section 7.2.2, there are many potential subsequent experiments that can be carried out to build on the work that has been conducted here. One specific line of study that is being continued at present but which has not been included in this thesis is a HiSeq (meta)transcriptomic examination of the pure cultures and co-cultures that were studied in Chapter 3. Transcriptomic work would give a more detailed insight into the genes that were actively responsible for pure culture and co-culture growth in the conditions tested in Chapter 3.

It is recommended that future experiments be conducted with granular sludge to analyse additional types of sludge in order to determine the relative composition of differently-sized granules. The results from the activity tests in Chapter 4 demonstrate that there are differences in methanogenic activities between the three granule sizes, so further studies are required to analyse the composition, characteristics and importance of granule size. Our research group is continuing this work on granular size by setting up separate lab-scale reactors containing only

small, medium and large granules, as the case may be, to follow their respective life cycles.

While the results presented in the μ SBR experiment provide a basis for obtaining comprehensive ecological information on individual granules longer-term trials are required in order to fully assess the impact of environmental stresses, especially with cobalt and other important trace metals that can be found widely in the environment. As well as testing the effects of more conditions on individual granules, it would also be beneficial for any such future experiments to test the conditions over a range of sizes. The next generation of μ SBR studies could utilise stable-isotope probing in order to follow the important species responsible for growth under various environmental conditions (Strous et al., 1998, Radajewski et al., 2000). Further studies could also implement some form of “in-line gas analysis” to monitor the effect of environmental stresses on CH₄ production. Continuous-flow reactors would allow real time analysis of these gas fluxes along with microfluidic studies which would enable the examination of microbial motility (Son et al., 2015).

Major lines of investigation in single cell microbiology, transcriptomics, synthetic ecology and microfluidics of pure cultures and co-cultures could build on the progress made throughout this work and make informative and important additions to the study of microbial ecology. In parallel to this important generation of data, a whole-ecosystems approach should also be taken for the individual anaerobic granules whereby the ecological data collected could then be transformed into robust mathematical modelling. These models could be used as tools for predicting how environmental stresses and climate change will impact on microbial communities.

With computational scientific modelling, microbial ecology is opened up to a wider premise of combining data from complicated networks observed in microbial and biofilm science (ADM1) and synthetic ecology. There is great potential to incorporate predictions from the disparate data already generated

It has been commented that the field of microbial ecology has been both driven but also limited by techniques (Prosser et al., 2007). High-throughput sequencing has

provided many opportunities for the study of microbial ecology. A vast quantity of data has been generated from pure cultures, natural mixed cultures and synthetically mixotrophic cultures, including their meta-genomics, meta-transcriptomics, meta-proteomics, biochemistries, metabolisms and metabolomics. Along with advancing this knowledge using the plethora of biological techniques, with the possibility of manipulating and integrating this deluge of data that comes from the new sequencing technology platforms into a condensed definitive understanding of the ways in which these microbes survive and function presents a great intellectual challenge. The task resides in the ways in which we interpret this data in a more integrative way. On a more applied level, the ways in which these processes can be used to beneficially intervene to mitigate the effects of climate change and to sustain the natural environment.

Sequencing has now progressed to the point that its computer power requirements may surpass what is available, generating a co-evolution between technology and researchers (Logares et al., 2012). Microbial biology studies now require both sound experimentation and bioinformatics data analysis.

7.3 Final comments and some outlooks on the future of anaerobic microbial ecology

In conclusion, the culmination of this work has provided some additional fundamental data on three key model microorganisms and the impacts of their interactions. The competitive interaction between the methanogens *M. barkeri* and *M. maripaludis* is novel within the literature. Although *M. barkeri* has been described as an acetoclastic methanogen, it has been shown here and in other studies that it is capable of consuming H₂-CO₂. This leads on to a recommendation of re-classing *M. barkeri* as a general methanogen / flexible methanogen. Moreover, a comprehensive study detailing the physico-chemical and ecological traits of anaerobic granules of different sizes and from different wastewater treatment sources provided a general insight into the differences that can occur in granular sludge.

These single granule ecological studies supported a preliminary conclusion that individual granules are replicated whole ecosystems and the μ SBR trial indicated that there are some initial high-throughput data supporting the hypothesis that the microbial communities in single granules adapt in the same way under specific environmental stresses.

Despite the many advances in the field of AD, there is a lack of understanding behind the fundamental microbiology underpinning AD. This is especially true of the dynamics and relationships between microbes in the AD system. There have been relatively few studies on the environmental parameters in AD (Appels et al., 2011, Tomei et al., 2009) the high-throughput single granule data under many environmental conditions can help to bridge this gap in knowledge. A combination of both pure cultures and high-throughput sequencing studies will help to enhance the complete understanding of complex systems such as AD. It is necessary to build predictive models to underpin the ecological understanding of complex microbial communities (Widder et al., 2016). Continued studies on a combination of pure cultures, synthetic co-cultures and existing microbial communities are required to achieve this goal toward fully understanding the ecophysiology of anaerobic communities in the future.

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