Ecology, Physiology and Performance in High-Rate Anaerobic Digestion

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Abstract

The design demands on water and sanitation engineers are rapidly changing. The global population is set to rise from 7 billion to 10 billion by 2083. Urbanisation in developing regions is increasing at such a rate that a predicted 56% of the global population will live in an urban setting by 2025. Compounding these problems, the global water and energy crises are impacting the Global North and South alike. High-rate anaerobic digestion offers a low-cost, low-energy treatment alternative to the energy intensive aerobic technologies used today. Widespread implementation however is hindered by the lack of capacity to engineer high-rate anaerobic digestion for the treatment of complex wastes such as sewage.

This thesis utilises the Expanded Granular Sludge Bed bioreactor (EGSB) as a model system in which to study the ecology, physiology and performance of high-rate anaerobic digestion of complex wastes. The impacts of a range of engineered parameters including reactor geometry, wastewater type, operating temperature and organic loading rate are systematically investigated using lab-scale EGSB bioreactors. Next generation sequencing of 16S amplicons is utilised as a means of monitoring microbial ecology. Microbial community physiology is monitored by means of specific methanogenic activity testing and a range of physical and chemical methods are applied to assess reactor performance. Finally, the limit state approach is trialled as a method for testing the EGSB and is proposed as a standard method for biotechnology testing enabling improved process control at full-scale.

The arising data is assessed both qualitatively and quantitatively. Lab-scale reactor design is demonstrated to significantly influence the spatial distribution of the underlying ecology and community physiology in lab-scale reactors, a vital finding for both researchers and full-scale plant operators responsible for monitoring EGSB reactors. Recurrent trends in the data indicate that hydrogenotrophic methanogenesis dominates in high-rate anaerobic digestion at both full- and lab-scale when subject to engineered or operational stresses including low-temperature and variable feeding regimes. This is of relevance for those seeking to define new directions in fundamental understanding of syntrophic and competitive relations in methanogenic communities and also to design engineers in determining operating parameters for full-scale digesters. The adoption of the limit state approach enabled identification of biological indicators providing early warning of failure under high-solids loading, a vital insight for those currently working empirically towards the development of new biotechnologies at lab-scale.

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.....a massive thanks to you all (and to those not mentioned you are so many), every one a star: my own wee constellation.

Author's declaration

I declare that no portion of the work in this thesis has been submitted in support of any application for any other degree or qualification from this or any other university or institute of learning. I also declare that the work presented in this thesis is entirely my own contribution unless otherwise stated.

Stephanie Connelly

Glasgow, June 2016.

List of abbreviations

1-D	one-dimensional
3-D	three-dimensional
AD	anaerobic digestion
ADR2	anaerobic digestion reactor 2 (of a set of 3)
ANOVA	analysis of variance
BPR	biogas production rate
COD	chemical oxygen demand
CSTR	continually stirred tank reactor
DNA	deoxyribonucleic acid
dx	d is a prefix used to indicate 'day' since commencement of the experiment, x is the number of days passed e.g. d25 is 25 days after the beginning of a reactor trial
EGSB	expanded granular sludge-bed bioreactor
FISH	fluorescent in-situ hybridisation
$\Delta G^{0'}$	Gibbs free energy under standard conditions
ID	identity: refers to either reactor identity or OTU identity
IQR	interquartile range
MPR	methane production rate
NBD	North British Distillery
NMDS	non-metric multidimensional scaling
NGS	next generation sequencing
OLR	organic loading rate
OTU	operational taxonomic unit: a proxy for species at 97% sequence similarity
pCOD	particulate COD (pCOD = COD – sCOD)

PCR	polymerase chain reaction
PSD	particle size distribution
QIIME	Quantitative Insights Into Microbial Ecology: and open-source bioinformatics pipeline
RDP	Ribosomal Database Project: provides ribosome related data services
RNA	ribonucleic acid
rRNA	ribosomal RNA
rtPCR	reverse transcription PCR
R'x'	R is a prefix indicating 'reactor', 'x' is a number defining the reactor unique ID e.g. R1 is reactor one (of a set of 12 tested)
Rxr	shorthand for 'reactor'
sCOD	soluble COD (passes 0.45um filter)
SMA	specific methanogenic activity
SYNTHES	synthetic sewage
TWP	Transforming Waste Project: a multidisciplinary research consortium
TSS	total suspended solids
UASB	upflow anaerobic sludge blanket
VFA	volatile fatty acids
tVFA	VFA as an acetate equivalent (VFA_asHac)
VLR	volumetric loading rate
VSS	volatile suspended solids

1 Introduction

The primary motivation for the work conducted in this thesis is the astounding reach and impact of the global sanitation crisis. In the Global North where access to sanitation is almost universal, it is estimated that as much as 5% of electricity generated is consumed in wastewater treatment processes (Logan 2008) calling into question the sustainability of current sewage treatment practices. In the Global South by contrast, pit latrines continue to act as the primary form of sanitation available to the urban poor (O'Riordan 2009, Jenkins, Cumming et al. 2015). Whilst pit latrines can provide a low-energy and low-water usage sanitation solution, poor pit-emptying practices and mismanagement of arising wastes are such that pit latrines frequently exacerbate the faecal-oral cycle they are intended to address (Uddin, Li et al. 2014). The socio-economic environments from which these problems stem are disparate however demonstrate the common need to find a sustainable, effective solution to waste-water-energy nexus.

Anaerobic digestion (AD) is a wastewater treatment method with the potential to provide a net energy gain. In AD, the organics in wastewater are degraded by a complex microbial consortia in a metabolic process described as methanogenesis. The result is an improved wastestream coupled with the production of biogas as a valuable end product. The complex microbial communities underpinning methanogenesis however are poorly understood such that the current capacity to engineer AD systems for less energy intensive treatment of complex wastes such as sewage is limited. New methods in molecular microbial ecology are for the first time enabling detailed insight to the microbial communities involved in AD and present the opportunity to improve understanding of methanogenesis and hence improve the capacity to engineer the process.

The work in this experimental thesis aims to improve the capacity to engineer AD biotechnologies for the treatment of sewage by improving understanding of the effect of engineered conditions in AD systems on the underlying biology. The experimental work uses a high-rate anaerobic digester type, the expanded granular sludge bed bioreactor (EGSB), as a model for study. The thesis commences with a review of literature in which the development history of the EGSB is documented and critiqued and key research questions are defined (Chapter 2). Experimental design methodology is then defined in relation to the hypotheses tested (Chapter 3). The hypotheses are tested in a series of experiments, the results and discussion of which form the main body of the thesis (Chapters 4-8). Finally, the original research contributions made to the field are outlined in the closing discussion (Chapter 9).

2 Review of Literature

2.1 Sewage Treatment: A changing paradigm

The role of the Engineer is to systematically assess a problem and design a solution with minimal negative impacts. This requires a thorough understanding of the problem, the range of solution options and some mechanism by which that range of solutions might be assessed. A simple example might be that a population wish to transport goods across a river to a neighbouring population and *vice versa*. The potential range of solutions is highly varied, some simple examples of which might be to design and construct a bridge, or a tunnel, or provide ferry terminals for ease of shipping. Detailed assessment of factors like, the site geology, weather conditions and frequency of use, enable the range of options to be narrowed according to practical feasibility. Beyond practical feasibility, the Engineer must then assess the options against a further range of *imposed factors*, that may include for example visual appearance of the structure, local acceptability in terms of practice, or environmental concerns with the key imposed factor likely to be cost. Having narrowed the options according to these imposed factors, the Engineer might then produce some preliminary designs for say, a tunnel as compared to a bridge. In producing those designs, the Engineer can draw on sound scientific principles to enable him or her to effectively size components of the designs for a given range of uses and to model the structural responses of the designs prior to construction. This enables final costing based on realistic material and construction costs, coupled with empirically obtained maintenance costs. The advantage to the population then in employing the Engineer is that he or she can effectively couple sound scientific principles with a standardised design philosophy to provide a solution that, within reason, they know will work. The Engineer can effectively apply scientific theory and design methodology to 'see ahead' to minimise risk. Environmental engineering is an emergent engineering discipline. Whilst biotechnologies have long been employed to tackle environmental problems such as water purification and wastewater treatment, today the capacity to truly engineer such systems is limited by a lack of both scientific and design theory.

The supply of clean drinking water coupled with the safe removal of wastewater for the protection of public health, became part of the remit of Civil Engineers in the Global North in the 1800s. Initially, the approach was simply to transfer wastes to a perceived 'safe' distance from the community served, for discharge to rivers and other water bodies. In many areas of the Global South, the practice of direct discharge to the environment is still commonplace today (Chambers 2009, Nyenje, Foppen et al. 2010, Tauseef, Abbasi et al.

2013). As population density in towns and cities increased, and the social and environmental impacts of direct discharge of waste to the environment became apparent, treatment of wastes prior to discharge became a pressing necessity (Orhon 2014). In the mid-1800s pioneering work by Snow, who demonstrated the link between polluted waters and the spread of disease, and by Koch and Pasteur, who demonstrated the microbial nature of contamination, helped to redefine the aim of sanitation from waste removal to waste treatment and pathogen control (Madigan, Martinko et al. 2009). Whilst Newtonian mechanics gave Engineers the capacity to design physical elements of the treatment scheme such as flow rates and pipe diameter, Engineers at that time had little capacity to design the treatment process itself. Early treatment schemes relied principally on physical settling and removal of solids wastes for drying, incineration or application to the land (Lofrano and Jeanette 2010). Latterly, downstream treatment for the bulk fluid after sewage settling was introduced. Recognising that the bulk water remains rich in fine particulate and dissolved organics, secondary biological treatments were deployed prior to discharge for the removal of organics, nutrients and inactivation of pathogens. The design and operation of the secondary treatment processes used then remains largely similar to that used now. Aerobic microorganisms are utilised to transform organic carbon along with nutrients in the wastestream to produce stabilised end products in the form of new cells that can be physically separated from the bulk water. The bulk water can then be discharged, perhaps with the requirement for some tertiary treatment, according to local or national water discharge standards, after the solids (new cells) have been settled for treatment along with the primary sludge. Whilst the design, development and operation of these biological systems remains largely empirical (Tchobanoglous, Burton et al. 2004), application has historically been viewed as successful when weighed against the remit of protecting public and environmental health (Henze 2008). What has changed since the early days of wastewater engineering however are the *imposed factors* on the design and operation of treatment schemes (Tchobanoglous, Burton et al. 2004), and attitudes towards 'risk' in engineering design.

Since the 1970s, the sustainability agenda has driven tightening environmental discharge standards (van Lier 2008). Energy and water availability have declined whilst demand, and consequently costs, have risen. The demand for safe practices in water supply and treatment has expanded globally and is rightly viewed as a human right rather than a privilege to be enjoyed by those living solely in wealthy countries (Hanjra and Qureshi 2010). Increasingly the operation of water and wastewater treatment plants has come under the control of private rather than public bodies and with that profitability becomes

ever more an issue (Scott 2012). With wastewater treatment estimated to consume as much as 5% of the electricity produced in the US (Logan 2008), the water and energy intensive waste collection and aerobic treatment systems of the past no longer meet the design requirements arising from these new imposed factors. Alternative solutions to the problem of wastewater treatment are now required. Compounding these wider issues, attitudes towards 'risk' have changed dramatically; empirical trial and error is no longer an acceptable approach in the eyes of either the public or of private enterprise. Vast amounts of public and private monies have been invested in trialling new alternative technologies to conventional aerobic wastewater treatment. Since the 1980s, an array of potential alternatives to aerobic treatments have been identified, most notably in the field of anaerobic digestion (AD), however as yet, laboratory successes have done little to increase the range of practical solutions available to the design engineer.

This study focuses on a single alternative treatment system described as the Expanded Granular Sludge-Bed bioreactor or EGSB. Under development since the 1990s, the EGSB is a biological anaerobic treatment system that has shown promise in the rapid and effective removal of organics from industrial and domestic wastewaters whilst simultaneously producing methane gas, a useful renewable energy source (Seghezzo, Zeeman et al. 1998). As an anaerobic technology, the treatment process eliminates the economic and energy costs associated with aeration and, as methane gas is produced as an end product, the system has the potential to run with a net energy gain improving both usefulness and profitability (Hulshoff Pol, de Castro Lopes et al. 2004, Shah 2014). As a high-rate technology, plant footprint is reduced and hence cost to the plant owner is minimised (Enright, McHugh et al. 2005). The ESGB has grown from, and in response to, the changing design paradigm in the field of wastewater treatment and is one of the most promising technologies arising from it. What remains to be fully addressed in the EGSB and other alternative biological treatment systems is the 'risk' factor for both design and operation. Even in long-established aerobic treatment schemes, there remains a lack of knowledge and understanding of the complex microbial consortium underpinning the wastewater treatment process that limits the capacity to truly engineer the treatment process. This knowledge gap effectively prevents the development of a design methodology that is founded in scientific theory, and that is ultimately both predictive and quantitative in terms of risk to plant owners and operators (van der Gast, Jefferson et al. 2006, Prosser, Bohannan et al. 2007). Closing this knowledge gap is essential if the contemporary imposed factors on the design process are to be attained and technologies such as the EGSB are to be widely adopted in the industry.

Simultaneously to the development of the EGSB, new molecular methods have arisen in the field of microbial ecology that are providing new insights into complex microbial communities in both natural and man-made systems. These exciting new tools have driven a surge of interest in lab-scale experimentation of EGSBs and other biotechnologies and currently represent the likeliest route to closing the knowledge gap (Enright, Collins et al. 2006). The popular adoption of molecular methods in EGSB trials has resulted in significant advancement of knowledge of the response of specific microbial communities to given conditions in experimental tests however currently, a lack of underlying theory around which to structure the collected data limits the applicability of this knowledge to both design and monitoring. Further, whilst monitoring of trials utilises methods that are in themselves ever more scientifically advanced, in the main, experiments conducted remain largely empirical in nature and the methodologies with which they are conducted disparate. If future trials are to inform design, and indeed to inform underlying theory, some unification of approach to the design of experiments themselves may be required such that data collected can be usefully utilised.

This literature review aims to highlight key advances made towards establishing scientifically founded design, monitoring and testing criteria of EGSB systems and to identify key areas where this might be improved. It is by no means the intent of the author to negate the other technologies currently under development that might prove equally or indeed more successful than the EGSB in the field. Rather, the intention is that by focussing on differences and similarities in the approaches to testing and analysis of a single system, general lessons might be learned that are adaptable to the testing and design of a broader range of biotechnologies. Further, the work that follows in the experimental chapters use the EGSB as a model for study. The review may be viewed as having four distinct parts. The first presents a brief chronological overview of the methodologies adopted in the field of wastewater treatment from the aerobic activated sludge process, to the development of high-rate anaerobic digesters such as the EGSB and outlines the current, empirically derived, design parameters of the EGSB. Secondly, the range of molecular monitoring tools available is assessed against the motivations of the experimental works in which they are applied. Thirdly, the ecologic and statistical methods available by which experimental data is currently interpreted are assessed. Finally, potential avenues of new research are summarised with a view to framing the experimental work conducted in this thesis.

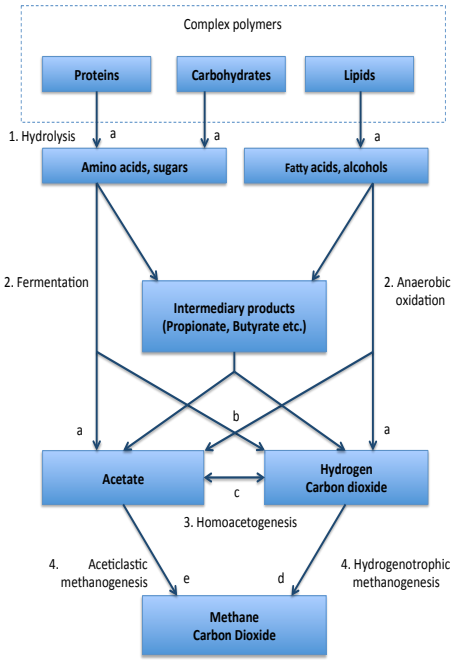
2.2 Anaerobic Digestion

Anaerobic digestion (AD) is a naturally occurring process in which organics are mineralised in the absence of oxygen to produce methane gas as the primary end product. In nature, AD is the final stage in the carbon cycle and is known to occur in a wide variety of environments and habitats including animal and human digestive tracts (Jensen 1996, Kim, Deepinder et al. 2012), lake sediments (Falz, Holliger et al. 1999), paddy fields (Conrad, Bak et al. 1989), arctic soils (Metje and Frenzel 2007, Høj, Olsen et al. 2008) and landfills (Gurijala and Suflita 1993). AD is underpinned by the process of methanogenesis and may be defined as the stepwise, syntrophic degradation of organics by a mixed microbial consortium in the absence of oxygen to methane gas and carbon dioxide as end products. Table 2-1 outlines the key stages in methanogenesis.

The first of the four steps is the hydrolysis of complex polymers such as cellulose, proteins and lipids. The process relies on exo-enzymes of hydrolytic bacteria that are excreted through the cell wall and degrade the complex compounds to smaller soluble compounds that can pass through the cell wall for intra-cellular degradation (Shah 2014). In anaerobic digestion of complex, high-solids wastes such as sewage in wastewater treatment plants, hydrolysis may be the rate-limiting step in the process (Noike, Endo et al. 1985). Hydrolysis rates are influenced by a range of parameters including pH and temperature, physical properties of the waste such as particle size of the organic matter (Ponsá, Ferrer et al. 2008), as well as microbial attributes such as enzyme production rates, and available surface area for contact between enzymes and particulate material (Shah 2014). Whilst this stage is crucial to the degradation of particulate matter, little is known of the growth rates or energetics associated directly with this step.

The second stage in the process is known as fermentation or acidogenesis and is conducted by hydrolytic and other fermentative bacteria. Here, the products of hydrolysis along with other dissolved components of the waste are degraded to produce volatile fatty acids, alcohols, carbon dioxide and hydrogen. In many cases, organisms responsible for hydrolysis additionally are responsible for acidogenesis. This stage in the degradation process is highly exergonic (van Lier 2008) enabling rapid growth of fermentative bacteria (Ghosh and Pohland 1974). In wastewater treatment over-growth of acidogenic consortia

Table 2-1: Schematic illustrating methanogenesis of complex organics with key process descriptors and associated microbial genera and families

Methanogenesis: Schematic Overview [1]	**Step-wise description of process**	**Prokaryotic groups involved** [1]	**Averaged kinetic properties and energy associated with each step** [1]	**Selected known family (F) or genus (G) associated with metabolic step**
 Reactive scheme for the anaerobic digestion of polymeric materials Four stage process, numbered Five key bacterial groups involved indicated by letters	**1. Hydrolysis:** Exo-enzymes used to convert complex material to smaller, dissolved compounds suitable for intra-cellular degradation. [2]	a) Hydrolytic and fermentative bacteria		Streptococcus (G) [2] Enterobacterium (F) [2,3,5] Ruminococcus (G) [2] Clostridium (G) [3,4,7] Baccillus (G) [5] Moraxella (G) [5] Pseudomonas (G) [5] Kleibsella (G) [5]
	2. Acidogenesis Intracellular degradation of dissolved compounds. Simpler molecules including VFAs, alcohols, CO_2, H_2 are excreted. New cell material formed. [3]	a) Hydrolytic and fermentative bacteria	**Kinetics:** Y = 0.15 gVSS/gCOD K_s = 200 mgCOD/l u_m = 2.00 l/d **Energy:** $\Delta G^{0'}$ = - 457.5 to - 610.5 kJ/mol, sucrose @ 25^{0}C	Pseudomonas (G) [2,6] Bacillus (G) [2, 5] Clostridium (G) [2] Micrococcus (G) [2] Flavobacterium (G) [2]
	3. Acetogenesis VFAs and other intermediary products converted to acetate, H_2 and CO_2. New cell material formed.	b) Acetogenic bacteria (use VFAs as substrate) [9,11] c) Homo-acetogenic bacteria (use H_2 CO_2 as substrates) [9,11]	**Energy:** $\Delta G^{0'}$ = + 76.1 kJ/mol (propionate), $\Delta G^{0'}$ = -70.3 kJ/mol (H_2-CO_2 > Acetate)	Syntrophomonas (G) [2,6,7] Syntrophobacter (G) [2] Syntrophomonodaceae (F) [4,7] Clostridium (G) [9, 10] Acetobacterium (G) [9, 10] Syntrophococcus [9]
	4. Methanogenesis Methane is formed from acetate or from H_2 plus CO_2, formate or methanol. New cell material formed.	d) Aceticlastic methanogens e) Hydrogenotrophic bacteria	**Ac Kinetics:** K_s = 30 mgCOD/l u_m = 0.12 l/d **Energy:** $\Delta G^{0'}$ = -31 kJ/mol **H_2CO_2 Kinetics:** K_s = 300 mgCOD/l u_m = 0.71 l/d **Energy:** $\Delta G^{0'}$ = -131 kJ/mol	Methanosarcinales (G) [2] Methanosaeta (G) [2] Methanosphaera [2] Methanobacteriales [2]

Kinetics and energetics parameters given: yield coefficient (Y), half saturation constant (K_s), maximum specific growth rate (u_m) and Gibbs free energy, standard conditions ($\Delta G^{0'}$)

Schematic adapted from [1] (van Lier 2008), [2] (Shah 2014), [3] (Muller 2001), [4] (Sousa 2008), [5] (Kalogo 2000), [6] (Pereira 2002), [7] (Sobieraj 2006), [8](O'Sullivan 2007) , [9] (Saady 2013), [10] (Diekert 1994)

has been known to cause reactor souring (low pH) resulting in low methane production and high COD in reactor effluent.

Acetogenesis follows fermentation and is conducted by acetogenic bacteria that form acetate, carbon dioxide and hydrogen from VFAs, or by homoacetogenic bacteria that form acetate from carbon dioxide and hydrogen. Homoacetogenesis is an exergonic reaction and may proceed independently from methanogens and indeed, proceeds competitively with hydrogenotrophic methanogens. Acetogenesis on the other hand is an endergonic reaction, that is, under standard conditions it requires energy input to proceed (Saady 2013). The metabolism of acetogenic bacteria is reliant on a syntrophic relationship with hydrogenotrophic methanogens, homoacetogens, or sulphate reducing bacteria to maintain a low hydrogen partial pressure such that the acetogenic degradation of VFAs is thermodynamically favourable and can sustain growth (O'Flaherty, Collins et al. 2006). Indeed, many species of syntrophic bacteria are found to be un-culturable in pure strains and rather must be co-cultured in the presence of hydrogen utilising species.

The final stage in the degradation process is methanogenesis. Whilst the preceding hydrolytic and fermentative stages of anaerobic digestion are undertaken by microorganisms belonging to the domain *Bacteria*, many of which may be facultative anaerobes, methanogenesis is mediated by obligate anaerobic microorganisms belonging to the domain *Archaea* (Liu 2008). Methanogens are typically delineated by the substrates they consume as either hydrogenotrophic or acetotrophic. Hydrogenotrophic methanogens reduce CO2 as a carbon source and utilise hydrogen as an electron donor and are the most genetically diverse of the methanogens. Acetotrophic methanogens utilise acetate as a substrate to form methane and carbon dioxide and are the most metabolically diverse of the methanogens, with species of the genus Methanosarcina able to both perform both acetotrophic and hydrogenotrophic methanogenesis. Whilst metabolically diverse they are the least genetically diverse with identified species belonging to only two known genera; Methanosarcina and Methanosaeta (Garcia 2000). In spite of slow growth and lower energy availability, it is estimated that ~70% of methane produced in anaerobic digestion is via the acetotrophic methanogens (O'Flaherty, Collins et al. 2006, Liu 2008) and as such they are amongst the most widely studied of the Archaea. Whilst hydrogenotrophic methanogenesis may be less productive in terms of methane yield, the associated methanogens have been linked with process stability in wastewater treatment schemes. Methanogens exhibit growth rates an order of magnitude lower than that of fermentative bacteria and as such methanogenesis is thought to be the rate-limiting step in the degradation process, particularly when soluble substrates are dominant in the substrate

composition (Lawrence 1969). Methanogens are particularly sensitive to pH and temperature of the environment, typically growing between pH 6-8.5 with optimal growth at temperatures between 25-40 degC. Methanogenesis has however been demonstrated to proceed at sub-optimal temperatures in both natural and engineered environments (Kotsyurbenko, Nozhevnikova et al. 1993, Bowen, Dolfing et al. 2014).

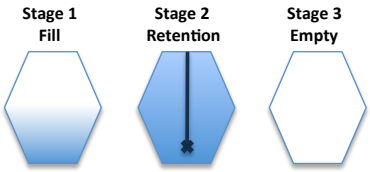
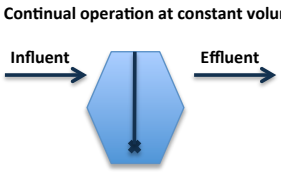
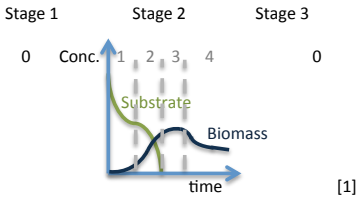
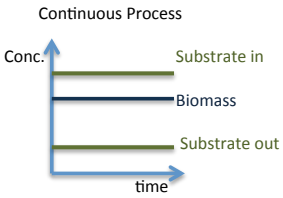
Whilst the genus and families identified in Table 2-1 are by no means inclusive of all that are able to perform the role attributed, it is interesting to note that some are associated with more than one step in the overall process. Of the examples given, the genus Pseudomonas is associated with both hydrolysis and fermentation, whilst Clostridium is reported to be involved with each of the three stages preceding methanogenesis. As such, assigning absolute roles to the various genus involved in the degradation process is extremely difficult for all but the methanogenic consortia. Further, many groups have been identified in mixed microbial consortia whose roles are not directly understood in relation to the 4-step process (O'Flaherty, Collins et al. 2006) a notable example being those of the Archaeal family Chrenarchaeota (Collins, O'Connor et al. 2005). Further work is required to elucidate the precise nature of the role of the many organisms involved in this vital process in both nature, and in engineered treatment schemes.

2.3 AD in WWT Design and Monitoring

Treatment in any biological wastewater treatment system is underpinned by the presence of an active microbial community, or biomass, in contact with organics in waste. The biomass may be viewed as a catalyst for the degradation of the organic waste to chemically stable end products (Shah 2014). In the case of aerobic treatment, organics and nutrients are metabolised using oxygen as an electron acceptor to produce new cells, water and CO_2 as end products. In anaerobic treatment, organics are mineralized to produce a relatively lower volume of new cells as compared to aerobic treatment equivalents, and methane gas and CO_2 as principal end products. The slow growth exhibited by microorganisms in anaerobic systems additionally supports the potential yield of scarce nutrients such as nitrogen and phosphorus as secondary end products that may be harvested from the reactor effluent. Whilst biological degradation processes in wastewater treatment plants are governed by the combined metabolism of complex mixed microbial consortia, much of current design and modelling of the treatment process hinges on the understanding of microbial behaviours in two simplified growth systems that are often used for the study of mono- or co-cultures in the laboratory. The first is described as a batch growth system,

and the second, as continuous growth system or chemostat. Table 2-2 describes the key features of each.

Table 2-2: Batch processing and continuous flow systems: schematics and key descriptors

Batch Processing	Continuous Flow
	 Flow in = Flow out
Hypothetical Relationship Between Substrate and Cell Concentrations	
 [1]	
Process Description	
• Assumes no treatment occurs during fill or emptying stages, the treatment occurs during retention in the tank • Substrate is supplied as a single 'event' in the bulk liquid, the tank volume is constant, the substrate is utilized at a variable rate until fully degraded. By-products of degradation remain in the reactor. • During retention there are **4 key phases**: • 1. Lag phase: low or no growth, acclimatization • 2. Exponential growth: biomass doubles at maximum rate, consumes substrate at maximum rate • 3. Stationary phase: substrate availability too low to maintain growth • 4. Death phase: biomass decay [1]	• The tank volume remains constant, inflow = outflow • Substrate is supplied continuously with the inflow and products including bacterial cells and any inhibitory by-products are removed with the outflow • Enables continuous study of microorganisms in the exponential growth phase provided: • The inflow volume and supply of substrate is balanced with microbial growth rates to enable constant substrate utilization and prevent washout of microorganisms

[1] adapted from (Comeau 2008)

In each case, the treatment rate is determined by the metabolic rate of microorganisms subject to a limiting substrate concentration. Growth in batch culture is one of the oldest methods of study of microorganisms. In batch processing, a new microbial community is established on each fill-retain-empty cycle and in sewage treatment batch processing, the community is established from microorganisms already present in the waste. Growth in such systems is characterised by distinct phases, the optimal of which is called exponential growth and represents the maximum growth rate and hence treatment rate in the system. The exponential growth rate declines when substrate availability declines below optimal levels or when by-products accumulate to inhibitory levels. Continuous flow or chemostat systems were developed in the 1950s to enable laboratory study of microorganisms in a constant growth phase. In chemostat systems, substrate is continually provided and

reactant continually removed hence there is the potential to sustain maximum growth of microorganisms and hence maximum treatment rates by balancing substrate concentration in the reactor volume with flow rates and microbial growth rates. Assuming completely mixed conditions, the chemostat effluent is identical to the bulk fluid in the reactor and hence has the same chemical and biological properties (Tchobanoglous, Burton et al. 2004). Understanding substrate limited growth in chemostat cultures enabled theory to be applied from physical and chemical sciences governing reactions rates to describe microbial growth and substrate use. These principles continue to apply in wastewater engineering today, key among which are summarised in Table 2-3.

Table 2-3: Summary of basic design principles applied in AD

Principle	Application	Formulae / Example
Volumetric Flow:	• Utilises continuity of flow to determine flow rates, flow velocity, reactor volume and hydraulic loading • Coupled with substrate concentration for calculation of organic loading	Qin = Qout = Q v = Q / A VLR = V / Q OLR = C / Q [1], [2]
Stoichiometry:	• Utilises conservation of mass and charge • Used to study fate of specific substrates in reaction • Enables substrate-product balance for e.g. cell growth utilising empirically derived formulae for cells • Used to determine half reactions for single electron equivalent as used in energy analyses • Enables calculation of COD equivalent for various chemicals • Used in determination of chemical reaction rates	Example: Aerobic oxidation of caesin to produce new cells: $C_8H_{12}O_3N_2 + 3O_2$ -> *(caesin)* $C_5H_7O_2N + NH_3 + 3CO_2 + H_2O$ *(New cells)* [2]
Kinetics:	• Determination of reaction rates for chemical or biologically mediated reactions • Determine the order of reaction • Central to the Monod equation: emprically derived mathematical model for determining substrate limited specific growth rate	Reaction rates & order: r = k zero order r = kC first order r = kC / (K+C) saturation or mixed [1] Monod Equation: $u = u_{max}(S / Ks + S)$ [3]
Energetics:	• Utilises stoichiometry and principle of Gibbs free energy, used to determine amount of energy available to microorganisms degrading a specific substrate • Used for yield estimates of biomass from specific substrates	**Example:** Energy available to cells from oxidation of glucose: Aerobic: $\Delta G^{0'}$ = -120.10 kJ/eeq Anaerobic: $\Delta G^{0'}$ = -17.85 kJ/eeq [2]
Mass Balance:	• Each of the above may be used to contribute to a mass balance determining the flow of single substrates, or energy / COD equivalents through a system	Example: COD balance in anaerobic process: $COD_{in} = COD_{out} + COD_{CH4} + COD_{cells}$ [1]

Symbols and abbreviations used in formulae: flow rate (Q), upflow velocity (v), volume (V), cross sectional area (A), volumetric loading rate (VLR), organic loading rate (OLR), concentration (C), reaction rate (r), reaction rate constant (k), specific growth rate (u), maximum specific growth rate (umax), substrate concentration (S)

[1] (Tchobanoglous, Burton et al. 2004), [2] (van Lier 2008), [3] (Henze 2008), [4] (Monod 1950)

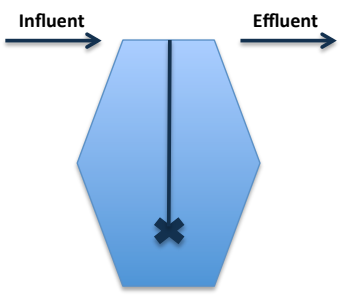
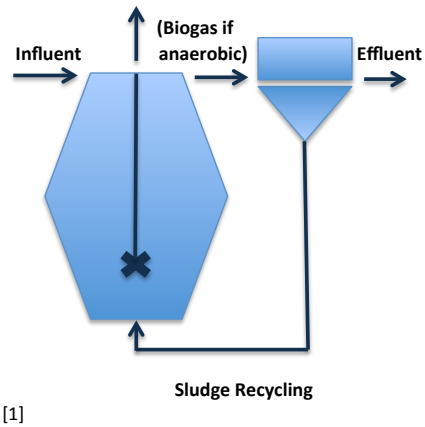
The Monod growth model was developed for the study of pure cultures in the laboratory and enables empirical fitting of biomass growth rates for a specific substrate concentration at a defined temperature, pH and pressure. In the 1960s, Perry McCarty proposed an analogous method for estimation of growth rates and biomass yield in anaerobic systems (Lawrence 1969). Recognising that a single stage in the degradation process will govern the reaction rate in a system, McCarty demonstrated the applicability of the Monod model for anaerobic degradation in which methanogenesis with acetate is the rate-limiting step. This enabled theoretical modelling of the biological process in anaerobic wastewater treatment for the first time. Today, application of Monod kinetics, as applied by McCarty, continues to form the biochemical basis for the vast majority of models of the AD process including that of the generalised Anaerobic Digestion Model, ADMI (Batstone, Keller et al. 2002) . Whilst ADMI and other models have been found useful for retrospective data fitting of reactor operation and for predicting model operation based on retrospective data, it has not yet been found useful in the design process. The kinetics approach relies on the assumption that the system remains in steady-state, which in real wastewater treatment plants is rarely if ever achieved. Further, the half-saturation constant, Ks, must still be determined empirically by specific measurement of a given biomass against a given substrate in either lab- or pilot-scale testing (Bhunia and Ghangrekar 2008). As such, the development and design of new technologies to meet the demands of the current wastewater treatment paradigm currently remains reliant on the experimental approach. Future research is required that begins to quantify the bounds within which steady-state assumptions hold true for a biological system, and further, that enables the design of microbial communities that can promote steady, predictable operation under a pre-determined range of fluctuating conditions. Due to variable influent and hence reactor conditions, and the lack of constancy in microbial community however, design of anaerobic systems remains largely based on empiric design procedures and the pilot plant approach (Switzenbaum 1995, Tchobanoglous, Burton et al. 2004, O'Flaherty, Collins et al. 2006).

2.4 From Activated Sludge to the EGSB

The continually stirred tank reactor (CSTR) is the most basic biological treatment process for sewage and may be operated in either batch or continuous flow. In the late 1800s, this type of reactor was widely used for the aerobic treatment of sewage in batch mode. In 1914, Ardern and Lockett recognised that in their batch operated aerobic sewage treatment tanks the degradation of organics improved with time. This they credited to what they

described as 'activated' sludge, a mixture of flocculent solids that accumulated in the treatment tank during the degradation process (Orhon 2014). To optimise treatment and reduce retention times a secondary settling tank was employed that enabled separation of this activated sludge from the effluent for return to the treatment tank to promote consistently higher treatment rates in the tank as shown in the Table 2-4. Ardern and Lockett additionally recognised that treatment rate was improved by improving contact between the activated sludge and the bulk fluid. This process, still described as the activated sludge process, became widely adopted in sewage treatment and is indeed still commonly applied today. The activated sludge they described was in fact adapted biomass functioning near optimum growth.

Table 2-4: CSTR and physical retention systems: schematic and key descriptors

Completely Stirred Tank Reactor	**Physical Retention**
	 [1]
Key Features	
• Simple design, few moving parts • Straightforward operation • Sub-optimal OLR required to prevent overloading with variable influent concentrations [2] • If influent is of variable substrate concentration, treatment rate is likely to be sub-optimal to suit growth rates at lowest concentration to prevent washout [2]	• Relatively simple design, require additional pumping for sludge return • Treatment rates may be controlled via sludge return >> higher rates >> reduced tank size, • Eliminate risk of washout in the system
Aerobic WWT Variants	
• Aerated ponds	• Activated sludge processes (developed 1914) [1] • Wider variants: fixed film reactors including trickling filter, rotating biological contactors
Anaerobic WWT Variants	
• Continually stirred anaerobic digester	• Anaerobic Contact Process (developed 1955) [1]
Relative Loading Capacity Under Anaerobic Conditions	
1	5 [2]

[1] adapted from (van Lier 2008), [2] (Tchobanoglous, Burton et al. 2004)

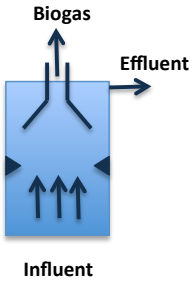
Understood in terms of the microbial growth models developed in the 1950s, what the activated sludge process had effectively done was to begin to decouple microbial growth rates from liquid retention times to enable utilisation of biomass at, or near, the exponential growth phase. Whilst the activated sludge process has proven successful in removal of organics and nutrients from settled sewage, the process requires the addition of energy to oxygenate the wastewater and results in large quantities of sludge that requires post-treatments for stabilisation.

In sewage treatment in the early part of the 19th century, AD was commonly applied in systems similar to the CSTR for the treatment of very high solids wastes at high retention times for the stabilisation of primary sludges. At that time, the slow growth exhibited by microorganisms in anaerobic conditions however had deemed AD unsuitable for treating lower strength wastes such as settled sewage at short retention times. It wasn't until 1955 that Schroepfer et al (Schroepfer 1955) developed an anaerobic system analogous to the aerobic activated sludge system. Described as the anaerobic contact process Schroepfers design again utilised a secondary settling tank and sludge return system and the method proved able to treat medium strength wastes at greatly reduced retention times as compared to traditional CSTR type AD processes. Whilst the anaerobic contact process had some success in the treatment of mid-strength wastes, it was not widely adopted, however, it is recognised as the precursor for treatments developed in the 1970's and 90's that built upon the idea retaining biomass in a system to decouple solids and liquids retention times (van Lier 2008). The best established amongst these are the UASB and the EGSB, the defining characteristic of each of these systems is shown in Table 2-5.

Both the UASB and the EGSB are based on an upflow configuration enabling biomass to settle in the lower half of the reactor and effluent to be drawn off from the upper portion. Each reactor type was developed as a fully retained biomass systems and the strongest commonality between them is that treatment in each is underpinned by the development of an established, well-adapted, self-immobilised granular biomass. Sludge granulation was first observed during the development of anaerobic filter treatment systems (Young and McCarty 1969) that utilised a support material for biofilm development and retention and in the Dorr'Oliver clarigesters in South Africa in 1979 (Hulshoff Pol, de Castro Lopes et al. 2004). In these systems it was observed that in addition to the planned biofilm, sludge flocs or granules also formed that were self-immobilised. In UASB reactors these naturally formed granules settle at the bottom of the sludge bed below the upper flocculent sludge layers. By contrast, in EGSB reactors, higher upflow velocities are utilised to wash out the flocculent material found in UASB reactors and to support development by

selective pressure of a sludge bed that exists almost solely of granular sludge. The higher upflow velocities in EGSB reactors further promote a high degree of mixing in the reactor volume, eliminating dead space in the reactor as is commonly associated with UASBs and improving contact between the waste and the granular sludge. Together these factors have been observed to promote treatment efficiencies in EGSB reactors as high as 75 times that found in traditional anaerobic CSTRs (Table 2-5), and 3 times that observed in the UASB.

Table 2-5: UASB and EGSB schematics and summary of key features

Immobilised Biomass	**Enhanced Contact**
	 [1]
Anaerobic Variants and Key Features	
Upflow Anaerobic Sludge Blanket: • Developed circa 1976, Lettinga • Upflow system • Fully retained biomass >> high-rate system • Gas-liquid-solid separation • Well settling sludge – granules and flocculent material • Activity in bed is gradated with depth • Suitable for high-strength wastes and very high solids wastes	**Expanded Granular Sludge-Bed:** • Developed circa 1992, van der Last & Lettinga • Upflow system • Fully retained biomass with enhanced mixing >> very high-rate system • Gas-liquid-solid separation • Well settling granular sludge, assumed fully mixed • Recirculation of liquor to promote bed expansion and improved mixing • Suitable for high-strength, low solids wastes
Relative Loading Capacity Under Anaerobic Conditions	
25	75 [1]

[1] Adapted from (van Lier 2008)

Granular sludge based technologies are advantageous over other forms of anaerobic contact processes for a variety of reasons. Firstly, the sludge is known to form naturally under appropriately engineered conditions without the requirement for addition of inert support materials as associated with fixed film reactors. Secondly, granular sludge has settleability characteristics that enable settling and retention in a single reactor (Hulshoff Pol, de Castro Lopes et al. 2004). Finally, granular sludge has been demonstrated to display improved activity over loosely flocculated sludges due to the close proximity of

syntrophic microorganisms in the granule matrix (Hulshoff Pol, de Castro Lopes et al. 2004). Sludge granulation then enabled complete decoupling of microbial growth rates and liquid retention times (Nachaiyasit and Stuckey 1997) (Zheng, Wang et al. 2012) and operation of reactors under continuous flow without risk of microbial washout for anaerobic treatment at truly high-rates.

2.5 Towards a Scientific Understanding of the EGSB

Whilst the EGSB is a relatively simple technology in terms of the hardware required to construct it, process design in an EGSB is somewhat more complex. A range of the parameters under the control of the engineer in operating an EGSB is described in Table 2-6. In presenting these parameters, an attempt has been made here to define 'limits' for each parameter listed based on the wealth of experimental data available in published literature. A key issue in defining limits of operation however is the complex range of interactions existing between each operating parameter. The lower limit for HRT for example is highly dependent on multiple factors including settleability of the sludge used, the composition and strength of the waste applied, and the required effluent COD concentration; the figure reported in the table is for a waste with soluble components only. As the 'limit' was determined using a readily degradable substrate it therefore is not universally applicable to wastes with a higher solids load. Similarly, the upper limit for organic loading reported here was not established at the lower limit for temperature operation. Adding to this, 'successful' operation itself is not universally defined, and 'failure' is rarely studied such that commonalities between studies might be established and mechanisms understood. Compounding these issues is a lack of repetition in tests conducted. The EGSB is a well-studied, and increasingly well-established technology, and a great number of papers are already published on the subject. Across the multitude of studies conducted however, frequently a single paper exists that tests a specific set of applied conditions. The reason for the lack of repetition in studies is difficult to ascertain. On the one hand, it is often the case in the field of sanitation that engineering design and application must, by necessity, precede scientific advancement and therefore broad knowledge over a range of conditions must be acquired quickly; perhaps at the expense of the repetition that would yield a measure of confidence by quantifying uncertainty. This approach does not always prove advantageous however as may be demonstrated by lag-time between scientific advancement and the application of technologies such as the UASB to municipal wastes which resulted in a situation where early failures in the system at full-scale has resulted in

Table 2-6: Key EGSB control parameters for process design, interactions, empirically derived limits and observations on implications for design

Parameter	Interactions with Biological Processes	Limits	Observations
Upflow velocity	• Controlled via recirculation flow, the applied upflow velocity interacts with gas production to combine to total velocity in reactor • Shear force influences granule structure especially during granulation [1] [2] • Influences degree of bed expansion, mixing and therefore substrate availability [3] • Reduces or eliminates dead space in reactor • Controlled to prevent granule washout [6] [7]	* Reported range in experiments ~3-15 m/h [4] * Commonly applied in practice ~4 m/h	Positive effects of mixing limited by tendency towards short-circuiting or washout at higher velocities. [5]
Temperature	Physical / Chemical • Decreasing temperature increases viscosity of reactor liquor impacting mixing and decreasing diffusion of soluble compounds [8] • Decreasing temperature increases solubility of gaseous end products in the reactor liquor [8] and impacting final methane yield Microbial • Selective influence on species abundances via influence on growth rates and metabolism, acclimation common for low-temperature adaptation [9] [10] • Mesophilic temperature associated with optimum growth rates for key methanogens [11] [12] [13] Process control • Lower temperature historically associated with process instability [14] [15]	Optimal reported as: Mesophilic: 25-37 ^{0}C Thermophilic: 45-60 ^{0}C * Problems < 20 ^{0}C [13] Low temperature in EGSBs at lab-scale successful: • 8 and above [13] • 4-10 after lengthy acclimation period [16]	Key issue with temperature is energy requirement and cost associated with heating a reactor. Energy and cost saved by operating at low temperature should be balanced against the impact of fluctuating performance in treatment.
pH	• Methanogenic consortia sensitive to extremes of pH [12] • Very low pH can result in reactor souring >> Influences methane production and COD removal	Operational problems out with the range pH 6.0 – 8.0 [12]	Commonly controlled by addition of buffers >> additional operating cost.
HRT	• Decreasing HRT associated poor contact between substrate and biomass [17] [18] • Low HRT associated with increasing granule diameter [19], very low HRT associated with washout of biomass [20]	Drop in performance observed at 1.5 h HRT [17] [20]	Upper limit not an issue, aim to minimise HRT, trial and error to determine for given sludge.

[1] (O'Flaherty, Lens et al. 1997), [2] (Alves 2000), [3] (Kato, Field et al. 1994), [4] (Karnchanawong 2009), [5] (Zheng, Wang et al. 2012), [6] (Lettinga 1991), [7] (Arcand, Guiot et al. 1994), [8] (Lettinga, Rebac et al. 2001), [9] (van Lier, Rebac et al. 1997), [10] (Connaughton, Collins et al. 2006), [11] (Bitton 2005), [12] (Liu and Tay 2004), [13] (Bowen, Dolfing et al. 2014), [14] (Matsushige, Inamori et al. 1990), [15] (Zeeman et al), [16] (McKeown, Scully et al. 2009), [17] (Kundu, Sharma et al. 2013), [18] (Girault, Rousseau et al. 2011), [19] (Seghezzo, Zeeman et al. 1998),

Table 2-6: Key EGSB control parameters for process design, interactions, empirically derived limits and observations on implications for design (Continued)

Parameter	Interactions with Biological Processes	Limits	Observations
SRT	• Influenced by settleability of granular sludge • Controlled by limiting upflow velocity (applied by recirculation and gas production) or HRT to prevent washout or promote selective washout of fine flocs [21] [22]	Min. of x3 that of doubling time of rate limiting organisms [23]	Beyond granulation typically aim to prevent sludge washout.
OLR	• Influences biogas production rate and COD removal rate depending on substrate strength and composition • Influences solids retention time via biogas production influence on upflow velocity	Upper limit: 40-45 kg COD / m^3.d [23] Ave: 20 kg COD/m^3.d [24]	Impact of solids composition of feed is key to determining optimum OLR. Upper limits often cited for low-solids substrates.
Substrate composition	• High VFAs and low solids may lead to souring of reactor >> methanogenesis is limiting step • High solids component >> hydrolysis may be rate limiting step [25] [26] • Presence of ammonia and alkalinity as found in sewage can buffer against pH changes • Various compounds known to inhibit methanogenesis, may be overcome via acclimation [27] [28]		Not under control of the engineer – typically other parameters designed to suit the substrate. High solids problematic for EGSB. [27]
Inoculum	• Assuming sterile feed supplied governs community richness and membership • May be manure, animal wastes but start-up minimised using existing granules as inoculum • Typically from existing digester – acclimation period allowed for new conditions of temperature etc. • Current investigation of alternative inoculum e.g. arctic sludges		

[20] (Yoochatchaval, Ohashi et al. 2009), [21] (Lettinga 1991), [22] (Arne Alphenaar, Visser et al. 1993), [23] (van Lier 2008), [24] (Frankin 2001), [25] (Vavilin, Fernandez et al. 2008), [26] (Palatsi 2011), [27] (Blum, Hergenroeder et al. 1986), [28] (Blum and Speece 1991)

scepticism in AD in the wastewater treatment industry (Switzenbaum 1995). On the other hand, the lack of repetition in studies may arise from additional costs and a perception that there is a limited gain in knowledge from the additional experiments. Alternatively, the drive for novelty in publishing may, to some extent, discourage repeat testing. Regardless of the precise cause, without repeat testing and some standardisation in test methods, development in the field remains not only entrenched in empiricism, but the capacity to draw useful conclusions from the empirical trials is greatly reduced.

A final factor that has hindered broader applicability of results from the extensive range of studies conducted in the EGSB is a lack of understanding of the underlying microbial community. The range of operating parameters described in the Table 2-6 previously were historically assessed in terms of the physico-chemical influence they have on the process e.g. the influence of upflow velocity is assessed by it's effect on COD removal or biogas production rate rather than the influence it has on the community that facilitates treatment in the system. As such, the potential to determine if the upflow velocity is influencing the treatment rate by, for example, inducing decreased productivity of the biomass at high upflow or, merely via physical effects on the retention of the biomass, was not possible. The use of specific methanogenic activity (SMA) testing of biomass was the first and most commonly applied method to circumvent this knowledge gap. The SMA of the biomass may be established by batch incubation of small quantities of biomass with specific substrates linked to the methanogenic metabolism such as acetate or hydrogen and recording the associated methane production using the pressure transducer method (Colleran, Concannon et al. 1992, Coates, Coughlan et al. 1996). In this way some understanding of the influence of external parameters on the functional capacity of the biomass is understood and variability between treatment levels achieved in differing studies might be quantified. Whilst a useful tool, the SMA cannot explain why the biomass response has changed, nor can it offer insights that enable reengineering of the process such that biomass response is more favourable. It is here that the revolution in molecular ecology is beginning to offer scientists and engineers the opportunity to adopt a truly holistic approach to process monitoring that might give rise to new engineering opportunities for process control and optimisation.

Overall, the complex interactions between design parameters, experimental methodologies and the underlying microbial community coupled with reliance on arbitrary empirical testing hinders the applicability of collected experimental data to full-scale reactor design at present. Indeed, in an ever-changing world with ever-changing social and industrial practices, and hence wastes, empirical determination of the influences for every

combination of every parameter is certainly not possible. What is required is the development of broad generalisations based on a scientifically determined hierarchy of parameter influence that might guide design. Two key strategies by which this might be achieved may be defined. The first remains largely empiric and might aim to improve the capacity to draw common conclusions across diverse studies. One method by which this could implemented is via the development of standardised experimental methods and procedures including the adoption of failure testing in laboratory experiments as is commonly applied in other areas of engineering. The second is to develop a methodology by which data pertaining to the underlying microbial community in EGSB reactors is collected, collated and reported to promote improved generalised understanding of the underlying microbial consortium in relation to engineered parameters.

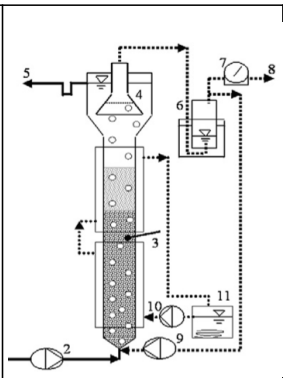
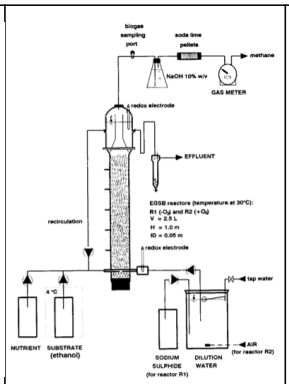
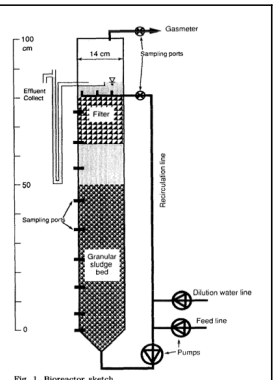
2.5.1 Experimental Design in Lab-Scale EGSB Trials

There is a huge degree of variability in experimental design and testing of EGSB type systems at lab-scale. To some extent this is natural in that each experiment will be designed to test a different hypothesis and will therefore require different test set-up and monitoring regimes. Some common features remain however, and therefore improving uniformity of experimental method and lab-scale reactor design might help unify results found such that broad findings may be extrapolated between multiple studies with varying end goals.

Lab-Scale Idealisation of the EGSB

One key area in which studies vary is the interpretation of the EGSB in terms of physical configuration in the laboratory. Typically there is broad agreement that an EGSB will comprise an upflow system housing granular sludge in which mixing and bed expansion is controlled via application of a recycle line. However, the geometries across which this is interpreted at lab-scale are highly varied and rarely does the geometric configuration specifically relate to the hypothesis tested. Table 2-7 shows some variations of lab-scale geometry in EGSB idealisation identified in the literature. In each of the examples given, the studies focussed on parameters that influence primarily the hydraulic regime in the reactor. In study [1] (Karnchanawong 2009) the influence of upflow velocity on treatment efficiency was investigated. Thus, the very tall lab reactor enabled large bed expansion without washing out the sludge. In the reactor geometry described in [2] above ((Zheng, Wang et al. 2012), the influence of upflow velocity and organic loading rate were examined simultaneously. Intuitively, the difference in geometry between the two reactor idealisations seems likely to result in very different hydrodynamics, and therefore, the

Table 2-7: Variation in lab-scale bioreactors idealisation: schematics and geometry

 [1]	 [2]	 [3]	 [4]
Volume / L			
39.3	4.4	2.5	13
Dimensions, diameter * height / mm			
100 * 5000	60 * 2000	50 * 1000	130 * 930
Aspect ratio, diameter : height			
1 : 50	1 : 33	1 : 20	1 : 7

[1] (Karnchanawong 2009), [2] (Zheng, Wang et al. 2012), [3] (Kato, Field et al. 1994),

[4] (Arcand, Guiot et al. 1994)

studies would potentially draw different conclusions despite of the similarities in the aims of the study. Indeed this is in part confirmed by the result that in study [1] optimal performance was achieved at an upflow of 4 m/h at an OLR of 11.3 g COD / L .d, whilst in study [2] 50% washout of biomass occurred at an upflow velocity of 3 m/h, albeit under a higher applied OLR of 26 gCOD / L .d. On the one hand, comparison of these studies confirms the interaction between OLR, gas production rate and upflow velocity whilst on the other the extreme difference in reactor idealisation muddies the potential to extrapolate a conclusion between the two. Studies [3] and [4] use differing idealisations again, but again both experiments focus primarily on hydraulic conditions. In study [3] (Kato, Field et al. 1994) granule washout was observed at velocities above 5.5 m/h at on OLR of 7 gCOD / L .d yet by contrast in the much shorter reactor used in study [4] (Arcand, Guiot et al. 1994), an upflow velocity of 6.6 m/h was comfortably maintained and accompanied by good COD removal at an OLR of 11.5 g COD / L .d. Whilst this is initially counterintuitive it is noted that in study [4] a filter layer consisting of plastic packing rings was included in the reactor design rather than the conventional gas-solid-liquid separator used in in study [3] hence tendency to washout at higher upflow velocity and OLR was resisted. Essentially, it is demonstrated in this small subset of studies that continually

reinterpreting the reactor at lab-scale limits the efforts to empirically determine operational limits.

Sampling Strategy

Between the reactor sets described in Table 2-7, a further key distinction can be made in the capacity for sampling. Reactors [1], [3] and [4] have sample ports distributed along the length of the reactor whilst reactor [2] does not. In each of the studies [1], [3] and [4] these sample ports were utilised to note physical differences in the sludge bed with height in the reactor. In studies [1] and [3] increased suspended solids were noted at lower ports in the sludge bed and study [3] identified that physical distinction could be made between the granules at the top and bottom of the sludge bed with respect to mean granule diameter. Neither of studies [1] and [3] utilised the sampling ports to investigate variability in biological sludge properties. In study [4] on the other hand, used sampling of sludge at different depths in the reactor to find that larger granules found at the bottom of the sludge bed were associated with increased specific methanogenic activity against acetate and propionate. Study [2] has only a single sample port and hence no variation with depth in the reactor is reported but it might be implied that if SMA had been measured here, the choice of position of the sampling port would influence the result; as indeed it would have in studies [1] and [3] were the ports utilised to support this measurement. Whilst other factors varied in each of these experiments such as temperature, source of biomass and composition of reactor influent, the implication from these studies is that reactor design might influence not only the physico-chemical results obtained in lab-scale trials but also those pertaining the underlying microbial community. The studies reported here were selected for their commonality as much as their contrast. Studies focussing on microbiological aspects of the EGSB are similarly conducted in vastly contrasting geometries and indeed this brief review does not encompass all the variation described in the literature. The influence of these varying geometries has yet to be fully understood either in terms of their influence on the underlying community structure or their impact on scaling-up results for design at full-scale. Determining this might provide valuable insights enabling the development of a common configuration that might be adopted, even if only as a control, in lab-tests such that more fundamental differences and commonalities between experimental results might be extrapolated.

Control Reactors:

The use of control reactors might seem like a prerequisite of any empirical study, yet in some studies it is seen as being unnecessary and others demonstrate a range of different

strategies for employing control reactors. Thus this is an area where a consistent approach might improve our ability to interpret empirically determined findings at lab-scale. The vagaries of control reactor configurations are rarely explained in terms of the ultimate goals of the experiments and one is left to guess whether the strategies are rational or reflect more prosaic considerations such as the cost and time of experiments. Table 2-8 below describes some of the key control strategies utilised in EGSB studies. The examples given here focus on acclimation of mesophilic sludges extracted from full-scale reactors to low-temperature applications in the laboratory:

Table 2-8: Commonly reported control strategies in lab-scale EGSB studies

Control Strategy	Aim	Observations
No control, single test reactor used, ramped decrease in temperature, all else constant [1]	To investigate stability and efficiency of long-term low-temperature treatment.	Granules disintegrated ~9.5degC, ~d584. Without control cannot tell if this is a result of duration at low temperature, the specific temperature applied or duration on soluble-only feed.
No control, duplicate test reactors, ramped OLR via reducing HRT, alternate addition of solvents, all else constant [2]	To investigate the feasibility of treatment of solvent contaminated wastes at low-temperature.	Granules disintegration ~d350. Again, difficult to ascertain cause without control. Duplicates did not perform identically, disintegration and acetate accumulation more severe in one reactor than the other.
Single parallel control at 37degC, duplicate test reactors at 15degC, ramping of upflow velocity and OLR via COD [3]	To investigate low-temperature granulation rate vs mesophilic granulation rate and identify populations associated with each phenomenon	The control reactor provides useful comparison of granulation rate and efficiency at 37degC, enables elucidation of temperature effect. Test reactor performance and community duplicated.
Duplicate parallel controls, duplicate test reactors		

[1] (McKeown, Scully et al. 2009), [2] (Enright, McHugh et al. 2005), [3] (O'Reilly, Lee et al. 2009)

Studies [1] and [2] represent the most common approach used in laboratory testing of EGSBs. No specific control reactor is employed but use of ramping of applied parameters to increasing extremes is conducted instead. To some extent the ramping strategy might imply that the authors regard the previous period of operation as the control for each ramping stage i.e. whilst no specific control reactor is used, the 'ramping' approach might be interpreted as an 'in-series' form of control. The success or otherwise of this type of control strategy is difficult to ascertain for several reasons. Firstly, it is not possible to extract changes in reactor operation that might have occurred due to on-going adaption to the laboratory reactor from that occurring due to the ramped applied changes. Whilst both papers [1] an [2] are studies in which acclimation of biomass to temperature effects are central to the study, the biomass used is additionally required to acclimate to alternative upflow velocities, substrate composition and reactor geometries. As such the granule disintegration, which is reported in each study in relation to low temperature treatment,

cannot be directly attributed to temperature effects. A second issue arises in that the time at which a ramped change takes place might impact the response in the underlying community. Microbial communities are known to be dynamic and relative abundances of microorganisms can fluctuate even in steady-state operation. Figure 2-1 below aims to illustrate this point:

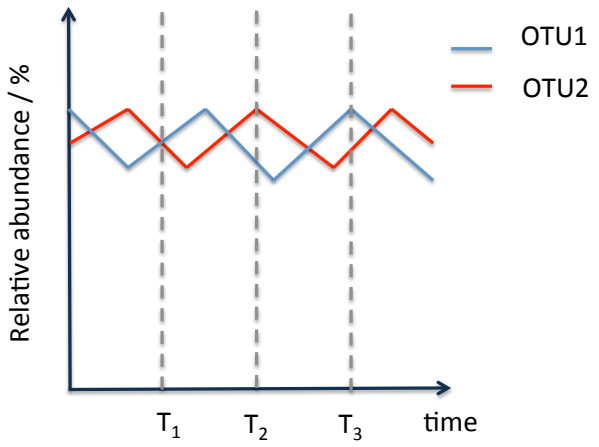


Figure 2-1: Schematic of arbitrary fluctuation in relative abundance of OTUs with time

The graph shows a hypothetical fluctuation between two organisms OTU1 and OTU2 and three arbitrary time points, T1-3. Suppose the aim was to impose a temperature change that would select for cold adapted organisms at a time when the putative cold adapted organisms were at their most abundant. Then from Figure 2-1, if it was assumed that OTU1 in is better able to function at low temperature than OTU2, then application of a temperature drop at arbitrary time T3 might be most favourable in terms of operation than at T2 which might be least favourable whilst at T1, there might be neutral effect of timing. Indeed, study [2] that utilised two test reactors subject to changes in substrate, demonstrated differential response to the applied change. It may be the case that underlying fluctuations in community structure contribute to this.

An alternative but less common approach is applied in study [3]. Again, temperature is central to the hypothesis and here an additional reactor is included in the study to enable elucidation of the effects of two different temperatures on the process of granulation. In this case, duplicate 'test' reactors are operated at 15degC whilst the control comprises a single reactor at 37degC. The logic in operating duplicate reactors at 15degC and a single reactor at 37degC presumably reflects the authors' confidence in the response of the microbial community at 37degC and lack thereof at 15degC. In this case, in contrast to the findings of study [2], the duplicate reactors performed similarly and a similar underlying microbial community was found at 15degC that was distinct from that established in the

37degC reactor. Here then, a parallel control strategy successfully elucidates temperature effects on the community although the degree of confidence in the result is limited by lack of duplication in the control. In summary, it is identified that the concept of the control is variously applied in EGSB studies and the most appropriate or informative strategy for control has not yet been established. As the running of control reactors contributes to the cost of a study in terms of time investment and additional reagent cost etc. as associated with reactor monitoring, a unifying strategy would be beneficial to the field in terms of the design of future experimental studies.

Failure

Empirical studies of ESGBs are entirely focused on optimising technologies for a range of conditions. The literature abounds with reports of successful adaptation strategies to, for example, cold conditions, pH increase or chemical contaminants. However, the limits of successful operation or thresholds in operational parameters that will induce failure in the system are rarely reported and are never the explicit goal of experiments. Defining the limits of failure is critical to many branches of engineering, especially where uncertainty in environmental variables exists; limit states for geotechnical materials is a prime example. So perhaps a more rigorous elucidation of failure in ESGB would be useful in design. Again taking the example of operation at low-temperature, the vast majority of research begins with the assumption that the treatment capacity of the sludge used is optimal at 37degC – the common temperature at which full-scale reactors are operated which in turn are the common source of sludges used in lab-scale trial. Working from this assumption, sludges are then acclimatised to lower temperatures, typically in gradual increments possibly to enable adaption of the sludge or possibly such that precise temperature beyond which failure occurs can be determined. In the vast majority of published works, limits for operation are determined as one or a combination of:

- the temperature at which either COD removal drops below a particular level (for low temperature >65% seems to be ‘good’ operation, in studies of high-solids removal, >50% seems accepted),
- the temperature beyond which methane content in the biogas drops below a particular level – sometimes assessed against theoretical maximum yield and sometimes against the yield achieved in the early stages of the ramping exercise

- the temperature at which biomass de-granulates and resultantly begins to wash out of the system. Response to degranulation is typically to adjust operating conditions such as organic loading rate or applied upflow velocity to enable biomass retention.

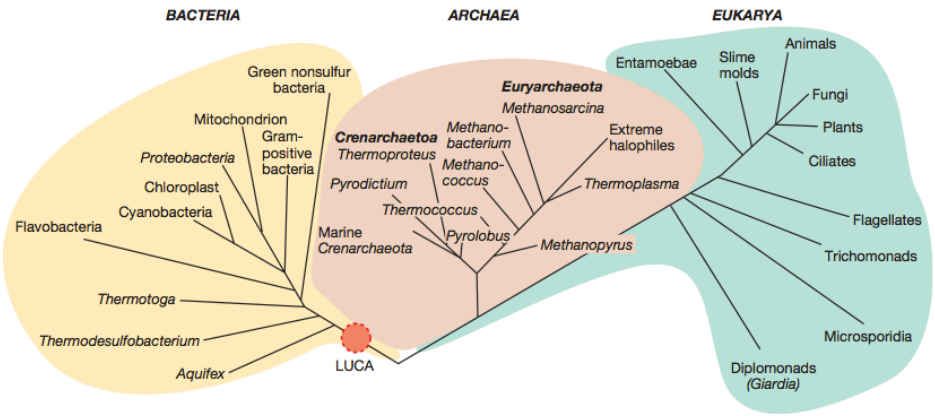
Whilst works conducted in this way have indeed begun to establish limits of sorts for the application of the EGSB, difficulty arises in the interpretation of the results due to subjectivity in defining success or failure. This subjectivity is in some ways inherent in the nature of wastewater treatment as wider field in that regulations vary regarding discharge standards and 'whole-plant-processes' will include variable downstream treatments to suit. In spite of this however, some universal guidelines against which success or otherwise of parameters tested might be assessed would be useful in enabling ready comparison of published results even if such assessment is only included as supplementary data for published works. Further, study of the failure mechanisms themselves is vital to determine if, for example, the failure point is consistently reproducible and the failure mode predictable. Without such information, the limits obtained in a study cannot be readily adopted to determine limits for operation and design of full-scale plants.

2.5.2 Microbial Ecology: A Historical Perspective

Wastewater treatment and effluent quality in the EGSB is fundamentally governed by the microbial composition and structure of the underlying granular biomass. Until recently, scientists have had little capacity to understand which microorganisms are present in the system, where they are and ultimately what they are doing. As a result, the, capacity to fully engineer the treatment process is hindered. Historically, microbial communities were studied using either microscopy or culturing and isolation methods and indeed both methods are still very relevant in terms of the physiological characterisation of microorganisms today. Both methods however have significant drawbacks in the study of complex communities. Microscopy frequently cannot distinguish between morphologically similar microbial populations and culturing methods are known to be highly selective (Gilbride, Lee et al. 2006) with culturable organisms representing less than 1% of complete community composition in certain environments (Amann, Ludwig et al. 1995). Despite these drawbacks, microscopy coupled with laboratory cultivation of microorganisms, has historically enabled taxonomic classification of microorganisms based on the microbial phenotype (Madigan, Martinko et al. 2009) providing a systematic framework for microbial characterisation. Simultaneously to the development of the EGSB, huge advances in the field of molecular microbial ecology have been made, driving a paradigm shift in the understanding and classification of microorganisms. The

development in the mid 1980s of readily applicable nucleic acid based approaches for the study of microbial communities has for the first time begun to enable insights to the in-situ role and function of un-culturable species in relation to the treatment process (Gilbride, Lee et al. 2006, O'Flaherty, Collins et al. 2006).

The cells of all living organisms contain deoxyribonucleic acid (DNA), a chemical whose structure defines and controls the nature of the cell and it's metabolic and reproductive capacities. The double stranded helical structure of DNA was famously identified by Watson and Crick in the 1950's however it's use in identifying microorganisms from mixed microbial communities was not widely applied for almost a further 50 years, in which time, a number of key findings were made. In 1977 Woese and Fox (Woese and Fox 1977) proposed a new method for classification of cellular life based on evolutionary genetics. The new system recognised three domains (kingdoms) of life based on classification according to sequence similarity of ribosomal ribonucleic acid (rRNA) giving rise to the system that underpins contemporary phylogenetic classification today. In phylogenetic classification cells are grouped according to the evolutionary history of the 18S rRNA small subunit (SSU) in Eukaryotes and the 16S rRNA SSU in Prokaryotes and phylogeny enabled for the first time identification of a third domain of life, Archaea, alongside previously determined Bacteria and Eukaryotes (Woese and Fox 1977, Fox, Stackebrandt et al. 1980). These three domains may be mapped graphically as the phylogenetic tree of life as shown in Figure 2-2:



From (Madigan, Martinko et al. 2009)

Figure 2-2: Phylogenetic Tree of Life, LUCA, Last Universal Common Ancestor

Whilst the structure of DNA was established in the 1950s and gene isolation methods developed in the 1970s enabled the development of phylogeny, studies of mixed microbial communities were hindered by the low quantities of DNA that could be extracted from environment samples. In the 1980s the Nobel Prize winning development of the

polymerase chain reaction (PCR) by Kary Mullis (Bartlett and Stirling 2003) opened the door to a new era in the study of microbial ecology. Using specifically designed primers which themselves are short sections of single stranded DNA, a process of repeated heating and cooling for denaturation, primer annealing and extension, enables synthesis of large volumes of short sections of copy DNA from template extracted from environmental samples. Thus PCR enables the study of genes of interest extracted from mixed microbial communities from specific environments. Automation of the PCR process along with the discovery of thermally stable polymerases meant that PCR could be applied with increased confidence using convenient and inexpensive bench top equipment such that the process became the foundation approach for a broad range experimental techniques. The gene encoding for 16S rRNA (Figure 2-3) may be found in all prokaryotes and consists of highly conserved regions whose sequence changes very slowly in an evolutionary time scale (Fox, Stackebrandt et al. 1980) such that it not only lends itself well to classification by phylogenetic means but to the design of primers for identification at of microorganisms at various taxonomic levels by utilising the highly conserved sequences flanking the hypervariable regions within it. As such the isolation and replication of the 16S rRNA SSU remains central to the many methods supported by the use of PCR that are central to the study of complex microbial communities today.

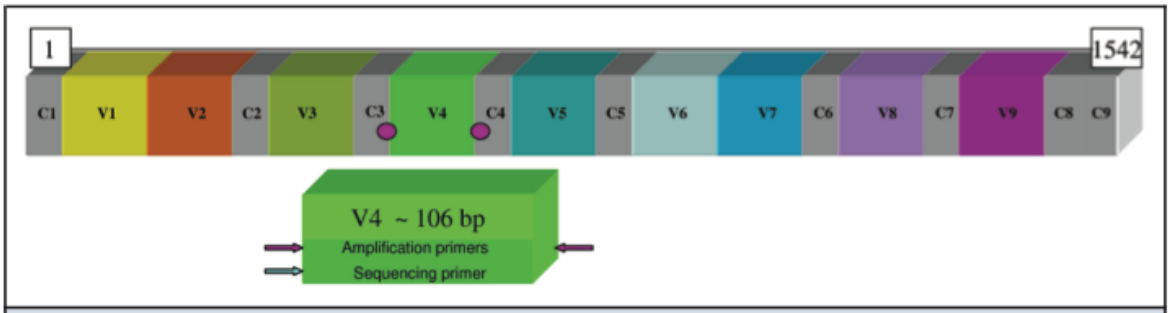


Fig. 2. Conserved and hypervariable regions in the 16S rRNA gene.
The interspersed conserved regions (C1–C9) are shown in gray, and the hypervariable regions (V1–V9) are depicted in different colors. Also illustrated is an example of primer selection for DNA amplification and sequencing-based microbial identification (V4 subregion with pink circles and arrows representing primer-binding sites).

From (Petrosino, Highlander et al. 2009)

Figure 2-3: Schematic of 16S rRNA SSU showing conserved and hypervariable regions

2.5.3 Microbial Ecology in EGSB Studies

A great number of techniques for studying mixed microbial communities are now well established with new methods constantly in development. This section of the report considers key among them as applied most commonly in the study of microbial diversity

and dynamics EGSB type reactors and highlights emergent methods that might provide deeper insight into microbial communities.

2.5.3.1 PCR, reverse-transcription PCR (rtPCT) and quantitative PCR (qPCR)

PCR in itself is typically used as a precursor for a variety of other techniques. PCR is most widely used in microbial ecology to amplify double stranded DNA, and for phylogenetic applications the genes coding for 16S rRNA are targeted as described previously. The amplified gene fragments are then used for downstream processes such as sequencing to enable analysis of microbial community composition. An alternative application for PCR is to extract RNA and to amplify the gene of interest using reverse transcription PCR (rtPCR). DNA is present in all cells regardless of whether they are active in a community and indeed DNA may linger in dead or dying cells thus a DNA based approach may not best describe community function. RNA, which is a single stranded molecule, is only produced in active cells and tends to degrade more rapidly in dying cells than does DNA thus rtPCR is a desirable option if functional community composition as opposed to total community composition is the priority of a study (Malik, Beer et al. 2008). Disadvantages of this method are that RNA degrades rapidly such that sampling strategy is known to influence results. Similarly to PCR, rtPCR typically involves downstream processing of the amplified product. A further alternative approach to PCR, and one that does not require downstream processing, is the use of qPCR. Here fluorescent probes are used to enable monitoring during the early stages of the synthesis process in PCR to provide best estimates of gene copy number of the original target template concentrations in a sample (Malik, Beer et al. 2008). As a quantitative method, qPCR has been widely applied however as with rtPCR, prior knowledge of targets required for primer design hinders applicability of the process at species level for rare or unknown organisms.

2.5.3.2 Fingerprinting Techniques

Fingerprinting techniques may be applied for the study of microbial community composition and dynamics and are advantageous in that their application is not reliant on sequencing. Rather, fingerprinting techniques use gel electrophoresis for size or sequence separated banding patterns that are identified to describe either total community fingerprints or species-specific fingerprints by using either universal primers or species-specific primers. Key methods among fingerprinting techniques used in EGSB studies are amplified ribosomal DNA restriction analysis (ARDRA), terminal-restriction length polymorphism (tRFLP), temperature gel gradient electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE). Whilst sequencing is not essential for these

techniques, it remains the only method that enables identification and classification of specific organisms in complex communities and as such is frequently applied in conjunction with them (Gilbride, Lee et al. 2006). Historically, the commonest sequencing approach applied in conjunction with such methods was the Sanger methodology. The advantages of Sanger sequencing are that lengthy sections of DNA may be sequenced such that for example the entire 16S gene consisting of >1000bp may be sequenced, and that error rates associated with the method are low. Problems associated with Sanger sequencing is that the process is not fully automated and the cost-per-base is high such that identification of rare species is expensive (Shendure and Ji 2008).

2.5.3.3 Next Generation Sequencing (NGS)

Sanger chemistry was introduced in 1977 and has been widely applied in EGSB 16S rRNA gene survey studies for sequencing genes isolated via clone library methods or from fingerprinting techniques. The Sanger process at best is able to process 96 unique samples simultaneously rendering it unsuitable for studies of ecological diversity in highly complex communities such as environmental samples that may contain DNA from thousands of unique species (Shokralla, Spall et al. 2012). NGS methods are now available however that offer considerable advantages over Sanger sequencing and resultantly great potential in new experimental design and monitoring opportunities. NGS technology developed in the last 10 years utilises massively parallel sequencing to read potentially tens of millions of reads simultaneously (Shokralla, Spall et al. 2012). As such, they enable experimental design with increased capacity for finely detailed sampling whilst enabling deeper insights to microbial community diversity. NGS technology spans a number of different platforms, each with it's own advantages and disadvantages in terms of read length, library preparation methods and costs. NGS technologies enable simultaneous sequencing of multiple samples in a single run using barcoded primers for sample identity. Whilst short read length has historically been an issue for many NGS platforms, the use of paired end reads has both improved the quality of the sequencing data and increased the read length such that specific hypervariable regions may be targeted in amplification for subject identification using phylogenetic methods. For the study of 16S rRNA genes from mixed communities in environmental samples, the Illumina Miseq platform coupled with a paired-end-read approach is identified as an effective tool for comparative analysis of microbial diversity between samples from a range of four of the most popular NGS platforms on the market (Amore 2015). A multiplexed barcoded primer approach may be used so that multiple samples can be sequenced in a single run with the potential to utilise as many as 500 barcodes, and hence samples, in a single sequencing run. As a result, the

cost associated with data retrieval from NGS sequencing is estimated to be two orders of magnitude lower than from Sanger sequencing (Shendure and Ji 2008) making NGS an increasingly appealing option for microbial community characterisation. The range of application of NGS technology extends far beyond 16S rRNA survey studies to include metagenomic and transcriptomic applications, alongside new emerging methods for sequencing without requirement for PCR. These methods are not considered in detail here as they are not applied in the context of this thesis. Rather, what is concluded is that in terms of diversity studies of complex microbial communities, NGS technologies generally, and the Illumina Miseq platform specifically, is a promising development in the field that potentially lends itself well for studies that aim to maintain fine-scale sampling using replicate controls without significantly increasing labour or cost as associated with fingerprinting methods.

2.5.3.4 Fluorescent In-Situ Hybridisation (FISH)

The methods described previously require DNA extraction as a precursor. A great number of DNA extraction methods exist, many of which are available in commercial kit form, but all of which are destructive at both the sample level and at the cell level. FISH is a technique that enables visualisation of targeted microorganisms without the requirement for DNA extraction. In FISH, samples are treated with a fixing agent and fluorescently tagged RNA probes used in conjunction with fluorescence microscopy to identify specific groups of organisms in a given sample without requirement to destroy either the cell or the spatial arrangement of the cells in the sample. As RNA rather than DNA is targeted, FISH also provides insight into viability in a community. An additional advantage of this method is that is may be regarded as quantitative. The key disadvantage that probes must be designed either blindly or based on prior knowledge of the community obtained through alternate means such as fingerprinting and sequencing methods described previously. As a visualisation method, FISH has been extensively used in EGSB studies to explore the complex architecture of the granule structure and to gain insight to syntrophic relationships between organisms in the biofilm by physical proximity and hence assumed association (Sekiguchi, Kamagata et al. 1999, Gonzalez-Gil, N. L. Lens et al. 2001, Collins, O'Connor et al. 2005, Diaz, Stams et al. 2006, Fernandez, Diaz et al. 2008, Kelly, Enright et al. 2011).

2.5.4 Microbial Ecology: Opening the Black Box

As the ability of researchers to acquire data from complex microbial communities continues to grow, so too does the demand for data sorting and analysis strategies that will

elucidate useful biological insights (Shendure and Ji 2008). Contemporary studies of wastewater treatment biotechnologies typically comprise monitoring of reactor operation and performance in conjunction with some form of monitoring of the underlying microbial community. The 16S rRNA gene survey continues to form the backbone of the majority of studies conducted, with multiplexed sequencing on high-throughput sequencing platforms increasingly the method of choice. The aim cited by most of this research is to 'unpack the black box' that is the underlying microbial community. In order to do so however, the researcher must first unpack the 'black box' that is the sequencing data. A common methodology for the processing of data arising from fingerprinting techniques has been proposed (Marzorati, Wittebolle et al. 2008, Carballa, Smits et al. 2011) however direct applicability of those methods to NGS is not yet established. Post processing for quality control, data from high-throughput sequencing of a 16S rRNA survey study essentially provides count data that quantifies the range and relative abundance of each species or OTUs present. The field of bioinformatics is therefore a rapidly developing field in its own right. Whilst open source pipelines including most notably QUIIME and MOTHUR exist no single strategy is currently adopted for the analysis of NGS data in EGSB studies. This said, two key strategies for data analysis from community survey studies may be identified. The first focuses on mapping the relative abundance profiles from collected data on established ecological frameworks. The second focuses on data visualisation methods coupled with statistical approaches to analysis. It is noted that these two approaches are not mutually exclusive however it is useful to consider them distinctly.

2.5.4.1 Experimental and Analytical Approaches to Microbial Ecology

Early studies of microbial communities using molecular methods often hinged on the reporting of microbial community composition in terms of simply which microorganisms were present in which abundances, and providing an overview of the phylogenetic relationships between those organisms. As the complexity of microbial communities became increasingly apparent with ever more sophisticated monitoring methods, the drive to characterise community data itself as a method of understanding community function became increasingly desirable. One avenue of exploration for the interpretation of data in this way comes from the study of ecology at the macro-scale (Horner-Devine, Lage et al. 2004). Survey studies at the macro-scale have been commonplace for well over a century. From these largely descriptive studies, common patterns emerge from count data that form the basis of contemporary ecology. From an engineering perspective, application of these patterns or models to the characterisation of micro-scale ecology is highly desirable for a number of reasons. Firstly, whilst two similarly operated biological wastewater treatment

technologies might perform highly similar functions, research increasingly tells us that these functions may be performed by microbial communities of disparate composition. Thus, generalised models may provide an improved method for understanding microbial community composition, over and above knowledge of community membership. Secondly, application of generalised models might enable more rapid assessment of the health of microbial communities in engineered systems in that establishing the 'shape' of data is a less lengthy procedure than is identifying community membership or complex between-species interactions. Finally, and perhaps most significantly, it is hoped that by providing structure to the data gained from such surveys, that models founded in theory might ultimately provide valuable predictive tools for use in bioreactor design and monitoring (Prosser, Bohannan et al. 2007). Thus far, ecological models have been fitted to microbial community data with varying degrees of success. On the one hand intriguing parallels have been drawn between the micro- and macro-scale ecology whilst on the other, contradictions exist between data from 'same-scale' data sets. Still, the number of data sets to which these models have been applied at the micro-scale are relatively few and indeed the size and quality of data sets currently being gathered using new and more effective methods, means that modelling in this way may still yield useful information into community structure and function.

2.5.4.2 Multivariate Analysis and Statistical Approach

An increasing number of multivariate and statistical methods are available for the assessment of gene survey data varying from data visualisation methods to more traditional significance testing of the data obtained. Data visualisation is a useful for tool particularly from the large data sets arising for NGS surveys. Data returned as count data from complex communities has the capacity to record changes in relative abundance for single OTUs between samples from complex communities comprising many thousands of unique OTUs. Traditional plotting methods such as linear and scatter plots are by necessity replaced by non-metric multidimensional scaling (NMDS) plots and heat maps in order that such large data sets can be compressed to readily viewable formats. The key advantage in so doing is to provide the researcher an overview of trends in the data gained which is difficult to ascertain from visual inspection of data tables in the traditional manner. The problems arising from such methods are that interpretation of for example 'similarity' or 'dissimilarity' is somewhat subjective. Nonetheless they provide a useful preliminary tool in data assessment. More statistical approaches are also widely applied to gene survey data, for example linear regression of count data against metadata to find correlation and the statistical significance thereof based on variance in the data. The

problem arising from such methods is that even with fine-scale sampling, sequencing data provides a snap-shot of a community at a specific point in time that may be subject to dynamic change which itself may or may not be driven by applied parameters identified in the experimental design i.e. the 'meaning' of significance in systems with dynamic capacity to fluctuate is weakened. The key to the application of statistical methods is to ensure that the approach adopted is hypothesis driven such that the result might be logically assessed and the null-hypothesis has genuine grounding from theory.

In tackling the problem of data analysis, similarly to the case for data collection for the study of such complex systems, a holistic approach is required. As yet neither statistics nor the ecology approach has been decisively established as better method for data analysis. Regardless of current issues, NGS technology and bioinformatics presents an exciting opportunity to researchers to explore new fields in greater depth than ever before. Whilst computationally complex, and by its nature at present somewhat subjective, it is likely that by collection and analysis of such data sets, new theory against which data may be assessed will arise.

2.6 Scope of this Thesis

The Transforming Waste Project (TWP) is a multidisciplinary research consortium spanning eight universities in the UK and Ireland. The aim of the consortium is to develop a socially appropriate, sustainable, emptying and treatment scheme for pit-latrine wastes in the peri-urban environment for improved sanitation, energy provision and quality of life for end-users. Central to the aims of the group is the design of a an anaerobic digester for pit latrine wastes. Currently pit latrines are frequently operated as 'holes in the ground'; once full, the latrine must be moved and the pit covered over, or the pit manually emptied. Pit latrines have been associated with ground water contamination, the spread of disease via insect vectors and so on, and pit latrine emptying and waste disposal practices are both physically and biologically unsafe for sanitation workers. The reactor proposed by TWP for the treatment of this high-solids, high-strength waste is a variant on the EGSB. The key variant is that the granules that are typically formed 'naturally' in the EGSB are specifically engineered to meet the challenges specific to pit latrine wastes. In brief the aims and objectives of the group may be defined as follows:

- to develop engineered microbial communities on density-gradated beads that enable:

- separation of the stages of methanogenesis spatially in the reactor for improved treatment opportunities
- retention in the system of the granules by density separation even under the action of high-solids, high-viscosity wastes such as sewage
- operation of small-scale plants that might be community owned and operated in the vicinity of homes in the peri-urban environment such that the requirement for a formal wastewater and energy grid is minimised.

The work conducted in this thesis acts as a precursor to the development and design of the microbial communities and density gradated beads. The work aims to define and answer some key experimental questions of 'naturally' occurring granules in EGSB reactors that might inform future trials towards this complex end goal. Further, the work aims to address some fundamental questions relating the test methods applied to development ad design of biotechnologies the results of which have relevance well beyond the scope of the Transforming Waste Project itself. The scope encompasses fundamental questions around the structure, activity and distribution of physiologies in complex AD communities under different conditions of temperature, organic loading and solids concentrations. The arising questions are defined in the following work packages and the results and recommendations form the remaining Chapters of the thesis:

Chapter 3: Materials and Methods

The literature reveals the requirement for a holistic approach to reactor monitoring. The materials and methods section lays out the range of operational parameters selected for monitoring highlighting their relation to theoretical perspectives on the EGSB. Further, the approach to experimental design is described and control strategies tested in each chapter are outlined.

Chapter 4: Scale-Down: A Spatio-Temporal Study of EGSB Type Bioreactors at Full- and Lab-Scale

The 'pilot plant' approach remains the dominant method to scaling up technologies from the bench to industrial application but as yet little is known of the influence of scale on either treatment capacity in the EGSB or the underlying microbial community. Further, lab-scale idealisations of the EGSB are highly varied and the influence of this idealisation is as yet unknown. Two lab-scale reactor types with differing geometries are developed and tested under near-identical conditions to an existing full-scale digester to investigate

simultaneously scale- effects and lab-scale idealisation effects on reactor function and microbial community composition.

Chapter 5: Microbial Community Adaptation with Substrate Type and Substrate Availability

A key aim of the Transforming Waste Project is to engineer specific microbial communities for the treatment of complex wastes such as high-strength high-solids pit latrine wastes and low-strength, high-solids wastes such as Global North sewage. Here triplicate reactor sets are operated with triplicate parallel controls i.e. nine reactors in total are operated under three conditions of substrate strength and composition. A range of physical and chemical monitoring methods are applied and the performance data is complemented with NGS profiling of 16S rRNA gene (V4 region) from whole community DNA during adaptation. Core organisms are identified that are common to the adapted communities and organisms are identified as unique to each condition and the replicate control strategy is discussed in relation to results gained.

Chapter 6: Application of the EGSB to Sewage Treatment in the Global North

The activated sludge process remains one of the most dominant sewage treatment methods used in the Global North today however, whilst an effective treatment method, aeration is estimated to account for over 50% of the energy budget in a sewage treatment plant. Poor solids removal efficiency reported in EGSB systems however limits the application of the technology to sewage treatment. Improved understanding of the microbial community underpinning the treatment of sewage in EGSB reactors might offer increased capacity to engineer the system to treat sewage at low temperatures sustainably and at lower cost than aerobic treatment alternatives. Triplicate 20-litre 1-D lab-scale EGSB reactors were subject to a series of engineered operating conditions aimed at enabling understanding of microbial community adaptation to the treatment of low-strength sewage (500mgCOD/L) at ambient temperatures (15degC). Whole community 16S rRNA gene (V4 region) amplicon sequencing was utilised to monitor microbial community dynamics both spatially and temporally under each condition.

Chapter 7: Failure Study

The Transforming Waste Project aims to develop a new variant on the EGSB for the treatment of pit-latrine wastes. Typically, empirical studies of ESGB are entirely focused on optimising technologies for a range of conditions. However, the limits of successful operation or thresholds in operational parameters that will induce failure in the system are

rarely reported and are never the explicit goal of experiments. This Chapter proposes the adoption of limit state theory to experimental design and uses the EGSB as a model for study. A staged ramping strategy is applied to replicate EGSBs, which are then pushed to failure to identify both predictors of failure and the failure mechanism under high-solids loading using a synthetic sewage.

Chapter 8: Granule Growth: Preliminary Study

Previous Chapters in the thesis point to stratification of function in the sludge-bed at lab-scale by size whilst the same is not seen in a full-scale digester. Here the relationship between size and density is explored in lab-scale granules to determine the separation mechanism. As no link was found between granule size and depth or between activity and depth at full-scale, granules of different size from the full-scale reactor are sequenced to determine if size is linked to community composition in full-scale digesters.

Chapter 9: Contributions, Discussion and Conclusion

Contributions made in this thesis are summarised and are discussed both in relation to the aims and objectives of the thesis and in relation to the wider field. Drawing from the conclusions future work is discussed in relation to both control strategies, the potential for failure testing, and the development of the EGSB for the treatment of high-strength, high-solids wastes such as sewage.

3 Materials and Methods

The aim of this chapter is threefold. Firstly, the approach to experimental design is outlined alongside a summary of the experiments conducted. The intention is that this will provide an overview to the structure of the thesis and make clear at the outset the logic applied to the arrangement of the experiments conducted and grouping of the data collected. Secondly, those experimental methods relevant to physical and chemical monitoring conducted in subsequent experimental chapters (Chapters 4-8) are outlined in detail. Each of the subsequent experimental chapters referring to this section additionally contains a brief re-introduction to the methods used and also outlines any amendments, or deviation from the methods as described here. Any method used in relation to a single experiment is described in detail in the Chapter in which that experiment is reported. Finally, the optimisation and application of molecular methods adopted are outlined.

3.1 Approach to Experimental Design

In the review of literature it was observed that the data arising from many experiments conducted on lab-scale EGSB type reactors may be difficult to interpret or lack statistical confidence for a variety of reasons. Key amongst those reasons is that EGSB-type reactors are underpinned by complex, diverse and dynamic microbial consortia capable of multifarious responses to a broad range of applied environmental conditions. The community underpinning treatment in such systems is so complex and so little understood that, at present, it cannot be stated with confidence that under conditions of external equilibrium whether the underlying microbial community would be expected to follow suit. As such, it was assumed that multiple observations of a community at multiple time points is advantageous over a single 'snapshot' of the community in attempting to describe the response to a given condition. To enable this, where possible, each experiment was conducted using replicate reactors, with experiments typically utilising triplicate reactor sets. Thus, multiple samples of were taken with respect to time and further, reproducibility of the result at a given time in a dynamic system might be attributed some statistical confidence. An additional difficulty in assessing data collected from EGSB type experiments reported in the literature was lack of availability of suitable external data that might enable direct comparison of the results obtained. To address this, where possible, triplicate reactors were run in parallel or in series with paired triplicate sets acting as a control. The aim of this 'controlled approach' was that a complementary data set could be collected that would have a single stepwise difference in operating condition for each parameter tested. To give an example for clarity, if temperature was the parameter of

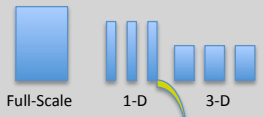
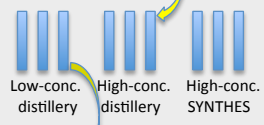
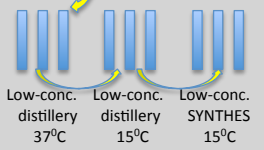
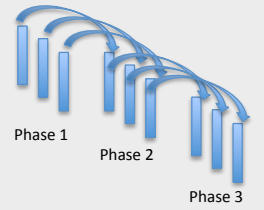
interest, three reactors would be operated at say 37degC and to provide a complementary data set for comparison, another three reactors would be operated at say 20degC under otherwise identical conditions. To some extent this approach negates the complex interactions between operating parameters described in the literature review. Again by example, lowering temperature in the reactor is likely to also alter solubility in the substrate such that the community response to operating at 20degC as compared to that at 37degC may have arisen from increased solids in the reactor rather than from temperature per se. To address those interactions, adopting a truer factorial design approach may have been beneficial. As the number of parameters influencing the community dynamics are so vast however, and the trials were conducted in triplicate, a true factorial trial was deemed physically impractical and rather, a 'single' parameter was changed between trials. That changing one parameter does in-fact alter others was addressed by identifying those parameters that intuitively appear to act as 'primary drivers' for change in conditions e.g. it whilst changing temperature in the reactor might increase solids in the liquor, increasing solids does not increase temperature therefore in that instance, temperature is the primary driver for change. In summary then, key logic to the design of the experiments conducted was:

- The use of replicate reactors to enable assessment of reproducibility of results and enable application of simple statistical measures to both physico-chemical and biological monitoring data.

- The use of control reactor sets to enable collection of data, either in parallel or in series, that might highlight microbial response to a given condition by enabling direct comparison with data sets collected with a 'single' difference in operating condition.

The aim in applying this logic to the design of the trials was to add value to the empirical data collected and the perceived success of each of the parallel and series control strategies is commented on in those chapters in which they are applied. The design of the experiments is distinct from the subject of the trials. The subject of each trial was identified either in relation to knowledge gaps identified in the existing literature or in response to the motives of the Transforming Waste Project as described previously. An overview of the trials conducted is given in Table 3-1.

Table 3-1: Overview of experimental approach, thesis structure, experimental design and aims

Experiment	Schematic	Experimental Design	Aims
Chapter 4: Scale-Down		- Single full-scale reactor, two sets of triplicate lab-scale reactors - Parallel control - Alternative lab-scale idealisations	- Determine whether lab-scale results reflect full-scale operation - Determine the influence of test rig at lab-scale on microbial community composition esp. in relation to spatial distribution in reactor
Chapter 5: Substrate type		- Parallel control - Triplicates - High strength distillery waste - Low strength waste - High strength sewage	- Identify influence of substrate availability and type on treatment efficiency and methane production - Identify organisms associated with low substrate availability and with high-solids loading
Chapter 6: Low-temperature sewage treatment (Global North)		- Series control - Triplicates - Low substrate availability, low-temperature, high-solids	- Determine performance of system in relation to global north sewage treatment - Identify organisms associated with low-temperature operation - Identify organisms associated with high-solids loading at low temperature
Chapter 7: Failure testing		- Stepped-series control - 'Triplicates' – not in synch - Limit-state testing	- Adopt the limit-state approach to testing used in civil engineering to a biological system - Determine the failure point under high-solids loading - Determine the failure mechanism under high-solids loading - Identify organisms associated with very high-solids loading
Chapter 8: Granule Growth and Density	**All reactors:** density measurements at end of run with depth **Full-Scale:-** **16S rRNA on:** 	- Four sets of triplicate lab-scale reactors for density measurements in biomass - Full-scale reactor used for sequencing of granules by size - Individual granules distinguished by size treated as triplicates	- To determine relationship between settleability of granules and size / density - To determine relationship between granule size and microbial community composition - Proposes a descriptive model of cyclical granule growth >> granule break-up >> granule re-growth

3.2 Design and Set-Up of Lab-Scale Bioreactors

A schematic of the defining features of a typical EGSB is given in Figure 3-1. In its simplest form the ESGB is an up-flow treatment system with influent delivered to the bottom of the granule bed combined with a recycle flow for improved treatment via increased bed expansion and hence improved contact between the influent and biomass. The EGSB is described as 'high-rate' in that it utilises a retained granular biomass that effectively decouples solids and liquid retention times. The formation of the granular biomass which is central to this technology is not fully understood; however, it is typically either formed 'naturally' during the start-up operation of a new reactor or alternatively a new reactor may be seeded using granular sludge from an existing reactor.

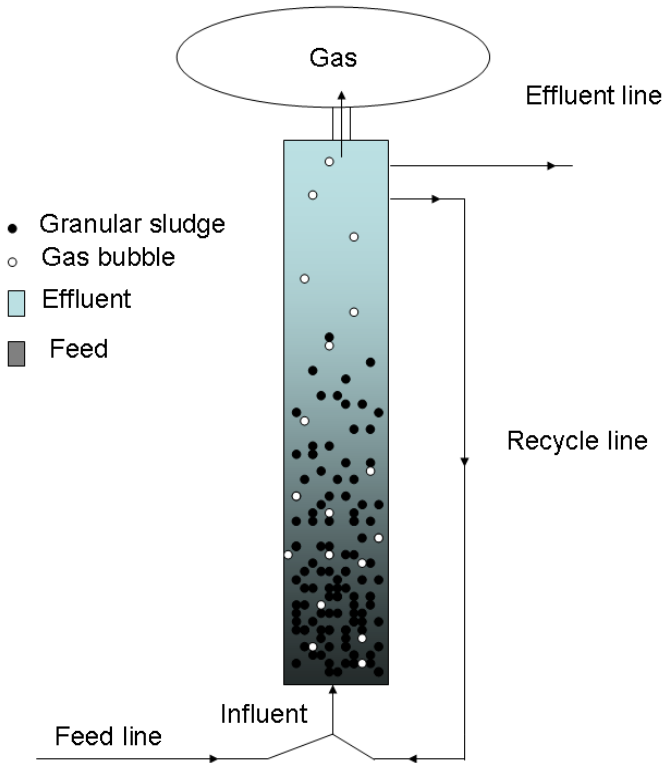


Figure 3-1: Schematic of Expanded Granular Sludge-Bed Bioreactor (EGSB)

Whilst elements of the EGSB such as the reactor volume and upflow velocity may be designed using engineering principles founded on fluid mechanics, currently the capacity to fully engineer the underpinning microbial community in the biomass is limited. The overall aim of the lab-scale trials here then is to: identify links, if any, between reactor performance and microbial ecology under varied engineered conditions; and to move inform future design of engineered microbial communities to improve treatment efficiency via increased specificity of the biomass to optimise treatment of a specific wastestream.

3.2.1 Lab-Scale Reactor Design

Design of EGSB reactors used at lab-scale is extremely varied and often is discussed only briefly in published works. From the review of literature it was noted that frequently in experimental work focussing on the impact of the hydraulic regime on ESGB reactor performance, lab-scale reactors that exaggerate the vertical length. By contrast, in those studies focussing primarily on microbial ecology in EGSB reactors are scaled in vertical and horizontal direction with a rule of thumb ~1:7 height : diameter aspect ratio employed. As such, two reactor types have been designed based on my interpretation of the two lab scale idealisations most commonly used, described here as 1-D and 3-D reactor models (Figure 3-2).

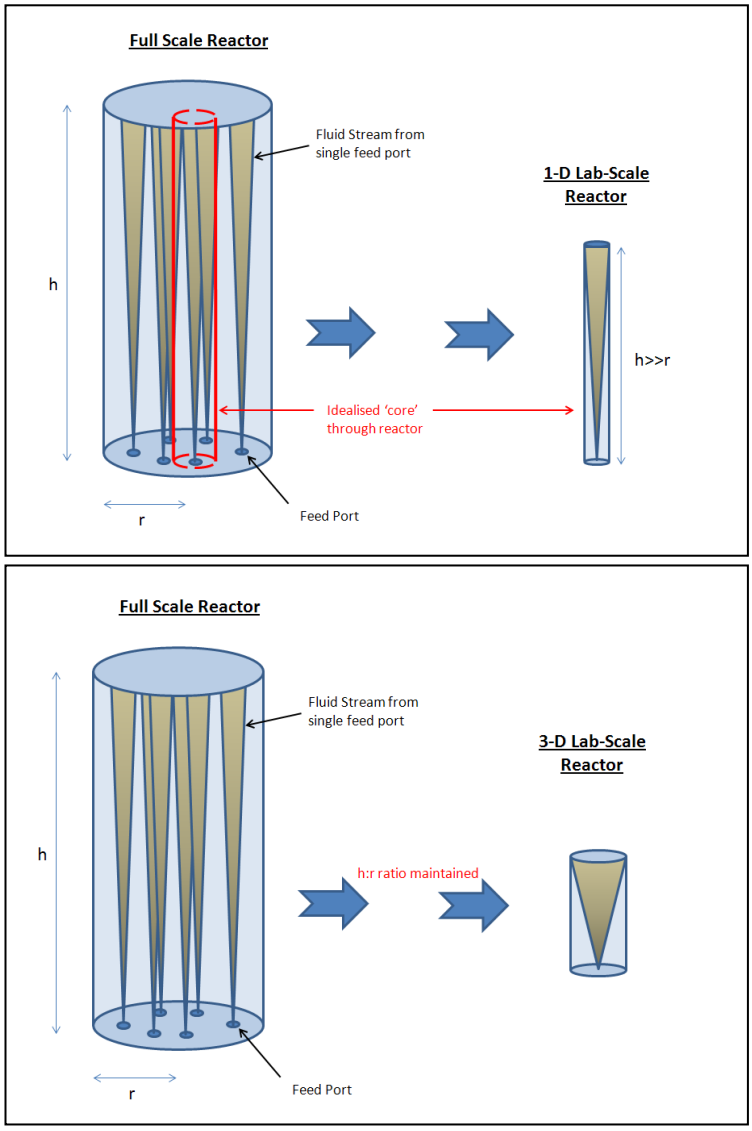


Figure 3-2: Schematic of scale-down logic for the design of the 1-D and 3-D laboratory-scale bioreactor geometry idealisations

The 1-D reactor shown at the top of Figure 3-2 is idealised as a 'core' through a full-scale reactor whilst the 3-D reactor is a more direct scaling of the original. Chapter 4, which looks at the impact of reactor scale and lab-scale reactor design on microbial community, utilises both reactor models. At the outset of the project however it was decided that twelve 20-litre lab scale reactors would be constructed such that four sets of triplicate reactors could be run in parallel. Resultantly, the decision as to which reactor type would best be used had to be made prior to the results of this first test. As spatial distribution of the microbial community with depth was of interest and intuitively the 1-D idealisation appeared to offer improved potential of measuring that distribution as compared to the 3-D idealisation, nine 1-D and three 3-D reactors were constructed. Design drawings of each reactor type are shown in Figure 3-3 alongside an image of each of the 1-D and 3-D reactor types used, and the full design detail is included in Appendix 1.

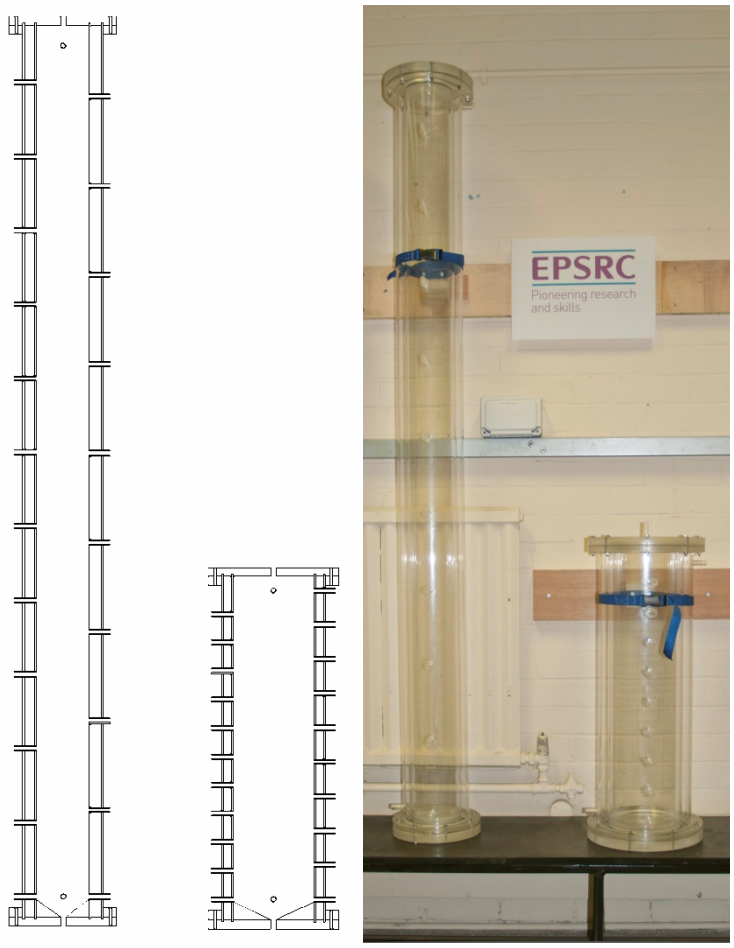


Figure 3-3: 1-D and 3-D laboratory-scale bioreactor design drawing, and, photograph of bioreactors as-built

The reactors were constructed using off-the-shelf diameters of extruded acrylic tubes (http://www.theplasticshop.co.uk) to act as the reactor body, external skin and feed ports, with turned, cast acrylic lids and bases manufactured in an in-house workshop. Each reactor had a single feed port at the base and a single port in the lid to enable gas collection. In each reactor, 10 ports were distributed evenly with height in the reactor body. Each reactor was of a double skinned design such that the void between the reactor vessel and the outer skin could be utilised as a water jacket for heating and cooling the reactor. The lid / base connection to the body of the reactor and to the skin was sealed using rubber o-rings to prevent air entering the system and to prevent transfer of fluids from the water jacket to the reactor vessel and vice-versa. Air-tightness was tested by application of compressed air to each sealed vessel and monitoring the change in pressure over time. Each reactor was additionally tested for water tightness under the influence of heating for a period of 7 days prior to seeding. Post-seeding (described below) a 3mm conical steel mesh was fitted in each reactor below the height of the recirculation line to promote granule retention and enable effective gas separation. Rubber gas-bags (10L volume), were used to measure gas production volume during measurement periods

(Figure 3-6) and rubber transfer tubing (Rapid Education, Catalogue No. 52-0139) was used to transport biogas produced to an external vent under its own production pressure at all other times. Each sampling port was fitted with a 6.6mm diameter stopcock (Fisher, Catalogue No. SYA-555-050K) connected to the sampling port using silicone transfer tubing (Fisher, FB50877).

3.2.2 Lab Scale Reactor Set-Up

Figure 3-4 shows the standard set up for a triplicate set of reactors. Hydraulics in each triplicate set was controlled using two peristaltic pumps each fitted with 3 extension pump heads (Watson and Marlow, 300 Series Peristaltic); one pump to control the delivery of substrate and the other to control the recirculation flow. An intermediate bulk container (IBC) feed-tank (640L, Smiths of the Forest of Dean) was used to supply influent to each reactor set to minimise variation in feed delivery between replicates (Figure 3-4 and 3-6). To prevent settling of solids in the feed tank, in-tank mixing was employed using an overhead stirrer (Fisher, Catalogue No.15169712) with extended rotary paddle.

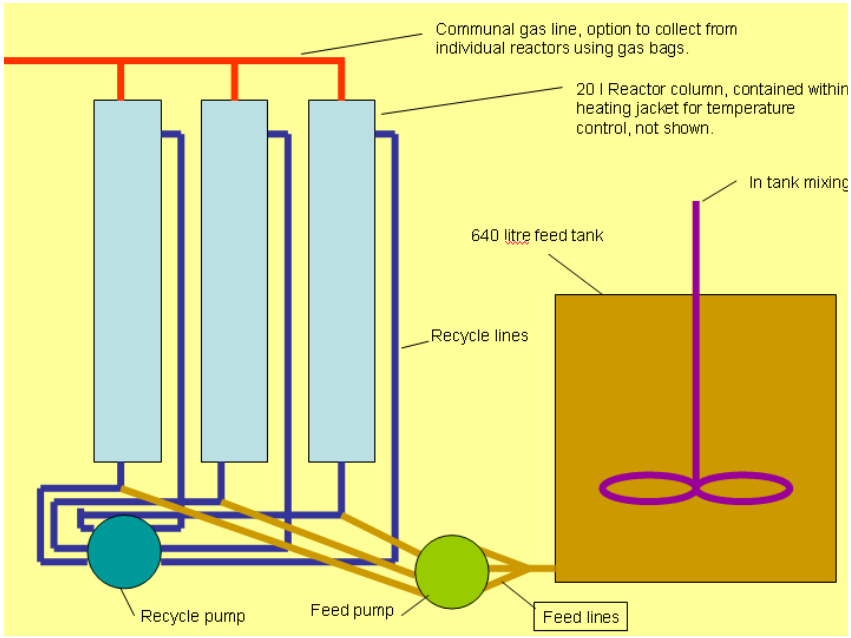


Figure 3-4: Schematic of lab-scale reactor set-up for a triplicate reactor set

The temperature in each reactor was controlled individually by supplying heated water to the water jacket. The water was heated using an electric domestic central heating boiler fitted with external electronic thermostat and controller. The water supply to each reactor water jacket was controlled using a manifold valve system (Figure 3-5) constructed from copper piping and ball valve taps. Each reactor was fitted with a light-proof insulation jacket to minimise the cost of heating and to discourage algal growth in the water heating system or in the reactor column itself. The reactors at the left of Figure 3-6 are under trial

and show the completed set-up for a triplicate reactor set, alongside the remaining nine reactors in-situ in the laboratory.

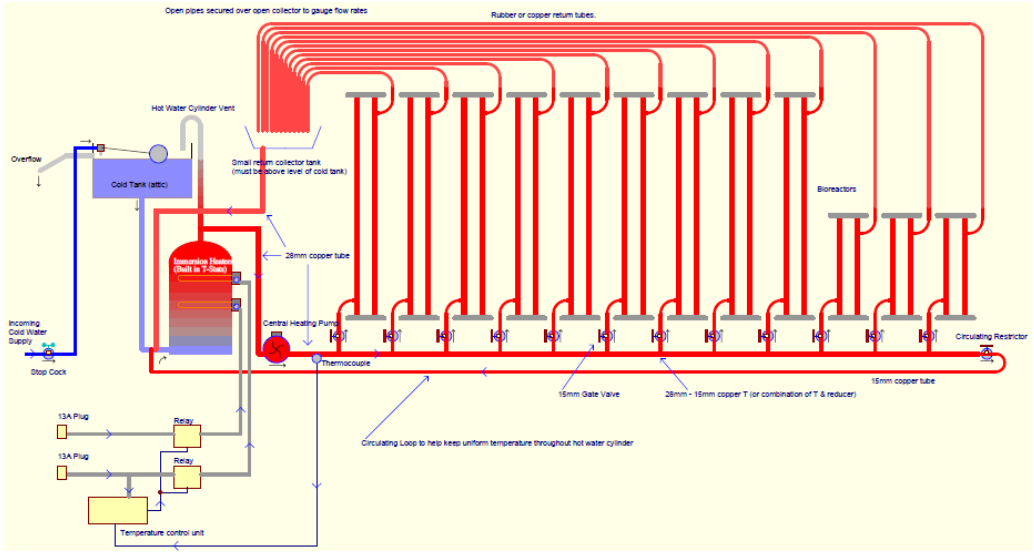


Figure 3-5: Schematic of design for temperature control system to laboratory-scale bioreactor water jackets

Figure 3-6: Laboratory-scale bioreactors R1-3 under trial and in-situ view of laboratory installation

3.2.3 Reactor Seeding and Start-Up Procedure

The experimental design assumed that to operate reactors in triplicate sets with, where possible, triplicate parallel controls would optimise data analysis potential. As such, 12 reactors were constructed in total: 9 No. 1-D reactors and 3 No. 3-3D reactors. To minimise differences in the underlying microbial community at the experiment offset and to enable parallel operation of the reactors, each of the 12 reactors were seeded simultaneously using a seed sludge extracted from a full-scale digester. The granular

biomass used was taken from a full-scale EGSB bioreactor (ADR2) used to treat spent waster from the distillation of maize at the North British Distillery on 10th of January 2013.

Total and volatile suspended solids (TSS & VSS) were measured for the seed sludge according to Standard Methods 2540B&E. In biofilm studies in engineered systems it is typically assumed that the volatile component of the solids measurement may be equated to the microbial component in the biofilm. The results for the measurement of TSS and VSS in the seed sludge are given in Table 3-2 below.

Table 3-2: TSS & VSS Measurement in seed sludge for laboratory-scale bioreactors

Pan (replicates)	Pan + Filter weight / g	Dry weight / g	Total suspended solids / g/L	Ash weight / g	Volatile suspended solids / g/L	Volatile solids as proportion total solids / %
A	3.5042	3.7025	49.6	3.5395	40.8	82.2
B	3.4285	3.6275	49.8	3.4676	40.0	80.4
C	3.4327	3.6303	49.4	3.4693	40.3	81.5
Mean			49.6g/L		40.3g/L	81.3%

>> Using 6 litres sludge per reactor, we have final VSS concentration of 12g/L

The results demonstrate that at a volumetric seeding of ~6L per reactor that each reactor had a starting VSS concentration of 12.1 g/l. This is a mid-low range concentration as identified from a review of literature (Table 3-3). At the point of collection however, the granular biomass in the full-scale reactor appeared to be in a growth phase and as such the reactors were seeded conservatively to avoid the requirement to remove biomass during the reactor run which might disrupt any naturally occurring stratification in the biomass with depth in the reactor.

Table 3-3: Range of seed sludge VSS for lab-scale EGSB reactors selected from literature

Seeding (g VSS / l)	**Subject of Trial**	**Reference**
6.4	Treatment of municipal waste leachate OLR 3.0 - 19.4 kgCOD/m^3 day	(Dang, Ye et al. 2013)
9.9	Treatment of swine manure waste OLR 2.4 kgCOD/m^3 day	(López-Fernández, Aristizábal et al. 2011)
23.0	Treatment of domestic wastewater OLR 6-16 kgCOD/m^3 day	(Xiao, Chunjuan et al. 2008)
43.0	Treatment of synthetic waste OLR 10 – 45 kgCOD/ m^3 day	(Zheng, Wang et al. 2012)

The seed granules were stored at room temperature in sealed containers for 7-days before being used to seed each reactor to a total volume of six litres per reactor. The final bed depth was approximately one third of the effective reactor volume. The bed was allowed to settle under action of gravity for 24 hours before low-flow recirculation was introduced to minimise wash-out the sludge during the start-up procedure. Recirculation was ramped up gradually to design flow over a 24 h period, again to minimise wash out. The start-up bed depths before and after the introduction of recycling were recorded for the purpose of comparison at the end of the study to allow observation of granule growth or washout. Feeding was introduced 48 hours after initial seeding. Sub-samples from the initial seed sludge batch were taken for molecular analysis and stored at -20degC on the day the sludge was sampled (d-31). The reactors were then operated for a period of 16 days under conditions unique to each rector set until biogas production was approximately regular for 3 consecutive HRTs.

3.2.4 TSS / VSS and Particle Size Distribution (PSD)

As an additional characterisation of the seed sludge, particle size distribution of the sludge was measured using a series of gradated sieves as is commonly used in soils characterisation (Figure 3-7). Wet sieving was used to minimise damage to granules and mesh sizes used were:

- 3.35, 2.8, 2.36, 2.0, 1.4, 1.0, 0.6, 0.355 (mm)

Figure 3-7: Particle Size Distribution; image shows equipment employed in wet sieving

Particle size distribution measurement was combined with VSS/TSS measurement (Standard Methods 2540B&E) for each size fraction to provide insight into the distribution of potentially active biomass in each size fraction. The result for T=0 PSD and VSS/Size fraction are given in Figure 3-8 below:

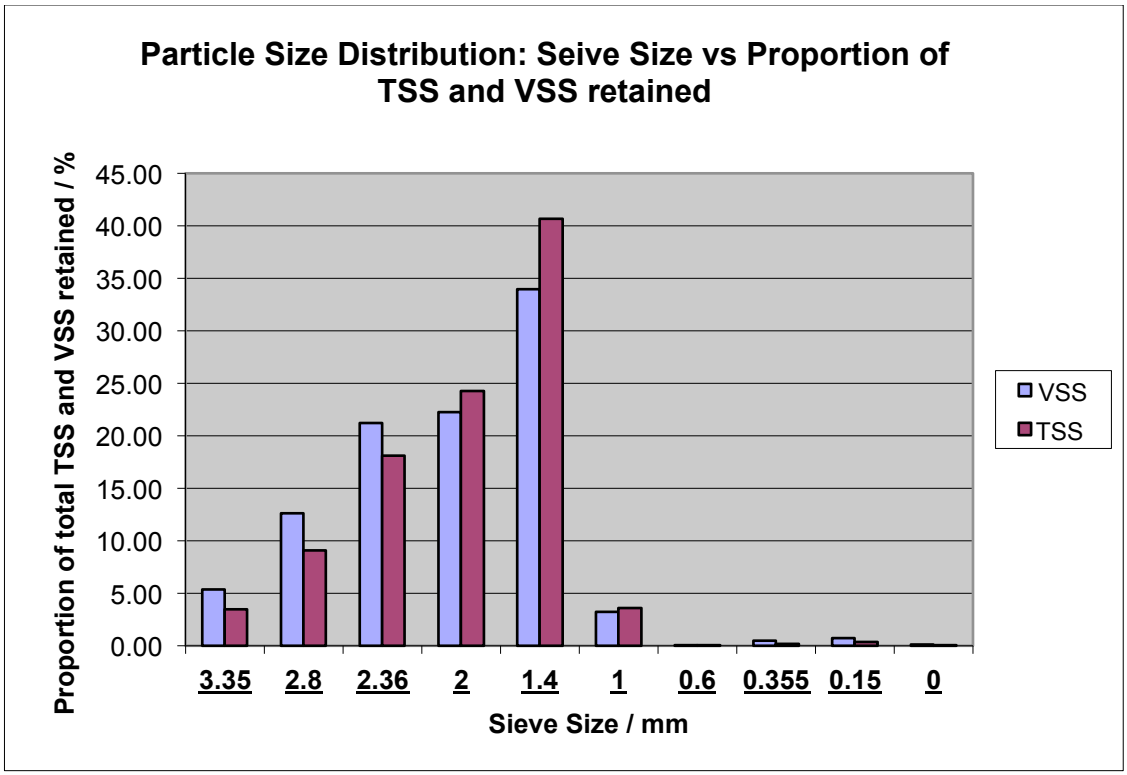


Figure 3-8: Particle Size Distribution of seed-sludge used in laboratory-scale bioreactors

It is noted that the majority of the granules are in the size range 1.4-2.0mm and that granules in the most common size fractions have the highest overall proportions of VSS : TSS of all granules measured. This procedure was applied to granules at the seeding stage and at the end of each experimental run. As such, this data is referred to not in each chapter as reported rather forms part of a separate chapter (Chapter 8) that focuses specifically on granule characterisation but is included here as the seed sludge was common to each reactor trial (Chapters 4-8).

3.3 Monitoring

Parameters were selected to form the basis of the monitoring program as summarised in Table 3-4. On the whole, the monitoring regime was applied consistently across each experiment. Where the program deviates, this is reported individually subsequent chapters.

Table 3-4: Overview of parameters monitored at laboratory-scale and frequency of monitoring

Parameter	Measurement / Sampling Frequency Lab-Scale
Specific Methanogenic Activity	Seed sludge and end of run
COD and sCOD, influent and effluent	Bi-weekly
pH influent and effluent	Bi-weekly
Biogas production	Daily
Methane content in biogas	Bi-weekly
Temperature	Daily in reactor liquor
Volatile Fatty Acids	Bi-weekly, 8 VFAs from C_2 to C_6 including iso-forms of C_4-C_6, and ethanol
Biomass samples	On a 3-weekly basis from each sample port in the sludge bed

3.3.1 Temperature

Temperature was controlled using an external water heating system with thermostat control and valve-controlled flow to the water jacket as described previously. Temperature was monitored on a daily basis by inserting a temperature probe (Tenma 72-2060) into the reactor liquor via Port 9 in the bioreactor gas headspace. Blockage of recycle lines was found to be the primary cause of temperature fluctuations. Blockages were rare but daily monitoring enabled quick clearance of recycle lines (within 16 hours) to resume flow rates and temperature typically returned to target temperature within ~6 hours. Temperature variations resulting from fluctuations in ambient room temperature were adjusted for by controlling the flow rate of hot water supplied to the water jackets using the ball valves described previously to limit or increase the flow of water to each jacket.

3.3.2 Substrate and Effluent Sampling Method

Reactor influent and effluent were sampled on a bi-weekly basis for COD testing, measurement of pH and quantification of VFAs. It was noted during operation that some loss of solids in the reactor feed occurred due to solids accumulation in the tubing upstream of the peristaltic pumps. Thus samples were taken by disconnecting tubing *downstream* of the pumphead rather than upstream to ensure samples were representative of substrate delivered to the reactors as opposed to substrate concentration in the storage tanks. The pumps were allowed to discharge feed for 2-3 minutes prior to collection to ensure that solids collected at the pump head had cleared prior to collection of the sample. Influent samples were collected to a total volume of 15ml in falcon tubes (Fisher, Cat. No. 10512802). Effluent samples were collected to a total volume of 15ml by temporary disconnection of the effluent line of each lab-scale reactor from the communal effluent drain. Again, effluent was allowed to run for 2-3 minutes prior to collection of the sample to ensure that biomass that may have settled in the effluent port was allowed to clear to ensure that samples were representative of typical effluent from the reactor. Samples were processed immediately up collection.

3.3.3 pH

The pH of bi-weekly reactor influent and effluent samples was measured using a digital meter and probe (Thermoscientific Meter, Eutech Instruments Probe). In instances where industrial waste was used as reactor influent, pH of the bulk waste was also measured prior to feeding to determine the necessity or otherwise of pH correction as is described in more detail in Chapter 4.

3.3.4 Chemical Oxygen Demand (COD)

Total and soluble COD measurement was made using the Closed Reflux, Colorimetric Method as described in Standard Methods 5220D. All chemicals used in preparation were laboratory grade. To ensure that measurements would most likely fall within the range of the assay, dilutions of both total and soluble samples were prepared prior to testing. The dilution factors used were based on recorded influent COD and removal rates as recorded for the full-scale reactor which were typically in the region 5000-7000mg/l COD and ~80% COD removal respectively. Dilutions used are given in Table 3-5. For preparation of soluble samples, 5ml sub-samples were taken from the 15ml collection tubes. To ensure good mixing of settled solids and retention of original solid : soluble ratio in the remaining sample, samples were mixed by inversion prior to drawing the 5ml. The 5ml fraction used for soluble measurement was then filtered using a sterile 0.45um syringe filter (Sartorius Stedim Biotech). Of the filtered sample, 2.5ml was used in the measurement of COD and the remaining 2.5ml of the sample was stored at -20degC for VFA measurement as described in Section 1.3.7 to follow. Total COD samples were taken direct from the sampling tubes and again care was taken to ensure good mixing of solids.

Table 3-5: Dilution Factors used for COD Testing

Sample Type	Dilution Factor	Anticipated measured COD after dilution / mg/L
Influent: Total and soluble COD measurements	x20	~250-350 (tCOD) ~175-245 (sCOD)
Effluent: Total and soluble COD measurements	x5	~200-280 (tCOD) ~140-195 (sCOD)

As outlined in the standard methods, the samples were cooked in pre-heated digester blocks at 150degC for 2 hours. Three standards were prepared at known COD concentrations of 0, 250 and 500mg/l. The '0' or blank standard was prepared using DI water. After cooking, samples were stored in the absence of light until COD was measured. Standards were used to prepare a calibration curve by taking readings on a spectrophotometer (Hach Lange DR2800) at 600nm, against which all subsequent measurements were made.

3.3.5 Biogas Production Rate

Biogas production volume was measured on a daily basis. Empty ten-litre gas collection bags were attached directly to the gas port at the top of each reactor for a measured time period typically in the region of two hours. Gas volume was then quantified by emptying the gas bags using a 1L gas syringe (JayTee Biosciences Ltd, Cat. No. SGE-009920) and hourly biogas production rates inferred as:

$$\text{Hourly rate} = \text{biogas volume} / \text{collection time}$$

3.3.6 Methane Content in the Biogas

The methane content of the biogas was measured bi-weekly to coincide with COD measurements. Biogas was subsampled to a total volume of 50ml per sample from the gas-bags, after collection as described above, using standard 50ml syringes fitted with two way valves. Care was taken to ensure that any maintenance required on the bioreactors that might disrupt the gas line was not conducted in the 24hours prior to gas sampling. Methane content in biogas was quantified using a gas chromatograph (7890A, Agilent, Palo Alto, CA) equipped with a GS-CarbonPlot capillary column and a flame ionization detector. Nitrogen was used as carrier gas at constant pressure of 15 psi (approximately 2.1 mL/min). The following conditions were applied: inlet temperature, 250 ^{o}C; oven temperature, 150 ^{o}C for 3 min; detector temperature, 250 ^{o}C; H_2 flow, 30 mL/min; air flow, 400 mL/min. It is noted that method development for methane measurement was conducted by Dr Seung Gu Shin.

3.3.7 Volatile Fatty Acids

Volatile fatty acids were measured at the end of the entire trial using samples stored at -20degC and prepared as per soluble COD samples as described previously. Volatile fatty acids (VFAs, C_2–C_6, including iso-forms of C_4–C_6) and ethanol were measured using a gas chromatograph (7890A, Agilent, Palo Alto, CA) equipped with a DB-FFAP capillary column and a flame ionization detector. Helium was used as carrier gas at constant flow of 1.5 mL/min. Samples were mixed with 3.0% (w/v) formic acid at 1:1 volume ratio before injection. The following conditions were applied: inlet temperature, 250 ^{o}C; injection volume, 1 µL; split ratio, 1:40; initial oven temperature, 70 ^{o}C for 2 min; ramp up to 210 ^{o}C at 20 ^{o}C/min and hold for 2 min; detector temperature, 250 ^{o}C; H_2 flow, 30 mL/min; air flow, 400 mL/min; makeup flow (N_2), 20 mL/min. It is noted that method development and measurement of VFAs was conducted by Dr Seung Gu Shin.

3.3.8 Biomass Sampling

Biomass samples were drawn from each lab-scale bioreactor typically on a 3-weekly basis. Samples of effluent (Port 8) and reactor liquor (Port 7) were taken along with samples from each port in the sludge bed (typically Ports P1-5). During the reactor trial period it was observed that biomass readily became trapped along the length of sample ports during operation as shown in Figure 3-9.

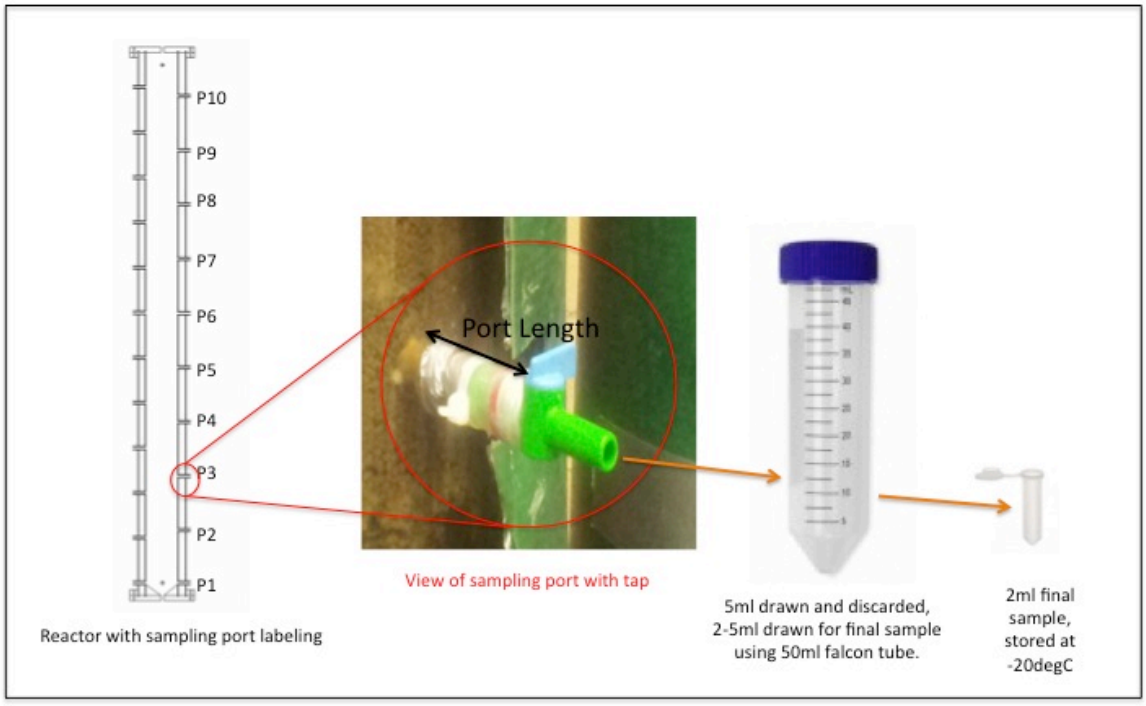


Figure 3-9: Schematic of sampling procedure for biomass in 1-D and 3-D lab-scale bioreactors

To ensure representative sampling of biomass, 5ml of biomass or reactor liquor was drawn and discarded immediately prior to drawing the final sample to account for the volume of biomass that was assumed to have been trapped in the port. Both the discarded and final samples were drawn by opening the tap in a given sampling port and collecting the flow-through in 50ml falcon tubes. Care was to minimise any wastage of biomass during this procedure to prevent destructive sampling of the sludge bed. Final volumes of 2ml of biomass or reactor liquor were transferred to Eppendorf tubes and centrifuged for 5 minutes at 3000rpm such that liquids could be removed prior to storage at -20dgC until processed further.

3.3.9 Specific Methanogenic Activity Testing (SMA)

The specific methanogenic activity of the biomass was measured against simple substrates; acetate, propionate, butyrate, and ethanol; according the protocol by Colleran et al (Colleran, Concannon et al. 1992). Additionally, activity was measured against hydrogen /

CO_2 as substrate according to the protocol by Coates et al (Coates, Coughlan et al. 1996) with the adaptation that the results presented here are by direct measurement of methane produced, as per the liquid substrates, rather than by theoretical calculation as described in the Coates et al protocol. Biomass sampling followed the methods described previously except that samples drawn were 5ml from each of the top two (P4&5), and bottom two (P1&2), ports in the sludge bed. To prepare the samples for testing, a pooled sample was then prepared representing the top and bottom of a reactor set e.g. for reactor set R1-3, samples from P1&2 from each of reactors R1-3 were combined to make a single sample representing the 'bottom' of that reactor set. Using this pooled sample, 0.5g of biomass was added to triplicate 30ml serum vials (Sigma Aldrich, Catalogue No. 33106) for each substrate tested, each of which contained 9.5ml anaerobic media. Weighing and biomass addition to bottles was conducted on the bench due to limited space in the anaerobic chamber and care was taken to ensure that time taken for weighing was kept to a minimum to reduce risk of oxygen damage to the granules. The serum vials were then sealed using rubber bungs and aluminium crimp caps (Sigma Aldrich, Catalogue No. 508500). The air in the headspace of the serum vials was exchanged to 1 atm with nitrogen and bottles left to acclimatise in incubators at 37degc overnight. Vials were then vented to 1 atm and liquid substrates added using a syringe before returning to the incubator to progress the assay. For hydrogen assays, the gas in the headspace was exchanged for Assays were conducted in triplicate for each substrate tested with triplicate blanks operated for each trial – 3 bottles with biomass only, no substrate addition. Hydrogen assays were conducted in 50m serum vials with initial preparation conducted similarly but for substrate addition which was performed by flushing the vial headspace with hydrogen / CO_2 80:20 mix for 1 min then over-pressuring to 2atm. Triplicate blanks were prepared comprising vials prepared with anaerobic media, biomass and nitrogen in the headspace but no applied substrate. Incubated vials were rotated at 40rpm for the duration of the test with hydrogen assays laid horizontally to increase liquid : gas contact area. The pressure change in the headspace of vials was recorded initially on a 2 hourly basis using a syringe probe and electronic pressure transducer. Subsequently, once an approximate rate of change of pressure was ascertained, pressure was recorded in each vial typically on a 4-hourly basis. Once pressure change had plateaued the gas in the headspace of each vial was sampled using a syringe and needle and the methane content of the gas ascertained using the GCMS as described previously. Total and volatile suspended solids of the biomass was then measured using filtration and drying and ashing methods described previously. The resultant methanogenic activity may then be calculated:

$$\text{Activity} = \frac{[\text{methane content (\%)}]*[\text{rate of change of pressure}*\text{headspace volume (mL / day)}]}{[\text{volatile solids (gVSS)}]}$$

The activity of the blanks was then subtracted from each of the liquid substrate assays, and added to the hydrogen assays, to calculate the final reported SMA activities for each substrate. For seed sludge the activity rates were recorded on a single sample for each reactor taken prior to seeding. At the end of reactor run SMA was recorded for each reactor individually at the top and bottom of the reactor bed. In instances where temperature was reduced during the reactor run, the end-of-run SMAs were conducted at both 37degC and at the most recent operating temperature.

3.4 Microbial Ecology

3.4.1 DNA Extraction

All biomass samples were stored at -20degC until the end of all the reactor trials. Care was taken to ensure that each sample went through only one freeze-thaw cycle as part of the extraction process to minimise degradation of DNA. The following section of the thesis details optimisation of the extraction procedure and outlines the final DNA extraction protocol.

3.4.1.1 Optimisation

Total genomic DNA was extracted from granule, liquor and effluent samples using the Fast DNA Spin Kit for Soil (MP Biomedical) and FastPrep-24 Instrument. The extraction process worked largely with manufacturers instructions for the Spin Kit with only quantity of biomass used, and lysis time, subject to optimisation. The key parameters used to assess the optimisation of DNA extraction were the quality of DNA extracted and DNA yield. Optimisation of the DNA extraction method for granular samples was conducted using biomass obtained from the full-scale reactor ADR2, Port 2. A pre-lysis step was introduced for granular samples whereby biomass was gently ground using a mortar and pestle prior to the bead-beating step for maximum cell lysis, which is explained in more detail in Section 1.4.1.2. For optimisation, a range of biomass volumes was then trialled during the lysis step and a range of lysis times applied as described in Table 3-6. All other steps in the extraction were conducted according to the manufactures instructions. Final DNA concentrations were determined using the Qubit 2.0 Fluorometer and Qubit dsBR Assay Kit (Invitrogen) according to manufacturers specifications.

Table 3-6: DNA yield (ng/ul) obtained using different biomass sample sizes (g) and lysis times (s) during the DNA extraction process

Weight of biomass used: / Lysis time:	0.05g	0.1g	0.3g	0.5g
40s	138	119	135	173
60s	128	136	158	474
80s	164	117	155	237

It was noted that DNA yield was high relative to that which would be required for downstream processes (PCR for next generation sequencing) for each combination of lysis time and biomass quantity trialled. As such, DNA quality rather than quantity was used as the determining factor for optimisation. Quality was assessed by running 10x dilutions of the resultant DNA samples on a 1% agarose using SYBR® Safe DNA Gel Stain (Invitrogen Life Technologies) for visualisation against a 1kb Plus DNA Ladder (Invitrogen Life Technologies) to check the size of extracted DNA (Figure 3-10). The images were then inspected for signs of shearing. The DNA sample circled in red in Figure 3-10 was thought to be of the highest quality, displaying the strongest band with minimum shear. The method applied to this sample (0.1g biomass with 40s lysis) was the adopted for all other granule extractions. Effluent and liquor samples were found to consist largely of light flocs and planktonic biomass and as such a separate optimisation was conducted. In this instance total sample volume available for extraction was found to very low (<0.1g) and as such the entire sample was used in each extraction. Following the same procedure of optimisation of lysis time, the optimum lysis time was found to be 20 seconds which was applied to all non-granular samples with all other steps conducted as per manufacturers instructions.

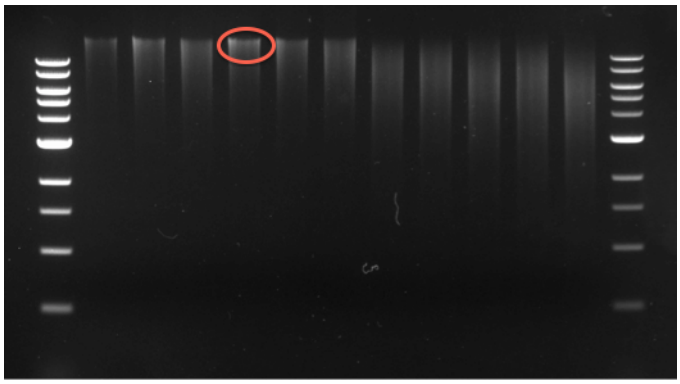


Figure 3-10: Gel electrophoresis image showing genomic DNA obtained from DNA yield optimisation trial run against a 1kb ladder (Invitrogen). Circled sample assumed to be best quality showing minimum shear and maximum yield.

3.4.1.2 Granule grinding optimisation

Gentle grinding of granular samples using a mortar and pestle before lysis is reported to improve cell lysis during DNA extraction (Collins, Woods et al. 2003), however the extra handling of the sample appeared likely to increase the potential for the introduction contamination to the samples. A range of grinding tools and methods were trialled with the aim of finding that method which produced a homogenous sample with minimum risk of contamination from external surfaces and minimum signs of shearing in the DNA sample obtained. The range of methods trialled is listed in Table 3-7 along with summary findings from the trials conducted.

Table 3-7: Summary of granule grinding methods trialled, and results obtained, for optimisation of DNA yield from granular sludge

Method	Comments	Yield / ng/ul
Mortar and pestle (MP): granules removed from storage tube, transferred to pestle for grinding	Removing granules from storage tube increases likelihood of contamination, cleaning of mortar laborious for large no. of samples	120
Mini pestle (F) for in-tube grinding (Fisherbrand)	Eliminates requirement to remove sample from storage tube and requirement for cleaning mortar. Expensive for large no. of samples as pestle is intended to be disposable and is difficult to clean.	72
Glass stirring rod (GR) for in-tube grinding	No sample transfer or mortar cleaning. Rod may be soaked in ethanol for re-use.	192

The samples were run on a 1% agarose gel as described previously to ensure no shearing occurred using alternative grinding methods (Figure 3-11). Grinding with a glass rod as a pestle and using the sample storage tube as a mortar was found to be the cleanest method of grinding with a high yield of DNA and minimum shear. This method was adopted on all granular samples.

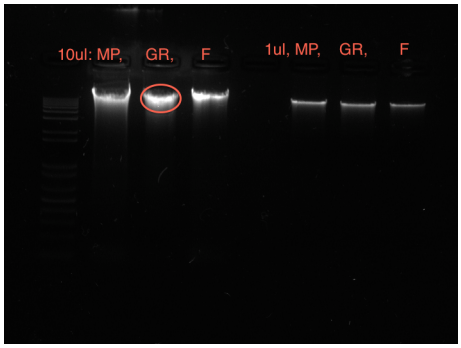


Figure 3-11: Gel image showing genomic DNA obtained during granule grinding optimisation using mortar and pestle (MP), glass rod (GR) and mini-pestle (F). Circle indicates best yield (brightest band) and quality (lowest shear) is obtained using GR.

3.4.1.3 Routine extraction from stored samples

Frozen biomass samples were allowed to thaw at room temperature for 30 minutes prior to processing. Granule grinding and DNA extraction were performed as described previously. The quality of DNA extracted was checked by running every tenth DNA sample on a 1% agarose gel at 100V for 45 minutes to check for signs of shearing. No shearing was observed on any sample checked and as such, quality was assumed acceptable on all samples. Extracted DNA concentration was quantified using 2ul samples on the Qubit following manufactures instructions for broad range measurement.

3.4.2 Preparation of Positive and Negative Controls for NGS

3.4.2.1 Positive control: Clone Library construction

A clone library was constructed to enable preparation of mock communities to be used as a positive control during NGS. The positive control is used in NGS to determine the extent to which NGS sequencing returns data that reflects the composition of sequenced samples in terms of:

- relative species abundances, and,
- to enable identification of contamination arising during the sequencing process from either carryover DNA from previous sequencing runs, or from barcode switching during sequencing.

The aim of preparation of the positive control is to produce triplicate barcoded samples by PCR from each of an evenly mixed and skewed mixed sample of known initial DNA template composition. To enable the preparation of the evenly mixed and skewed DNA template, plasmids were prepared by construction of a clone library using a sample of sludge from the full-scale reactor ADR2 as the starting point. An overview of the work-flow for clone library preparation is given in Figure 3-12.

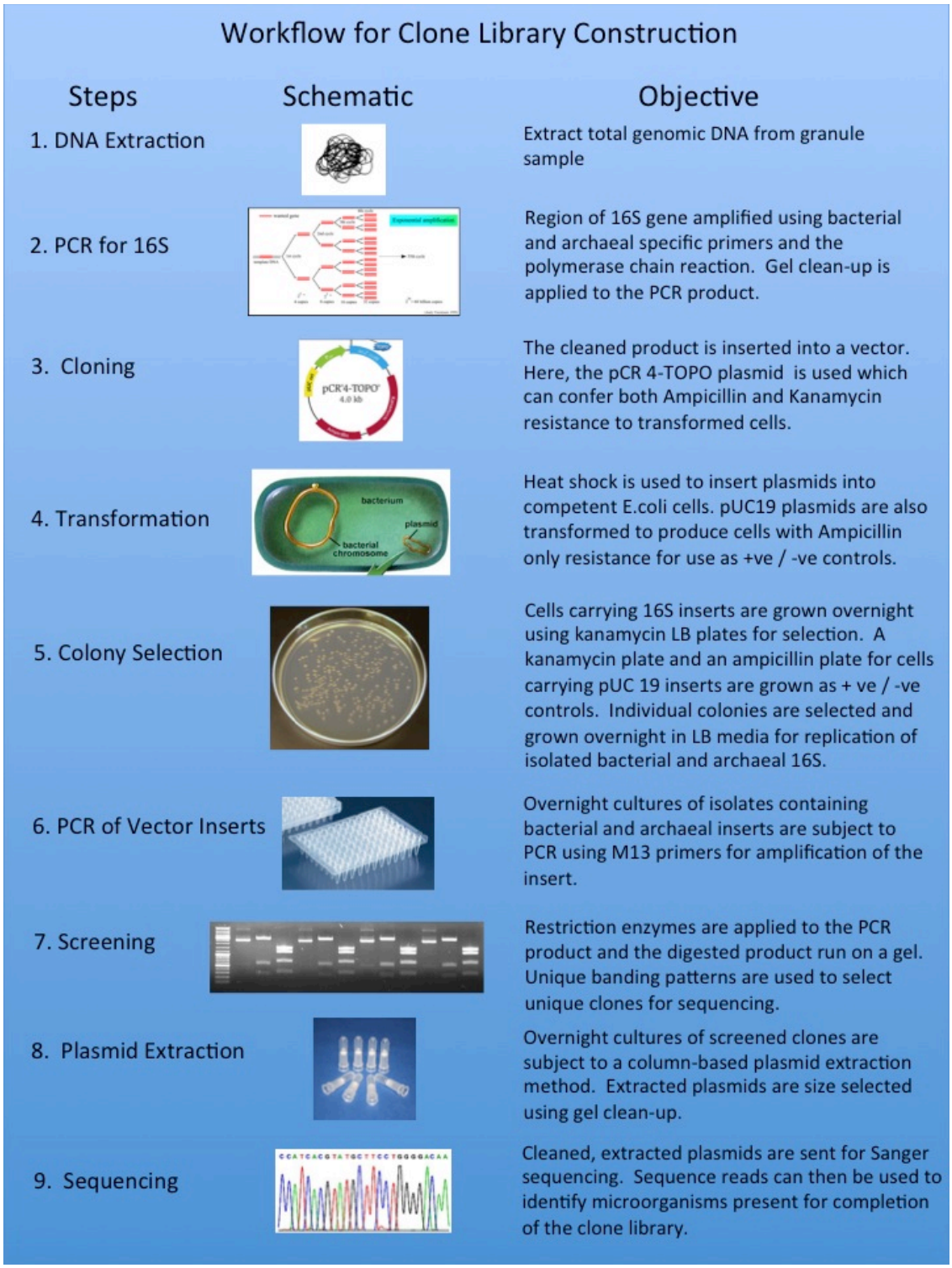


Figure 3-12: Schematic of work-flow for clone library construction outlining key steps and objectives for each step in the process.

Genomic DNA was extracted from full-scale distillery granules extracted from ADR2, Port 2 as described previously. Primers used for isolation and amplification of the 16S rRNA gene were 338F, ACTCCTACGGGAGGCAG (Yu, Lee et al. 2005), and 9F, CYGGTYGATCCYGCCRG (Collins, Woods et al. 2003), for bacteria and archaea respectively, and the universal primer 1392R, ACGGGCGGTGTGTRC (Collins, Woods et al. 2003) as the reverse primer in each case. Prior to use each primer pair was tested for

efficacy in-silico using the ProbeMatch function on the RDP classifier database. Efficacies determined at the point of use were 92% (1888533/2061117) and 71% (3239/4578) for bacteria and archaea respectively. PCR optimisation was conducted by testing a gradient of annealing temperatures across the calculated melting temperatures and a range cycle times for each primer pair to establish maximum yield of PCR product using a minimum number of cycles to minimise PCR error. A gradient of starting DNA concentration in the PCR reaction was also tested.

The final PCR reaction contained 25ul Biomix Red (Bioline), 2ul each of the forward and reverse primers at 10uM concentration, 1ul template and 20ul PCR-grade water (Qiagen Nuclease Free water, Cat No. 129115) to a total of 25ul. Reactions were held at 95^0C for 5 minutes, with amplification proceeding for 30 cycles of 94degC for 1 minute, annealing of 60^0C for bacterial primer pair and 58^0C for the archaeal primer pair for 1 minute and 72degC for 1 minute extension. The PCR product was run on an 1% agarose gel using SYBR® Safe DNA Gel Stain (Invitrogen Life Technologies) for visualisation against a 1kb Plus DNA Ladder (Invitrogen Life Technologies) to ensure the correct length of product was obtained in high quantity without production of secondary banding. The PCR product was then purified using the QIAquick PCR Purification Kit (250 reactions) according to the manufacturers instructions. The cleaned product was then used as a vector and cloned in competent cells using the TOPO-TA cloning kit (Life Technologies) according to manufacturers instructions. Cloned cells were grown overnight on kanamycin / LB plates at 37degC. In total 192 and 95 colonies were selected for screening from bacterial and archaeal plates respectively. Selected colonies were grown overnight in 96 well plates in LB and kanamycin media. Each overnight culture was then subject to PCR sing the vector-specific primers M13F (5'GTTTTCCCAGTCACGAC-3') and M13R (5'-CAGGAAACAGCTATGAC-3') and insert of the correct size were subject to restriction digest. Restriction digest was conducted using the tetrameric restriction endonuclease HaeIII (Promega) following the manufacturers protocol. The digests were run on a 0.7% gel and OTUs identified by inspection for unique banding patterns. Of the clones screened 95 and 16 banding patterns of bacterial and archaeal digests were identified. Overnight cultures of selected clones were prepared in deep well 96 well plates and plasmids extracted using the Quick Plasmid Miniprep Kit according to manufacturers instructions and sent for Sanger sequencing. Sequencing data indicated that of plasmids sent, 72 unique bacterial OTUs were identified along with 4 unique archaeal OTUs. Plasmids from each clone sequenced were stored in 25% glycerol solution at -80degC for preparation of the mock community.

The skewed mock community template was prepared by mixing concentrations of the most abundant OTUs identified from the clone library in approximate molar proportions to the relative abundances identified by the clone library. In all, 33 bacteria and 4 archaea were used to prepare the skewed mock community at an overall proportion of 80% bacteria and 20% archaea. The even mock community template was prepared by mixing equimolar concentrations of the same OTUs used for the skewed community. Those samples were then subject to NGS library preparation as described in the Section 1.4.3 to follow.

3.4.2.2 Negative Control: 'Blank' DNA extractions

NGS sequencing for the first time is allowing sufficiently deep insight to microbial communities such that the rare microbiome in many environments is now becoming revealed. With that, new challenges are arising in terms of quality control of data collected. The aim of the blank DNA extraction is to enable preparation of the negative control for sequencing. Recognising that DNA extraction kits themselves may be a source of contamination (Peters, Mohammadi et al. 2004), the DNA extraction applied to granule samples as described previously, was conducted in triplicate using the same extraction kit but without addition of biomass i.e. 3 blank samples extracted in total. The DNA concentration of the resultant sample was quantified using the high sensitivity kit of the Qubit, which determined the sample too low to quantify, and no band was seen when the final 'elution' was run on a gel. The prepared samples were then subject to NGS library preparation as described in Section 1.4.3 to follow.

3.4.3 Preparation of barcoded amplicons for NGS

The sequencing platform selected for use was the Illumina Miseq benchtop sequencer which has been demonstrated to produce high quality reads with low error rates at low cost per base (Amore 2015). Further, using the barcoded primer method for amplicon sequencing developed as part of the Earth Microbiome Project, in excess of 200 samples may be processed in a single sequencing run further reducing the cost and enabling ultra-high throughput of samples. The method utilises PCR to target the V4 region of the 16S rRNA gene using Golay primers each of which is synthesised with a unique barcode gene sequence and Illumina sequencing adaptor (Caporaso, Lauber et al. 2012). The primer pair used for amplification was F515 and R806 (Caporaso, Lauber et al. 2011). An additional degeneracy on the Golay primers was utilised; base-N substituted for base-C in third position of F515 forward primer; for improved detection of Archaea. For PCR an optimisation procedure was conducted for both the PCR reaction and subsequent clean up.

3.4.3.1 PCR Optimisation and Processing

All biomass samples were stored at -20degC until the end of the sampling campaign prior to DNA extraction. DNA extraction and purification was conducted using the Fast DNA Spin Kit for Soils (MP Biomedical) according to manufacturers instructions as described previously. Extracted DNA was quantified using the Broad-Range Qubit Assay (Life Technologies) and stored at -20degC until used in NGS library preparation. Prior to PCR preparation, each DNA sample was thawed, normalised to 1ng/ul template DNA by dilution in PCR grade water (Qiagen Nuclease Free water, Cat No. 129115), and stored at -20degC until PCR preparation to minimise the concentration of any PCR inhibitors present in the extracted DNA elution. The primer melting temperature calculated was 54degC and a temperature gradient PCR was conducted using both the KAPA HiFi HotStart ReadyMix PCR Kit and the KAPA HiFi HotStart PCR Kit with dNPTs (both KAPA Biosystems) to test both the performance of the kits and to determine the annealing temperature for the PCR amplification. Each reaction used was 50ul volume prepared to the manufacturers instructions and the reaction contained 5ng of DNA. The resulting PCR products were run on a 1% agarose gel as described previously. The results are seen in Figure 3-14. It is seen that the Kit was superior to the ReadyMix system and as such was selected as the appropriate chemistry for use in the remaining optimisation steps. The annealing temperature selected was 62degC due to production of a clean band of PCR product of the correct length and minimum production of primer dimers. Whilst this is higher than would be indicated by the melting temperature calculated for the primers, the temperature is consistent with the recommended annealing temperature for the Kapa chemistry due to high salt concentrations in the buffers.

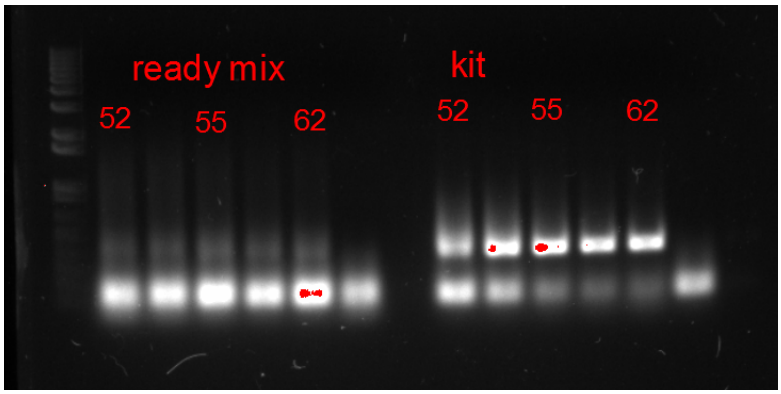


Figure 3-13: Gel image showing PCR product obtained trialling annealing temperatures of 52^{O}C to 62^{O}C and the Kappa Ready Mix and Kit chemistries. Samples are shown against a 1kb ladder in left hand lane; reduced primer dimer demonstrates in Kit samples demonstrates greater efficacy of Kit chemistry.

Each sample processed was amplified in triplicate 25ul reactions to maximise the final amount of final PCR product whilst minimising PCR error. PCR amplification reactions

were prepared using reagents from the KAPA HiFi HotStart PCR Kit with dNPTs (KAPA Biosystems) and a single 25ul reaction contained: 11ul PCR grade water (Qiagen as previous), 5ul 5X KAPA HiFi Fidelity Buffer, 0.75ul dNTP Mix, 0.75ul of forward and reverse primers at 0.3uM concentration, 0.5ul KAPA HiFi Taq, 6.25ul template DNA at 1ng/ul concentration. Reactions were held at 95^0C for 5 minutes for initial denaturation of the DNA, with amplification proceeding for 25 cycles of 98^0C for 20 seconds denaturation, 60^0C for 15 seconds for annealing, and 72^0C for 40 seconds for extension, followed by 72^0C for 1 minute final extension. Triplicate reactions were pooled and PCR product was gel-purified (Zymoclean Gel DNA Recovery Kit) and quantified prior to sequencing using the High-Sensitivity Qubit Assay (Life Technologies). In instances where the yield of PCR product was below 5ng/ul from the triplicate PCR after the gel clean up then the PCR would be repeated, again in triplicate and the cleaned samples pooled.

Each purified PCR product was normalised to 5ng/ul DNA in nuclease free water. 2ul of each normalised sample was then added to a clean 2ml tube to create a single pooled sample for sequencing. Positive and negative control samples were included in triplicate. The pooled sample was vortexed thoroughly to ensure good mixing of all samples and the pool concentration was measured using the Quibt as described previously. The concentration of the final pool was checked by quantitation using the High-Sensitivity Qbit Assay (Life Technologies) and the purity of the final pool was checked by visualisation on a 2% agarose gel. A 50ul subsample was then frozen at -20degC and stored on dry ice until delivered to the sequencing centre. The pooled multiplexed library was sequenced using the Illumina Miseq bench-top sequencer at the Centre for Genomic Research, Liverpool. Arising sequencing data was de-multiplexed at the sequencing centre and processed according to the *Illumina Amplicons Processing Workflow* by Dr Umer Ijaz (Ijaz 2015) to create OTU and taxa tables compatible with R Software for analysis. Samples with low read counts (<5000 reads per sample) were re-sequenced in a further sequencing run following the same preparation protocol but with alternative sequencing barcodes assigned to the sample. A single abundance profile for each sample was then constructed for re-run samples by collating abundance profiles (determined as described below) by collating abundance profiles according to one-way subject ANOVA (www.tinyurl.com/JCbioinformatics).

The amplicon processing workflow begins with quality trimming of reads using Sickle software which applies a windows based approach, trimming regions in which the quality score drops below 20. This is followed by overlapping the paired end reads with minimum overlap of 50bp using PANDAseq paired-end assembler, which has been demonstrated to

reduce substitution error rates in Illumina platform by an average of 93% for amplicon data sets (Schirmer, Ijaz et al. 2015). Overlapped reads are then multiplexed, dereplicated and filtered for singletons. Chimeras are then removed by employing both de-novo (removing chimeras by mapping against more abundant reads within) and a reference based approach (using the gold database http://drive5.com/uchime_download.html derived from ChimeraSlayer reference database to remove chimeras missed in the previous step as a result of parents in lower abundance) using UPARSE pipeline. Two alternate approaches were then applied. The first approach may be described as a reference based approach in which the nonchimeric reads were classified at phylum, class, order, family and genus level using RDP classifier to obtain abundance tables at each taxonomic level, latterly described in this thesis as 'taxa tables'. The second approach is a de-novo clustering approach in which the nonchimeric reads were clustered at 97% sequence similarity using UCLUST to generate operational taxonomic units (OTUs) that serve as a proxy for species level assignment to generate an abundance table latterly described as an 'OTU table'. The representative OTUs were also taxonomically classified using RDP classifier. The inherent problem with the reference-based approach is that the reference databases are not complete and therefore a sizable number of reads end up as "unassigned". The OTU clustering-based approach directly addresses this problem by ensuring that those unassigned sequences are still differentiated as distinct species. Thus OTUs offer greater statistical power in downstream analysis as compared to reference-based approach. The only caveat being that the underlying platform generating the sequencing must have a low error rate otherwise an inflated number of OTUs will be observed. The Illumina platform however is sufficiently accurate (99.7%) after the previously mentioned quality steps in the post-sequencing data workflow (Amore 2015). Multiple sequence alignment was then performed on representative OTU sequences using MUSCLE and a phylogenetic tree was then generated using FastTree all within the QIIME framework.

The final quality check of the data prior to analysis was inspection of OTU tables arising from the even and skewed mock communities and confirmation that no sequences arose from the blank samples. Quantification of DNA from the blank extractions indicated no yield had arisen and PCR of the same samples again indicated no yield post clean-up. Post-sequencing it was observed that none of the blank samples yielded any reads during sequencing. This confirmed that sample contamination did not occur during the DNA extraction process and that PCR primer dimer or other contaminants did not yield false reads. Thus, the blank samples confirmed the efficacy of the library preparation method applied. The OTUs generated from the even mock community had a 95.1% match with the

known plasmid sequences used in preparation (standard deviation 0.6%), whilst those arising form sequencing of the skewed community showed 97.0% (standard deviation 0.4%) match. Thus, whilst lower than the reported accuracy for the platform, the mock communities demonstrate a high level of accuracy in sequencing these samples for cases of both even and skewed ecology.

4 Scale-Down: A Spatio-Temporal Study of EGSB Type Bioreactors at Full- and Lab-Scale

Abstract

Feasibility of new biotechnologies inevitably begins with lab-scale trials however rarely do we determine whether lab-scale results reflect full-scale performance or ecology. This study aims to bridge that knowledge-gap using the expanded granular sludge-bed bioreactor (EGSB); a high-rate anaerobic digester; as a model system for study.

Two laboratory-scale idealizations – a one-dimensional and a three- dimensional scale-down of a full-scale design – of the EGSB were designed and operated in triplicate under near-identical conditions to a full-scale EGSB. The lab-scale bioreactors were seeded using biomass obtained from the full-scale bioreactor and distillery wastewater was applied as substrate at both scales. Over 70 days, performance measures were monitored and microbial ecology was monitored over time at various depths in the sludge-beds.

Particulate COD removal efficiency at lab-scale was significantly higher than at full-scale (74% and 50% respectively) however removal efficiency in the lab-scale reactors was less stable. Illumina Miseq sequencing data (V4 region of whole community 16S rRNA) demonstrated significantly greater abundance of families associated with carbohydrate degradation; *Veillonellaceae*, *Porphyromonadaceae* and *Armatimonadetes;* at full-scale. Specific methanogenic activity in the seed-sludge suggested dominance of the hydrogenotrophic pathway, which appeared to be associated with dominance of *Methanobacterium* in the seed-sludge. Each of these distinctions we propose arose from increased variability in operation at full-scale.

Further, we identify that laboratory-scale idealization influences the distribution of microbial populations throughout the sludge-bed. Significantly increased relative abundance of the genera *Geobacter*, *Syntrophomonas*, *Methanomethylovorans* and *Methanosarcina* were associated with significantly increased methanogenic activity in replicated, stratified communities in 1-D type reactors.

4.1 Introduction

Development of new biotechnologies for the treatment of wastewater typically follows the empirical route from lab-scale through to pilot- and full-scale trials (Switzenbaum 1995, Tchobanoglous, Burton et al. 2004, O'Flaherty, Collins et al. 2006, Shida, Sader et al.

2012). However, few published papers track the success of a trial across each of the lab-, pilot- and full-scale development stages. Thus, knowledge of the applicability of lab-scale studies to the design and operation of full-scale treatment plants is limited. Further, whilst lab-scale trials typically report operational success, reported failure of anaerobic technologies at full-scale continues to hinder the widespread adoption of AD in the industry, especially for the treatment of high-solids and variable wastes such as sewage. As the 'pilot-plant-approach' is both costly and time-consuming, it is proposed here that a study of scale-down, as opposed to scale-up, might provide valuable insights to the apparent disparity of scale. Further, as lab-scale trials frequently use highly varied idealisations of the biotechnologies tested, here it is proposed that two distinct methods of scale-down be applied such that the influence on lab-scale results might be established. The aims of this experiment then were threefold:

- Evaluate the operation of three replicate full-scale reactors such that operation can be mimicked at lab-scale.
- Design, build and operate triplicate lab-scale reactors of two distinct designs as are commonly reported in the literature, and evaluate performance under near identical operating conditions to the full-scale digesters.
- Evaluate differences in performance and associated spatio-temporal microbial community dynamics between,
    i. The full and lab-scale reactors
    ii. The alternative lab-scale idealisations.

4.2 Experimental Design

The following section provides an overview of the experimental design. The use of a full-scale reactor as a 'control' for lab-scale work is outlined alongside the proposed resolution of arising technical issues in relation to the full-scale study. The logic for the sampling schedule is provided and an overview of the timeline of works conducted is presented.

4.2.1 Full-Scale Reactor Study

The full-scale reactors selected for study are operated at the North British Distillery (NBD) in Edinburgh for the treatment of spent water from the distillation of alcohol from maize. The reactors are variants on the EGSB described as the External Circulation Sludge Bed reactor (ECSB). The ECSB is one of a range of commercially available reactor types

produced by Hydrothane; a group of companies associated with the design, construction and operation of high-rate anaerobic digesters for industrial application. A schematic of the ECSB is given in Figure 4-1 below:

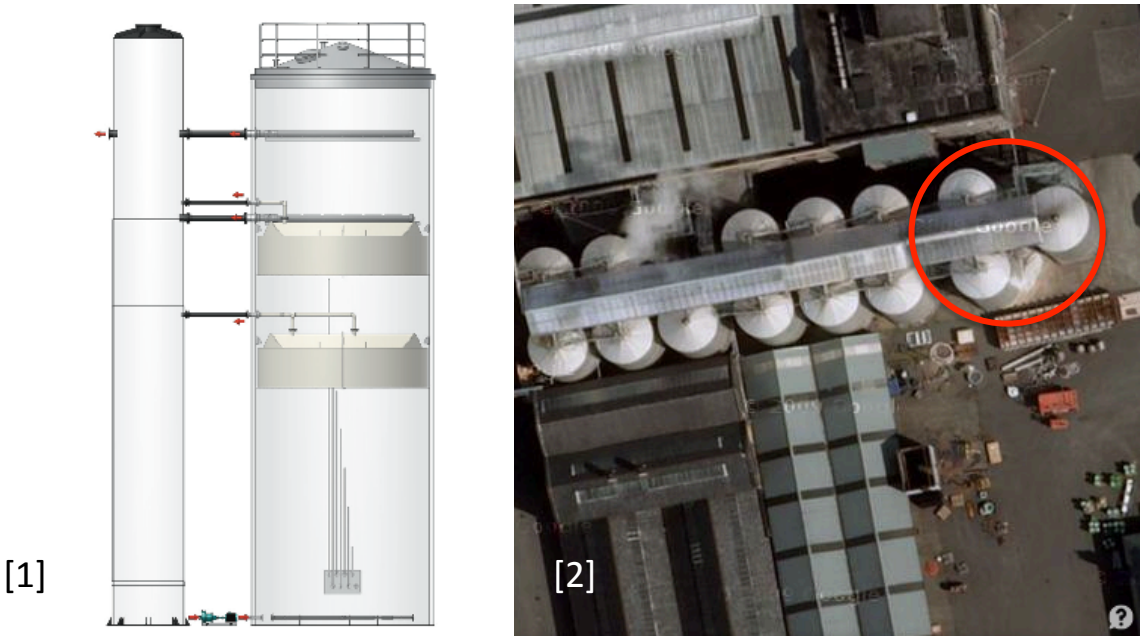


Figure 4-1: [1] Schematic showing ECSB with two gas-solid-liquid separators and distributed sampling ports, and, [2] Satellite image of full-scale bioreactors in-situ at NBD

[1] (http://www.hydrothane-stp.com/HydroThane-ECSB.php).

[2] Satellite image obtained from Google Maps 25/03/15

The ECSB is a second-generation EGSB, the key variant being the inclusion of two gas-solid-liquid separators within the reactor aimed at improving process stability and biomass retention under high organic loading rates. The ECSB is reported to be suitable for the treatment of non-viscous wastes with relatively high solids components at loading rates in the region of 15-35 kgCOD/m3.d (http://www.hydrothane-stp.com/HydroThane-ECSB.php). The key defining features of the EGSB are still adhered to i.e. the ECSB is an upflow anaerobic system utilising liquor recycling to promote mixing, and treatment is underpinned by the microbial activity of the granular sludge-bed. Whilst the ECSB is physically a more complex reactor type than the EGSB, the North British Distillery was approached as a partner for this study for the primary reason that each of their three ECSB reactors are operated in parallel under highly similar operating conditions and treating the same waste representing, thus a unique opportunity to provide statistical significance to the study at full scale. Additionally, the Distillery conducts detailed physico-chemical monitoring on reactor control and performance, the results of which was able to be shared as part of the study. Finally, each of the three Distillery reactors has eight sample ports

distributed vertically enabling sampling of biomass at different depths in the reactor sludge bed to determine variation in the microbial community not only in time but with depth in the reactor.

4.2.2 Determining Operating Parameters for Lab-Scale Reactors from Full-Scale Monitoring Data

In order to determine the impact of scale on EGSB performance and microbial community, we aimed to mimic the operating parameters of the full-scale digesters. The Distillery reactors ADR1-3 were constructed using existing tanks on site that were adapted to purpose by Hydrothane. ADR1 was brought on-line in 2010 and following one year of successful operation ADR2&3 were constructed. The design and operational parameters for the Distillery reactors ADR1-3 from the 6-month operating period prior to commencement of the lab-scale reactors are summarised in Table 4-1.

Table 4-1: Full-scale bioreactor geometries and operational parameters for ADR1 and ADR2&3 at NBD

Parameter	ADR1	ADR2&3
Internal diameter (m)	7.2	6.7
Height (m)	12	12
Aspect ratio d:h	1 : 1.7	1 : 1.8
Effective volume (m^3)	485	425
Volumetric load rate (m^3/hr)	47	27
Feed : Recirculation ratio	1:3	1:3
HRT (hr)	10.3	15.7
Recirculation rate (m^3/hr)	150	100
Upflow velocity (m/hr)	4.8	3.6
Mean influent COD (mg / l)	6230	6230
Mean influent sCOD (mg / l)	4393	4393
Mean OLR (kgCOD/Lrxr.d)	14.5	9.5
Mean reactor pH	6.84	6.97
Reactor temperature (degC)	37	37

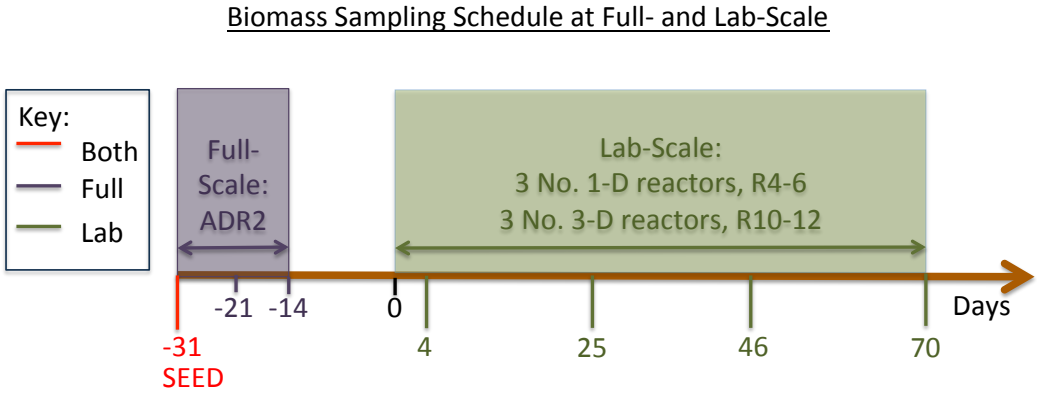
The key physical difference between the reactors is volume; ADR1 has 14% greater working volume at 485m^3, whilst ADR2&3 are each 425m^3 working volume. Whilst the Distillery initially reported similar operation between the three reactors, inspection of the data provided for the three-month period prior to commencement of the experiment showed distinct operational differences between ADR1 and ADR2&3. OLR and upflow

velocity are 52% and 33% higher on ADR1 respectively as compared to ADR2&3. The key biological difference between the reactors is that ADR2&3 were seeded one year later than ADR1, using biomass from a pre-existing off-site digester and as such the microbial community in those reactors may be expected to show less similarity to ADR1 than to each other. As a result, the reactors do not represent true triplicates however ADR2&3 may be regarded as duplicate reactors. Thus, ADR2&3 were selected as the reactors on which to base the experiment design parameters at lab-scale.

4.2.3 Alternative Approach to Full-Scale Study

The aim at the offset of the laboratory work was to sample biomass from each of the full-scale reactors and from the lab-scale bioreactors on a three-weekly basis for the same duration. The rationale was that the sequencing data collected at each scale would be subject to the same conditions and therefore might be directly comparable. Unfortunately, this aim was not fully realised due to unexpected breakdown of ADR2 during the lab-scale trial. ADR2 suffered a physical failure caused by cracking of the reactor vessel and, as ADR2&3 are of the same reactor vessel type and age, both ADR2&3 were shut down for a period of 80 days for inspection and repair. During that 80-day period, all waste produced in the Distillery was directed to ADR1, resulting in large differences in operating parameters for ADR1 compared to the lab-scale reactors. Thus an alternative strategy was adopted for the full-scale study such that the opportunity to investigate disparity of scale was not lost.

Biomass samples from each port in ADR2 were obtained at three time points prior to breakdown and prior to commencement of the lab-scale experiment (Figure 4-2). The time points were at d-31, d-21 and d-14, spanning 17 days in total. 16S rDNA gene amplicons were sequenced from pooled samples from the top two ports and bottom two ports of the sludge bed at each time point. Whilst these samples do not represent an ideal comparison for the lab-scale trials, they are thought to represent the best option from those available (Figure 4-2).



Parameter	Measurement / Sampling Frequency Full-Scale	Measurement / Sampling Frequency Lab-Scale
Specific Methanogenic Activity	Seed sludge only	Seed sludge and end of run
COD and sCOD, influent and effluent	Daily	Bi-weekly
pH influent and effluent	Daily	Bi-weekly
Biogas production	Daily	Daily
Methane content in biogas	Daily	Bi-weekly
Temperature	Daily in influent	Daily in reactor
Volatile Fatty Acids	Daily as Hac	Bi-weekly, 8 VFAs from C2 to C6, EtOH
Biomass samples	3 Sample times at top and bottom of sludge bed, in 17 day period as shown.	4 Sample times at 5 depths in 1-D reactors and 4 depth in 3-D reactors over 70 day period as shown

Figure 4-2: Schematic overview of experiment showing experiment duration and relative timings of biomass sampling of full- and lab-scale bioreactors; and summary of physico-chemical monitoring parameters.

4.2.4 Design of Lab-Scale Trial

The degree of variability in lab-scale idealisation of the ESGB was reported in the literature review with aspect ratios of the reactors discussed varying from 1:50 to 1:7. This led us to design, two reactor types based on two alternative interpretations of these diverse lab-scale idealisations and are described here as 1-D and 3-D reactor types (Figure 4-3). The 1-D reactor type shown at the top of Figure 4-3 is idealised as a 'core' through a reactor and is greatly exaggerated in the vertical direction. The 3-D reactor type by contrast is a more direct scaling of the volumetric dimensions of a full-scale digester. The diameter to height aspect ratios of the reactors utilised in this work are 1:15 and 1:4 for the 1-D and 3-D reactors respectively.

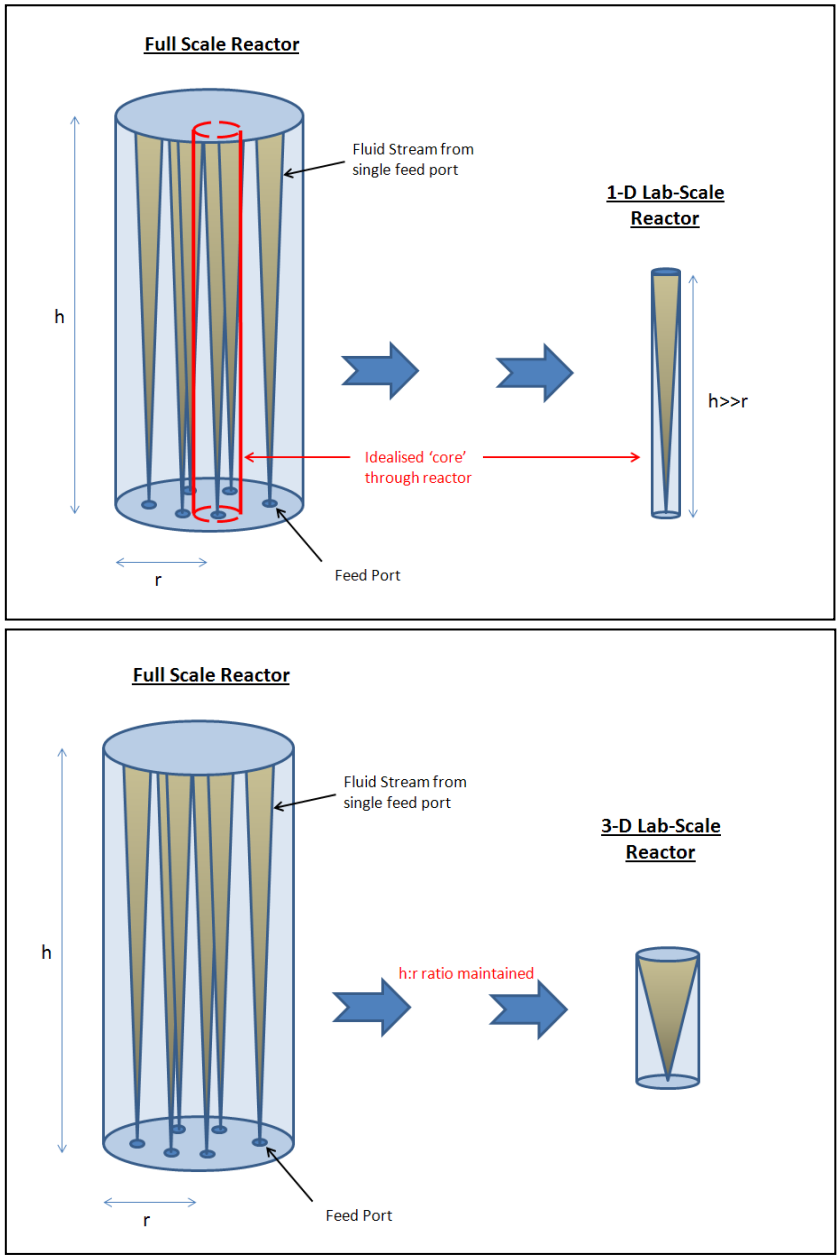


Figure 4-3: Schematic of scale-down logic for the design of the 1-D and 3-D laboratory-scale bioreactor geometry idealisations

Both reactor types have a total working volume of 20 litres and detailed design drawings of each reactor type as shown in Figure 4-4 are included in Appendix 1. To facilitate spatial as well temporal data collection for the microbial biomass as was possible at full-scale, biomass sampling ports were distributed along the length of the reactor to include 8 sampling ports accessing the sludge bed and reactor liquor as seen at full-scale. Each reactor type was built and operated in triplicate. In each reactor type the same operating conditions were maintained throughout the study such that as much as possible, physical configuration was the sole difference between the reactor types with the aim that the influence of reactor configuration on both performance and the underlying microbial community might be elucidated.

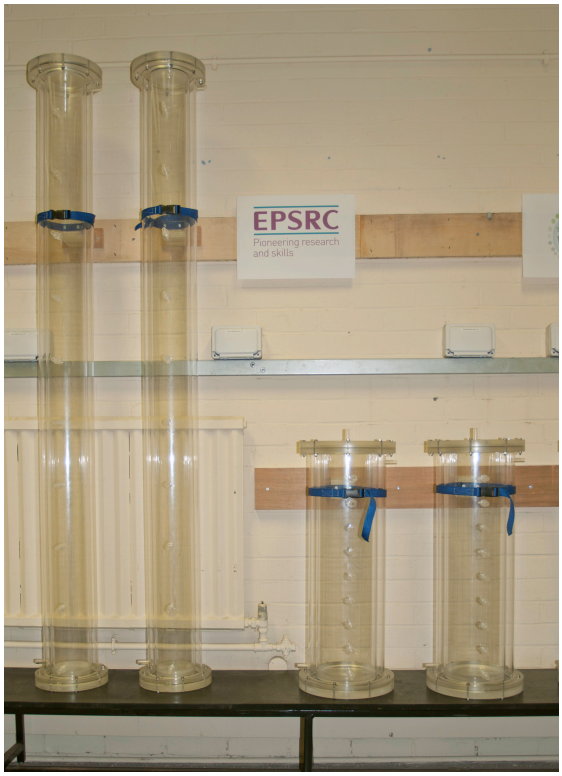


Figure 4-4: Photographs of 1-D and 3-D lab-scale bioreactors as-built in the laboratory

Design parameters for operation as defined by the study of the full-scale digester ADR2 and summarised in Table 4-2. To ensure similar substrate provision for the biomass at lab-scale, reactor influent was collected and transported from the Distillery on a weekly basis to serve as influent for the lab-scale digesters. The primary difference between the lab-scale reactor types is aspect ratio enabling investigation of the influence of lab-scale reactor design on the underlying microbial community. The sampling and monitoring schedule at lab-scale is provided in Figure 4-2.

Table 4-2: Design Operating Parameters at Lab-Scale

Parameter	Design Value or Condition
OLR	Governed by COD of Distillery waste during the trial
HRT (hours)	16
Upflow Velocity (m/h)	3.5
Influent pH	6
Temperature (degC)	37
Feed Type	Distillery waste transported to laboratory on weekly basis.

4.3 Materials and Methods

Detailed materials and methods for all the research included in this thesis are described in Chapter 3 of this report. The following section provides a brief summary of those methods applied in relation to the work conducted within this chapter.

4.3.1 Substrate Preparation and Feeding

Distillery waste was transferred to the laboratory in two 640 litre water containers on a weekly basis and stored at room temperature until used. The sampling port from which the waste was drawn at the Distillery was upstream of the neutralisation tank that feeds the full-scale reactors and as such the waste collected was of low pH (<5). In our laboratory, the wastewater was neutralised using a low-grade caustic, and supplemented with trace metals, to mimic the process at the Distillery (Appendix 2). Stored reactor feed was not neutralised or supplemented until immediately prior to use of the feed to ensure minimum substrate degradation during storage. All six reactors were fed from a single tank utilising in-tank mixing to promote solids delivery to the reactors and to ensure replicated feeding conditions.

4.3.2 Physical and Chemical Monitoring

All sampling and measurements at lab-scale were conducted according to the materials and methods described in Chapter 3. The operation and performance indicators measured at full- and lab-scale were the same with two exceptions: firstly, biogas production rates, and methane content in the biogas, were measured for each reactor at lab-scale in contrast to at full-scale, where measurements were made collectively for the three digesters; secondly, at lab-scale, VFA measurements distinguished individual acids in contrast to the distillery monitoring which utilised a single measurement for total VFAs (TVFA). To improve comparison between lab- and full-scale data sets, total VFA at lab-scale was calculated as an acetate equivalent based on measurements of individual VFAs and is presented as such in the results section of the report. Distillery employees conducted monitoring at full-scale on-site and, as such, methods are not described here. As different measurement methods may have an effect of the absolute values recorded, where possible trends established within the full- and lab-scale data sets are compared rather than comparing the absolute values between the data sets.

4.3.3 Specific Methanogenic Activity Testing

Specific methanogenic activity (SMA) testing was conducted on a single sample of the seed sludge at d-31 representing activity at full-scale. SMA was additionally conducted on samples from the top and bottom of the sludge beds in the 1-D and 3-D reactor types at the end of the lab-scale trials i.e. after microbial adaption to reduced scale and alternative reactor geometries. Full methodology for this work is described in Chapter 3; briefly, however, sub-samples of sludge were incubated in sealed serum vials with specific substrates and activity monitored using the pressure transducer method. Substrates tested were acetate (Ac_C2), propionate (Pr_C3), butyrate (Bu_C4), ethanol (Et_OH) and hydrogen (H2) and each test was conducted at mean reactor temperature (37degC).

4.3.4 Biomass Sampling and Storage

Distillery employees conducted sampling at full-scale and care was taken to flush sampling lines prior to bottling samples. Sludge samples (50 ml) were stored in pre-sterilised air tight containers at room temperature for up to three days prior to transfer to the laboratory. Subsequently, 50ml sludge was re-suspended by inverting and sub-samples (2 ml) were taken. Sub-samples were then centrifuged and stored at -20degC until the end of the experiment when DNA was extracted (as described in Chapter 3). Biomass sampling at lab-scale was conducted on a three-weekly basis at each port in the sludge-bed using the method described in Chapter 3. Samples taken at lab-scale were transferred to storage at -20degC within 2 hours of sampling.

4.3.5 NGS Library Preparation

All biomass samples were stored until the end of the experiment. DNA extraction and purification was conducted using the Fast DNA Spin Kit for Soils (MP Biomedical). Extracted DNA was quantified using the Broad-Range Qubit Assay (Life Technologies) and stored at -20degC until used in NGS library preparation. NGS library preparation involved PCR amplification of the V4 region of the 16S rRNA gene using Golay barcoded primers (Caporaso, Lauber et al. 2012) with an additional degeneracy on the forward primer for improved detection of Archaea. PCR product was gel-purified and quantified prior to sequencing using the High-Sensitivity Qubit Assay (Life Technologies). The pooled multiplexed library normalised to 5ng/ul DNA was sequenced using the Illumina Miseq bench-top sequencer. The arising data was processed and quality assessed according to the (http://userweb.eng.gla.ac.uk/umer.ijaz#bioinformatics) *Illumina Amplicons Processing Workflow*. Samples with low read counts (<5000 reads per sample)

were re-sequenced in a further sequencing run following the same preparation protocol but with alternative sequencing barcodes assigned to the sample. A single abundance profile for each sample was then constructed for re-run samples by collating abundance profiles according to one-way subject ANOVA (www.tinyurl.com/JCbioinformatics). The sequencing was de-multiplexed at the sequencing centre and all other quality control methods applied to the sequencing data are described in detail in Chapter 3.

4.3.6 Qualitative and Statistical Data Analysis Methods

Data pertaining to operation and performance of the reactors at both full- and lab-scale is summarised to provide mean values for each parameter recorded. Standard deviation is presented alongside minimum and maximum values in each data set to enable qualitative assessment of stability of the mean values. Where important trends are identified in the data results are presented graphically, again to enable qualitative comparisons of the conditions in the reactors. Statistical evaluation of difference in operation and performance data with scale and reactor type is conducted using the one-way analysis of variance (ANOVA, aov()) function in the R software package and P-values for significance stated.

To account for differences in sequencing depth between remaining samples, sequencing data was normalised by calculation of relative abundance for each OTU or taxa in the sample. For calculation of ecological indices, each sample was rarefied to a common minimum, which was the minimum read count sample for the whole sample set for the experiment. Ecological indices calculated were rarefied richness, Simpsons Index of Diversity and Pielou's Evenness Index with each calculated using the R-Vegan software package. Phylogenetic distances between samples were calculated using a QIIME based phylogeny file in the Phyloseq package in R with taxonomic identification of OTUs conducted by sequence comparison with the RDP Classifier database. A qualitative analysis of community membership at each scale drawn from published literature is provided for those taxa identified at family level in the most abundant 100 OTUs identified in the sample set. NMDS plots and supporting analysis of variance (PERMANOVA, adonis(), R-Vegan) were conducted two alternative distance metrics to describe the sequencing data. Firstly, Bray-Curtis similarity matrices were utilised to determine variance based on percentage abundances in the count data. Secondly, distance matrices were obtained using the generalised Unifrac (GUnifrac) method, alpha=0.5. GUnifrac is a measure that incorporates phylogenetic distance between OTUs in addition to distance calculated using the abundance data with weighting between the two given by the alpha

parameter; using alpha=0.5 both are given equal weighting. Both NMDS plotting methods were conducted using the Phyloseq package in R. To identify trends in specific taxa in relation to reactor scale and sampling position in the reactor, the Kruskal-Wallis test for non-parametric data was applied (with Benjamini-Hochberg correction for multiple tests applied on the P-values to obtain the adjusted P-values) to describe the significance of results and was again calculated using the R-Vegan software package.

4.4 Results and Discussion

The results section is divided into three key components. (1) The first presents the reactor operation and the physico-chemical characteristics of reactor inputs. (2) The second presents performance data, and aims to establish the degree of replication between full- and lab-scale reactors, and between the data sets collected for the two lab-scale idealisations. (3) The third presents the results of the sequencing data arising from the biomass samples extracted throughout the experiment.

4.4.1 Reactor Operating Conditions

Reactor operating conditions monitored were operating temperature and substrate pH, COD and VFA characteristics. Operation at each scale is summarised and ANOVA used to determine the extent of replication in operation between

- Reactors within triplicate sets
- Reactor type at lab-scale
- Full and lab-scale operation.

4.4.1.1 Operation of Full-Scale Reactor ADR2

Data pertaining to influent pH, temperature and COD obtained from the full-scale digester prior to long term shutdown arising from cracking of the reactor vessel was analysed from days d-85 to d0 as summarised in Table 4-3.

Table 4-3: Summary operating data from full-scale bioreactor ADR2 from days -85 to 0

	pH_NT2	temp_NT2 / degC	VLR / m3/h	CODin / kgCOD/m3	sCODin / kgCOD/m3	pCODin / kgCOD/m3	VFA_asHac / mg / L
Mean	7.00	36.1	28	6538	4385	2108	1359
Min	6.43	30.0	12	5021	3303	1455	905
Max	8.00	37.7	50	7844	5752	2650	1706
St.Dev	0.19	1.0	6	723	598	324	239

(Operating parameters given are: pH of NT2 which is the influent tank at full-scale, temperature of NT2 which is the assumed temperature of the full-scale digester, volumetric loading rate, and influent COD and VFA characteristics)

It is seen that influent pH and temperature are relatively stable throughout but that influent substrate concentrations are more variable. The minimum recorded influent temperature, 30degC, was recorded for a single day after a brief 5-day interval during which the distillery plant was closed for maintenance and the reactors unfed (d-27 to d-21). The maximum and minimum values for VLR were recorded on two subsequent days immediately after shutdown suggesting an attempt at readjusting the reactor after a period of starvation. Neither extreme of loading rate was sustained for more than two HRTs nor repeated during the period of interest. As granular sludge has been demonstrated to be active after storage of up to four years without feeding, it can be assumed that the brief period of 5 days without feeding is unlikely to impact COD removal efficiency in the longer term. The impact of such events on the dynamics and diversity of the underlying microbial community however cannot be directly ascertained. Standard deviation for COD, sCOD and pCOD is low relative to the mean for each and the durations of the application of high and low strength wastes was less than 2 HRTs in each case. Qualitatively, the analysis of operating conditions at full-scale suggests that ADR2 was stably operated in the period prior to acquisition of the seed sludge for the lab-scale trial and throughout the period of the full-scale study.

4.4.1.2 Operation of Lab-Scale Reactors

The biomass used to seed the reactors was stored at room temperature in airtight containers for a period of 14 days prior to commencement of the experiment. Subsequent to seeding the reactors and commencement of feeding, biogas production was monitored for a 16-day period until stable production rates were observed. Operational data for the period Day 0-70 of the 1-D and 3-D reactors are summarised in Table 4-4.

Table 4-4: Summary of operating data for 1-D and 3-D lab-scale bioreactors

	Influent pH	Temperature / degC						CODin / mg/L	sCODin / mg/L	pCODin / mg/L	VFA_asHac / mg/L
		1-D			3-D						
Reactor	ALL	R4	R5	R6	R10	R11	R12	ALL	ALL	ALL	ALL
Mean	6.2	37.2	37.1	32.0	37.2	36.9	37.0	4707	3505	1203	766
Minimum	5.4	36.3	35.6	15.9	33.4	25.0	35.2	3729	2589	202	476
Maximum	7.0	38.4	38.7	37.6	40.2	40.6	40.9	5878	5112	2131	1202
St.Dev	0.4	0.5	0.5	8.3	1.4	1.9	1.4	733	598	600	157

(Temperature presented on by-reactor basis, all other parameters reported are common to both the 1-D and 3-D bioreactor sets.)

As all six lab-scale reactors were fed from a single feed tank, the only variant in terms of operation between the triplicate 1–D and 3-D sets was temperature, which was controlled via a hot water jacket on each reactor. On day 46 of the lab-scale trial, cracking occurred in the water jacket that controlled temperature occurred in R6 and was not able to be remedied. One-way-ANOVA was performed on temperature data excluding that from R6 from day d46 to d70 and no significant difference was found either between the 1-D and 3-D reactor types, nor between triplicate reactor sets. Standard deviation for each of the substrate variables is low and maximum and minimum values were not maintained for more than 4 HRTs therefore qualitatively, the reactor operation may be described as stable.

4.4.1.3 Comparative Analysis of Full- and Lab-Scale Operation

ANOVA was conducted to compare the full-scale data set with each of the lab-scale sets for the operating parameters temperature, pH, COD and TVFA. Significant difference ($P<0.001$) was found in each case. In the case of temperature, whilst the difference is significant, in real terms the difference in means is low (<1degC). In the case of parameters describing substrate characteristics however, the magnitude of the differences established are greater. Figure 4-5 shows the mean and standard deviations for each of these at both full- and lab-scale graphically.

In each case it is observed that the full-scale reactor loading was greater than at lab-scale. These differences arise from the fact that the lab-scale experiment was run subsequently to the full-scale study and is reflective of seasonal variation in productivity at the Distillery. Whilst the loading on each reactor type is different the degree of variability to which each reactor type is subject appears similar in terms of the magnitude of the standard deviations.

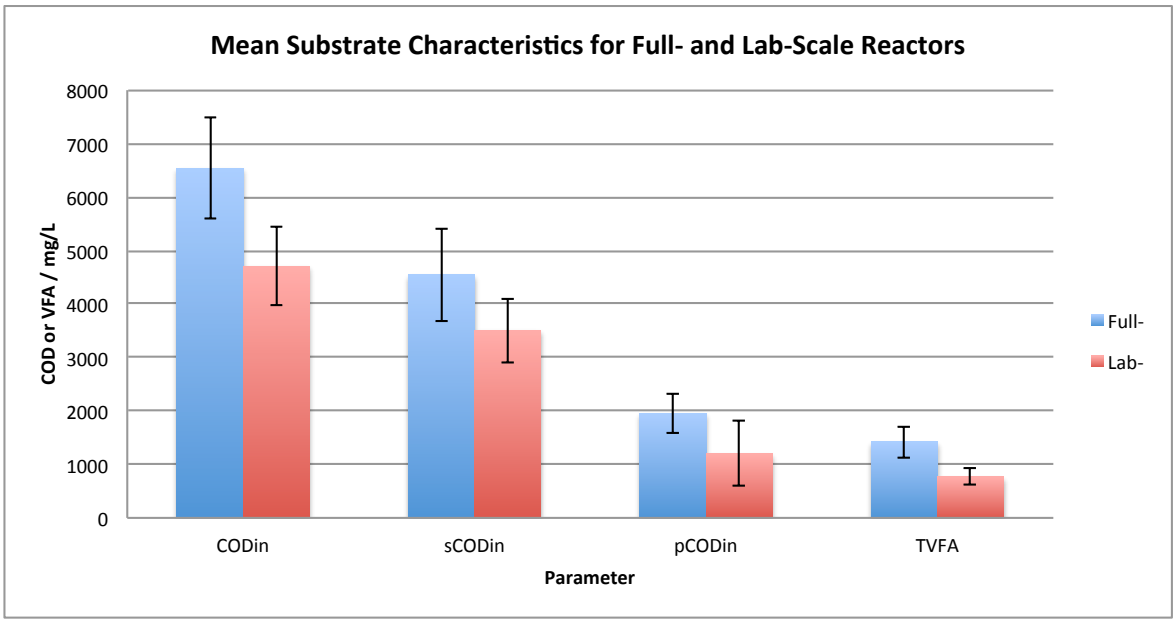


Figure 4-5: Grouped barplot for comparison of influent substrate COD and VFA characteristics at full- and lab-scale. Bar height indicates mean values recorded over 85 days at full-scale and 70 days at lab-scale; error bars show standard deviation.

4.4.2 Reactor Performance

Performance indicators were monitored throughout the trial as described previously. Again, the results at each scale are summarised and analysis of variance was used to determine the extent of replication in operation between

- Reactors within triplicate sets
- Reactor type at lab-scale
- Full and lab-scale performance.

4.4.2.1 Performance at Full-Scale

Key performance indicators for the full-scale digester ADR2 are effluent pH, VFA accumulation in the effluent, and COD removal efficiencies. Table 4-5 summarises key statistics for each of these performance indicators for the period d-85 to d0.

Table 4-5: Summary of data describing performance of full-scale bioreactor ADR2 d-85 to d0

	pH	VFA_asHac / mg / L	CODrem / %	sCODrem / %	pCODrem / %
Mean	7.11	283	72.9	82.0	51.2
Minimum	6.98	165	56.4	66.9	26.3
Maximum	7.19	549	81.2	89.1	72.2
St. Dev	0.05	94	5.7	4.6	10.7

(Data describes effluent pH, VFA as acetate equivalent and COD profile from full-scale bioreactor.)

Reactor pH is seen to be relatively stable with low standard deviation. VFA accumulation in the reactor is demonstrated by detectable VFA in the reactor effluent. VFA accumulation suggests that the reactor is not functioning optimally however the mean removal efficiencies for COD and sCOD removal are high in relation to those reported in the literature for industrial wastes. Removal of solids as described by pCOD removal efficiency is lower than that for total and soluble COD removal and is consistent with the literature regarding poor solids treatment in EGSB type reactors (Chan, Chong et al. 2009). It is noted that the minimum removal efficiency for pCOD is recorded two days after the highest pCOD:sCOD ratio recorded in the influent. This suggests that pCOD may initially accumulate in the reactor but then is washed out only partially degraded. Overall, the results qualitatively describe a reactor that is performing stably and well in response to the operating conditions.

Biogas production rates and methane content in the biogas are also useful indicators of reactor performance. However they are not included in the summary results here because whilst biogas production is recorded at the Distillery, the measurements are for the plant as a whole i.e. a single biogas production rate is measured collectively for the three digesters on-site. As such, precise methane production rates for the ADR2 individually cannot be established. It is noted however that for the period of study mean methane content in the biogas was 70% across the plant and was relatively stable (standard deviation 2.9%) for that period indicating good performance of the reactors and a good yield of methane in the biogas as compared to data published in the literature. Mean biogas production rate for ADR2 inferred as a proportion of the total was produced across the plant was 2.5 $m^3/m^3rxr.d$. Whilst methane content in the biogas was stable, biogas production volume was highly unstable with variation as much as 139% from the mean with no clear correlation with COD in the influent.

4.4.2.2 Performance at Lab-Scale

Performance indicators monitored at lab-scale were effluent pH, VFA accumulation in the effluent, COD removal efficiencies, biogas production rates and methane content in the biogas. The performance results are summarised in Tables 4-6 and 4-7 for each of the 1-D and 3-D reactor sets. One-way analysis of variance was conducted to determine replication between reactors in the 1-D and 3-D lab-scale triplicate sets for each parameter tested. No significant difference was found for any parameter with the exception of VFA in the effluent of the 1-D reactor group ($P<0.001$). The reactor performance then may be regarded as well replicated within each triplicate reactor set.

Table 4-6: Performance Indicators 1-D Lab-Scale Reactors

	pH	TVFA / mg /L			CODrem /	sCODrem /	pCODrem /	BPR /	CH4 /
		R4	R5	R6	%	%	%	L/Lrxr.d	%
Average	7.15	25.7	5.4	6.9	91.1	93.7	77.9	2.95	74.5
Minimum	6.78	12.8	0.0	0.0	67.3	84.9	-48.5	0.51	61.6
Maximum	8.17	73.7	12.8	48.8	96.4	97.4	125.7	7.87	82.8
St.Dev	0.24	14.9	3.6	8.4	5.1	2.3	28.3	0.91	3.5

Table 4-7: Performance Indicators 3-D Lab-Scale Reactors

	pH	TVFA / mg / L	CODrem / %	sCODrem / %	pCODrem / %	BPR / L/Lrxr.d	CH4 / %
Mean	7.28	29.7	88.1	92	69.3	3.01	72.8
Minimum	6.82	0.0	69.1	73.3	-21.4	1.96	42.5
Maximum	7.76	376.1	93.8	96.2	119.4	4.96	83.3
St.Dev	0.23	58.3	4.8	4.9	26	0.61	6.4

(Data describing performance of 1-D and 3-D laboratory bioreactors including effluent pH, total volatile fatty acids as an acetate equivalent, COD profiles, biogas production rate and methane content in the biogas. Data are presented as a mean for the reactor set where no significant difference was determined between replicates and individually for each reactor in cases where a significant difference was found (ANOVA, $p<0.05$).)

In each case low standard deviation for pH in reactor in the effluent indicates that pH in the reactors was stable. Further, reactor pH is comfortably within the reported range for maintenance of syntrophic relationships enabling full progression to methanogenesis (O'Flaherty, Collins et al. 2006). Similarly to full-scale results, VFA is seen to accumulate albeit at low-levels indicating sub-optimal performance, however, again COD and sCOD removal efficiencies are high and performance appears relatively stable. Solids removal efficiency is least stable, which again points to problems with pCOD removal as reported in the literature (Chan, Chong et al. 2009). Standard deviation of both biogas production rates and methane content in the biogas also indicate stable performance.

ANOVA was additionally used to determine the degree of replication of results between the two lab-scale reactor types. No significant difference was found between reactor pH, pCOD removal efficiency, or biogas production rates. However, significant differences were found between total VFA accumulation ($p = 0.0036$), CODrem ($p = 0.0014$), sCODrem ($p = 0.018$) and methane content in the biogas ($p = 0.014$) with the 1-D reactor types performing marginally better in real terms in each case.

4.4.2.3 Comparative Analysis between Full- and Lab-Scale Reactor Performance

Operation of the full- and lab-scale reactors was demonstrated to be significantly different in terms of reactor loading and as such, direct comparison of performance is not

appropriate. Rather, a qualitative comparison of performance trends between scales is applied and is considered in relation to operating trends. Figure 4-6 shows time-series for

- COD influent characteristics: graphs a) Full-Scale and b) Lab-Scale, and for,
- COD removal efficiencies: graphs c) Full-Scale and d) 1-D Lab-Scale, e) 3-D Lab-Scale reactors.

Figure 4-6: Scatter plots showing time-series of COD profile in bioreactor influent and COD removal efficiencies at full-scale (a, c) and lab-scale (b, d, e).

Qualitatively, it is seen that each of COD, sCOD and pCOD in the influent to the full-scale reactor (graph a) is reasonably constant over time. For the same period, whilst COD and sCOD removal efficiencies are reasonably constant, pCOD removal efficiency declines over time at full-scale (graph c). During the period for which there are biomass samples, and hence sequencing data for the full-scale reactor (blue box in graphs a and b), influent COD concentrations decrease and as do COD removal efficiencies. The decline in

performance follows the brief period of shutdown from day d-27 to d-21 described previously and performance is seen to recover in the subsequent period of operation.

At lab-scale by contrast, both total COD and the ratio of pCOD:sCOD decreases over time (graph b). Similarly however whilst COD and sCOD removal efficiencies appear highly stable in both the 1-D and 3-D lab-scale reactors, and more so than in the full-scale reactor, pCOD removal efficiency is seen to decline in both cases (graphs d and e). Speculatively, the decline in pCOD removal at lab-scale may arise from gradual solids accumulation in the reactor during the period of operation reaching saturation, and beginning to washout. This is supported by the 'negative' pCOD removal efficiencies reported in both the 1-D and 3-D reactors towards the end of the study in which higher solids were measured in the reactor effluent without observation of a corresponding increase in solids in the influent. Both the lab-scale reactor types demonstrate higher COD, sCOD and pCOD removal efficiencies than the full-scale reactor (Tables 4-5 to 4-7) however as influent characteristics were different, the improved performance cannot be directly attributed to scale.

4.4.3 Community Physiology at Full- and Lab-Scale: Specific Methanogenic Activity Testing

Specific methanogenic activity in the biomass was measured using substrates Acetate (Ac), Propionate (Pr), Butyrate (Bu), Ethanol (Et), and Hydrogen / CO_2 (H2). Samples tested were biomass from the seed sludge representing activities in the sludge at full-scale and in the seed-sludge, and in samples extracted from the top and bottom of the sludge-bed for each of the lab-scale reactor types. Results are summarised graphically for each trial in Figure 4-7 with standard deviation of triplicate (tests) shown using error bars. The results at full-scale are somewhat unexpected in that at full-scale, hydrogen represents the most active methanogenic pathway. Acetotrophic methanogenesis is commonly assumed to be the most dominant pathway in both engineered systems (O'Flaherty, Collins et al. 2006) and in the natural environment. Frequently in the literature on low-temperature methanogenesis in the laboratory however a trend towards hydrogenotrophic methanogenesis is common at increasingly low temperatures (McKeown, Scully et al. 2009, O'Reilly, Lee et al. 2010) with selective pressure on the microbial community cited as a possible cause. As temperatures in this instance are not low, it is possible that variable loading, and in particular variable solids loading, is inducing a similar stress on acetotrophic methanogens by for example ammonia toxicity (Westerholm, Dolfing et al. 2011). Alternatively, the activity of microorganisms governing the earlier stages of

methanogenesis, specifically those involved in acetogenesis in which VFAs are reduced to acetate, hydrogen and carbon dioxide, may be dictating the route to methanogenesis perhaps by competition with acetotrophic methanogens. Further consideration of the data collected here appears to support the argument that solids loading may induce selective pressure favouring hydrogenotrophic methanogenesis. The data show a significant decline (ANOVA, $P<0.001$) in hydrogenotrophic activity at lab-scale particularly in the 1-D reactor types during the 70 day trial. By inference then, at lab-scale, where solids delivery was significantly lower than at full-scale, the 1-D reactor types appear to switch to a more balanced activity profile between acetotrophic and hydrogenotrophic methanogenesis. Activity recorded using ethanol as substrate was also seen to be significantly lower at lab-scale ($P<0.1$) though less markedly than with hydrogen.

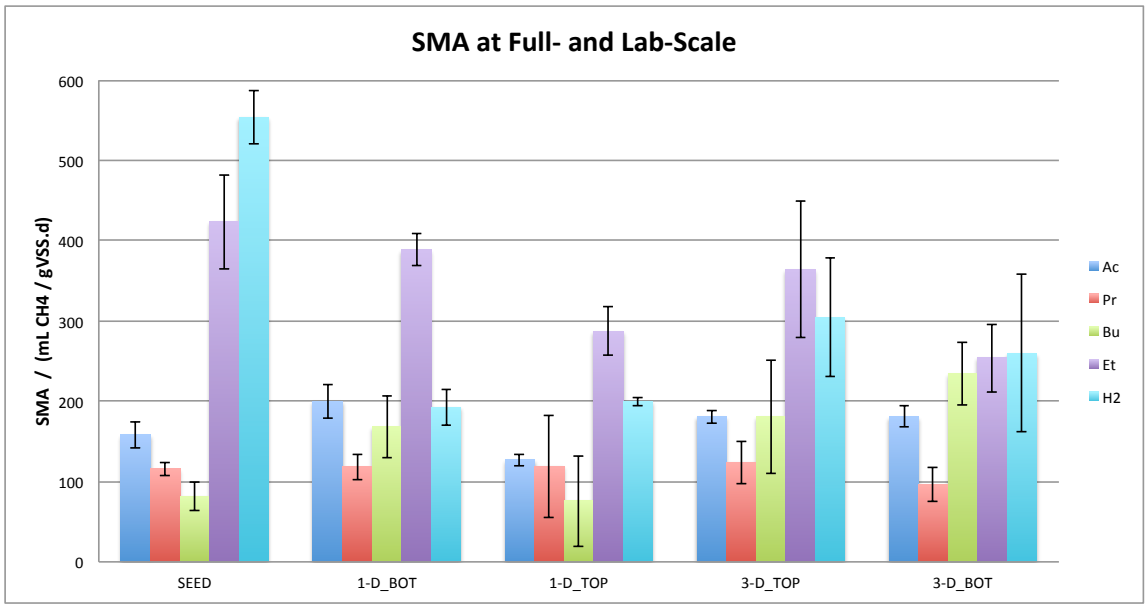


Figure 4-7: Grouped bar plot showing SMA recorded in biomass from full-scale (SEED) and lab-scale bioreactors at the beginning (SEED) and end of the trial against specific substrates: acetate (Ac), propionate (Pr), butyrate (Bu), Ethanol (Et) and hydrogen (H2). Bars show blank-adjusted mean and error bars show standard deviation of triplicate measurements.

On data collected at lab-scale, one-way analysis of variance was conducted to determine the difference in activity between the 1-D and 3-D reactor types, and between activity in the biomass at the top and bottom of the sludge bed in each reactor type. Between the 1-D and 3-D reactor types, no significant difference was found for any substrate except hydrogen ($P<0.05$), which shows decreased activity in the 1-D type reactors. Interestingly, the 1-D reactor type is more commonly applied at lab-scale than the 3-D reactor type is and as such it could be inferred that lab-scale reactor design may influence the common finding that acetate is the more dominant route to methanogenesis as at lab-scale. Comparing activities at the top and bottom of the sludge-bed in the 1-D type reactors, it is found that activity is higher in sludge extracted from the bottom of the reactor for both acetate and

ethanol ($P<0.001$). This suggests that not only might reactor configuration influence the route to methanogenesis at lab-scale but that the depth at which a biomass sample is taken might influence the inferred dominant route in 1-D lab-scale reactors. No significant difference in activity with depth was found in the 3-D type reactors for any substrate tested.

4.4.4 Analysis of Microbial Community using 16S rRNA NGS Results

Library construction for sequencing consisted of 107 unique barcoded samples and with 9 additional samples as positive and negative controls as described in Chapter 3. For the entire library 4035 unique OTUs were identified at 97% similarity from a total of 7080898 reads excluding control samples. The range of reads per sample was 621 to 453230 and median read count was 36031. Two samples (R6:P2:d46, R6:P1:d25) had total read counts <5000 and were excluded from the analysis such that the lowest read count per sample is 5232 reads. Of the samples included in the analysis, the minimum read count sample identified 382 OTUs in a single sample as compared to 1479 OTUs identified in the maximum read count sample. A collectors curve and rarefaction curves (R software) demonstrates that saturation was not reached with respect to sequencing depth and as such two data normalisation methods are applied in the analysis; rarefaction and species relative abundance.

4.4.4.1 Qualitative Overview of Microbial Community at Full- and Lab-Scale

The Phyloseq package in R was applied using a QIIME phylogeny file to generate a phylogenetic tree for the most abundant 100 OTUs identified across all samples in the set, accounting for a mean of 78.9% of the total community population (standard deviation 4.3%) as shown in Figure 4-8. The leaves in the tree are labelled at family level where identification has been possible using RDP classifier to map OTU IDs. In-spite of significant differences in operating conditions, each of the top 100 OTUs identified are present in each of the full-scale, and lab-scale reactor types suggesting strong phylogenetic similarity between the communities at each scale and in each reactor type. It is noted that of the most abundant OTUs, the majority cannot be mapped to an identity of a known or characterised microorganisms using publicly accessibly databases. This points to the fact that whilst NGS sequencing is useful as an exploratory tool for complex microbial communities, further work culturing and characterising microorganisms is necessary if the data gleaned is to be fully interpreted. Some interesting observations may be made however from those OTUs whose identities are tracked. As is often reported, Archaea

appear to be less varied than Bacteria in terms of community membership and to be most consistent in terms of relative abundances.

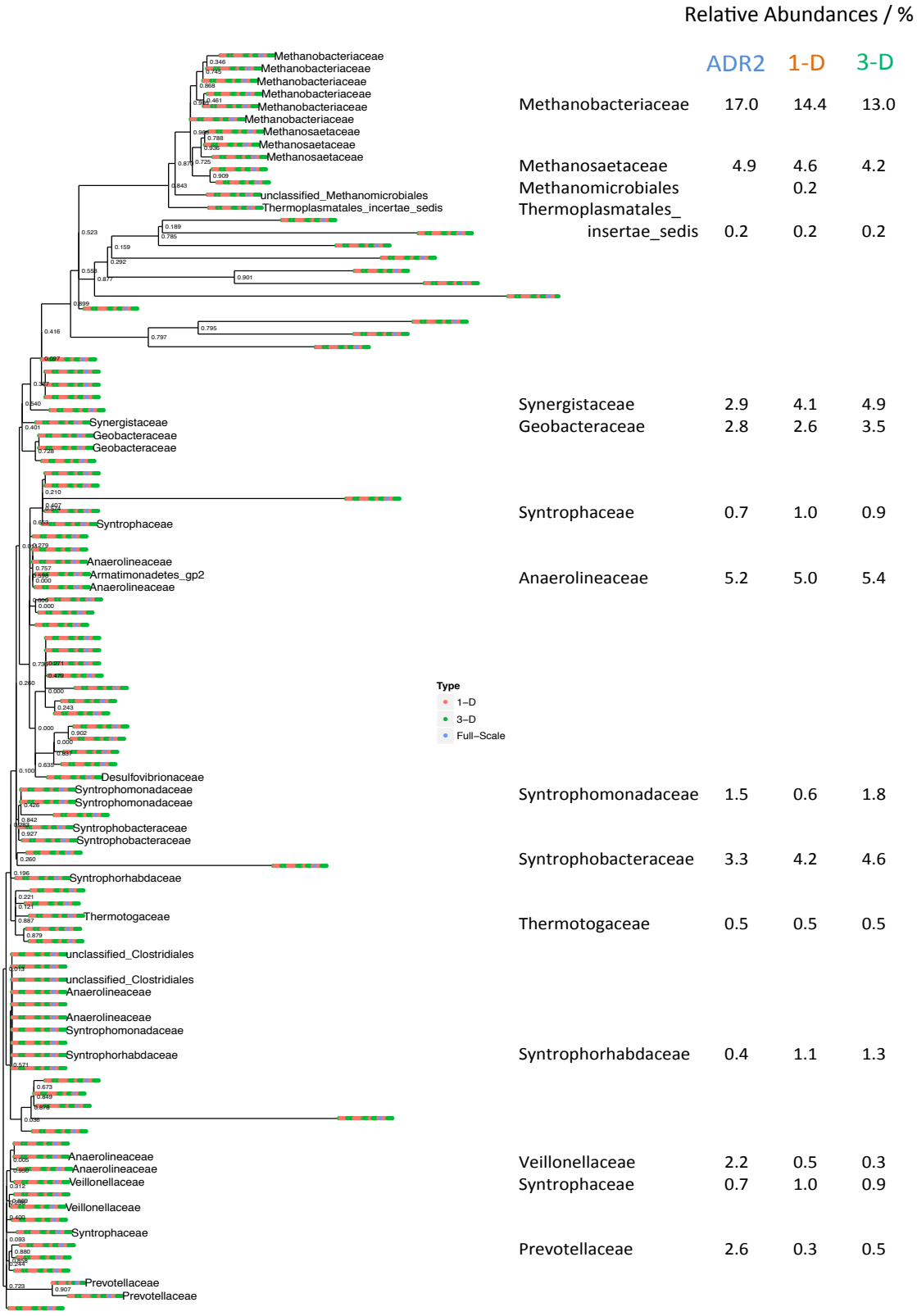


Figure 4-8: Phylogenetic tree plotted using Phyloseq using QIIME phylogeny file in Newick format. Leaves represent the 100 most dominant OTUs across the full- and lab-scale sample set and are labelled with taxonomy at family level; mean relative abundance of the most dominant 15 families is provided for each of the full-scale and 1-D and 3-D lab-scale reactor sets.

The proportion of Archaea in the total community was found to be 24.5%, 20.7%, and 19.5% in each of the full-scale and 1-D and 3-D lab-scale reactor types respectively. Of Archaeal OTUs in the top 100 most abundant species, a decline in Methanobacterium at lab-scale accounts for the majority of the difference in Archaeal community composition with scale. *Methanobacterium* are the most dominant of any family identified and are H2, CO2 and formate utilising methanogens (Madigan, Martinko et al. 2009). Whilst this result is somewhat unexpected as acetate utilising bacteria typically reported as most dominant in EGSB sludges it is strongly supported by the methanogenic activity measurements reported previously that demonstrated the dominance of the hydrogenotrophic pathway at both full- and lab-scale. The second most abundant Archaeal family identified was *Methanosaetaceae*, comprising 3 OTUs each of which was identified as of the genus *Methanosaeta*. *Methanosaeta* are filamentous, acetate-utilising methanogens and are associated with granule formation and maintenance, and are thought to form the core of anaerobic sludge granules (Hulshoff Pol, de Castro Lopes et al. 2004). *Thermoplasmatales Incertae sedis*, is identified as present at low abundance at each scale. Of the order *Thermoplasmatales*, this genera is known to be moderately thermophilic and growth is promoted in highly acidic environments including acid mine drains and acidic hot springs (Itoh and Iino 2013). As reactor pH is close to neutral at both full- and lab-scale, the cause of the proliferation of such organisms is unknown but could point towards gradients in pH within the granule. A single hydrogenotrophic methanogen, *Methanomicrobiales*, is seen to be amongst the most abundant OTUs in the 1-D lab-scale reactor but absent in the 3-D lab-scale and full-scale reactors at very low abundance.

Of OTUs identified at family and genus level, dominant Bacterial families may be identified whose relative abundance appear reasonably consistent at each scale including *Anaerolineaceae*, *Synergistaceae*, *Geobacteraceae*, *Syntrophomonadaceae* and *Syntrophobacteraceae*. *Anaerolineaceae* belong to the phylum *Chloroflexi* and have been associated with carbohydrate fermentation in anaerobic systems (Yamada, Sekiguchi et al. 2006, Narihiro, Terada et al. 2012) such that functionally these OTUs may be involved in the earlier stages of methanogenesis. Species of the family *Synergistaceae* have been associated with EGSB reactors treating waste with a high protein load (Delforno, Okada et al. 2012) and tentatively with high lipids loading (Palatsi, Affes et al. 2012) and again may be involved in early stages of degradation of organics in the waste stream. The family *Synergistaceae* is of the phylum *Synergystes*, an isolate of which has been found to produce VFAs, CO2 and H2 from amino acids from a mesophilic anaerobic digester treating municipal waste (Ganesan, Chaussonnerie et al. 2008). Two OTUs were identified

as of the family *Geobacteraceae* and genus *Geobacter*. *Geobacter* species have recently been identified with the capacity for direct electron transfer with Methanosaeta species (Rotaru, Shrestha et al. 2014) enabling *Methanosaeta* to produce methane via reduction of carbon dioxide. *Methanosaeta* is the second most dominant methanogenic family identified here. Two OTUs are identified as of the family *Syntrophomonadaceae* that have been associated with the degradation of long-chain fatty acids in methanogenic chemostat cultures and EGSB reactors treating oleic acid (Pereira 2002, Shigematsu, Tang et al. 2006). Finally, of reasonably constant relative abundance at each scale is the family *Syntrophobacteracea*, members of which have been variously isolated from sewage sludge, marine and freshwater sediments and have been demonstrated to grow via fermentation or syntrophically with H2 utilisers (Kuever 2014) such as *Methanobacterium,* which are abundantly present at each scale in this experiment.

Whilst a core of consistently abundant species may be identified across each scale, some families are noted to have pronouncedly different abundances in the full-scale digester as compared to the lab-scale reactor types. Of the bacterial families identified, the most apparent differences associated with reactor scale are of the families *Prevotellaceae* and *Veillonellaceae*, which are substantially more abundant at full-scale than in the laboratory reactors. *Prevotellaceae* constitute 2.6% of the total microbial community in the full-scale digester whilst accounting for only 0.3% and 0.5% of the microbial communities in each of the 1-D and 3-D lab-scale reactor types respectively. Genus of the family *Prevotellaceae* have been associated with the human gut microbiome of those with carbohydrate-rich diets (Wu, Chen et al. 2011) and with degradation of plant residues and carbohydrates to produce VFAs (Garcia-Peña, Parameswaran et al. 2011). Increased relative abundance at full-scale then may reflect the higher component of pCOD in the full-scale reactor substrate and more stable pCOD removal efficiencies at full-scale. The family *Veillonellaceae* is identified for four of the top 100 OTUs at full-scale across two known genus, and two unknown genus: *Megasphaera* (1.1%), *Selenomonas* (0.5%), unknown (0.6%); and accounts for 2.2% of the total community in the full-scale digester. Of these, only *Megasphaera* is present in the lab-scale digesters at relative abundances of 0.48% and 0.34% for the 1-D and 3-D reactor types respectively. The genus *Megasphaera* is associated with anaerobic degradation of long-chain fatty acids (C12-C19) to VFAs including acetate, propionate and butyrate (Marchandin and Jumas-Bilak 2014) and again may point to the full-scale reactor community having adapted to higher pCOD concentrations than were delivered at lab-scale. As the relative abundances of key methanogens appeared reasonably consistent between the full- and lab-scale digesters,

these bacterial families whose abundances vary so greatly with scale might be linked to the dominance in hydrogenotrophic pathway displayed at full-scale. Some species of the family *Prevotellaceae* are known to produce hydrogen in small quantities and both *Prevotellaceae* and *Veillonellaceae* have been identified as amongst the dominant species in laboratory studies of mixed microbial communities for hydrogen production (Mariakakis, Bischoff et al. 2011, Shida, Sader et al. 2012).

4.4.4.2 Ecological Spatio-Temporal Dynamics with Scale, Reactor Type and Depth

Ecological indices were calculated based on count data for each sample in the sequencing set and are used to describe the structure of the microbial community in relation to ecological theory. Indices used were; species richness rarefied to minimum sampling depth (5232 reads); community diversity using the Simpsons Index of Diversity; and community evenness expressed as Pielou's Index. Each index was calculated with time and depth for each of the full-scale and lab-scale reactor types and was calculated using the R-Vegan software package. Figure 4-9 shows the variation of richness, diversity and evenness with time and depth at each scale graphically. Overall the data describes a species rich community that is both highly diverse and strongly even in distribution. The plot for rarefied richness shows an increasing trend with time in the 1-D and 3-D lab-scale reactor types that was found to be significant ($P<0.001$, 1-D &$P<0.01$, 3-D) as compared to full-scale reactor. Further, the community in the 1-D reactor has a significantly higher mean richness ($P<0.01$, ANOVA) than either the full-scale or 3-D lab-scale reactor types. No significant trend was identified for either evenness or diversity statistics with reactor type in time or spatially. That the community richness would increase at reduced scale is unexpected as ecology theory suggests that increasing scale tends to increase the number of species present. Scale however is not the only difference between the lab- and full-scale reactors; operational differences occurred too. As such, no direct conclusion may be drawn.

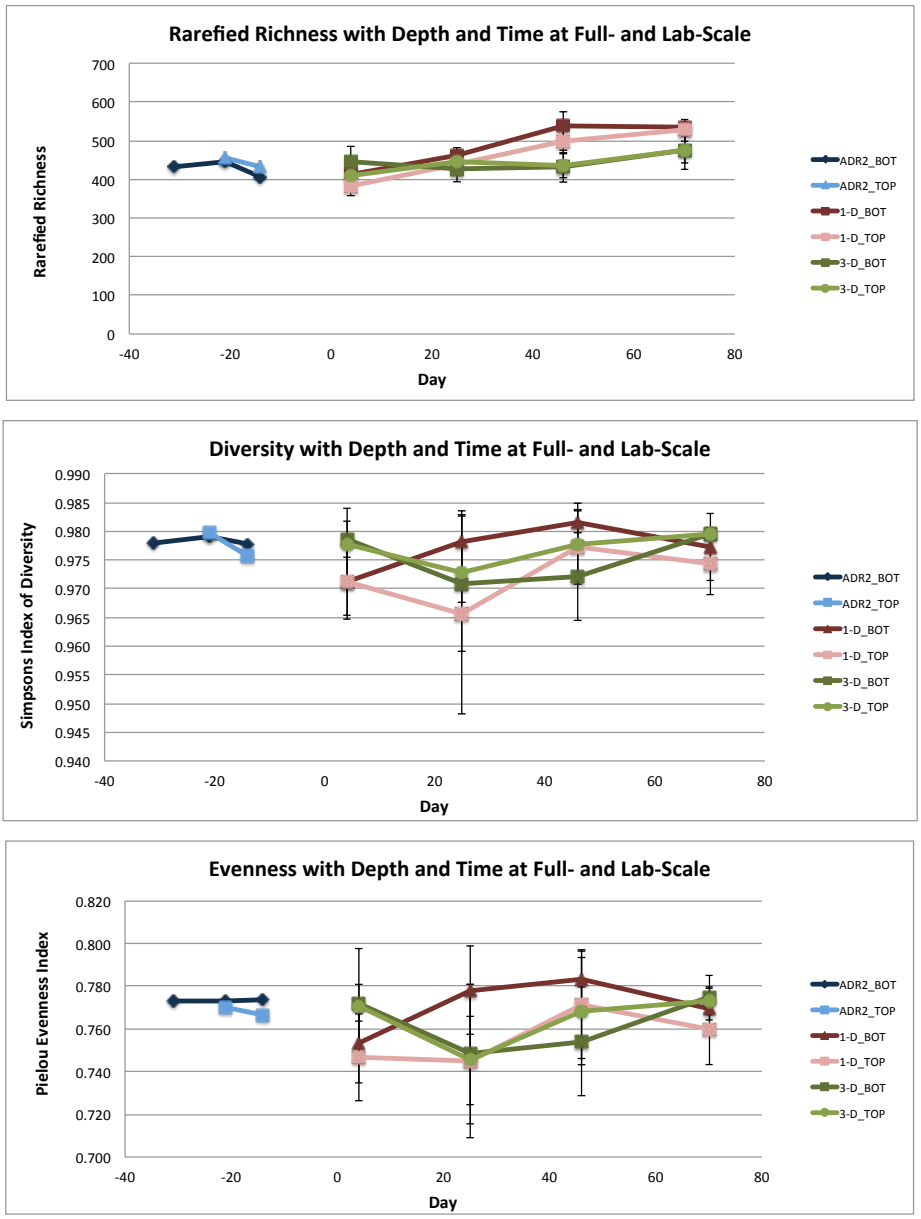


Figure 4-9: Scatter plots showing time-series for mean rarefied richness, diversity and evenness in each of the full-scale and lab-scale sample sets; error bars show standard deviation. In each case, statistics are calculated using samples grouped to represent the microbial community at the top (_TOP) and bottom (_BOT) of the sludge bed.

4.4.4.3 Similarity in Microbial Community within Reactor Type

To determine the extent of replication in the microbial community between replicates in each of the 1-D and 3-D reactor sets, multivariate analysis of variance (Adonis, R-Vegan) was conducted using dissimilarity matrices to represent the sequencing data. Whilst no significant difference was found between the communities in the 1-D reactors using either method, a significant difference ($P<0.001$) was found between the 3-D reactors measured by Bray-Curtis similarities with reactor estimated to account for 16% of variance in the sequencing samples. As a single entity, the full-scale digester was not included in this analysis. The result at lab-scale however demonstrates that replicate testing is important when running lab-scale experiments in that the 3-D reactors appeared highly replicated

both in terms of operation and performance yet are significantly different in terms of underlying community. Further, the results might imply that interpretation of composition in microbial community and association with environmental factors should not be assumed to be absolute when examining full-scale digesters where replication of the outcomes is not possible.

NMDS plots were then used to provide insight into clustering of the OTUs for each of the full-scale and 1-D and 3-D lab-scale reactor types and again, two alternative distance metrics were used. Bray-Curtis distance was plotted (Figure 4-11) to investigate clustering based on relative abundance of the most dominant species and GUnifrac, alpha=0.5, distances were plotted (Figure 4-12) to determine clustering based on phylogenetic distances as shown in Figures below. In both instances plots were based on the most abundant 100 species across the sample set.

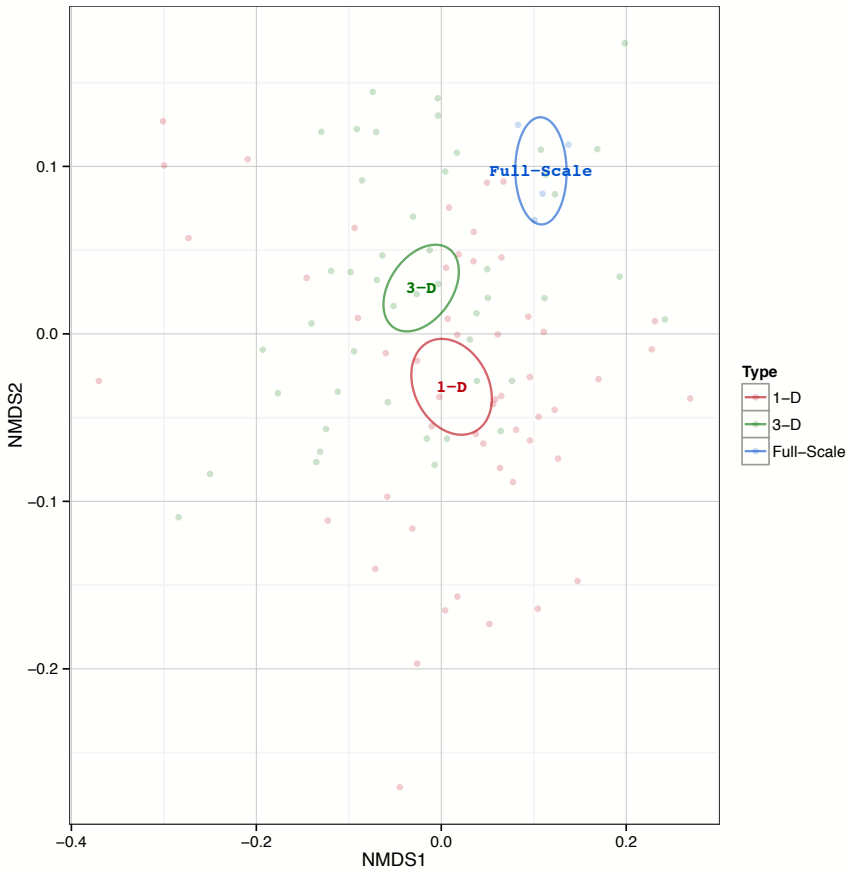


Figure 4-10: Two-dimensional NMDS ordination plot (Stress = 0.181) of the 100 most abundant OTUs in the sample set, grouped by reactor type and plotted using Phyloseq and Bray-Curtis similarity. In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

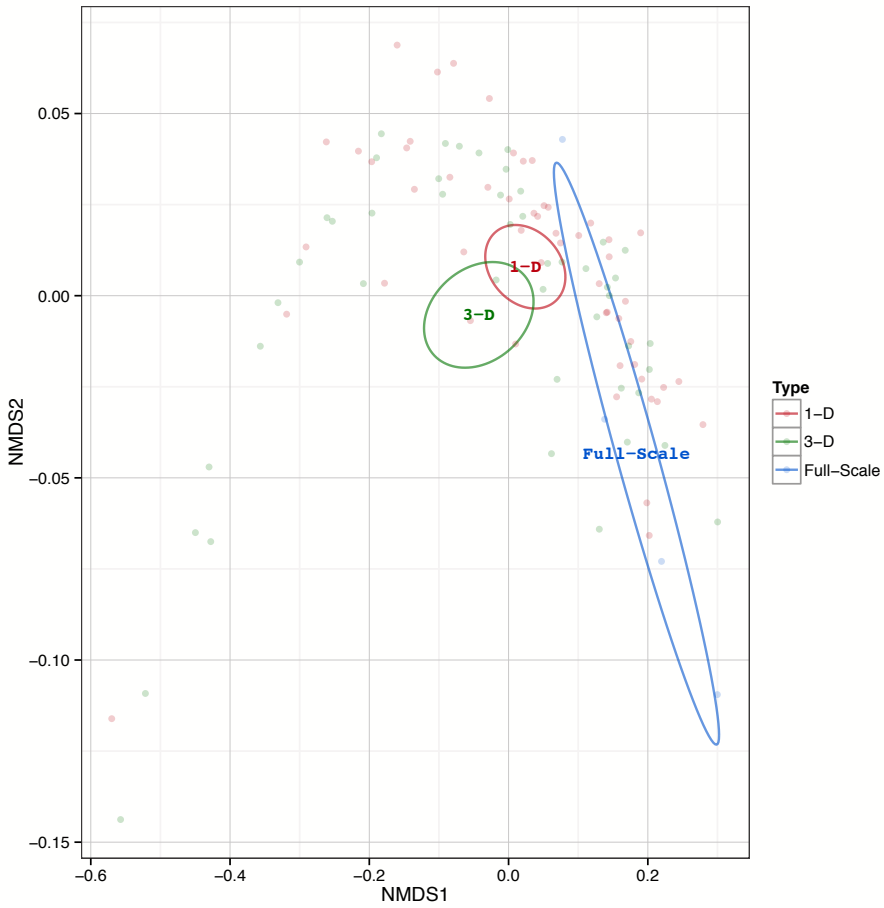


Figure 4-11: Two-dimensional NMDS ordination plot (Stress = 0.024) of the 100 most abundant OTUs in the sample set, grouped by reactor type and plotted using Phyloseq and GUnifrac distances (alpha = 0.5). In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

Both plotting methods show that the lab-scale reactor communities cluster more closely to each other than to that of the full-scale reactor from which the seed sludge was drawn. The plot using count data (Figure 4-10) appears to enable more distinct clustering than that plotted using phylogenetic distances (Figure 4-11) suggesting similar community membership across scales but distinct community structure. PERMANOVA was applied to confirm this interpretation. It was found that no significant difference between communities could be found using phylogentic distances but that significant differences were found for community variation with reactor scale, type and port depths using count data ($P<0.001$ in each case). This suggests that the variation arising from each factor influences the relative abundances of species within the communities present rather than membership of the communities present. Indeed, this result might be predicted in that each reactor type was seeded from the full-scale digester and assuming external migration to the digester community was minimal, and extinction unlikely due to similar operating conditions, phylogeny should have been maintained in each case.

NMDS plots were additionally drawn using Bray-Curtis distances to investigate OTU clustering with time and depth for each of the lab-scale bioreactors types. The full-scale digester was again omitted from this analysis due to lack of replicate sampling. Figures 4-12 and 4-13 show sample clustering in time:

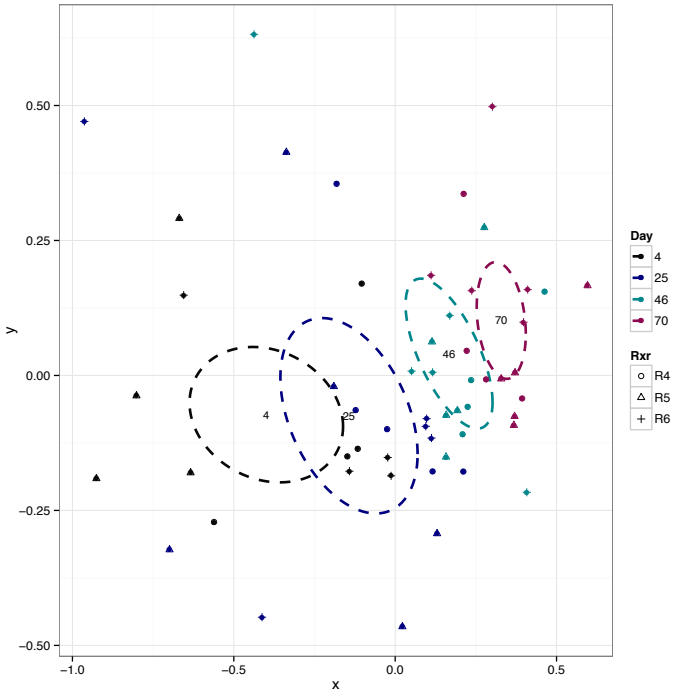


Figure 4-12: Two-dimensional NMDS ordination plot (Stress = 0.127) of the 1-D lab-scale sample set, grouped by sampling day and plotted using Bray-Curtis similarity. In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

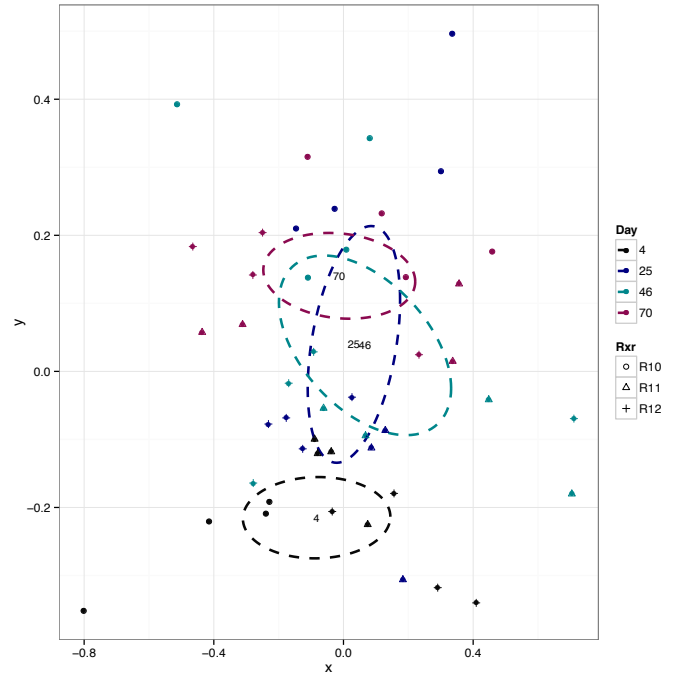


Figure 4-13: Two-dimensional NMDS ordination plot (Stress = 0.169) of the 3-D lab-scale sample set, grouped by sampling day and plotted using Bray-Curtis similarity. In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

The microbial community in the 1-D reactors appears to continually evolve in terms of community composition with differences reducing towards the end of the experiment. The trend in the 3-D reactors is less clear but suggests an initial change between days d4 and d25 and minor changes subsequently. That the community in each case appears to evolve with time might be anticipated by inspection of the operating data for the reactor set, in particular for data pertaining to influent COD. COD in the reactor influent continually fluctuated in the reactors as did the relative component of soluble and particulate COD. Figures 4-14 and 4-15 show clustering with port (depth) in each reactor type.

In the 1-D type reactors (Figure 4-14), OTUs representing the microbial community at ports P1 and P2 at the bottom of the sludge-bed cluster distinctly from ports P3-P5 at the top of the sludge-bed. In the 3-D reactor type by contrast, little distinction in clustering is seen (Figure 4-15). This directly supports the earlier findings of the SMA testing. SMA tests indicated that biomass at the bottom of the 1-D reactor type was significantly more active using substrates acetate and ethanol than that at the top of the sludge-bed whilst no significant difference was found in activity at the top and bottom of the 3-D reactor types. ANOVA confirms the trend is statistically significant with 15.4% variation ($P<0.01$) associated with depth in the 1-D reactors and no significant trend found in the 3-D reactors. Again, this points to the importance of both the influence of lab-scale idealisation on community structure both spatially and temporally, and of ensuring an appropriate sampling strategy in a reactor to adequately describe microbial community composition.

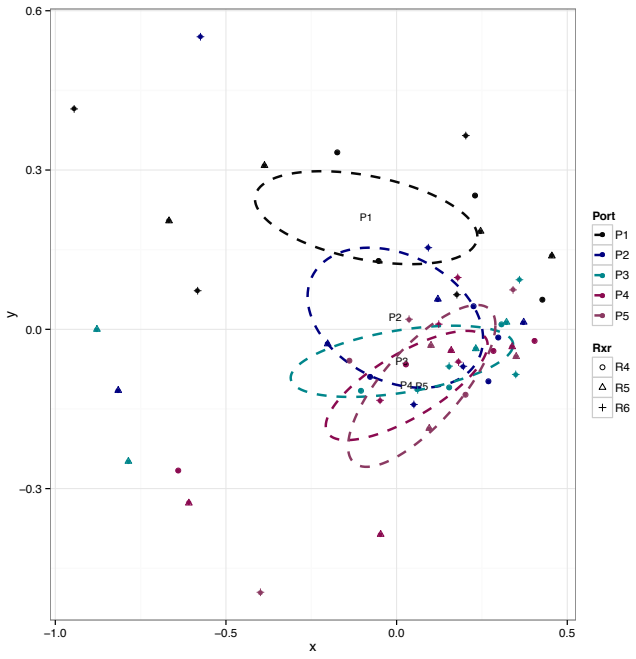


Figure 4-14: Two-dimensional NMDS ordination plot (Stress = 0.118) of all samples in the 1-D lab-scale sample set, grouped by sampling depth and plotted using Bray-Curtis similarity (ports P1 - P5 where P1 is at the bottom of the sludge-bed and P5 is at the top). In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

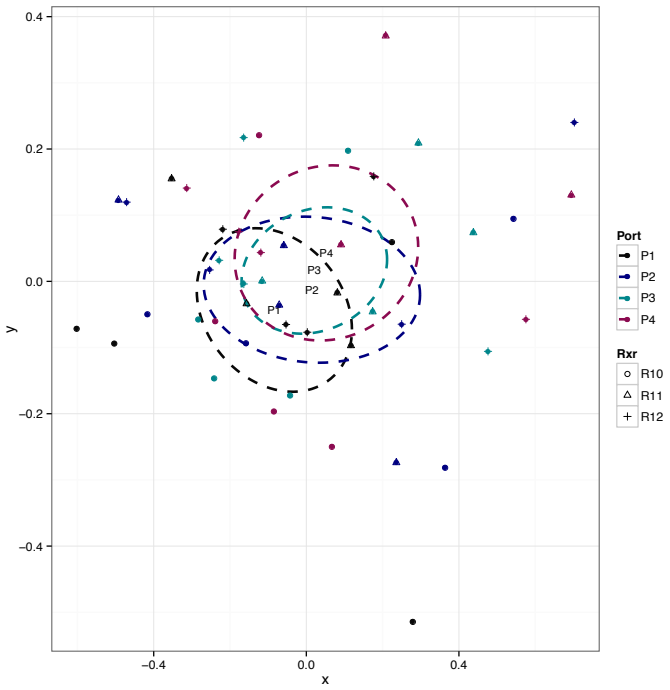


Figure 4-15: Two-dimensional NMDS ordination plot (Stress = 0.129) of all samples in the 3-D lab-scale sample set, grouped by sampling depth and plotted using Bray-Curtis similarity (ports P1 - P5 where P1 is at the bottom of the sludge-bed and P4 is at the top). In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

4.4.4.4 Key Families and Genus Associated with Reactor Type and Depth

The Kruskal-Wallis was applied to using taxa tables to determine significant variation in relative abundance of key taxa both with reactor type and depth. The analysis with reactor type was conducted at family level and results demonstrate significant variance ($P<0.05$) in the relative abundance of 21 families. Of those, five families show a decrease in relative abundances at lab-scale whilst 3 show an increasing trend. Figure 4-16 shows graphically the trends in of the most abundant 4 families whose relative abundances decreased at lab-scale; (a) *Veillonellaceae*, (b) *Porphyromonadaceae*, (c) *Armatimonadetes gp2*, and (d) *Bifidobacteriaceae*. *Veillonellaceae* was highlighted previously as more abundant in the top 100 OTUs at full-scale than at lab-scale and was noted to be associated with long chain fatty acids degradation. *Porphyromonadaceae* are associated with the degradation of carbohydrates and proteins and have been found in mesophilic anaerobic digesters for the treatment of distillers grains (Ziganshin, Schmidt et al. 2011) and in microbial communities established in biomethane potential tests for the treatment of whey and tomato wastes (Merlino, Rizzi et al. 2012). This again suggests that key differences in microbial community with scale may arise from differential solids loading. The family *Armatimonadetes* has only 3 cultivated strains with varying physiology of which two are known to degrade carbohydrates and C5-C6 sugars (Lee, Dunfield et al. 2014). Finally, *Bifidobacteriaceae* is of the class *Actinobacteria*, which is again associated with

carbohydrate degradation, but is less commonly reported in anaerobic digestion studies and more frequently reported in studies pertaining to the gut microbiome of humans and animals (Kämpfer 2010, Gosalbes, Durbán et al. 2011, Hooda, Vester Boler et al. 2013).

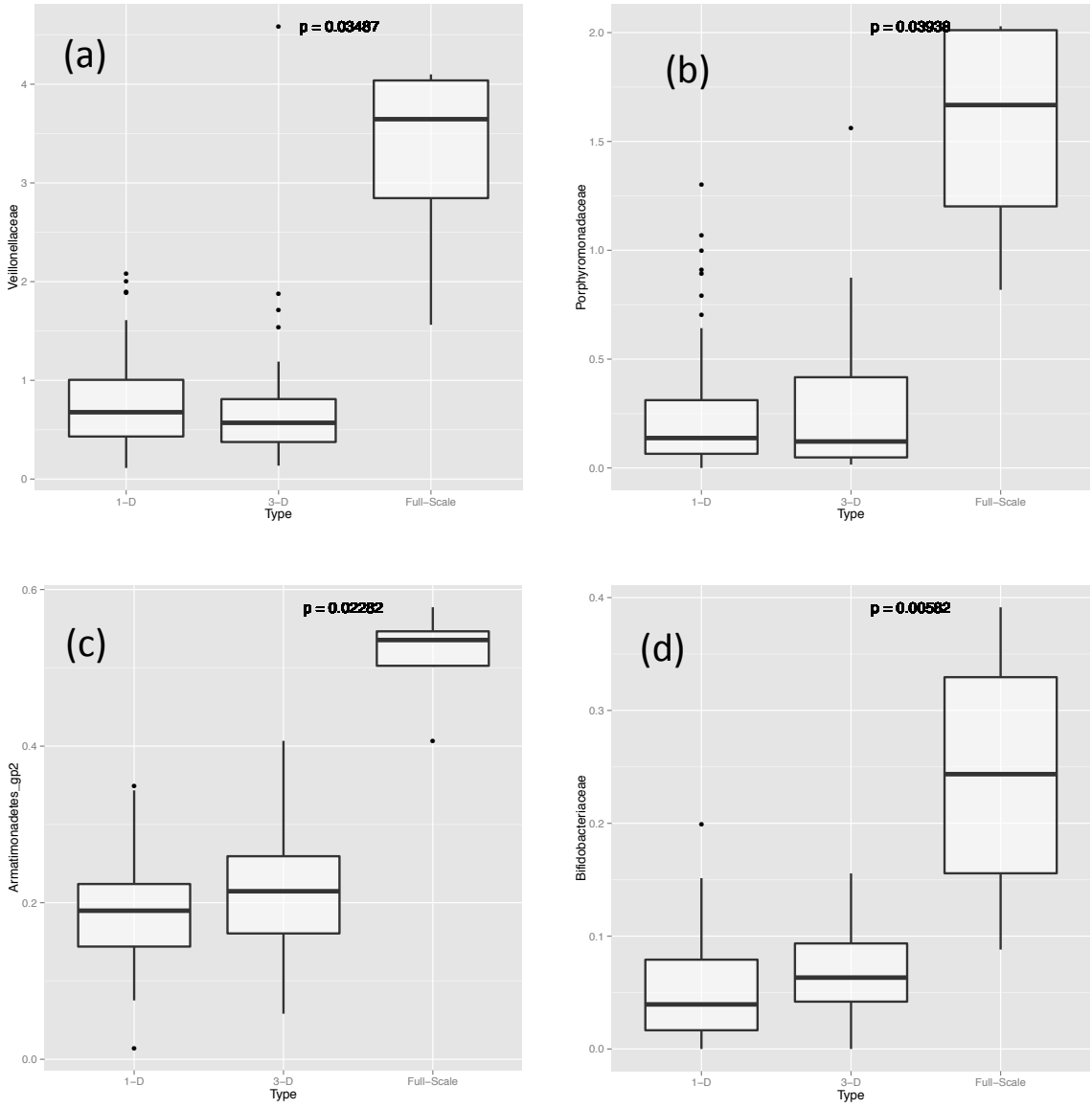


Figure 4-16: Boxplots showing distribution of relative abundance of families in the sample set showing significant variation (Kruskal-Wallis with Benjamini-Hochberg correction on p-value, p<0.05) in abundance between reactor types. The bands show the median value for each group; bottom and top of boxes show the first and third quartiles; and whiskers show maximum and minimum values with 1.5 of IQR of upper and lower quartiles.

Organisms were also identified whose relative abundance was found to be significantly increased at lab-scale (P<0.05) including the genera *Subdivision 3 Genera Incertae Sedis*, and two families of the order *Methanomicrobiales*. *Subdivision 3 Genera Incertae Sedis* was recently identified as part of a core community of microorganisms in a study spanning 14 full-scale wastewater treatment systems in China (Wang, Hu et al. 2012) although their function at present is not clearly defined. The mean relative abundance of *Methanomicrobiales* in the lab-scale digesters is 0.15% and 0.19% for the 1-D and 3-D

reactors respectively as compared to 0.12% in the full-scale digester. As such whilst the variation is found to be significant the increase is small and appears not to reflect the shift in activity profile from dominantly hydrogenotrophic at full-scale towards a more balanced profile with acetotrophic methanogenesis at lab-scale.

Kruskal-Wallis analysis was additionally conducted at Genus level to enable determination of organisms whose relative abundance significantly varied with depth. Results show that no organism was identified as varying significantly with depth at full-scale nor in the 3-D lab-scale reactors. In the 1-D lab-scale reactors by contrast significant variance was seen for 12 organisms in total, ten of which showed increased relative abundance towards the bottom of the sludge-bed whilst two showed decreased relative abundance. The trends for the four most abundant organisms shown to vary are summarised in Figure 4-17.

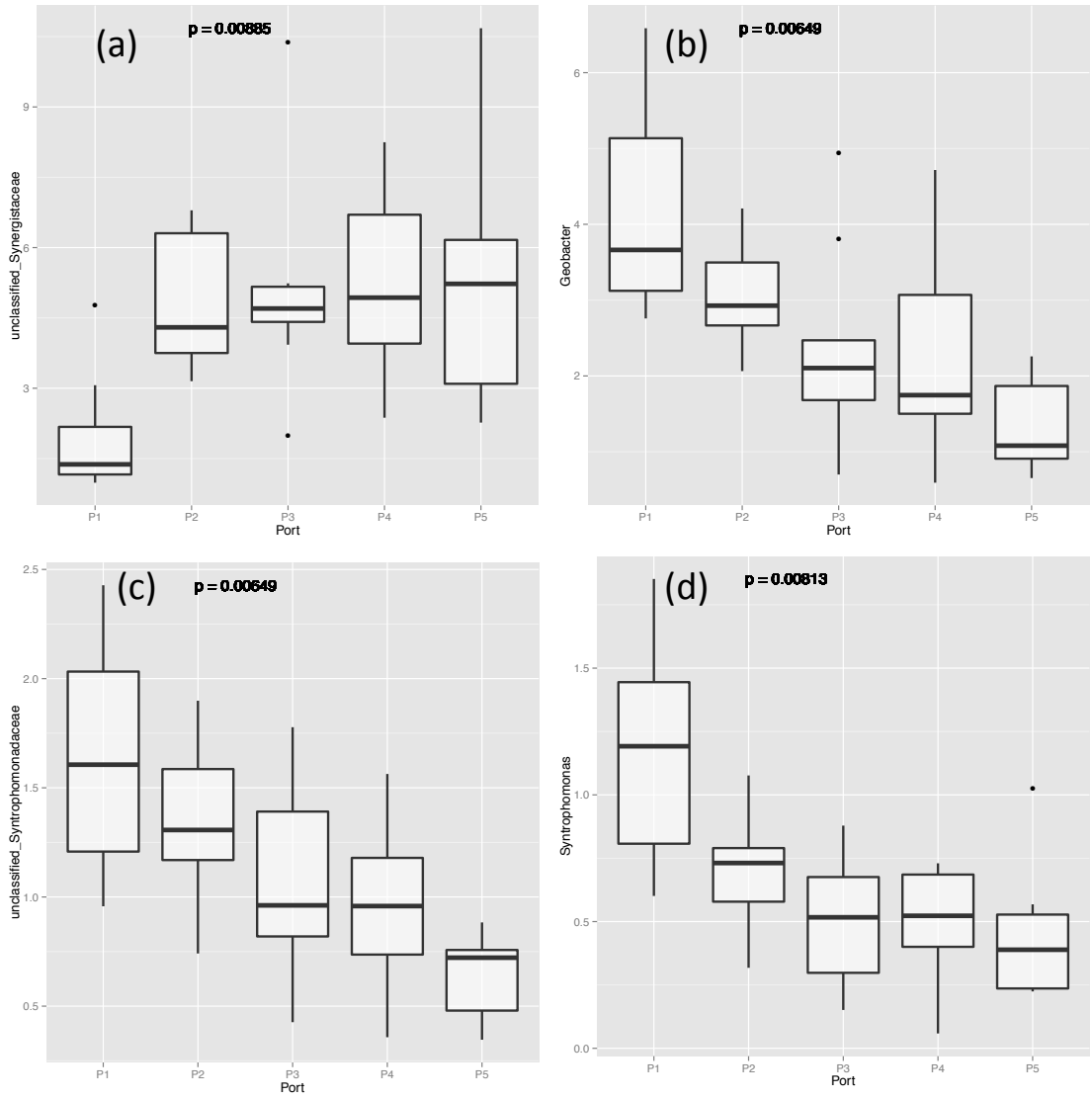


Figure 4-17: Boxplots showing distribution of relative abundance of genera showing significant variation (Kruskal-Wallis with Benjamini-Hochberg correction on p-value, $p<0.05$) in abundance with depth in the 1-D lab-scale bioreactors. The bands show the median value for each group; bottom and top of boxes show the first and third quartiles; and whiskers show maximum and minimum values with 1.5 of IQR of upper and lower quartiles.

Those organisms displaying an increasing trend with depth at genus level are unclassified *Ruminococcaceae*, *unclassified Veillonellaceae*, Geobacter (Figure 4-17(b)), *unclassified Syntrophomonadaceae* (Figure 4-17(c)), *Sporobacter*, *Syntrophomonas* (Figure 4-17(d)), *Methanomethylovorans*, *unclassified Peptococcaceae*, *Methanosarcina* and *Clostridium IV*. Strains of *Sporobacter* are known to be homoacetogenic (Grech-Mora, Fardeau et al. 1996), and as such increased abundance at the bottom of the sludge-bed may support the finding that acetate utilisation was greater at the bottom of the reactors as demonstrated by the SMA testing at depth. *Peptococcaceae* are fermentative organisms that produce VFAs, H2, CO2 and ammonia from amino acids (Rogosa 1971). *Veillonellaceae* and *Syntrophomonadaceae* are known to be associated with LCFA degradation. *Syntrophomonas* grows in syntrophy with H2 utilising microorganisms such as hydrogenotrophic methanogens (Sieber, Sims et al. 2010) which continue to present the dominant methanogenic pathway throughout the 1-D digesters despite increased acetate affinity in biomass at this position in that reactor type. Two genera were found to decrease with depth: unclassified Synergistaceae and Bellilinea. Synergistaceae is associated with VFA production from amino acid degradation, and Bellilinea is of the order Chloroflexi and is associated with fermentation of sugars. In terms of metabolism then, the variation in abundances is not decisive towards a particular stage in the methanogenic process, rather, variation appears to occur across each stage in the process.

The methanogenic genera identified to have increased relative abundance with depth, *Methanomethylovorans* and *Methanosarcina*, are both of the order *Methanosarcinales*. *Methanosarcinales* is the most metabolically diverse order of the methanogens with members able to utilise one or more of each of the three methanogenic pathways: CO2 reduction with hydrogen, cleavage of acetate, or from methyl groups (Kendall and Boone 2006). *Methanomethylovorans* strains have been identified to grow solely on methyl compounds (Cha, Min et al. 2013) whilst *Methanosarcina* are capable of using each of the three known pathways (Madigan, Martinko et al. 2009). The increase in *Methanosarcina* at the bottom of the 1-D reactors supports the previous finding that the biomass from the bottom of the 1-D reactors was more active using acetate than at the top. No SMA was conducted for methyl compounds however the increased abundance of *Methanomethylovorans* at the bottom of the 1-D reactors further supports the finding that the inferred methanogenic pathway in lab-scale reactors might be influenced by the position from which biomass samples are drawn.

4.4.5 Summary Findings and Discussion

4.4.5.1 Disparity of Scale

A key aim of this trial was to provide insight to the apparent disparity of scale in the performance of EGSB reactors. Whilst this aim was not fully realised due to disrupted running of the full-scale digesters involved in the study, some important findings were made in relation to the operation of EGSB type reactors at full-scale. Firstly, reactor loading at full-scale was noted to be variable not only in terms of fluctuating COD and substrate composition related to variability in daily processes conducted at the distillery, but also seasonally according to production rates and hence waste production, which were driven by demand. Secondly, at full-scale, feeding was not continual as is commonly applied in laboratory studies but again reflected upstream activity at the plant including regular plant closures for maintenance. This resulted not only in regular periods of starvation for the full-scale reactor but periods of disturbance to reactor temperature as no heating was applied to the EGSB digesters during periods of plant closure. The impacts of such variable operating conditions on reactor performance and associated microbial community are little understood. In lab-scale trials such conditions are rarely replicated. Even in studies conducted using industrial wastes, or those in which shock-loading is applied, such extreme disturbances as starvation coupled temperature fluctuation are rarely studied. The reductive approach, in which operational parameters are either held constant or gently ramped, as is common in lab-scale trials indeed makes sense. A key reason for this is that in studies of such complex microbial communities as are found in anaerobic systems, extracting meaning from results of environmental and sequencing data analysis in even the simplest and most highly controlled studies is still difficult at present. The disparity between lab- and full-scale operating conditions however should not be ignored if lab-scale trials are intended to inform full-scale design. It is noted here that in recognition of the requirement to understand variable loading and operating conditions, the study on the three full-scale digesters was extended to a period of 1 year. Biomass samples were taken on a 3-weekly basis during periods of reactor operation to enable a unique long-term spatio-temporal study at full-scale. DNA from those samples has been used to create both a 16S rRNA library for the sample set and a metagenomic library to provide fuller insight into microbial community function in response to full-scale operating conditions. Results of this study are currently in preparation for publication however is not reported in the context of this thesis.

4.4.5.2 Solids Loading

Hydrolysis is frequently described as the rate-limiting step in anaerobic digestion (Noike, Endo et al. 1985) and solids loading is known to be particularly problematic in high-rate digesters such as the EGSB. In this study, this was confirmed in both the full- and lab-scale trials. In the lab-scale trials pCOD removal efficiency was lower than that for sCOD or COD removal and standard deviation for pCOD removal was high. The trend at full-scale was similar. At full-scale, mean pCOD removal efficiency was 49.8% as compared to 77.9% and 69.3% in the 1-D and 3-D reactor types respectively. The cause of this cannot be linked directly to scale however the full-scale digester was more highly and more variably loaded with solids in the influent. Further, whilst the mean pCOD removal was lower at full-scale, times series for the data for both of the lab-scale reactor types showed a declining trend in pCOD removal efficiency. This suggests that solids gradually accumulate in 'young' digesters, only to be released as the reactor ages and saturation of solids in the reactor liquor is reached. Whilst pCOD removal was lower in the full-scale digester, solids removal was more stable than in the lab-scale digesters as evidenced by lower standard deviation of pCOD removal efficiency. This suggests a better-adapted community for stable solids treatment may develop with higher solids loading as was applied at full-scale. This is supported by analysis of sequencing data which showed that the families *Veillonellaceae*, *Porphyromonadaceae* and *Armatimonadetes,* which are associated with carbohydrate degradation, were found to have significantly greater relative abundance in the full-scale digester than at lab-scale. The response of microbial communities to high-solids loading requires further study to ascertain upper limits for solids loading and to better understand community adaptation to high-solids applications. These issues are addressed in more detail in Chapter 6 of this thesis.

4.4.5.3 Dominance of the Hydrogenotrophic Pathway

The dominance of the acetotrophic pathway is a common assumption in AD in both natural and engineered settings. Increasingly however, reports are emerging in the literature of dominance of the hydrogenotrophic pathway particularly in relation to low-temperature engineered systems. Here, the full-scale digester, which was maintained in the mesophilic range for the duration of the three-month study of operating conditions, was found to be dominantly hydrogenotrophic both by SMA testing and by analysis of sequencing data. *Methanobacterium* was the dominant methanogenic family in the digester accounting for 17.0% of the total community. The dominance of that family was maintained at lab-scale but at a lower level: 14.4% and 13.0% for the 1-D and 3-D reactor types respectively,

which was reflected in decreased activity recorded using hydrogen at lab-scale. As operating conditions were not identical between the full- and lab-scale digesters the absolute cause of this trend cannot be ascertained. Putatively however, it is suggested that the more variable operation with less favourable feed types found at full-scale may influence the methanogenic pathway.

4.4.5.4 Zoned Activity and Microbial Community in 1-D Lab-Scale Reactor Types

The design of bioreactors adopted in lab-scale EGSB trials is highly varied. A key aim of this study was to ascertain the influence of two distinct lab-scale idealisations on both reactor performance and the microbial community. Analysis of performance data found that the 1-D lab-scale reactor type significantly out-performed the 3-D lab-scale reactor type with higher recorded COD and sCOD removal efficiencies, lower VFA accumulation and higher methane content in the biogas produced. Whilst these improvements were significant it is noted that in real terms the differences were small. Further differences were established between the two reactor types however that demonstrates that lab-scale idealisation significantly influences both microbial community function and distribution in reactors at lab-scale. SMA testing found that biomass at the bottom of the sludge-bed in the 1-D digesters was more active using both acetate and ethanol than biomass extracted from the top of the sludge bed. Although it is noted that the hydrogenotrophic pathway remained dominant throughout the 1-D reactors, it was seen that at the bottom of the 1-D reactors, this dominance was reduced and the activity profile more balanced. Zoned activity in the 1-D reactor types was linked with zoned microbial community composition by use of NMDS plots that showed distinct clustering of the OTUs with depth in the 1-D digesters, and by analysis of variance that attributed 15.6% of variance in the 1-D digester community to depth. Finally, 12 genus were identified whose relative abundance varied significantly with depth in the 1-D digester. Of those, two belonged to the family *Methanosarcinaceae,* the most metabolically varied family of the methanogens. *Methanosarcinales* were found to be most abundant at the bottom of the 1-D type reactors and proliferation there may be linked to the apparent shift in metabolic pathway measured in the biomass. No similar trend was found in the 3-D lab-scale reactor type. As such, lab-scale idealisation is found to influence microbial community composition, structure, and metabolic activity at lab-scale. The drivers for zoned activity and microbial community composition are investigated in further detail in Chapters 5 and 7 of this thesis.

4.5 Conclusions and Recommendations

The aim of this study was two-fold. Firstly, the work aimed to evaluate the influence of scale on reactor operation, performance and underlying microbial community. This aim was not fully realised. What can be concluded from this aspect of the work however is that the reality of full-scale reactor operation does not fit well with typical studies at lab-scale. If lab-scale studies are to inform full-scale reactor design, testing must reflect the varied conditions to which a full-scale reactor must adapt. The second aim was to ascertain whether lab-scale idealisation influenced performance, microbial community structure and spatial distribution at lab-scale. The results conclude that lab-scale idealisation strongly influences each of these. In order that scale-up of biotechnologies investigated in the laboratory is successful, the influence of lab-scale reactor design must be better understood. The lab-scale trials demonstrated the importance of adequate sampling of biomass not only to develop time series studies but also in relation to spatial distribution of communities that might be wrongly assumed to be homogeneous. Finally, the findings of this work particularly in relation to microbial community analysis highlights the advantages offered by NGS sequencing methods in enabling fine scaled sampling of microbial communities at lower cost as compared to conventional DNA sequencing methods.

5 Chapter 5: Microbial Community Adaptation with Substrate Type and Substrate Availability

Abstract

The high water and energy demands of sewage treatment methods in the Global North are such that technologies applied are unfit for direct transfer to the Global South, and, increasingly their application is unsustainable even in water and energy rich economies. As such, the development of sustainable sewage treatment alternatives is imperative. The EGSB is a high-rate anaerobic wastewater treatment method with the potential to couple wastewater treatment with renewable energy generation. Application of the EGSB to sewage treatment however is hindered by lack of understanding of the complex microbial community underpinning treatment in the system.

This study investigates the adaptation of a single seed sludge to the treatment of both low- and high-strength, high-solids wastes. Nine 20-litre 1-D lab-scale EGSB reactors were seeded using granular sludge from a full-scale EGSB. The reactors were operated in parallel as triplicate reactor sets which were used to treat one of; (i) industrial wastewater, (ii) low-strength industrial wastewater and (iii) high-strength synthetic sewage; at 37degC. COD removal efficiencies, VFA accumulation and methane production were used to assess reactor performance under each condition. Specific methanogenic activity assays were used to monitor community physiology, and whole community 16S rRNA gene (V4 region) amplicon sequencing was utilised to monitor microbial community dynamics under each substrate condition.

Good COD removal efficiency was determined in the treatment of both high- and low-strength industrial wastewaters and high-strength synthetic sewage (85.9%, 91.1% and 82.6% respectively). Treatment of synthetic sewage was found to result in reduced evenness and diversity in the microbial community and high solids loading was tentatively associated with hydrogenotrophic methanogenesis. Methanogenic activity in the sludge was found to be disproportionately depressed in the treatment of low-strength industrial waste as compared to the treatment of high-strength industrial waste. A core group of organisms belonging to three families; *Methanobacterium*, *Synergistaceae* and *Syntrophobacter*; were found to be relatively highly abundant under each substrate condition. OTUs belonging to the classes *Clostridia* and *Bacteriodetes* and the phylum *Spirochaetes* were identified as associated with the treatment of high-strength synthetic sewage.

5.1 Introduction

Today, sewage treatment presents itself as both a challenge and an opportunity in terms of sustainable infrastructure development in the Global North and South alike. In the Global South an estimated 2.5 billion people have no access to improved sanitation (UNDESA 2015) and a further 1.3 billion have no access to electricity (UNDP 2015). In the global North by contrast, sanitation provision is almost universal, as indeed are wastewater treatment and energy provision. It is estimated however that in the Global North as much as 5% of total electricity production is used in wastewater treatment (Logan, 2010) rendering current developed world solutions unsustainable in the long term and, therefore, unsuitable for direct transfer to the Global South. Biological treatment of municipal waste in the Global North is dominated by the application of aerobic treatments such as the activated sludge process (ASP) for the removal of dissolved and particulate organics (Orhon 2014). However, aeration required for treatment by technologies such as the ASP in a typical municipal wastewater treatment plant is estimated to consume over 50% of the total energy budget for the plant (McCarty, Bae et al. 2011). Thus for municipal waste treatment to become more sustainable then there is a need to drive industry away from energy intensive methods and to seek new more sustainable technologies for sewage treatment. The EGSB is a high-rate, anaerobic treatment system underpinned by the activity of a well-settling granular sludge (van Lier 2008). As an anaerobic treatment method, the energy requirements for aeration of waste are eliminated (Chong, Sen et al. 2012). Further, organics in wastewater are digested by a mixed microbial consortium to produce not only an improved waste stream but a renewable energy source (methane gas), and an effluent rich in scarce nutrients such as phosphorus and nitrogen that have economic value as a recyclable resource (Lettinga, van Velsen et al. 1981, O'Reilly, Lee et al. 2009). The EGSB is an increasingly popular technology for the treatment of high-strength, low-solids industrial wastes, however the technology remains unpopular as a direct treatment for high-solids effluents such as sewage (Aiyuk, Odonkor et al. 2010) due to low treatment efficiency of particulate materials (Chan, Chong et al. 2009).

In order that such a technology is adopted for sewage treatment globally, it must first be recognised that sewage itself differs drastically between the Global North and South (Aiyuk and Verstraete 2004). In the Global North, sewage collection systems rely on extensive networks of sewers to deliver sewage over many miles to treatment plants. This in turn relies on heavy water usage such that sewage is suitable for conveyance under gravity flow in pipe networks and, resultantly, Global North sewage tends to be a low-

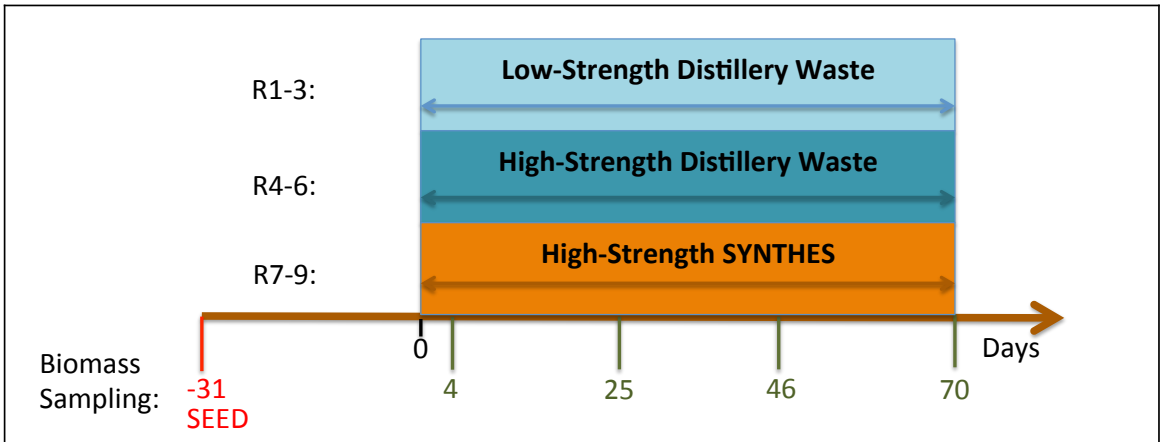
strength, high-solids waste (van Lier 2008). In the Global South on the other hand, in the absence of formal sanitation provision, many people are reliant on pit latrines for sewage collection with little or no facility for sewage treatment. As pit latrines are typically operated without a water supply, pit latrine waste is both a high-solids, and a high-strength waste. To satisfy treatment demands of both such wastes then, the EGSB must be adaptable to treatment of proportionately high-solids wastes at both low and high substrate concentrations. Recognising this, the aim of experiment conducted here is two-fold:

1. To determine the difference in performance of lab-scale EGSBs treating two distinct wastes types:
    a. Firstly, a high-solids, low-strength industrial waste approximately simulating the Global North low-energy sewage scenario,
    b. Secondly, a high-strength, high-solids synthetic sewage simulating Global South Sewage
    c. And each of these is assessed in contrast to the same system performance treating a 'traditional' industrial mid-strength waste-type.
2. To identify key microorganisms and microbial community response associated with each substrate condition applied.

It is noted here that further issues arising around the adaptation of sewage treatment are further investigated in two subsequent chapters (Chapters 6 & 7) in which problems specific to the Global North case and the Global South case are addressed in isolation.

5.2 Experimental Design

The experiment utilises nine lab-scale EGSB bioreactors to investigate performance and microbial community adaptation under three distinct feeding regimes. The lab-scale reactors were operated in parallel as three triplicate reactor sets with a single step-wise difference in feeding condition between each set (Figure 5-1). Each reactor used was of the 1-D lab-scale reactor type, constructed and operated as described in Chapter 4, and had a total working volume of 20L per reactor. Each of the nine reactors was seeded using sludge extracted from a full-scale mesophilic (37degC) EGSB operated at the North British Distillery (ADR2) for the treatment of spent water from the distillation of maize. The wastes used to simulate each condition were a dilute industrial wastewater, a full-strength industrial wastewater and a high-strength synthetic sewage. The industrial wastewater was collected on a weekly basis from the North British Distillery as described previously.



Parameter	Measurement / Sampling Frequency Lab-Scale
Specific Methanogenic Activity	Seed sludge and end of run
COD and sCOD, influent and effluent	Bi-weekly
pH influent and effluent	Bi-weekly
Biogas production	Daily
Methane content in biogas	Bi-weekly
Temperature	Daily in reactor
Volatile Fatty Acids	Bi-weekly, 8 VFAs from C2 to C6, EtOH
Biomass samples	4 Sample times at 5 depths in R1-3 and R4-6 and at top and bottom of the sludge bed in R7-9 over 70 day period as shown

Figure 5-1: Schematic showing experiment duration, relative timings of biomass sampling and summary of physico-chemical monitoring parameters for lab-scale triplicate bioreactor sets. Reactor sets are R1-3 treating low-strength distillery waste, R4-6 treating full-strength distillery waste and R7-9 on high-strength distillery waste.

The reactor set R4-6, treating full-strength industrial waste, was intended to act as an overall 'control' for the other two reactor sets. The logic supporting this is that the microbial community in that reactor set was well-established in the treatment of distillery waste prior to commencement of the study thus, microbial community adaptation in this reactor set should give 'background' information on community adaptation to the change of scale in going to lab set-ups; as distinct from adaptation to feeding condition. To enable the use of those reactors processing the full-strength distillery wastewater as a control, the design operating parameters adopted across the experiment were based on the operating parameters applied at the Distillery for the 3-month period prior to commencement of the lab-scale study thus minimising adaptation to the lab-scale set-up. Table 5-1 details the design operating parameters for each reactor set.

Table 5-1: Design operating parameters for each of the lab-scale triplicate bioreactor sets: R1-3 treating low-strength distillery waste, R4-6 treating full-strength distillery waste and R7-9 on high-strength distillery waste

Parameter	**Design Value or Condition**		
	R1-3	**R4-6**	**R7-9**
HRT (h)	16	16	16
Upflow Velocity (m/h)	3.5	3.5	3.5
Influent pH	6	6	6
Temperature (degC)	37	37	37
Feed-Type	Low-strength distillery waste	Full-strength distillery waste	SYNTHES
Feed-Strength (mg COD / L)	500	5000	5000

5.3 Materials and Methods

The following section details those materials and methods specific to this Chapter. Further, a summary of methods applied in this chapter but described in full in the Materials and Methods chapter.

5.3.1 Substrate Preparation and Feeding

The feed-type described as full-strength distillery waste was collected on a weekly basis from the North British Distillery as described previously and stored in airtight IBC containers until used. The distillery wastewater was neutralised prior to use to ~pH 6 by dosing with a low-grade caustic and supplemented using a trace minerals solution (Table 5-2) as was applied in the full-scale digester ADR2. The feed-type described as low-strength distillery waste was made by 10x dilution of the full-strength distillery wastewater using tap water to dilute the waste. The low-strength waste was similarly treated with caustic for pH correction and trace nutrients were supplied at the same concentrations as were applied to the full-strength waste. The feed-type described as high-strength SYNTHES was prepared in the laboratory as per Aiyuk et al (Aiyuk and Verstraete 2004) using tap water as a solute. SYNTHES was applied to the reactors at COD of 5000mg/L similarly to that of the full-strength distillery waste and mimicking a dilute pit-latrine waste. For each feed type, in-tank mixing was used to promote solids delivery to the reactors and minimise variation in feed composition for the duration of feeding from a single tank.

Table 5-2: Trace mineral solution for low and high-strength distillery waste

Micronutrient	Dosing Concentration (g/m3)
FeSO4 7H2O	6.042
CoCl2 6H2O	0.015
CuCl2 2H2O	0.051
MnSO4 H2O	0.053
NiSO4 6H2O	0.080
Al2(SO4)3 18H2O	0.277
ZnCl2	0.047
Na2SeO3 5H2O	0.003

5.3.2 Physical and Chemical Monitoring

All methods are described in detail in Chapter 3 and a brief overview is repeated here. Sampling of both influent and effluent for each reactor was performed on a bi-weekly basis for the duration of the 70-day experiment and was subject to a range of monitoring methods (Figure 5-1) as follows. Total and soluble COD was measured using the Closed Reflux, Colorimetric Method, Standard Methods 5220D. Influent and effluent pH was measured using a digital meter and probe (Thermoscientific Meter, Eutech Instruments Probe). Volatile fatty acids (VFAs, C_2–C_6, including iso-forms of C_4–C_6) and ethanol concentrations were measured using a gas chromatograph (7890A, Agilent, Palo Alto, CA) equipped with a DB-FFAP capillary column and a flame ionization detector.

Biogas production rate was recorded on a daily basis using 20L volume rubber gasbags attached to each reactor gas line to collect the biogas produced for timed periods of approximately 2 hours for high COD feed and12 hours for low COD feed. Biogas volume was quantitated using a gas-tight syringe to empty gasbags of the collected volume and biogas production rates was calculated as volume produced per unit time. Methane content in the biogas was quantified on a biweekly basis to coincide with influent and effluent monitoring. Methane content was measured using a gas chromatograph (7890A, Agilent, Palo Alto, CA) equipped with a GS-Carbon Plot capillary column and a flame ionization detector. Reactor temperature was measured on a daily basis using digital meter and probe inserted into the reactor liquor.

5.3.3 Physiological Monitoring: Specific Methanogenic Activity Testing

Specific methanogenic activity was measured on a single sample of the seed sludge at d-31 representing the starting activity in the biomass for each of the triplicate reactor sets. SMA

testing was additionally conducted at the end of the 70-day trial to determine the effect of each substrate on underlying microbial activity. The SMA tests at the end of the trial were conducted by reactor set i.e. biomass was extracted from the top and bottom of the sludge bed from each reactor in a set and pooled to make a two samples representing each feeding condition; one from the top and one from the bottom of the sludge-bed. Sub-samples of each pool were incubated in sealed serum vials at mean reactor temperature (37degC) with a specific substrate and methanogenic activity monitored using the pressure transducer method. Substrates tested were acetate (Ac_C2), propionate (Pr_C3), butyrate (Bu_C4), ethanol (Et_OH) and Hydrogen (H2).

5.3.4 Biomass Sampling and Storage

Biomass was sampled at each port in the sludge bed on days 4, 21, 46 and 70 for reactors R4-6 treating full-strength distillery wastewater and on days 4, 21, 46 and 67 for reactors R1-3 treating low-strength distillery wastewater, and R7-9 treating high-strength SYNTHES. Biomass samples were taken and stored according the method outlined in Chapter 3.

5.3.5 NGS Library Preparation

All biomass samples were stored at -20degC until the end of the experiment. DNA extraction and purification was conducted using the Fast DNA Spin Kit for Soils (MP Biomedical). Extracted DNA was quantified using the Broad-Range Qubit Assay (Life Technologies) and stored at -20degC until used in NGS library preparation. For reactors R1-3 and R4-6, library construction was applied to DNA samples from each port in the sludge bed. For reactors R7-9, DNA samples extracted from sludge at the bottom two ports and top two ports in the sludge bed were combined to make a single ‘bottom’ and ‘top’ sample for each reactor at each time point. PCR amplification of the V4 region of the 16S rRNA gene using Golay barcoded primers (Caporaso, Lauber et al. 2012) with an additional degeneracy on the forward primer for improved detection of Archaea was conducted on each DNA sample in the sample set. PCR product was gel-purified (Zymoclean Gel DNA Recovery Kit) and quantified prior to sequencing using the High-Sensitivity Qubit Assay (Life Technologies). The pooled multiplexed library, normalised to 5ng/ul DNA, was sequenced using the Illumina Miseq bench-top sequencer. Arising sequencing was de-multiplexed at the sequencing centre and all other quality control methods applied to the sequencing data are described in detail in Chapter 3.

5.3.6 Qualitative and Statistical Analysis Methods

Data pertaining to operation and performance of the reactors at both full- and lab-scale is summarised for the 70-day time period of the experiment to provide mean values for each parameter recorded. Standard deviation is presented alongside minimum and maximum values in each data set to enable qualitative assessment of stability of both reactor operation and performance. Where important trends are identified in the data, results are presented graphically, again to enable qualitative assessment of the conditions in the reactors. Statistical evaluation of difference in operation and performance data with feed type is conducted using ANOVA (aov(), R software) and P-values for significance stated. Where performance indicators are reported as percentage efficiency direct comparison is possible hence ANOVA was conducted across each of the three sets. Where performance data is reported as absolute values e.g. biogas production rates and TVFA in the reactor effluent, ANOVA was conducted between the reactor set of interest and the control reactor set only. Correlation analysis was additionally applied to the environmental metadata set (corr(), R, library corrplot) to identify correlations between operating parameters and performance across the data set.

Low-read count samples were excluded from analysis of sequencing data with a cut-off threshold of 5000 reads per sample. To account for differences in sequencing depth between remaining samples, sequencing data was normalised by calculation of relative abundance for each OTU or taxa in the sample. For calculation of ecological indices, each sample was rarefied to a common minimum, which was the minimum read count sample for the whole sample set for the experiment. Ecological indices calculated were rarefied richness, Simpsons Index of Diversity and Pielous Evenness Index and each was calculated using the R-Vegan software package. Phylogenetic distances between samples were calculated using a QIIME based phylogeny file in the Phyloseq package in R with taxonomic identification of OTUs conducted by sequence comparison with the RDP Classifier database. A qualitative analysis of community membership under each substrate condition is provided for those OTUs in the most abundant 100 OTUs identified in the sample set whose abundances appear to be distinct according to feed-type. Two-dimensional NMDS plots and supporting multivariate analysis of variance were conducted using two alternative distance metrics to describe the sequencing data. Bray-Curtis similarity matrices were utilised to determine variance based on percentage abundances in the count data. Secondly, phylogenetic distances were obtained using the generalised Unifrac method, alpha=0.5, which calculates distances based on difference in branch

lengths of the phylogenetic trees between samples. Both methods were conducted using the Phyloseq package in R. Finally, correlation analysis coupled with Kruskal-Wallis test for non-parametric data (with Benjamini-Hochberg correction for multiple tests applied on the P-values to obtain adjusted P-values) was conducted to determine the significance of differences arising from the qualitative assessment of variance between reactor communities.

5.4 Results and Discussion

The results section is divided into four components. The first presents data pertaining to reactor operation and the physico-chemical characteristics of reactor input. The second presents results and analysis of reactor performance under each of the feeding conditions. The third presents the results of SMA testing on biomass after adaptation to each substrate type. Finally, a qualitative overview of phylogeny under each substrate type is presented and key taxa whose relative abundances show significant variation with both substrate type and reactor performance are identified. Results are presented with some initial discussion and a full discussion follows subsequently in Section 1.5.

5.4.1 Reactor Operation

5.4.1.1 Summary Operating Statistics Within Sample Sets

Reactor operation here is defined as the input parameters for the lab-scale reactors. Input parameters monitored were operating temperature, and substrate characteristics; pH, COD profile and VFA profile. At lab-scale, each reactor in a triplicate set was fed from a single feed tank therefore substrate characteristics are assumed to be identical for each reactor in that set. Temperature at lab-scale was controlled on a per-reactor basis and as such, recorded temperatures were compared within each reactor set using ANOVA such that the degree of replication for this parameter can be determined. Tables 5-3 to 5-5 below show the summary statistics for operation of the low- and high-strength reactors sets, and the SYNTHES reactor set.

Table 5-3: Summary statistics of operational data collected for R1-3, Low-Strength Distillery Waste

	Influent pH	Temperature / degC			CODin / mg / L	sCODin / mg / L	pCODin / mg / L
		R1	R2	R3			
Mean	6.3	36.9	37.3	37.0	966	511	455
Minimum	5.6	35.4	36.7	35.5	305	63	45
Maximum	9.2	38.4	38.4	38.0	2277	1093	1883
St. Dev	0.7	0.6	0.4	0.5	537	188	542

Table 5-4: Summary statistics of operational data collected for R4-6, Full-Strength Distillery Waste

	Influent pH	Temperature / degC			CODin / mg / L	sCODin / mg / L	pCODin / mg / L
		R4	R5	R6			
Mean	6.1	37.2	37.1	37.2	5010	3555	1455
Minimum	5.6	36.1	35.6	36.6	3729	2589	202
Maximum	6.6	38.3	37.9	37.6	10453	5112	6000
St. Dev	0.3	0.6	0.5	0.3	1498	621	1245

Table 5-5: Summary statistics of operational data collected for R7-9, SYNTHES

	Influent pH	Temperature / degC			CODin / mg / L	sCODin / mg / L	pCODin / mg / L
		R7	R8	R9			
Mean	6.5	37.3	37.5	37.1	5066	3102	1963
Minimum	4.5	36.3	36.4	35.2	2957	2480	295
Maximum	8.4	39.1	40.6	40.1	8999	3967	6068
St. Dev	1.1	0.6	0.9	1.2	2056	450	1961

(Temperature is presented on by-reactor basis, all other parameters reported are common to across the triplicate bioreactor set.)

ANOVA demonstrates that the difference in operating temperature seen in R1-3 is significant ($P<0.05$) however in real terms the difference in each of the mean, standard deviation and range is small (<1degC). No significant difference was found between operating temperatures for either the reactor set R4-6 or R7-9. As such, qualitatively each of the three reactor sets are thought to have been operated well as replicates within each set for the 70 day duration of the experiment.

5.4.1.2 Reactor Operation: Comparison Between Sample Sets

ANOVA was additionally applied to data between sample sets to determine the degree of variation in operation between each alternative feed-type and the control reactor set. No significant difference was found for operating temperature between reactor sets such that substrate conditions were the sole difference in operation. Mean COD in the influent for the low-strength reactors R1-3 is seen to be approximately one fifth that of the full-strength and SYNTHES reactors which is higher than anticipated by dilution rates. The suspected reason for this is that in spite of the use of in-tank maxing for reactor feed, solids components of the waste tended to settle rapidly in the tanks such that the portion of waste that was diluted to prepare the low-strength distillery feed was of higher strength than the mean for the tank. This is confirmed by the observation that proportionately, the low-strength distillery feed was higher in solids than the full-strength feed. The full-strength distillery waste and SYNTHES mean influent COD is similar in magnitude and it is noted that the particulate fraction is 39% for SYNTHES as compared to only 29% for the distillery waste. Thus, SYNTHES does represent a high-solids waste for this reactor type and is therefore a suitable substrate for determination of microorganisms associated with

high solids loading. The percentage of solids in the low-strength distillery waste is the highest at 47% of total COD however in real terms, the amount of solids delivered to those reactors is substantially lower than that for each of the other two reactor sets.

5.4.1.3 VFA Profile in Reactor Influent by Substrate Type

Figures 5-2 to 5-4 show influent VFA profiles as a time series for the duration of the experiment for each reactor set.

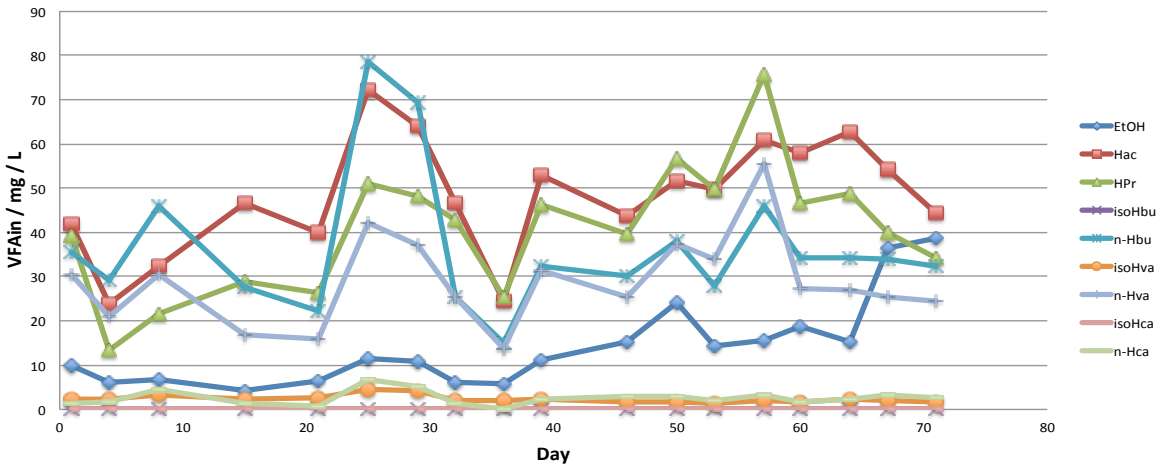


Figure 5-2: Scatter plot showing time-series of VFA profile of low-strength distillery wastewater

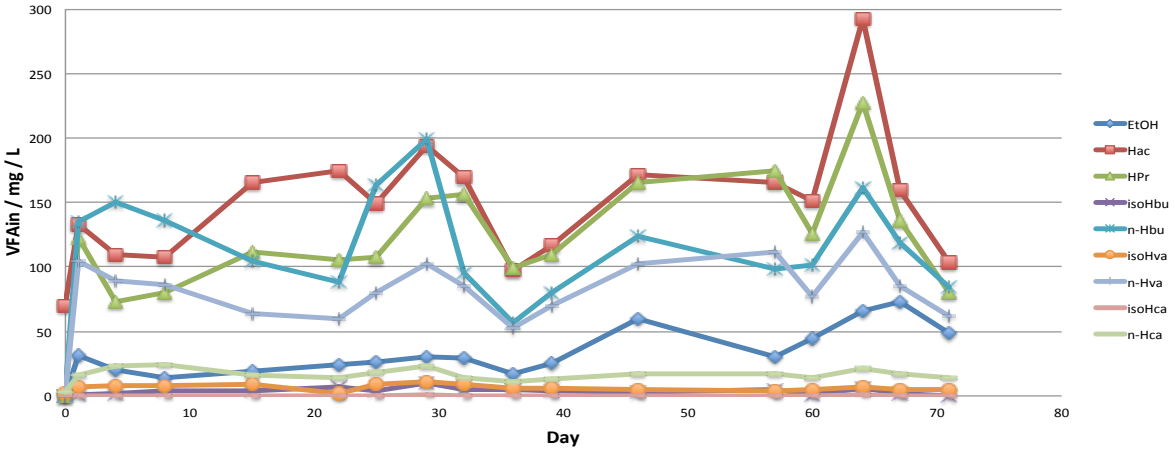


Figure 5-3: Scatter plot showing time-series of VFA profile of full-strength distillery wastewater

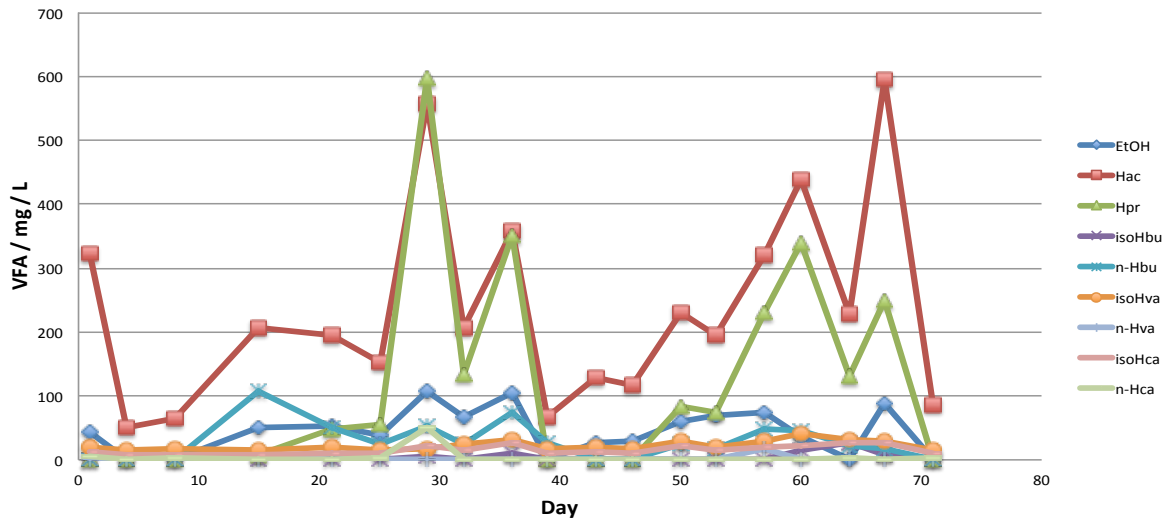


Figure 5-4: Scatter plot showing time-series of VFA profile of SYNTHES

(In each of Figures 5-2 to 5-4, VFAs measured are: ethanol (EtOH), acetate (Hac), propionate (Hpr), isobutyrate (isoHbu), butyrate (n-Hbu), isovalerate (isoHva), valerate (n-Hva), isocaproate (isoHca), caproate (n-Hca)).

Inspection of the graphs for low- and full-strength distillery wastes shows that whilst VFA concentrations are somewhat variable, the relative proportions of each VFA in the waste tends to remain reasonably constant. The VFA profile for SYNTHES by contrast is highly variable in terms of both the relative proportions of the component VFAs and the absolute quantities delivered to the reactors. Two notable spikes are seen in the delivery of acetate and propionate between days d25 and d39, and days d57 to d68 which is contrary to what was expected from the delivery of a synthetic waste in which substrate composition is more highly controlled than in a 'real' waste. To extrapolate the cause of these spikes, COD data for the reactor set R7-9 treating SYNTHES is plotted (primary vertical axis) along with pH data for the reactor set (secondary vertical axis) in Figure 5-5). Qualitatively sCOD data, which would be anticipated to reflect VFA composition, remains relatively stable during the time points for which VFA was seen to peak in the SYNTHES reactor influent (green areas on plot). COD data at those time points by contrast show increasing trends for both COD and pCOD however solids loading would not be anticipated to impact VFA concentrations. Further, during those same time periods no clear trend is observable with pH and the increase in VFA suggesting that whilst acetate may have been more concentrated in reactor feed at various time points, buffering of the reactor feed was adequate to prevent pH spiking also.

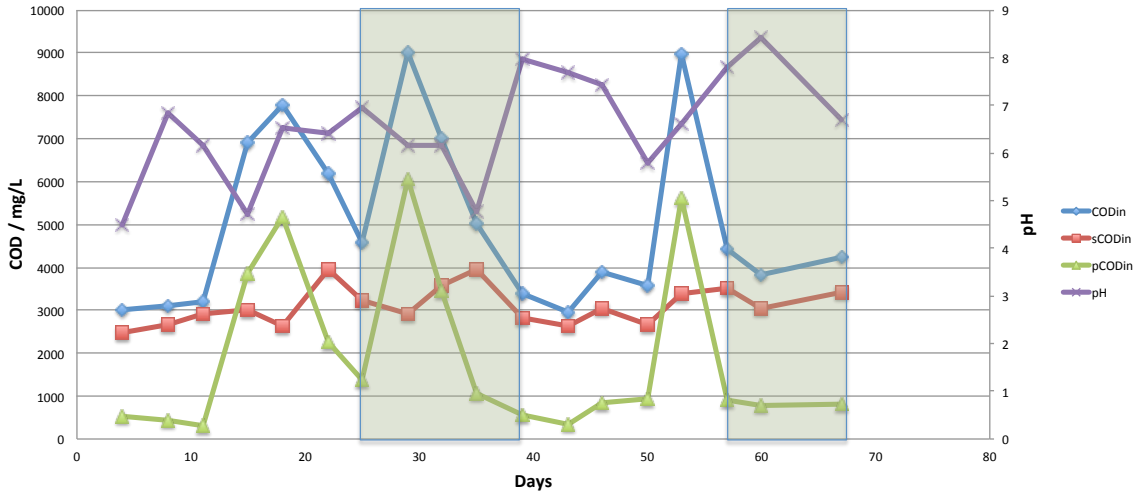


Figure 5-5: Scatterplot showing COD profile in SYNTHES (R7-9 influent) as a time series. Green boxes overlaid on scatterplot indicate time periods of peak VFA concentration in the influent SYNTHES.

What can be determined from the plot is that in spite of carefully controlled and consistent preparation of SYNTHES, that COD and pCOD composition in the reactor influent was highly variable indicating inadequate mixing in the feed. By extrapolation, it may be possible that inadequate mixing could have additionally contributed to the variable VFA delivery by enabling separation of VFA fractions from the bulk liquid by density however the absolute cause of the spikes in VFA remains unknown. As each of the three reactors in the triplicate set R7-9 treating SYNTHES were fed from a single feed tank for the duration of the study, it may be concluded that whilst the SYNTHES feeding regime was less stable than anticipated, that the instability will have affected each reactor in the set such that replication in terms of operation of the reactor set is not impacted.

5.4.2 Reactor Performance

5.4.2.1 Reactor Performance: Summary Statistics with Reactor Sets

Reactor performance indicators used were COD removal efficiencies, effluent pH, TVFA accumulation, biogas production rates and methane content in the biogas. Tables 5-7 to 5-9 show summary data for performance for each reactor set across the 70-day period of operation. One-way analysis of variance was used to determine the degree of replication of performance between reactors within each triplicate set. Where no significant difference was found for a specific parameter, then mean is reported for the reactor set.

Table 5-6: Summary of data describing performance of reactor set R1-3 treating Low-Strength Distillery Waste

	Effluent pH	TVFA / mg / L	CODrem / %	sCODrem / %	pCODrem / %	BPR / L/Lrxr.d	CH4 / %		
							R1	R2	R3
Mean	6.91	0.8	85.9	83.9	77.9	0.353	66.9	71.6	72.2
Minimum	6.51	0.0	57.6	10.5	-111.8	0.090	44.0	49.5	51.5
Maximum	7.51	17.7	97.7	96.1	110.1	0.571	78.9	82.0	82.4
St. Dev	0.27	2.7	8.6	17.1	36.3	0.102	9.0	6.9	7.3

Table 5-7: Summary of data describing performance of reactor set R4-6 treating Full-Strength Distillery Waste

	pH	TVFA / mg /L			CODrem / %	sCODrem / %	pCODrem / %	BPR / L/Lrxr.d	CH4 / %
		R4	R5	R6					
Average	7.15	25.7	5.4	6.9	91.1	93.7	77.9	2.95	74.5
Minimum	6.78	12.8	0.0	0.0	67.3	84.9	-48.5	0.51	61.6
Maximum	8.17	73.7	12.8	48.8	96.4	97.4	125.7	7.87	82.8
St.Dev	0.24	14.9	3.6	8.4	5.1	2.3	28.3	0.91	3.5

Table 5-8: Summary of data describing performance of reactor set R7-9 treating SYNTHES

	Effluent pH	TVFA / mg / L	CODrem / %	sCODrem / %	pCODrem / %	BPR / L/Lrxr.d	CH4 / %
Mean	7.74	33.2	82.6	92.1	39.5	2.12	78.2
Minimum	7.22	18.1	27.7	78.4	-344.8	1.14	59.9
Maximum	8.23	167.9	94.5	97.3	95.5	6.33	84.2
St. Dev	0.25	27.4	11.6	2.8	77.6	0.98	4.9

(Data describing performance of triplicate bioreactor sets including effluent pH, total volatile fatty acids as an acetate equivalent, COD profiles, biogas production rate and methane content in the biogas. Data are presented as a mean for the reactor set where no significant difference was determined between replicates and individually for each reactor in cases where a significant difference was found (ANOVA, $p<0.05$).)

Methane content in the biogas was found to vary significantly between the reactors in the set R1-3 treating low-strength waste ($P<0.05$) whilst no significant difference was found between any other performance indicator for that reactor set. As COD removal rates were similar, the likeliest cause of the variance in methane content in the biogas in R1-3 arises from reactor configuration whereby maintenance on R1 was more frequent than on the other two reactors in the set due to slight leakage of the reactor water jacket. TVFA accumulation in the reactor set R4-6 treating full-strength distillery waste was found to vary significantly ($P<0.001$) whilst significant difference was not found on other performance parameters. The cause of variance in TVFA for this reactor set is not known at this point. No performance indicator was found to vary for the reactor set R7-9. Qualitatively, variance on one or fewer of the performance parameters tested for each reactor set was thought to indicate good replication of performance within each set. Further, each reactor set performed well and stably throughout the study in terms of COD removal and methane production rates.

5.4.2.2 Reactor Performance: Comparison Between Reactor Sets

ANOVA was additionally conducted for each parameter tested to determine difference in performance *between* reactor sets. Reactor pH was found to be significantly different between the reactor sets with mean low-strength reactor pH of 6.91 and mean SYNTHES reactor pH at a mean of 7.74 which is the highest recorded for any of the three reactor sets. To some extent this is counterintuitive in that TVFA accumulation in the SYNTHES reactors is seen to be lowest in the low-strength reactors, and, VFA accumulation is often the cause of low pH in EGSB type reactors. The likeliest cause of elevated pH in the SYNTHES reactor is the production of ammonia from peptone in the reactor, which is known to occur under high-solids loading (Kayhanian 1994). Optimum pH for methanogenic communities is typically assumed to be between pH 6-8. The SYNTHES reactors however operated stably throughout even though maximum pH was as high as 8.23. Speculatively, this suggests that a pH gradient may occur in the granule such that methanogens experienced a lower pH than found in the reactor liquor, or that sensitivity to pH at the upper rather is less than at the lower end of the empirically derived limits.

COD removal efficiencies were noted to be significantly lower for both the low-strength reactors and the SYNTHES reactors than for those treating full-strength waste ($P<0.001$) however when sCOD and pCOD removal efficiencies are considered, no clear trend was established. Qualitatively, with the exception of pCOD removal in the SYNTHES reactors, COD removal efficiencies are high and the reactors performed well (>70% removal in each case). In the case of pCOD removal in the SYNTHES reactors, the low removal rate (39.5%) demonstrates that solids removal in EGSB type reactors is poor, even after a 70-day acclimation to high-solids feed.

Qualitative assessment of biogas production rates in the low-strength reactors shows that whilst COD in the substrate delivered was one fifth that delivered to the full-strength reactors, biogas production rates for the low-strength reactors was closer to one tenth that produced by the full-strength reactors. This suggests that low-strength feed disproportionately impacts biogas production rates. It was noted previously however that the low-strength feed contained proportionately higher solids load as compared to the full-strength feed. As solids removal efficiencies were lowest for each substrate condition, it might be implied that the low biogas production rates in the low-strength reactors arises from delivery of less readily degradable substrate. ANOVA was conducted between reactor the low- and full-strength reactor sets for methane content in the biogas and demonstrated that biogas from the low-strength reactors R1-3 had a significantly lower

proportion of methane in the biogas (P<0.001) than that of the full-strength reactors R4-6. This again indicates that low substrate availability may metabolically alter the microbial community activity in a way that is unfavourable to methane production. ANOVA between the full-strength and SYNTHES fed reactors reveals that the SYNTHES reactors produced a significantly lower volume of biogas (P<0.001) suggesting that high solids loading might also negatively impact metabolism in methanogenic communities. ANOVA between the SYNTHES and full-strength reactors however demonstrated that the SYNTHES reactors produced significantly higher methane content in the biogas than the full-strength reactors (P<0.001). Expressing biogas production rate coupled with methane content in the biogas as methane production rate however demonstrates that the full-strength reactors produce 2.19 LCH4 / Lrxr / d as compared to only 1.66 LCH4 / Lrxr / d. In summary then, whilst COD removal efficiencies are high under low substrate availability (R1-3), biogas production volumes and methane content in the biogas are disproportionately low. Further, whilst COD removal efficiencies are similar in the treatment of wastes of similar strength (R4-6 and R7-9), methane production rates appear to be impinged under high solids loading (R7-9).

5.4.2.3 Correlation Analysis of Operating Data with Performance Data

Results from each reactor set were combined to a single data set to enable generalised analysis of correlation between operating and performance data (Figure 5-6). It was found that as control parameters COD, sCOD and pCOD increase so too does the effluent pH. This again is thought to point to ammonia production under higher loading rates generally and in particular under high-solids loading rates. Increase of the same parameters is also strongly correlated with VFA accumulation particularly for acetate, isovaleric acid, isocaprionic acid and caprionic acid. As ammonia is inhibitory to methanogens at high concentrations, the likely ammonia increase under solids loading may be the causative factor for increased acetate in reactor effluent. From the correlation analysis it is also seen that whilst an increase in sCOD in reactor substrate is associated largely with increased acetate accumulation in the reactor effluent, an increase in pCOD is associated with increased levels of acetate, isovaleric acid, isocaprionic acid and caprionic acid. This trend is consistent with the literature, which suggests that high soluble wastes can put pressure on methanogenic communities therefore resulting in the presence of methanogenesis precursors such as acetate in the reactor effluent. By contrast, high-solids wastes tends to put pressure on organisms higher in the methanogenic ‘food-chain’ resulting in the release of partially degraded solids in the form of longer chain acids, and as suggested previously, ammonia (Kayhanian 1994).

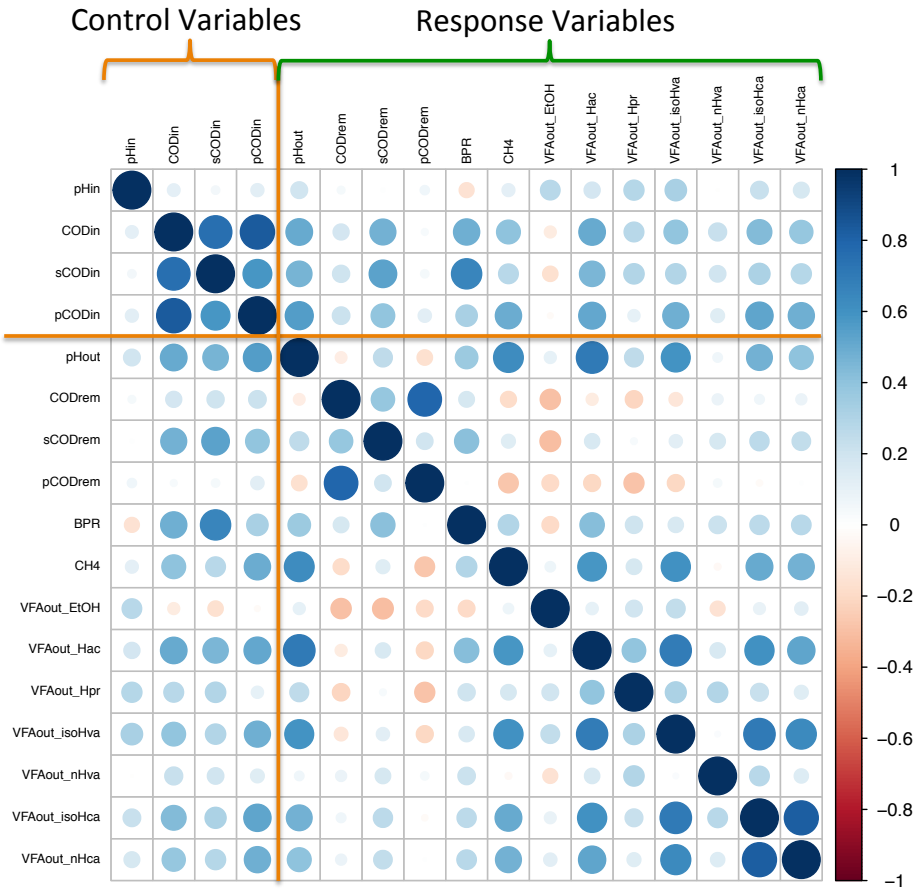


Figure 5-6: Correlogram showing Kendall correlation computed from combined meta data from each of the triplicate reactor sets R1-3, R4-6 and R7-9. Depth of colour and size of circle are proportional to the correlation coefficients i.e. indicate strength of correlation between parameters. Parameters are compartmentalised in the visualisation only to indicate those variables under direct control of reactor operator as opposed to response variables.

It is noted however that increases in each of COD, sCOD and pCOD are correlated positively with biogas production, methane content in the biogas and COD removal efficiencies which suggests that whilst VFA accumulation was observed to occur, and ammonia may have accumulated in the SYNTHES reactors, each reactor set may be described as functioning well and stably during the 70-day trial.

5.4.3 Community Physiology: SMA

Specific methanogenic activity was measured for both the seed sludge at the experiment offset and for representative samples of sludge from the top and bottom of the sludge-bed in each reactor set at the end of the 70-day trial (Figure 5-7).

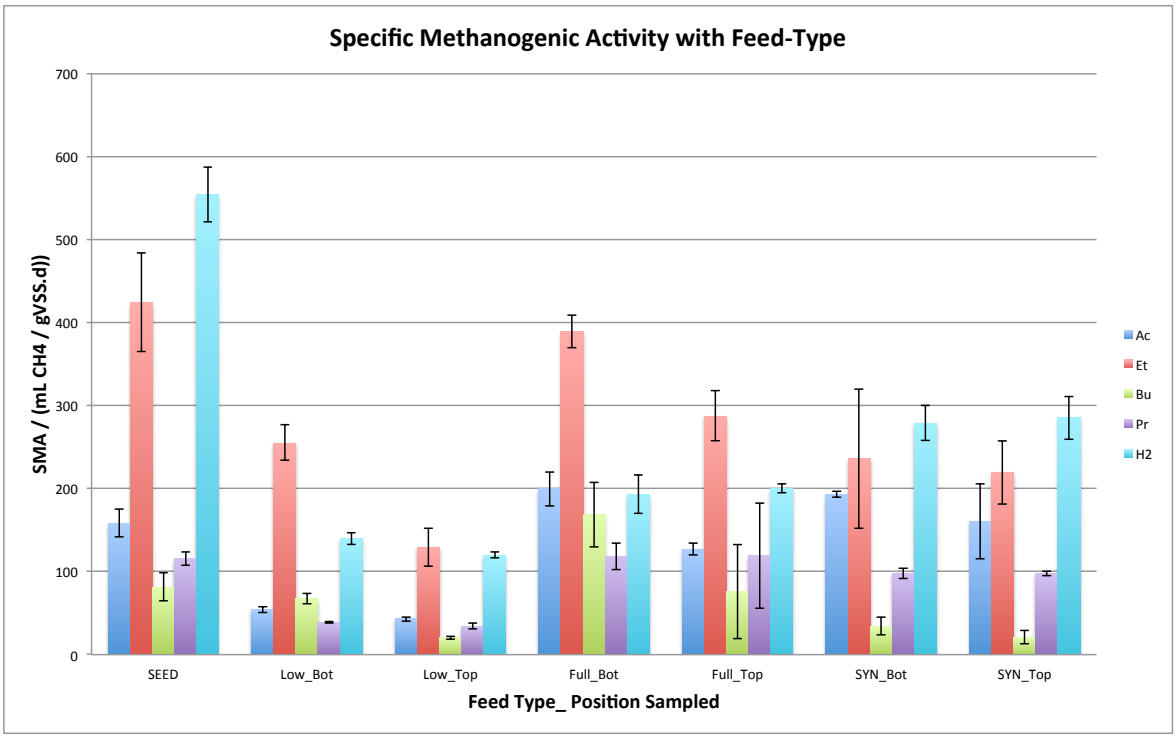


Figure 5-7: Grouped bar plot showing SMA recorded in biomass at the offset of the reactor trials (SEED) and at the top (_Top) and bottom (_Bot) of the sludge bed end of the trial for each of the low strength distillery waste (Low), high strength distillery waste (Full) and SYNTHES (SYN) fed reactor sets. SMA was measured against specific substrates: acetate (Ac), propionate (Pr), butyrate (Bu), Ethanol (Et) and hydrogen (H2). Bars show blank-adjusted mean and error bars show standard deviation of triplicate measurements.

It was established previously that treating full-strength distillery wastewater in 1-D lab-scale reactor types resulted in gradated activity with depth, with significantly higher activity recorded using substrates acetate and ethanol ($P<0.001$) at the bottom of the reactor (Chapter 4). ANOVA was applied to data collected here to determine whether feed-type influenced the activity profile with depth for each of the alternate feed-types applied. For the low-strength distillery waste reactor set, activity was found to be significantly higher at the bottom of the reactor for each of the substrates tested ($P<0.05$). For the SYNTHES reactor set by contrast, no significant difference in activity was found for any of the substrates tested between the top and bottom of the sludge bed. Thus, feed type is found to influence the activity profile in 1-D lab-scale reactors. The increased variation of activity with depth in the low-strength reactor set as compared to the full-strength reactor set might suggest that diminished gas production resulting from low substrate availability has resulted in decreased mixing in the reactor and hence increased stratification in the sludge-bed. However, biogas production rates were lower in the SYNTHES reactors than in the full-strength reactor set and no variation in activity profile was seen in the SYNTHES reactors. This suggests that mixing via gas production, or lack thereof, cannot be the sole cause of activity stratification and points to more complex interactions between activity in the biomass, reactor-type and feed-type.

ANOVA was additionally applied to determine whether significant differences in activity were found between either the low- and high-strength distillery reactor sets or between the high-strength distillery and SYNTHES reactor sets. It was determined that activity was significantly higher ($P<0.05$) for each substrate tested in the full-strength distillery waste reactor set as compared to the low-strength set. Whilst intuitively decreased productivity as a response to decreased substrate availability makes sense, the mechanism by which this occurs is at present unknown. Speculatively, microbial community composition may have shifted to include larger numbers of organisms with lower metabolic rates, or alternatively it might be implied that the same or similar community is present but is functioning at depressed rates. Fuller analysis of this finding is given in relation to the sequencing data results to follow. Between the full-strength and SYNTHES reactor sets, it is seen that activity using substrates ethanol and butyrate is significantly higher ($P<0.05$) in the full-strength distillery reactors whilst activity using hydrogen / CO_2 is significantly higher ($P<0.001$) in the SYNTHES reactors. Increased activity using substrates ethanol and butyrate in the full-strength distillery reactor set may reflect influent substrate concentrations given that concentrations of both were higher in the reactor feed than in the SYNTHES reactors. Higher activity using hydrogen in the SYNTHES reactor set than in the full-strength distillery reactors on the other hand might reflect the higher solids composition in the reactor feed. Previously it was demonstrated the hydrogenotrophic pathway was dominant in the full-scale digester from which the seed sludge was drawn both in terms of sequencing data on the biomass and SMA data (Chapter 4). This tendency was tentatively linked to selective pressure on acetotrophic methanogens induced by higher solids loading. That the SYNTHES reactors, which were subject to the highest solids loading in this case, show the same trend strengthens the case that pressure on the community induced by high solids loading promotes hydrogenotrophic methanogenesis.

5.4.4 Analysis of Microbial Community Using 16S rRNA NGS Results

Library construction for sequencing consisted of 129 unique barcoded samples with 6 additional samples as triplicate positive and negative controls. Across each of the 129 samples, 3014 unique OTUs were identified at 97% similarity from a total of 7699168 reads excluding control samples. The range of reads per sample was 3872 to 453230 with a median read count of 35285 reads. Six samples had total read counts below 5000 and are listed in Table 5-9.

Table 5-9: Low Read Count Samples

Sample Name	Read Count	Reactor	Port	Day
S125	3872	R6	P2	d46
S23	3886	R6	P1	d25
S86	4031	R9	P1	d4
S161	4432	R2	P1	d67
S88	4527	R9	P3	d4
S84	4793	R8	P3	d4

As the reactors were operated in triplicate, and the low read count samples reasonably distributed between reactors, the low read count samples were removed from further analysis such that 123 samples were included subsequently. Of those samples included in the analysis, the minimum read count sample identified 374 OTUs whilst the maximum read count sample identified 1479 OTUs. Rarefaction curves plotted using R-Vegan demonstrated that saturation was not reached, even for maximum read count sample indicating a highly diverse microbial community. As such, two data normalisation methods were applied in the following analysis: rarefaction and species relative abundance as described previously.

5.4.4.1 Qualitative Overview of Microbial Community with Substrate Type

The sequencing data for the entire sample set identifies 3014 unique OTUs across 28 phyla, 63 classes, 96 orders, 185 families and 404 genus such that the microbial community underpinning treatment in the reactors may be described as highly diverse. NMDS plots were used to investigate clustering of the microbial community associated with each feeding condition using data for the 100 most abundant OTUs in the sample set. The plots (Figures 5-8 and 5-9) utilised two distinct distance measures to describe the sequencing data; Bray-Curtis distance, and phylogenetic distance (GUnifrac, alpha=0.5, Phyloseq R).

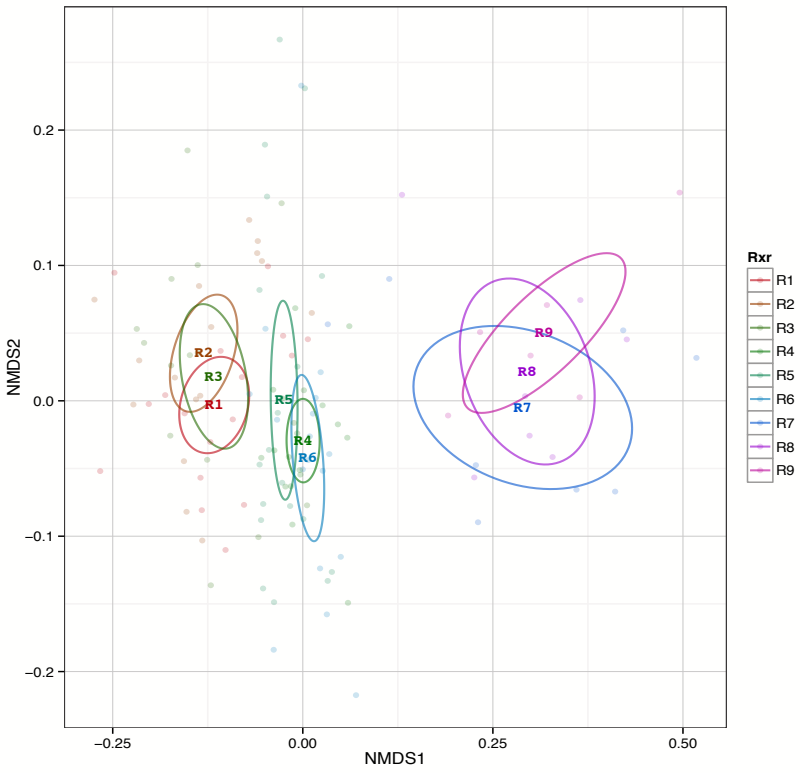


Figure 5-8: Two-dimensional NMDS ordination plot (Stress = 0.125) of the 100 most abundant OTUs in the sample set, grouped by individual reactor ID (Rxr) and plotted using Phyloseq and Bray-Curtis similarity. In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

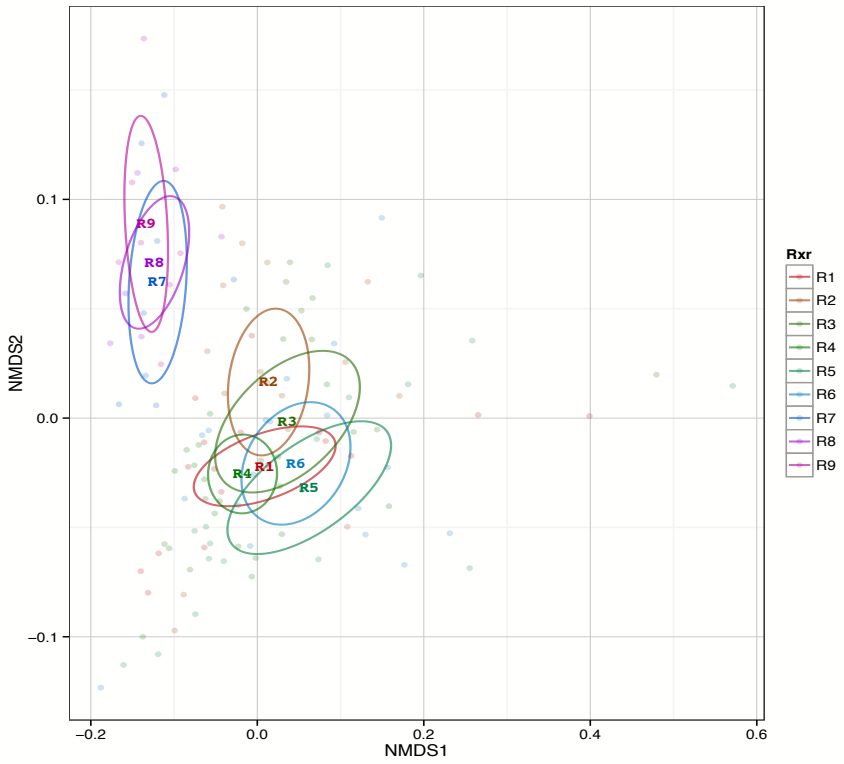


Figure 5-9: Two-dimensional NMDS ordination plot (Stress = 0.106) of the 100 most abundant OTUs in the sample set, grouped by individual reactor ID (Rxr) and plotted using Phyloseq and GUnifrac distances (alpha = 0.5). In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean

It is seen that using Bray-Curtis distances (Figure 5-8), which utilise count data only, the communities cluster distinctly by feed-type. Greater distance in clustering is seen between reactor sets than within reactor sets indicating good replication of the microbial community within each reactor set. Further, those communities treating low- and full-strength distillery waste (R1-3 and R4-6) cluster more closely to each other than to the community treating SYNTHES (R7-9). This implies that substrate composition rather than substrate availability is the key driver of variance in species relative abundance within a reactor community. The plot utilising phylogenetic distance (Figure 5-9) shows that the communities for the low- and full-strength waste reactors cluster together as a common group whilst the community for the SYNHTES reactors cluster distinctly. Given that using count data each reactor set clusters distinctly but phylogenetically, the low- and full-strength reactor sets cluster as a group, these results suggest that substrate availability influences relative abundance amongst the most dominant species in a community whereas substrate type influences community membership amongst the most dominant species in a community. Interpreted in relation to the SMA data, this suggests that small shifts in relative abundance in a community can have large impacts on community productivity. Whilst SMA data for the low-strength reactor set showed significantly depressed activity as compared to the full-strength reactor set the NMDS plots suggest that the communities driving each activity profile are not highly distinct i.e. the same or similar community is functioning differently according to substrate availability. By contrast, distinct grouping between the full-strength and SYNTHES reactor sets seen in the phylogenetic plot suggests that provision of alternative substrate drives change not only in relative abundances but also of community membership amongst the 100 most abundant OTUs in the data set.

5.4.4.2 Ecology Indices with Substrate-Type

Ecology indices based on relative abundance in the count data were calculated to further explore the disparity in community structure suggested by the NMDS plots. Rarefied richness, evenness and diversity were calculated at each biomass sampling time point and are plotted in Figures 5-10 to 5-11. Rarefied richness was previously demonstrated to increase significantly with time ($P<0.001$) in the 1-D lab-scale reactor set treating full-strength distillery waste (Chapter 4). The cause of this was not fully determined but was tentatively linked to both stratification of the microbial community with depth in the reactor and with relatively reduced solids loading as compared to the full-scale reactor ADR2 from which the seed sludge was drawn.

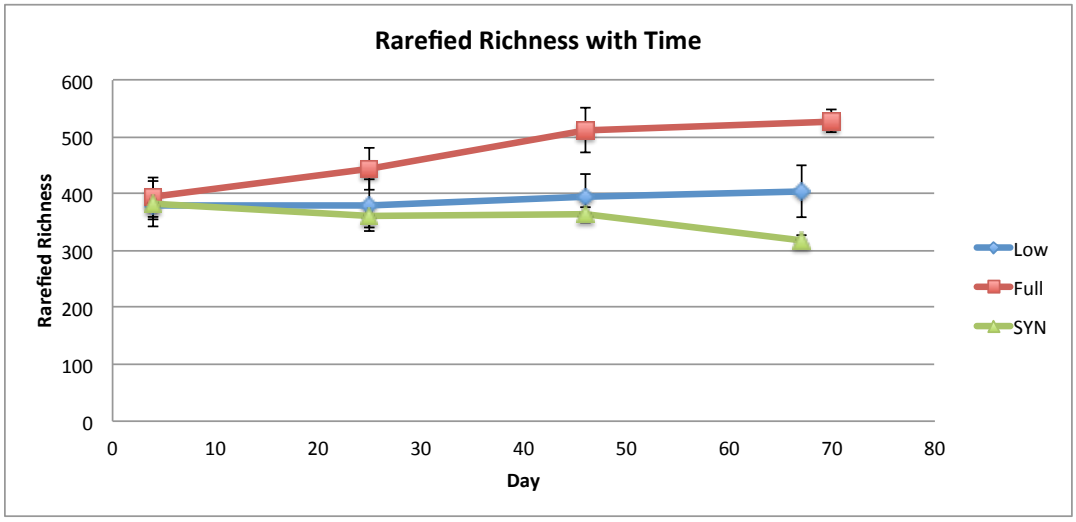


Figure 5-10: Scatter plot showing time-series for mean rarefied richness in each of the low-strength (Low), full-strength (Full) and SYNTHES (SYN) sample sets; error bars show standard deviation.

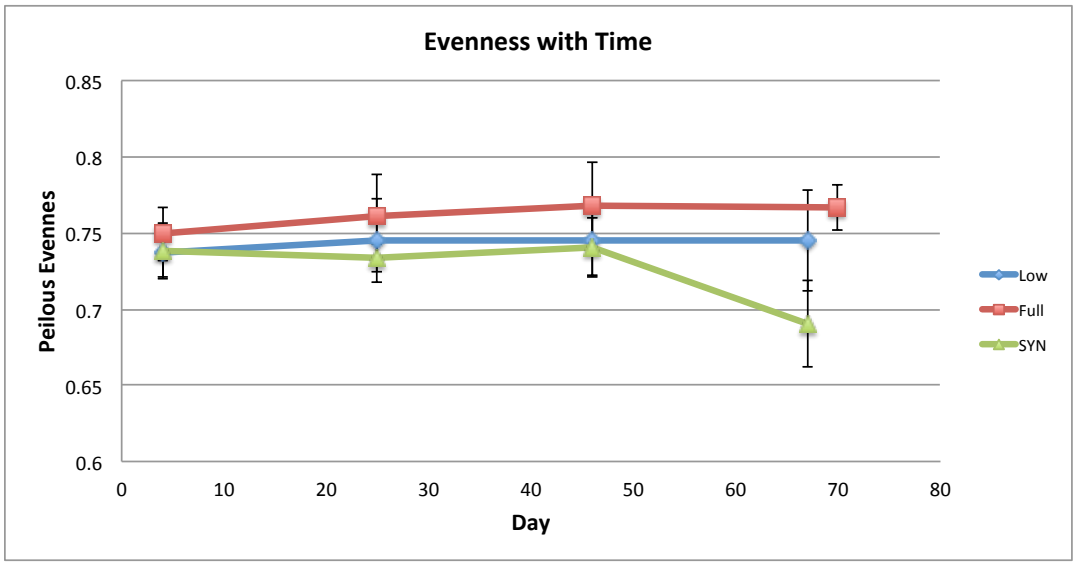


Figure 5-11: Scatter plot showing time-series for mean community evenness in each of the low-strength (Low), full-strength (Full) and SYNTHES (SYN) sample sets; error bars show standard deviation.

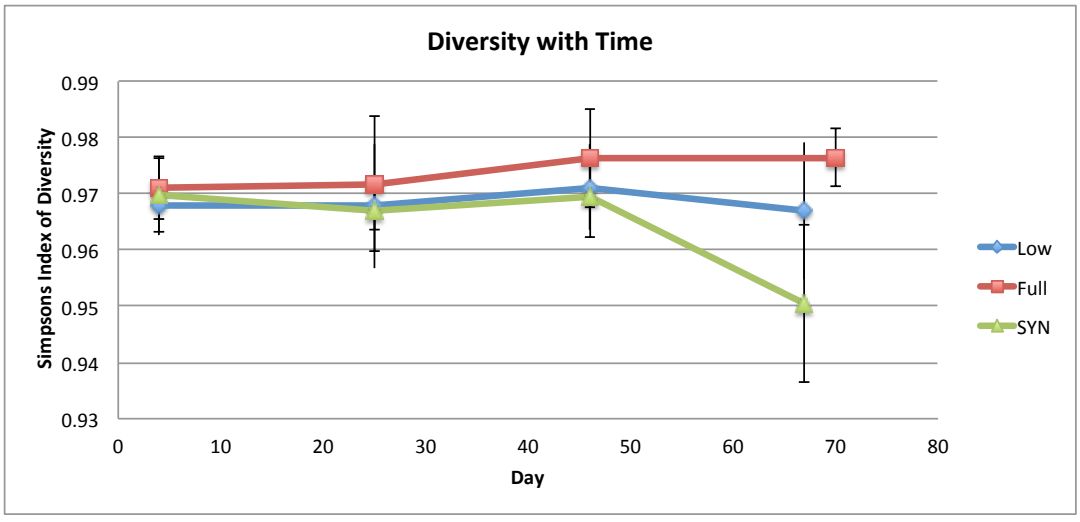


Figure 5-12: Scatter plot showing time-series for mean community diversity in each of the low-strength (Low), full-strength (Full) and SYNTHES (SYN) sample sets; error bars show standard deviation.

Rarefied richness in the SYNTHES reactor set here is seen to decrease with time with a dramatic decrease in richness occurring between d46 and d70. SMA results show that no significant stratification of activity with depth was found in the SYNTHES reactor set however this alone seems unlikely to drive a reduction in community richness. With regards solids loading however it is noted that here solids loading is proportionately higher in the SYNTHES reactor set than in either the full-strength distillery waste reactor set at lab-scale or the full-scale reactor from which the seed sludge was extracted. That richness was reduced in the SYNTHES reactor set then appears to confirm the previous finding that solids loading acts as a driver for reduced richness in the microbial community. Rarefied richness however is not an absolute measure of richness; rather it is a function of both community evenness and diversity. Both diversity and evenness are seen to decline in the SYNTHES reactor set particularly at time d70, suggesting that the apparent decrease in richness may be an artefact of differing community structure rather than a real reduction in species numbers per se. Regardless, as evenness and diversity have variously been associated with robustness in microbial communities and bioreactor performance, it would appear that ecologically, the microbial community is weakened by application of high solids loading. This is not reflected in reactor performance however, which was seen to be relatively stable in the SYNTHES reactor set throughout this experiment. Tentatively, the reduced richness, evenness and diversity in the treatment of SYNTHES as compared to the treatment of a real wastewater may arise from lack of complexity in the synthetic waste, which may in turn reduce the number of niche functions available to the microbial community. In a short reactor run such as this (70 days in total) it is not possible to determine the longer-term impact of this result. This reactor set however was run for a greater period than is described in the context this chapter and as such, this is returned to in more detail in Chapter 7 to follow. By contrast, whilst community productivity was demonstrated to have significantly reduced in the low-strength reactor set as compared to both the full-strength distillery waste reactor set and that of the seed sludge each of richness, evenness and diversity in the low-strength distillery waste reactor set is seen to seen to remain relatively stable with time. It is suggested then that whilst ecology measures might be informative of underlying trends in the community, ecology measures alone do not provide adequate information on reactor performance to act as a monitoring tool.

5.4.4.3 Statistical Analysis of Sequencing Data

The aim of the statistical analysis was to identify taxa associated with each of the two alternative substrate types applied. Kruskal-Wallis testing was applied to determine those genera whose relative abundances significantly varied with feed-type. In total, 67 genera

distributed amongst 22 distinct phyla were identified as significantly different with feed-type ($P<0.001$) indicating that substrate type has acted as a strong driver for change in the underlying microbial community. To enable visualisation of the extent of change within the most abundant species within the community, a heat map was plotted for the 100 most abundant OTUs in the set. Collectively the 100 most abundant species account for 78.6% of the total community sampled. In the heat map plotted, tiles are coloured white if the species is absent, blue if more abundant, and are shaded according to relative abundance in the whole data set (Figure 5-10). Organisms of interest (I-IV, and (a)-(m)) were identified by qualitative assessment of the heat map, and the nearest known taxa for each OTU identified by mapping the sequence for that OTU against known taxa using the BLAST database (Table 5-10).

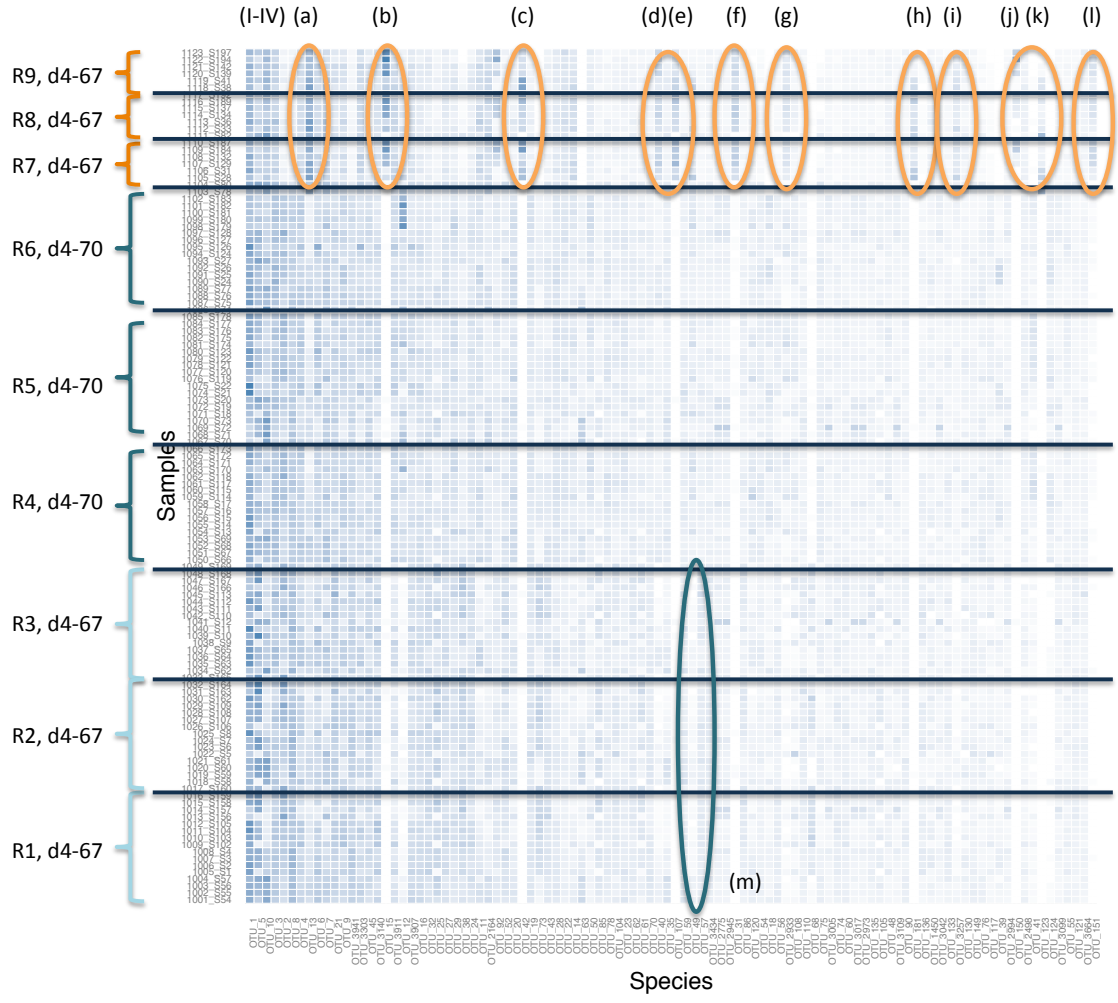


Figure 5-13: Heatmap showing the relative abundance of the 100 most abundant OTUs across the whole sample set whereby depth of colour of squares represents abundance with dark blue indicating the relatively most abundant OTUs and white indicating relatively least abundant OTUs. OTUs labelled (I-IV) are qualitatively identified as core organisms present in each reactor set. Yellow ellipses indicate organisms (a-l) that are dominant in SYNTHES reactors. The blue ellipse highlights OTU (m) that is notably absent in the low-strength distillery waste reactors.

Four highly abundant OTUs were identified as common to the community in each reactor set (Figure 5-10, I-IV). OTU1 was the most abundant organism in the sample set and was identified as *Methanobacterium* (100%), a genera of H2, CO2 and formate utilising methanogens (Madigan, Martinko et al. 2009). The relative abundance of *Methanobacterium* was 8.2%, 8.1% and 6.17% of the total community in each of R1-3, R4-6 and R7-9 respectively. The dominance of hydrogenotrophic methanogens is consistent with the SMA tests conducted at the end of the trial for both low-strength distillery waste reactors R1-3, and for the SYNTHES reactors R7-9. The SMA test for high-strength distillery waste by contrast, showed a shift towards acetotrophic methanogenesis by the end of the trial as compared to that measured for the seed sludge that does not appear to be reflected in the relative abundance of this specific OTU. The dominance of the hydrogenotrophic pathway in the low-strength reactor set and the SYNTHES reactor set is somewhat unexpected as it is commonly assumed that 70% of methane produced by methanogenic communities is via the acetotrophic pathway. Frequently in literature on low-temperature AD, a shift towards hydrogenotrophic methanogenesis seen at increasingly low temperature (McKeown, Scully et al. 2009, O'Reilly, Lee et al. 2010), and this shift may be interpreted as selective pressure or stress response in the microbial community. That the low-strength and SYNTHES reactors maintain the hydrogenotrophic pathway as dominant could suggest that both low substrate availability and high-solids loading similarly induce stress in the community. In the case of SYNTHES, it may be that protein degradation resulted in ammonia toxicity to which acetotrophic methanogens are sensitive. It has been demonstrated that ammonia can promote growth of acetate oxidising bacteria that are able to compete with acetotrophic methanogens for acetate as a substrate (Westerholm, Dolfing et al. 2011). In the low-strength case, the cause of 'stress' on acetotrophic methanogens is unknown.

Table 5-10: OTUs distinct to feed-type: mean relative abundance and taxonomic identify (RDP classifier).

Relationship	OTU	Mean Relative Abundance / %			Position on Heatmap	Significant Difference / Pa-value	Closest identifier, RDP classier with 80% confidence threshold	Similarity / %
		R1-3 Low-Strength	R4-6 Full-Strength	R7-9 SYNTHES				
Abundant, common across substrates	OTU_1	8.22	8.11	6.17	I	0.22246395	Methanobacterium (G)	100
	OTU_5	4.1	7.28	2.44	II	3.03E-05	Synergistaceae (F)	100
	OTU_10	3.72	3.09	4.91	III	0.00016794	Synergistaceae (F)	73
	OTU_3	3.97	2.93	2.44	IV	2.70E-06	Syntrophobacter (G)	100
Abundant SYNTHES only	OTU_13	0.93	0.94	7.56	a	8.75E-11	Clostridia (C)	34
	OTU_15	0.002	0.004	7.62	b	9.51E-11	Clostridia (C)	29
	OTU_42	0.0003	0.002	3.97	c	1.98E-10	Sphingobacteriia (C)	28
	OTU_40	0.23	0.1	1.34	d	1.38E-13	Candidatus Cloacamonas	100
	OTU_107	0.001	0.002	2.08	e	2.61E-10	Synergistaceae (F)	35
	OTU_31	0.005	0.002	1.63	f	2.14E-08	Bacteroidales (O)	41
	OTU_2933	0.3	0.041	0.6	g	2.37E-10	Methanobacteriaceae (F)	63
	OTU_181	0.0003	0.0003	1.11	h	1.89E-11	Trichococcus (G)	96
	OTU_3257	0.104	0.032	0.7	i	5.72E-16	Synergistaceae (F)	88
	OTU_150	-	0.0004	0.98	j	2.65E-07	Synergistaceae (F)	43
	OTU_123	0.0004	0	0.95	k	7.15E-11	Acetoanaerobium (G)	99
	OTU_151	0.015	0.001	0.87	l	1.50E-12	Clostridia (C)	25
Less abundant, low-strength	OTU_49	0.07	0.49	0.56	m	4.92E-14	Aminobacterium (G)	56

Also similarly dominant across all reactor sets were OTU5 and OTU10, which were both identified as of the family *Synergistaceae* at 100% and 73% similarity respectively (BLAST). Cumulatively, *Synergistaceae* accounts for 7.82%, 10.37% and 7.35% in each R1-3, R4-6 and R7-9 respectively. Species of the family *Synergistaceae* have been associated with EGSB reactors treating waste with a high protein load (Delforno, Okada et al. 2012) and tentatively with high lipids loading (Palatsi, Affes et al. 2012) and may be involved in early stages of degradation of organics in the waste stream. That relative abundance of this family is decreased in R1-3 and R7-9 might suggest that protein and lipids content in those feed types may be lower than that of full-strength distillery waste, or that other organisms fulfil this role in the alternative feed-type reactor sets. Finally, common across each reactor feed-type is OTU3, which was identified as of the Genus *Syntrophobacter* (100%), and of the family *Syntrophobacteraceae*. Members of the family *Syntrophobacteraceae* have been variously isolated from sewage sludge, marine and freshwater sediments and have been demonstrated to grow via fermentation or syntrophically with H_2 utilisers (Kuever 2014) such as *Methanobacterium* as are abundantly present with each feed type in this experiment.

Whilst similarities in community membership and abundance are seen across reactors treating each feed type, some strong distinctions are seen in the community treating SYNTHES. Twelve OTUs were identified whose abundances were notably greater in the SYNTHES reactors than in the low- and high-strength waste reactors. For each OTU identified, significance ($P<0.001$) was established using the Kruskal-Wallis test. Of those, OTU2933 was the only identified Archaea and was found to be of family *Methanobacteriaceae* (67% similarity) belonging to the class *Methanobacteria*. *Methanobacteria* are H2/CO2 and formate utilising methanogens (Madigan, Martinko et al. 2009) further supporting the finding that the hydrogenotrophic pathway was dominant in the SYNTHES reactors. Of bacteria identified three OTUs (OTUs 13, 15 & 151) belonged to the class *Clostridia* and cumulatively account for 16.05% of the total community in the SYNTHES reactors as compared to only 0.95% in each of the low- and high-strength reactor sets. Species of the class *Clostridia* have been associated with long chain fatty acid degradation (Sousa 2008), and the degradation of cellulose (O'Sullivan 2007) suggesting that here, those OTUs contribute to hydrolysis and early stages of fermentation and that their elevated abundance in the SYNTHES reactors may be linked with solids loading. Further, *Clostridia* have been associated with ammonia production (Vince and Burridge 1980), which may account for the increased pH of the SYNTHES reactors as suggested previously. OTU42 is identified as of the class *Sphingobacteria*,

species of the are known to grow on peptone (Yano, Tomiyasu et al. 1982), again suggesting the community has adapted to increased hydrolytic activity under high solids loading. OTU40 was identified as of the *Candidatus Cloacamonas* (41%), a member of the bacterial candidate subdivision WWE1 that branches from the phylum *Spirochaetes*. *Candidatus Cloacamonas acidominovorans* has been identified in-vitro using metagenomic samples from a low-rate mesophilic anaerobic digester and is thought to be a syntrophic hydrogen producer utilising acetate, sugars and amino acids as substrates (Pelletier, Kreimeyer et al. 2008). Members of the subdivision WWE1 are thought to be ubiquitous in anaerobic digestion (Pelletier, Kreimeyer et al. 2008) but have been associated with cellulose degradation (Limam, Chouari et al. 2014), and, the phylum *Spirochaetes* has been linked with long chain fatty acid degradation (Hatamoto, Imachi et al. 2007) in AD. This suggests that OTU40 might contribute towards hydrolysis and further, putative syntrophic hydrogen production by OTU40 might support the hypothesis that the hydrogenotrophic pathway might be driven selectively under high solids loading. OTU31 was found to be of the order *Bacteroidales* (41%), which is of the class *Bacteroidetes*. *Bacteroidetes* have been associated with long chain fatty acid degradation (Carballa and Vestraete 2010) and with degradation of olive mill residue at high organic loading rates (Rincón, Borja et al. 2008) and again points to elevated hydrolytic activity in the SYNTHES community. OTU181 was identified as of the genera *Trichococcus* (96%), an isolate of which have been associated with bulking sludge in an activated sludge system(Scheff, Salcher et al. 1984). Whilst no bulking was observed during the trial, and indeed this trial was anaerobic rather than aerobic, it is noted that *Trichoccocus flocculiformis*, the isolate from bulking sludge was grown on a media rich in peptone and urea, two dominant components in SYNTHES. OTU123 was found to be of the genera *Acetoanaerobium* (99%), which is of the class *Bacilli*. *Acetoanaerobium* are homoacetogenic microorganisms, producing acetate from H2 and CO2. Finally, three OTUs (OTUs 107, 3257 & 150) found to be of the family *Synergistaceae* were noted to be more abundant in the SYNTHES reactors than in the distillery waste reactors however, the same family was identified as amongst the most common across the three reactor sets. As such, it may be suggested that this species of this family may be more ubiquitous in anaerobic digesters.

Interestingly, whilst qualitative analysis of the heat map suggests the SYNTHES and full-strength distillery reactor communities show clear distinction from each other particularly in relation to hydrolysis, little distinction is seen between the microbial communities in the low- and full-strength distillery waste reactor sets. This qualitative assessment supports

the finding of the NMDS plots shown previously which suggested that phylogenetically the top 100 OTUs for each of the low- and full-strength reactor types were highly similar and that in terms of relative abundances were reasonably similar. Putatively, together with the SMA data, these results suggest that low substrate availability can induce low productivity in a methanogenic community without drastically altering community composition. As such, whilst community composition may be indicative of reactor function, an understanding of the environmental conditions by which productivity levels are bounded would provide an enhanced insight to sequencing data were it applied as a monitoring tool for reactor performance.

5.5 Summary Findings and Recommendations

5.5.1 Low Substrate Availability

The adaptation of a granular biomass treating a low-strength industrial wastewater in EGSB type lab-scale bioreactors was investigated with the aim of assessing the impact of low substrate availability on reactor performance, community physiology and microbial community composition. The primary aim in so doing was to glean information relevant to the case of Global North sewage treatment i.e. treatment of low-strength, high-solids wastes. Reactor performance was found to be satisfactory in terms of COD removal efficiencies however biogas production rates were lower than was anticipated by scaling of results from the treatment of full-strength distillery waste. On balance however, as a wastewater treatment method, the result is positive in terms of improving a waste stream whilst producing energy in the form of methane gas rather than requiring energy input as in the case of aerobic treatment alternatives. The finding that biogas production was lower than might have been anticipated was confirmed by SMA testing of the biomass, which recorded depressed activity of the biomass for each substrate tested as compared to activity of biomass from reactors treating full-strength distillery waste. Both qualitative and ecological analysis of the sequencing data suggests that whilst microbial activity is depressed, neither community composition or structure is greatly affected by low substrate availability. The suggestion then is that a given microbial community can exist at a given equilibrium whilst functioning at various level of productivity. This finding is highly significant in terms of the application of NGS technologies to assessment of microbial communities by 16S rRNA surveys in isolation. At present, the capacity to interpret the subtleties of such surveys is inadequate to provide an accurate assessment of microbial communities in relation to the performance of specific functions.

5.5.2 High Solids Loading

The adaptation of the same biomass, in the same reactor type, to a high solids loading condition was investigated with the aim to determine the suitability of application of the EGSB reactor to the treatment of high-strength, high-solids wastes such as pit latrine wastes in the Global South. In terms of performance, whilst sCOD removal efficiencies remained high, solids removal efficiency was low. SMA testing indicated that high-solids loading promoted dominance of the hydrogenotrophic pathway in the SYNTHES reactors which was consistent with previous findings in the study of the full-scale reactor ADR2 (Chapter 4). Ecological analysis of the sequencing data suggested that microbial community structure shifted unfavourably over time in relation to high solids loading however no similar effect was noted in performance. As such, longer trials should be conducted to establish whether the deteriorating impact on community structure continues, or reaches equilibrium, in relation to solids loading. Statistical analysis of microbial community structure in relation to high solids loading identified a number of taxa associated with hydrolysis key amongst which belong to the classes *Clostridia* and *Bacteriodetes* and the phylum *Spirochaetes*. These taxa warrant further study in relation to both solids degradation and the influence on the route to methanogenesis. The influence of substrate type rather than substrate availability was found to have a greater impact on the underlying microbial community composition and structure. In terms of the appropriateness of the EGSB for the treatment of high solids wastes, further trials are required over longer time periods to enable more accurate assessment of community response to the stresses associated this feeding condition.

5.5.3 Application of Parallel Control Reactors

In each of the substrate cases, the reactor set treating full-strength distillery waste was used as a reference or control reactor set against which data collected on the alternate substrates might be assessed. The strategy was found to be extremely useful in enabling identification of specific taxa associated with each feeding condition. The use of triplicate reactors in each set further enabled statistical confidence in the results obtained. Under each substrate condition applied however, good replication of the underlying microbial community was seen. Running replicate reactors and control reactors is both time-consuming and costly at lab-scale and indeed impractical at full-scale. As such, whilst further work regarding the extent of replication in microbial communities under a range of given operating conditions should be investigated.

5.6 Conclusions and Future Work

The study aimed to investigate the potential to adapt a given biomass established in the treatment of an industrial wastewater to the treatment of both Global North and Global South sewage. In the case of Global North sewage the study demonstrated promising performance. The waste utilised however was not truly reflective of Global North sewage and indeed, the temperature conditions applied are not that of Global North sewage. As such, adaptation of the biomass in the low-strength distillery waste reactor set was investigated under the treatment of low-strength SYNTHES at low temperatures (15 degC). The results of this investigation are reported in Chapter 6 of the thesis. In the case of Global South sewage treatment, the waste applied was not of the solids content as might be anticipated of true pit latrine waste. Further, the results implied that whilst performance at the solids loading applied was satisfactory, ecological analysis indicated that community structure had shifted unfavourably under solids loading. As such, the biomass in that reactor set was subject to increasing solids loading by ramping up the solids component of SYNTHES to induce microbial failure in the reactors. The results of this experiment are reported in Chapter 7 of the thesis.

6 Application of the EGSB to Sewage Treatment in the Global North

Abstract

The activated sludge process remains one of the most dominant sewage treatment methods used in the Global North today. Whilst an effective treatment method, aeration is estimated to account for over 50% of the energy budget in a sewage treatment plant. The EGSB is a high-rate anaerobic wastewater treatment system with the potential to couple removal of dissolved organics in a waste stream with the production of methane gas for an effective net energy gain. Poor solids removal efficiency reported in EGSB systems however limits the application of the technology to sewage treatment. Improved understanding of the microbial community underpinning the treatment of sewage in EGSB reactors might offer increased capacity to engineer the system to treat sewage at low temperatures sustainably, and at lower cost than aerobic treatment alternatives.

Triplicate 20-litre, 1-D lab-scale EGSB reactors were operated and monitored during phased adaptation from the treatment of low-strength industrial waste (600mgCOD/L) at mesophilic temperature (37degC) to the treatment of low-strength synthetic sewage (500mgCOD/L) at ambient temperature (15degC). Phased operation included: treatment of low-strength industrial waste at 37degC, 22degC and 15degC and treatment of low-strength synthetic sewage at 15degC. COD removal efficiencies, VFA accumulation and methane production rates were used to assess reactor performance under each condition. Specific methanogenic activity assays were used to monitor community physiology after adaptation to each condition. Whole community 16S rRNA gene (V4 region) amplicon sequencing using the Illumina Miseq platform was utilised to monitor microbial community dynamics both spatially and temporally under each condition.

Reactor monitoring data indicated that whilst good COD and pCOD removal efficiency (80.0%) was achieved in the treatment of low-strength synthetic sewage at ambient temperatures, methane production rates were reduced. This was supported by specific methanogenic activity assays that indicated the development of a psychrotolerant rather than psychrophilic microbial community. Analysis of both sequencing data and specific methanogenic activity profiles indicated a highly stratified microbial community with depth in the reactors under each condition tested. Throughout the trial, the apparent dominance of hydrogenotrophic methanogenesis observed in the seed sludge was maintained indicating that successful application of the EGSB to the treatment of sewage

at ambient temperatures requires a deeper understanding of this metabolic pathway at the community level.

6.1 Introduction

The development of sewers and sewage treatment processes in the late 1800s is one of the greatest achievements in the field of Civil Engineering. The methods applied for the treatment of municipal sewage today however have remained largely unchanged for well over a century. Central to most sewage treatment plants in the Global North is a form of the activated sludge process; a method by which the degradation of organics by aerobic microorganisms in the post-settled bulk liquor, is promoted by aeration of the waste. The arising sludge (new microbial cells) along with the settled sewage solids is then treated separately: most commonly by drying and incineration, anaerobic digestion or application to land. The effectiveness of this chain of processes is well proven however the energy demands are high. It is estimated that in the Global North as much as 5% of electricity produced is used in water and wastewater treatment processes (Logan 2008) with a significant portion of that consumed in the process of aeration.

Post treatment of solids presents a further energy demand. Anaerobic digestion of settled solids typically occurs in low-rate anaerobic digesters operated at mesophilic and even thermophilic temperatures. These design temperatures arise from two key assumptions. Firstly, that higher temperatures will support improved treatment efficiency by promoting physically favourable conditions for solids breakdown. Secondly, and perhaps most importantly, these temperatures are assumed optimal for the growth and function of acetotrophic methanogens who are understood to be responsible for promoting the majority of methane produced in biogas systems. The energy demand associated with heating digesters however has a negative impact on any energy gain from methanogenesis and indeed may negate energy generation completely.

The development of retained biomass anaerobic digesters such as the EGSB in the 1990's enabled decoupling of microbial growth rates from liquid retention times in a reactor. Thus the potential to apply the EGSB to low-strength wastes such as sewage was presented such that both the energy requirement associated with aeration *and* the energy costs of post-treatment of settled solids might be eliminated by enabling anaerobic treatment of sewage as a single waste-stream and at low-temperatures. Indeed, the EGSB was demonstrated to be applicable to the treatment of low-strength, low-solids wastes such as unsettled municipal sewage at mid-low temperatures with some success. Poor treatment of suspended solids in the EGSB (Seghezzo, Zeeman et al. 1998) coupled with low capacity

to fully engineer treatment in such systems however means that the application of EGSBs to treat municipal wastes remains low and the energy input of treatment remains high.

Increased understanding of the microbial processes underpinning methanogenesis in retained biomass systems at low temperature may present the opportunity for a truer engineering approach to design and provide a much needed solution to the energy demands of sewage treatment. Next generation, high-throughput sequencing technologies are enabling improved insights into microbial communities than has previously been viable. Further, application of multiplexing strategies is lowering the cost of such methods, expanding opportunities to run replicate reactors with increasingly fine time-series to describe microbial community dynamics. The aim of this experiment then is to:

- Evaluate the performance of triplicate lab-scale reactors treating a low-strength synthetic sewage (SYNTHES) at low temperature (15degC) by adopting phased operation from treatment of low-strength industrial waste at 37degC, 22degC and 15degC to treatment of low-strength synthetic sewage at 15degC. The phased operation is treated as an 'in-series' control approach the success of which is also evaluated.

- Utilise NGS and specific methanogenic activity assays to provide new insights to microbial adaptation both spatially and temporally to the treatment of sewage at low-temperature in 1-D lab-scale EGSB bioreactors.

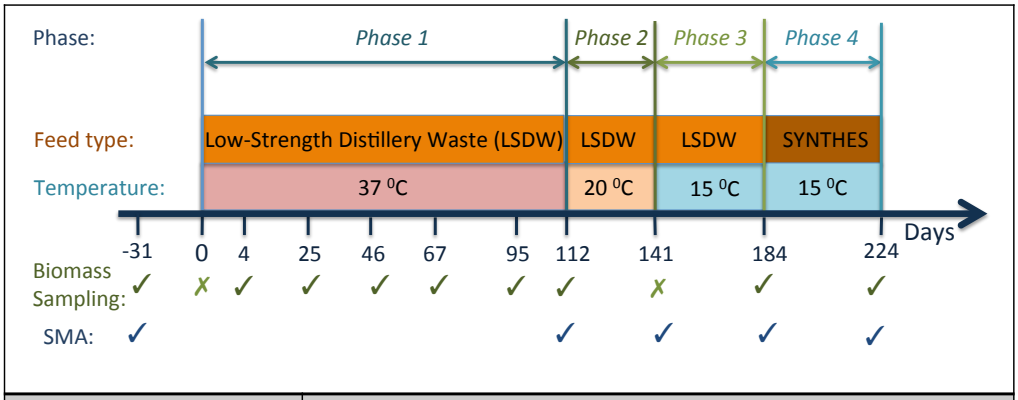
6.2 Experimental Design

The experiment utilises three lab-scale EGSB reactors, each of which was seeded using a granular biomass extracted from a full-scale mesophilic (37degC) digester used to treat spent water from the distillation of maize. Each reactor was of the 1-D reactor type (as described in Chapter 4) with a total working volume of 20L per reactor. Each reactor, R1-3, was operated identically for the duration of the experiment. To investigate the adaptation of the microbial community to the treatment of low-strength, high-solids synthetic sewage at ambient temperature (15degC), the biomass was subjected to a series of step-wise engineered changes in operating condition with time. In so doing it was intended that the previous period of operation would act as the control for each new condition. This approach is described here as an 'in-series' control strategy and the success of such as compared to the parallel control methods applied in previous chapters of this thesis is assessed alongside the arising experimental results. The introduction of each change in operating condition defines a phase of reactor operation as outlined in Table 6-1:

Table 6-1: Key operating conditions applied during Phase 1-4 of reactor operation and operating parameters common across each phase (ALL).

Phase	Operating conditions
1	Treating low-strength distillery waste (600mg/l COD) at 37degC for 12 weeks
2	Treating low-strength distillery waste (600mg/l COD) at 22degC for 3 weeks
3	Treating low-strength distillery waste (600mg/l COD) at 15degC for 10 weeks
4	Treating low-strength SYNTHES (500mg/l COD) at 15 degC for 6 weeks
ALL	HRT: 16 hours, Upflow Velocity: 3.5 m/h, Influent pH 6

The microbial community adaptation to low-substrate availability during Phase 1 of operation of the reactor set R1-3 was described in Chapter 5 previously in which lab-scale reactors treating full-strength distillery waste acted as a parallel control for the initial adaptation to low substrate conditions. For continuity in operation, the same hydraulic operating parameters used in that Phase (Phase 1 here) were maintained throughout the trial and are given in Table 6-1 (ALL). The design operating conditions for each phase of the experiment are illustrated in the schematic in Figure 6-1 alongside the monitoring regime applied throughout the duration of the trial.



Parameter	Measurement / Sampling Frequency Lab-Scale
Specific Methanogenic Activity	Seed sludge and end of each phase
COD and sCOD, influent and effluent	Bi-weekly
pH influent and effluent	Bi-weekly
Biogas production	Daily
Methane content in biogas	Bi-weekly
Temperature	Daily in reactor
Volatile Fatty Acids	Bi-weekly, 8 VFAs from C2 to C6, EtOH
Biomass samples	9 Sample times at 5 depths in each 1-D reactor over 224 day period as shown

Figure 6-1: Schematic showing operating conditions and duration of each of Phases 1-4, relative timings of biomass during each phase, and summary of physico-chemical monitoring parameters.

6.3 Materials and Methods

6.3.1 Substrate Preparation and Feeding

Phases 1-3

Low-strength distillery waste was prepared by tenfold dilution of a full-strength distillery waste to a target COD of 500mg/L to reflect an approximate COD strength of municipal sewage using tap water as a solute. The distillery waste was collected on a weekly basis from the North British Distillery as described previously and stored in airtight containers until used. Once diluted, the distillery wastewater was neutralised prior to use to ~pH 6 by dosing with a low-grade caustic and supplemented using a trace minerals solution (Table 6-2) as was applied at the distillery.

Table 6-2: Trace mineral solution for Low-Strength Distillery Waste

Micronutrient	Dosing Concentration (g/m3)
FeSO4 7H2O	6.042
CoCl2 6H2O	0.015
CuCl2 2H2O	0.051
MnSO4 H2O	0.053
NiSO4 6H2O	0.080
Al2(SO4)3 18H2O	0.277
ZnCl2	0.047
Na2SeO3 5H2O	0.003

Phase 4

A synthetic sewage (SYNTHES) and was applied as substrate to the reactor set during Phase 4 of the experiment. The SYNTHES was prepared in the laboratory as per Aiyuk et al (Aiyuk and Verstraete 2004) using tap water as a solute. SYNTHES was applied to the reactors at COD of 500mg/L similarly to that of the low-strength distillery waste and was intended to mimic the strength and composition of Global North sewage. For each feed type, in-tank mixing was used to promote solids delivery to the reactors and minimise variation in feed composition for the duration of feeding.

6.3.2 Temperature Control

Three temperature conditions were applied during the experiment. In Phase 1, the reactors were operated in the mesophilic temperature range with a target operating temperature of 37degC. The temperature was controlled using an external heating system and water jackets on each reactor. In Phase 2 the reactors were operated at ambient temperature and

therefore no specific temperature control strategy was applied. In Phases 3 and 4 of the experiment the reactors were operated with a target temperature of 15degC. The temperature was again controlled via the use of the reactor water jackets however this time a cooling system was used to chill water and enable reactor liquor temperature to drop. During each Phase reactor temperature was monitored on a daily basis using an electronic temperature meter and probe inserted into the reactor liquor.

6.3.3 Physical and Chemical Monitoring

All methods are described in detail in Chapter 3 and a brief overview is repeated here. Sampling of both influent and effluent for each reactor was made on a bi-weekly basis for the duration of the experiment. Liquid samples were then used to monitor key operation and performance parameters using a range of experimental methods (Figure 6-1). Total and soluble COD was measured using the Closed Reflux, Colorimetric Method, Standard Methods 5220D. Influent and effluent pH was measured using a digital meter and probe (Thermoscientific Meter, Eutech Instruments Probe). Volatile fatty acids (VFAs, C_2–C_6, including iso-forms of C_4–C_6) and ethanol concentrations were measured using a gas chromatograph (7890A, Agilent, Palo Alto, CA) equipped with a DB-FFAP capillary column and a flame ionization detector.

Biogas production was recorded on a daily basis using 20L volume rubber gasbags attached to each reactor gas line to collect the biogas produced for timed periods of approximately 12 hours. Biogas volume was quantified using a gradated gas-tight syringe to empty gasbags of the collected volume and biogas production rates were calculated as volume produced per unit time. Methane content in the biogas was quantified on a biweekly basis to coincide with influent and effluent monitoring. Methane content was measured using a gas chromatograph (7890A, Agilent, Palo Alto, CA) equipped with a GS-Carbon Plot capillary column and a flame ionization detector.

6.3.4 Physiological Monitoring: Specific Methanogenic Activity Testing

Specific methanogenic activity was measured on a single sample of the seed sludge at d-31 representing the starting activity in the biomass in each reactor. SMA testing was additionally conducted at the end of each phase of operation to determine the effect of each applied temperature condition and feed-type on the methanogenic activity of underlying microbial community. Biomass used in the SMA was extracted from the top and bottom of the sludge bed from each of the reactors R1-3 and pooled to make two samples for the

reactor set; one from the top and one from the bottom of the sludge beds. The SMA was then conducted either at:

- 37degC only (end of Phase 1),

or, in instances where the reactors were operated at lower temperatures the SMA was conducted both at

- mean reactor temperature for that phase and also at 37degC (Phases 2-4).

The aim of this was to determine whether the microbial community had developed a psychrophilic or psychrotolerant activity profile. Substrates tested were acetate (Ac_C2), propionate (Pr_C3), butyrate (Bu_C4), ethanol (Et_OH) and Hydrogen (H2).

6.3.5 Biomass Sampling and Storage

Biomass was sampled at each port in the sludge bed from each reactor during Phase 1 (Figure 6-1). During subsequent phases, the same sampling strategy was maintained for R2 whilst an alternative strategy was adopted on R1 and R3. On those reactors, samples were taken from each port in the sludge bed however DNA from the top 2 ports and bottom 2 ports for each reactor was combined at equal concentrations (ng/ul) to make a sample representing the 'top' and bottom' of the sludge bed in each. The aim in so doing was to reduce the total number of samples sequenced to allow a higher degree of resolution in sequencing in terms of the number of reads per sample. In each case, biomass was sampled according the method outlined in Chapter 3 and stored at -20degC until the end of the end of the experiment before further processing for NGS library preparation.

6.3.6 NGS Library Preparation

DNA extraction and purification was conducted using the Fast DNA Spin Kit for Soils (MP Biomedical). Extracted DNA was quantified using the Broad-Range Qubit Assay (Life Technologies) and stored at -20degC until used in NGS library preparation. Library construction was applied to DNA samples from each port in the sludge bed. PCR amplification of the V4 region of the 16S rRNA gene using Golay barcoded primers (Caporaso, Lauber et al. 2012) with an additional degeneracy on the forward primer for improved detection of Archaea was conducted on each DNA sample in the sample set. PCR product was gel-purified (Zymoclean Gel DNA Recovery Kit) and quantified prior to sequencing using the High-Sensitivity Qubit Assay (Life Technologies). The pooled multiplexed library, normalised to 5ng/ul DNA, was sequenced using the Illumina Miseq bench-top sequencer. Arising sequencing was de-multiplexed at the sequencing centre and

all other quality control methods applied to the sequencing data are described in detail in Chapter 3.

6.3.7 Qualitative and Statistical Analysis Methods

Data pertaining to operation and performance of the reactors during each phase of the experiment are summarised to provide mean values for each parameter recorded. Standard deviation is presented alongside minimum and maximum values in each data set to enable qualitative assessment of stability of both reactor operation and performance. Where important trends are identified in the data, results are presented graphically, again to enable qualitative assessment of the conditions in the reactors. Statistical evaluation of difference in operation and performance data during each phase was conducted using the one-way analysis of variance (aov()) function in the R software package and P-values for significance stated.

Low-read count samples were excluded from the analysis of sequencing data with a cut-off threshold of 5000 reads per sample. To account for differences in sequencing depth between the remaining samples, sequencing data was normalised by calculation of relative abundance for each OTU or taxa in the sample. For calculation of ecological indices, each sample was rarefied to a common minimum, which was the minimum read count sample for the whole sample set for the experiment. Ecological indices calculated were rarefied richness, Simpsons Index of Diversity and Pielous Evenness Index and each was calculated using the R-Vegan software package. Phylogenetic distances between samples were calculated using a QIIME based phylogeny file in the Phyloseq package in R with taxonomic identification of OTUs conducted by sequence comparison with the RDP Classifier database. Two-dimensional NMDS plots and supporting multivariate analysis of variance were conducted using two alternative distance metrics to describe the sequencing data. Bray-Curtis similarity matrices were utilised to determine variance based on percentage abundances in the count data. Secondly, phylogenetic distances were obtained using the generalised Unifrac method, alpha=0.5, which calculates distances based on difference in branch lengths of the phylogenetic trees between samples. Both methods were conducted using the Phyloseq package in R. Correlation analysis was utilised to investigate potential syntrophic relationships between key organisms. Finally the Kruskal-Wallis test was utilised to determine organisms whose relative abundance varied significantly between phases and with depth in the sludge bed.

6.4 Results and Discussion

The results section is divided into four key components. Firstly, recorded operating data are assessed against design operating parameters for each of the replicate reactors (Section 1.4.1). Performance monitoring data is then presented and analysed to assess replication between reactors during each phase, and between mean performances of the triplicate set between each phase (Section 1.4.2). The development of community physiology under each operating condition is then presented and discussed (Section 1.4.3). Finally, data from the NGS 16S rRNA gene survey is used to assess microbial community ecology and dynamics (Section 1.4.4).

6.4.1 Reactor Operation

6.4.1.1 Reactor Operation: Replication Between Reactors

Operating parameters monitored were reactor temperature, and substrate characteristics; pH, influent COD profile and influent VFA profile. Table 6-3 shows the summary statistics for operation averaged for each phase of reactor operation.

Table 6-3: Summary operating data for bioreactor set R1-3, Phases 1-4

Parameter	Statistic	Phase 1	Phase 2	Phase 3	Phase 4
Influent pH	Mean	6.36	6.07	5.95	6.42
	Minimum	5.40	5.82	5.25	5.95
	Maximum	11.16	6.54	6.53	7.33
	St. Dev	1.18	0.24	0.33	0.43
Temperature / degC	Mean	36.9	23.2	17.5	15.3
	Minimum	34.5	20.1	13.9	14.2
	Maximum	38.4	37.5	25.6	16.2
	St. Dev	0.5	5.6	3.6	0.4
CODin / mg/L	Mean	942	684	735	453
	Minimum	305	492	340	286
	Maximum	2277	907	1320	841
	St. Dev	468	148	241	179
sCODin / mg/L	Mean	537	531	536	263
	Minimum	63	413	198	92
	Maximum	1093	817	836	428
	St. Dev	189	143	220	107
pCODin / mg / L	Mean	405	153	199	190
	Minimum	36	49	-22	-9
	Maximum	1883	309	598	413
	St. Dev	465	91	192	149

As each reactor in a triplicate set was fed from a single feed tank, substrate characteristics are assumed to be identical for each reactor in the set. Temperature was controlled on a per-reactor basis and ANOVA used to assess replication. A significant difference

($P<0.001$) was found between mean reactor temperature during Phase 1 only however the difference in mean temperature was small. Mean operating temperature recorded during Phase 1 for each of R1-3 was 36.9degC, 37.1degC and 36.8degC respectively. As no significant difference was found for operating temperature during subsequent phases of operation the reactors are thought to have been operated well as replicates for the duration of the experiment.

6.4.1.2 Reactor Operation: Qualitative Analysis of Operational Parameters Between Phases

A key aim of the experiment was to introduce step-wise changes in engineered parameters such that a single change was introduced between each phase as follow:

- Phase 1-2: Temperature change from 37degC to ambient temperature, all else constant
- Phase 2-3: Temperature change from ambient temperature to 15degC all else constant
- Phase 3-4: Substrate change from low-strength distillery waste to low-strength SYNTHES, all else constant including temperature

The aim in so doing was to enable each phase to act as a control for that which followed such that the influence of each step change could be elucidated by direct comparison with the previous phase. This was unfortunately not achieved. The following section of the report identifies the errors incurred by both qualitative and statistical analysis of the operating data (Table 6-3) and aims to identify the arising strategy for subsequent data analysis.

Influent pH

Mean influent pH ranged from 5.95 to 6.49. It was intended that influent pH should be held constant throughout the trial. Whilst mean influent pH was not absolutely constant across each phase the difference between phases was not found to be significant.

Temperature

Temperature was a test variable and the nearness to target temperature at each phase is considered. During Phase 1 target temperature was 37degC, and as mean reactor temperature achieved was 36.9degC, temperature control was deemed satisfactory for Phase 1. The design temperature change from Phase 1 to Phase 2 was from 37degC to

ambient temperature (~22degC). No ramping was used between Phase 1 and Phase 2 such that the temperature change was both instantaneous (<12hrs) and consistent across the reactor set as per the experimental design. The design temperature between change between Phase 2 and Phase 3 was from ambient temperature to 15degC and no subsequent temperature was intended to occur i.e. 15degC was to be maintained throughout Phases 3&4. Here error was incurred. Mean operating temperature during Phase 3 was 2.5 degC above target. The reason for this was difficulty experienced in controlling reactor temperature when the cooling system was initially brought on-line. Those problems were overcome by trial and error such that by the end of Phase 3, target operating temperature was achieved. As target temperature was applied consistently during Phase 4 but not during Phase 3, a single stepwise change was *not* achieved between Phases 3 and 4 in that both substrate type and mean operating temperature changed in a single step. Thus, whilst good replication between reactors is maintained the notion of the control by response in the system during the previous phase is somewhat weakened.

Influent COD Profile

It was intended that the absolute value of COD should remain constant throughout each Phase of the experiment i.e. only temperature or substrate type would vary between phases. This was not achieved. Both total and particulate COD in the influent declined in the region of 50% over the duration of the experiment. The primary reason for this was inaccurate dilution of the industrial waste during Phases 1-3. Dilutions were prepared based on calculations from historic rather than current COD data. This resulted in under-dilution of the waste during Phase 1 particularly, and also in Phases 2 and 3 albeit to a lesser extent. Thus, recorded COD declined simultaneously with operating temperature in the reactors. This renders the series control strategy somewhat notional in that at each phase, more than a single change occurred such that the response of the microbial community in terms of composition and performance is less readily attributed to a single cause.

Influent VFA Profile

In addition to the tabulated monitoring data, the VFA profile in the feed was also monitored (Figure 6-2).

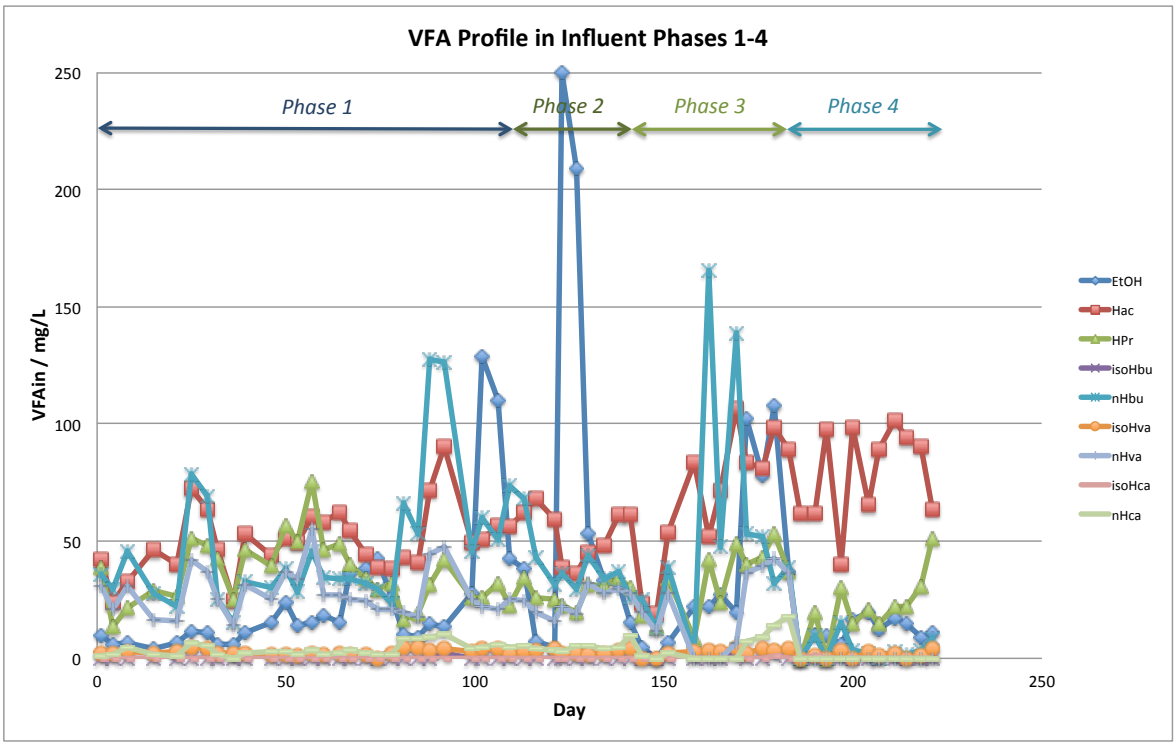


Figure 6-2: Scatterplot showing time-series of influent VFA profile during Phases 1-4. VFAs measured are: ethanol (EtOH), acetate (Hac), propionate (Hpr), isobutyrate (isoHbu), butyrate (n-Hbu), isovalerate (isoHva), valerate (n-Hva), isocaproate (isoHca), caproate (n-Hca).

Qualitatively, the VFA profile was most stable during Phase 1 when the reactors were operated at 37degC and were notionally under least pressure from either unfavourable temperature or putatively unfavourable substrate type. An extreme spike influent ethanol concentration was observed during Phase 2 however the spike was maintained for only a brief period and no associated decline in performance was observed. During Phase 3 both acetate and butyrate concentrations increase in the reactor influent. Finally, in Phase 4, it is noted that elevated acetate concentrations are approximately maintained in a period in which pCOD in the influent is low. Indeed, during Phase 1, acetate comprised just 10% of the sCOD in the influent as compared to 31.9% of sCOD (and 18.5% of total COD) during Phase 4. As acetate is an immediate precursor to methanogenesis, this may impact the dominant route to methanogenesis. Again the variable VFA profile confirms that the intended single step change between phases was not achieved.

Approach to Subsequent Data Analysis

In interpreting the data gathered during this experiment it is important to note that the design operating conditions for the experiment were not achieved. The experiment was subject to human error in both temperature control and influent substrate profiles such that two conditions were changed at the transition between each phase. As such, arising changes in the reactor performance and microbial community profile is less readily associated with a single applied condition. It is noted however that in interpreting complex

data sets of this sort, it must be acknowledged that within a given data set, it is likely that not all determining factors are measured and evaluated such that even had satisfactory control been achieved for each monitored parameter, underlying variability in operating conditions (e.g. alkalinity, trace metals) may have occurred that was simply not documented. As interesting findings appear to have arisen in the data collected over the duration of this study in spite of poor experimental control, the performance and ecological monitoring data is presented and evaluated qualitatively against the measured conditions for each phase and statistically where appropriate. Interpretation of trends seen in relation to the applied conditions is offered which whilst tentative is thought to contribute to the field in spite of difficulties encountered in operating the reactors.

6.4.2 Reactor Performance

Reactor performance during each Phase is summarised in Table 6-4. Values of both mean and standard deviation for each parameter are presented to enable qualitative assessment of performance stability in relation to each engineered condition. The degree of replication in performance between is both statistically (ANOVA) and qualitatively to assess differences in performance arising in association with each phase.

Table 6-4: Summary statistics describing performance of reactor set R1-3 during Phases1-4

Parameter	Statistic	Phase 1	Phase 2	Phase 3	Phase 4
Effluent pH	Mean	6.87	6.63	6.45	7.04
	Minimum	6.45	6.37	6.20	6.69
	Maximum	7.59	7.08	6.72	7.34
	St. Dev	0.29	0.18	0.12	0.18
TVFA / mg / L	Mean	0.53	0.22	1.21	0.11
	Minimum	0.00	0.00	0.00	0.00
	Maximum	17.71	3.15	14.38	1.08
	St. Dev	2.16	0.69	3.30	0.29
CODrem / %	Mean	87.0	84.6	80.3	80.0
	Minimum	57.6	56.2	59.9	64.7
	Maximum	97.7	93.9	91.5	90.9
	St. Dev	7.4	7.5	7.6	7.1
sCODrem / %	Mean	86.1	87.9	84.3	73.9
	Minimum	10.5	80.4	62.2	30.1
	Maximum	96.1	94.3	100.0	89.4
	St. Dev	13.9	4.3	8.5	16.0
pCODrem / %	Mean	80.0	65.0	67.1	86.5
	Minimum	-111.8	-61.4	-28.2	-83.3
	Maximum	110.1	101.4	115.5	223.1
	St. Dev	30.9	34.2	28.7	52.3
BPR / L / Lrxr / d	Mean	0.40	0.31	0.23	0.13
	Minimum	0.09	0.13	0.02	0.02
	Maximum	0.70	0.51	0.44	0.33
	St. Dev	0.12	0.10	0.11	0.05
CH4 / %	Mean	72.4	70.8	60.2	57.2
	Minimum	33.2	54.4	19.5	18.6
	Maximum	82.4	78.8	83.4	78.9
	St. Dev	7.8	4.6	15.2	13.3

6.4.2.1 Reactor Performance: Analysis of Replication Between Reactors

No significant difference was found between reactors for any performance parameter during any phase with a single exception; biogas production rate (BPR) which was significantly different between the replicates during Phase 4. Recorded production rates during Phase 4 were 0.15, 0.13, 0.10 $L_{biogas}/L_{rxr}/d$ for each of R1-3 respectively. The cause of this difference is unknown however, as a single difference across a range of parameters at a range of operating conditions, it is concluded that the reactors performed reasonably well as replicates for the duration of the experiment.

6.4.2.2 Reactor Performance: Analysis of Difference in Performance Between Phases

As reactors performed well as replicates, the data is subsequently analysed for the reactor set rather than on a 'by reactor' basis. In contrast to the analysis of replication between *reactors*, it was determined that there *was* significant difference in performance between each *phase* ($P<0.05$, ANOVA) for each parameter tested with the exception of TVFA. It is noted that during each phase there was negligible fatty acid accumulation indicating that despite applied stresses to the system via temperature reduction or substrate type, the reactors performed well throughout the trial.

pH

Qualitatively, mean effluent pH is noted to vary non-linearly with time. Higher pH was seen to occur during Phases 1 and 4 in which proportionately higher solids load was applied to the reactors. This is concurrent with the findings of both Chapter 4 and 5 in which increased effluent pH was tentatively proposed to result from increased ammonia production during solids degradation.

COD Removal

Overall, COD removal efficiency remains relatively stable throughout the trial however a slight decline occurs as each additional stress is induced in the system. Data for soluble COD shows that sCOD removal efficiency remains relatively constant across the temperature change from 37degC to 17.5 degC (Phases 1-3) but drops considerably during the temperature decrease to 15degC. As this temperature change occurred simultaneously with the switch to SYNTHES as a substrate, the exact cause of this decline cannot be fully elucidated. In Chapter 5 however it was observed that at mesophilic temperatures, high-strength (5000 mgCOD/L) SYNTHES appeared no less readily degradable than the distillery waste used in terms of soluble COD removal efficiency. As such, it seems

unlikely that the substrate composition itself was unfavourable in terms of sCOD removal efficiency in this instance. Rather, it may be the case that at low temperature the microbial community was less able to adapt to the change in substrate applied. An alternative interpretation may be drawn from the VFA profile in the reactor influent. It was observed that during Phase 4 in which COD removal efficiency declined, the VFA profile in the reactor influent shifted to be dominated by acetate, which comprised 32.9% of soluble COD in the reactor influent as compared to only 10-14% in previous phases. It may be the case that when substrate composition is dominated by a direct precursor to methanogenesis that a stress is placed on the methanogenic community that negatively impacts sCOD removal. It is noted however that no acetate accumulation occurred. A final interpretation might be that the decline in removal efficiency relates not to the change in applied conditions specifically but to the length of time at which the reactors have been operated below the mesophilic temperature range and here it is noted that the series control strategy presents a serious weakness in relation to that of the parallel control. Of the COD removal efficiencies is it noted that particulate COD removal efficiency was the least stable, which was again highlights issues with solids degradation in the EGSB. Assessed by qualitative comparison of the range of removal efficiencies recorded, the least stable period of pCOD removal efficiency occurred during Phase 4 of operation (SYNTHES at 15degC). This is not reflected in the mean removal efficiency for pCOD however, which is higher during Phase 4 than at other periods of the trial. The variability in pCOD removal is not reflective of either the stability of pCOD in the reactor influent or the absolute quantity of pCOD in the reactor influent and as such is assumed to reflect the temperature condition rather than the substrate type. That solids may be less readily soluble at lower temperatures may have influenced this result however no firm conclusion can be drawn here at present.

Biogas and Methane

Both biogas production rate and methane content in the biogas are seen to decline as the experiment progresses and the decline in biogas production rate is noted to be disproportionate to the decline in COD removal efficiency. As the absolute quantities of COD delivered to the reactors also decreased over the course of the experiment however, direct comparison of biogas production rates at each phase is not appropriate in this instance. As an alternative approach to understanding the relationship between COD removal and biogas production, here the results are presented graphically expressed as litres of *biogas* per gram COD removed and litres of *methane* per gram COD removed at each phase (Figure 6-3). To provide a comparison for these results, the same parameters

are plotted for the lab-scale reactors treating full-strength distillery waste at 37degC as reported previously (Chapter 5) which is shown on the plot as Phase '0'.

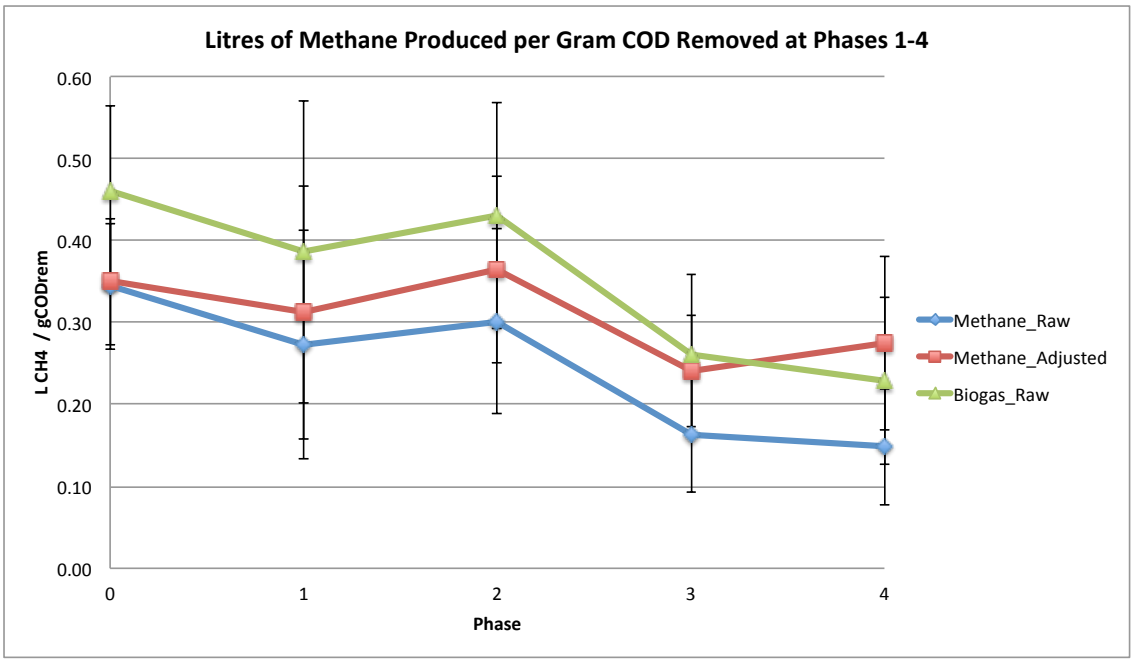


Figure 6-3: Scatterplot showing total biogas and methane generated per gram of COD removed during Phases 1-4. Data plotted as mean for the reactor set at each phase and with error bars showing standard deviation. An adjusted estimate of methane generated is also provided to account for methane lost due to increased solubility at low temperatures.

It is seen that during Phase 1, at low substrate availability as compared to the seed sludge (Phase '0'), lower volumes of both biogas and methane are produced per gram COD removed. This implies that a lower portion of the COD removed is utilised in biogas production under low substrate conditions. Extrapolating that idea, the result suggests that a greater proportion of carbon is going to the biomass under low substrate conditions than in higher substrate conditions perhaps promoting microbial growth as the community adapts to this condition. Previously in Chapter 5 however this hypothesis was discredited in that little change was seen in the microbial community between low and high substrate availability. As such, an additional plot was made here (methane adjusted) to account for the solubility of methane in water to investigate the possibility that the decrease in methane production rate seen here was related to methane losses in the system rather than to decreased productivity. Calculation of these adjusted values assumed maximum possible solution of methane in the reactor effluent at each temperature and losses arising due to passage of 30L/d effluent through the reactor (VLR). In so doing, it is seen that the apparent decline in productivity is greatly reduced. As the total volume of methane produced in the low-strength reactors is low, it appears that as the relative proportion washed out in solution in the reactor effluent is increased which disproportionately impacts the apparent productivity of the low-strength reactors. As 'real world' operation of low-

strength reactors would likely involve process optimisation via increasing VLR and hence OLR, this effect may be more exaggerated in full-scale applications of low-strength digesters. Thus it is noted that in terms of methane recovery from low-strength systems, methane content of the reactor liquor should be in the least monitored and preferably recovered to improve methane yield and to minimise environmental degradation via methane emission from the effluent. During Phase 2 of operation (low-strength distillery waste at 22degC) the reactor productivity as assessed by the adjusted biogas plot are seen to recover to the same level of productivity of those reactors treating full-strength distillery waste at 37degC (as reported in Chapter 5 previously). This is an informative finding in that it suggests that ambient temperature treatment of low strength wastes in EGSB reactors is viable. Again considering the adjusted plot, productivity in the reactors is seen to decline with the shift to 17.5deg but to recover somewhat at the shift to 15degC. The recovery may relate to the length of time for which the reactors were operated at low temperature or alternatively may be a response to changing substrate. Either way, if future work demonstrates this recovery to be maintained in the longer term, then in terms of treatment of sewage at low temperatures recovery this is a positive finding.

Performance Summary

In all it is thought that good COD removal (>80%) was maintained at low temperature under low levels of substrate availability. It is noted however that the removal of particulates appears to be increasingly problematic at low temperatures, which in real world applications would have impacts on downstream treatments. Further it is noted that whilst methane produced per gram of COD removed decreased only slightly at low temperature, the total amount of COD removed also declines such that both water treatment and methane yield are negatively impacted. For real world applications a full energy balance should be conducted specific to temperature, biomass and waste stream to determine whether methane lost at low temperature is more or less than that required to increase reactor temperature. Finally it is noted that the length of the trial here is perhaps insufficient to conclude absolutely the success or failure of the low-temperature SYNTHES case, particularly in the absence of parallel controls.

6.4.3 Community Physiology: Specific Methanogenic Activity

The specific methanogenic activity in the biomass was recorded for the seed sludge at the experiment outset. Subsequently biomass was sampled from the top and bottom of the sludge bed as described previously at the end of each phase. Activity was measured at 37degC at the end of each phase, and additionally at mean reactor temperature during those

phases in which reactor temperature was below 37degC (Phases 2-4). Mean activity is presented in Figure 6-4 (37degC) and Figure 6-5 (mean reactor temperature) with error bars showing standard deviation calculated from the triplicate biomass samples tested for each substrate.

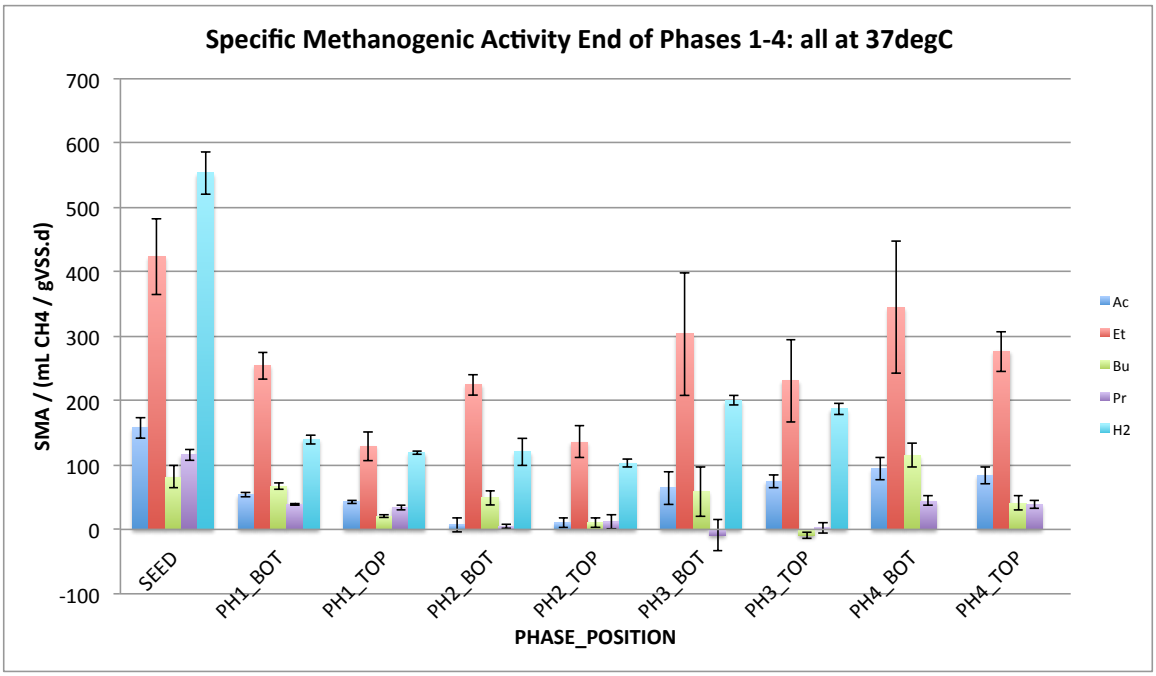


Figure 6-4: Grouped bar plot showing SMA recorded at 37^{O}C in seed sludge (SEED) and in biomass from the top and bottom of the sludge bed at the end of each phase in the trial against specific substrates: acetate (Ac), propionate (Pr), butyrate (Bu), Ethanol (Et) and hydrogen (H2). Bars show blank-adjusted mean and error bars show standard deviation of triplicate measurements.

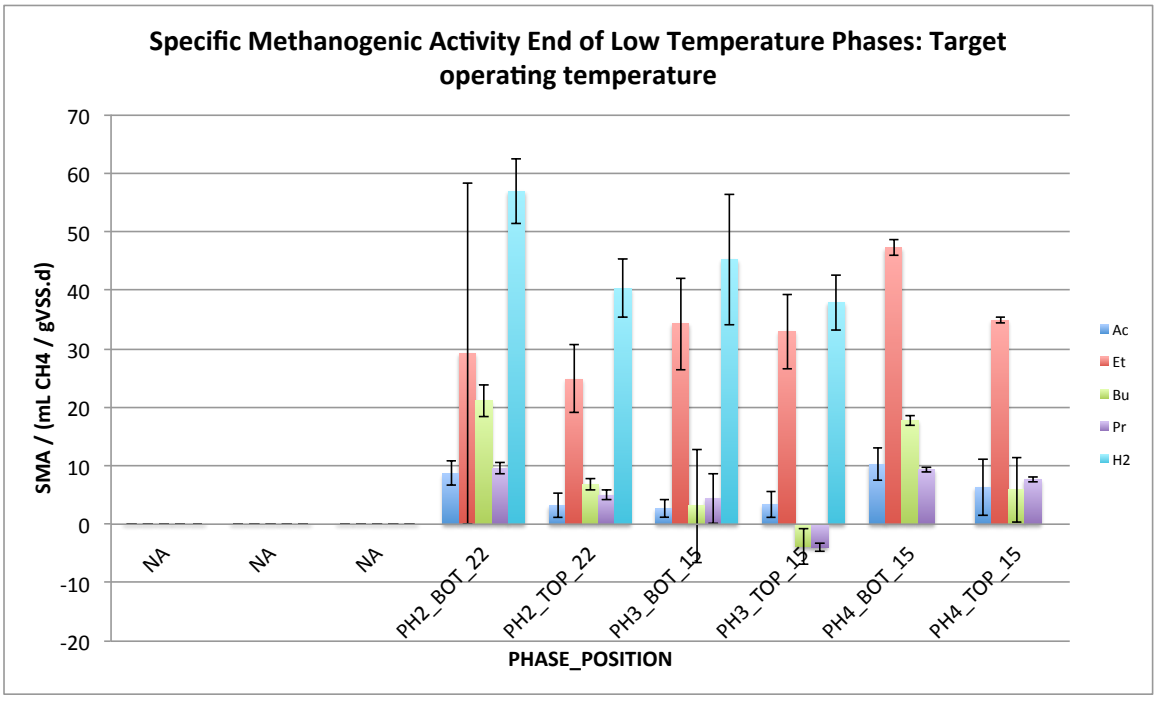


Figure 6-5: Grouped bar plot showing SMA recorded mean reactor temperature in biomass from the top and bottom of the sludge bed at the end of each phase in which the reactors were operated below 37^{O}C i.e. Phases 2-4 (PH2_ to PH4_) against specific substrates: acetate (Ac), propionate (Pr), butyrate (Bu), Ethanol (Et) and hydrogen (H2). Bars show blank-adjusted mean and error bars show standard deviation of triplicate measurements.

6.4.3.1 Route to Methanogenesis

During Phase 1 of the experiment good replication was seen between the triplicate samples used to assess the SMA as is evidenced by low standard deviation from the mean. In the SMA trials conducted at the end of lower temperature phases (2-4) the standard deviation tended to increase suggesting that granules may have adapted to new conditions at different rates hence increasing variability in the biomass. Qualitative assessment of activity measured during each phase using acetate and hydrogen as substrates, each of which is assumed to act as an immediate precursor to methanogenesis, shows that activity is higher using hydrogen during each of Phases 1-3 at both 37degC and at mean reactor temperature. This may indicate that hydrogenotrophic methanogenesis is the dominant metabolic route in the biomass and is concurrent with the activity profile of the seed sludge. SMA using hydrogen was not measured at the end of Phase 4; however, as activity using acetate remains low whilst methane content in the biogas remained stable, it might be assumed that no shift occurs during this phase.

In both natural and engineered environments, the majority of methane produced has historically been assumed to occur via the acetotrophic pathway (Müller, Sun et al. 2013). An increasing body of evidence however suggests that under specific conditions such as stress induced by high acetate concentration or distinct community composition, syntrophy between acetate oxidising bacteria and hydrogenotrophic methanogens can enable dominance of the hydrogenotrophic pathway (Hao, Lü et al. 2010, Muller 2013). Acetate here was a dominant VFA in the reactor influent however concentrations were not thought to be sufficient to stress acetotrophic methanogens. The seed sludge however was noted to be significantly more active in relation to hydrogen ($P<0.001$) as compared to acetate suggesting that an inherited community structure may have influenced the pathway in this instance. It was observed in Chapters 4 and 5 that in relation to the treatment of full-strength distillery waste and SYNTHES in lab-scale reactors, that acetotrophic activity in the same biomass gradually increased over a 3-month period suggesting a ‘recovery’ to ‘normal’ carbon flow in the system. That the hydrogenotrophic pathway is maintained in these reactors may suggest that either low substrate availability in combination with low temperatures, or variable operation of reactors, is sufficient to stress the acetotrophic methanogens and maintain dominance of the hydrogenotrophic pathway.

6.4.3.2 Community Physiology with Phase of Operation

Scatter plots indicate that a substantial decline in activity occurred between the seed sludge ('Phase 0') and the sludge at the end of Phase 1 of operation in response to low substrate availability (Figure 6-6).

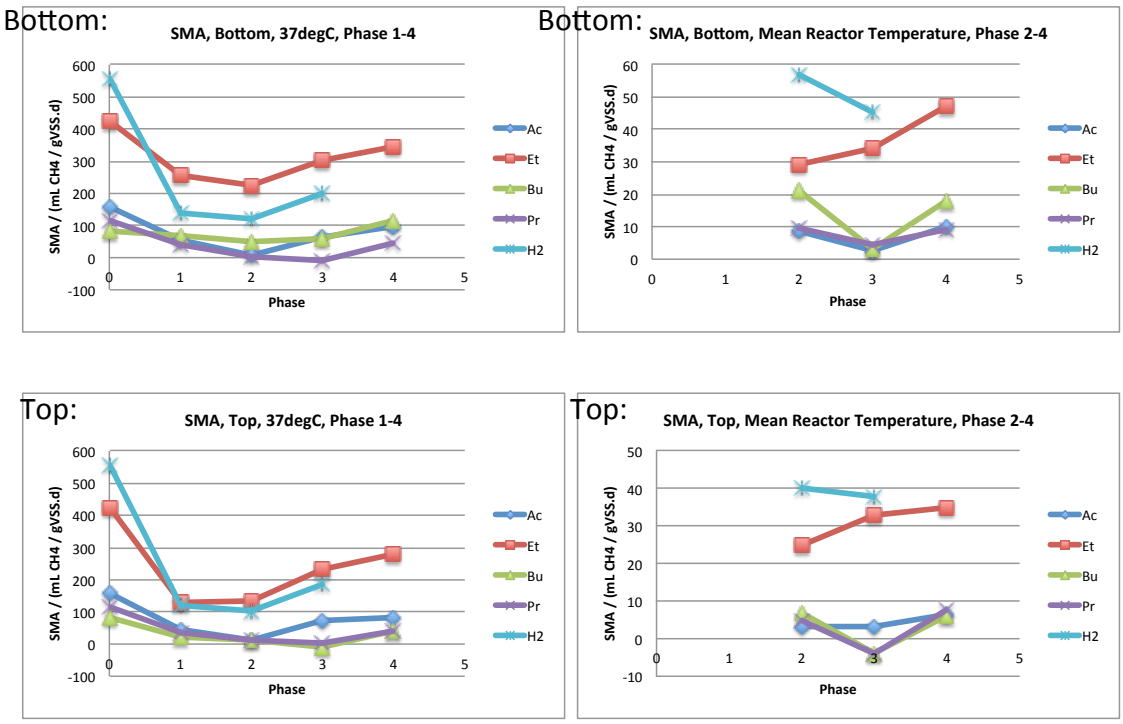


Figure 6-6: Scatterplots showing SMA trends for activity in the biomass with phase and depth at 37^{O}C and at mean reactor temperature.

The decline in activity continued although less markedly during Phase 2 (22degC) as demonstrated in activities measured at both 37degC and at 22degC. A similar trend is observed again during Phase 3 at 15degC. During Phase 4 and the treatment of SYNTHES at 15degC however, activity measured at mean reactor appears to show some recovery albeit to levels well below that seen in the seed sludge. The influence of substrate change on community activity cannot be distinguished from the influence of time available for community adaptation in this instance again highlighting a weakness in the in-series control method. To some extent this data appears to conflict the reactor performance data shown previously that demonstrated little decline in COD removal efficiency at lower temperatures. It is noted however that the SMA measures the maximum potential activity in the biomass such that a truer interpretation of the result might be that reduced potential productivity induced in the biomass by low temperature does not impact reactor performance under low loading conditions (OLR).

It is also observed that in each test, activity measured at 37degC is as much as ten-fold higher than that measured at mean reactor temperature. This phenomenon is widely

reported in the literature (Connaughton, Collins et al. 2006, Enright, Collins et al. 2007, Madden, Chinalia et al. 2009) and is typically used to evidence the fact that the biomass is not developing a psychrophilic community, rather that the community developed may be described as psychrotolerant. Indeed this might be expected as no new species were introduced to the system that might confer psychrophilic metabolism. The increase in activity recorded when temperature is temporarily elevated for the SMA measurement, potentially offers an interesting opportunity in terms of low-temperature reactor operation. The SMA is conducted over a relatively short time period such that the duration of this increased productivity is not known. Further research into this effect to determine whether fluctuating temperature in a reactor might promote productivity in the biomass whilst reducing the overall energy costs as compared to mesophilic operation may be of value.

6.4.3.3 Community Physiology with Depth in the Reactor

Finally, similarly to the stratification of activity in the biomass with depth in the reactor demonstrated in Chapter 4 for the treatment of distillery waste in 1-D reactors, the SMA tests again indicate a relationship between the depth from which the biomass is sampled and the relative activity recorded for the sample. During each phase and at each temperature, mean activity measured is greater in biomass from the bottom of the sludge bed than that measured in sludge from top of the sludge bed. This suggests that some consistent structure exists within the sludge bed possibly underpinned by a gradient in the microbial community membership or structure. Table 6-5 summarises the result of one-way ANOVA for each substrate at each phase and temperature tested between the top and bottom sludge samples. Qualitatively activity was higher in biomass from the bottom of the sludge bed for each substrate tested at each phase, and statistically, this difference was in many instances determined to be significant (ANOVA). It is seen that the difference in activity with depth in the sludge bed is least pronounced during Phase 3, which may relate to a recorded flux in bed-depth during initial operation at 15degC. Subsequent to the temperature drop from 22degC to 15degC, it was observed that the height of the sludge bed in each of the three reactors increased by ~20% as compared to the previous period. This expansion was observed to decline again during the subsequent period. The increase in height recorded was attributed to increased bed expansion rather than growth of the biomass for two reasons. Firstly, during Phase 3 the sludge bed was visibly more fluid in each reactor, and secondly, the subsequent recovery to the previous bed depth during Phase 4 indicates that growth did not occur to the same magnitude of the bed expansion as no wash out of biomass was observed.

Table 6-5: Comparison of sludge activity with depth for each of specific substrates: acetate (Ac), propionate (Pr), butyrate (Bu), Ethanol (Et) and hydrogen (H2).

Test	Ac	Et	Bu	Pr	H2
Phase1_37	*	**	***	N	*
Phase2_37	*	N	**	**	*
Phase2_22	N	**	**	N	N
Phase3_37	N	N	*	N	*
Phase3_17.5	N	N	N	*	N
Phase4_37	N	N	**	N	NA
Phase4_15	N	***	**	***	NA

Key:		
	N	No significant difference
	*	P<0.05
	**	P<0.01
	***	P<0.001

(Stars indicate that a significant difference (ANOVA) was found between SMA on a specific substrate between sludge from the top of the sludge bed as compared to that measured against the same substrate at the bottom of the bed at each phase and temperature (e.g. Phase 1 recorded at 37^{O}C is Phase1_37). Where no significance was found, 'N' is reported in the table. Where no measurement, and therefore no comparison was made, 'NA' is reported in the table.

During operation it was suspected that the increase in bed expansion was a physical response to reduced temperatures resulting from increased buoyancy of the granules. The motivation for this hypothesis was that at lower temperatures, increased solubility of biogas in the reactor liquor might reduce the diffusion gradient of gas leaving the granule such that it's relative density declined. If this hypothesis held true then the cause of recovery of the original bed depth during Phase 4 is unknown. Tentatively, the recovery might indicate that the granules structurally altered to enable improved evacuation of biogas from the granule such that previous buoyancy of the granules was restored. These explanatory hypotheses are somewhat speculative and indeed no formal measurement was made of either methane content in the reactor liquor or diffusion rates within the granules. This said, the phenomenon of sudden increased bed expansion did occur, and did occur simultaneously in each of the three replicate reactors. Further, with that expansion, the distinction between activity in the top and bottom of the sludge bed declined. In relation to low temperature treatment in EGSB reactors then the expansion phenomenon may be worthy of further study in that if uncontrolled such it might lead to reactor washout, or alternatively, if understood and fully controlled then the same phenomenon might be used to engineer mixing in granular sludge reactors. Further to this, that the sludge is markedly more active at the bottom of the sludge bed than at the top presents an interesting opportunity in the context of this work in that sequencing data collected with depth might inform something of the community composition or structure that is associated with elevated levels of activity. This is explored further in Sections 1.4.5 and 1.4.6 of this Chapter.

6.4.4 NGS Data Analysis: Spatial and Temporal Microbial Community Dynamics

Library construction for sequencing consisted of 108 unique barcoded samples with 6 additional samples as triplicate positive and negative controls. Across each of the 108 samples, 2942 unique OTUs were identified at 97% similarity from a total of 6306294 reads excluding control samples. The range of reads per sample was 4337 to 509212 with a median read count of 29782 reads. Three samples had total read counts below 5000 and are listed in Table 6-6.

Table 6-6: Illumina Miseq samples with total read count below 5000

Sample Name	Read Count	Reactor	Port	Day
S219	4337	R2	P4	d95
S161	4432	R2	P1	d67
S220	4730	R2	P5	d95

Each of the low read count samples are noted to be from a single reactor (R2). Further, two of the low read count samples are noted to be from the same reactor on the same day (R2, d95) and together those samples represent the 'top' of the sludge bed in that reactor. During Phase 1 however, the reactors were sampled with greater frequency than during subsequent phases, and each of the triplicate reactor set was sampled similarly during this phase. As such, it is thought that excluding those samples would influence the analysis less than rarefying all samples to the read count of the lowest samples therefore those samples were excluded from the remaining analyses. Thus there were a total of 105 samples included subsequently and each sample in the set had read counts of 5000 per sample or more. Of those samples included in the analysis, the minimum read count sample identified 374 OTUs whilst the maximum read count sample identified 1479 OTUs. Rarefaction curves plotted using R-Vegan (rarecurve()) demonstrated that saturation was not reached, even for the maximum read count sample indicating a highly diverse microbial community. As such, two data normalisation methods were applied in the following analysis: rarefaction and relative abundance as described previously.

6.4.4.1 Qualitative Assessment of Dominant Organisms

The 20 most abundant OTUs across the entire data set were determined by calculation of mean relative abundance across all samples and those OTUs were taxonomically identified using the RDP classifier database. Mean relative abundance of the OTUs and their identity are presented in Table 6-7, and those OTUs belonging to domain *Archaea* are highlighted

in blue. Cumulatively, the 20 most abundant OTUs from the set of 2942 OTUs identified across the entire data set comprises a mean of 50.9% of the total microbial community sampled. Thus, assuming the data to be semi quantitative, the sequencing data describes a skewed distribution whereby fewer than 1% of all species present account for half of the total population present.

Table 6-7: OTU ID, mean relative abundance and RDP Taxonomic classification of the 20 most relatively abundant OTUs across the whole sample set

OTU	Relative Abundance (%)	Domain	(%)	Phylum	(%)	Class	(%)	Order	(%)	Family	Genus	(%)
OTU_5	7.49	Bacteria	1	Synergistetes	0.99	Synergistia	0.99	Synergistales	0.99	Synergistaceae	unclassified_Synergistaceae	0.99
OTU_2	7.18	Bacteria	0.97	unclassified_Bacteria	0.97							
OTU_1	6.14	Archaea	1	Euryarchaeota	1	Methanobacteria	1	Methanobacteriales	1	Methanobacteriaceae	Methanobacterium	1
OTU_8	3.47	Bacteria	1	unclassified_Bacteria	1							
OTU_9	2.50	Bacteria	1	Bacteroidetes	1	unclassified_"Bacteroidetes"	1					
OTU_21	2.34	Bacteria	1	Chloroflexi	1	Anaerolineae	1	Anaerolineales	1	Anaerolineaceae	unclassified_Anaerolineaceae	1
OTU_3	2.02	Bacteria	1	Proteobacteria	1	Deltaproteobacteria	1	Syntrophobacterales	1	Syntrophobacteraceae	Syntrophobacter	1
OTU_4	1.92	Archaea	1	Euryarchaeota	1	Methanobacteria	1	Methanobacteriales	1	Methanobacteriaceae	Methanobacterium	1
OTU_3941	1.87	Bacteria	1	unclassified_Bacteria	1							
OTU_7	1.84	Bacteria	1	Proteobacteria	1	Deltaproteobacteria	1	Desulfuromonadales	1	Geobacteraceae	Geobacter	1
OTU_25	1.84	Bacteria	0.96	unclassified_Bacteria	0.96							
OTU_45	1.80	Bacteria	1	Chloroflexi	1	Anaerolineae	0.99	Anaerolineales	0.99	Anaerolineaceae	unclassified_Anaerolineaceae	0.99
OTU_10	1.64	Bacteria	0.99	unclassified_Bacteria	0.99							
OTU_6	1.60	Archaea	1	Euryarchaeota	1	Methanomicrobia	1	Methanosarcinales	0.95	Methanosaetaceae	Methanosaeta	0.92
OTU_38	1.58	Bacteria	1	unclassified_Bacteria	1							
OTU_3140	1.26	Archaea	1	Euryarchaeota	1	Methanobacteria	1	Methanobacteriales	1	Methanobacteriaceae	Methanobacterium	1
OTU_16	1.11	Archaea	0.98	Euryarchaeota	0.96	unclassified_"Euryarchaeota"	0.96					
OTU_3907	1.11	Archaea	1	Euryarchaeota	1	Methanomicrobia	1	Methanosarcinales	0.92	Methanosaetaceae	Methanosaeta	0.85
OTU_3303	1.11	Archaea	1	Euryarchaeota	1	Methanomicrobia	1	Methanosarcinales	0.96	Methanosaetaceae	Methanosaeta	0.96
OTU_32	1.09	Bacteria	1	unclassified_Bacteria	1							

Of the 20 most abundant OTUs, seven are classified as Archaea. Three of those seven OTUs are identified as of the genera *Methanobacterium* which are H2/CO2 and formate utilising methanogens (Madigan, Martinko et al. 2009). Collectively the three *Methanobacterium* OTUs account for 9.32% of the entire community sampled such that Methanobacterium is the most dominant *Archaeal* genera. Qualitatively, this supports the hypothesis that the dominant route to methanogenesis is via hydrogenotrophic methanogenesis as was derived from the SMA results. A further 3 of the 7 *Archaeal* OTUs identified amongst the most dominant 20 OTUs are of the genera *Methanosaeta*. *Methanosaeta* collectively account for 3.82% of the total community sampled and are strictly acetotrophic methanogens (Madigan, Martinko et al. 2009). A single *Archaeal* OTU was not classified beyond phylum level. *Methanosaeta* are frequently identified in EGSB reactors reported in the wider literature and are associated with granule formation as filamentous organisms central to the 'tree-ring' theory of sludge granulation (Hulshoff Pol, de Castro Lopes et al. 2004). Collectively, all *Archaea* identified in the 20 most abundant OTUs accounts for 14.3% of the microbial community sampled. Assuming the data to be quantitative, as *Archaea* across the whole community sampled collectively account for 22.3% of the total population, it is demonstrated that the most abundant 20 OTUs describes the majority of *Archaea* sampled.

The most dominant organism identified from the set (OTU 5) is bacterial and of the genera *Synergistaceae* comprising 7.49% of the total community sampled. Species of the family

Synergistaceae have been associated with EGSB reactors treating waste with a high protein load (Delforno, Okada et al. 2012) and tentatively with high lipids loading (Palatsi, Affes et al. 2012). As such, OTU 5 may be involved in early stages of degradation of organics in the waste stream. An alternative interpretation of the function of OTU 5 however may be derived. Bacteria belonging to the Phylum *Synergistetes* have been demonstrated to function as acetate oxidising bacteria even in the presence of *Methanosaeta* like organisms given the presence of an active hydrogen utilising population (Ito, Yoshiguchi et al. 2011). The high abundance of this OTU then may support the hypothesis that the hydrogenotrophic pathway is dominant in this community. This hypothesis is further supported by the high relative abundance of OTUs 3 and 7. OTU3 is of the genera *Syntrophobacter* and accounts for 2.02% of the total community population. *Syntrophobacter* is of the family *Syntrophobacteraceae*, members of which have been variously isolated from sewage sludge, marine and freshwater sediments and have been demonstrated to grow via fermentation or syntrophically with H2 utilisers (Kuever 2014) such as *Methanobacterium* as are abundantly present in the reactor sludge. Finally OTU 7 is of the genera Geobacter and comprises 1.84% of the total community population. *Geobacter* species have recently been identified with the capacity for direct electron transfer with Methanosaeta species (Rotaru, Shrestha et al. 2014) enabling *Methanosaeta* to produce methane via reduction of carbon dioxide. Together, dominance of each of OTUs 5, 3 and 7 which collectively account for 11.35% of the community appear to further support the assertion that the hydrogenotrophic pathway is dominant in this biomass.

Of the remaining bacterial OTUs identified amongst the 20 most abundant, 3 are identified as of the family Anaerolineaceae and collectively comprise 4.14% of the total community population. *Anaerolineaceae* belong to the phylum *Chloroflexi* and have been associated with carbohydrate fermentation in anaerobic systems (Yamada, Sekiguchi et al. 2006, Narihiro, Terada et al. 2012) such that functionally these OTUs may be involved in the earlier stages of methanogenesis. Seven of the most abundant OTUs are bacteria that are unclassified beyond Domain level using the RDP classifier database. Collectively, unidentified bacteria found amongst the top 20 OTUs comprise 18.67% of the total community population; a relatively large proportion that serves to highlight the necessity for further work isolating and characterising microbial species.

6.4.4.2 Qualitative Assessment of Replication Between Reactors

Having determined good replication in reactor performance during each phase of the experiment, the extent of replication in the response of the microbial community during each phase is also of interest. The mean relative abundances of the 20 most dominant OTUs in each reactor during the 37degC (Phase 1) and 15degC (Phases 3 & 4) temperature conditions are plotted in Figure 6-7.

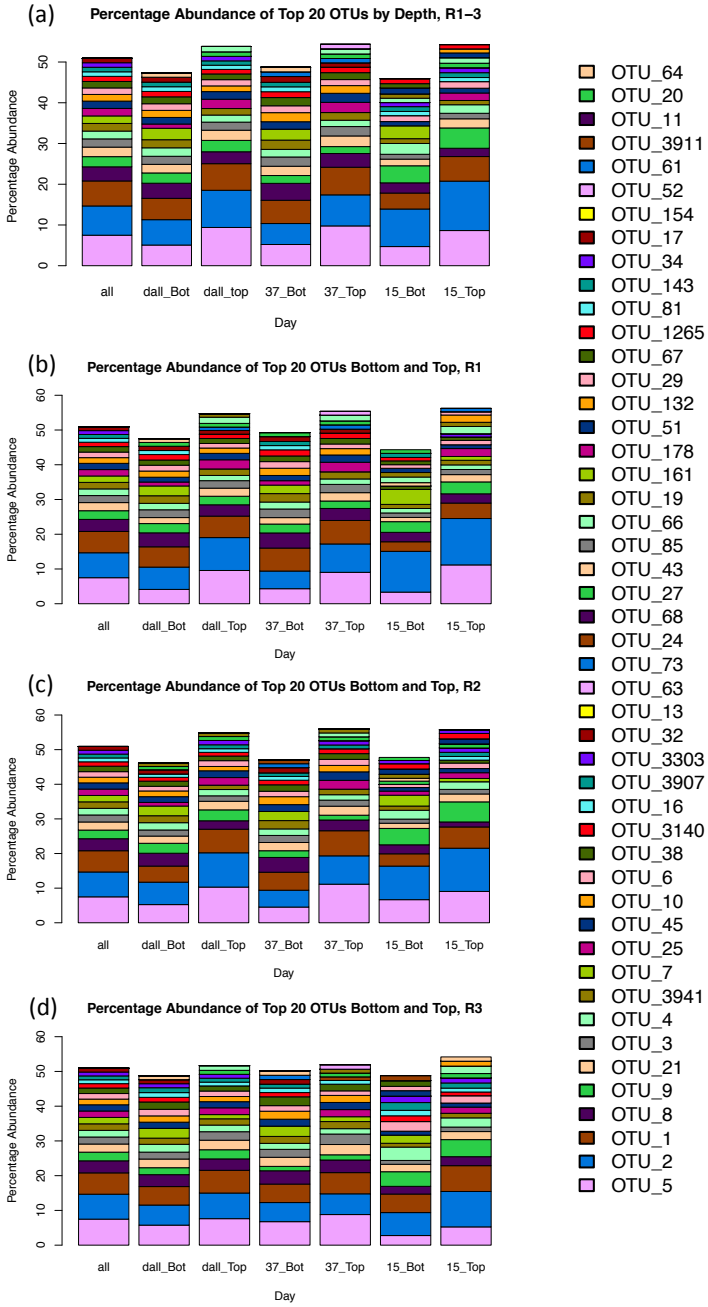


Figure 6-7: Stacked bar plots showing mean relative abundance of the 20 most dominant OTUs across the sample set for (a) the reactors as a set and (b-c) for R1-3 as individual reactors. Means are presented for all time points and depths (all), and; all time points with binning as samples from bottom (dall_Bot) and top (dall_Top) of sludge bed; time points from mesophilic operation binned as samples from bottom (37_Bot) and top (37_Top) of sludge bed; and samples from operation at 15^{O}C binned as samples from bottom (15_Bot) and top (15_Top) of sludge bed.

Qualitatively, the graphs indicate that amongst the most abundant OTUs a high degree of replication is seen between the microbial communities in each reactor at each temperature condition and in relation to depth in the reactors. This may suggest that whilst operating triplicate reactor sets enables statistical confidence in results, that little extra information is gleaned if the most dominant OTUs are of primary interest. Thus, when designing such experiments, statistical confidence must be balanced with the additional costs associated with running replicates. Further, that a single reactor seems likely to provide reproducible information on changes in the abundant community composition in response to changing conditions in a reactor suggests that if there is necessity to choose between replicate in-series trials from singular but parallel trials that the parallel method is likely to offer more insight to the microbial community response to a given circumstance. This may be evidenced here in that the community structure amongst the dominant community members appears to have changed with reduced temperature in the reactor but in the absence of a parallel control, this change cannot be distinguished from that which would have occurred in the reactor communities in response to other 'arbitrary' influences such as, for example, time.

Amongst the 20 most abundant OTUs, qualitatively OTUs 5, 2, 1, 8, 9, 21, 3, 4, 3941, 7 and 6 of the Phyla *Synergistia*, *Methanobacteria*, *Bacteroidetes*, *Anaerolineae* and *Methanomicrobia* demonstrate both persistence in the system and high relative abundances (>1%). Collectively, these OTUs may be considered to form a core community in the sludge that is relatively stable with respect to both temperature and time. The core OTUs however appear to play an interesting role in distinguishing community structure between the sludge from the top and bottom of the sludge bed. The graphs show that the top 20 OTUs at the bottom of the sludge consistently accounts for a lower proportion of the total community than do the top 20 OTUs from the top of the reactor – this trend holds true when each sampled time point is plotted without averaging as applied here. In other words, qualitative assessment of the dominant community structure at the *bottom* of the reactor suggests that the community is less skewed, or more evenly distributed, that the community found at the *top* of the reactor. That the community structure appears to vary with depth may provide some insight to the variance in activity with depth measured via the SMA tests whereby activity was highest at the bottom of the reactor in what appears to be a more evenly distributed community. OTUs contributing to this effect are most notably are OTU 5, 2 and 1. Identified previously, OTU 5 and 1 are of the families *Synergistaceae* and *Methanobacteriaceae* respectively whilst OTU 2 is an unclassified bacterium. That those OTUs increase in relative abundance at the top of the reactor, which

was previously associated with lower activity levels, does not suggest that their increased abundance reduces activity per se. More likely, increased abundance of the most dominant OTUs is likely to indicate diminished numbers of rarer OTUs such that the relative proportion of dominant species is amplified. The structural variation with depth in the reactor is considered more fully using ecology measures and NMDS plots, and is investigated statistically using Kruskal-Wallis tests in the following section of this Chapter.

6.4.4.3 Community Development in Relation to Engineered Conditions and Depth in the Reactor

Analysis of variance (adonis(), R-Vegan) was used to statistically assess the extent of replication in microbial community between the replicate reactors. It was found that reactor identity (i.e. R1, R2 or R3) *was* a significant predictor ($P<0.1$) for microbial community using Bray-Curtis similarity however reactor identity accounted for only 1.7% of the difference in the microbial community assessed across all time points. Given the complexity of the underlying community, this is thought to indicate a high degree of replication between reactors. Difference between the group reactor community was also assessed across Phases and with depth in the reactor. It was determined that feed type and temperature together accounted for 18.3% ($P<0.001$) of the difference in microbial community composition whilst the depth at which a sample is drawn accounted for 16.0% ($P<0.001$). This highlights that the engineered conditions applied drove shifts in relative abundance in the community and adds significance to the finding that the community is strongly stratified with depth under these conditions. The variable 'Day' (time) was also used as a predictor in the analysis and was attributed 32.2% ($P<0.001$) of the variance in the community. This suggests that whilst engineered conditions here have driven changes in the community that the community is dynamic and fluid in composition under it's own momentum. This highlights the importance of sampling biotechnologies at multiple time points such that community composition is better reflected in sequencing results. The time and depth relationships are further investigated using NMDS plots to visualise community development. Two distance measures are employed: Bray-Curtis similarity and GUnifrac. Figure 6-8 shows the clustering of OTUs by reactor and with feed-type using Bray-Curtis distance. It is noted that 'feed-type' here is a compound variable with temperature as SYNTHES was introduced after the temperature drop.

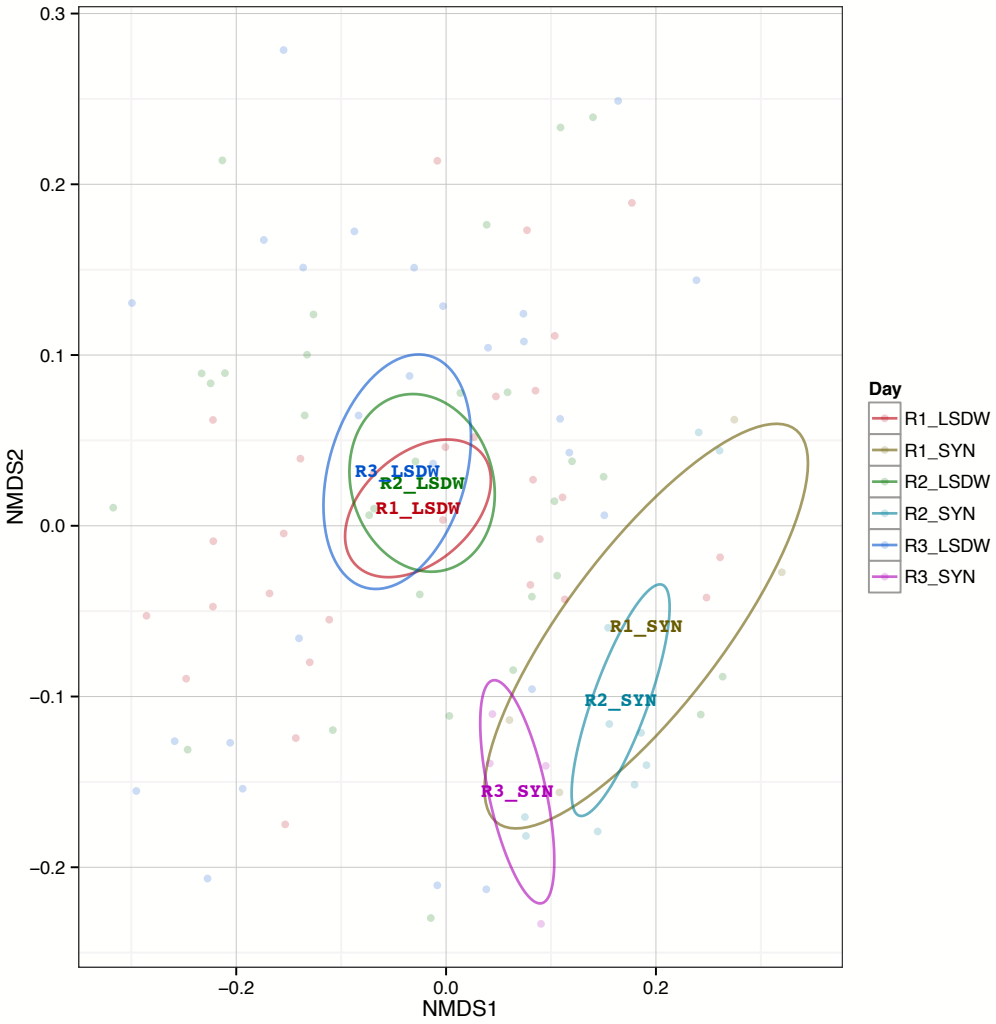


Figure 6-8: Two-dimensional NMDS ordination plot (Stress = 0.181) of the 100 most abundant OTUs in the sample set plotted using Phyloseq with Bray-Curtis similarity and grouped by reactor ID (R1-R3) and feed-type (LSDW is low-strength distillery wastewater and SYN is SYNTHES) and. In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

The reactor communities cluster distinctly by feed type. Whilst treating SYNTHES at 15degC, however R1 and R2 appear more closely replicated than R3. As no difference was found in operating condition between the triplicate reactors, this result suggest complex microbial communities subject to shifting operating conditions have the potential to exhibit multiple responses to the same shift. The ongoing shift in reactor community is visualised in Figure 6-9. It is observed that a continual progression in community composition occurred even during Phase 1 (d4-d112) in which both temperature and feed type were held constant. Indeed, the shift in community during this period appears to be greater than that occurring after the engineered changes. An explanatory hypothesis for 'slowing' of the progressive development of the community after engineered changes were introduced is that slower growth rates of the organisms at low temperature may restrict the rate of overall community development. Further, this may be compounded by slower degradation of DNA of dead organisms at low temperature, which would have the effect of

'stabilising' the community composition. Even at low temperatures however the plot indicates that communities underpinning EGSB type reactors are dynamic under relatively stable conditions and that to truly understand microbial community dynamics in relation to changing external conditions, parallel control reactors should be operated.

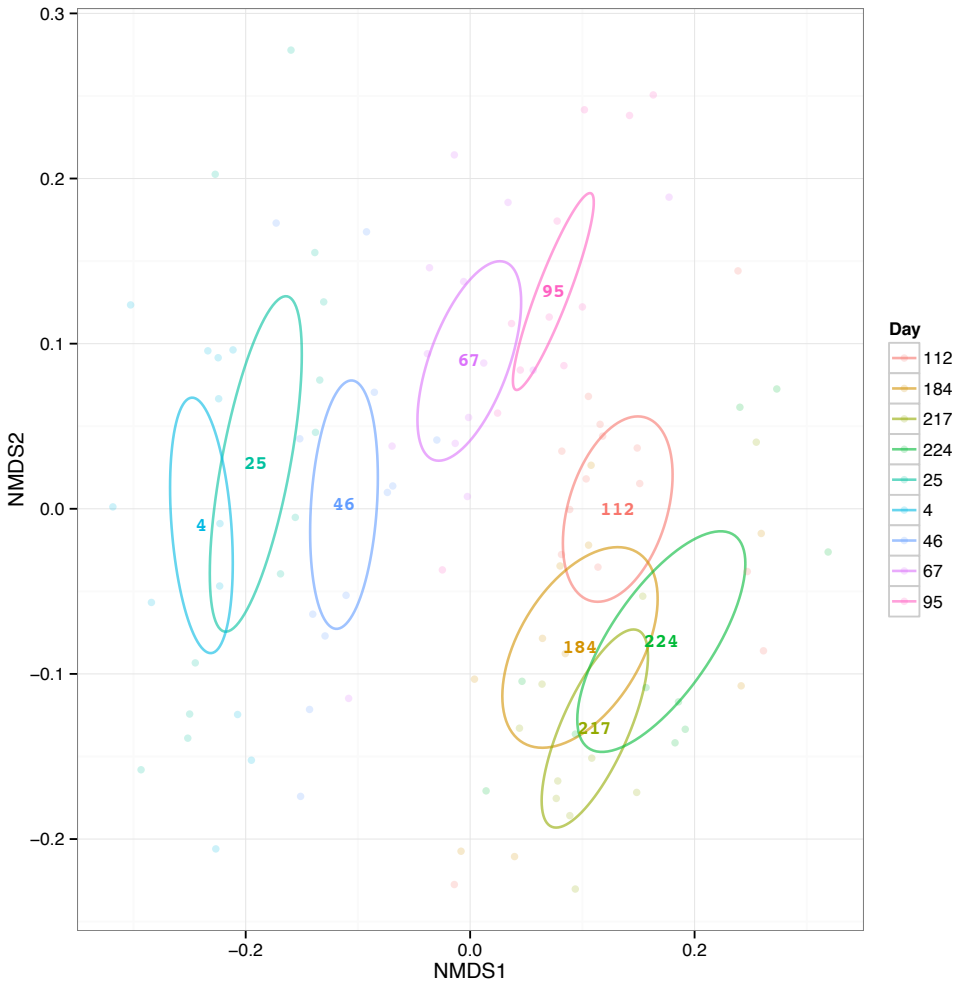


Figure 6-9: Two-dimensional NMDS ordination plot (Stress = 0.180) of the 100 most abundant OTUs in the sample set, grouped by sampling time (Day) and plotted using Phyloseq with Bray-Curtis similarity. In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

Depth resolved NMDS plots during Phase 1 at 37degC and Phases3&4 at 15degC were plotted (Figure 6-10) to investigate temperature effects on stratification in the reactors. During Phase 1, using Bray-Curtis similarity (stress=0.164) it is seen that ports P2-4 cluster as a group whilst P1 and P5 cluster distinctly. During the same phase, using GUnifrac distances (stress=0.086) no distinction for clustering with depth is seen. This suggests that whilst the community varies with depth, that the variation is in the relative abundance of species present rather than in identity of species present which is consistent with the barplots shown previously (Figure 6-7). During Phases 3 and 4 at low temperature, the Bray-Curtis similarity (stress=0.173) shows distinct clustering with each

depth and here the progression of clustering more strongly reflects the depth of sampling i.e. clusters in order P1-P5. Using GUnifrac distances here (stress=0.100), the samples cluster more distinctly than during Phase 1. This suggests stratification is maintained in the reactor at low temperature and further that if stratification is maintained in a reactor over an extended period of time that community membership, in addition to relative abundance of organisms, may also become stratified influenced.

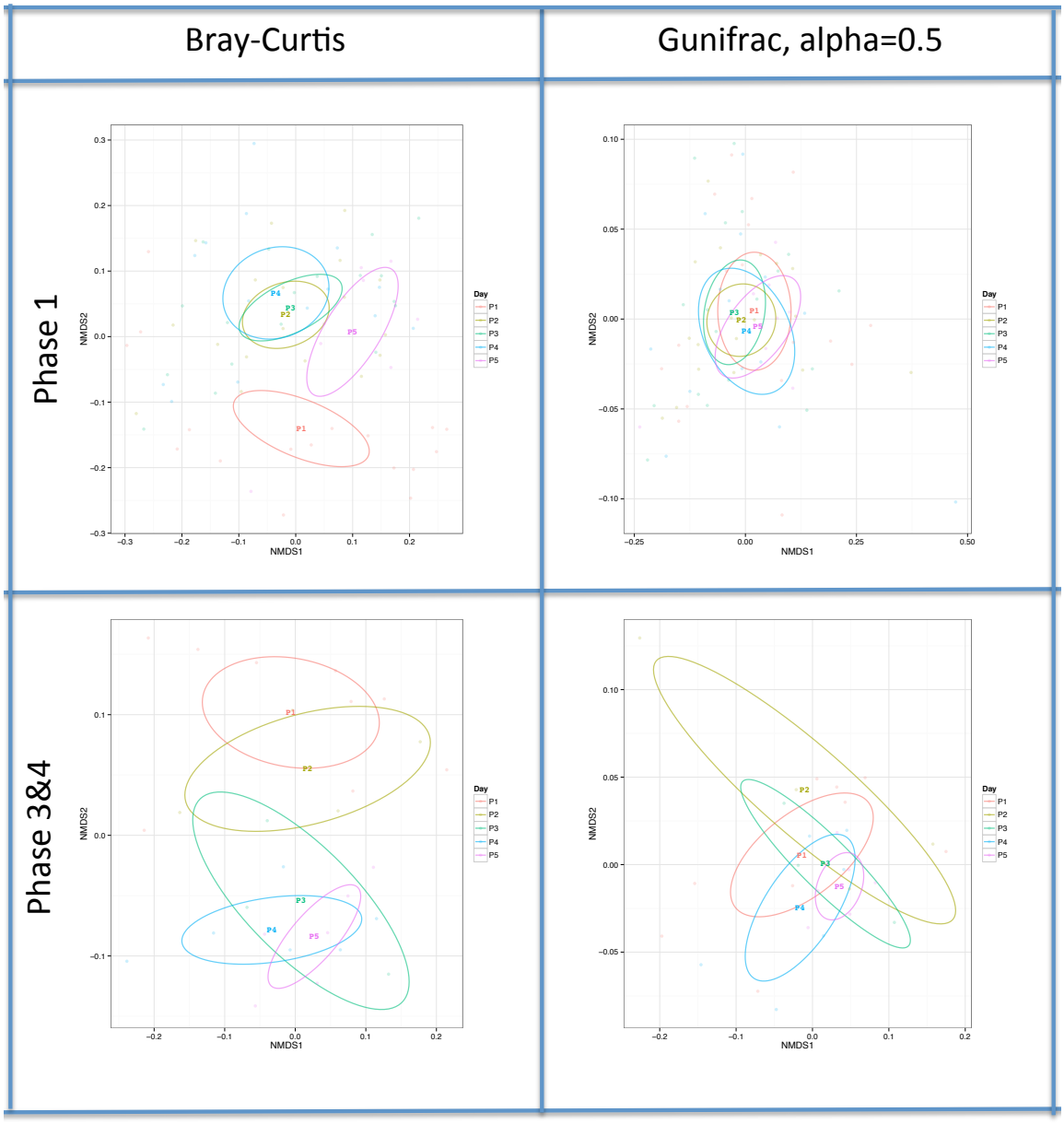


Figure 6-10: Two-dimensional NMDS ordination plots of the 100 most abundant OTUs in the sample set, grouped by sampling depth and plotted using Phyloseq with Bray-Curtis similarity (depths are shows as P1-P5, where P1 is at the bottom of the sludge bed and P5 is at the top of the sludge bed). In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

6.4.4.4 Ecology Indices

Rarefied richness, Pielous Evenness Index and Simpsons Index of Diversity, were used to investigate structural changes in community composition both in time and with depth in the reactors (Figures 6-11 to 6-13).

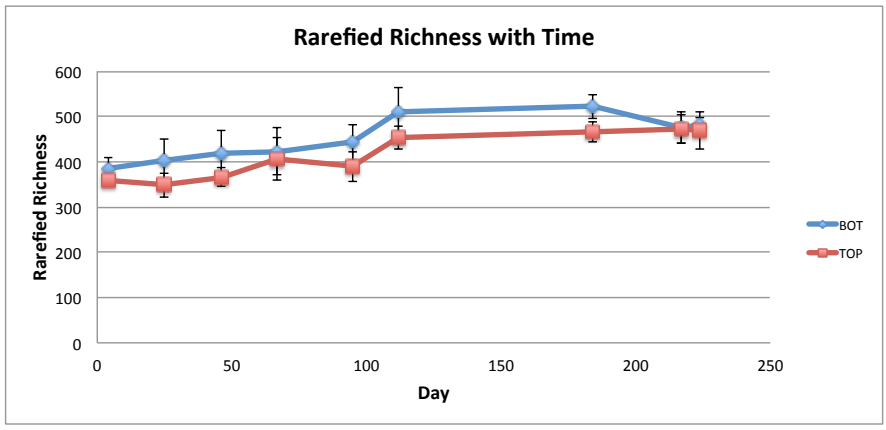


Figure 6-11: Scatterplot showing change in rarefied richness with time

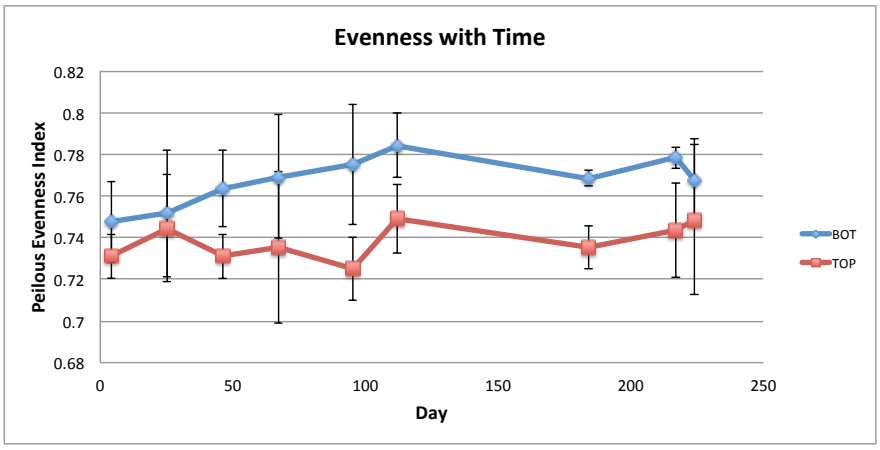


Figure 6-12: Scatterplot showing change in microbial community evenness with time

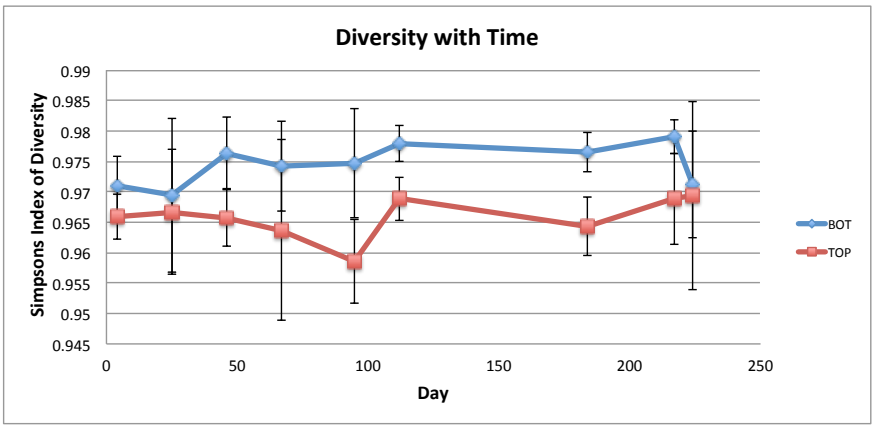


Figure 6-13: Scatterplot showing change in microbial diversity with time

(In each case, richness, evenness and diversity at each sampling time point are given as mean values calculated for the reactor set using samples from the bottom (BOT) and top (TOP) of the bioreactor sludge beds. Error bars show standard deviation.)

It is observed that rarefied richness, evenness and to a lesser extent diversity in the community tends to increase during operation at 37degC and to stabilise somewhat during low temperature operation. Tentatively, then, by assessment of COD removal and biogas production rates during the trial, increasing richness, evenness and diversity may be associated with well functioning reactors whilst stable or declining richness and evenness may be associated with less well functioning reactors.

A similar trend may be identified with depth as with time. The microbial community at the bottom of the sludge bed is characterised by a community structure in which evenness and diversity are significantly higher in each case ($P<0.001$) as compared to the same measures describing the microbial community at the top of the sludge bed. Further, it was demonstrated previously using the SMA data that sludge at the bottom of the reactor was significantly more active than at the top of the sludge bed. Thus the data describes a sludge bed in which both ecology and physiology are stratified with depth and additionally one in increased richness, evenness and diversity is associated with increased productivity in the biomass.

6.4.5 Identification of Key Taxa with Phase and Depth

The Kruskal-Wallis test was applied to determine that the relative abundance of 26 of the 36 phyla identified in the sample set were found to vary significantly with time ($P<0.001$). Plotting the relative abundances of those 26 Phyla to enable qualitative assessment of trends in the data against engineered conditions in the reactors, two distinct trends are identified (Figure 6-14). The first appears to enable identification of organisms associated with applied engineered changes whereby after d112, significantly increased relative abundance suggests that growth of those Phyla may be associated either with low temperature treatment or with treatment of SYNTHES (Figure 6-14 a-c). Phyla identified this way are *Firmicutes*, *Bacteroidetes* and an unclassified *Archaea* whose abundance all increases during the low-temperature phase of operation (d184-d224). Of the others whose relative abundances vary significantly with time however, the trends plotted may be described as approximating sin waves (Figure 6-14 d-f) and this general trend is seen in ten of the 26 phylum identified using this test. This could be interpreted to suggest that the abundance of organisms in complex communities exist in an on-going state of flux that is not directly driven by, or at least readily attributable to, external influences such as engineered operating conditions in the reactor.

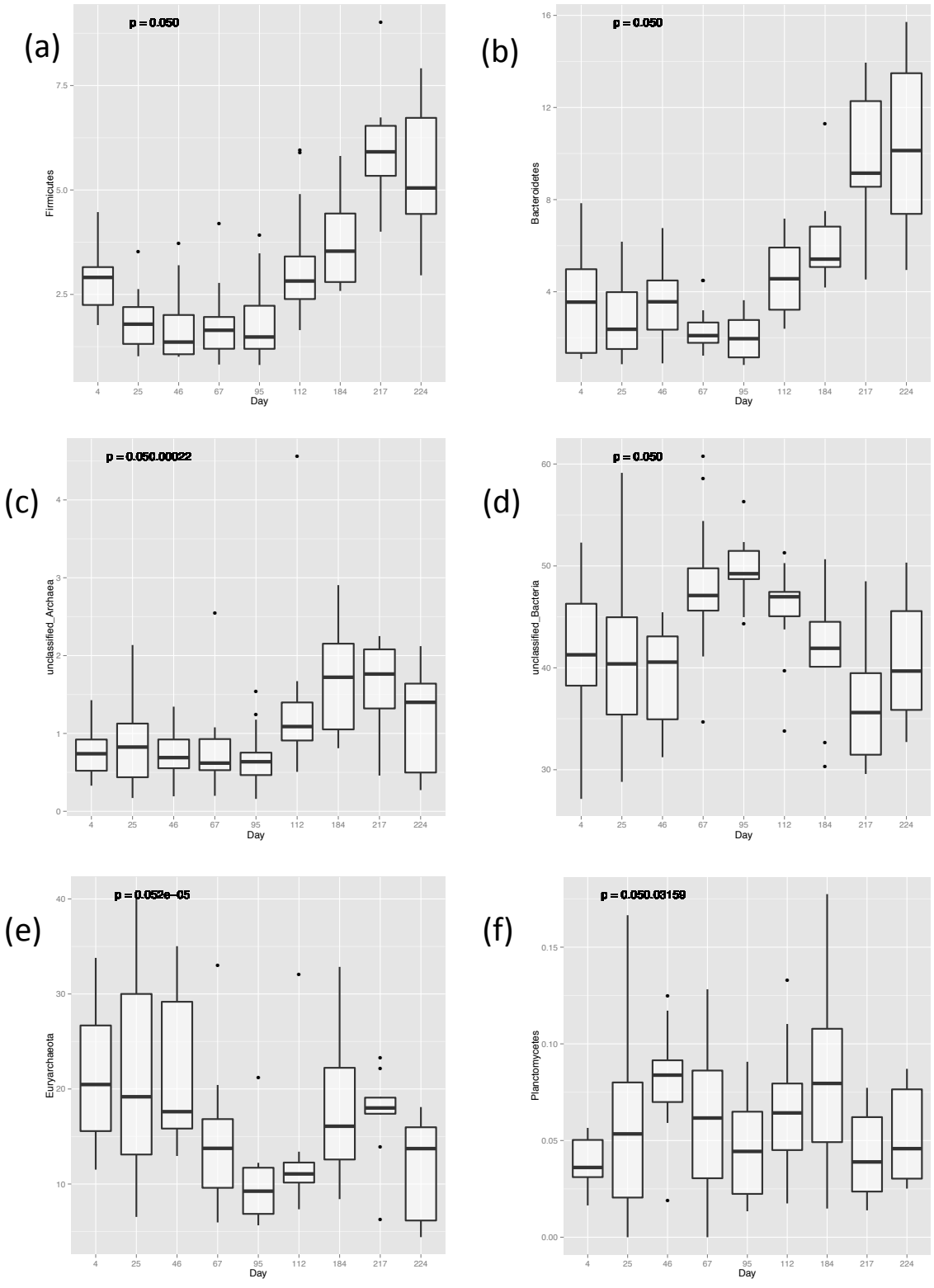


Figure 6-14: Boxplots showing relative abundance of the most dominant phyla in the sample set whose relative abundance varied significantly with time (Kruskal-Wallis with Benjamini-Hochberg correction on p-value, $p<0.05$). The bands show the median value for each group; bottom and top of boxes show the first and third quartiles; and whiskers show maximum and minimum values with 1.5 of IQR of upper and lower quartiles.

From the figure shown it is seen that the 'period' of the putative 'sin waves' is not regular across taxa. It may be the case that this arises from difference in growth rates or in competitiveness between other taxa. That the period of the sin waves is variable casts

some doubt on the association of those phyla associated with changes in operating condition as here, it may be the case the period of flux in those phyla coincides with the engineered changes applied. Again, this emphasises the importance of sampling at multiple time points in a given community such that mean abundances may be used to infer community composition for a given time period and stresses the value of operating parallel control reactors.

The Kruskal-Wallis test was also applied to identify genera whose relative abundance varied significantly with depth at each of Phase 1 (LSDW at 37degC) and Phases 3&4 (LSDW & SYN at 15degC). The samples taken during Phase 1 identify 16 genera whose relative abundance varies significantly ($P<0.001$) with depth, whereas only 5 are identified during Phases 3 and 4. As with those phyla identified as varying with time, the relative abundances of the genera identified here were plotted to enable analysis of the trends displayed. In relation to depth, the trends plotted are more immediately identifiable with the position in which the samples are drawn in that plots of median relative abundance with port tends to plot a smooth curve for each genera identified (Figure 6-15). The genera identified during Phase 1 whose abundance increases with depth are *Syntrophomonas*, unclassified *Syntrophomonadaeae*, *Geobacter* (Figure 6-15a), *Thermogymnomonas*, *Methanoregula* (Figure 6-15b), *Sulphurovum*, *Desulphovibrio*, unclassified *Desulfovibrionaceae*, *Thermotogaceae*, *BCR1 genera incertae sedis* and *Meniscus*. Those decreasing with depth are unclassified *Synergistaceae*, *WS3 genera incertae sedis*, whilst those showing no clear trend are *Clostridium sensu stricto* and unclassified *Rhodocyclaceae*. During Phases 3&4 no organism was found to significantly decrease with depth however 5 were identified whose relative abundance was found to increase with depth. Of those, *Geobacter* (Figure 6-15c) and *unclassified Syntrophomonadaceae* (Figure 6-15d) show common trends with Phase 1 whilst *Blastochloris*, *Rhodospirillaceae* and *Peptococcaceae* are distinct to the low-temperature phases. Tentatively, when considered in relation to the SMA, *Geobacter* and *Syntrophomonadaceae* whose relative abundance consistently increases significantly with depth may be associated with increased activity in granular sludge. Indeed, *Geobacter* and *Syntrophomonaceae* were additionally identified in Chapter 4 in relation to the treatment of full-strength distillery waste in 1-D lab scale reactors as more abundant at the bottom of the reactor where again the sludge was significantly more active. Given the large number of genera present however this is certainly not proven here. What can be concluded however is that stratification of activity exists with depth in 1-D type lab-scale reactors that is consistently associated with both certain taxa and with the structure of the ecology data.

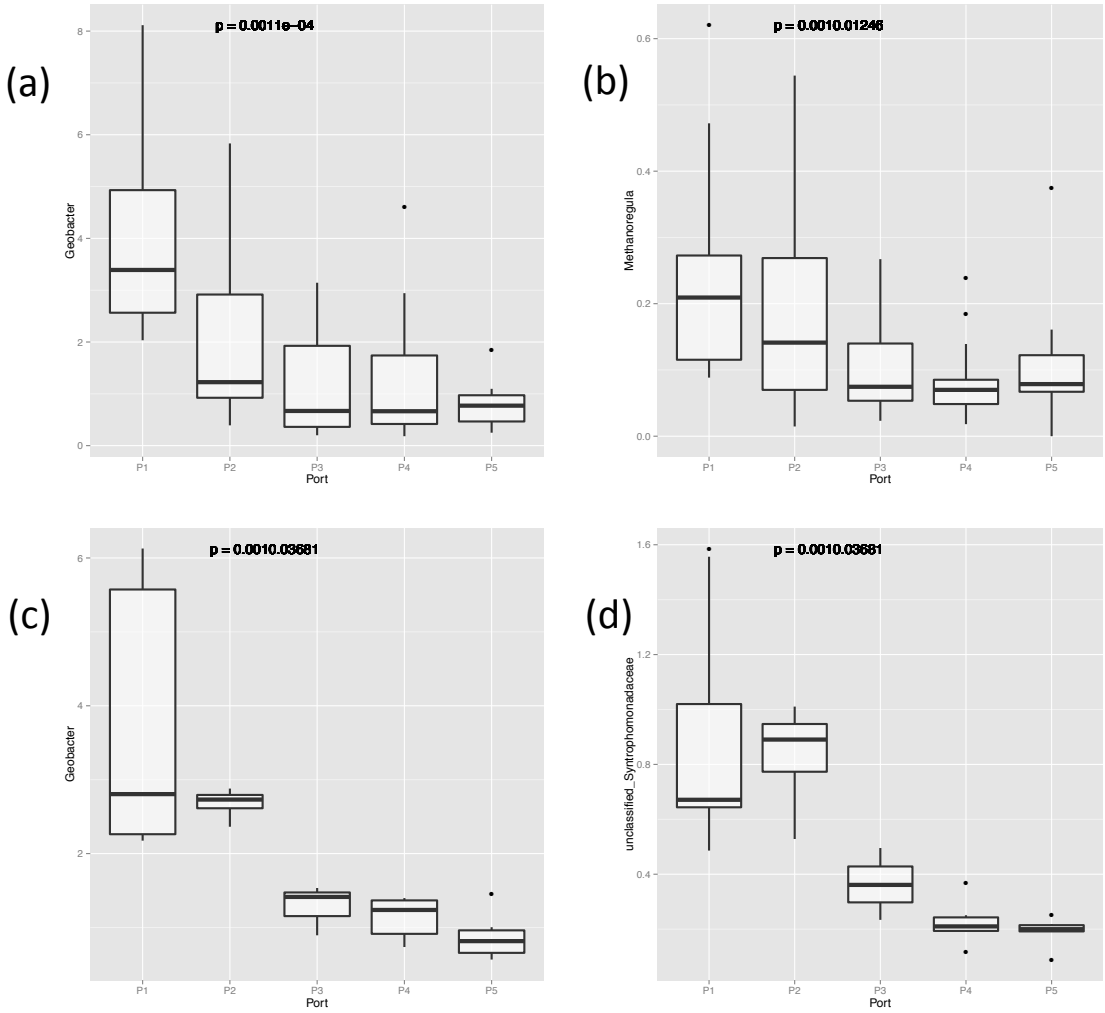


Figure 6-15: Boxplots showing relative abundance of the most dominant genera in the sample set whose relative abundance varied significantly with sampling depth (Kruskal-Wallis with Benjamini-Hochberg correction on p-value, $p<0.05$). The bands show the median value for each group; bottom and top of boxes show the first and third quartiles; and whiskers show maximum and minimum values with 1.5 of IQR of upper and lower quartiles.

6.4.5.1 Route to Methanogenesis: Correlation of Methanogenic Consortia with Putative Syntrophic Acetate Oxidising Bacteria

The high relative abundance of *Methanobacterium* in the sludge appears to support the hypothesis arising from the SMA data that the hydrogenotrophic pathway is dominant in the treatment of low-strength SYNTHES at low-ambient temperatures. As relatively high numbers of the acetotrophic genus *Methanosaeta* are also present in the consortia, correlation analysis was conducted to identify possible competitive or syntrophic relationships between the methanogens present and putative syntrophic acetate oxidising bacteria. Kendall's correlation was used to indicate the nature of the relationship between methanogens and bacteria tested using the assumption that strong positive correlation would indicate syntrophy whilst strong negative correlation would indicate competition. The analysis was conducted using the Genus level taxa table. All methanogens present in the study were included in the analysis along with those whose genera has been associated

with syntrophic acetate oxidation the wider literature: *Clostridium*, *Thermoacetogenium*, *Thermotoga*, *Syntrophaceticus*, (Westerholm, Roos et al. 2010), *Synergistetes*, (Ito, Yoshiguchi et al. 2011), *Geobacter*, *Anaeromyxobacter* and *Syntrophus* (Hattori 2008). The resulting correlation plot shows only those organisms from this set that were determined to be present in the sludge samples (Figure 6-16).

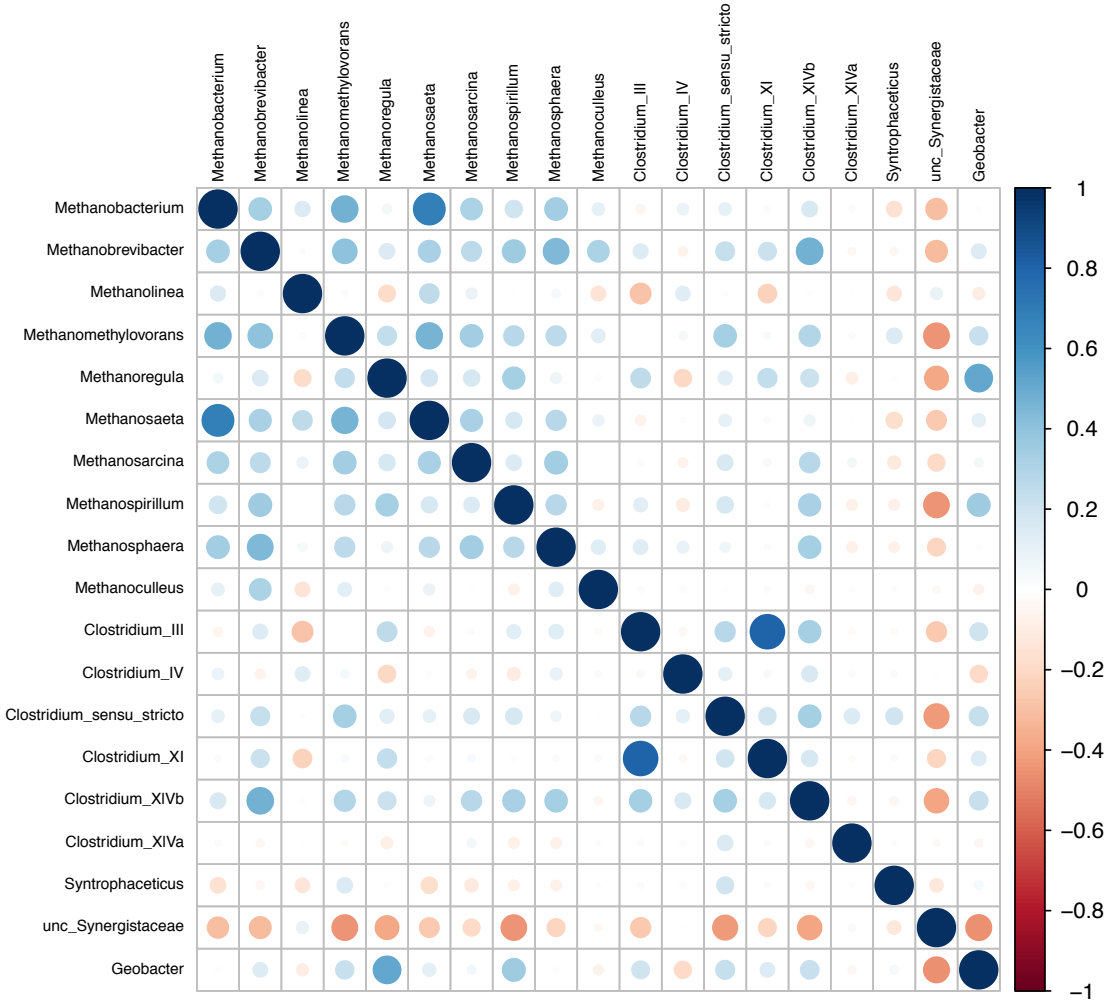


Figure 6-16: Correlogram showing Kendall correlation between methanogens and putative acetate oxidising bacteria calculated using relative abundances across all sampling times and depths, and the genus level taxa table. Depth of colour and size of circle are proportional to the correlation coefficients i.e. indicate strength of correlation between parameters.

No strong methanogen-bacteria relationship is identified from this analysis for each of the genera tested. A possible reason for this is that only single species from each genera identified as syntrophic acetate oxidisers have been identified as capable of syntrophic acetate oxidation i.e. analysis based on abundances assigned to genera level rather than to species level may mask true relationships. Future work both in-vivo via culturing and in-vitro by, for example, metagenomics should be conducted to identify more species associated with this important pathway. Analysis at species level using the OTU table was not possible as the V4 region is insufficient for identification of organisms at species level. It is noted from the plot (Figure 6-16) that on the whole, the relative abundance of the

methanogens tends to correlate positively across the 7 methanogenic OTUs identified. The change in of the relative proportion of Archaea in the in the community is shown in Figure 6-17 and the changing relative abundance of the most dominant genera (relative abundance > 0.5%) with time is shown in Figure 6-18.

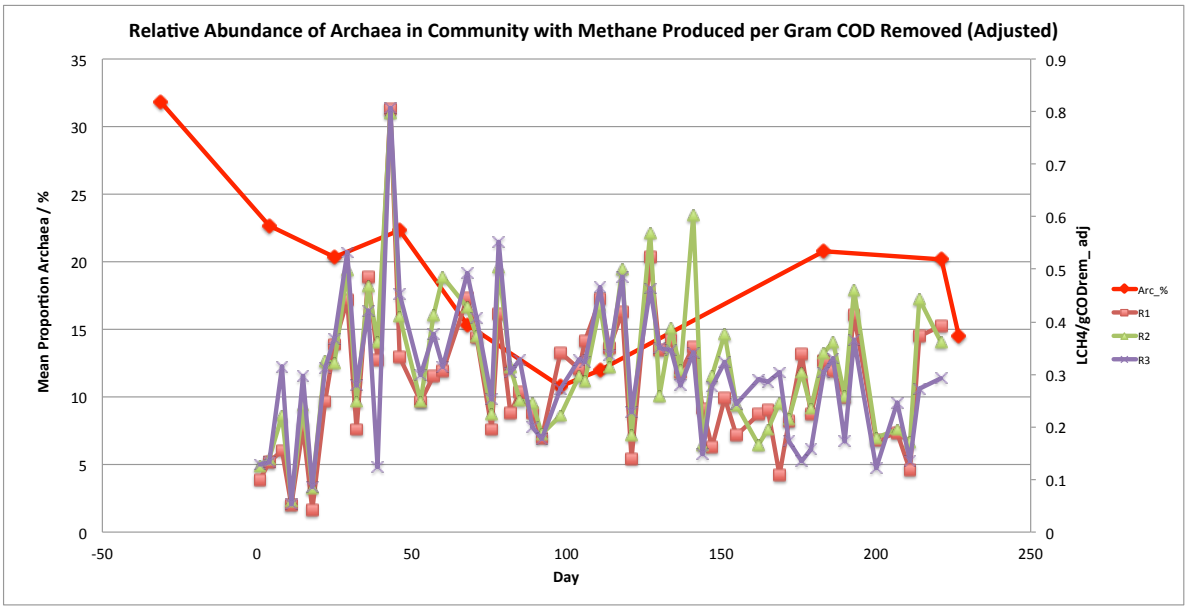


Figure 6-17: Scatterplot contrasting time-series of mean relative abundance of Archaea in the microbial community with methane production as a function of influent COD.

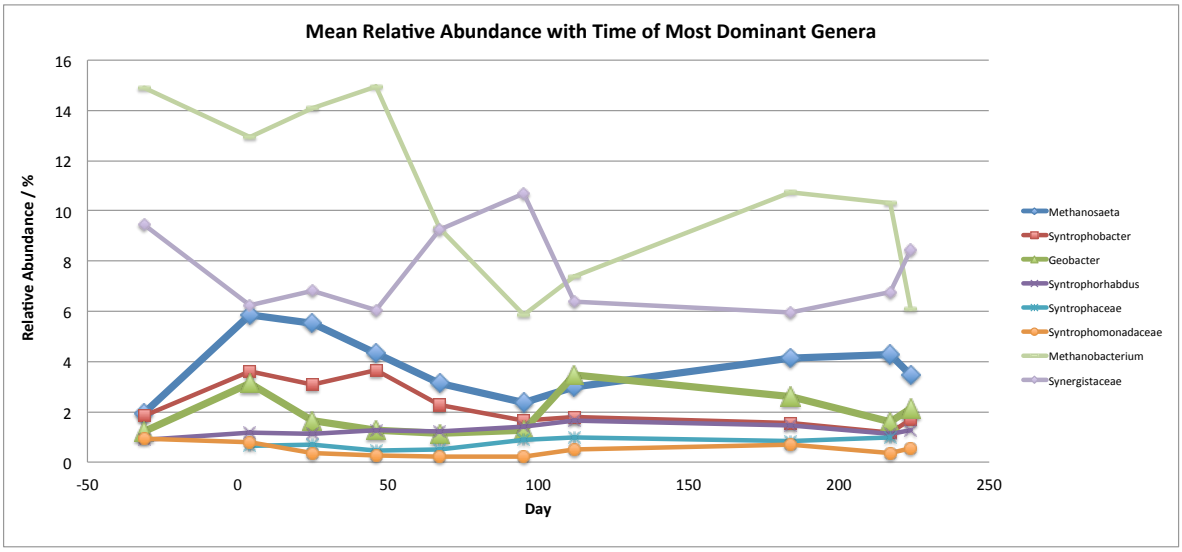


Figure 6-18: Scatterplot showing variation of the mean relative abundance of the most dominant genera with time

The relative proportion of Archaea in the community is seen to decline sharply from 31.8% in the seed sludge to only 10.77% on day d95 during treatment of low-strength distillery waste at 37degC at lab-scale. Interestingly, this decline is not reflected in the productivity of the community as measured by quantity of methane produced per gram of COD removed (Figure 6-9). Whilst a brief decline in productivity coincides with the lowest measured relative abundance of Archaea in the population a recovery follows that is not

accompanied by an increase in relative abundance of Archaea. Indeed overall, productivity in this period tended to increase as the community adapted to low-substrate conditions at 37degC. Speculatively, this suggests that the decline in the proportion of Archaea might not be reflected in actual population numbers and that rather, the bacterial population increased in real terms during this adaptation period. As no quantitative measures were made on the respective Archaeal and Bacterial populations however, this cannot be confirmed. To enable enumeration of organisms present, application of qPCR may be beneficial as a quantitative tool in future works however method development would be required to ensure biomass sample volumes were sufficiently standardised prior to DNA extraction such that absolute comparison between samples was appropriate. What may additionally be inferred from this plot however is that whilst COD removal efficiency and biogas production remained relatively stable throughout reactor operation, these functions were delivered by a highly dynamic population. At genus level (Figure 6-18) a dynamic relationship is also seen between dominant acetotrophic and hydrogenotrophic Archaea. The dominant hydrogen utilising population of Methanobacterium appears to decline most rapidly during the initial adaptation period enabling a partial recovery of Methanosaeta towards the assumed acetotrophic dominance. That this trend is reversed during low temperature operation appears to strengthen the hypothesis that the hydrogenotrophic pathway is dominant under stress conditions reactors.

6.5 Summary Findings and Discussion

6.5.1 Reactor Performance at Low-Temperature

COD removal efficiency was observed to decline as each new stress was introduced in the system. Even so, during low-temperature treatment of SYNTHES, COD removal efficiency of 80% was achieved suggesting that the technology may be successfully applied to the treatment of sewage assuming SYNTHES is a good substitute for ‘real world’ sewage. The decline in total COD removal arose largely from a decline in sCOD removal rather than pCOD as might have been predicted. A possible reason for this was that during low temperature treatment of SYNTHES, acetate comprised 50% of the VFAs measured in the influent such that this may have added additional stress to temperature sensitive acetotrophic methanogens. It is noted however, that the decline in sCOD removal was not reflected in measures of VFA in the reactor effluent in which neither acetate or propionate accumulation was recorded therefore indicating well functioning reactors. It is assumed therefore that the duration of operation at low temperature negatively impacted sCOD removal.

Methane production rates were seen to be lower during treatment at lower temperatures than during either the 37degC period of operation even after adjustment for decreased COD removal or increased solubility of methane at low temperature. This is attributed largely to the lower proportion of methane in the biogas rather than the quantity of biogas produced. The decreased proportion of methane in the biogas produced may indicate that low temperature operation additionally stresses hydrogenotrophic methanogens such that CO2 produced during acidogenesis less efficiently reduced to methane than under mesophilic operating conditions. An alternative interpretation might be that other organisms competing for hydrogen such as sulphate reducing bacteria that may be less affected by low temperatures however H2S in the biogas was not recorded.

Whilst COD removal efficiency was satisfactory at low-temperature, specific methanogenic activities measured at the end of each Phase indicated that the maximum potential activity in the biomass was approximately ten-fold lower at low temperature (22 or 15degC) than at 37degC for each substrate tested. Thus it was determined that the microbial community developed was psychrotolerant rather than psychrophilic. The decline in activity measured in the SMAs was not reflected in reactor performance however. As such, it might be assumed that the reactors were operated below maximum capacity during the trial. Were an optimisation process were conducted such that the reactors were operated at or near capacity the suggestion here is that retention times would be substantially greater at 15degC than required at 37degC. In real-world applications this would imply that low temperature operation might require larger reactor vessels, increasing the construction cost of a full-scale plant and increasing plant footprint as compared to a mesophilic reactor treating the same waste.

Regardless, with relatively low retention times the reactors performed well at low temperature. The decision to heat the reactor or not in real world applications would require an energy balance to determine if reduced methane yield at low-temperature was balanced by the reduced requirement for heating the reactors. Further, the increased cost of the plant due to increased retention times should be considered in an energy-cost budget. In the absence of these wider analyses, it is thought that the reactors performed well for the treatment of SYNTHES at low temperatures and offer a promising alternative to multi-stream liquid / solids treatment underpinned by aerobic removal of dissolved organics as are currently practised.

6.5.2 Replication Between Reactors

Reactor Performance

It was determined that during each phase of the experiment the reactors performed well as replicates with the single exception of biogas production during Phase 4 of the experiment in which R1 outperformed R2&3 by a margin of 13.3% and 33.3% respectively in terms of biogas production volume. This suggests that if no extreme change is introduced in a system that reactors seeded with similar communities will retain similar communities such that performance might be somewhat predictable. This is a significant finding for experimental practice. In lab-scale trials, the suggestion is that replicate testing might not be essential given that relatively stable operation of reactors is maintained.

Microbial Community

Replication in performance was strongly echoed in the microbial community composition of the most dominant organisms in the system. This was evident particularly during Phase 1 in which no applied disturbance was applied to the system. Qualitatively, during that phase, the 20 most dominant organisms in the system varied similarly in each of the 3 reactors. This was confirmed statistically for the 100 most dominant species in the reactor amongst which it was determined that reactor identity was responsible for only 1.6% dissimilarity in the microbial community across the duration of the experiment.

The variability in biogas production during Phase 4 of the experiment was reflected in the clustering patterns of the NMDS plots in which it appeared that R1 clustered distinctly from either R2 or R3. The suggestion here is that under the influence of external disturbances in the system, that a single community might respond dissimilarly, or that minor differences in a community might be amplified. A possible cause for this was identified from the statistical analysis of relative abundance of each phylum with time. Of 36 phyla identified in the system, 26 were found to significantly vary in proportion with time. Plotting the shift in relative abundances here, qualitative analysis of the trends shown strongly suggests that the microbial community is in a state of continual flux with shifts in relative abundance putatively described as sinusoidal. This implies that a given microbial community has an internal dynamic that acts somewhat independently of external influences. Were external pressures applied at an unfavourable time within a periodic flux for specific organisms, the wider system may be affected. In all, despite latent differences in the reactor microbial communities, the core reactor community appeared well replicated throughout the trial. For real world applications this suggests

there may be value in developing seed sludges specific to certain applications for which predictable community response might be elucidated for a range of perturbations.

6.5.3 Series Control Strategy

NGS data indicates that even under relatively stable operating conditions, the microbial community exists in a state of flux in terms of the relative abundance of species. As such, interpretation of data in relation to engineered conditions in the reactor is difficult in the absence of parallel control in which no change is made. As the community was well replicated for the majority of the trial for the most dominant community members, it is recommended that a parallel control be prioritised over operation of triplicate reactors in situations where both is not possible and where no major adjustment to engineered conditions is made.

6.5.4 Depth Profile: Association of Productivity and Community Structure

It was determined that both community physiology and microbial community composition were stratified with depth in the reactor. SMA profiles recorded at the top and bottom of the sludge bed at the end of each Phase indicated significantly increased activity rates in sludge samples from the bottom of the reactor. Analysis of NGS data determined that the position of sampling accounted for 16% of the variance in microbial community sampled (ANOVA) and NMDS plots indicated that difference was not phylogenetic, rather arose from varying relative abundance of species with depth. Significantly increased abundance of the genera Geobacter and Synotrophomonadaceae in sludge at the bottom of the reactor was observed for the duration of the trial and as such tentatively, these genera may be associated with increased productivity in granular sludge. Indeed this finding is consistent with the findings of Chapter 4 in which stratified activity was observed in 1-D lab-scale reactors treating full-strength distillery waste at 37degC. Perhaps more interestingly, structural differences in the community profile were also observed with depth in the reactor. Both diversity and evenness were found to be elevated in sludge from the bottom of the reactor and as such each might be associated with increased activity. Not only does this finding feed into wider field of microbial ecology but also suggests that if confirmed as a general rule, then monitoring of microbial community structure may provide useful information on reactor health in full-scale technologies. Further, the finding that those communities that are more diverse and more even appear more productive might provide a useful starting point for those aiming to engineer synthetic microbial communities in biotechnologies.

6.5.5 Dominance of the Hydrogenotrophic Pathway

The acetotrophic pathway is typically assumed dominant in both natural and engineered environments and indeed bioreactor design is often geared towards operating conditions believed to promote growth and activity of sensitive acetotrophic populations. Here, the seed sludge used in the trial came from a full-scale digester and was found to be both more active using hydrogen than acetate by SMA testing, and to have a higher relative abundance of hydrogenotrophic methanogens than acetotrophic. In Chapter 4 of this thesis where full-scale operation was mimicked in the lab, it was seen that the apparent dominance of the hydrogenotrophic pathway declined gradually during the trial. The proposed reason for this was that mode of operation of the reactors in the lab-scale trials was more stable and that this reduced stress on sensitive acetotrophic methanogens. At full-scale, feeding was irregular in terms of substrate applied and further the full-scale digester was subject to periodic shut-down in which no feeding was applied and temperature fluctuated. In this trial it was seen that the putative dominance of the hydrogenotrophic pathway also initially declined during the 'steady-sate' operating phase of the trial as evidenced by declining relative abundance of Methanobacterium coupled with increased relative abundance of Methanosaeta. The recovery of the acetotrophic community was not maintained however and after introducing a new stress to the reactors; a reduction in temperature; the hydrogenotrophic methanogens retained dominant abundance and activity in the reactors at low temperature. From this it is proposed that under stress conditions as may be experienced during routine operation of a full-scale digester, and that may be the design case for treatment of municipal sewage, the proposed metabolic pathway in the community may be described as shown in Figure 6-19.

Further testing is required to confirm this hypothesis and indeed alternative test methods may be better applied. Specific methanogenic activity assays typically assume acetate as an immediate precursor to methanogenesis however recent developments in understanding the role of syntrophic acetate oxidisers suggests that in some instances acetate will in fact function as an intermediary. The extent to which this occurs could be examined using radio-labelled acetate in SMA reactions (Karakashev, Batstone et al. 2006). Further, metagenomic analysis coupled with meta-transcriptomics may provide more insight into true abundances in the system and the role undertaken by specific organisms.

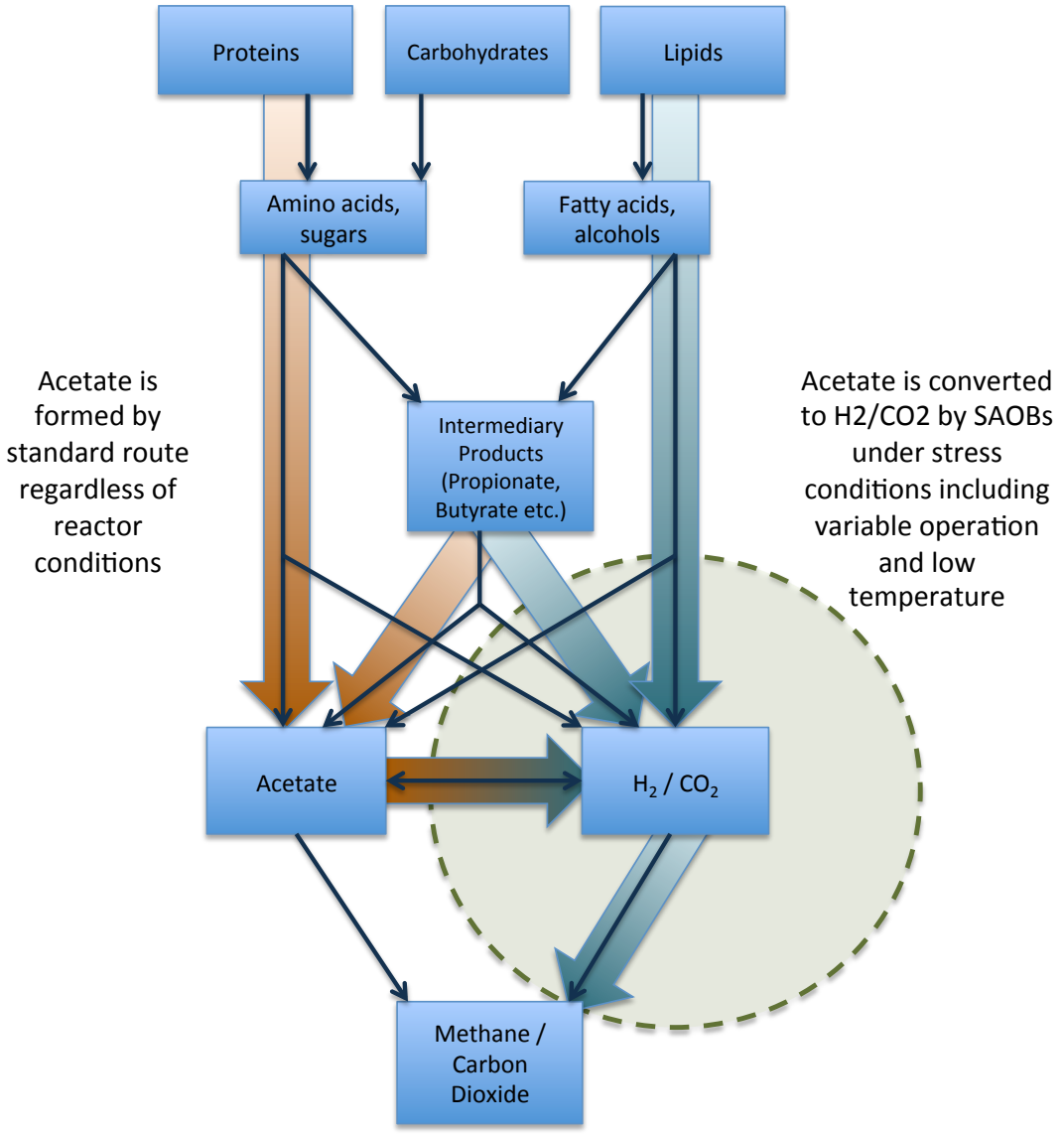


Figure 6-19: Proposed pathway to methanogenesis during low-temperature treatment of SYNTHES. Green circle highlights reliance on hydrogenotrophic methanogenesis in these conditions and the proposed interaction with syntrophic acetate oxidising bacteria.

6.6 Conclusions and Recommendations

The experiment aimed to determine the suitability of the EGSB for treatment of low-strength, high-solids wastes such as Global North sewage at ambient temperatures. It was found that treatment of SYNTHES at the strength of municipal sewage was possible at 15degC and that whilst particulate COD removal was low, granularity was maintained in the sludge, biogas production remained relatively stable and no VFA accumulation was recorded in the reactor effluent.

In addition to that, application of new, low-cost DNA sequencing methodologies during the trial provided insights that may contribute more widely to the field of microbial

resource management. The biomass in the reactors displayed a distinctly stratified structure as was determined in Chapter 4 previously. Via observation of that stratified structure, ecology indices in combination with SMA data were found to indicate that activity in a biomass may be driven by, or result in, structural changes in community composition. The biomass at the bottom of the reactors consistently showed higher maximum productivity as measured by SMA assays and this more active biomass was characterised by a community structure that was more diverse and more even, than less productive sludge at the top of the sludge bed. This result is significant both in the use of next generation sequencing methods as a monitoring tool for biotechnologies and for the design of synthetic microbial communities intended to perform specific functions. The drivers for stratification in the bed at this stage appear to be associated with physical mixing properties in the sludge bed and this is explored in more detail in Chapter 9 of this thesis.

Finally, the data collected indicated dominance of the hydrogenotrophic pathway during low-temperature treatment of synthetic sewage. The data here is not conclusive however points towards potential reward to be gained from re-visiting some of the basic assumptions on which anaerobic technologies are designed. Design of anaerobic technologies typically focuses on the promotion of activity and growth of acetotrophic methanogens. Arising from these ideals is the requirement for reactor heating, typically in the mesophilic temperature range, and continual feeding of reactors. If however, those design ideals are no longer fitting of either the nature of the waste to be treated or the mode in which the waste is produced it makes sense to investigate more fully those pathways that enable good, stable treatment under a broader range of conditions. Design of both reactor and feeding regime for alternate metabolic pathways including that in which syntrophy between acetate oxidising bacteria enables dominance of hydrogenotrophic methanogens should be investigated further such their potential may be exploited in full in meeting todays engineering challenges.

7 Failure Study

Abstract

High-solids wastes such as pit-latrine waste present something of a challenge for high-rate anaerobic technologies such as the EGSB. Perhaps a greater challenge however is testing, developing and promoting new sustainable biotechnologies to meet such challenges in the absence of a formal framework for engineering the system. Limit state testing and design is recognised as best practice in a wide range of engineering disciplines from structural to geotechnical design. This trial adopts a limit state methodology to test the EGSB to failure under high-solids loading to determine the failure point and associated mechanisms, and to identify 'early warning' indicators of impending failure in the system.

Three 20L lab-scale EGSB bioreactors were seeded using a granular sludge obtained from a full-scale EGSB. The reactors were operated at 37degC treating a high-strength, high-solids synthetic sewage at increasing OLR. COD removal efficiencies, VFA accumulation and methane production were used to assess reactor performance under each condition. Specific methanogenic activity assays were used to monitor community physiology after adaptation to each condition. Whole community 16S rRNA gene (V4 region) amplicon sequencing was utilised to monitor microbial community dynamics in both the reactor sludge bed and in the reactor effluent under each condition.

Whilst the precise loading rate inducing failure was not elucidated, two distinct failure mechanisms were observed enabling identification of two potential early warning predictors of failure in EGSB reactors under high-solids loading. Failure was observed to occur at both the reactor level and the granule level. At the reactor level, increased biogas production coupled with proposed increased viscosity of the reactor liquor resulted in washout of the granular sludge by physical lifting of the granules. At the granule level, failure by disintegration under high-solids loading was observed with granules developing increasingly indistinct surfaces at high OLR and under sustained treatment of high-solids wastes. Prior to failure in each of the reactors, microbial community evenness and diversity was seen to increase in the reactor effluent. Further, relative abundance of *Methanobacterium* in the reactor effluent was greatly elevated prior to failure. As such it is proposed that limit state testing should be applied to biotechnologies and that microbial community monitoring of reactor effluent shows promising signs of providing as early warning signals for failure in EGSB reactors.

7.1 Introduction

Currently, an estimated 2.5 billion people live without access to improved sanitation (UNDESA 2015) with pit latrines acting as the primary form of sanitation used by the urban poor in many developing regions (O'Riordan 2009, Jenkins, Cumming et al. 2015). In the peri-urban environment, where land is at a premium, pit-latrines may be emptied before the contents are microbially stabilised and mismanagement of the arising solids can serve to exacerbate the faecal-oral cycle they are intended to address (Uddin, Li et al. 2014). As the global population continues to shift from rural to urban environments (UNDESA 2014), new technological solutions are required for the treatment of pit-latrine wastes in the peri-urban environment. The EGSB is a high-rate anaerobic digester type typically applied for the treatment of mid-high strength, low-solids wastes. As a high-rate digester type, EGSB reactors are compact such that land requirement and construction costs are low (Enright, McHugh et al. 2005). Further, as an anaerobic technology, the EGSB produces not only an improved wastestream but also methane gas; a useful energy source that may be used for cooking and heating without the health risks associated with combustion of traditional biomasses. Currently, low solid removal efficiencies reported in EGSB type reactors (Seghezzo, Zeeman et al. 1998) hinders application to the treatment of high-strength, high-solids wastes such as pit latrine waste. This study aims to apply a 'limit-state' approach to experimental design in relation to solids treatment in the EGSB to investigate the solids loading limit.

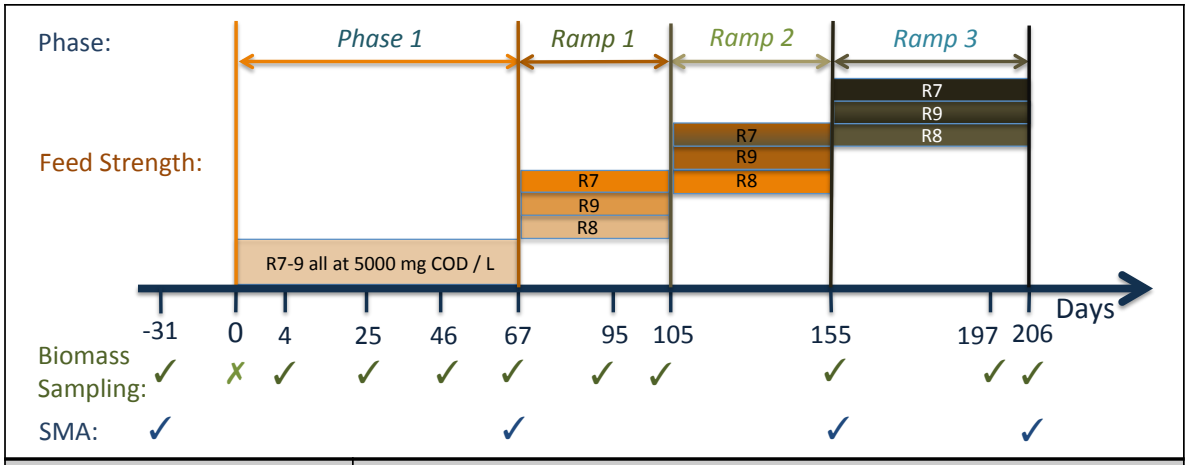
Conceived in the 1930s in the former USSR, and developed in the 1960's in Europe, limit state design is a rationale applied to ensure that within acceptable probabilities, a structure will function safely and predictably during its design life (Arya 2009). Underpinned by Newtonian mechanics and empirical failure testing of structures and materials, the approach may be defined as a design methodology founded on the application of partial safety factors to both loading and material parameters enabling design to both serviceability (acceptable deformation) and ultimate limit (failure) state. Limit state design is widely applied as best practice across Civil Engineering disciplines including steel, timber, concrete, and geotechnical design and acts as the basis for the Structural Eurocodes in Europe, yet no such unifying design code exists at present for the design of biotechnologies. Without testing biotechnologies to failure such that limits of operation are identified and failure modes are understood, application will remain firmly entrenched in empiricism and the associated risks will continue to hinder widespread adoption of new technological advances. In this study, failure in EGSB type bioreactors was induced at lab-

scale under an increasing organic load rate whilst maintaining volumetric load rate in order explore the appropriateness of a limit state testing approach in this reactor type. Four key aims were identified for the experiment as follows:

1. To adopt a limit state methodology for EGSB testing under high-solids loading by increasing OLR i.e. to test the system to failure.
2. To determine both the failure point and the failure mechanism in EGSB reactors treating a high-solids waste.
3. To identify potential indicators of failure in EGSBs under high-solids loading.
4. To use the limit state approach to assess the suitability of EGSB type reactors for the treatment of pit-latrine wastes.

7.2 Experimental Design

Three lab-scale 1-D type EGSB reactors were used in the experiment and each had a total working volume of 20L. The reactors were initially operated in triplicate and in parallel with second set of triplicate EGSBs as reported in Chapter 5. The reactors reported in detail in this trial (R7-9) treated a high-strength synthetic sewage and the parallel reactor set treated an industrial wastewater at equivalent COD strength (5000mg/l) for a period of 10 weeks to determine COD removal efficiencies under 'normal' loading regimes as reported previously. This period, described here as Phase 1, enabled identification of organisms specifically associated with the treatment of mid-high strength synthetic sewage. Subsequent to Phase 1, the sewage treating reactors R7-9 were subject to a series of ramped increases in organic loading by increasing the quantities of solids components of the synthetic sewage. The ramping strategy is described in the schematic in Figure 7-1 and the target OLR's are outlined in Table 7-1. Figure 7-1 shows that subsequent to Phase 1, the reactors were operated singly, with a new increased OLR applied to each i.e. the reactors were no longer operated as replicates. Ramping of OLR took place at equal time intervals for each reactor whilst the magnitude of the incremental rise in OLR was different for each reactor. The aim in so doing was to investigate a possible 'failure envelope' for the reactors. It was intended that the lowest OLR at each stage be equivalent to the highest OLR from the previous stage such that a degree of replication of operating conditions would occur.



Parameter	Measurement / Sampling Frequency Lab-Scale
Specific Methanogenic Activity	Seed sludge and end of each phase
COD and sCOD, influent and effluent	Bi-weekly
pH influent and effluent	Bi-weekly
Biogas production	Daily
Methane content in biogas	Bi-weekly
Temperature	Daily in reactor
Volatile Fatty Acids	Bi-weekly, 8 VFAs from C2 to C6, EtOH
Biomass samples	9 Sample times as shown. Samples at top and bottom of sludge bed in each reactor plus effluent sample at each time point for each reactor.

Figure 7-1: Schematic of experiment overview including duration, relative timings of biomass sampling and summary of physico-chemical monitoring parameters for reactor set R7-9. Schematic shows reactors are operated as replicates during Phase 1 but as individual reactors during the Phases Ramp 1-3.

Table 7-1: Design COD and OLR for each stage of Phase of operation

Phase	Reactor	COD / mg / L	OLR mg / d
Phase 1	R8	5000	7.5
	R9	5000	7.5
	R7	5000	7.5
Ramp 1	R8	10000	15
	R9	13333	20
	R7	16667	25
Ramp 2	R8	16667	25
	R9	20000	30
	R7	23333	35
Ramp 3	R8	23333	35
	R9	26666	40
	R7	30000	45

The reactor ramping strategy was such the 'strongest' reactor as determined by Phase 1 COD removal efficiencies was ramped most slowly, and the 'weakest' was ramped with the largest increments in OLR. The aim in so doing was to attempt to ensure that failure occurred slowly enough that the biological and physical response might be adequately recorded in the least ramped reactor. As such R8 was ramped least, R7 ramped most and

R9 was subject to intermediate ramping. As in previous chapters, the design operating parameters utilised in the experiment were the same as those applied at the North British Distillery and, again, the motivation for this was to enable each engineered condition during Phase 1 of the trial to be studied in relation to a control reactor set. Key design operating parameters are detailed in Table 7-2 below:

Table 7-2: Design operating parameters common to the reactor set for the duration of the trial

Parameter	Design Value or Condition
Temperature (degC)	37
HRT (h)	16
Upflow Velocity (m/h)	3.5
Influent pH	6

7.3 Materials and Methods

The following section details those materials and methods specific to this Chapter. Further, methods applied in this chapter but described in full in the Materials and Methods chapter of the report are given in summary form.

7.3.1 Substrate Preparation and Feeding

During Phase 1 of the experiment, SYNTHES was prepared to that of the stock concentration as described by Aiyuk et al (Aiyuk and Verstraete 2004) by dilution with tap water and applied to the reactors at a final dilution of 5000mg/L again using tap water to dilute. The SYNTHES was prepared in 640L batches on a weekly basis and a single IBC was used as the feed tank for each of the three reactors to minimise variation between the substrate applied to each. In-tank mixing was applied to promote solids delivery and to ensure that the influent contents were homogeneous throughout the weekly feeding period. Throughout Phase 1, trace metals were applied at the concentrations described by Aiyuk et al i.e. in relative proportion to the COD of the influent. During subsequent ramping periods of the trial, only those compounds contributing to the solids load in the SYNTHES were increased to ensure that increasing COD additionally increased the relative proportion of solids in the reactor influent. Trace metals were not increased in proportion to the COD, rather, they were applied at the same concentration throughout the trial. To provide a numeric example of this strategy, Table 7-3 shows the chemical composition of SYNTHES at target strengths of 5000 mgCOD / L and 23333 mgCOD / L. In each case, those

components of the influent highlighted in grey remained constant throughout the trial whilst all other were scaled accordingly. The full substrate preparation details for each ramped condition applied are given in detail in Appendix 3. It is noted that during Ramped phases, each reactor was fed from a separate feed tank of 60L volume and feed was prepared every 48 hours to ensure continuous operation.

Table 7-3: SYNTHES Preparation. Table shows published recipe for SYNTHES and demonstrates approach to increasing OLR by increasing Food and selected Chemical component concentrations (white) whilst maintaining all others (grey)

Reactor: / Strength:	*(Stock, as per Aiyuk et al. 2004)*	R7-9 S1	R9 S6
Chemical compounds			
Urea	1600	1000	4667
NH4Cl	200	125	583
Peptone	300	188	875
K2HPO4 3H2O	400	250	1167
K2HPO4	305	191	891
Na-acetate 3H2O	2250	169	169
MgHPO4 3H2O	500	37.5	37.5
FeSO4 7H2O	100	7.5	7.5
CaCl2	100	7.5	7.5
Food ingredients			
Starch	2100	1313	6125
Milk powder	2000	1250	5833
Dried yeast	900	563	2625
Soy oil	500	313	1458
Trace metals			
Cr(NO3)3 9H2O	15	1.125	1.125
CuCl2 2H2O	10	0.75	0.75
MnSO4 H2O	2	0.15	0.15
NiSO4 6H2O	5	0.375	0.375
PbCl2	2	0.15	0.15
ZnCl2	5	0.375	0.375
Overall parameters			
COD total	8000	5000	23333
COD sol	2500		
COD particulate	5500		
pH	7.1		
Sum			

7.3.2 Physical and Chemical Monitoring

Sampling of both influent and effluent for each reactor was made on a bi-weekly basis for the duration of the experiment. Liquid samples were then used to monitor key operation and performance parameters (Figure 7-1) using a range of experimental methods. Total and soluble COD was measured using the Closed Reflux, Colorimetric Method, Standard Methods 5220D. Influent and effluent pH was measured using a digital meter and probe (Thermoscientific Meter, Eutech Instruments Probe). Volatile fatty acids (VFAs, C_2–C_6, including iso-forms of C_4–C_6) and ethanol concentrations were measured using a gas chromatograph (7890A, Agilent, Palo Alto, CA) equipped with a DB-FFAP capillary

column and a flame ionization detector. Total VFA is calculated as acetate equivalent using measured values.

Biogas production volume was recorded on a daily basis using 20L volume rubber gasbags attached to each reactor gas line to collect the biogas produced for timed periods of typically 2 hours. During times of high biogas production rates, collection time was reduced to a period of 1 hour. Biogas volume was quantified using a gradated gas-tight syringe to empty gasbags of the collected volume and biogas production rates was calculated as volume produced per unit time. Methane content in the biogas was quantified on a biweekly basis to coincide with influent and effluent monitoring. Methane content was measured using a gas chromatograph (7890A, Agilent, Palo Alto, CA) equipped with a GS-Carbon Plot capillary column and a flame ionization detector. Reactor temperature was measured on a daily basis using a digital meter and probe inserted into the reactor liquor.

7.3.3 SMA

Specific methanogenic activity was measured on a single sample of the seed sludge at d-31 representing the starting activity in the biomass in each reactor. At the end of Phase 1, the SMA was determined for the reactor group as a single entity. Biomass used in the SMA was extracted from the top and bottom of the sludge bed from each of the reactors R7-9 and pooled to make two samples for the reactor set: one from the top and one from the bottom of the sludge-bed. During subsequent phases each reactor was treated individually such that SMA testing was conducted for each reactor and, again, the SMA was conducted at the top and bottom of the sludge bed to account for stratification in activity with depth. Substrates tested were acetate (Ac_C2), propionate (Pr_C3), butyrate (Bu_C4), ethanol (Et_OH) and Hydrogen (H2).

7.3.4 Biomass Sampling and Storage

Biomass was sampled from the seed sludge and at each port in the sludge bed from each reactor at nine time points over the course of the experiment (Figure 7-1). Similarly to work conducted in previous chapters, biomass was sampled at each port in the sludge bed. Unlike the previous research however, DNA extracted from the sludge at the top two, and bottom two, ports in each reactor was combined in equal concentration (ng/ul) to make a single sample representing the community at the ‘top’ or ‘bottom’ or the sludge bed. Further, as granule disintegration appeared a likely failure mechanism under high-solids loading, a 2ml effluent sample was taken from each reactor at each biomass sampling time

point. Those samples were centrifuged at 13000 rpm for 5 minutes, the liquid fraction discarded and the solids fraction stored for DNA extraction. Granular biomass was sampled according the method outlined in Chapter 3 and stored at -20degC until the end of the end of the experiment.

7.3.5 NGS Library Preparation and Sequencing

DNA extraction and purification was conducted using the Fast DNA Spin Kit for Soils (MP Biomedical). Extracted DNA was quantified using the Broad-Range Qubit Assay (Life Technologies) and stored at -20degC until used in NGS library preparation. Library construction was applied to DNA samples from each port in the sludge bed. PCR amplification of the V4 region of the 16S rRNA gene was conducted on each DNA sample in the sample set using Golay barcoded primers (Caporaso, Lauber et al. 2012) with an additional degeneracy on the forward primer for improved detection of Archaea. PCR product was gel-purified (Zymoclean Gel DNA Recovery Kit) and quantified prior to sequencing using the High-Sensitivity Qubit Assay (Life Technologies). The pooled multiplexed library, normalised to 5ng/ul DNA, was sequenced using the Illumina Miseq bench-top sequencer. The arising sequencing data was de-multiplexed at the sequencing centre and all other quality control methods applied to the sequencing data are described in detail in Chapter 3.

7.3.6 Qualitative and Statistical Analysis Methods

Data pertaining to operation and performance of the reactors during each phase of the experiment is summarised to provide mean values for each parameter recorded. Standard deviation is presented alongside minimum and maximum values in each data set to enable qualitative assessment of stability of both reactor operation and performance. Where important trends are identified in the data, the results are presented graphically, again to enable qualitative assessment of the conditions in the reactors.

Low-read count samples were excluded from the analysis of sequencing data with a cut-off threshold of 3000 reads per sample. To account for differences in sequencing depth between the remaining samples during the analysis of community membership, sequencing data was normalised by calculating the relative abundance of each OTU or taxa in the sample. In calculating the ecological indices, each sample was rarefied to a common minimum, which was the minimum read count sample for the whole sample set for the experiment. The ecological indices calculated were rarefied richness, Simpsons Index of Diversity and Pielous Evenness Index and each was calculated using the R-Vegan software

package. A qualitative analysis is given for the time series of relative abundance of the most dominant 20 OTUs in the reactors. Two-dimensional NMDS plots were constructed using two alternative distance metrics to describe the sequencing data. Firstly, Bray-Curtis similarity matrices were utilised to determine variance based on percentage abundances in the count data. Secondly, phylogenetic distances were obtained using the generalised Unifrac method, alpha=0.5, which calculates distances based on the difference in branch lengths of the phylogenetic trees between samples. Both methods were conducted using the Phyloseq package in R. As the plots using each metric were similar, only Bray-Curtis similarity plots are shown.

7.4 Results and Discussion

The results section is split into four key components. Section 7.4.1 presents data describing the operation of the reactors and assesses that data in relation to the initial experimental design. Section 7.4.2 presents monitoring data, analyses reactor performance throughout the trial and qualitatively describes the failure mode of the reactors. The mode of granule disintegration is also discussed in relation to wider literature on granule structure. Community physiology is described in section 7.4.3 where data from SMA testing is presented. Finally, in section 7.4.4, NGS data is used to describe microbial community composition, structure and dynamics throughout the trial and links are made between the community, the wider metadata set and the mode of failure in the reactors.

7.4.1 Reactor Operation

Reactor operating data is summarised in Table 7-4. Mean operating values are presented alongside the range for each parameter to enable qualitative assessment of the stability of operation. Temperature control during the trial was thought to be satisfactory in that the reactors performed at near target temperature (37 degC) for the duration of the trial. The pH of the reactor influent was observed to decrease at the onset of Ramp 2. At no stage in the trial was the pH of the influent amended and as such the change in pH in the influent is associated with increased COD. The change in pH was not thought to influence the failure point in the reactors as it was not reflected in the pH of the reactor effluent suggesting sufficient buffering capacity in the reactor liquor (Table 7-5) i.e. the reactors were not noted to acidify in response to decreased influent pH. Further, as the change in influent pH was simultaneous for each reactor in the set, the ramping of OLR was the primary difference in operation of the reactors, which was consistent with the experimental design.

Table 7-4: Reactor operation summary data: data shown key operating statistics for each reactor (Rx) during Phase of operation and substrate condition (_Sx)

Parameter	Statistic	Phase 1			Ramp 1			Ramp 2			Ramp 3		
		R7_S1	R8_S1	R9_S1	R7_S4	R8_S2	R9_S3	R7_S6	R8_S4	R9_S5	R7_S7	R8_S4	R9_S6
Influent pH	Mean	6.48	6.48	6.48	7.10	7.14	7.01	4.08	3.94	3.74	4.13	4.01	3.98
	Minimum	4.34	4.34	4.34	6.35	6.33	6.57	3.58	3.34	3.46	3.87	3.78	3.57
	Maximum	8.43	8.43	8.43	7.83	8.28	7.69	4.89	4.80	4.14	4.58	4.38	4.46
	St. Dev	1.03	1.03	1.03	0.74	1.01	0.60	0.38	0.38	0.20	0.23	0.21	0.24
Temperature / degC	Mean	37.2	37.3	37.1	36.9	37.1	37.2	36.5	36.5	36.6	36.8	37.0	36.7
	Minimum	35.5	35.5	34.0	34.8	35.4	35.1	35.1	36.0	35.0	35.8	36.2	36.2
	Maximum	39.1	40.6	40.1	37.9	37.8	38.3	37.6	37.0	38.1	38.0	38.6	37.8
	St. Dev	0.7	0.8	1.0	0.6	0.5	0.6	0.7	0.4	0.8	0.6	0.9	0.5
CODin / mg/L	Mean	5066	5066	5066	13137	8788	10525	17523	12621	14033	21981	12254	17007
	Minimum	2957	2957	2957	9234	5851	5829	6645	7570	9646	16146	9999	12968
	Maximum	8999	8999	8999	19267	15219	16569	35133	23797	24883	26674	14781	23458
	St. Dev	2056	2056	2056	4176	3883	3584	6468	4236	4003	3258	1267	2843
sCODin / mg/L	Mean	3102	3102	3102	4759	3616	5373	8299	5908	7621	10050	7250	8106
	Minimum	2480	2480	2480	1131	1176	1277	3823	2378	5630	7316	4756	4803
	Maximum	3967	3967	3967	12037	7350	11163	14892	9482	11392	17457	14949	10513
	St. Dev	450	450	450	3910	1877	2934	2661	1922	1545	2567	2320	1696
pCODin / mg / L	Mean	1963	1963	1963	8378	5173	5152	9224	6713	6412	11931	5004	8901
	Minimum	295	295	295	2332	1983	1186	404	1180	2650	6741	-554	6064
	Maximum	6068	6068	6068	18136	14043	7331	24788	16524	18315	19217	7939	14033
	St. Dev	1961	1961	1961	5273	4768	1933	6592	4056	3778	3082	1932	2151
TVFAin mg / L	Mean	590	590	590	1153	1615	1285	696	2387	2388	2944	3625	3634
	Minimum	116	116	116	361	417	405	85	285	201	60	1144	1550
	Maximum	1707	1707	1707	2092	3823	2129	4225	7652	5210	6923	5387	5976
	St. Dev	441	441	441	864	1545	837	933	1806	1746	2573	1284	1453

high solids SYN appears to have lower soluble COD
3 observations using SYN at ~12.5gCOD/L
2 observaions using SYN at ~17 gCOD/L

During Phase 1 the reactors were operated as replicates. Feeding for the reactors was from a single feed tank such that substrate characteristics were common to each reactor and mean COD and temperature were very close to design values for this Phase of the trial for each reactor in the set. As such, it may be concluded that good replication in operation was achieved during Phase 1. Subsequently, during the Phases described as Ramp 1 to Ramp 3, the OLR in each reactor was stepped up by increasing the COD of the reactor influent. As described previously, the increase in OLR during each ramp was different for each reactor i.e. the reactors were *not* operated as parallel replicates which was consistent with the experimental design. Ramping of OLR took place at equal time intervals for each reactor whilst the magnitude of the incremental rise in OLR was different for each reactor. The aim in so doing was to investigate a possible 'failure envelope' for the reactors. This strategy was adhered to during the trial but it is noted that the target OLRs applied were adjusted during the trial in response to reactor performance, and further with the exception of Phase 1, the target OLR was not achieved. The actual applied OLRs are summarised in Figure 7-2.

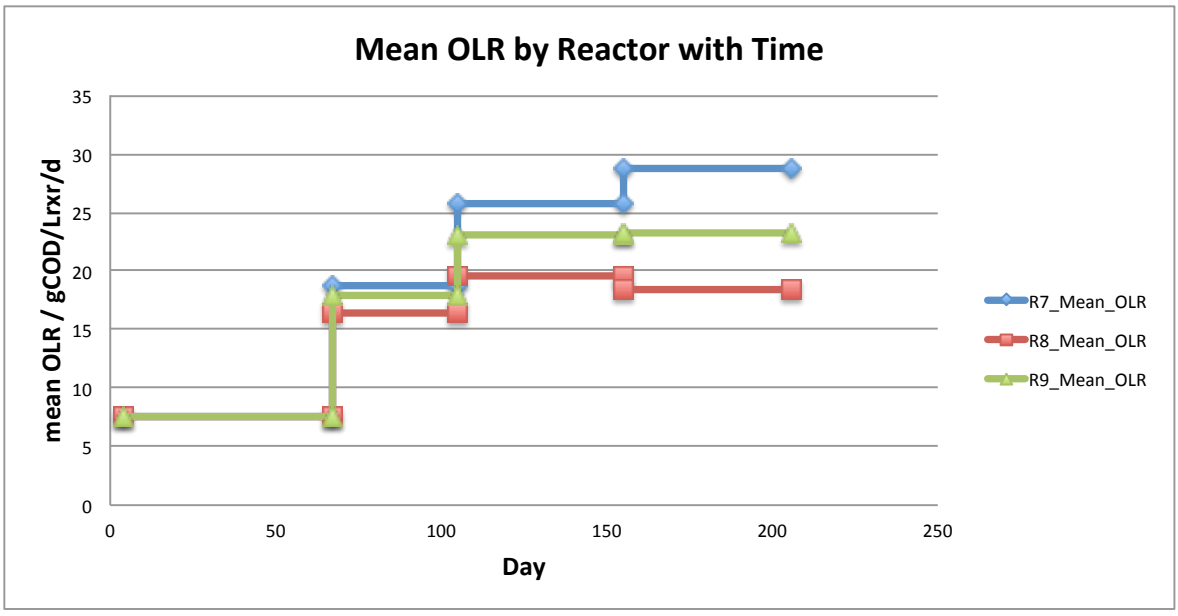


Figure 7-2: Scatterplot showing mean OLR for each reactor with time

During Phase 1 the COD of the SYNTHES was as anticipated by the chemistry of the recipe. During Ramp 1-3 however it was observed (Table 7-4) that the measured COD of the influent was typically lower than intended. The primary reason for this may be attributed to inadequate in-tank mixing. At high concentrations of particulates, the mixing applied was not sufficient to deliver all the solids in the tank to the reactor. A further reason that the intended OLR was not applied was difficulty in transferring solids from the

feed tank to the reactors using peristaltic pumps. Particulates caught at the peristaltic wheel mechanism tended to clog the feed line causing a partial blockage of the line. Whilst this did not interrupt feeding immediately, blockages tended to preferentially transfer liquids to the reactor as further solids collected at the blockage until the line had to be manually cleared. In addition to these technical difficulties, deviation from the original plan for the trial was made during Ramp 3 to reflect performance of the reactors. Reactor performance is considered in greater detail in Section 1.4.2 to follow but it is noted that partial failure of R8 and R9 during Ramp 2 prompted the decision not to increase the applied OLR on those reactors during Ramp 3. Whilst the absolute values of OLR were not as intended, the measured values demonstrate that in spite of both feeding problems and adjusted targets, an incremental ramping strategy *was* implemented such that reactor operation did reflect the intention of the experiment.

7.4.2 Reactor Performance

Summary performance data for the reactor set is given in Table 7-5 and again the range of measured values is given alongside standard deviation to indicate stability of performance. As these reactors were not operated in steady-state, nor allowed to fully recover performance at each ramping of the OLR, much of the data is also presented graphically. Each performance indicator is considered in turn in relation to either reactor effluent characteristics or methane production.

7.4.2.1 Effluent pH and TVFA

It is noted that the effluent pH remains relatively stable throughout the trial, even after the reactors begin to fail and in spite of the apparent acidification of the reactor influent in the latter stages of the experiment. Further, it is noted that the pH of the reactor effluent is little influenced by increasing VFA accumulation in the effluent. The reason for this is unknown however it is assumed that SYNTHES and bi-products created during digestion form sufficient buffering capacity to prevent acidification in the reactor. Whilst the exact cause of the high and stable pH in the reactor effluent is unknown, the data does appear to confirm that souring of the reactor is not the failure mode in this case.

Table 7-5: Summary performance data: data shown key operating statistics for each reactor (Rx) during Phase of operation and substrate condition (_Sx)

Parameter	Statistic	Phase 1			Ramp 1			Ramp 2			Ramp 3		
		R7_S1	R8_S1	R9_S1	R7_S4	R8_S2	R9_S3	R7_S6	R8_S4	R9_S5	R7_S7	R8_S4	R9_S6
Effluent pH	Mean	7.58	7.60	7.64	7.59	7.42	7.76	7.62	7.37	7.63	7.42	7.24	7.41
	Minimum	6.86	7.02	6.92	7.28	6.99	7.59	7.25	6.96	7.16	7.09	6.80	7.25
	Maximum	8.23	8.18	8.17	7.93	7.74	7.82	8.21	7.82	8.09	7.79	7.60	7.62
	St. Dev	0.26	0.28	0.26	0.30	0.35	0.11	0.26	0.28	0.23	0.20	0.28	0.12
TVFA / mg/L	Mean	35.1	40.0	25.5	4015.7	324.5	2640.1	4521.1	3197.6	4172.3	8185.7	6874.0	6138.1
	Minimum	18.2	19.5	18.1	1415.7	70.9	2060.6	2907.5	72.9	1424.2	4067.9	4633.2	3234.2
	Maximum	167.9	124.7	68.9	5185.8	1774.0	4081.6	6623.6	6702.2	6152.9	12604.8	10005.3	8342.8
	St. Dev	35.0	31.2	11.8	1240.3	591.2	760.7	988.8	2331.3	1143.7	3051.3	1119.5	1729.2
CODrem / %	Mean	84.2	81.2	82.6	58.4	81.9	60.9	20.6	37.7	11.5	26.0	1.6	27.8
	Minimum	68.1	41.7	27.7	48.8	54.7	43.5	-58.0	-173.4	-109.5	-12.6	-33.4	-1.6
	Maximum	93.3	93.5	94.5	67.0	93.6	81.0	72.9	88.5	71.8	57.6	38.1	57.5
	St. Dev	7.1	11.6	15.1	6.7	13.1	13.8	36.5	62.4	48.6	24.3	23.4	15.1
sCODrem / %	Mean	91.3	92.4	92.5	-12.6	88.6	59.6	-2.6	40.0	26.5	2.2	-4.2	28.7
	Minimum	78.4	88.9	90.0	-262.4	80.6	51.7	-433.7	-8.1	-29.7	-48.5	-51.9	-3.0
	Maximum	95.9	97.3	96.7	81.1	95.0	75.2	59.5	98.6	62.6	57.6	47.2	78.6
	St. Dev	3.9	2.3	1.9	119.0	4.9	8.3	117.9	40.6	22.3	34.2	28.3	19.2
pCODrem / %	Mean	53.0	32.0	33.4	57.7	67.6	50.1	-24.1	12.7	-25.2	45.0	37.7	23.8
	Minimum	-51.8	-248.8	-344.8	8.4	-8.4	-45.9	-344.4	-460.4	-436.3	-4.9	-73.8	-74.2
	Maximum	95.5	93.3	94.8	93.6	93.2	98.9	91.3	83.0	86.2	97.1	459.0	77.4
	St. Dev	43.8	79.2	101.4	34.9	35.9	48.1	120.8	132.3	130.6	31.0	129.8	43.4
BPR / L / Lrxr / d	Mean	2.20	2.31	2.16	2.28	3.22	2.87	3.87	3.30	3.53	3.99	1.64	5.61
	Minimum	0.85	0.66	0.72	0.58	0.95	1.07	1.44	0.40	0.30	0.30	0.00	0.00
	Maximum	5.70	6.33	7.61	3.68	5.48	5.43	8.26	7.84	5.38	7.23	3.36	57.97
	St. Dev	0.87	0.98	1.04	0.83	1.04	1.24	1.40	1.70	1.20	1.79	0.82	8.61
CH4 / %	Mean	78.3	77.7	79.5	66.8	74.9	72.6	68.1	69.7	63.0	57.8	52.7	61.3
	Minimum	68.0	62.9	59.9	56.4	64.4	61.5	49.6	56.7	41.6	45.8	37.1	48.6
	Maximum	83.1	83.4	84.2	81.2	90.2	80.3	86.9	81.4	73.6	67.7	65.0	69.5
	St. Dev	2.7	3.5	4.0	7.1	5.6	5.2	7.4	6.6	7.0	5.9	7.5	5.3

7.4.2.2 Soluble COD Removal Efficiency and Effluent VFA Profile

Removal efficiencies for sCOD are plotted for each reactor in Figure 7-3. During Phase 1 in which the reactors were operated as replicates, a high degree of replication is seen in overall performance. Removal efficiency for sCOD is both well replicated between reactors and remarkably stable during this phase. This suggests the reactors are underpinned by well functioning microbial communities that are suitably adapted to the treatment of SYNTHES at COD of 5000 mg/l and OLR of 7.5 gCOD/Lrxr/d. Qualitatively, during Ramp 1 the effect of the initial increase in OLR showed some linearity with the response in that the magnitude of the increase in OLR reflects the rank order for decline in COD removal efficiency. Reactor R8 in which the OLR was approximately doubled on d65, continued to function both well and stably in terms of sCOD removal efficiency with a mean for the period of 81.9% and standard deviation of 13.1%. For reactor R9 in which OLR increased by a factor of ~2.5, an initial decline in sCOD removal was however by day d104 the efficiency recovered to around 75%. For R7 by contrast sCOD removal was initially seen to plummet immediately in response to an increase in OLR by a factor of ~3.3 with a mean of -12.6% reported for the period. This indicates that whilst solids may be digested in the reactor, that full progression of the arising soluble products to methane did not occur in this reactor.

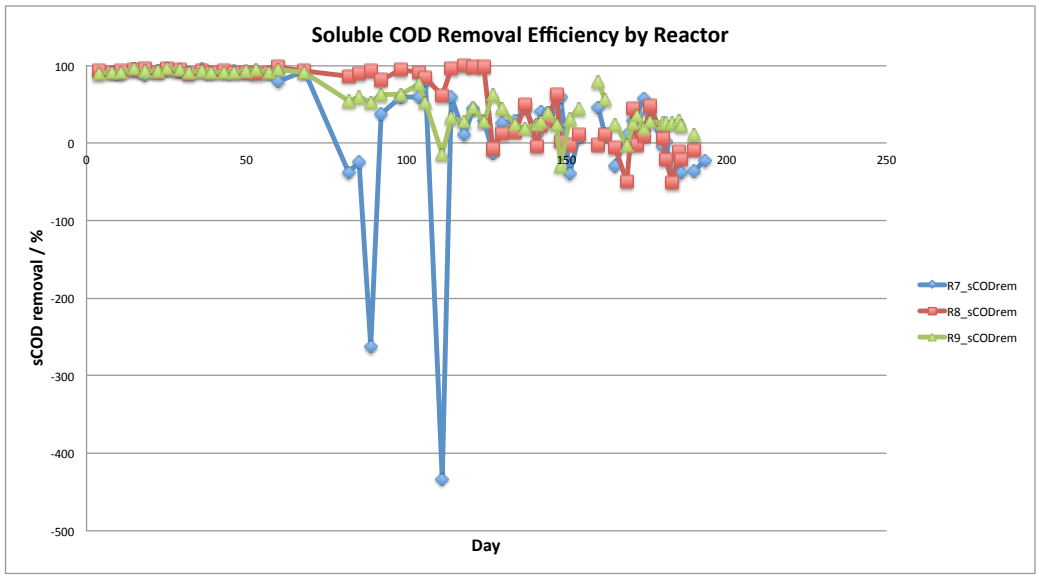


Figure 7-3: Scatterplot showing time series of sCOD removal efficiency calculated for each of reactors R7-9

For R7, the quantity of sCOD recorded in the reactor effluent was initially far greater than that in the reactor influent. The source of the additional soluble fraction is assumed to have arisen from partially degraded solids. By the end of Ramp 1 however, sCOD removal efficiency in R7 had recovered to 75% efficiency. The arising VFA profile for each of the reactors is given Figures 7-4 to 7-5.

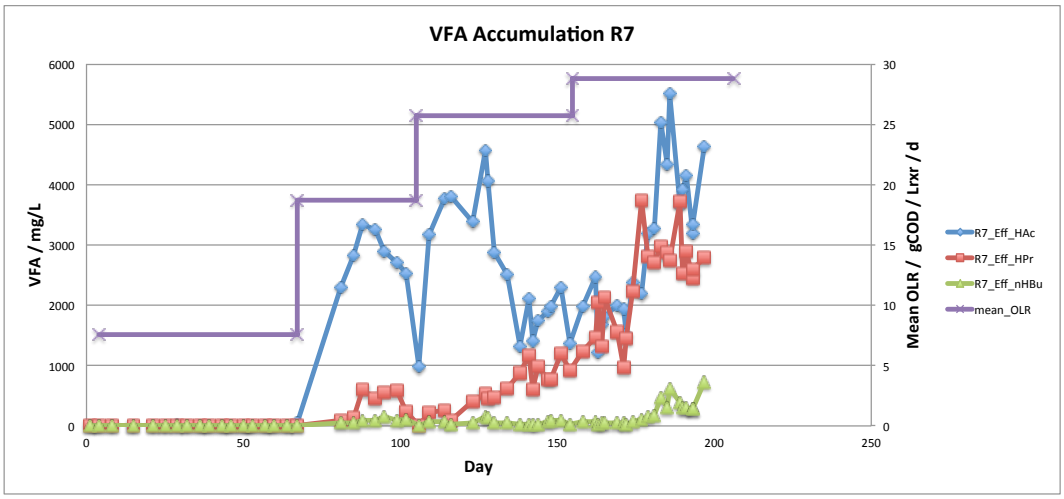


Figure 7-4: Scatterplot showing VFA accumulation with time in R7 (Ramped most) and mean as-measured OLR during each Phase

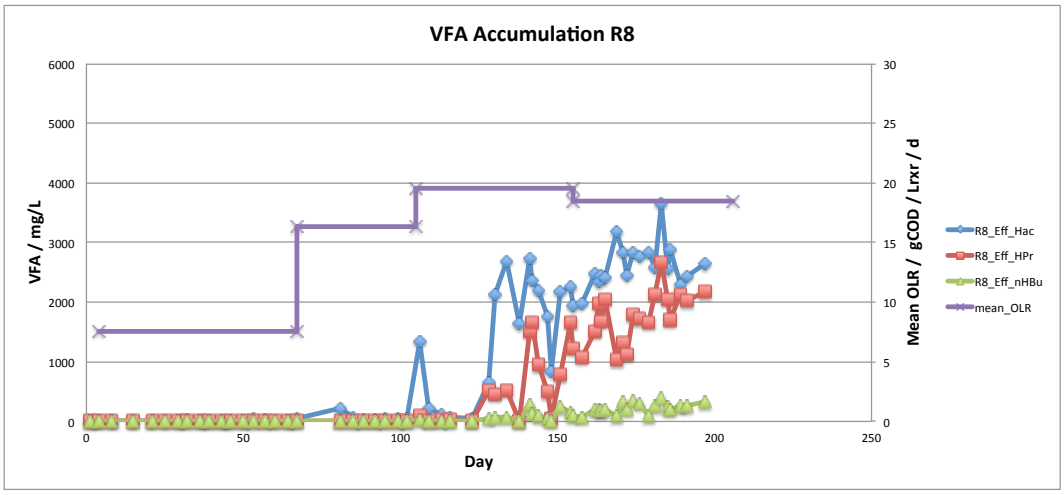


Figure 7-5: Scatterplot showing VFA accumulation with time in R8 (Ramped least) and mean as-measured OLR during each Phase

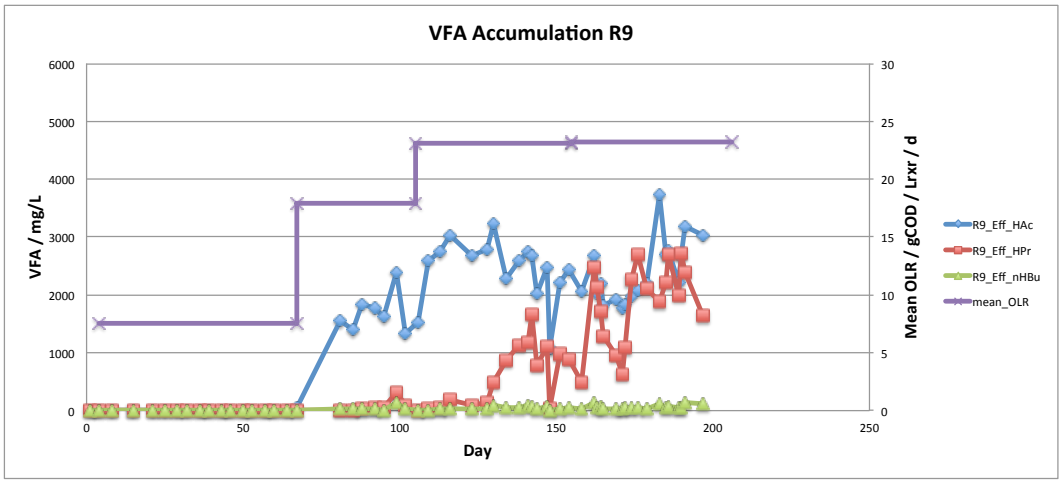


Figure 7-6: Scatterplot showing VFA accumulation with time in R9 (Intermediate ramping) and mean as-measured OLR during each Phase

Reactor R7 was ramped most during Ramp 1. The VFA profile for R7 during Ramp 1 shows a spike in acetate in the reactor effluent that was sustained for a period of over 20 days. An accompanying increase in propionate accumulation was observed however was

of far lower magnitude and of lesser duration. This suggests that during Ramp 1 the bacterial processes of hydrolysis, fermentation and acetogenesis proceeded well but the methanogenic community was effectively overwhelmed by the rapid increase in OLR and was unable to rapidly adapt to the increase in acetate produced. For R7 by the end of Ramp 1, the capacity for acetate utilisation in the system was recovered and propionate accumulation was almost eliminated suggesting good recovery from both methanogenic and bacterial populations. In R9, which was subject to 'intermediate' ramping, little propionate accumulation was seen during Ramp 1 however significant acetate accumulation was recorded. It is observed however that the recovery of the methanogenic population as indicated by acetate accumulation was both slower and less complete in R9 than for R7. For R8, little accumulation of VFAs was seen during Ramp 1 with the exception of a single peak in acetate in the effluent towards the end of the period and which was sustained only briefly. Tentatively, that acetate accumulation was more sustained in R9 than in R7 suggests that the microbial community may have responded differently to the different levels of stress in those reactors with that reactor which was 'stressed most' responding best in terms of full progression towards methanogenesis.

Immediately after Ramp 2, R7 responded similarly to during Ramp 1 with a sharp increase in acetate accumulation sustained this time for 30 days rather than 20 days, followed by something of a recovery though not to full acetate removal. Dissimilarly to Ramp 1 however, from d116 onwards in R7, propionate accumulation gradually increased for the duration of the period indicating the onset of failure in the reactor. The trend for acetate accumulation suggests that the methanogenic population was both slower to adapt than during the initial ramp, and slower to adapt to change than the bacterial population which is consistent with literature on growth rates of methanogenic and bacterial organisms (van Lier 2008, Shah 2014). That propionate accumulation progressively increased however suggests a decline in the capacity for the bacterial population to maintain acetogenic activity. Interestingly, R9, which was subject to intermediate ramping and appeared to recover less than R7 during the previous period of operation, again responds more poorly than does R7. Resultantly the apparent 'linearity' in response to ramping suggested by the summary data (Table 7-5) during Ramp 1 is lost. The recovery of acetate conversion during Ramp 2 occurs over 45 days in R9 as compared to only 30 days in R7 suggesting that methanogens are responding more slowly in this reactor. Further, from the onset of propionate accumulation on d128, the accumulation occurs more rapidly and to a greater magnitude in R9 than in R7 suggesting that bacterial populations in this reactor are also less able to adapt in this reactor. This may reflect a breakdown in the syntrophic

interactions between acetogens and the apparently stressed methanogenic consortia in R9. During Ramp 2, R8, which was ramped least, initially showed little response to the increased OLR. This may be contrasted with R9 during the previous phase in which R9 was subject to a similar OLR but via a more sudden increase. Towards the end of Ramp 2 however, both acetate and propionate accumulation in R8 exceed that of R9 under similar loading. Whilst not conclusive, it appears that a sudden increase in OLR as was applied to R7 and R9 during Ramp 1 has improved the capacity of those reactors to respond to the increased applied loading. By the end of this phase in each reactor, acetate and propionate accumulation was recorded. This coincided with decreasing bed-depth in each reactor that occurred during this phase (described in Section 1.4.2.3) and indicating the onset of failure in the reactors, which is confirmed during Ramp 3.

During Ramp 3, the metabolic response in each reactor community indicated by the VFA profile in each reactor was distinct to that determined in the previous phases. No immediate spike in acetate accumulation was seen on commencement of the new OLR in R7, rather a gradual increase in both acetate and propionate accumulation was observed. This trend was also observed in each of R8 and R9 in which no ramping was applied. Whilst no ramping was applied to those reactors it is noted that due to the substantial washout occurring during the previous phases, the granules remaining were effectively subject to ongoing increases in OLR. This cumulative trend for acetate and propionate in the reactor effluent coupled with diminished bed-depth was thought to indicate reactor failure in each case. The modes of washout that led to failure are considered in more detail in relation to pCOD removal to follow and activity of the biomass remaining in the system is considered in the Section 1.4.2.3.

7.4.2.3 Particulate COD Removal Efficiency, Granule Characteristics, Biogas Production and Failure Modes

The removal efficiencies for total and particulate COD are given as time series in Figures 7-7 and 7-8. The approximate bed depth in each reactor with time is given in Figure 7-9. A descriptive overview of the development of the physical nature of the granules (based on visual inspection) throughout the trial is given in relation to these results. During Phase 1 and for each reactor, COD removal efficiency appears relatively stable. This stability arises from the extremely stable removal of soluble COD recorded during this phase coupled with the relatively high proportion of soluble COD in the reactor influent (mean of 61.2%). Particulate COD removal by contrast is seen to sharply decline after 30 days treatment of the high-strength SYNTHES. The decline pCOD removal might best be understood in relation to the physical characteristics of the granules during this phase.

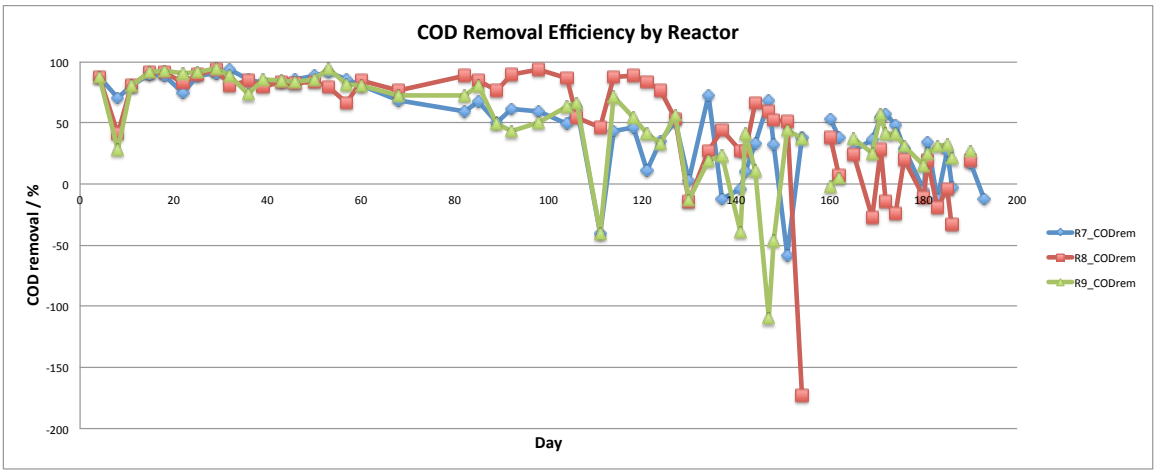


Figure 7-7: Scatterplot COD removal efficiency as a time series of each of reactors R7-9

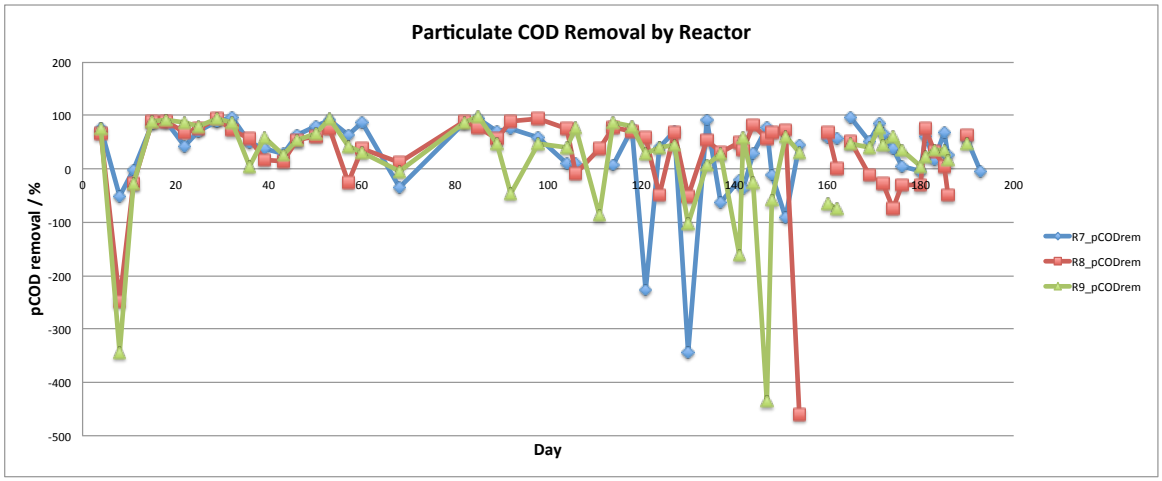


Figure 7-8: Scatterplot pCOD removal efficiency as a time series of each of reactors R7-9

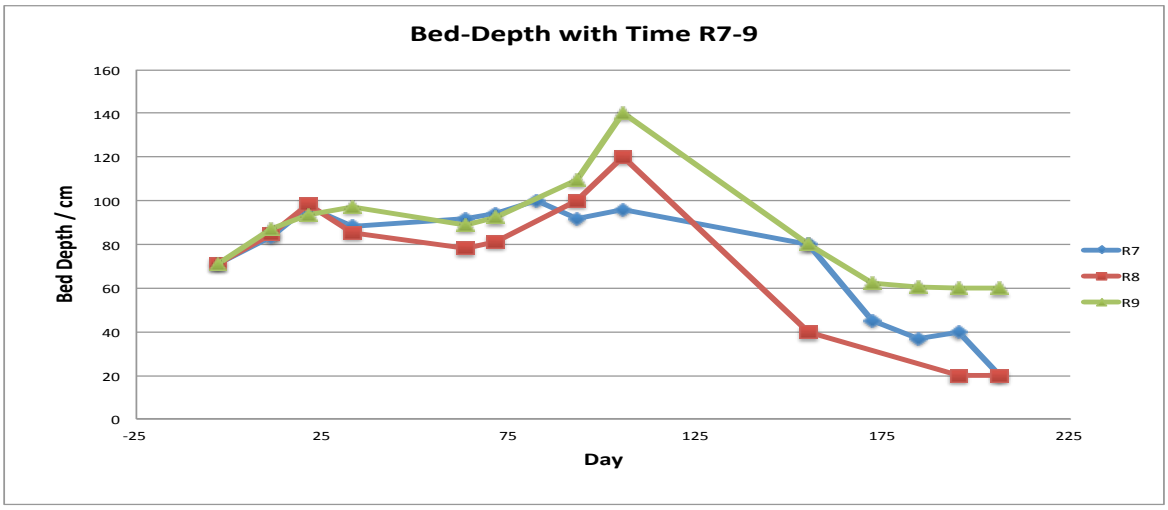


Figure 7-9: Estimated bed-depth in each of reactors R7-9 with time

Within 14 days of treating SYNTHES the sludge granules changed colour from olive-brown (as exhibited in the seed sludge) to very dark black-brown. The change in colour affected those granules at the bottom of the reactor first and the phenomenon spread upwards to the top of the sludge bed over a number of days. By day d17 all granules visible in each of the reactors appeared black-brown in colour. This same phenomenon

was similarly observed in the treatment of low-strength SYNTHES reported in Chapter 6 previously. By day d33 of this trial, the surface texture of the granules was also observed to have changed. The granules in the seed sludge (Figure 7-10) visually appeared to comprise two distinct 'materials' typically arranged in three or more layers. The first material formed the outer shell or 'skin' of the granule. The skin appeared smooth, was olive-brown in colour and appeared 'fat-like' in texture. The skin was able to resist gentle pressure applied with a blunt instrument. The second material, which formed the inner parts of the granule, was brown-black in colour and comprised a material that appeared more fibrous than the outer skin. The fibrous material typically appeared to form to one or two sub-layers with the distinction between layers visible due to different 'packing' or structure of the fibres. The layers closest to the skin were more tightly packed and solid. The material at the core appeared 'fluffy' with fibres typically more loosely packed than near the granule surface. In contrast to the outer layers, the core of the granule readily deformed when granules were cut open. By day d33 of the trial, the granules appeared not only to have changed colour but the smooth skin was no longer visible and granules appeared more 'fluffy' on the surface.

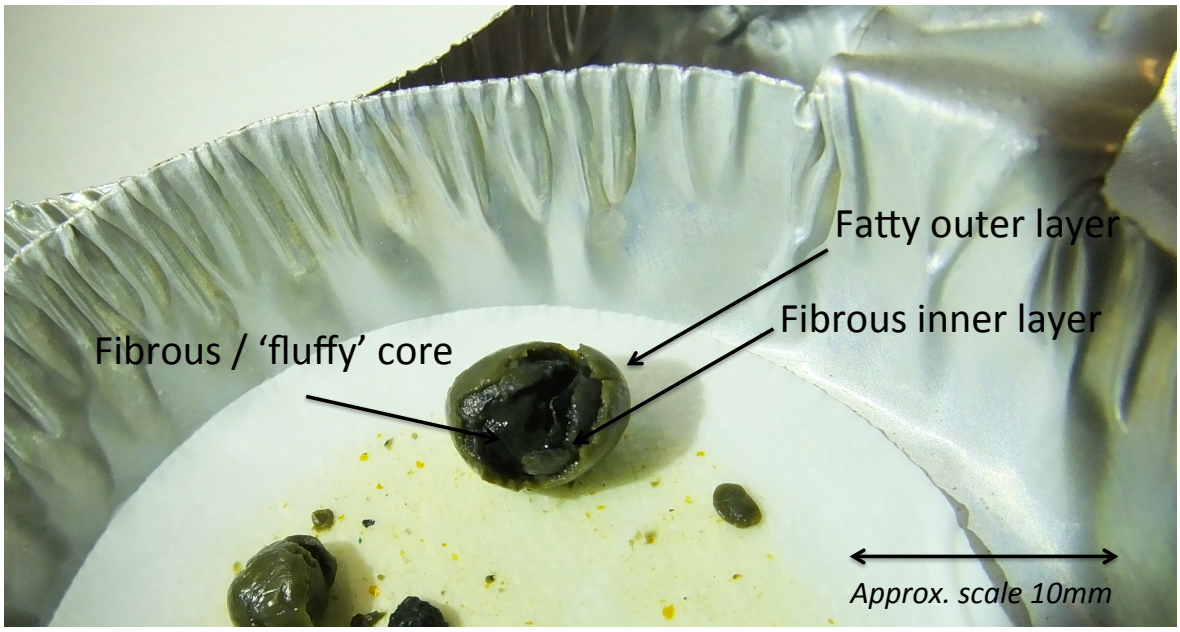


Figure 7-10: Digital photograph showing visible characteristics of granule structure in the seed sludge (note: non-typical granule diameter was used for photograph for improved image resolution, median granule diameter in seed sludge was 1.4 - 2.0mm)

At the same time, the reactor liquor and effluent, which had previously been white-cream in colour similarly to the untreated SYNTHES itself, became 'grey' in colour and appeared to contain small, fine, black-brown flocs possibly from the granule surface. Whilst insufficient analysis was conducted on the reactor effluent, it would appear that the decline in particulate COD removal efficiency recorded at that stage in the trial occurred not from

the passing of untreated influent solids through the reactors per se but from a change in the physical granule structure possibly stimulated by a microbial response to prolonged treatment of high-strength SYNTHES. In assessing failure in the reactors it is noted that whilst the occurrence could be described as a form of washout, that the bed-depth was maintained and even increased during this phase such that the process might best be considered as an adaptation in the community. That methane production rates (Figure 7-11) did not decline in this period supports the assertion that substantial washout was not taking place.

During Ramp 1, COD removal efficiencies showed similar 'linearity' in response to the increase in OLR as was shown during in sCOD removal in that R8 which was least ramped showed the smallest decline in removal efficiency, whilst R7 showed the greatest decline. The bed depth in each of reactors R7 and R9 increased by ~35% and that increase was attributed to increased mixing via increased biogas production in response to the increase in OLR. During this period, the increased bed expansion occasionally resulted in the passage of granules to the recycle line. In turn, the peristaltic action at the pumphead resulted in manual grinding of some granules such that the greying appearance of the effluent increased. In the lab-scale reactors this contributed to minor washout in the reactors and this was attributed to biological formation of biogas that promoted physical uplift of the granules. The decline in pCOD removal strongly reflects the degree washout in the reactors i.e. at the early stages of ramping, negative pCOD is indicative of granule washout in floc form rather than the passage of untreated solids through the reactors per se.

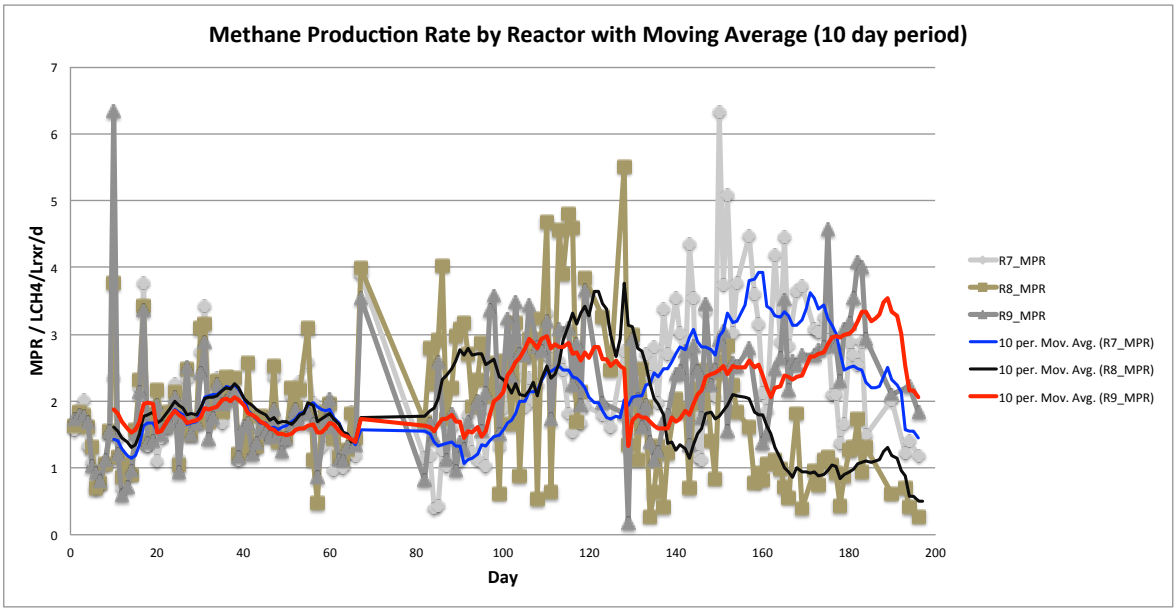


Figure 7-11: Scatterplot showing as-calculated time series of methane production rates for each of reactors R7-9. A 10-observation moving window average is also plotted for each reactor to improve interpretation of overall methane production trends.

During Ramp 2, R7 which was subject to the highest increase in OLR, suffered two incidents of mass washout of biomass reflected in the highly negative pCOD removal efficiencies recorded on days d121 and d130 of -225.7% and -344.4%. It is noted that these washout events occurred shortly after peak acetate accumulation events in R7 on days d114 and d127. During peak washout events, whole granules were found in the reactor effluent however the volume of 'whole' granules found in the effluent was not thought to reflect the volume of biomass lost. The negative pCOD values recorded were for samples containing liquor only i.e. whole granules appearing in the effluent did not account for the high values recorded. The granules in R7 at this time were found to be increasingly indistinct from the granule liquor i.e. disintegration of the granules themselves also contributed to the loss. Others, including those found in the effluent appeared 'coated' with white material that appeared to be solidified solids from the reactor influent (Figure 7-12).

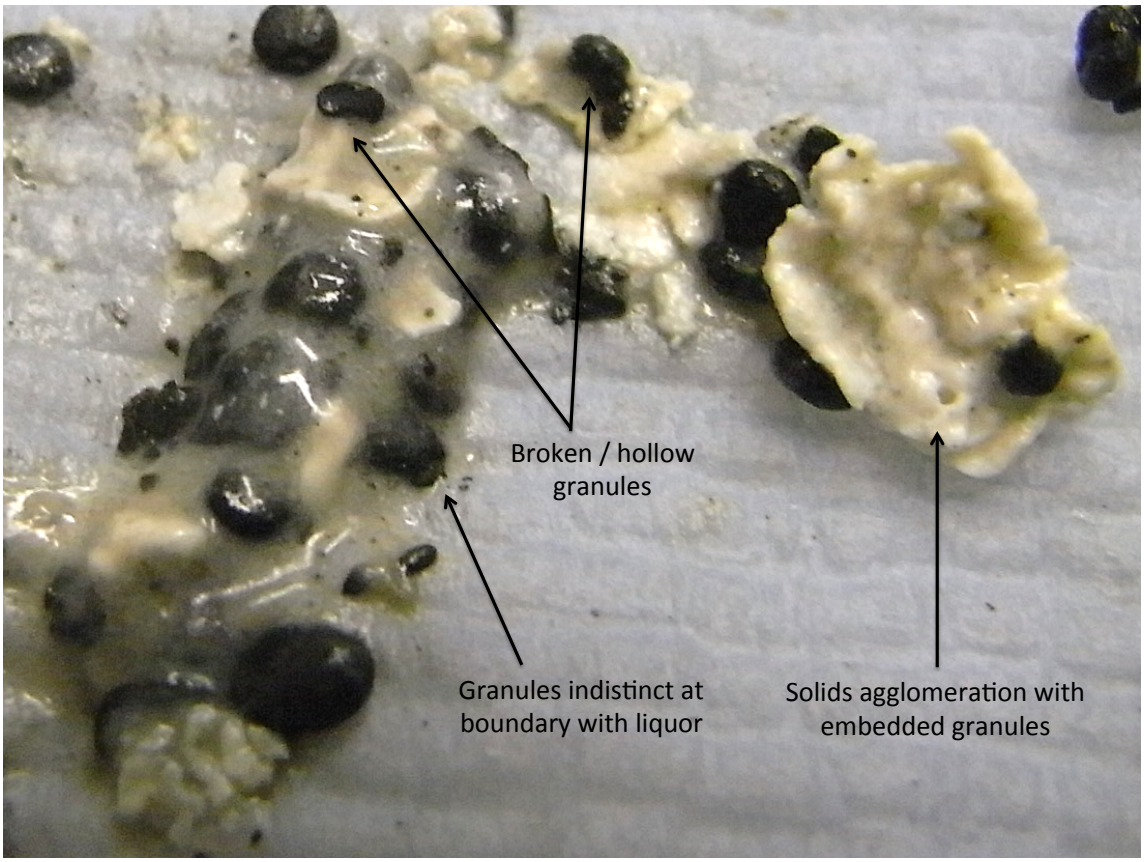


Figure 7-12: Digital photograph of granules found in effluent from reactor R7 showing indistinct granule surface and washed out solids from reactor influent.

Whilst some mechanical grinding as reported previously had occurred, this process naturally hit equilibrium at the point in which the diminished bed ceased to expand above the recycle line. As such, disintegration was assumed to be occurring for physico-chemical or biological reasons rather than purely mechanical reasons. That the disintegration process appears to occur simultaneously with acetate accumulation suggests that pressure in the

system on acetotrophic methanogens such as Methanosaeta as are associated with granule formation may result in loss of integrity in the granule structure. This is revisited in more detail in Section 1.4.4.3. Interestingly, biogas production rates were high in R7 during though the methane content in the biogas tended to decline.

R9 responded similarly during this phase but experienced a larger number of washout events although of lower magnitude. Granules in the reactor effluent appeared less coated with white solids but the reactor effluent was again suggestive of granule disintegration during that period. Two of the four washout events coincided with peak acetate accumulation in R9 on days d130 and d141. R8, which was ramped least, initially performed well with positive pCOD removal efficiency of 12.7%. This value reflects that only slight disintegration was seen in this reactor for the majority of Ramp 2. At the end of Ramp 2 however, a catastrophic washout event occurred in R8 towards the end of the phase. The cause of this washout is unknown and represents a distinct mode of failure as compared to the more progressive failure seen in R7 and R9. Extreme turbulence in the granules was visible in R8 reactor liquor during the failure period and granule settling appeared hindered by both biogas production and increased 'viscosity' in the reactor liquor arising from granule disintegration (Figure 7-13). The extent of washout in R8 and R9 prompted the decision not to increase OLR on those reactors during Ramp 3.

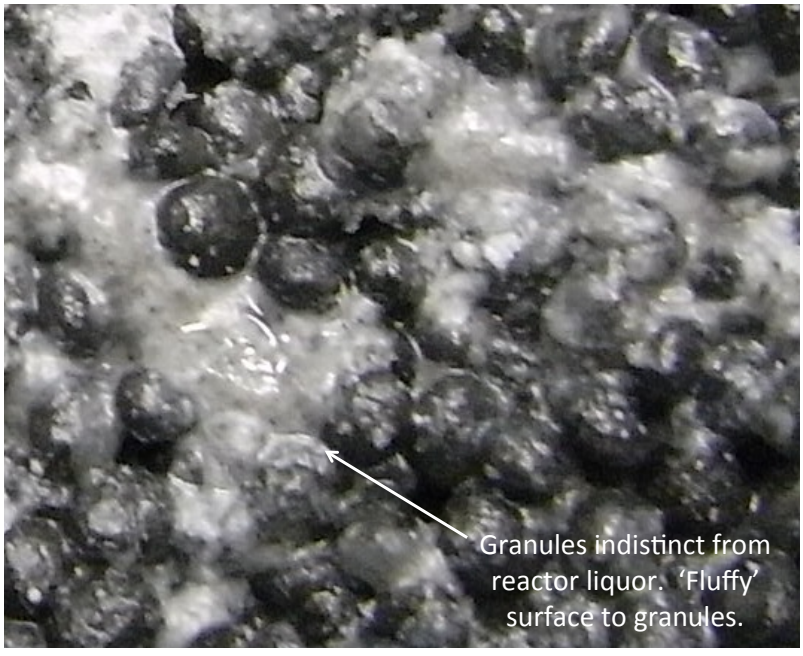


Figure 7-13: Digital photograph of granules found in the effluent of R8 showing indistinct granule surface.

During Ramp 3, fewer major washout events occurred in any of the reactors however the bed depth in both R8 and R9 continued to decline under the action of granule disintegration. The bed-depth in R7 by contrast appeared to stabilise however assessment

of COD removal efficiencies recorded for that period is difficult due to the large quantities of solids accumulation from the reactor influent occurring in the sludge bed. Biogas production for that period tended to decline in each of the three reactors (Figure 7-11). The experiment was stopped at this stage as each reactor appeared to have exhibited behaviour that would necessarily be described as failure if occurred during operation of a full-scale plant and indeed during Ramp 2 and no signs of recovery were seen in any of the reactors after the 50 day period of Ramp 3.

In summary, three distinct failure modes were observed:

1. Mechanical grinding of granules occurred due to interference with the recycle line, which arose from increased bed expansion under the action of biogas production.
2. Physical removal of the granules via the effluent line occurred under the action of biogas production.
3. Disintegration of the granules at increasing OLR and with increased time to which the granules were subject to high OLR, which was associated with periods peak acetate accumulation in the reactors.

Both granule settleability and liquor viscosity are considered in more detail in Chapter 9.

7.4.3 Community Physiology

The change in specific methanogenic activity measured for the sludge in each reactor with time is shown in Figure 7-14. The first time point plotted on each graph in Figure 7-14 shows the activity of the seed and is applied at both top and bottom of the bed under the assumption that the activity profile in the bed was initially homogeneous. The remaining time points show the methanogenic activity recorded in at the end of each Phase of the trial. At the end of Phase 1, in which the reactors were operated as replicates, a single measurement was made for each of a pooled sample of sludge for the top and the bottom of the sludge bed for the reactor set. Once ramping of OLR commenced, SMA was measured at the top and bottom of the sludge bed for each reactor individually until such time that washout meant sludge could only be sampled at the bottom of the bed.

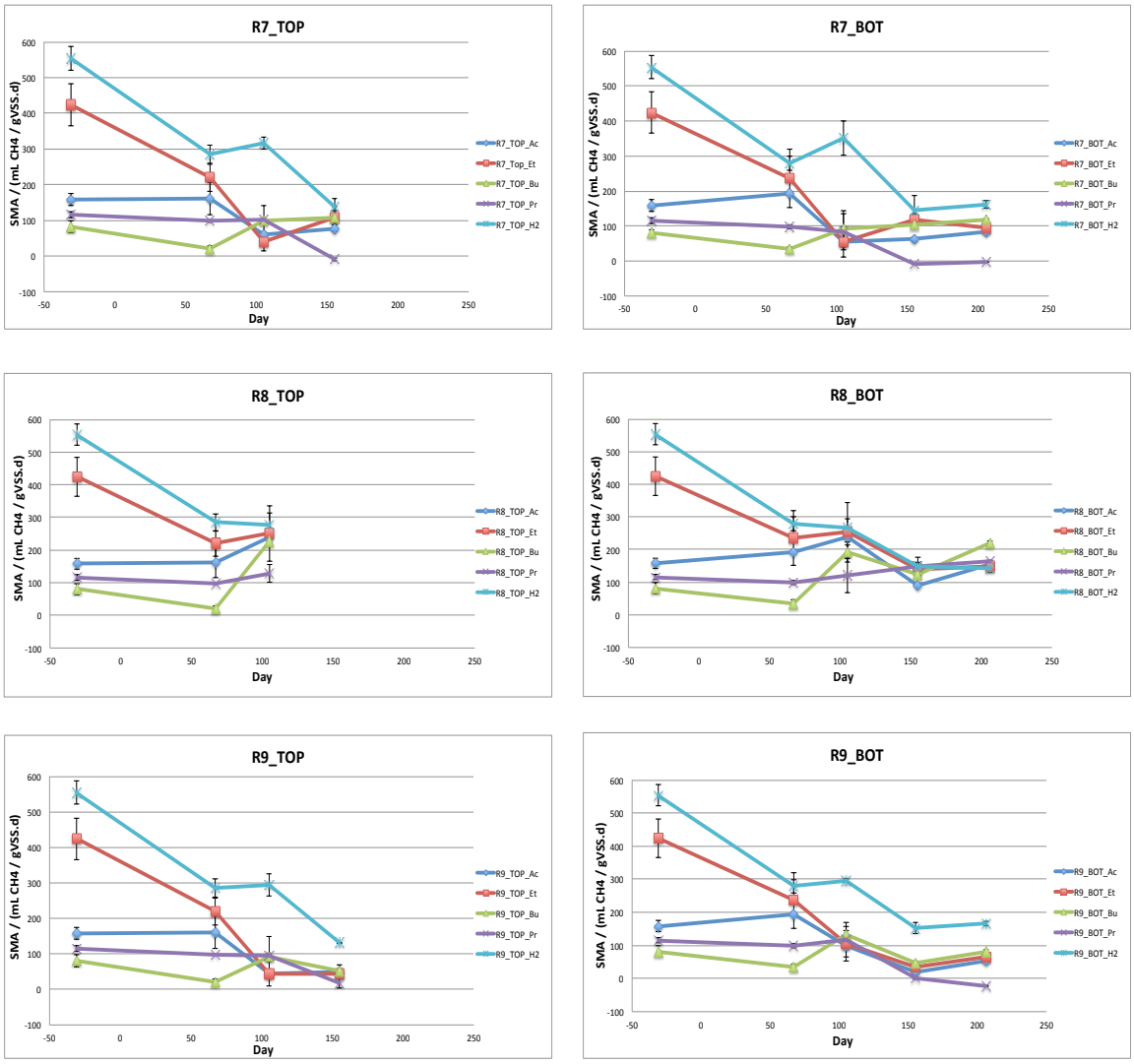


Figure 7-14: Scatterplots showing time series of SMA at the top (_TOP) and bottom (_BOT) of the sludge-bed in reactors R7-9 against specific substrates: acetate (Ac), propionate (Pr), butyrate (Bu), Ethanol (Et) and hydrogen (H2). Data points show blank-adjusted mean and error bars show standard deviation of triplicate measurements.

It was previously determined (Chapter 5) that no significant difference could be found in activity between the sludge at the top and bottom of the bed in high-strength SYNTHES reactors indicating a different mixing regime in these reactors as compared to either those reactors treating similar strength distillery waste or low-strength SYNTHES. As such the improved mixing was linked directly to the treatment of high-strength SYNTHES. At the end of Phase 1 the granules in the reactors had significantly declined in activity measured using both ethanol and H_2/CO_2 as substrates ($P<0.05$, AOV). This decline in activity measured using H_2/CO_2 as substrate was also seen at lab-scale using full-strength distillery waste as a substrate and was thought to arise from a decrease in stress on the system due to reduced solids loading (Chapter 4). Tentatively then, the decline in activity recorded using H_2/CO_2 here may indicate 'comfortable' treatment of SYNTHES during Phase 1. Activity measured using longer chain VFAs; propionate and butyrate; declined but to a lesser extent. Activity measured using acetate as substrate was seen to increase during Phase 1

particularly in those granules from the bottom of the reactor (P<0.01, AOV), which was again similar to the reactors treating distillery waste at similar COD as reported in Chapter 4. At the end of Phase 1 however, similarly to the both the seed sludge and the sludge acclimatised to alternative substrates at lab-scale, activity using H_2/CO_2 as substrate was significantly greater than that recorded for acetate (P<0.01) suggesting dominance of the hydrogenotrophic pathway.

SMA for R7: Largest Increments in Ramping

For R7 at the end of Ramp 1, activity in the biomass using acetate shows a highly significant decrease as compared to that recorded at the end of Phase 1 (P<0.001, AOV). This is reflected in the VFA profile for that Phase in which substantial acetate accumulation was recorded in R7. Activity using H2/CO2 at the end of Ramp 1 by contrast is seen to significantly increase (P<0.05, AOV). This trend appears to support the hypothesis that when stress is applied to granular sludge reactors that sensitive acetotrophic populations are most negatively impacted but that an active hydrogenotrophic population is able to thrive in such conditions enabling ongoing degradation of wastes. This is significant for the design of digesters treating variable strength, high-solids wastes such as pit latrine waste. Activity using propionate remains relatively stable during this period and again this is reflected in the VFA profile for the reactor effluent, which showed some initial propionate accumulation followed by an apparent recovery in degradation.

During Ramp 2, R7 was subject to granule washout both by uplift of granules and by granule disintegration as described previously. At the end of Ramp 2, conversely to the trend seen in response the initial ramping, activity measured using H2/CO2 significantly declines (P<0.001) whilst activity using acetate as substrate is approximately maintained. In Chapter 6, the dominance of the hydrogenotrophic pathway in the sludge as observed for the treatment of low-strength SYNTHES was putatively associated with the activity of acetate oxidising bacteria in syntrophy with hydrogen utilising methanogens. As the sludge granules began to lose structure and disintegrate, it may be the case that these syntrophic populations were no longer favourably positioned spatially to enable such syntrophy to proceed rendering the acetate oxidisers unable to compete with acetate utilising methanogens. That acetate consumption in the reactor improved during this phase is confirmed by the effluent VFA profile shown previously. Activity measured using propionate was also seen to decline during Ramp 2 and again this is confirmed by the VFA profile of the reactor. This additionally suggests that as the granule integrity was weakened that syntrophy between acetate oxidisers and hydrogen utilising methanogens

may have been spatially disturbed. At the end of Ramp 3, observation of activity was only possible for sludge from the bottom of the reactor due to substantial washout in the previous phase. Activities observed here appear reasonably consistent with those recorded at the end of Ramp 2 although there is some suggestion that the hydrogenotrophic pathway was beginning to recover. During Ramp 1 and prior to failure it appears that the hydrogenotrophic pathway is dominant and promotes robust performance in the reactor. During Ramp 2, granule disintegration began to occur in R7, which might have been assumed to indicate a decline in acetotrophic populations such as Methanosaeta as are associated with granule formation. The SMA data does not appear to suggest this is the case. Tentatively then, it is proposed that hydrogenotrophic activity declined as a result of changes in granule structure possibly due disruption of the spatial distribution of hydrogenotrophic methanogens and syntrophic acetate oxidising bacteria.

SMA for R9: Mid-Increments in Ramping

The response to the applied ramping of OLR was highly similar in R9 to that seen in R7. An increase in hydrogenotrophic activity accompanied by a decline in activity using acetate as substrate was seen at the end of Ramp 1, which again suggests that the hydrogenotrophic pathway may be important during periods of applied stress in the bioreactors. At the end of Ramp 2 after significant degranulation, hydrogenotrophic activity was again seen to decline during Ramp 2 although in this case, no recovery of acetotrophic activity was observed. Similarly again to R7, activity using propionate was maintained during Ramp 1 but declined during Ramp 2. For both acetate and propionate activities, the VFA profile in the effluent confirms the activities measured.

SMA for R8: Smallest Increments in Ramping

Conversely to the trends seen for either R7 or R9, at the end of Ramp 1, activity using H2/CO2 as substrate declined in R8 whilst activity using acetate increased. This is consistent with the effluent VFA profile for R8 in which no acetate accumulation was recorded during Ramp 1. At the end of Ramp 2 activity measured in the H2/CO2 assays again declined whilst activity using acetate was approximately maintained. During Phase 2, less granule disintegration was observed in R8 as compared to either R7 or R9. Disintegration of the granules may have been prevented by the maintenance of the acetotrophic population of methanogens, in particular of Methanosaeta as are often associated with granule integrity. That the failure mode exhibited by R8 was distinct and appears to be strongly reflected in the activity profile for the reactors. The catastrophic failure by granule uplift under the action of biogas production during Ramp 2 in R8

however remains unexplained. It is noted that activities recorded at the end of Ramp 2 are similar regardless of the ramping method applied. It may be that washout in R7 and R9 appeared less catastrophic purely because disintegration meant that washout was able to happen more continually and that had integrity been maintained the granules of those reactors that the failure mechanism would have been more similar across the reactors.

7.4.4 NGS data analysis

The next generation sequencing data is assessed in relation to the metadata to determine

- Which organisms are dominantly present in the treatment of high-strength, high-solids SYNTHES.
- Whether there are microbial indicators associated with any of the failure modes identified.

7.4.4.1 NGS Data: Basic Analysis

The sequencing data collected during the experiment was processed as described in Chapter 3 to produce a de-multiplexed sample set for analysis. Prior to analysis, summary statistics were produced on the full data set for the experiment to ensure consistency between samples. The data set consists of 73 samples in total, across which 2364 OTUs are identified. Table 7-2 below shows the distribution of reads across samples in the set.

Table 7-6: Distribution of reads across the sample set generated from Illumina Miseq amplicon sequencing.

Statistic	Read Count	Sample	Sample ID
Minimum	3321	S287	R8-P1, d105
Maximum	306117	S367	R9-EFF, d197
Mean	54084	NA	NA
Median	31839	S189	R8-P1, d67
Standard Deviation	60176	NA	NA

It is noted that the sample with lowest read count in this instance is well below the target threshold of 5000 reads per sample. Indeed, inspection of the data shows that 9 samples out of the full set of 73 samples sequenced. Table 7-3 below lists those samples and identifies the reactors and time points they represent. Additionally the first samples representing both an effluent and a biomass sample that exceeds the threshold are listed:

Table 7-7: Samples with total read-count below the threshold target of 5000 reads/sample.

Sample	Read Count	Reactor	Port	Day
S287	3321	R8	P1	105
S360	3712	R7	EFF	197
S86	4031	R9	P1	4
S293	4048	R8	EFF	105
S88	4527	R9	P3	4
S359	4538	R7	EFF	197
S453	4703	R9	P1	206
S513	4737	R9	EFF	95
S84	4793	R8	P3	4
S511	5330	R7	EFF	95
S291	5539	R8	P5	105

That more samples are below the threshold as compared to previous data sets may reflect the effect of the influent feed composition on PCR contaminants such as humic acids that may subsequently affect the potential for sequencing. Alternatively the lower read counts may simply have arisen from the reduced potential for repeat sequencing of this sample set whereby some low read count samples from Pool 1 were re-sequenced in both Pool 2 and 3 whereas R7-9 samples were sequenced for the first time in Pool 2. For the purpose of calculating ecology indices, samples were rarefied to a common minimum of 3321 reads to enable all samples to be included in those measures and most dominant organisms only are considered in analysis of community membership.

7.4.4.2 Qualitative Assessment of Microbial Community Membership

The mean relative abundance for the twenty most dominant OTUs in the sample set are given in Table 7-8 and along with taxonomic assignment using RDP Classifier. Two OTUs in the set (OTU_1 and OTU_3911) are classified as *Methanobacterium*, which is the most dominant Archaeal genus present. Indeed OTU_1 represents the second most dominant species present in the system contributing 6.47% of the overall community. *Methanobacterium* are hydrogen utilising methanogens (Madigan, Martinko et al. 2009) and have been reported to be dominant both in the seed sludge used in these reactors and under apparent stress conditions in Chapter 6 of this thesis. By contrast *Methanosaeta*, the only acetotrophic methanogen present in the most abundant twenty organisms, comprises only 1.32% of the total community population. This finding again suggests dominance of the hydrogenotrophic pathway in EGSB reactors during stress conditions.

OTU	Relative Abundance (%)	Domain	(%)	Phylum	(%)	Class	(%)	Order	(%)	Family	Genus	(%)
OTU_15	8.56	Bacteria	1	unclassified_Bacteria	1							
OTU_1	6.47	Archaea	1	Euryarchaeota	1	Methanobacteria	1	Methanobacteriales	1	Methanobacteriaceae	Methanobacterium	1
OTU_42	3.86	Bacteria	1	Bacteroidetes	0.9	unclassified_"Bacteroidetes"	0.9					
OTU_10	3.86	Bacteria	0.99	unclassified_Bacteria	0.99							
OTU_13	3.85	Bacteria	0.99	unclassified_Bacteria	0.99							
OTU_31	2.84	Bacteria	1	Bacteroidetes	1	unclassified_"Bacteroidetes"	1					
OTU_17	2.65	Bacteria	1	Bacteroidetes	1	Bacteroidia	1	Bacteroidales	1	Prevotellaceae	Prevotella	1
OTU_3	2.14	Bacteria	1	Proteobacteria	1	Deltaproteobacteria	1	Syntrophobacterales	1	Syntrophobacteraceae	Syntrophobacter	1
OTU_98	2.05	Bacteria	1	Spirochaetes	1	Spirochaetes	1	Spirochaetales	1	Spirochaetaceae	Treponema	1
OTU_5	1.85	Bacteria	1	Synergistetes	0.99	Synergistia	0.99	Synergistales	0.99	Synergistaceae	unclassified_Synergistaceae	0.99
OTU_106	1.76	Bacteria	1	Firmicutes	1	Clostridia	1	Clostridiales	1	Gracilibacteraceae	Lutispora	0.9
OTU_107	1.72	Bacteria	1	unclassified_Bacteria	1							
OTU_92	1.57	Bacteria	0.98	unclassified_Bacteria	0.98							
OTU_3303	1.32	Archaea	1	Euryarchaeota	1	Methanomicrobia	1	Methanosarcinales	0.96	Methanosaetaceae	Methanosaeta	0.96
OTU_150	1.32	Bacteria	1	unclassified_Bacteria	1							
OTU_91	1.30	Bacteria	1	Firmicutes	1	Clostridia	1	Clostridiales	1	Eubacteriaceae	unclassified_Eubacteriaceae	0.98
OTU_7	1.27	Bacteria	1	Proteobacteria	1	Deltaproteobacteria	1	Desulfuromonadales	1	Geobacteraceae	Geobacter	1
OTU_2164	1.16	Bacteria	1	unclassified_Bacteria	1							
OTU_3911	1.12	Archaea	1	Euryarchaeota	1	Methanobacteria	1	Methanobacteriales	1	Methanobacteriaceae	Methanobacterium	1
OTU_40	1.07	Bacteria	0.99	unclassified_Bacteria	0.99							

Table 7-8: Mean relative abundance of the 20 most relatively abundant OTUs in R7-9 sludge and OTU taxonomic classification found using RDP classifier. Blue highlight indicates organisms belonging to the domain Archaea.

The relative abundance of the 20 most abundant organisms at each time point in each reactor is shown in Figure 7-13. Qualitatively, during Phase 1 in which the reactors were operated as replicates, fluctuations in composition of dominant community members appears reasonably well replicated. Subsequent to ramping however the communities begin to diverge. *Methanobacterium* (OTU_1) is consistently dominant in the community and along with OTU_15 and OTU_10, both unidentified bacteria, and appear to form something of a dominant core in the community. OTU_13 appears abundant during healthy operation in Phase 1 however declines in subsequent phases but is again an unidentified bacteria. During Phase 1 OTU_42, which is of the class *Bacteroidetes*, appears dominant and increasingly so in R8 during Ramp 1 on day d95. Organisms of the phylum *Bacteroidetes* have been found to form a core group of organisms in the digestion of municipal sewage in full-scale digesters along with those of the phylum *Synergistetes* (Riviere, Desvignes et al. 2009) which is also abundant here (OTU_5) during Phase 1. OTU_31 and OTU_17 are also identified as of the phylum *Bacteroidetes*, suggesting that this phylum is central not only to treatment of sewage at full-scale but to the treatment of synthetic sewage in lab-scale digesters. Of the *Bacteroidetes*, OTU_17 which is further identified as of the genera *Prevotella*, appears highly abundant in the granule community on days d155 and d197 for R8, and on d197 for reactors R7 and R9 after the onset of failure in the digesters. Species of the genera *Prevotella* are associated with protein degradation and have been identified in anaerobic digesters treating food wastes (Garcia-Peña, Parameswaran et al. 2011). The increased abundance in the latter stages in this trial may reflect increasing peptone concentrations in the reactor influent. Also after the onset of failure, OTU_98 of the genera *Treponema*, is emergent in the most dominant community post-failure and is found to very highly abundant in each of the three reactors on d197. Species of the genera *Treponema* are identified as metabolically and spatially

associated with cellulose degraders on the surface of cellulolytic food in animal rumen (Kudo, Cheng et al. 1987) suggesting that here they may play a role in aiding hydrolysis and fermentation.

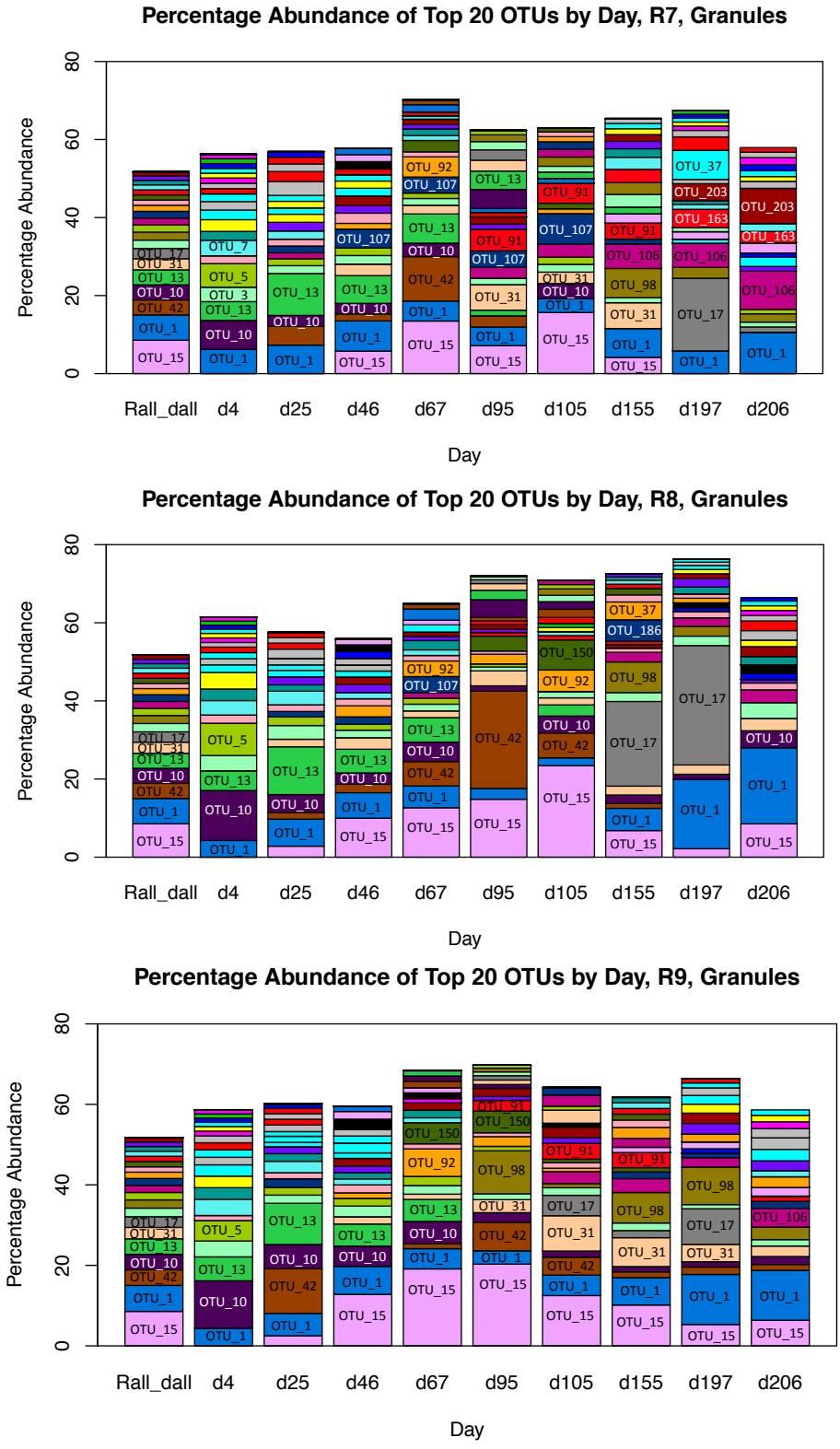


Figure 7-15: Stacked bar plots visualising the change in relative abundance of the 20 most abundant OTUs in the reactor sludge of each of bioreactors R7-9 with time against a mean for the reactor set at all time points (Rall_dall). Most dominant OTUs or organisms whose relative abundance shows a large increase between sampling points are labelled with unique OTU ID (e.g. OTU_1 etc).

The bar plots also point to a structural change in community with time in that the 20 most dominant species tend to comprise a greater portion of the community as the OLR is increased. As evenness has been associated with stability in mixed microbial communities (Wittebolle, Marzorati et al. 2009), this would suggest that as stress is increased in the system via increasing OLR, the community structure tends to one that is less favourable for stable reactor operation. In all, the composition of the most dominant OTU's in the reactor sludge is found to be highly variable however a core group of organisms including OTU_1 of the genera *Methanobacterium* appears to persist even under stress conditions. That these organisms persist even after 'failure' was deemed to occur in the reactor indicates that those granules remaining post-washout have not in themselves failed rather failure arises due to loss of granules by washout and disintegration. This is confirmed by activities recorded in the final SMA. As such, the sludge granules themselves may not act as the best source of information on reactor failure, rather reactor effluent samples may be more informative.

7.4.4.3 Identifying Indicators for Failure in NGS Data

During this trial failure was deliberately induced in each of three lab-scale EGSB reactors by incrementally increasing OLR. The metadata was analysed in relation to observed failure modes to determine association between failure and physico-chemical monitoring data. Here, a similar approach is taken using the NGS data.

Relative Proportion of Archaea in Sludge and Effluent Communities

The NGS data was inspected to determine the changing relative proportion of Archaea in the community in both the reactor sludge and the reactor effluent (Figures 7-16 and 7-17).

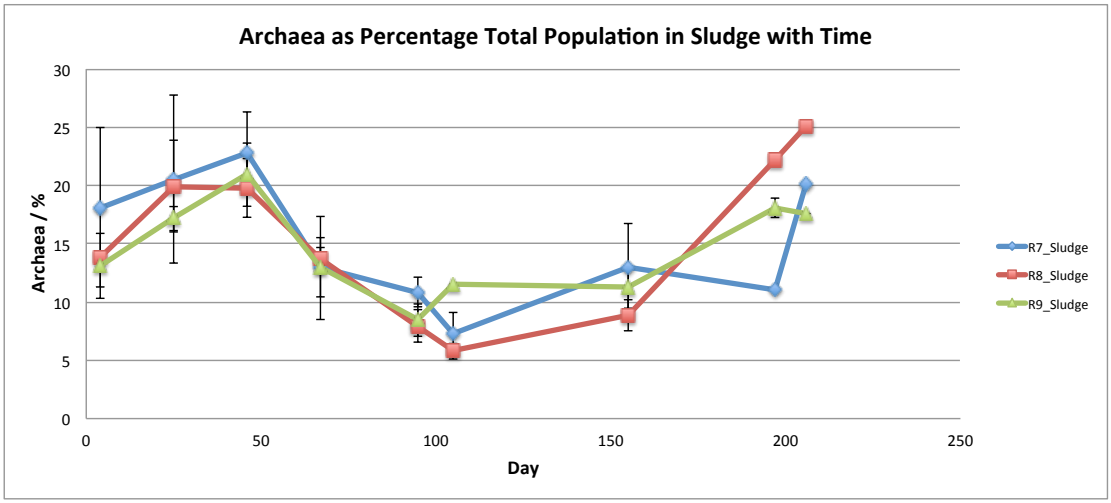


Figure 7-16: Scatterplot showing change in relative abundance of Archaea in sludge community with time in each of reactors R7-9. Points plotted are mean of the top and bottom reactor samples at that time point and error bars show standard deviation.

An initial increase in relative abundance of Archaea is seen in each reactor during Phase 1 until day d46. Subsequently, a gradual decline in the relative abundance of Archaea in the sludge community for each reactor is observed with a minimum occurring at the end of Ramp 1 for each reactor type in the treatment of high-strength SYNTHES. As absolute numbers of Archaea were not determined, it cannot be known whether this decline arises from a decrease in the number of Archaea present, or from an increase in the absolute number of bacteria present. Whilst bed depth was noted to increase during the trial, the increase was minimal in each reactor during the period to day d95 and any increase measured visually appeared to arise from increased expansion in the sludge bed as opposed to granule growth. As such it is assumed that the declining population of Archaea reflects increasing stress due to increasing OLR in the reactors. During Ramp 2 and Ramp 3 however the relative abundance of Archaea is seen to recover. During these stages, the granules appeared less distinct from the reactor liquor. It may be the case that bacterial populations typically found at the outside of granules were diminished by washout during partial disintegration of the granules during these stages.

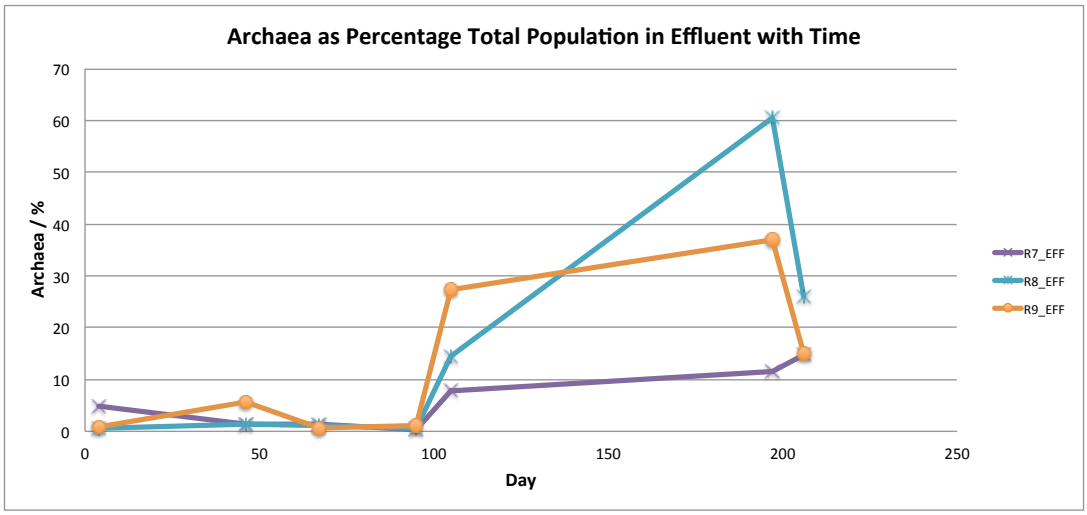


Figure 7-17: Scatterplot showing change in relative abundance of Archaea in reactor effluent community with time in each of reactors R7-9. Single samples were taken at each time point hence error bars cannot be plotted.

Perhaps of greater interest in relation to determining possible predictors of reactor failure, is the changing relative proportion of Archaea in the effluent for each reactor. It is observed that at on d105 at the end of Ramp 1, the relative proportion of Archaea in the effluent of each reactor sharply increases (Figure 7-17). This occurs subsequently to the period of acetate accumulation in the reactor effluent for each reactor, but prior to propionate accumulation i.e. prior to the onset of failure in the reactors. Further, the increase in Archaea in the effluent occurred prior to substantial granule loss via either granule disintegration or mass washout events. As such it is proposed that a spike in

acetate accumulation accompanied by a sharp increase in Archaea detected in reactor effluent may act as an indicator for the onset of failure of EGSB reactors.

Indicator Organisms: Relative Abundance of *Methanosaeta* and *Methanobacterium* with Time in Sludge and Reactor Effluent

The metadata recorded for this experiment suggests that the hydrogenotrophic pathway is dominant under stress conditions arising from increasing OLR in lab-scale EGSB reactors. Further, the metadata suggests that acetate accumulation acts a precursor to sludge degranulation, and acetate accumulation is associated with a decline in activity in the sludge using H2/CO2 as substrate. As it was previously determined that degranulation is additionally preceded by an increase in the abundance of Archaea in the reactor effluent, the data was analysed to determine whether dominant acetotrophic or hydrogenotrophic methanogens in the reactor effluent might act as indicator organisms for the onset of reactor failure. The relative abundance of *Methanosaeta* with time in both the sludge and reactor effluent is plotted for each reactor alongside the relative abundance of *Methanobacterium* (Figure 7-18). As acetotrophic methanogens are more commonly associated with sludge granulation is was anticipated that *Methanosaeta* may have appeared most dominant in the effluent during periods in which granule disintegration was observed. The data collected here however suggests that in fact the relative abundance of *Methanobacterium* sharply increases in the reactor effluent immediately before and during reactor failure. This increase occurs a minimum of nine days prior to propionate accumulation in the reactors and prior to significant loss of granules by either washout or disintegration. As such, Methanobacterium are proposed here as indicators for failure that may provide early warning of propionate accumulation in EGSB type reactors treating high-strength SYNTHES. This is a significant finding if determined to be a consistent failure mode across a range of anaerobic sludges and reactor types. Methanobacterium and other Archaea have been demonstrated to autofluoresce due to the presence of F420 enzymes (Solera 2001). As such, as predictors of failure they may be detected rapidly, and without the requirement for DNA based assays making them ideal indicator organisms for failure in-line and in real-time in full-scale anaerobic plants. The mechanism by which Methanobacterium becomes dominant in the effluent cannot be fully ascertained from this trial. A speculative mechanism is outlined however and future works to assay this mechanism are proposed.

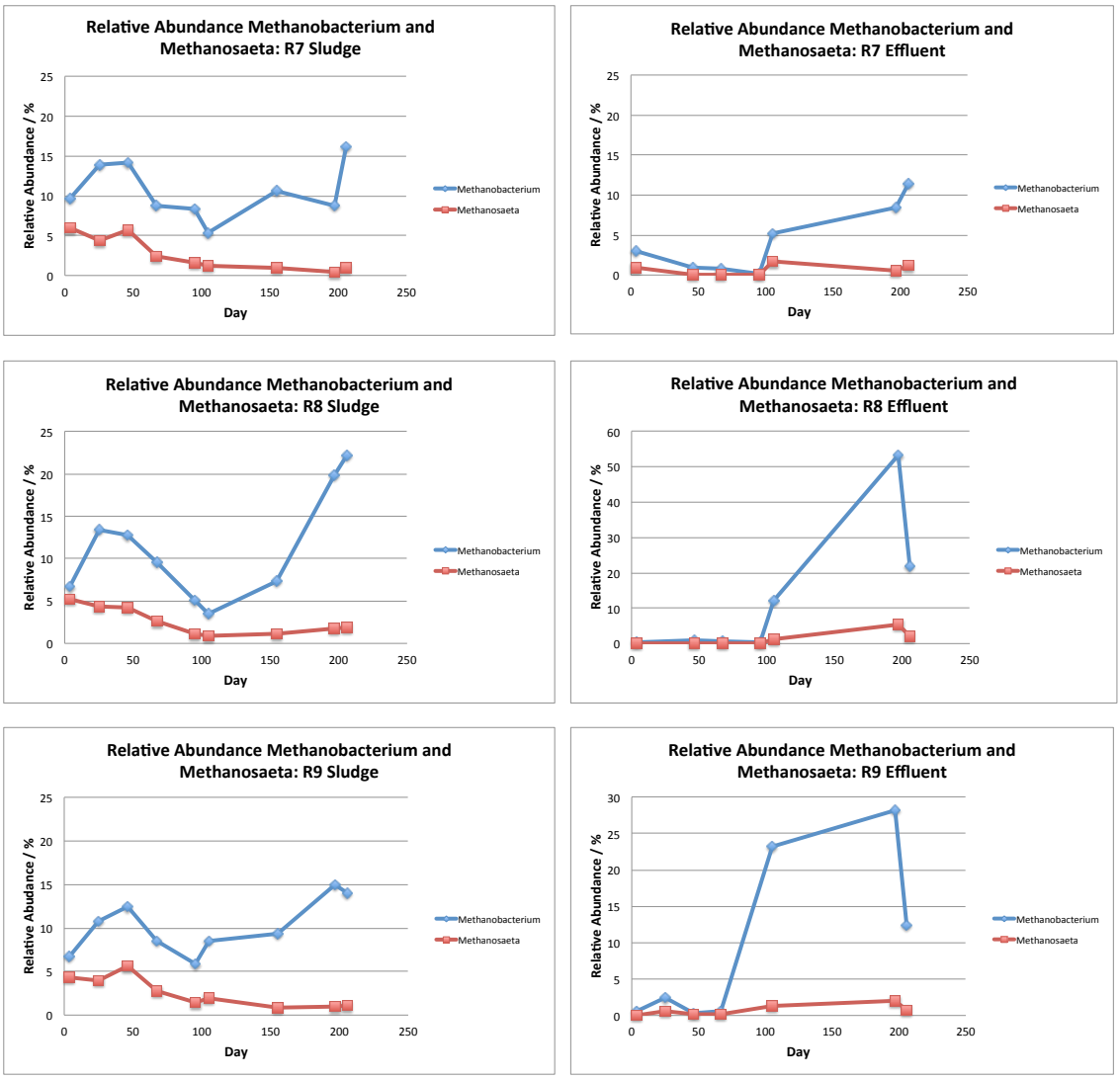


Figure 7-18: Scatterplots showing relative abundance of Methanosaeta and Methanobacterium in reactor sludge beds (mean of two observations per reactor) and reactor effluent (single observation per reactor) with time in each of the reactors R7-9 sludge beds.

Whilst a great number of papers are written on the subject of granulation in upflow anaerobic digesters such as the EGSB, fewer are written on either granule development or granule disintegration. Dominant amongst those works on granule formation is the observation that Methanosaeta, a filamentous acetotrophic methanogen, is responsible for forming the core of the granule (Schmidt and Ahring 1996, Hulshoff Pol, de Castro Lopes et al. 2004). Fluorescent In-Situ Hybridisation (FISH) has been utilised to verify this mode of formation by inspection of spatial distribution of methanogens in granules believed to be in different stages of growth (Figure 7-19). It is seen that in 'young' (small) granules, Archaea dominate the inner core of the granule. In 'older' (mid-large) granules that represent the bulk of those present in a functioning digester however it is seen that Archaea are also distributed in colonies near or at the surface of the granule. Indeed, Macario et al report in mature thermophilic granules that whilst acetotrophic methanogens do dominate the core of a granule, that hydrogenotrophic methanogens such as *Methanobacterium* are

found in microcolonies near the granule surface (Macario, Visser et al. 1991). Assuming a similar formation here, as granules were seen to become increasingly indistinct at the granule surface it may be the case that during the disintegration processes that these colonies became dislodged from the granule surface hence the sudden increase *Methanobacterium* in the reactor effluent. Hydrogen utilising methanogens are required to maintain a low hydrogen partial pressure for the syntrophic degradation of VFAs and dislocation of sufficient numbers of such colonies might then be sufficient to disrupt the spatial distribution of syntrophs necessary for propionate degradation. That propionate accumulation occurred shortly after the increase in Methanobacterium in reactor effluent appears to support this hypothesis.

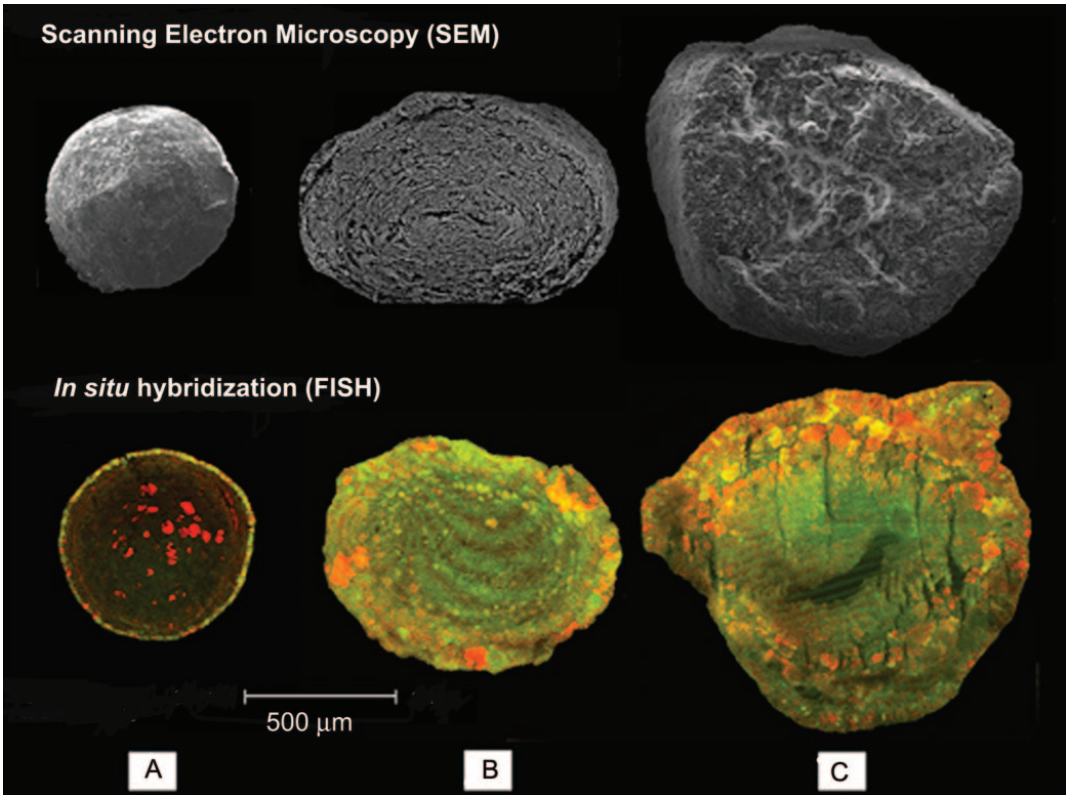


Figure 7-19: Change in granule structure with granule age / size demonstrated using FISH (Diaz, Stams et al. 2006). Image shows that in mature / large granules Archaea (Arch915-Cy3, red, universal probe for Archaea) may be distributed close to the granule surface rather than at the granule core, whilst Bacteria are more uniformly distributed (Eub338- fluorescein, green, universal probe for Bacteria).

To ascertain such a mechanism for granule disintegration FISH should be utilised on samples of sludge from the reactors taken before and after failure, and from those granules found in the reactor effluent. Samples of such have been stored however time constraints prevented that work being conducted as part of this thesis. Whilst potentially informative, the proposed mechanism describes the result of disintegration rather than the cause. Further work on the interaction of the material properties of the granular biofilm and the influent solids should also be conducted.

Microbial Ecology in Reactor Sludge and Effluent

Ecology indices for rarefied richness, Pielous Evenness Index and Simpsons Index of Diversity were calculated on samples from reactor sludge and effluent at each sampling time point with all samples rarefied to a common minimum read count of 3000 as described previously. It is observed that rarefied richness and evenness tends to follow a similar trend in the sludge granules for each reactor regardless of the ramping strategy applied (Figure 7-20).

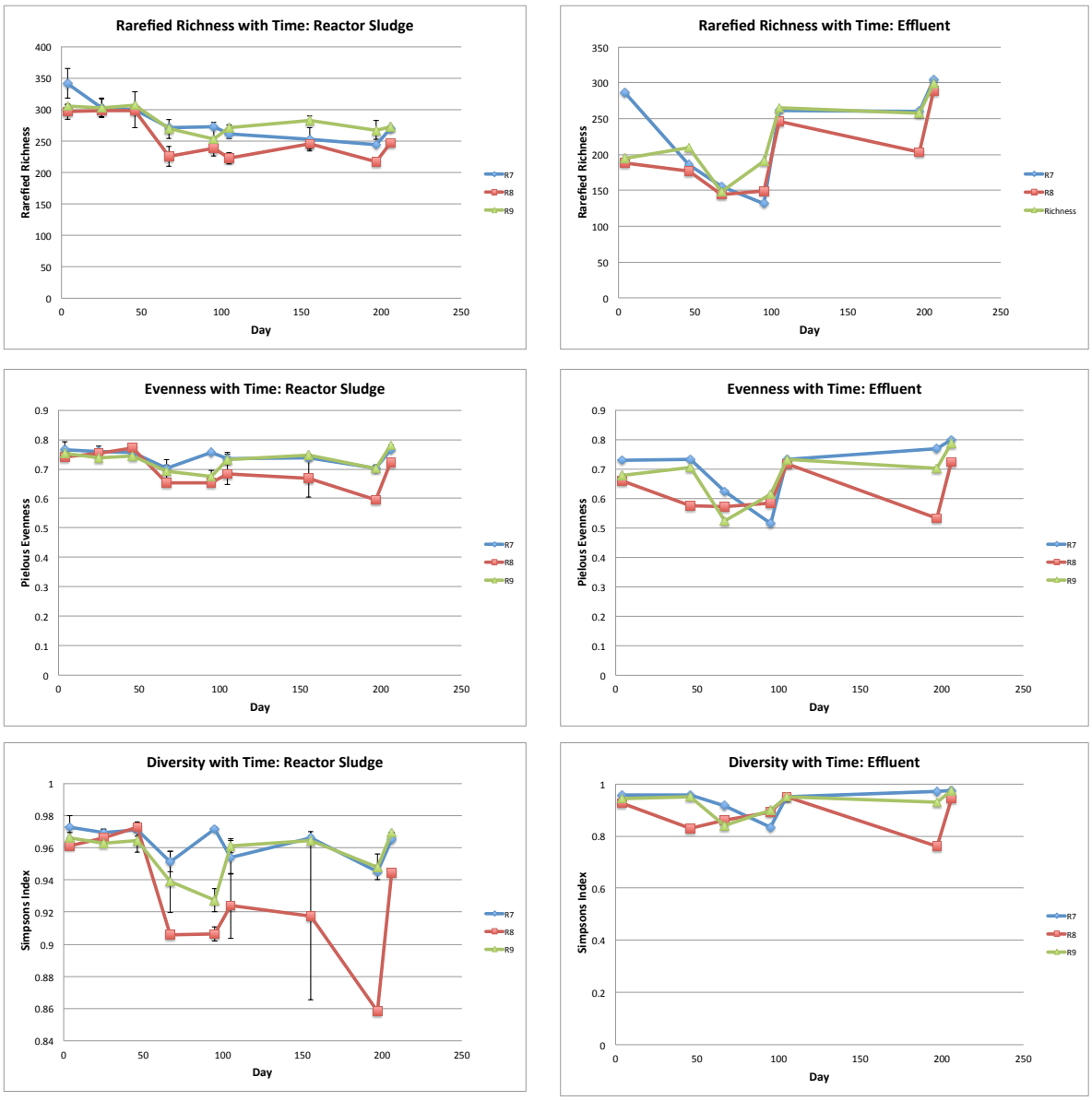


Figure 7-20: Scatterplots showing time-series of rarefied richness, microbial community evenness and microbial diversity in reactor sludge and effluent for each of reactors R7-9. Sludge samples are mean of two observations and error bars show standard deviation whilst effluent samples are single observation for each reactor at each time point.

Richness is found to diminish after d46 of the trial during Phase 1 in response to the treatment of high-strength SYNTHES. Thereafter, richness in the sludge granules remains relatively stable with time. Evenness follows a generally similar trend but shows some sign of recovery towards the end of the trial in those granules remaining in the reactor post-washout. This may suggest that the granules washing out of the reactor were of lower evenness or alternatively, that the granules remaining in the reactor have adapted to the increased OLR. Diversity in the sludge granules shows more distinct trends in each reactor. It was suggested previously that the magnitude of ramping in both R7 and R9 might have resulted in improved responses to stress in those reactors. Diversity and to a lesser extent richness indices however would indicate that R8 which was ramped least may have been less rich and less diverse than R7 and R9 prior to the onset of ramping. This may in part have contributed to the difference in failure mode in R8 in which ramped OLR was applied most gradually.

Ecology indices for the reactor effluent by contrast to those for the reactor sludge granules appear more indicative of failure in the reactor. The VFA profiles for reactor effluent indicated failure by the onset of irrecoverable propionate accumulation in each of R7, R8 and R9 on days d114, d123 and d128 respectively. The ecology indices describing the community structure in the reactor effluent show a distinct increasing trend on day d105 most notably for richness and evenness. That this increasing trend for richness and evenness in reactor effluent occurs prior to propionate accumulation strongly suggests that in addition to the indicator organism identified previously, that evenness and richness in reactor effluent might function as an early warning signal for failure in a reactor under high solids loading. If verified in further studies, this finding may offer a crucial monitoring tool for operation of full-scale EGSB reactors. Whilst currently DNA based assays are somewhat expensive and take significant time to process from sample though to extraction, PCR, sequencing and bioinformatics and processing even with the use of NGS methods, other simpler methods for the analysis of community structure are under development. Flow cytometry is one such method capable of detecting shifts in microbial community structure in real time (Bombach 2010, De Roy, Clement et al. 2012). Coupled with increased understanding of the implication of changes in ecology in a reactor effluent, this could offer an inexpensive method for monitoring reactor operation and predicting failure in sufficient time that it can be successfully mitigated.

Microbial Community Development and Granule Disintegration

Increased richness, evenness and diversity in the reactor effluent were identified as precursors to granule disintegration. Further, richness and evenness were not only maintained at a high level during the reactor failure period but also tended towards those values found for the sludge granule. Tentatively, this supports the somewhat subjective assertion that granule disintegration was occurring in the bioreactors. The relative abundance of the most abundant 20 OTUs for each effluent sampling time point are plotted in Figure 7-21.

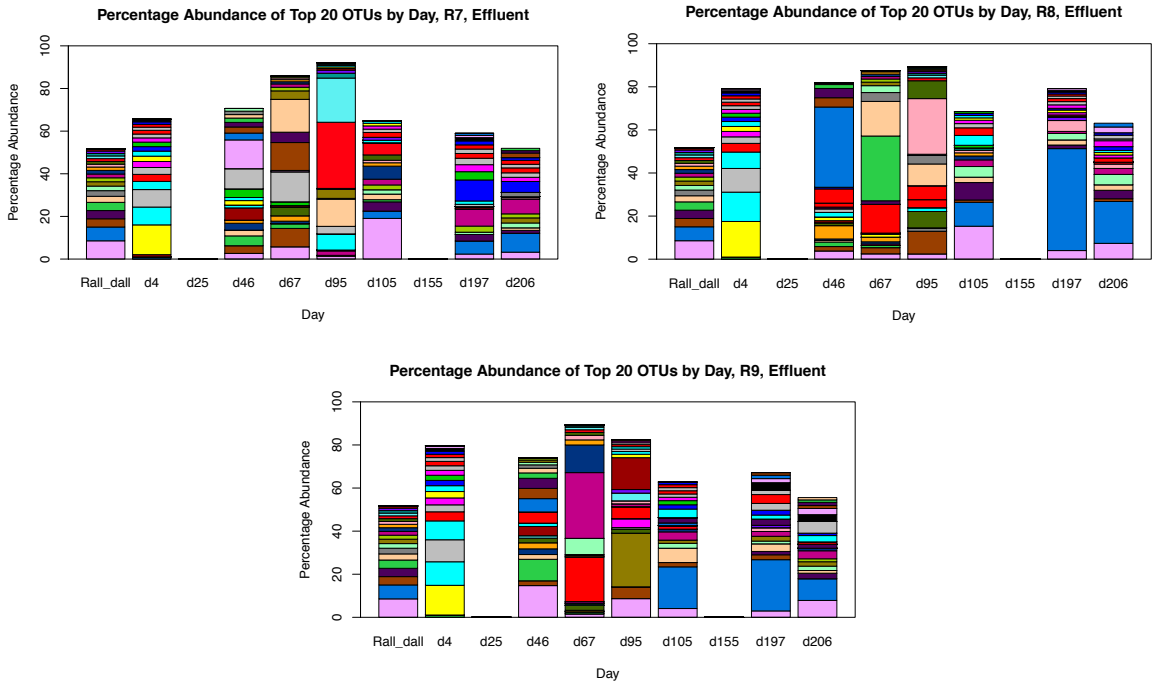


Figure 7-21: Stacked bar plots visualising the change in relative abundance of the 20 most abundant OTUs in the reactor effluent of each of bioreactors R7-9 with time against a mean of the sludge bed community for the reactor set at all time points (Rall_dall). No bars are plotted for days d25 or d155 as no effluent sample was taken at these times.

Here the identity of the individual organisms in not considered in detail, rather the plot is given to evidence the increasing similarity in composition of the reactor effluent to that of the reactor sludge during the failure period. In each effluent sample, no whole granules were present; only reactor liquor was centrifuged for DNA extraction. In Figure 7-21, the plot in each case shows the mean composition of the reactor granules over the duration of the trial in the first bar (Rall_dall). It is observed that during periods of successful operation the reactor effluent community is compositionally and structurally distinct from the reactor sludge. On d105 and on subsequent days in which granule disintegration was visibly occurring in the reactors, the dominant composition of effluent community tends towards that of the granules both in terms of membership and community structure. This is

confirmed for the 100 most abundant OTUs by plotting Bray-Curtis similarities (Figure 7-22) for periods defined as 'healthy' (d4-d95), 'warning' (d105) and 'failing' (d155-d206) for the reactor set.

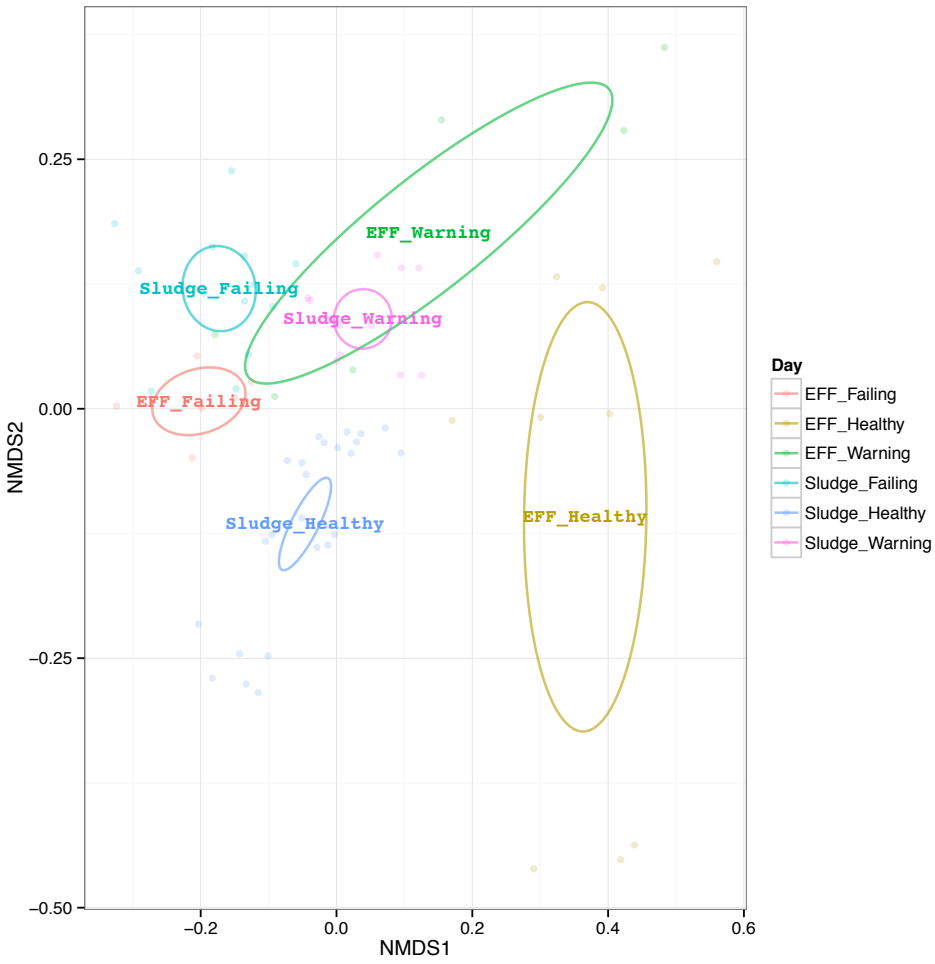


Figure 7-22: Two-dimensional NMDS ordination plot (Stress = 0.110) of the 100 most abundant OTUs in the sample set, grouped by sample type (EFF_ indicates effluent samples, Sludge_ are samples from reactor sludge bed) and reactor condition (Healthy, warning and failing) and plotted using Phyloseq and Bray-Curtis similarity. In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

It is observed that during 'healthy' operation of the reactor, the effluent community is both distinct from that of the reactor sludge and more variable than that of the reactor sludge. During the warning period it is observed that the effluent and sludge communities tend to merge but that the effluent community maintains some degree of increased variability as compared to that of the sludge granules. During failure, the sludge and effluent communities remain similar and the effluent community tends to contract in terms of variability confirming that after the onset of granule disintegration, the reactor effluent is dominated by organisms from the sludge bed.

7.5 Conclusions and Recommendations

The proposed failure mechanisms found in this trial are summarised in Figure 7-23.

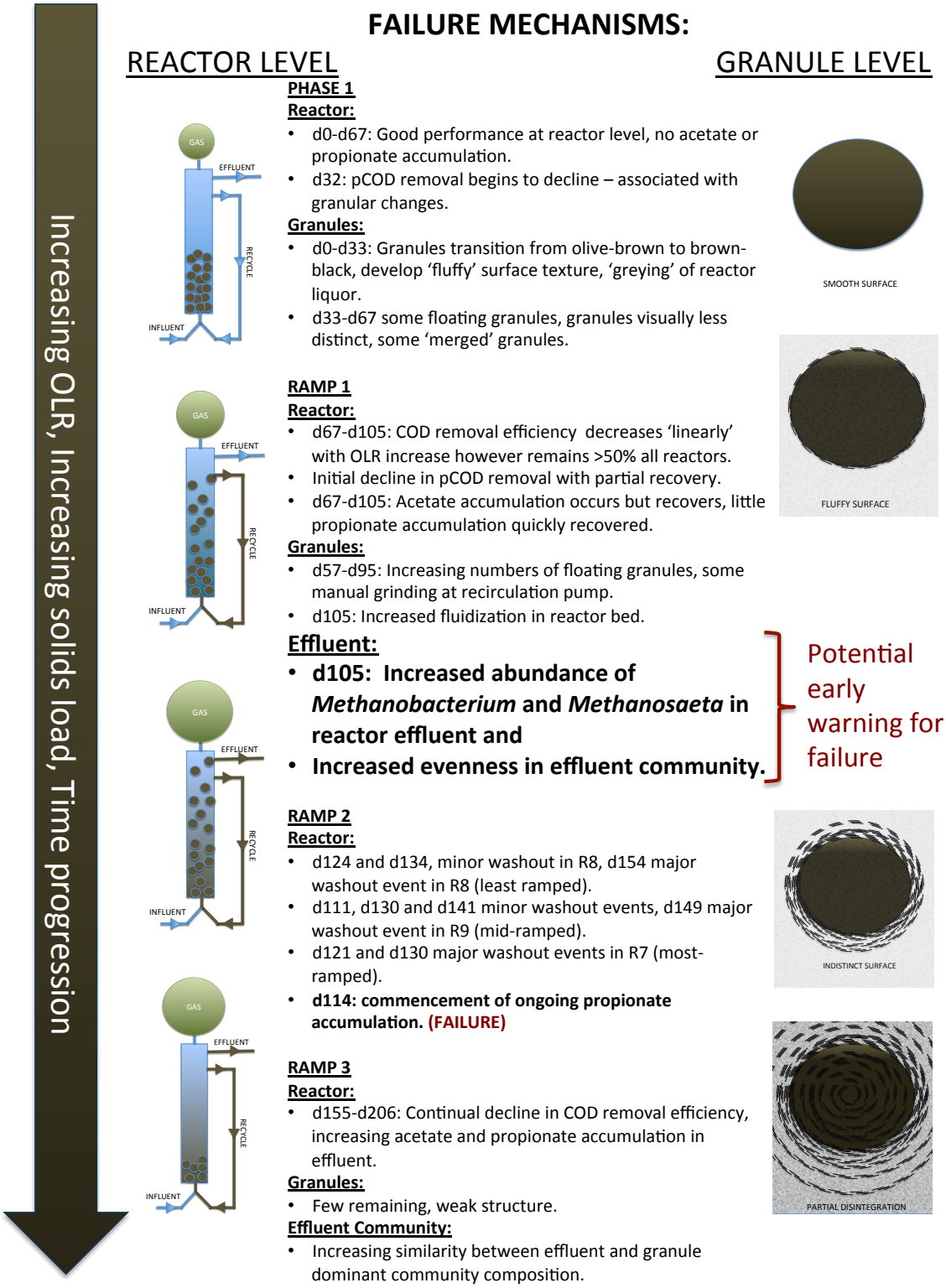


Figure 7-23: Schematic of proposed failure mechanisms of the EGSB under high-solids loading

The key failure mechanisms observed were washout under the action of increased biogas production and washout due to granule disintegration. In terms of the utilisation of EGSB

reactors for the treatment of pit-latrine wastes, the study suggests that without significant adaptation of the biofilm to high solids environments, that significant dilution of wastes would be required for treatment function successfully. Whilst plausible, such an approach is likely to negate the advantages of small reactor footprint for application in the peri-urban environment. As such, further work is recommended to determine methods to increase granule density and integrity for this purpose and the EGSB is not recommended for this application at present. Application of FISH to granules to confirm or reject the proposed disintegration method is recommended as a starting point in this work.

Whilst the study concludes negatively in terms of applying the EGSB to the treatment of pit latrine wastes, the adoption of the limit state approach to experimental design produced extremely positive results. Deliberately inducing failure in the reactors under increasing OLR, enabled identification of *Methanobacterium* as a possible indicator organism in reactor effluent predicting the onset of propionate accumulation in EGSB reactors. Further, the method identified increased evenness and richness in reactor effluent as indicators of granule disintegration. As such, NGS data suggests that monitoring microbial community structure and composition in the effluent of EGSB type reactors, can provide early warning of failure in a reactor stressed under high-solids loading such that failure might be mitigated. By identifying not only indicator organisms for failure but wider ecological indicators, the study suggests that in-line monitoring of reactor effluent community may be an inexpensive and realistic option for managing biotechnologies in the near future. The study concludes that by deliberately inducing failure in biotechnologies that not only might the limits of application be better understood but that process control in application of biotechnologies might be greatly improved.

8 Granule Growth: Preliminary Study

Abstract

The development, maintenance and retention of a diverse and resilient microbial community in well-settling granular sludge is vital to the operation of high-rate anaerobic digestion technologies such as the EGSB. Granulation is an extensively studied phenomenon yet agreement on granulation mechanisms is yet to be reached. Furthermore, granule growth and maintenance remain poorly understood. Here a growth cycle for sludge granules is proposed arising from observation and analysis of granules during experiments conducted in 1-D lab-scale EGSB bioreactors.

Specific methanogenic activity assays in combination with 16S rRNA (V4 region) amplicon sequencing of sludge granule samples indicated stratification of physiology and ecology in the sludge bed in the 1-D lab-scale EGSBs. Particle size distribution and granule settling velocity are used to investigate potential physical mechanisms for biological stratification in the sludge bed. In addition, the relationship between granule size and microbial community composition is used to propose a sludge growth cycle.

It was determined that for sludge sampled from a fully mixed full-scale EGSB, granule size may be associated with community physiology. Larger granules are found to be significantly ($P<0.01$) more active in acetate, butyrate and propionate assays than small granules; whilst small granules are significantly more active ($P<0.01$) in H2/CO2 and ethanol assays. It is also shown that granules in 1-D lab-scale reactors are stratified by size and settleability in the reactor. Finally, a means for testing the proposed growth cycle, for which the work presented may be viewed as a preliminary study, is outlined and discussed.

8.1 Introduction

Anaerobic sludge granulation was first observed over 40 years ago in the study of anaerobic filter treatment systems and clarigesters for the treatment of sewage (Young and McCarty 1969, Hulshoff Pol, de Castro Lopes et al. 2004). The discovery inspired the development of a range of high-rate anaerobic wastewater treatment systems including the UASB, the EGSB and the fluidised bed bioreactor, broadening the range of applications for AD and ensuring that AD remains a growing market today. In each of these technology types, high-rate treatment depends on the growth, development and maintenance of a diverse and resilient microbial community in well settling sludge granules, the retention of which enables decoupling of solids and liquid retention times in

the system (Nachaiyasit and Stuckey 1997, Zheng, Wang et al. 2012). As such, sludge granulation is an extensively studied phenomenon. Numerous theories describing sludge granulation have been proposed however agreement on the exact mechanism by which granules are formed is yet to reached (McHugh, O'Reilly et al. 2003, Hulshoff Pol, de Castro Lopes et al. 2004, Lim and Kim 2014). Further, whilst granulation is extensively studied, granule growth and maintenance, as are essential in a reactor beyond start-up, remain relatively less well understood.

This Chapter proposes a growth cycle for anaerobic granular sludge and draws on data collected during the course of the PhD in combination with data gathered to specifically enable development of the hypothesis. In 1-D lab-scale EGSB bioreactors treating low solids wastes, gradation of granules by size and settleability with depth in the reactor have been reported in the literature (Arcand, Guiot et al. 1994, Aiyuk and Verstraete 2004, Karnchanawong 2009). Further, studies have reported variability in spatial microbial community distribution and composition within granules of differing size (Diaz, Stams et al. 2006). Within the context of the work conducted here, gradation of both community composition and physiology with depth in the sludge bed was reported in relation to the treatment of high-strength (Chapter 4) and low-strength (Chapter 6) industrial wastewaters in 1-D lab-scale bioreactors. The drivers for this gradation in microbial community composition and physiology with depth in lab-scale EGSBs are investigated in relation to granule size and granule settleability. Further, gradation in community composition and physiology with granule size and depth in a full-scale digester are investigated. Contrasting the data between the full-scale and lab-scale digester studies, the growth cycle and a methodology by which it might be tested are proposed. This proposal is now being implemented by a PhD candidate Anna Trego, NUI Galway, who is co-supervised by Dr Gavin Collins and I.

This study reported here aims to:

1. Establish whether spatial distribution of microbial communities exists in the full-scale digester ADR2.
2. Determine the extent to which granules distribute by size in:
    a. 1-D and 3-D lab-scale digesters used for the treatment of industrial waste.
    b. The full-scale digester ADR2.

3. Determine whether the difference in community composition and physiology with depth in 1-D lab-scale reactors is related to size of the granule or is driven by the depth-associated position of the granule in the sludge bed.
4. Qualify the value in a 'granule-growth' study by providing preliminary data associating trends in microbial community composition, structure and physiology with granule size in full-scale reactor sludge.

8.2 Hypothesis Outline

It was observed in Chapters 4 and 6 that both microbial community composition and community physiology was gradated with depth in the sludge bed in the treatment of industrial wastewater in 1-D lab-scale EGSBs. During these trials, visual observation of granules with depth in the sludge bed was facilitated both in-situ, by virtue of the transparent reactor vessel, and via depth distributed sampling ports. Visual inspection of the granules suggested that granule size within the reactors was gradated with depth with larger granules collecting at the bottom of the bed and smaller granules at the top. These observations are illustrated in Figure 8-1 and outlined in as a step-wise progression as follows:

Size and Settleability Hypothesis:

- Granules form as small, densely packed structures.
- As microorganisms embedded in the granule multiply and new microorganisms attach, granules increases in effective volume – it is the extent of this growth, and the accompanying accumulation of mass, that will determine the position in the 1-D reactors by increasing settleability of the granules.
- As granules grow / mature, the community improves yielding better degradation of waste through increased methanogenic activity.

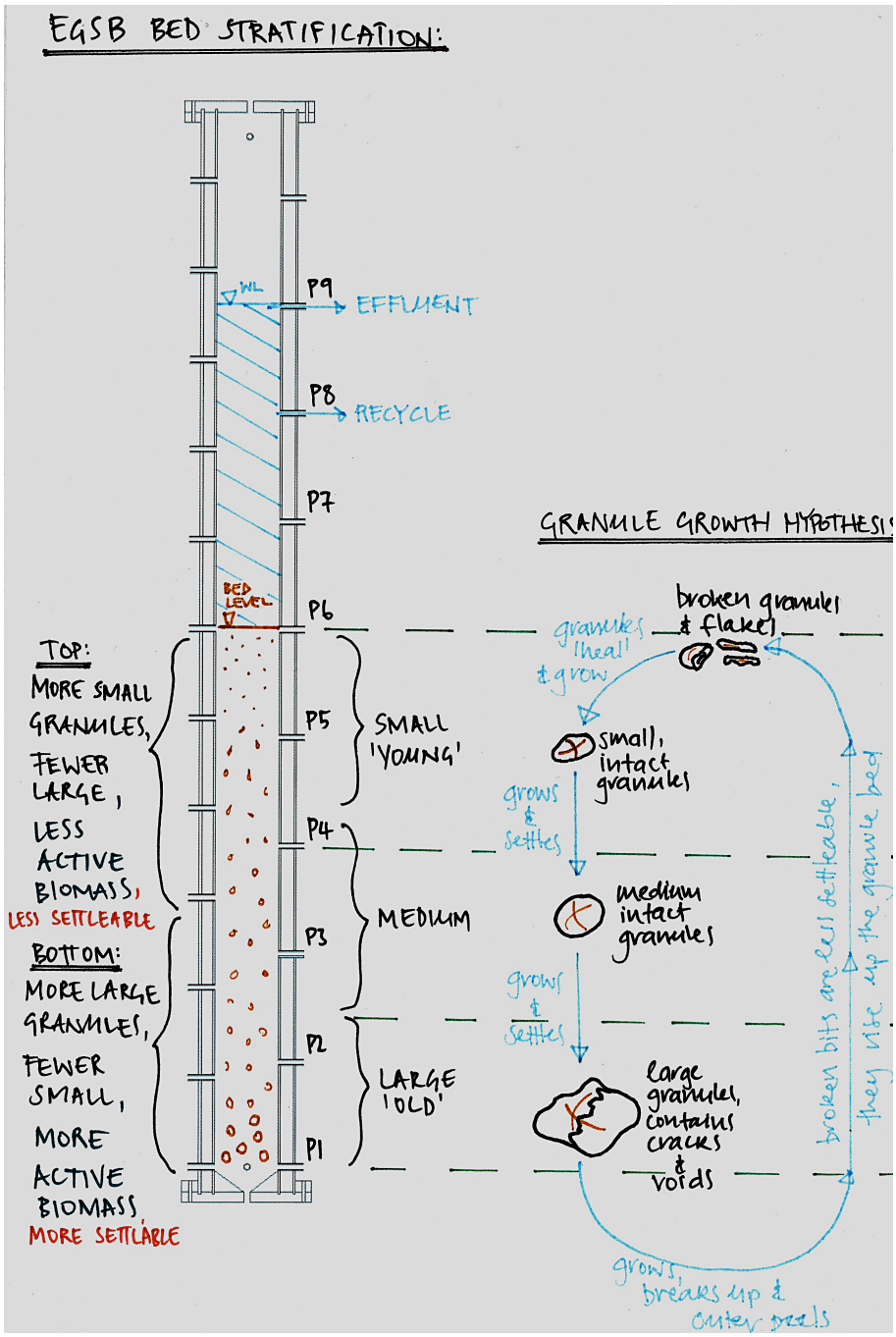


Figure 8-1: Schematic showing the primary hypothesis tested in this work – the proposed granule growth cycle

Visual observation of large granules at the bottom of the reactor during sampling suggested that growth was not indefinite as the largest granules seen were in the region of 4-6mm in diameter. Frequently, during sampling, large granules were observed that appeared partially fractured with void space at the granule core. Inspection of the sludge at the top of the reactor suggested that broken 'bits' of larger granules rose through the bed. Of these biofilm 'bits', some were angular at the edges and looked freshly broken others whilst others appeared to be smoothing and reforming into new granules. These observations gave rise to a secondary hypothesis as follows:

Growth Cycle Hypothesis:

- Larger granules are structurally weaker than small granules, containing more void space.
- Eventually large granules develop large voids and break due to forces from collisions with other granules or by accumulation of biogas in the central void spaces.
- The 'broken bits' of the large granules comprised dense biofilm sections that previously formed the exterior of the granules. These remain in the reactor and are smoothed due to collisions / shear, become small granules again and re-grow – the process is cyclical.

A sketch of the proposed growth cycle is included in Figure 8-1 in relation to depth in the reactor and two modes of size limited growth are given in Figure 8-2, both of which were visually observed in the reactors.

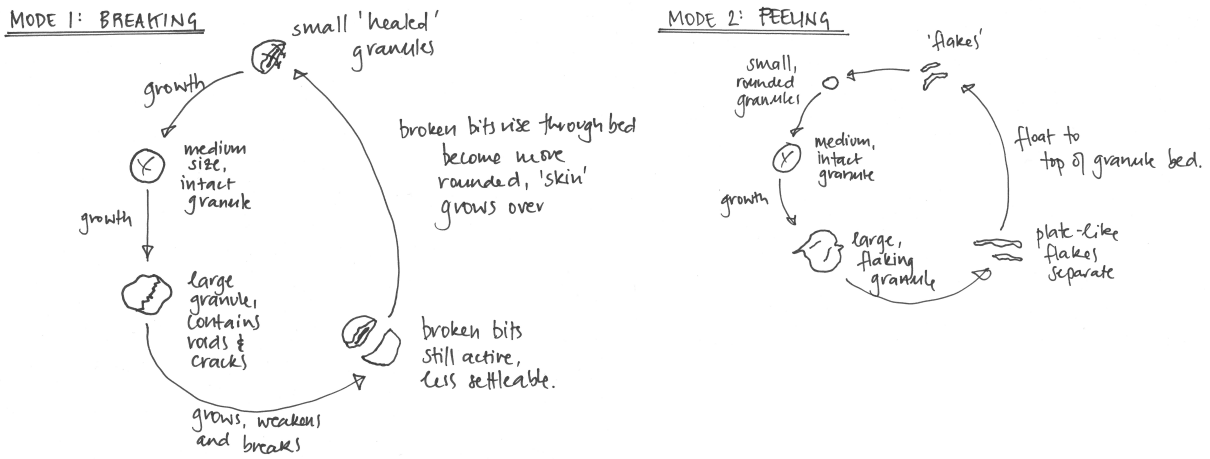


Figure 8-2: Schematics showing proposed growth modes in growth cycle: Mode 1 Breaking and Mode 2 Peeling

8.2.1 Data Sources

This Chapter contains results from experiments that are new to the thesis along with data collected from a range of other experiments that have been discussed previously. NGS and community physiology data demonstrating gradation of microbial community in 1-D lab-scale reactors is represented (Chapters 4 and 6). This data is then reassessed in light of

new data presented here including particle size distribution and granule settleability, size specific community physiology and NGS data on single granules of different size fractions. The data sets are drawn together to enable address of the key questions outlined in the introduction of this Chapter. The data refers to both lab-scale and full-scale reactor studies as follows (Figure 8-2):

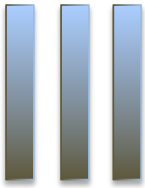
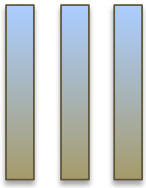
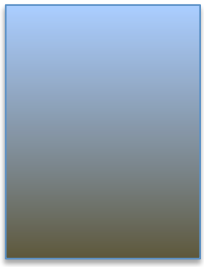
Lab-Scale		**Full-Scale**
 **R4-6: Full-Strength Distillery Waste @ 37^{0}C**	 **R1-3: Low-Strength Distillery Waste @ 37^{0}C**	 **ADR2: Full-Strength Distillery Waste @ 37degC**
Particle Size Distribution and Settling Velocity		
• Measured on seed sludge on day d-31, *and* • At top and bottom of sludge bed on day d122		• Measured at port P2 on day d-31, *and* • At top and bottom of sludge bed on day d267
Community Physiology (SMA)		
• Measured on seed sludge on day d-31, *and* • At top and bottom of sludge bed on day d70		• Measured at port P2 on day d-31, • And • On size gradated samples (small, medium and large) at top and bottom of sludge bed on day d410
Next Generation Sequencing (16S)		
• Mixed size samples from individual reactors in each set sampled at ports P1-P5 on days d4, d25, d46 and d70		• Mix size sludge samples sampled at top and bottom of sludge bed on days d189, d210, d238 and d266 • **Individual granules:** 3 granules each of sizes large, medium, small and very small from reactor ADR2 pot P2 on d459

Table 8-1: Schematic of experiment overview and outlining the physical, physiological and ecological monitoring methods applied.

Lab-Scale Data

NGS and SMA data collected from the 1-D lab-scale reactor trials reported in Chapter 4 and Chapter 6 are revisited. Further, sludge from those trials is subject to assessment of particle size distribution with depth and particle settleability measurement.

Full-Scale Data

A sub-sample of NGS data collected from a year long study of the full-scale digester ADR2 is used to assess the microbial community distribution with depth at full-scale. Particle size distribution and settleability with depth are also reported for sludge at the top and bottom of the sludge-bed in ADR2. Specific methanogenic activities in granules belonging to specific size fractions (small, medium and large) extracted from the top and bottom of the sludge bed in ADR 2 are reported. Finally, NGS data specific to granule size fractions (very small, small, medium and large) from a single port in the sludge-bed in ADR2 is reported. It is noted that the data describing the distribution of community composition and physiology with granule size in the full-scale reactor are not concurrent. Whilst regrettable, this arises from the developmental nature of this work and, hopefully, is appropriately reflected in the analysis and discussion of the data.

8.3 Materials and Methods

As elsewhere in this thesis, a detailed description of the materials and methods used is given in Chapter 3. A brief description of those methods specific to this chapter is given for completeness.

8.3.1 Granule Sampling for Particle Size Distribution and Settleability

1-D Lab-Scale, Full-Strength Distillery Waste (R4-6)

Granule samples of approximately 50ml total volume (mixed granules and liquor) were drawn from the top and bottom of the sludge-bed in each of the 1-D lab-scale reactors R4-6 on day d122. It is noted that d122 is beyond the bounds of the trial reported in Chapter 4. Day d122 marked the end of a 42 period in which the reactors R4-6 treated full-strength distillery waste at ambient temperature. No notable increase in bed-depth was noted on the temperature change from 37degC to 22degC and no increased mixing was visually observed. As such, the samples are thought to sufficiently represent the size distribution of the sludge at the end of the 37degC period (d70) for which SMA and NGS data are reported.

1-D Lab-Scale Reactors, Low-Strength Distillery Waste, R1-3

Sampling for this reactor set also occurred on d122 during which time the study reported in Chapter 6 was underway. As larger samples were required for PSD and settleability measurements than for NGS, these reactors were treated as a set rather than as individual reactors to prevent destructive sampling in the sludge bed during an active experiment. Two 50ml samples representing the sludge at (i) the top and (ii) the bottom of the sludge bed for the reactor set R1-3 were made by pooling smaller samples from the top and bottom of the bed in each of the individual reactors R1-3. Similarly to R4-6, this reactor set was being operated at ambient temperature at the point of sampling. Again, no significant increase in bed depth was observed at this point. As such, the samples are taken as a proxy for a typical distribution during operation in the mesophilic temperature range for these reactors.

Full-Scale Reactor ADR2

A 25ml subsample of the seed sludge, which was drawn from port P2 in full-scale digester ADR2, was subject to particle size distribution on day d-31 of the trial. For subsequent PSD measurement and size gradated SMA (day d267), and NGS (day d410) granule samples of approximately 1 litre were drawn from the top and bottom of the sludge bed in ADR2. Sub-samples (50ml) for particle measurement were then made in the laboratory taking care to ensure thorough mixing of the sludge prior by inversion prior to sub-sampling.

From each of the 50ml samples drawn at either full- or lab-scale, 25ml was used for measurement of particle size distribution and 25ml was used for measurement of settleability.

8.3.2 Particle Size Distribution

Particle size distribution was measured on 25ml samples of the seed biomass by gently washing biomass through a range of sieves (0.335mm, 1.0mm, 1.7mm, 2.0mm, 2.36mm, 2.8mm and 3.35mm) taking care not to damage granules.

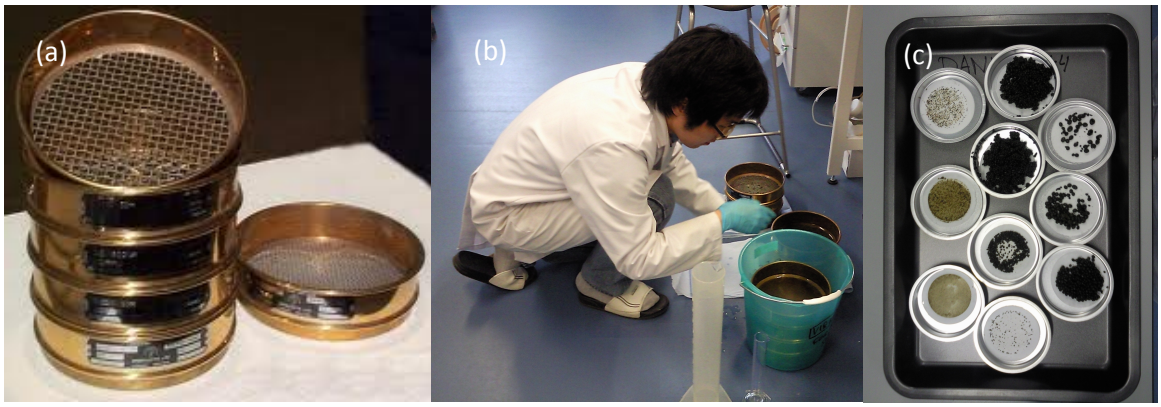


Figure 8-3: (a) Sieves used for size separation, (b) Wet sieving, (c) Filtered size fraction for TSS / VSS measurement

The granules were then gently backwashed from each sieve size using tap water to make size gradated sub-samples. The resultant water / granule mix for each size gradated subsample was filtered onto glass filter pads for TSS and VSS measurement (Standard Methods 2540B&E). The quantity of biomass in each size fraction within a 25ml sample is reported as a proportion of the total TSS as follows:

$$\text{granules represented by size fraction (\%)} = \frac{\text{TSS within that size fraction (g)}}{\Sigma\ \text{TSS for all size fractions (g)}} * 100$$

8.3.3 Settling Velocity and Density

Biomass samples (25ml) were sieved according to the protocol described previously. Single granules from each size fraction were placed into the top of a vertically levelled, water-filled, transparent acrylic tube and allowed to settle under the action of gravity. The settling tube had distance markings distributed at 10cm intervals along its length and the time taken to pass 3 consecutive 10cm intervals was recorded to ensure that terminal velocity had been reached. Settling velocity for each granule was then calculated according to equation:

$$\text{Settling velocity (m/s)} = \frac{\text{Distance settled (m)}}{\text{Time taken (s)}}$$

Fifteen granules in each size fraction were selected at random from each sieved fraction for velocity measurement and mean settling velocity for that size fraction is reported.

8.3.4 Community Physiology

1-D Lab-Scale

Specific methanogenic activity in each of the 1-D lab-scale reactor sets R1-3 (low-strength distillery waste) and R4-6 (full-strength distillery waste) was measured for pooled samples at the top and bottom of the sludge bed as described previously. For the purpose of comparison, results at the end of day d70 are reported for each reactor set during which time the sole difference between the reactor sets was the strength of the substrate treated.

Full-Scale

A size-gradated SMA was conducted on samples from the top and bottom of the sludge bed in the full-scale reactor ADR2. Samples were sieved as described previously except that x1 PBS solution was used to rinse samples through the sieves and to backwash with the aim of minimising damage to cells in the biofilm during handling. Due to restriction on the transfer of sieves to the anaerobic cabinet, the sieving work was conducted on the bench top i.e. conditions during sieving were not anaerobic. To quantify the impact of washing in an aerobic environment on granule activity, two additional SMA assays were conducted using mixed biomass samples; one washed, the other unwashed. No significant difference was found between the activities measured for any substrate tested and as such it was assumed that the sieved SMA would provide a true record of activity in the different size fractions.

8.3.5 Biomass Sampling for NGS Library Preparation

Distillery employees conducted sampling at full-scale and care was taken to flush sampling lines prior to bottling samples. Sludge samples (50 ml) were stored in pre-sterilised air tight containers at room temperature for up to three days prior to transfer to the laboratory. Subsequently, 50ml sludge was re-suspended by inverting and sub-samples (2 ml) were taken. Sub-samples were then centrifuged and stored at -20degC until the end of the experiment when DNA was extracted (as described in Chapter 3). Biomass sampling at lab-scale was conducted on a three-weekly basis at each port in the sludge-bed using the method described in Chapter 3. Samples taken at lab-scale were transferred to storage at -20degC within 2 hours of sampling.

8.3.6 NGS Library Preparation

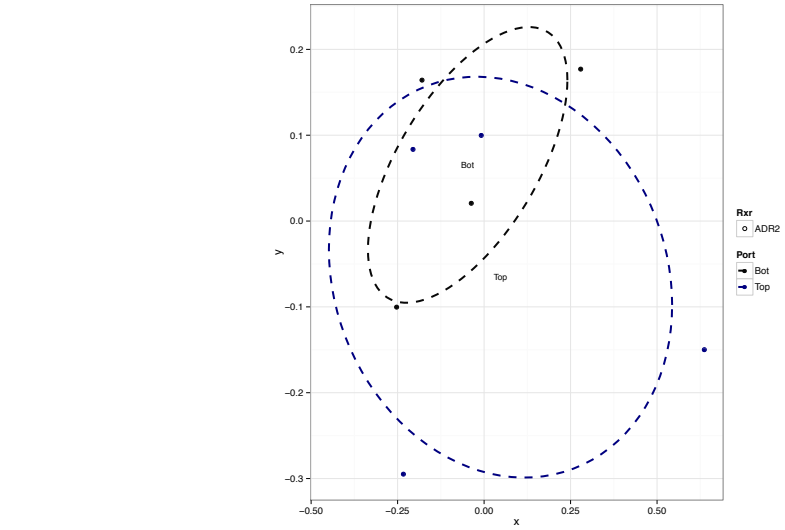
All biomass samples were stored until the end of the experiment. DNA extraction and purification was conducted using the Fast DNA Spin Kit for Soils (MP Biomedical). Extracted DNA was quantified using the Broad-Range Qubit Assay (Life Technologies) and stored at -20degC until used in NGS library preparation. NGS library preparation involved PCR amplification of the V4 region of the 16S rRNA gene using Golay barcoded primers (Caporaso, Lauber et al. 2012) with an additional degeneracy on the forward primer for improved detection of Archaea. The PCR products were gel-purified and quantified prior to sequencing using the High-Sensitivity Qubit Assay (Life Technologies). The pooled multiplexed library normalised to 5ng/ul DNA was sequenced using the Illumina Miseq bench-top sequencer. Arising sequencing was de-multiplexed at the sequencing centre and all other quality control methods applied to the sequencing data are described in detail in Chapter 3.

8.4 Results and Discussion

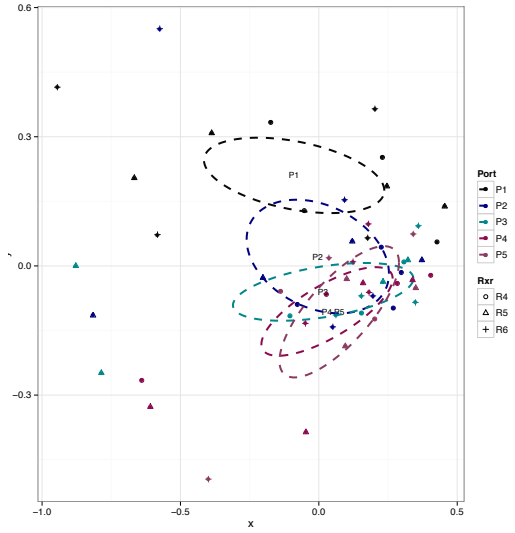
The results section is divided into 4 key components. Firstly, evidence for gradation in the microbial community in 1-D lab-scale EGSB reactors using NGS data from Chapters 4 and 6 previously are re-presented and key findings summarised. Further, a sub-set of data drawn from the year long study of ADR2 is used to enable comparison between with microbial community distribution with depth at full-scale. Secondly, the results of particle size distribution and settleability measurements with depth in each of the 1-D lab-scale and full-scale sample sets are presented and discussed. Thirdly, community physiology data collected from specific size fractions collected from the top and bottom of the sludge bed at full-scale is contrasted with the depth resolved SMA data from the 1-D lab-scale trials. Finally, NGS data collected from size-gradated granules extracted from the full-scale digester ADR2 is presented and discussed in relation to future work.

8.4.1 Gradation of Community Composition and Structure with Depth at Full- and Lab-Scale

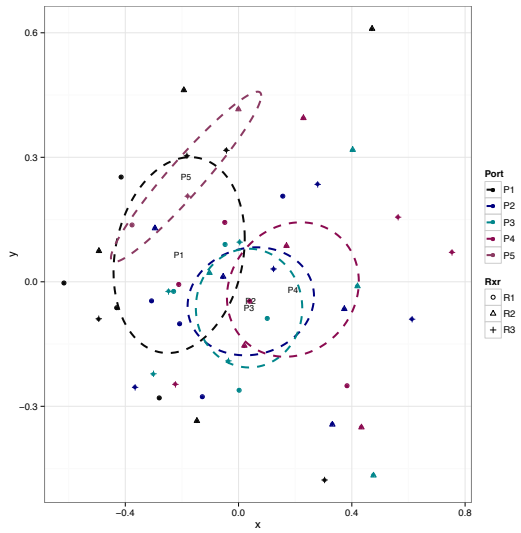
Data gathered on 1-D lab-scale EGSBs in Chapters 4 and 6 are re-represented alongside a subset of data collected from a year-long study of the full-scale digester ADR2. NMDS plots of Bray-Curtis similarity between samples were used to provide insight into clustering of OTUs for each of the 1-D lab-scale reactor sets and the full-scale reactor set with depth (Figure 8-4).



Full-Scale: ADR2



Lab-Scale: R4-6



Lab-Scale: R1-3

Figure 8-4: Two-dimensional NMDS ordination plots of all OTUs in each of the full-scale (ADR2), and high- (R4-6) and low-strength (R1-3) lab-scale sample sets, grouped by sampling depth and plotted using Bray-Curtis similarity. In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean (stresses: ADR2: 0.016, R4-6: 0.118, R1-3: 0.148).

It is observed that at lab-scale, the microbial community appears to distribute with depth in the reactor whilst at full-scale it does not. At full-scale there is strong overlap in the communities at the top and bottom of the sludge bed. It noted however that those samples at the bottom of the reactor cluster more tightly than those at the top, which may suggest a greater degree of consistency or stability in the community at the bottom of the reactor. In the lab-scale reactors R4-6 that treated full-strength distillery waste it is observed that ports P1 and P2 at the bottom of the reactor cluster distinctly from the ports P3-5 at the top of the reactor i.e. strong gradation with depth occurs in this reactor set. Interestingly, in the lab-scale reactors R1-3 treating low-strength distillery waste, by contrast to R4-6 it is seen that samples from port P1 at the bottom of the reactor cluster most closely with samples from P5 at the top of the sludge bed. Indeed, this observation may support the growth

cycle hypothesis. Visual inspection of the sludge bed in R1-3 indicated there were more small granules at the top of the sludge bed in R1-3 than in R4-6 (confirmed by measurement in Section 1.4.2 to follow). Further, these small granules appeared not to be fully formed granules, rather they appeared to be broken 'bits' of larger granules. Tentatively then, the similarity in community between the top and bottom of the sludge bed in R1-3 may result from broken granules from the bottom of the sludge bed rising to the bed surface due to reduced settleability via reduced mass and is suggestive of a cyclical relationship between granule size and community composition. Multivariate ANOVA using Bray-Curtis similarities determined that 15.4% and 18.6% ($P<0.001$) of community variation may be attributed to position in the reactor in R4-6 and R1-3 respectively. No significant difference was found with depth in the reactor in ADR2.

8.4.2 Particle Size Distribution and Settling Velocity

Particle Size Distribution

Particle size distribution was measured using the sieve range 0.335mm, 1.0mm, 1.7mm, 2.0mm, 2.36mm, 2.8mm and 3.35mm for sludge samples at full- and lab-scale with the exception of the seed sludge. The samples subject to PSD measurement were:

a) The reactor seed sludge (port P2, ADR2, d-31) with sieve range 0.15mm, 0.36mm, 0.6mm, 1.0mm, 1.4mm, 2.0mm, 2.36mm, 2.8mm and 3.35mm,

b) Pooled samples of sludge representing the top and bottom of the sludge bed in the lab-scale reactor set R1-3, low-strength distillery waste on d122,

c) Samples from the top and bottom of the sludge bed in each of lab-scale reactor set R4-6, full-strength distillery waste on day d122,

d) Samples from the top and bottom of the sludge bed in the full-scale digester ADR2 on day d267.

The results are presented graphically in Figure 8-5.

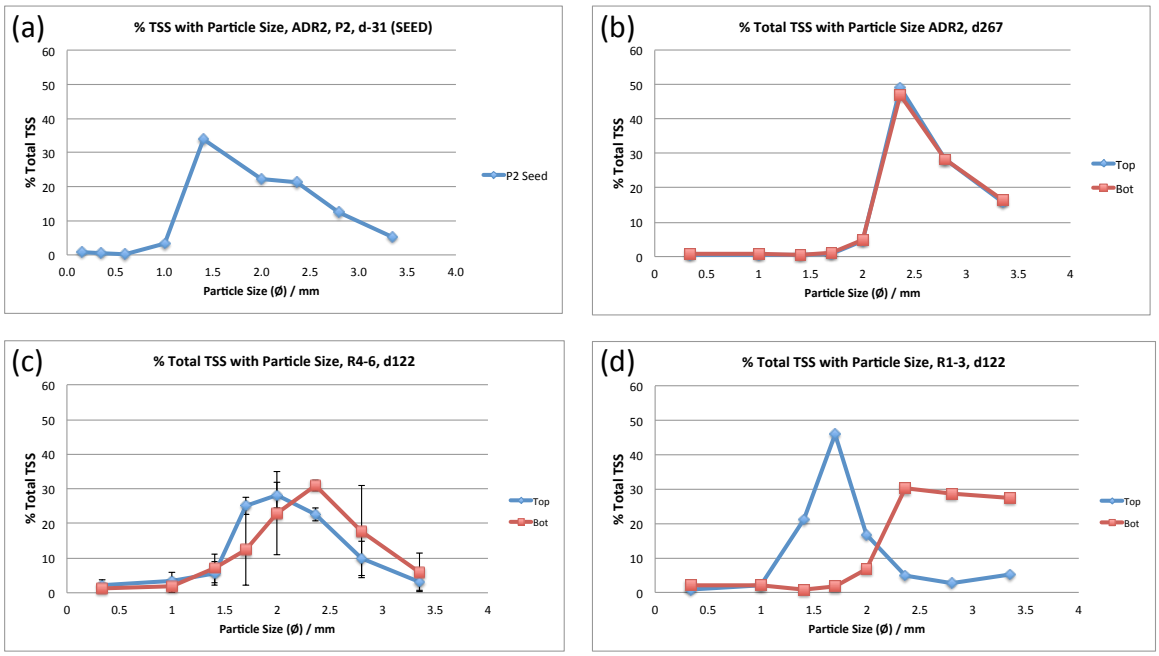


Figure 8-5: Scatter plots showing particle size distribution in sludge samples of the lab-scale seed sludge (ADR2, P2, d-31 SEED) and from the top and bottom of the sludge-bed in each the full-scale reactor (ADR2, d267), the lab-scale reactors treating high- (R4-6, d122) and low-strength (R1-3, d122) distillery wastewater.

In the seed sludge (Figure 8-5a), which was extracted from the full-scale digester ADR2 at day d-31, the largest single proportion of granules (33.9%) retained on any one sieve, were retained on the 1.4mm sieve. By day d267 in ADR2 (Figure 8-5b) by contrast, only 0.4% of granules were retained in the 1.4mm sieve whilst the largest proportion of the granules (47.1%) was retained in the 2.4mm sieve. The measured increase in diameter appeared similar at both top and bottom of the sludge bed i.e. mean granule diameter increased by over 70% over a period of 10 months. Assuming the sludge bed consisted of the same absolute number of granules throughout this time period, a crude calculation based on mean diameter in the most dominant size fraction suggests a volumetric increase in the sludge bed of ~250%. Perhaps unsurprisingly, this was not observed to occur. Subsequent to manual correction of bed-depth on day d133 to approximately 4.8m (granules were transferred to failing reactor ADR1), the recorded bed depth in ADR2 increased to approximately 6.8m by day d267. This represents a volumetric increase of around ~42% over 4.5 months. If extrapolated across the 10-month study period, the volumetric bulking is less that half of that which might be anticipated by the diameter increase. Further, measured volumetric change was concurrent with an increase in COD (~28%) in the reactor influent, which would be anticipated to increase bed expansion even in the absence of growth. Resultantly it is not possible to exactly correlate increased bed-depth with granule growth. What is clear however is that the increase in granule diameter in ADR2 sludge did not result in a proportionate increase in sludge volume. As no

significant washout was seen to occur, two possible modes of granule growth at full-scale are proposed:

- Mean granule diameter may have increased by the merging of smaller granules to make larger ones thus increased diameter would simultaneously reduce the total number of granules in the system.
- Increased granule diameter may be associated with increased granule settleability thus the sludge bed may have increased in 'density' of granule packing thus minimising effective volume change arising from increased diameter.

In all likelihood, some combination of these two mechanisms suppressed the bulking of sludge as would be predicted by mean granule diameter. It is noted that the trend for increased granule diameter continued until day d410 (ADR2 T=END) by which time mean diameter exceeded 3.35mm, which was the largest sieve size used at the beginning of the trial (Figure 8-6). Again, no proportional bulking of the sludge bed was observed supporting the assertion that granule merging might account for some of the increase in granule diameter.

At lab-scale, granules were both observed to increase in mean diameter during the test period, and to gradate according to size in the reactors (Figure 8-5 c&d). In reactors R4-6 treating full-strength distillery wastewater the dominant size fractions from the top and bottom of the sludge bed were retained on the 2.0mm (28.2%) and 2.36mm (30.9%) sieves respectively at day d122 as compared to 1.4mm throughout on day d-31. Volumetrically, assuming dominant mean diameters for each fraction and constant number of granules in the system, this represents an approximate increase in sludge volume of 111% and 249% at the top and bottom of the bed respectively.

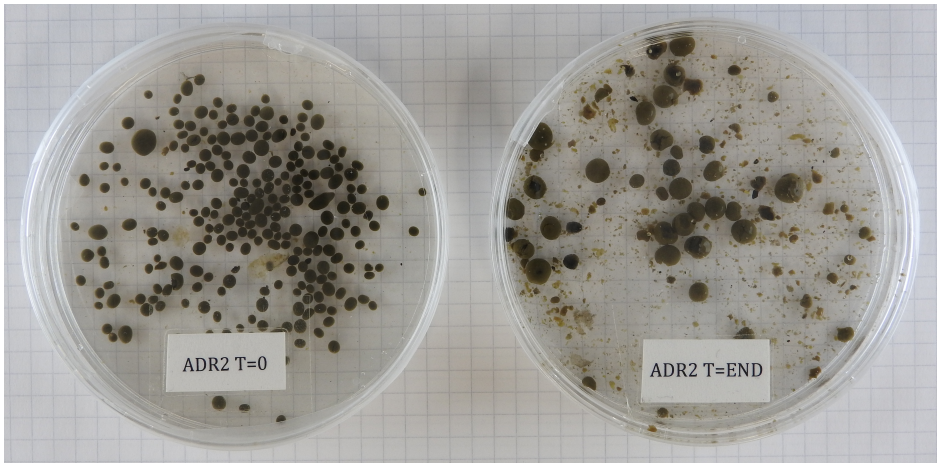


Figure 8-6: Photograph of seed sludge (ADR2, d0) and sludge sampled at the end of trial (ADR2, d410) against 5mm grid.

Again, no such bulking of the sludge was observed to occur in the reactors. Rather, sludge bed volume in the reactors R4-6 was estimated to have increased by 20% from the beginning of the trial until the end of the trial on d122 using comparison of unfed granule depth with zero flow in the reactor for beginning and end measurements. Again no significant washout of sludge was recorded in these reactors and as such, some system of granule growth coupled with granule merging appears the likeliest explanation for increased granule diameter occurring independently of a volumetric increase in the sludge bed.

The lab-scale reactor set R1-3 treating low-strength distillery wastewater was the most size-gradated with depth. The dominant size fractions from the top and bottom of the sludge bed were retained on the 1.7mm (46.0%) and 2.36mm (30.4%) sieves respectively. Given that the applied COD in R1-3 was approximately one fifth that of R4-6 and that a larger number of smaller granules were retained in R1-3 than in R4-6 suggests that the growth rate is driven by availability of substrates as might be anticipated. Similarly to R4-6, the degree of bed expansion in in R1-3 did not reflect the volumetric increase in the size of the granules. The driver for size-gradation with depth is considered in relation to settling velocity to follow.

Settling Velocity

Ten granules from each size fraction in each sample processed for particle size distribution (except that of the seed sludge) were subject to measurement of settling velocity in a no-flow water column. In ADR2 on day d267 no gradation was seen with size in the reactors (Figure 8-5b). The settling profiles for the sludge at the top and the bottom of the sludge bed in ADR2 on d267 (Figure 8-6b) appear similar for the most dominant size fractions and a relatively linear relationship between mean diameter and settling velocity for granules at the top of the reactor (R2=0.91) is observed. Some difference in settleability was seen for size fractions <2mm with those granules at the bottom of the reactor settling with greater velocity than at the top. This suggests that whilst small granules at the bottom of the reactor are similarly sized and appear with similar frequency to small granules at the top of the reactor, that those at the bottom of the reactor are of greater density. That settling velocity increases somewhat linearly with particle size rather than particle volume suggests that larger granules are proportionately less dense than small granules and that granule growth may be accompanied by a restructuring of the biofilm. Overall then it appears that the sludge bed in ADR2 is fully mixed in terms of granule size

but that some small granules may maintain specific depths in the reactor driven by granule density.

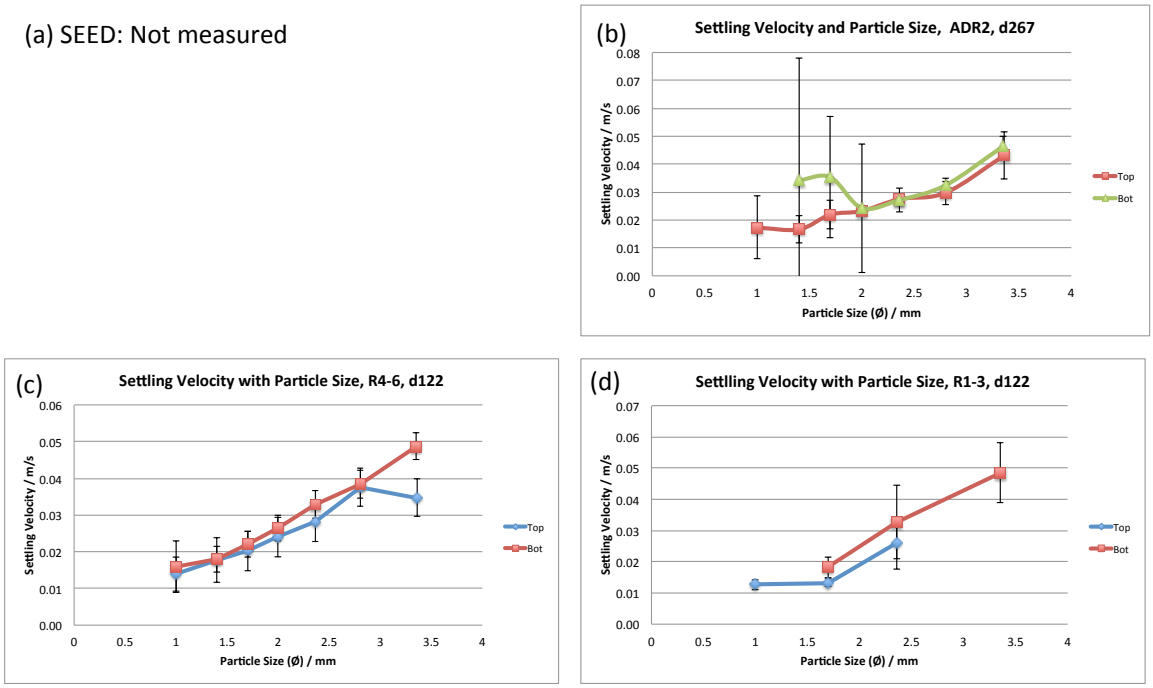


Figure 8-7: Scatterplots showing mean (n=10) settling velocity with particle size. Error bars show standard deviation.

For the lab-scale reactor set R4-6, settling velocity appears similar for similarly sized granules at the top and bottom of the sludge bed except for very large granules (>3.36mm). For granules at the bottom of the reactor, the increase in settling velocity with size is highly linear (R^2=0.98). The relationship between size and velocity in granules at the top of the reactor is similar (R^2=0.91) except for very large granules (>3.35mm). Very large granules at the top of the sludge bed are found to settle less rapidly than equivalently sized granules at the bottom of the reactor again implying that density may play a role in the position of the granules in the reactor. In the lab-scale reactors R1-3, settleability was measured in fewer size fractions. A highly linear relationship between diameter and settling velocity is seen in granules at the bottom of the reactor (R2=0.99). Again, the relationship is less linear for granules at the top of the reactor with very small granules settling very slowly. Overall, it was observed that in lab-scale bioreactors the granules show a relatively linear relationship between diameter and settling velocity in the dominant size fractions in the reactor. That the granules show a linear relationship between diameter and settling velocity, rather than granule volume and settling velocity, suggests that granule growth does not mean an increase in the absolute amount of biofilm in the reactors but that the biofilm is restructured in large granules.

These results appear to support the assertion that in 1-D lab-scale EGSBs, particle size is the driver for gradation of community with depth. The full-scale reactor ADR2 did not show significant difference in community with depth in the sludge bed, and further, did not show gradation of granule size with depth in the sludge bed. Each lab-scale reactor set by contrast was found to show significant variation in community composition with depth in the sludge bed, and these reactors also show gradation of granule size with depth. As such, this data strongly suggests a relationship between granule size and community composition. The stratification of granules by size in 1-D lab-scale reactors is assumed to be promoted by the reactor geometry i.e. that the narrow reactor vessel enabled the structured bed by constraining lateral movement of granules and promoting settling of larger granules. To further investigate the relationship with granule size, community composition and position in the sludge-bed an alternative interpretation of the data is considered. It could be suggested that the narrow body of the 1-D lab-scale reactors encourage plug flow of substrates rather than fully mixed conditions in the sludge bed. This may in turn mean that those granules situated at the bottom of the sludge bed have improved access to nutrients thus promoting growth. That the full-scale reactor appeared fully mixed enables some further investigation of these two hypotheses by investigating community physiology in granules of different size.

8.4.3 Community Physiology at Full- and Lab-Scale

In Chapters 4 and 6 previously it was observed that granules from the bottom of the sludge bed were typically more active than granules from the top of the sludge bed in the treatment of both high- and low-strength distillery waste (Table 8-2). PSD measurement indicated that these same reactors displayed size-gradated sludge beds suggesting that granule size, and granule function, may be linked. By this logic, it could be suggested that larger, more ‘mature’ granules are more active than small granules. Alternatively however it could be suggested that increased activity in the sludge and increased growth rate / granule size at the bottom of the reactor may arise not as a result of granule size but from the position in the reactor in relation to the feed port. To this end the apparently fully mixed reactor ADR2 presents something of an opportunity to investigate which, if either, of these hypotheses appeared most true. It may be assumed that:

- if granule size or ‘age’ rather than position in the reactor determined community physiology then granules of the same size from the top and bottom of ADR2 should display similar activities regardless of position in the reactor,

  and

- that large granules in ADR2 should be more active than small granules regardless of their position in the reactor.

As such a size gradated SMA was conducted on sludge samples from the top and bottom of ADR2 on day d410 (Table 8-2) using three distinct size fractions: small (1.0-1.7mm), medium (2.0-2.26mm) and large (2.8-3.35mm). It was observed that in the full-scale digester ADR2, generally granules of the same size show no significant difference in activity regardless of position in the reactor. Three exceptions to this are activity measured using butyrate in both small and large granules which perform significantly better at the bottom of the reactor, and, activity measured using propionate in large granules which again perform significantly better at the bottom of the reactor ($P<0.05$). This suggests that position in the reactor may have some influence on activity possibly via availability of substrates but that overall, ADR2 has a well-mixed sludge bed with no significant difference in activity between granules of the same size at the top and bottom of the sludge bed in 12 out of the 15 tests conducted.

*Table 8-2: SMA with depth and particle size at Lab- and Full-Scale (ANOVA P-values for 'top vs bottom', * <0.05, ** <0.01, *** <0.001)*

Rxr / Rxr set	Day	Position	Granules	SMA / (mL CH4 / gVSS.d)				
				Acetate	Ethanol	Butyrate	Propionate	Hydrogen
R1-3	d70	Top	Mix	43.0	129.4	20.8	34.1	119.4
R1-3	d70	Bot	Mix	54.5 *	254.8 **	67.1 ***	39.1	140.1 *
R4-6	d70	Top	Mix	127.1	287.3	76.0	119.2	199.8
R4-6	d70	Bot	Mix	199.8 ***	388.6 ***	168.5	118.4	192.8
ADR2	d410	Top	Small	83.2	404.0	136.9	40.8	225.1
ADR2	d410	Bot	Small	88.4	448.9	187.2 *	57.0	228.3
ADR2	d410	Top	Medium	75.5	271.2	141.1	73.4	116.9
ADR2	d410	Bot	Medium	97.0	338.2	146.1	77.6	111.4
ADR2	d410	Top	Large	129.9	333.3	177.8	114.8	103.5
ADR2	d410	Bot	Large	177.5	365.1	217.8 *	134.0 *	83.5

Having established that on the whole, activity in granules is not driven by position in the reactor, the relationship between granule size and activity is considered. The data demonstrates that granule size has a strong influence on activity in the sludge. Whilst no significant difference with depth for any granule size fraction was found for activities measured using either acetate or H2/CO2, a highly significant difference was found for activity measured using both acetate and H2/CO2 with granule size:

- Activity recorded using acetate as substrate is significantly greater in large granules as compared to small or medium granules ($P<0.0001$),

and,

- Activity using H_2/CO_2 is significantly greater in small granules as compared to medium or large granules ($P<0.0001$).

Additionally, small granules are significantly more active using ethanol as substrate ($P<0.001$) whilst large granules are significantly more active using butyrate ($P<0.01$) and propionate as substrates ($P<0.0001$).

The strength of the relationships between substrate utilisation and granule size as implied from the significance of findings suggests that granule size rather than position in the reactor is a key predictor for community physiology. It appears that granule size influences both methanogenic activity and the route to methanogenesis in anaerobic granules whereby small granules promote hydrogenotrophic methanogenesis whilst large granules promote acetotrophic methanogenesis. That both lab-scale reactor sets show increased activity at the bottom of the sludge bed using acetate as substrate where larger granules are more abundant appears to support this finding as a general rule. By extrapolation from the full-scale finding that granule size, rather than position in the reactor, links with granule function it is proposed that the mechanism for community and physiologic gradation with depth in 1-D reactors occurs as follows:

- granule size influences settling velocity and hence position in 1-D lab-scale EGSB reactors,

and

- gradation of community physiology in 1-D lab-scale EGSB reactors arises from gradation of granules by size rather than by position in the reactor.

Thus, the categorical variable 'Port' in the data for the 1-D reactors *could* be interpreted as a proxy for granule size. Arising from these findings a 'growth cycle' study was designed and some preliminary data collected which is outlined in Section 1.4.4.

8.4.4 Growth Cycle Study and Preliminary Data

Data collected during this study gave rise to the following hypotheses:

- Granule size influences settleability by accumulation of mass in 1-D lab-scale EGSBs which in turn influences position in the sludge bed.

- Granules experience a 'life cycle' from:
    - small granules consisting of flakes and fractured sections of biofilm (least settleable), to,
    - medium sized granules that are rounded by impact and shear in the sludge that grow and possibly merge, to,
    - large granules (most settleable) which eventually peel and fracture to become small granules again.
- Granule size or age influences both methanogenic activity and the route to methanogenesis.

To test these hypotheses a study was designed in which four bench-scale EGSBs (250ml) would be used to treat industrial wastewater for an indeterminate time period. Each of the four test reactors would be seeded with sludge of a specific size fraction (Figure 8-8) from ADR2 and a fifth reactor seeded with mixed granules would act as a control.

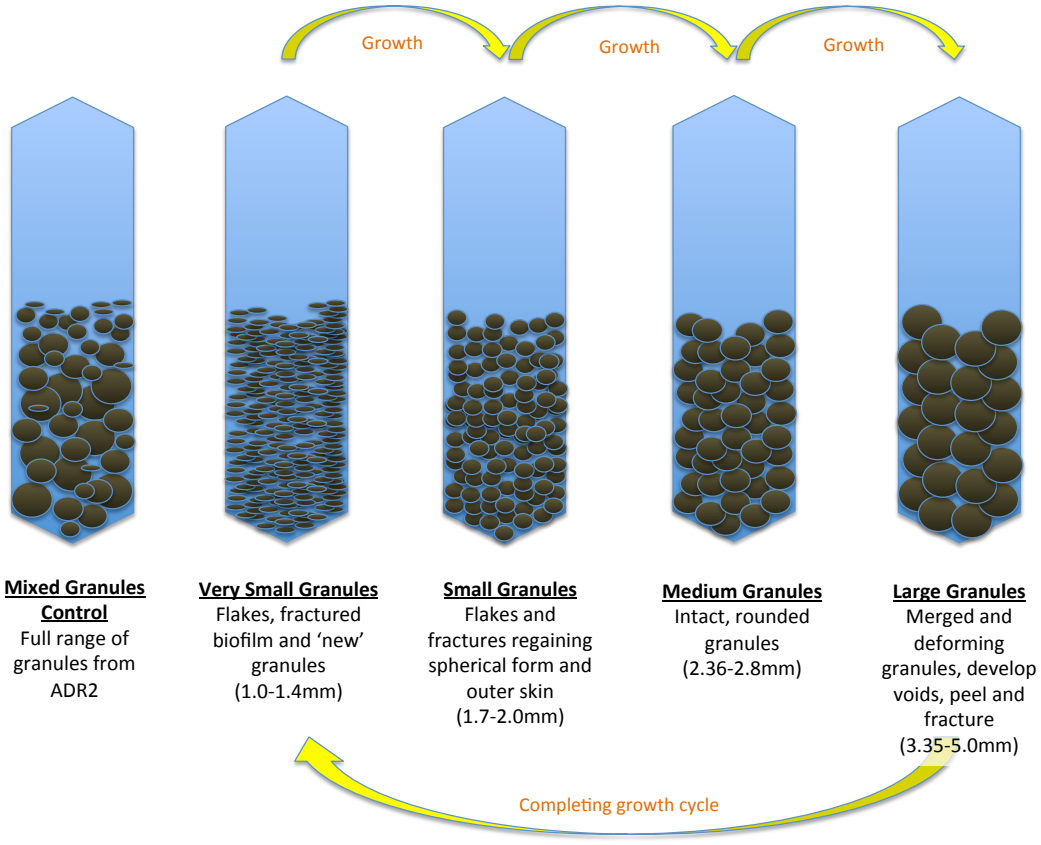


Figure 8-8: Schematic showing overview of set-up for the granule growth cycle trial and outline of hypothesis tested

The study aimed to use monitoring of biogas production rates, COD and VFA profiling, and methane production rates to determine activity of each size fraction of the biomass. Further PSD would be applied at the end of the study after visible signs of growth to determine whether growth had followed predicted routes in the reactors. SMA would be

conducted using the size fractioned seed and sub-samples of the 'new' size fractions at the end of the trial to describe changes in community physiology. Samples of biomass at T=0 and T=END in each size fraction would be sampled for DNA, RNA and FISH studies.

The experiment was set up in the lab (Figure 8-9) however mechanical failure of the gas-solid-liquid separators in the reactors meant that biomass was ground at the recycle pump. As such the trial was stopped and, due to time constraints of the PhD, the trial was not restarted. DNA from biomass from each size fraction was sequenced using the multiplexed 16S genomic approach applied elsewhere in this thesis. Three granules were sequenced from each of the small, medium and large size fractions and three replicate samples of very small and mixed samples were also sequenced. Preliminary analysis of this is reported in section 8.4.4.1.

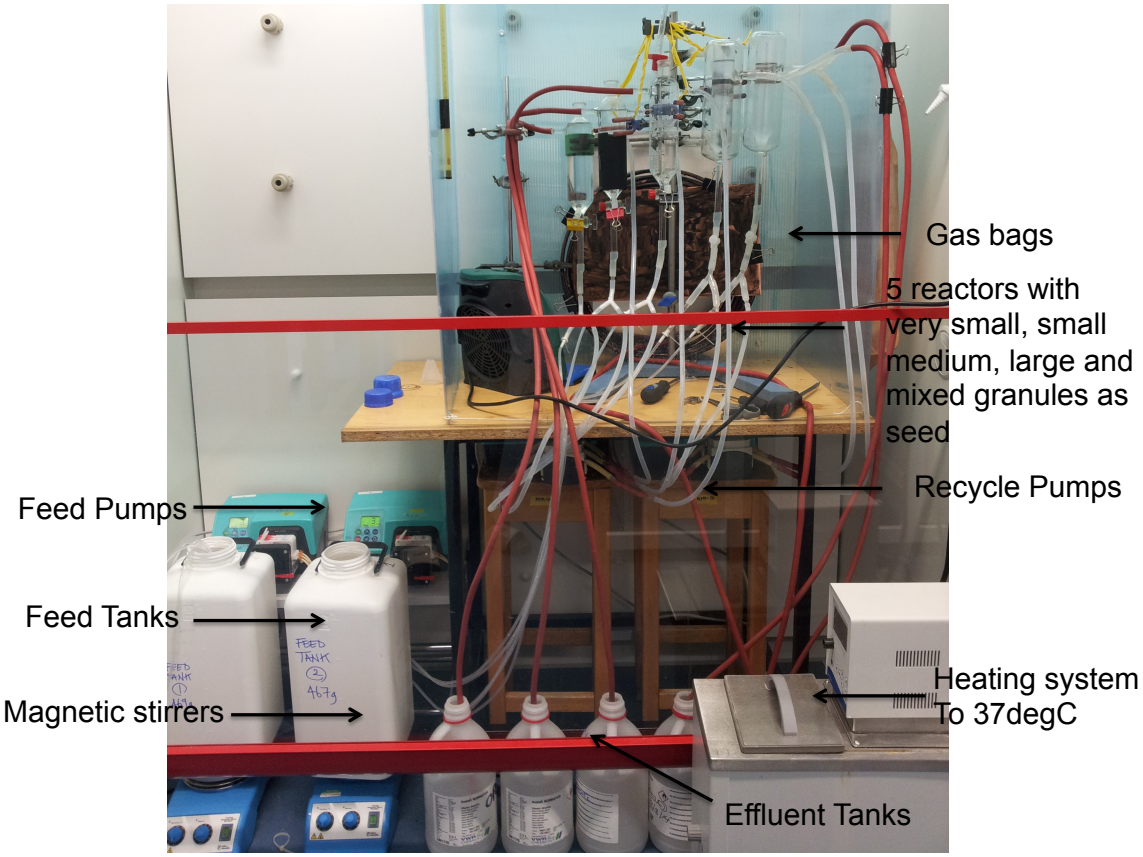


Figure 8-9: Photograph showing the granule growth cycle experimental set-up in the laboratory

8.4.4.1 NGS Data Analysis: Granule Size

The Phyloseq package in R was used to plot two-dimensional NMDS plots (Figure 8-10) by size fraction using both Bray-Curtis similarities and GUnifrac distances (alpha=0.5) for the 100 most abundant OTUs in the samples. Using Bray-Curtis similarity, strong clustering is seen by size with small and very small granules grouping distinctly from both each other, and from medium and large granules. Medium and large granules are observed

to less distinctly from each other with some overlap between the communities in these size fractions. The mixed sample group is seen to cluster most closely with the large size fraction, which was anticipated given a highly skewed PSD towards large granules in ADR2 at the time this study was conducted (data not shown). Using GUnifrac distances, very small and small granules show a high degree of overlap as do medium and large granules. Each of these pairings however cluster distinctly from the other. The suggestion then is that based on relative abundances amongst the most dominant OTUs in the community, each size fraction has a distinct community whilst a phylogeny based approach suggests 'pairing' of small/very small and medium/large granules.

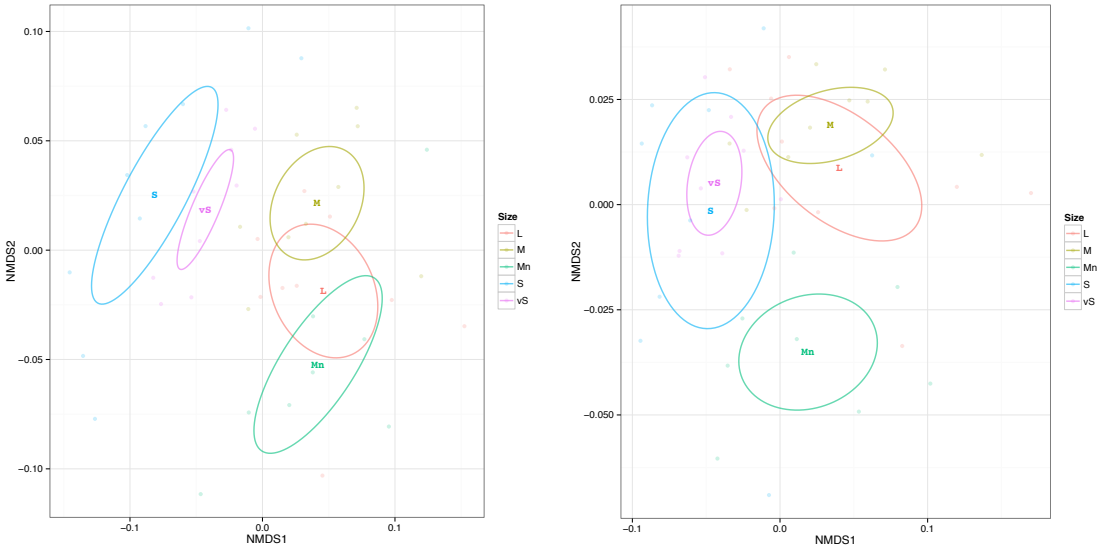


Figure 8-10: Two-dimensional NMDS ordination plot of the 100 most abundant OTUs in the sample set, grouped by granule size and plotted using Phyloseq and Bray-Curtis similarity (left. stress=0.161) and GUnifrac distance, alpha=0.5 (right, stress=0.160). In each case ellipses show 95% confidence interval of the standard error of the samples in each group around the population mean.

This analysis is confirmed using PERMANOVA. Using Bray-Curtis similarity, size is attributed 42.5% of variance in the samples whilst using GUnifrac distances size is attributed 29.6% of variance in the samples ($P<0.001$ in each case). This result appears to confirm that community composition and phylogeny are strongly related to granule size. Returning to the data for depth-associated variance in the 1-D lab-scale EGSBs using Bray-Curtis similarity, depth was attributed 15.4% and 18.6% of variance in the samples for each of R4-6 and R1-3 respectively. Whilst these values are lower than the 42.5% reported here it is noted that whilst granule size was gradated with depth, granules of each size fraction were found at each depth but in differing proportions such the reduction in variance is most likely accounted for by varying degree of stratification by size in each reactor set.

As PERMANOVA results determine that relative abundance in the community is distinct by granule size, the Kruskal-Wallis test was applied to determine *which* genera were significantly associated with each size fraction. In all, 22 genera were identified as varying significantly by granule size (P<0.01).

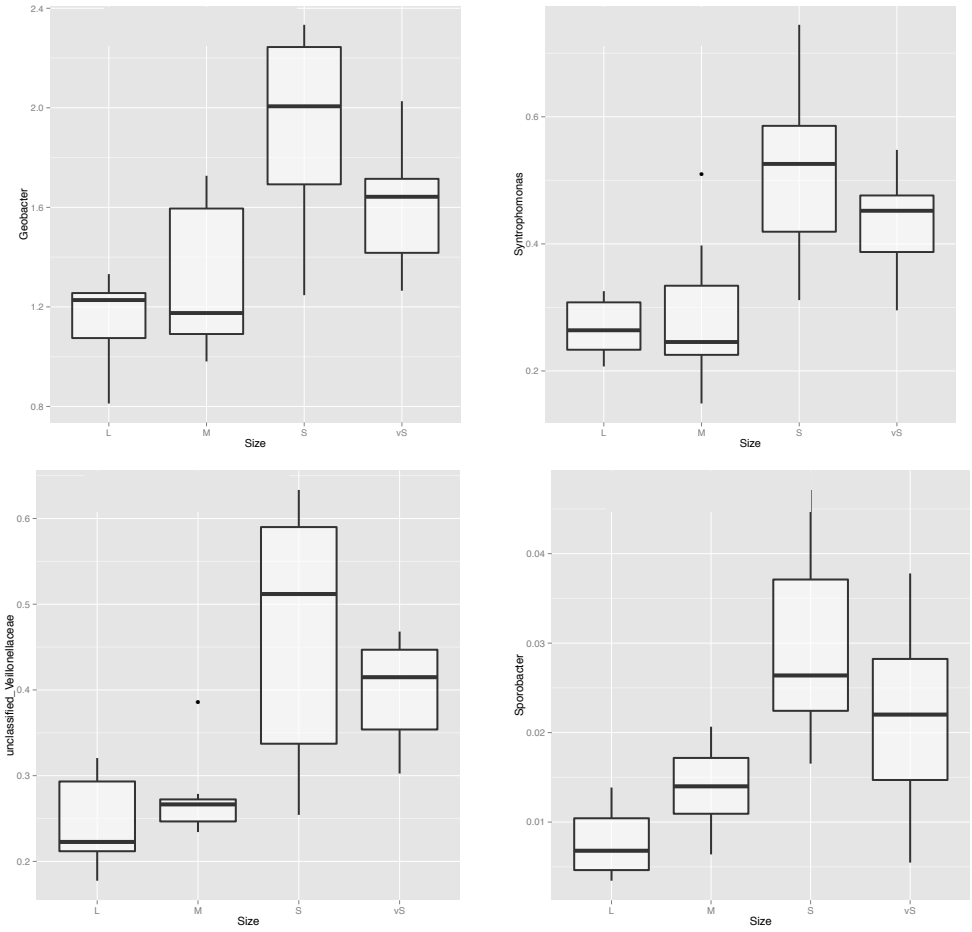


Figure 8-11: Boxplots showing distribution of relative abundance of genera in the sample set (both full- and lab-scale) showing significant variation (Kruskal-Wallis with Benjamini-Hochberg correction on p-value, p<0.05) in abundance with granule size. The bands show the median value for each group; bottom and top of boxes show the first and third quartiles; and whiskers show maximum and minimum values with 1.5 of IQR of upper and lower quartiles.

Typically, those results reflect the 'pairing' suggested by the NMDS plots in that those genera that were significantly more abundant in small granules were also highly abundant in very small granules, whilst those abundant in medium granules were also abundant in large granules. Of those, three were methanogenic Archaea. *Methanobacteriaceae* and *Methanobacterium* were found to be more abundant in large and medium granules whilst *Methanospirillum* were more abundant in small and very small granules. Each of these methanogenic genera is associated with hydrogenotrophic methanogenesis (Madigan, Martinko et al. 2009) and as such this finding provides little insight to the community physiology determined previously whereby acetotrophic activity increased with granule size at full-scale and tentatively also at lab-scale. Indeed, *Methanobacterium,* a

hydrogenotrophic methanogen, was the most dominant Archaea present in each size fraction and was more abundant in large (25%) rather small granules (18.5%) as might have been expected from the SMA results. Interestingly however, several genera were identified as most abundant in the size fraction classified here as 'small' (mean 2.6mm dia) that were identified as significantly abundant at the bottom of the lab-scale digesters where the mean granule diameter was estimated as 2.4mm including *Geobacter*, *Syntrophomonas*, *unclassified Veillonellaceae* and *Sporobacter* (Figure 8-11). Whilst far from conclusive this may suggest that that these bacterial genera are key to physiology in the biofilm at different stages of the life-cycle contributing to distinct physiology with granule size.

Community ecology was also investigated in relation to granule size (Figure 8-12). It is observed that similarly to both NMDS plots and Kruskal-Wallis tests that community ecology suggests a pairing between small and very small granules and medium and large granules. It was found that whilst rarefied richness was relatively constant with size, that both evenness and diversity was higher in very small and small granules (labelled 1 & 2 on plots) than in medium and large granules (labelled 3 & 4 on plots). Interestingly, those size fractions showing lowest diversity and lowest evenness (medium and large) were found to be most active using 3 of five substrates tested in the SMA. As it was previously suggested that ecology indices might infer reactor health it should be noted that this result implies that comparison of ecology indices may only be appropriate for sufficiently similar communities (here the communities have been demonstrated to be very distinct in terms of both relative abundance and phylogeny). Also interesting is that large granules appear most variable in terms of evenness and diversity. It was observed visually that large granules may be described as physically intact or showing signs of physical deterioration. Tentatively, this variability may arise from varied ecology associated with these differing physical states although to draw any such conclusion with any certainty a far greater number of observations would be required and granules should be categorised prior to sequencing. Also tentatively, it might be suggested that tendency for the smallest size fraction tends to have the highest evenness and diversity supports the assertion that this size fraction consists largely of the densest parts of the biofilm from broken large granules.

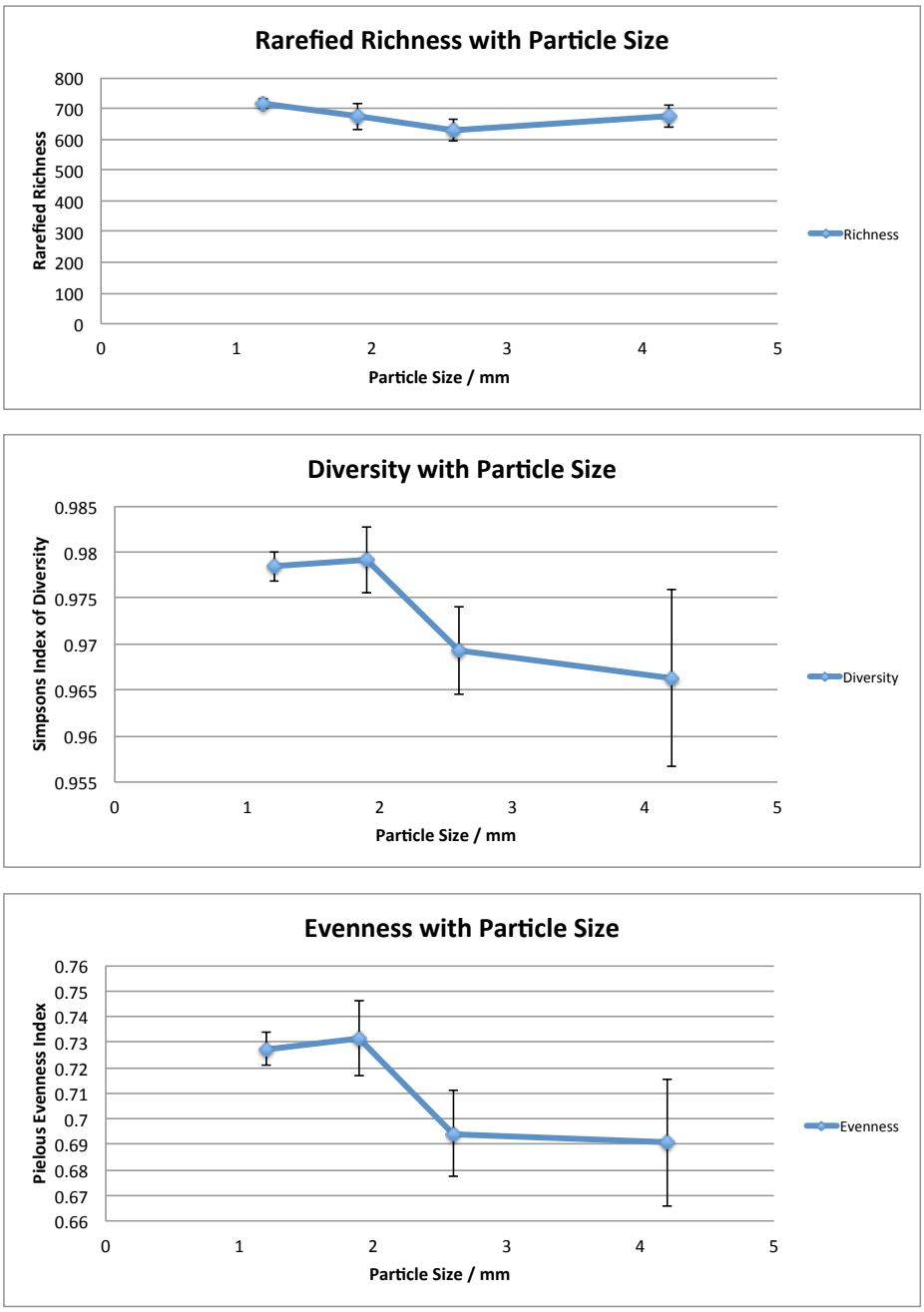


Figure 8-12: Scatterplots showing mean rarefied richness, community diversity and community evenness with mean granule diameter. Error bars show standard deviation.

8.5 Summary Findings and Future Work

In Chapters 4 and 6 previously in the study of industrial wastewater treatment in 1-D lab-scale EGSB bioreactors it was determined that community composition and physiology tended to vary with depth in the bioreactor. In this study it was determined that the biomass at the bottom of these reactors which was to be most active tended to be formed of larger more settleable granules than that at the top of the reactor. Further, SMA testing and NGS data collected on size-gradated granules from the full-scale reactor ADR2 confirmed that granules of different sizes are distinct in terms of physiology, community composition and community ecology. The study concludes that granule size dictates position in 1-D lab

scale bioreactors and that size acts as a predictor for community composition and function. This is a vital finding for assessing the suitability of specific seed sludge for specific applications. Understanding the relationship between granule size and community physiology could present great opportunities for engineering specific granular biofilms for specific treatments in future. Further, the findings confirm the importance of understanding mixing of the sludge bed both in terms of reactor operation and for sampling of full- and lab-scale bioreactors. By both observation of the physical nature of granules in the 1-D lab-scale EGSBs and the physiological and ecological data presented in this study, the granule-growth cycle hypothesis at present seems well supported. The proposed growth study would allow a firmer conclusion to be drawn. As such, a growth study similar to that outlined in this Chapter is recommended as a future work package as is application of the sludge characterisation methods used here to other granular biofilms.

9 Contributions, Discussion and Conclusion

This Chapter is used to summarise the contributions made in this thesis. These contributions are discussed both in relation to the aims and objectives of the thesis and in relation to the wider field. Future work is proposed that might build upon the trials conducted here.

9.1 Scale-Down

Development of new biotechnologies for the treatment of wastewater typically follows the empirical route from lab-scale studies through to pilot- and full-scale trials. Few papers however track the success of a trial across each of these three scales. Thus, knowledge of the applicability of lab-scale studies to full-scale implementation is limited. The work conducted in Chapter 4 aimed to bridge this knowledge gap. Understanding the disparity of scale is vital to scientists and engineers working in research environments in terms of analysis and reporting of lab-scale studies. Further, this work is of value to plant designers and operators in the industry in terms of assessing which literature is relevant to real-world applications.

The Disparity of Scale

Central to Chapter 4 was the highly novel approach of attempting to scale-down and mimic operation of an existing reactor in order to assess the impact of scale on reactor operation, performance and ecology. The approach enabled the observation that, whilst lab-scale trials typically strive to attain 'steady-state' operation, full-scale reactor operation may be highly *variable* not only in terms of substrate composition, strength and loading rate but *intermittent* in terms of shutdown of both feeding and heating systems. It might be tempting to conclude that the full-scale digester under study was poorly managed or inappropriately operated. Here an alternative perspective is taken which is that the onus should be on those developing new technologies for wastewater treatment, even at laboratory scales, to take serious consideration of the variable modes of waste generation, and hence reactor operation, when designing trials. It is proposed that variable operation was the key driver for the differing performance and ecology found between scales. It was determined from both specific methanogenic activity testing and 16S rRNA gene survey data that hydrogenotrophic methanogenesis was the dominant pathway in the reactor sludge at full-scale. By contrast it was found that in the more stably operated lab-scale reactors, community physiology in the sludge at lab-scale become more balanced between acetotrophic and hydrogenotrophic methanogenesis over time. Further it was determined

that organisms belonging to the families *Veillonellaceae, Porphyromonadaceae* and *Armatimonadetes*, that have been associated with carbohydrate degradation, were more abundant at full-scale and that this may have been associated with more stable pCOD removal efficiency recorded at full-scale than at lab-scale. These are highly significant findings. Anaerobic digesters are, in the main, designed and operated such that reactor conditions are favourable to acetotrophic methanogens. Recognising that the apparent dominance of the acetotrophic pathway in anaerobic digestion may in fact be an artefact of experimental design at lab-scale could lead to great opportunities for improving full-scale digester design and operation.

Zoned Physiology and Ecology in 1-D Reactors

The work conducted in Chapter 4 offered a further contribution to both the design of lab-scale trials and to monitoring regimes for full-scale reactors. In the experimental design for this work it was recognised that, whilst reactor geometry is frequently a secondary consideration in lab-scale EGSB studies, geometry is likely to have an influence on experimental results. Two lab-scale reactor types were developed; the 1-D and 3-D lab-scale reactors. The results indicated that lab-scale reactor type has a significant impact on both reactor performance and underlying microbial community. Both community physiology and ecology were found to be zoned in 1-D lab-scale reactor types, while the sludge bed in the 3-D reactors appeared to be well-mixed. Additionally, the 1-D lab-scale reactors were found to perform significantly better than the 3-D reactor type in terms of COD removal efficiencies and biogas production. These observations highlight the importance of understanding that lab-scale idealisations *do* have an effect on the system studied which should in the least be recognised by ensuring adequate sampling and appropriate reporting of sampling strategies utilised if not in the design of experimental reactors. Further, the results suggest value in designing multiple access sampling points in bioreactors at full-scale.

9.2 Substrate Type

The water and energy crises are such that sewage treatment presents a challenge for civil engineers in the Global North and South alike however sewage characteristics are highly variable across geographical locales. In the Global North, sewage may be deemed a low-strength wastewater whilst in the Global South by contrast sewage, especially that collected in pit latrines, is a high-solids, high-strength waste. The UK Transforming Waste Project proposed a potential solution to this binary problem. The TWP research consortium aimed to develop synthetic microbial communities specific to each waste type.

The aim of the work conducted in Chapter 5 of this thesis was to monitor the 'natural' adaptation of a single seed sludge to two alternative waste types; a low-strength industrial waste, and a high-strength synthetic sewage; to enable identification of organisms and microbial community structure associated with the treatment of each.

Low-Substrate Availability

Qualitative assessment of the relative abundance of the 100 most abundant OTUs in the sample set indicated highly similar community membership and relative abundance in samples from each of the high- and low-strength wastewater reactor sets. Physiology data by contrast suggested activity in the sludge was significantly lower than that from the full-strength waste reactor set. Further, activity in the sludge from the low-strength wastewater reactor set appeared to be disproportionately affected by the low-substrate conditions. The conclusions drawn were somewhat tentative but the suggestion was that a given microbial community can exist at a given ecological equilibrium whilst functioning at different levels of productivity. This is a potentially significant finding for those aiming to use NGS as a monitoring tool. The implication is that the characterisation of the community by membership and structure alone might not be sufficient to describe reactor health as community composition was shown to be robust despite significantly different concentrations of substrate availability and activity measured in the biomass. To investigate this more fully, more quantitative measures of the numbers of organisms present in each of the sludges, such as those obtained by qPCR, would be useful. Further, application of metatranscriptomics might provide insight into variable gene expression between the two sludge types to inform this apparent decline in productivity at reduced substrate levels. Results arising from such a study might inform the selection of organisms for synthetic communities by ensuring a broad gene complement is available in the community enabling function at both levels of productivity. Finally, as full-scale reactors are frequently subject to loading at variable substrate concentration, it would be advantageous to investigate if, or ,how, communities shift between productivity states and how rapidly they are able to do so.

High Strength Synthetic Sewage

Organisms belonging to the classes *Clostridia* and *Bacteroidetes* and the phylum *Spirochaetes* were identified as significantly more abundant in the sludge from reactors treating high-solids, high-strength synthetic sewage than in those reactors treating an equivalent strength of industrial wastewater. These taxa warrant further study in relation to hydrolysis in anaerobic systems, and, as the route to methanogenesis appeared to remain

dominantly hydrogenotrophic, study of the syntrophies promoted by these taxa should also be investigated. In this respect, application of traditional culturing methods may prove valuable. Whilst reactor performance in the treatment of high-strength synthetic sewage was satisfactory, it was observed that community richness, diversity and evenness declined in relation to both the seed sludge and the industrial water control reactor set. Further work should be conducted to ascertain whether this phenomena is related to high-solids loading, or, whether synthetic sewage represents a less complex waste type than 'real world' wastes, which may have negatively impacted community structure by limiting the number of niche roles in the community.

Parallel Replicate Controls

Three reactor sets consisting of triplicate reactors were operated in parallel during this trial. Whilst operation was somewhat cumbersome and costs associated with monitoring and sampling increased, the approach was extremely useful in terms of analysis and interpretation of the data. As microbial communities appear to be somewhat dynamic under their own momentum (Chapter 6) the parallel control method was useful in ascertaining forced change as opposed to intrinsic changes in community composition. Further the use of triplicates was found to provide insight into variability in community response to engineered changes. These finding are useful for others working in the field of research and development of biotechnologies in terms of the design of future experimental trials. Additionally, in the work conducted here, good replication in both performance and ecology was determined, which is a promising finding for those aiming to engineer and control synthetic communities as it implies predictable community response to variable loading.

9.3 Global North Sewage

Sewage treatment in the Global North today is dominated by energy intensive aerobic biological treatment methods. The adaption of a high-rate anaerobic technology to the treatment of sewage has the potential not only to eliminate as much as 50% of the energy required in current sewage treatment plants, but to contribute to renewable energy generation via the production of biogas. Microbial community adaptation to the treatment of low-strength, high-solids synthetic sewage at ambient temperature was investigated using triplicate lab-scale bioreactors and an in-series control strategy.

Reactor Performance

COD removal efficiency was found to decline in the treatment of low-strength SYNTHES at low-temperature as compared to the treatment of similar strength industrial waste at 37degC. SMA tests indicated activity was approximately ten-fold lower at low temperature (15degC) thus community physiology suggested that the community adapted to lower temperatures was psychrotolerant rather than psychrophilic. Organisms belonging to the bacterial phyla *Firmicutes* and *Bacteriodetes* were found to be significantly more abundant during operation at low temperatures. An unclassified Archaeal phylum was also found to be more abundant at low temperature. The capacity to truly identify organisms whose abundance was associated with each phase of operation was hindered by both problems operating the reactors and by lack of a parallel control. As good replication of both reactor performance and associated community was found in each phase of operation, the use of parallel controls is recommended as priority over replicate reactors in situation where use of both is not possible.

Association of Productivity with Community Structure

It was observed that both community physiology and community composition were highly stratified in the treatment of low-strength wastes in 1-D lab-scale bioreactors. Further it was determined that community structure was also stratified whereby the sludge was significantly more even and diverse at the bottom of the sludge bed as compared to that at the top of the reactors. As sludge at the bottom of the reactors was also found to be significantly more active than sludge at the top of the reactors, the tentative conclusion was drawn that increased evenness and diversity may be associated with increased productivity in anaerobic sludge. Further work is required to ascertain the drivers for stratification in the sludge bed (Chapter 8). Additionally, more in depth investigation to the relationship between microbial community structure and productivity is required. This work could be conducted by studies of activity and ecology of a broad range of anaerobic sludges. Alternatively co-culturing organisms in synthetically manufactured microbial communities might enable truer correlation between community composition, structure and productivity. As it was observed previously in relation to low- and high-strength wastewaters (Chapter 5) that two very similar communities appear to be able to exist in different states of productivity, it is recommended that this work should also use substrate concentration as a variable.

Hydrogenotrophic Methanogenesis

Under a series of applied stresses to the system, community physiology and community composition, as indicated by NGS data, appeared to suggest dominance of the hydrogenotrophic pathway. This is consistent with the findings of previous studies operating the EGSB at low temperatures reported in the literature. In light of the finding that this same pathway also appeared to associated with variable operation at full-scale at 37degC, it is proposed here that the pathway is not strictly associated with low temperature but is a response to stress in the microbial community. Further, the results suggest that a stable treatment system dominated by hydrogenotrophic methanogenesis is viable. As such, detailed study of hydrogenotrophic methanogenesis, especially that mediated by syntrophic acetate oxidising bacteria, should be of high importance to AD researchers.

9.4 Failure Study

Low or unstable solids removal efficiency was observed throughout the work conducted in this thesis and indeed this has been reported for EGSB type reactors in the literature. As a potential treatment for pit latrine wastes, understanding of the ecology and limits of applicability under high solids loading is vital if the EGSB is to be adapted to this purpose. This was addressed in Chapter 7 of the thesis, which proposed the adoption of the limit state approach to the development and design of biotechnologies.

Limit State Approach

The review of literature (Chapter 2) highlighted that the development and design methodologies for biotechnologies generally, and the EGSB specifically, are deeply entrenched in empiricism. Further it posed that this empirical approach hinders the widespread adoption of sustainable technologies such as the EGSB for the treatment of complex wastes. To enable widespread adoption of new biological treatment processes in a risk averse economy, a truer engineering approach is required. Limit state design is the gold standard for Civil Engineering in Europe and is applied in the design of steel and concrete structures and in geotechnical engineering. The approach may be defined as a design methodology founded on the application of partial safety factors to both loading and material parameters enabling design to both serviceability (acceptable deformation) and ultimate limit (failure) state. In Chapter 7 it was proposed that the limit state approach be applied to the development and design of biotechnologies. This marks a novel and valuable contribution to the field by constructively challenging the status quo and

proposing a starting point for the development of a unifying engineering philosophy in biotechnology testing and design.

Identification of Monitoring Targets for Predicting Failure

In EGSB bioreactors, propionate accumulation in a previously well functioning reactor is assumed to arise from the breakdown of key syntrophic relationships between acetogens and methanogens that enable full degradation of VFAs to methane. As such propionate accumulation is frequently cited as a failure indicator in EGSB bioreactors. However as propionate accumulation occurs *after* syntrophic relations are disturbed, propionate accumulation indicates that failure is already underway. In managing the operation of anaerobic digesters there is a need to establish *predictors* as opposed to *descriptors* of failures. The limit state approach was applied by inducing failure in three EGSB reactors using a 'failure envelope' approach. Application of NGS to reactor effluent samples enabled identification of two distinct microbial phenomenon that appear to occur prior to propionate accumulation in a failing bioreactor. The first failure predictor was the identification of an indicator organism for failure in the reactor effluent. In the sludge tested, *Methanobacterium* was found to be a potential indicator organism whose increased abundance in reactor effluent might serve as a predictor of failure in an EGSB under solids loading giving advance warning of impending propionate accumulation in this instance by 9 days, or 13.5 HRTs. The second failure predictor was identification of structural change in the microbial community in the reactor effluent. Increased evenness and diversity was detected in the reactor effluent community, again 9 days in advance of propionate accumulation in the reactor effluent. This finding is particularly significant in that changes in effluent community structure may be identified using relatively inexpensive monitoring methods such as fluorescence microscopy or flow cytometry rather than by application of molecular methods which are currently more expensive and are not able applicable for in-line monitoring. In terms of contribution to the field, to the knowledge of this author is the first time that predictive indicators for failure in EGSB systems have been identified by the application of NGS. Further, each of these potential indicators were identified due to application of the limit state methodology to experimental design and themselves indicate that this approach may yield promising results in future trials.

To ascertain true contribution to the field however further work is required to validate each of these finding. The time lapse between the indicators for failure and propionate accumulation should be confirmed in other sludges to ensure that the result is universally reproducible. The methods applied here might suffice for this work but ideally would

utilise a finer time series to determine the absolute point of failure in different systems. Further, the reversibility of the trend must be identified in order to be certain that these phenomena are truly failure predictors rather than merely failure descriptors. This could be ascertained by, for example, adjusting solids loading at the onset of these failure signals and determining the capacity to avoid propionate accumulation and reactor failure. Finally, as the failure in each case appeared to arise from structural changes in the granules, spatially sensitive methods such as FISH should be applied to granules to enable more detailed study of the mechanisms of granule disintegration under high-solids loading.

9.5 Granule Growth Cycle

The development, maintenance and retention of a diverse and resilient microbial community in well-settling granular sludge is vital to the operation of high-rate anaerobic technologies such as the EGSB. Whilst granulation is an extensively studied phenomenon, granule growth and maintenance are less-well studied and remain poorly understood. In Chapter 8 a growth cycle is proposed for sludge granules in 1-D lab-scale EGSB bioreactors.

Granule Size and Position in Sludge Bed

Stratification of both community physiology and microbial ecology in the sludge bed in 1-D bioreactors was reported in this thesis (Chapters 4 and 6) and indeed stratification of both granule size and activity has been reported elsewhere in the literature on 1-D EGSB bioreactors. Here, the position of granules in the sludge bed was found to be associated with granule settleability, which appears to be a function of granule size. These observations led to the hypothesis that granule size may be linked to granule function in EGSB bioreactors.

Granule Size and Function

No ecological stratification was observed in the full-scale digester ADR2 however a range of granule sizes was found to occur in the reactor sludge. This presented the opportunity to test whether granule size or position in the reactor was most closely associated with granule function. It was determined that there was a highly significant relationship between granule size and function for each substrate tested. Further, it was determined that granule size appeared to be associated with the route to methanogenesis: the activity rate measured using hydrogen as a substrate was significantly greater for small granules whilst the rate using acetate was significantly greater for large granules. Further, these findings informed the development of a growth cycle hypothesis.

Growth Cycle Hypothesis

It was proposed that subsequent to formation, granules experience a growth cycle. Visual inspection of the sludge during sampling enabled observation that large granules tended to crack and flake, frequently revealing void space at the core of the granule. Flakes and broken granules were most frequently observed at the top of the sludge bed suggesting that diminished mass was involved in the stratification. In the central portion of the bed, granules were observed that appeared to be re-formed from these flaked and broken granule sections possibly under the effect of shear and impact with other granules. Thus, granule growth and development was proposed be cyclical. NGS data from a 16S rRNA gene survey on different size fractions from the full-scale digester ADR2 revealed that whilst size appeared to influence the route to methanogenesis that this was driven by bacterial rather than methanogenic populations.

The work conducted in this chapter forms a preliminary study for a full granule growth study. The proposed method for testing the hypothesis was to operate bench-scale reactors containing granules of single size fractions to enable observation of growth mechanisms. Whilst inconclusive in the context of this thesis, this work is on-going in collaboration with PhD candidate Anna Trego at NUI Galway.

9.6 Concluding Remarks

The work in this thesis was motivated by the urgent need for engineers to respond to the ongoing sanitation crisis in the Global South by the development of new, more sustainable wastewater treatment methods than are utilised in the Global North. Recognising that one avenue by which this might be achieved is by engineering microbial communities to treat specific wastes two key aims were developed:

- To contribute to the current understanding of the complex microbial consortia involved in high-rate anaerobic digestion using the EGSB as a model for study, and,
- To improve the relevance of lab-scale studies to the design and implementation of full-scale systems.

In attempting to understand the microbial communities underpinning treatment in the EGSB, the work conducted here sheds only a little light on what remains a vast unknown. In order that survey studies of this sort are able to contribute more to the development of engineered microbial communities fundamental theory against which community data can

be assessed is required. In this respect, association of ecology indices describing both sludge and effluent community with community productivity and reactor health appear to offer the most promising line of research. In order to develop such theory it is likely that two strands of research might provide further insight. The first is to utilise a broader 'omics approach to gain an understanding of for example diversity or evenness in terms of the functional gene complement of a community rather than just community membership. The second might be to revisit laboratory culturing of organisms as a method to increase the range of organisms available for studies aiming to utilise pared down community composition to test ecology theory. If the goal of engineering and predictive modelling of microbial communities is to be realised, it is likely that the development of this fundamental theory must come first.

In terms of improving the relevance of lab-scale studies to full-scale design and implementation perhaps greater steps forward were made. Monitoring the full-scale reactor, and contrasting the mode of operation and associated ecology with that at lab-scale, highlighted that testing technologies under 'ideal' conditions with the expectation that this will transfer to a non-ideal world may be of limited value. Further, looking outside the traditional testing methods for biotechnologies to well-established methods in the wider field of civil engineering, adoption of the limit-state methodology proved promising in terms of identifying predictors of failure that might enable responsive reactor operation. In the short term, until such fundamental theory is established as might be required for truly predictive monitoring and control, it is likely that wider application of such unifying test methods might bring engineers closer to designing low-cost sustainable technologies in the near future.

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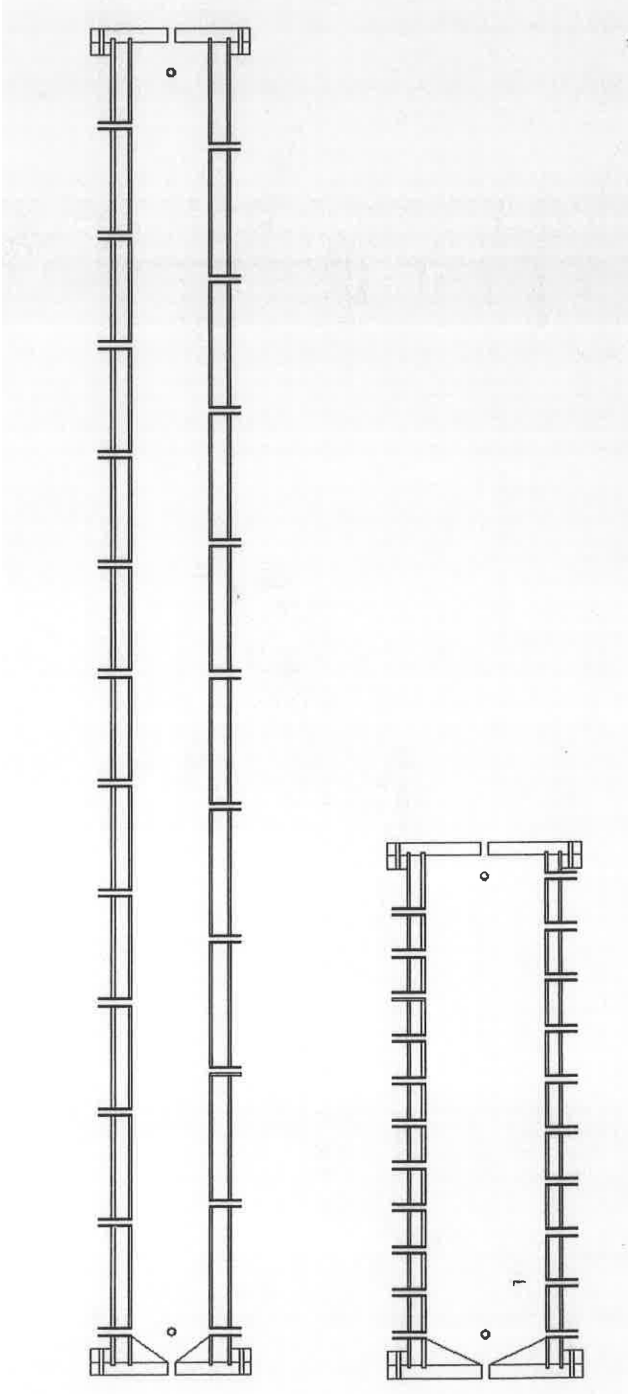
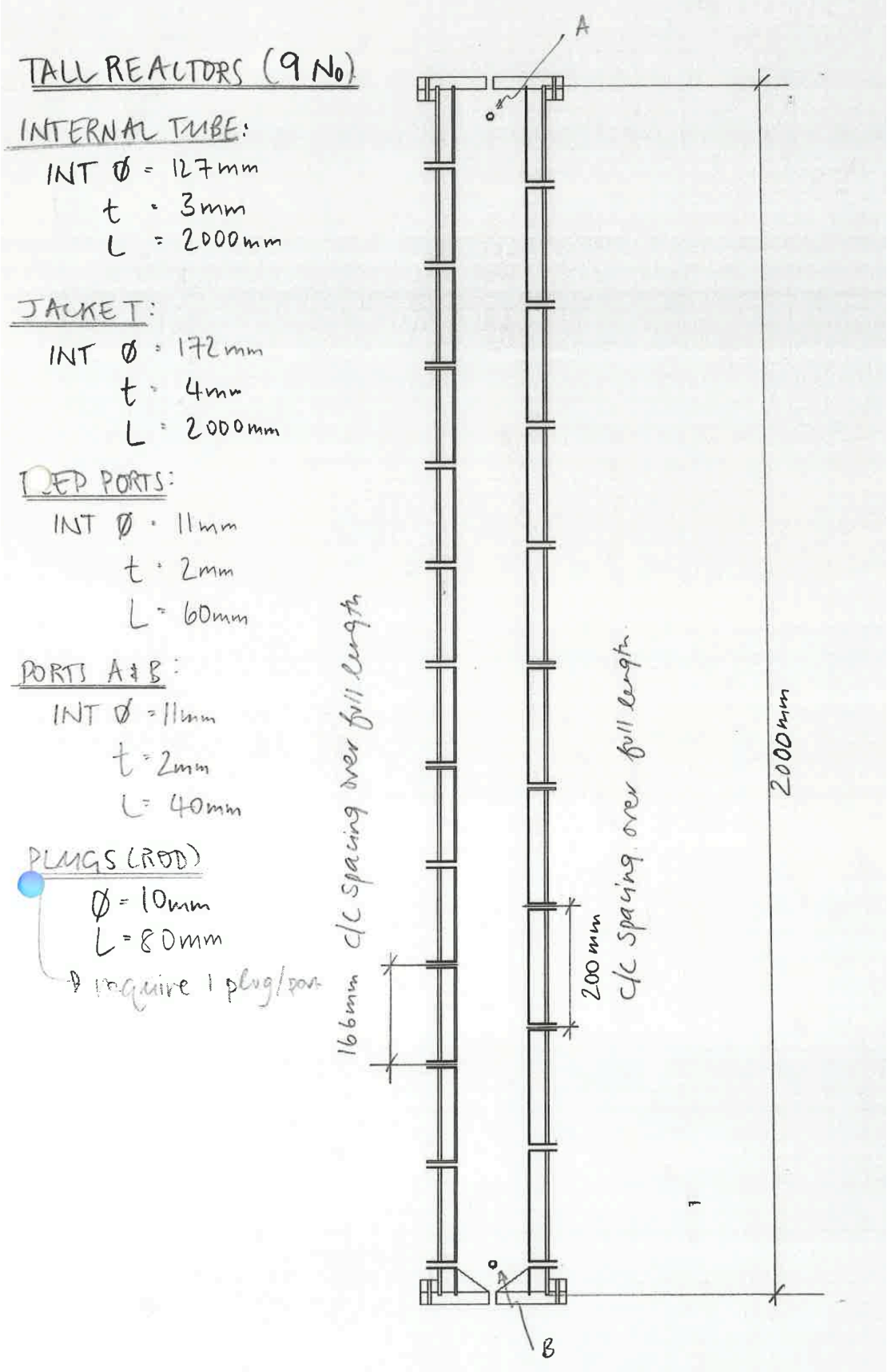
Appendices

Appendix 1: Design Detail for Lab-Scale Bioreactors

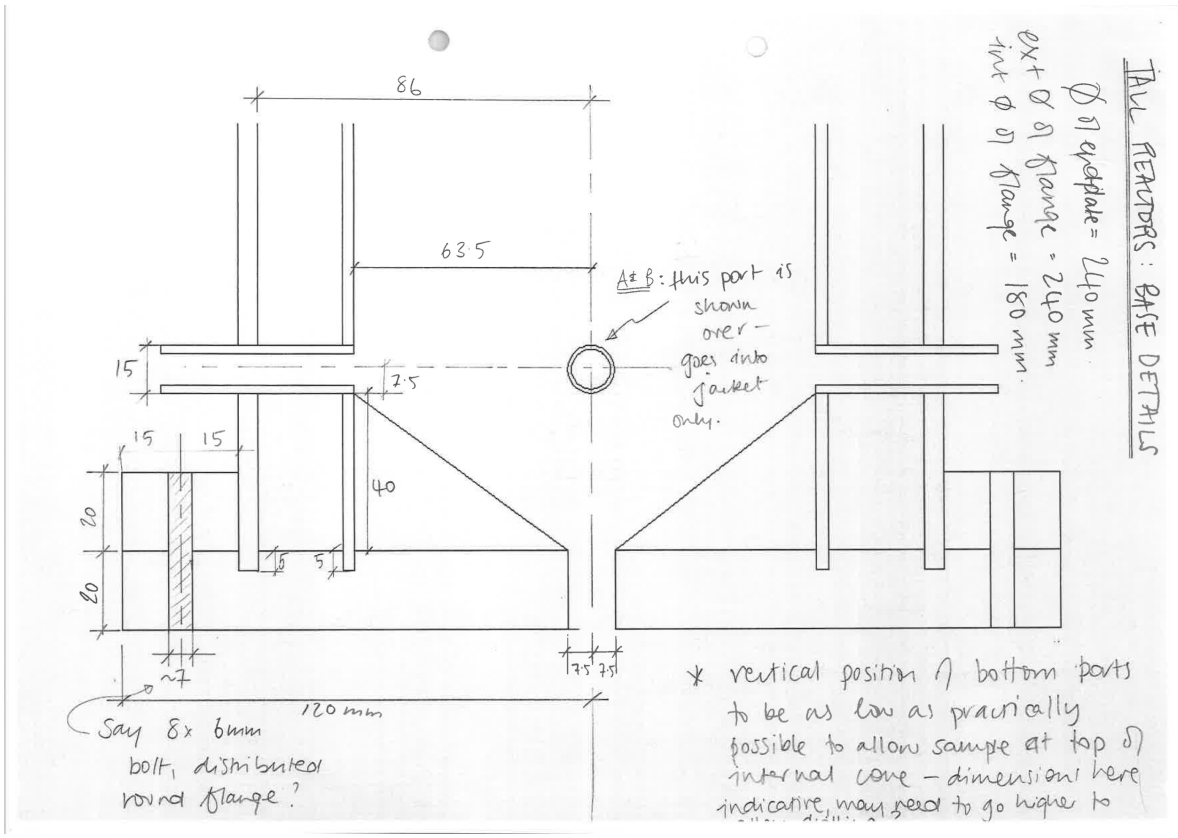


1-D and 3-D Reactors Types

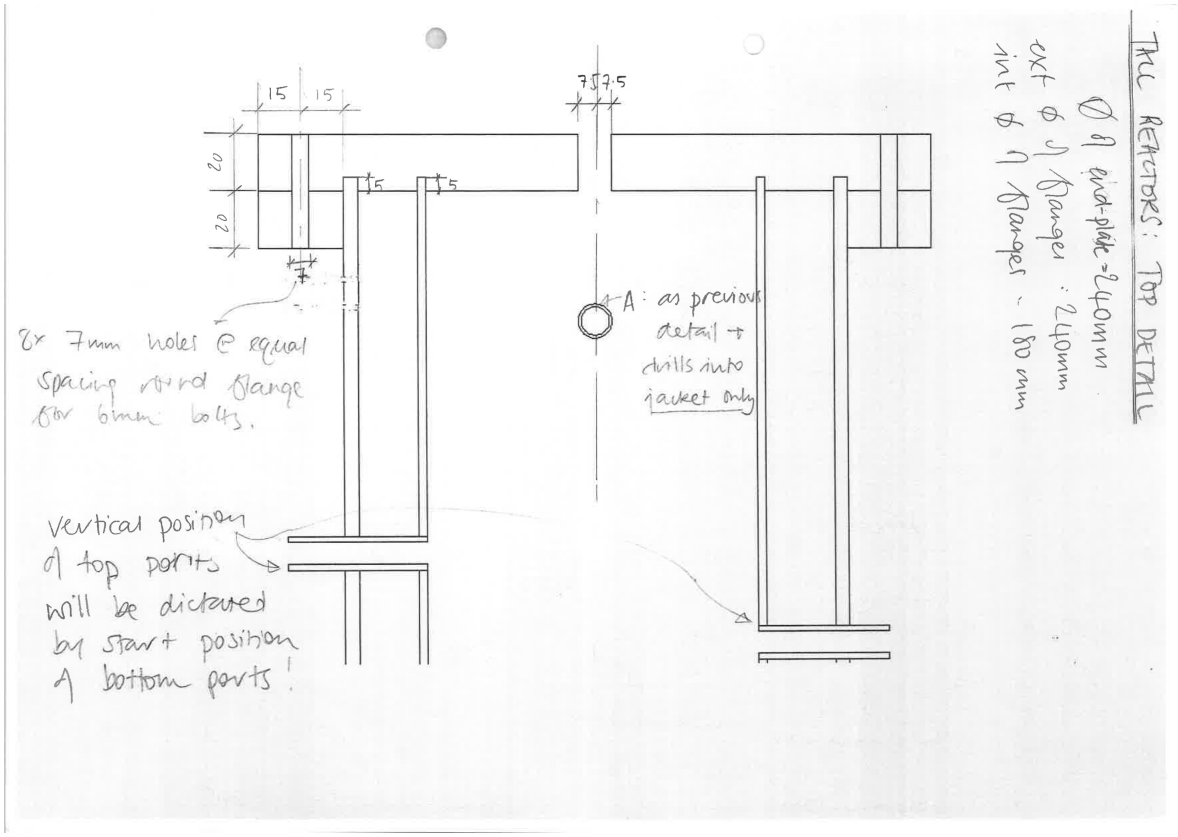


1-D Reactor Design Detail

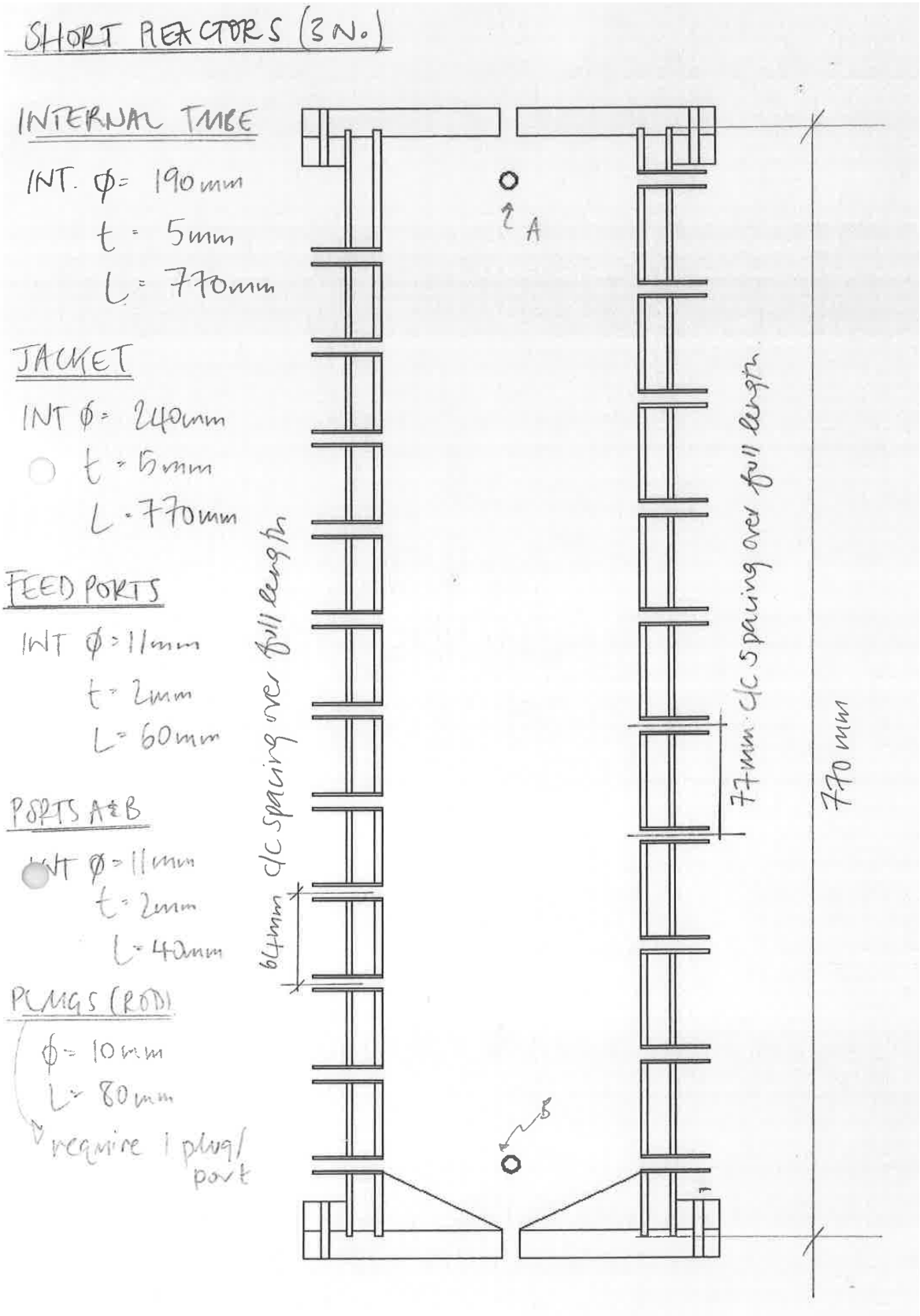
N.B only ports with 200mm c/c spacing were included in final working models



1-D Reactor Base Plate Detail

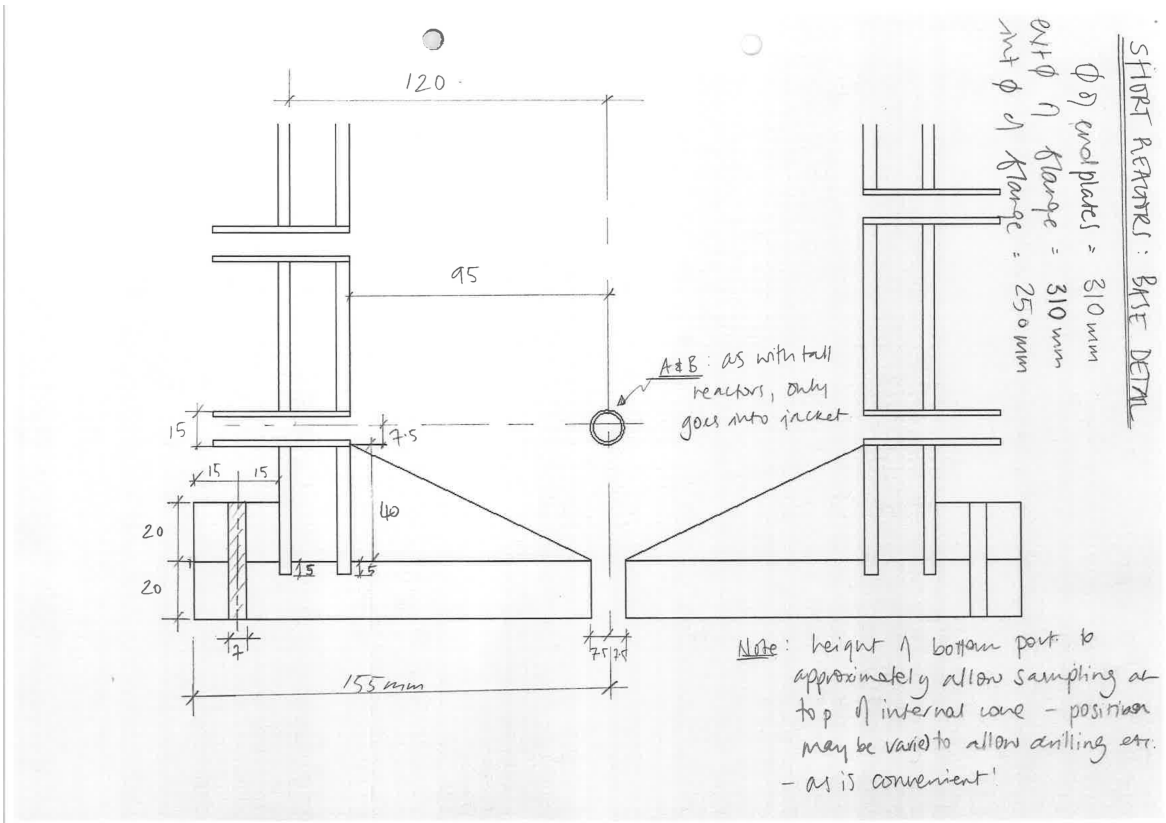


1-D Reactors Top Plate Detail

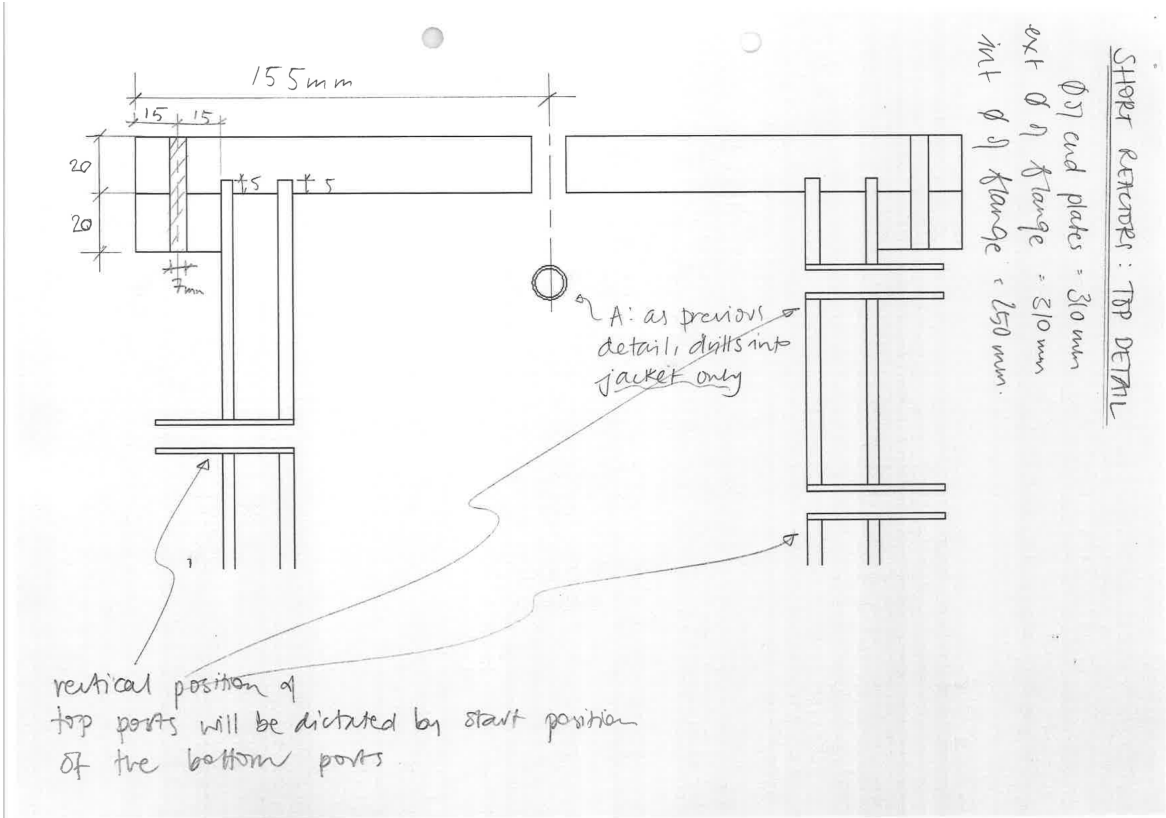


3-D Reactors Design Detail

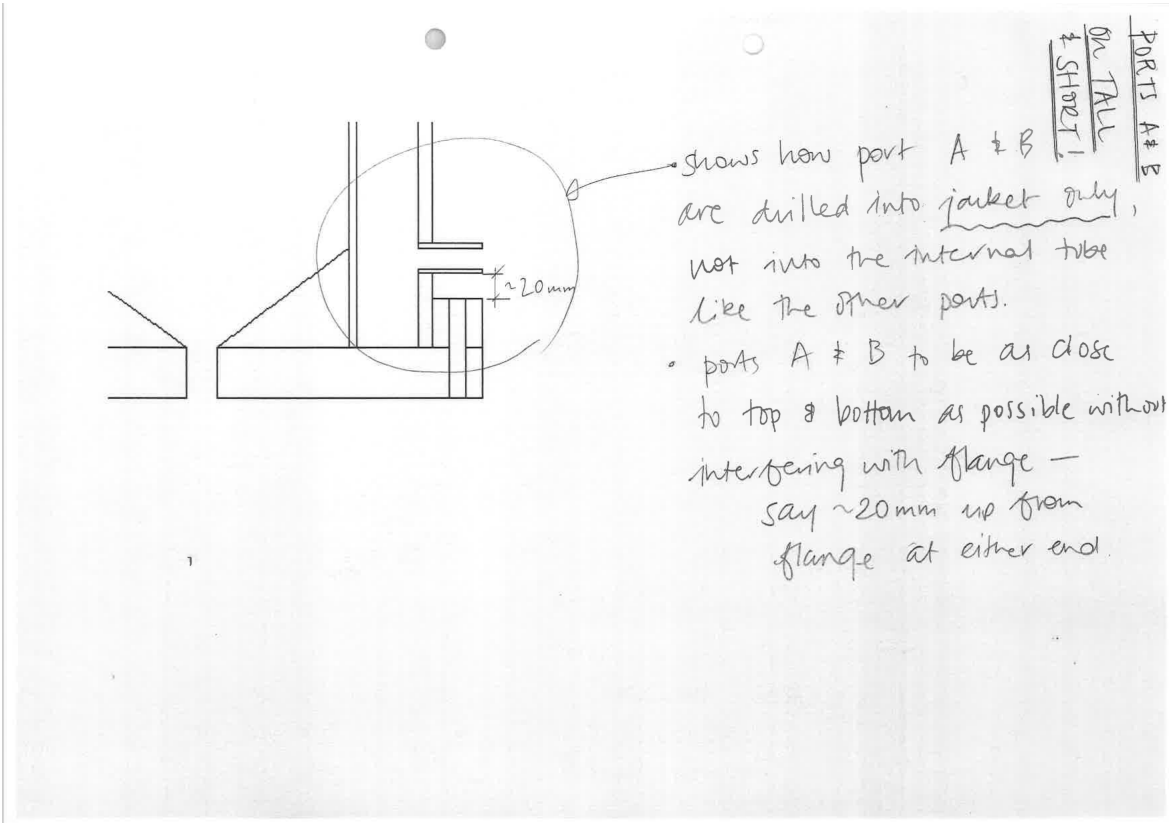
N.B. Only ports with 77mm c/c spacing were included in final working models



3-D Reactor Base Plate Detail



3-D Reactor Top Plate Detail



1-D and 3-D Reactor Water Jacket Detail

The Clear Plastic Shop: http://clearplastictube.co.uk/index.php?route=product/category&path=35

Tall Reactors:

Part	Length (mm)	Int. Diameter / Width (mm)	Ext. Diameter / Width (mm)	Thickness (mm)	Eff. Length (mm)	Eff Vol. (litres)	Eff Area (m2)	Off the shelf length (mm)	Off the shelf area (m2)	No. of Parts Req	No. of Units Req	Unit Cost (£)	Ttl Cost (£)
Internal tube	2000	127	133	3	1990	25.2		2000		9	9	40.00	360.00
Jacket	2000	172	180	4	2000	46.5		2000		9	9	59.00	531.00
Sample ports	56.5	11	15	2	60			2000		216	7	3.76	26.32
Port plugs	76.5		10		80			2000		216	9	5.60	50.40
Base plate			240	20			0.0576		3	18	0.3456	0.00	0.00
Flange		180	240	20			0.0576		3	18	0.3456	0.00	0.00
Cone		15	127	40			use the plastic cut from inside flanges			9	9	0.00	0.00
Depth in plate	10												
												Total: £	967.72

Volume of recirculation fluid: 18.7 litres

Short Reactors:

Part	Length (mm)	Int. Diameter / Width (mm)	Ext. Diameter / Width (mm)	Thickness (mm)	Eff. Length (mm)	Eff Vol. (litres)	Eff Area (m2)	Off the shelf length (mm)	Off the shelf area (m2)	No. of Parts Req	No. of Units Req	Unit Cost (£)	Ttl Cost (£)
Internal tube	770	190	200	5	760	21.5		2000		3	2	84.92	169.84
Jacket	770	240	250	5	770	34.8		2000		3	2	137.54	275.08
Sample ports	60	11	15	2	60			2000		72	3	3.76	11.28
Port plugs	80		10		80			2000		72	3	5.60	16.80
Base plate			310	20			0.0961		3	6	0.1922	0.00	0.00
Flange		180	310	20			0.0961		3	6	0.1922	0.00	0.00
Cone		15	127	40			use the plastic cut from inside flanges			3	9	0.00	0.00
Depth in plate	10												
												Total: £	473.00

Volume of recirculation fluid: 10.6 litres

1-D and 3-D Reactors Acrylic Sizes

Appendix 2: Trace Minerals for Distillery Waste

Stock (1000x).

Composition of micronutrient minerals		Dosing conc.		Molecular Weight	MW from metal	Alternative	MW	New Dosing		For 640 L		1000x	
FeCl3	40%	3.530	g/m3	162.35	55.85	FeSO4 7H2O	277.85	6.042	g/m3	3.867	g	6.0419	g/L
CoSO4	<1%	0.009	g/m3	155	59	CoCl2 6H2O	238	0.015	g/m3	0.009	g	0.0145	g/L
CuSO4	<5%	0.047	g/m3	159.5	63.5	CuCl2 2H2O	170.5	0.051	g/m3	0.032	g	0.0506	g/L
MnSO4	<5%	0.047	g/m3	151	55	MnSO4 H2O	169	0.053	g/m3	0.034	g	0.0530	g/L
NiSO4	<5%	0.047	g/m3	154.7	58.7	NiSO4 6H2O	262.7	0.080	g/m3	0.051	g	0.0804	g/L
Al2(SO4)3	<15%	0.142	g/m3	342	54	Al2(SO4)3 18H2O	666	0.277	g/m3	0.177	g	0.2766	g/L
ZnCl2	<5%	0.047	g/m3	136.4	65.4	ZnCl2	136.4	0.047	g/m3	0.030	g	0.0473	g/L
SeO2	trace			111	79	Na2SeO3 5H2O	173	0.003	g/m3	0.002	g	0.0030	g/L

Use 32 mL for 640 L

Appendix 3: SYNTHES Preparation Ramps 1-3, Concentrations S1-S7

SYNTHES: Final Concentrations of Feed Strengths S1-S7

Reactor Strength	*(Stock, as per Aiyuk et al)*	R7-9 S1	R8 S2	R9 S3	R7 S4	R9 S5	R9 S6	R7 S7
Chemical compounds	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Urea	1600	1000	2000	2667	3333	4000	4667	6000
NH4Cl	200	125	250	333	417	500	583	750
Peptone	300	188	375	500	625	750	875	1125
K2HPO4 3H2O	400	250	500	667	833	1000	1167	1500
K2HPO4	305	191	382	509	636	763	891	1145
Na-acetate 3H2O	2250	169	169	169	169	169	169	169
MgHPO4 3H2O	500	37.5	37.5	37.5	37.5	37.5	37.5	37.5
FeSO4 7H2O	100	7.5	7.5	7.5	7.5	7.5	7.5	7.5
CaCl2	100	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Food ingredients								
Starch	2100	1313	2625	3500	4375	5250	6125	7875
Milk powder	2000	1250	2500	3333	4167	5000	5833	7500
Dried yeast	900	563	1125	1500	1875	2250	2625	3375
Soy oil	500	313	625	833	1042	1250	1458	1875
Trace metals								
Cr(NO3)3 9H2O	15	1.125	1.125	1.125	1.125	1.125	1.125	1.125
CuCl2 2H2O	10	0.75	0.75	0.75	0.75	0.75	0.75	0.75
MnSO4 H2O	2	0.15	0.15	0.15	0.15	0.15	0.15	0.15
NiSO4 6H2O	5	0.375	0.375	0.375	0.375	0.375	0.375	0.375
PbCl2	2	0.15	0.15	0.15	0.15	0.15	0.15	0.15
ZnCl2	5	0.375	0.375	0.375	0.375	0.375	0.375	0.375
Overall parameters								
COD total / mg/L	8000	5000	10000	13333	16667	20000	23333	30000
COD sol / mg/L	2500							
COD particulate / mg/L	5500							
pH	7.1							