Anaerobic digestion of freshwater microalgae: effects of reactor type, operation and cultivation conditions

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Abstract

This thesis evaluates the technical potential of using microalgae as a substrate for anaerobic digestion. Investigating the control and operation of different reactors, under different operating conditions (OLR, SRT, HRT) to determine potential of microalgae as a feedstock and determine whether improvements in performance can be achieved.

Thermophillic digestion offers higher methane yields compared to mesophilic digestion in simple reactor systems at 25 day SRT, being able to cope with higher organic loading rates. Low C:N ratio in microalgae has the potential to result in high levels of ammoniacal nitrogen within anaerobic systems with levels as high at 754 mgTAN/L observed at maximum loading rates. No apparent inhibition was observed in any reactor, with free ammoniacal nitrogen levels of 100 mg/L achieved without any drop in methane yield.

While a UAnMBR system offered improved yields compared to CSTR systems, its performance was still relatively poor compared to theoretical maximum yields. The UAnMBR system did however cope with high hydraulic throughput (low HRT) without a significant drop in methane yield demonstrating that this system is potentially suitable for simultaneous harvesting and digestion.

The microalgal biomass was inherently resistant to degradation, and over the duration of a lengthened growth cycle, can change its intracellular and cell membrane structures, changing its susceptibility to enzymatic attack and subsequent methane yield. Nutrient depletion in batch microalgae culture results in intracellular lipid and carbohydrate accumulation, which potentially could have resulted in a higher methane yield of 0.283 LCH_4/gVS_{in} (equivalent to 0.184 - 0.201

$LCH_4/gCOD_{in}$) when compared to microalgae harvested during nutrient replete conditions. Allowing cultures to mature for longer periods in the stationary phase of growth under nutrient depleted conditions resulted in a significant reduction in methane yield to 0.174 LCH_4/gVS_{in} (0.124$LCH_4/gCOD_{in}$). The selection of microalgal species appears to significantly affect the methane potential and degradation rates, with methane yield as high as 0.313 LCH_4/gVS_{in} (0.222 $LCH_4/gCOD_{in}$) and as low as 0.130L CH_4/gVS_{in} (0.092 $LCH_4/gCOD_{in}$) found in different pure cultures. The difference in yield was considered to stem from a wide variability in intracellular and cell wall structures. Poor correlation existed between gross biochemical content (protein, lipid, carbohydrate) and the methane yield, and confirms that variability in methane yield is not solely dependent on the biochemical composition (e.g. lipid content).

Acknowledgements

The road towards producing this PhD has been long and difficult, but I have enjoyed the experience, learning and absorbing as much information and knowledge as possible.

For all the students, staff and visitors who I have encountered along the way I thank you for the contribution you have made.

Liz, your support, encouragement and faith in me both academically and personally is something I will never be able to repay. To my coffee buddies Beate and Paola; at times we have argued and disagreed but the friendship and fun we have had along the way has made this journey worthwhile.

My housemate Kate, together we pushed ourselves to the limit and then a little bit further but we got there in the end.

To Marta, together towards the end, you provided me the happy foundation and love to overcome the final hurdles and finally submit.

To my family and friends outside of University, I cherish your love and emotional support over a few too many beers.

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

"*Forsan miseros meliora sequentur*"

Better things will follow indeed!

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Nomenclature

AD	Anaerobic digestion/digester	STP	Standard temperature and pressure
ANOVA	Analysis of Variance	TAN	Total ammonia nitrogen
BBM	Bolds basal media	TC	Total carbon
BBM+VIT	Bolds basal media plus vitamins	tCOD	Total chemical oxygen demand
BMP	Bio-methane potential	TKN	Total Kjeldahl Nitrogen
C	Celsius	TN	Total nitrogen
Ch-a	Chlorophyll-a	TOC	Total organic carbon
Ch-b	Chlorophyll-b	TP	Total phosphorous
CH_4	Methane	TS	Total solids
COD	Chemical oxygen demand	TSS	Total suspended solids
CO_2	Carbon dioxide	UASB	Up-flow anaerobic sludge blanket
CSTR	Continually stirred anaerobic reactor	UAnMBR	Up-flow anaerobic membrane bioreactor
DOC	Dissolved organic carbon	VFA	Volatile fatty acid
FAN	Free ammonia nitrogen	VS	Volatile solids
FID	Flame ionisation detector	VSS	Volatile suspended solids
GC	Gas chromatograph	v:v	Volume: volume
GCV	Gross calorific value	w:v	Weight: volume
HCL	Hydrochloric acid	WW	Wastewater
HRT	Hydraulic retention time		
H_3PO_4	Phosphoric acid		
LCFA	Long chain fatty acid		
MSAR	Manual stirred anaerobic reactor		
NADPH	Nicotinamide adenine dinucleotide phosphate		
OLR	Organic loading rate		
RPM	Revolutions per minute		
sCOD	Soluble chemical oxygen demand		
SD	Standard deviation		
SE	Standard error		
SMA	Specific methanogenic activity		

Chapter 1 Introduction

The world is facing unparalleled challenges of climate change, fossil fuel depletion and rapid population growth. As a result, energy prices have risen and the need for new carbon neutral sources of energy has become paramount to our future. The European Union (EU) has committed through the renewable energy directive to producing 20% of renewable energy by 2020 (2009/28/EC). Energy from biomass is seen as a critical route to the decarbonisation of future energy supplies, increasing production from the existing 50 EJ per year generated across the EU, and is expected to contribute up to two thirds of the 20 % required by 2020

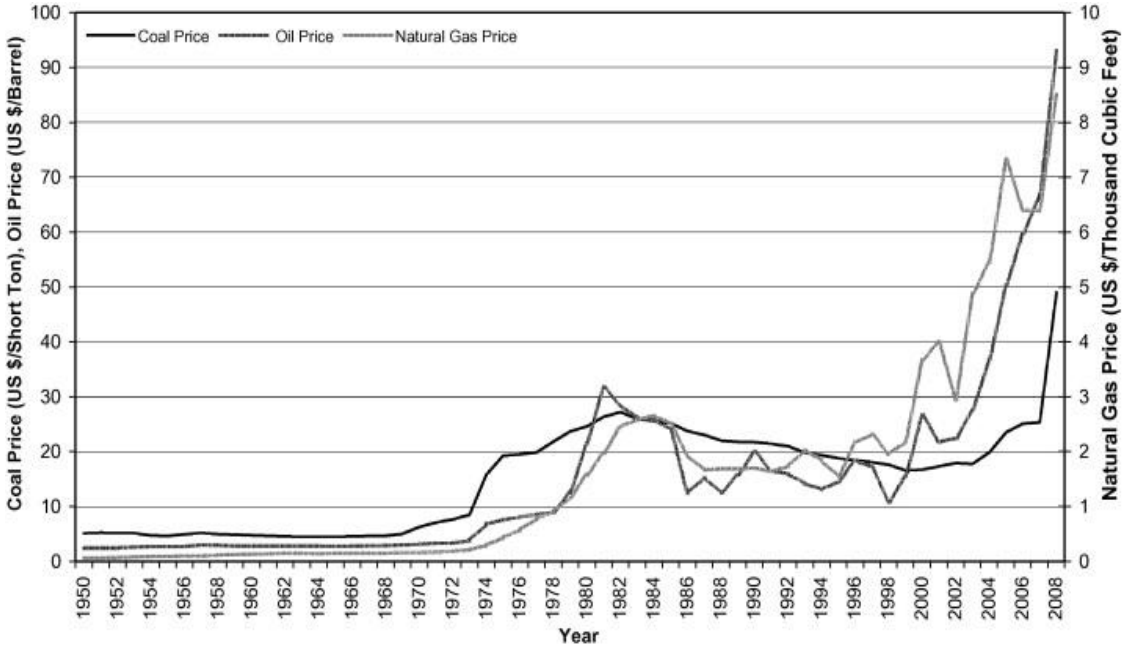

Figure 1-1 Wholesale energy prices (Shafiee *et al.,* 2010)

(2009/28/EC). The UK has set additional targets to reduce the carbon emissions by 60% by 2050 (Yassin *et al*., 2009), with biomass expected to contribute significantly to this carbon reduction target.

It is envisaged that by 2050 up to half of the world's primary energy consumption can be met by biomass energy (McKendry, 2002).

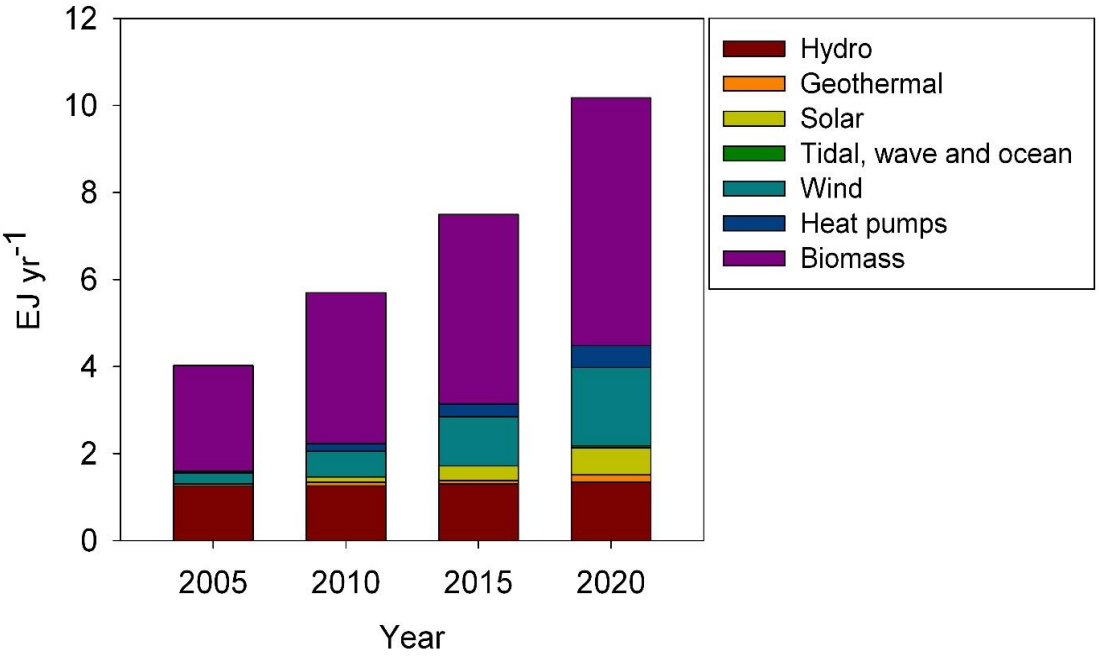

Figure 1-2 Projected renewable energy contributions in the European Union based on national renewable energy action plans (Bentsen and Felby, 2012).

Biomass energy refers to any source of heat energy produced from biological materials through conversion of CO_2, light and water in photosynthesis (Field *et al*., 2008). This can be from both terrestrial and marine sources. While the annual total solar energy received at the earth's surface is approximately 2,500,000 exajoules (EJ) only 2,200,000 EJ is in the 400 – 700nm wavelength range which is available for photosynthesis (Larkum, 2010). Theoretical conversion efficiencies can exceed 11.3%, while true practical yields are typically less than 1% of solar energy to stored chemical energy (McKendry, 2002; Brennan and Owende, 2010). With even the lowest conversion efficiency this available energy resource can still far exceed the global energy consumption of 3.2 EJ/year (Rittmann, 2008), whilst offering a

significant net carbon sequestration potential compared to current fossil fuel consumption (Figure 1-2). Traditional and second generation biofuels can be produced from a number of different sources including: oil palm, rapeseed, soybean, sugarcane, organic wastes, grasses lingo-cellulosic forestry wastes and marine based resources such as macro- and microalgae. The different conversion technologies are large, and can be separated into thermochemical and biological processes (Mckendry, 2002). Thermochemical technologies include combustion, pyrolysis, gasification, Fischer-Tropsch and trans-esterification. Biological conversion processes include: anaerobic digestion and fermentation in single/multiple fermentation steps. The conversion technologies can produce a range of liquid and solid based fuels for direct use or storage including heat, methane, syngas, bio-oil, biodiesel, bioethanol, bio-methane and chars. Terrestrial biomass has been the focus of primary research as a bioenergy source, but significant concerns remain regarding its true net GHG reduction potential including the impact of widespread use would have on food security following the conversion of agricultural land to land for biofuels (Field *et al*., 2008),; and the actual land area available for large scale cultivation of bioenergy crops being lower than many estimates (Singh *et al*., 2011); the water and nutrient consumption of terrestrial biomass, and the true GHG emissions from converting land to biofuel use.

This has led to the marine environment being heralded as an untapped resource for a wide variety of different products (Ryther, 1959). Micro- and macro-algal biofuels have a real potential to meet some of the existing and future demand for energy without the same compromises and impacts that land-based bioenergy has (Wile *et al*., 2011). Microalgae can convert between 3% - 8% of incoming solar

energy into stored chemical energy, significantly higher than those of most terrestrial biomass at 0.5 – 2%, and closer the theoretical limits of photosynthesis of 11.3 % reported (Brennan and Owende, 2010). The higher conversion results in significantly higher yields per unit area than terrestrial biomass (Lardon *et al*., 2009; Larkum, 2010). They can be cultivated on land not suitable for food production (Chisti and Yan, 2011), in waters including wastewaters not suitable for terrestrial biomass, and their cultivation can be coupled with industrial processes to sequester large quantities of CO_2 (Hansen *et al*., 2004; Doucha *et al*., 2005). Approximately 183 tonnes of CO_2 consumed for every 100 tonnes of biomass produced (Benemann, 1997, Chisti, 2007).

While their growth can be maximised and manipulated to achieve a large number

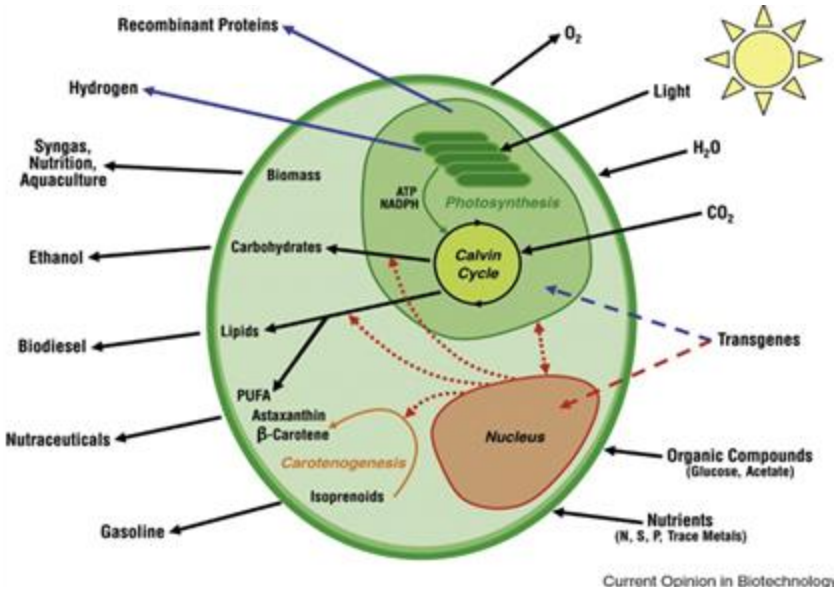

Figure 1-3 Potential products from microalgae cell. (Rosenberg *et al*., 2008)

of different commercial products including biodiesel (Mata *et al*., 2010), bioethanol (John *et al*., 2011), bio-methane (Sialve *et al*., 2009), bio-hydrogen (Chisti, 2008; Brennan and Owende, 2010), food, pharmaceuticals (Borowitzka, 1995) and

cosmetics (Chisti, 2008) through manipulation and exploitation of different metabolic processes and different chemical and biological conversion technologies. Interest first stemmed in the *potential* of microalgae to produce high value products in the 1950's (Spolaore et al., 2006), with commercial cultivation taking place in the past 20 years (Borowitzka, 1999). Current commercial production is estimated at 10^7 tons, produced by approximately 60 - 70 commercial companies worldwide each year (Walker *et al.,* 2005).

Although microalgae-derived biodiesel remains the primary focus, there are a number of concerns relating to its wide scale use and suitability as a replacement for liquid transport fuels that have meant it has not translated into full scale production, and remains firmly in research and development. Although huge productivities with high lipid yielding species are reported, the translation to large yields at a feasible scale, with low economic cost, remains a significant hurdle to overcome (Aguirre *et al.,* 2013). Current yields in advanced photo-bioreactors can achieve upwards of 1.535 kg/m^3.d, but in more economically ,viable cultivation in raceway ponds yields achieve only 0.117 kg/m^3.d (Chisti 2007), with yields being both spatially and temporally variable. Reported lipid content in these systems can achieve up to 40 -70 % of dry weight (Illman *et al*., 2000), but these levels occur under idealised cultivation conditions, with high light intensities (Gordillo *et al*., 1998), CO_2 addition (Chiu *et al*., 2009), and nutrient deficiency used as strategies to maximise lipid yield (Dragone *et al*., 2011), strategies that impose reduced growth rates and lower total biomass yields. In addition to the costs and energy requirements of cultivation, there remain other significant hurdles to biodiesel production becoming economically favourable, these include the harvesting and concentration of algae, low cost cell lysis, low cost catalysts for *in-situ* trans-

esterification; and the acceptability of biodiesel to meet the EN 14214 and ASTM D6751 standards for road transport use (Knothe *et al*., 1997).

To overcome current limitations, significant R&D is needed. With these current bottlenecks to wide scale production, the costs can be extremely high and highly variable, with estimated cost per litre of biodiesel ranging between <1 $/L to 298 $/L, values typically above current fossil fuel or other biofuel production costs. While research focus continues on biodiesel from algae, due to its higher economic potential, its current limitations far exceed the current status.

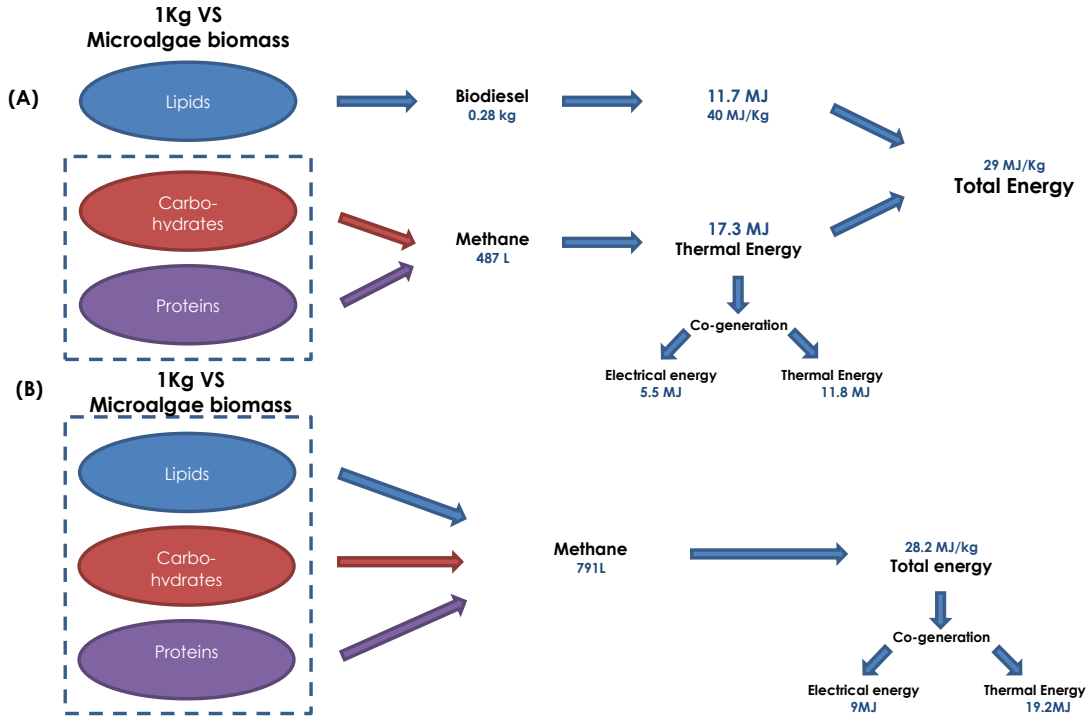

Figure 1-4 Energy potential of microalgae using two different options: A, biodiesel production from lipids followed by anaerobic digestion of residual particulates; B, use of whole cells for anaerobic digestion (Tor*eci et al*., 2009).

The anaerobic digestion of whole cell microalgae could offer a unique alternative conversion processes that gives similar or potentially higher energy yields to either biodiesel or a combined biodiesel/biogas production system (Figure 1-4).

First proposed as an energy solution in the 1950's (Golueke and Oswald, 1959), energy from algal biomass has received limited attention until the past decade. The whole cell AD of microalgae can achieve a "closed loop" system for carbon and nutrients (Harun *et al*., 2011, Wiley *et al*. 2011), and offer a number of different process benefits compared to biodiesel or bioethanol. Grown on wastewater in open or closed systems, microalgae can uptake large quantities of nutrients and carbon dioxide prior to being converted to methane in anaerobic digestion. There is no need for energy intensive harvesting or significant pre-concentration that is required in biodiesel/bioethanol production, with no extraction procedure, other than the biological conversion in the digester, theoretically being required. Unlike bioethanol and biodiesel, where only a proportion of the microalgae cell energy is utilised, AD can potentially convert almost all of the biomass to methane. When lipid levels are low it has been shown to be more favourable to use whole cell anaerobic digestion (Sialve *et al*., 2009) over biodiesel production, while when lipid levels are high, microalgae can be integrated into a combined biodiesel and biogas production operation, whereby the biodiesel production residues (proteins, carbohydrates, glycerol, methanol) can be valorised further through anaerobic digestion (Eihmen *et al*., 2009). The digestate from anaerobic digestion, being rich in nitrogen, phosphorous and potassium, can be further valorised as commercial fertilizer, and the liquid fraction returned for the cultivation of more microalgal biomass. The flue gas from electricity generation containing $CO_{2,}$ can be re-circulated back into the cultivation system to enhance biomass productivity and

sequester carbon (Figure 1-5). In theory, all nutrients and carbon dioxide should be constantly recirculated without loss, in practice, there would be inherent losses from the system, with nitrogen uptake by anaerobic biomass, only partial conversion of microalgae to methane, and less than 100% conversion of CO_2 to microalgal biomass through inefficient mixing and gas transfer in cultivation, and gas volatilisation. Despite these limitations, the potential for increased use of microalgal biomass as a feedstock for anaerobic digestion is large, and the infrastructure is already in place for utilising the product (methane) without the significant capital expenditure that biodiesel and bioethanol requires. Research to date has shown varying degrees of degradation (i.e. breakdown of biomass into soluble carbon compounds that support methanogenic archaea) exists between studies. Furthermore, to date there has been insufficient research to allow an adequate understanding of the factors that govern the efficient production of microalgal biomass, and the efficient conversion of microalgal biomass to methane. Research must now focus on trying to engineer new alternative technologies for microalgae energy extraction and optimisation.

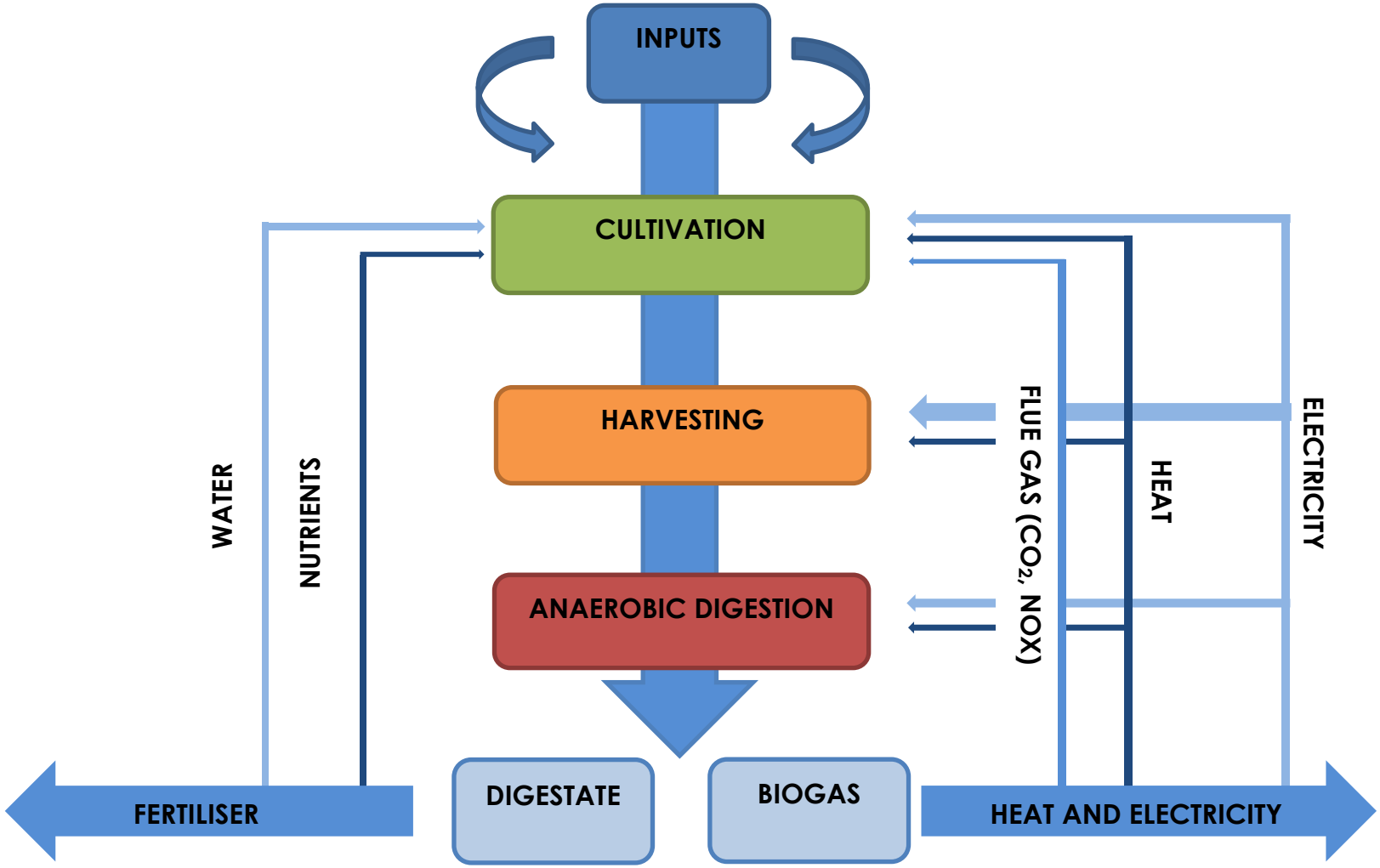

Figure 1-5 Integration of microalgal wastewater treatment and biogas production facilities.

Chapter 2 Research gaps

2.1. Aims

This thesis aims to evaluate the technical potential of freshwater microalgae as a feedstock for anaerobic digestion (Figure 2-1).

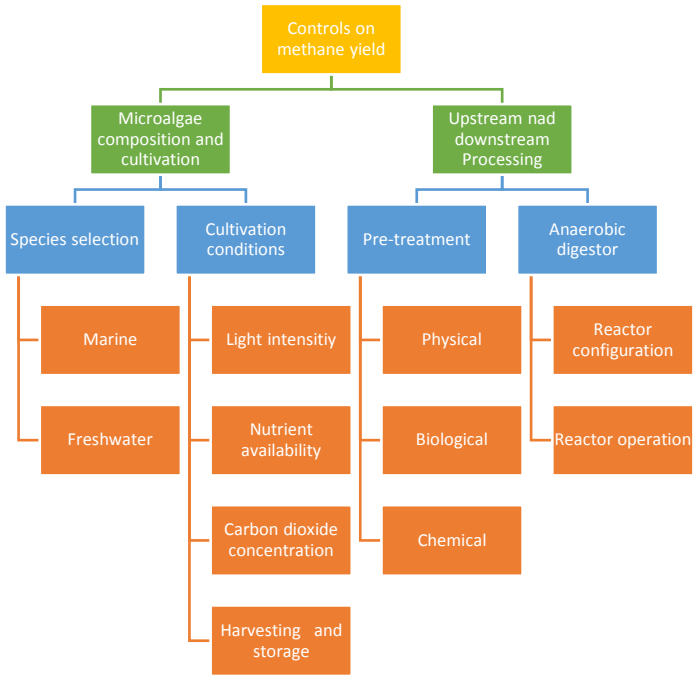

Figure 2-1 Identified research areas

2.2. Objectives

- Evaluate the influence of difference operating parameters (temperature, organic loading rate, solid retention time) in simple continuous laboratory scale anaerobic digesters fed on mixed culture freshwater microalgae.
- Identify the optimum microalgae species based on methane potential and methane production rate.

- Evaluate the performance of a novel up-flow anaerobic membrane reactor fed on mixed culture microalgae.
- Evaluate the influence of cultivation and storage conditions (light, nutrients, harvesting time, storage time and temperature) on methane yield from mixed culture freshwater microalgae.
- Evaluate potential of anaerobic membrane reactor effluent as a suitable growth media from microalgae to close the biomass production and cultivation loop.

Chapter 3 Literature review

3.1. Microalgae

3.1.1. Classification, and general structure

Microalgae are unicellular microorganisms from both the Eukaryotic and Prokaryotic kingdoms. They include Cyanobacteria, photosynthetic prokaryotes that have similar photosynthetic functions to Eukaryotes, and contain only chlorophyll–a as the primary photosynthetic pigment.

Algae are classed as organisms that produce oxygen in photosynthesis, contain chlorophyll-a, and have wide ranging cell composition and structure, although lacking the complexity in structure of plant cells (Slade and Bauen, 2013). They range in size from 0.8µm to 200µm and are found in both marine and freshwater environments. Their biodiversity is large, with estimates as high 800,000 species believed to be in existence, and with over 30,000 cultured and identified strains (Makooi *et al.,* 1976; Parmar *et al*., 2011). Algae can be classified based on cell structure, life cycle, cell wall composition and storage structures. Algae, including macro- and micro-varieties, can be separated into 11 different divisions: Cyanophyta, Glaucocystophyta, Rhodophyta, Cryptophyta, Keterokontophyta, Haptophyta, Dinophyta, Euglenophyta, Apicomplexa, Chlorophyta and Chlorachniophyta (Croft *et al.,* 2006). Microalgae, and the dominant group Chlorophyta, are the focus of research in this thesis, and are described below in more detail.

Eukaryotic cells, are surrounded by a cell wall typically composed of polysaccharides a protein matrix, and a lipid matrix (Figure 3-1). Inside the cell wall is the plasmalemma, which surrounds the main part of the cell, and controls what can pass through into the

protoplasm where important metabolic functions take place (Lee, 1999). In prokaryotic cells an additional mucilage exists on the outside of the cell. Prokaryotic organelles are not membrane bound, while in Eukayroyotic cells the DNA and photosynthetic organelles (nucleus and thylakoids) are enclosed in a membrane. Under certain conditions both lipids and starch can accumulate within the cell walls and internal regions of the cell, including the chloroplasts. The thylakoids reside inside the chloroplast, and are the location where light dependent reactions of photosynthesis take place, and ATP/NADH are generated. The nucleus is bound by another matrix, and contains all of the cell genetic material and is where cell replication is driven.

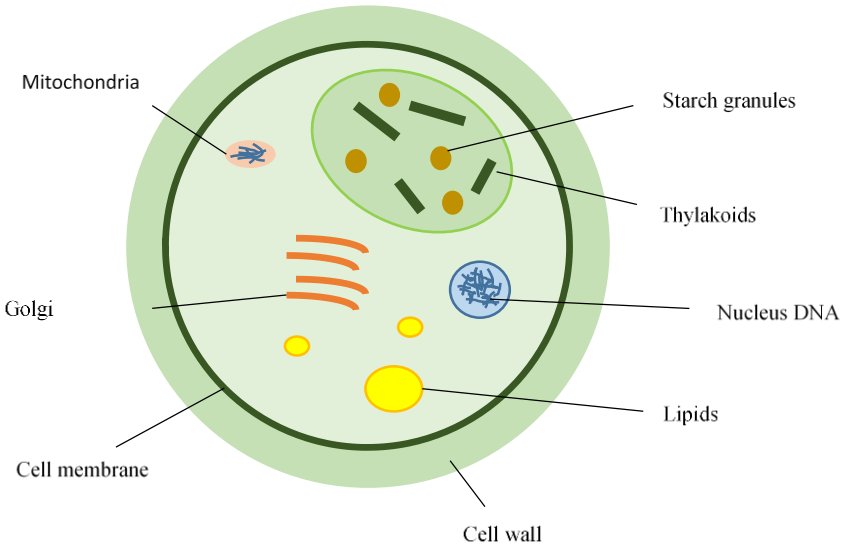

Figure 3-1 Typical cell wall structure of green microalgae (Aguirre *et al*., 2013).

In Chlorophyta, the exact cell wall composition and structure can vary significantly between species and groupings of microalgae (Van Den Hoek *et al*., 1995). Typically assumed average composition is approximately 25 – 30% cellulose, 15 – 25% hemicellulose, 35% pectin and 5 – 10% glycoprotein (González-Fernández *et al.*, 2012) and is believed to be separated into two main components, the fibrillary

component and the amorphous component. The fibrillary component is the skeleton structure of the cell wall, while the amorphous component is where the fibrillary component is encased (Lee, 1999). Initially the fibrillary component was believed to be composed of polymers of linked glucose, primarily cellulose (Baldan *et al.,* 2001). This has now been shown to be significantly different between different clades of green algae, and can include acid sugars, neutral sugars, glycoproteins, cellulose and different resistant biopolymers broadly termed algaenans (Domozych *et al.,* 2012). The composition and presence of different cell walls and extracellular structures are described in (Table 3-1).

Table 3-1 Cell coat characteristics of different microalga taxa.

Taxon	Notable species	Cell coat	Composition	References
Prasinophyceae	*Ostreococcus*	Scales, coatings	2-Keto sugars (e.g., DHA), mannans, glycoproteins	(Moestrup and Walne, 1979) (Becker *et al.,* 1994)
Chlorodendrophyceae	Tetraselmis	Wall of fused scales	2-Keto sugars (e.g., DHA), proteins	(Becker *et al.,* 1991)
Trebouxiophyceae	Chlorella	Cell walls	Cellulose,algaenan, β-galactofuranan	(Rodrigues and da Silva Bon, 2011)
Chlorophyceae	Dunaliella	Crystalline glycoprotein walls; fibrillar cell walls	Hyp-rich glycoproteins, cellulose pectins, AGP, extensin	(Voigt *et al.,* 2001)

Adapted from (Domozych *et al.,* 2012)

The biochemical composition of these cell wall and extra cellular surfaces vary, as do the structure and alignment of the chemical compounds that confer extra structure and resistance (Baldan *et al.*, 2001; Voigt *et al*., 2001; Domozych *et al.*; 2012). *Chlorella*

luteoviridis was shown to have a glucose-mannose cell wall (Takeda, 1991), while *Chlamydomonas Volvox* and *Chamydomona reinhardtii* was shown to contain no cellulose in the cell wall, but instead had a crystalline glycoprotein structure (Domozych *et al*., 2012). The presence or absence of cellulose based cell walls can be directly linked to their ability to resist biological degradation, but there are a number of other compounds that can have the same effect. *Nanochloropsis,* a marine alga which is of great interest for its ability to accumulate large quantities of lipids has a complex polymer cell wall called a sporopollenin, or broadly classified under the term algaenans. Algaenans are resistant biopolymers which are believed to form on the outer surface of the cell wall of some marine algae (Kodner *et al*., 2009). Their distribution and occurrence is widely reported in *Chlorophyceae* and *Eustigmatophyceae*. While their exact structure is not singularly defined, they are believed to be a class of aliphatic biopolymers that have been broadly categorized into one grouping that confers significant resistance to biological and chemical degradation (Gelin *et al.*, 1999; Simpson *et al.,* 2003), and improved physical strength to the algae (Cooney *et al.,* 2009).

3.1.2. Metabolism and growth

In principal, algal metabolism and growth can be autotrophic or heterotrophic, using a variety of different sources of energy and carbon for growth (Table 3-2)

Table 3-2. Types of nutrition found in microalgae

Type of nutrition	Principle source of energy for growth	Principal source of carbon for growth
	Autotrophic	
Photoautotrophic	Light	Carbon Dioxide
Chemoautotrophic	Oxidation of organic compounds	Carbon dioxide
	Heterotrophic	
Photoheterotrophic	Light	Organic compounds
Chemoheterotrophic	Oxidation of organic compounds	Organic compounds

Adapted from Lee, 1999

Photoautotrophic alga uses light as their principle source of energy for growth and inorganic carbon (CO_2) for their source of carbon (Primary photosynthesis). Chemoautotrophic alga obtains energy through the oxidation of organic compounds, and use inorganic carbon as the carbon source, primarily CO_2. Photo-heterotrophic alga use light as their principle energy source, and use organic compounds as their carbon source. Chemoheterotrophic algae oxidise organic compounds for energy, and organic compounds as their carbon source. An additional form of microalgal metabolism and nutrition, mixotrophy, can also exist (Shi *et al.,* 2002), in which algae utilise both inorganic and organic carbon sources to synthesise new cellular material but use light as their energy source.

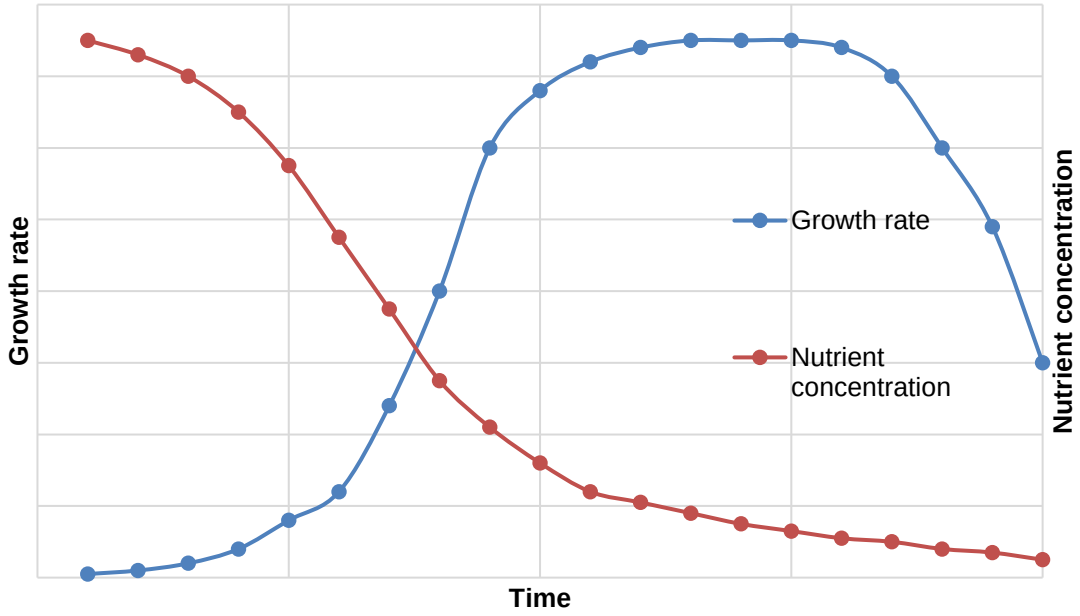

Figure 3-2 Relationship between microalga growth and nutrient concentration in a typical batch culture over time.

In the typical photoautotrophic growth systems, two important functions take place for energy and growth which makes up photosynthesis and respiration. These are primarily undertaken in photosystems I and II. Photosystem II catalyses the light dependent reactions. Light is utilised to activate catalyst pigments (chlorophyll-a) to produce NADP and ATP within the chloroplasts (Photosynthesis). In photosystem I the light independent reactions take place. These uses the ATP and NADP produced in combination with a carbon source (CO_2), to produce carbohydrates for biomass. The direct equation for photosynthesis is described in **(3-1**).

$$6CO_2 + 12H_2O + Photons \rightarrow C_6H_{12}O_6 + 6O_2 + 6H_2 \quad (3\text{-}1)$$

Algal growth can be separated into 5 distinct phases, with different reproductive cells present at different stages (Fogg, 1978). These are lag, exponential, declining rate,

stationary phase and death (Figure 3-2). Algal doubling time is typically 1 day, but can be below this during exponential phase when cell replication is at its quickest (Mata *et al.,* 2010). During stationary phase one or a number of requirements for cell growth is limiting, reducing cell replication and under certain conditions forcing cells to drive carbon synthesis away from cell growth. During these growth phases a number of reproductive cells form. The different reproductive cells which have different structure and function can be broadly classified into spores or gametes (Lee, 1999). Aplanospores are non-motile spores, hypnospores or hypnozygotes are similar in form but have a significantly thicker cell wall. The main function of hypnospores is for survival when growth conditions are not ideal and serve as a mechanism to protect the cell (Van Den Hoek *et al.,* 1995). Gametes are cells that have formed a zygote, and can be motile (planogametes) or non-motile (aplanogametes) (Lee, 1999). Akinetes are cells that are inactive and have very thick cell walls, and are usually a result of unfavourable environments. The difference in reproductive cell types can have significant effects on the suitability of microalgae for biotechnological use and processing.

3.1.3. General biochemical composition

During phototrophic photosynthesis using light, nutrients and water the primary products formed are carbohydrates, proteins and lipids which are utilised for cell replication and growth. The relative quantity and distribution of these products is closely related to the environmental conditions in which they are formed in. Sunlight, CO_2, macro- and micronutrients, and temperature all play important roles in determining how and where each product is formed and stored (Juneja *et al.*, 2013).

3.1.3.1. Proteins

Proteins are a major component microalgal cell, important for the growth and synthesis of new material, as well as repair of existing cells. They form up to 60% of microalgal cell, depending on the species, and have been shown to vary depending on the growth conditions and the point in the cell lifecycle. Proteins are distributed throughout the cell, forming an integral part of the cell wall, and also form important intracellular components. It is the high protein content of microalgae which has led stimulated interest in them as a future world protein source (Breure *et al.,* 1986).

3.1.3.2. Carbohydrates

Carbohydrates form a ubiquitous group of compounds that have a varying degree of importance in cell function and durability. Algae contain a combination of simple reducing sugars (glucose, manose) and polysaccharides such as starch and cellulose. The concentration of carbohydrates varies significantly depending on microalgal species and cultivation conditions. Starch is present in significant quantities, contained in intracellular granules that provide cell energy storage mechanisms, while cellulose is present in the cell wall giving structural strength and creating a physical protective barrier to the external environment. The quantity of cellulose can vary significantly with levels as low as 7.1% of dry weight reported (Ververisu *et al.*, 2007) and up to 70% in some macroalgal species (Baldan *et al.*, 2001). The production and synthesis of intracellular starch is an important intracellular process required for the production of bioethanol (John *et al.*, 2011).

3.1.3.3. Lipids

Lipids are primarily composed of hydrocarbons, fatty acids, phospholipids, sterols, wax esters and glycolipids, and form both structural and non-structural components of the alga cell. The intracellular content can be as high as 50% of the alga cell content (Chisti, 2007; Juneja *et al.*, 2013), while the quantity and relative distribution of these lipids is significantly affected by cultivation conditions and choice of species (Sydney *et al*., 2011; Aguirre *et al.,* 2013). Lipids serve as intracellular carbon and energy storage mechanisms for when cell growth and stress conditions arise. It is the triglycerides which are of primary focus for biodiesel production. These can accumulate under different stress conditions, and are not bound to the cell wall, but instead form in different locations within the cytoplasm (Chisti, 2007). Accumulating the right kind of lipids without compromising growth rates, accessing these lipids and converting them efficiently to high quality biodiesel remain a significant engineering challenge to the use of these for bioenergy (Aguirre *et al*., 2013).

3.1.4. Effect of environmental conditions on microalgae composition

3.1.4.1. Light

Light is a primary requirement for photoautotrophic growth and involves two sets of reactions, light dependent and light independent (3.1.2). Increasing light intensity up to photo-saturation point will result in higher growth rates and greater synthesis of new cellular material, while above this level will result in negative changes in rate. The impact of light on photosynthesis is strain and temperature specific, but typically changes in cell volume, number of thylakoid membranes, chloroplast membranes and chloroplast lamellae can occur (Juneja *et al*., 2013), while adaption and acclimation mechanisms can result in significant changes to cell metabolism (Berner *et al.,* 1989).

Elevated light intensities beyond tolerable levels result in a disruption of chloroplast lamellae and inactivity of key functional enzymes in carbon synthesis (Brody and Vatter 1959; Iqbal and Zafar, 1993) primarily by the production of free radicals resulting in cells spending more energy on cell repair than reproduction and growth (Gordon and Polle, 2007). The threshold value varies but theoretical limits are between 500 - 2000 µmol photons/m^2.s (Hu *et al.,* 1998; Melis, 2009).

Light intensity has been shown to have a number of different effects on macromolecular composition of algae. Increasing light intensity has been shown to result in increases in lipid fraction, with a decrease in protein observed in *Dunaliela tertiolecta* (Cuhel *et al*., 1984) and *Phaeodactylum tricornutum* (Iqbal and Zafar, 1993). The opposite response was observed in *Dunaliela Virdis* where darkness reduced quantities of free fatty acids, alcohols and sterols (Smith *et al.*, 1993) and in *Nanochloropsis* which showed a higher lipid content under low light intensities (Sukenik *et al.*, 1989). The differing responses observed indicate that different metabolic pathways exist between species for the synthesis of different compounds between species, while different stress mechanisms in different studies may be a result of interactions between more than just light intensity alone. Light wavelengths and pulsation as well as light intensity have been shown to play an important role in cell synthesis and production rates (Blair *et al.,* 2014). Blue wavelengths (400 – 480 nm) directly impact growth rates and cell division in *Chlamydomonas reinhardtii,* while individual red and blue light has been shown to lead directly to starch synthesis and polysaccharide production, the combination of the two can lead to lipid and insoluble carbohydrate fractions (Miyachi and Kamiya, 1978). Changing the photoperiod can result in increased growth and reduced inhibition at these high light intensities (Gordon and Polle, 2007).

3.1.4.2. Nitrogen

Nitrogen forms the basis of all cellular protein and can account for significant proportions of cell weight (Hu, 2004). The preferred nitrogen source is ammonium, and it has been shown to effect growth rate and biochemical composition when nitrate is used instead (Allen *et al.,* 2011) primarily due to the requirement of algal cells to first convert nitrate into the reduced form ammonium before uptake (Joy and Hageman, 1966). Nitrogen deficiency in cultures has been shown to result in increased triglycerides and lipid synthesis, with a subsequent reduction in cell protein content (Li *et al*., 2008; Griffiths and Harrison, 2009). Nitrogen deficiency has also been shown to lead to carbohydrate/starch synthesis (Dragone *et al.,* 2011), and a subsequent reduction in photosynthetic pigments such as chlorophyll-a (Juneja *et al*., 2013). The diversion away from cell protein synthesis leads to a reduction in growth rates (Converti *et al.*, 2009).

3.1.4.3. Phosphorous and Iron

Phosphorous, like nitrogen is essential for the synthesis of ATP by photosystem II. Deficiency has been shown to increase total lipids in *Scenedesmus sp*. (*Li et al*., 2010), while a limitation of bioavailable phosphorous can lead to a reduction in photosynthetic efficiency through a reduction in phosphatidylglycerol concentrations, found in the chloroplasts that drive cell growth and synthesis of chlorophyll-a/protein complexes.

Trace metals such as iron are essential in low quantities for cell production and growth, iron being one of the most important as it serves as a catalyst in photosynthesis and nitrogen assimilation. Limited levels of iron can reduce NADPH production and subsequently rates of photosynthesis.

3.1.4.4. Carbon

Carbon is essential for photosynthesis, for respiration, energy and cell synthesis, and without sufficient quantities cell growth is inhibited, while cell composition can also be directly or indirectly affected. The use of mixotrophic growth conditions allows direct exploitation of both photo and hetero trophic growth, switching carbon utilisation from carbon dioxide in light periods to organic carbon in dark periods to maximise potential cell yields.

In photoautotrophic growth, elevated levels of CO_2 have been shown to have a number of different effects primarily on the lipid fraction of algae, including: a shift from 14:0 fatty acids to 22:6 (n-3) poly unsaturated fatty acids (Riebesel *et al.*, 2000); an increase in total and unsaturated fatty acids and biomass (Tsuzuki *et al.*, 1990, Jeon *et al.*, 2013); and a decrease in protein content and subsequent proportional increases in carbohydrate content in *Dunaliella viridis* (Gordillo *et al*., 1998). The mechanisms for carbohydrate accumulation remain poorly understood.

While primarily studied for the effect on lipid and starch synthesis the effects of different carbon sources were shown to directly affect cell wall structure, primarily the abundance of cellulose (Makooi *et al*., 1976).

3.1.4.5. pH

Microalgae are able to live in water with extremely low pH (acidophillic) and high pH (alkaliphillic) conditions. Acid conditions have been shown to significantly affect cell biology, including the development of a cell barrier that is extremely impermeable to protons, a less fluid membrane from higher amounts of saturated fatty acids, and a number of glycerol derivatives in the protoplasm (Tatsuzawa *et al.*, 1996), while alkaline conditions have been shown to cause an increase in intracellular protoplasm

sodium concentrations, without this sodium sequestration rapid cell lysis can occur (Schlesinger *et al.,* 1996).

3.1.4.6. Temperature

Temperature plays an important role in controlling the rate of all chemical/biological reactions within the cell. Growth rate has been shown to increase up to a certain optimal growth temperature, above and below this number maximum growth rate μmax is limiting. The optimal temperature varies between species (Passos *et al.,* 2014), but during optimum temperature conditions cell size is at a minimum, with maximum carbon and nitrogen utilization observed (Juneja *et al.*, 2013). Outside the optimal temperature condition several effects can be observed: CO_2 utilisation is reduced, subsequent cell growth diminished; and protein synthesis is impeded, eventually resulting in damage to photosystem II.

Low temperatures can result in decreased fluidity in the cell membrane, which invokes a response to increase fatty acid content. These fatty acids can stabilise and enhance cell membrane function to protect the vital photosystems against damage (Nishida and Murata, 1996). This response has been observed in *Dunaliella salina and Botryococcus braunii* which have been observed to increase fatty acids at sub optimal temperatures (Sato *et al.*, 1979; Lynch and Thompson 1982; Kalacheva *et al.,* 2002)

The response to elevated temperatures is species dependent, and suggests that different acclimatisation or evolutionary growth temperatures of alga play a role in how they respond. *Nanochloropsis sp*. showed an increased lipid content between 25°C and 30°C, while *Chlorella Vulgaris* showed the opposite response with a decreases in lipid content (Converti *et al.,* 2009). Starch content was shown to decrease at elevated temperatures, attributed to enzymatic degradation and conversion of starch produced.

This response to stress is reversible when temperatures are reduced again (Nakamura and Miyachi, 1982). Increased temperatures have also been shown to increase carotenoid concentrations in the cells, a direct response to counter oxidative damage of the photosystem (Tjahjono *et al.,* 1994, Juneja *et al.,* 2013).

3.1.5. Microalgal cultivation systems

The two primary cultivation systems used for microalgae are classified into open and closed systems (Figure 3-3).

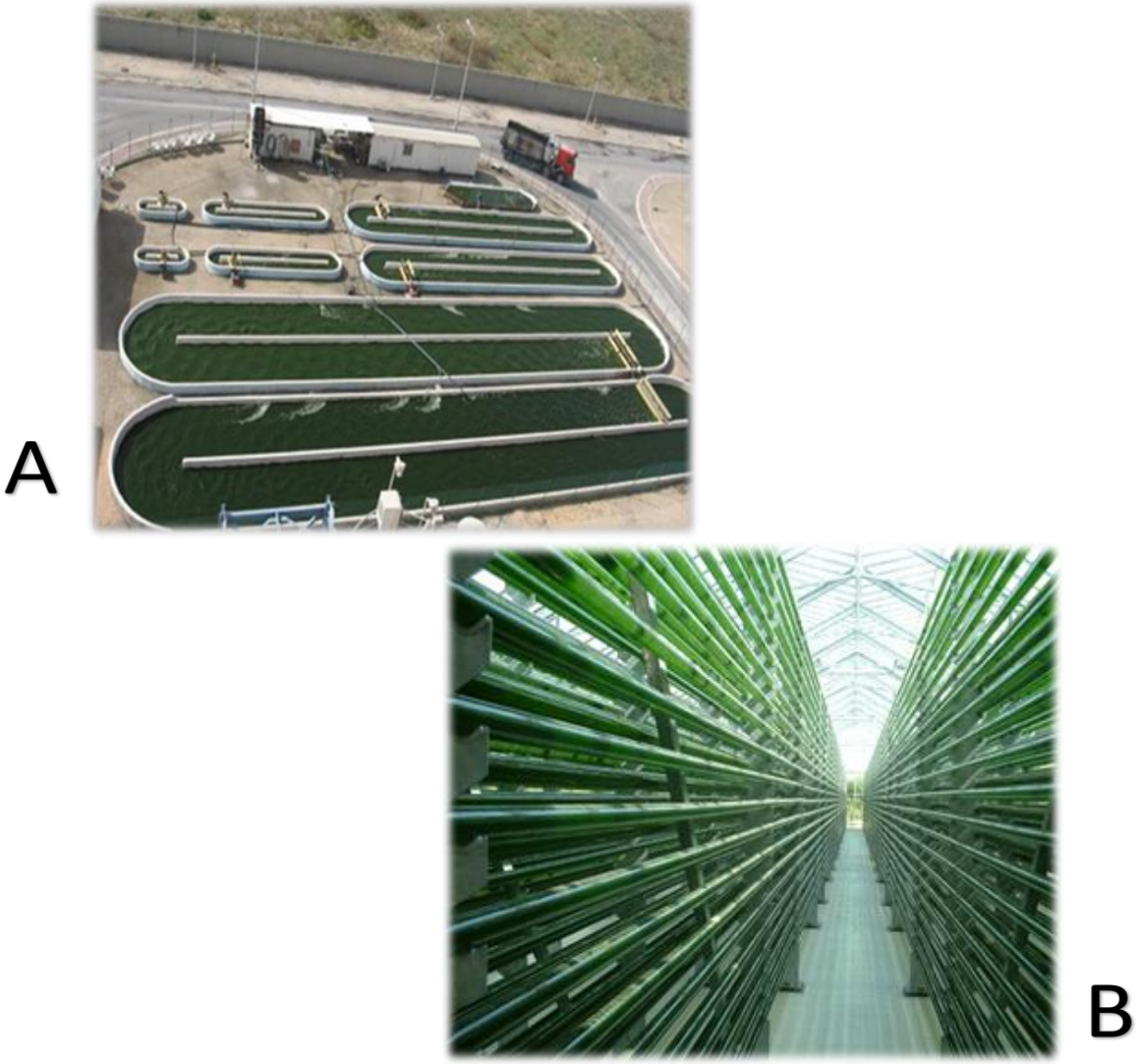

Figure 3-3 Microalgal cultivation in: A, an open pond system (source: www.makebiofuel.co.uk); B, a photo-bioreactor system (source: http://www.et.byu.edu/)

Open pond systems are the cheapest and most common form of low cost microalgal culture, in operation since the 1950's (Brennan and Owende, 2010). These are typically outdoor large area cultivation systems, operating low energy mixing with paddle wheels/stirrers, and artificial addition of carbon dioxide gas derived from flue gases or other industrial processes (Benemann, 1997; Doucha *et al.,* 2005). Maximum yield in open systems varies depending on growth medium, system used and geographical location (sunlight hours and temperature), but typically range between 20 g/m^2 d (Posten and Schaub, 2009) to 35 g/m^2 d (Chisti, 2007). These systems are typically constructed out of low cost materials such as sand and, or cement, while higher rate open systems use lined PVC or glass at additional cost. Closed systems, such as photo-bioreactors (PBRs) are enclosed high rate systems which offer greater control over the environmental conditions that affect growth. These systems come in a number of different configurations, sizes and designs, e.g.: (1) tubular or flat plate systems; (2) horizontal or vertical systems; (3) serpentine systems (Schwed *et al.*, 2013), and have been used successfully to cultivate specific microalgae species for a range of commercial products (Pulz, 2001). They are specifically designed to optimise carbon dioxide supplementation, maximise light usage and nutrient uptake through more efficient gas transfer, mixing, and optimised light intensity and frequency. Biomass yields can be as high as 72g/m^2.d (Pulz, 2001; Chisti, 2007), while unit area required to generate higher yields is lower than in open systems. The relative production efficiencies of open and closed systems are described by Chisti (2007) for a set annual production rates (Table 3-3)

Open cultivation systems are significantly cheaper to build and operate than closed photo-bioreactors, but have a number of disadvantages including susceptibility to contamination, limited light penetration, inefficient gas transfer and mixing, and large

land requirements. Furthermore, limited numbers of microalga strains are suitable for open cultivation. Closed photo-bioreactors have better control over environmental conditions, such as light and temperature, more efficient gas transfer and effective mixing, which results in high productivities, lower susceptibility to culture crashes through infection, and a reduced footprint size. However, these benefits come at a cost, primarily economic, through increased capital and operational expenditure which is estimated at 3 times higher than open systems (Mata *et al.,* 2010), mainly because energy consumption increases significantly. The predicted cost of producing biomass in photo bioreactors and open systems was estimated by Chisti (2007) to be $2.95 and $3.80, respectively. The costs are expected to drop with further improvements in yields, while the economics of production may improve if cultivated on wastewater with savings in energy taken into account. These systems can be used to successfully remediate wastewater and industrial effluents by sequestering nutrients and trace metals (McGinn *et al.,* 2011). It is the potential of using anaerobic digestate and biogas as a source of nutrients and carbon dioxide for cultivation, respectively, that has garnered recent interest (Cordoba *et al..* 2008; Ras *et al.,* 2011). Corodoba et al., (2008) demonstrated a relatively high growth rate of 0.96/d when cultivating *Chlorella Zofingiensis* on olive mill digestate, albeit this rate was lower than when grown on a synthetic media used as a control. This is in contrast to a study by Marcilhac *et al*., (2014) which showed inhibition of algal growth due to digestate colour interference and potential chemical inhibition. It is clear that the use of digestate for algal cultivation needs further investigation and is dependent on species of algae cultivated and the

type of digestate used (McGinn *et al.,* 2011). The use of highly colour digestate would potentially require additional mixing due to reduced light penetration.

Table 3-3. Comparison of open and closed systems for microalgae production.

Variable	Closed system (PBR	Open system
Annual production (kg)	100000	100000
Volumetric productivity ($g/m^3.d$)	1535	117
Productivity per area ($g/m^2.d$)	48.0[a]	35.0[b]
Biomass concentration (g/L)	4.00	0.140
Area required m^2	5681	7828
Annual CO_2 consumption (kg)	183333	183333

a Based on facility area

b Based on pond area

*Adapted from Chisti, 2009

3.2. Anaerobic digestion

3.2.1. Anaerobic biochemistry

Anaerobic digestion is the breakdown of organic matter by microorganisms in the absence of molecular oxygen. It consists of a series of biologically driven reactions that are undertaken by a number of different microorganisms, working syntrophically through the exchange of by-products and metabolites in a series of independent metabolic process, which ultimately uses carbon dioxide as the electron acceptor for the production of methane. The process has been adapted and engineered to maximise the production of methane, while stabilising waste and purpose grown materials such as energy crops.

Anaerobic digestion can be separated into four biological processes: hydrolysis; acidogenesis; acetogenesis; and methanogenesis.

Hydrolysis is the first stage, and involves the breakdown of complex polymeric compounds into simpler water soluble molecules. Reactions are undertaken by extra-cellular enzymes (proteases, amylases, cellulases and lipases) produced by facultative bacteria and is typically the rate limiting process in the breakdown of complex wastes (Appels et al. 2008) although is not always the overall limiting step. The end products of hydrolysis of complex polymers i.e. proteins, carbohydrates and lipids are the simpler compounds of amino acids, sugars and fatty acids, respectively. The conversion of complex molecules to glucose and hydrogen is demonstrated in (3-2).

$$C_6H_{10}O_4 + 2H_2O \rightarrow C_6H_{12}O_6 + 2H_2 \quad (3\text{-}2)$$

Acidogenesis is a process driven by acidogenic bacteria (e.g. *Clostridia, Bacteroides* etc). These organisms ferment the degradation products from the first stage to fatty acids such as acetic, propionic and butyric acid, hydrogen, alcohols, carbon dioxide and hydrogen sulphide. The proportion of these products is dependent on the composition of the waste material broken down in the first stage, and the relative abundance of the different acidogenic bacteria. The fermentation of glucose conversion to ethanol is shown in Eq. 3-3.

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3 + 3H_2 \quad (3\text{-}3)$$

Acetogenesis converts the organic fatty acid products from acidogenesis to acetate, hydrogen and carbon dioxide. Typically the process has hydrogen production included in the metabolic process diagrams, but hydrogen is produced primarily through the dehydrogenation of fatty acids, with only a small proportion directly produced in acetogenesis stage dependent on the oxidative state of the organic compound. Acetate can also be formed through the acetate oxidization and homoacetogenic pathway, both of which require a specific community of bacteria to utilise this metabolic pathway (Batstone *et al.,* 2002). The conversion of propionic acid to acetic acid is shown in Eq. 3-4.

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3 + 3H_2 \quad (3\text{-}4)$$

Methanogenesis is the end process of anaerobic digestion, forming methane and carbon dioxide. The process is undertaken by Methanogens, a group of Archaea. These organisms utilise the end products of acetogenesis and some products of acidogenesis for the production of methane. Two primary pathways exist, acetotrophic methanogenesis, which uses acetate (Eq. 3-5), and hydrogenotrophic methanogenesis (Eq. 3-6), which uses hydrogen and carbon dioxide. In addition to the two dominant pathways other substrates can be used by methanogens such as formate, methanol and carbon monoxide.

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (3\text{-}5)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (3\text{-}6)$$

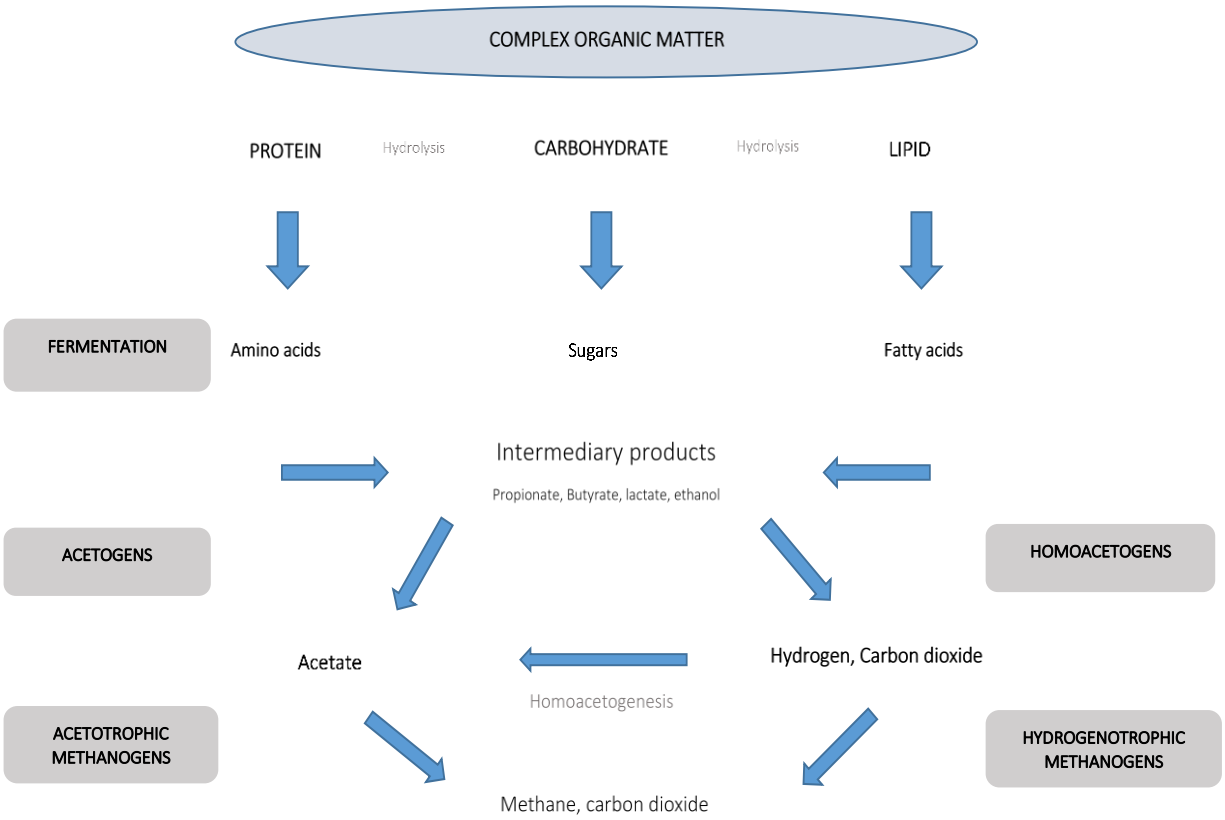

Figure 3-4 Schematic diagram showing the conversion processes in anaerobic digestion (Adapted from Gujer and Zehnder, 1983).

3.2.2. Operating conditions

3.2.2.1. Temperature

The microbial community responsible for anaerobic digestion is extremely temperature dependent, with distinct but overlapping microbial communities formed at different temperatures (Figure 3-5). These organisms operate in different environments and at different rates, the higher the temperature, the faster the kinetics and degradation rates observed until the upper temperature tolerance of each group of organisms is reached. Typically engineered systems operate at pyschrophillic (< 20°C), mesophillic (30 – 40°C) or thermophillic (50 - 60°C) temperature.

3.2.2.2. Solid retention time (SRT) and Hydraulic retention time (HRT)

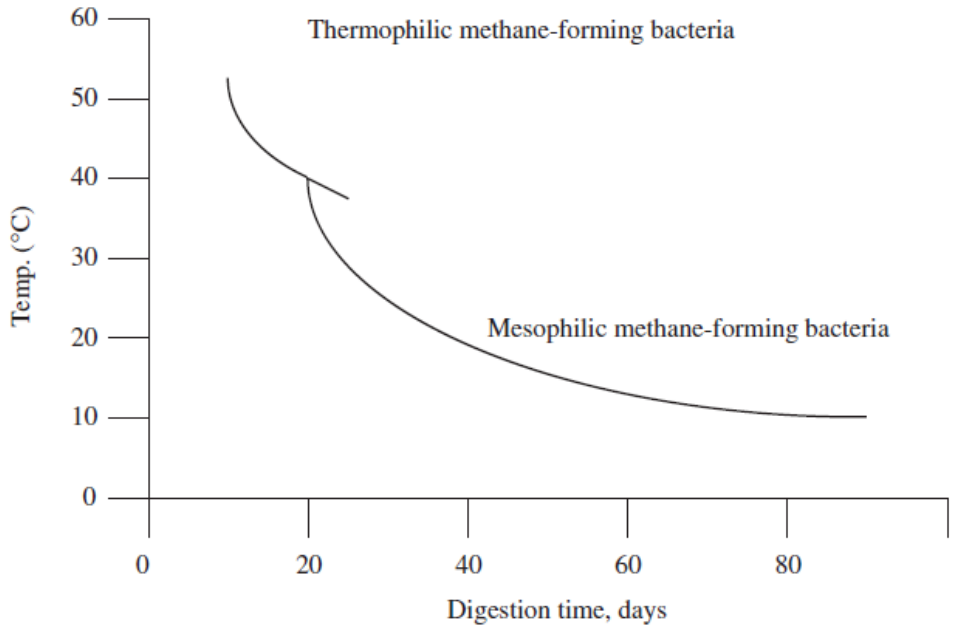

Figure 3-5 Effects of operating temperature on digestion time (Geradi, 2003)

Solid retention time (SRT) and Hydraulic retention time (HRT) are important process parameters in anaerobic digestion, and their effect on anaerobic digestion has been

been studied extensively (Miron *et al.,* 2000, Lee *et al.,* 2011). Solid retention time determines the duration that the substrate and anaerobic biomass spends in the digester, while hydraulic retention time is the time for complete hydraulic throughput. The relationship between these can be 1:1 or can be decoupled depending on the reactor system. The retention time is critical to the microbial community composition. Methanogens have the slowest growth rates out of all the organisms, and so for adequate methanogenesis, the retention time must be set above this level.

Solid retention times at ≤ 8 days result in the predominance of acidogenic conditions in primary sludge digestion, while retention times above 8 days permit stable methanogenic conditions to develop (Miron *et al.,* 2000). Under typical methanogenic conditions, hydrolysis is normally the rate limiting step.

3.2.2.3. pH

pH within the anaerobic digestion is a function of reactor CO_2, VFA, NH_4 bicarbonate concentrations within the system. Those bacteria responsible for hydrolysis, acidogenesis and acetogenesis can tolerate significantly lower pH environments compared to methanogens, directly a result of their VFA production, with performance optima at pH 5–7. The methanogens are more sensitive to pH conditions, with decreases in their performance at decreased pH. Optimum pH for methanogenesis is pH 7 – 8 (typically operated at pH 6.5 – 8.5). The reactor pH can be strictly related to relative concentrations of carbon dioxide, fatty acids and alkalinity within the reactor system. Elevated pH does not directly affect methanogenesis but results in increased quantities of CO_2 dissolved in the liquid phase and can indirectly affect methanogens through shifts in equilibrium between the inhibitory NH_3 and the less inhibitory NH_{4+},

increasing proportions of the inhibitory unionised form, and resulting in reduced methane production (Chen *et al.*, 2008).

3.3. Microalgae as a substrate for anaerobic digestion

Microalgae theoretically offer great potential as a substrate for anaerobic digestion when compared to other algal biofuels, and digestion of other energy crops (Sialve *et al.,* 2009).

Methane potential of microalgae was first evaluated by Golueke and Oswald (1959). Since then there have been a number of different studies which have used different techniques to evaluate different factors such as SRT, HRT, temperature, loading and species showing a large variation in methane yield across different conditions (Sialve *et al.,* 2009). All have reported varying methane yields from 0.137 LCH_4/gVS for an un-identified mixed algal culture (Passos *et al.,* 2014) to as high as 0.395 LCH_4/gVS for a mixed microalgal culture of *Chlorella, Scenedesumus and Nanochloropsis* (Alzate *et al.,* 2012) using different experiment configurations and, operating conditions. The different effects of some key variables are detailed below.

3.3.1. Effect of temperature

Temperature plays an important role in the anaerobic digestion of microalgae and can result in improved degradation rates. The first study by Golueke *et al.,* (1959) demonstrated that methane yields can be increased from 0.17 to 0.32 LCH_4/gVS_{added} when operating temperature is increased to 50°C. Although a significant improvement it was noted that only 2% of solar energy was converted to methane indicating relatively low conversion efficiency, attributed to un-degradable components and potential ammonia inhibition. The organic loading rates were low and the solid retention extremely conservative. Since this study a number of other researchers have

investigated the effect of digestion temperature and found conflicting results. De Schamphelaire and Verstraete (2009) found no observed difference in methane yield when temperature was increased from 35°C to 41°C, while Zamalloa *et al*., (2012) found results that agree with both previous findings. Using a high rate hybrid filter it was demonstrated that increasing process temperature from 35 – 50°C improved methane yield in both *Scenedesmus obliquus* and *Phaeodactylum tricornutum*, but the effect was more pronounced on *Scenedesmus* species, with an increase from 0.14 to 0.17 $LCH_4/gCOD_{added}$ reported. These reported yields for *Scendesmus sp*. are in contrast to Mussgnug *et al.* (2010) who showed that even after 6 months ultimate methane yield did not exceed 0.08 LCH_4/gVS_{added}, well below the values reported even when taken into an approximate COD/VS conversion ratio of 1.4 . The effect of temperature on microalga degradation may be related to both improvements in the activity of the degrading organisms, but also the impact of elevated temperature on the microalgal cell by exposing to temperatures outside its normal growth conditions (González-Fernández *et al.*, 2012). Removing microalgae from typical growth conditions would potentially result in reduced growth rates, and changes in intra-cellular composition, and cell structure. The results by De Schamphelaire and Verstraete (2009) indicate no increase in methane yield when digester temperature is increased from 35 to 41°C. These results may suggest that elevated process temperatures do not improve yield, or alternatively, the small 6°C increase was not significant enough increase to process temperature to have any effect on the microalgae itself.

3.3.2. Effect of solid retention time

Solid retention time (SRT) plays an important role in the process rates in anaerobic digestion, directly determining the residence time of the substrate and microorganisms within the system. A number of studies have evaluated the effect of different SRTs on methane yield with 10 – 30 days reporting a wide range of results (Figure 3-6). Ras *et al*., (2011) showed that an SRT of 28 days resulted in a methane yield of 0.240 $LCH_4/gVSS_{in}$, while observing a significant drop in yield to 0.147 $LCH_4/gVSS$ when operating a SRT of 16 days. Golueke and Oswald (1959) reported that retention times of 30 days or more resulted in no further improvements degradation observed and suggested this as the maximum, while the lower limit of 10 days was reported by Ehimen *et al.,* (2009) to be critical to prevent washout of microorganisms when fed on algae. The effects of retention time can also be related to different degradation rates that potentially exist between different microalgae, with some species such as *Scenedesmus obliquus* demonstrating particularly resistant degradation rates under idealised batch anaerobic digestion conditions for length periods of time (Mussgnug *et al.,* 2010).

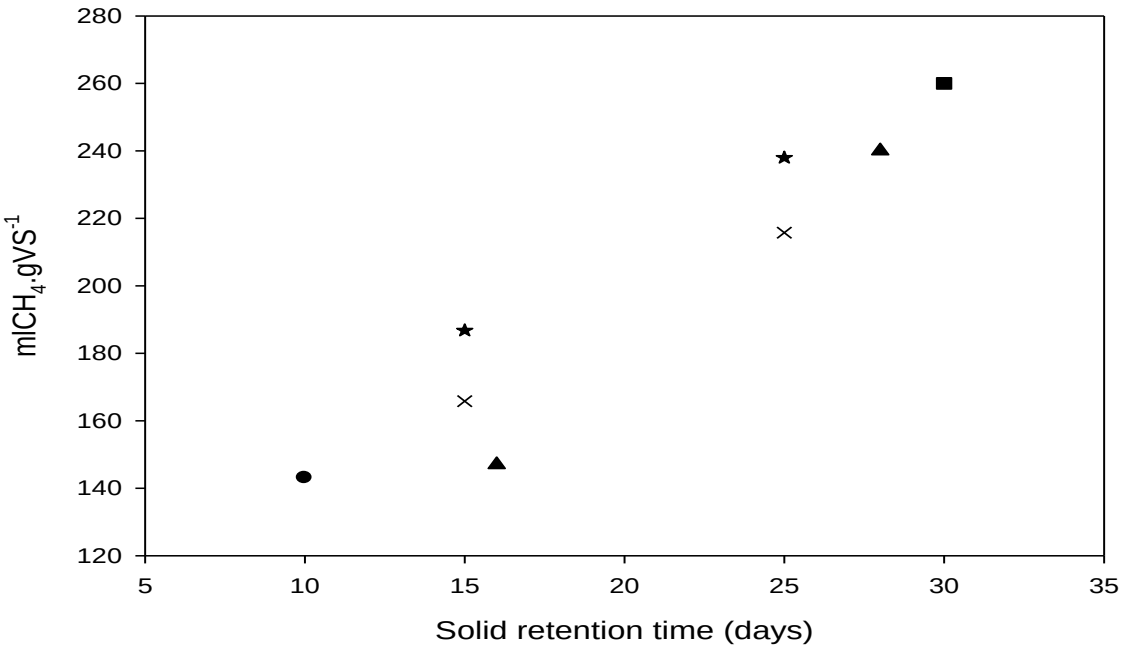

Figure 3-6 Effect of different solid retention times on methane yield of microalgae: (•), denotes Brune *et al.,* 2007; (▲), denotes Ras *et al.,* 2011; (■), denotes Golueke and Oswald, 1959

Table 3-4 Summary of previous experiments on batch or simple CSTR digestion experiments of microalgae

Study	Species	Reactor type (Size L)	Temperature °C	SRT (d)	OLR (gVS/L.d)	CH_4 production (LCH_4/gVS)	CH_4 (%)
(Alzate *et al.*, 2012)	*Chlorella, Scenedesmus, Nanochloropsis*	Batch	35	Batch (60)	Batch	0.395 ± 0.01	77
	Acutodesmus, Oocystis, Phormidium, Nitzschia	Batch	35	Batch (60)	Batch	0.188 ± 0.01	52
	Microspira	Batch	35	Batch (60)	Batch	0.329 ± 0.01	52
(Foree and Mccarty, 1970)							
(Golueke and Oswald, 1959)	*Chlorella, Scenedesmus*	CSTR (11)	30 - 50	30	1.44	0.170 – 0.320	61 – 62.4
(Hidaka *et al.*, 2014)	*Chlorella Vulgaris*	Batch (0.5)	35	Batch	Batch	0.200 – 0.320	N/D
(Mottet *et al.*, 2014)	*Dunaliella Salina*	Batch (0.5)	35	Batch	Batch (0.5)	0.36 ± 0.008	N/D
(Mussgnug *et al.*, 2010)	*Arthrospira platensis*	Batch (0.06)	38	Batch (32)	Batch	0.293 ± 0.006	61
	Chlamydomonas reinhardtii	Batch (0.06)	38	Batch (32)	Batch	0.387 ± 0.006	66
	Chlorella kessleri	Batch (0.06)	38	Batch (32)	Batch	0.218 ± 0.005	65
	Dunaliella salina	Batch (0.06)	38	Batch (32)	Batch	0.323 ± 0.002	64
	Euglena gracilis	Batch (0.06)	38	Batch (32)	Batch	0.324 ± 0.002	
	Scenedesmus obliquus	Batch (0.06)	38	Batch (32)	Batch	0.178 ± 0.006	62
(Passos *et al.*, 2014)	*N/D*	CSTR (1.5)	35	15 - 20	0.76 – 0.99	0.130 – 0.170 (0.02 – 0.03)	68.1 – 68.5
(Ras *et al.*, 2011)	*Chlorella Vulgaris*	CSTR (1)	35	16 – 28	0.70**	0.147 – 0.24*	
(Samson and Leduy, 1982)	*Spirulina*	Semi	30	33	0.97	0.260	68-72
(Sukias and Craggs, 2011)	*N/D*	Unmixed (1-2)	20		5.00 – 6.56	0.224 – 0.399	69.7 – 80.6
(Yuan *et al.*, 2011)	*Blue algae*	CSTR (4.5)	35	Batch (30)	Batch	0.189	36.7

N/D Not disclosed

** Denotes gVSS/L.

3.3.3. Effect of reactor configuration and operation

The design and operation of different reactors are primarily to improve process degradation and ultimately methane yield (3.2.2.3). The dominant method for evaluating methane potential is batch methane potential tests, while a number have used CSTRs to evaluate methane potential. Two studies to date have attempted to study the effect on methane yield of reactor configuration (Table 3-5). Zamalloa *et al.,* (2012) evaluated the use of a hybrid flow through reactor on the methane yield of *Scenedesmus obliquus* and *Phaedactylum tricornutum*. The hybrid was a novel system comprising an anaerobic sludge blanket with an anaerobic filter in the upper part. The flow was vertical at 1m/hr, similar to a UASB. The benefit to this system was its ability to be fed on relatively dilute concentrations of algae, something that until this study had only not happened with some form of pre-concentration of microalgae required to enable a typical OLR for anaerobic digestion to be achieved. Conversion in *Scenedesmus* species was lower than *Phaeodactylum species*, these results agree with a previous study which demonstrated lower ultimate methane yields for *Scenedesmus species* (Mussgnug *et al.,* 2010). The lower yields/higher degree of resistance can be attributed to the ability of the microalgae species to grow and survive in elevated temperatures, meaning that it is more suited to surviving in mesophillic temperatures in AD. The increase in process temperature from mesophillic to thermophillic appeared to improve digestion in both species tested, showing promise to achieving the most out of the algal methane potential at elevated organic loadings. The reactors however were still operated at conservative loading, while the effluent solids and COD was high indicating continued washout of both anaerobic biomass and

microalgae biomass. This study again indicated that algal biomass retains a significant component that is not degraded in anaerobic digesters, but with optimised reactor configuration improvements in yield can be achieved. It also indicates that under the same reactor conditions species variance can play an important part in methane yield.

The second study by Zamalloa *et al*., (2012b) used a novel anaerobic membrane bioreactor to aid retention of anaerobic and microalgal biomass, and de-couple the relationship between SRT and HRT. The de-coupling of SRT/HRT is important to reduce the requirements for costly harvesting or pre-settling while offering improvements in anaerobic microbial community performance. Feeding *Phaedactylum tricornutum* in a laboratory scale digester equipped with internal an membrane operating at extremely low hydraulic retention times of 2.5 days, and solid retention times of 10 – 20 days while varying organic load up to 5.9 gCOD/$L_{reactor}$ d. Conversion efficiencies of 48% were observed, while an effluent low in solids and COD were reported demonstrating efficient use of the microalga components that were available. Although performance was excellent it was again observed that irrespective of reactor conditions degradation rates were relatively low and while it worked well at high loading conditions, its performance was again hindered by microalgal degradation potential. The microbial community response to being fed microalgae was shown to be stable with *Methanosaeta sp*, the dominant methanogen indicating a primarily acetoclastic pathway to methanogenesis. The performance of the membrane was variable with an accumulating level of solids decreasing membrane flux and increasing transmembrane potential until the SRT was dropped to 8 days. Following the reduction in retention time there was no drop in methane yield indicating that in

membrane systems operating with a SRT below 10 days is achievable without process performance dropping. While performance was relatively poor in terms of CH_4 yield, the reactor itself appeared to access a larger proportion of the available methane potential than other studies had previously done. As microalgae have such unique substrate characteristics the impact of feeding different microalga onto the membrane reactor may elucidate some performance characteristics such as membrane fouling not previously observed with *Phaedactylum tricornutum.*, Increasing organic loading and reducing hydraulic retention further to feed high OLR with dilute cultures is important to evaluating the operational limits of these systems when fed with microalga. The use of a lyophilised marine culture instead of a freshly cultivated culture may have resulted in improved reactor performance, though, e.g. increasing membrane or cell wall permeability from the drying process, or through changes in osmotic membrane potential when exposing the microalgae to non-saline reactor conditions. The additional impact of salinity on methanogenesis in the reactor was not evaluated. These merit further investigation.

Table 3-5 Effect of anaerobic reactor configuration on the methane yield of different mixed microalgae cultures.

Study	Species	Reactor type	Size (L)	Temperature (°C)	Retention time (d)	OLR ($gVS/L_{reactor}d$)	CH_4 yield (LCH_4/gVS_{added})	CH_4 (%)
(Zamalloa *et al.*, 2012a)	*Scenedesmus obliquus*	Hybrid flow through sludge blacket	2.0	33 – 54	2.2 ± 0.4 2.2 ± 0.3	2.7 ± 0.7 (M) 2.8 ± 0.6 (T)	0.13 ± 0.05 0.17 ± 0.08	74.3 ± 2.5 77.1 ± 3.9
(Zamalloa *et al.*, 2012a)	*Phaeodactylum tricornutum*	Hybrid flow through sludge blacket	2.0	33 - 54	2.1 ± 0.3 2.3 ± 0.6	1.9 ± 0.5 (M) 2.0 ± 0.7 (T)	0.27 ± 0.09 0.29 ± 0.11	75.1 ± 8.9 78.6 ± 5.0
(Zamalloa *et al.*, 2012b)	*Phaeodactylum Tricornutum*	AnMBR	8.0	35	2.6 ± 0.4	1.3±0.4 - 5.8±0.9	0.31	75.3±2.6

*AnMBR: Anaerobic membrane bioreactor

3.3.4. Species variance

Most studies are dominated by two predominant freshwater species, *Chlorella sp*. and *Scenedesmus* sp.

It is known that composition of microalgae varies widely between species (Becker, 1994), based on this composition it would be expected that methane yield per cell would vary (Siale *et al*., 2009). Based on gross composition of different components (lipids, proteins, carbohydrates) those with greatest quantity of lipids should produce the highest amount of methane. On this assumption species like *Nanochloropsis* with high lipid contents reported should yield the highest methane content, however previous studies have shown *Nanochloropsis* to be poorly degradable with methane yields in the region of 0.15 -0.2 L/gVSS (Schwede *et al*., 2013) compared to other microalgae such as *Chlorella vulgaris* 0.24 L/gVSS (Ras *et al*., 2011). Comparisons between studies can offer limited insight into which species is more degradable than others due to different anaerobic reactor conditions (temperature, retention time, inoculum) while growth conditions of the microalgae itself are also significant to effecting composition and potentially methane yield. Studies which have directly compared methane yield under identical conditions are most useful when attempting to assess the effect of species. Mussgnug *et al.,* (2010) provided a comprehensive evaluation of 7 different species for methane production, including one cyanobacteria species. They observed variability in methane content from 0.178 LCH_4/gVS for *Scenedesmus obliquus* to 0.366 LCH_4/gVS for *Chlamydomonas reihardtii*. This is lower than the 0.210L/gVS observed for *Scenedesmus sp* in a previous study (Zamalloa *et al.,* 2012a).

Species variation appears to affect both ultimate methane potential but also the hydrolysis or breakdown rates of the algae during batch methane potential test. Mussgung *et al.,* (2010) observed quicker disintegration in marine species (*D.Salina)*, over freshwater species (*C. reinhardtii, E. gracilis, C. kessleri*). While this study showed elevated disintegration, this did not translate into elevated methane yields, suggesting that the components although are broken down, they are then not subsequently converted to methane. This study indicates a need for a further work to identify the best yielding algae, and the growth conditions suitable for biomass yield, and then breakdown in anaerobic digestion

3.3.5. Co-digestion

Microalgae typically contain a high protein content, meaning that the carbon to nitrogen ratio is below optimal required for efficient digestion. Co-digestion has been suggested as mechanism to improve methane yield from two substrates complementary to each other. The use of co-substrates is designed to provide the nutrients lacking in one substrate by digesting with a substrate that has a high content of the deficient nutrient, allowing a balance of nutrients in digester liquid (Mata-Alvarez *et al.,* 2000). Other additional advantages are controlling moisture content using two contrasting substrates and economic advantages associated with economies of scale and existing anaerobic assets.

One previous study has evaluated the combination of microalgae and waste paper with the aim to balance the C:N ratio with different combinations of paper and algal sludge (Yen and Brune, 2007). This study observed that a 50:50 W:W paper to algal biomass, with a C:N ratio of 18:1 resulted in higher methane yields than algal sludge and waste paper alone. Although co-digestion has been previously shown

to be promising for other substrates, evidence from BMP tests in numerous studies have shown that resistance to breakdown is the key factor, and so co-digestion can only benefit the co-substrate, with limited success at improving methane yield. A second study evaluated the co-digestion of microalgal biomass with pig manure residues. This showed limited degradability of microalgae used (*Scenedesmus and Chlorella* sp.), and suggested the need for pre-treatments due to the recalcitrant nature of microalgae (González-Fernández *et al.,* 2011).

The co-digestion with sewage sludge is seen as a logical step, with microalgae cultivation likely used as a nutrient removal wastewater treatment step post aerobic treatment, followed by the co-digestion with activated sludge in existing digestion facilities (Wang *et al.,* 2013). This study showed that co-digestion of microalgae with activated sludge enhanced digestion potential of each substrate, while it was suggested that de-watering capability of the sludge is enhanced when co-digesting these substrates.

3.3.6. Pre-treatments

Pre-treatments of substrates for anaerobic digestion have been established for over thirty years (Hendriks and Zeeman, 2009; Carlsson *et al.,* 2012). The primary aims of pre-treatments are to improve rate, or ultimate methane yield, while potentially reducing inhibitory or poorly degradable components of the substrate. For microalga biofuels cell disruption has been the primary focus of research, developing and testing new and existing technologies designed to completely lyse or partially disrupt alga cells (Halim *et al*., 2012; Passos *et al*., 2014). For microalga biomass these technologies can be broadly characterised into: thermal, chemical, physical/mechanical and biological technologies detailed below.

3.3.6.1. Thermal

Thermal treatment involves the input of additional thermal energy categorised into thermal, hydrothermal and steam explosion technologies (Passos *et al.,* 2014). At low temperatures cell walls are affected but not completely solubilised, while at high temperatures the cellulose and hemi-cellulose components in the cell walls are potentially solubilised (Garrote *et al.,* 1999) and full cell lysis occurs. A number of different studies have been undertaken for microalgae specifically for anaerobic digestion under various operating conditions.

Thermal treatment typically involves low temperature treatment (up to 100°C) for extended durations (2 – 24 hours) at near atmospheric pressures. The results show a varying degree of improvement in microalga solubilisation and methane yield. The original work by Chen and Oswald (1998) demonstrated a 33% increase in methane yield at 100°C for 8 hours, with temperature being shown to be the dominant mechanism for improved yields when combined with chemical treatments such as acids and alkalis. The effect of temperature seems the dominant mechanism with a threshold temperature needed to be reached before significant methane yield improvements occur. Gonzalez-Fernandez *et al.,* (2012) demonstrated that while temperatures as low as 70°C for 3 hours can result in increases in solubilisation 7 fold, the impact on methane yield was only by 12%, yet when exposed to 90°C *Scenedesmus* sp. had a 11 fold increase in soluble components with a 220% increase in methane yield. This study indicated that while improving soluble components is a good thing it does not necessarily directly lead to improved ultimate yields. It is likely that components solubilised are ones that would have degraded anyway, and only at 90°C do previously un-degraded components break down. The threshold appears to be approximately 80°C with a

further study showing an improvement in methane yield between 70 and 80°C of 57% (Gonzalez-Fernandez *et al.,* 2012). These results are in agreement with Alzate *et al.,* (2012) who demonstrated a drop in yield at 55°C. However, other studies have shown only a marginal increase in methane yield at 80°C, albeit at very low contact times of 30 minutes (Cho *et al.,* 2013). The differences between studies are likely a result of differences in species structure and upper temperature tolerances, the concentration of solids used in pre-treatment and the contact times which can vary significantly.

Elevated temperature or hydrothermal treatment involves subjecting microalgae to temperatures between 100 and 200°C for shorter contact times than that of low temperature treatment. A number of studies have evaluated the potential of temperatures up to 170°C, at 6 bar for as long as 30 minutes. Increases in methane yield of between 60 and 120% were observed after subjecting alga to 120°C to 140°C for up to 30 minutes (Alzate *et al.,* 2012; Cho *et al.*, 2013). Mendez et al., (2013) showed increases in yield by 93% when exposed to 120°C for up to 40 minutes, with a significant increase in hydrolysis rates (0.1 – 0.23 d^{-1}) observed. The effect of contact time appears significant as the increase in contact time up to 2 hours at 120°C improved methane yield in *Nanochloropsis* sp. by 108% (Schwede *et al.,* 2013). The impact of different elevated treatments again appears significant with the cell wall appearing to be damaged at more elevated treatment temperatures and contact times (Passos *et al.,* 2014). Temperatures of 120°C appeared to only solubilise cellulosic cell wall materials, with the presence of glucose, a direct degradation product of cellulose indicating this (Mendez *et al.,* 2013). The lack of hemicellulose degradation at these temperatures is consistent with the understanding that hemicellulose components need higher temperatures

to degrade (Garrote *et al.,* 1999). At lower temperatures it is likely that complete solubilisation of cellulose does not take place, but does result in a disruption of the hydrogen bonds between cellulose and hemicellulose in the cell wall (Laureano-Perez *et al.,* 2005). There was no significant change in protein solubilisation at elevated temperatures.

The effect of inhibitory compounds produced from thermal treatment has not been studied, but with low humic/fulvic acids and lignin related components in the cell wall inhibitory compounds that can be produced from thermal degradation are perceived to be unlikely.

Steam explosion otherwise known as thermal hydrolysis is the rapid de-pressurisation following elevated temperatures, typically in excess of 160°C where pressure can be between 6 – 8 bar. Sudden decompression results in cell wall rupture and improved solubilisation. Thermal hydrolysis at 170°C and 8 bar for 30 minutes prior to decompression reduced in an 81% improvement in methane yield, and 10 fold solubilisation (Keymer *et al.,* 2013).

While all thermal pre-treatment studies shown significant effects in biodegradability the additional energy requirement can be considerable at elevated temperatures and pressures (Passos et al. 2014). Low temperature pre-treatments have been shown to be the most viable option, with net positive energy reported of > 2 GJ/d at 75°C treatment time, with 20 day HRT critical (Passos and Ferrer, 2014). The effectiveness of these temperatures is highly species related, but at low temperatures it was shown to work well without significant prior concentration of biomass. Further work at scale is required to fully evaluate process performance.

3.3.6.2. Chemical

Chemical pre-treatments involve the addition of acids, alkalis and oxidising agents to disrupt cell walls and oxidise inhibitory or recalcitrant components. Typically these result in the solubilisation of cell materials but with potentially important implications for formation of inhibitory compounds and corrosion of digesters and equipment. Chemical treatment normally is interrelated with thermal treatment as excess heat is generated from most of the chemical reactions, and so improvement in solubilisation can be partially related to increases in temperature.

While NaOH treatment has been shown to improve methane yield by 33% it was the pre-treatment temperature that had the largest effect on methane yield (Chen and Oswald, 1998). Mendez *et al.,* (2013) demonstrated a similar trend showing that thermal treatment alone increased methane yield to 0.267LCH_4/gCOD_{in}, demonstrating an increase of 93%. Acid and alkali treatment increased soluble protein and carbohydrates by 2 and 7 fold respectively, but achieved a lower methane yield than thermal pre-treatment. The increase in soluble carbohydrates has been observed with the use of H_2SO_4 at 160°C, with only 33.7% intact cells remaining (Halim *et al.*, 2012). While the use of alkali alone demonstrated improved solubilisation, but limited effectiveness for improving methane yield when compared to combined thermal chemical pre-treatments (Bohutskyi *et al.,* 2014). The exact mechanism behind improved solubilisation without methane yield improvements is likely attributed to either the production of inhibitory compounds or the solubilisation products still not easily degradable to anaerobes. Although many methods demonstrate some improvement the treatment effectiveness is poor in comparison to thermal treatment while the ability to use dilute feedstocks remains unanswered. The additional drying costs for prior pre-treatment with

chemicals would be prohibitive to the cost effectiveness digestion of algae, while drying biomass has been shown to potentially reduce methane yields (Mussgnug *et al.,* 2010).

3.3.6.3. Physical

Physical pre-treatment includes grinding/crushing, chopping/macerating and high pressure homogenisation, all designed with the same purpose, to disrupt cell structure and walls through combinations of pressure, translation or rotational energy (Barjenbruch and Kopplow, 2003). The effectiveness of physical treatments on microalgae is likely limited due to the small nature of algal cells meaning that shear forces generated are unlikely to be large enough on particulate cells. Most studies on physical pre-treatments have evaluated ultrasound technology. Ultrasound pre-treatment involves the rapid compression and decompression of sonic waves at different wavelengths and frequencies. The rapid movement creates tiny bubble to form, which are trapped within the cells. The rapid compression/decompression of these bubbles creates cell damage or possible cell lysis (Kim *et al.,* 2013). A number of studies to date have evaluated the use of this technology on algae for anaerobic digestion using a number of different microalga strains, applied energies and biomass concentrations with varying degrees of success. Methane yield increases were observed in most studies with ultrasound. However, baring one study, energy inputs below 75 MJ/kg DW resulted in a maximum 33% improvement in methane yield (Alzate *et al*., 2012; Gonzalez-Fernandez *et al.,* 2012; Passos *et al.,* 2014). Increasing energy input to 200 MJ/kg resulted in significantly higher increases in yield of 80 – 90% (Gonzalez-Fernandez *et al.,* 2012). The net energy payback at such elevated levels is poor, but it was

shown that if improvements to VS concentrations exceed 8%, then energy balance may be positive.

The second most evaluated physical pre-treatment option is microwave technology, a technology extensively studied for waste activated sludge (Toreci *et al.,* 2009). The application of this technology for the disruption of microalga cell walls for biofuels is relatively new, while its specific application for AD of produced biomass is in real infancy (Passos *et al.,* 2014). Microwave radiation induces water to boil through short waves of electromagnetic energy, typically at 2450Hz frequency (Passos *et al.,* 2014), resulting in a change in the structure of proteins and lipids which effects cell damage and potentially lysis (Park *et al.,* 2010). The application of microwave radiation to algae was shown to improve methane yield in mixed culture microalgae grown on wastewater from 0.17 LCH_4/gVS_{in} to 0.27 CH_4/gVS_{in} at 900W, 3 min treatment time representing an improvement of 60% in daily yield (Passos *et al.,* 2014). . With the current energy balance being exceptionally poor (typical energy inputs of 70 MJ/Kg used to generate significant yield) it is evident further work is required.

3.3.6.4. Biological

Biological pre-treatment offers a suitable alternative to traditional mechanical and thermochemical technologies. The use of biological or enzyme pre-treatments offers the potential for low energy inputs, and reduction in potential inhibitory compounds produced when excess heat or chemical treatments are applied (Mahdy *et al.,* 2014a). The use of different enzymes for pre-treatment has been studied considerably for waste activated sludge and in-situ anaerobic digestion improvements, while there are a growing number of studies evaluating the

supplementation for biofuel yield improvement from microalgae (Gerken *et al.,* 2013). Mahdy *et al.,* (2014a) demonstrated the use of proteases for hydrolysing or disrupting *Chlorella* species cell components. This study demonstrated 73% increases in methane yield (0.253 $LCH_4/gCOD_{in}$ compared to 0.147 $LCH_4/gCOD_{in}$) when applying enzyme dosage of 0.585 gDW_{enzyme} per 16g DW_{algae}/L. While no hydrolysis constant was reported it was observed that up to 90% of methane was produced in the first 6 days. Although this study demonstrated some benefits of protease disruption it did not evaluate whether there was any impact on the anaerobic biomass from enzyme addition with inclusion of enzyme/inocula controls. Other studies by the same authors have shown the relative benefits of using a commercial mix of enzymes containing b-glucanase, arabanase, cellulase, b-glucanase, hemicellulase and xylanase (Mahdy *et al.,* 2014b). The application of enzyme mixtures would be beneficial to microalgal degradation, which has been shown to contain a wide range of different cell compounds and cell wall structure intra and inter species. This study demonstrated that the application of multi enzyme cocktails is not as important as targeted specific enzyme addition. Carbohydrates were solubilised, potentially from the cell walls but only 14% improvements in the methane yield of 0.196 $LCH_4/gCOD_{in}$ were observed achieving a conversion of 0.223 $LCH_4/gCOD_{in}$. The application of proteases (Mahdy *et al*., 2014) proved to be more successful in improving yields.

The poorer performance of cellulases alone (Gerken *et al*., 2013), and good performance of proteases on both species corresponds with the observed lack of cellulose, and presence of glycoproteins in cell walls (Adair *et al*., 1990). The combination effect with both enzymes observed indicates that potentially some small amounts of cellulose or carbohydrate compounds do exist either in the cell

wall, or in parts of the cell that are previously un-degraded under normal anaerobic conditions. Potentially these exist in the thylakoids where starch has been observed to accumulate and would correlate with the observation of efficient cellulose degradation of starch for bioethanol production previously seen (Fu *et al.,* 2010).

The application of enzymes to algal AD technology could prove important, but the efficiency is strongly dependent on matching the right enzymes with the right algal species based on cell wall characteristics and intracellular compounds. It is also likely that the impact of algae cultivation conditions such as light intensity, nutrient concentration and temperature on cell composition and structure could dramatically change the efficacy/efficiency of specific enzymes. While pre-treatment with enzymes proved effective the additional costs of production and reaction of enzymes with algae are unlikely to be favourable, although no cost comparison has been undertaken. This leads to the suggestion that only direct addition of enzymes to digesters, or feedstock balance tanks, without temperatures beyond those found in digesters could prove economically viable. The impact on digestion needs further investigation.

3.3.7. Toxicity/Inhibition

Microalga contains a number of different compounds that could potentially be inhibitory to the anaerobic digestion process.

3.3.7.1. Salinity/Sulphates

The presence of significant quantities of light metals and cations such as those present in sea water (potassium, calcium, magnesium, sodium) can have inhibitory effects on the AD process (Chen *et al.,* 2008). Although anions are associated with cations, it is only the presence of the cation that is of primary concern (McCarty and McKinney, 1961). In low concentrations these cations have been proven to be beneficial to anaerobic digestion (Sumper and Brunner, 2006), but in significant quantities they lead to a variety of different performance related problems. The presence of sodium ions at concentrations found in seawater, 12g Na^+/L (Feijoo *et al.,* 1995) can cause cells to suffer from dehydration due to osmotic pressure, while the sulphate present allows a growth of sulphate reducing bacteria (SRB) and for them to predominate over methanogens in the utilisation of acetate and hydrogen (Equation 3-7, and Equation 3-8).

$$H_2 + SO_4^{2-} \rightarrow H_2S + H_2O \quad \textbf{(3-7)}$$

$$CH_3COOH + SO_4^{2-} \rightarrow H_2S + CO_2 + H_2O \quad \textbf{(3-8)}$$

Several different strategies exist for overcoming potential saline inhibition, including the use of halophilic inocula from marine sediments (Nishida and Murata, 1996), adaption of anaerobic biomass to elevated saline levels (Lynch and Thompson, 1982; Schwede *et al.*, 2013, Bohutskyi *et al.,* 2014), and the use of

solutes such as glycine betaine to reduce inhibitory effects of cations (Chavan *et al.,* 2014). The inhibitory effects have been shown to be specific to different trophic groups of methanogens (Liu and Boone, 1991) and have been shown to be overcome in algal digestion technologies through pre-acclimatisation and use of correct inocula (Mottet *et al.,* 2014).

Potential sulphate inhibition and production of H_2S can be overcome using a number of different strategies. Cleaning and re-injecting biogas constantly will result in the removal of H_2S from the gas phase and a shift in equilibrium drawing it out of the liquid phase. Injecting small quantities of oxygen in the headspace can reduce the H_2S concentration, but at a cost to the energy content of the biogas. While the most common mechanisms involves the addition of iron hydroxide to precipitate out the H_2S.

The use of extremely halo tolerant algal species would need further investigation, and while pre-concentration can potentially lead to a reduction in total salt levels, the use of dilute feedstocks in membrane systems would not. This problem would obviously be eliminated through the use and cultivation of freshwater species.

3.3.7.2. Ammonia

Anaerobic digestion of nitrogenous compounds such as proteins results in the production of ammonia (NH_3 and NH_4^+), with only small quantities utilised for cell synthesis. Ammonia is known to inhibit microorganisms at high concentrations, with methanogens known to be particularly sensitive to inhibition. Both hydrogenotrophic (Wiegant and Zeeman, 1986) and acetoclastic methanogens (Angelidaki and Ahring, 1993) have previously shown particular sensitivity to high levels of ammonia, dependent on conditions such as operating temperature and

substrate load. The production of volatile fatty acids (VFAs) appears to not be affected by high levels of ammonia nitrogen (Koster and Lettinga, 1988).

The toxicity of ammonia is primarily related to the unionized free ammonia form NH_3 dissolving across the cell membrane of the cell changing intracellular pH, increases in energy maintenance requirements, proton unbalance and potassium deficiency (Sprott and Patel, 1986; Wittmann *et al.,* 1995). The proportion of each form of ammonia (NH_3 and NH_4^+) is governed by the equilibrium constant of the reaction which contains a $[H^+]$ term, so is pH dependent.

The concentration of total ammonia at which inhibition of either groups of methanogens occurs varies widely in the literature from as little as 1.5 gN/L (McCarty, 1964) to levels as high as 4 gN/L without inhibition (Angelidaki and Ahring, 1993). Previous studies have shown total ammonia tolerances up to 7.8 gN/L (Debaere *et al.,* 1984). The reason for these differences are likely related to type of reactor, temperature of the reactor, pH and the degree of acclimatisation the reactor sludge has had to high levels of total ammonia nitrogen (Vanvelsen, 1979).

With high protein content the potential for ammonia inhibition has been briefly summarised before (Sialve *et al.,* 2009). Previous studies involving the anaerobic digestion of microalgae have shown elevated levels of 1.8 gN/L (Golueke *et al.,* 1957) exceeding the reported pH dependent threshold of 1.7 gN/L (McCarty and Michinney, 1964) being reported in thermophillic reactors. However, as no exact pH was reported other than “lower than 8”, the calculation of exact free ammonia concentration cannot be made but it further highlights the need for effective pH control in reactors treating substrates with high nitrogen content, especially reactors operating at thermophilic conditions and pH 8.0. At that pH, free ammonia

nitrogen concentrations would be 8 times the concentration of a reactor with a pH of 7.0 and would likely result in inhibitory levels previously reported for FAN (McCarty and McKinney, 1961). It should be appreciated that reported inhibitory ammonia levels vary widely in the literature, studies that have shown higher tolerances attributing this to acclimatisation of biomass with slowly increasing quantities of ammonia, meaning the effects are reduced. It is likely that the start-up of such facilities would be important, slowly increasing the substrate concentration so as not to overload reactors. It should be noted that based on a theoretical ammonia release of 54mg NH_3-N/gVS for *Chlorella vulgaris* (Sialve *et al.,* 2009), loading rates in the order of 4-8 gVS/$L_{reactor}$d would need to be achieved to bring ammonia within the average inhibitory range reported, however, with control of pH in the digester the free ammonia levels could be controlled more reliably. Another ammonia mitigation strategy is the type of reactor used, with membrane and fixed film bioreactors both washing out and diluting ammonia levels more effectively through removal of the predominant liquid phase ammonium. This flushing-out of any toxic component by these reactor designs is one of their major benefits, and they can allow relatively high ammonia loads to be tolerated.

3.4. Conclusion

While there is a large number of studies evaluating the potential of microalgae feedstock for anaerobic digestion the effort has been somewhat disjointed, filling in gaps left by previous researchers while undertaking different methodologies to evaluate these gaps. This has resulted in a large quantity of conflicting results with no clear indicator on the exact optimum process parameters for anaerobic digestion of microalgae. The relationship between microalgae composition,

species and methane yield is needed to better identify which microalgae species is optimal, while the impact of long term operation of anaerobic digesters fed on microalgae increasing loading rates and reducing retention times is needed to fully optimise algal AD and determine the anaerobic communities response to this unique substrate.

Chapter 4 Materials and Methods

4.1. Algae Cultivation and harvesting

4.1.1. Photo-bioreactor

A mixed freshwater culture of predominantly *Chlorella and Scenedesmus* species isolated from a freshwater pond (.9840° N, 1.6150° W, Newcastle upon Tyne, UK) *w*as grown in 12 - 14 * 22L bioreactors under constant aeration (4 L_{air}/min) and light/dark cycles of 16/8 hours using a mixture of 6ft cool white tubes and warm white tubes (Royal Phillips Electronics, The Netherlands). The lights were chosen to provide a mixture of light wavelengths suitable microalgae and were placed vertically to maximise coverage of the reactors. Incoming light energy was measured at 70.9 – 110.8 µmol/m^2s using a LI-250 Light meter connected to LI-192 Quantum sensor (LI-COR, USA). The frame holding the reactors was covered in a reflective material for higher light intensities.

Each individual photo-bioreactor was constructed using polyethylene lay-flat tubing sealed at one end and hung at the other end from the top of the frame (Figure 4-1B). Fresh sterile media was pumped into the top of the photo-bioreactors through polyvinyl chloride tubing (3mm I.D, VWR, UK) at 20 ml/min using a Watson Marlow 520s peristaltic pump equipped with Marprene tubing (4mm ID, Watson Marlow, UK).

Aeration was provided through an air stone located at the bottom of the reactor supplied with a constant supply of air at a controlled flow rate 4 L_{air}/min using a variable flow rotometer (RS components, UK).

The culture was harvested manually or through pumps via a small opening at the bottom of the reactor system, and drained into sterile 20 L Nalgene containers (Thermofisher Scientific, USA) and stored at 4 ± 1.6°C in the dark prior to use.

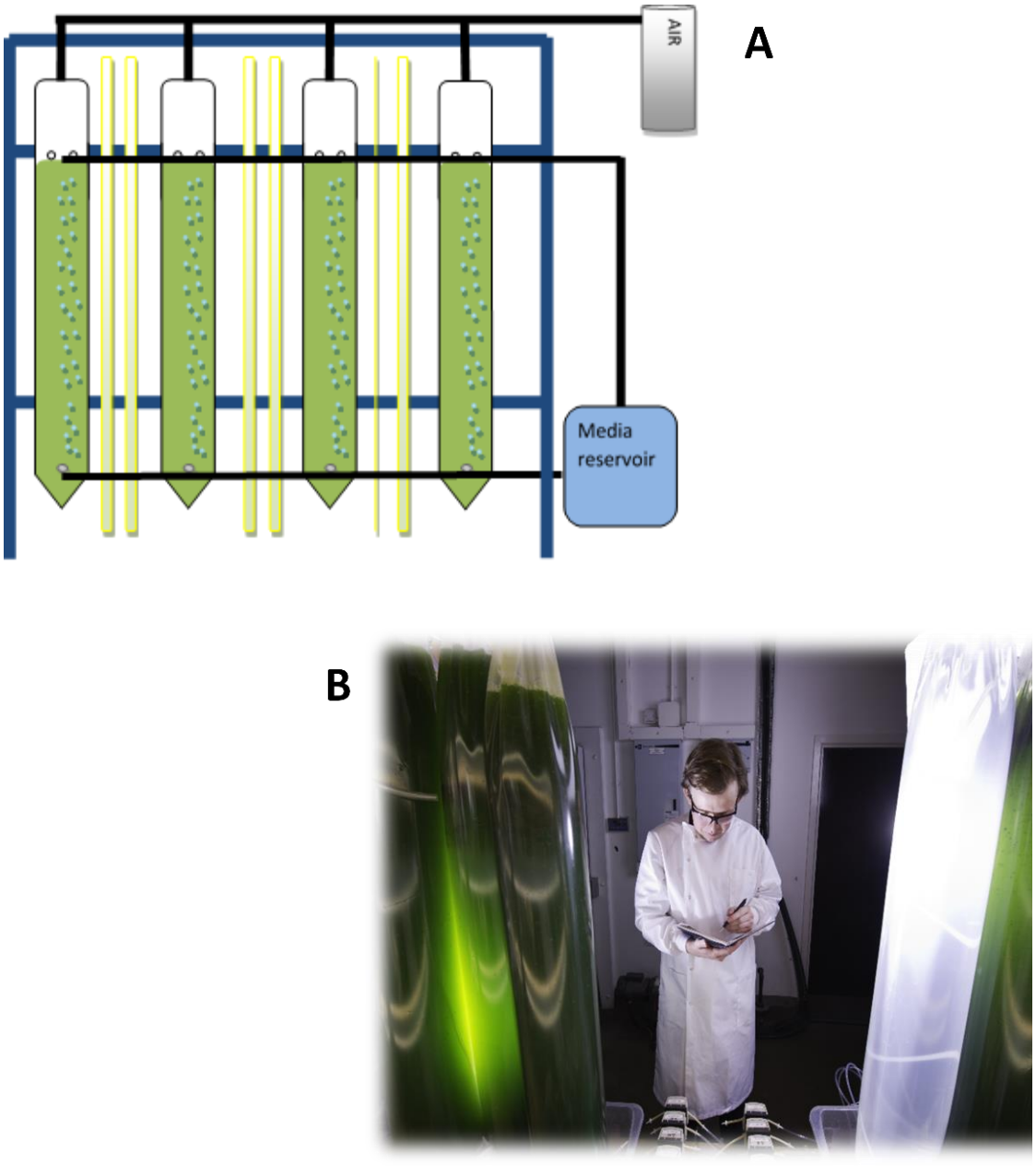

Figure 4-1 Microalgal laboratory scale photo-bioreactors: A, illustration of 22L photo-bioreactor for continuous microalgae cultivation under artificial light and aeration; B, Photograph of photo-bioreactor in operation with S Edwards

Table 4-1. Bolds basal media (BBM) chemical concentrations

Constituent	Final concentration (µMol)
$NaNO_3$	2941.3
$MgSO_4$	304.4
NaCl	427.8
K_2HPO_4	328.6
KH_2PO_4	1286.0
$CaCl_2$	170.1
$ZnSO_4$	30.7
$MnCl_2$	3.6
MoO_3	10.9
$CuSO_4$	2.0
$Co(NO_3)_2$	1.7
H_3BO_3	184.4
$EDTANa_2$	134.3
KOH	552.5
$FeSO_4$	17.9

* Bischoff and Bold (1963)

Table 4-2. Vitamins Concentration

Vitamin	Final concentration (µMol)
Vitamin B1 (Thiamine Hydrochloride)	$29.7 * 10^{-3}$
Vitamin H (Biotin)	$1.02 * 10^{-3}$
Vitamin B12 (Cyanocobalamin)	$1.11 * 10^{-3}$

Table 4-3. F/2 microalgae media chemical concentrations

Constituent	Concentration (mg/L)
Na_2 EDTA	4.16
$FeCl_3.6H_2O$	3.15
$CuSO_4.5H_2O$	0.01
$ZnSO_4.7H_2O$	0.022
$CoCl_2.6H_2O$	0.01
$MnCl_2.4H_2O$	0.18
$Na_2MoO_4.2H_2O$	0.006
Cyanocobalamin (Vitamin B12)	0.0005
Thiamine HCl (Vitamin B1)	0.1
Biotin	0.0005
$NaNO_3$	75
$NaH_2PO_4.2H_2O$	5.65

* Adapted from (Guilard and Ryther, 1962)

4.1.2. Algae harvesting

Different harvesting methods were employed for different experiments. For the majority of experiments through this thesis centrifugation was used for speed and yield. Microalgae was centrifuged at 4,400 RPM for 30 minutes in a 1L Nalgene® (ThermoFisher, USA) sterile centrifuge tubes, using a Heraeus cyrofuge 5500i (ThermoFisher, USA). The supernatant was discarded and the pellet was washed with distilled water then re-centrifuged for a further 30 minutes to remove any traces

of media on the algal culture. The supernatant was discarded and the pellet recovered for use. This was stored at 4 ±1.6°C prior to use for up to 2 weeks to prevent prior degradation before entering the reactors. No significant changes in soluble carbon occurred during the harvesting process.

4.1.3. Cultivation assessment

Understanding photo-bioreactor performance and the optimal time to harvest the microalgae when in batch production mode was vital to efficient cultivation and production. The point at which algae was harvested in the growth cycle played an important role in the algae's composition, and ability to settle effectively and ultimately the digestibility of the algae (this variance is addressed in a results Chapter 7).

Different methods were employed to monitor microalgae productivity. These were total suspended solids (TSS) analysis, chlorophyll-a and b, absorbance and total chemical oxygen demand (TCOD). The methods of analysis are described below in analytical methods. These methods were employed daily at a set time every day. Absorbance at 685 nm was employed as the main method for culture monitoring on a routine basis. The peak absorbance was determined using a spectral scan between wavelengths 400nm and 1000nm on a UV-1700 UV-VIS spectrophotometer (Shimadzu, Japan). A calibration curve was made between absorbance at 685nm and TSS, COD, cell counts and chlorophyll-a –allowing absorbance as a rapid tool to determine biomass quantity, and health of culture. These are included in the appendices.

Algae is expected to follow under batch cultivation conditions phases typical of a unicellular organism where substrate/nutrients or light are limiting at one point.

According to Becker (1994) these phases are adaption, accelerating growth phase, exponential growth phase, decreasing log growth, stationary phase, accelerated death and logarithmic death phase. At exponential growth phase the maximum or specific growth rate (μ_{max}) can be calculated based on cell concentration/biomass changes over a set period of time using the equation based on monod kinetics (Eq. 4-1)

$$\mu = \frac{\ln 2}{g} \quad \textbf{(4-1)}$$

Where μ is specific growth rate, and g is generation/double time.

The generation or doubling time (g) can be calculated according to the equation below (Equation 4-2)

$$\boldsymbol{M_t = M_i 2^{\frac{t}{g}}} \quad \textbf{(4-2)}$$

Where M_t is concentration at time *t*, M_i is initial/start concentration and *g* is generation time.

As the culture started to reactor stationary phase, the culture was harvested as previously described. Leaving for extended periods of time in stationary phase was believed to affect viability of both the culture and the chemical composition of the algae cells could vary.

4.2. Analytical procedures

4.2.1. pH

pH was measured according to APHA standard method 4500-H^+B (APHA 2005) using a Jenway 3010 pH-meter (Jenway, UK) equipped with double junction electrode (VWR, UK), calibrated prior to use with commercial certified standards, pH 4 and pH 7 (VWR, UK).

4.2.2. Total and Soluble chemical oxygen demand

Total chemical oxygen demand (tCOD) was undertaken according to APHA closed reflux titrimetric method 5220C (APHA 2005) in triplicate, using a range of dilutions. Dilutions are prepared using volumetric flasks and distilled water.

Soluble chemical oxygen demand (sCOD) was undertaken by first obtaining the soluble component. This was obtained by centrifuging at 13,000 RPM for 10 minutes in a microfuge (Sanyo, Japan) then filtering the supernatant through a 0.22 µm polyethersulfone membrane syringe filter (VWR, UK). The filtrate was then analysed for COD using the same method as tCOD.

All samples and blanks were undertaken in triplicate, periodically a prepared standard as described by APHA (APHA 2005) which was used to confirm all reagents were precise.

4.2.3. Total Kiejdahl nitrogen and Total Ammoniacal Nitrogen.

Total Ammonia Nitrogen (TAN) NH_3-N was determined using a Vadopest 30S steam distillation unit (Gerhardt, United Kingdom) according to APHA standard method 4500-NH_3 B and 4500-NH_3C (APHA 2005).

The accuracy of the distillation/titration method was tested using an ammonia calibration standard prepared according to 4500-NH_3C Sample 1 test (APHA, 2005).

Total Kiejadahl Nitrogen (TKN) was undertaken by acid digestion followed by steam distillation. Acid digestion was undertaken using a Turbotherm digestion unit (Gerhardt, UK) with reagent blanks and a commercial standard (Sigma Aldrich, USA). All samples and blanks were undertaken in triplicate with averages and standard error presented.

4.2.4. Gas Concentration analysis

For the reactor systems CH_4 and CO_2 was sampled regularly using a 100 µl gas tight syringed (SGE, Australia). The sample was injected directly into a SRI 8610C gas chromatograph equipped with a 6' x 1/8" silica gel packed column (SRI, USA) connected to a flame ionisation detector (FID, held at 306°C). The carrier gas was Hydrogen at 20 PSI with a flow rate of 15 ml/min with an oven temperature held isothermally at 80°C.

Calibration was undertaken using two calibration standards (Scientific Technical Gases, UK) injecting different volume of calibration gas to represent different concentrations of CH_4 and CO_2. All analysis was undertaken in triplicate with a minimum calibration coefficient of determination (R^2) required of 0.99 before analysis was undertaken. Periodic standards were injected to check the stability of the run.

For bio-methane potential tests only CH_4 was analysed. 100 µl of sample was extracted directly from the headspace of the BMP bottle using a pressure lock gas tight syringe (SGE, Australia) or from a larger syringe used to equalise/determine

pressure. This sample was then injected directly into a Carlo Erba HRGC S160 GC, equipped with an Agilent HP-PLOTQ column (0.32 mm diameter, 30m length and 20µm film, Agilent, UK) connected to a Flame ionisation detector (FID). The carrier gas was hydrogen (250 ml/min) with an oven temperature held isothermally at 35°C.

4.2.5. Volatile Fatty acid analysis

Volatile fatty acids (VFAs) were prepared first by filtering aqueous samples using 0.22 µm poylethylene sulphone syringe filter (VWR international, UK). This was then diluted 1:1 V:V with 0.1N Octane sulfonic acid (Thermoscientific, UK) prior to sonication for 40 minutes to drive of carbonate (50/60 Hz, Decon Ultrasonics Ltd, UK). The VFAs were then measured in duplicate using liquid Ion Chromatography (Dionex ICS-1000, equipped with an Ionpack ICE ASI column, with heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant). Calibration was undertaken using a range of VFAs prepared to a range of concentrations. Detectable range was >2ppm and to a maximum of 500 ppm without dilution. Concentrations above this were diluted with deinoised water.

4.2.6. Anion analysis

Samples for anion analysis were prepared using filtration described above. The anion content of anaerobic sludge and microalgae substrate was measured in duplicate using liquid Ion Chromatography on a Dionex ICS-1000 fitted with an AS40 auto sampler (Thermo scientific, UK). The column is an Ionpac AS14A, 4x250 mm analytical column with a flow rate is 1 ml/min. The eluent is 8.0 mM

Na_2CO_3/1.0 mM $NaHCO_3$ solution. Samples were prepared in duplicate alongside a number of pre-prepared standards. Detectability was > 5ppm.

4.2.7. Dissolved organic carbon analysis

Dissolved organic carbon was run on a Shimadzu 5050A (Shimadzu, Japan) Total organic carbon analyser, equipped with an ASI-5000A auto sampler. The carrier gas is zero grade air, and the inorganic catalyst solution is 25% phosphoric acid. This machine measured both organic and inorganic carbon via combustion and measuring the CO_2 given off. Samples were prepared first by obtaining the soluble component as described above, then 7 ml of sample was placed into borosilicate glass vials pre washed with 5% (V:V) HCL to remove any inorganic material. Samples were analysed in duplicate, and run alongside external calibration standards and a series of blanks. Detectability was > 1ppm.

4.2.8. Total organic carbon

Total organic carbon (TOCD) was analysed using a LECO CS244 carbon analyser (LECO Ltd, UK). All samples were undertaken in triplicate and calibrated against a known commercial standard.

4.2.9. Total solids and volatile solids

Total (TS) and volatile solids (VS) were undertaken in triplicate according to the APHA standard method (Eaton 2005).

Total suspended solids and volatile suspended solids (TSS and VSS) were again undertaken in triplicate using glass microfiber papers (GFA, Sartorius, UK) according to APHA standard methods (Eaton 2005).

4.2.10. Alkalinity

Alkalinity on anaerobic sludge was measured according to standard methods (Eaton 2005). The sample was first filtered through a glass microfiber paper to remove solids, then 50 ml or an aliquot diluted to 50 ml was used for analysis. If the pH was below 8.3 only a one stage titration was required using methyl orange titrating from high to low to the endpoint required. Analysis was undertaken in minimum duplicate.

Total and partial alkalinity were analysed to give an indication of reactor stability using a two stage titration previously described (Ripley et al. 1986).

The difference between total and partial alkalinity is known as intermediate alkalinity, and is related to volatile fatty acid presence. Partial alkalinity, is primarily bicarbonate alkalinity, but includes alkalinity provided by OH^-, NH_3, HCO_3^- and CO_3. The ratio has been described as a method to determine process stability in anaerobic digesters using the formula below (Eq. 4-3), with values < 0.3 indicating a stable process.

$$\propto (\boldsymbol{Alkalinity\ ratio}) = \frac{\boldsymbol{IA}}{\boldsymbol{PA}} \qquad \textbf{(4-3)}$$

4.2.11. Calorific value

Gross calorific value (GCV) was calculated using an adiabatic bomb calorimeter (Parr, 6100, Parr, USA). 1g of air dried sample was placed in a crucible and ignited under 100% oxygen conditions with the aid of benzoic acid sample to boost calorific values above detection. All samples were undertaken in triplicate, with benzoic acid

used as the calibration standard and where energy content of the microalgae was not sufficient to meet internal quality checks of the calorimeter, mixed with the microalgae in a known proportion.

4.2.12. Chlorophyll analysis

Chlorophyll-a and b were determined according to Becker (1994) modified with an additional extraction step to improve chlorophyll content. 40 ml of microalgae was first mixed 4:1 v/v with 100% methanol and incubated at 50°C for 2 hours (Stuart Scientific Ltd, UK) followed by sonication at 50/60 hz (Decon Ultrasonics ltd, UK) for 30minutes. The samples were then centrifuged at 4400 RPM for 10 minutes (Sigma Aldrich, United Kingdom). 100µl of the supernatant was mixed with 900 µl of methanol pre filtered using a 0.2 µm PTFE syringe filter (VWR, UK). The absorbance was then determined in a quartz glass cuvette using an ATI Unicam UV-VIS spectrophotometer at 665 and 643nm for chlorophyll-a and chlorophyll b. Chlorophyll concentrations were calculated as Equation 4-4, 4-5 and 4-6 below.

$$\text{Chlorophyll a} = (12.7 \times A_{663}) - (2.69 \times A_{645}) \quad (4\text{-}4)$$

$$\text{Chlorophyll b} = (22.9 \times A_{645}) - (4.64 \times A_{663}) \quad (4\text{-}5)$$

$$\text{Chlorophyll a + b} = (8.02 \times A_{663}) + (20.2 \times A_{645}) \quad (4\text{-}6)$$

4.2.13. Protein, Carbohydrate and Lipid extraction

Lipids, proteins and carbohydrate were first extracted using an optimised method of the sonication, lysis buffer and heating previously reported (Lee et al. 2010) modified with process development included in Appendices. 1L of sample was first concentrated by centrifugation at 4400 RPM for 30 minutes at 15°C in a Heraeus cyrofuge 5500i (ThermoFisher Scientific, USA). The supernatant was disposed of and the algal pellet was re-suspended in 100 ml of distilled water. The sample was then frozen at -80°C until use. The frozen sample was then transferred to a Modulyod vacuum freeze drier (ThermoScientific, USA) held at -53°C with 849 mbar of vacuum to remove all the remaining water from the samples. The freeze dried sample was transferred to glass vial with stopper with 100 ml of lysis buffer water, then incubated at 50°C for 1 hr. Post incubation, an aliquot of the sample was sonicated using a 500w ultrasonic processor equipped with ¼inch probe at 20kHz (Colepalmer, USA). Any evaporation was corrected using DI water up to original volume. Chlorophyll should now be removed wit protein and carbohydrate ready to be quantified. Further extraction is required for lipids as below.

Lipids were then extracted from the sample by adding a methanol: chloroform mixture (1:1 v/v) to the sample at a ratio of 1:1 v/v. The mixture was then shaken vigorously for 30 minutes at 150 – 200 RPM (Stuart scientific, UK). This was then filtered through a glass microfiber filter (GFA, Sartorius, UK) to remove particulates. The sample was transferred to a 200 ml glass separation funnel fitted with a glass stopper. The sample was then mixed vigorously for 5 minutes, then with the stopper removed and allowed to separate until clear separation is evident of the different fractions. The lipid fraction was drawn off into a pre- weighed clean 250 ml round

bottomed flask and allowed to evaporate under a fume cupboard. The sample was further dried at 40°C then the flask plus dried contents were weighed to four figures. Extractions were undertaken in duplicate or triplicate. All glassware was thoroughly cleaned by subsequent cleaning steps; tap water, de-ionised water, furnace at 400°C for 2 - 4 hours and finally with methanol and allowed to air dry.

4.2.14. Protein quantification

Protein quantification was taken using the previously described protocol (Bradford 1976) using Bovine Serum Albumin (BSA) as the standard. BSA has been shown to be suitable standard for use when quantifying protein in microalgae (Barbarino and Lourenco 2005). The reaction is based on the interaction between proteins and Coomassie Brilliant Blue G-250, which provides a brown-blue solution that can be determined spectro-photometrically. Samples were reacted for 50 seconds and were then stable for 30 minutes. Analysis was undertaken using a spectrophotometer at 595 nm (ATI Unicam Spectrophotometer, ATI, UK).

4.2.15. Carbohydrate quantification

Total carbohydrate quantification was undertaken using a modified Phenol-Sulphuric acid method previously described by Dubois et al (1951). 10 mg of the extracted biomass suspended in 10 ml of de-ionised water. 1ml of this was reacted with 1 ml of 5 % Phenol (w/v) with 3 ml of Sulphuric acid (72% conc.) for 10 minutes in an oven set to 90°C. Samples were allowed to cool in darkness to room temperature (22 ± 5°C) then the absorbance was measured at 490nm.

To determine the concentration, a standard curve (Appendices) was prepared in the same way as the sample with known concentrations of a defined carbohydrate,

primarily glucose (Dubois et al. 1951). The values were then converted to COD equivalent using glucose, $C_6H_{12}O_6$ as a reference material.

4.2.16. Microbial cell counts

Total microbial cell counts were undertaken periodically during the different phases of operation. 1 ml of mixed sample was stored with 1 ml of 0.22µm filter sterilised ethanol (VWR, UK) then frozen at -20 °C to preserve morphological shape of cells. 10 µL of preserved sample was added to 990 µL of filter sterilised phosphate buffer saline (Oxoid Media, UK). To provide cell concentrations between 30 – 300 cells a series of dilutions was undertaken using phosphate buffer. Cells were stained by adding 50 µL of SYBR Gold nucleic acid stain (Invitrogen Ltd, UK) then wrapped in aluminium foil and incubated at 25°C for 30 minutes. These were then filtered onto a sterile 0.22 µm pore size black polycarbonate filter (EMD Millipore, USA). The filters were then placed on microscope slides containing 0.1 ml of Citifluor antifadent (Citifluor Ltd, UK) with a further 0.1 ml of Citifluor placed between the top of the filter and cover slip. Total cell counts were undertaken using an Olympus BX40 Epifluorescence microscope (Olympus, Japan). 20 - 30 random separate fields of view were taken for statically valid counts.

4.3. Performance calculations

4.3.1. General biogas calculations

Conversion of gas volume to standard temperature and pressure (STP)

All biogas should be reported under the same conditions, typically Standard temperature and pressure. This can be done using Eq.4 – 7.

$$V_{STP} = \frac{P_{gas}}{P_{STP}} \times \frac{T_{STP}}{T_{gas}} V_{gas} \quad (4\text{-}7)$$

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

4.3.2. Bio-methane potential test calculations

A number of specific calculations are required for bio-methane potential tests.

Correction for sludge control methane production

A number of different sludge controls are used. The main sludge control is the use of the anaerobic inoculum without any substrate addition. This is used to demonstrate the residual methane potential produced by the anaerobic sludge without substrate.

$$BMP = \frac{V_{substrate} - V_{blank}\frac{mts}{mtb}}{mss} \quad (4\text{-}8)$$

$V_{substrate}$ is the accumulated methane volume from substrate, v_{blank} is the accumulated volume from the inoculum, mts is the organic material of inoculum in substrate bottle, mtb is the organic material of inoculum in blank bottle, mss is the organic material of substrate in substrate bottle.

First order kinetic model (Hydrolysis rate) (Angelidaki et al., 2009)

Assuming hydrolysis is the limited step in the conversion of a particulate substance to methane then BMP data can be used to obtain the rate using first order principles using Eq. 4-9

$$-K_h S = \frac{dS}{dt} \quad (4\text{-}9)$$

Where *t* is time, K_h is first order hydrolysis constant, *S* is biodegradable substrate.

Taking into account the relationship between biodegradable substrate and the methane generated the first order hydrolysis rate can be calculated according to Eq. 4-10.

$$-k_h t = ln \frac{B_\infty - B}{B_\infty} \quad (4\text{-}10)$$

Where t is time, B_∞ is the value of the ultimate methane potential, B is the methane produced at time t and k_h is the hydrolysis constant. This is calculated from the slope of the curve.

4.3.3. Reactor operation and performance calculations

For Chapters 5 and 6 different anaerobic reactors were used. There are a number of important calculations for the design, operation and monitoring of the reactor.

Organic loading rate, B_v

The organic loading rate is the quantity of substrate added per unit of reactor per day. This can be calculated using Eq. 4 – 11.

$$B_v \left(\frac{\text{gCOD}}{L_{reactor}\text{d}}\right) = \frac{\text{gCODadded}}{\text{vol}_{reactor}\cdot\text{d}} \quad (4\text{-}11)$$

$gCOD_{added}$ is the quantity of COD added. $Vol_{reactor}$ is the volume of reactor, and d is day

Solid and hydraulic retention time

The solid retention time determines the duration of the solids/biomass retention in the system. This can be calculated using Eq. 4 - 12.

$$SRT = \frac{V}{Q} \quad (4\text{-}12)$$

Where V = volume of reactor (L or m^3), Q = influent flow rate (L/d, or m^3/d), SRT = solid retention time in days (d).

COD and VS Destruction

COD and VS destruction are two important parameters for measuring the performance of the reactor, and will allow a mass balance of influent and effluent COD/methane to be calculated. COD destruction can be calculated a number of different ways demonstrated in Eq. 4 – 13.

$$\text{COD destruction } \% = \frac{\text{CODin (day x to day y)} - COD eff\ (day\ y - day\ x)}{CODin\ (Day\ x\ to\ Day\ y)} \times 100 \quad (4\text{-}13)$$

COD_{in} is influent (mg COD/L), COD_{eff} is effluent COD (mg COD/L). The same can be can be applied for VS destruction.

Volumetric methane production

Daily volumetric methane production can be calculated using Eq. 4 – 14.

$$\text{Volumetric. } CH_4 \text{ production } (\text{L } CH_4/\text{L}_{\text{reactor}} \cdot \text{d}) = \frac{V_{Biogas} \times CH_4\ conc.}{V_{Reactor}} \quad (4\text{-}14)$$

Where V_{Biogas} is the volume of biogas produced per day (L) normalised to STP and vapour, CH_4 is the concentration of methane in biogas, and $V_{Reactor}$ is the volume of reactor (L).

Theoretical Ammonia yield

Theoretical ammonium yield based on the breakdown of organic matter can be calculated according using elemental analysis based on Sialve *et al.,* (2009), derived from (Buswell and Neave, 1930).

$$\text{Theoretical ammonia Yield } (\text{mg N} - NH_3/\text{gVS}) = \frac{d \times 17 \times 1000}{12a+b+16c+14d} \quad (4\text{-}15)$$

Nitrogen mineralisation

Nitrogen mineralisation is the conversion of influent organic nitrogen to ammonia nitrogen, and can be calculated based on the theoretical ammonia yield calculated using Eq. 4 – 16.

$$\text{Nitrogen mineralisation } \% = \frac{Ammonia\ release\ (\frac{mg\ N-NH_3}{gVS})}{Theoretical\ Ammonia\ release\ (\frac{mg\ N-NH_3}{gVS})} \times 100 \quad (4\text{-}16)$$

Dissociation constant for ammonium ion

The dissociation constant for ammonium ion can be calculated according to Calli, Mertoglu *et al*., (2005) using Eq. 4 – 17.

$$\mathrm{pk_a} = 0.09018\frac{2729.2}{\mathrm{T}+273.15} \quad (4\text{-}17)$$

Where T = Temperature (C), and pK_a is the dissociation constant.

Free Ammoniacal nitrogen (FAN)

The free ammoniacal nitrogen levels can be calculated based on the dissociation constant for the ammonium ion (Eq. 4 – 18). This can be calculated using Eq. 4 – 18 (Hansen *et al*., 1998; Calli *et al*., 2005).

$$\mathrm{FAN\ (mg\ FAN} - \frac{\mathrm{N}}{\mathrm{L}}) = \frac{[\mathrm{TAN}]}{1+10^{(\mathrm{pK_a}-\mathrm{pH})}} \quad (4\text{-}18)$$

Where TAN is total ammonia nitrogen (mg TAN- N/L) and pk_a is dissociation constant for ammonium ion.

Chapter 5 Anaerobic digestion of mixed culture microalgae in manually stirred anaerobic reactors: effect of reactor temperature, organic loading rate and solid retention time.

5.1. Introduction

Previous reactor studies have evaluated different process parameters such as reactor temperature (Golueke *et al.,* 1957; Zamalloa *et al.,* 2012a), organic loading rate (Ras *et al.*, 2011) and solids retention time (Ras *et al*., 2011), investigating their effect on methane yield, solids destruction and short term reactor stability. All these studies have been undertaken with different laboratory-grown or environment-grown microalgae, at conservative organic loading rates (OLR), and without a full comparison of different operational conditions such as temperature, solids retention time and microalgae species together in one experiment/system. Neither have they varied operational parameters over the duration of a long experiment that achieve near steady state operating conditions. This study investigates the combined effects of different retention times, temperatures and organic loadings using classical CSTR systems to determine the baseline theoretical potential of microalgae as a feedstock for anaerobic digestion, and determine which parameters (SRT, temperature, OLR) are the most important to further optimise anaerobic digestion and maximise yield.

5.2. Aim and objectives

Evaluate the operational performance of anaerobic digestion of microalgae in simple anaerobic reactors systems under different operating conditions.

- Evaluate the effects of reactor operating temperatures on methane yield from microalgae.
- Evaluate the effects of different organic loading rates on methane yield from microalgae.
- Evaluate the effects of different solid retention times on methane yield from microalgae.

5.3. Methods

5.3.1. Analytical procedures

General analytical procedures are as described in Analytical procedures

5.3.2. Manually stirred anaerobic reactor configuration

Anaerobic 1L reactors were operated at two temperatures (35°C and 50°C) and were configured according to (Figure 5-1). The reactors were 1L Duran bottles (Duran®, Germany), adapted with a gas outlet and inlet feed pipe inserted into the GL45 cap using Silicone tubing (60 mm length, 2 mm wall thickness, VWR International, UK). The silicone tubing had a 40 mm long piece of stainless steel tube (304 grade, ID: 8mm) inserted inside that ensured a seal was made against the plastic cap. To prevent any leakage, silicone sealant (RS components, UK), was used externally over the joint. Leak testing of the reactors was carried out by filling with 100 % N_2 (BOC gases, UK) with a slight positive overpressure, and submerged under water to check for bubble formation.

Attached to the silicone tubing was a section polyvinyl chloride tubing (ID: 6mm, PVC, VWR international, UK). This was attached in line to an optical bubble counter (made in house, Newcastle University) followed by a 1L Tedlar® gas bags (Sigma

Aldrich, UK). The bubble counter uses an infra-red counter to count each gas bubble rising in an oil-filled tube and breaking the IR light beam. This was calibrated by injecting a known volume of gas and periodically compared to the volume of gas collected in the gas bag. Optical counters were calibrated regularly to ensure accurate gas measurements. Gas was correct for STP (Eq. 4 – 7).

Reactors were wrapped in aluminium foil to prevent photosynthesis, and held at two temperatures (35 ±1°C and 50 ±2°C) using two Grant water baths (Grant Instruments, UK). The temperature inside the reactors was periodically checked using a manual hand held thermometer. Reactors were manually stirred four times a day including pre- and post-feeding.

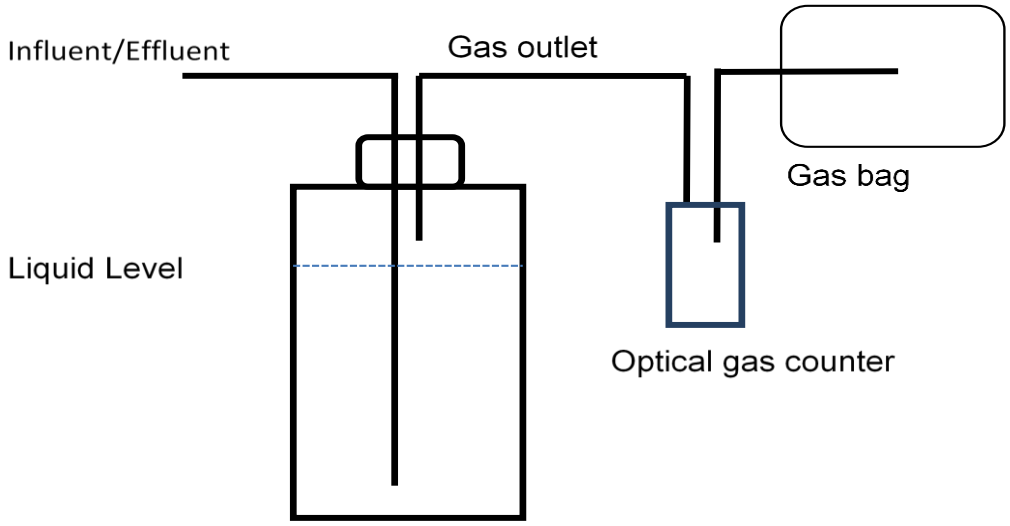

Figure 5-1 Manually stirred anaerobic reactor configuration.

5.3.3. MSAR operation

Reactors were operated with the solids retention time (SRT) and organic loading rate (OLR) as shown in (Table 5-1) for a total period of 400 days. During the course of the experiment, SRT and OLR were varied to gain an insight into the effect on reactor performance at two temperatures.

Table 5-1. Different operation conditions (OLR, HRT/SRT) for mesophilic and thermophilic reactors.

Operation condition	Phase 1	Phase 2	Phase 3	Phase 4
OLR (gCOD/$L_{reactor}$.d	1	2	4	4
HRT/SRT (d)	25	25	25	15
Duration (d)	75	71	54	100

Reactors were operated using daily batch feed procedure, whereby a volume of sludge was extracted from the reactor each day, the volume being determined by the SRT. This sludge was then used for analysis according to analytical procedures detailed previously (4.2). Following sludge removal, the same volume of pre-concentred microalgae substrate (predominantly *Scenedesmus Quadricauda* and *Chlorella Vulgaris* species as identified by light microscopy, grown according to Chapter 4) as well as tap water, was fed daily into each reactor using a second plastic syringe, equivalent to the volume of reactor sludge removed. The reactor feed tube was then sealed, and the contents thoroughly mixed by shaking inversion before having the headspace sparged with 100 %N_2 for 5 minutes (BOC Gases, UK). The same procedure was undertaken for all reactors. Adjustments to reactor

pH were made using 1M hydrochloric acid or 1M sodium hydroxide, or to provide greater alkalinity, sodium bicarbonate was added to raise pH and provide improved pH stability (VWR International, UK).

5.3.4. Anaerobic sludge and sludge conditioning

Anaerobic sludge comprised a 50:50 (v:v) mix of granular anaerobic sludge taken from a citric acid digesting sludge and anaerobic sewage sludge, both previously operated at 35°C. Prior to the experiments the sludge was crushed and sieved and decanted into two 2L bottles and mixed with a small quantity of microalgae. One bottle held at 35°C and the other at 50°C until both showed methane production and low VFA/stable pH indicating both reactors were working at their designated temperatures.

5.4. Results and Discussion

5.4.1. Microalgae composition

Typical microalgae composition is shown in Table 5-2. Elevated protein content is reflected by high TKN, and the low C:N ratio observed. The VS content of the microalgae was high, while it was observed that the biochemical composition in terms of protein, carbohydrate and lipid varied significantly across the duration of the research. This is addressed further in Chapter 7.

Table 5-2. Typical mixed culture microalgae composition feed (standard deviation in parenthesis)

Parameter	Value	Parameter	Value
TS:VS Ratio	1.2 (0.2)	pH	7.4 (0.9)
COD:VS Ratio	1.5 (0.2)	Protein %VS	64.4 (15.5)
COD:TKN Ratio	11.3 (0.1)	Carbohydrate %VS	16.3 (10.2)
TKN %	9.9 (0.3)	Lipid %VS	19.3 (1.2)
TP %	6.5 (0.4)	CV (MJ/kg)	20.1 (0.8)
NH_4^+ mg/L	0.0		

The C:N ratio is typical of microalgae grown in nutrient replete conditions (Ras *et al.,* 2011; Zamalloa *et al.*, 2012a), significantly below that of terrestrial based crops and is outside of the recommended C:N ratio of 25 to 30:1 that has been shown to be optimum for anaerobic digestion (Kayhanian and Tchobanoglous, 1992). The low C:N ratio has the potential at high loading rates to result in elevated ammonia levels, potentially resulting in inhibition of the methanogens in the system (Angelidaki *et al.,* 1993; Chen *et al.,* 2008). The lipid content is typical of *Chlorella*

Vulgaris, which dominates this mixed culture, and has the potential to result in higher methane production due to higher theoretical methane yields from lipids, over carbohydrates and proteins.

Gross calorific values are typical of freshwater microalgae (Scragg *et al*., 2002) and were shown to vary dependent on growth conditions and point in harvest. This variation is addressed in Chapter 7.

5.4.2. Mesophilic and thermophilic anaerobic digester performance between Phase 1 and 4.

The average methane yield for Phase 1 was 0.147 $LCH_4/gCOD_{in}$ (0.221 LCH_4/gVS_{in}) at thermophilic and 0.109 $LCH_4/gCOD_{in}$ (0.164 LCH_4/gVS_{in}) at mesophilic temperatures (Figure 5-2), with similar variability observed at both temperatures. These yields are slightly lower than previously reported for microalgae digestion at different temperatures (Golueke *et al.*, 1957). Methane yield at thermophilic temperatures varied towards the end of the Phase 1, with a noticeable increase in yield at thermophilic temperatures on days 46, and 61 – 72. At mesophilic temperatures the yield dropped on day 16 until day 31. Methane concentration remained relatively constant across Phase 1 at both temperatures, with the thermophilic reactors averaging slightly higher concentration (71% vs. 74%). The high methane concentration is typical of microalgae fed digesters (Zamalloa *et al*., 2012a), partly due to the biochemical composition of microalgae rich in lipids and partly due to the elevated nitrogen content from protein degradation which acts as a weak base resulting in higher pH . The high pH results in increase CO_2 in liquid phase producing a biogas rich in CH_4.

Table 5-3 Summary performance data for mesophilic and thermophilic reactors across different operational phases

REACTOR	MESOPHILIC (35°C)				THERMOPHILIC (50°C)			
PHASE	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Duration (d)	75	71	54	100	75	71	54	100
OLR ($gCOD/L_{reactor}$ d)	1.1±0.1	2.1±0.2	3.9±0.3	4.1 ±0.4	1.1±0.1	2.1±0.2	3.9±0.3	4.1±0.4
SRT (d)	25	25	25	15	25	25	25	15
$LCH_4/gCOD_{in}$	0.109	0.134	0.119	0.103	0.147	0.140	0.136	0.116
LCH_4/gVS_{in}	0.164	0.208	0.179	0.154	0.221	0.210	0.204	0.174
pH	7.4	7.3	7.4	7.5	7.5	7.3	7.6	7.6
Methane (%)	71 ± 3	70 ± 2	70 ±3	70 ± 5	74 ± 4	73 ± 4	71 ± 3	70 ± 3

Increasing OLR in Phase 2 initially resulted in no noticeable change in methane production at either temperatures, but after day 81 improvements in methane production was observed in thermophilic reactors, and in mesophilic reactors after day 92. Fluctuating levels of biogas production were observed at both temperatures, but the general trend was an increase in biogas production from day 81 and day 92 at thermophilic and mesophilic conditions respectively. The thermophilic reactors reached steady state quicker than the mesophilic reactors, with thermophilic digestion producing an average of 0.311 $LCH_4/L_{reactor}$.d at steady state conditions (day 92), while mesophilic took longer to reach steady state (day 114), and produced less biogas, averaging of 0.281 LCH_4/day. Both reactor

temperatures showed consistent performance at Phase 2 with gas production being statistically different at the two temperatures, and between Phase 1 and 2. Methane yield at thermophilic initially dropped after the OLR was increased, but recovered to similar levels (0.140 $LCH_4/gCOD_{in}$) to that of the previous phase (0.147 $LCH_4/gCOD_{in}$). At mesophilic temperatures there was a noticeably higher average methane yield in Phase 2, with 0.134 $LCH_4/gCOD_{in}$ observed at steady state (day 102 – 144) compared to 0.109 $LCH_4/gCOD_{in}$ during Phase 1. This difference between Phase 1 and 2 was statistically significant (Paired sample t test, $p<0.05$).

The increase of OLR at Phase 3 resulted in increased volumetric methane production with thermophilic and mesophilic reactors averaging 0.550 LCH_4/day and 0.474 LCH_4/d respectively. This was statistically significant (Paired sample T test, $p<0.05$). The mesophilic reactors took longer to reach steady state at the higher OLR, showing stability after day 178, compared to day 166 for the thermophilic reactors. A similar period was required to establish steady state at Phase 2, when OLR was doubled. Methane production peaked on day 168 in thermophilic reactors at 0.590 LCH_4/day, but dropped to 0.530 LCH_4/day after day 168. It is evident that methane yield at both mesophilic and thermophilic temperatures decreased from Phase 2 to Phase 3, with steady state yields being 0.119 $LCH_4/gCOD_{in}$ and 0.136 $LCH_4/gCOD_{in}$, respectively, although the drop was more pronounced under mesophilic conditions.

The response of mesophilic and thermophilic digesters to changes in organic loading showed a need to slowly introduce changes in operating conditions allowing time to adapt to elevated levels of available substrate. The variability in performance when increasing loading is in contrast to Zamalloa *et al*., (2012b)

which demonstrated good response to increasing OLR in a membrane bioreactors, likely a result of the prior acclimation strategy adopted that exposed digesters to high organic loading of glucose to increase biomass concentrations.

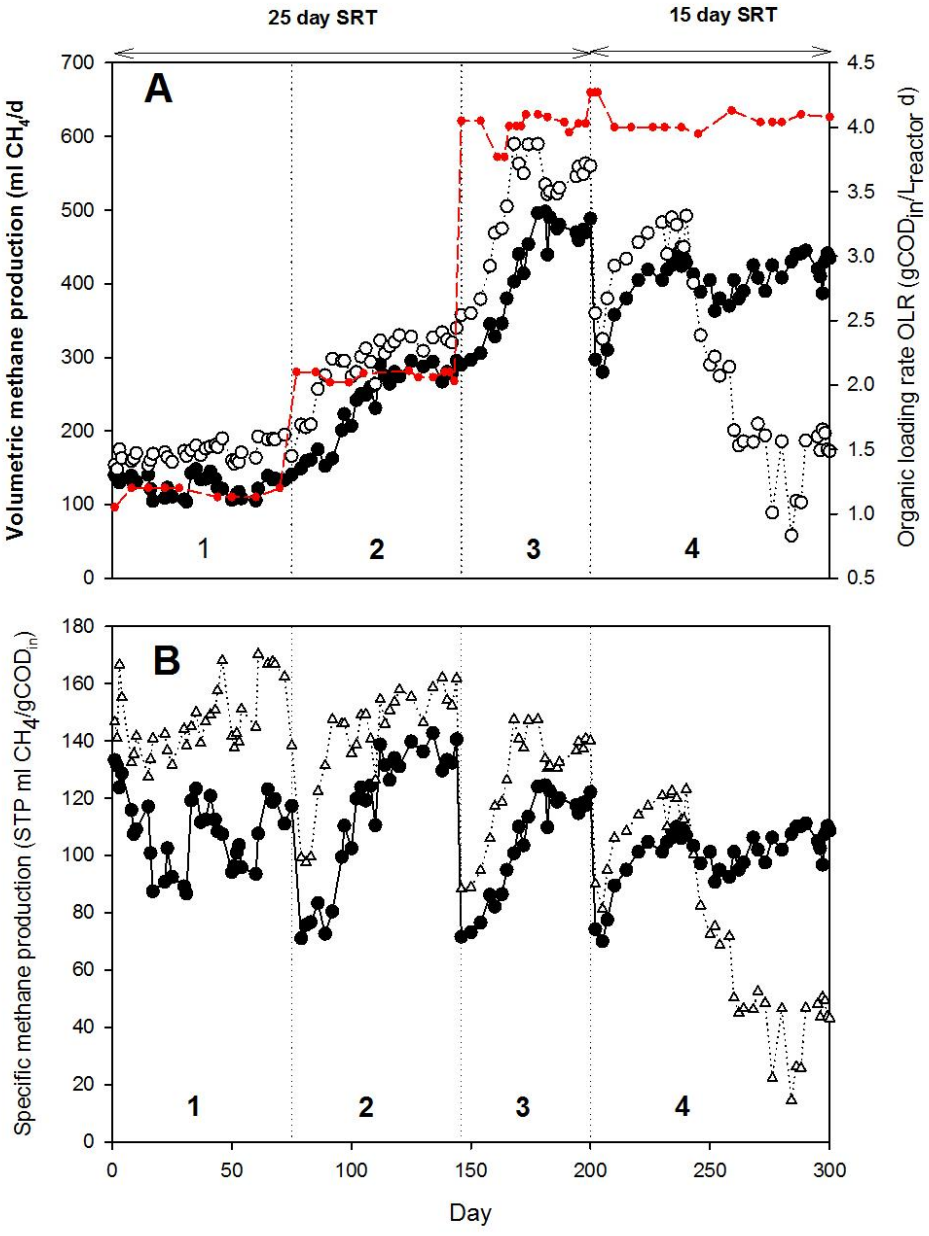

Figure 5-2. Methane production at 35 °C and 50°C with different OLR and SRT: A, (•) denotes volumetric methane production at 35°C; (•), denotes OLR; (○) denotes volumetric methane production at 50°C; B, (•), methane yield at 35°C; (○), denotes methane yield at 50°C.

Phase 4 applied a reduction in SRT from 25 to 15 days, while maintaining a fixed target OLR of 4 gCOD/$L_{reactor}$.d. This reduction in SRT gave a significant drop in methane production at both temperatures (Figure 5-3). Initially the methane production at both temperatures steadily increased between 4 – 8 days after the reduction in SRT, but lower steady state production was eventually shown after day 215 at thermophilic and 220 at mesophilic temperatures. The average volumetric methane production between days 215 and 240 at thermophilic temperatures was 0.466 LCH_4/d and between days 220 and 300 at mesophilic temperatures was 0.413 LCH_4/d. These rates were lower than observed at Phase 3, whilst the average methane yield was lower at 0.116 $LCH_4/gCOD_{in}$ and 0.103 $LCH_4/gCOD_{in}$ in thermophilic and mesophilic reactors respectively (Figure 5-2).

After day 240, volumetric methane production declined rapidly at thermophilic temperatures stabilising around day 260. Between day 260 and 300, although there were extremely low values of 0.058 LCH_4/d, the average rate was 0.164 LCH_4/d, resulting in an average methane yield of 0.041L $CH_4/gCOD_{in}$. This was significantly below that observed between day 215 and 240 (Paired sample T test, $p<0.05$), whereas the mesophilic reactors appeared to remain relatively stable, maintaining a consistent gas production and yield between day 240 to 300. Furthermore, methane concentration in thermophilic reactors decreased over a similar period, following a trend similar to the overall production rate.

During Phase 1 total VFA (tVFA) levels remained very low in reactors at both temperatures, with only a slightly more elevated level present in thermophilic reactors (53mg/L) compared to mesophilic reactors (35 mg/L). Propionic acid levels were very low at both temperatures and was typically below detection limits

of the instrument (< 5 mg/L, Figure 5-3). The VFA levels at both temperatures increased progressively during Phase 1 from 28 mg/L at 35°C and 61 mg/L at 50°C on day 72 to 268 mg/L in mesophilic reactors and 328 mg/L in thermophillic reactors on day 142. The observed differences between Phase 1 and Phase 2, and between mesophilic and thermophillic were not statistically significant ($p>0.05$).

During Phase 3, increasing tVFA and propionic acid concentrations were observed in thermophilic reactors, while the levels in mesophilic reactors appeared to stabilise to average 150 mg/L of tVFAs. The increase in VFA levels was significant, but no significant correlation existed between VFA concentration and methane yield at either reactor temperature was observed.

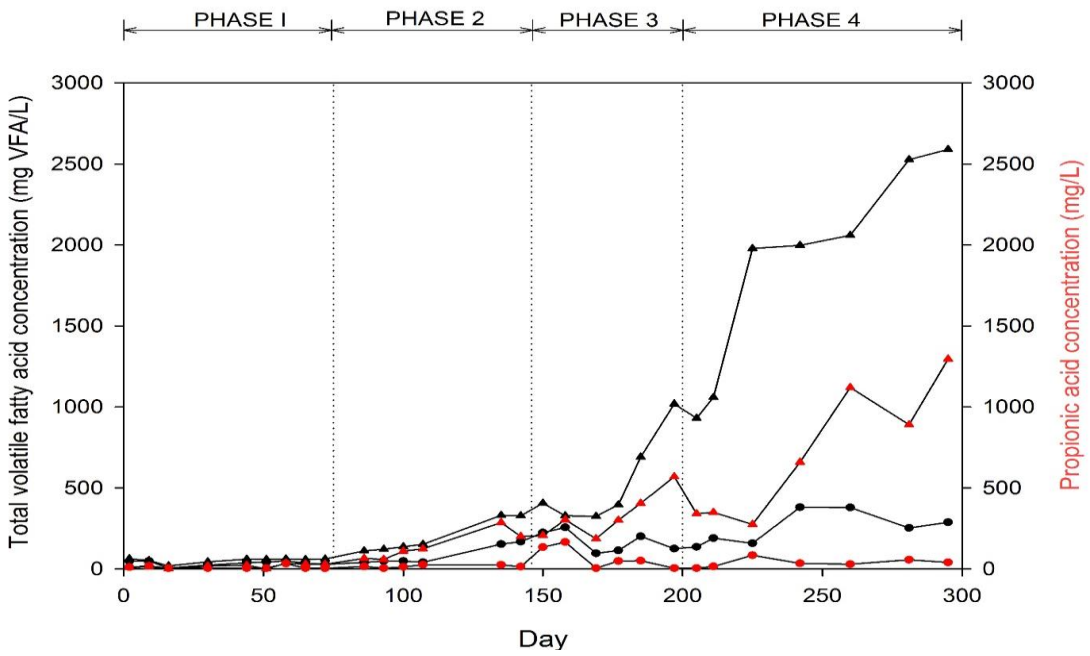

Figure 5-3. Volatile fatty acid concentrations in mesophilic and thermophilic digesters during Phase 1 to 4; (•), total VFA at 35 °C; (•), propionic acid concentration at 35 °C; (▲), total VFA at 50 °C; (▲), propionic acid concentration at 50 °C.

During Phase 4, immediately after changing the SRT, the tVFA and propionic levels dropped in both sets of reactors, but after day 205 tVFA levels continue to rise at

50°C reaching 2590 mg/L before the end of the experiment. Propionic acid levels within these reactors reached a peak of 1295 mg/L on day 295 indicating a destabilisation in the reactors. At 35°C there was a small increase in tVFA levels to average 325 mg/L, but propionic acid remained low and only made up a small proportion of this tVFA level at this temperature.

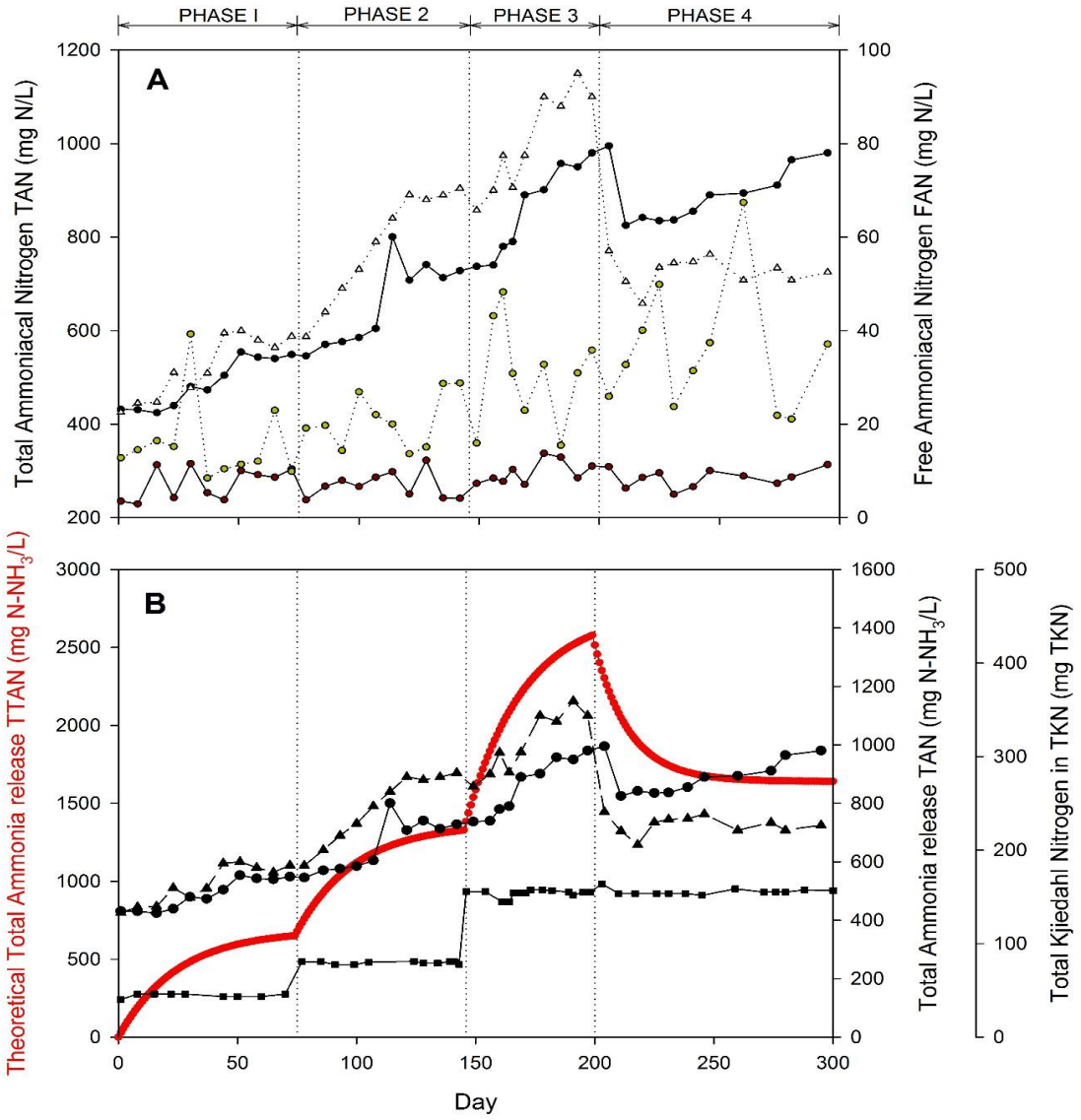

Figure 5-4 Total ammoniacal nitrogen (TAN) and free ammoniacal nitrogen (FAN) in mesophilic and thermophillic reactors across phases 1 – 4: (A), (•) TAN at 35 C; (▲), TAN at 50 C;(•), FAN at 35 C; (•), FAN at 50; (B), (•) Theoretical total ammonia release; (•) Total ammonia release at 35 C; (▲), total ammonia release at 50 C; (■) Influent TKN.

During Phase 4, the relationship between tVFA levels and methane production was significant with increase VFA consistent with reduction in methane produced. This indicated a destabilisation of the methanogens within the system.

Thermophilic reactors contained higher levels of TAN than mesophilic reactors across all phases of operation (655 compared to 754 mgTAN/L). This is a direct result of higher levels of organic nitrogen (protein) degradation in thermophilic reactors and potentially indicates that more protein was being converted to ammonia and utilized for methane production in the thermophilic digesters. With higher TAN concentration and higher temperatures the FAN levels were predictably higher at 50°C than 35°C. Nitrogen levels increased with both reactors across Phases 1, 2 and 3 as the OLR was increased (Figure 5-5). Steady state conditions were achieved in Phases 1, 2 and 3 within 2 – 3 retention times, consistent with theory, with thermophilic achieving steady state faster than mesophilic reactors.

As the OLR increased, ammonia production increased as a response to increase in biomass (and nitrogen) entering the reactors. Thermophilic reactors produced more ammonia with levels achieving 1081 mgTAN/L during Phase 3, while in mesophilic reactors the maximum TAN levels observed were 936 mg TAN/L. FAN levels fluctuated within all reactors due to the pH effects and constant buffering of the system on ionization equilibrium shifting. FAN reached a maximum concentration of 68 mg N-FAN/L at 50°C and 12 mg FAN/L at 35°C. Increased TAN production in thermophillic digestion is a consequence of increased hydrolysis rates of organic nitrogen in thermophilic digestion, while the increased FAN levels is a consequence of the shift to free ammonia with higher temperatures. During phase 4, TAN levels dropped with a reduction in HRT increasing washout of

nitrogen produced. During this period thermophilic ammonia levels dropped below that of mesophilic levels, and while mesophilic TAN levels continued to increase, thermophilic TAN levels remained constant at around 658 – 753 mg TAN/L, significantly below levels previously observed in Phase 3. The reduction in TAN is a direct result of reduced breakdown of microalgae indicating process instability.

5.5. General discussion

5.5.1. Effect of temperature on performance

Reactors at both temperatures showed consistent performance across Phases 1 and 2. Thermophilic digestion gave higher methane yield than mesophilic digestion across all phases, both at different organic loading rates and solid retention times applied, albeit thermophilic reactors accumulating greater quantities of VFA and ultimately reaching a period of instability towards the end of the study after a reduction in SRT.

Higher methane production at elevated temperatures has previously been observed (Golueke *et al.,* 1957; Zamalloa *et al.,* 2012a), indicating higher breakdown and conversion efficiency of substrates, a potentially positive benefit from operating digesters at increased temperature. The ultimate methane yield observed in bio-methane potential (BMP) test is the same at mesophilic and thermophilic temperatures, but rate of conversion is higher at thermophilic temperatures, a response demonstrated previously with other substrates (Veeken and Hamelers, 1999; Angelidaki and Sanders, 2004; Zamalloa *et al.,* 2012a). The results both support and contrast the results obtained by Zamalloa *et al.,* (2012a).

This study showed improved gas yield at thermophillic compared to mesophilic temperatures when digesting *Scenedesmus obliquus* in a hybrid flow through reactor, with yields of 0.13 ± 0.05 LCH_4/gVS_{in} at 33°C compared to 0.17 ± 0.08 LCH_4/gVS_{in} at 54°C reported and their work used algae of the same genus as this thesis, encouragingly showed similar yields. However the same study observed no significant differences when digesting *Phaeodactylum tricornutum* in the same reactor configuration at 33°C and 54°C, with reported methane yields of 0.27 ± 0.09 LCH_4/gVS_{in} and 0.29 ± 0.11 LCH_4/gVS_{in}, respectively. The differences in methane yields in these studies likely can be attributed to the effects of different species composition and cell structure (Foree and Mccarty, 1970), the effect of cultivation and harvesting conditions, and reactor configuration and operation. In the study by Zamalloa *et al*., (2012a) a hybrid reactor used was likely to have been very efficient, and so will have digested any hydrolysed carbon relatively quickly to methane irrespective of temperature. With different degradation rates observed for the different algae species (Foree and Mccarty, 1970). P*haeodactylum* species had a faster degradation rate than *Scenedesmus* species, and so even at mesophilic temperatures algal breakdown rates were close to their ultimate degradability as measured in an earlier study of 0.35 LCH_4/gVS_{in}. This is further illustrated by the low levels of VFAs and soluble COD exiting the reactors, indicating good conversion of all soluble material. With low OLR coupled with an efficient reactor, the variability between operating temperatures in hydrolysis rates is difficult to detect. Only a further increase in OLR to its maximum would demonstrate this hypothesis.

Reactor temperature has two other effects specific to the substrate itself. It is likely to affect both the stability of microalgal cell including cell wall fluidity and structure, alongside the accessibility and solubility of the intracellular components. Typically optimal growing temperatures for microalgae are between 10°C and 35°C (Becker, 2004). When exposed to prolonged periods of elevated temperatures, algal metabolism and cell systems would start to shut down, causing cell disintegration, and releasing cell contents. By introducing microalgae into an environment with an elevated temperature above normal culture conditions, it is likely that there would be a net positive effect on cell disruption and thus methanogenic performance.

The effect of temperature on solubility and breakdown of different components for different substrates has been addressed in previous studies (Gujer and Zehnder 1983), with differences observed in methane yield between lipids, carbohydrates and proteins being attributable to the variation in carbon content and molecular structure, but also the bioavailability and water solubility. These effects are likely to be partly responsible for different hydrolysis rates observed between species (Foree and Mccarty, 1970) and under both similar and different operating conditions. Both temperature and solids retention time play an important role that is inextricably linked to the degradation of algal components, with higher temperatures and longer retention times generally showing elevated rates of breakdown of different complex components. The three main components of complex organic matter found in algae are lipids, carbohydrates and proteins. It is expected that changing the conditions may affect one component to a greater extent than the others. Hydrolysis of proteins is generally slower than the hydrolysis rate of carbohydrates and lipids (Eastman and Ferguson, 1981). The hydrolysis of

proteins is undertaken by extracellular enzymes called proteases and peptidases converting the proteins to polypetides, oligopeptides and amino acids. These are then converted by a different group of fermentative bacteria or through anaerobic oxidation reactions to VFA's, H_2, CO_2 and NH_4^+ (Pavlostathis and Giraldogomez, 1991). The rate of this conversion is a function of temperature, concentration of substrate and by-product and retention time. It has been shown that reduced pH improved the solubilisation of particulate substrates, including nitrogenous compounds (Pavlostathis and Giraldogomez, 1991) with a pH change from 5.14 to 6.67 improving nitrogenous COD conversion from 0.28 to 0.69. The effects of pH above this point have not been shown, and so whether there was any improved performance of running anaerobic reactors at the pH values observed in this study on protein solubilisation are unknown. The high organic nitrogen mineralisation observed in this study demonstrates efficient protein degradation with elevated pH both a benefit to protein degradation, but also a result of conversion of protein to ammonia nitrogen which is a weak base.

Soluble protein concentration was found to be lower in thermophillic reactors compared to mesophillic reactors, indicating either poor solubilisation of proteins, or efficient hydrolysis of proteins to amino acids and ammonia. The presence of elevated levels of TAN indicates the second mechanism to be more likely. To confirm this, a more detailed investigation into the extent of protein breakdown is required, but nitrogen mineralisation was high in both sets of reactors indicating that protein degradation was unlikely to have been limiting.

The other main component in microalgae is lipids, a large proportion of these being polyunsaturated fatty acids. It is these fatty acids that are used to produce biodiesel

through the trans-esterification process, but are potentially vital to sustainable whole cell algae anaerobic digestion. Lipids, being rich in carbon have the highest methane yield (Gujer and Zehnder, 1983) and can make up a significant proportion of the algae cell constituents. Degradation of lipids occurs through hydrolytic enzymes (lipases), producing fatty acids, phosphoric acid, and glycerol as products. Triglycerides are first hydrolysed to glycerol and long chain fatty acids (LCFA) and these LCFA are then further oxidised to acetate and propionate and hydrogen via β-oxidation (Weng and Jeris, 1976). It has been previously shown that low concentrations of LCFA such as oleate and stearate can be inhibitory to anaerobic processes, causing an accumulation of acetic acid and propionic acid and reduction in methane yield (Angelidaki and Ahring, 1992). Lipid levels of greater than 1000 mg/L appear to cause inhibition to both the conversion of LCFA and n-butyrate to acetate, and hydrogen production, which subsequently affects both acetogenic and hydrogenotrophic methanogesis (Hanaki *et al.,* 1981). Concentrations observed in this study are unlikely to have reached these inhibitory levels due to the maximum loading rates not being extremely high. However levels of individual LCFA still have the potential to cause inhibition when exposed to shock loads and overload conditions, with concentrations of oleate and stearate of 200 mg/L and 500 mg/L respectively reported to be inhibitory to methanogenesis (Angelidaki and Ahring, 1992). The study by Angelidaki and Ahring (1992) suggested the inhibitory process to be irreversible, and in order to reduce the potential for inhibition, wastes should be introduced slowly to prevent shock loads and overloading of biomass, allowing cell numbers of organisms capable of degrading these toxic compounds to increase. The shock increases in OLR from

Phase 2 – 3, and the reduction in SRT from Phase 3 to Phase 4 may have led to instability directly related to the LCFA levels.

In addition to the impact of temperature on biochemical components and their individual degradability, it has a significant effect on the nitrogen levels within the reactor – with these being linked. Nitrogen accumulation in the form of ammonia is expected as a result of organic nitrogen breakdown. High ammonia levels are typical of substrates that have low C:N ratio and high protein content (Table 5-2) and are a direct concern for microalga AD (Sialve *et al.,* 2009). Elevated nitrogen levels can significantly impact the AD process through inhibition of the microorganisms and increases in reactor pH. Ammonia is a weak base, and results in higher reactor pH. This directly leads to higher CO_2 solubility in the aqueous phase, resulting in lower CO_2 and higher methane concentrations in the biogas. With increases in organic loading more protein/nitrogen is added to the system, which results in higher levels of free ammonia with maximum concentrations observed during Phase 3 at both temperatures. Importantly, at higher temperatures, and elevated pH there would have been a greater shift from ionised ammonium to unionised ammonia (Eq. 4- 21). In its free unionized form, ammonia exerts a toxic effect on microorganisms (Chen *et al.,* 2008), specifically, methanogenic systems are known to be less tolerant of free ammonia (Angelidaki and Ahring, 1994). The concentration at which free ammonia exerts a toxic effect varies between studies but measured values (Figure 5-4) were always below the threshold values reported in the literature for reactors at thermophilic temperatures (Chen *et al.,* 2008).

During Phase 3 the conversion efficiency of organic nitrogen to ammonia is greater than the overall COD conversion efficiency, indicating those compounds higher in nitrogen are more readily degradable than carbon rich compounds. Total

ammoniacal nitrogen levels peaked within all reactors, but were still below the inhibitory threshold previously shown at 1.5 – 3g N-TAN/L (Vanvelsen, 1979; Angelidaki and Ahring, 1994). At thermophilic temperatures, pH fluctuations occurred between 6.6 and 7.8, indicating some potential process instability within the reactor. The poor pH control was linked to fluctuating free ammonia concentrations at thermophillic temperatures. As retention time dropped during Phase 3 to Phase 4 the levels of nitrogen in both sets of reactors dropped during to lower HRT/higher hydraulic throughput. While retention time can impact methane yield, it can benefit any potential nitrogen inhibition through wash out of ammoniacal nitrogen and should be a consideration for process configuration when operating at high organic loads/high nitrogen levels.

5.5.2. Effect of OLR on performance

Changing the organic loading rate from 1 to 2g COD/$L_{reactor}$.d resulted in an increase in methane production across all reactors, with no obvious drop in methane yield. Methane yield was higher in thermophilic reactors compared to mesophilic reactors, and when substrate concentration doubled, the thermophilic reactors responded quicker to the higher loading, showing increased biogas production almost immediately. COD destruction in both sets of reactors dropped due to the higher quantity of COD entering the system, with the hydrolytic organisms needing time to adapt and grow with higher substrates. Encouragingly, after a period of growth and adaption at both temperatures, methane yield stayed similar to previous levels at lower OLR. The proportion of intact algae cells present in the effluent increased at a similar proportion to the increase in algal biomass

introduced as feed, indicating resistance to degradation and incomplete digestion (Appendices, Pg Vi, Figure 4-2).

By increasing OLR further, a similar trend was observed, with thermophilic biogas production showing a quicker response. The methane yield, after a period of variability at thermophilic temperatures, seemed to improve straight after organic loading rate increased from 2 to 4 gCOD/d. This peak is attributable to the accumulation of previously un-degraded components, plus the introduction of increased soluble components with the new substrate addition. Soluble carbon was shown to significantly change in the feedstock over the duration of the storage conditions, while length of storage was also shown to significant impact methane yield in the concentration microalgae (7.4.4).

The improvement and stability of thermophilic reactors during Phase 3 could be related to the longer operation of reactors by this point in the sequence, leading to a stabilisation (acclimation) of the microbial community. The original thermophilic inoculum had not been taken from a working thermophilic digester, but had been adapted from mesophilic sludge by short-term exposure of algae and substrate to elevated temperatures prior to the experiment.

Increasing organic loading from 1 gCOD/$L_{reactor}$.d to 4 gCOD/$L_{reactor}$.d did not result in overload conditions (high tVFA, high nitrogen, high solids), something previously reported at similar loading rates (Ras *et al.,* 2011; Gonzalez-Fernandez *et al.,* 2013), but it appeared that solids accumulation and nitrogen accumulation in the reactors (Figure 5-4) might present an issue with further increases in OLR. Although the thermophilic reactors produced more biogas, it was the higher

hydrolytic efficiency that might have led to higher VFA and ammonia nitrogen levels, which ultimately would become a serious limitation at longer operational periods with continued higher OLR, and so should be carefully considered when assessing the future potential of microalgal AD. The changes experienced with increasing OLR further highlights the need to take a more systematic approach to testing microalgal biomass as a substrate for anaerobic digestion, where most studies have only ever run under extremely conservative loading rates for short periods of time, neither of which would have allowed solids accumulation or nitrogen accumulation to have become problematic. Consideration should then be made for how changes in operating conditions are undertaken in experiments, with shock changes used in this study evidently impact reactor performance, primarily at thermophilic temperatures where the microbial community are more sensitive to change.

In addition, it also demonstrates the need to use more than just ultimate methane potential as a guide for testing substrate potential, with use of dynamic reactor systems being vital to a gaining fuller understanding of microalgal biomass as a potential feedstock for AD.

5.5.3. Effect of SRT/HRT on reactor performance

Methane yield at 25 days SRT for mesophilic temperatures was similar tothat previous studies (Golueke *et al.,* 1957; Ras *et al.*, 2011) while higher methane yields were observed for thermophilic reactors at 25 day SRT/HRT, similar to the work of Golueke *et al.,* (1957). The results are consistent with other studies which showed an increasing methane yield with increasing retention time (Ras *et al.,*

2011). Retention times greater than 30 days are unlikely to result in any greater methane yield, with BMP studies showing that the a large proportion of degradable components reside in the system less than 20 – 25 days.

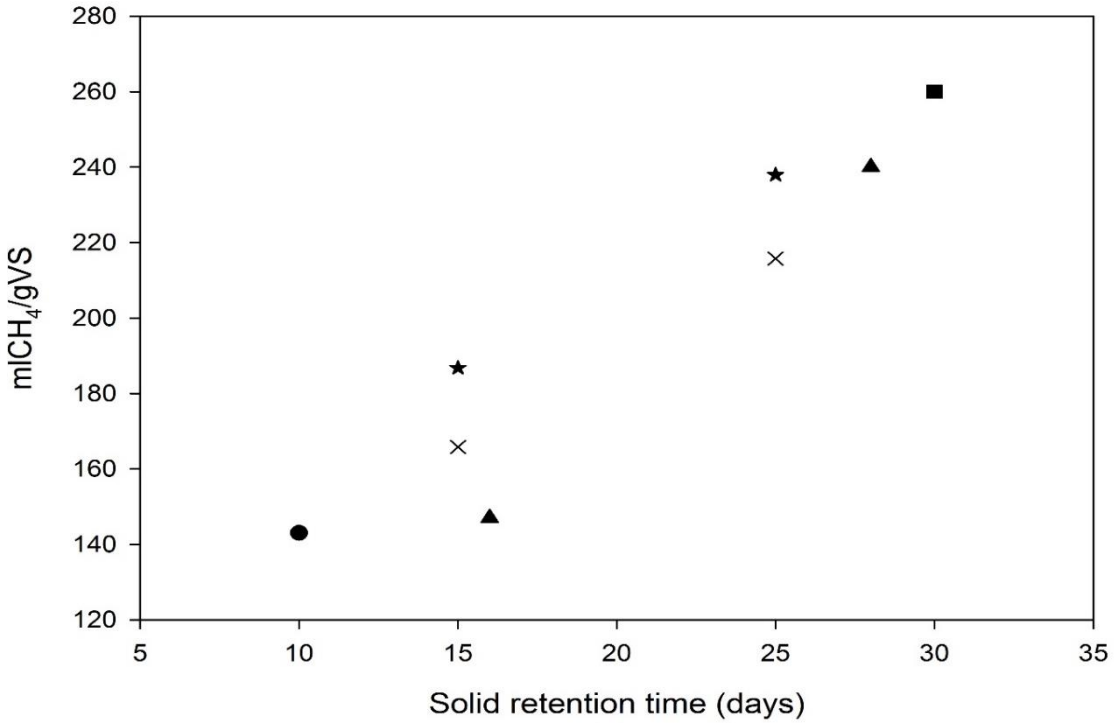

Figure 5-5 Comparison of studies investigating the effect of solid retention time on methane yield from microalgae. (★), this study at 35°C; (X), this study at 50°C; (•), is Yen and Brune, 2007, (▲) is Ras *et al.,* 2011; (■) is Golueke *et al.,* 1957.

The change in SRT from 25 day to 15 day during Phase 4 showed contrasting results between reactor temperatures. For mesophilic reactors, the drop in retention time first showed a reduction in ammonia, VFA levels consistent with a removal of 50% more solids/liquid from the reactor during each daily feed. This was followed by a period of reduced COD destruction, consistent with the greater removal of active anaerobic biomass that occurred with each feed. This marked a period of minor instability in the reactor, with fluctuating gas levels and COD destruction efficiency in the reactor. After 3 – 5 days the reactor started to stabilise,

with improvement in methane yield evident. By day 220 the reactor had obtained similar gas production, methane yield and COD destruction efficiency as previously found during Phase 3, indicating that there had been only minimal effects of reducing SRT from 25 to 15 days. This is in disagreement with Ras *et al.*, (2011), who showed a reduction in SRT from 28 to 16 days caused a reduction in methane conversion from 48 % to 29 %. The reasons for this could be due to the duration that their experiment ran, not giving biomass sufficient time to acclimatise and stabilise. Furthermore, differences in algal species and composition, and differences in overall reactor performance and control may have affected the different reactor performance.

The drop in SRT from 25 to 15 days in the thermophilic reactors resulted initially in a similar trend to the mesophilic reactors, with a reduction in ammonia, VFAs and COD destruction. However, the thermophilic reactors appeared to recover faster than mesophilic reactors, with gas production returning quickly to similar levels as observed in Phase 3. This is consistent with thermophilic reactors being able to respond quickly to a reduction in SRT due to higher microbial growth rates, meaning less washout of active biomass. However, as the operational period continued, the thermophilic reactors became increasingly unstable, with greater fluctuations in pH, reducing COD destruction rates and methane yield. At day 234 methane production started to drop off considerably, with large fluctuations in pH evident. Process instability was evident at this phase, with increasing quantities of VFAs, with a large proportion of this due to propionic acid accumulation. Until this period, the thermophilic reactors had been outperforming the mesophilic reactors for gas production, but to a lesser degree than they had at 25 day SRT. This contradicts the results observed by De Schamphelaire and Verstraete (2009) who

observed no effect on the anaerobic digestion of Chlorella by increasing temperature from 34 °C to 41 °C at HRTs between 14 and 25 days of operation. Once again, the reason for the difference in their results could be related to reactor operation, duration of reactor runs, variability in substrate and loading, or most probably, the fact that their maximum temperature was still within the mesophilic range, and so any effect of true thermophilic temperature on substrate degradation would have been unclear. Exposing microalgae to the true thermophilic conditions that were investigated in the current research may have resulted in temperature-related breakdown of microalgae, or solubilisation of microalgal components, these being more likely to occur when the microalgae were subjected to temperatures outside their normal growth temperature range.

A reduction in HRT resulted in reduced methane yield, COD destruction and ammonia production, which is consistent with previous studies (Golueke *et al.,* 1957; Ras *et al.*, 2011), with both algae and anaerobic biomass being retained in the reactor for shorter periods of time the rate of degradation would decrease. As SRT is tied to HRT the reduction in HRT resulted in reduced substrate retention within reactors, and a smaller community of microorganisms to maintain reactor performance. This has been shown to increase the chances of process instability. The HRT change resulted in a reduction in ammonia and VFAs within all four reactors; this was likely due to increasing washout of both components and a reduction in the bacteria capable of producing and consuming these products. After a period of 5-10 days accumulation of both ammonia and VFAs started to take place again indicating that the process had overcome the initial shock of changing conditions. Nitrogen mineralisation with reduced HRT dropped at 35°C and 50°C to 36% and 40% respectively. COD conversion in thermophillic reactors after day

246 dropped rapidly with conversion only achieving a maximum 19% but continued to drop until reactor analysis was stopped on day 300. The drop in total COD destruction in thermophillic reactors is followed by a rapid increase in tVFA's. This is indicating that hydrolysis and acidogenesis is taking place at similar levels before day 246, but the conversion to acetic acid and methane in acetogenesis and methanogenesis is not taking place. Typically, excess VFA production results in reducing pH but the increased ammonia levels which results in raised pH may have offset these effects in a so called inhibited steady state (Chen *et al.*, 2008). HRT/SRT alone is unlikely to be the main cause of failure in thermophillic reactors as it successfully ran for 40 days without such significant drop off in gas production, but it is probably likely to be a contributory factor coupled with ammonia accumulation and temperature.

5.5.4. Process stability

Between Phases 1 and 3 both mesophillic and thermophillic reactors worked well, with low VFA levels and consistent gas production and concentration. A reduction in HRT/SRT eventually caused the thermophillic reactors to fail, with a reduction in both volume of biogas and methane concentration. This is inconsistent with previous studies that have shown thermophillic digestion to perform better at reduced HRT than mesophillic digestion (Golueke *et al.,* 1957). Although failure occurred in Phase 4, it did not occur until after day 240, 40 days after the SRT change, and only after 2 full SRT periods had been completed. Failure or imbalance in thermophillic reactors was also evidenced by high VFA levels (1979 mg Acetate eq.L^{-1}) observed, This amount of VFAs had been accumulating from when the organic loading rate had been increased to 4 gCOD.L^{-1} (Phase 3). Volatile

fatty acid accumulation has been shown to be a good indicator of process imbalance in anaerobic digesters (Ahring *et al.,* 1995). The large quantity of both acetic acid and other VFAs such as propionic, butyric and valeric acid indicated imbalance in two primary reaction mechanisms; acetogenesis resulting in the conversion of propionic, butyric and other VFAs to acetic acid, and methanogenesis; resulting in the conversion of acetic acid into methane and CO_2. The uncoupling of the relationship between producers and consumers of VFAs is typical for communities under stress and is the first signs of impending reactor failure (Hill *et al.,* 1987; Ahring *et al.,* 1995). Importantly, the start of VFA accumulation will lead to further inhibition, more impaired performance, and further VFA accumulation (Boone and Xun, 1987).

VFA concentrations that can be tolerated in anaerobic systems without impact on methane production vary due to reactor configuration, temperature, organic loading rates, solids and hydraulic retention times, and importantly seed acclimatisation effects (Angelidaki *et al.,* 1993). Levels up to 50mM have been shown to have no effect on methane production rates, but were good indicators of imbalance, and not direct inhibition (Ahring *et al.*, 1995). The levels observed in the current study have been tolerated in other systems without leading to failure, and so although accumulation indicates an imbalance, the exact basis of this imbalance lies elsewhere. Normally the accumulation of VFAs results in reduced pH which can further impair the process (Chen *et al.*, 2008). This did not occur here, with elevated pH being observed in the failing thermophilic reactors.

It has previously been shown that thermophilic reactors have reduced stability when compared to mesophilic reactors, likely due to the lower phylogenetic

diversity, with a smaller functional redundancy being observed at thermophilic temperatures (Guo *et al.,* 2014). Mesophilic reactors have been shown to have higher diversity (Karakashev *et al.*, 2005), with the microbial composition and diversity shown to be different in granules between mesophilic and thermophilic reactors (Sekiguchi *et al.,* 1998). With lower diversity, any perturbations or change in operating conditions is more likely to result in process imbalance, and cause thermophilic digestion to fail. The production of ammonia and VFA, with a concomitant reduction in methane levels, is a likely indicator that the methanogens were most probably, the most affected group of microorganisms.

Previous studies on food waste digestion (Banks *et al.,* 2012) have shown that after long periods of operation trace elements become deficient (primarily selenium). Selenium is required for propionate oxidation and syntrophic hydrogenotrophic methanogenesis, as well as the oxidation of formate. At higher OLRs (Banks *et al.,* 2012) and higher VFA levels (Kim *et al.,* 2002) the need for additional trace elements such as selenium, molybdenum, cobalt, nickel and iron has been observed.

The intermittent mixing of the reactors might have been a mechanism that improved methane production and reactor stability, whereby discrete pockets of relatively unmixed sludge would have allowed microbial consortia to have been in close proximity to each other, and to transfer metabolites efficiently, whilst at the same time exposing them less to new feedstock's, and providing reduced impact from toxic intermediate compounds such as ammonia, VFA accumulation or the trace oxygen levels inherently present, or formed from the new substrate soon after feeding (Guo *et al.,* 2014).

5.6. Conclusions

- Microalgae contain elevated levels of nitrogen due to high protein content that result in low C:N ratios. C:N ratios which are below the 25:1 reported as optimum for AD, and potentially result in elevated ammonia levels, and inefficient digestion.
- Thermophilic conditions resulted in increased methane yield from microalgae at conservative loading rates (1 – 4 $gCOD/L_{reactor}.d$).
- Mesophilic reactors were more stable at elevated OLR and shorter HRT/SRT than thermophilic reactors.
- Long term stability has been demonstrated for both mesophilic and thermophilic reactors operating on microalgal biomass feedstock, but ultimately the failure of the thermophilic reactors draws concern for the use of thermophilic microalgae AD without further investigation into the basis of this instability.
- Substrate hydrolysis appeared to be the main rate limiting step at 25 day SRT in all reactors, but at a 15 day SRT the methanogenic reactions at thermophilic temperatures became limiting, while hydrolysis remained limiting at mesophilic temperatures.
- Shock change in retention time resulted in some destabilisation in the microbial community in thermophilic reactors and led to wash out of biomass.
- Increased ammonia production due to the higher hydrolysis rates in thermophilic digestion, coupled with the increased quantity the more toxic free ammonia mean that while thermophilic digestion can improve methane

yields, it potentially can be more susceptible to toxicity and shock changes in the system.

- The effects of microbial community dynamics in response to changes in the operation and performance of AD reactors digesting microalgal biomass warrants further investigation using modern molecular techniques, in order to understand whether the lower functional redundancy and diversity expected within thermophilic reactor, results in a greater tendency to fail under stressed conditions such as elevated nitrogen, VFA or lower HRT and SRT, or trace element depletion.
- Improving biomass retention at lower SRTs through different reactor design such as membrane systems, and anaerobic filters could enable higher yields, while also limiting any potential effects of inhibitory compounds.

Chapter 6 Anaerobic digestion of mixed culture microalgae using an Up-flow Anaerobic Membrane Bioreactor

6.1. Introduction

The use of a membrane in anaerobic systems has been proposed as a way to vastly improve reactor performance, primarily to remove biological constraint or recalcitrant compounds (Grundestarn and Hellstrom, 2007). The guiding principle is that by utilising a membrane the bacterial and archaea biomass in the reactor is retained alongside the substrate for longer periods of time (Anderson *et al.,* 1986), preventing wash-out of microorganisms, and essentially decoupling the SRT and HRT relationship, as well as potentially decreasing reactor size (Ho and Sung, 2010). These systems provide a high effluent quality, removal of pathogens and viruses, and typically result in a smaller footprint (Gander *et al.,* 2000).

The main operational concern with the use of membrane systems is related to membrane fouling (Drews, 2010). Fouling results from an accumulation of particulate material, or polymeric substances on the membrane surface over time. Typically, fouling itself is very hard to predict with wastewaters and sludge's because the physiological characteristics of the sludge and biomass (including particle size distribution, extracellular polymeric substances and MVLSS concentration) change (Le Clech *et al.,* 2003). There are several strategies that have been used in aerobic systems to reduce the effects of fouling, including operating the membrane surface under high shear stress (Yang *et al.,* 2011).

In addition to concerns relating to fouling, and the associated costs of cleaning, the other main disadvantages include additional energy consumption in pumping and

pressurising the membrane. Infinite SRT can eliminate washout, but is not practical due to solids and biomass accumulation over time, so typically, depending on organic loading rates and methane potential of the substrate, SRT is still set above zero, but at an order of magnitude higher than the typical 10 – 30 days observed in most conventional CSTR systems. In addition to increased energy costs, plant complexity is greater, and with complexity comes significant capital cost and maintenance implications. The only way these increased costs can be justified is through achieving improved performance, or the need for consistently high levels of performance.

Typical substrates include industrial wastewaters high in carbon, wastewaters that include compounds toxic to the receiving environment, like pharmaceuticals, or compounds with low biodegradability that require long residence times or a specific microbial community capable of degrading them. Very little research has been carried out on the use of these systems fed on microalgae.

The use of a membrane offers unique advantages. Firstly, the unicellular algal biomass is fully retained in the system, increasing solids residence time and importantly removing the need to use any energy intensive systems to harvest the biomass. Secondly, having high protein content, algae have the potential to produce large quantities of nitrogen in the form of ammonia, a known toxicant to anaerobic micro-flora (Sung and Liu, 2003; Calli *et al.,* 2005; Chen *et al.,* 2008). As ammonia/ammonium is soluble, it can pass directly through the membrane, removing contact and reducing its ability to express toxicity, primarily with the methanogens. Thirdly, from a holistic view, the production of no-solids effluent rich in ammonium, phosphorous and trace metals from breakdown of algae could be directly recycled to the cultivation system or valorized as a fertilizer.

Previous studies have focussed on the use of batch and continuous stirred reactors to understand and evaluate methane potential of microalgae (Golueke *et al.,* 1957; Vergara-Fernandez *et al.,* 2008; Mussgnug *et al.,* 2010; Ras *et al.,* 2011; Sukias and Craggs, 2011; Zamalloa *et al.*, 2012a). Design and utilisation of different reactor systems to improve performance and reduce any potential inhibitory effects from LCFAs and NH_3 has not been widely investigated. Optimising different systems to increase biomass and substrate retention could yield improvements in overall energy efficiency needed to make algal AD feasible.

High rate reactors such as membrane bioreactors, anaerobic filters, baffled anaerobic reactors and hybrid two stage systems usually offer improved methane yields, with higher rates of substrate destruction, better effluent quality and other performance benefits. However, limited studies have been undertaken on the use of high rate reactors fed on microalgae. Zamalloa et al., (2012b) studied a laboratory-scale membrane bioreactor fed on *Phaeodactylum tricornutum* under two different OLRs and SRTs. Results showed 52% COD destruction, higher than other studies have shown with similar OLRs (this thesis), but remained reasonably low for such an intensive and high rate system. Their research also used pre-dried algae, something that removed any pre-concentration requirements, but it failed to demonstrate whether the use of pre-dried algae had any impact on the bio-methane potential, compared to fresh algae. The current research aims to test the performance of a high rate membrane bioreactor using a feedstock of fresh algae harvested by gravity. It goes further to test the performance under different hydraulic regimes and organic loading rates, in order to identify the optimal performance possible without any requirement for harvesting and concentration of the algal culture, other than by that provided by the bioreactor membrane itself.

The work uses the UAnMBR system as a tool to investigate the mechanisms behind algal cell degradation, and the influences of SRT and HRT on the activity of key hydrolytic enzymes involved in anaerobic digestion, while also comparing whether improvements in performance can be made utilising membrane based anaerobic systems compared to simple CSTR systems.

6.2. Aims and Objectives

- Evaluate the performance of a novel Up-flow anaerobic membrane bioreactor (UAnMBR) fed solely on microalgae.
    - Subject the UAnMBR to increasing organic loading rates and determine reactor performance.
    - Test the UAnMBR under different hydraulic retention times and feed the reactor system on dilute microalgae feedstocks.
    - Evaluate the effect of changing reactor operating conditions on enzyme activity.
    - Evaluate potential of UAnMBR effluent for cultivation of microalgae and to “close the loop”.

6.3. Methods and reactor configuration

6.3.1. Analytical procedures

The general analytical procedures have been described in Analytical procedures.

6.3.2. Up-flow anaerobic membrane bioreactor configuration

The reactor was configured according to Figure 6.1.

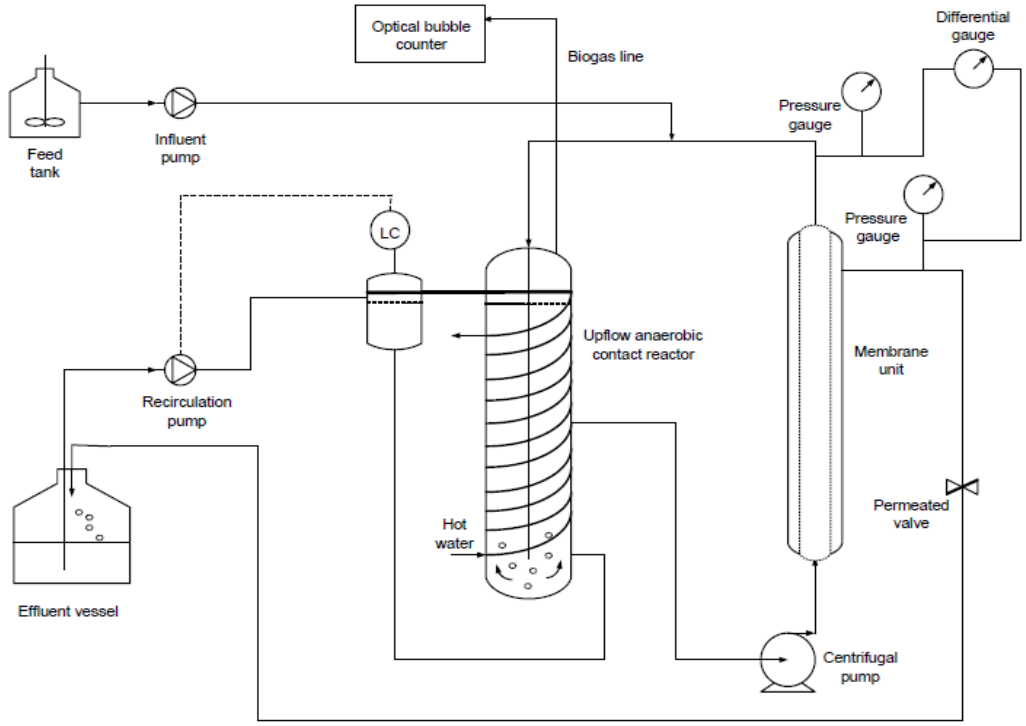

Figure 6-1 Up-flow anaerobic membrane bioreactor (UAnMBR) fed on microalgae configuration 1

The reactor consisted of an up-flow anaerobic contact reactor illustrated in (UAnCR) with a working volume of 2.7 L made from polyvinyl chloride (PVC, Normplast plastics, UK). The UAnCR was coupled using PVC tubing (30 mm I.D, UK VWR) through a variable speed centrifugal pump DC (Totton Pumps, UK) to a

modified polyehtersulphone (PES) hollowfibre membrane filtration unit which had a total surface area of 0.2 m^2 and a nominal pore size of 0.2 -0.5 µm (Milleniumpore, UK). The filter itself was made up of 6 individual hollow fibre filters. The pump speed was set using an analogue voltage controller at 70 % capacity (Mitsubishi Freqrol 0100, Japan). The total reactor itself will be known as a UAnMBR. Internal pressure on the membrane was controlled using a 20mm Stainless steel ball valve (Worcester, UK) and measured on a pressure gauge (Bundeberg Pressure Instrumentation, UK) aiming to maintain a membrane pressure of 41.4 kPA with the trans-membrane pressure differential, set at 17.2 kPA, being controlled by a secondary effluent valve (1/4" Needle valve, Swagelok, USA). Daily recordings of the membrane differential were taken. Over time it was expected that the pressure would vary due to membrane fouling so a solenoid valve was attached the permeate side of the membrane that was designed to periodic stop effluent flow and build up back pressure to scour the membrane. To overcome potential membrane fouling periodic stopping and re-starting of the centrifugal pump was used to create a turbulent flow that would strip the membrane of biomass and microalgae.

The bioreactor had a water-jacket made from coiled PVC tubing (10 mm I.D, VWR, UK) which was connected to a thermal circulator (Grant FH15, Grant Instruments, UK).

Influent was fed initially at rate of 0.72 ml/min using a peristaltic pump (Watson Marlow 520s, United Kingdom) equipped with Marprene™ peristaltic pump tubing (3.2 mm ID, Watson Marlow, UK). The peristaltic pump was manually calibrated weekly to maintain accurate flow. To prevent settling and maintain consistent feed characteristics, the influent tank was stirred constantly using a magnetic stirrer

(Stuart SB162, UK). Effluent flow rate was monitored daily and adjusted to maintain a consistent daily flow rate.

The level within the reactor was originally controlled using a type 4 conductivity sensor (Hawker Electronics, UK), which controlled a recirculation pump (Watson Marlow 313s, United Kingdom) that returned permeate (effluent) back into the reactor (Figure 6.2).

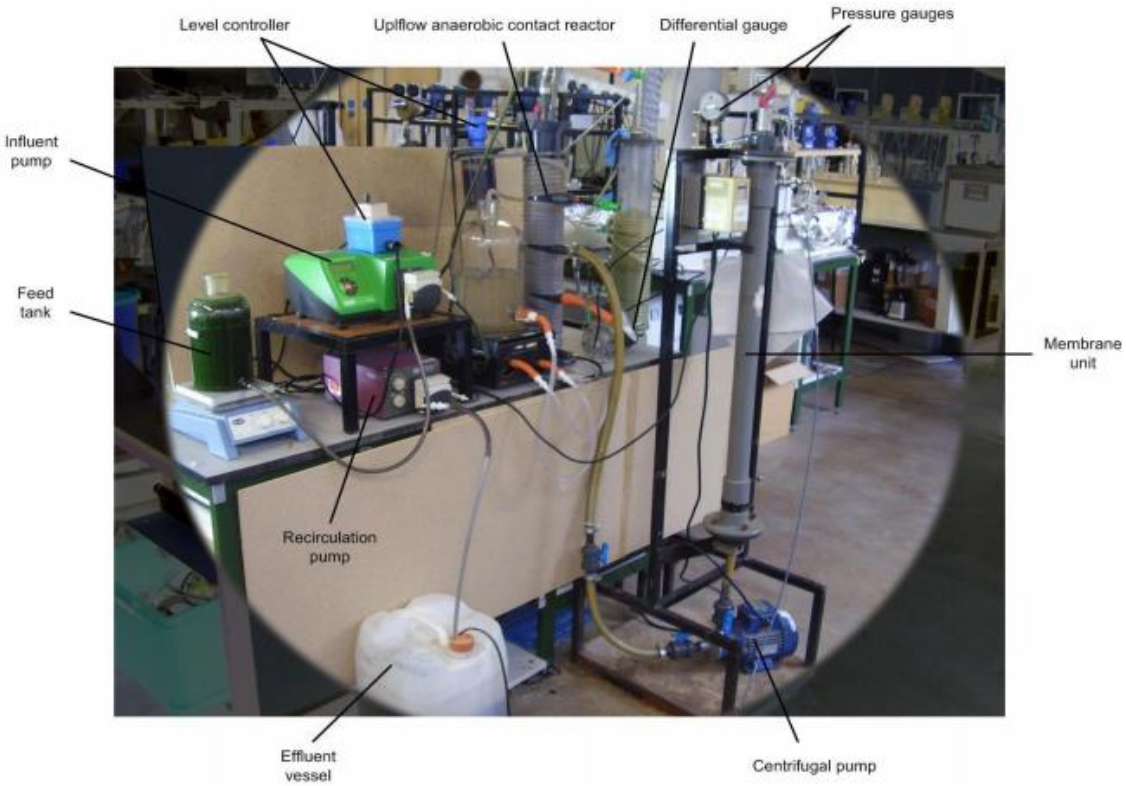

Figure 6-2 Illustration of UAnMBR 1 in operation at Newcastle University (Picture taken by N.G.Novas, 2012).

The biogas outlet was attached to an optical bubble counter (Challenge Respirometer, CES, USA) that continuously recorded volumetric gas production. Calibration was undertaken regularly using an injection of a known volume of biogas. Biogas was collected periodically for compositional analysis in a 1L

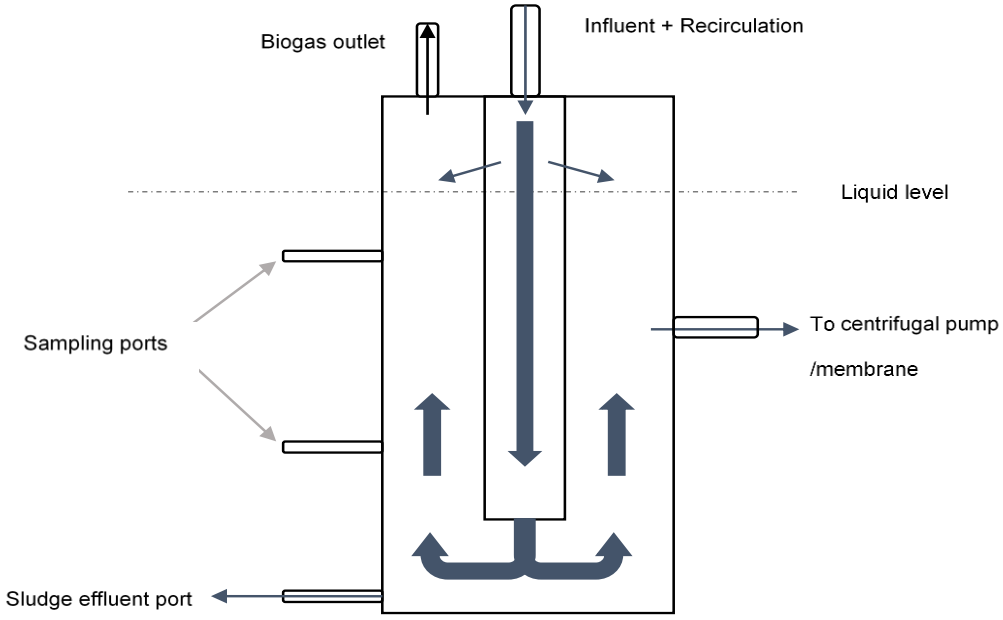

Figure 6-3 Schematic drawing of Up-flow anaerobic reactor component of UAnMBR system.

Supel™Inert Multi-Layer Gas Sampling Bags with thermogreen® LB-2 Septa (Sigma Aldrich, UK).

6.3.3. Inoculum

Anaerobic digester sludge used for inoculum was a mixture 50:50 (V:V) mixture of two active anaerobic sludge's. The first was taken from a mesophilic laboratory CSTR that had been investigating the anaerobic digestion of macro-algae (Hinks *et al.,* 2013), the second comprised granules taken from a full scale UASB treating paper mill effluent (Smurfit Kappa, UK). The granules were washed with phosphate saline buffer (pH 7.0, Sigma Aldrich, UK) and crushed through a sieve (200 microns). The sludge's were combined and diluted with tap water to give an approximate VSS of 6 – 10 g/L. The sludge was added directly into the top of the reactor and allowed to acclimatise at 35 °C for a period of 2 days prior to pumps

being started. The use of different inocula to Chapter 5 was primarily down to practical availability of sufficient inoculum to seed the reactor, whilst retaining enough for Chapter 7 where acclimated biomass is critical to correct batch results. The use of mixture of UASB and macro-algae fed CSTR inoculum was to enable both a consortia rich in methanogens (UASBs), whilst also providing a mixed microbial population believed to have sufficient quantities of bacteria capable of degradading complex polymers like cellulose, and proteins. Salinity of the sludge was not a significant issue as any presence of high levels of cations present in the seed sludge would be diluted and removed through the membrane system.

6.3.4. Operational Periods

The reactor was subjected to increasing organic loading and decreasing hydraulic retention time over the experimental period (Table 6-1). Prior to the experiment the reactor sludge was conditioned through feeding of different microalgae/brewery waste mixes to stimulate and acclimate the sludge to the microalga substrate. This was done to ensure sufficient activity remained in the system while being acclimated to microalgae.

The operation of the reactor was split up into 2 themes;

- Test the effect of increasing organic loading rate on reactor performance (Phases 1 – 4)
- Test the effect of reducing HRT and switching to dilute cultures on reactor performance with changing organic loading (Phases 5 – 7).

Table 6-1 Phases of operation of the UAnMBR (SRT, HRT and OLR)

Phase	Duration (d)	Solid retention time (d)	Hydraulic retention time (d)	OLR (gCOD/ $L_{reactor}$.d)
1	24	44	2.7	1.3 – 1.5
2	31	44	2.7	2.1-2.5
3	16	44	2.7	2.7-3.1
4	10	44	2.7	3.4-3.9
5	23	44	1.35	2.9-3.3
6	18	44	0.3	2.5-2.8
7	18	44	0.2	2.7-3.6

6.3.5. Specific methane activity test

Specific methane activity tests were undertaken once in each operational period. The test was based on the protocol previously described (Soto *et al.,* 1992; Hutnan *et al.,* 1999). The test targeted the acetoclastic methanogen activity of the sludge using acetate as the model substrate. The test was run over a 48 hour period where methane was sampled and measured regularly according to previously described analytical methods. Methane produced was calculated on a gCOD CH_4 basis.

6.3.6. Microalgae effluent cultivation

Mixed microalgae of the same species that was fed into the reactor (Methods 3.4) was used to test the potential of the membrane reactor effluent as a cultivation source for microalgae, in order to demonstrate the feasibility of “closing the water

cycle". The effluent from the membrane reactor was used for microalga cultivation under the same light/dark cycles and light intensity as the photobioreactor used for growth of the microalga feedstock. The cultivation experiment was undertaken in sterile 500ml Erlenmeyer flasks fitted with a bug stopper (VWR, UK) with 250ml of effluent or a dilution of effluent along with 30 ml of mature microalgae culture giving an approximate dry weight starting concentration of 0.05 gDW/L. The flasks were constantly stirred at 100 - 150 rpm using a magnetic stirrer with the flask being rotated periodically through the positions on the stirrer to maintain equal conditions. All cultivation experiments were undertaken in duplicate on two separate occasions. The different concentrations of effluent that was pre-diluted with distilled water were 10%, 5%, 2.5% of original digestion effluent.

Dry weight and NH_4^+-N mg/L analysis was employed at the start and end of these batch cultures, photometric absorption (Absorbance 685 nm) being used to determine biomass concentration and growth rates according to Methods 3.1.4

Two batch cycles were operated where the biomass generated from cycle 1 was used to inoculate cycle 2. This was undertaken to demonstrate any potential effects of acclimatisation.

6.4. Results and Discussion

6.4.1. Microalgae characteristics

The general characteristics of microalgae are shown in Table 5.2.

6.4.2. Biogas production

During Phase 1 average daily methane production was 0.272 $LCH_4/L_{reactor}.d$, which gave an average methane yield of 0.160 $LCH_4/gCOD_{in}$ (0.244 LCH_4/gVS_{in}) corresponding to an average COD conversion efficiency based of 45.7 %.

As OLR was increased volumetric methane production increased reaching approximate steady state at day 40, with an average daily methane production for this Phase of 0.356 $LCH_4/L_{reactor}.d$. This gave a reduction in methane yield to 0.153 $LCH_4/gCOD_{in}$ (0.228L CH_4/gVS_{in}), corresponding to a COD to CH_4 conversion efficiency of 43.8 %.

Increasing organic loading further between day 55 and 71 gave an increase in methane production, but no steady rate was observable until day 68, where methane production averaged 0.375$LCH_4/L_{reactor}.d$, with a methane yield of 0.124$LCH_4/gCOD_{in}$ (0.186 LCH_4/gVS_{in}). As OLR increased further in Phase 4 the methane production increased further to 0.448 LCH4/Lreactor.d, which gave an average yield of 0.114LCH4/gCODin (0.171 LCH4/gCODin). A large drop in biogas production was observed in Phase 5 when hydraulic retention time was halved, with average methane production of 0.396LCH4/Lreactor.d. Methane production rates were consistent in the first half of Phase 5 while started to fluctuate during the final half. Peak production was observed directly after Phase 4 on day 82 at 0.475 LCH4/Lreactor.d, and on day 97 at 0.445 LCH4/Lreactor.d. The average

yield for this phase was 0.131LCH4/gCODin (194 LCH_4/gVS_{in}). Phase 6 saw a further reduction in HRT from1.35 days to 0.3 days, reflecting being fed on dilute cultures without concentration.

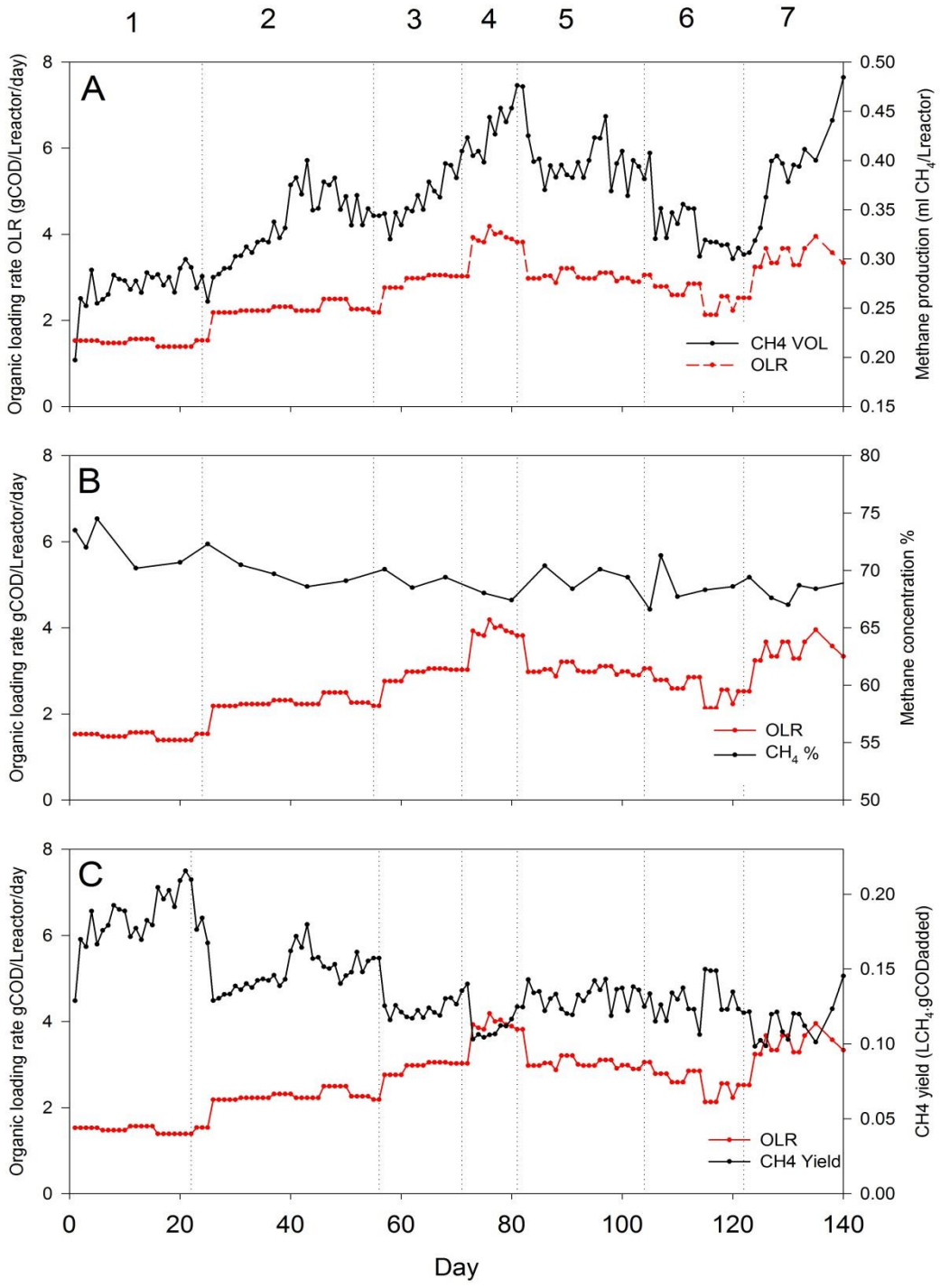

Figure 6-4. Effects of organic loading rate on UAnMBR across different phases of operation; (A), Methane volume; (B), Methane concentration; (C), Methane yield.

The variability in OLR associated with Phases 4 - 7 are a result daily variability in algal biomass productivity of the feedstock cultures. Phase 6 had two stages of OLR, the first until Day 112 where OLR averaged 2.81 gCOD/$L_{reactor}$.d then from Day 112 to 121 OLR averaged 2.27 gCOD/$L_{reactor}$.d. The average performance observed for this whole phase was 0.327 $LCH_4/L_{reactor}$.d, with a yield of 0.130 $LCH_4/gCOD_{in}$ (0.195 LCH_4/gVS_{in}). The final phase, Phase 7, attempted to increase OLR with dilute microalgal cultures by reducing HRT further to 0.2 days, whilst being fed at the same influent COD concentration. Methane production responded well to increasing OLR with an increase in methane production rate to 0.411 $LCH_4/L_{reactor}$.day, while the yield of 0.120 $LCH_4/gCOD_{in}$ (0.180 LCH_4/gVS_{in}) was lower during Phases 5 and 6.

Methane concentration in the biogas remained high across all phases of operation averaging 69.5 % with a gradual drop over Phase 1 – 7 from 72.2% to 68.2 %. Methane yield from the UAnMBR during Phase 1 of 0.160 $LCH_4/gCOD_{in}$ exceeds the 0.110 $LCH_4/gCOD_{in}$ at 35°C observed in the MSAR used in5.4.2 with the same mixed microalgae culture used. The yield represents 45.7% conversion of added total COD to methane, and efficiency of 82.0% of the maximum methane yield observed from the BMP (0.195 $LCH_4/gCOD_{in}$/0.292 LCH_4/gVS_{in}). The use of UAnMBR potentially demonstrates that by increasing SRT beyond the 25 days using in Chapter 5 improvements in yield can be achieved. The increase in retention can combat the slow hydrolysis rates of microalgae, and preventing the washout of organisms that occurred in Chapter 5, albeit this was with thermophilic digestion systems. Whilst in-direct comparisons to the previous studies in the literature can give some indicator of relative performance, the different species used, variable cultivation techniques mean direct comparisons are almost

impossible. The yield reported by Ras et al. (2011) of 0.180 $LCH_4/gCOD_{in}$ using a reported similar species mix to this study had higher methane yields with a CSTR type system. The biochemical composition of the microalgae was not reported by

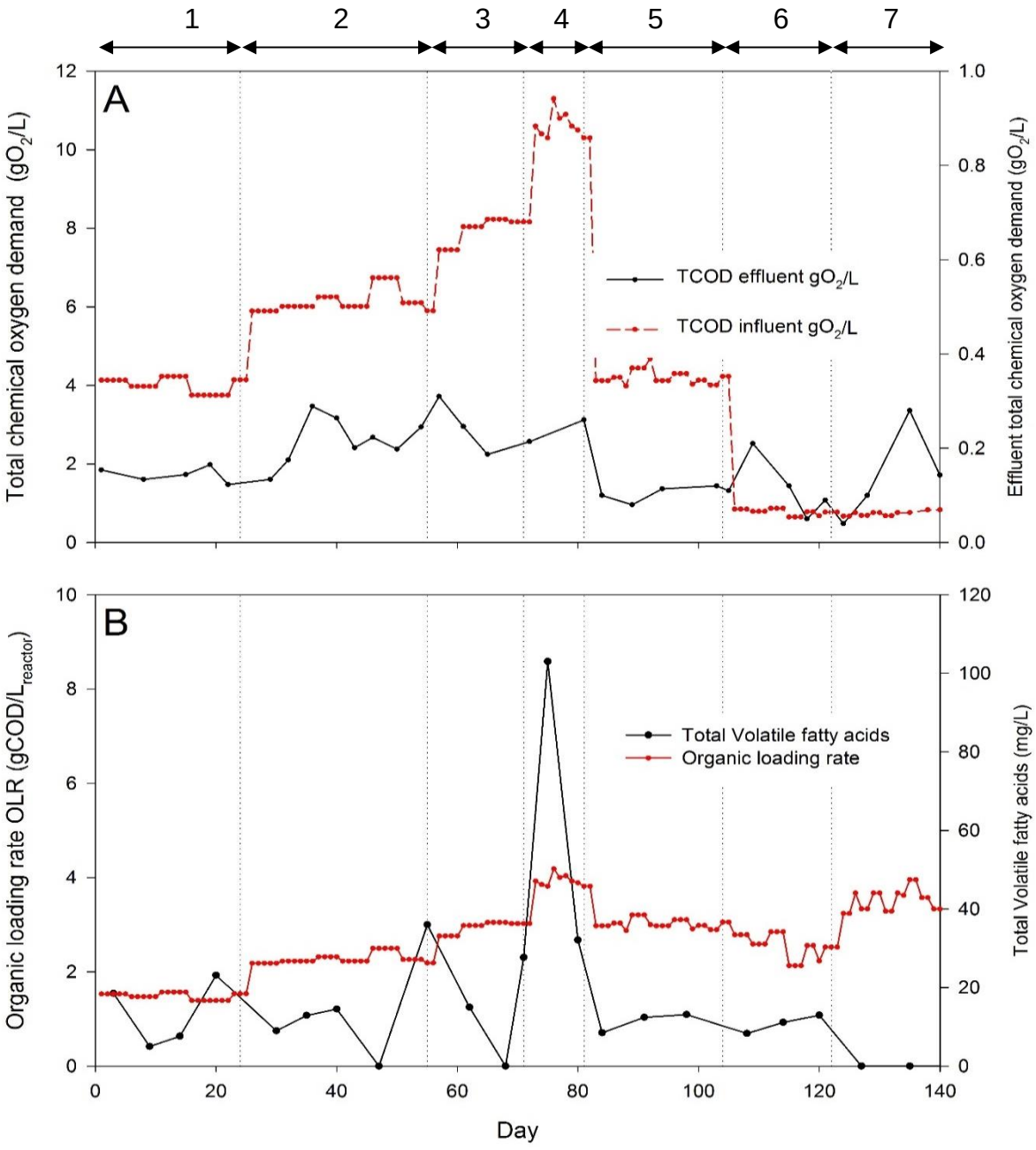

Figure 6-5 Total chemical oxygen demand and volatile fatty acid in influent and effluent in UAnMBR; (A), TCOD influent and Effluent. (•) denotes TCOD influent, (•) denotes TCOD effluent; (B), (•) denotes VFA levels, (•), denotes OLR.

Ras et al. (2011) which is likely a more appropriate means of comparing performance, than species alone.

6.4.3. COD destruction

Levels of effluent COD and volatile fatty acids remained very low across all Phases of operation. Effluent COD did not exceed 0.320 gCOD/L with no observed trend in effluent COD when OLR increased. As HRT was reduced in Phase 5 there was an observed reduction in effluent COD from 0.260 g COD/L on day 81 prior to HRT change, to 0.105 gCOD/L on day 84 following the shift in hydraulic state, a result of dilution of the reactor soluble COD from greater quantities of influent media. No change in overall COD removal was observed as total mass of COD leaving the system per day remained steady. COD levels fluctuated across Phases 5, 6 and 7 but still remained low until day 135 where there was an increase to 0.281 gCOD/L. COD in the effluent remained low and remained consistent indicating algal biomass was retained within the system, and the majority of the available soluble carbon was utilised, even at increased organic loading rates.

Volatile fatty acids leaving the system are very low across all phases of operation reaching below detection limit on a number of occasions. The VFA levels agree with effluent COD levels which remained similarly low indicating that the process is stable and has the potential, based on effluent levels to allow an increase in OLR further

The low levels of both COD and VFAs in the effluent indicate that methanogenesis was not limiting, with hydrolysis likely to be the limiting process. A recalcitrant component of the cell (assumed to be cell wall) remains un-degraded, similar to results in Chapter 5 which show that even with increased SRTs of 44 days, cell

material could not be degraded completely. This is consistent with existing literature which demonstrates in BMP/degradation experiments that up to 41% of the algal cell can be resistant or poorly degradable (Foree and McCarty, 1970), and shows significant variability in degradation between species.

6.4.4. Solids accumulation and destruction

Solids levels increased within the reactor across Phases 1 – 4, indicating incomplete degradation of algal cells in the system, with an indication of significant quantities of cell material remaining in the reactor (Figure 6-6A).

The theoretical solids accumulation was calculated based on a reactor starting concentration of 0 g/L and used the influent solid rates fed into the reactor. The model assumes no degradation and is used to illustrate the difference in theoretical accumulation and actual solids levels within the system. With it being a membrane system there is retention of 100% of algal biomass, which only leaves the system either through biodegradation to methane/soluble components, or undegraded solids removal which was affected by solids retention time. Reactor solids concentrations are approximately 5 times lower than the theoretical limit of the MBR system based on the influent feed and SRT removal rate. Comparing the expected solids destruction based on gas yield with the actual solids destruction observed in the reactor based on VSS measurements indicates a large difference between the two. This discrepancy in values could be related to; accumulation of solids in the system without measurement, components being solubilised but not converted to methane and leaving the system through the membrane, alternative aerobic or anoxic processes resulting in no biogas but degradation of algal

biomass, formation of cake on the membrane surface binding biomass, or biogas is leaking from the system.

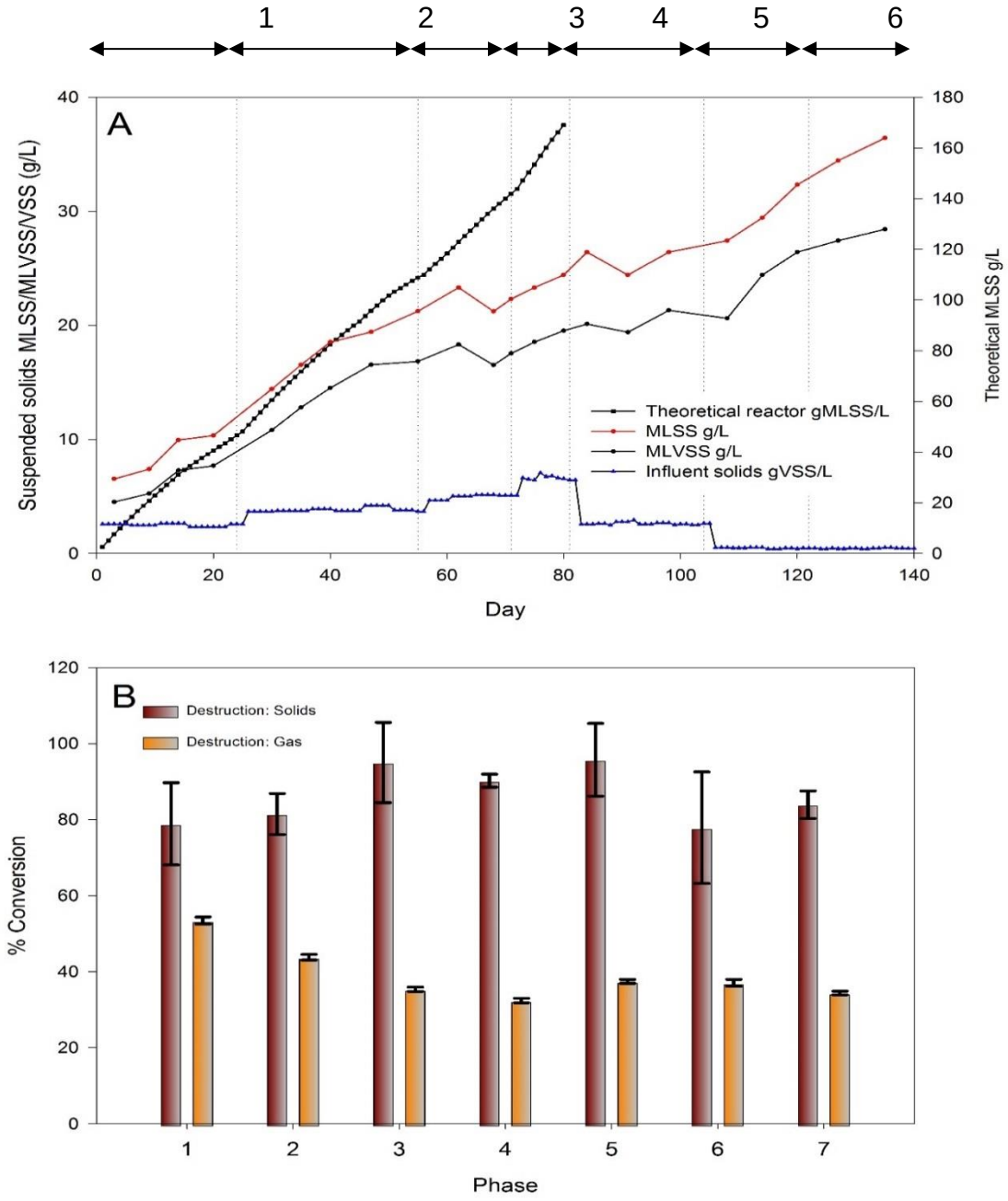

Figure 6-6A Observed reactor influent solids (TSS) and mixed liquor solids (MLSS and MLVSS). • denotes MLSS, • denotes MLVSS and ▲denotes Influent TSS. ◘ denotes theoretical solids accumulation based on no degradation and a starting concentration of 0 g/L. Figure 6-6B. Comparison of calculated solids destruction based on reactor solids data, and reactor solids destruction based on gas data. Error bars denote standard deviation, n =3 varies between Phases**.**

The method of measurement and sampling of solids from the reactor, coupled with the configuration of system could also be partly responsible for the differences observed. The membrane reactor had previously been used in other research and showed changes in structure of sludge from flocculated to free suspended cells (Yuzir *et al.,* 2013). It was proposed that the cells are larger than the membrane used (0.2 µm) but small enough to pass through the GFC type filter papers used for TSS/VSS measurements. It is possible that the differences observed in this research result from this phenomenon, and so the change in structure of flocs/cells could have accounted for this large disparity. Microalgal cell solubilisation to by-products larger than the pore size of the membrane, but smaller than the average pore size of the GFC filters for TSS measurement, could also have resulted in retention of products in the systems that were not degradable, but too small to register during TSS/VSS measurements. Reactor TCOD measurements correlate further with this hypothesis, while SCOD measurements indicate a fractionation of different “soluble” components based on filter size used for measurement.

The final mechanism for differences in destruction rates is the loss of soluble components either through the membrane or by other competing processes. Although soluble components left the system as VFAs and COD (Figure 6-5) the quantity was relatively small (<0.32 gCOD/L, <100 mg/L VFAs) compared to the differences in destruction rates observed, meaning solubilisation without conversion through to methane is unlikely. Alternative processes that utilise soluble by-products such as acetate could have co-existed in the system, including aerobic and anoxic processes through different specific inorganic acceptors such as nitrate, sulfate or sulphite. The introduction of oxygen in the system through influent, or photosynthesis by remaining whole cell algae could have led to

preferential use of acetate by aerobic metabolism due to the greater associated Gibbs free energy (Rittmann, 2001). The same mechanisms exist for potential anoxic processes, such as denitrification which could have utilised any nitrate in the influent (Akunna *et al.,* 1992), or similarly with sulphate reduction (Rittmann, 2001). The latter process would produce compounds in the liquid phase such as HS^- which are inhibitory to methanogens, and would have reduced methane production rates further (see Section 6.4.6). These mechanisms do not fully explain the differences between solid destruction observed and theoretical solids destruction based on gas production. At the end of the experiment when decommissioning the reactor system, it was clear that un-degraded solids had been accumulating in the upper part of the reactor, and in the reactor dead spaces. These solids had a noticeable green appearance, were extremely thick and contained some granules of anaerobic sludge biomass. The solids concentration of these solids was too high to measure using TSS, but approximately 124 g TS was collected from the head of the reactor. The locations of solids accumulation in the reactor is shown in Figure 6-7. The accumulation of solids within the reactor system demonstrates that mixing was not optimised for operation with microalga feedstocks. The natural buoyancy of microalgae presents unique challenges to reactor design, which in this research might have resulted in underperformance of the reactor, and lower methane yields compared to other studies (Zamalloa *et al*., 2012b), and a drop in performance at elevated OLRs. Residual methane potential test (BMP) was carried out on this accumulated solids with a BMP of 0.150 $LCH_4/gCOD_{in}$ demonstrating that there was considerable methane potential remaining in the accumulated sludge.

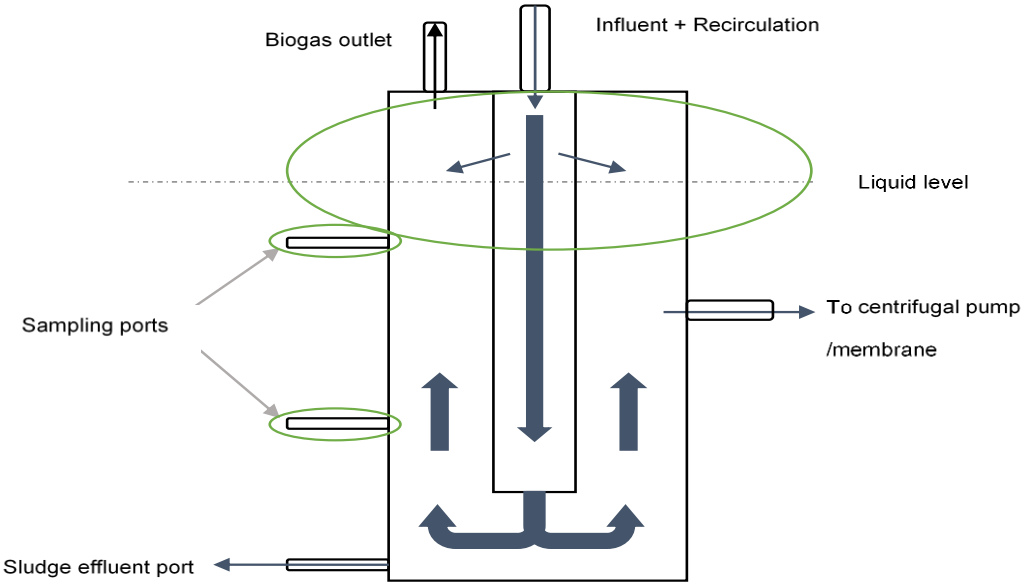

Figure 6-7 Illustration of position of un-degraded accumulated microalgal solids in the UAnMBR. Green circles illustrate location of solids deposits. Diagram not to scale.

6.4.5. Nitrogen mineralisation

Influent TKN concentrations increased across Phases 1 – 4 with a peak in influent levels of 850 mgTKN-N/L achieved at Phase 4 (Figure 6-8). Total ammonia levels increased over time, with effluent levels reaching as high as 554 mgTAN-N/L reported. Effluent ammonium levels were slightly lower than expected and observed previously in Chapter 5 (Figure 5-7), likely due to the accumulation of solids observed in Figure 6-7, but as consistent with levels calculated based on VSS destruction previously calculated (Sialve et al. 2009). The levels of total ammonia nitrogen leaving the reactor were well below 1.7 g – 14 g/L reported to be inhibitory (Chen *et al.,* 2008).. The high levels of nitrogen in the system offer a

real potential for recirculation back into the micro-algal cultivation (Uggetti et al., 2014).

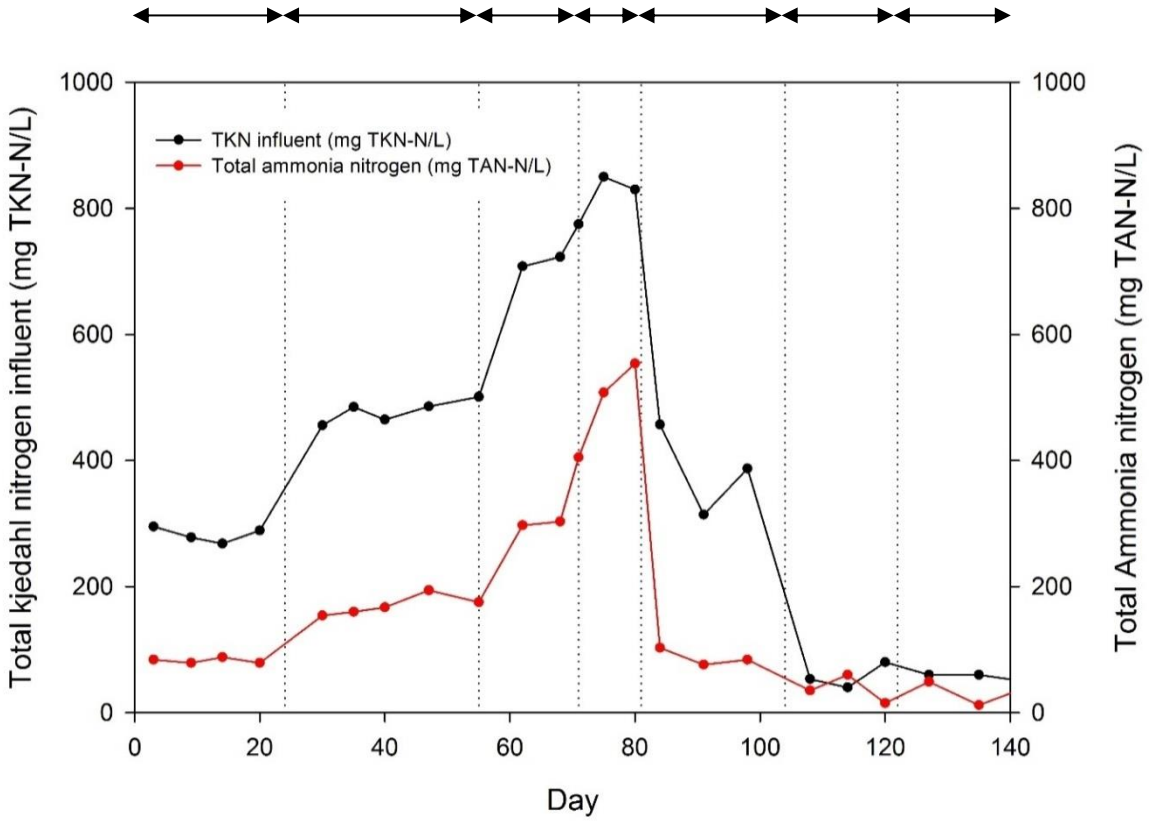

Figure 6-8 Total Kjedahl nitrogen influent and Total ammonia nitrogen effluent across different OLRs and HRTs. •denotes influent TKN, • denotes effluent TAN.

The difference in reactor pH and potential inhibition between Chapter 5 and 6 can be attributed to the increase solid retention time, allowing greater abundance of microbes tolerant of elevated ammonium, while greater residual buffering capacity present in the UAnMBR influent from the residual cultivation media which was present in higher quantities than concentrated feed for Chapter 5.

The drop in the effluent concentration of total ammonia nitrogen after Phase 5 is a result of a drop in hydraulic retention time, alongside small reductions in organic load. The drop in HRT resulted in a dilution of the liquid phase ammonia to well

below previous levels, and offered a suitable mechanism to reduce any potential inhibitory effects that might have existed from high levels. Phase 6 was where the hydraulic retention time was dropped further to 0.3 days, with the feedstock being switched to a dilute culture without any pre-concentration (settling) treatment. Organic load was kept the same as Phase 5, while effluent levels of TAN dropped further averaging 95.5 mg TAN-N/L, indicating increased hydraulic throughput and greater dilution of nitrogen levels.

6.4.6. Hydrogen sulphide content of biogas.

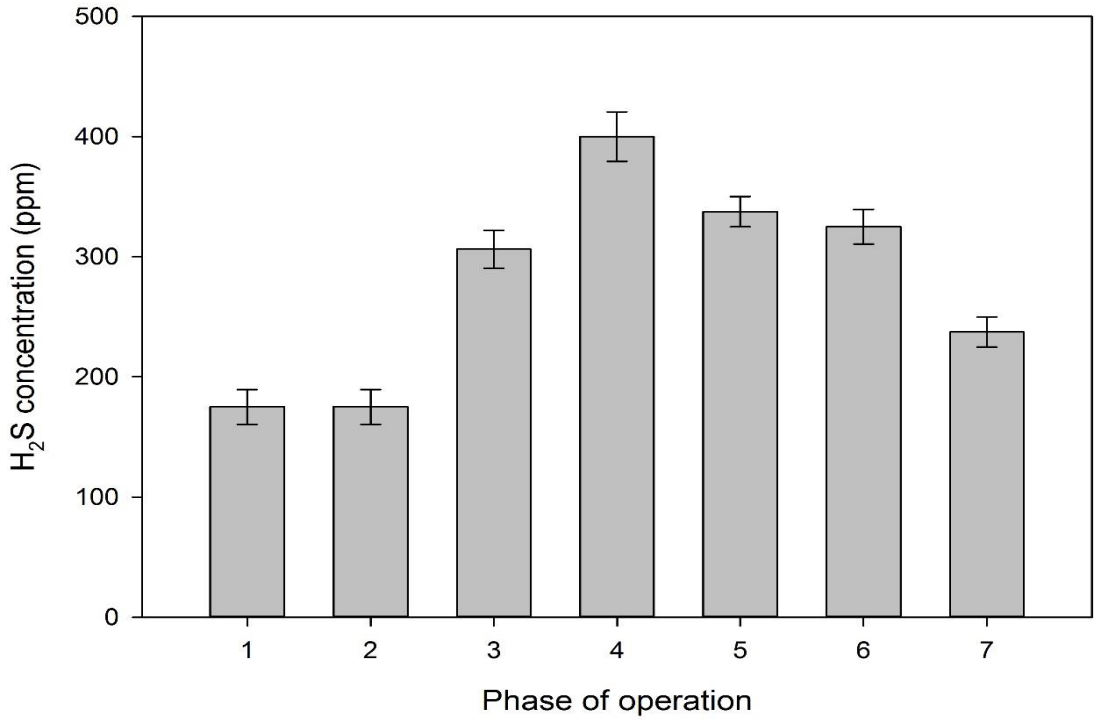

Figure 6-9 Hydrogen sulphide concentration in UAnMBR biogas during different phases of operation. Error bars denote standard error n=4.

Hydrogen sulphide concentration in the biogas remained relatively low across all phases of operation. Between Phase 2 and 4 the concentration increased from 175 ppm to 400 ppm, concentrations being significantly below the 20,000 ppm

observed in the digestion of macroalgae (Briand and Morand, 1997), or the 4,100 ± 500 ppm previously reported for microalgae digestion (Zamalloa *et al.,* 2012a). Microalgae are known to contain relatively low levels of sulphurated amino acids (Becker, 1988), and so hydrogen sulphide has not been addressed as a significant issue for microalgal AD (Sialve *et al.,* 2009). Although lower than reported in macroalgae AD, the 400ppm reported in this research is still above the 100 ppm recommended maximum concentration required for combined heat and power (CHP) units to prevent corrosion and odours (Peu *et al.,* 2011), meaning the biogas would require treatment before use in CHP plants.

The relatively low observed concentrations of H_2S than theoretical values can be attributed to a number of factors:

- Precipitation of what appeared to be elemental sulphur on the walls and headspace of the reactor, potentially through biological light driven desulphurisation (Basu *et al.,* 1994). The precipitation of sulphur might have contributed to the accumulation of solids observed in the headspace (Figure 6-7), and indicates that oxygen was present in the headspace of the digester. The presence of oxygen likely through introduction in the media, and some continuing photosynthesis reactions from whole live cell microalgae.
- The presence of residual trace elements from the microalgal cultivation media resulted in the precipitation of H_2S from liquid phase to metal sulphides, resulting in a shift in gas phase equilibrium. A mechanism which has been proposed for improving macroalgal digestion through additional metal dosing (Nkemka and Murto, 2010). Utilising microalgae to remediate

metal rich wastewaters may effectively add to H_2S control when algae is digested. This mechanism may have had beneficial effects on reactor performance through trace metal and sulphide supplementation to methanogens when limited (Daniels *et al.,* 1986), while offering a reduction in numbers of SRBs present and the immobilisation of any potentially metals found to be toxic to methanogens (Jin *et al.,* 1998)

- The presence of residual nitrate used in the cultivation media. Nitrate has been shown to affect H_2S with the precipitation of metal sulphides, whist also out-competing SRBs thermodynamically (Cirneet al. 2008). This may have led to de-nitrification, and utilisation of COD in the system producing the low values observed.
- Variation in intracellular sulphur concentration between species (Becker, 1988) led to differences in H_2S in this study with other studies (Zamalloa *et al.,* 2012b). Zamalloa *et al*., (2012b) used marine microalgae, which contained higher quantities of sulphate in the growth media which may have contributed to higher levels of H_2S reported. This provides a justification for use of freshwater microalgae over marine systems when using a membrane system fed on dilute cultures to reduce any impact of H_2Smicroalgae.

Between Phase 5 and 7, a drop in gas H_2S concentration was observed. The trend of reducing H_2S levels cannot be related to a significant change in conversion efficiency as this was not observed between Phases 5 and 6 (Figure 6-9), and so must be related to the effect of changing hydraulic retention time. Decreasing the hydraulic retention, meant increased flow through of cultivation media, increasing the supply of trace metals and nitrates, and potentially increasing the precipitation

of sulphur. This mechanism can also help explain the difference in H_2S concentration compared to elevated concentrations found in Chapter 5 when the reactor was fed on the same microalgae, but in concentrated pre-washed form. The increased hydraulic throughput could also have resulted in an equilibrium between liquid phase and gas phase H_2S different from that seen in other studies. Further investigation is needed to understand the balance between the different forms of sulphur, and the individual toxicity, during the operation of microalgae-fed reactors, with a mass balance approach to further quantify and understand the effects of low hydraulic retention times on H_2S formation. This is also relevant to the operation of anaerobic membrane reactors fed on other sulphur rich substrates.

6.4.7. Membrane performance

No noticeable drop in membrane performance occurred during the course of this study (Figure 6-10). Increasing solids concentrations and very low hydraulic retention times exposed the membrane to large fluctuations in operating conditions, but it appeared to perform extremely well, producing a stable and clear effluent.

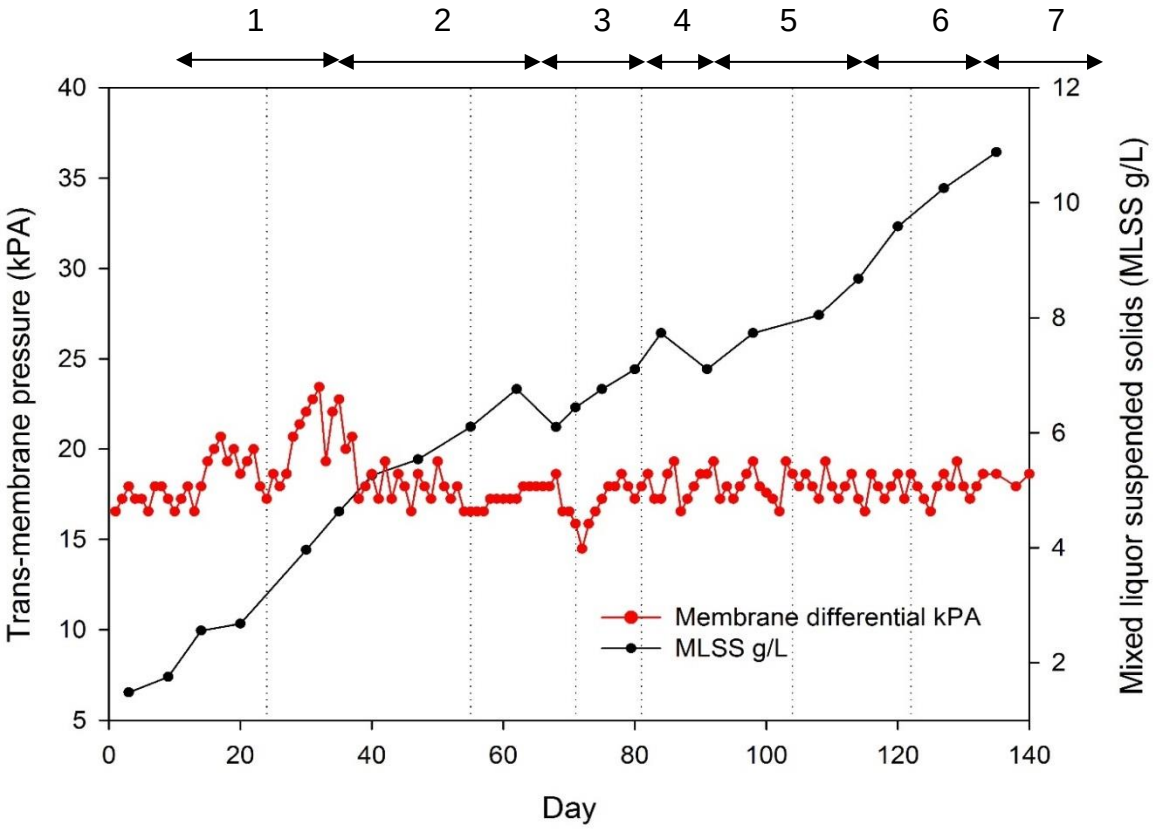

Figure 6-10 Comparison of trans-membrane differential pressure and mixed liquid suspended solids in the UAnMBR over the duration of operation; (•), denotes membrane differential (kPA); (•) denotes MLSS (g/L).

The membrane itself was not investigated to see whether a cake was forming, partly due to its construction restricting easy access, and partly due to the absence

of any reduction in performance over time, with the pressure differential averaging an almost constant 18.13 kPa (± 1.39). When reactor gas production had stabilised, effluent was always clear, low in soluble carbon and solids, and stable in pH. Given more time, the performance of the membrane could have been investigated extensively over more prolonged periods, but the period for the experiment was relatively short.

6.4.8. Reactor activity assessment

Specific methanogen activity (SMA) tests were undertaken over the course of the experiment to evaluate acetoclastic methanogen performance *(*Figure 6-11), the perceived dominant pathway of methanogens in microalgal anaerobic digestion. SMA appeared to drop from Phase 1 to 7, with initial Phases 1 and 2 showing values at the lower end the normal range 0.1 – 1.0 $gCODCH_4/gVSS/d$ (Soto *et al.,* 1992; Angelidaki *et al.,* 2009). Between Phase 3 and Phase 7 SMA dropped to levels well below those previously reported for laboratory systems. The drop in activity appears to indicate that the sludge became less active over time, and potentially indicates an imbalance in the process, or switch in dominant metabolic pathway. However, because of the nature of the substrate and membrane reactor, the drop is more likely a function of the accumulation of the microalgal biomass material reducing the proportion of anaerobic bacterial biomass in the sludge which was measured as total solids (TS, gVSS/L). Under these circumstances, comparing SMA using “activity per volume” or as a function of the whole reactor system, would be a more accurate means to determine changes in methanogenic activity.

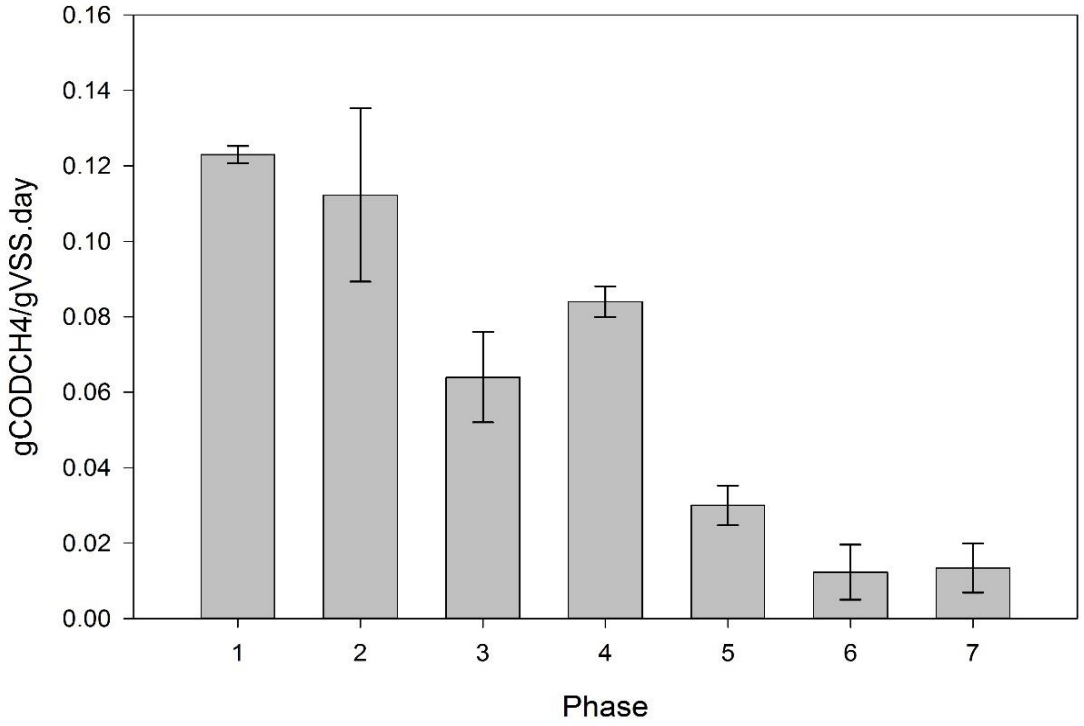

Figure 6-11 Specific methane activity (SMA) of the reactor sludge at different phases of operation. Error bars denote standard deviation, n =3.

6.4.9. Closing the loop: Microalgae cultivation potential

Coupling the microalgal cultivation system with the anaerobic digestion is an important consideration for the application of microalgal anaerobic digestion, and for the UAnMBR system in particular. Previous studies have demonstrated an ability to cultivate microalgae on anaerobic effluents (Olguin et al. 1994, Ras et al. 2011, Bjornsson et al. 2013).

Table 6-2 UAnMBR effluent characteristics at Phase 4, OLR of 12.2 gCOD/d.

Parameter	UnAMBR effluent
pH	7.2 (0.4)
Dissolved oxygen (mg/L	N/D
NH_4^+ (mg NH_4^+-N/L)	508.0 (45.4)
TKN (mg TKN-N/L)	595.3 (34.3)
NO_2^- (mg NO_2 – N/L)	N/D
NO_3^- (mg NO_3-N/L)	N/D
Alkalinity (mg $CaCO_3$/L)	1754.4 (334.4)
PO_4^{3-} (mg PO_4^{-3}- P/L)	190.4 (20.1)

N/D: Not detected (<5ppm)

Standard deviation in parenthesis

The typical effluent characteristics of the UAnMBR are shown in (Table 6-2) with stable pH, and high ammonium nitrogen dominating the TKN. No NO_3^- and NO_2^- were detected during this phase, but were present (< 20 mg NO_3^-) during Phases 5, 6 and 7 when fed on more dilute cultures cultivated with an excess of nitrate in the growth source. The presence of effluent nitrate could have had contributed to

lower levels of H_2S present (6.5.6) and low levels of soluble COD through de-nitrification.

The effluent was used in different dilutions for microalgal cultivation over a 10 - 12 day period. Growth rates during Batch 1 showed only a small difference in maximum growth rates of 0.33 and 0.38 $days^{-1}$ (Table 6-3), giving a doubling time of 1.82 – 2.1 days. The growth rates across all 3 concentrations during Batch 1 are lower than the growth rates observed in previous studies using anaerobic effluents (McGinn *et al.,* 2011), and in the main photo-bioreactor used to cultivate microalgae for the UAnMBR feedstock.

Total biomass yield for 10%, 5% and 2.5% concentrations of AD effluent (diluted with water) was 0.24, 0.19 and 0.28 gDW/L, respectively. All cultures showed good removal of ammonium, with the 2.5% effluent condition having the highest removal

Table 6-3. Growth rate, doubling time, biomass yield, initial ammonia, and ammonia usage of microalgae cultivated on UAnMBR effluent from Phase 4 over two batch cycles (Standard deviation in parenthesis).

Batch cycle	Effluent concentration (%)	UnAMBR (mg NH_3 - N/L)	Growth rate ($days^{-1}$)	Doubling time (days)	Total biomass yield (g/L)	NO_3^- (mg/L)	NH_3-N Remova %
1	10.0	53.23 (5.4)	0.33	2.10	0.24 (0.1)	0.0	43.4
1	5.0	22.21 (4.4)	0.38	1.82	0.19 (0.1)	0.0	59.5
1	2.5	10.12 (3.2)	0.34	2.03	0.28 (0.1)	0.0	96.4
2	10.0	50.12 (1.9)	0.55	1.26	0.38 (0.1)	0.0	61.8
2	5.0	20.16 (2.3)	0.61	1.14	0.33 (0.1)	0.0	83.5
2	2.5	12.32 (1.3)	0.66	1.05	0.29 (0.1)	0.0	100.0

(Standard deviation in parenthesis)

efficiency compared to other conditions, albeit with the lowest starting concentration of nitrogen. The long lag phase seen in Batch 1 suggests that a period of acclimatisation to the new culture media was required – specifically, the nitrogen source and concentration was switched from nitrate to ammonia at more elevated concentrations.

Batch 2 showed a shorter lag phase (Figure 6-12), with much higher growth rates for all 3 concentrations, improved biomass yields (0.29 – 0.38 gDW/L) and higher nitrogen uptake. The reduction in lag phase can be attributed to an acclimatisation of microalgae culture used to the different growth conditions. The doubling time ranged from 1.05 – 1.26 d, close to the 1 day reported for healthy cultivation systems (Chisti 2007). The growth rates observed in this research are lower than the 1.58 $days^{-1}$ previously reported for AD and algal AD effluents (Bjornsson *et al.,* 2013). The differences can be attributed to variability in species growth rates and to the different cultivation systems employed.

The effect of higher than normal nitrogen conditions does not appear to limit cultivation growth, however further investigation is needed to evaluate the cumulative effects of microalgal cultivation under elevated nitrogen concentration. Concerns relating to inhibition of growth or reduction in light transmission from anaerobic digestates raised by some researchers (Marcilhac *et al.,* 2014) were not observed in this study. This can be attributed to the dilute, low solids nature of the membrane reactor effluent operating at low HRT, when compared to normal CSTR systems, and is possibly also due the nature of microalgal culture itself, with low concentrations of humic and fulvic compounds (suspected) that could result in dissolved colour formation. The impact of this ecological shift on the methane potential and reactor performance is discussed in further detail in Chapter 7.

Further work is needed to translate the results from these simple batch cultivation tests to continuous open cultivation systems or enclosed photo-bioreactors at large-scale, to determine the true potential of reusing AD digestate for microalgal biomass production.

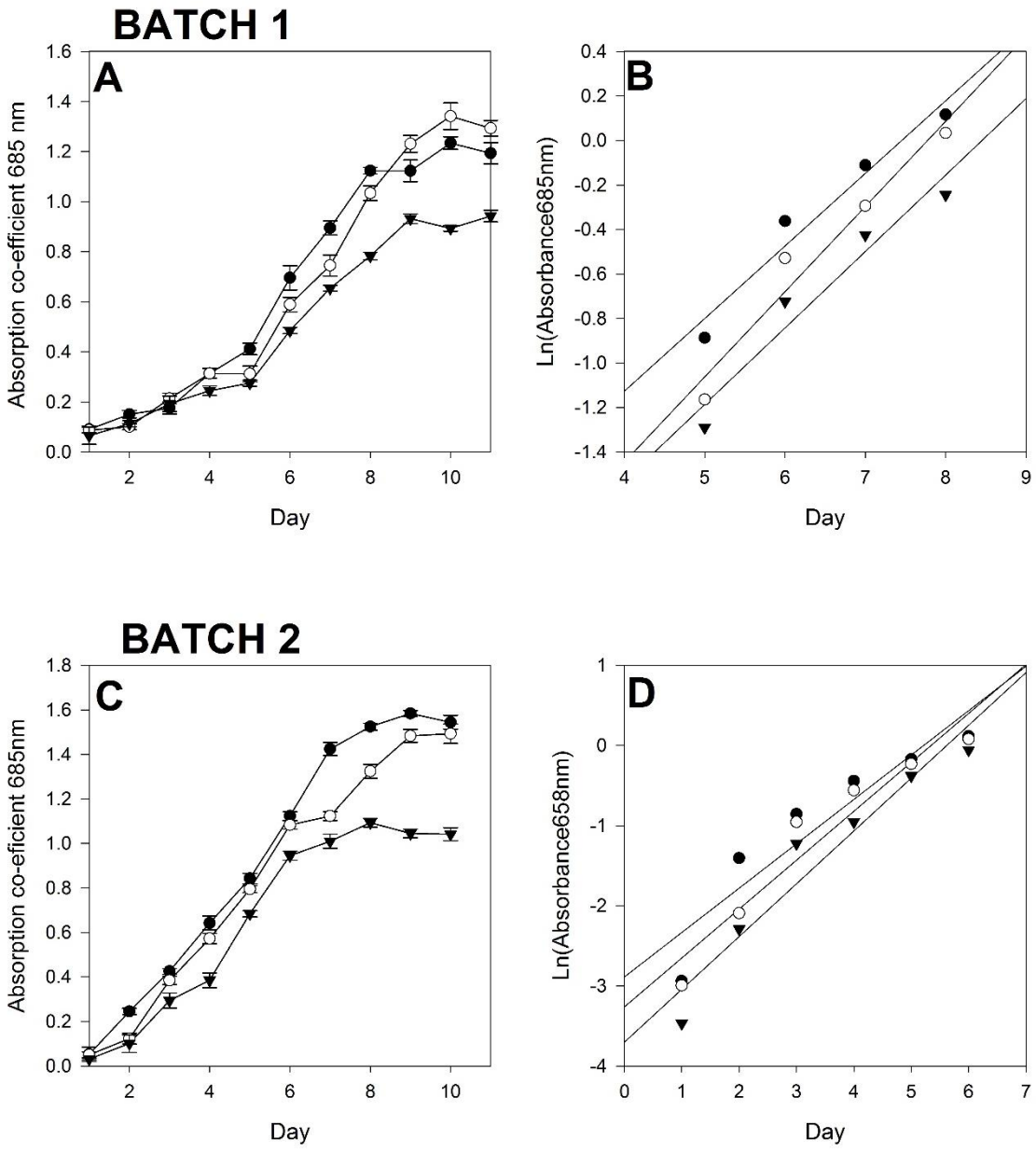

Figure 6-12. Growth curves of microalgae cultured on different concentrations of anaerobic effluent: (• 10%; ○ 5%; ▲ 2.5%); A, growth curve from Batch 1; B, linear regression of log transformed data for Batch 1; C, growth curve from Batch 2; D, linear regression of log transformed data for Batch 2

6.5. General Discussion

6.5.1. Effect of increasing organic loading rate

Increases in organic loading rate resulted in a drop in conversion efficiency of microalgal biomass to methane, and greater accumulation of microalgal solids in the reactor (Table 6-4). Not all solids were degraded with considerable amounts remaining in the system, especially in the dead spaces of the reactor. Although a solid retention time of 44 days compared to the simple system used in Chapter 5 (<25 days) showed some improved conversion of the algal cells to methane the conversion efficiency was still relatively poor, albeit consistent with existing literature Increased SRT allows the retention of more algal biomass, containing components that take longer to degrade and are typically washed out of the system in lower SRTs, while also allowing a potentially larger community of anaerobes to exist. The difference in microbial community for the UnAMBR and the CSTRs (Chapter 5) was not evaluated and could be used to prove or disprove this hypothesis. Improvements to the design of the up-flow reactor configuration, flow distribution and feed mechanisms, would likely reduce the accumulation of solids observed at higher OLRs, and allow the MBR to achieve closer to the ultimate methane potential on microalgal biomass under continuous operation, but there still remains a large component that may not be achievable without pre-treatment of the biomass. Creating more turbulent flow regime at the top of the system with baffles and high flow velocity, minimising oxygen present in the influent, repositioning of the reactor outlet/membrane inlet line to the top of the liquid level could encourage better solids distribution.

Table 6-4 Summary of the UAnMBR performance at different organic loading rates.

Phase	OLR (gCOD/ Lreactor.d)	Average methane Yield $LCH_4/gCOD_{in}$	Average COD conversion to CH_4 (% theoretical)
1	1.3 - 1.5	160.1	45.7
2	2.1 - 2.5	153.4	43.8
3	2.7 - 3.1	124.7	35.4
4	3.4 - 3.9	114.5	32.4
5	2.9 - 3.3	131.5	37.5
6	2.5 - 2.8	130.0	37.1
7	2.7 - 3.6	120.2	34.4

6.5.2. Effect of changing hydraulic retention times

Reducing the HRT appeared to have no effect on methane yield during Phases 5 and 6, but started to show some effect during Phase 7. The methane yield was lower during these phases compared to Phase 1, but this can be mainly attributed to the accumulation of un-digested microalgal solids in the dead space of the reactor or potential washout of trace elements and vitamins

The reduction in HRT allows significant dilute feedstock's to be fed in, but potentially introduces trace amounts of nitrate residual in cultivation media, alongside dissolved oxygen. These potentially cause precipitation of sulphur, and de-nitrification to take place within the digester, reducing available soluble carbon products available for methane production. The impact of HRT reduction on microbial community should be further investigated, alongside better chemical

composition of the AnMBR effluent to determine whether the system retains enough trace elements for optimum digestion.

The use of synthetic microalgal growth media at low HRTs in much of this research may have contributed a number of other process benefits including; supplementation of trace elements and vitamins present within the BB media that may not be present in real growth mediums (industrial wastewaters)

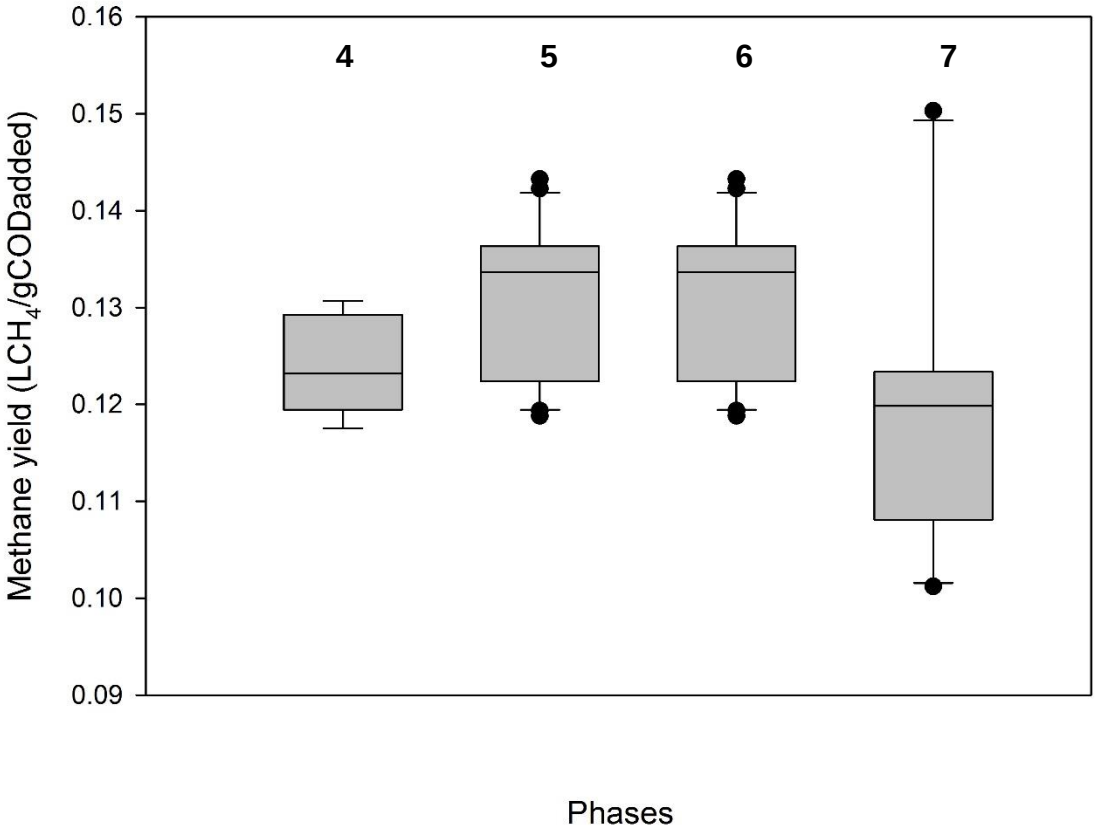

Figure 6-13. Box plot showing the effect of HRT on specific methane yield in UAnMBR. Median and standard deviation are shown.

6.6. Conclusion

The UAnMBR offers some potential for slightly higher methane production compared to mesophilic CSTR systems (Figure 5-2) due to higher retention of anaerobes, and microalgal biomass. Although methane yield at low OLRs was improved compared to that observed in Chapter 5, higher OLRs and low HRT appeared to result in a reduced overall performance with exact reasons unknown. There appeared to be no negative effect from the microalgal biomass on the membrane performance, and membrane fouling was not evident over the course of the experiment. The ability to operate the UAnMBR with dilute feedstock's, and at elevated OLR is a promising feature, reducing the need for energy intensive harvesting or prior concentration of microalgal feedstock's. Generating an effluent high in nitrogen, with no suspended solids, stable pH, and sufficient buffering, meant that the UAnMBR offers good potential for recycling effluent for microalgal cultivation or for other uses. The growth yield of microalgae from this system was comparable to that of microalgae grown in domestic wastewater, with no evidence of inhibited growth rates from use of anaerobic digestion effluent, as observed in previous studies. The next stage in this research would be to attempt a full integration of these systems with cultivation through to AD processing. Such research might include a hybrid high rate algal pond/activated sludge system that provided a mixture of bacteria and microalgae which could be concentrated and co-digested in a membrane bioreactor system. Overcoming issues such as oxygen introduction from live cultures, higher levels of metals and other compounds in growth mediums, and some potential for inhibitory compounds from the degradation of co-digestates like activated sludge.

Chapter 7 Strategies to improve and control methane production from microalgae: effect of cultivation conditions, species selection and harvesting processes.

7.1. Introduction

Methane yield from microalgae has been shown to be limiting and although anaerobic reactor operating conditions can have some moderate improvement in yield (Chapter 5, and 6), without engineered intervention to drastically improve yield, the energy yields remain unfavourable. An assessment of the energy balance and economic potential of the whole systems demonstrates a required conversion efficiency of 75% (VS to CH_4), alongside requiring a number of ambitious feed in tariffs €0.133kwh^{-}1 and carbon credits of €30 ton$^{-1}CO_{2\ (eq)}$ to justify the economics of the system (Zamalloa *et a.,* 2011). Bottlenecks to the process are cultivation yields, harvesting to produce a concentrated feedstock, and microalgal biomass conversation to methane.

There are a number of different strategies that have been proposed to achieve better methane yields including: different chemical, thermal and biological pre-treatment technologies (Gonzalez-Fernandez et al., 2013; Ciudad *et al.*, 2014; Mahdy *et al.,* 2014a); identification of the optimum microalgal species (Mussgnug *et al.,* 2010), and optimised reactor configuration (Zamalloa *et al.,* 2012a & b). The biotechnological manipulation of cultures to enhance certain desired microalgal characteristics has been proposed as a method to control and manipulate microalgae for product enhancement (Barra *et al.,* 2014).

Most work to date explores the impact of environmental factors such as macro- and micro nutrients concentration, light intensity and carbon dioxide concentration to modify and improve yields of carbohydrates for bioethanol (Dragone *et al.,* 2011) and lipids for biodiesel (Lv *et al.,* 2010; Juneja *et al.*, 2013). Only limited work has been undertaken to explore the manipulation of cultivation, and pre- and post-conditioning of cultures for improved yields (Mussgnug *et al.*, 2010; González-Fernández *et al.,* 2012).

Trying to understand and improve conversion of microalgal biomass to methane through culture manipulation, species evaluation and post-cultivation conditioning is vital to understanding and improving the potential of the microalgal AD technology. Improvements need to be balanced against any additional process requirements and associated economic costs.

7.2. Aims and Objectives

- Improve methane yield from a mixed culture of microalgae through cultivation manipulation and digestion strategies.
    - Evaluate the methane potential of different species and identify any taxonomic link with methane yield.
    - Evaluate any change in biochemical composition of the algal culture, and methane yield over a batch cultivation cycle.
    - Evaluate the effects of nitrogen concentration on biochemical composition of algal culture and subsequent methane yield.
    - Evaluate the effects of storage conditions of microalgal biomass (temperature, concentration) on the methane yield over time.

- Evaluate the effects of drying microalgal biomass before anaerobic digestion on the methane yield.

7.3. Methods

7.3.1. Analytical procedures

As described in previously (4.2).

7.3.2. Bio-methane potential test method

The bio-methane potential test used throughout Chapter 7 as a tool to determine both ultimate methane yield and hydrolysis rates between a number of different experimental factors. The method was a modification of the methods described by Owen *et al.,* (1979), Angelidaki *et al* (2004) and Angelidaki *et al* (2009).

Prepared active inoculum (7.3.3) was placed in a glass 125 ml (160 ml total volume) serum bottles (Wheaton, USA) with de-gassed revised anaerobic mineral media prepared according to (Owen et al. 1979). The final algal biomass/substrate concentration did not exceed 2g tCOD/L, with a target inoculum substrate ratio (ISR) of (2:1 or 3:1) depending on the activity of the inoculum measured using a SMA test. The inoculum substrate ratio was determined previously and is an important parameter to ensuring an accurate and reproducible BMP test. The liquid level was then topped up to a final volume of 60 - 80 ml using distilled water depending on concentration of sludge inoculant. BMP bottles were then sealed with butyl rubber septum and aluminium crimp caps (Sigma Aldrich, UK) and degassed using 80 % N_2: 20 % CO_2 (BOC gases, UK) for 10 minutes. The BMP test was performed in triplicate, plus a number of different controls. The controls were outlined previously (Angelidaki *et al.,* 2009). To summarise:

- Sludge control containing only sludge, media and water known as the blank or sludge control.
- Cellulose control, containing sludge, media, water and 1 g/L of amorphous cellulose (Sigma Aldrich, UK). This was designed to test the hydrolytic activity of the sludge.

The microcosm bottles were kept inverted at 35 ± 1.0C and mixed at 150 RPM in an orbital Incubator for a period up to 60 days (Stuart Scientific, UK). Temperature was logged on a manual alcohol thermometer, or later in the study using a digital data logging thermometer attached to a K type thermocouple (Lascar electronics, UK).

Figure 7-1 Batch bio-methane potential test bottles

7.3.3. Anaerobic sludge preparation for BMP test

Original inoculum for was taken from a 50:50 (v:v) mixture of citric anaerobic sludge and an on farm mesophillic digester treating cow and pig manure (Newcastle University Cockle Park farm, UK). The sludge was sieved through a 2 mm sieve to remove large fibrous debris, and then stored in a sealed container at 4 ± 1.6°C until use. Prior to the BMP test sludge was warmed at 35°C for a period of 5 – 10 days with a small quantity of mixed culture microalgae to acclimatise sludge to new substrate conditions, followed by a period without substrate to remove any trace VFAs present and “degas” the inocula. Subsequent BMP tests were undertaken with acclimated microalgae AD sludge taken from reactors in Chapter 5 and 6. Prior to use they followed the same acclimation and degassing procedure as the original BMP inocula above

7.3.4. Gas sampling

Gas was sampled directly from the headspace of the bottle using a 100 µl gas tight syringe equipped with a pressure lock (SGE, Australia) according to Hansen *et al.,* (2004) and is illustrated in Figure 7-1. The sample was then directly injected into a GC-FID as described previously (4.2.4).

The concentration calculated was then converted to quantity by multiplying volume produced by concentration, and normalised to STP and corrected for water vapour as previously described Methane produced from the substrate was determined by removing the inocula methane production and then first order hydrolysis rates were determined.

7.3.5. Effect of microalgal species on methane yield

Different microalgal cultures were obtained from external and internal sources, including both marine and freshwater species. Marine based microalgae were cultivated in F/2 + Vitamins media (Table 4-3), whilst freshwater microalgae were cultivated in BBM + Vitamins media (Table 4-1, 4-2). Cultivations were prepared as previously described Section 4.1.2. Microalgal biomass was cultivated in either a 10L clear Nalgene container (Thermofisher, UK), or a 10 L glass aspirator (Pyrex, UK). The culture vessels were placed under an illumination of 16/8 hr light/dark cycles at an approximate illumination intensity of 70 - 100 µMol Photons.m^2/s, provided by fluorescent lights as previously described (Section 4.1.1), and provided with constant aeration at (2 – 4 L_{air}/min), controlled by a variable area flow indicator (RS components, UK). Measurements of biomass were taken through optical density at 685 nm measurements (OD_{685}) as previously described (4.1.3), with the microalgal biomass harvested using centrifugation (4.1.2).

7.3.6. Effect of cultivation cycle in the biochemical composition and methane potential of mixed culture microalgae.

The life cycle experiment was designed to test how microalgal composition, and subsequently methane yield, changes over the life cycle of a batch culture. The microalgae were a mixed population culture used in Chapters 5 and 6, grown on BBM+VIT media in the 22 L photo-bioreactors with constant aeration (4 L/min) as described earlier. The mixed culture was chosen due to difficulties in growing sufficient biomass using a single culture in a mixed use laboratory. Multiple photo-bioreactors were used to provide sufficient harvested biomass for compositional analysis, and the biomass yields and bio-methane potential tests. The inoculum for

the photo-bioreactors was taken from another photo-bioreactor in early exponential growth phase growing on the same media. The aeration was turned off and algae were allowed to settle and was used to inoculate each photo-bioreactor with approximately 2 g of pre-concentrated algal biomass, based on TSS, per photo-bioreactor to give an approximate starting concentration of 100 mg/L. Samples were taken regularly for VS, TOC, TKN, BMP, LHV, protein, carbohydrate and chlorophyll-a analysis as previously described. Lipids were assumed to be the remaining balance between the sum of proteins + carbohydrates + ash, and normalised to VS content. Microalgae were harvested by centrifugation using two stages to improve biomass yield from centrate during the early stages of the microalgal culture.

7.3.7. Effect of nitrogen concentration on the bio-chemical composition of microalgal cultures, and subsequent methane yield.

Three different starting nitrogen conditions were trialled to explore the effects of this cultivation strategy on microalgal biomass growth rates, biomass yield and the impact on the methane yield. Light intensity, aeration were kept identical to earlier cultures, while multiple batches were run simultaneous to provide sufficient biomass for analysis.

The experiment used the same mixed microalgal culture used throughout this thesis (*Chlorella and Scenedesmus sp.)* with BBM + VIT as the base media, with varying concentrations of nitrate, this experiment being based on two previous studies, which evaluated the effects of nitrogen concentration on lipid production in *Chlorella* and *Nannochloropsis* species of microalgae (Converti *et al.,* 2009; Lv *et al.,* 2010). The nitrate concentrations investigated were 0.58 mM, 2.9 mM and

11.6mM $NaNO_3$/L, the range being set higher (4X) and lower value (X0.25) than the standard nitrate concentration used in BBM+VIT media (2.9 mMol). The media was prepared as previously described Section 4.1.2, and cultures were harvested routinely for chemical analysis as described in Section 7.3.4. Biomass samples from replicate growth bags were pooled to ensure an adequate quantity of homogeneous material was obtained for analysis. The cultures were harvested after 7 days, a growth period previously shown to give high methane yields (7.4.2). Harvesting was undertaken immediately to prevent change, and samples prepared for bio-methane potential tests straight after concentration and solids determination.

7.3.8. Effect of post-harvest storage conditions on methane yield.

A mixed culture of microalgae was harvested from 22L photo-bioreactors in early exponential phase as previously described (4.1.3). Two storage concentrations were tested, in the first, biomass was pre-concentrated using centrifugation, then stored in 250 ml closed bottles, while in the second, the dilute culture was stored in 20 L Nalgene closed storage containers (ThermoScientific, UK) without concentration. The bottles and containers were stored at two temperatures in the dark: room temperature (18 ± 6°C) and cold room temperature (4 ± 1.1°C). Samples were taken for TCOD, DOC, TOC Chlorophyll-a, soluble carbohydrate, soluble protein and bio-methane potential at set intervals. Routine in-situ measurements of pH and dissolved oxygen were taken, while anions were evaluated at the start and end of storage. All analytical procedures were as previously described (4.3).

7.3.9. Statistical analysis

All statistical analysis (Students two sample t test, and One-way ANOVA and Mann Whitney, and Kruskall Wallis) was undertaken in Minitab (Minitab, USA). Significance was deemed to be at 95 % confidence ($p<0.05$).

7.4. Results and Discussion

7.4.1. Effect of different microalgae species on methane yield.

Different microalgae were chosen to compare the methane yield grown under similar light, nutrient and CO_2 conditions. All microalgae species showed relatively poor degradation under test conditions compared to theoretical maximum yields (Figure 7.2) but the wide variation in yield between species is consistent with previous work which has shown considerable variation in methane potentials of microalgae (Table 3-4). The highest methane yield was achieved by *Dunalia Salina* at 0.270LCH_4/gVS_{in}, and *Phaedactylum tricornutum at 0.231*LCH_4/gVS_{in} whilst the lowest was *Nanochloropsis Oculata* at 0.105 CH_4/gVS_{in}

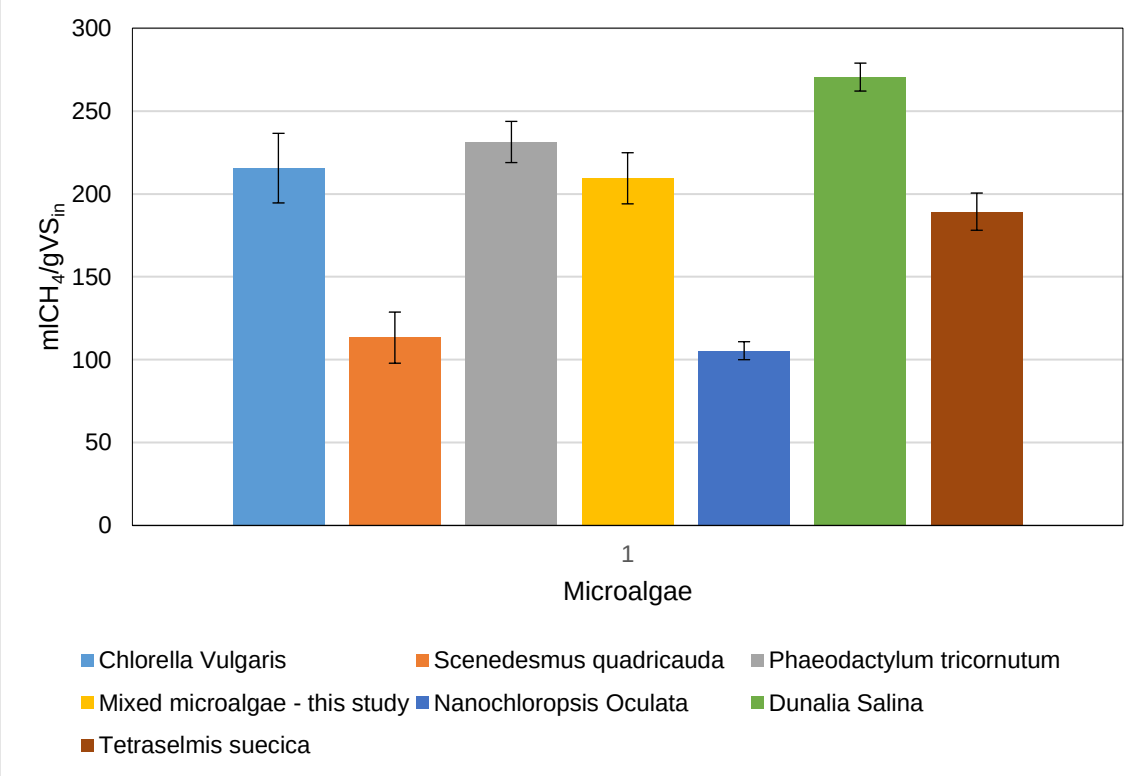

Figure 7-2 Net bio-methane potential of different microalgae species (marine and freshwater). Error bars denote standard deviation.

Table 7-1 Summary of the first order hydrolysis rate constants (k) for different species of microalgae in BMP tests.

Microalgae Species	First order rate k (d^{-1})
Chlorella Vulgaris	0.114
Nanochloropsis Oculata	0.099
Scenedesmus quadricauda	0.079
Dunaliella Salina	0.140
Spirulina maxima (Arthrospira	0.100
Phaeodactylum tricornutum	0.104
Tetraselmis suecica	0.078
Mixed microalgae (Chlorella and Scenedesmus) sp.)	0.123

Calculated according to Eq. 4-14 (Angelidaki *et al.,* 2009). Further details are included in Appendix.

These results are consistent with previous work which has shown *Phaedactylum sp.* to be a relatively high methane yielding species compared to other species such as *Scenedesmus* sp, (Zamalloa *et al.,* 2012a), while the methane yield of 0.215 LCH_4/gVS_{in} for commonly used species Chlorella vulgaris was a little lower than previously reported under continuous reactor conditions (Ras *et al.,* 2011; Wang *et al.,* 2013). Dunaliela Salina was another high yielding microalgae producing 0.270 LCH4/gVSin, albeit lower than the 0.323 LCH4/gVSin previously reported for the same species (Mussgnug *et al.,* 2010).

The different first order constants (hydrolysis rates) are shown in Table 7-1. The mixed microalgae culture used throughout this thesis had the highest rate at 0.123 d^{-1}, closely followed by *Chlorella Vulgaris* at 0.114 d^{-1}. The small difference and close relationship was expected as *Chlorella sp.* was the dominant part of the

mixed microalgae culture. The lowest kinetic values were obtained for *Scenedesmus and Tetraselmis* sp.at 0.079 and 0.078 d^{-1}, respectively. The first order values obtained are in a similar range to those observed by Zamalloa *et al*., (2012) who calculated values of 0.11 and 0.14 d^{-1} for *Scenedesmus* and *Phaeodactylum* sp., respectively, and demonstrated that *Phaeodactylum* is one of the more readily degraded microalgae, and with a high methane potential. The values obtained for microalgae hydrolysis are well below those observed for wastes, such as activated sludge, which have been shown to have first order hydrolysis constants of 0.169 d^{-1} (Ferreiro and Soto, 2003). The addition of enzymes was shown to significantly improve hydrolysis of sludge with rates of up 0.576 d^{-1} observed when using mixed enzyme addition at optimum temperatures (Yang *et al.,* 2010), and could present a suitable method to improving methane yield from microalgae (González-Fernández *et al.,* 2012).

Comparing the methane yield and hydrolysis constants between marine and freshwater cultures shows average methane yields between marine and freshwater environments was 0.170 LCH_4/gVS_{in} and 0.192 LCH_4/gVS_{in}, respectively, with a greater range observed for freshwater species (Figure 7-3). This was shown to be not significant ($p>0.05$), suggesting either the cultivation environment (between marine and freshwater medias) does not significantly affect the methane yield, or the genetic differences between microalgae in marine and freshwater environments do not control ultimate methane yield. No significant difference was observed between hydrolysis constants ($p>0.05$). The lack of significance between marine and freshwater species digestion results is in direct contrast to Mussgnug *et al.,* (2011) and Zamalloa *et al.*, (2012a) who showed that marine species *Dunaliella and Phaeodactylum sp.* disintegrate faster than freshwater species such

as *Scenedesmus* sp. Their proposed mechanism for improved degradation was a rapid switch between saline and freshwater environments causing cells to rupture and release cell contents. This is logical, and it is likely that rapid shifts in salinity does play a role in degradation rates. But the response to rapid changes in salinity is likely to be species related, primarily cell structure and the individual alga's response to stress. This is highlighted by the significant difference between the methane yield observed for *Dunaliella Salina* observed here, and the methane yield of *Dunaliella tertiolecta* (Lakaniemi *et al.,* 2011).

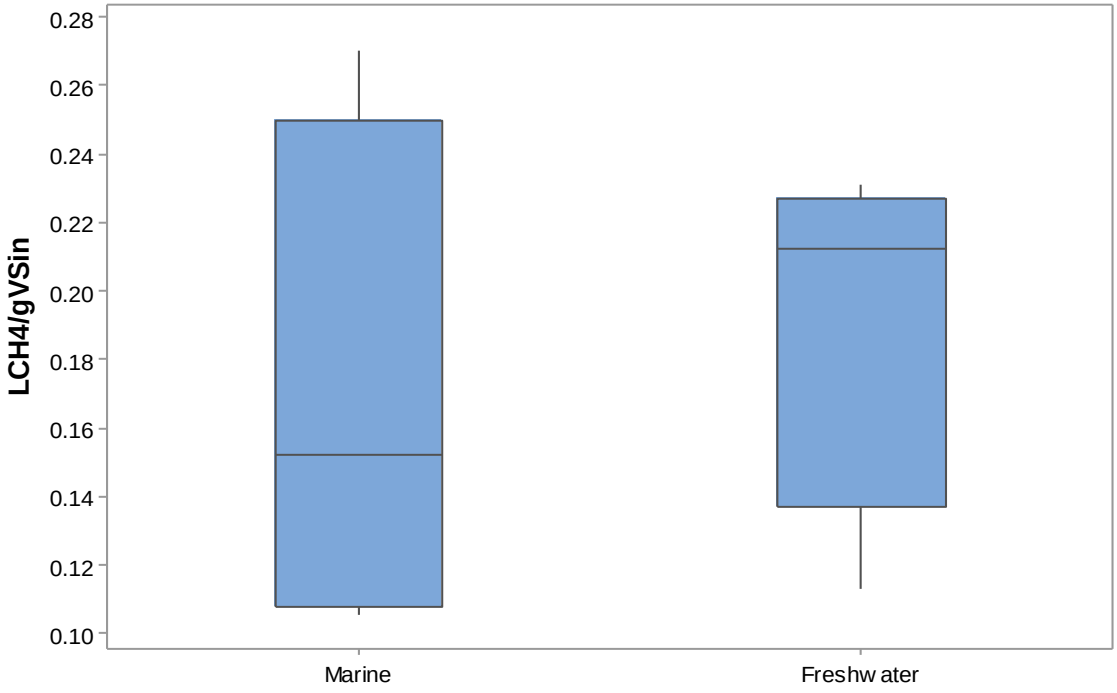

Figure 7-3 Boxplot comparison of methane yield of freshwater and marine microalgal species used in this study.

The second factor affecting marine cultures is the potential for sodium and sulphate inhibition. Sodium inhibition and dominance of SRBs are of real concern for anaerobic digestion of substrates having high sodium or sulphate ion content (Chen *et al.,* 2008). The difference in yields observed between marine and

freshwater in this research and by Zamalloa *et al.,* (2012a) does not show any evidence of inhibition. However, cultures were concentrated significantly prior to digestion and any residual media was washed prior to use removing excess sodium and sulphates. Further work is required to evaluate whether acclimatisation strategies can be used to overcome elevated saline concentrations.

A comparison of the calorific value of the microalgae feedstock to the methane yield indicates a poor correlation. This demonstrates that a microalgae species which has a high energy value does not necessarily indicate a high methane potential (Figure 7-4). The same poor correlation exists between C:N ratio and methane yield (Figure 7-5).

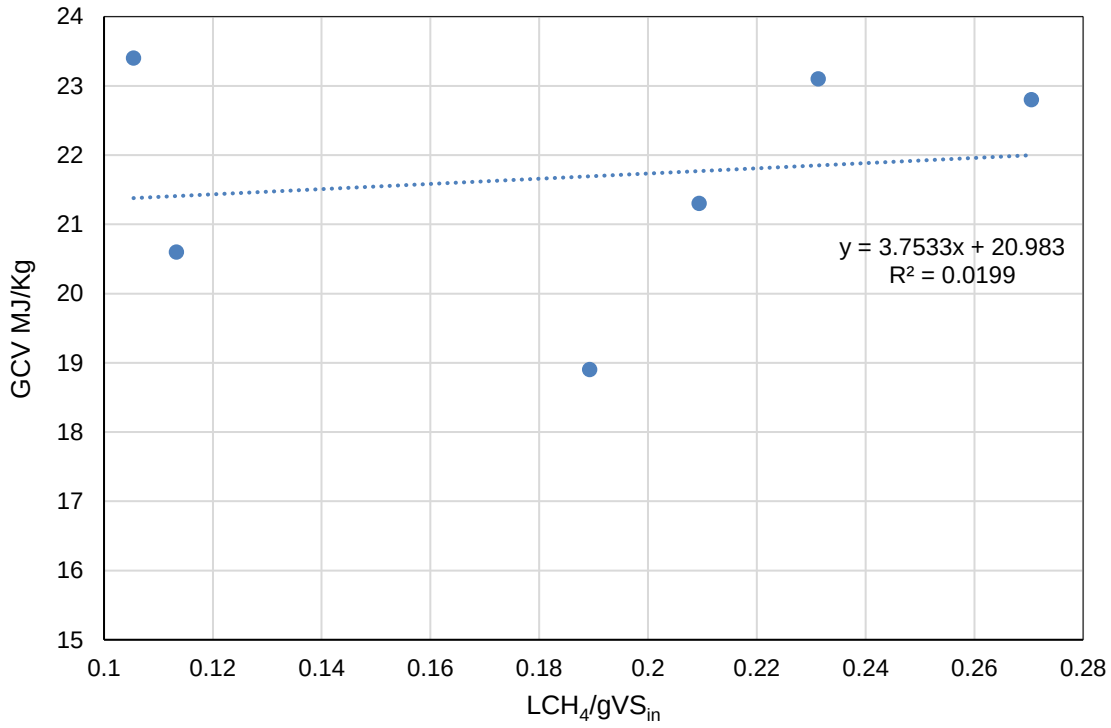

Figure 7-4 Correlation between methane yield and calorific value of microalgae.

The differences in methane yield observed between different microalgal species can be attributed variation in microalgae species composition and structure.

Increasing the composition of lipids could be an effective strategy to improving methane yield, whist accumulation of intra-cellular starch granules may present a method to providing a quick release of available sugars. Comparing the proportion of lipids, proteins and carbohydrates may yield further information, and be a predictive tool to assessing methane yield. *Chlorella, Dunaliella and Phaeodactylum* are species that have been demonstrated to accumulate intracellular lipids, and this may be responsible for both the high C:N ratio and calorific value observed in this study, as well as the higher than average methane yield. The high levels of lipids reported for *Nannochloropsis oculata* previously indicate that lipid yield alone does not determine high methane yields, but that other controls exist. The second control on methane yield is the structure of the microalga itself. Components of the cell (e.g. cell wall) that are poorly degradable under anaerobic conditions vary significantly between species, with not just one component being responsible. The resistance of the cell wall is the primarily mechanism which allows microalgae to resist degradation (Gerken *et al.,* 2013), and varies significantly between the species tested here. *Nannochloropsis oculata* has been shown to have a rigid cellulose encased walls, containing the resistant biopolymer algaenan (Gelin *et al.,* 1997; Simpson *et al.*, 2003), while *Scenedesmus sp.* has been shown to contain a strong crosslinked hydroxyl fatty acid monomer cell wall complexes, and be particularly difficult to degrade (Blokker *et al.,* 1998). The presence of cell wall structures that are difficult to degrade are in contrast to those of *Dunaliella Salina* which is known to have a limited cell wall structure (Sheffer *et al.,* 1986). Conflicting evidence to the presence or lack of cellulose or biopolymers in the cell wall of *Chlorella* sp. (Takeda, 1991; Baldan *et al.,* 2001), indicates either a variability of detection based on different analytical

methods, or significant variations in cell wall exist between species type. The lack of efficiency of cellulase's in disrupting these species either confirms the lack of cellulose in the cell wall, or that its presence can vary over time (Gerken, *et al.,* 2013). The variability observed for methane yields indicates that even for the same species cultured under similar conditions, methane yield can vary significantly, indicating that bio-chemical composition can influence methane yield, albeit to a lesser extent than cell structure.

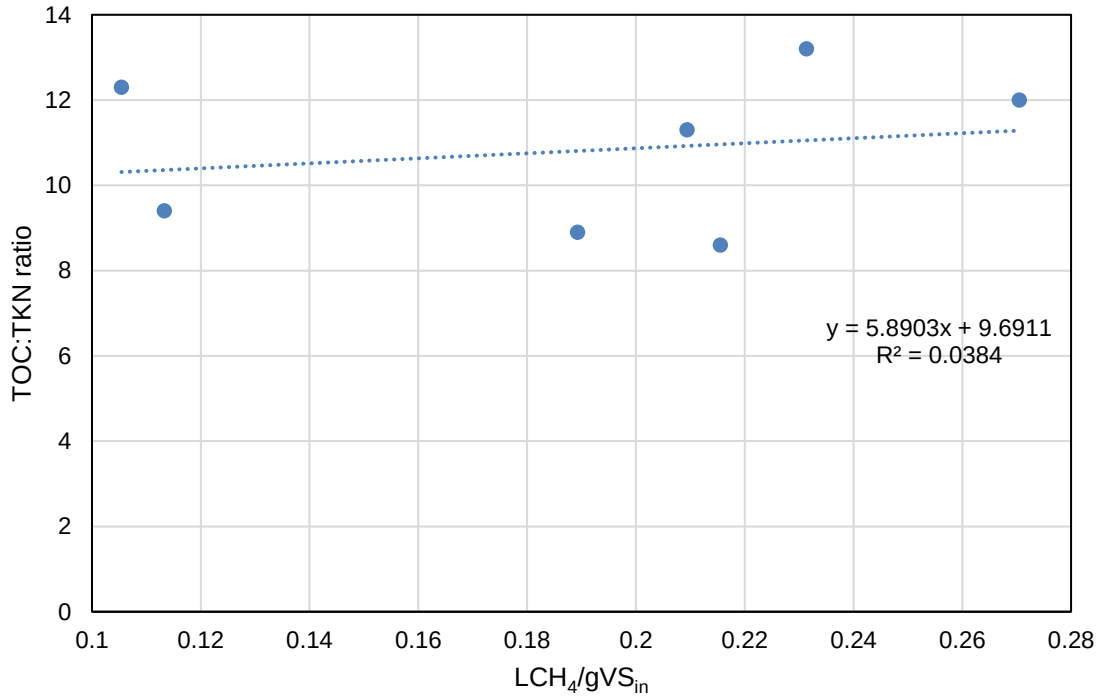

Figure 7-5. Correlation of C:N ratios of different microalga species with methane yield.

7.4.2. Effect of algal cultivation cycle on methane yield.

Table 7-2 Summary of the effect of growth cycle on the biomass production, biochemical composition and methane yield.

Days	Biomass (gTSS/L)	VS %	CH_4 Yield ($mLCH_4/gVS_{in}$)	Protein (%)	Carbohydrate (%)	Lipid (%)	C/N ratio	GCV (MJ/kg)	Theo. GCV* (MJ/kg)
0	0.099	88.4	223.6	66.4	21.2	14.4	6.1	17.8	23.7
4	0.334	90.1	230.5	62.1	22.4	15.5	7.3	18.8	23.4
7	0.410	87.5	283.3	54.5	27.5	18.04	8.9	19.6	23.4
11	0.445	92.3	236.3	46.6	29.3	24.1	11.3	22.3	24.2
14	0.466	94.6	175.1	37.8	32.8	29.4	11.9	22.8	24.8

The mixed microalgal culture reached a maximum biomass concentration 0.466 gDW/L entering a stationary phase at approximate 5 days, although the culture continued to increase in biomass concentration slowly after this period of exponential growth was over (Table 7-2). The maximum specific growth rate was $0.450 d^{-1}$, giving a generation time of 1.47 days. The growth rate was well below, and doubling time well above, those reported elsewhere, with $0.62\ d^{-1}$ observed in similar studies with *Chlorella Vulgaris* (Ras *et al.,* 2011), and the typical doubling times of 1 day reported by Chisti (2007). The differences can be attributed to the different operating conditions (algal strain, nutrient conditions, light intensity, CO_2 enrichment and transfer efficiency). Daily biomass yields found in the current research during exponential phase were 0.046 – 0.062 gDW/L/d, well below the 0.2 – 0.4 gDW/L/d observed in other studies (Chiu *et al.,* 2009). The lower biomass yields indicates that the system used in this research was poorly optimised for maximum biomass productivity.

Gross biochemical composition changed significantly across the experiment duration (Figure 7-6). At day 0, normalised protein content was greatest at 66.4%, whilst carbohydrate and lipids were 19.2 and 14.4%, respectively. Protein to TKN ratios were approximately 5.15, below the 5.95 reported for microalgal conversion (Gonzalez Lopez *et al.,* 2010). The TOC/TKN ratio was 6.1 (Table 7-2) a ratio at the lower end of the normal range (Geider and La Roche, 2002), reflecting the elevated protein content of the microalgal cell (Lourenco *et al.,* 2004) compared to typical algal cultures. Low C:N ratios at the start are typical of microalgal cells undergoing rapid cell reproduction where protein and biomass synthesis is dominant. Chlorophyll-a content was 0.54%, whilst the calorific value of microalgae

at 17.8 MJ/kg showed a reasonably high energy content, but lower than typical biomass energy content. The calorific value is a little lower than the average reported elsewhere but is still within typical reported ranges (Scragg *et al.,* 2002), and has been shown to vary significantly between species (Paine and Vadas, 1970).

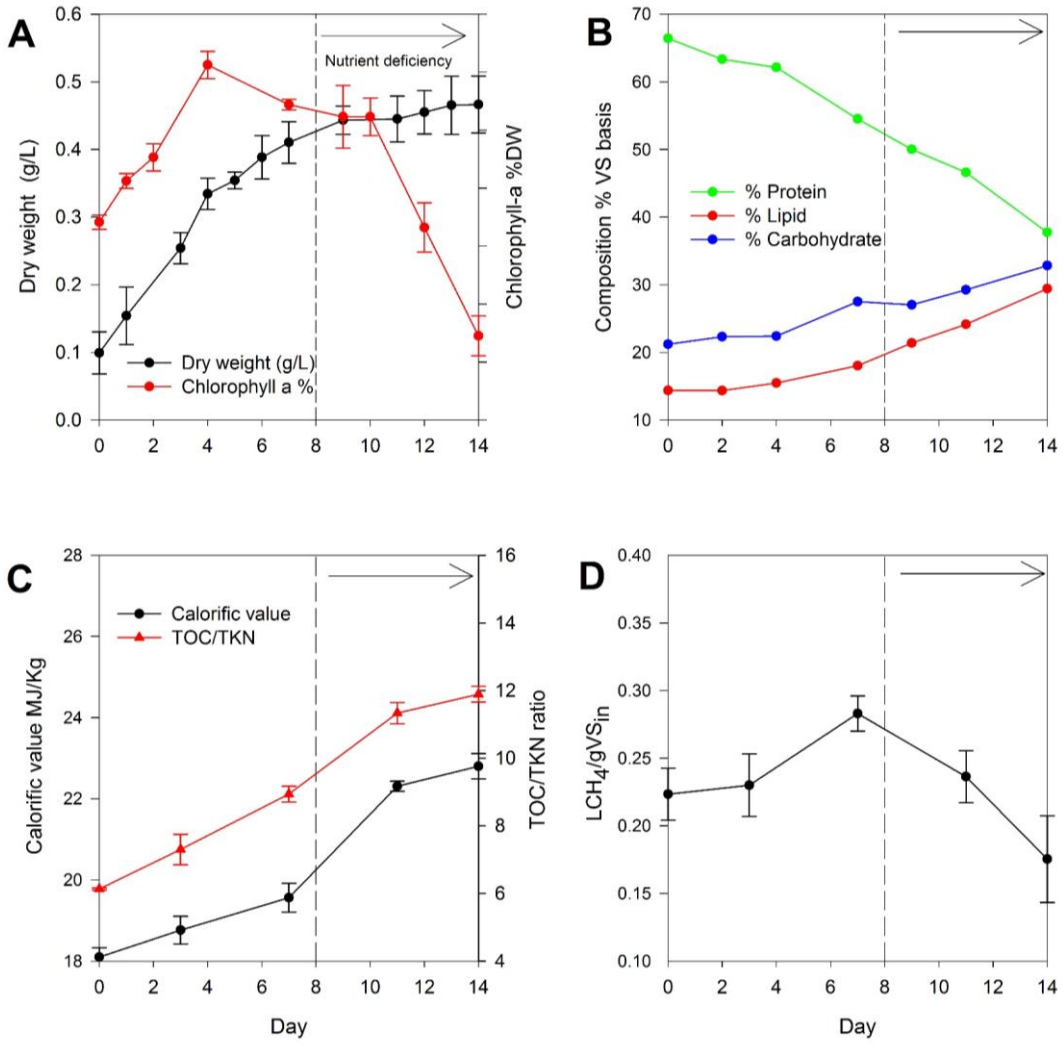

Figure 7-6 Impact of growth cycle of mixed culture microalgae on biochemical composition, energy content and bio-methane potential. A; Microalgae biomass production and chlorophyll-a content; B: Biochemical composition (Lipids, Carbohydrates and Protein content); C: Calorific value and TOC/TKN ratio; D; Bio-methane potential. Error bars denote standard deviation. Dashed line denotes point at which Nitrogen (NO_3) becomes limiting.

At day 4, when the culture was in mid- exponential phase of growth, the protein content dropped slightly to 62.1%, whist carbohydrate and lipid content increased to 22.4 and 15.5% respectively. The microalgal biomass being in the exponential phase of growth showed maximum daily biomass yields, with 0.080 gDW/L/d being measured. The TOC/TKN ratio increased to 7.3, in parallel with an increase in Chloropyll-a content to 0.812%. The calorific value was only slightly higher at 18.8 MJ/kg, potentially reflecting the small increase in lipid content compared to the start conditions.

At day 7 the algal culture was at the end of exponential growth, with a slower growth rate at 0.06d^{-1}, indicating the cultures were entering stationary phase. Biomass concentration had reached 0.413 g/L. Protein content had dropped to 54.5%, and both carbohydrate and lipids had increased significantly to 27.4% and 18.0%, respectively. Chlorophyll-a had dropped to 0.72%, while the TOC/TKN ratio had increased to 8.9, and calorific value had increased to 19.6 MJ/kg. Nitrate was almost exhausted by this point in the culture cycle, and was below the detection limits of the IC, indicating that cultures were experiencing nitrogen deficiency

At 11 days the algal culture was in stationary phase of growth, with biomass concentration relatively steady at 0.455 g/L. Protein content had dropped further to 46.6 %, while lipid and carbohydrate concentrations were 24.1% and 29.3%, respectively. The TOC/TKN ratio had increased further to 12.3, while calorific value was at 22.4 MJ/kg. Biomass concentration was 0.445 g/L while chlorophyll-a at 12 days was 0.5%

At the end of the experiment (day 14) microalgal cultures were in late stationary phase/early death phase, with a biomass concentrations of 0.480 g/L. Protein was

37.8%, while carbohydrate and lipid content had stabilised at 32.8% and 29.4%, respectively. Chlorophyll-a concentration was at its lowest at 0.310 %. Calorific value reached its highest value of 22.8 MJ/kg, while TOC/TKN increased slightly to 11.89, a value exceeding those in typical cultures, and indicative of nutrient stress and carbon driven growth (Geider and La Roche, 2002).

The increase in lipid and carbohydrate concentration over the course of the culture cycle, and concomitant decrease in protein over time was significant ($p>0.05$), and is reflected by an increase in TOC/TKN ratio, showing a shift in nitrogen content in the cell towards more elevated carbon content (reduction in protein with preferential accumulation of carbon rich compounds like lipids). The final lipid content observed is consistent with previous studies which have demonstrated *Chlorella Vulgaris sp*. can have lipid concentrations as between 20 – 40% of cell weight when cultures are exposed to nitrogen deficiency (Illman *et al.,* 2000; Griffiths and Harrison, 2009). The shift in nitrogen accumulation to carbon accumulation is reflected in calorific values which increased steadily from 7 days onwards as nitrogen was depleted from the media, with none being detectable by 9 days. Lipid accumulation is primarily thought to occur through deficiency in one of a number of different environmental requirements for cell growth; primarily nitrogen limitation has been shown to force microalgae to shift lipid metabolism away from membrane lipid synthesis towards the storage of intracellular lipids, thereby increasing total lipid content and potentially changing the structure of the cell membrane and its susceptibility to enzyme degradation (Hu, 2004; Chiu *et al.,* 2009). Growth rate and cell reproduction are significantly reduced when this switch in lipid metabolism occurs (Illman *et al.,* 2000; Li *et al.*, 2008).

The same response to nutrient deficiency has been demonstrated for carbohydrate content of the microalgal biomass. Carbohydrates accumulated from 22.4% at 4 days to 32.8% at 14 days. Levels being consistent with previous studies which have shown nutrient depletion can drive starch synthesis in *Chlorella Vulgaris* to between 37 – 41% (Hirano *et al.,* 1997; Dragone *et al.*, 2011). This phenomenon is also related to the diversion of carbon away from cell growth and towards intracellular storage products (Hu, 2004; Takeshita *et al.*, 2014). This directly impacts culture growth rates. Although starch was not directly assayed in this thesis it believed that a large proportion of the carbohydrate accumulated could have been a result of intracellular starch granules. This accumulation mechanism and the location of either carbohydrate or starch should be further investigated as the location has significant implications for the potential biotechnological use of microalgae.

Chlorophyll-a concentration changed significantly over the growth cycle (Figure 7-6). Increasing chlorophyll-a concentration reflects both an increase in cell numbers, but also an increase in intracellular chlorophyll concentration. During exponential phase of growth cells increase chlorophyll content to maximise light harvesting, while at higher cell densities it becomes part of a shelf-shielding mechanism typical in high density cultures. The drop in chlorophyll-a is related to nutrient depletion, reducing the ability and need to produce chlorophyll a. The reduction in chlorophyll-a corresponds with both a reduction in proteins and an increase in the C:N ratio of the algal cell, all pointing towards nutrient depletion as seen in the nitrate data. By day 14 the culture was showing clear signs of being a culture under growth stress, with turbid biofilm accumulation in the upper layer, and signs of foaming as a result

of extracellular polymeric substances being released from cells. The growth experiment was discontinued at this point.

Methane yield from the microalgal biomass varied significantly across the growth cycle ($p<0.05$) (Figure 7-6). The inocula biomass gave a methane yield of 0.223 LCH_4/gVS_{in}, and this yield remained consistent early in the growth (day 3). At day 7, towards the end of the exponential phase of growth methane yield increased 0.283 LCH_4/gVS_{in}, the highest yield observed during the course of the batch cycle. However, at 11 days methane yield had dropped again to 0.236 LCH_4/gVS_{in} and dropped further by the end of the experiment to 0.175 LCH_4/gVS_{in}. The change in the methane yield potential of the biomass over the duration of cultivation cycle is significant ($p < 0.05$). The wide range of values observed across the batch growth cycle are consistent with the wide range of yields reported previously for *Chlorella vulgaris* (Golueke and Oswald, 1959; Hernandez and Cordoba, 1993; Ras *et al.,* 2011), with most studies not disclosing exact culture conditions, or when they harvested. The increase in methane yield between the start and 7 days was expected due to observed accumulation of lipids (Figure 7-6). Lipids have a higher theoretical methane content compared to proteins and carbohydrates (Cirne *et al.,* 2007) and so any accumulation should result in higher methane yields(Sialve *et al.,* 2009; González-Fernández *et al.,* 2012). In parallel with higher lipid content, the higher C:N ratio observed could potentially improve digestion efficiency, and reduce any potential detrimental impacts of excess nitrogen accumulation. Furthermore, the rapid cell replication seen in exponential phase may have resulted in a reduction of cell strength wall, or cell wall components, which would have left the cell more susceptible to disintegration during subsequent anaerobic

degradation. There was a moderate increase change in first order hydrolysis rates across the growth cycle The drop in methane yield between day 7 and day 11 that occurred, while the concentration of lipids was increasing, was unexpected. The weak correlation between gross biochemical composition and methane yield (Figure 7-7) indicates that more than these biochemical parameters are important for methane production. This is in contrast to the link between calorific value of microalgae and their total lipid content, which showed a very good correlation (R^2 = 0.939), and is consistent with that previously observed (Scragg *et al.,* 2002).

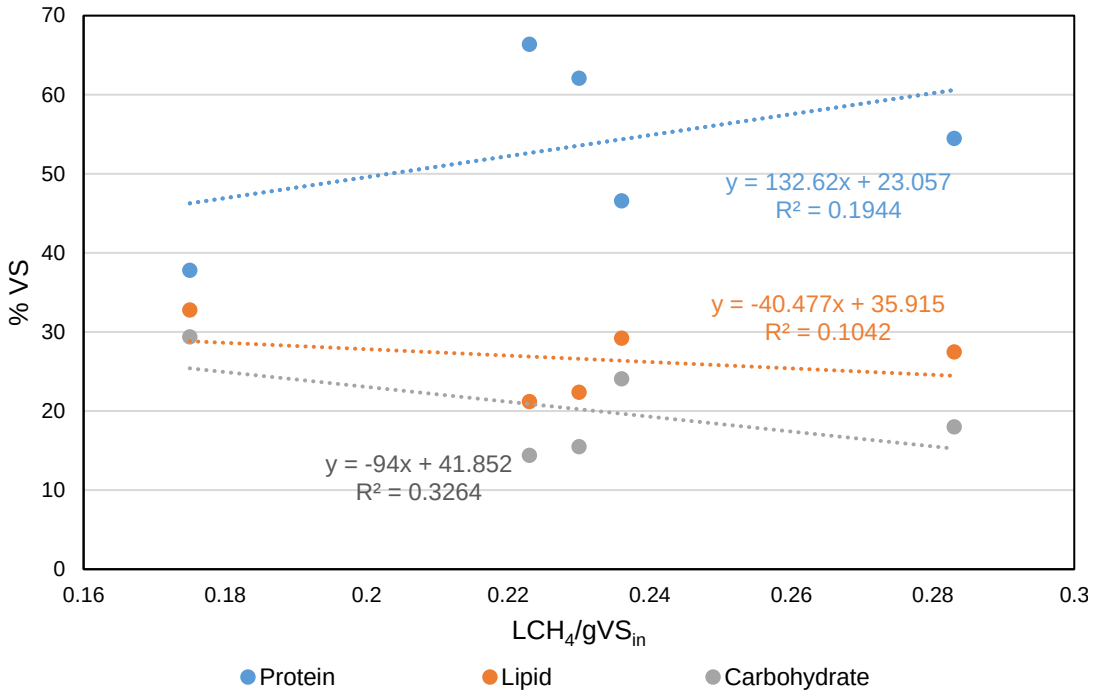

Figure 7-7 Correlation between biochemical composition and methane yield: (•) denotes Carbohydrate Vs Methane yield; (•) denotes lipid Vs methane yield; (•) denotes Protein Vs Methane yield.

The decrease in methane yield of microalgal biomass harvested between 7 and 14 days can be attributed to a number of different responses that microalgae show

when responding to stress conditions or biological degradation. These responses to stress can include:

- A switch in the chemical composition of accumulated compounds. Biochemical tests used here may indicate no significant change in mass of lipids or carbohydrates but the chemical composition can change which results in more or less digestible compounds. Low carbon dioxide concentrations have been shown to increase quantities of 22:6 (n−3) PUFA, while greater quantities of 14:0 fatty acids were found to predominate at higher CO_2 concentrations (Riebesell *et al.,* 2000). An opposite mechanism was observed where phosphorous limitation led to a reduction in the synthesis of n-3 PUFA (Reitan *et al.,* 1994), demonstrating a wide range of responses to stress that could significantly impact the anaerobic digestion of the biomass.
- Change in the location of storage compounds reducing the bioavailability of lipids or carbohydrates. Stress conditions have been shown to drive production and synthesis of lipids and carbohydrates, but their bioavailability can be restricted, and is strongly related to the location of these compounds (VanDonk *et al.,* 1997). Delayed cell division could result in these compounds being shifted to cell wall structures, so while an increase in total lipid might seem beneficial, the location could mean that they are essentially inaccessible. Cell wall thickening has been observed in micro algal cells over extended growth cycles, and when exposed to nutrient deficiency (VanDonk *et al.,* 1997; Gerken *et al.*, 2013). This mechanism has been

shown to directly reduce the susceptibility of cells to enzymatic attack from daphnia, and reduce the potential digestibility (VanDonk *et al.,*1997).

- Production of different cell wall components similar to cell thickening mechanisms. Resistant biopolymers such as algaenans (Simpson *et al.,* 2003; Rodrigues and da Silva Bon, 2011), or the formation of a new structural compound could occur through changes in growth conditions or environmental stress. Certain compounds are known to be highly resistant to bacterial degradation (Gunnison and Alexander, 1975), but the mechanism of synthesis and occurrence, like that of most other structural components in microalgal cells, is poorly understood (Popper and Tuohy, 2010). This mechanism could also explain why such a varying cell wall structure is reported for microalgae of the same species, even though genetic 18s RNA studies cannot distinguish between them (Takeda, 1991). This same potential mechanism of cell wall thickening was observed in *Botryococcus braunii*, and was directly linked to nutrient stress which prevented degradation (Gelin *et al.,* 1997). However, while the presence of cellulose in *Chlorella vulgaris* is disputed, there is a possibility that under certain environmental conditions, increases or shifts in cellulose characteristics to form a more rigid cell wall structure might take place. These results indicate that high levels of nutrients can stimulate higher biomass yields, and as long as nutrient levels remain high there is no significant drop in protein levels, and as a result methane yield is not maximised through preferential accumulation of lipids.

- Production of extra cellular exudates from microalgal cells inhibiting biological degradation. During nitrogen deficient conditions, diatoms have been shown to produce extracellular polysaccharides that have been shown to prevent enzymatic degradation (Malej and Harris, 1993). These extracellular products have been shown to prevent degradation of microalgae in Daphnia. The observation of foaming and biofilm/scum formation in the culture at 14 days correlates with the onset of this potential inhibitory mechanism, but no further investigation into products was carried out.

Table 7-3 Effect of nitrogen concentration on microalgal growth rates, total lipid concentration and ultimate methane yield, at 7 days.

Nitrogen media (mM)	Specific Growth rate ($\mu_{max}d^{-1}$)	Biomass conc. (g DW/L)	Max. Chlorophyll-a (%)	Protein content (%)	Lipid (%)	Carbohydrate (%)	Methane y ($mLCH_4/g$V
11.6	0.428	0.413	2.13	69.4	8.5	25.3	210.6
2.9	0.430	0.394	1.03	50.3	19.0	30.5	268.4
0.58	0.400	0.231	0.65	42.1	24.5	33.4	213.3

Methane yield changes significantly across the growth cycle, and could have implications for the expected yield from microalgal feedstock's in anaerobic systems. Therefore, the impact of environmental stress, forcing greater lipid accumulation, could ultimately cause either higher or lower methane yields from the biomass.

7.4.3. Effect of nitrogen cultivation conditions on microalgal growth and methane potential

Nitrogen concentrations of 0.58 mM $NaNO_3$ resulted in lower biomass yields than higher nitrogen concentrations conditions, reaching approximate steady state at approximately 3.5 - 4 days, although steady state was difficult to determine as no clear exponential phase or steady state was visible (Figure 7-9). Total biomass yield was low at 0.231 gDW/L while the maximum growth rate was 0.401 d^{-1} (Table 7-4 Increasing nitrogen concentration to 2.9 mM $NaNO_3$ resulted in higher biomass yields, with no distinct stationary phase apparent after 7 days, although it was envisaged that the stationary phase would have occurred very soon afterwards based on previous experiments (Figure 7-9). Total biomass yield was 0.401gDW/L, while growth rate was 0.410 d^{-1}. Increasing the nitrogen concentration further resulted in a higher biomass yield of 0.412 gDW/L, and a growth rate of 0.431 d^{-1}. The biochemical composition of the inoculum showed distinct differences with biomass at these elevated nitrogen concentrations (Table 7-3). As nitrogen concentrations decreased, carbohydrate concentration increased significantly, from 25.3 % at 11.6 mM $NaNO_3$, to 33.4 % at low concentration (0.580 mM $NaNO_3$). Lipid concentrations increased with reduction in nitrogen concentration, from 8.54 % to 24.54 % at 11.6mMol $NaNO_3$ to 0.580mMol $NaNO_3$, respectively. At both medium and low concentrations, nitrate levels at the end of the growth phase were below the detection levels (Appendix E), indicating that nitrogen limitation existed in a similar manner to that described previously (7.4.3). The biochemical composition at the highest nitrate concentration was very similar to that of the start inoculum, indicating that with excess nutrients no major changes occur in

biochemical composition over cultivation cycle when nutrients are present in excess. The composition of medium and low differs significantly to that of the inoculum on day 0 and the high nutrient concentration on day 7. Lipid concentrations are significantly higher in both medium and low nutrient conditions compared to both the inoculum and the high condition. Low nutrient conditions have a lower protein and carbohydrate fraction, similar to day 11 in 7.4.3.

Microalgae grown at medium nitrate concentrations achieved similar methane yields to microalgae grown in 7.4.2, at 0.268 LCH_4/gVS_{in}, while high and low conditions gave similar yields to the inoculum. The results were unexpected, as it was hypothesized that under low nitrogen conditions an accumulation of intracellular lipids and carbohydrates might have led to improved methane yields. Low nitrogen conditions gave low biomass productivity, and the culture reached stationary phase quicker than under other nutrient conditions. The lipid concentrations at day 7 were comparable to those at seen in 7.4.2 (for 11 d growth), but the methane yield of the microalgal biomass did not reflect this abundance of lipids. The reason behind this may be related to the point at which the biomass was harvested for analysis. Under low nitrogen conditions, the cells had clearly entered stationary phase by the time they were analysed for methane yield and biochemical composition. This may have resulted in changes to cell structure similar to those observed described earlier, and seen at 11 d in Section 4.1.1, due to nutrient depletion and an environmental stress mechanism happening earlier in this culture. From this investigation, the impact of carbohydrate accumulation is unclear, and

more research is needed to identify the location and type of the carbohydrates being synthesised and stored.

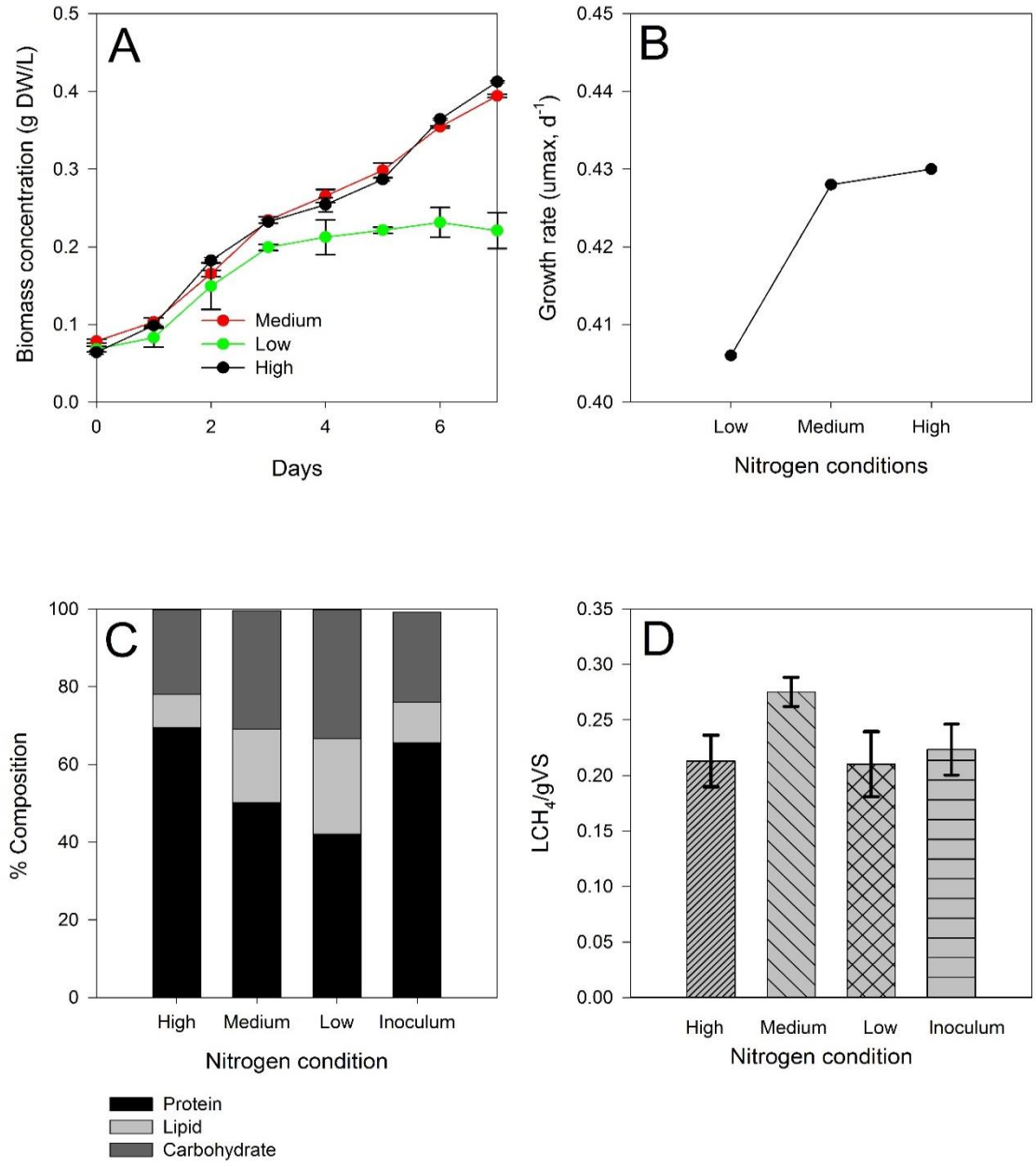

Figure 7-8 Effect of nitrogen concentration on the growth and bio-chemical composition of a mixed microalgal culture: A, biomass concentration; B, growth rate; C, biochemical composition of microalgae at 7 d; D, methane yield at 7 d. High, medium, low and inoculum concentration of nitrate, was 11.6, 2.9, 0.58 mM respectively.

7.4.4. Effect of storage duration and temperature on the methane yield from microalgal biomass

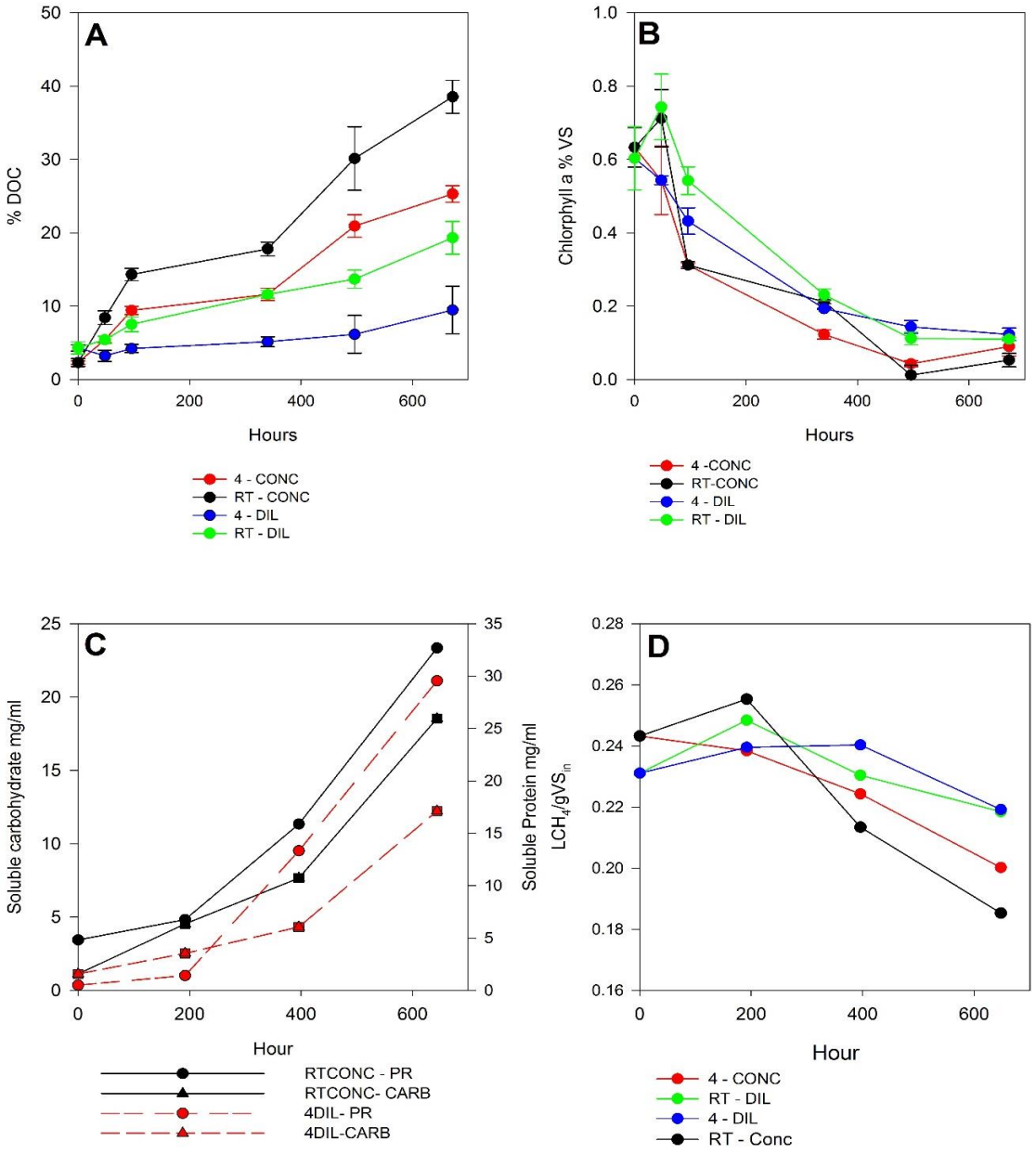

Figure 7-9 Effect of storage temperature, storage duration, and biomass concentration on the composition and methane yield of biomass from a mixed microalgal population: A, DOC; B, chlorophyll-a concentration; C, soluble protein and carbohydrate concentration; D, methane yield. Abbreviations: RT, room temperature; 4 , 4 C temperature; Conc, concentrated biomass; Dil, diluted biomass; PR,protein; Carb, carbohydrate.

Storage conditions have been indicated to be an important consideration for microalgal digestion (González-Fernández *et al.,* 2012b).

Over the duration of the experiment (4 weeks) there was a significant change in the biochemical composition of the stored microalgae (Figure 7-10). The percentage of soluble organic carbon (DOC) as a proportion of total carbon increased in all four temperature conditions (Figure 7-10A), with the largest increase observed in concentrated forms of microalgae. Chlorophyll-a decreased in all four conditions (Table 7-3) resulting from a shutdown of the photosynthetic apparatus, and release and/or breakdown of chlorophyll-a. This is likely due to storage in the dark, and any remaining nutrients being depleted forcing degradation of any chlorophyll-a as no cellular energy was available from photosynthesis.

Soluble protein and carbohydrate content increased in both concentrated and dilute forms (Figure 7-9C), while the total amount of carbohydrate and protein decreased, albeit at a low rate. Concentrating the biomass appeared to have a greater effect on the soluble protein and soluble carbohydrate mass, and corroborates increases in DOC observed at the same time, however the mass balance was not completely consistent with these changes. Methane yield at hour 0 was consistent in both storage concentrations, indicating that concentration of the biomass by centrifugation alone does not affect algal cells significantly, something previously observed for marine microalgae. Methane yield increased at 196 hours in the samples stored at room temperature (Figure 7-10D) while at lower temperatures only dilute cultures showed an increase. These differences were not statistically significant. By 396 hours, methane yield in the concentrated cultures

had dropped at both temperatures, being more pronounced at room temperature, with concentrated algal samples significantly below hour 0 methane yield.

The increase in soluble components (Figure 7-9A) is a result of both chemical and biological processes affected by the change in environmental conditions. This results in cell autolysis, release of extracellular polymeric substances, oxidative stress on fatty acids, and microbial degradation of key components by bacteria/enzymes (Montaini *et al.,* 1995). The effect on soluble carbon is more pronounced when stored at room temperature, consistent with the typical effect of temperature on chemical and biological reaction kinetics. However, even at low temperature there is a clear change in the chemical composition, contradicting previous work by Montaini et al., (1995) which showed high cell viability and no change in fatty acid composition for *Tetraselmis Suenica* biomass stored at 4°C. The elevated level of biomass solubilisation was more pronounced in the concentrated samples than the dilute samples. This results from the major shift in environments rather than the pre-concentration method itself. Concentrating the biomass resulted in removal of all excess growth medium, which buffered the algae against any shift in pH which would have been induced by the stress and degradation mechanisms discussed above.

The mechanism for microbial degradation of cellular protein and carbohydrate is unclear. Solubilisation of proteins and carbohydrates occurred (Figure 7-9C) but it is unclear whether this was microbial mediated. The chemical tests used are non-specific, and would have detected simple sugars and amino acids, by-products of carbohydrate and protein degradation. The increase in soluble protein indicates that protein may have been degraded preferentially over carbohydrate and lipids.

Methane yield appeared to be affected to a greater extent at room temperature after 196 hours of storage. This increase in yield could have been related to a change in cell composition through production of, or solubilisation of, cell compounds that were not normally degradable.

The small reduction in methane yield after 196 hours of storage primarily in room temperature storage could be related to the increased solubilisation of material and subsequent in-situ aerobic or anaerobic degradation prior to a bio-methane potential testing.

Storage of microalgae at room temperature results in elevated degradation and cell lysis. This can potentially benefit anaerobic degradation in the short term, but in the long term can impact methane production with decreases in yield . These effects can be reduced if stored at 4°C and the use of cryo-protectants at industrial scale could potentially reduce these compositional changes (Gwoet al. 2005). The effects of storage at different stages of microalgal growth should be investigated further to determine whether storage can aid or reduce degradation, in a similar way that late growth stage harvesting and nutrient depletion can both aid and hinder biomass breakdown during anaerobic digestion.

Storage of dilute cultures in their growth medium appears to enable better long term stability, with improved buffering capacity, and dilution of any bacteria or grazers that may reduce biomass yields.

7.4.5. General discussion

The original aim was to optimise cultivation conditions to improve methane yield through the manipulation of microalgal composition. It is clear that preferentially

accumulating lipids resulted in improved C:N ratio and calorific content. It is not clear whether this directly improved methane yield when cultures reached the end of exponential phase, as the results were inconclusive. However, harvesting mixed cultures towards the end of the exponential phase, irrespective of its relationship with biochemical content, did improve methane yield above the level that was observed in early exponential phase, and importantly, when culture have been left to enter long stationary phase, this had a negative impact on anaerobic degradability. The mechanism behind the poor degradability observed in the nitrogen deficient growth phase (stationary) can be attributed to a defence mechanism of algal cells to low nutrient stressed environments. These defence mechanisms can include thickening of cell walls/membranes that can further resist breakdown/protect important cellular components

Feeding of fresh cultures to an anaerobic digester is recommended in order to reduce storage capacity required, but if storage were possible up to 7 – 10 days, then small scale improvements might potentially be observed in methane yields. Long term storage is not recommended as solubilisation and bacterial degradation occur, with carbon losses of real concern. The impact on cell structure and composition following storage was not evaluated, but potentially could have a significant impact in the methane yield from microalgae.

Chapter 8 Conclusions and Future work

8.1. Conclusions

The use of microalgae as a feedstock for anaerobic digestion offers a unique alternative to traditional terrestrial based energy crops. Thermophilic digestion of mixed microalgae cultures offers marginally higher methane yields of 0.147 LCH_4gCOD_{in} (0.220 LCH_4/gVS_{in}) when compared to mesophilic temperatures yield of 0.134 $LCH_4/gCOD_{in}$ (0.164LCH_4/gVS_{in}). While the thermophillic reactors offered some improvements in yields they were more susceptible to rapid changes in reactor operation, with the loss of biomass from SRT reduction resulting in an unbalanced system and increasing VFA levels. While the shock change in SRT affected thermophillic reactors, mesophillic reactors appeared to cope without complete failure, and so can be operated safely, albeit with significantly lower yields at 15 day SRT. If reactor SRTs were needed to be reduced then smaller and slower stepwise reductions in SRT, especially at thermophilic temperatures would reduce any potential for reactor failure.

Both reactors reached higher organic loading rates than other previous studies, and until day 260 no signs of inhibition were observed. Consideration for the potential for inhibitory effects should be made in the future if any further increases in organic loading rates or any changes in reactor operation such as increases in temperature, or retention time are undertaken.

The operation of a UnAMBR offered other potential advantages when fed on microalgae, allowing operation at high SRT, and low HRT reducing any potential inhibitory effects of free ammonia nitrogen. At SRTs of 44 days the highest

methane yields were recorded, while the process appeared stable with no evidence for membrane fouling, VFA accumulation, or drop off in specific methane potential. The ability to feed dilute microalgae feeds at very low HRTs is extremely promising, and has not previously been observed. Typical previous systems have required energy intensive harvesting technology, while the UnAMBR here demonstrated that there is no reduction in methane yield when feeding un-concentrated microalgae through the system.

While anaerobic reactor process conditions can be optimised it is clear that the limiting factor is the ultimate methane potential of the microalgae. A large fraction of the microalgae remains un-degradable under anaerobic conditions, irrespective of reactor temperature, retention time and loading rate and so some form of pre-treatment is required to increase methane yields further.

In addition to pre-treatments, the selection and cultivation of microalgae could also yield increases in methane potential. Microalgae are diverse organisms and choosing the right species for energy production was shown to be vital to ensuring maximum biogas production. *Dunalia Salina* was shown to have the highest methane potential out of all microalgae tested, while *Nanochloropsis Oculata* the lowest, both marine species. These yields are consistent with previous studies. The methane yields should be balanced against the relative growth rates of the microalgae prior to understanding which offers the best overall energy potential.

Classical techniques for measuring composition such as lipids, carbohydrates and protein assays; alongside energy content (calorific value) do not directly correlate with methane yields. The use of other intracellular compositional measurements and cell wall structure determination are likely better for assessing methane

potential. In addition to choosing the correct species, it became evident that growth conditions, and the point when harvesting in the growth cycle play a vital role in determining methane potential. Nitrogen deficiency in mixed microalgae cultures drove lipid and carbohydrate accumulation potentially improving methane yield if harvested at the end of the exponential phase of growth, but with prolonged exposure to these nutrient deficient conditions came at a significant reduction in methane potential. The cause of this reduction is not clear, but careful monitoring of biomass growth kinetics/yields, and culture nutrient levels would be advised to obtain the highest methane yield possible. This is in contrast to bio-diesel production where allowing further nitrogen deficiency can enhance lipid, and subsequent bio-diesel yields.

Following cultivation, the harvesting and storage of the microalgae impact the methane potential of microalgae. Exposing microalgae to elevated temperatures, results in higher breakdown/pre-hydrolysis, which can impact ultimate methane potential through loss of available carbon. Prolonged exposure can lead to changes in intracellular composition, with short term storage benefiting methane yields marginally, but long term impacting biogas yields. Concentration of biomass enhances degradation, and again can impact on methane yields from microalgae. If storage is required prior to digestion then storing in the less concentrated forms and at lower temperatures can retain methane potential. However, this is at the expense of land requirements, and energy for any additional cooling.

8.1. Future work

Microalgal degradation, irrespective of optimal species selection and reactor configuration, remains a significant hurdle to overcome before microalgae become a feasible feedstock for anaerobic digestion. Work needs to be undertaken to improve methane conversion efficiency, and should cover two distinct areas: technical laboratory research; and desk based feasibility assessments.

The technical research should focus on two aspects, continuing the theme of this thesis: the algal cell itself, involving manipulation of its growth and metabolism for optimal breakdown; and the anaerobic digestion conditions, to improve breakdown using improved novel systems and engineered approaches.

Research into the structure and composition of microalgal cells may yield greater improvements than optimising existing reactor digestion systems alone. This could be achieved by identifying the exact components resistant to degradation under anaerobic conditions. Research would use a mixture of classical biochemical techniques (lipid, carbohydrate, and protein analyses) and anaerobic degradation tests, in combination with more advanced methods such as Fourier transform infrared spectroscopy (FTIR), solid state nuclear magnetic resonance (SS – NMR), and GC/LC - MS. Providing more in-depth understanding of microalgal cell composition, how these components resist degradation, and where these components are located within the cell. Combining this information to identify which enzymes are responsible for anaerobic degradation of microalgal components, and which enzymes are lacking in the process, is vital to increasing yield.

The second stage of work would evaluate how the microalgal cell composition/cell structure and growth rates respond under different controlled environmental

conditions/stresses (light intensity; photo-period; light wavelength; nutrient concentration; CO_2 concentration; pH; salinity; carbon source). Optimising growth environment to manipulate cell composition to preferentially increase cell compounds which have previously been identified as degradable, and reduce the compounds that have been shown to be poorly degradable. The accumulation of readily biodegradable compounds would need to be balanced against the effects that different growth conditions have on biomass yields, carbon sequestration potential and nutrient uptake, so that a balance can be found to maximise energy production. This should be done on a number of different suitable microalgal strains as each one is likely to have different responses to environmental conditions and offer different methane yields.

Focus should then be shifted to improving the downstream processing and operation of algae through isolation and use of new enzymes that can specifically target the poorly degradable components identified above. The use of commercial enzyme mixes for pre-treatment studies have proven to have some limited success. In the natural environment algae is consumed by a number of different organisms such as: snails; marine worms; filter feeders such as clams; fish; protozoans. Isolation of the enzymes used/inside these organisms and replication/production of an enzyme mixture capable of improving anaerobic degradation could potentially lead to much greater solubilisation and utilisation of algal biomass for methanogenesis. This may involve isolating a number of different enzymes, that can be used in combination to breakdown the different complex components that exist in microalgae. The efficacy of these enzymes may also be species related, so using the compositional tools developed previously will enable

a database of enzyme suitability to each microalgal strain/growth condition to be developed. The isolated enzymes would then need to be tested and optimised, specifically for use as a pre- or *in-situ* treatment for improving methane yield in anaerobic digestion.

In parallel with enzyme development and preferential accumulation of more readily biodegradable intracellular components, there is still a need to test different existing, or develop new anaerobic digestion technologies. Two-stage anaerobic reactors with a high temperature acidogenic first stage could lead to improve hydrolysis rates, or reactors which impose higher physical stresses on algal cells, have yet to receive significant attention.

Following improvements in methane yield, a comprehensive evaluation of technological factors and economic potential is required to fully evaluate the microalgal AD technology. Modelling the energy balance of the entire system incorporating different cultivation systems and potential for wastewater treatment; new and novel pre-treatment technologies; novel anaerobic reactor systems; and post digestion digestate uses to produce an optimal scenario for energy production. By modelling all of the different feedback mechanisms from changing cultivation conditions, and how this affects both microalgal composition, biomass yields and methane production will be important to gaining a better understanding on whether microalgal AD is workable, how the potential will vary under different scenarios, and which scenario is best. This modelling exercise could then be translated to the construction and operation of a pilot test facility using the best scenario, which would enable a more thorough energy balance to be evaluated. This system would

help identify further bottlenecks to wide scale application, and would be the model system on which to perform a life cycle analysis

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Zamalloa, C., De Vrieze, J., Boon, N. and Verstraete, W. (2012b). Anaerobic digestibility of marine microalgae Phaeodactylum tricornutum in a lab-scale anaerobic membrane bioreactor. *Applied Microbiology & Biotechnology* 93(2): 859-869.

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Appendix A

List of Figures

Tables

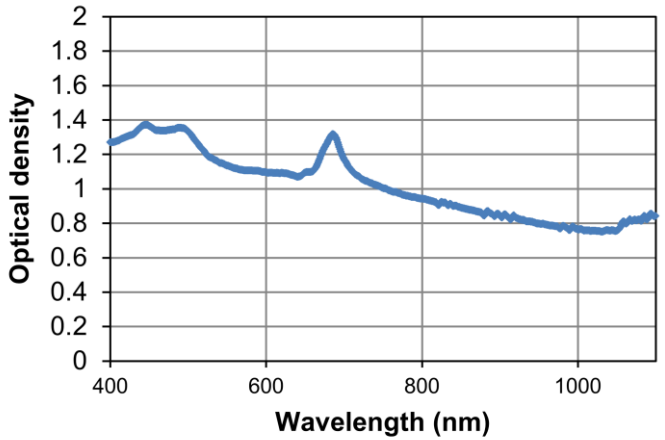

Figure 1-1. Spectral scan of freshwater mixed culture microalgae. Peak absorbance at 680nm

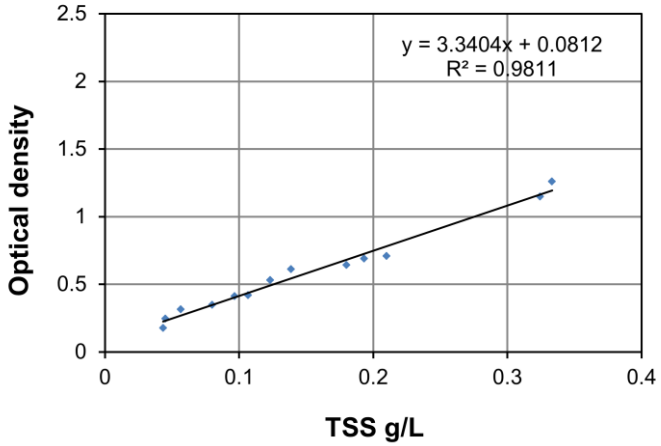

Figure 1-2. Typical TSS/Optical Density calibration curve for low yield cultures. OD set at 680nm

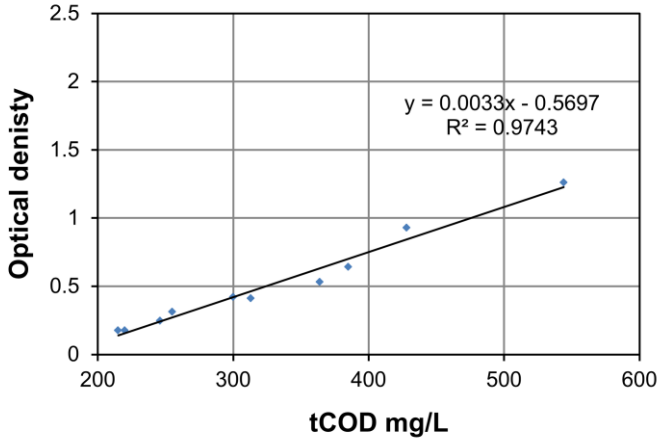

Figure 1-3. Typical COD/Optical density calibration curve for low yield cultures. OD set at 680nm

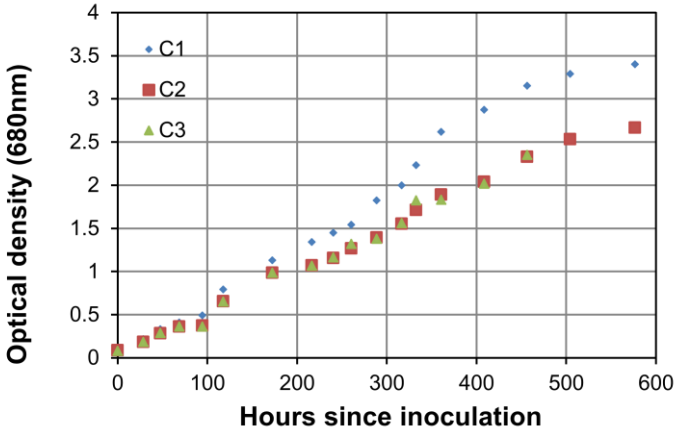

Figure 1-4. Typical photo-bioreactor growth curve using mixed microalgae cultures at low volume inocula (1% V:V) and light intensity. OD 680nm

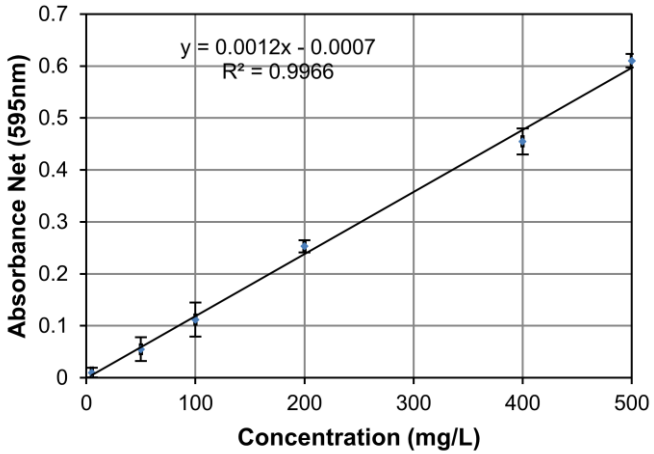

Figure 2-1. Bradford assay example calibration curve using BSA as standard. Error bars denote standard deviation

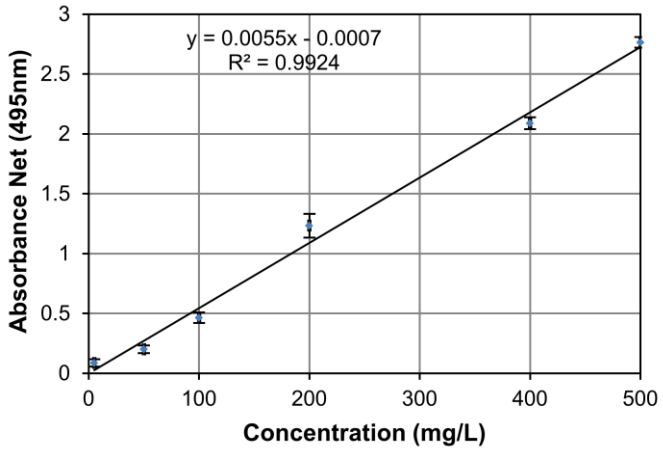

Figure 2-2. Dubois assay calibration curve using glucose as standard. Error bars denote standard deviation

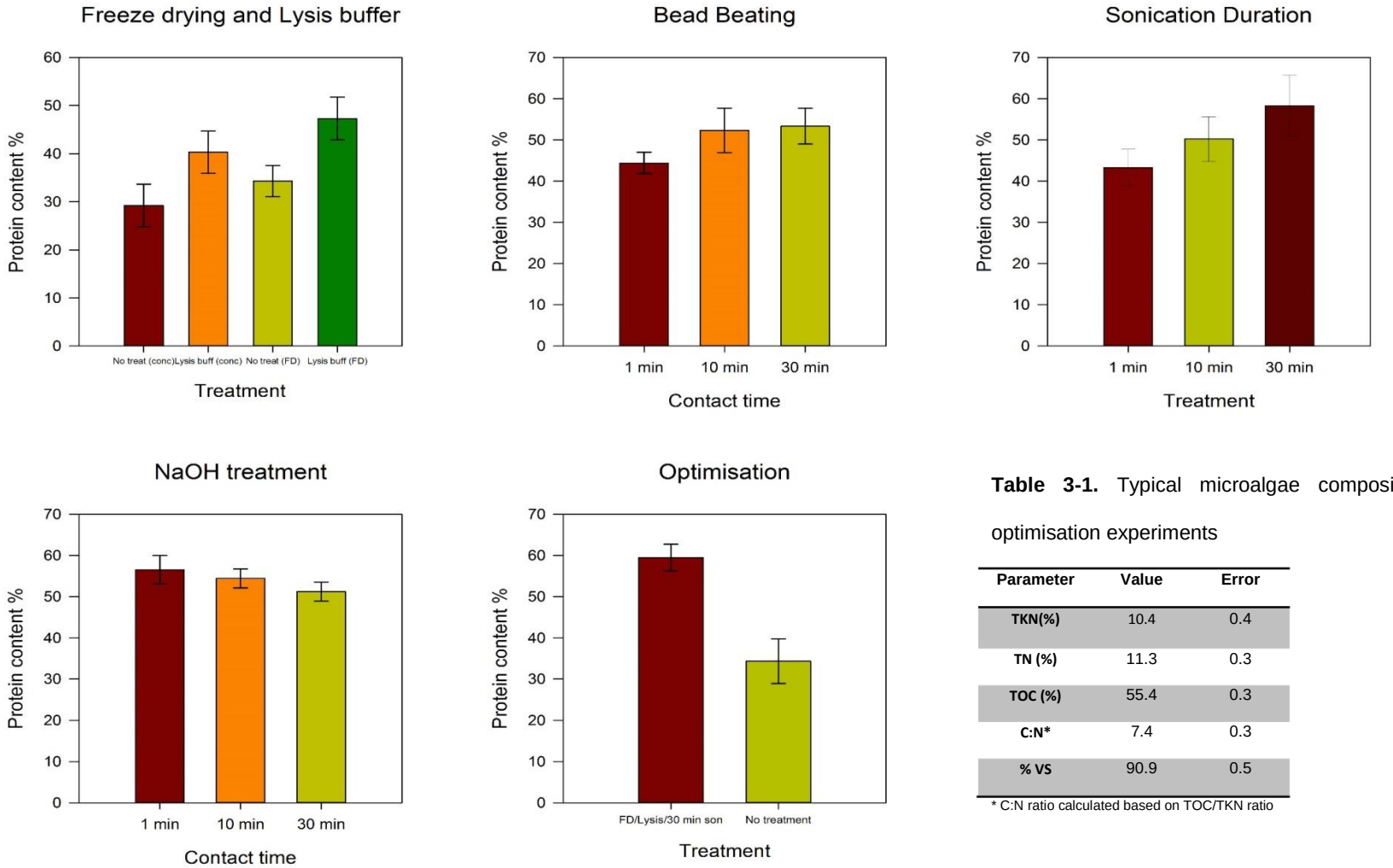

Figure 3-1. Bio-chemical assay extraction optimisation (Protein extraction efficiency comparison)

Table 3-1. Typical microalgae composition for optimisation experiments

Parameter	Value	Error
TKN(%)	10.4	0.4
TN (%)	11.3	0.3
TOC (%)	55.4	0.3
C:N*	7.4	0.3
% VS	90.9	0.5

* C:N ratio calculated based on TOC/TKN ratio

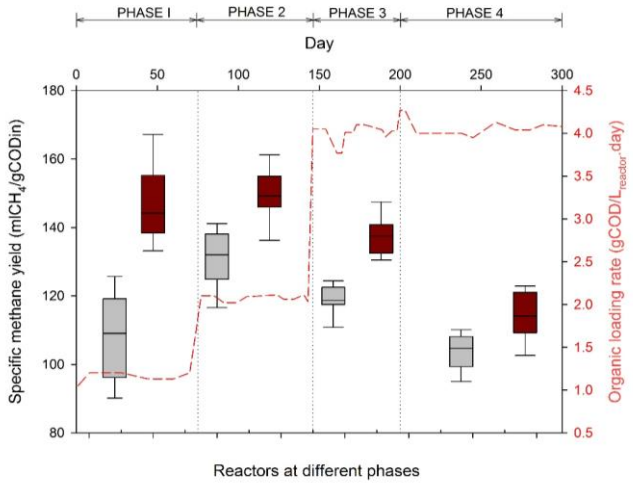

Figure 4-1. Box plot of steady state average CH_4 yield at different OLR and SRT. Red dashed line denotes OLR. Grey boxes denote mesophilic reactors, and Dark red boxes denote thermophilic reactors. Phase 4 50 °C yield is before d 260.

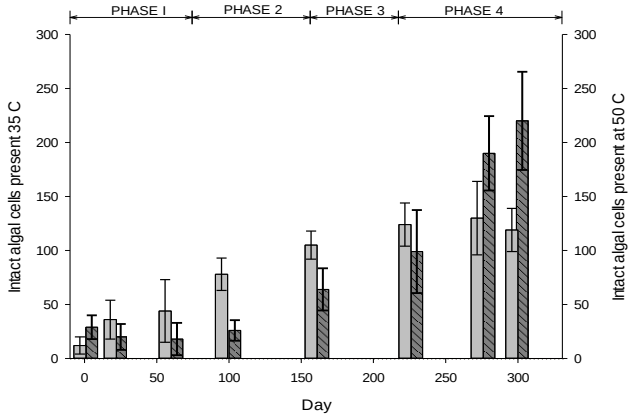

Figure 4-2. Cell counts of intact microalgal cells present in reactor effluents at 35°C and 50°C: Grey bars denote cell counts at 35°C; hatched dark grey bars denote cell counts at 50°C. Error bars denote standard error.

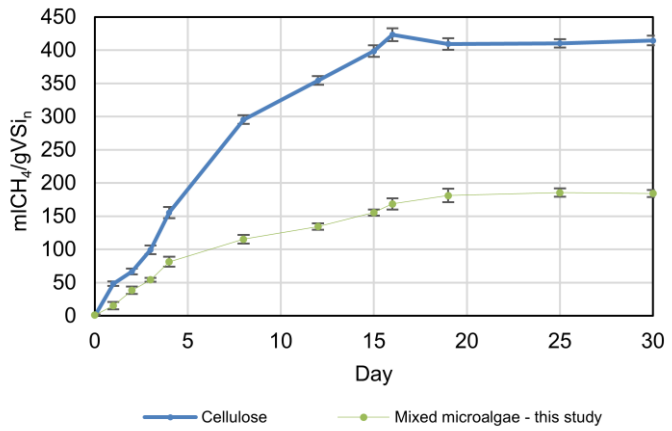

Figure 5-1. Bio-methane potential test B1 of mixed microalgae cultures and cellulose control. Error bars denote standard error N=3

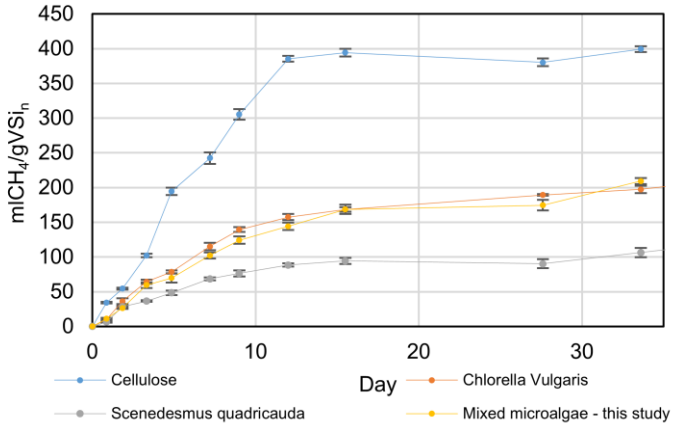

Figure 5-2. Bio-methane potential test B2a of different freshwater single and mixed microalgae plus cellulose control. Error bars denote standard error N = 3

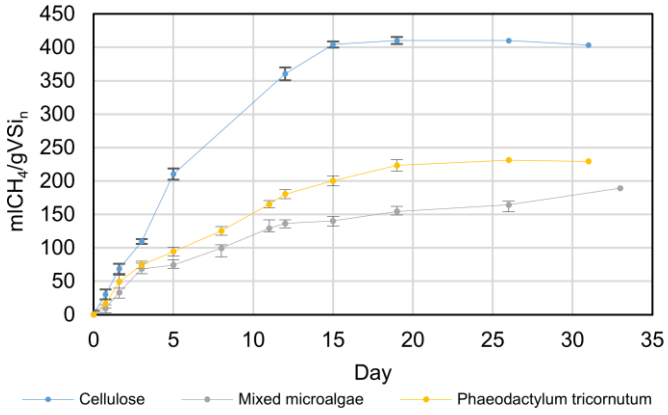

Figure 5-3. Bio-methane potential test B2b of different freshwater single and mixed culture microalgae. Error bars denote standard error N = 3

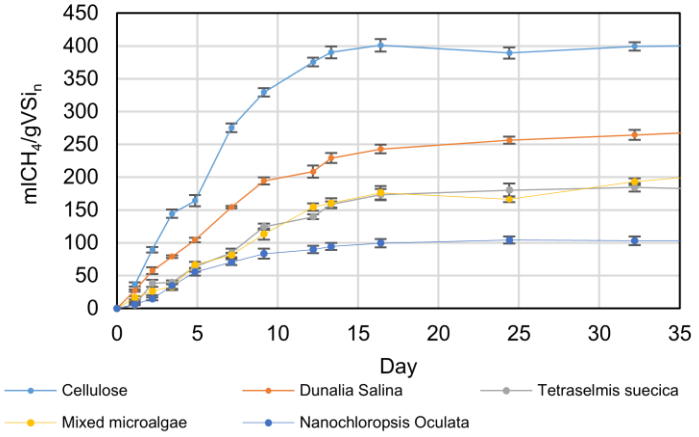

Figure 5-4. Bio-methane potential test B2c of different freshwater and marine microalgae strains. Error bars denote standard error N=3

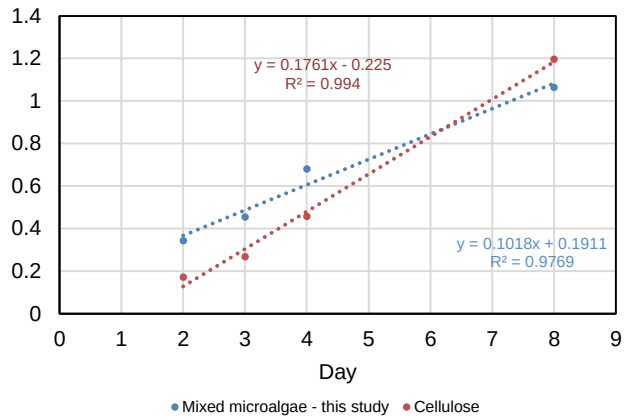

Figure 5-5. First order hydrolysis rates from BMP1 for mixed microalgae and cellulose

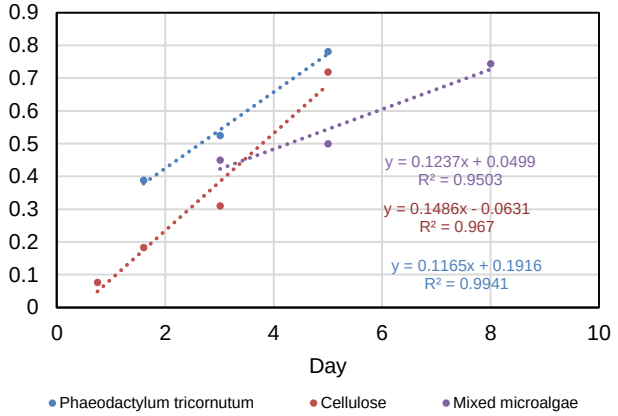

Figure 5-7. First order hydrolysis rates from BMP2b for mixed microalgae, *Phaedoctylum* sp

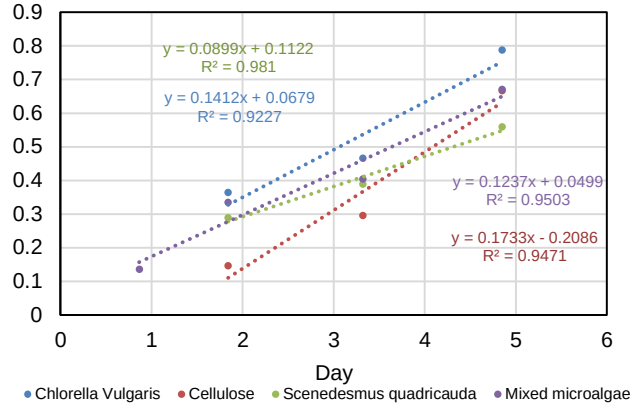

Figure 5-6. First order hydrolysis rates from BMP2a for mixed microalgae, *Chlorella* and *Scenedesmus* sp

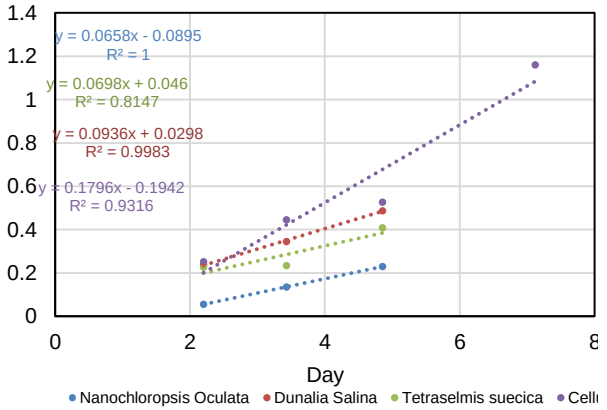

Figure 5-8. First order hydrolysis rates from BMP2c for mixed microalgae, *Nanochloropsis, Dunalia, Tetraselmis*

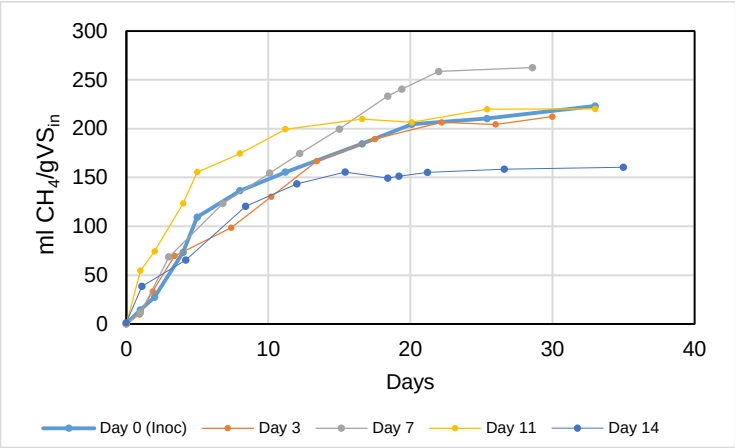

Figure 6-1. Bio-methane potential test of mixed microalgae harvested at different points in the algae cultivation/growth cycle. Corrected for blanks. Error bars denote standard error, N=3

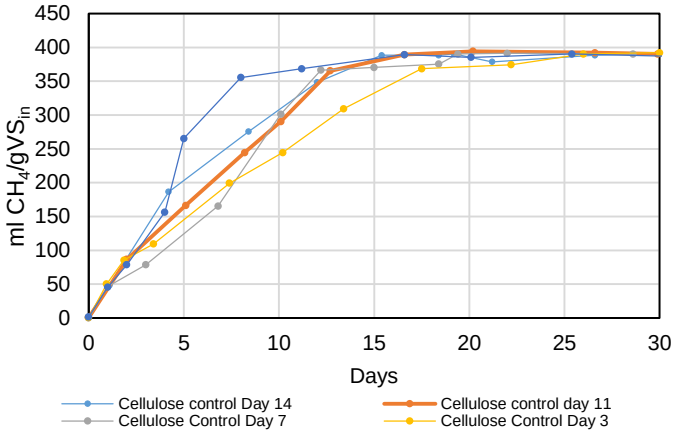

Figure 6-2. Bio-methane potential test (Cellulose controls). Error bars denote standard error.

Table 6-1. Pre and post BMP digestion conditions for cultivation cycle experiment

BMP condition	Start pH	Post pH	VS% Destruction	Pre Total VFAs mg/L	Post Total VFAs mg/L
Day 0	7.12 (0.0)	7.22 (0.0)	52.5 (4.0)	23.3	38.4
Day 3	7.23 (0.10)	7.23 (0.0)	55.4 (3.9)	48.4	65.5
Day 7	7.11 (0.0)	7.20 (0.0)	58.2 (2.9)	65.5	44.8
Day 11	7.19 (0.1)	7.11 (0.1)	53.1 (3.3)	204.4	38.1
Day 14	7.05 (0.0)	7.1 (0.0)	44.2 (4.4)	51.9	29.5

* Standard deviation in parenthesis

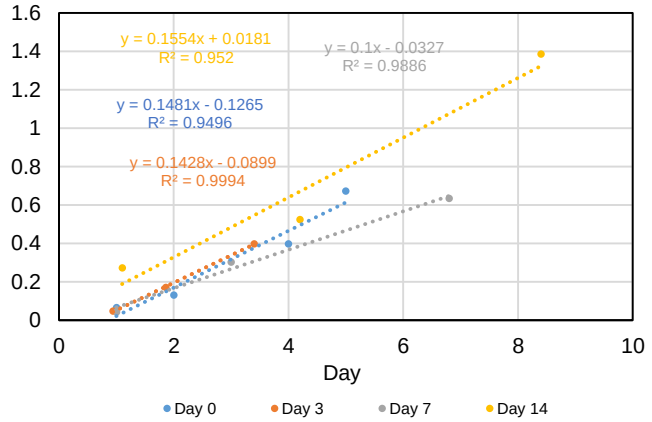

Figure 6-3. First order kinetics (hydrolysis constants d^{-1}) of mixed culture mixed harvested at different points in algae cultivation growth cycle.

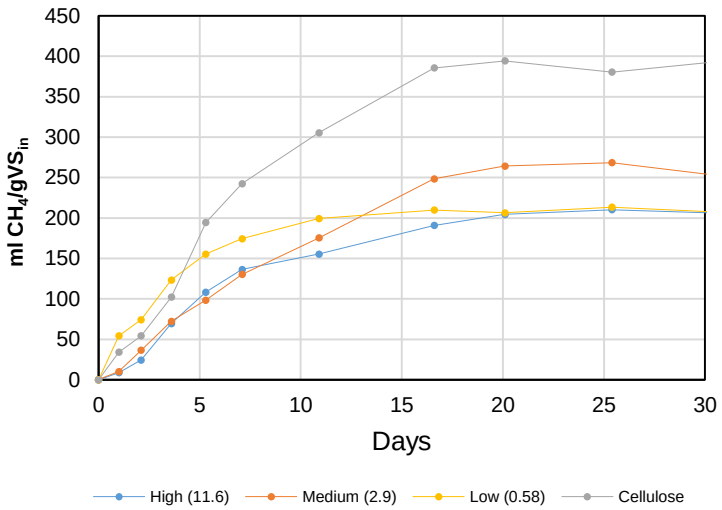

Figure 7-1. Bio-methane potential of freshwater mixed microalgae culture grown in different nutrient concentrations. Error bars denote standard error, N=3.

Table 7-1. Pre and post BMP digestion conditions for nitrogen deficiency experiment

BMP condition	Start pH	Post pH	VS% Destruction	Pre Total VFAs mg/L	Post Total VFAs mg/L	Post NH_4^+ (mg/L)
High	7.34	7.40	46.4 (10.2)	22.4	21.4	409 (8.8)
Medium	7.23	7.34	59.4 (2.9)	18.7	65.4	448 (10.4
Low	7.19	7.44	50.2 (3.4)	28.5	38.5	388 (5.5)

* Standard deviation in parenthesis

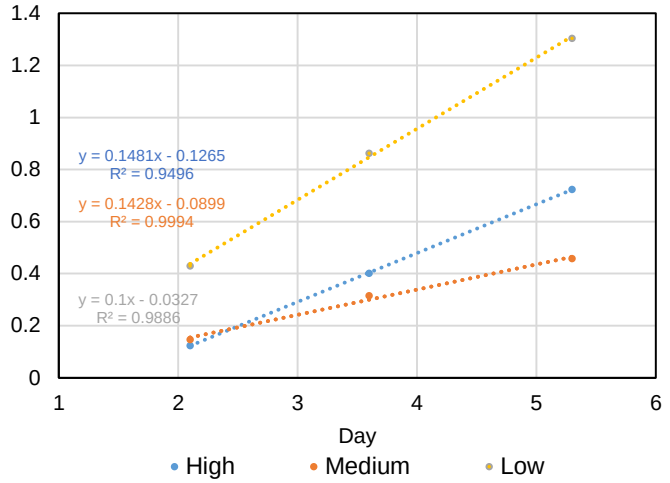

Figure 7-2. First order kinetics (hydrolysis constant d^{-1}) of freshwater mixed microalgae culture grown in different nutrient concentrations.

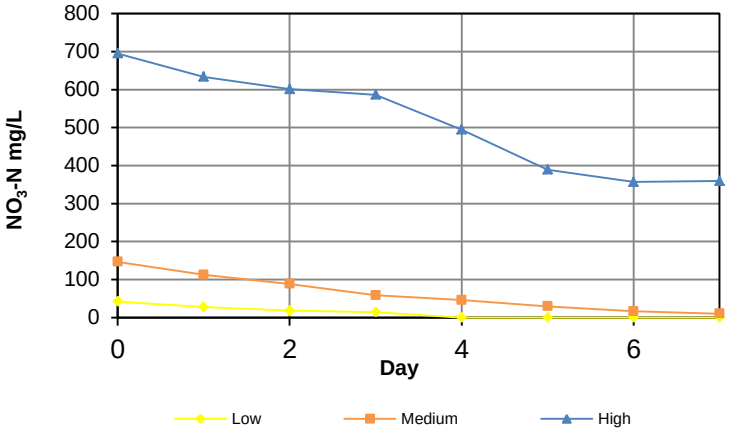

Figure 7-3. Nitrate concentration in freshwater mixed microalgae cultures grown with different nutrient starting conditions.

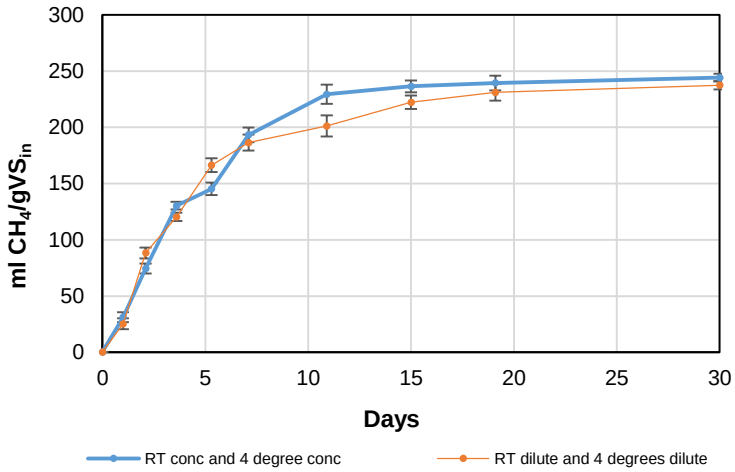

Figure 8-1. Bio-methane potential of freshwater mixed culture microalgae stored under different conditions (temperature and concentration) at time 0hr

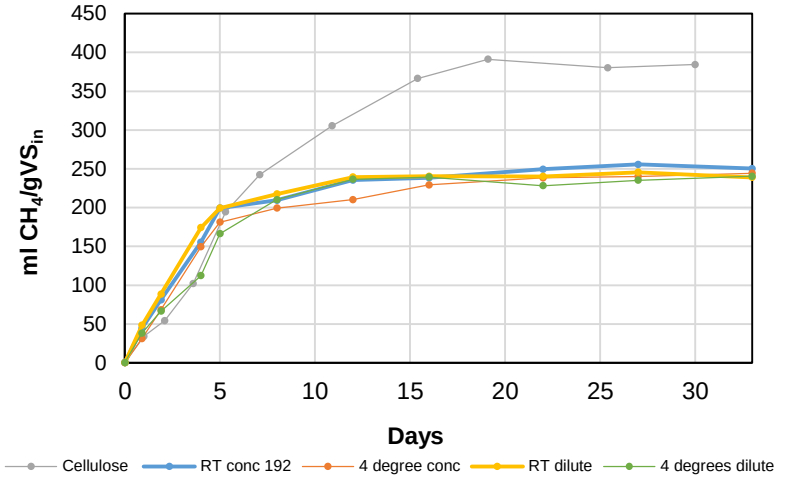

Figure 8-2. Bio-methane potential of freshwater mixed culture microalgae stored under different conditions (temperature and concentration) at time 192 hr

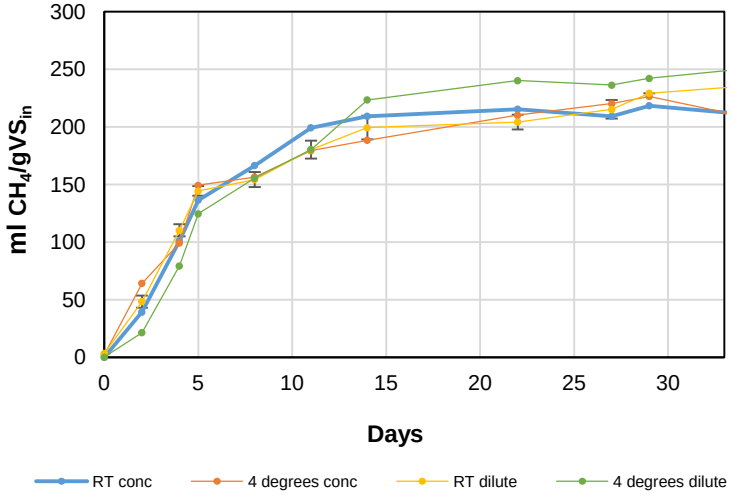

Figure 8-3. Bio-methane potential of freshwater mixed culture microalgae stored under different conditions (temperature and concentration) at time 396 hr

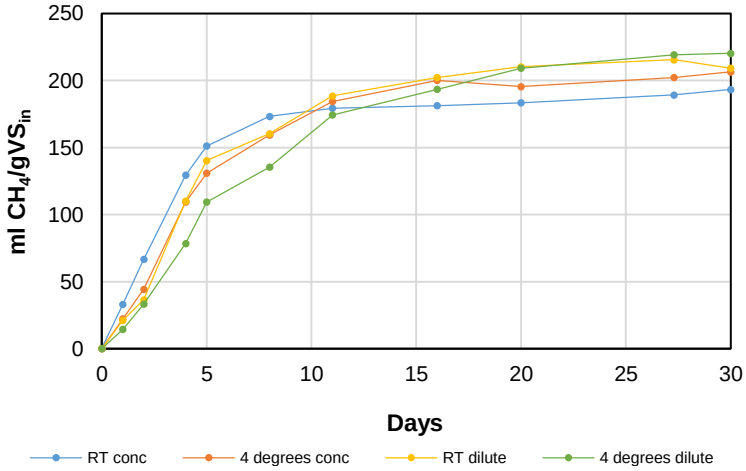

Figure 8-4. Bio-methane potential of freshwater mixed culture microalgae stored under different conditions (temperature and concentration) at time 648 hr