UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING AND THE ENVIRONMENT

Bioenergy and Organic Resources Research Group

Anaerobic digestion of microalgal biomass: effects of solid concentration and pre-treatment

by

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ABSTRACT

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ANAEROBIC DIGESTION OF MICROALGAL BIOMASS: EFFECTS OF SOLID CONCENTRATION AND PRE-TREATMENT

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Microalgae have recently attracted considerable attention as a potential substrate for biofuel production. Through the anaerobic digestion process, microalgal biomass can be converted to biogas. Although the first work on anaerobic digestion of microalgae appeared in the 1950s, for a long time further information on AD of microalgae was scarce. This study assessed the potential for energy recovery from microalgal biomass grown in two large-scale systems in Spain: a closed tubular photobioreactor (PBR) and an open raceway.

A series of digestion trials was carried out using freeze dried microalgae (FDA) from the PBR and fresh frozen microalgae (FFA) from the open raceway system as feedstock. Results from biochemical methane potential (BMP) assays showed that both feedstocks were poorly degraded and gave low methane yields. The specific methane yields of FDA and FFA were 0.161 and 0.220 L CH_4 g^{-1} VS , respectively, which is only about 30% and 44% of the Theoretical Methane Potential (TMP) of these substrates based on their elemental composition.

Digestion of FDA under semi-continuous conditions was stable at feedstock concentrations of up to 10% VS, equivalent to a hydraulic retention time of 20 days. Specific methane yields (SMY) were 0.11 - 0.12 L CH_4 g^{-1} VS, corresponding to 69 - 75% of the value obtained from BMP. Digestion of FDA at 20% VS concentration gave only 0.09 L CH_4 g^{-1} VS which is 56% of the value from BMP, or ~21% of the measured calorific value (CV). The digesters operating at 20% VS were able to achieve meta-stable operation at very high total ammonia nitrogen (TAN) concentrations of up to 12 g L^{-1} while showing reasonable methane production. They therefore showed a degree of adaptation to high TAN, but no evidence of improved biomass degradation even after operating periods in excess of 800 days. Results from the isotope labelling experiment indicated that syntrophic methanogenic pathway was the major route in high TAN concentration digesters.

Digestion of FFA was stable at feedstock concentration of 4.33% VS and OLR up to 3.5 g VS L^{-1} day^{-1}. SMY obtained under semi-continuous conditions was ~0.13 L CH_4 g^{-1} VS, corresponding to 23% of the measured CV. A series of pretreatments were carried out on FFA, and the combined thermal-alkaline pretreatment (dosage of 3% w/w NaOH and incubated in water bath at 80 ^{o}C for 2 hours) enhanced SMY by 42% compared with that of untreated FFA obtained under batch condition. The results from semi-continuous condition indicated that some improvement was achieved with the thermal alkaline pretreatment, but there were also signs of inhibition due to the high alkaline dosages of 3% NaOH (w/w) required. There is clearly scope for optimisation of the treatment of feedstock and adaptation of the inoculum.

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I, Khanh Cong Tran declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

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ACRONYMS AND ABBREVIATIONS

AD	Anaerobic Digestion
BMP	Biochemical Methane Potential
C/N	Carbon Nitrogen Ratio
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred-Tank Reactor
CV	Calorific Value
DW	Dry Weight
FDA	Freeze dried microalgae
FFA	Fresh frozen microalgae
FMP	Fresh microalgal paste
GC	Gas Chromatograph
HPTH	High Pressure Thermal Hydrolysis
HRAP	High Rate Algal Pond
HRT	Hydraulic Retention Time
IA	Intermediate Alkalinity
MSW	Municipal Solid Waste
OLR	Organic Loading Rate
PA	Partial Alkalinity
SMY	Specific Methane Yield
SRT	Solid Retention Time
STP	Standard Temperature and Pressure
TA	Total Alkalinity
TAGs	Triacylglycerols
TAN	Total Ammonia Nitrogen
TCV	Theoretical Calorific Value
TE	Trace Element
TKN	Total Kjeldahl Nitrogen
TMP	Theoretical Methane Potential
TS	Total Solids

UASB	Upflow Anaerobic Granular Sludge Bed
VBP	Volumetric Biogas Production
VFA	Volatile Fatty Acid
VS	Volatile Solids
WW	Wet Weight
WWTW	Wastewater Treatment Work

1. INTRODUCTION

1.1 Background

The fact that fossil fuel supplies are finite combined with an increasing global demand for energy has led to substantial interest in developing biologically produced renewable fuels. Biofuels are renewable fuels made from organic matter and most biofuels used presently are derived from land crops, including sugar cane, maize and vegetable oil. There has been criticism, however, of some of these so-called “first generation” biofuels for competing with food production for land and water resources, motivating the search for alternatives. These include “second” and “third generation” biofuels from wood, wastes, agricultural residues, and microalgal biomass (Brennan and Owende, 2010, Singh and Dhar, 2011, Behera et al., 2015, Maity et al., 2014). Microalgae have been seen as an attractive source of renewable biofuels for several reasons. Firstly, microalgae are highly efficient sunlight-driven cell factories that convert carbon dioxide, water and nutrients to biomass. They grow rapidly and can double their biomass within 24 hours, with some strains able to double their biomass every 3.5 hours during peak growth (Chisti, 2007). It has been shown that the biomass of these autotrophic microorganisms is rich in proteins, lipids, carbohydrates and other complex compounds that can be utilised for various purposes (Spolaore et al., 2006, Becker, 2007, Milledge, 2010). Secondly, microalgae can be grown in variable climates and non-arable land including marginal areas unsuitable for agricultural purposes (e.g. desert and seashore lands), in non-potable water or even as a waste treatment purposes; they generally use less water than traditional crops and do not displace food crop cultures; their production is not seasonal and can be harvested daily (Gouveia and Oliveira, 2009, Bohutskyi and Bouwer, 2013, Chisti, 2007, Chisti, 2008). Furthermore, they may not need pesticides or herbicides, and can be cultivated using nutrients from wastewater (Salerno et al., 2009, Rodolfi et al., 2009).

Microalgae can be used to produce different types of biofuels. The high content of proteins, lipids, and carbohydrates in algal biomass make them very promising feedstock for producing biodiesel, bioethanol, biohydrogen and biogas (Chisti, 2007, Borowitzka and Moheimani, 2010, Singh and Dhar, 2011). Production of biodiesel from algae has attracted considerable interest because of one main reason that the bulk of the natural oil made by microalgae is in the form of triacylglycerols (TAGs), which is the right kind of oil for producing biodiesel. Production of biogas from algal biomass has also gained increasing interest, however, with a growing number of studies in this area in recent years.

The energy contained in algal biomass can be converted to biogas in a process known as anaerobic digestion. This process has long been used with various applications and proved to be a good solution for waste treatment and power generation. Through anaerobic digestion, biogas can be produced from unprocessed algae, or from residues left after other products have been extracted, including oils. A relatively small number of studies has been carried out to date on biogas production from the anaerobic digestion of microalgal biomass, and there are still many unknowns that need to be discovered to make this promising substrate more realistic and efficient, especially at industrial scale.

1.2 Research aims and objectives

1.2.1 Aims

The aims of this research are:

- To quantify the methane yields of microalgal biomass grown in large scale production systems;
- To investigate factors influencing the anaerobic digestion of microalgal biomass grown in large scale production systems;
- To investigate potential methods that may be able to enhance the anaerobic biodegradability and methane yield of microalgae.

1.2.2 Objectives

In order to fulfil these aims, the following objectives have been identified:

- To carry out experiments under batch and semi-continuous conditions to determine the anaerobic biodegradability of specific microalgal feedstocks;
- To investigate the effect of solid concentrations and organic loading rates on anaerobic digestion of specific algal feedstock under semi-continuous condition;
- To investigate the impact of ammonia concentration and any potential for acclimatisation under long term exposure;
- To carry out batch screening tests to determine the effectiveness of thermochemical pretreatments to enhance methane yields;
- To carry out digestion trials under semi-continous condition with the most promising thermochemical pretreatments

2. LITERATURE REVIEW

2.1 Overview of the anaerobic digestion process

Anaerobic digestion (AD) is a series of biological processes in which microorganisms break down complex organic materials and convert them into biogas in the absence of oxygen. Biogas, which is a mixture of methane CH_4 and carbon dioxide CO_2, can be combusted to generate electricity and heat, or can be processed into renewable natural gas and transportation fuels. The digestate produced from the AD process can be utilised to produce valuable by-products such as soil conditioner or fertiliser.

AD is a complex process in which various groups of microorganism form a balanced ecosystem and each group plays a specified role (Chernicharo, 2007). The AD process occurs naturally in warm and wet environments such as swamps, river sediments and marshes and in the digestive tract of ruminants (Evans, 2001). AD is usually considered a two-stage process in which complex organic compounds are converted into simpler organic materials, mainly volatile fatty acids, carbon dioxide and hydrogen gases in the first stage; and these products of the first stage are subsequently converted into methane and carbon dioxide in the second stage (Chernicharo, 2007). The two stages of AD can be subdivided into four interrelated steps, consisting of the following:

(i) Hydrolysis,
(ii) Acidogenesis,
(iii) Acetogenesis, and
(iv) Methanogenesis.

The four steps of AD and their interrelationship are presented in Figure 2.1.

2.1.1 Hydrolysis

The first step in the biodegradation of organic matter is termed hydrolysis, in which complex insoluble organic material is solubilised by exoenzymes excreted by hydrolytic microorganisms. This is an essential step to allow the transport of macromolecules into microbial cells for further steps to take place. Hydrolysis is commonly referred to in the literature as the rate-limiting step in the process (Veeken et al., 2000, Vavilin et al., 2008, Parkin and Owen, 1986).

2.1.2 Acidogenesis

Acidogenesis, also referred to as fermentation, is the second step in which soluble materials produced by the hydrolysis process are broken down further into a mixture of organic acids, hydrogen and carbon dioxide. Acidogenesis is the generation of volatile fatty acids (C>2), such as propionic and butyric acid. Fermentation is generally considered to be the fastest of the individual steps in the anaerobic process (Mata-Álvarez, 2003).

2.1.3 Acetogenesis

The third step, acetogenesis, is performed by acetogenic bacteria, and forms the link between acidogenesis and methanogenesis. During acetogenesis, the fermentation products of the previous step, such as volatile acids or long chain fatty acids, are converted into acetic acid, hydrogen and carbon dioxide. The breakdown of the volatile fatty acids is called β-oxidation. This step is important because higher volatile acids like proprionate or butyrate cannot be directly utilised by methanogenic archea. Acetate is normally the most common precursor in methanogenesis. Homoautotrophic acetogenesis is the production of acetate from hydrogen and carbon dioxide (Madigan et al., 2003).

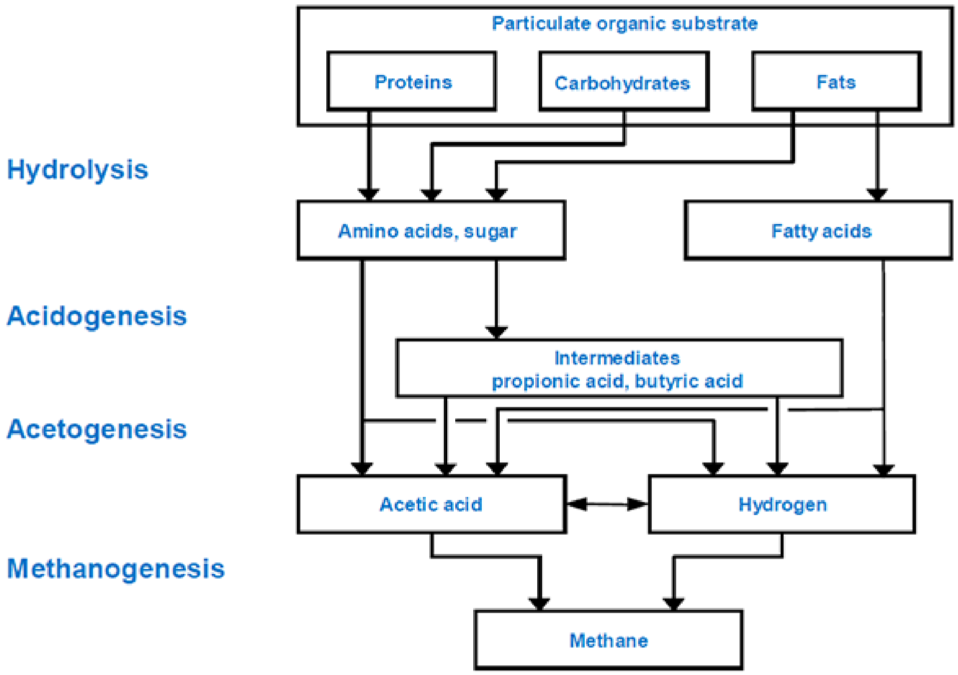


Figure 2.1. Four steps of AD and their interrelationship (adapt from Demirel and Scherer, 2008)

2.1.4 Methanogenesis

The final step in the overall anaerobic degradation process of organic compounds into methane and carbon dioxide is carried out by a group of organisms known collectively as methanogens. Two groups of methanogenic organisms are involved in methane production. Those involved in the generation of methane from cleavage of acetate are known as acetotrophic or acetoclastic methanogens. The second group converts hydrogen and carbon dioxide to methane and are termed hydrogenotrophic methanogens. It is generally believed that about 70% of the produced methane results from the degradation of acetic acids (Gerardi, 2003, Melville et al., 2014).

Methanogens have a lower growth rate than acidogens and acetogens (Angelidaki et al., 1999, Gerardi, 2003), and therefore are slower to react to changes in operational conditions and are more sensitive to unfavourable conditions in the systems than the bacterial populations.

Table 2.1 presents some of the main reactions involved in the steps of AD with glucose as an example substrate (Conrad, 1999, Stams et al., 2005).

Table 2.1. Main reactions involved in the steps of AD with glucose as an example substrate

- Acidogenic and fermentative reactions

$C_6H_{12}O_6 \rightarrow 2/3\ CH_3CH_2CH_2COO^- + 2/3\ CH_3COO^- + 2\ CO_2 + 8/3\ H_2O$

$C_6H_{12}O_6 \rightarrow 4/3\ CH_3CH_2COO^- + 2/3\ CH_3COO^- + 2/3\ CO_2 + 2/3\ H_2O$

$C_6H_{12}O_6 \rightarrow 3\ CH_3COOH$

$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH$

- Acetogenic reactions

$CH_3CH_2COOH + 2\ H_20 \rightarrow CH_3COOH + CO_2 + 3\ H_2$

$CH_3CH_2CH_2COOH + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ H_2$

$CH_3CH_2OH \rightarrow CH_3COOH + 2\ H_2$

$2\ CH_3CH_2COOH \rightarrow CH_3COOH + CH_3CH_2CH_2COOH$

$4\ H_2 + 2\ HCO_3^- + H^+ \rightarrow CH_3COO^- + 4\ H_2O$

- Methanogenic reactions

$4\ H_2 + CO_2 \rightarrow CH_4 + 2H_2O$

$CH_3COOH \rightarrow CO_2 + CH_4$

$4\ HCOOH \rightarrow 3\ CO_2 + CH_4 + H_2O$

$4\ CH_3OH \rightarrow CO_2 + 3\ CH_4 + 2\ H_2O$

2.1.5 Microbiology of the AD process

As noted above, AD can be considered an ecosystem where several groups of microorganisms work interactively in the conversion of complex organic matter into final products, such as methane, carbon dioxide, ammonia, and new bacterial cells (Chernicharo, 2007). The group of microorganisms responsible for hydrolysis and acidogenesis consist of facultative and obligate anaerobic bacteria. Some of the representatives of this group are *Clostridium*, *Desulfovibrio*, *Lactobacilus*, *Actinomyces*, *Staphylococcus* and *Escherichia coli*. The microorganisms responsible for methane production, classified as archaea, consist of strict obligate anaerobes. The main genera of methanogens that have been identified include *Methanobacterium*, *Methanobacillus*, *Methanococcus*, *Methanothrix*, and *Methanosarcina* (Tchobanoglous et al., 2004).

These microorganisms form a symbiotic relationship in which fermentation end products such as hydrogen, formate, and acetate produced by acidogens are converted to methane by the methanogens. This symbiotic relationship keeps the anaerobic system well balanced.

2.1.6 Factors influencing anaerobic digestion

In an anaerobic digestion system, anaerobic microorganisms are highly susceptible to changes in environmental conditions. Some of the environmental conditions are temperature, pH, and toxicity. In addition, other factors relating to feedstock characteristics (e.g. substrate concentration or composition, C/N ratio), or to operational conditions (e.g. retention time, loading rate, nutrients supplements) can also contribute to the efficiency and stability of an AD system.

2.1.6.1 Temperature

Anaerobic systems, like most other biological systems, are strongly temperature dependent. Although methane production can take place at a wide range of temperatures, there are three optimal temperature ranges for methanogenesis: psychrophilic (5 - 15 ^{o}C), mesophilic (35 - 40 ^{o}C), and thermophilic (50 - 60 ^{o}C) (Khanal, 2008, Chernicharo, 2007). Anaerobic conversion rates generally increase with temperature up to 60 ^{o}C, and biological activity doubles for every 10 ^{o}C increase in temperature within the optimal temperature range (Khanal, 2008). Previous studies have reported the strong temperature dependence of the maximum substrate utilisation rates of microorganisms (van Lier et al., 1997, Lettinga et al., 2001, Komemoto et al., 2009). According to Angelidaki et al., temperature not only influences the growth of anaerobic microorganisms, but also physical parameters such as viscosity, surface tension and transfer properties which also affect the overall rate of reaction (Angelidaki et al., 2003a).

2.1.6.2 pH

Microorganisms can rarely tolerate pH of below 4.0 or greater than 9.5. The optimum pH for AD ranges from 6.8 - 7.4 with neutral being the ideal (Khanal, 2008, Melville et al., 2014). Methanogens are more susceptible to pH variation than other microorganisms in the anaerobic consortium (Grady et al., 2010). Low pH reduces the activity of methanogens, causing accumulation of acetic acid and H_2. It has also been reported that the hydrolysis rate constant is pH dependent (Veeken et al., 2000, Grady et al., 2010).

2.1.6.3 Toxic materials

A variety of substances can cause toxicity in anaerobic digesters. The most common cause of toxicity is due to the high ammonia concentration produced during anaerobic digestion of organic nitrogen compounds. Ammonia inhibition has been addressed by many authors (Angelidaki and Ahring, 1993a, Hansen et al., 1998, Mata-Alvarez et al., 2000, Sterling et al., 2001, Koster and Lettinga, 1984). Sterling et al. (2001) suggested the toxicity threshold limit should be 3000 mg N L^{-1} , whilst Angelidaki and Ahring (1993) stated an ammonia concentration exceeding 4000 mg N L^{-1} could inhibit biogas yield and promote high VFA concentrations during AD of cattle manure. The consequences of ammonia toxicity in relation to AD of microalgal biomass are discussed in more detail in section 2.2.3.1 below. Sulfide, which is produced during the degradation of sulphur-containing organic matter, can also be toxic and it is believed that unionised sulphide (H_2S) is more toxic to methanogens than the ionised forms (HS^-) (Khanal, 2008). Salts, heavy metals, organic compounds, long chain fatty acids are also reported to be toxic to anaerobic processes (Stronach et al., 1986, Feijoo et al., 1995, Sawyer et al., 2003, Chen et al., 2008).

2.1.6.4 C/N ratio

The Carbon/nitrogen (C/N) ratio of the feedstock is an important factor for the stability of an AD process. A low C/N ratio could result in inhibition due to an excess of nitrogen, whilst a very high C/N ratio could lead to failure due to nitrogen deficiency for biomass synthesis. It is believed that the optimum C/N ratio in feedstock for the anaerobic digestion ranges between 20/1 - 30/1 (Angelidaki et al., 2003b, Yen and Brune, 2007) .

2.1.6.5 Substrate composition

The performance of an AD process is much dependent on the type and the composition of the material to be digested (Murto et al., 2004). All types of biomass which contain proteins, carbohydrates, lipids, celluloses and hemicelluloses as main components can be used as substrate for biogas production (Weiland, 2010). Results from a study of the influence of carbohydrate and lipids on anaerobic degradation of bovine serum albumin (BSA) in a horizontal-flow anaerobic immobilized biomass reactor fed with BSA-based substrates showed that the initial protein degradation rates were negatively affected by the presence of other organic compounds such as carbohydrates (Tommaso et al., 2003). Substrates rich in lipids and easily degradable carbohydrates were indicated to yield the highest methane potential, whilst more recalcitrant substrates with a high lignocellulosic fraction have the lowest (Labatut et al., 2011). In many cases, the physical association between lignin and cellulose is the limiting factor for AD (Mata-Alvarez et al., 2000).

2.1.6.6 Retention Time

Retention time is an important factor in achieving degradation of the feedstock. Retention time has a significant effect on the microbial populations of methanogens, homoacetogens and sulfate-reducing bacteria and on the composition of fermentative products (Zhang and Noike, 1994). It has been reported that the digestion stability and methane yield were significantly influenced by different hydraulic retention times (Kim et al., 2006). Shorter hydraulic retention time can be one of the causes for a decline in methane yield (Salminen and Rintala, 2002).

2.1.6.7 Organic Loading Rate

Organic loading rate (OLR) is an important parameter due to its effect on the performance of AD. The OLR is dependent on the type of substrate and the operational capacity of the reactor (Sánchez et al., 2005, Rincón et al., 2007). Overloading or shock loading in feeding may cause instability in AD due to an accumulation of VFA and finally to a pH breakdown (Wheatley et al., 1997), or biomass washout may lead to process failure (Rajeshwari et al., 2000), as indicated by a reduction in biogas and methane yields (Gómez et al., 2006, Wheatley et al., 1997). When slaughterhouse wastewater was anaerobically treated in a fluidised-bed reactor, it was observed that the volume of biogas produced per day increased with increased OLR over the range tested, however the methane content decreased when the OLR increased (Borja et al., 1995).

2.1.6.8 Mixing

Mixing can improve the contact between the microorganisms and substrate inside the digester, thus enhancing the microbial population's ability to assimilate the nutrients. Mixing also prevents the formation of scum and the development of temperature gradients within the digester (Speece, 2008). For example, in AD of primary sludge or a mixture of primary sludge and the fruit and vegetable fraction of municipal solid wastes, the absence of mixing resulted in a reduction in specific biogas production (0.3 and 0.5 L g^{-1} VS) due to reduction of the contact between the substrate and the microorganisms, whilst a low mixing rate (80 rpm) gave high specific biogas production of 0.5 and 0.61 L g^{-1} VS, respectively (Gómez et al., 2006). Excessive mixing, however, can disrupt the microorganisms and negatively influence the performance of the digester (Stroot et al., 2001).

2.1.6.9 Nutrients

Macro- and micro-nutrients are required for the stable growth of anaerobic microorganisms. The macro-nutrients required in highest concentration are nitrogen and phosphorus, where the phosphorus requirement for bacterial growth is about 14-20% of the nitrogen requirement (Parkin and Owen, 1986). A deficiency in macro-nutrient may cause inadequate microbiological activity, indicated by a reduction in biogas production and yield (Vintiloiu et al., 2012). Generally the approximate ratio of macro-nutrients should be in the range of 600:15:5:3 for C:N:P:S (Fricke et al., 2007) or 600:15:5:1 for C:N:P:S (Weiland, 2010).

Micro-nutrients or trace elements (TE), although required in very low concentrations, are essential for growth of bacterial biomass and maintaining a stable anaerobic digestion process (Florencio et al., 1994, Zhang et al., 2011, Zhang and Jahng, 2012). Climenhaga and Banks (2008) reported that regular addition of trace elements allowed stable digestion at high total volatile fatty acid concentrations. It was observed in their study that reactors on HRT of 25, 50 and 100 days with no micronutrient supplementation exhibited methanogenic failure after approximately 40, 100 and 90 days respectively, while duplicate reactors with micronutrient supplementation maintained stable digestion (Climenhaga and Banks, 2008). Banks et al. demonstrated that addition of selenium and cobalt could prevent propionic acid accumulation in anaerobic digesters treating food waste operating at high ammonia concentrations (Banks et al., 2012).

2.2 Review of AD of microalgae

2.2.1 Overview

The idea of using algal biomass as substrate for biogas production first appeared in the 1950s. The instability in global oil supply in the subsequent decades once again drew attention to the use of algal biomass as substrate for biofuel production.

Macroalgae was considered a good source of substrate for biofuels due to its prolific growth in eutrophic coastal waters, leading to fouling of beaches and coastal waterways (Ward et al., 2014). Anaerobic digestion has been proposed as a solution to process this material and at the same time produce biogas (Hughes et al., 2012, McKennedy and Sherlock, 2015). Experimental results have shown that converting macroalgal biomass to methane is possible (Costa et al., 2012, Habig and Ryther, 1983, Tedesco et al., 2014, Tedesco et al., 2013, Bruhn et al., 2011); however problems associated with the AD of marine macroalgae have also pointed out. For example, in terms of biochemical composition, macroalgal biomass contains a high percentage of polyphenols which are recalcitrant components in AD process (Bird et al., 1990, Briand and Morand, 1997). High concentrations of Na and S in macroalgal biomass can also inhibit anaeorobic microbes in converting this type of substrate to biogas (Moen et al., 1997, Peu et al., 2011). Furthermore, since macroalgal aquaculture is very much dependent on climate conditions, the stability of biomass supply is also a factor that significantly influences the viability of using macroalgae as substrate for biofuel production (Dębowski et al., 2013). Anaerobic digestion of macroalgae has been extensively reviewed (Hughes et al., 2012, McKennedy and Sherlock, 2015, Chen et al., 2015, Milledge et al., 2014, Montingelli et al., 2015, Ward et al., 2014), and the following section mainly focuses on the AD of microalgal biomass.

Microalgae are a diverse group of photosynthetic aquatic organisms that can survive and thrive in all types of water bodies. In natural conditions, they can be found in fresh water, brackish or saline environments. When growing microalgae in artificial conditions, the two most common large-scale systems are the open raceway and the closed photobioreactor. Each system has its own advantages and disadvantages, and the choice of system for the large-scale cultivation of microalgae depends on a number of factors. For example, closed photobioreactors present low risk of contamination, low water utilisation, high volumetric productivity and high cell density in comparison with open pond systems. However open pond systems have lower capital and running costs, and therefore are still extensively adopted (Richardson et al., 2012, Ugwu et al., 2008, Resurreccion et al., 2012).

In recent years, the integration of microalgal cultivation and wastewater treatment has been seen as a sustainable approach. As autotrophic organisms, microalgae have the ability to assimilate nutrients in wastewater effluent for their growth, at the same time producing oxygen through photosynthesis, and thus facilitating the biological treatment of wastewater by bacteria present in wastewater. Microalgal biomass produced from this integrated system can be used as substrate for the production of biofuels, e.g. biodiesel, biohydrogen or biogas (Park et al., 2011, Pittman et al., 2011).

As mentioned in section 1.1, microalgae have some potential advantages compared to the common subtrates for first and second generation biofuels. They are fast growing and are capable of doubling their biomass within 24h (Chisti, 2007). Productivity of between 10 - 25 g m^{-2} day^{-1} can be obtained in open raceway ponds, or between 25-50 g m^{-2} day^{-1} can be achieved in closed photobioreactors (Wiley et al., 2011). In regard to biochemical composition, some microalgae species contain high concentrations of proteins, lipids and carbohydrates. For example, *Arthrospira maxima* contains 60 - 71% protein, *Spirogyra* sp. contains 33 - 64% carbohydrates (Becker, 2007), *Scenedesmus dimorphus* contains 16 - 40% lipids (Becker, 1994). For these reasons, microalgae are considered a potential substrate for biofuel production, particularly bio-methane production through AD because unlike other processes such as bio-hydrogen production which requires high content of carbohydrate or bio-diesel production which requires high lipid content, AD can convert all digestible components in microalgal biomass to methane-rich biogas.

The first reported work on AD of microalgal biomass was by Golueke et al. (Golueke et al., 1957). In their study, AD experiments were carried out using biomass of *Chlorella vulgaris* and *Scenedesmus* which were grown as part of a wastewater treatment process. Research on AD of microalgal biomass was continued by the authors and other researchers (Golueke et al., 1957, Golueke and Oswald, 1959) and was reported in a series of scientific publications describing the role of microalgae in sewage treatment using “Advanced Integrated Wastewater Pond Systems” (Ward et al., 2014). The early work by Golueke, Oswald and co-authors identified several key factors that could hinder the digestion of microalgal biomass. These factors are discussed in the sections below.

For a period of several decades after this, further information on AD of microalgal biomass was scarce. After the first report by Golueke et al (1957), only a limited number of publications on AD of microalgal biomass became available in literature. These included the report by Foree and McCarty on the anaerobic decomposition of microalgae under simulated natural conditions (Foree and McCarty, 1970), and a study by Gunnison and Alexander on the resistance and susceptibility of algae to decomposition by natural microbial communities (Gunnison and Alexander, 1975). In the next decade some work on AD of *Spirulina maxima* was published (Samson and Leduy, 1982, Samson and Leduy, 1983a, Samson and Leduy, 1983b, Samson and LeDuy, 1986, Varel et al., 1988). The only notable work available during the nineties was the report by Chen and Oswald on the effect of thermochemical treatment on algal biomass grown in high-rate sewage stabilisation ponds (Chen and Oswald, 1998). The number of publications on AD of microalgae started to increase in the latter half of the previous decade, however, and reports have become more abundant in the past five years.

Experimental results on AD of microalgal biomass that have been reported in the scientific literature are summarised in Table 2.2. As can be seen, the microalgal strains used as substrate for AD experiments are rather diverse. Except for *Euglena gracilis* and *Pavlova_cf* sp. which are the only representatives of the phylum Euglenozoa and Haptophyta, respectively, the remaining microalgae listed in Table 2.2 belong to the three phyla Cyanobacteria, Chlorophyta and Heterokontophyta. The most-used microalgae in digestion experiments are species and strains under the phylum Chlorophyta including *Chlorella, Scenedesmus* (also known as *Acutodesmus*) and *Dunaliella*; under the phylum Cyanobacteria including *Spirulina* (also known as *Arthrospira*); and under the phylum Heterokontophyta including *Nannochloropsis* . Most studies in Table 2.2 used biomass of single microalgal species as substrate, while a few studies used mixtures consisting of two or more microalgal species, or in some cases also consisting of bacteria and other impurities due to the fact that the biomass was cultivated in open raceway ponds, or harvested from natural water bodies or sedimentation ponds. Except for *Dunaliella, Isochrysis, Nannochloropis, Nanofrustulum, Tetraselmis, Pavlova_cf* and *Phaeodactylum* which are halophytic microalgae, all the others are freshwater microalgae.

Most studies, as can be seen in Table 2.2, were carried out under batch conditions, while a few were conducted under continuous or semi-continuous conditions. With respect to reactor type, besides the more commonly-used reactors including the static batch reactor and the continuously stirred tank reactors (CSTR), some other types were also utilised such as the accumulating volume reactor (Kinnunen et al., 2014b), hybrid flow-through reactor (Zamalloa et al., 2012a), anaerobic membrane bioreactor (AnMBR) (Zamalloa et al., 2012b), advanced flow-through anaerobic reactor (AAR), advanced flow-through anaerobic digester with an integrated recirculation loop microbial fuel cell (ADMFC) (Inglesby and Fisher, 2012), upflow anaerobic sludge bed reactor (UASB) (Tartakovsky et al., 2015). In regards to temperature, apart from three studies that reported results at a digestion temperature of 15 - 25 ^{o}C (Samson and LeDuy, 1986, Ehimen et al., 2011, Kinnunen et al., 2014b), all other studies were carried out in mesophilic or thermophilic temperatures with the range of 35 - 38 ^{o}C being predominant.

Most studies reported in the literature have investigated the AD of whole cell microalgae biomass. Another approach which is the use of microalgal residues after extraction process for macromolecules including lipids, carbohydrates or nucleic acids as substrate for AD has been adopted in the recent five years. Many authors have stated that the methane potential of microalgae is species-specific (Mussgnug et al., 2010, González-Fernández et al., 2012, Passos et al., 2014c). As can be seen in Table 2.2, methane yield from AD of microalgae varies significantly. The lowest methane yield reported is 9.87 mL g^{-1} VS from

Isochrysis galbana (Santos et al., 2014). According to the authors, this very low methane yield was due to the inhibitory sodium concentrations for AD; thus it does not truly represent the methane potential of the tested biomass. The lowest methane yield reported for fresh water microalgae is 30 mL g^{-1} VS from AD of *Spirulina maxima* (Samson and LeDuy, 1986). This yield was obtained when *Spirulina maxima* was digested at 15 ^{o}C, however, and when digested at 35 ^{o}C, the methane yield was 200 mL g^{-1} VS. The lowest methane yield reported for AD of freshwater microalgae at 35 ^{o}C is 54 mL g^{-1} VS when digesting *Scenedesmus* sp. (Inglesby et al., 2015). The highest methane yield recorded is 600 mL g^{-1} VS for the digestion of *Chlorella protothecoides* (Bohutskyi et al., 2014b).

The very low or very high BMP values reported in some studies (Santos et al., 2014, Lakaniemi et al., 2011, Mahdy et al., 2015b, Fernández-Rodríguez et al., 2014, Bohutskyi et al., 2014b, Zhao et al., 2014) problably do not truly represent the methane yield of the test biomass. In practice, many factors are likely to affect reported BMP values, ranging from the test method used and the competence of researcher to the growth conditions of the microalgal biomass and even the type of inoculum. There is as yet no single widely-accepted protocol or duration for a BMP assay for biomass samples, although the International Water Association's Task Group on Anaerobic Biodegradation, Activity and Inhibition (ABAI) has provided guidelines on key requirements for a test protocol (Angelidaki et al., 2009). BMP assays over fixed or short intervals do not necessarily provide reliable comparative values, especially for microalgal material which may continue to be degraded over long periods (Roberts et al., 2016a). The very wide range of values quoted therefore is problably more indicative of the limitations and inconsistencies of the test procedures.

Table 2.2. Anaerobic digestion of different microalgae species under different conditions

Substrate	**Reactor type**	**Temperature (^{o}C)**	**HRT (days)**	**OLR (g VS L^{-1} day^{-1})**	**VS$_{red}$ (%)**	**CH$_4$ (mL g^{-1} VS)**	**CH$_4$ (%)**	**References**
Anabaena planctonica	Batch	35	30			284.2	74.1	(Mendez et al., 2015, Bohutskyi et al., 2014b)
Aphanizomenon ovalisporum	Batch	35	30			287.7	74.2	(Mendez et al., 2015)
Borzia trilocularis	Batch	35	30			255	75.9	(Mendez et al., 2015)
Botryococcus braunii *(a)*	Batch	35	70			404		(Neumann et al., 2015)
Botryococcus braunii	Batch	35	34-50			343-370		(Frigon et al., 2013)
Chlamydomonas debaryana-AMB1	Batch	35	34-50			302		(Frigon et al., 2013)
Chlamydomonas sp.-AMLS1b	Batch	35	34-50			333		(Frigon et al., 2013)
Chlamydonomas reinhardtii	Batch	35	22			263.1 *(nn)*		(Mahdy et al., 2014a)
Chlamydonomas reinhardtii	Batch	38	32			387	66	(Mussgnug et al., 2010)
Chlamydonomas reinhardtii *(b)*	Batch	38	40			478		(Klassen et al., 2015)
Chlamydonomas reinhardtii *(c)*	Batch	38	40			290		(Klassen et al., 2015)
Chlorella kessleri	Batch	38	32			218	65	(Mussgnug et al., 2010)
Chlorella kessleri (P. kessleri)- *(b)*	Batch	38	40			449		(Klassen et al., 2015)
Chlorella kessleri (P. kessleri) *(c)*	Batch	38	40			240		(Klassen et al., 2015)
Chlorella minutissima	Batch	36	30			163	48	(Prajapati et al., 2014b)
Chlorella protothecoides (A. protothecoides)	Batch	35	60			600		(Bohutskyi et al., 2014b)
Chlorella protothecoides (A. protothecoides) *(a)*	Batch	35	60			375-408		(Bohutskyi et al., 2014b)
Chlorella protothecoides *(a)*	CSTR	35	20-40	0.97-2.05	55-73	180-250	47-52	(Bohutskyi et al., 2015)
Chlorella pyrenoidosa	Batch	36	30			264	57	(Prajapati et al., 2014b)
Chlorella sorokiniana	Batch	35	34-50			283		(Frigon et al., 2013)
Chlorella sorokiniana	Batch	37	50			172-518	71.9-77.0	(Hernández et al., 2013)
Chlorella sorokiniana	Batch	38	35			118	62	(Ometto et al., 2014)

Substrate	**Reactor type**	**Temperature (°C)**	**HRT (days)**	**OLR (g VS L^{-1} day^{-1})**	**VS $_{red}$ (%)**	**CH$_4$ (mL g^{-1} VS)**	**CH$_4$ (%)**	**References**
Chlorella sorokiniana (wet)	Batch	40-41				98		(Polakovicova et al., 2012)
Chlorella sorokiniana-RBD8	Batch	35	34-50			331		(Frigon et al., 2013)
Chlorella sp.	Batch	35	50			310-360		(Hidaka et al., 2014)
Chlorella sp.	Batch	37	45			123	47.4	(Wang et al., 2013)
Chlorella sp.	Batch	37	20		40	230	55.4	(Wang and Park, 2015)
Chlorella sp.	Batch	35	90-95			340	74	(Bohutskyi et al., 2014a)
Chlorella sp. (68%) and Scenedesmus sp. (28%)	Batch *(ff*	mesophilic	20	2		172	59.6	(Zieliński et al., 2014)
Chlorella sp. (70%) and Scenedesmus sp. (30%)	Batch	35	23			336	56-60	(Cho et al., 2013)
Chlorella sp. *(d)*	CSTR	25	15	5		192	62	(Ehimen et al., 2011)
Chlorella sp. *(d)*	CSTR	30	15	5		208	61.7	(Ehimen et al., 2011)
Chlorella sp. *(d)*	CSTR	35	15	5		295	65.3	(Ehimen et al., 2011)
Chlorella sp. *(d)*	CSTR	40	15	5		265	63.1	(Ehimen et al., 2011)
Chlorella sp. *(e)*	CSTR	25	15	5		188	64.5	(Ehimen et al., 2011)
Chlorella sp. *(e)*	CSTR	30	15	5		227	68.3	(Ehimen et al., 2011)
Chlorella sp. *(e)*	CSTR	35	15	5		302	67.9	(Ehimen et al., 2011)
Chlorella sp. *(e)*	CSTR	40	15	5		308	69.2	(Ehimen et al., 2011)
Chlorella sp. *(a)*	Batch	37	21-35			222-268		(Ehimen et al., 2009)
Chlorella sp. Island-R	Batch	35	34-50			302		(Frigon et al., 2013)
Chlorella sp. RB1a	Batch	35	34-50			309		(Frigon et al., 2013)
Chlorella vulgaris	Batch	37	49			286		(Lakaniemi et al., 2011)
Chlorella vulgaris	CSTR	35	16-28	1		147-240	70	(Ras et al., 2011)
Chlorella vulgaris	Batch	28-31	64			315-350	67.8-76.1	(Sánchez-Hernández and Travieso-Córdoba, 1993)
Chlorella vulgaris	Batch	36	30			195	53	(Prajapati et al., 2014b)

Substrate	Reactor type	Temperature (^{o}C)	HRT (days)	OLR (g VS L^{-1} day^{-1})	VS $_{red}$ (%)	CH_4 (mL g^{-1} VS)	CH_4 (%)	References
Chlorella vulgaris	Batch	35	34-50			361		(Frigon et al., 2013)
Chlorella vulgaris	Batch	37	21			245		(Wieczorek et al., 2014)
Chlorella vulgaris	Batch	55	33			317.8		(Lu et al., 2013)
Chlorella vulgaris	Batch	35	22			190.6 *(nn)*	72	(Mahdy et al., 2014a)
Chlorella vulgaris	Batch	35	30			108.2 *(nn)*	67	(Mahdy et al., 2015a)
Chlorella vulgaris	CSTR	35	15	1.5 *(mm)*		50 *(nn)*	73	(Mahdy et al., 2015b)
Chlorella vulgaris	Batch	35	30			147.4-160.4 *(nn)*		(Mahdy et al., 2014b)
Chlorella vulgaris	Batch	35	28			142 *(nn)*	69.5	(Mahdy et al., 2015c)
Chlorella vulgaris	Batch	35	32			150.2 *(nn)*		(Mendez et al., 2014a)
Chlorella vulgaris	Batch	35	29			156.4 *(nn)*		(Mendez et al., 2014b)
Chlorella vulgaris	Batch	35	30			138.9 *(nn)*	69.9	(Mendez et al., 2013)
Chlorella vulgaris	Batch	35	30			240.3	75.8	(Mendez et al., 2015)
Chlorella vulgaris	Batch	37	90			307		(Roberts et al., 2016a)
Chlorella vulgaris UTEX 265	Batch	35	25			228	62.5	(Park et al., 2013)
Chlorella vulgaris UTEX 395	Batch	35	30		66.06	337	63.82	(Zhao et al., 2014)
Chlorella vulgaris UTEX 395 (a)	Batch	35	30		64.21	314	59.13	(Zhao et al., 2014)
Chlorella vulgaris-FGP1	Batch	35	34-50			263		(Frigon et al., 2013)
Chroococcus sp.	Batch	36	45			317.31		(Prajapati et al., 2014a)
Chroococcus sp.	Batch	37	30			267.6		(Prajapati et al., 2015)
Dunaliella salina	Batch	38	32			323	64	(Mussgnug et al., 2010)
Dunaliella salina	Batch	35	25			63		(Fernández-Rodríguez et al., 2014)
Dunaliella tertiolecta	Batch	37	49			24		(Lakaniemi et al., 2011)
Dunaliella sp	Batch	37	90			276		(Roberts et al., 2016a)
Ettlia sp. (a)	Batch	35	117			125		(Suresh et al., 2013)

Substrate	**Reactor type**	**Temperature (°C)**	**HRT (days)**	**OLR (g VS L^{-1} day^{-1})**	**VS_{red} (%)**	**CH_4 (mL g^{-1} VS)**	**CH_4 (%)**	**References**
Euglena gracilis	Batch	38	32			325	67	(Mussgnug et al., 2010)
Glossomastix chrysoplasta	Batch	35	34-50			227		(Frigon et al., 2013)
Hydrodictyon reticulatum	Batch	35	45			170		(Lee et al., 2014)
Isochrysis galbana	Batch	30	15			9.87	79.4	(Santos et al., 2014)
Isochrysis galbana	Batch	37	90			349		(Roberts et al., 2016a)
Isochrysis sp.	Batch	35	34-50			408		(Frigon et al., 2013)
Micractinium sp.	Batch	37	20		42	209	55.1	(Wang and Park, 2015)
Micractinium sp.-RB1b	Batch	35	34-50			360		(Frigon et al., 2013)
Microalgae and bacteria mixture	Batch	35	44			105.6		(Passos et al., 2015)
Microalgal biomass (g)	CSTR	37	15-20	0.71-0.88	21.3-35.7	100-180	67.8-68.3	(Passos and Ferrer, 2014)
Microalgal biomass (g)	Batch	35	29			120		(Passos and Ferrer, 2015)
Microalgal biomass (g)	CSTR	37	20	0.7	28	120	68	(Passos and Ferrer, 2015)
Microalgal biomass (g)	Batch	35	26			143.7-158.5	68.2-69.1	(Passos et al., 2013a)
Microalgal biomass (g)	CSTR	35	15	0.99	28.3	130	68.5	(Passos et al., 2014b)
Microalgal biomass (g)	CSTR	35	20	0.76	29.4	170	68.1	(Passos et al., 2014b)
Microalgal biomass (g)	Batch	35	46			117.63	68.2	(Passos et al., 2013b)
Microalgal biomass (g)	Batch	35	58			147.7		(Passos et al., 2014a)
Microalgal mixture (h)	plug flow	34-41	14-45			350-600 (oo)	40-65	(De Schamphelaire and Verstraete, 2009)
Microalgal mixture (i)	Semi-continuous	45	20			600 (oo)	60-74	(Golueke and Oswald, 1959)
Microalgal mixture (j)	Fed-batch operated digester	38	28	1.2		240		(Chen and Oswald, 1998)
Microalgal mixture (k)	Batch	35	22			287.6		(Miao et al., 2013)
Microalgal mixture (l)	Batch	35	22			233.8		(Miao et al., 2013)

Substrate	**Reactor type**	**Temperature (^oC)**	**HRT (days)**	**OLR (g VS L^{-1} day^{-1})**	**VS $_{red}$ (%)**	**CH_4 (mL g^{-1} VS)**	**CH_4 (%)**	**References**
Microalgal mixture (m)	Batch	35	22			181.7		(Miao et al., 2013)
Microalgal mixture (n)	Batch	35	22			225.7		(Miao et al., 2013)
Microalgal mixture (o)	Batch	35	22			199.7		(Miao et al., 2013)
Microalgal mixture (p)	AVR (gg)	20		1.7-0.3	40	180		(Kinnunen et al., 2014b)
Microalgal mixture (p)	AVR (gg)	37		1.7-0.3	47	273		(Kinnunen et al., 2014b)
Microalgal mixture (p)	AVR (hh)	20		1.2-0.3	36	179		(Kinnunen et al., 2014b)
Microalgal mixture (p)	AVR (hh)	ambient (8-20)		1.2-0.6	24	121		(Kinnunen et al., 2014b)
Microalgal mixture (p)	CSTR	37			32	179		(Kinnunen et al., 2014b)
Microalgal mixture (q)	AVR (hh)	20		1.2-0.5	42	227		(Kinnunen et al., 2014b)
Microalgal mixture (q)	CSTR	37			39	205		(Kinnunen et al., 2014b)
Microalgal mixture A (r)	Batch	35	35			395	70	(Alzate et al., 2012)
Microalgal mixture B (s)	Batch	35	35			188	72	(Alzate et al., 2012)
Microalgal mixture C (t)	Batch	35	60			329	66	(Alzate et al., 2012)
Microcystis sp.	Batch	35	30			94-140	35.92-45.19	(Zeng et al., 2010)
Microcystis sp.	CSTR	35	10	2	49.67	113	71.25	(Zhong et al., 2013)
Microcystis sp.	CSTR	35	10	4	45.19	145	69.47	(Zhong et al., 2013)
Microcystis sp.	CSTR	35	10	6	42.24	160	68.76	(Zhong et al., 2013)
Microcystis sp.	CSTR	35	10	8	39.28	108	57.39	(Zhong et al., 2013)
Microcystis sp.	CSTR	35	30			189.89	36.72	(Yuan et al., 2011)
Microcystis sp. (C/N = 6.35)	Batch	55	26			302 (pp)		(Zhao and Ruan, 2013)
Microcystis sp. (C/N=6)	Batch	35	30		41.26	201	51.39	(Zhong et al., 2012)
Nannochloropsis gaditana	Batch	35	34-50			228		(Frigon et al., 2013)
Nannochloropsis gaditana	Batch	35	53			300		(Alzate et al., 2014)

Substrate	Reactor type	Temperature (^{o}C)	HRT (days)	OLR (g VS L^{-1} day^{-1})	VS $_{red}$ (%)	CH_4 (mL g^{-1} VS)	CH_4 (%)	References
Nannochloropsis gaditana	Batch	35	30			109.37		(Munoz et al., 2014)
Nannochloropsis gaditana (a)	Batch	35	41			331		(Alzate et al., 2014)
Nannochloropsis oculata	Batch	55	52-66			204		(Buxy et al., 2012)
Nannochloropsis oculata	Batch	37	12			206-237 (qq)	73.9	(Marsolek et al., 2014)
Nannochloropsis oculata	Batch	37	90			231		(Roberts et al., 2016a)
Nannochloropsis salina	Batch	35	30		78.5	557	66.63	(Zhao et al., 2014)
Nannochloropsis salina	Batch	35	19			430	61.8	(Quinn et al., 2014)
Nannochloropsis salina (a)	CSTR	37	40	2		130	33-69	(Park and Li, 2012)
Nannochloropsis salina (a)	Batch	35	30		73.83	383	74.95	(Zhao et al., 2014)
Nannochloropsis salina (a)	Batch	35	19			140	49.5	(Quinn et al., 2014)
Nannochloropsis sp.	Batch	35	90-95			360	72	(Bohutskyi et al., 2014a)
Nannochloropsis sp.	Batch	35	30		65.9	357	66.6	(Zhao et al., 2014)
Nannochloropsis sp. (u)	CSTR	35	30	3		128		(Kinnunen et al., 2014a)
Nannochloropsis sp. (u)	CSTR	55	61	1.5		220		(Kinnunen et al., 2014a)
Nannochloropsis sp. (v)	Batch	35	39			194		(Kinnunen et al., 2014a)
Nannochloropsis sp. (v)	CSTR	35	36	2		156		(Kinnunen et al., 2014a)
Nannochloropsis sp. (a)	Batch	35	30		64.41	399	67.62	(Zhao et al., 2014)
Nannochloropsis sp. (w)	Batch	35	77			482		(Kinnunen et al., 2014a)
Nanofrustulum sp.	Batch	35	30		76.41	507	73.16	(Zhao et al., 2014)
Nanofrustulum sp. (a)	Batch	35	30		59.33	304	69.56	(Zhao et al., 2014)
Neochloris oleoabundans	Batch	35	34-50			308		(Frigon et al., 2013)
Pavlova_cf sp.	Batch	35	90-95			510	73	(Bohutskyi et al., 2014a)
Phaeodactylum tricornutum	AnMBR (ii)	33	2.5	2.6-5.9 (mm)		350 (nn)	75.3	(Zamalloa et al., 2012b)
Phaeodactylum tricornutum	Batch	30	30	2		350		(Zamalloa et al., 2012a)

Substrate	Reactor type	Temperature (°C)	HRT (days)	OLR (g VS L^{-1} day^{-1})	VS $_{red}$ (%)	CH_4 (mL g^{-1} VS)	CH_4 (%)	References
Phaeodactylum tricornutum	HFTR *(jj)*	33	2.1	1.9		270	75.1	(Zamalloa et al., 2012a)
Phaeodactylum tricornutum	HFTR *(jj)*	54	2.3	2		290	78.6	(Zamalloa et al., 2012a)
Phaeodactylum tricornutum	Batch	35	30		70.6	337	64.81	(Zhao et al., 2014)
Phaeodactylum tricornutum *(a)*	Batch	35	30		60.2	339	69.32	(Zhao et al., 2014)
Porphyridium aerugineum	Batch	35	34-50			352		(Frigon et al., 2013)
Rhizoclonium sp.	Batch	53	28			62-97		(Ehimen et al., 2013)
Scenedesmus almeriensis *(a)*	Batch	38	65			203		(Hernández et al., 2014)
Scenedesmus dimorphus	Batch	35	34-50			397		(Frigon et al., 2013)
Scenedesmus dimorphus *(a)*	Batch	37	12			101	40-45	(Sarat Chandra et al., 2014)
Scenedesmus obliquus	Batch	30	30	2		210		(Zamalloa et al., 2012a)
Scenedesmus obliquus	HFTR *(jj)*	33	2.2	2.7		130	74.3	(Zamalloa et al., 2012a)
Scenedesmus obliquus	HFTR *(jj)*	54	2.2	2.8		170	77.1	(Zamalloa et al., 2012a)
Scenedesmus obliquus	Batch	38	32			178	62	(Mussgnug et al., 2010)
Scenedesmus obliquus	Batch	38	35			88	60	(Ometto et al., 2014)
Scenedesmus obliquus *(b)*	Batch	38	40			401		(Klassen et al., 2015)
Scenedesmus obliquus *(c)*	Batch	38	40			213		(Klassen et al., 2015)
Scenedesmus sp.	Batch	35	33		22	37 *(nn)*		(Gonzalez-Fernandez et al., 2012b)
Scenedesmus sp.	CSTR	35	23	1		84 *(nn)*	58-60	(Gonzalez-Fernandez et al., 2012b)
Scenedesmus sp.	Batch	37	40		48.2	140.3	79.1	(Ramos-Suárez et al., 2014b)
Scenedesmus sp.	Batch	35	30			81.8 *(nn)*	62.3	(Gonzalez-Fernandez et al., 2012a)
Scenedesmus sp.	CSTR	35	15	1		33 *(nn)*	63-68	(González-Fernández et al., 2013)
Scenedesmus sp.	Batch	35	90			220		(Tran et al., 2014)

Substrate	**Reactor type**	**Temperature (^{o}C)**	**HRT (days)**	**OLR (g VS L^{-1} day^{-1})**	**VS $_{red}$ (%)**	**CH$_4$ (mL g^{-1} VS)**	**CH$_4$ (%)**	**References**
Scenedesmus sp.	CSTR	35	12.4-21.7	2-3.5	30	131-139	62	(Tran et al., 2014)
Scenedesmus sp.	Batch	35	28			141.6 *(nn)*		(Mahdy et al., 2015c)
Scenedesmus sp.	Batch	35	32			154 *(nn)*		(Mendez et al., 2014a)
Scenedesmus sp.	Batch	37	32-40		48.2	140.3	79.1	(Ramos-Suárez and Carreras, 2014)
Scenedesmus sp.	Batch	35	67			163		(Astals et al., 2015)
Scenedesmus sp.	Batch	37	50		13	54		(Inglesby et al., 2015)
Scenedesmus sp.	Batch	38	35			180		(Keymer et al., 2013)
Scenedesmus sp.	Batch	37	90			261		(Roberts et al., 2016a)
Scenedesmus sp. (x)	Batch	37	32-40		58.1	272.8	68	(Ramos-Suárez and Carreras, 2014)
Scenedesmus sp. (y)	CSTR	37	40	1.85		247.5	62.1	(Ramos-Suárez et al., 2014a)
Scenedesmus sp. (y)	CSTR	37	20	3.8		201.8	59.7	(Ramos-Suárez et al., 2014a)
Scenedesmus sp. (y)	CSTR	37	40	1.85		293.5	68.6	(Ramos-Suárez et al., 2014a)
Scenedesmus sp. (y)	CSTR	37	20	3.85		291.5	71.2	(Ramos-Suárez et al., 2014a)
Scenedesmus sp. (z)	CSTR	37	115-120	1.1		284.2	51.5	(Ramos-Suárez et al., 2014a)
Scenedesmus sp. (a)	Batch	37	32-40		63.8	212.3	58.3	(Ramos-Suárez and Carreras, 2014)
Scenedesmus sp. (a)	Batch	35	67			223		(Astals et al., 2015)
Scenedesmus sp. (a)	Batch	38	35			240		(Keymer et al., 2013)
Scenedesmus sp. (aa)	Batch	37	50			323		(Yang et al., 2011)
Scenedesmus sp. (bb)	Batch	37	37.5			393		(Yang et al., 2011)
Scenedesmus sp. (cc)	Batch	35	67			211		(Astals et al., 2015)
Scenedesmus sp. (dd)	Batch	35	67			222		(Astals et al., 2015)
Scenedesmus sp. AMDD	Batch	35	35			350		(Tartakovsky et al., 2015)
Scenedesmus sp. AMDD	UASB	35	1.81-7.29	1.04-3.57		80-220	63.5-80.0	(Tartakovsky et al., 2015)
Scenedesmus sp. AMDD	CSTR	35	16-58	0.19-0.64		150-370		(Tartakovsky et al., 2013)

Substrate	Reactor type	Temperature (°C)	HRT (days)	OLR (g VS L^{-1} day^{-1})	VS $_{red}$ (%)	CH$_4$ (mL g^{-1} VS)	CH$_4$ (%)	References
Scenedesmus sp. AMDD	Batch	35	34-50			306-410		(Frigon et al., 2013)
Scenedesmus sp. and Chlorella sp.	Fed-batch operated digester	35	30	1.44	43.1	248	61	(Golueke et al., 1957)
Scenedesmus sp. and Chlorella sp.	Fed-batch operated digester	50	30	1.44	54	314	62	(Golueke et al., 1957)
Scenedesmus sp. and Chlorella sp.	CSTR	35	10	2.0-6.0		90-136	68-71	(Yen and Brune, 2007)
Scenedesmus sp.-PN2	Batch	35	34-50			258		(Frigon et al., 2013)
Spirulina maxima	CSTR	15	20	2.02	5.6	30	54.1	(Samson and LeDuy, 1986)
Spirulina maxima	CSTR	25	20	2.02	17.6	120	69	(Samson and LeDuy, 1986)
Spirulina maxima	CSTR	35	20	2.02	39.2	200	72	(Samson and LeDuy, 1986)
Spirulina maxima	CSTR	52	20	2.02	6.6	40	53.3	(Samson and LeDuy, 1986)
Spirulina maxima	Batch	35	105			330		(Varel et al., 1988)
Spirulina maxima	CSTR	35	8.0-16.0	22.5	27.8-39.8	75-225	61.2-65.6	(Varel et al., 1988)
Spirulina maxima	CSTR	55	8.0-16.0	22.5	22.6-31.8	75-165	48.7-63.2	(Varel et al., 1988)
Spirulina maxima (A. maxima)	CSTR	35	10	0.5		90		(Inglesby and Fisher, 2012)
Spirulina maxima (A. maxima)	AAR *(kk)*	35	2.0-4.0	0.5-3.0		136		(Inglesby and Fisher, 2012)
Spirulina maxima (A. maxima)	ADMFC *(ll)*	35	2.0-4.0	0.5-3.0		173		(Inglesby and Fisher, 2012)
Spirulina maxima (A. maxima)	Batch	38	35			60		(Ometto et al., 2014)
Spirulina platensis	Batch	35	40		75.09	354.98	70.07	(El-Mashad, 2013)
Spirulina platensis	Batch	50	40		79.04	358.35	65.9	(El-Mashad, 2013)
Spirulina platensis	Batch	50	30		73.71	371.58	70.54	(El-Mashad, 2013)
Spirulina platensis (A. platensis)	Batch	38	32			293	61	(Mussgnug et al., 2010)
Spirulina platensis (A. platensis) *(ee)*	CSTR	35	30	0.36-0.41*(mm)*		203 *(nn)*		(Markou et al., 2013)
Spirulina sp.	Batch	37	50		23	113		(Inglesby et al., 2015)

Substrate	**Reactor type**	**Temperature (^oC)**	**HRT (days)**	**OLR (g VS L^{-1} day^{-1})**	**VS $_{red}$ (%)**	**CH_4 (mL g^{-1} VS)**	**CH_4 (%)**	**References**
Synechocystis sp.	Batch	35	30			380	75.4	(Mendez et al., 2015)
Tetraselmis sp.	CSTR	35	14	2		250-310	72-74	(Asinari Di San Marzano et al., 1983)
Tetraselmis sp.	Batch	35	90-95			420	79	(Bohutskyi et al., 2014a)
Tetraselmis sp.	Batch	37	30		66	252	58	(Ward and Lewis, 2015)
Tetraselmis sp. [(a)]	Batch	38	65			236		(Hernández et al., 2014)
Tetraselmis sp. [(a)]	Batch	37	30		66	122	34	(Ward and Lewis, 2015)
Tetraselmis suecica	Batch	37	30			127-174	59.6-73.2	(Santos-Ballardo et al., 2015)
Tetraselmis suecica	Batch	55	30			91	68.1	(Santos-Ballardo et al., 2015)
Thalassiosira pseudonana	Batch	37	90			435		(Roberts et al., 2016a)
Thalassiosira weissflogii	Batch	35	34-50			265		(Frigon et al., 2013)
Thalassiosira weissflogii	Batch	35	90-95			380	74	(Bohutskyi et al., 2014a)

(a) lipid extracted biomass
(b) cultured in low nitrogen conditions
(c) cultured in replete conditions
(d) residues after ACIST; C/N = 12.44
(e) residues after ACIST; C/N = 8.53
(f) mainly composed of Stigeoclonium sp, Monoraphidium sp, Nitzschia sp and Navicula sp
(g) grown in a pilot High Rate algal pond (HRAP) treating urban waste water
(h) Chlorella sp, Pseudokirchnerialla sp and Chlamydomonas sp
(i) Chlorella sp, Scenedesmus sp, Euglena sp and Oscillatoria sp
(j) grown in the effluent of high-rate sewage stabilisation ponds
(k) Microcystis 42.6%, Cyclotella 21%, Cryptomonas 12.7%, Scenedesmus 8.3% - after 15 days storage
(l) Microcystis 42.6%, Cyclotella 21%, Cryptomonas 12.7%, Scenedesmus 8.3% - after 30 days storage
(m) Microcystis 42.6%, Cyclotella 21%, Cryptomonas 12.7%, Scenedesmus 8.3% - after 60 days storage
(n) Microcystis 42.6%, Cyclotella 21%, Cryptomonas 12.7%, Scenedesmus 8.3% - after 7 days storage
(o) Microcystis 42.6%, Cyclotella 21%, Cryptomonas 12.7%, Scenedesmus 8.3% – fresh
(p) Pediastrum sp, Micractinium sp, Scenedesmus sp
(q) Pediastrum sp, Micractinium sp, Scenedesmus sp - freeze-thaw
(r) 40% Chlamydomonas sp, 20% Scenedesmus sp, 40% unknown algae (tentatively Nannocloropsis sp)

(s) 58% Acutodesmus obliquus, 36% Oocystis sp, 1% Phormidium sp, 5% Nitzschia sp
(t) mainly Microspora
(u) rinsed (dried, oil extracted)
(v) dried, oil extracted, Na$^+$ 2.9 g^{-1} L
(w) wet extracted
(x) amino acid extracted
(y) amino acid extracted; TS = 10.5%
(z) amino acid extracted; TS = 17.6%
(aa) lipid extracted; one stage process
(bb) lipid extracted; two stage process
(cc) protein and lipid extracted
(dd) protein extracted
(ee) raw, contain 60% carbohydrate
(ff) oxitop respirometer
(gg) 2 L accumulating volume reactor
(hh) 20 L accumulating volume reactor
(ii) Anaerobic Membrane Reactor
(jj) Hybrid flow-through reactor
(kk) Advanced Flow-through Anaerobic Reactor
(ll) Advanced Flow-through Anaerobic Digester with an Integrated Recirculation Loop Microbial Fuel Cell
(mm) expressed as g COD g^{-1} day^{-1}
(nn) expressed as mL CH_4 g^{-1} COD$_{in}$
(oo) expressed as mL biogas g^{-1} VS
(pp) on TS basis
(qq) calculated from given biogas and %CH_4

2.2.2 Challenges for AD of microalgae

2.2.2.1 Low concentration of digestible substrate

Most of the studies listed in Table 2.2 used concentrated microalgae as feedstock for their anaerobic digestion experiments. Many authors stated that the harvesting and concentrating of microalgal biomass are among the key factors that determine the economic viability of a system using microalgal biomass as substrate for AD or alternative biofuel production (Golueke et al., 1957, De Schamphelaire and Verstraete, 2009, Mussgnug et al., 2010, Ward et al., 2014).

In the early study on AD of microalgae by Golueke et al. (1957), low VS loading rate was identified as the factor influencing the performance of the anaerobic digester (Golueke et al., 1957). The low VS rate is due to the low concentration of microalgal biomass present in large volume of water. De Schamphelaire and Verstraete (2009) observed that an AD unit which was originally operated in continuous mode failed to produce any methane during the experimental period. According to the authors, the required VS loading rates comprising of microalgal biomass was too dilute and contained excessive water, leading to the washout of the anaerobic bacteria community (De Schamphelaire and Verstraete, 2009). Mussgnug et al. also indicated that microalgal biomass after concentration still contains a high degree of water: specifically in their report, a typical *Chlamydomonas* pellet after centrifugation contained approximately 6% VS and 94% water (Mussgnug et al., 2010). These authors therefore concluded that a concentrating step would be required for optimal performance of the AD process.

Chemical coagulation, flocculation and centrifugation have been applied as a means of havesting and concentrating microalgal biomass (Benemann et al., 1977, Harun et al., 2010, Golueke et al., 1957). Golueke et al. noted that alum flocculation was an essential step in one of the most economical methods of harvesting algae (Golueke et al., 1957). Results from their experiments indicated that digester performance was not affected by the use of alum-flocculated microalgal biomass. It was reported that concentrations of up to 4% aluminium in the feedstock had no effect on digester stability or gas production (Golueke and Oswald, 1963). It has been shown that the use of commercially available chemical coagulants also has very few detrimental effects to the AD process (Krishnan et al., 2006, Campos et al., 2008), or even in some cases improves the performance of the anaerobic digesters (Kalyuzhnyi et al., 1998, Callander and Barford, 1983). Flocculation and centrifugation as a means of havesting microalgal biomass, however, are energy-intensive techniques, and the high costs associated with energy consumption could result in the inefficiency of the AD system (Benemann et al., 1977, Harun et al., 2010). More recently, researchers have tried flotation techniques for harvesting of biomass with successful results being reported at demonstration scale in the ALL-Gas project (Arbib et al., 2014). Flotation is also energy intensive, however, and can have high investment and operational costs (Milledge and Heaven, 2013).

Microbial washout due to the low VS content of the feed and excessive water could be addressed by decoupling the hydraulic retention times and the solids retention times. For example, Zamalloa et al. demonstrated that the use of an anaerobic membrane reactor (AnMBR) for digestion of microalgal biomass is possible. In their experiment, the addition of the membrane to the reactor gave an HRT of 2.5 days, whilst the SRT was increased to between 10 and 20 days depending on the solids loading rates (Zamalloa et al., 2012b). The decoupling of the HRT and the SRT can also be achieved by using upflow anaerobic sludge blanket (UASB) reactors, anaerobic filter (AF) and anaerobic fluidised bed reactors (AFBR) (Inglesby and Fisher, 2012).

2.2.2.2 Degradability of microalgal cell wall

The cell walls of certain microalgal species are known to be highly resistant to microbial attack (Gunnison and Alexander, 1975). Several authors have observed intact, green microalgal cells in good condition in digestate after a 30 day HRT (Golueke et al., 1957), or after 45 days of anaerobic digestion (Zhou et al., 2009). Sanchez-Hernandez and Travieso-Cordoba reported that chlorophyll concentrations increased within the digester during the first two weeks of the experiments and was still detectable after nine weeks of digestion when biomass of *Chlorella vulgaris* was used as substrate for anaerobic digestion (Sánchez-Hernández and Travieso-Córdoba, 1993). Intact cells of *Scenedesmus* sp were also found in an anaerobic digester even six months after the start of the experiment (Mussgnug et al., 2010).

The role of the microalgal cell wall in AD was highlighted in the work of Mussgnug et al. (2010). In this study, six different microalgal species were investigated for their potential as substrate for AD. Methane yields were recorded in the range from 287 to 587 mL CH_4 g^{-1} VS. Noteworthily, the authors observed that higher methane yield was associated with the microalgal species that had either no cell wall (*Dunaliella salina*) or a protein-based cell wall containing no cellulose or hemicellulose (*Chlamydomonas reinhardtii*, *Arthrospira platensis*, *Euglena gracilis*), whilst lower methane yields were seen in species that had a carbohydrate-based cell wall containing hemicellulose (*Chlorella kessleri*, *Scenedesmus obliquus*). The lowest methane yield recorded was from species *Scenedesmus obliquus* which is characterised by having a particular rigid cell wall containing sporopollenin-like biopolymers (Mussgnug et al., 2010). The presence of biopolymers resistant to bacterial degradation was also reported in the outer cell wall of microalgae species *Botryococcus braunii* (Templier et al., 1992, Banerjee et al., 2002, Nguyen and Harvey, 2003, Nguyen et al., 2003). The tri-laminar sheat (TLS) structure, which serves as a protective outer wall (Derenne et al., 1992, Afi et al., 1996), and algaenan compound, which is characterised as non-hydrolysable resistant polymer, found in the cell walls of many microalgae (Afi et al., 1996, Gelin et al., 1997, Gelin et al., 1999, Nguyen et al., 2003, de Leeuw et al., 2006, Kodner et al., 2009) are believed to be the main factor that hinders the biodegradation of this type of biomass.

The relation between the cell wall degradation and the amount of gas produced that was reported in the work of Mussgnug et al. (2010) has shown that biogas production potential is dependent on the microalgal species used as substrate for AD, and also on the growth conditions. Many authors have suggested that a pretreatment step is necessary to disrupt the microalgal cell wall in order to increase bacterial hydrolysis and achieve higher biogas production (Golueke and Oswald, 1959, Samson and Leduy, 1983b, Chen and Oswald, 1998, González-Fernández et al., 2012, Gonzalez-Fernandez et al., 2012b, Marsolek et al., 2014, Ometto et al., 2014, Passos et al., 2014c, Montingelli et al., 2015, Passos et al., 2015, Ras et al., 2011).

2.2.2.3 The C/N ratio

As mentioned in Section 2.2.1, C/N ratio is an important factor affecting the stability of anaerobic digestion. Many studies report that the C/N ratio in microalgal biomass varies from 4.16 to 14.87 (Ras et al., 2011, Inglesby and Fisher, 2012, Yen and Brune, 2007, Park and Li, 2012, Samson and LeDuy, 1986, Zhao et al., 2014), which is unfavourable for AD. A C/N ratio ranging from 20 to 30 is considered optimal for AD (Yen and Brune, 2007, Khanal, 2008). The C/N ratio below 20 often results in an imbalance between carbon and nitrogen requirement for the anaerobic microorganisms inside the digester (Speece, 2008, Khanal, 2008). Due to this imbalance, nitrogen will be released and accumulated in the form of ammonia during digestion and leads to the accumulation of VFA in digester, which can become inhibitory to methanogens (Speece, 2008, Khanal, 2008). Ammomia-nitrogen and VFA are important intermediate products in the AD process but can also become a potential inhibitors when allowed to accumulate (Parkin and Owen, 1986).

The value of C/N ratio in biomass can be manipulated by applying specific growth conditions. For example, a C/N ratio of between 24-26 was obtained for *Chlamydomonas reinhardtii, Parachlorella kessleri* and *Scenedesmus obliquus* (Klassen et al., 2015) when these algal species were cultivated under nitrogen starvation. Applying selected growth conditions can lead to higher C/N ratios, however a lower algal production rate is also often associated with this technique (Ras et al., 2011).

A measure commonly applied in AD to overcome the problematically low C/N ratios is co-digestion. In order to balance the carbon and nitrogen requirement for anaerobic microbials and to avoid excessive ammonia accumulation, carbon-rich materials are used to co-digest with microalgal biomass. A recent study by Beltral et al. (2016) reported significant improvement in SMY when waste activated sludge (WAS) was co-digested with *Chlorella sorokiana* biomass under batch condition. It was observed that the highest SMY (442 mL CH_4 g^{-1} VS) was obtained for the mixture of 75% WAS and 25% microalgae, which was 22% and 39% higher than the SMY obtained from digestion of WAS and microalgae as sole substrate, respectively (Beltrán et al., 2016). Another study by Wang et al. (2013) reported that mesophilic anaerobic co-digestion of *Chlorella* sp. biomass with varying amounts of WAS under batch condition increased the biogas yields by 73 - 79% compared to the digestion of *Chlorella* sp. as sole substrates (Wang et al., 2013). The use of other materials such as paper waste, glycerol, or corn straw has also shown positive results in methane yield when they are co-digested with microalgal biomass (Yen and Brune, 2007, Zhong et al., 2012, Zhong et al., 2013, Ehimen et al., 2011).

2.2.2.4 Lipid and microalgae

High lipid content in some microalgal species makes them a promising substrate for biofuel production (Chisti, 2007, Mata et al., 2010, Williams and Laurens, 2010, Nascimento et al., 2012). Compared to proteins and carbohydrates, lipids have higher theoretical methane potential and thus are an attractive substrate for AD (Zamalloa et al., 2011). However, intermediate products of lipids such as long chain fatty acids (LCFAs) and volatile fatty acids (VFAs) produced during AD can have an inhibitory effect on the anaerobic consortium if the alkalinity and buffering capacity in the digester is low (Park and Li, 2012). For high lipid content microalgae, the residual microalgal biomass after lipid extraction for liquid biofuel production has been considered a suitable substrate for AD, because high lipid concentrations can be inhibitory (Cirne et al., 2007, Park and Li, 2012), and the lipid extraction itself is a pretreatment making the organic matter in the microalgal biomass residues more easily accessible for anaerobic microorganisms. Nevertheless, it should be noted that AD of residual microalgal biomass can be significantly influenced by the lipid extraction methods applied. For instance, the residual chloroform from the Blight and Dyer extraction process was believed to be the cause for a significant decrease in biogas production from microalgal biomass residues, even though heat treatment had been applied to remove residual entrained solvents after the extraction process (Bligh and Dyer, 1959, Ward et al., 2014). Butanol, hexane and methanol have been shown to have no detrimental effects on AD when residual solvents are removed by heating (Ehimen et al., 2009).

2.2.3 Inhibition of AD of microalgae

2.2.3.1 Ammonia nitrogen toxicity

Ammonia-nitrogen is produced from the biological degradation of proteins, urea and nucleic acids (González-Fernández and García-Encina, 2009). The high protein content in microalgae can lead to significant release of ammonia during AD. For example, when cyanobacterium *S. maxima* containing up to 60% of proteins was subjected to AD, ammonia concentrations of 3.0 - 5.0 g N L^{-1} (Samson and Leduy, 1983a), and up to 7.1 g N L^{-1} (Samson and LeDuy, 1986) were found during the AD process.

Ammonia-nitrogen at low concentrations is essential for the growth of anaerobic microorganisms; however high concentrations of ammonia may be toxic to the microbial community (Rajagopal et al., 2013). Ammonia inhibition during AD can be triggered by several factors including ammonia concentrations, pH, temperature, presence of other ions and the absence of acclimatisation (Chen et al., 2008). The main forms of inorganic ammonia nitrogen in aqueous solution are ionised ammonium (NH_4^+) and free ammonia (NH_3) (Chen et al., 2008). The equilibrium between ionised ammonium and free ammonia can be affected by a change in pH or temperature within the anaerobic digester. An increase in pH or temperature can be very detrimental to the anaerobic microbial consortium as the equilibrium shifts to free ammonia which is the more toxic form (Parkin and Owen, 1986, Yenigün and Demirel, 2013, Rajagopal et al., 2013).

For these reasons, reported inhibitory concentrations of ammonia range widely from 1.7 to 14 g N L^{-1} (Angelidaki and Ahring, 1993b). Ammonia concentrations between 1.5 - 3.0 g N L^{-1} show a moderately inhibitory effect, while above 3 g N L^{-1} the inhibitory effect is strong (Parkin and Owen, 1986). Results from a study by Ramos-Suarez et al. of the digestion of highly concentrated (17.6% TS) *Scenedesmus* residues after amino acid extraction indicated that inhibition was due to the TAN concentrations of up to 4.2 g N L^{-1} and free ammonia concentration of 200 mg N L^{-1} (Ramos-Suárez et al., 2014a). Kinnunen et al. reported unstable operation of a thermophilic reactor at ammonia concentrations in the range of 1.7 - 2.9 g N L^{-1} when digesting *Nannochloropsis* residues (Kinnunen et al., 2014a). Schwede et al. also indicated that the release of ammonia from degradation of *N. salina* biomass significantly affected methane yield and process stability by accumulation of VFA (Schwede et al., 2013).

2.2.3.2 Sodium toxicity and effect of salinity

As can be seen in Table 2.2, some halophilic microalgae have been investigated and shown potential as a substrate for biogas production. BMP values for *Nannochloropsis salina* and *Nanofrustulum* sp. reached 557 and 507 mL CH_4 g^{-1} VS, respectively (Zhao et al., 2014), that of *Pavlova_cf* sp. and *Tetraselmis* sp. reached 510 and 420 mL CH_4 g^{-1} VS, respectively (Bohutskyi et al., 2014a). Methane yield from BMP of *Nannochloropsis* sp. and *Isochrysis* sp. was 482 mL CH_4 g^{-1} VS (Kinnunen et al., 2014a) and 408 mL CH_4 g^{-1} VS (Frigon et al., 2013), respectively. However, the lowest methane yields presented in Table 2.2 are also from halophylic microalgae. The lowest methane yield of 9.87 mL CH_4 g^{-1} VS was reported for *Isochrysis galbana* (Santos et al., 2014), and only 24 mL CH_4 g^{-1} VS was recorded for *Dunaliella tertiolecta* (Lakaniemi et al., 2011). The main reason for these low methane yields is probably the high salinity of the microalgae slurry, and they do not in fact represent true BMP values.

At low concentrations sodium is essential for microbial metabolism, but higher concentrations can be extremely toxic to methanogenic microorganisms. Sodium concentrations in the range of 100 - 200 mg L^{-1} have been reported as stimulatory for the anaerobic consortium (McCarty, 1964). At high concentrations, sodium could readily affect the activity of microorganisms and interfere with their metabolism (Mendez et al., 1995, Balslev-Olesen et al., 1990). The degree of inhibition depends on the concentration of sodium ions. Sodium concentrations between 3.5 - 5.5 g L^{-1} are moderately inhibitory, and above 8.0 g L^{-1} can be severely inhibitory (McCarty, 1964, Chen et al., 2008). High salt concentrations cause dehydration in microorganisms due to osmotic pressure (Chen et al., 2008).

Several authors have indicated that acclimatisation may significantly improve the biomass activity under saline conditions (Chen et al., 2008, Kimata-Kino et al., 2011). Experimental results from a recent study have shown that halophilic sediment was able to produce methane efficiently up to salinity conditions of 35 g L^{-1} (Mottet et al., 2014). According to the authors, after an acclimatisation period involving the three successive additions of substrate, the performance was very close to the methane production in normal condition. The authors also indicated that in high salinity of 75 g L^{-1} and above, only methanogens of industrial origin showed a capacity of acclimatisation (Mottet et al., 2014). Roberts et al. carried out a long-term semi-continuous study looking at acclimatisation of inoculum from mesophilic digestion of municipal wastewater biosolids to high concentrations (31 g L^{-1}) of marine salt and found that specific methane production was 6 - 7% less than in controls using the same feedstock without saline addition (Roberts et al., 2016b).

2.2.4 Pretreatment for improvement of methane yields from AD of microalgae

Two strategies which have been adopted for enhancing methane production from AD of microalgae are pretreatment and codigestion. Whist the former attempts to enhance the hydrolysis rate by disrupting the microalgal cells, the latter aims at balancing the C/N ratio of the medium to improve the degradative microorganism activity by avoiding potential inhibition and limitations. Codigestion of microalgae with other substrates has been reviewed by several researchers (González-Fernández, 2012, Sialve, 2009, Bohutskyi, 2013, Dębowski, 2013), and the following sections mainly focus on pretreatment of microalgae.

Many authors have indicated that pretreatment is a necessary step to enhance biogas production from microalgae (Samson and Leduy, 1983b, Chen and Oswald, 1998, Alzate et al., 2012, Marsolek et al., 2014, Passos et al., 2014c, Spiden et al., 2015). The effectiveness of pretreatment methods on biogas production is very much dependent on the characteristics of the microalgal feedstock such as the structure of the cell wall and the biochemical composition of the cells. Experimental results have shown that methane yields of microalgae species without cell wall (e.g. *Dunaliella* sp. and *Pavlova_cf* sp.) or containing a glycoprotein cell wall (e.g. *Chlamydomonas* sp., *Tetraselmis* sp.) are higher than that of those species with more complex cell wall or containing recalcitrant compounds (e.g. *Scenedesmus* sp., *Chlorella* sp.) (Mussgnug et al., 2010, Bohutskyi et al., 2014a). Most authors who worked on the AD of *Scenedesmus* have reported low methane yield and poor biodegradability of this microalgal biomass. The cell walls of *Scenedesmus* are composed of multilayers of cellulose and hemicellulose on the inside, and sporopollenin and politerpene on the outside (Bisalputra and Weier, 1963, Blokker et al., 1998, Burczyk and

Dworzanski, 1988, Takeda, 1996). This complex cell wall structure of *Scenedesmus* is likely to confer resistance to biological attack, explaining the poor methane yield and biodegradability of this microalgal biomass.

In general, pretreatments can be divided into four groups: mechanical, chemical, thermal and biological methods. Each group can also consist of several techniques. For example, mechanical pretreatment can be carried out by milling, ultrasound or microwave. Similarly, chemical pretreatment can be carried out using either acid or alkaline. Thermal pretreatment, depending on the temperature applied and pressure involved, can be subdivided into thermal and hydrothermal pretreatment. Pretreatments can be applied separately as single techniques, but in many cases the combination of two or more techniques has been used to bring about positive results.

2.2.4.1 Mechanical pretreatment

Mechanical pretreatments work by disrupting microalgal cells through a physical force. Most studies that adopted mechanical methods are associated with improving the lipid extraction for biodiesel production because these techniques are less dependent on microalgal species and are less likely to contaminate the lipid product (Lee et al., 2012). Although mechanical pretreatments are effective at disrupting the cell wall of microalgae, they have a high energy demand. Milling, ultrasounds and microwave pretreatments are the three main mechanical methods that have been used to improve AD of microalgae.

a. Milling

A suspension of *Isochrysis galbana* biomass was mechanically pretreated by placing it in a glass tube with screw cap with glass beads and submitted to vortexing (Santos et al., 2014). Assays were performed by varying the mass of glass beads and the stirring time. Stirring of 5 mL microalgal biomass suspension with 1 g of glass beads for 1 min and 5 min resulted in an increment of 61.7% and 63% in soluble COD compared to the initial soluble COD, respectively. However, the biogas volume of the mechanically pretreated biomass observed on day 15 of a batch experiment was only 12.7 mL compared to the 22.0 mL of the untreated biomass. The specific methane production was 24.8 and 43.1 mL CH_4 g^{-1} COD for the pretreated and untreated biomass, respectively (Santos et al., 2014). According to the authors, membrane disruption alone is not sufficient to promote anaerobic digestion: the very low reported gas volumes and BMP values indicate, however, that the experimental results may not be reliable.

Batch phase bead milling was also used as the mechanical pretreatment to rupture the cells of *Spirulina* sp. and *Scenedesmus* sp.(Inglesby et al., 2015). Milling resulted in a significant increase in soluble COD (2.26-fold for *Spirulina* sp. and 1.8-fold for *Scenedesmus* sp.). Reported methane yields were 166 mL g^{-1} VS_{loaded} for *Spirulina* sp., and 97 mL g^{-1} VS_{loaded} for *Scenedesmus* sp., an increase of 47% and 79.6% respectively compared to the control.

b. Ultrasound

Ultrasound is acoustic energy in the form of waves that have a frequency above the human hearing range. The continuing cycles of rapid compression and decompression of sonic waves generate cavitation inside the cells, forming regions containing liquid vapour, so-called microbubbles. These microbubbles are formed by the movement of liquid molecules in the acoustic waves. Depending on the ultrasound intensity, they are compressed and implode, producing heat, free radicals, high pressure, shockwaves and thus damaging the cell wall (Kim et al., 2013).

Ultrasound can be applied at low (<50 kHz) and high (>50 kHz) frequencies. Low frequencies favour mechanical effects, whilst high frequencies favour the formation of free radicals (Passos et al., 2014c). Once the frequency is fixed, output power and exposure time are controllable parameters that influence ultrasound pretreatment. The effect of ultrasound pretreatment is also influenced by the biomass concentration because the specific energy is a function of applied power, exposure time and biomass concentration. Biomass concentration should be high enough to increase the probability of contact between cells, but not so high as to increase biomass viscosity (Lee et al., 2012, Kim et al., 2013). Temperature may also play an important role, since it affects the vapour pressure inside the cell: the lower the temperature, the lower the pressure and effectiveness of the pretreatment (Lee et al., 2012, Kim et al., 2013).

Ultrasound pretreatment on microalgae biomass results in cell wall disruption and organic matter solubilisation. The results depend on the microalgae species and pretreatment conditions. For example, Alzate et al. (2012) applied four energy levels of between 10 and 57 MJ kg^{-1} TS to pretreat three microalgal mixtures and obtained significant increases in soluble COD in all three. The authors observed that the degree of solubilisation increased with increasing ultrasound energies. The highest increases in soluble COD recorded for mixture A (consisting of 40% *Chlamydomonas* sp., 20% *Scenedesmus* sp., 40% unknown algae), mixture B (58% *Acutodesmus obliquus*, 36% *Oocystis* sp., 1% *Phormidium* sp., 5% *Nitzschia* sp.) and mixture C (mainly *Microspora* sp.) at 57 MJ kg^{-1} TS of applied energy were 32%, 60% and 62%, respectively.The improvement in methane yield, however, did not correspond to the increase in soluble COD. The authors reported methane yield improvements of 12%, 13% and 22% for mixtures A, B and C respectively (Alzate et al., 2012). In another study, five energy levels were applied for ultrasound pretreatment of *Scenedesmus* sp. No improvement in methane yield was observed with energy level 35.5 and 47.2 MJ kg^{-1} TS, but at higher energy levels increments of 14%, 75% and 88% in methane yield were reported for energy levels of 76.5, 100.7 and 128.9 57 MJ kg^{-1} TS, respectively (Gonzalez-Fernandez et al., 2012a).

Biomass of *Chlorella sorokiniana*, *Scenedesmus obliquus* and *Arthrospira maxima* was subjected to ultrasound pretreatment with five different specific energy inputs from 0.35 to 35 MJ kg^{-1} TS. The results showed that whilst *C.sorokiniana* and *S.obliquus* reached their highest sCOD increase of 5-fold and 10-fold respectively at energy inputs of 35 MJ kg^{-1} TS, *A.maxima* reached 82% solubilisation at a lower energy input of 10 MJ kg^{-1} TS (Ometto et al., 2014). All energy inputs increased biogas production; however the pattern of improvement was not the same for three microalgae treated. Specifically, the highest biogas increases observed were 53% and 38% for *C.sorokiniana* and *A.maxima* at energy input 35 MJ kg^{-1} TS, whilst the best result for *S.obliquus* was 27% at energy input 10 MJ kg^{-1} TS.

Various specific energy levels from 16.0 to 67.2 MJ kg^{-1} TS were applied to pretreat microalgal biomass grown in a pilot high rate algal pond (HRAP) treating real urban wastewater (Passos et al., 2014a). Biomass solubilisation was fairly low (16 - 17%) with applied specific energy below 22.4 MJ kg^{-1} TS. It increased with the output power and reached a maximum of 101% at an energy level of 48 MJ kg^{-1} TS. According to the authors, exposure time seemed to have a higher impact on biomass solubilisation than output power. Overall, the methane yield was increased by 6 - 33%. The authors noted that the increase in methane yield did not exceed 20% with specific energy below 30 MJ kg^{-1} TS. Other researchers have reported that methane yield increase did not exceed 20% with applied specific energy below 75 MJ kg^{-1} TS (Alzate et al., 2012, Gonzalez-Fernandez et al., 2012a) but it increased by 80-90% when specific energy of 100 - 200 MJ kg^{-1} TS was applied (Gonzalez-Fernandez et al., 2012a).

Experimental results from the above mentioned studies show that there is considerable variation between reported values, but the energy inputs in each case are relatively high in relation to the potential energy likely to be recovered from the algal biomass. The calorific value of some of the freshwater microalgae that have been used as substrate for AD has been found to be between 18 - 35 MJ kg^{-1} VS (Illman et al., 2000, Scragg et al., 2002, Ghayal and Pandya, 2013, Chia et al., 2013, Tran et al., 2014). It should be noted that this is the maximum energy that can be obtained from these microalgae. For ultrasound pretreatment, it was reported that the specific energy below 30 MJ kg^{-1} TS did not significantly improve methane yield (Passos et al., 2014). Higher energy levels (eg. 50 MJ kg^{-1} TS and above) utilised in other studies to pretreat microalgal biomass (Alzate et al., 2012, Gonzalez-Fernandez et al., 2012, Cho et al., 2013), even though reported to give some improvement in methane yield, do not make sense in terms of the overall energy balance as the energy input for pretreatment exceeds the total energy that can be recovered from the algal biomass. Hence, it can be concluded that ultrasound is not an effective pretreatment for microalgae.

c. Microwave

Microwaves are short wavelength electromagnetic energy varying in frequency from 0.3 to 300 GHz. Domestic and industrial microwave ovens typically operate at a frequency of 2.45 GHz. Microwave heating is a consequence of the rapidly oscillating electric field of a polar or dielectric material, which induces heat by the friction forces of molecules in movement. These waves increase the kinetic energy of the water leading to a boiling state. The quantum energy applied by microwave irradiation is not capable of breaking down chemical bonds but can break hydrogen bonds (Passos et al., 2014c).

Similar to ultrasound, the main controllable parameters influencing microwave pretreatment are output power and exposure time. The main advantage of this technique is the quick penetration of irradiation into the biomass (Kim et al., 2013), but it is also highly energy intensive. Biomass concentration and temperature are also important parameters influencing the effectiveness of pretreatment, since the biomass is heated by movement of water molecules.

Microwave pretreatment has been applied to different types of substrates to enhance methane production (Chi et al., 2011, Kuglarz et al., 2013, Saifuddin and Fazlili, 2009, Ariunbaatar et al., 2014, Jackowiak et al., 2011, Zhu et al., 2005). Microalgal biomass grown in a HRAP for wastewater treatment was subjected to microwave pretreatment (Passos et al., 2013b, Passos et al., 2014b). Experimental results indicated that the effect of the pretreatment on biomass solubilisation and methane yield increased with the applied specific energy. In BMP assays, the best result was obtained when the highest specific energy of 65.4 MJ kg^{-1} TS was applied with 8% biomass solubilisation and 78% methane yield increase (Passos et al., 2013b). In continuous conditions, methane yield increased by 30% and 58% when operating at 15 and 20 day HRT, respectively (Passos et al., 2014b). According to the authors, although some microalgae cells were still found intact under microscopic observation, cell organelles were damaged beyond repair after pretreatment, improving the anaerobic degradability as intracellular constituents were more readily available to anaerobic bacteria.The authors also noted, however, that despite the improved methane production rate and methane yield, no positive energy balance was obtained, suggesting that microwave pretreatment consumed more energy than the extra energy it produced (Passos et al., 2014b).

Although some improvements are seen, it is clear that the energy inputs considerably exceed the energy recoverable as methane from the treated biomass. Results of research into mechanical pretreatments applied to microalgal biomass are summarised in Table 2.3.

Table 2.3. Mechanical pretreatment for microalgae biogas production

Pretreatment	**Substrate**	**Reactor**	**Methane yield (mL g^{-1} VS)**	**Pretreatment conditions**	**Methane yield post treatment (mL g^{-1} VS)**	**Improvement (% CH_4 increase)**	**Energy recoverable as methane (MJ kg^{-1} VS)**	**References**
Bead Milling	*Isochrysis galbana*	Batch	43.1 $^{(e)}$	stirring of 1-5 g Glass beads (3 mm diameter) for 1-5 min	24.8	-42	0.99	Santos et al. 2014
	Scenedesmus sp.	Batch	54	stirring of 1 L of concentrated algal slurry with glass beads (1 mm diameter) for 4 h	97	80	3.9	Inglesby et al. 2015
	Spirulina sp.	Batch	113	stirring of 1 L of concentrated algal slurry with glass beads (4 mm diameter) for 1 h	166	29	6.6	Inglesby et al. 2015
Ultrasound	*Chlorella* sp. (70%) *and Scenedesmus* sp. (30%)	Batch	336	39, 117, 234 MJ kg^{-1} TS	356, 368, 385	6, 10, 15	14.2, 14.7, 15.3	Cho et al., 2013
	Microalgal biomass$^{(a)}$	Batch	148	8-34 MJ kg^{-1} TS; 19 g TS L^{-1}	156 - 196	6 - 33	6.2, 7.8	Passos et al., 2014a
	Microalgal biomass$^{(a)}$	Batch	106	26.7 MJ kg^{-1} TS	114	8	4.5	Passos et al., 2015
	Mixed microalgal culture A$^{(b)}$	Batch	272	10, 27, 40, 57 MJ kg^{-1} TS	310, 309, 309, 305	14, 14, 14, 12	12.4, 12.3, 12.3, 12.2	Alzate et al., 2012
	Mixed microalgal culture B$^{(c)}$	Batch	198	10, 27, 40, 57 MJ kg^{-1} TS	209, 214, 223, 223	6, 8, 13, 13	8.3, 8.5, 8.9, 8.9	Alzate et al., 2012
	Mixed microalgal culture C$^{(d)}$	Batch	255	10, 27, 40, 57 MJ kg^{-1} TS	314, 301, 301, 310	23, 18, 18, 22	12.5, 12.0, 12.0, 12.4	Alzate et al., 2012
	Scenedesmus	Batch	82 $^{(e)}$	100 - 130 MJ kg^{-1} TS	144, 154	75 - 90	5.7 - 6.1	Gonzalez-Fernandez et al., 2012a
Microwave	Microalgal biomass$^{(a)}$	Batch	117	21.8, 43.6, 65.4 MJ kg^{-1} TS	150, 167, 210	12 - 78	6.0, 6.7, 8.4	Passos et al., 2013b
	Microalgal biomass$^{(a)}$	CSTR, 20 days HRT	170	900W, 3 min, (70 MJ kg^{-1} TS), 26 g TS L^{-1}	270	60	10.8	Passos et al., 2014b
	Microalgal biomass$^{(a)}$	Batch	106	34.3 MJ kg^{-1} TS	128	21	5.1	Passos et al., 2015

(a) Microalgal biomass grown in wastewater treatment open pond

(b) 40% *Chlamydomonas* sp., 20% *Scenedesmus* sp., 40% unknown algae (tentatively *Nannocloropsis* sp.)

(c) 58% *Acutodesmus obliquus*, 36% *Oocystis* sp., 1% *Phormidium* sp., 5% *Nitzschia* sp.

(d) mainly *Microspora*

(e) expressed as mL CH_4 g^{-1} COD_{in}

2.2.4.2 Chemical pretreatment

Acid and alkali reagents are commonly used to solubilize the hemicellulose and lignin present in terrestrial biomass, making them more available for enzymatic attacks (Rodriguez et al., 2015). It has been indicated that acid pretreatment is more effective in hemicellulose solubilisation, whilst alkali pretreatment is more efficient in lignin removal (Bohutskyi and Bouwer, 2013, Cho et al., 2013, Mendez et al., 2013). Chemical pretreatments are commonly used in terrestrial plant biomass and also can be used for hemicellulose and potentially for the other complex cross-linked molecules that perform the same role in microalgae. In regard to pretreatment of microalgae, mostly alkaline pretreatments have been used and often combined with heat.

Cho et al. (2013) subjected a microalgal mixture of 70% *Chlorella* sp. and 30% *Scenedesmus* sp. to alkali pretreatment in which the pH of the sample biomass was adjusted to 9, 11 and 13 using 5 N NaOH. At pH 13 it was reported that the methane content of biogas decreased to 43.6%, whereas it was in the range 56-60% for untreated biomass (Cho et al., 2013). The authors suggested that alkali pretreatment to pH 13 could have reduced the activity of the inoculum as the initial pH of the mixture reached 10.2. Only alkali pretreatment at pH 9 showed a small improvement of about 8% in methane yield (363 mL CH_4 g^{-1} VS compared to 336 mL CH_4 g^{-1} VS from untreated biomass).

In work by Mendez et al. (2013), acid and alkali pretreatment was carried out on biomass of *Chlorella vulgaris*. The pH of the microalgal suspension was adjusted to 2 by adding 4 M H_2SO_4 and to 10 by adding 4 M NaOH. An increment in carbohydrates and proteins solubilized was observed, but methane yields for both acid (119.8 mL CH_4 g^{-1} COD) and alkali (113.1 mL CH_4 g^{-1} COD) pretreatments were lower than that obtained from untreated biomass (138.9 mL CH_4 g^{-1} COD). Significant improvements of between 59 - 73% in methane yield were obtained, however, when these chemical pretreatments were combined with heat treatment at 120 ^{o}C (Mendez et al., 2013).

Similarly, alkali pretreatment alone was found ineffective when five microalgae (*Chlorella* sp., *Nannochloropsis* sp., *Thalassiosira weissflogii*, *Tetraselmis* sp., and *Pavlova_cf* sp.) were pretreated at different NaOH concentrations (from 0 to 21 g L^{-1}). The effect of alkali pretreatment was negligible on biogas and methane production for all species except *Nannochloropsis* sp., where biogas production decreased by 10-30% depending on the NaOH dose. Combining alkali with high temperature pretreatment resulted in a 30 - 40% increase in methane yield for *Chlorella* sp. and *Nannochloropsis* sp., but it had no effect on the other species (Bohutskyi et al., 2014a)

2.2.4.3 Thermal pretreatment

Thermal pretreatments involve the application of heat to solubilize microalgae biomass. These techniques have long been adopted for enhancing particulate organic matter disintegration in other AD feedstocks at temperatures from 50 to 270 ^{o}C (Hendriks and Zeeman, 2009, Carrere et al., 2010, Carrere et al., 2016); however the optimal temperature range depends on the substrate characteristics (Passos et al., 2014c). For example, lignocellulosic biomass starts to solubilise when the temperature increases above 150 - 180 ^{o}C, but temperatures of 250 ^{o}C and higher should be avoided as unwanted pyrolysis reactions start to take place in this range (Hendriks and Zeeman, 2009). Thermal pretreatment of sewage sludge at temperatures above 180 ^{o}C may lead to the formation of recalcitrant compounds, reducing biomass biodegradability (Wilson and Novak, 2009). In regards to microalgae, temperatures from 55 to 170 °C have been employed to investigate the effect of thermal pretreatment on AD of this type of biomass (Chen and Oswald, 1998, Alzate et al., 2012, Gonzalez-Fernandez et al., 2012b, Keymer et al., 2013, Passos and Ferrer, 2014, Passos and Ferrer, 2015, Passos et al., 2013a, Schwede et al., 2013). As noted above, thermal pretreatments can be divided into thermal and hydrothermal depending on the conditions applied.

a. Thermal pretreatment

Thermal pretreatment, sometimes also referred to as low temperature pretreatment, consists in heating to temperatures below 100 °C at atmospheric pressure. The effect of thermal pretreatment at two temperatures (70 and 90 °C) on *Scenedesmus* biomass was investigated by Gonzalez-Fernandez et al. (2012b). In their study, the temperatures were achieved by means of a water bath; hot water was pumped into the reactor jacket and therefore the effect of heat is quantified without a change in pressure. The soluble organic matter increased 2.1 and 2.4-fold at 70 and 90 °C respectively after 30 min of pretreatment, and up to 4-fold (70 °C) and 4.4-fold (90 °C) after 180 min of pretreatment. Results of a 33-day batch test revealed that no significant effect was observed with substrate pretreated at 70 °C, whereas a 2.2-fold in methane yield was obtained with substrate pretreated at 90 °C. The authors also reported that no cell wall disruption was taking place under thermal pretreatment at 70 °C, whilst at 90 °C it was observed after 30 min and the impact increased with pretreatment time (Gonzalez-Fernandez et al., 2012b).

The effect of low temperature pretreatment on the AD of microalgal biomass grown in a pilot HRAP treating wastewater was investigated by Passos et al. (2013a). It was reported that the biomass was mainly composed of

Chlamydomonas and *Nitzchia*, although other microalgae species like *Chlorella*, *Ankistrodesmus*, *Monorraphidium* and *Scenedesmus* were also present. Thermal pretreatment was carried out at 55, 75 and 95 ^{o}C for 5, 10 and 15 h. The results showed that biomass solubilisation was enhanced with pretreatment temperature and exposure time up to 10 h. The methane yield was improved by 14%, 53% and 62% at 55, 75 and 95 ^{o}C, respectively; and was correlated with the increased solubilisation. Pretreatment at 95 ^{o}C for 10 h increased VS solubilisation by 1188%, the initial methane production rate by 90% and final methane yield by 60% compared to untreated microalgae (Passos et al., 2013a). The authors noted that solids concentration is a key factor in the energy balance, because microalgal biomass pretreated at 95 ^{o}C at a concentration of 1.17% VS resulted in a negative energy balance. When the feedstock was concentrated to 2% VS, however, the energy balance was neutral, and further concentration until 3% resulted in a positive energy balance.

Marsolek et al. (2014) investigated the effect of thermal pretreatment on AD of *Nannochloropsis oculata* following two scenarios. For scenario 1, the microalgal slurries were pretreated in an oven at 30, 60 and 90 ^{o}C for 4 h (constant time with variable temperature). For scenario 2, pretreatment was at 90 ^{o}C for 1, 3.5 and 12 h (constant temperature with variable time). No significant increase in biogas production relative to the control was observed at 30 or 60 ^{o}C, but a significant increment of 41% was achieved for the sample pretreated at 90 ^{o}C (Marsolek et al., 2014). According to the authors, this was attributed to the increase in soluble COD. For scenario 2, increases of 26.8%, 35.2% and 35.8% in biogas production were reported for 1, 3.5 and 12 h pretreatment time, respectively. The authors noted that these results are comparable to the increase of 33% in methane production when microalgal biomass was thermally pretreated at 100 ^{o}C for 8 h by Chen and Oswald (Chen and Oswald, 1998). It was considered that the process without pretreatment is still more favorable, however, because despite enhanced biogas production, the energy balance is negative for thermal pretreatment (Marsolek et al., 2014).

A similar conclusion was also drawn by Cho et al. (2013). In their study, mixed microalgal biomass consisting of *Chlorella* sp. (70% ww^{-1}) and *Scenedesmus* sp. (30% ww^{-1}) was thermally pretreated for 30 min at 50 and 80 ^{o}C in a water bath, and at 120 ^{o}C in an autoclave. Solubilisation, calculated as a function of changes in COD, was used to evaluate the efficiency of pretreatment methods. The authors reported that solubilisation increased by 1.5, 16.9 and 28.9% for the three temperatures adopted, respectively; however the increased methane production was not proportional to the increase in sCOD. Specifically, the increase in sCOD was 1.2, 3.6 and 5.5-fold for sample pretreated at 50, 80 and 120 ^{o}C, respectively, compared to the untreated sample, whereas methane production showed a 1.04, 1.14 and 1.20-fold increase, respectively. Calculations showed only biomass pretreatment at 120 ^{o}C produced positive net energy (6.3 kJ g^{-1} VS), whilst it was negative for biomass pretreated at 50 ^{o}C (-5.6 kJ g^{-1} VS) and 80 ^{o}C (-4.4 kJ g^{-1} VS). The improvement in methane production by the pretreatment was not greater than the input energy cost, and therefore it was recommended that raw microalgae be used directly as substrate for AD (Cho et al., 2013).

b. Hydrothermal pretreatment

Hydrothermal pretreatment is applied at temperatures above 100 ^{o}C, with consequent increase of pressure. In the study by Alzate et al. (2012), three microalgal mixtures, namely microalgae A (composed of 40% *Chlamydomonas* sp., 20% *Scenedesmus* sp., 40% unknown algae tentatively characterised as *Nannochloropsis*), microalgae B (58% *Acutodesmus obliquus*, 36% *Oocystis* sp., 1% *Phormidium* sp., 5% *Nitzschia* sp.) and microalgae C (mainly *Microspora* sp.) were subjected to hydrothermal hydrolysis by direct 9 bar steam injection at 110, 140 and 170 ^{o}C followed by a sudden decompression. This pretreatment process resulted in higher increases in COD solubilisation and methane yield at increasing temperatures for microalgae A and B. The highest methane yields recorded for microalgae A and B were 398 and 307 mL g^{-1} VS, respectively, both obtained at 170 ^{o}C. In contrast, the highest methane yield of 413 mL g^{-1} VS for microalgae C was obtained at 110 ^{o}C, whilst the lowest methane yield of 359 mL g^{-1} VS was observed with pretreatment at 170 ^{o}C despite the fact that higher solubilisation was reached at the highest temperature for all three microalgae. These results indicated that COD solubilisation might not be the key factor in methane production (Alzate et al., 2012) and confirm similar findings in studies with other pretreatment methods (Cho et al., 2013, Gonzalez-Fernandez et al., 2012b, Alzate et al., 2012).

High pressure thermal hydrolysis (HPTH) was employed as a pretreatment method in a study by Keymer et al. (2013). A pressure vessel containing raw *Scenedesmus* biomass and lipid extracted biomass was heated to 170 ^{o}C at 800 kPa for 30 min, after which the contents were vented to release pressure. The HPTH treatment increased the methane yields by 81% (150 mL g^{-1} VS) and 58% (140 mL g^{-1} VS) compared to that for the raw and lipid extracted microalgae, respectively. Lipid extraction itself had an effect on methane yield and gave an increment of 33% over that of the raw algae. The combined pretreatment of lipid extraction followed by HPTH resulted in a methane yield increase of 110% over raw material. In this study, the increase in methane yield followed a similar trend to that observed for the solubilisation of COD (Keymer et al., 2013).

The effect of HPTH on organic matter solubilisation and biogas production from microalgae was also investigated by Mendez et al. (2014b). *Chlorella vulgaris* biomass was subjected to three temperatures (140, 160 and 180 ^oC) and two heating times (10 and 20 min), giving pressures of approximately 3, 6 and 10 bar at 140, 160 and 180 ^oC, respectively. Results from the HPTH treatment showed that carbohydrate solubisation increased with increasing temperatures. Results from the 29-day BMP test indicated that all pretreatments showed significant increases in methane yield compared to that from raw biomass. Methane yields of samples pretreated at 140, 160 and 180 ^oC were 223, 257 and 228 mL g^{-1} COD_{in}, respectively, which were 1.4, 1.6 and 1.5 times higher than that of the raw sample (156.4 mL g^{-1} COD_{in}), respectively. Even though highest degree of solubilisation was obtained at 180 ^oC, the highest improvements in methane yield and anaerobic biodegradability were actually achieved at 160 ^oC (Mendez et al., 2014b). The authors reported that no notable differences in methane yield were observed between samples treated for 10 and 20 min. In their previously published paper, however, the effect of heating time was found to be significant. In that study, *C.vulgaris* biomass was thermally pretreated at 120 ^oC in an autoclave for 20 and 40 min. The results indicated that compared to the untreated sample (138.9 mL CH_4 g^{-1} COD_{in}), methane yield increased by 30% for 20 min pretreatment (180.3 mL CH_4 g^{-1} COD_{in}), and by 93% for 40 min (267.7 mL CH_4 g^{-1} COD_{in}) (Mendez et al., 2013).

Heating time was also reported to have an effect by Schwede et al. In this study, *Nannochloropsis salina* biomass was thermally pretreated at 100 and 120 ^oC for 2 h and 8 h. Compared to the untreated sample, cell concentrations decreased by 22% and 39% for samples pretreated at 100 ^{o}C for 2 h and 8 h, respectively. At 120 ^{o}C, the cell number decreased by 33 and 59% for 2 h and 8 h pretreatments, respectively. Results from a batch assay showed significant increases in methane yields for pretreated samples compared to the untreated. After 49 days of digestion, methane yields of samples pretreated at 2h and 8h were 510 and 570 mL CH_4 g^{-1} VS respectively, and were about 3 times higher than the untreated sample.

Mendez et at. investigated the effect of thermal pretreatment on two different microalgae *Chlorella vulgaris* and *Scenedesmus* sp. Biomass of *Chlorella vulgaris* and *Scenedesmus* sp. were diluted to reach 1.6%, 3.2%, 6.5% and 13% (weight per unit volume, w v^{-1}) and subjected to autoclaving at 120 ^{o}C for 40 min. Carbohydrates and proteins were analysed to evaluate the effect of the thermal pretreatment on organic matter solubilisation. Soluble carbohydrate content in the medium increased together with increasing biomass loads, and the trend was similar for both microalgae. Protein solubilisation showed a different profile than carbohydrates, however: specifically no remarkable differences were observed among the different biomass loads thermally pretreated when compared to the untreated material. In general, the degree of solubilisation was higher for carbohydrates than for proteins for both microalgae. Thermal pretreatment showed enhanced methane yield and biodegradability but the trend was not the same for each microalgal species. For *Chlorella vulgaris*, thermal pretreatment resulted in methane yields of 225.4, 215.4, 198.5 and 225.1 mL CH_4 g^{-1} COD_{in} at biomass concentrations of 1.6%, 3.2%, 6.5% ad 13%, respectively, which were 1.5, 1.4, 1.3 and 1.5 times higher than methane yield from the untreated biomass (150.2 mL CH_4 g^{-1} COD_{in}). For *Scenedesmus* sp., methane yields after pretreatment were 186.6, 194.7, 191.6 and 186.7 mL CH_4 g^{-1} COD_{in} at biomass concentrations of 1.6%, 3.2%, 6.5% ad 13%, respectively, which were 1.2, 1.3, 1.2 and 1.2 times higher than from the untreated biomass (154 mL CH_4 g^{-1} COD_{in}). Anaerobic biodegradability of pretreated biomass thus increased by 32 - 50% for *Chlorella vulgaris*, and 21 - 27% for *Scenedesmus* sp. This difference was attributed to the different cell wall compositions of the microalgae (Mendez et al., 2014a).

Bohutskyi et al. (2014a) investigated the effect of thermal pretreatment on five different microalgae: *Chlorella* sp., *Nannochloropsis* sp., *Thalassiosira weissflogii*, *Tetraselmis* sp., and *Pavlova_cf* sp. Microalgal samples were autoclaved at 120 ^oC and 10 bar for 30 min. The authors reported that thermal pretreatment had a minimal effect on solubilisation of *Chlorella* sp., and a moderate effect on *Nannochloropsis* sp. No effect on *Thalassiosira weissflogii* solubilisation was observed, whilst thermal pretreatment did enhance the solubilisation of *Tetraselmis* sp., and *Pavlova_cf* sp. Results from BMP tests showed that thermal pretreatment was beneficial for methane production for *Nannochloropsis* sp. and less evident for *Thalassiosira weissflogii.* In contrast , it did not significantly affect methane production from *Tetraselmis* sp., and was unfavourable for methane production fomr *Chlorella* sp. and *Pavlova_cf* sp. (Bohutskyi et al., 2014a).

Ometto et al. used thermal pretreatment on three microalgae *Chlorella sorokiniana*, *Scenedesmus obliquus* and *Arthrospira maxima* at 105, 120, 145, 155 and 165 ^oC, with associated saturated pressures close to 1, 2, 3, 5 and 7 bar respectively. Increases in biogas production were achieved in all cases except for *A.maxima* pretreated at 105 and 120 ^oC where the biogas production was inferior to the control. The highest increases in biogas production were 44%, 44% and 72%, all attained at highest temperature applied (165 ^oC), for pretreated

S.obliquus, *C.sorokiniana* and *A.maxima*, respectively. However, the biogas increment was not proportional to increasing temperatures (Ometto et al., 2014).

Defatted biomass of *Scenedesmus dimorphus* at various concentrations (1, 3 and 5 g L^{-1}) was thermally pretreated at 100, 120 and 150 ^{o}C for 20, 40 and 60 min (Sarat Chandra et al., 2014). The results showed that the solubilised organic matter increased with increasing intensity of pretreatment. A maximum of up to 71 mg sCOD L^{-1} (6.2% solubilisation) was observed at a biomass concentration of 5 g L^{-1}, temperature of 150 ^{o}C and treatment duration of 60 min. Results from the BMP assay showed that compared to the untreated biomass, higher methane yields were obtained from the pretreated samples. Methane yield from pretreated biomass ranged between 137 - 162 mL g^{-1} VS_{in} whereas from untreated biomass was 101 mL g^{-1} VS_{in}. Overall, the pretreatment increased methane yield up to 60% (Sarat Chandra et al., 2014).

Results of research into thermal pretreatments applied to microalgal biomass are presented in Table 2.4.

Table 2.4. Thermal pretreatment for microalgae biogas production

Pretreatment	**Substrate**	**Reactor**	**CH_4 yield of untreated material (mL g^{-1} VS)**	**Pretreatment conditions**	**Improvement (% CH_4 increase)**	**References**
Thermal pretreatment	*Chlorella* sp. (70%) *and Scenedesmus* sp. (30%)	Batch	336	50, 80 ^{o}C; 30 min	4, 14	(Cho et al., 2013)
	Microalgal biomass (a)	Fed-batch	300	60 ^{o}C; 1, 2, 3 h; algae conc.3, 6, 9%	13, 27, 17	(Chen and Oswald, 1998)
	Microalgal biomass (a)	Fed-batch	300	80 ^{o}C; 1, 2, 3 h; algae conc. 6, 9, 3%	7, 10, 10	(Chen and Oswald, 1998)
	Microalgal biomass (a)	Fed-batch	300	100 ^{o}C; 1, 2, 3 h; algae conc.9, 3, 6%	-3, 3, 17	(Chen and Oswald, 1998)
	Microalgal biomass (a)	Fed-batch	300	100 ^{o}C; 8 h; algae conc. 3.7%	33	(Chen and Oswald, 1998)
	Microalgal biomass (a)	CSTR, 20 days HRT	180	75, 95 ^{o}C; 10 h	67, 72	(Passos and Ferrer, 2014)
	Microalgal biomass (a)	Batch	105-111	55, 75, 95 ^{o}C; 5-15 h	11, 48, 60	(Passos et al., 2013a)
	Microalgal biomass (b)	20 L AVR (g)	179	60 ^{o}C; 3.8 h	23	(Kinnunen et al., 2014b)
	Microalgal biomass (a)	Batch	105.6	95 ^{o}C; 10 h	72	(Passos et al., 2015)
	Microalgal biomass (a)	CSTR, 28 days HRT	240	100 ^{o}C; 8 h	33	(Chen and Oswald, 1998)
	Nannochrolopsis oculata	Batch	206 (i)	30, 60, 90 ^{o}C; 4 h	-3, -4, 43	(Marsolek et al., 2014)
	Nannochrolopsis oculata	Batch	237 (i)	90 ^{o}C; 1, 3, 5 h	24, 34, 39	(Marsolek et al., 2014)
	Scenedesmus sp.	Batch	76 (j)	70, 90 ^{o}C; 3 h	12, 124	(Gonzalez-Fernandez et al., 2012b)
	Scenedesmus sp.	Batch	81.8 (j)	70, 80 ^{o}C; 25 min	9, 56	(Gonzalez-Fernandez et al., 2012a)
	Scenedesmus sp.	CSTR	33 (j)	90 ^{o}C; 1h	194	(González-Fernández et al., 2013)
	Spirulina maxima	CSTR	190	50, 100 ^{o}C; 1 h	5, -5	(Samson and Leduy, 1983b)
Hydrothermal pretreatment	*Arthrospira platensis (20% carbohydrate)*	Semi-continuous	123 (j)	Autoclave; 20 min	-22	(Markou et al., 2013)
	Arthrospira platensis (60% carbohydrate)	Semi-continuous	203 (j)	Autoclave; 20 min	-8	(Markou et al., 2013)
	Chlorella sp. (68%) *and Scenedesmus* sp. (28%)	Batch (h)	172	120 ^{o}C; 30, 60, 120 min	7, 35, 37	(Zieliński et al., 2014)
	Chlorella sp. (70%) *and Scenedesmus* sp. (30%)	Batch	336	120 ^{o}C; 30 min	20	(Cho et al., 2013)

Pretreatment	**Substrate**	**Reactor**	**CH_4 yield of untreated material (mL g^{-1} VS)**	**Pretreatment conditions**	**Improvement (% CH_4 increase)**	**References**
	Chlorella vulgaris	Batch	156.4 (j)	140 °C; 3 bar; 10, 20 min	40, 44	(Mendez et al., 2014b)
	Chlorella vulgaris	Batch	156.4 (j)	160 °C; 6 bar; 10, 20 min	83, 65	(Mendez et al., 2014b)
	Chlorella vulgaris	Batch	156.4 (j)	180 °C; 10 bar; 10, 20 min	45, 48	(Mendez et al., 2014b)
	Chlorella vulgaris	Batch	138.9 (j)	120 °C; 20, 40 min	30, 93	(Mendez et al., 2013)
	Chlorella vulgaris	Batch	150.2 (j)	120 °C; 40 min	50, 43, 32, 50 (k)	(Mendez et al., 2014a)
	Microalgal biomass (a)	Batch	122	110, 130 °C; 15-30 min	24, 17, 39, 33	(Passos and Ferrer, 2015)
	Microalgal biomass (a)	CSTR, 20 days HRT	120	130 °C; 15 min	42	(Passos and Ferrer, 2015)
	Microalgal biomass (a)	Batch	105.6	130 °C; 1.7 bar; 30 min	28	(Passos et al., 2015)
	Mixed microalgal culture A(c)	Batch	272	110, 140, 170 °C; 15 min	19, 33, 46	(Alzate et al., 2012)
	Mixed microalgal culture B(d)	Batch	198	110, 140, 170 °C; 15 min	11, 31, 55	(Alzate et al., 2012)
	Mixed microalgal culture C(e)	Batch	255	110, 140, 170 °C; 15 min	62, 50, 41	(Alzate et al., 2012)
	Nannochloropsis salina	Batch	200	100-120 °C; 2h, 8 h	185	(Schwede et al., 2013)
	Nannochloropsis salina	CSTR	130	100-120 °C; 2h, 8 h	108	(Schwede et al., 2013)
	Scenedesmus sp. (f)	Batch	380	170 °C; 8 bar; 30 min	111	(Keymer et al., 2013)
	Scenedesmus sp.	Batch	154 (j)	120 °C; 40 min	21, 26, 24, 21(k)	(Mendez et al., 2014a)
	Scenedesmus sp.	Batch	180	170 °C; 8 bar; 30 min	81	(Keymer et al., 2013)
	Spirulina maxima	CSTR	190	150 °C; 1 h	-5	(Samson and Leduy, 1983b)

(a) Microalgal biomass grown in wastewater treatment open pond
(b) *Pediastrum* sp., *Micractinium* sp., *Scenedesmus* sp.
(c) 40% *Chlamydomonas* sp., 20% *Scenedesmus* sp., 40% unknown algae (tentatively *Nannocloropsis* sp.)
(d) 58% *Acutodesmus obliquus*, 36% *Oocystis* sp., 1% *Phormidium* sp., 5% *Nitzschia* sp.
(e) mainly *Microspora*
(f) lipid extracted biomass
(g) 20 L accumulating volume reactor
(h) Oxitop respirometer
(i) calculated from biogas and CH_4 content presented in paper
(j) expressed as mL CH_4 g^{-1} COD_{in}
(k) corresponds to biomass concentration of 1.6, 3.2, 6.5 and 13%, respectively

2.2.4.4 Biological pretreatment

Bohutskyi and Bouwer argued that enzymatic hydrolysis of microalgal biomass presented a promising alternative to energy-demanding pretreatments (Bohutskyi and Bouwer, 2013). Moreover, enzymatic pretreatment does not involve inhibitory compounds. The most important parameters that influence the pretreatment effect include enzyme dose, temperature and exposure time. The main disadvantage of this pretreatment is that it is a slow and time-consuming process. Other factors including the enzyme to substrate specificity, the enormous diversity of microalgae cell wall, the enzyme production costs, and the large space required to carry out biological pretreatment are the issues that need to be considered before applying this method at industrial scale (Bohutskyi and Bouwer, 2013, Passos et al., 2014c, Sindhu et al., 2016, Montgomery and Bochmann, 2014).

Alzate et al. incubated three microalgae mixtures in the dark in a roller shaker at 55 ^{o}C for 12 h and 24 h in aerobic conditions. The biological pretreatment resulted in a reduction in methane production for both mixture A (40% *Chlamydomonas* sp., 20% *Scenedesmus* sp., 40% unknown algae) and mixture B (58% *Acutodesmus obliquus*, 36% *Oocystis* sp., 1% *Phormidium* sp., 5% *Nitzschia* sp.), but showed a 4% and 5% increase of methane production when mixture C (mainly *Microspora* sp.) was pretreated for 12 h and 24 h, respectively (Alzate et al., 2012). According to the authors, the microaerophilic biological pretreatment applied in their study requires the presence of microorganisms capable of secreting the hydrolytic enzymes needed for the degradation of complex organic matter. The authors suggested that the slight increase in methane production recorded for mixture C compared to the reduction observed for mixture A and B indicated the presence of a larger population of bacteria capaple of excreting hydrolytic enzymes in mixture C (Alzate et al., 2012).

Biomass of *Rhizoclonium* was pretreated with five different enzymes (α-amylase, protease, lipase, xylanase and cellulase) in an enzyme dosage of 1% (w/w) (Ehimen et al., 2013). The highest methane yield of 145 mL CH_4 g^{-1} TS was obtained when using a mixture of enzymes (20% of each of the five enzymes) , whilst lower methane yields of 115, 118, 121, 116 and 133 mL CH_4 g^{-1} TS were achieved when lipase, xynalase, amylase, protease, and cellulase were used separately, respectively. The methane yield obtained from use of the enzyme mixture was reported to be 31% and 21% higher than the methane yield from samples subjected to pretreatment in a Waring blender pretreatment and by sonication, respectively.

Five commercial enzymes were used in different combinations at different concentrations to pretreat three microalgae *A.maxima*, *C.sorokiniana* and *S.obliquus* (Ometto et al., 2014). Microalgae were incubated with the enzymes for 24 h at 50 ^{o}C. Results from the batch test showed positive effects from enzymatic pretreatment. Specifically, biogas production post treatment was 1461 - 1996 mL g^{-1} VS_{add} for *A.maxima*, 868 - 1292 mL g^{-1} VS_{add} for *C.sorokiniana*, and 1065 - 1669 mL g^{-1} VS_{add} for *S.obliquus*. Compared with the values from the control, biogas production increased by 8 - 10-fold for *A.maxima*, 3.2 - 4.7-fold for *C.sorokiniana*, and 4.0 - 6.3-fold for *S.obliquus*. It should be noted, however, that the values for biogas production post treatment reported by Ometto et al. (2014) are far higher than theoretical maximum biogas production. As reported in this paper, biogas production of *S. obliquus* after pretreatment with enzyme E1 and the mixture of E1 and E2 was 1425 ± 224 and 1669 ± 63 mL g^{-1} VS_{add} respectively. With the reported methane content of 63%, the methane production post treatment is 898 ± 141 and 1051 ± 40 mL CH_4 g^{-1} VS_{add} respectively, which is higher than the maximum yield of 497 mL CH_4 g^{-1} VS_{add} that is calculated theoretically (given the typical COD/VSS ratio = 1.42, and 350 mL of CH_4 is produced per each gram of COD). Therefore, these data can be considered untrustworthy and their conclusion about the positive effect of enzyme pretreatment is misleading.

2.2.4.5 Combined pretreatment

The effect of pretreatments which are the combination of more than one technique on AD of microalgae has been investigated by several researchers. One of the rare studies on pretreatment of algae in the 1980s was carried out by Samson and Leduy. In their study, *Spirulina maxima* biomass was subjected to mechanical (ultrasonic and mechanical disintegration) and thermochemical pretreatment prior to anaerobic digestion. Samples of algal biomass were adjusted to pH 1.0, 3.0, 11.0 and 13.0 by adding HCl and NaOH solution, then thermally pretreated at 50, 100 and 150 ^{o}C for 1h. The results showed that the thermochemical pretreatment significantly enhanced COD solubility, with a more pronounced effect from alkali addition than the acid. The maximum COD solubilisation of 78% was obtained when algal biomass was treated at pH 13 and 150 ^{o}C. Thermo-acid pretreatments resulted in negative effects on methane yield, whilst thermo-alkali pretreatments showed an increase of 11, 16 and 26% in methane yield when algal biomass was adjusted to pH 11 and pretreated at 50, 100 and 150 ^{o}C, respectively (Samson and Leduy, 1983b).

A similar study by Chen and Oswald in 1998 investigated the influence of themochemical pretreatment on the conversion efficiency of algal biomass to methane. Algae used for the experiment were harvested from the effluent of high-rate sewage stabilisation ponds. The algal biomass was thermo-chemically processed at different temperatures (60, 80, 100 ^oC), chemical (NaOH) doses (0 - 200 g NaOH kg^{-1} algae) and concentrations (3 - 9%) before being fed to the digestion system. The authors reported that best efficiency was attained at 100 ^oC for 8 hours at 3.7% biomass concentration and without NaOH. They stated that in comparison with untreated algae, pre-treatment improved the efficiency of methane fermentation a maximum at 33% (Chen and Oswald, 1998).

Biomass of *Rhizoclonium* was mechanically pretreated in a Waring blender followed by sonication for 10 min (Ehimen et al., 2013). The combined pretreatment increased methane yields by up to 27% compared to that for the samples subjected to mechanical pretreatment alone. Similarly, when enzymes were used in combination with mechanical pretreatment, methane yields were improved by >20% compared to the use of a mechanical size reduction method alone.

A microalgal suspension of *Chlorella vulgaris* was adjusted to pH 2 and 10 by adding 4 M H_2SO_4 and 4 M NaOH solutions, then subjected to thermal pretreatment at 120 ^oC for 20 and 40 min. After the pretreatment, the samples were neutralised before being tested in a BMP assay. The effects of thermochemical pretreatment were determined in terms of carbohydrates and protein solubilsation. The addition of H_2SO_4 to reach pH 2 enhanced carbohydrate solubilisation by 5 and 7-times for 20 and 40 min of heating time, respectively. Soluble carbohydrate concentration increased 5 times when NaOH was used, regardless of heating time. The only remarkable effect for protein solubilisation was attained with NaOH addition with an increase of 1.7 and 1.9-fold for 20 and 40 min of heating time, respectively. Results from the BMP test showed that the effect of heating time was negligible in the range used, as methane yields were 221.8 and 228.8 mL g^{-1} COD_{in} for thermochemical acid pretreatment, and 237.9 and 240.6 mL g^{-1} COD_{in} for thermochemical alkali pretreatment for 20 and 40 min heating time, respectively. These results showed the thermochemical pretreatments increased methane yield by 60 - 73% compared to the untreated biomass (138.9 mL g^{-1} COD_{in}). However the maximum yield of 267.7 mL g^{-1} COD_{in} (93% increase) was obtained when the biomass was pretreated at 120 ^{o}C for 40 min without using chemicals (Mendez et al., 2013).

The combination of NaOH addition followed by thermal pretreatment (autoclaving at 120 ^{o}C for 30 min) had a dramatic impact on COD solubilisation for four microalgae *Chlorella* sp., *Nannochloropsis* sp., *Tetraselmis* sp., and *Pavlova_cf* sp., but no significant effect was observed on *T. weissflogii*. Soluble COD of *Nannochloropsis* sp., *Tetraselmis* sp., and *Pavlova_cf* sp. all reached nearly 90%, probably as a result of disruption of microalgal cells and release of intracellular matter into surrounding solution, solubilisation of macromolecules, and partial hydrolysis of organic polymers (Bohutskyi et al., 2014a). Results from the BMP assay indicated, however, that a positive effect on methane yield was only achieved for *Chlorella* sp. and *Nannochloropsis* sp. with an increase of up to 30 - 40% for both microalgae relative to the untreated samples.

Results of effect of combined pretreatments applied to microalgal biomass are presented in Table 2.5.

Table 2.5. Combined pretreatment for microalgae biogas production

Pretreatment	**Substrate**	**Reactor**	**CH_4 yield of untreated material (mL g^{-1} VS)**	**Pretreatment conditions**	**Improvement (% CH_4 increase)**	**References**
Mechanical + untrasound	*Rhizoclonium*	Batch	62 – 97	Waring blender + sonication	27	(Ehimen et al., 2013)
Thermochemical	*Chlorella* sp.	Batch	340	NaOH (various conc.); 121 °C; 10 bar; 30 min;	30-40	(Bohutskyi et al., 2014a)
	Chlorella vulgaris	Batch	138.9 [(a)]	pH 2; 120 °C; 20, 40 min	60, 65	(Mendez et al., 2013)
	Chlorella vulgaris	Batch	138.9 [(a)]	pH 10; 120 °C; 20, 40 min	71, 73	(Mendez et al., 2013)
	Isochrysis galbana	Batch	9.27	0.2 % (v/v) H_2SO_4; 40 °C; 16 h	71.5	(Santos et al., 2014)
	Nannochloropsis sp.	Batch	360	NaOH (various conc.); 121 °C; 10 bar; 30 min;	30-40	(Bohutskyi et al., 2014a)
	Pavlova_cf sp.	Batch	510	NaOH (various conc.); 121 °C; 10 bar; 30 min;	No effect/ negative effect	(Bohutskyi et al., 2014a)
	Spirulina maxima	Semi continuous anaerobic fermentation	190	pH 3.0; 50, 100, 150 °C	-42, -16, -16	(Samson and Leduy, 1983b)
	Spirulina maxima	Semi continuous anaerobic fermentation	190	pH 11.0; 50, 100, 150 °C	11, 16, 26	(Samson and Leduy, 1983b)
	T. weissflogii	Batch	420	NaOH (various conc.); 121 °C; 10 bar; 30 min;	No effect /negative effect	(Bohutskyi et al., 2014a)
	Tetraselmis sp.	Batch	380	NaOH (various conc.); 121 °C; 10 bar; 30 min;	No effect/ negative effect	(Bohutskyi et al., 2014a)

(a) expressed as mL CH_4 g^{-1} COD_{in}

2.2.4.6 Comparison between pretreatment methods

Comparing pretreatment techniques is not a simple process because most studies to date have been conducted with different microalgae species and under different conditions. Nevertheless, some authors applied different pretreatment techniques to the same microalgal biomass that was used as a substrate for their experiment. For example, Alzate et al. (2012) compared the effects of thermal hydrolysis, ultrasound and biological pretreatment on three mixtures of microalgal biomass. Thermal pretreatment (170 °C and 6.4 bars) showed the highest increases in methane yield of 46% and 55% for microalgae mixture A (composed of *Chlamydomonas* sp., *Scenedesmus* sp., and *Nannocloropsis* sp.) and B (composed of *Acutodesmus obliquus*, *Oocystis* sp., *Phormidiu*m sp., *Nitzschia* sp.), respectively. Microalgae C, which consists mainly of *Microspora*, showed the best result of 62% increase in methane yield when pretreated at 110 °C and 1 bar. In all cases, ultrasound pretreatment resulted in a relatively low increase in methane yield (6 - 24%), whilst biological pretreatment showed either negligible improvement (4 - 5%) or negative results (-13 - -3%) (Alzate et al., 2012). In another study, Gonzalez-Fernandez et al. (2012a) compared ultrasound and thermal pretreatment of *Scenedesmus* biomass and observed the highest methane yield increases (75 - 88%) after ultrasound pretreatment with a specific energy higher than 100 MJ kg^{-1} TS. Cho et al. (2013) compared thermal, hydrothermal, ultrasound and alkali pretreatment on *Chlorella* sp. and *Scenedesmus* sp. biomass. Hydrothermal pretreatment at 120 °C for 30 min was the best method with a 20.5% increase in methane yield (Cho et al., 2013). Mendez et al. (2013) investigated the effect of thermal and thermochemical pretreatment on *Chlorella vulgaris* biomass. Thermoalkali pretreatment (120 °C, 20 - 40 min, pH 10) and thermoacid pretreatment (120 °C, 20 - 40 min, pH 2) showed significant methane yield increases of 71 - 73% and 60 - 65%, respectively as compared to the raw material.The best result obtained in this case, however, was achieved by thermal pretreatment without chemical addition (120 ^{o}C, 40 min), which enhanced methane yield by 93% (Mendez et al., 2013). Kinnunen et al. (2014b) reported that the freeze-thaw pretreatment and thermal pretreatment at low temperature (50 - 56 ^{o}C) enhanced the digestibility of microalgal biomass consisting of *Pediastrum* sp., *Micractinium* sp. and *Scenedesmus* sp.. However, no significant difference in ultimate methane yield between the two pretreatment methods was observed (Kinnunen et al., 2014b). Mahdy et al. (2014a) investigated the effect of autohydrolysis at 50 ^{o}C and its combination with NaOH at different dosages on *Chlorella vulgaris* and *Scenedesmus* sp. biomass. Despite the positive effect of the pretreatment on organic matter solubilisation, only a slight increase in methane yield was obtained. The best result for *Chlorella* biomass was achieved with thermoalkali pretreatment (2% w/w NaOH, 50 ^{o}C, 48 h) which enhanced methane yield by 17%; likewise *Scenedesmus* biomass pretreated with 5% NaOH at 50 ^{o}C for 24 h resulted in 20% methane yield enhancement (Mahdy et al., 2014a). A recent study by Passos et al. (2015) evaluated the effect of thermal (95 ^{o}C, 10 h), hydrothermal (130 ^{o}C, 15 min), microwave irradiation (900 W, 3 min, 34.3 MJ kg^{-1} TS) and ultrasonication (70 W, 30 min, 26.7 MJ kg^{-1} TS) on the mixed microalgal biomass consisting of *Stigeoclonium* sp., *Monoraphidium* sp., *Nitzschia* sp. and *Navicula* sp.. The methane yield was increased by 72% for thermal pretreatment, 28% for hydrothermal and 21% for microwave pretreatment, whilst no significant increase was observed for ultrasonication as compared to control (Passos et al., 2015).

As can be seen in Tables 2.3, 2.4 and 2.5, some authors achieved significant methane yield improvements when applying heat-related pretreatments to microalgal biomass. More specifically, all improvements with over 100% methane yield increase are associated with heat pretreatment methods, while this type of increase is not observed in any other non-heat employing method. For example, thermal pretreatment of *Scenedesmus* sp. at 90 ^{o}C in 1 h and 3 h increased methane yield by 194% (González-Fernández et al., 2013) and 124% (Gonzalez-Fernandez et al., 2012b), respectively. Hydrothermal pretreatment (100-120 ^{o}C, 2-8 h) enhanced methane yield of *Nannochloropsis salina* by 185% under batch condition, and by 108% under semi-continuous condition (Schwede et al., 2013). Similarly, hydrothermal pretreatment (170 ^{o}C, 8 bar, 30 min) enhanced methane yield by 110% for lipid extracted residual biomass of *Scenedesmus* sp. (Keymer et al., 2013). It should be noted, however, that the most significant methane yield improvements as a result of thermal pretreament were associated with the application of higher temperatures (80 ^{o}C and above). In contrast, thermal pretreatment at lower temperatures (50 - 70 ^{o}C) did not result in significant methane yield increase. The highest reported methane yield increase achieved by thermal treatment in this temperature range is 23% when pretreating biomass of *Pediastrum* sp., *Micractinium* sp., and *Scenedesmus* sp. mixture at 60 ^{o}C for 3.8 h (Kinnunen et al., 2014b). Other studies employing thermal pretreatment with a temperature range of 50 - 70 ^{o}C reported between 4% decrease and 12% increase in methane yield compared to the yield of untreated material (Gonzalez-Fernandez et al., 2012b, Gonzalez-Fernandez et al., 2012a, Cho et al., 2013, Passos et al., 2013a, Marsolek et al., 2014).

Pretreatment performance may be influenced by a number of factors. In regard to thermal pretreatment, temperature seems to be the most important factor for biomass disintegration and anaerobic digestibility. Thermal pretreatment of *Scenedesmus* sp. for 25 min at 70 and 80 ^{o}C resulted in 9% and 56% methane yield increases, respectively (Gonzalez-Fernandez et al., 2012a). Similarly, thermal pretreatment of *Scenedesmus* sp. for 3 h at 70 and 90 ^{o}C increased methane yield by 12% and 124%, respectively (Gonzalez-Fernandez et al., 2012b). The effect of temperature and exposure time was more clearly illustrated in the study by Masolek et al. (2014) in which treatment of *Nannochloropsis oculata* for 4 h at 60 and 90 ^{o}C led to 4% decrease and 43% increase in methane yield, whereas the improvement was almost the same (34% and 39%) for 3 and 5 h of thermal pretreatment at 90 ^{o}C.

The effect of pretreatments on AD of microalgae depends not only on the pretreatment conditions but also on microalgal species. In the study by Alzate et al. (2012), pretreatment at 170 ^{o}C for 15 min resulted in the maximum methane yield increase of 46% and 55% for microalgae mixture A (consisting of *Chlamydomonas* sp., *Scenedesmus* sp., and *Nannocloropsis* sp.) and B (consisting of *Acutodesmus obliquus*, *Oocystis* sp., *Phormidium* sp., and *Nitzschia* sp.), respectively. The same pretreatment condition led to an increase in methane yield of only 42% for microalgae C (mainly *Microspora*), whereas the highest increment of 62% in methane yield was obtained when biomass of microalgae C was pretreated at 110 ^{o}C for 15 min. In another study, Bohutskiy et al. (2014a) observed 30 - 40% methane yield increase for *Chlorella* sp. and *Nannochloropsis* sp., but no improvement or negative effect for *Pavlova_cf* sp., *T.weissflogii* and *Tetraselmis* sp. when these microalgae were subjected to the same pretreatment (NaOH, 120 ^{o}C, 10 bar, 30 min).

Some authors have proposed that extraction of macromolecules (lipids, proteins, nucleic acids) present in the cells of microalgae can be considered as a pretreatment because through this process the cell walls are broken down, making inner materials more easily accessible which favours the AD process. In some cases, AD of microalgal biomass subjected to extraction process showed higher methane yield compared to the raw material. For example, methane yields of lipid extracted biomass of *Scenedesmus* sp. (Keymer et al., 2013, Astals et al., 2015) and *Nannochoropsis gaditana* (Alzate et al., 2014) were reported to be higher than that of raw material.The opposite behavior was also observed, however: ie. methane yields of microalgal biomass subjected to extraction process lower than that of the raw material, as in the case of *Chlorella protothecoides* (Bohutskyi et al., 2014b), *Chlorella vulgaris* UTEX 395, *Nannochloropsis salina* and *Nanofrustulum* sp. (Zhao et al., 2014), or *Tetraselmis* sp. (Ward and Lewis, 2015). The cause for this lower methane yield, as discussed earlier, could be due to the residual reagents presented in the microalgal biomass after extraction process.

2.3 Conclusions from the literature review

Preliminary studies have shown the potential of microalgae as a substrate for renewable energy production in the form of biogas from anaerobic digestion. In addition to biogas which can be converted into heat and electricity or used directly as a fuel with or without gas upgrading to biomethane, digestate produced from AD of microalgae usually contains high ammonia concentrations which can be used as biofertiliser, or can be returned to cultivation system as a source of nutrient for the growth of microalgae.

Reported methane yields from digestion of microalgal biomass vary across a wide range from 9.87 to 600 mL CH_4 g^{-1} VS. The methane yield is strongly dependent on the individual microalgae species and specific microalgal biomass that is used as substrate for anaerobic digestion. Experimental studies have indicated that apart from operational parameters such as temperature, hydraulic retention time, organic loading rate, AD of microalgae is also significantly influenced by other factors including solid concentrations, biochemical composition and cell wall characteristics of microalgae. Ammonia toxicity and the poor degradation of the cell have been identified as factors limiting performance of AD of microalgae, and recommended as topics requiring further study.

Some researchers have carried out pretreatments in an attempt to enhance biodegradabity and methane yield of microalgae. Among the pretreatment techniques that have been used, heat-employing methods appear to be most effective with significant improvement in some cases. However, the efficiency of pretreatment methods strongly depends on such factors as pretreatment conditions, and particularly the specific microalgal biomass used as substrate for AD. Nevertheless, work on pretreatment of microalgae to date is still inadequate and thus more studies are required in order to suggest appropriate pretreatments or optimum pretreatment conditions for a particular microalgal biomass. To draw firm conclusions on the optimum strategies to enhance anaerobic degradation, further fundamental work is required looking both at the composition and molecular structure of micro-algal cells, and at factors affecting this.

Integrating microalgae cultivation in wastewater with anaerobic digestion has become a trend in recent years. This has been regarded as a sustainable approach because on the one hand microalgae can utilise nutrients in wastewater for their growth, on the other hand, the microalgal biomass produced can be used as a substrate for biofuel production. At large scale, microalgae are cultivated in HRAP and the biomass obtained is usually a mixture of different microalgae with varying species compostition as well as biochemical composition depending on the cultivation conditions, seasons etc. To date little work has been done to investigate the potential of this specific microalgal biomass as substrate for biogas production.

Most researchers employed BMP assays to investigate the AD of microalgae. BMP assays are an excellent method to determine methane potential of a substrate. However, the scalability of a substrate can only be estimated based on the results from CSTR experiments. In regard to microalgae, only a limited number of studies has been done reporting results from CSTR trials.

The following chapters address these points through a series of laboratory experiments and discussion of the results obtained.

3. MATERIALS AND METHODS

3.1 General Analytical Methods

3.1.1 Chemical Reagents and Glassware Used

Reagents

Except where otherwise stated all chemicals used were of laboratory grade and obtained from Fisher Scientific (Loughborough, UK)

Water

Solutions and standards were prepared using ultra-pure deionised (DI) water obtained from a Barnstead Nanopure ultrapure water purification system (Thermo Scientific, UK)

Laboratory practice

All laboratory operations were carried out using good laboratory practice, and having first carried out the appropriate risk assessments and, where necessary, COSSH assessments. All equipment, laboratory apparatus, and analytical instruments were operated in accordance with the manufacturer's instructions unless noted. All glassware was washed using washing detergent followed by rinsing with tap water and deionised water. The glassware used for the acid digestion was soaked in a 10% nitric acid bath for a 24 hour period after which the glassware was rinsed with deionised water.

3.1.2 Total Solids (TS) and Volatile Solids (VS)

TS and VS determination was based on Standard Method 2540 G (APHA, 2005). After thorough agitation, approximately 10 g of sample was transferred into a weighed crucible by pipetting (digestate samples) or spatula (substrate samples). Samples were weighed to an accuracy of 10 g (to ± 0.001 g) (Sartorius LC6215 balance, Sartorius AG, Gottingen Germany) and placed in an oven (Vulcan laboratory oven, LTE Scientific Ltd., Oldham UK) for drying overnight at 105 ± 1 °C. After drying the samples were transferred to a desiccator to cool for at least 40 minutes. Samples were then weighed again with the same balance, transferred to a muffle furnace (Carbolite Furnace 201, Carbolite, UK) and heated to 550 ± 10 °C for two hours. After this ashing step, samples were again cooled in a desiccator for at least one hour before weighing a third time.

After all analyses, crucibles were washed with detergent, rinsed with deionised water, and stored in an oven until required for the next analysis. Crucibles were transferred from the oven to a desiccator for cooling to room temperature before each analysis. Total and volatile solids were calculated according to the following equations:

$$\%\,\mathrm{TS} = \frac{\mathrm{W_3 - W_1}}{\mathrm{W_2 - W_1}} \,\mathrm{x}\, 100 \qquad \text{Equation 3.1}$$

$$\%\,\mathrm{VS}\ (\text{on a wet weight basis}) = \frac{\mathrm{W_3 - W_4}}{\mathrm{W_2 - W_1}} \,\mathrm{x}\, 100 \qquad \text{Equation 3.2}$$

$$\%\,\mathrm{VS}\ (\text{on a TS basis}) = \frac{\mathrm{W_3 - W_4}}{\mathrm{W_3 - W_1}} \,\mathrm{x}\, 100 \qquad \text{Equation 3.3}$$

Where: W_1 = weight of empty crucible (g)

W_2 = weight of crucible containing fresh sample (g)

W_3 = weight of crucible and sample after drying at 105 °C (g)

W_4 = weight of crucible and sample after heating to 550 °C (g)

3.1.3 pH

pH was measured using a Jenway 3010 meter (Bibby Scientific Ltd, UK) with a combination glass electrode, calibrated in buffers at pH 4, 7 and 9.2. The pH meter was temperature compensated and had a sensitivity of ±0.01 pH unit and accuracy of 0.01±0.005 pH units. Buffer solution used for calibration was prepared from buffer tablets (Fisher Scientific, UK) prepared according to the supplier's instructions. During measurements, the sample was stirred to ensure homogeneity. In addition, the pH probe was rinsed with DI water in between measurements and placed into a mild acid solution to avoid cross-contamination. Digestate samples were measured immediately after sampling to prevent changes in pH due to the loss of dissolved CO_2.

3.1.4 Alkalinity

Alkalinity was measured by titration based on Standard Method 2320B for Alkalinity (APHA, 2005). Digestate was sieved to obtain a homogenous sample and 2-5 g of this was added to 40 mL of DI water. Titration was done using a Schott Titroline Easy automatic digital titration burette system (Schott, Mainz, Germany), with the samples being magnetically stirred while the titration was carried out. A 0.25 N H_2SO_4 titrant was used to determine endpoints of pH 5.7 and 4.3, allowing calculation of total (TA), partial (PA) and intermediate alkalinity (IA) (Ripley *et al.*, 1986). PA is a measurement of bicarbonate buffering while IA is attributed to the buffering capacity of Volatile Fatty Acids (VFA).

The pH probe was calibrated before titration using buffers as described before and washed with DI water between subsequent samples to avoid cross contamination. Alkalinity was calculated according to the following equations:

$$\mathrm{TA} = \frac{(\mathrm{V}_{4.3} + \mathrm{V}_{5.7}) \times \mathrm{N} \times 50000}{\mathrm{Ws}} \qquad \text{Equation 3.4}$$

$$\mathrm{PA} = \frac{\mathrm{V}_{5.7} \times \mathrm{N} \times 50000}{\mathrm{Ws}} \qquad \text{Equation 3.5}$$

$$\mathrm{IA} = \frac{\mathrm{V}_{4.3} \times \mathrm{N} \times 50000}{\mathrm{Ws}} \qquad \text{Equation 3.6}$$

Where:

TA = total alkalinity (mg $CaCO_3$ L^{-1})

PA = partial or bicarbonate alkalinity (mg $CaCO_3$ L^{-1})

IA = intermediate or volatile fatty acid alkalinity (mg $CaCO_3$ L^{-1})

W_s = wet weight of sample (kg)

$V_{subscript}$ = volume of titrant required to reach the pH value indicated in the subscript (mL)

N = normality of the H_2SO_4 titrant, or the theoretical normality multiplied by a correction factor for the specific batch of titrant

3.1.5 Total Ammonia Nitrogen

Total ammonia nitrogen (TAN) analysis was based on Standard Method 4500-NH3 B and C (APHA, 2005). A sample aliquot of between 2 - 3 g was weighed (i201, My Weigh Europe, Huckelhoven Germany) into a digestion tube and 50 mL of DI water added. Blanks (50 mL DI water) and standards (containing 10 mL of 1000 mg L^{-1} NH_4Cl with 40 mL DI water) were also prepared in digestion tubes. 5 mL of 10 M sodium hydroxide (NaOH) was added to each digestion tube to raise the pH above 9.5 and the samples were distilled using either a Foss Tecator Kjeltec system 1002 distillation unit (Foss Tecator A-B, Hoganas, Sweden) or a Büchi K-350 Distillation Unit (Büchi, UK). Erlenmeyer flasks previously filled with 25 mL of boric acid as an indicator were used to collect the distillate and progress of the distillation was indicated by a colour change from purple to green. The distillate was titrated manually with 0.25N H_2SO_4 using a digital titration system (Schott Titroline, Gerhardt UK Ltd) until an endpoint was reached as indicated by a colour change to purple at which point the volume of titrant added was recorded. Standards and blanks were distilled in the same way. The TAN concentration was calculated according to the following equation:

$$\mathrm{TAN} = \frac{(\mathrm{A} - \mathrm{B})\ \mathrm{x}\ 14.0\ \mathrm{x}\ \mathrm{N}\ \mathrm{x}\ 1000}{\mathrm{W_s}} \qquad \text{Equation 3.7}$$

Where:

TAN = total ammonia nitrogen (mg kg^{-1} wet weight)

A = volume of titrant used to titrate the sample (mL)

B = volume of titrant used to titrate the blank (mL)

N = normality of the H_2SO_4 titrant, or the theoretical normality multiplied by a correction factor for the specific batch of titrant

W_s = wet weight of sample (kg)

3.1.6 Total Kjeldhal Nitrogen

Total Kjeldhal Nitrogen (TKN) analysis was carried out on duplicate samples alongside blanks and controls as follows: 3 - 5 g (weighed to ± 1 mg) of sample was placed in a glass digestion tube. Two Kjeltab Cu 3.5 catalyst tablets were added to facilitate acid digestion by lowering the activation energy of the reaction. 12 mL of low nitrogen concentrated H_2SO_4 was added carefully to each digestion tube and agitated gently to ensure that the entire sample was completely exposed to acid. The digestion tubes were then placed into the heating block with exhaust system using either a Foss Tecator 1007 Digestion System 6 (Foss Analytical, Hoganas Sweden) or a Büchi K-435 Digestion Unit (Büchi, UK) for approximately two hours until the solution colour became a clear blue-green. Both systems operated at 420 ± 5 ^{o}C and once the reaction was completed the tubes were cooled to around 50 °C and 40 mL of DI water slowly added to the digestion tube to prevent later crystallisation on further cooling. Samples, blanks and standards were then distilled and titrated as for Total Ammonia Nitrogen.

$$\mathrm{TKN} = \frac{(\mathrm{A} - \mathrm{B})\ \mathrm{x}\ 14.0\ \mathrm{x}\ \mathrm{N}\ \mathrm{x}\ 1000}{\mathrm{W_s}} \qquad \text{Equation 3.8}$$

Where:

TAN = total ammonia nitrogen (mg kg^{-1} wet weight)

A = volume of titrant used to titrate the sample (mL)

B = volume of titrant used to titrate the blank (mL)

N = normality of the H_2SO_4 titrant, or the theoretical normality multiplied by a correction factor for the specific batch of titrant

W_s = wet weight of sample (kg)

3.1.7 Gas Chromatograph determination of volatile fatty acid (VFA)

The method used was based on SCA (1979): Determination of Volatile Fatty Acids in Sewage Sludge (1979). Samples were prepared for analysis by centrifugation at 14,000 g (micro-centrifuge, various manufacturers) for 15 minutes. 0.9 mL of the supernatant was transferred by pipette to vials with 0.1 mL formic acid to give a final concentration of 10% formic acid. Where dilution was necessary, deionised water was used and formic acid was added to give a concentration of 10% of the total volume for analysis. If the samples at this point were turbid they were centrifuged again at 14,000 rpm to obtain a clearer supernatant. The supernatant after acidification and centrifugation was transferred into the vials and loaded onto the GC auto-sampler ready for the VFA measurement.

A standard solution containing acetic, propionic, iso-butyric, n-butyric, iso-valeric, valeric, hexanoic and heptanoic acids, at three dilutions to give individual acid concentrations of 50, 250 and 500 mg L^{-1} respectively, was used for calibration and also loaded onto the GC.

Quantification of the VFA was by a Shimazdu GC-2010 gas chromatograph (Shimadzu, Milton Keynes, UK), using a flame ionization detector and a capillary column type SGE BP-21. The carrier gas was helium at a flow of 190.8 mL min^{-1} and a split ratio of 100 to give a flow rate of 1.86 mL min^{-1} in the column and a 3.0 mL min^{-1} purge. The GC oven temperature was programmed to increase from 60 to 210 ^{o}C in 15 minutes with a final hold time of 3 minutes. The temperatures of injector and detector were 200 and 250 ^{o}C, respectively.

3.1.8 Gas composition

The gas produced during anaerobic digestion of wastes contains methane and carbon dioxide (CO_2) as its major components with minor quantities of hydrogen (H_2), hydrogen sulphides (H_2S), nitrogen (N_2), and oxygen (O_2).

Methane and carbon dioxide

Biogas composition was quantified using a Varian Star 3400 CX gas chromatograph (Varian Ltd, Oxford, UK). The GC was fitted with a Hayesep C column and used either argon or helium as the carrier gas at a flow of 50 mL min^{-1} with a thermal conductivity detector. The biogas composition was compared with a standard gas containing 65 % CH_4 and 35% CO_2 (v/v) for calibration. A sample of 10 mL was taken from a Tedlar bag used for sample collection and was injected into a gas sampling loop.

3.1.9 Gas volume

Gas bags. Unless noted, biogas was collected in gas-impermeable sampling bags. Gas bag volumes were measured using a weight-type water displacement gasometer (Walker et al. 2009). The measurement procedure was as follows: the initial height of solution in the gasometer (h_1) was recorded before the collected gas was introduced into the column through the top valve. After the bag was empty, the final height (h_2) and the weight of water (m) were recorded, as well as the temperature (T) and pressure (P) in the room. All gas volumes reported are corrected to standard temperature and pressure of 0^{o}C, 101.325 kPa as described by Walker et al. (2009) according to the following equations:

Height Gasometer Governing Equation Equation 3.9

$$V_{stp} = \frac{T_{stp}A}{T_{atm}p_{stp}}\Big(\big(p_{atm} - p_{H_2O}(T_{atm}) - \rho_b g\left(h_{t2} - h_{c2}\right)\big)h_{c2} - \big(p_{atm} - p_{H_2O}(T_{atm}) - \rho_b g\left(h_{t1} - h_{c1}\right)\big)h_{c1}\Big)$$

Weight Gasometer Governing Equation Equation 3.10

$$V_{stp} = \frac{T_{stp}A}{T_{atm}p_{stp}}\left[\left(\left(P_{atn} - p_{H_2O}(T_{atm}) + \rho_b g\left(H - h_1 - \frac{m_b}{A\rho_b}\right)\right)\left(h_1 + \frac{m_b}{A\rho_b}\right)\right) - \left(p_{atn} - p_{H_2O}(T_{atm}) + \rho_b g\left(H - h_1\right)\right)h_1\right]$$

Where:

V = gas volume (m^3)

P = pressure (Pa)

T = temperature (K)

H = total height of column (m)

h = distance to liquid surface from a datum (m)

A = cross-sectional area of gasometer (m^2)

m_b = mass of barrier solution (kg)

ρ = density pf barrier solution (kg m^{-3})

g = gravitational acceleration (m s^{-2})

$^{1,\ 2,\ stp,\ atm,\ b,\ t,\ c}$ subscripts refer to condition 1 (before addition of gas to column), condition 2 (after gas addition to column), standard temperature and pressure, atmospheric, barrier solution, collection trough and column respectively. Weight measurements were used for gas volumes with height measurements used as a back up and check on accuracy of data recording.

3.1.10 Calorific value (CV)

Calorific value was quantified using a bomb calorimeter (CAL2k EC, Digital Data System Ltd, South Africa) according to the manufacturer's instructions. The sample was pre-dried in an oven overnight at 105 ºC. Then 0.5 g (weighed with an accuracy of 0.1 mg) was added to the crucible and placed in position in the calorimeter. A cotton firing thread was attached to the ignition wire and fed to the crucible. The bomb vessel was then assembled and pressurised, using the filling station, with oxygen until the pressure reached 3 MPa. The bomb was then placed in the calorimeter and fired and temperature changes logged on the computer. A blank was also run to account for the energy released in burning the fuse and a standard was run using benzoic acid (around 1 g with an accuracy of 0.1 mg) with an higher heat value (HHV) of 26.454 MJ kg^{-1} TS.

Theoretical calorific value (TCV) was calculated from the elemental composition using the Dulong equation according to the method in Combustion File 24 (IFRF, 2014):

$$\text{HHV (MJ kg}^{-1}\text{ VS)} = (34.1C + 102H + 6.3\,N + 19.1S - 9.85O)/100 \quad \text{(Equation 3.11)}$$

3.1.11 Elemental Composition

Carbon, hydrogen and nitrogen contents of samples were determined using a FlashEA 1112 Elemental Analyser (Thermo Finnigan, Italy). Samples were air dried and milled to obtain a homogenous sample. Sub-samples of approximately 0.3 - 0.4 mg were weighed into standard weight tin disks using a five decimal place analytical scale (Radwig, XA110/X, Poland). These were placed in a combustion/reduction reactor held at 900°C then flash combusted in a gas flow temporarily enriched with oxygen resulting in a temperature greater than 1700 ^{o}C and the release of N_xO_x, CO_2, H_2O and SO_2 (depending on the composition of the sample). The gas mixture was then analysed by GC with the different components are measured by appropriate detectors. The working conditions of the elemental analyser were as described in the manufacturer's technical literature and method sheets. Standards used in this analysis were atropine, nicotinamide and birch leaf.

3.2 Materials

During the study, three types of microalgal feedstock were used, namely freeze dried microalgae (FDA), the fresh frozen microalgae (FFA), and the fresh microalgal paste.

The first type of feedstock was grown in a tubular photobioreactor in the experimental station of the University of Almeria at Las Palmerillas, El Ejido Spain. This is a new strain of microalgae which is classified as *Scenedesmus almeriensis*. After being harvested, the biomass was freeze-dried and a batch with a total dry weight of around 20 kg was transported to the University of Southampton, UK. Another batch of FDA grown under the same conditions with a total dry weight of around 7 kg was also transported to the University of Southampton and used for digestion trials.

The second type of feedstock was fresh-frozen microalgae (FFA) which were grown in a 100 m^2 open raceway at the Las Palmerias experiment station of Fundacion Cajamar in El Ejido, Spain. The material was not a pure culture but was mainly comprised of *Scenedesmus* sp and *Chlorella* sp, two microalgal species which are very typical of the green algae (Chlorophyta) that inhabit waste stabilisation pond systems and nutrient-enriched waters. The biomass was then centrifuged and frozen for shipping to the UK. Once it got to the University of

Southampton, it was thawed and mixed to give a single homogenous sample, then was distributed to 2 L containers and stored at -18 ^{o}C before usage.

The third type of feedstock was fresh microalgal paste (FMP) which was grown under the same conditions as the FFA mentioned above but was from a different batch. After being harvested, around 5 kg of the fresh microalgal paste was sent by courier from Spain to the University of Southampton and was used immediately for digestion experiments.

The freeze dried algae were prepared for feeding to the digesters by mixing with an appropriate quantity of deionised water to give the desired solids concentration.

Both freeze drying and freezing of microalgae can be considered as pretreatments, as they may affect the specific methane yield of the material. The effects of these processes are discussed in chapter 4.

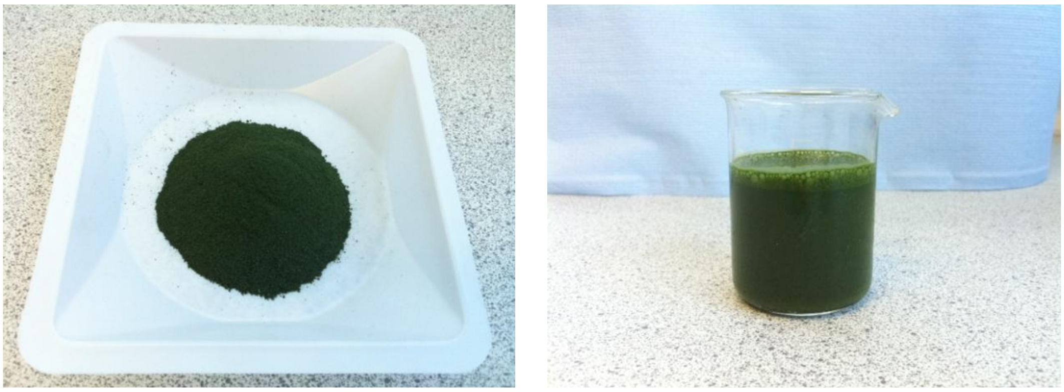


Figure 3.1. Freeze dried microalgae (left) and fresh frozen microalgae after being thawed (right)

3.3 Digesters

Two types of digester were used in this study: continuously stirred tank reactors (CSTR) and static biochemical methane potential (BMP) digesters. The following is the description of construction and operation these digesters.

3.3.1 Continuously stirred tank reactors (CSTR)

3.3.1.1 Construction

Each digester had a total volume of 2 litres and was operated at a working volume of 1.5 litres. A schematic drawing of a pair of digesters is shown in Figure 3.2. The digesters were constructed in PVC with a top flange to which a top plate was secured using stainless steel bolts and wing nuts. A gas tight seal between the top plate and the digester flange was maintained using a closed pore neoprene gasket. The top plate was fitted with a gas outlet connector and a feed port sealed with a rubber bung. On the top plate a DC motor was mounted which was coupled to the digester stirrer through a draught tube water gas seal, the draught tube itself being secured in a gas tight compression seal. The digester contents were continuously stirred at 40 rpm by means of an asymmetric stirrer. Digestate was removed from the digester via a 15 mm diameter outlet port at the base of the digester. Digester temperature was maintained at 35 ^{o}C +/- 0.5; by water circulating through an external heating coil around the digesters. When assembled, and before filling, each digester was tested for gas leaks by applying a positive pressure to the digester and submerging in water to ensure there was no gas escape when all ports were sealed. The digesters were connected to gas counters, which continuously measured gas production throughout the digestion period; the gas counters operated by the alternate filling and discharging of a calibrated tipping bucket cell which logged each discharge via a labjack (labjack ltd) computer interface. (Walker et al, 2009). The calibration of each gas counter was checked twice a week by attaching a 10-litre gas collection bag (Tedlar SKC 232, SKC Ltd, Blandford Forum, UK) to the gas vent of the gas counter and measuring the collected volume.

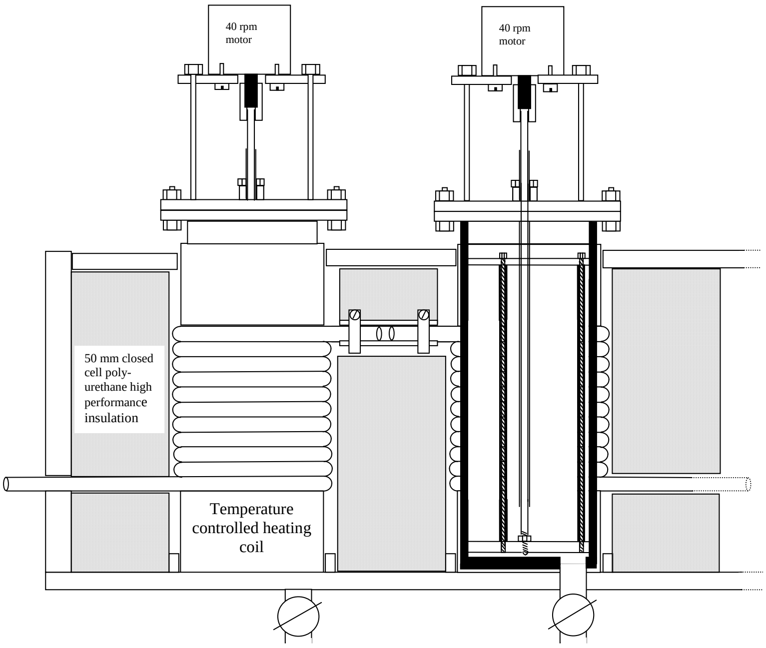


Figure 3.2. Schematic of 2-L CSTR digester

2-L digester

Set of 2-L digesters in temperature controlled box

Figure 3.3. Laboratory-scale digesters used in the research

3.3.1.2 Digester operation and calculations

The digesters were operated in semi-continuous mode i.e. fed daily with a specific amount of feedstock and with digestate removed to maintain a constant volume in the digesters. The organic loading rate (OLR) was determined according to equation 3.12.

$$OLR = \frac{mVS_{substrate}}{V_{reactor}} \qquad \text{(equation 3.12)}$$

Where:

m is the mass of substrate daily added to the reactor (g day^{-1})

VS $_{\text{substrate}}$ is the volatile solid content of feedstock (% wet weight)

V $_{\text{reactor}}$ is the volume of reactor (L)

The Hydraulic Retention Time (HRT) of the digester was calculated as in equation 3.13.

$$HRT = \frac{V_{reactor}}{Q} \qquad \text{(equation 3.13)}$$

Where:

V $_{\text{reactor}}$ is the working volume of each reactor (mL)

Q is the daily flow of material (substrate added and digestate removed) through the reactor (mL day^{-1})

The amount of substrate and digestate was measured in g but for ease of calculation it was assumed that both the substrate and digestate had a specific gravity of 1.0. Therefore, 1g of substrate and digestate was considered to be equivalent to 1 mL.

The performance of bioreactors was monitored in terms of specific biogas and methane production and VS destruction which were calculated using equations 3.14, 3.15 and 3.16

Specific biogas production = $\dfrac{V_{biogas}}{OLR \times V_{reactor}}$ (equation 3.14)

Where:

V_{biogas} is the volume of biogas produced daily (L day^{-1})

OLR is the organic loading rate (g VS L^{-1} day^{-1})

$V_{reactor}$ is the volume of reactor (L)

Specific methane production = $\dfrac{V_{CH4}}{OLR \times V_{reactor}}$ (equation 3.15)

Where:

V_{CH4} is the volume of methane produced daily (L day^{-1})

OLR is the organic loading rate (g VS L^{-1} day^{-1})

$V_{reactor}$ is the volume of reactor (l)

The destruction of volatile solids expressed as a % was calculated as follows:

$$VS_{reduction} = \frac{VS_{added}(g) - VS_{removed}(g)}{VS_{added}(g)} \quad \text{(equation 3.16)}$$

3.3.2 Static BMP test

The BMP apparatus consisted of glass reaction bottles each with a capacity of 550 mL and a working volume of 400 mL sealed with a rubber bung through which a stainless steel metal tube was inserted. The bottles were maintained at 35 ^{o}C in a temperature-controlled water bath at 35^{o}C with each one connected by PVC tubing from the stainless steel tube to liquid displacement gasometers. The gasometers themselves were sealed acrylic cylinders with graduated markings and were partially immersed in a trough filled with a barrier solution of acidified saline (75% saturated solution of sodium chloride (NaCl) at pH 2), designed to minimise losses of CH_4. The barrier solution in the tubes when displaced by biogas was maintained at a constant head in the trough by means of a constant head overflow. The volume of biogas collected was corrected to a STP of 0°C and 101.325 kPa (Walker et al., 2009). Biogas samples were taken from the gas collection cylinder via a 3-way valve and a syringe and analysed for gas composition (section 3.1.9). The gas collection tubes were refilled with the barrier solution when required: this was done using a vacuum pump connected to the 3 way valve at the top of each cylinder.

(a) Reactors in water bath (b) Detail of gasometers

Figure 3.4. Photographs of BMP apparatus

Before starting the test, the BMP apparatus was checked for leaks by filling the trough in which the gasometers were mounted with the acidified saline solution, and pulling it up in the gasometer and making sure that the level had not dropped in a 48-hour period. On the day of test, fresh inoculum and substrate were collected. Control reactors were filled with 400 mL inoculum whereas; the test reactors were filled with a mixture of inoculum and substrate in a ratio according to the experimental design. Temperature, pressure and level of barrier solution in the gasometers was noted every hour during working hours in the first week, every 2 - 3 hrs in the 2nd and 3rd week and once a day over the remaining period of the test. TS and VS of digestate was determined at the end of BMP test. The results were expressed in terms of cumulative biogas and specific methane yield per gram of substrate added and the VS destruction.

Theoretical methane potential (TMP) and biogas composition was calculated based on the Buswell equation (Symons and Buswell, 1933)

$$C_aH_bO_c + \left(a - \frac{b}{4} - \frac{c}{2}\right)H_2O \rightarrow \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4}\right)CO_2 + \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4}\right)CH_4 \qquad \text{(equation 3.17)}$$

3.3.3 Digester Inocula

The 2-litre digesters used for the semi-continuous trial were initially seeded with digestate from a mesophilic digester treating municipal wastewater biosolids at Millbrook Wastewater Treatment plant, Southampton, UK. Acclimated digestate from previous experimental runs was used as the inoculum in succeeding digestion trials. Digestate from Millbrook Wastewater Treatment plant was also used as inoculum for all BMP assays in this study.

3.3.4 Digester supplements

The trace element (TE) solutions used, one composed of cations and the other oxyanions (see Table 3.1) were based on a modified TE recipe developed by University of Southampton (Banks et al., 2012). TE were supplemented by weekly addition of the two solutions at a rate of 0.5 mL of each solution for every 1 kg of microalgal biomass added to give a steady state minimum concentration of TE in the digester.

Table 3.1. Concentration of trace elements in stock solution

Trace element	**Compound use**	**Element concentration in the working condition (mg L^{-1})**	**Compound concentration in stock solution (g L^{-1})**
Cation			
Aluminium (Al)	$AlCl_3.6H_20$	0.1	1.790
Boron (B)	H_3BO_3	0.1	1.144
Cobalt (Co)	$CoCl_2.6H_2O$	1.0	8.076
Copper (Cu)	$CuCl_2.2H_2O$	0.1	0.536
Iron (Fe)	$FeCl_2.4H_2O$	5.0	35.6
Manganese (Mn)	$MnCl_2.4H_2O$	1.0	7.204
Nickel (Ni)	$NiCl_2.6H_2O$	1.0	8.100
Zinc (Zn)	$ZnCl_2$	0.2	0.718
Oxyanion			
Molybdenum (Mo)	$(NH_4)_6Mo_7O_{24}.4H_2$	0.2	0.736
Selenium (Se)	Na_2SeO_3	0.2	0.876
Tungsten (W)	$Na_2WO_4.2H_2O$	0.2	0.718

3.4 Experimental plan

The work involved experimental studies at laboratory scale to investigate the methane yield of microalgal biomass grown in large-scale production systems, and to investigate potential methods of enhancing this through pre-treatments. The work involved semi-continuous studies aimed at establishing the digestibility of the material, the effect of factors such as concentration and ammonia inhibition, and any potential for acclimatisation under long-term exposure. These are complemented by batch screening tests to determine the effectiveness of thermal acid and alkaline pre-treatments to enhance methane yields, with the most promising options tested in semi-continuous trials. The experimental work carried out as follows:

- Biochemical methane potential (BMP) assays to investigate the anaerobic digestion kinetic and ultimate methane yield two microalgal substrates (freeze-dried *Scenedesmus almeriensis* and fresh frozen mixture of *Scenedesmus* and *Chlorella*).
- Semi-continuous digestion trials of freshwater microalgae in continuously stirred tank reactor (CSTR) to investigate the digestibility and methane yield of these microalgal substrates.
- Batch screening tests to find out the optimum pretreatments on anaerobic digestion of microalgal mixture of *Scenedesmus* and *Chlorella*
- Semi-continuous digestion trials of microalgal mixture after pretreatment to evaluate the effect of pretreatment under continuous conditions

Full details of the experiments are presented in Chapter 4.

4. RESULTS AND DISCUSSIONS

4.1 Substrate characterisation

Detailed characterisation was carried out on the fresh-frozen microalgae (FFA) and on the main batch of freeze-dried microalgae (FDA) used in the research (batch 1). The results are shown in Table 4.1. A second batch of FDA grown under the same conditions was also used and give average results for TS and VS.

Table 4.1. Characteristics of freeze-dried microalgae (FDA) and fresh-frozen microalgae (FFA)

Parameters	**FDA**	**FFA**
TS (g kg^{-1} WW)	943.2 [a]	108.5[b]
VS (g kg^{-1} WW)	767.7[a]	43.3[b]
VS (as %TS)	81.4	39.9
Elemental analysis (%, on a VS basis)		
C	54.2	45.5
H	6.8	9.0
O	28.6	35.8
N	9.6	8.7
S	0.8	1.0
Other substrate parameters		
C/N	5.6	5.2
TKN (g N kg^{-1} WW)	73.69[a]	3.77[a]
Measured Calorific Value (CV) (MJ kg^{-1} VS)	23.1	21.4
Theoretical CV (MJ kg^{-1} VS)	23.36	21.91
Theoretical Methane Potential (TMP) (L CH_4 g^{-1} VS)	0.536	0.495

[a] measured as dry matter
[b] measured as wet matter

As can be seen in Table 4.1, the TS and VS content of freeze-dried microalgae (FDA) were 940.4 and 764.7 g kg^{-1} WW, respectively, corresponding to a VS/TS ratio of 81.4%. The fresh frozen algal mixture (FFA) had a lower TS and VS content of 108.5 and 43.3 g kg^{-1} WW, respectively. These values show that FFA has high proportion of inorganic matter (VS/TS ~0.4). The difference in values for the two algal materials may reflect the different cultivation systems, with the FFA subject to both ingress of wind-blow grit and increases in salt concentration due to evaporation. The Total Kjeldahl Nitrogen (TKN) of FDA and FFA was 73.69 and 3.77 g N kg^{-1} WW, respectively. Calorific values measured by bomb calorimetry and expressed on a VS basis were 23.1 and 21.4 MJ kg^{-1} VS for FDA and FFA respectively, showing good agreement with the theoretical CV values and thus giving confidence in the elemental analysis results. The C/N ratio of the two algal materials are 5.6 and 5.2, both much lower than the ratio of 20 - 30/1 that is often considered optimum for AD in literature (Yen and Brune, 2007).

4.2 Anaerobic digestion of non-pretreated algal biomass

4.2.1 Biochemical methane potential of FDA and FFA

Objective

The purpose of the BMP test was to obtain a value that represents the maximum possible methane yield which can be obtained under non-limiting conditions: this provides a baseline value against which the methane yield in semi-continuous digestion trials can be compared.

Summary method

A BMP assay was carried out to determine the methane potential and biodegradability of different microalgal substrates including FDA, FFA and six other laboratory-grown microalgae samples. The BMP was carried out jointly by Keiron Roberts and myself, using inoculum from Millbrook WWTW. The inoculum to substrate ratios used were around 4:1 based on the VS content of the materials. The test was run against blank and positive controls at 37 ±1 ^{o}C over a period of 90 days with gas volume and composition measured at regular intervals. A detailed description of BMP set-up and implementation is presented in section 3.3.2.

Results

This section presents BMP results for the two substrates that were used in this study: some of these have also been published in Roberts et al. (Roberts et al., 2016a).

The results of the 90-day BMP test for the two algal materials FDA and FFA are shown in Table 4.2 and Figure 4.1. Data are presented as average value of triplicate samples. Biomass concentration and other characteristics of the two microalgal materials are shown in Table 4.1.

Table 4.2. Specific methane yields of FDA, FFA and cellulose positive control (BMP test values)*

Samples	Measured BMP (L g^{-1} VS)	TMP (L g^{-1} VS)	BMP as % of TMP	BMP as % of measured CV
FDA	0.161 ± 0.017	0.536	30.0	26.4
FFA	0.220 ± 0.007	0.495	44.4	41.0
Cellulose control	0.415 ± 0.004	0.415	100.0	-

**data expressed as average value of triplicate samples*

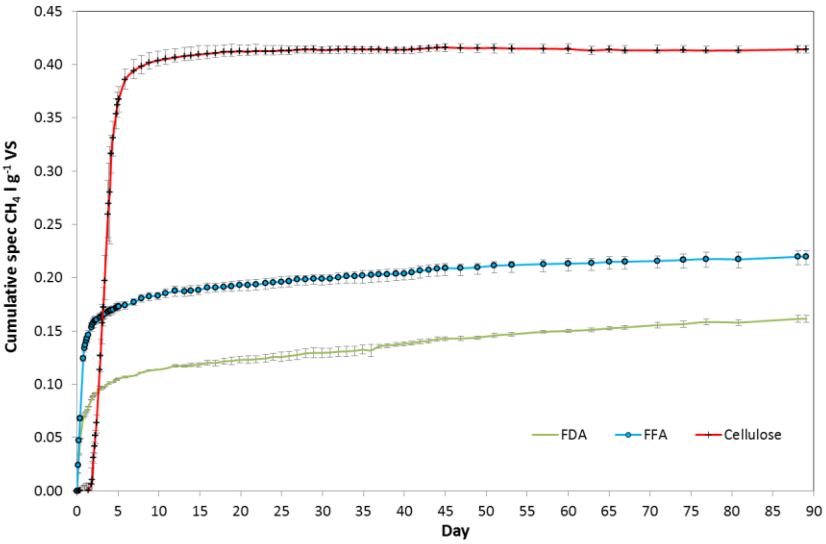


Figure 4.1. Cumulative net specific methane production of FDA, FFA and the cellulose positive control

As can be seen in Figure 4.1, after a short time lag the cellulose positive control had a rapid methane production and reached a plateau after 9 days: the specific methane yield (SMY) of the cellulose control at the end of the test was 0.415 ± 0.004 L CH_4 g^{-1} VS: this is in good agreement with the theoretical value and confirms that the inoculum and test conditions used were suitable.

For algal samples, the cumulative specific methane yield of FFA by day 90 was significantly higher than that of FDA, at 0.220 ± 0.007 and 0.161 ± 0.017 L CH_4 g^{-1} VS respectively. It can be seen, however, that the cumulative SMY of the two samples was still increasing very slightly at the end of the test, even after 90 days.

The change in slope of the cumulative SMY curves occurred within the first 2 days of the test for both samples, indicating that most readily degradable biomass was broken down quickly in the first 2 days, and the more difficult to degrade biomass is broken down gradually in the subsequent days. By day 2, the SMY for FFA was 0.158 L CH_4 g^{-1} VS accounting for 72% of the final SMY, whilst it was 0.089 L CH_4 g^{-1} VS for FDA, accounting for 55% of the final SMY. This suggests that FFA has a higher proportion of readily biodegradable material compared to FDA.

The measured BMP value for FDA was considerably lower than that for FFA. Compared to the theoretical methane potential calculated from the Buswell equation (equation 3.17), the measured BMP value for FDA was only 30% of the TMP whilst it was 44% for FFA, indicating a significantly different degree of VS breakdown between the two materials. The efficiency of conversion of the substrate, expressed in terms of the proportion of the measured CV recovered as methane, was 41.0% for FFA and only 26.4% for FDA. The low measured BMP values could be due to the rigid cell wall structure of *Scenedesmus* that hinders microalgae degradation (Roberts, 1974, Gerken et al., 2013, Torres et al., 2013). A possible cause for the lower measured BMP value of FDA is the loss of more readily degradable volatile organic compounds through sublimation during the freeze-drying process. Possible support for this hypothesis comes from Mussgnug et al. who observed reductions in BMP values after oven drying of *Chlorella kessleri* and *Chlamydomonas reinhardtii* (Mussgnug et al., 2010), and attributed this to loss of volatile compounds and/or decreased accessibility of the dried residue.

The SMY of FDA from this BMP test is comparable to the value of 0.163 L CH_4 g^{-1} VS reported by Astals et al. (2015), 0.170 L CH_4 g^{-1} VS reported by Zamaloa et al. (2012b), and 0.178 L CH_4 g^{-1} VS reported by Mussgnug et al. (2010), but is considerably lower than the range of 0.203 - 0.401 L CH_4 g^{-1} VS reported by other researchers who also used *Scenedesmus* biomass as substrate for their anaerobic digestion experiments (Frigon et al., 2013, Hernández et al., 2014, Zamalloa et al., 2012, Klassen et al., 2015, Tartakovsky et al., 2015). Lower methane yields for *Scenedesmus* biomass are also reported by some other authors, including the value of 0.54 L CH_4 g^{-1} VS by Inglesby et al. (2015), 0.88 L CH_4 g^{-1} VS by Ometto et al. (2014), 0.140 L CH_4 g^{-1} VS by Ramos-Suarez and Carreras (2014). Some of these very low values are suspect, however, and may reflect poor practice or non-optimum conditions during measurement; whereas in the current test the good agreement of the positive control indicates that the low values obtained are valid.

The SMY of FFA achieved in this BMP is close to the middle of the rather wide range of 0.172 - 0.336 L CH_4 g^{-1} VS reported for digestion of mixture of *Chlorella* and *Scenedesmus* biomass (Golueke et al., 1957, Cho et al., 2013, Zieliński et al., 2014).

4.2.2 BMP kinetic model

Biodegradability of the two microalgal samples was assessed from BMP kinetic data using the pseudo-parallel first-order model shown in equation 4.1 below. This model was proposed by Rao et al. (2000) for municipal garbage, and has been successfully used of a range of substrates with more readily and less readily biodegradable fractions (Rao et al., 2000, Roberts et al., 2016a)

$$Y = Y_m \left(1 - Pe^{-k_1 t}\right) - (1 - P)e^{-k_2 t}) \qquad \text{[equation 4.1]}$$

Where:

Y is the cumulative methane yield at time t

Y_m is the ultimate methane yield

k_1 is the first order rate constant for the proportion of readily degradable material

k_2 is the first order rate constant for the proportion of less readily degradable material

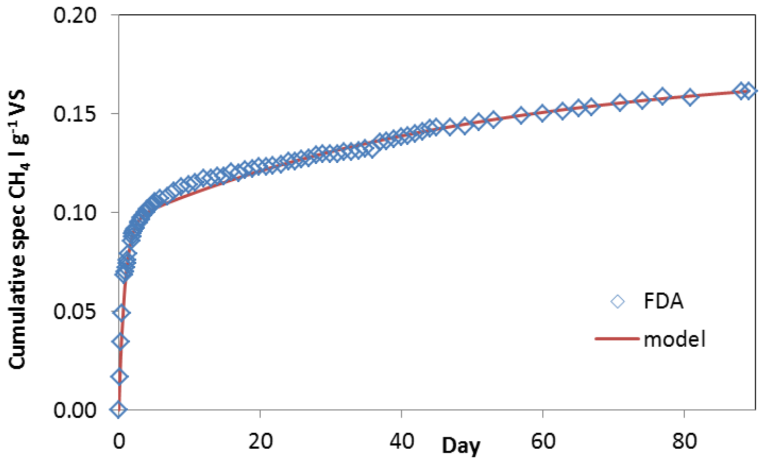
P is the proportion of readily degradable material

Values of kinetic parameters were estimated manually by sequential variation of P, k_1 and k_2 to 2 decimal places to give the maximum coefficient of correlation (R^2) with the experimental data using the method of least squares. The results of the modelling approach are shown in Figure 4.2 and kinetic coefficients obtained are shown in Table 4.3.

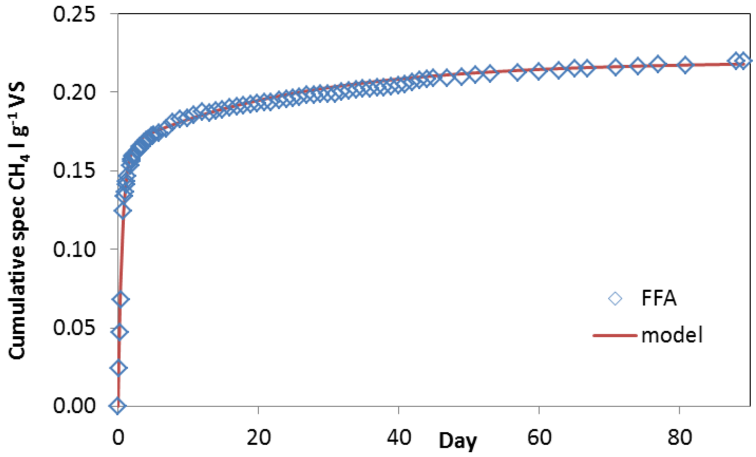
Table 4.3. Kinetic coefficients from modelling

	FDA	FFA
Y_m	0.175	0.220
P	0.54	0.75
k_1	1.21	1.66
k_2	0.02	0.04
R^2	0.9945	0.9956

Table 4.3 shows that the proportion of anaerobically biodegradable material that was readily degradable was 54% for FDA and 75% for FFA. Compared to the FFA, the FDA had lower values for k_1 and k_2. With R^2 values of 0.9945 and 0.9956 for FDA and FFA respectively, the model gives a good fit to the experimental data. As these kinetic coefficients were obtained from BMP test, they cannot be used directly for continuous or semi-continuous digestion, but they do indicate the amenability of the material to digestion and thus may suggest the most suitable type of reactor and retention time for use in a full-scale system. In both cases although the k_1 values were reasonably high, the proportion of readily degradable material was low, indicating a long retention time and therefore a relatively high solids content may be beneficial if a conventional CSTR system is to be used. Reliable design values, however, can only be obtained by carrying out a continuous or semi-continuous fed trial over a period of several retention times and this therefore formed the next stage of the work.



a) FDA



b) FFA

Figure 4.2. Kinetic models showing methane production from FDA (a) and FFA (b)

4.2.3 Semi-continuous Digestion Trials of non-pretreated materials

A series of three semi-continuous digestion trials was carried out to investigate the AD performance of the two microalgal samples under semi-continuous conditions. FDA was used as feedstock for digestion trial 1 and 2 and FFA as feedstock for digestion trial 3.

4.2.3.1 Digestion Trial 1 (DT-1)

Summary method

Ten 2 L (1.5 L working volume) digesters were utilised, with inoculum from Millbrook WWTW. At the beginning, the ten digesters were fed with FDA at a feed solids concentration of 20% VS and an organic loading rate of 2 g VS L^{-1} day^{-1}, which gave a HRT of 100 days. This relatively high concentration was chosen in order to provide an extended period for biodegradation, similar to that used in the BMP tests.

On day 116, six digesters (K3, K4, K5, K6, K7 and K8) were transferred to Digestion Trial 2 (DT-2) while two pairs of digesters(K1-K2 and K9-K10) continued in operation in DT-1. From day 116, the OLR for the pair K1-K2 was raised to 3 g VS L^{-1} day^{-1} , with a HRT of 67 days, whilst the OLR for the pair K9-K10 remained unchanged at 2 g VS L^{-1} day^{-1} as a control. The digesters were supplemented with trace elements by weekly addition as described in section 3.3.4.

The FDA feedstock used in DT-1 came from different cultivation batches. The first batch of material was used to feed the digesters from day 1 to day 570. From day 571 to the end of the run, the digesters were fed with FDA from the second batch. TS and VS of first batch FDA were 943.2 and 767.7 g kg^{-1} WW. TS and VS of second batch FDA were 948.2 and 678.2 g kg^{-1} WW.

Due to a shortage of feedstock, feeding of digesters K2 and K10 was stopped on day 822, while K1 and K9 continued to run normally until the end of digestion trial DT-1 on day 853.

Operational conditions for DT-1 are shown in Table 4.4.

Table 4.4. Notation and operational conditions of DT-1

Digester	Operational condition
All	Started day 1. VS of feed 20%. OLR 2 g VS L^{-1} day^{-1} until day 115.
K1 and K2	OLR increased to 3 g VS L^{-1} day^{-1} from day 116. K2 stopped on day 822.
K3 and K4	Stopped day 116.
K5 and K6	Stopped day 116.
K7 and K8	Stopped day 116.
K9 and K10	OLR remained at 2 g VS L^{-1} day^{-1} throughout. K10 stopped on day 822.

Experimental results

Start up

Figure 4.3 shows the main monitoring parameters in the first 115 days of operation when all 10 digesters were running under the same conditions.

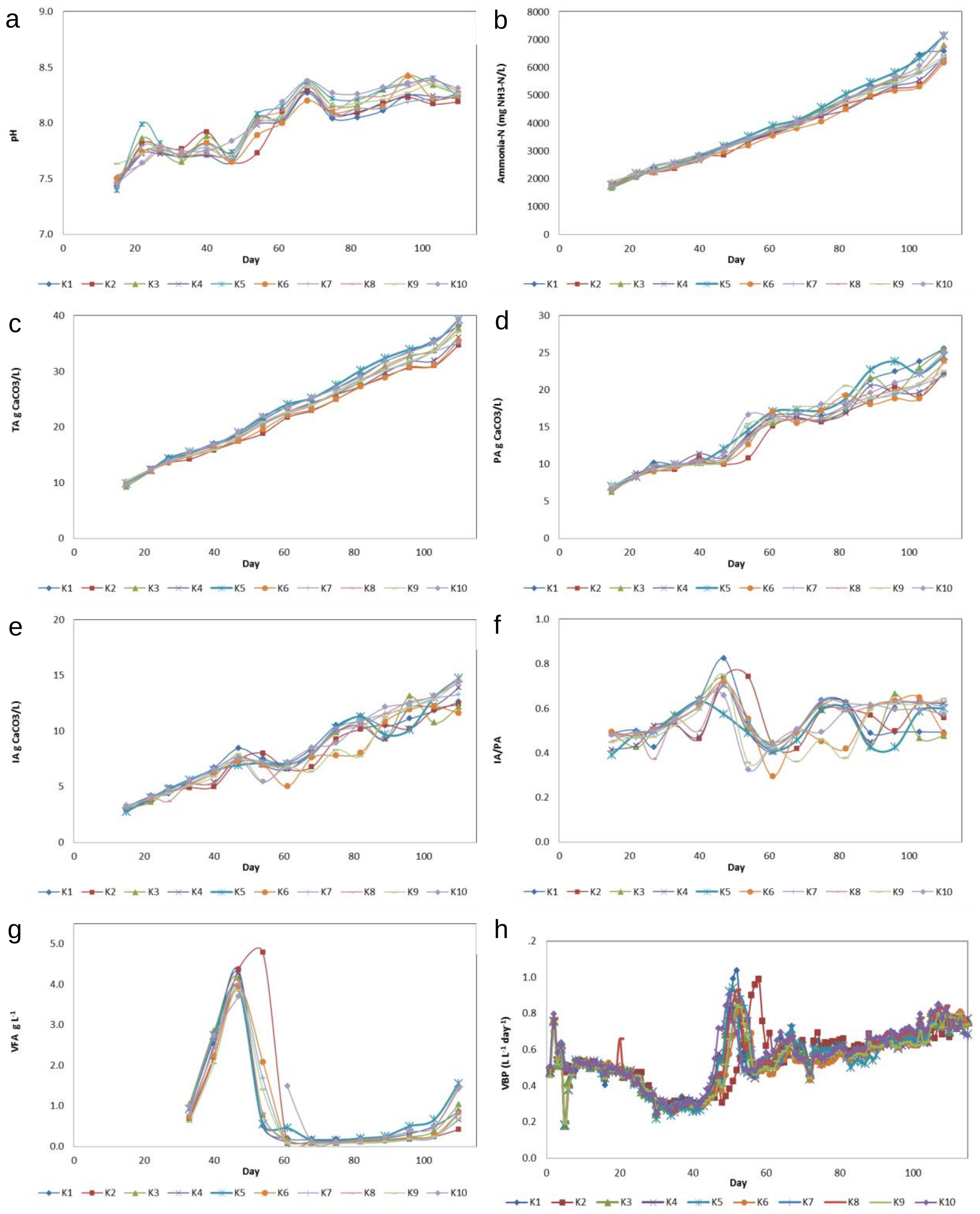


Figure 4.3. Semi-continuous digestion of FDA at a VS concentration of 20%: pH (a), TAN (b), Total alkalinity (c), Partial alkalinity (d), Intermediate alkalinity (e), IA/PA (f), VFA (g) and VBP (h) during first 115 days of operation

It can be seen that, although conditions in the digesters were changing fairly rapidly in response to the new feedstock, there was generally very good agreement between replicates. pH appeared to have stabilised at around 8.3 from day 60 onwards. TAN concentrations were rising rapidly but in step in all digesters. The increase in TAN was reflected in a rise in TA. PA and IA also increased, but there was a slight increase in the IA/PA ratio between days 40 - 60, which then stabilised at 0.5 - 0.65. The cause of this shift was a transient peak in VFA concentrations which was seen in all digesters in this period. Similar transient VFA peaks have previously been observed during acclimatisation of this inoculum to new feedstocks (Roberts et al., 2016). The increase in VFA concentrations by day 100 could be attributed to the high TAN concentrations which were around 5.2 - 6.4 g N L^{-1} and are in the inhibitory range (Chen et al., 2008). TAN and VFA concentrations continued to increase in the subsequent days whilst biogas was still produced, it is thus hypothesized that there was a shift in methanogenic population to the more ammonia tolerant hydrogenotropic type. Volumetric biogas production fell during the period of VFA accumulation then increased while the VFA was consumed; but by the end of the period appeared to have stabilised at around 0.75 L L^{-1} day^{-1}. Biogas methane concentration (not shown) was only measured from day 62 but appeared to have stabilised at around 62% giving a specific methane productivity of around 0.22 L CH_4 g^{-1} VS, well above the BMP value of 0.175 L CH_4 g^{-1} VS. At the end of the first HRT it therefore appeared that the digesters were showing good replicability in adaptation to the new feedstock.

Continuation of experiment

Figure 4.4a shows the gradual accumulation of both TS and VS in the digesters over the course of the experiment. It is clear that both TS and VS did not reach a stable value until around day 320 (3.2 HRT in K9 and K10) with the concentration of TS at ~17.5% and VS at ~12.50%. After day 571, TS gradually increased to ~19% while VS decreased and stabilised at ~11%, indicating that the feedstock used from day 571 was slightly more degradable than that used in the first period. The degradation of the volatile solids fed to the digester in both periods is less than 50%, however, indicating that the algae are not fully degraded even at a HRT of 100 days.

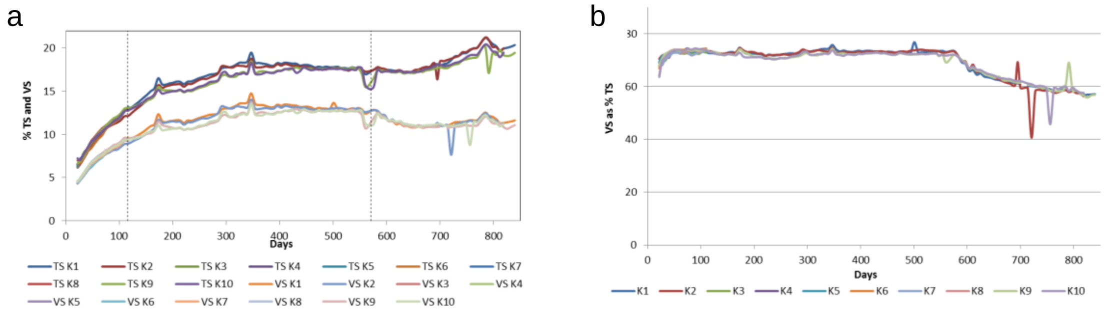


Figure 4.4. Semi-continuous digestion of FDA at a VS concentration of 20%: (a) TS & VS; (b) VS as a % of TS. Vertical dotted lines indicate end of feeding for digesters K3-K8 on day 116 and the use of second batch FDA material from day 570.

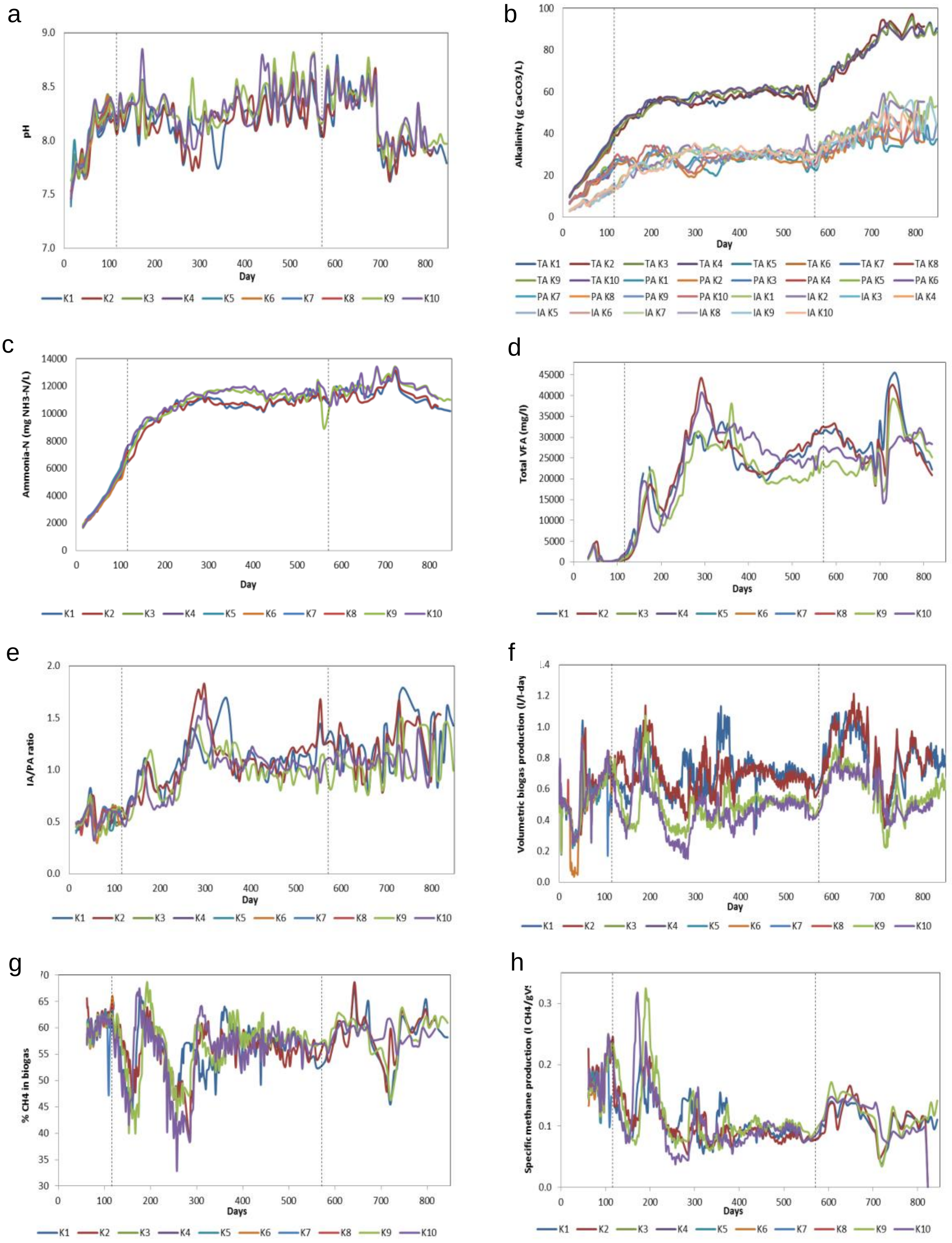


Figure 4.5. Semi-continuous digestion of FDA at a VS concentration of 20%: (a) TS & VS; (b) pH; (c) Total alkalinity; (d) TAN; (e) Total VFA; (f) IA/PA ratio; (g) Volumetric biogas production; (h) CH_4 concentration; (i) Specific methane production. Vertical dotted lines indicate stopping of feeding for digesters K3-K8 on day 116 and the use of second batch FDA material on day 570.

The pH in the digesters (Figure 4.5a) also showed an increase over time and generally fluctuated between 8 and 8.5 from day 50 onwards. There were periods when pH dropped below 8.0. The first period occurred between day 160 and 180, the second between day 200 and 300. The pH values in these periods ranged between 7.72 and 8.03 which is still within the optimum range for methanogens (Khanal, 2008).

As can be seen in Figure 4.5a, on day 690 pH dropped drastically and ranged between 7.63 - 8.35 until the trial ended. The reason for this pH drop was due to an incident that happened on the night before day 690: all the tubes connecting the digesters with the gas counters became blocked by foaming digestate causing an increase in digester pressure and a subsequent explosion. Because of the blast, an amount of digestate was forced out of the digesters through the feeding holes which were sealed by rubber bungs. Although the digesters were returned back to normal operation after this incident, the system had suffered from a serious shock that was clearly illustrated in the drastic drop in pH.

The fluctuation of pH at high values suggests that the digesters have high alkalinity. This was confirmed through the alkalinity measurements which are shown in Figure 4.5b. Total alkalinity (TA) reached a value of around 55 g L^{-1} as $CaCO_3$ by day 200, and stabilised in the range 55 - 60 g L^{-1} as $CaCO_3$ until day 571 when the second batch of FDA feedstock was introduced. After this, TA continued to increase to ~90 g L^{-1} as $CaCO_3$ and stabilised at this value until the trial ended. Partial alkalinity (PA) and intermediate alkalinity (IA) in the digesters also followed similar trends to TA (Figure 4.5b). It is noteworthy, however, that initially the PA exceeded the IA, but there were two periods during which this situation was reversed (around days 175 and 250).

The high alkalinity is in part a result of the build-up of ammonia in the digesters, released from the degradation of proteinaceous biomass. As can be seen in Figure 4.5c, like the alkalinity, the TAN concentration in the digesters increased dramatically until it reached 11 g N L^{-1} by day 250, and stabilised around this value until it slightly increased after the FDA from batch 2 was used from day 571. It is well known that high TAN concentrations in a digester can be inhibitory to the digestion process, although the precise concentrations at which this occurs depend on the operating temperature, the pH and the availability of the trace nutrients essential for different metabolic routes to methane formation. The first signs of ammonia inhibition in the current trial are reflected in the increase in volatile fatty acid concentration in the digesters. The total VFA concentration increased from around day 100, showing a first peak between days 160 - 180, followed by a gradual fall in concentration then a rapid increase after day 200 reaching the second peak of ~44 g L^{-1} in digester K2 and ~40 g L^{-1} in digester K10 by day 291. Digester K1 and K9 also followed similar trends, however the VFA concentration was lower, at around ~30 g L^{-1} for both K1 and K9. This is indeed a very high VFA concentration and there may be potential for extraction as a product in this situation. VFA produced from the acidogenic fermentation process has been considered as a potentially valuable substrate for a variety of applications such as the production of biodegradable plastics (eg. polyhydroxyalkanoates PHA), production of bio-hydrogen via electrohydrolysis process, or production of microbial lipids for biodiesel (Fei et al., 2011, Tuna et al., 2009, Lee et al., 2014, Park et al., 2014).

The peaks in VFA correspond to the points where the partial alkalinity exceeded the intermediate alkalinity (Figure 4.5d), and the ratio of these two values (IA/PA, sometimes known as the Ripley ratio) is a good indicator of digester stability. The

IA/PA ratio is plotted in Figure 4.5e and it can be seen that it increased over time and fluctuated around 1.0 after day 300. The three peaks around day 50, 180 and 290 corresponded to the VFA peaks during these periods.

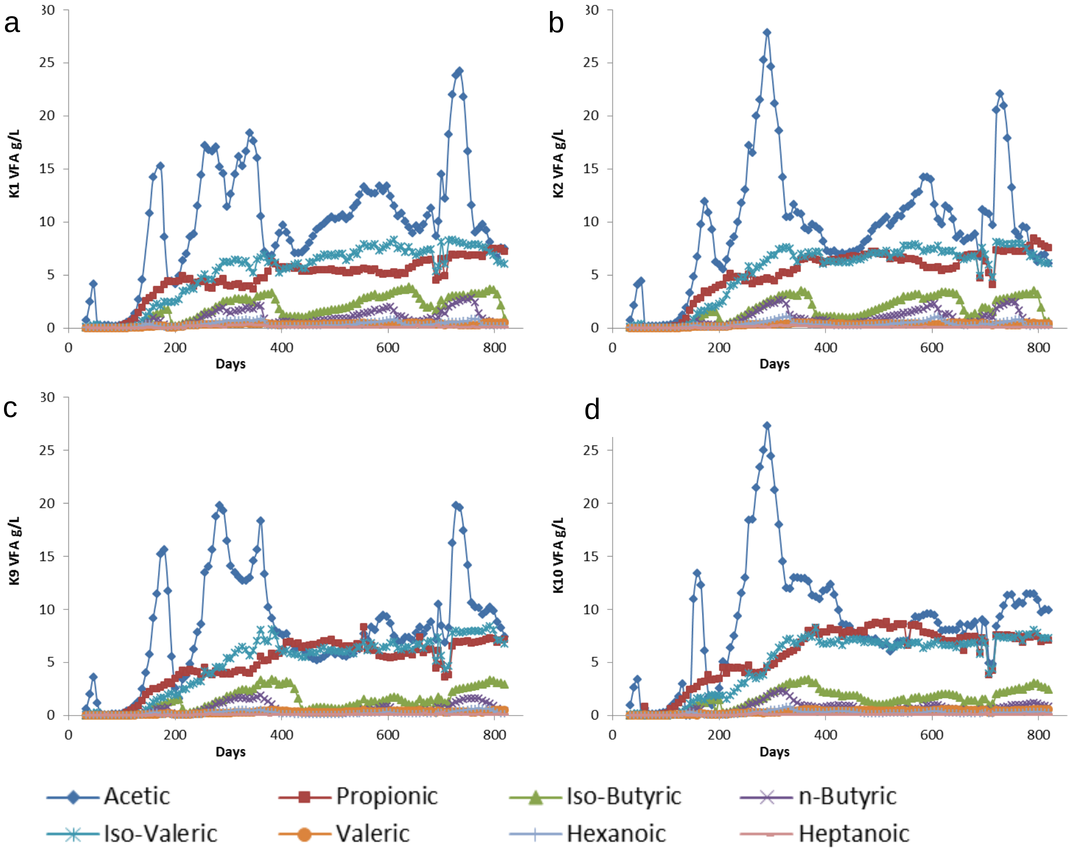


Figure 4.6. Semi-continuous digestion of FDA at a VS concentration of 20%: VFA profiles of digesters K1 (a) and K2 (b) at maximum OLR 3 g VS L^{-1} day^{-1}, and digesters K9 (c) and K10 (d) maximum OLR 2 g VS L^{-1} day^{-1}

Figure 4.6 shows the VFA profiles for the digesters. The main peaks in total VFA were caused by changes in the acetic acid concentration. Propionic acid started to accumulate soon after day 100 in all cases, followed by iso-valeric acid. Once these two VFAs reached concentrations of around 6 - 8 g L^{-1} they remained relatively stable. Accumulations of iso-butyric and n-butyric acid were also seen when the overall VFA concentration, driven up by acetic acid, became too high. K9&10 at OLR 2 g VS L^{-1} day^{-1} showed high but relatively stable VFA concentrations between days ~440 - 690, while in the same period K1&2 first showed increasing concentrations of acetic, iso-butyric and n-butyric acids followed by a fall from around day 632 until the time of the digestate loss at day 690.

The volumetric biogas production (VBP) as shown in Figure 4.5f reflected the observations made above regarding volatile solids (VS) destruction and VFA accumulation. After day 116 the OLR to digesters K1 and K2 was increased to 3 g VS L^{-1} day^{-1}, leading to small increase in the VBP compared to the pair K9 and K10. The decline and increase in VBP in all digesters after day 116 again corresponded to the fluctuation of accumulated VFA. No general trend could be drawn for VBP, however there was a period between day 400 and 571 where the digesters appeared to be in steady state. During this period, the VBP of the pair of digesters at a loading rate of 3 g VS L^{-1} day^{-1} (K1&2) was around 0.7 L L^{-1} day^{-1}, similar to the value at the end of the start-up period, and in the pair fed at 2 g VS L^{-1} day^{-1} (K9&10) was lower at around 0.5 L L^{-1} day^{-1}. After day 571, the VBP increased in all digesters indicating a higher proportion of degradable material in FDA feedstock used in this period.

The fluctuation in VBP indicates that the digesters were not performing in an optimal way. The periods of declining biogas productivity are also reflected in the biogas methane concentration as shown in Figure 4.5g, where methane concentration fell as low as 33 - 40%. In periods of relatively stable performance (between day 405 and 543), the average biogas methane content was around 57%.

The specific methane yield (SMY) reflects both the biogas productivity and the methane concentration of the biogas. The SMY should not be influenced by the applied loading except when the digester is under stress and the solids applied are not being converted through to biogas. The results show that even under the most favourable conditions of low VFA and low applied organic loading the SMY was less than 0.25 L CH_4 g^{-1} VS_{added}; peaks shown in Figure 4.5h which exceed this value are most likely due to the accumulated VFA being subsequently degraded. The average value over the stable operating period between day 400 and day 571 was ~0.090 L CH_4 g^{-1} VS_{added} in both pairs of digesters. This indicates that the OLR of 3 g VS L^{-1} day^{-1} was not in itself sufficient to cause stress on the reactors.

Conclusions from long-term FDA digestion experiment

It is clear that digestion at a feed solids concentration of 20% in a continuous stirred tank reactor (CSTR) design digester is not optimal even at a low loading rate of 2 g VS L^{-1} day^{-1}. This is possibly due to the high TAN concentration and the likely toxicity that this exerts on the methanogenic population. It is likely that the reduction in VFA peak after day 175 was due to a shift in methanogenic population to the more ammonia-tolerant hydrogenotrophic type but even these are inhibited when TAN concentrations increase above 8 - 9 g N L^{-1}. The results also show that less than 50% of the algal biomass is being broken down, but a significant part of the biomass broken down remains as VFA, and only a small fraction is converted to gaseous products: this low value is reflected in the specific methane yield of the algae.

The digesters did show adaptation to the high TAN concentrations but, despite the very long operating period which would presumably have allowed full acclimatisation to the feedstock, there was no evidence of improved VS degradation. The fact that the digesters are still capable of methanogenic activity suggests that the low conversion rates are almost certainly due at least in part to lack of hydrolysis of primary substrate.

There was clearly no advantage of operating at this high feedstock solids concentration; and as microalgal biomass is typically harvested at rather low solids concentrations and every concentration step normally requires an energy input, there are definite advantages to operating at lower concentration. The BMP results indicated that the freeze-dried material (FDA) had a lower methane yield and was possibly more recalcitrant to degradation, and/or more difficult to hydrolyse in conditions of high TAN and VFA concentration. For these reasons, it was decided to carry out later trials with fresh material at lower VS concentration.

4.2.3.2 Radioactive labelling experiment for determination of the methanogenic pathway in DT-1

The isotope labelling experiment was kindly carried out by Dr Ying Jiang following the method reported in his PhD thesis (Jiang, 2012). The following is a brief description of the methodology.

High ammonia digestate samples were taken from the four digesters K1, K2, K9 and K10 on day 593 (TAN in the range of 11.1 - 12.0 g N kg^{-1} WW). A low nitrogen digestate sample (TAN = 1.58 g N kg^{-1} WW) taken from Millbrook WWTW was used for comparison. Each sample was mixed with anaerobic medium as described by Jiang (2012) in the ratio of 1:2. 2.5 KBq of ^{14}CH3COONa was added into 45 mL of the sample/medium mixture and incubated in 119 mL crimp top serum bottles under 37 ^{o}C for 24 hours. The CO_2 and CH_4 produced in the headspace were separately collected in alkali traps containing 20 mL 1M NaOH solution. After absorption, 1 mL NaOH solution in each alkali trap and 0.5 mL of the sample/medium mixture (after centrifugation) were added into 15 mL Gold Star multi-purpose liquid scintillation cocktail (Meridian Biotechnologies Ltd, Surry, UK) and counted in a Beckman Coulter LS6500 scintillation counter (Beckman Coulter, Inc., UK).

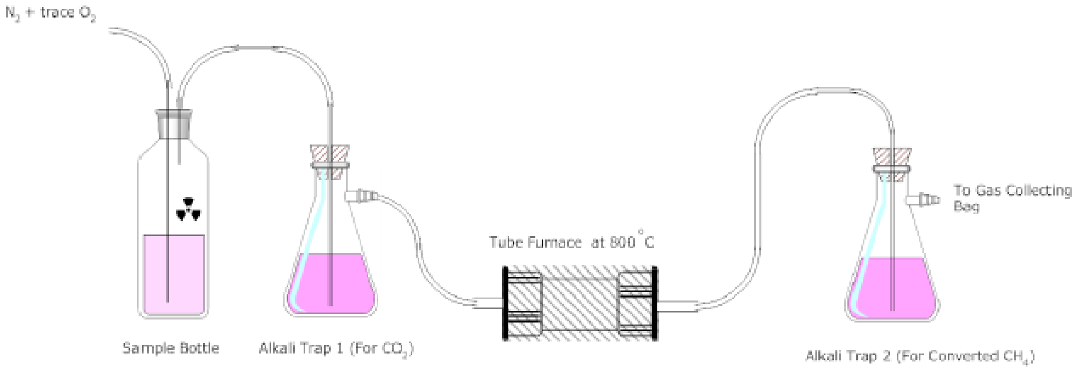


Figure 4.7. Schematic illustration of the radioactive ^{14}C labelling experiment

After incubation, carbon dioxide and methane labelled with radioactive isotope produced from each digestate sample were sequentially captured in NaOH solution. The radioactivity of each was subsequently determined by scintillation counting. Despite very high ionic strength and alkalinity, a transparent solution was formed and a counting efficiency between 84 - 92% was achieved in all samples (Table 4.5). Any ^{14}C in the digestate/culture medium mix in the culture bottles was also accounted for following a centrifugation step.

The TAN concentration in the two pairs of digesters K1&2 and K9&10 was in the range 10 - 12 mg N L^{-1} after three HRTs. It is assumed that under such high TAN concentrations, the dominant metabolic pathway shifts to hydrogenotrophic methanogenesis due to the higher tolerance of hydrogenotrophic methanogens to ammonia. If this assumption is valid, a much higher quantity of $^{14}CO_2$ molecules would be detected in the biogas produced compared to those from low nitrogen sewage digestate where the acetoclastic route is believed to predominate.

The results from the ^{14}C acetate labelling experiment are summarised in Table 4.5. For the control samples, the $^{14}CO_2$:$^{14}CH_4$ ratios were 0.060 and 0.056 respectively, indicating that the major methanogenic pathway was acetoclastic. In contrast, the $^{14}CO_2$:$^{14}CH_4$ ratios for microalgal digestate samples were between 2.46 - 3.61 which strongly suggests that the dominant pathway in the four reactors K1, K2, K9 and K10 was via syntrophic methanogenesis.

Jiang conducted an isotope labelling experiment using similar method to determine the methanogenic pathway in food waste digesters (Jiang, 2012). The author reported that the $^{14}CO_2$:$^{14}CH_4$ ratios were between 1.94 - 3.07 for the digesters with high ammonia concentration (TAN in the range of 3.5 - 5.3 g NH_3-N kg^{-1} WW), indicating a route to methane formation by the hydrogenotrophic methanogens.

The findings from the above mentioned works together with results from this study therefore strongly support the hypothesis that under high ammonia concentration, microalgal digestion adopts a syntrophic methanogenic pathway as the major route for methane production.

Table 4.5. Results from ^{14}C labelling experiment

Sample	Subsample	Counting Efficiency (%)	^{14}C (kBq)	Total ^{14}C recovered (KBq)	Total ^{14}C added (kBq)	Recovery rate (%)	^{14}CO2:^{14}CH4
Control 1	Sludge	94.5	0.45				
	CO_2	94.02	0.57				
	CH_4	94.05	9.4	10.41	10	104	0.06
Control 2	Sludge	94.51	0.33				
	CO_2	93.88	0.48				
	CH_4	94.09	8.57	9.38	10	94	0.056
K1.1	CO_2	95.47	0.06				
	CH_4	95.41	0.02				
	Digestate	92.81	4.53	4.61	5	92	2.72
K1.2	CO_2	95.43	0.05				
	CH_4	95.4	0.01				
	Digestate	92.41	4.21	4.27	5	85	3.34
K2.1	CO_2	95.43	0.03				
	CH_4	95.42	0.01				
	Digestate	92.59	4.46	4.51	5	90	3.11
K2.2	CO_2	95.48	0.03				
	CH_4	95.37	0.01				
	Digestate	92.61	4.34	4.38	5	87	3.61
K9.1	CO_2	95.37	0.03				
	CH_4	95.36	0.01				
	Digestate	93.6	4.4	4.45	5	89	2.52
K9.2	CO_2	95.46	0.03				
	CH_4	95.43	0.01				
	Digestate	92.96	4.17	4.21	5	84	2.46
K10.1	CO_2	95.37	0.04				
	CH_4	95.37	0.01				
	Digestate	92.66	4.36	4.41	5	88	3.34
K10.2	CO_2	95.37	0.04				
	CH_4	95.33	0.01				
	Digestate	92.73	4.41	4.46	5	89	3.6

4.2.3.3 Digestion Trial 2 (DT-2)

Summary method

At day 116 of DT-1, the digestate from six of the digesters was removed and mixed with fresh inoculum from Millbrook WWTP to reduce the TAN concentration to around 2.3 g N L^{-1}. Pairs of digesters were then fed on FDA at VS concentrations of 6, 8 and 10% at an OLR of 3 g VS L^{-1} day^{-1}. At these lower feed VS concentrations the HRT was reduced to 20, 26.7 and 33.3 days for feed VS concentrations of 6, 8, and 10% respectively. The TKN per kg of material added was also reduced due to the dilution with water and was equivalent to around 5.9, 7.9 and 9.9 g N L^{-1} day^{-1}. The digesters were supplemented with trace elements by weekly addition as described in section 3.3.4.

DT-2 was run for 148 days. The operational conditions for DT-2 are shown in Table 4.6.

Table 4.6. Notation and operational conditions of DT-2

Digester	Feedstock concentration (VS %WW)	OLR (g VS L^{-1} day^{-1})	HRT (days)
K3 and K4	6	3	20.0
K5 and K6	8	3	26.7
K7 and K8	10	3	33.3

Experimental results

TS and VS in the digesters fed at VS concentrations of 6 and 8% reached stable values after ~42 days of feeding, while the TS and VS in the pair fed at 10% VS (K7&8) still increased slowly and stabilised at around day 100 (figure 4.8a). The digestate VS concentration in the digesters fed at the lowest concentration was

~4.5% VS and in that at the highest VS feed concentration was ~7.2%, with TS values correspondingly higher. These values indicated that the % degradation of the algal biomass was low, and corresponded to between 25 - 28% VS destruction. These low values may reflect the short retention time and the difficult nature of this type of biomass. The VS/TS ratio was similar and stable at around 75% in all digesters after day ~50 (Fig 4.8b), indicating a similar degree of VS degradation in each case.

The pH in the digesters stabilised between 7.4 and 8 with higher values associated with the higher VS feed concentrations (Figure 4.8c). This pH was lower than in DT-1, and its relationship to the feedstock %VS reflects the TAN concentration in the digestate and the alkalinity this provides.

Digestate TAN concentrations are shown in Figure 4.8d. It can be seen that at the lower feed VS concentration, TAN stabilised at around 1.8 g N L^{-1} and for the higher feed concentration at around 3.3 g N L^{-1} where it could still potentially pose a threat to the methanogenic population. It is interesting to note that the relationship between feedstock VS and TAN concentration is strongly linear between 6-10% VS (R^2 = 0.9989, $p < 4.5 \times 10^{-7}$), but this relationship breaks down if the values from DT-1 at 20% VS are added, indicating a change in VS destruction rates and consequently in ammonia release.

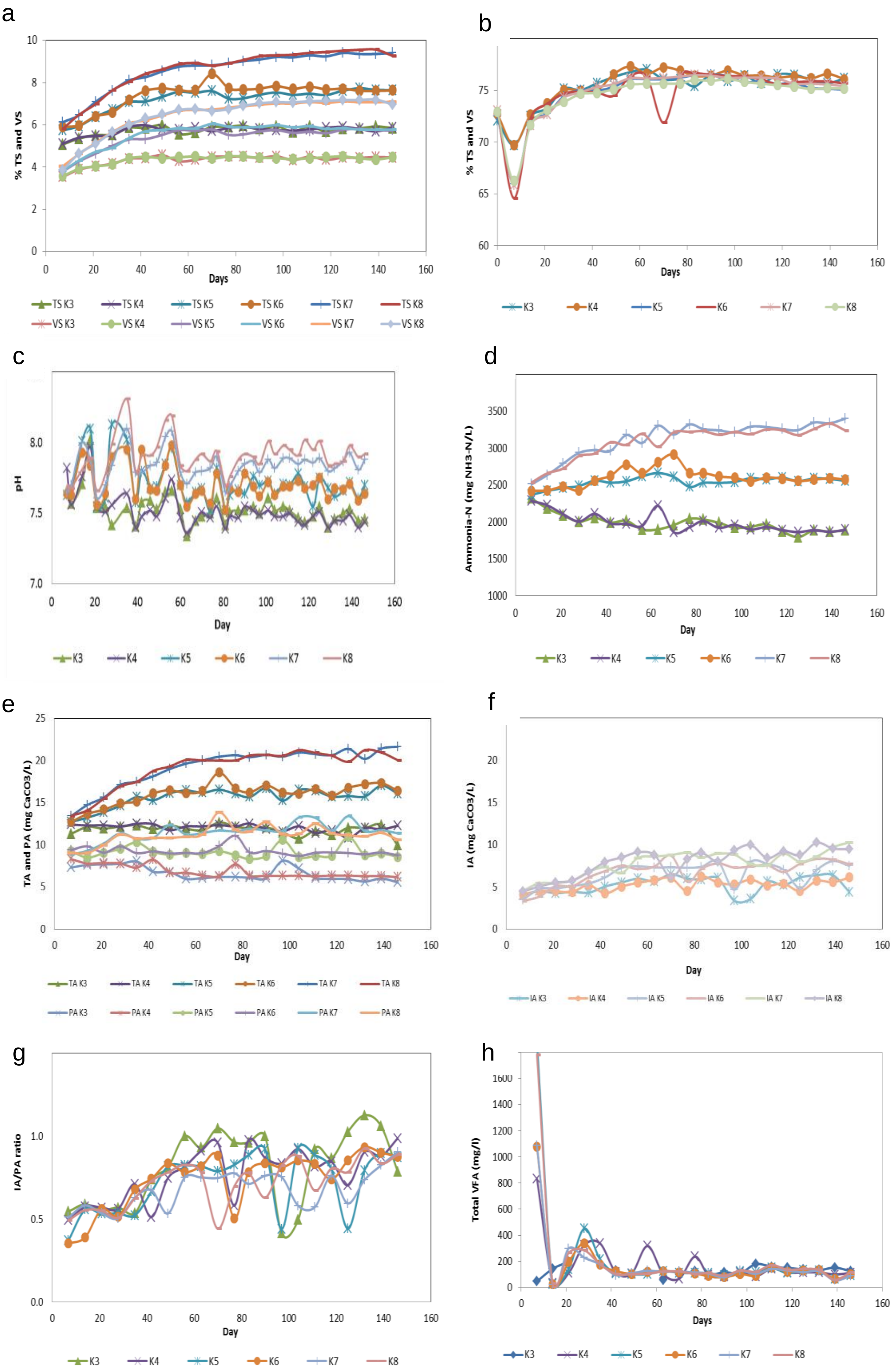


Figure 4.8. Semi-continuous digestion of FDA at a VS concentration of 6, 8 and 10%: (a) TS & VS; (b) vs % TS; (c) pH; (d) TAN; (e) TA and PA; (f) IA: (g) IA/PA ratio; (h) total VFA.

The lower ammonia concentrations correspond to a reduction in the digestate alkalinity as can be seen in Figure 4.8e with Total Alkalinity (TA) ranging from 12 to 20 g $CaCO_3$ L^{-1} for the three different feed VS concentrations. Overall there is an increase in the proportion of intermediate (IA) to partial alkalinity (PA) leading to a ratio in the region of 0.4 - 1 after operation for more than 60 days (approximately 2 - 3 HRT) (Figure 4.8g). Although these values are higher than those sometimes regarded as ideal, digester operation appeared to be stable. VFA concentrations (Figure 4.8h) remained below 200 mg L^{-1} in all digesters from day 83 on.

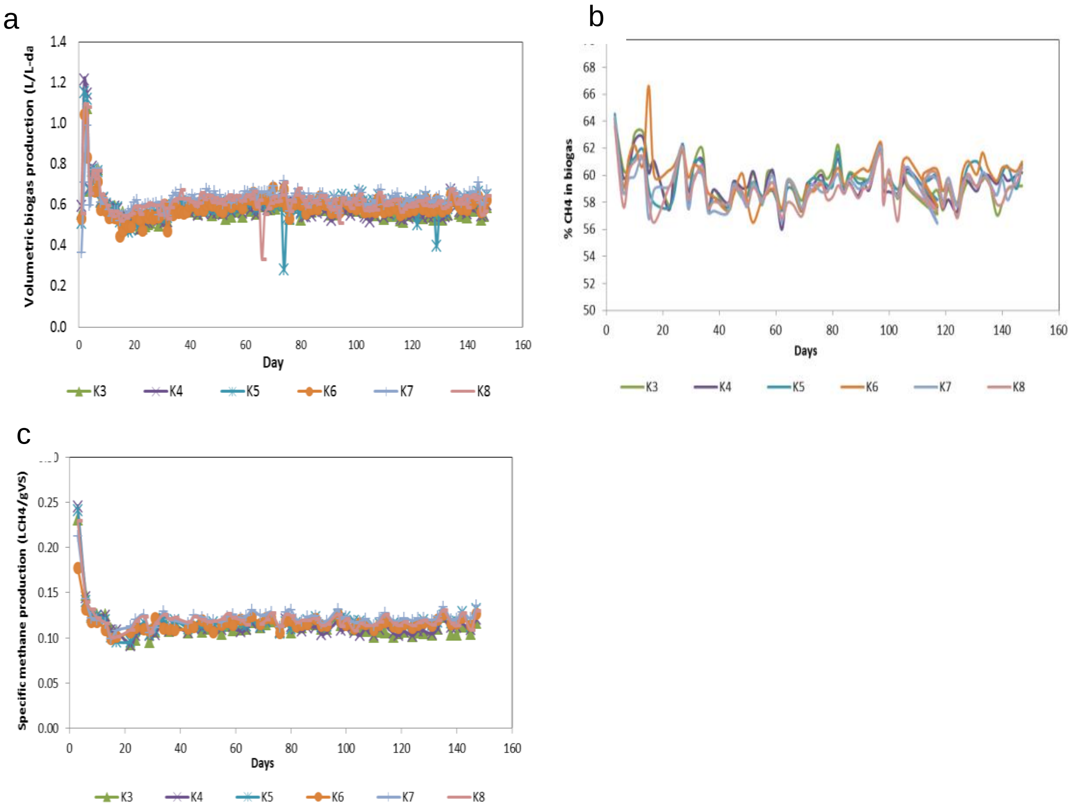


Figure 4.9. Semi-continuous digestion of FDA at a feedstock VS concentration of 6, 8 and 10%: (a) VBP; (b) Biogas methane content; (c) SMY

Figure 4.9a shows the volumetric biogas production (VBP) over the course of the experiment. Despite the moderate OLR of 3 g VS L^{-1} day^{-1}, VBP was low and fluctuated around 0.6 L L^{-1} day^{-1} in all 6 digesters irrespective of the feed VS concentration, with a biogas methane content between 57 - 61% (Figure 4.9b).

As shown in Figure 4.9c, the specific methane yield (SMY) was stable between 0.11 - 0.12 L CH_4 g^{-1} $VS_{added,}$ and showed little variation between the digesters fed at the different %VS concentrations despite the differences in HRT.

Conclusions from digestion trial DT-2

The digesters could be operated in a very stable manner using freeze dried algae at a lower %VS than in DT-1, and the values up to 10% appeared satisfactory in terms of ammonia concentration at the observed VS destruction rates. If the VS destruction were improved then this may no longer be the case, as the ammonia concentration at a feedstock VS of 10% was around 3.5 g N L^{-1} and doubling the solids destruction could potentially double this value taking it well into the region of methanogenic toxicity. Based on the same argument, a 75% destruction of VS at a feedstock concentration of 6% could result in ammonia concentrations less than 6 g N L^{-1}. It has been shown that digesters operating on food wastes can be successfully operated at this concentration if given the correct trace element supplementation (Banks et al., 2012).

The results of the current trial indicated that high TAN concentrations were not the main reasons for the low VS degradation, and that biomass recalcitrance was a likely factor. In the next trial it was therefore decided to use FFA.

4.2.3.4 Digestion Trial 3 (DT-3)

Summary method

At the end of DT-2, digestate from the six digesters (K3-K8) was removed, mixed well with each other to homogenise it and then redistributed equally back to the digesters as the inoculum for a new digestion trial (DT-3). The DT-3 was run for 165 days and can be divided into 3 phases:

- Phase 1: all digesters were fed with FDA at a solids concentration of 6% VS and OLR of 3 g VS L^{-1} day^{-1} for the first 28 days;
- Phase 2: by day 29 FDA was no longer used as feedstock, instead FFA was used to feed the digesters at a solids concentration of ~4.33% VS and OLR of 1.24 g VS L^{-1} day^{-1}. The amount fed daily was gradually increased so that by day 42 the OLR for all six digesters reached 2 g VS L^{-1} day^{-1}. This OLR was kept unchanged for 60 days (from day 42 to day 102).
- Phase 3: from day 102, the OLR for the pairs K5&6 and K7&8 was gradually increased to 2.75 g VS L^{-1} day^{-1} and 3.5 g VS L^{-1} day^{-1}, respectively. The OLR for the pair K3-K4 remained at 2 g VS L^{-1} day^{-1} as a control until the end of DT-3.

Operational conditions for DT-3 are shown in Table 4.7.

Table 4.7. Notation and operational conditions of DT-3

All digesters	Started day 1. FDA was used as feedstock until day 28 then was replaced by FFA with VS of feed 4.33% from day 29
K3 and K4	OLR of 2 g VS L^{-1} day^{-1} kept unchanged from day 102 until the end.
K5 and K6	OLR of 2.75 g VS L^{-1} day^{-1} kept unchanged from day 105 until the end.
K7 and K8	OLR of 3.5 g VS L^{-1} day^{-1} kept unchanged from day 107 until the end.

Experimental results

Digester pH during the course of DT-3 is shown in Figure 4.10a. In phase 1 and 2 of DT-3, pH values in all digesters fluctuated following similar trend, and the variation between them was very small. This is because all six digesters were operated under exactly the same conditions (i.e. same feedstock and OLR); the day-to-day variations suggest either that the calibration of the pH probe varied, or that minor differences in the time between sampling and measurement may have had an effect on pH due to changes in temperature, dissolved CO_2 content etc. In phase 3, however, although the fluctuation in pH still shows similar trends, it can be seen there is a clearer variation in pH values between the digesters. This is likely to be due to the different OLRs that were applied to each pair of digesters in this phase. Overall, pH values fluctuated between 7.07 - 7.65 which is within the optimum range for AD. The average pH value for all digesters was 7.2 in the last 100 days of experiment.

TS and VS concentrations during the course of DT-3 are shown in Figure 4.10b. In general, the trend was quite similar for all three pairs of digester for both TS and VS. In phase 1, the trends in TS and VS and the TS and VS values of all digesters were almost identical, mainly because they were all fed with a very homogeneous feedstock at the same solids concentration (6% VS) and OLR (3 g VS L^{-1} day^{-1}). In comparison with the results from the pair fed at 6% VS in the previous experiment (DT-2), however, the TS and VS values in DT-3 were a slightly higher with average 6.4% TS and 4.8% VS. The reason could be that the digestate from three pairs of digesters in the previous experiment was removed and mixed and used as inoculum for this one. The TS values in phase 1 decreased slowly from 6.5 to 6.2 % and would probably have stabilised at ~5.8% as in DT-2 if feeding with FDA had continued for a little longer. Based on the feed concentration of 6% VS, and an average digestate VS concentration of 4.8% over 28 days, the calculated VS destruction rate in this phase was ~20%.

Figure 4.10. Semi-continuous digestion of FFA: (a) pH; (b) TS & VS; (c) TAN; (d) Total alkalinity; (e) IA/PA ratio; (f) Total VFA; (g) Volumetric biogas production; (h) CH_4 concentration; (i) Specific methane production. Vertical dotted lines indicate the change of feedstock from FDA to FFA on day 28 and the change of OLR on day 102

Digestate TS values increased rapidly in phase 2 and stabilised at around 10% by day 60 of DT-3. Although there were variations in TS values between digesters, the trend in TS is similar for all digesters.

Unlike the TS, digestate VS content showed a gradual decrease in phase 2 and stabilised at ~3% in phase 3. It is interesting to note that, although the solids concentration of the feed in phase 2 and 3 was lower than that of the feed used in phase 1 (4.33% versus 6%), the VS destruction rate in phase 2 and 3 was higher than that of phase 1. Specifically, the VS destruction rate at the end of phase 2, and in phase 3 was ~30%, whereas it was only ~20% in phase 1. This is understandable, because FFA had higher proportion of readily degradable material compared to FDA, as indicated in the BMP test and the model discussed in section 4.2.

As shown in Figure 4.10c, the TAN concentration of the 6 digesters decreased from 2.4 g N L^{-1} to about 2 g N L^{-1} by the end of phase 1. This was because the inoculum was a mixture of digestate removed from digesters fed at 6%, 8% and 10% VS concentration as discussed earlier. If feeding with the FDA had continued for longer, the TAN concentration would be expected to decrease to 1.8 g N L^{-1} as in the case of the pair fed at 6% VS concentration in DT-2.

TAN concentrations increased quickly at the start of phase 2, reaching their highest values of 2.55 g N L^{-1} in K3 (day 42), and 2.54 g N L^{-1} in K5 (day 56), then despite some day to day variations showed a gradually decline towards the end of DT-3. The increase in TAN concentration in the first 14 days of phase 2 could be explained by the proportion of algae that is readily degradable. At the end of the experiment, TAN content in the six digesters ranged between 1.6 - 2.0 g N L^{-1}. The digestate TKN concentration at the end of the run was around 4.1 g N L^{-1} corresponding to a breakdown rate for organic nitrogen-containing compounds of around 44%, slightly above the VS destruction rate but still quite low.

Total alkalinity decreased slightly from 15 to 13 g $CaCO_3$ L^{-1} in phase 1, then increased rapidly in the first 28 days of phase 2 to 40 g $CaCO_3$ L^{-1} (Figure 4.10d). After that it stabilised, ranging between 32 - 40 g $CaCO_3$ L^{-1} at the end of the run. The high alkalinity is as a result of the presence of carbonates, and of decomposition of organic compounds during digestion. Again, the rise of total alkalinity in phase 2 could be explained by the proportion of readily degradable biomass contained in the FFA. This high alkalinity level ensured a sufficient buffering capacity that neutralise organic acids produced during the biochemical reactions and keep pH values stable at around 7.2 - 7.3 as discussed above. The whole system was therefore kept operating at a steady state.

As shown in Figure 4.10e, the IA/PA ratio of all six digesters in phase 1 was quite stable at around 1. However, when moving to phase 2, this ratio began to increase and fluctuated significantly, in amplitude as well as between the digesters. High values of up to 4.09 were observed in K3 on day 91 (phase 1) and day 126 (phase 3), or 3.38 on day 91 in digester K5, 3.34 on day 70 in digester K7. According to Ripley et al (1986), IA/PA ratio values higher than 0.3 indicate the occurrence of disturbances in the AD process. In this experiment, however, despite abnormally high and fluctuating IA/PA ratio, the system operated stably, as reflected in VFA concentration, biogas production and specific methane yield which are discussed below. The reason for this high value for the IA/PA ratio under apparently stable conditions is unknown.

The VFA concentration is an important indicator of the health of an AD system. As shown in Figure 4.10f, total VFA concentration in the digestate of all six digesters remained below 310 mg L^{-1} during the course of DT-3. Total VFA quickly fell from a starting concentration of 265 mg L^{-1} in phase 1 to below 110 mg L^{-1} for the two pairs of digesters K5&6 and K7&8 until the end of the run. In phase 1 and 2, total VFA for digesters K3&4 was higher than in the other digesters but declined and remained below 100 mg L^{-1} in phase 3. The higher VFA concentrations in K3 and K4 in phase 1 and 2 were probably an artefact, due to fact that the sampling syringe of the Shimadzu GC-2010 gas chromatograph was not cleaned completely after analysing previous samples (specifically K1 and K2 in DT-1, which had extremely high VFA concentrations at this point), resulting in carry-over of peaks and incorrect results for K3 and K4. Overall, however, total VFA concentrations were within the range of 50 - 250 mg L^{-1} which is an indicator of a healthy AD system (Sawyer et al, 2003).

The volumetric biogas production of the six digesters during the course of DT-3 is shown in Figure 4.10g; the impact of different OLRs can be clearly seen. In phase 1 and 2 where six digesters were fed at the same OLR, the volume of biogas produced was almost identical for all digesters. VBP fluctuated between 0.50 - 0.65 L L^{-1} day^{-1} in phase 1, and was stable at 0.67 L L^{-1} day^{-1} in phase 2. It is noteworthy that, even though the OLR applied in phase 2 was lower than in phase 1 (2 vs. 3 g VS L^{-1} day^{-1}), the average VBP in phase 2 was about 12% higher than that in phase 1. This can again be attributed to the higher proportion of readily degradable material in FFA.

In phase 3, the effect of different OLRs applied between the three pairs of digester pair was clearly reflected in VBP. The VBP of the pair K3&4 was very stable at ~0.67 L L^{-1} day^{-1} from phase 2 until the end with unchanged OLR of 2 g VS L^{-1} day^{-1}. At higher OLR of 2.75 g VS L^{-1} day^{-1} for K5&6 and 3.5 g VS L^{-1} day^{-1} for K7&8, the VBP was also higher at ~0.87 L L^{-1} day^{-1} and ~1.06 L L^{-1} day^{-1}, respectively. Overall, the biogas yield during the run of DT-3 was low but stable, with methane content ranging between 57-66% (Figure 4.10h).

Figure 4.10i shows that SMY values in phase 2 and 3 were very stable at ~0.13 L CH_4 g^{-1} VS regardless of the OLR applied. This value was considerably below the BMP value of around 0.220 found in the 90-day batch test (section 4.2), but relatively similar to the specific methane yield of around 0.137 L CH_4 g^{-1} VS achieved by the second day of the BMP test, and corresponding to the change of slope in the cumulative gas production curve. This confirms indications from the batch test that a proportion of the biomass is not readily degradable, and was thus probably not being broken down due to the relatively short HRTs.

Conclusions from digestion trial DT-3

The digestion of FFA was very stable at a solids concentration of 4.33% VS and at OLR of up to 3.5 g VS L^{-1} day^{-1}. Both VFA and TAN concentrations were low, in the latter case probably due to both the poor feedstock degradation and the relatively high dilution. VBP was higher at higher OLR, but overall the specific methane yield was similar at all OLR at around 0.13 L CH_4 g^{-1} VS_{added}. This value is only slightly higher than the SMY from the digestion of FDA, but still far below the specific methane yield of the 90-day BMP test. The VS destruction rate was low at ~30%, which is similar to the rate observed in the case of digesting FDA at 10% VS concentration. If the VS destruction were improved, e.g. if the solids destruction were doubled, the estimated ammonia released in digestion FFA would be less than 4 g N L^{-1} which is significantly lower in comparison with ~7 g N L^{-1} in case of FDA, and therefore anaerobic digestion of FFA with 60% VS degradation could still be possible.

4.2.4 Overall discussion and conclusions from AD of non-pretreated algal biomass

Experimental results from the three digestion trials are summarised in Table 4.8. As can be seen, SMY from digesting the two feeds FFA and FDA are low. SMY was in the range of 0.09 and 0.121 L CH_4 g^{-1} VS for FDA, and 0.127 - 0.131 L CH_4 g^{-1} VS for FFA. Compared with the value achieved from BMP, the SMY value are 56 - 75% for FDA and ~59% for FFA. When comparing with theoretical methane potential (TMP), SMY obtained under semi-continuous conditions were only 17 - 22% for FDA, and 26% for FFA (Table 4.9). This indicates that the two microalgal samples were very poorly degraded. Even under favourable conditions (e.g in BMP), the highest SMY values for FDA and FFA were only 30 and 44% of the TMP for FDA and FFA, respectively.

As can be seen from Table 4.8, the effect of solid concentration was reflected in DT-1 and DT-2. Digestion was stable for substrate at 6, 8 and 10% VS concentration. 10% VS feedstock gave highest SMY (0.121 L CH_4 g^{-1} VS). Total ammonia obtained when digesting feedstock at 10% was stable at 3.25 g L^{-1}. Although this value, according to several researchers, could strongly inhibit the methanogenic polulation, no harmful effect was observed. Digestion of FDA at 6 and 8% VS concentration were also stable. The SMY values for feedstock with 6 and 8% VS concentration were slightly lower than that of FDA at 10%, which is 0.111 and 0.117 L CH_4 g^{-1} VS.

No clear general trend could be drawn from digesting FDA at 20% VS concentration, except for the period between day 400 and day 572 which could be considered relatively stable. The SMY during this period fluctuated around 0.09 L CH_4 g^{-1} VS. It should be noted that the ammonia concentration in digestate was extremely high, ranging between 10 - 12 g L^{-1} after three consecutive HRTs.

VFA concentration was also very high (14.3 - 44.3 g L^{-1}), however due to the high total alkalinity, the system was well buffered. It was reported that the methanogenic population lost 56.5% of its activity when ammonia concentration exceed 4 g L^{-1} (Koster and Lettinga, 1988). It can be seen from this experiment, however, that there was an acclimatisation of the microorganisms to high ammonia concentration. Once adapted, the microorganisms can retain viability at concentrations far exceeding the initial inhibitory concentrations (Chen et al., 2008). Koster and Lettinga (1988) reported that whilst unacclimated methanogens failed to produce methane at 1.9 - 2 g N L^{-1}, they produced methane at 11 g N L^{-1}. Successful operation of anaerobic filters has been achieved at 6 g N L^{-1} and 7.8 g N L^{-1} after adaptation (Chen et al., 2008). The results from DT-1 are in agreement with the view that methane yield from acclimatised digesters are lower than that for digesters with lower ammonia load (Koster and Lettinga, 1988, Borja et al., 1996)

Digestion of FFA in DT-3 was stable with ammonia concentration between 1.5 - 2.5 g L^{-1} and total VFA below 100 mg L^{-1}. Effect of different OLRs was clearly reflected in the VBP. Three different OLRs of 2, 2.75 ad 3.5 g VS L^{-1} day^{-1} were applied which gave VBP values of 0.67, 0.87 and 1.06 L L^{-1} day^{-1} respectively. The SMY value of 0.127 - 0.131 L CH_4 g^{-1} VS obtained for all three OLRs was in good agreement with the result reported by Yen & Brune (2007). These authors obtained between 0.09 - 0.136 L CH_4 g^{-1} VS when digesting mixture *Scenedemus* and *Chlorella* under semi-continuous condition.

The BMP values for FDA and FFA obtained in this study are typical of median values found in literature, and are in good agreement with the values reported for digestion of *Scenedesmus* biomass under batch conditions (Golueke et al., 1957, Zamalloa et al., 2012, Mussgnug et al., 2010, Keymer et al., 2013, Astals et al., 2015). The very high or very low BMP values reported by some researchers, as shown in Table 2.2, are suspect and probably do not truly represent the methane yield of the tested biomass. For example, values of 0.054 and 0.088 L CH_4 g^{-1} VS were reported for *Scenedesmus* biomass by Inglesby et al. (2015) and Ometto et al. (2014), respectively. Gonzalez-Fernandez et al. (2012b) reported the value 0.037 L CH_4 g^{-1} COD for *Scenedesmus* sp. in one of their papers, but in another publication they reported the value 0.082 L CH_4 g^{-1} COD for the same substrate under the same digestion conditions (Gonzalez-Fernandez et al., 2012a). The variation in reported BMP values could therefore be attributed to inconsistencies in the test procedures.

As mentioned in section 4.2.1, the two substrates FDA and FFA were included in two BMP tests and compared with other laboratory-grown microalgae samples. The tests were conducted jointly with a colleague and the results were partially published in Roberts et al. (2016a). The two BMP assays were run consecutively, in each case using fresh inoculum taken from the same source within a one-month period. The BMP value for the cellulose controls in the 28-day and 90-day tests were 0.412 ± 0.007 and 0.415 ± 0.004 L CH_4 g^{-1} VS respectively, both very close to the theoretical value, with the difference of <0.5% giving confidence in the validity of results from the two assays. The tests indicated that the two large scale microalgae (FDA and FFA) had considerably lower methane potential than the value of 0.307 and 0.261 L CH_4 g^{-1} VS obtained for the two individual laboratory-grown freshwater species *Chlorella* sp. and *Scenedesmus* sp., respectively. The distinct BMP values could be attributed to the differences in characteristics between the tested microalgal samples. Of the four microalgal samples, FFA had the lowest VS content, with only 39.9% as TS, whereas FDA, laboratory-grown *Chlorella* and laboratory-grown *Scenedesmus* the values were more than 2 times higher, at 81.4, 94.7 and 83.9% as TS, respectively. Interestingly, however, the BMP value for FFA was significantly higher than that for FDA, and relatively close to the value of 0.261 L CH_4 g^{-1} VS for laboratory-grown *Scenedesmus* sp. Compared to the measured CV, FDA had the lowest apparent conversion efficiency into methane, at 26.4% whereas the conversion efficiencies for FFA, laboratory-grown *Chlorella* and laboratory-grown *Scenedesmus* were considerably higher at 41%, 49% and 48.3%, respectively. The significantly different degree of VS breakdown between FDA and the other three samples could partly be influenced by storage technique. Several authors have reported that techniques of storage have a strong impact on the biochemical composition of microalgae. For example, storage at -20°C causes a loss of organic cell contents (Cordero and Voltolina, 1997); carbohydrates and proteins content was reported to decrease during freezing (Babarro et al., 2001). Loss of total organics after two months of storage was approximately 20% for air- and freeze-dried algae, but lower in the samples stored in a commercial freezer (Esquivel et al., 1993). The reduction in BMP values were attributed to loss of volatile compounds and/or decreased accessibility of the dried residue (Mussgnug et al., 2010).

The low VS destruction rates for both FDA and FFA as shown in Table 4.8 indicate that a proportion of the organic matter is either non-biodegradable or protected from biodegradation by incorporation into structural cell material: this is similar to the situation in terrestrial plants, in which the cellulose in woody biomass is protected by a non-degradable lignin fraction (Roberts et al., 2016a). Although lignin is not a component of algal cells, similar cross-linked macromolecular structures exist in the cell walls offering protection to the cell contents or simply making the cell wall carbon non-digestible (Roberts, 1974, Gerken et al., 2013). The cell walls of the fresh water green microalgae *Tetraedron minimum*, *Scenedesmus communis* and *Pediastrum boryanum* are composed of highly resistant, non-hydrolyzable aliphatic biopolymers (Blokker et al., 1997, Blokker et al., 1998). *Chlorella kessleri* and *Scenedesmus obliquus* have been characterised as having carbohydrate-based cell walls containing hemicellulose (Takeda, 1991, Takeda, 1996). The cell wall of *S. obliquus* has been described as particularly rigid because it contains a sporopollenin-like biopolymer (Burczyk and Dworzanski, 1988). The polyether nature of these algaenans makes them highly resistant to degradation. In a detailed study of the susceptibility of *C. vulgaris* and other microalgae to enzymatic degradation Gerken et al. (2013) noted that major changes in cell wall composition might depend on very small differences in growth conditions, as well as on factors such as the culture age. The greater recalcitrance to degradation of the FDA and FFA samples, as indicated by their low BMP values, may reflect the more challenging growth conditions in large-scale systems, including factors such as turbulence-induced shear forces and the presence of predators (Roberts et al., 2016a).

The three digestion trials DT-1, DT-2 and DT-3 confirmed that methane production was low even at low loading rates and after long periods of acclimatisation; and the following work therefore focused on finding ways to enhance the anaerobic degradability of the microalgal biomass with the aim of improving specific methane yields and optimising net energy production potential.

Table 4.8. Performance parameters for semi-continuous digestion of microalgal biomass

Trial	**Solids conc.** (VS as % WW)	**OLR** (g VS L^{-1} day^{-1})	**HRT** (days)	**Digestate pH**	**Digestate TS** (%)	**Digestate VS** (%)	**VS destruction** (%)	**TAN** (g L^{-1})	**Alkalinity** (g $CaCO_3$ L^{-1})	**IA/PA**	**VFA** (g L^{-1})	**VBP** (L L^{-1} day^{-1})	**CH_4 conc.** (%)	**SMY** (L CH_4 g^{-1} VS_{added})
DT-1	20	3	66	7.4 - 8.8	17.94$^{(a)}$	13.18$^{(a)}$	34$^{(a)}$	10.8$^{(a)}$	57.9$^{(a)}$	0.88-1.69	19.6-38.5	0.70$^{(a)}$	47 - 64	0.09$^{(a)}$
		2	100	7.4 - 8.8	17.42$^{(a)}$	12.61$^{(a)}$	37$^{(a)}$	11.5$^{(a)}$	60.4$^{(a)}$	0.80-1.45	18.8-38.1	0.48$^{(a)}$	51 - 64	0.09$^{(a)}$
DT-2	6	3	14.44	7.3 - 8.0	5.82$^{(b)}$	4.44$^{(b)}$	26$^{(b)}$	1.93$^{(b)}$	11.89$^{(b)}$	0.4 - 1.1	<0.2	0.58$^{(b)}$	57 - 61	0.111$^{(b)}$
	8	3		7.5 - 8.1	7.63$^{(b)}$	5.78$^{(b)}$	28$^{(b)}$	2.60$^{(b)}$	16.47$^{(b)}$	0.4 - 0.9		0.60$^{(b)}$		0.117$^{(b)}$
	10	3		7.6 - 8.3	9.21$^{(b)}$	6.98$^{(b)}$	30$^{(b)}$	3.25$^{(b)}$	20.65$^{(b)}$	0.4 - 0.9		0.62$^{(b)}$		0.121$^{(b)}$
DT-3	4.33	2	21.66	7.1 - 7.5	9.73$^{(c)}$	2.98$^{(c)}$	31$^{(c)}$	1.6 - 2.5	25-39	1.23- 4.09	<0.1	0.67$^{(c)}$	57 - 66	0.131$^{(c)}$
		2.75	15.75		9.66$^{(c)}$	3.03$^{(c)}$	30$^{(c)}$	1.5 - 2.5	24-40	1.23- 3.38		0.87$^{(c)}$		0.129$^{(c)}$
		3.5	12.37		9.69$^{(c)}$	3.07$^{(c)}$	29$^{(c)}$	1.6 - 2.4	24-41	1.23- 3.34		1.06$^{(c)}$		0.127$^{(c)}$

(a) verage values during periods of stable digestion of DT-1 between day 405 and 543
(b) Average values during periods of stable digestion of DT-2 between day 62 and 147
(c) Average values during periods of stable digestion of DT-3 between day 42 and 164

Table 4.9. Specific methane production from BMP and CSTR in comparison with theoretical methane potential

Samples	**Theoretical methane potential (L CH_4 g^{-1} VS)**	**Theoretical CV (MJ kg^{-1} VS)**	**Measured CV (MJ kg^{-1} VS)**	**Measured BMP (L CH_4 g^{-1} VS)**	**BMP as % of TMP**	**Energy value of CH_4 from BMP (MJ kg^{-1} VS)**	**% of CV from BMP converted to CH_4**	**Measured CSTR (MJ kg^{-1} VS)**	**CSTR as % of BMP**	**CSTR as % of TMP**	**Energy value of CH_4 from CSTR (MJ kg^{-1} VS)**	**% of CV from CSTR converted to CH_4**
FDA	0.536	23.36	23.1	0.161	30	6.4^{a}	28^{b}	0.09 - 0.121^{c}	56 - 75	17 - 22	3.6 - 4.8^{a}	16 - 21^{b}
FFA	0.495	21.91	21.4	0.22	44	8.8^{a}	41^{b}	0.13^{c}	59	26	5.2^{a}	24^{b}

[a] Calculated with the higher heat value of CH_4 taken as 39.84 MJ m^{-3} at STP.
[b] Based on measured CV
[c] Average values during periods of stable digestion: for DT-1 between day 405 and 543; for DT-2 day 62 - 147; for DT-3 day 42 - 164.

4.3 Anaerobic digestion of pretreated algal biomass

4.3.1 Batch pretreatment trials to improve anaerobic degradability of microalgal biomass

A series of batch trials were carried out to test possible pretreatment methods that may be able to improve the anaerobic biodegradability and methane yield of microalgal biomass. Apart from the fresh microalgal paste (FMP) that was used in the initial assay, these trials used the fresh frozen microalgae (FFA) i.e. centrifuged mixed microalgal culture taken from the same source as the material used in the previous semi-continuous digestion study DT-3.

4.3.1.1 Initial assay

Summary method

An initial assay was carried out using fresh microalgal paste (FMP) with a solids content of 78.24 g VS kg^{-1} wet weight (WW). Samples of the paste were tested fresh; frozen at -18 ^{o}C overnight and thawed; autoclaved (120 ^{o}C for 15 mins); and sonicated (Soniprep, 5 mins, at specific energy level of 20 MJ kg^{-1} TS), in a 35-day BMP assay.

Results

The results of this initial assay are presented in Table 4.10 and Figure 4.11.

Table 4.10. 35-day BMP values for pretreated FMP samples

L CH_4 g^{-1} VS	Fresh	Frozen	Autoclaved	Sonicated	Freeze dried
Average	0.16	0.17	0.163	0.206	0.134*
Max	0.165	0.18	0.166	0.209	0.138*
Min	0.157	0.159	0.158	0.201	0.130*
RSD	2.10%	4.50%	0.30%	1.50%	2.85%
% Fresh BMP	100%	106%	102%	129%	84%

* values recorded by day 35 from a subsequent assay running on freeze-dried FMP sample

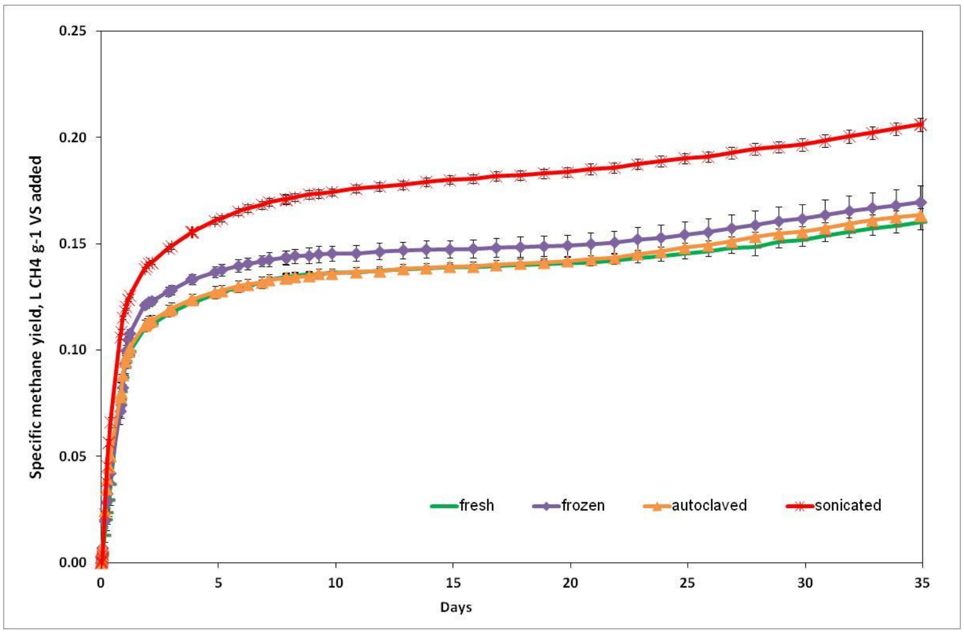


Figure 4.11. Cumulative specific methane yield for fresh, frozen, autoclaved and sonicated FMP samples (35-day results)

As can be seen in Figure 4.11, the results of the 35-day BMP test showed good agreement between quintuplicates. Sonication appeared to give an increase of around 30% in the BMP value, while freezing and autoclaving had relatively little effect with increases of no more than 6 and 2% respectively. A subsequent batch assay (results shown in Figure 4.13) was also run on a freeze-dried sample of the algal paste and gave the values shown in Table 4.11, indicating a ~16% decrease in BMP. This confirmed the hypothesis that freeze-drying can reduce BMP value, and may be in part responsible for the low BMP values obtained for the FDA in section 4.2.1. On this basis it was decided that fresh frozen material would be used for the following tests.

The above assay was kept running until day 104. The results are shown in Table 4.11 and Figure 4.12.

Table 4.11. 104-day BMP values for pretreated FMP samples

L CH_4 g^{-1} VS	Fresh	Frozen	Autoclaved	Sonicated	Freeze dried
Average	0.191	0.2	0.196	0.244	0.159*
Max	0.198	0.219	0.206	0.253	0.164*
Min	0.183	0.183	0.188	0.233	0.153*
RSD	3.60%	6.80%	3.40%	3.10%	3.32%
Increase on 35-day value	119%	118%	120%	118%	119%
% Fresh BMP	100%	105%	102%	128%	83%

* value recorded at the end (day 95) of the subsequent assay running on freeze-dried FMP sample

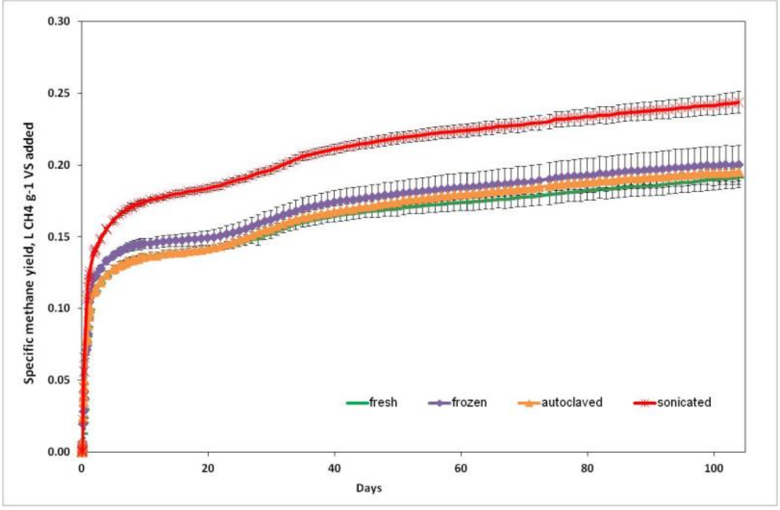


Figure 4.12. Cumulative specific methane yield for fresh, frozen, autoclaved and sonicated FMP samples (104-day result)

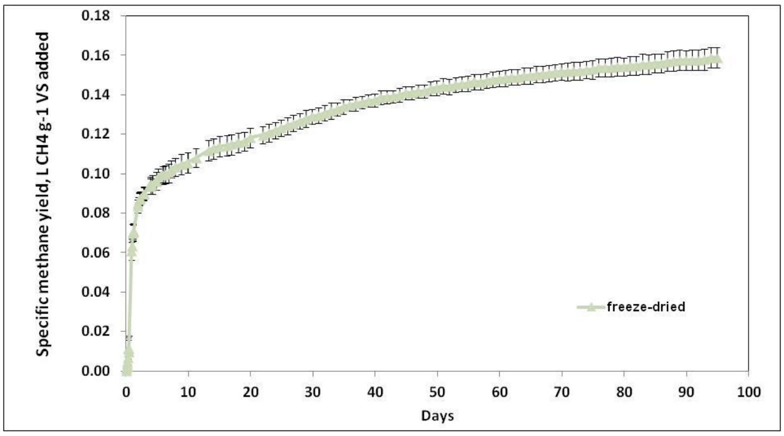


Figure 4.13. Cumulative specific methane yield for freeze-dried FMP samples (95-day result)

As shown in Figure 4.12, there was still good agreement between quintuplicates by the end of the assay. The final methane yield values for fresh, frozen, autoclaved, sonicated and freeze dried FMP samples were 0.191, 0.200, 0.196, 0.244 and 0.159 L CH_4 g^{-1} VS respectively, or about 18 - 20% higher than the 35-day values in each case; but the improvement in methane yield produced by each pretreatment at the end of the assay was very similar to the results recorded by day 35 (Table 4.10 and 4.11). The final methane yield for frozen FMP samples from this assay was 10% lower than that for FFA reported section 4.2.1. The negative effect of freeze drying technique was confirmed once again in the lower methane yield for freeze dried FMP sample compared to the fresh sample.

On the basis of the results obtained from the initial assay, a series of six trials were then carried out in which the biomass of FFA was pretreated by the addition of either H_2SO_4 or NaOH at different dosages with incubation at 80 ^oC in a water bath for different durations. After treatment the material was allowed to cool to room temperature. A series of batch assays were carried out to quantify the effect of the pre-treatments on specific methane yield (SMY) from the treated material.

4.3.1.2 Assay No.1

Summary method

Assay No. 1 was an initial screening test which compared treatments with alkaline and acid at different heating durations. After being thawed and mixed well, 200 g of FFA was distributed to 0.5 L Erlenmeyer conical flasks. The initial pH of FFA sample was adjusted to 10.2 and 3.5 by adding 1% (w/w) solutions of 4M NaOH and 4M H_2SO_4 respectively. The flasks were then incubated at 80 ^oC in water bath for durations of 12, 24, 36 and 48 hours. After treatment the material was cooled down to room temperature before being used. The assay ran for 35 days in total.

Results

Results after 35 days are shown in Table 4.12 and Figure 4.14.

Table 4.12. Cumulative methane production from thermal and chemical pretreated FFA samples in assay No.1

	Cumulative methane production (L)				
pH	12 h	24 h	36 h	48 h	Control
3.5	1.154	1.188	1.212	1.23	
6.8	1.298	1.311	1.332	1.362	1.2
10.2	1.381	1.403	1.359	1.425	

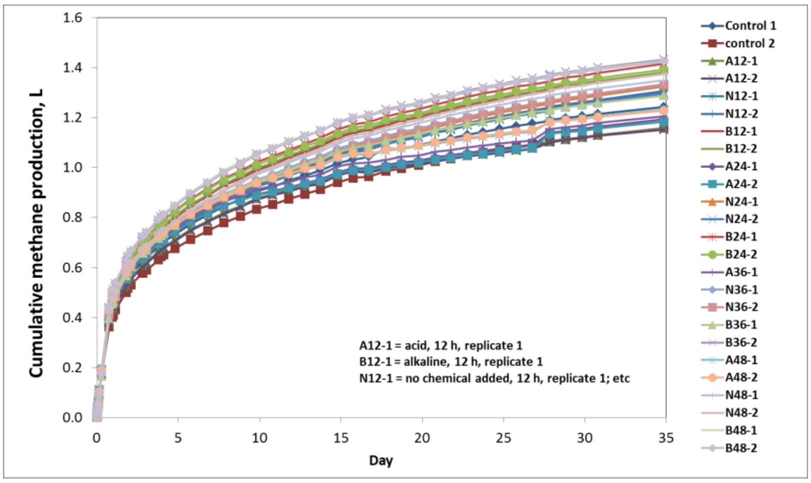


Figure 4.14. Cumulative methane production from acid and alkaline pretreated FFA biomass

Results from assay No.1 showed that cumulative methane production from thermal-acid pretreated samples was either lower (as in sample A12, A24) or only 1 - 2% higher (as in A36, A48) than that from the control. Thermal-only pretreated samples did show increases in cumulative methane production, with higher increases associated with longer pretreatment durations. The maximum increase, however, was only 13% as observed in sample N48 compared to the control.

It can be seen that more methane was produced by samples pre-treated with NaOH than with H_2SO_4 or by the control samples. The highest cumulative methane production after 35 days was from sample B48 (1.425 L), but this was only ~3% higher than from sample B12 (1.381 L), and ~18% higher than in the control (1.20 L). This suggested that thermal pre-treatment for longer than 12 hours was not necessary, as methane production was only slightly improved while the additional energy consumption is likely to be significant.

4.3.1.3 Assay No.2

Summary method

FFA samples contained in six 0.5-L Erlenmeyer conical flasks were dosed with 1% and 10% (w/w) NaOH and thermally pretreated at 80 ^{o}C in water bath for 4, 8 and 12 hours. After treatment the FFA samples were cooled down to room temperature. Assay No. 2 ran for 7 days.

Results

The results are shown in Table 4.13 and Figure 4.15.

Table 4.13. Effect of 4, 8 and 12 hour pretreatment at 80 ^{o}C with 1% NaOH in Assay No.2

Duration	Hours	0	4	8	12
7-day SMY	L CH_4 g^{-1} VS	0.145	0.215	0.217	0.208
Increase	% Control	100%	148%	149%	143%

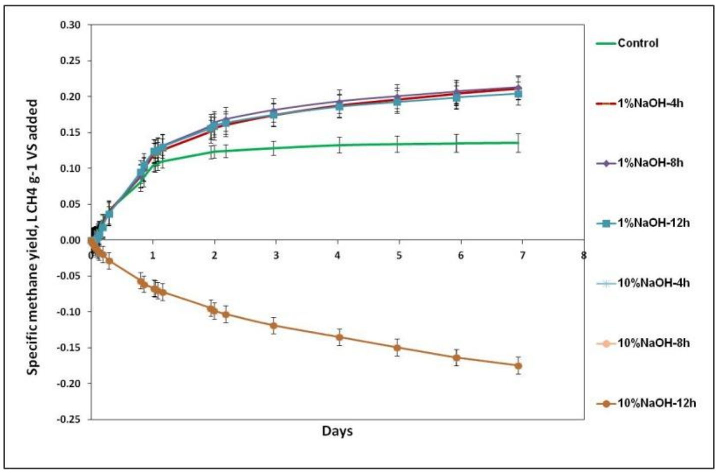


Figure 4.15. Effect of 4, 8 and 12 hour pretreatment at 80 °C with 1% NaOH in Assay No.2

Result showed that the SMY of samples at a lower NaOH dose (1% w/w) was 43 - 49% higher than in the control. The difference in the 7-day SMY for samples after 4, 8 and 12 hours of treatment was insignificant (Table 4.13), however, indicating that pre-treatment for longer than 4 hours is unnecessary. At 10% NaOH no methane was produced from the samples, clearly indicating the toxic effect at this dose.

4.3.1.4 Assay No.3

Summary method

In Assay No. 3, all FFA samples were dosed with 1% w/w NaOH and pretreated at 80 °C for up to 4 hours. The test ran for 7 days.

Results

Results of assay No.3 are shown in Table 4.14 and Figure 4.16.

Table 4.14. Effect of 1-4 hour pretreatment with 1% NaOH at 80 °C in Assay No.3

Duration	Hours	0	1	2	3	4
7-day SMY	L CH_4 g^{-1} VS	0.125	0.184	0.193	0.192	0.194
Increase	% Control	100%	147%	154%	154%	155%

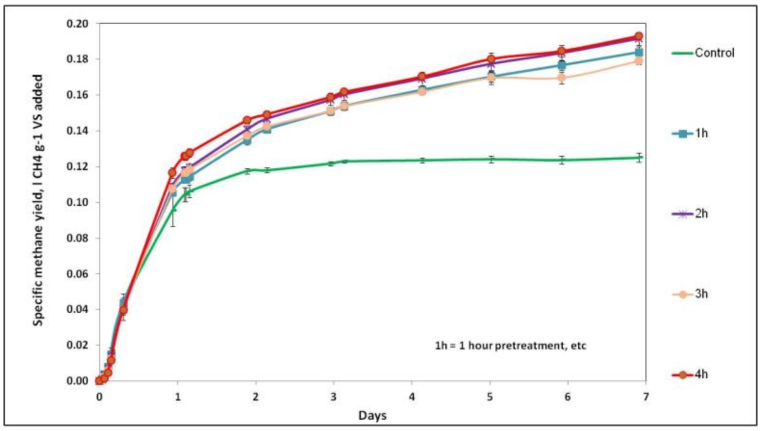


Figure 4.16. Effect of 1 - 4 hour pretreatment with 1% NaOH at 80 °C in Assay No.3

Results from the 7-day assay showed that 1 - 2 hours appeared to be the optimum treatment duration, with a 54.5% improvement in SMY in comparison to the control.

4.3.1.5 Assay No.4

Summary method

In Assay No. 4 all FFA samples were pretreated at 80 °C for 2 hours but received different NaOH dosages (0 - 3% w/w). The tests were allowed to run for 6 - 14 days depending on the initial degree of inhibition.

Results

Results of assay No.4 are shown in Table 4.15 and Figure 4.17.

Table 4.15. Effect of 2-hour pretreatment in Assay No. 4

6-day SMY L CH_4 g^{-1} VS	Control	Heat only (80 °C)	1% NaOH only (no heat)	0.5%NaOH + 80 °C	1%NaOH + 80 °C	2%NaOH + 80 °C	3%NaOH + 80 °C
Average	0.131	0.144	0.143	0.165	0.183	0.165	0.273
Max	0.135	0.147	0.149	0.172	0.208	0.17	0.293
Min	0.129	0.141	0.141	0.161	0.152	0.159	0.239
% Control	100%	110%	109%	126%	139%	126%	208%

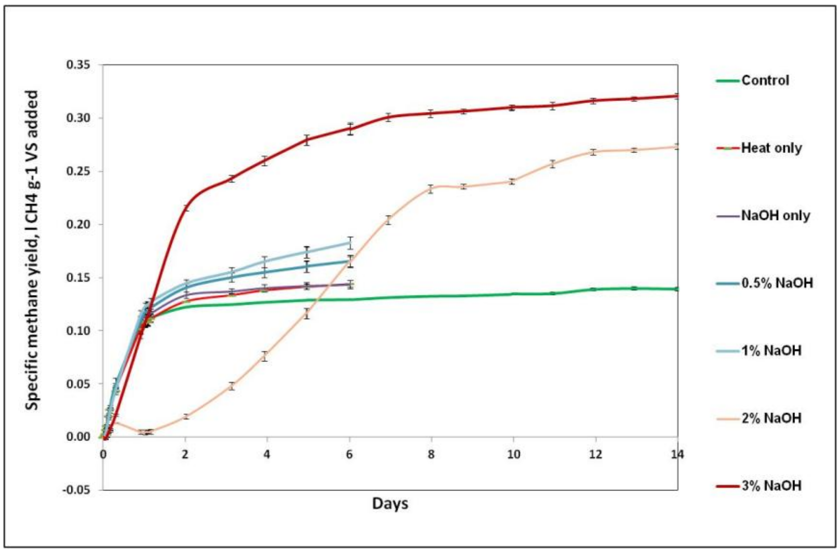


Figure 4.17. Effect of 2-hour pre-treatment in Assay No. 4

As can be seen in Figure 4.17, all treatments showed some increase in SMY compared to the control. The largest increase was at 3% NaOH which gave a 7-day SMY of 0.301 L CH_4 g^{-1} VS_{added} or 130% higher than the control. 2% NaOH showed initial inhibition, but recovered to give a 14-day SMY of 0.273 L CH_4 g^{-1} VS_{added}, compared to 14-day values of 0.140 and 0.321 L CH_4 g^{-1} VS_{added} for the control and 3% NaOH respectively. It is thought that the intermediate NaOH dosage of 2% produced rapid acidification without sufficient buffering to prevent a pH drop and temporary loss of methane production until the accumulated acid products were reduced. The 3% NaOH provided sufficient buffering to overcome this.

4.3.1.6 Assay No.5

Summary method

In Assay No. 5 all FFA samples were pre-treated at 80 ^{o}C for 2 hours and received different NaOH dosages (1.5 - 3.0% w/w) which were then made up to 3% NaOH after the heat treatment. The tests ran for 7 days.

Results

The results of assay No.5 are shown in Table 4.16 and Figure 4.18 where it can be seen that there was an increase in 7-day SMY for all treatments, which was higher with additions of 2% NaOH or more.

Table 4.16. Effect of 2-hour pre-treatment in Assay No. 5

7-day SMY L CH_4 g^{-1} VS	Control	1.5% NaOH + 80 ^oC	2.0% NaOH + 80 ^oC	2.5% NaOH + 80 ^oC	3.0% NaOH + 80 ^oC
Average	0.15	0.193	0.226	0.241	0.259
Max	0.155	0.213	0.242	0.276	0.275
Min	0.146	0.178	0.212	0.214	0.24
% Control	100%	129%	150%	160%	172%

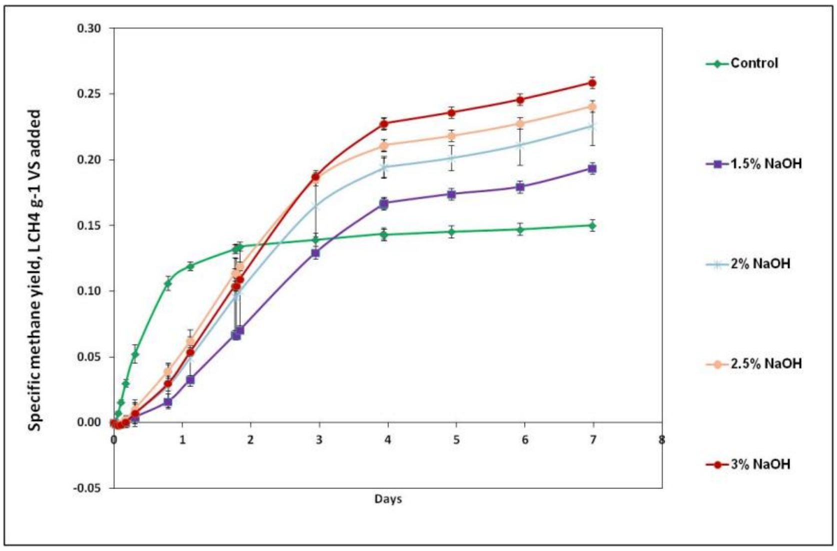


Figure 4.18. Effect of 2-hour pre-treatment in Assay No. 5

4.3.1.7 Assay No.6

Summary method

In Assay No. 6 all FFA samples were pretreated at 80 ^oC for 2 hours and received different NaOH dosages (1 - 5% w/w), to provide a check on and extension to the conditions in Assay No. 4. The assay was continued for 208 days to allow estimation of the ultimate BMP value.

Results

Results are shown in Table 4.17 and Figure 4.19. NaOH additions of >4% (w/w) showed some initial inhibition (Figure 4.19) which was variable in degree but followed the same trend in each case.

As can be seen from Table 4.17 the initial inhibition at NaOH concentrations of 4% and 5% was eventually overcome, with these variants showing the highest ultimate BMP values. 1 - 3% NaOH gave similar ultimate BMP values but the 7-day SMP increased with increasing alkali addition.

Table 4.17. Effect of 2-hour pre-treatment in Assay No. 6

L CH_4 g^{-1} VS		Control	0%	1%	2%	3%[a]	4%	5%
7-day SMY	Average	0.149	0.16	0.208	0.249	0.3	0.313	0.097
	Max	0.153	0.163	0.212	0.254	0.308	0.323	0.175
	Min	0.145	0.158	0.203	0.247	0.292	0.296	0.032
	% Control	100%	107%	139%	167%	201%	210%	65%
BMP	Average	0.27	0.308	0.346	0.357	0.382	0.39	0.389
	Max	0.276	0.324	0.374	0.369	0.396	0.405	0.416
	Min	0.263	0.292	0.32	0.341	0.368	0.379	0.376
	% Control	100%	114%	128%	132%	142%	145%	144%

[a] One replicate omitted

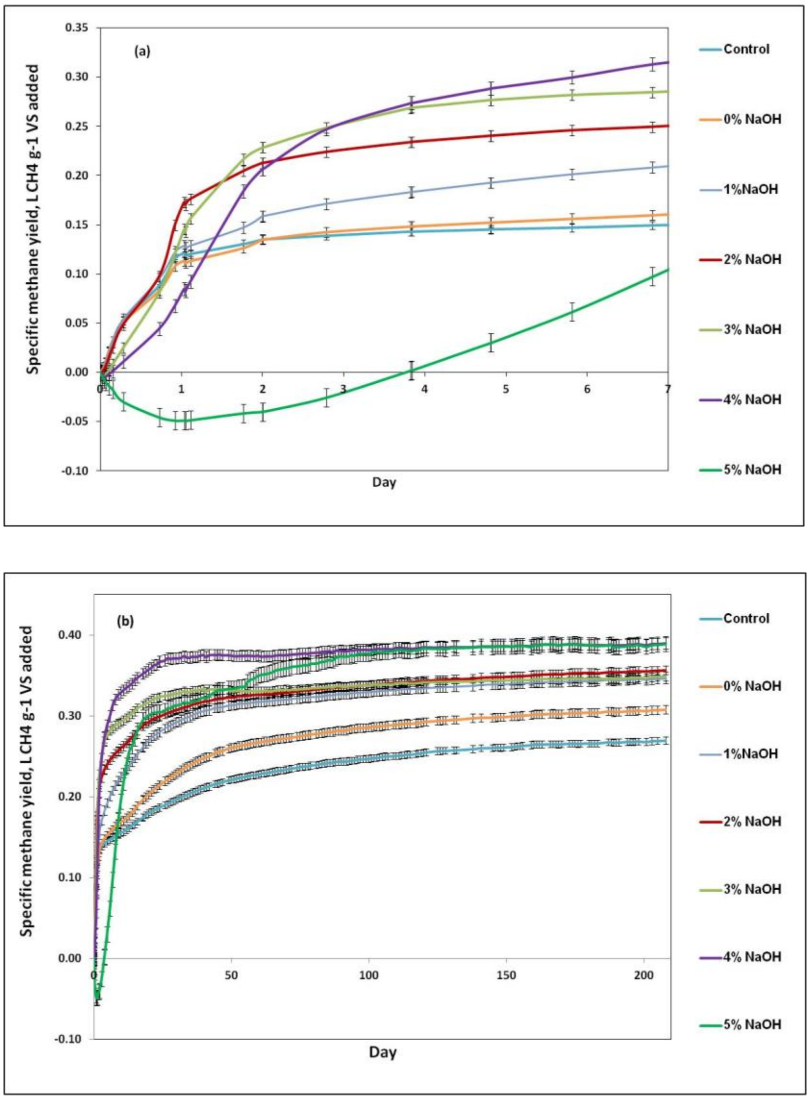


Figure 4.19. Effect of 2-hour pretreatment in Assay No. 6: day 1-7 (a) and day 1-208 (b)

Conclusion from batch pretreatment trials

Combined treatment of microalgae with NaOH and heat gave a significant improvement in specific methane yield under batch conditions. Dosage of 3% NaOH (w/w) in combination with 2-hour treatment at 80 ^{o}C appeared to be the optimum, and showed improvements of up to 101% in 7-day SMY, and 42% in 208-day BMP. This treatment was therefore decided to be applied for the following semi-continuous digestion trial.

4.3.2 Semi-continuous digestion of pretreated microalgal biomass

Summary method

A semi-continuous digestion trial was carried out in duplicate in mesophilic digesters (K1-10) each with a working volume of 1.5 L as described in section 3.3.1.1. Three pairs of digesters were initially inoculated with digestate from digesters fed on a synthetic substrate made up to marine concentration of 31.1 g L^{-1} (Roberts et al., 2016b), while the remainder received fresh inoculum from Millbrook WWTW. This trial used FFA i.e. centrifuged mixed algal culture taken from the same source as the material used in the previous semi-continuous digestion study DT-3. Untreated FFA was fed to reactors K1&2 as control. FFA pretreated with dosage of 1% and 3% NaOH (w/w) at 80 ^{o}C in water bath for 2 hours was used as feedstock for other reactors. Details of the conditions used in this trial are summarised in Table 4.18.

Table 4.18. Experimental conditions in pre-treated algal digestion trial

Reactor	OLR g VS L^{-1} day^{-1}	HRT day	Inoculum	Feedstock	Comment
K1&2	2.5	23	Millbrook	Control (untreated FFA)	Stopped feeding on day 75
K3&4	2.5	23	Millbrook	3% NaOH	Stopped feeding on day 44
K5&6	2.5	23	Acclimated	Untreated + 3%NaOH;	Day 0-9: fed with untreated algae plus 3% NaOH without pre-treatment
				Untreated;	From day 10: fed with untreated algae only
				3%NaOH + HCl	From day 44 fed with algae pre-treated with 3% NaOH and neutralised with HCl
K7&8	2.5	23	Acclimated	3% NaOH	From day 0-44: fed with algae pre-treated with 3% NaOH
			Millbrook	1% NaOH	On day 45: reactors re-inoculated and fed with algae pre-treated with 1% NaOH
K9&10	3	19	Acclimated	3% NaOH	Day 0-25: fed with algae pre-treated with 3% NaOH Day 26-44: fed with algae pre-treated with 3% NaOH then neutralised with HCl
	2.5	23	Millbrook	1% NaOH + HCl	Day 45: re-inoculated and fed with algae pre-treated with 1% NaOH (w/w) and neutralised with HCl. OLR adjusted to 2.5 g VS L^{-1} day^{-1}

Results

Monitoring parameters during continuous digestion of pretreated FFA biomass are shown in Figure 4.20. As can be seen in Figure 4.20a and 4.20b, VBP and SMP in duplicate reactors followed closely similar trends, with slightly better agreement for SMP. The average SMP in the control reactors during the final 15 days of operation was stable at 0.139 L CH_4 g^{-1} VS or about 52% of the 208-day BMP, reflecting the shorter retention time. All of the reactors fed on treated algal biomass showed a sharp drop in SMP from the start of feeding: this was especially clear in K5&6, indicating that the untreated NaOH addition was causing inhibition. After day 45 gas production in the reactors with treated feedstock fluctuated, but by the end of the trial appeared to stabilise. Average SMP for the last 10 days of the run was 0.175, 0.156 and 0.135 L CH_4 g^{-1} VS for K5&6 (3%NaOH +HCl), K7&8 (1%NaOH) and K9&10 (1%NaOH + HCl) respectively: corresponding to improvements of 26% and 12% over the control for the first two treatments. The 3%NaOH + HCl sample achieved 65% of the control BMP and 46% of its own BMP (Table 4.17), while the equivalent results for 1%NaOH were 58% and 45%. These results are indicative only as gas production had not fully stabilised by the end of the trial: they suggest, however, that while the thermal alkaline pre-treatment achieved some improvement in SMY the increase was not as marked as in batch testing. The use of acclimated inoculum may have been beneficial in K5&6 after the change of feedstock on day 44. Neutralisation of the 1% NaOH treatment with HCl did not appear to be beneficial with unacclimated inoculum.

pH in the control digesters stabilised at around 7.5, while in the digesters with treated feedstocks it fluctuated reflecting the degree of buffering from ammonia and alkali addition and the build-up of VFA. IA/PA ratios fell towards the end of the run indicating that some degree of stabilisation had been achieved. The higher TAN concentrations in the digesters with pre-treated feedstocks may reflect the greater degree of breakdown of the algal material. VS concentrations in K5&6 were slightly below those in the other digesters, supporting the view that improved degradation had occurred.

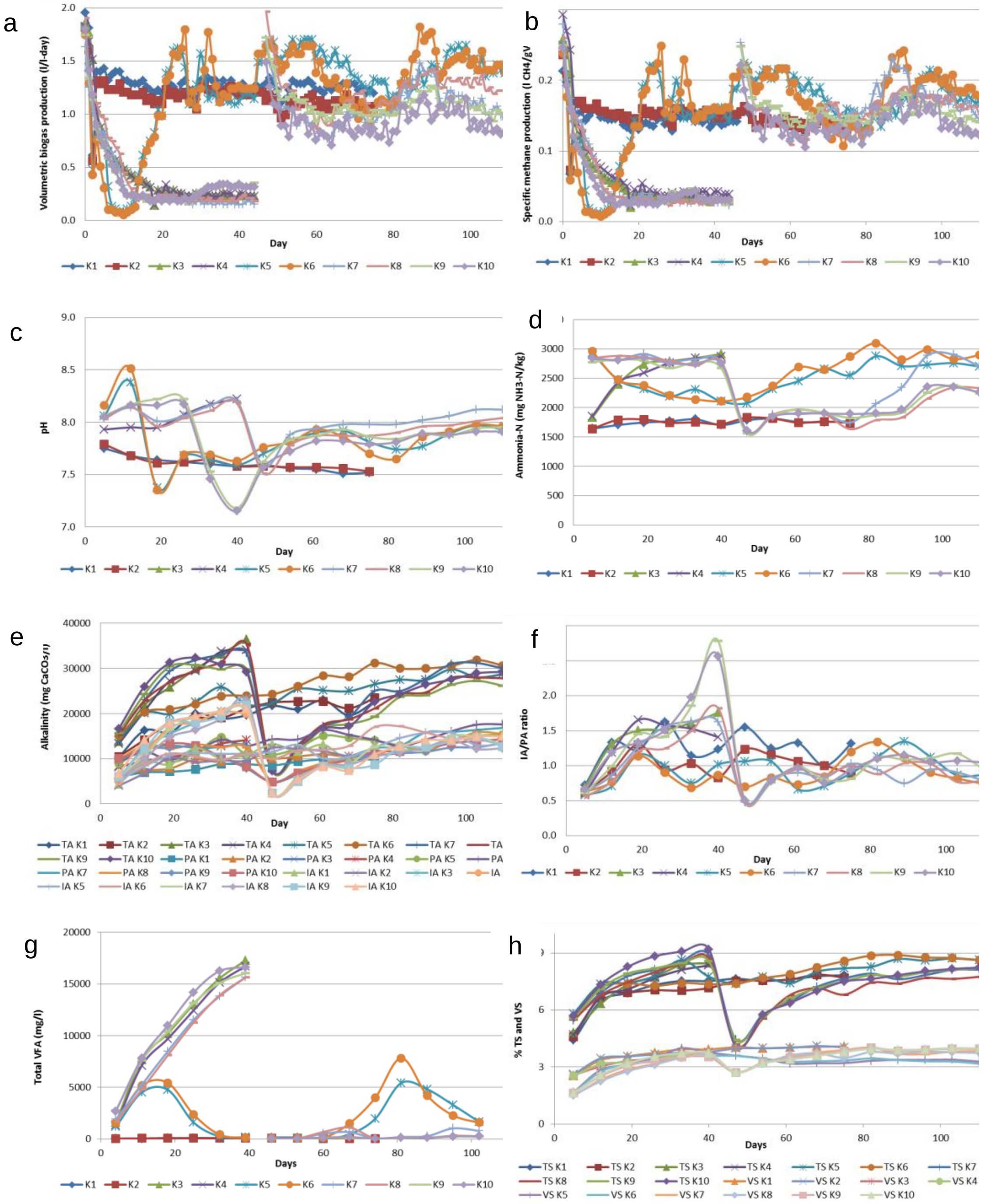


Figure 4.20. Monitoring parameters during continuous digestion of pretreated FFA biomass.

Conclusion

The results indicated that some improvement was achieved with the thermal alkaline pretreatment, but these may be limited by hydraulic retention times and there is clearly scope for optimisation of the treatment of feedstock and inoculum.

4.3.3 Discussion

Biogas production from microalgal biomass through AD has been confirmed as technically feasible over the last decades. It has been noted, however, that the poor biodegradability of this type of substrate is one of the major hurdles that needs to be overcome for the AD of microalgae to be economically viable. For some common freshwater microalgae that have been used as substrate for AD studies, many researchers have pointed out that the rigid cell wall structure is the likely cause of their low biodegradability. For this reason, pretreatments have been investigated to enhance the biodegradability and methane production of this type of subtrate. As presented in the literature review, to date there have been many studies employing heat pretreatments (Table 2.4), and fewer studies investigating mechanical, chemical, biological and combined pretreatments (Tables 2.3, Table 2.5). Some positive effects have been reported, yet in general it is difficult to propose an optimum pretreatment because the effectiveness of a method is very much dependent on a number of factors including pretreatment conditions and especially on specific microalgal biomass. For instance, Kinnunen et al. (2014b) obtained an increment of 23% in methane yield when pretreating a mixture of *Pediastrum* sp., *Micractinium* sp. and *Scenedesmus* sp. at 60 ^{o}C in 3.8 hours, whereas Marsolek et al. (2014) reported that methane yield decreased by 4% after biomass of *Nannochloropsis oculata* was pretreated at 60 ^{o}C in 4 hours.

In most cases, heat-employing methods appears to be most effective with certain degree of improvement in methane yield. The resulting energy balance is not always positive, however, since some of these pretreatments are very energy-intensive (Passos et al., 2014b, Ward et al., 2014, Jankowska et al., 2017). Pretreatments that require minimal energy input, such as low and mild temperature, biological, and chemical methods have therefore been attracting more interest recently (Passos and Ferrer, 2014, Passos et. Al., 2013, Passos et al., 2016, Kinnunen and Rintala, 2016, Kinnunen et al., 2014).

The recalcitrant nature of some common freshwater microalgae (eg. *Scenedesmus* sp, *Chlorella* sp.) is generally considered to be due to the complex cross-linked macromolecular structures of their cell walls (Roberts, 1974, Burczyk and Dworzanski, 1988, Blokker et al., 1997, Gerken et al., 2013). This is similar to the situation in terrestrial plants, in which the cellulose in woody biomass is protected by a non-degradable lignin fraction (Roberts et al., 2016a). Alkaline pretreatment has been employed extensively to make lignin-containing biomass amenable to enzymatic reactions, and proven to be one of the most viable process options due primarily to its strong pretreatment effect and relatively simple process scheme (Kim et al., 2016). Although microalgae do not contain lignin (Pandey et al., 2014), some benefits have also been reported in the application of an alkaline pretreatment to microalgae. A recent study has indicated that treatment of *Microcystis aeruginosa* biomass with CaO before acid and/or enzymatic hydrolysis enhanced the degradation of algal cells (Khan et al., 2017). A certain degree of improvement in solubilisation and methane yield was obtained after a microalgal mixture of *Chlorella* sp. and *Scenedemus* sp. was adjusted to pH 9 using 5N NaOH (Cho et al., 2013). Nevertheless, alkaline pretreatments of microalgal biomass have not been extensively investigated and the results are sometimes contradictory. Indeed, the combination of thermal and alkaline pretreatments applied to different microalgal biomass has been reported to be more effective than thermal or alkaline pretreatments applied separately (Bohutskyi et al., 2014a). Thermo-alkali pretreatments have been tested at low (<100 ^{o}C) and high (>100 ^{o}C) temperatures. It has been indicated, however, that high temperatures may lead to the production of refractory organic compounds or inhibitory intermediates generated through intramolecular reactions (Stuckey and McCarty, 1984). The use of low temperatures might therefore be more appropriate.

The results from the series of screening tests in this study showed that thermo-alkaline pretreatment was more effective in enhancing the methane yield of FFA material than separate thermal pretreatment and alkaline pretreatment. As shown in Assay No. 4, compared to untreated material, methane yield was enhanced by only 10 % for thermal pretreatment (80 ^{o}C for 2 hours), and 9% for alkaline pretreatment (dosage of 1% w/w NaOH to raise substrate pH to 10.2). In contrast, thermo-alkaline pretreatment gave significant increases in methane yield (26 - 108%) for various dosages of alkali (0.5 - 3% w/w NaOH) in combination with 2-hour treatment at 80 ^{o}C (Table 4.15). Similar results were also obtained in Assay No.6, in which thermal pretreatment only enhanced methane yield by 7% at 7-day SMY and 14% by the end of 208-day BMP, whereas thermo-alkaline pretreatment increased methane yield by 39 - 110% (except for the dosage 5% w/w NaOH which reduced methane yield by 35%) for 7-day SMY, and 28 - 45% for 208-day BMP (Table 4.17).

As shown in Table 4.17, methane yield was enhanced in proportion to increasing alkali dosage. The increase in methane yield by the end of 208-day BMP, however, was almost similar for dosages of 3%, 4% and 5% w/w NaOH. Indeed, the difference between these three dosages was the inhibition exhibited at dosage 4% and 5% w/w NaOH in the first few days of the test, whilst no inhibition was observed for dosage 3% w/w NaOH. The inhibition present at higher alkali dosages (ie. 4% and 5% NaOH w/w) could have been caused by the rapid conversion of excess soluble matter produced after the thermo-alkali pretreatment into VFAs, resulting in a pH drop and a temporary loss of methane production. Thermo-alkali pretreatment has been reported significantly to enhance the organic matter solubilisation for microalgae (Bohutskyi et al., 2014, Passos et al., 2013). It has also been observed that the degree of carbohydrate solubilisation increases in proportion to the increasing dosage of alkali used for pretreatment (Mahdy et al., 2014). Nevertheless, the increase in sCOD has been found not always proportional to methane yield enhancement (Alzate et al., 2012, Bohutskyi et al., 2014). For instance, the sCOD of *Nannochloropsis* sp., *Tetraselmis* sp. and *Pavlova_cf* sp. reached nearly 90% after thermo-alkaline pretreatment, but no improvement in methane yield was observed (Bohutskyi et al., 2014). One possible explanation for this is that a large fraction of the particulate matter could be hydrolysed and biodegraded by anaerobic microorganisms even without any pretreatment. Moreover, a combination of high alkali concentrations and temperature may result in the formation of refractory compounds (Stuckey and McCarty, 1984) and in partial decomposition of the organic matter (Samson and Leduy, 1983). With an increase in methane yield of 101% for 7-day SMY, and 42% for 208-day BMP, the dosage of 3% w/w NaOH combined with 2-hour treatment at 80 ^{o}C was considered optimum for FFA material. When applied in continuous trials, however, the methane yield improvement was not as marked as in batch testing. Digestion of FFA material pretreated with 3% w/w NaOH neutralised with HCl under continuous condition achieved a SMY of 0.175 L CH_4 g^{-1} VS, which was a 26% increase compared to the control. A dosage of 1% w/w NaOH was also tested with and without HCl neutralisation. SMY post treatment was 156 L CH_4 g^{-1} VS (12% increase) for a dosage of 1% w/w NaOH without HCl neutralization, whilst it was 135 L CH_4 g^{-1} VS (3% decrease) for dosage of 1% w/w NaOH with HCl neutralisation. It should be noted that these values were obtained for the last 10 days of the run, and due to the shortage of material, the trial was stopped by day 113. The results are therefore indicative only as gas production has not fully stabilised by the end of the trial.

Results of thermo-alkali pretreatment from this study were compared with findings from other researchers and are presented in Table 4.19. Improvement of between 12.2 - 25% in methane yield was reported in a recent work in which CaO was used as alkaline reagent and tested at two temperatures of 55 ^{o}C anf 72 ^{o}C (Solé-Bundó et al., 2017a). The best improvement in methane yield of up to 86% was reported by Passos et al. (2016). The authors investigated the effect of thermo-alkali pretreatment on AD of a microalgal biomass harvested from a stabilisation pond. The material was pretreated with various dosages of KOH at 80 ^{o}C for 2 hours, but methane yield improvement was not proportional to increasing amount of alkali used. Dosage of 0.5% w/w KOH gave the best results with 86% methane yield increase, whilst dosage of 1.25% and 2% w/w KOH enhanced methane yield by only 28% and 3%, respectively. Moreover, the SMY of untreated material was only 0.078 L CH_4 g^{-1} VS, considerably lower than the values obtained from this study. Mendez et al. (2013) reported increases of 71 and 73% in methane yield after biomass of *Chlorella vulgaris* was thermo-alkali pretreated for 20 minutes and 40 minutes respectively. The authors stated, however, that thermal pretreatment only was even more effective: thermal pretreatment of microalgal biomass at 120 ^{o}C for 40 minutes enhanced methane yield by up to 93%. Chen and Oswald (1998) also reported that the best result of maximum 33% increase in methane yield was obtained with thermal pretreatment at temperature of 100 ^oC for 8 hours, whereas thermo-alkali pretreatment did not result in any improvement. Bohutskyi et al. (2014) found thermo-alkali pretreatment ineffective for *Pavlova_cf* sp., *T. weisflogii* and *Tetraselmis* sp., but it enhanced methane yield by 30 - 40% for *Chlorella* sp. and *Nannochloropsis* sp. In short, to date thermo-alkaline pretreatments on microalgal biomass have not been extensively investigated, however results from the existing available papers are rather diverse and are sometimes contradictory. Nevertheless, a combination of thermal and alkaline pretreatments can be promising.

Table 4.19. Results of thermo-alkaline pretreatment of microalgal biomass from several studies

Substrate	**Digestion conditions**	**CH_4 yield of untreated material (mL g^{-1} VS)**	**Pretreatment conditions**	**Methane yield post treatment (mL g^{-1} VS)**	**Methane yield increase (%)**	**References**
Microalgal biomass[a]	Batch, 35 °C, 7 day HRT	149	3% NaOH (w/w), 80 °C, 2h	300	101	this study
Microalgal biomass[a]	Batch, 35 °C, 208 day HRT	270	3% NaOH (w/w), 80 °C, 2h	382	42	this study
Microalgal biomass[a]	CSTR, 35 °C, 23 day HRT, 2.5 g VS L^{-1} day^{-1} OLR	139	3% NaOH (w/w) + HCl, 80 °C, 2h	175[f]	26	this study
Microalgal biomass[a]	CSTR, 35 °C, 23 day HRT, 2.5 g VS L^{-1} day^{-1} OLR	139	1% NaOH (w/w), 80 °C, 2h	156[f]	12	this study
Microalgal biomass[a]	CSTR, 35 °C, 23 day HRT, 2.5 g VS L^{-1} day^{-1} OLR	139	1% NaOH (w/w) + HCl, 80 °C, 2h	135[f]	-3	this study
Microalgal biomass[b]	Fed-batch operated digester, 38 °C, 28 day HRT, 0.93 g L^{-1} day^{-1} OLR	240	20 - 200 g NaOH kg^{-1} algae, 3% TS solid, 100 °C, 8h	unspecified	No effect/ negative effect	(Chen and Oswald, 1998)
Microalgal biomass[c]	Batch, 35 °C, 105 day HRT	260	55 °C; 4% CaO; 24h	255	-2.1	(Solé-Bundó et al., 2017a)
Microalgal biomass[c]	Batch, 35 °C, 105 day HRT	260	55 °C; 10% CaO; 24h	292	12.2	(Solé-Bundó et al., 2017a)
Microalgal biomass[c]	Batch, 35 °C, 105 day HRT	260	72 °C; 4% CaO; 24h	287	10.3	(Solé-Bundó et al., 2017a)
Microalgal biomass[c]	Batch, 35 °C, 105 day HRT	260	72 °C; 10% CaO; 24h	325	25	(Solé-Bundó et al., 2017a)
Microalgal biomass[d]	Batch, 35 °C, 32 day HRT	78	0.5% KOH (w/w), 80 °C, 2h	145.10	86	(Passos et al., 2016)
Microalgal biomass[d]	Batch, 35 °C, 32 day HRT	78	1.25% KOH (w/w), 80 °C, 2h	100.14	28	(Passos et al., 2016)
Microalgal biomass[d]	Batch, 35 °C, 32 day HRT	78	2.0% KOH (w/w), 80 °C, 2h	80.78	3	(Passos et al., 2016)
Chlorella sp.	Batch, 35 °C, 90-95 day HRT	340	NaOH (various conc.); 121 °C; 10 bar; 30 min	unspecified	30 - 40	(Bohutskyi et al., 2014)
Chlorella vulgaris	Batch, 35 °C, 38 day HRT	unspecified	2% NaOH (w/w), 50 °C, 24h	unspecified	13	(Mahdy et al., 2014)
Chlorella vulgaris	Batch, 35 °C, 38 day HRT	unspecified	2% NaOH (w/w), 50 °C, 48h	unspecified	17	(Mahdy et al., 2014)
Chlorella vulgaris	Batch, 35 °C, 30 day HRT	138.9[g]	pH 10; 120 °C; 20 min	237.9[g]	71	(Mendez et al., 2013)
Chlorella vulgaris	Batch, 35 °C, 30 day HRT	138.9[g]	pH 10; 120 °C; 40 min	240.6[g]	73	(Mendez et al., 2013)
Nannochloropsis sp.	Batch, 35 °C, 90 - 95 day HRT	360	NaOH (various conc.); 121 °C; 10 bar; 30 min	unspecified	30 - 40	(Bohutskyi et al., 2014)
Pavlova_cf sp.	Batch, 35 °C, 90 - 95 day HRT	510	NaOH (various conc.); 121 °C; 10 bar; 30 min	unspecified	No effect/ negative effect	(Bohutskyi et al., 2014)
Scenedesmus sp.	Batch, 35 °C, 38 day HRT	unspecified	5% NaOH (w/w), 50 °C, 24h	unspecified	20	(Mahdy et al., 2014)
Spirulina maxima	SCAF[e], 35 °C, 20 day HRT, 2.0 g VS L^{-1} day^{-1} OLR	190	pH 11.0; 50 °C	210	11	(Samson and Leduy, 1983)
Spirulina maxima	SCAF[e], 35 °C, 20 day HRT, 2.0 g VS L^{-1} day^{-1} OLR	190	pH 13.0; 50 °C	90	-53	(Samson and Leduy, 1983)
Spirulina maxima	SCAF[e], 35 °C, 20 day HRT, 2.0 g VS L^{-1} day^{-1} OLR	190	pH 11.0; 100 °C	220	16	(Samson and Leduy, 1983)
Spirulina maxima	SCAF[e], 35 °C, 20 day HRT, 2.0 g VS L^{-1} day^{-1} OLR	190	pH 13.0; 100 °C	140	-16	(Samson and Leduy, 1983)
Spirulina maxima	SCAF[e], 35 °C, 20 day HRT, 2.0 g VS L^{-1} day^{-1} OLR	190	pH 11.0, 150 °C	240	26	(Samson and Leduy, 1983)
Spirulina maxima	SCAF[e], 35 °C, 20 day HRT, 2.0 g VS L^{-1} day^{-1} OLR	190	pH 13.0; 150 °C	80	-58	(Samson and Leduy, 1983)
T. weissflogii	Batch, 35 °C, 90 - 95 day HRT	420	NaOH (various conc.); 121 °C; 10 bar; 30 min	unspecified	No effect/ negative effect	(Bohutskyi et al., 2014)
Tetraselmis sp.	Batch, 35 °C, 90 - 95 day HRT	380	NaOH (various conc.); 121 °C; 10 bar; 30 min	unspecified	No effect/ negative effect	(Bohutskyi et al., 2014)

[a] mainly *Scenedesmus* sp. and *Chlorella* sp. cultivated and harvested from a 100 m^2 raceway in Almeria, Spain

[b] grown in the effluent of high-rate sewage stabilization pond at Richmond Field Station, USA

[c] mainly *Scenedesmus* sp. and *Chlorella* sp. harvested from a pilot raceway pond (17 m^3) in Narbonne, France

[d] harvested from a stabilization pond in a full-scale WWTP in Ouro Blanco (Minas Gerais, Brazil)

[e] Semi continuous anaerobic fermentation

[f] average SMY for the last 10 days of the run

[g] expressed as mL CH_4 g^{-1} COD

5. CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

Although the number of studies on AD of microalgae has increased in recent years, the reported results are still limited, especially data obtained from continuous and semi-continuous digestion conditions. This study examined the methane production of two microalgal feedstocks: freeze-dried algae and fresh frozen microalgae under mesophilic and semi-continuous conditions. This is the first study ever reporting on the long run digestion of microalgal biomass under semi-continuous condition which can be served as a baseline for scale-up in the future. Both substrates were successfully converted to biogas; however the biodegradability was very poor and the methane yields were low. The research therefore also examined the effect of pre-treatments on methane production from fresh frozen microalgae, focusing specifically on thermo-alkaline pretreatment.

The following main findings and conclusions can be drawn from the work carried out:

5.1.1 Digestion of freeze-dried algae

- Semi-continuous trials with FDA showed that at feed solids concentrations of 6, 8 and 10% VS, digestion was stable without any observable negative effects on the methanogenic consortium that have been highlighted within the literature.
- At a feed concentration of 20% VS, the digesters were able to achieve meta-stable operation at very high TAN concentrations of up to 12 g N L^{-1}. A reasonable methane production was obtained showing a degree of adaptation to high TAN.

- Despite a very extended operating period, in excess of 800 days at 20% feedstock VS, there was no improvement in the degree of anaerobic biodegradation achieved as indicated by the VS destruction or the specific biogas and methane production in periods of relatively stable operation. The results therefore indicate that while acclimatisation to high TAN concentrations may be possible, no acclimatisation to the feedstock itself has occurred.
- A radio isotope experiment conclusively proved the dominant metabolic pathway for methane formation in digesters at high ammonia concentrations was via the syntrophic route.

5.1.2 Digestion of fresh frozen algae

- The FFA was successfully converted to biogas via semi-continuous anaerobic digestion at a solids concentration of 4.33% and at OLR of up to 3.5 g VS L^{-1} day^{-1}. The VS destruction was low at ~30%, however, and only ~24% of the measured calorific value could be recovered in the form of methane, suggesting that the substrate could benefit from a pretreatment step to improve the energy recovery.

5.1.3 Digestion of pretreated fresh frozen algae

- Thermo-alkaline pretreatment of FFA significantly improved specific methane yield under batch conditions. Up to 201% increase was achieved in 7-day SMY when FFA was pretreated with dosage of 3% NaOH (w/w) in combination with heat treatment at 80 ^{o}C for 2 hours. The improvement, however, was less significant compared with results from the batch trials when pretreated FFA was digested under semi-continuous condition. This highlights the importance of conducting continuous operation.

5.2 Further work

Batch and continuous digestion studies both from the literature and from this study have indicated the recalcitrant nature of the two microalgal substrates harvested from large scale cultivation; both were very poorly biodegradable and failed to reach their maximum methane potential.

Further work should focus on understanding the cell wall composition of microalgae, to determine which parts of the microalgae are recalcitrant and why. This could be done by employing scanning electron microscope imaging to locate areas of the cell that remain postdigestion. These regions can then be isolated prior to digestion and analysed for their composition by biochemical analysis methods and/or state-of-the-art biotechnology.

Storage techniques have shown strong impact on the quality of substrate. It would be interesting to conduct digestion trials using microalgal biomass that is freshly harvested or frozen at different temperatures to find out the most appropriate condition for storage and best substrate condition for AD.

Thermo-alkaline pretreatments have shown significant improvement in methane yield under batch testing, but not that effective in continuous condition. Further work could focus on optimising pretreatment conditions: eg. using of different reagents, testing at lower temperatures etc. There is also scope for optimisation of the treatment of feedstock and inoculum.

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