Anaerobic digestion of *Spirulina* sp. and *Scenedesmus* sp.: a comparison and investigation of the impact of mechanical pre-treatment

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Abstract

Anaerobic digestion (AD) is a unit process that integrates beneficially and sustainably into many bioprocesses. This study assesses and compares the production of methane from the biomass of the microalga *Scenedesmus* sp. and the cyanobacterium *Spirulina* sp. in batch anaerobic digesters. Anaerobic digestion of whole cell *Spirulina* resulted in a substantially higher methane productivity (0.18 L $CH_4.L_{reactor}^{-1}.day^{-1}$) and methane yield (0.113 L $CH_4.g^{-1}$ volatile solids (VS)) compared to the digestion of whole cell *Scenedesmus* (0.12 L $CH_4.L_{reactor}^{-1}.day^{-1}$ and 0.054 L $CH_4.g\ VS^{-1}$). *Spirulina*, possibly due to a combination of osmotic shock, the filamentous nature of the cells and lower mechanical strength of the non-cellulosic cell wall, was more readily degraded by hydrolytic and acidogenic microorganisms, resulting in the generation of a greater amount of acetic acid. This in turn provided greater substrate for methanogens and hence higher methane yields. In addition, *Spirulina* cells could be disrupted mechanically more quickly (1h) than *Scenedesmus* cells (4h) in a bead mill. Mechanical pre-treatment improved the final methane yields (L $CH_4.g\ VS^{-1}$) obtained from digestion of both substrates, however, the improvement was greater for *Scenedesmus*. Mechanical pre-treatment resulted in a 47% increase in methane production for *Spirulina* compared to 76% increase for *Scenedesmus* fed digesters. The more substantial increase observed for *Scenedesmus* was due to the relatively inefficient digestion of the whole, unruptured cells.

Key words

Anaerobic digestion, biogas, methane, *Scenedesmus*, *Spirulina*, algae

Introduction

Microalgae have great potential as biocatalysts in the fields of wastewater treatment, bioremediation and carbon dioxide sequestration, as well as for the production of food, feeds, biofuels and high value products such as proteins, pigments and lipids. They have been reported to be more productive than higher plants and can be grown in poor quality or non-potable water (Illman et al., 2000). Major challenges in the application of microalgae in a variety of fields, particularly in the production of renewable fuels, include the sustainable and economic provision of nutrients such as nitrogen, phosphorous and CO_2, and the minimisation of energy use (Borowitzka and Moheimani, 2013; Chisti, 2013). Anaerobic digestion (AD) is a simple, low-cost unit operation that could be advantageously integrated into many bioprocesses. Either whole algal biomass, or the biomass residue after product extraction, could be fed to an AD reactor, either as the sole feed or in combination with other substrates. AD offers the opportunity to recover and recycle nutrients, carbon dioxide and energy from the substrate, thereby yielding a more sustainable, cost effective and energetically feasible process (Borowitzka and Moheimani, 2013; Ward et al., 2014, Harrison et al. 2014).

AD involves a series of reactions, including hydrolysis, acidogenesis, acetogenesis and methanogenesis, during which microorganisms break down biodegradable material in the absence of oxygen to produce biogas, as well as a liquid effluent rich in ammonia and phosphate (Borowitzka and Moheimani, 2013). Biogas from AD is usually rich in methane (65-70%) and carbon dioxide (30-35%), with small amounts of hydrogen and hydrogen sulphide (Gunaseelan, 1997; McKendry, 2002). The biogas can be utilised directly or indirectly to derive energy in a number of processes. These include use in a combined heat and power unit (CHP), where the gas is combusted to produce heat and electricity; direct compression or liquefaction to produce a transport fuel (De Schamphelaire and Verstraete, 2009) or purification for use in the production of traditional transport fuels such as petroleum or diesel (De Schamphelaire and Verstraete, 2009). The CO_2 in the biogas, as well as that generated by combustion of the methane, if carried out on site, could be recycled to algal ponds. The liquid effluent can be recycled to the algal cultures, reducing the requirement for fresh nutrients (Ward et al., 2014).

There are several challenges associated with AD of algal biomass, including ammonia and sulphur toxicity due to a high protein content, salinity of the biomass, if marine algal species are used, and recalcitrance of algal cells walls, leading to slow hydrolysis and low

digestibility (Ras et al. 2011; Ward et al., 2014). AD of algal biomass has typically resulted in a lower performance than that of traditional energy crops (Braun et al., 2010), yet values of 0.5 m^3 biogas per kg algal organic dry matter have been obtained (62.5% CH_4) (Golueke et al., 1957). The anaerobic digestion of *Spirulina maxima* resulted in a biogas yield of 0.3–0.37 m^3 biogas.kg^{-1} volatile solids (VS), with 70% methane and conversion efficiencies up to 48% (Samson and Leduy, 1982; 1983a,b; 1986). Maximum yields were obtained with a retention time of 30 days and an initial algal concentration of 20 kg VS.m^{-3}. In contrast to the study of Golueke et al. (1957), a mesophilic temperature (35 °C) was found most preferable for the degradation of the algal biomass (Samson and Leduy, 1986). Biochemical methane potential (BMP) tests with *Scenedesmus* have suggested a potential yield of 0.4 m^3 biogas.kg VS^{-1} (McGinn et al., 2012).

The degradability of algal biomass in anaerobic digestions is species dependent. In some cases, cell walls resistant to degradation can be one of the major limiting factors in the digestion of algal biomass (Ward et al., 2014; Sialve et al., 2009). Chen & Oswald (1998) reported that up to 60% of whole, untreated algal biomass could remain undigested in an AD reactor. More recently, Tartakovsky et al. (2013) reported that only 52-53% of the algal biomass fed to an anaerobic digester was degraded at a retention time of 16 days, largely due to the limited hydrolysis of the algal biomass. Algal biomass is typically characterised by a relatively high cellulose (7.1%) and hemicellulose content (16.3%) (Ververis et al., 2007). Hydrolysis of cellulose does occur during AD, but is slow and may be rate limiting if present in high concentrations (Yen and Brune, 2007). Algal cell walls protect the cell from extracellular enzymes produced by the anaerobic microbes, creating a strong resistance to hydrolysis. Golueke and co-workers (1957) and Sanchez and Travieso (1993) reported the presence of whole cells in their digesters after long periods of time, confirming the resistance to degradation.

Mechanical and thermochemical pre-treatments have been applied to algal biomass to increase the biodegradability (Golueke and Oswald, 1959; Chen and Oswald, 1998; Gonzalez-Fernandez et al., 2012a&b; Samson and Leduy, 1983a). Chen and Oswald (1998) investigated pre-treatment with various combinations of heat and sodium hydroxide. All pre-treatment scenarios yielded better results than untreated controls, with a maximum increase in methane production of 33% with pretreatment at 100°C for 8 h. Gonzalez-Fernandez et al. 2012a) reported an increase in methane production of 9% with thermal pretreatment at 70°C and 59%

at 90°C for 1h in *Scenedesmus* sp. Samson and Leduy (1983b) investigated the thermo-chemical and mechanical pre-treatment of *Spirulina maxima* biomass. They report that ultrasonic degradation for 10 min was as efficient as thermal pre-treatment at 150°C for 1 h. They also showed that freezing of the biomass could result in enhanced solubilisation.

Algal cell wall composition and degradability are species dependent (Mussgnug et al., 2010). *Scenedesmus* sp. has a recalcitrant cell wall with a highly resistant trilaminar structure, composed of cellulose, algaenan and glucosamine-containing biopolymers and glycoproteins (Voigt et al., 2014). *Spirulina* has a much more easily degraded cell wall, composed of protein, polysaccharides and peptidoglycans (Van Eykelenburg, 1977). This study assesses and compares the efficiency of digestion and the production of methane from the biomass of the microalga *Scenedesmus* sp. and the cyanobacterium *Spirulina* sp. in batch anaerobic digesters. The conversion of solid to soluble chemical oxygen demand (COD) as well as the evolution of volatile fatty acids (VFAs) were analysed with and without mechanical pre-treatment of the biomass in order to better understand the effect of species choice and biomass pre-treatment on the efficiency of digestion.

Most studies conducted on AD of algal biomass have used a semi-continuous system, in which feed is added to the digesters at a specific load of volatile matter and desired retention period. However, few studies consider fundamental batch data before proceeding to operate a semi-continuous or continuous mode digester. By conducting these studies in batch reactors, with rigorous analytical analysis, the effect of the treatments on the different stages of AD can be better elucidated in order to inform continuous AD process design. One of the potential limitations of batch experiments is that they may not identify problems from the build up of inhibitory substances, e.g. sulphur and ammonia, that can occur during the continuous operation of anaerobic digesters. Inhibitory levels may not be reached during a batch experiment when compared to the continuous operation of digesters.

Methods

Algal cultivation Scenedesmus sp. (isolated from algal ponds in Uppington, South Africa) and *Spirulina* sp. (isolated from tannery effluent ponds, Wellington, South Africa) were maintained in 3N BBM medium (Bold, 1949) and Zarrouk's medium (Zarrouk, 1966) respectively. Cultures were grown in 3.2 L airlift photobioreactors and a 50 L raceway pond. The glass and steel photobioreactors were 600 mm high, with a 100 mm diameter column and

a 50 mm diameter draught tube. Reactors were sparged at 2 $L.min^{-1}$ with either regular air (for *Spirulina* sp.) or air enriched with 2900 ppm CO_2 (for *Scenedesmus* sp.). Light was supplied continuously by three Osram 18 watt cool white fluorescent bulbs at a distance of 3 cm from the column surface, providing 300 μmol $photon.m^{-2}.s^{-1}$. A 50 L Perspex raceway pond with a paddle wheel was used for larger-scale algal cultivation. *Scenedesmus* sp. cultures were sparged with air (25 $L.min^{-1}$) through a 6.35 mm stainless steel tube; with 1 mm holes drilled every 20 cm, positioned on the reactor floor. *Spirulina* sp. cultures were not sparged, as the primary carbon source was bicarbonate (HCO_3^-), not CO_2. Circulation of the media was achieved using a four bladed paddle wheel at 20 rpm for *Scenedesmus* sp. and at 10 rpm for *Spirulina* sp. The liquid depth was 10 cm and the total surface area was 0.51 m^2. Six 58 watt and two 36 watt fluorescent bulbs provided continuous light to the cultures. Algal biomass concentration was determined daily by measuring optical density at 750 nm with a Helios spectrophotometer and converting these to dry mass concentration using a calibration curve. Biomass dry weight was measured by filtration of a 5 mL sample through a pre-weighed 0.22 μm filter, which was then dried at 80°C overnight before being re-weighed.

Mechanical pre-treatment of algal biomass Batch phase bead milling was used as the mechanical pre-treatment to rupture the algal cells. One litre of concentrated algal slurry at concentrations of 40 $g.L^{-1}$ or 20 $g.L^{-1}$ was loaded into a cylindrical 1 L glass reactor with four 10 mm wall baffles. Glass beads (1 mm diameter beads for the smaller *Scenedesmus* sp. and 4 mm diameter beads for the filamentous *Spirulina* sp. cells) were loaded at 35% vol/vol. The mixture was agitated at 900 rpm, using a 20 mm diameter Rushton turbine, for 1 h for *Spirulina* and 4 h for *Scenedesmus.* Both *Spirulina* and *Scenedesmus* were milled until no whole cells could be identified by light microscopy and the increase in soluble COD had stabilised. Milling of *Spirulina* resulted in a significant increase in soluble COD (18250 mg $COD.L^{-1}$ to 41250 mg $COD.L^{-1}$), and decrease in solid COD (46400 mg $COD.L^{-1}$ to 24400 mg $COD.L^{-1}$). The soluble COD of the *Scenedesmus* slurry increased from 5700 to 10300 mg $COD.L^{-1}$, whilst that of the solid matter decreased (43300 to 39000 mg $COD.L^{-1}$) during milling.

Anaerobic digester inoculum preparation A mixture of two different inocula was used to provide the required anaerobic microbes in all anaerobic digesters. The first inoculum was obtained from an anaerobic digester treating brewery effluent, located at South African Breweries (SAB), Newlands, Cape Town, South Africa. The second component of the

inoculum was obtained from a 1 L stock reactor maintained on *Spirulina* sp. and sodium acetate (1 $g.L^{-1}$). The stock reactor was inoculated with 20% (vol/vol) activated sewerage sludge. The stock digester was operated in fed batch mode with intermittent loading of *Spirulina* sp. biomass. Gas production and composition were monitored to ensure that the digester was operating efficiently. The digester was harvested when necessary and the volume gradually increased back to the operating volume of 1 L by addition of *Spirulina* sp. slurries. The method of inoculation utilised in this study ensured a relatively consistent initial population of microorganisms in each digester.

Anaerobic digester operation The digestion experiments were carried out in continuously mixed bench-top batch reactors constructed from 1 L Duran Schott bottles. The reactors were operated with minimal headspace, at 37 ± 2°C, and were continuously mixed at 140 rpm on an orbital shaker. Biogas was collected using water displacement vessels filled with saturated sodium chloride (NaCl) solution to minimise the dissolution of CO_2. To prevent any backflow of NaCl into the reactors, 1 L trap bottles were inserted on all biogas lines. These trap bottles were fitted with a secondary biogas sampling point. The volume of biogas produced was corrected for normal temperature and pressure (STP). The digester pH was controlled by injecting 5 M sodium hydroxide (NaOH).

Triplicate digesters were loaded with 20 g DW of the appropriate substrate (either whole, wet biomass or a slurry of ruptured cells with an initial loading of 20 g DW). Tap water was added to a total volume of 800 mL. The reactor was inoculated with 100 mL of the stock (digesting *Spirulina*) inoculum and 100 mL of the SAB inoculum. The reactor was made airtight using a custom cast silicone seal, which fitted inside the screw cap. The sampling and gas collection port were connected and the reactor prepared for anaerobic operation by sparging with nitrogen for 5 min. All ports, with the exception of the gas exhaust port, that fed the gas collection system, were sealed.

Analytical techniques The methane content of the biogas was determined using flame ionisation detection gas chromatography (FID GC). All FID GC measurements were conducted on a Perkin Elmer Autosystem Gas Chromatograph using a Supelco wax column (1.2 mm x 37 m), a detector temperature of 280°C, an oven temperature of 50°C and an injection volume of 100 µl. Nitrogen, at a flow rate of 1.5 $mL.min^{-1}$, was used as the carrier gas. The chromatograph was calibrated with a standard gas containing 52.8% CH_4 vol/vol.

All COD measurements were carried out using the Merck reagent test protocol for high (1500 - 10000 $mg.L^{-1}$) concentrations. A full volatile fatty acid (VFA) profile analysis was conducted to quantify the concentration of lactic, acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids present in all digesters over the duration of digestion. The concentration of each VFA was determined using HPLC on a Waters Breeze 2 HPLC system equipped with a Bio-Rad Organic Acids ROA column and a UV (210 nm wavelength) detector. The system was run isocratically using a mobile phase of 0.01 M H_2SO_4 at a flow rate of 0.6 $mL.min^{-1}$. The pressure in the column did not exceed 2000 psi. Sample injection volumes of 100 µL were used. Total solids (TS), volatile solids and pH were measured using the standard methods (American Public Health Association, 1992).

Results

Substrate analysis Characteristics of the *Spirulina* and *Scenedesmus* biomass relevant to their anaerobic digestion are listed in Table 1. The key differences were in the different C/N ratios and the difference in cell morphology.

Biodegradability of whole cell biomass The progress of hydrolysis in AD reactors fed with whole cell *Spirulina* and *Scenedesmus* biomass was monitored by measuring solid and soluble COD concentrations (Figure 1). *Spirulina* solid COD decreased to 50% of the starting concentration within the first four days. This was accompanied by an increase in soluble COD. Both solid and soluble COD then decreased slowly to a residual concentration of approx. 4000 $mg.L^{-1}$. *Scenedesmus* solid COD remained at a level equivalent to the starting concentration for the first 12 days, then decreased slowly to day 30 and more rapidly thereafter. Relatively high concentrations of solid COD remained in the reactor after 48 days of AD. *Scenedesmus* soluble COD reached a maximum between days 6 and 14 and then decreased to negligible values.

VFA profiles for whole cell biomass The two types of unruptured algal biomass had very different VFA production profiles (Figure S1a, supplementary information). Reactors loaded with *Spirulina* sp. had much higher total VFA accumulation (8000 mg Total $VFAs.L^{-1}$) compared to those loaded with *Scenedesmus* sp. (2800 mg Total $VFAs.L^{-1}$). This indicates that hydrolysis is the rate-limiting step in *Scenedesmus* digestion, while methanogenesis is rate limiting in the *Spirulina*-fed reactors. The ratio in which the key acids (acetate:butyrate:propionate) were accumulated was 8:4:1 in the *Spirulina* digesters and 3:2:1 in the *Scenedesmus* digesters at the maximum total VFA concentration.

Methane production of whole-cell biomass The biogas productivity profiles (Figure S2, supplementary information) for the digestion of *Spirulina* and *Scenedesmus* followed a similar trend. Biogas production was maximum within the first two days, however, the methane concentration of the biogas was very low (10-20%) (Figure S3, supplementary information). Gas production then ceased until day 10 for *Scenedesmus* and day 16 for *Spirulina*. Between days 10 and 30, biogas production increased and corresponded to high methane contents and elevated rates of acetic acid consumption (Figure S1). Methane production was delayed in *Spirulina* relative to *Scenedesmus*.

Combining the methane content and biogas productivity data yielded the methane productivity of the AD systems (Figure 2). The *Spirulina* AD reactors showed an increase in both biogas production and methane content after approximately 15 days. After 18 days the biogas productivity started to increase substantially, reaching a peak after 27 days at 0.23 L $biogas.L_{reactor}^{-1}.day^{-1}$. At this same point the methane content was at a maximum of 84%,

resulting in a maximum methane productivity of 0.18 L $CH_4.L_{reactor}^{-1}.day^{-1}$. After this point, both the methane content and biogas productivity steadily decreased, resulting in lower methane productivities. The *Scenedesmus* digesters followed a similar trend. The key difference, which related directly to the biogas productivity, was that the maximum methane production occurred earlier (16 days) and at a lower rate (0.12 L $CH_4.L_{reactor}^{-1}.day^{-1}$).

Impact of mechanical pre-treatment The main purpose of performing a mechanical pre-treatment was to release the intracellular contents, such that digestion was not limited by hydrolysis. The disruption efficiency was determined by microscopy and through quantifying the increase in soluble COD of the slurry. Milling had a proportionally greater effect on the solid and soluble COD values for *Spirulina* (see methods section for details of COD values before and after milling).

The VFA profile obtained for the ruptured *Spirulina* (Figure S4a, supplementary information) was very similar to that of the unruptured biomass (Figure S1a, supplementary information), both in terms of VFA production and consumption. AD of the ruptured *Scenedesmus* cells resulted in significantly different VFA profiles (Figure S4b, supplementary information) compared to whole cell digestion (Figure S1b, supplementary information). After 15 days, the ruptured cell digesters averaged approximately 5600 mg total $VFA.L^{-1}$ whereas the whole cell digesters averaged 2800 mg total $VFA.L^{-1}$. Mechanical pre-treatment resulted in significantly higher VFA concentrations during AD for *Scenedesmus*, but not for *Spirulina*.

For *Spirulina* sp. the liberation of key organics through disruption resulted in a greater maximum methane productivity of 0.26 L $CH_4.L_{reactor}^{-1}.day^{-1}$ compared to 0.18 L $CH_4.L_{reactor}^{-1}.day^{-1}$ for the whole cell digesters during the linear VFA consumption phase. With the exception of the higher peak, rupturing did not significantly alter the profile.

The methane productivity profiles for the *Scenedesmus* sp. digesters indicated that the disruption of the algal cells resulted in a significant rise in methane production across digestion. However, the maximum average productivity remained lower than that for the *Spirulina* sp. digesters (0.14 L $CH_4.L_{reactor}^{-1}.day^{-1}$ compared to 0.26 L $CH_4.L_{reactor}^{-1}.day^{-1}$). The final methane yield for *Spirulina* sp. increased from 0.113 L $CH_4.g\ VS^{-1}$ loaded to 0.166 L $CH_4.g\ VS^{-1}$ loaded with the inclusion of mechanical pre-treatment. The methane yield from *Scenedesmus* sp. digestion increased from 0.054 L $CH_4.g\ VS^{-1}$ to 0.097 L $CH_4.g\ VS^{-1}$ with

disruption (Figure 3). There was a significant lag in methane production, which was not reduced by cell disruption. This may have been caused by the relatively high initial substrate loading rate.

pH The optimum pH for digestion is between 6.5 and 7.6 (Parkin and Owen, 1986). During the initial days of the experiment, the pH of the whole-cell *Spirulina* sp. culture decreased from pH 6.5 to pH 6.1 and the *Scenedesmus* sp. culture from pH 6.6 to pH 6.2. This indicates an accumulation of VFAs, as the acids formed at a higher rate than acetic acid consumption by the methanogenic microbes. The additional alkalinity in the *Spirulina* sp. substrate may have buffered the system, resulting in a lower pH drop despite a higher concentration of VFAs. In the digesters fed with disrupted *Scenedesmus*, there was an initial decrease in pH from pH 6.60 to pH 4.59. The *Spirulina* digesters did not suffer from a decrease in pH, presumably since residual alkalinity buffered the pH. After this initial decrease in pH, active control using 5 M NaOH was initiated to maintain the pH above 7.0 and ensure maximum methanogen activity. This drop in reactor pH could indicate that the initial loading rate of the batch reactors may have been too high, although Samson and LeDuy (1986) reported maximum methane yield (0.35 $m^3.kg^{-1}$ VS) at a loading of 20 kg $VS.m^{-3}$. If the reactor pH had not been manually controlled, the reactors may have crashed due to low pH. This could explain the long lag times in methane production (Figures 2, S2 and S5), and could indicate that an equivalent loading rate in continuous culture would be too high.

Efficiency of digestion The efficiency of AD can also be represented by the overall destruction of COD (both solid and soluble), VFAs and VSs (Table 2). The values for VS destruction are low compared to those for COD destruction. This could be because the calculation employed (Varel's equation) relies on accurate measurement of CO_2 and CH_4 evolution. It is possible that some output carbon data could be missing due to dissolved CO_2 trapped in the liquid portion of the reactors and other soluble products not converted into biogas.

Discussion

Spirulina and *Scenedesmus* represent quite different substrates for AD. The differences in cell size and morphology, as well as the differences in cell wall composition suggest that *Spirulina* would be more easily digested and more susceptible to mechanical breakage than *Scenedesmus*. The optimum C/N ratio for AD is reported to be between 20 and 30 (Yen and Brune, 2007), therefore *Spirulina* has a less favourable C/N ratio (5.33) compared to

Scenedesmus (11.5). Digestion of the *Spirulina* biomass with a relatively high protein content (60%) and low lipid content (3%) could be expected to lead to ammonia accumulation in the digester, causing inhibition of the methanogens (Ward et al., 2014). This inhibition was not experienced in the batch experiments conducted here (at no stage for the whole-cell or ruptured biomass of either algal species did accumulation of any indicator VFA for ammonium inhibition occur), however, inhibition may only become apparent in continuous studies.

The culture medium used for *Spirulina* (Zarrouk's medium) had a high salt concentration (conductivity ± 20 $mS.cm^{-1}$, compared to only 2 $mS.cm^{-1}$ for the 3N BBM medium used for Scenedesmus), therefore, resuspension of the slurry in pure water could cause osmotic damage to the cells. The alkalinity of the *Spirulina* slurries was higher, probably due to residual media transferred along with the biomass. The increased alkalinity can act as a buffer to stabilise the pH and reduce the possible inhibition by VFAs and NH_3 (Samson and LeDuy, 1986).

The progress of AD was monitored by measuring solid and soluble COD in the reactors over time. For the *Spirulina* digesters, a large initial decrease in solid COD was accompanied by an increase in soluble COD (days 0 to 4, Figure 1) indicating that insoluble, complex organic molecules were broken down into their component parts by hydrolytic enzymes. The cyanobacterium *Spirulina* has a soft cell wall made of complex sugars and proteins, unlike the cellulosic walls of most algae, as well as a filamentous structure, making it more easily disrupted and digested (Van Eykelenburg, 1977). This was confirmed by the significant decrease in solid COD from day 0 to 30. The residual soluble COD suggests the presence of soluble components that are not readily utilised by methanogenic organisms.

The solid COD in the *Scenedesmus* digesters only decreased significantly at a later stage (day 26, Figure 1), indicating lower biodegradability, than *Spirulina*. The cell walls of *Scenedesmus* are composed of degradation resistant, polyether, non-hydrolysable aliphatic biopolymers (Blokker et al., 1998). Further, the cell walls contain large amounts of hemicelluloses that hydrolyse slowly in AD systems (Yen and Brune, 2007). The soluble COD concentration of the *Scenedesmus* digesters was decreased to the detection limit. This related directly to the slow biomass degradation, which was the rate-limiting step.

The higher concentration of VFAs produced from *Spirulina* biomass was directly related to the availability of easily fermentable organics and readily degradable biomass within the digesters (Angelidaki et al., 1999). *Scenedesmus* sp. has a strong cell wall made up of complex biopolymers resistant to hydrolysis, therefore, fewer readily available organics were present in the aqueous phase of the slurry for conversion to VFAs during the initial stages of digestion. After the initial high rate of production of these specific acids, the total VFA concentrations stabilised. The high initial concentration of VFAs, as well as the initial drop in pH, indicated that the initial substrate loading rate may have been too high. This would have affected the activity of the microbial consortia, requiring it to acclimatise to the new environment (Angelidaki et al., 1999), and resulting in the long lag phase before significant methane production (Figure 2). The methanogenic activity increased after a shorter period (10 days) for the *Scenedesmus* sp. digesters than the *Spirulina* sp. digesters (18 days). The latter produced three times the total VFAs during the initial stages of digestion, resulting in a longer stabilisation or acclimatisation period.

Biogas production peaked during the first two days after inoculation, after which it ceased. This was consistent with the VFA concentration profiles that showed a "lag" period, where minimal changes in VFA concentration occurred (Figure S1, supplementary information). When methanogenic activity began to increase, biogas production increased simultaneously. The subsequent peak in biogas productivity related directly to the maximum rate of consumption of VFAs in both species. The higher maximum biogas productivity of the *Spirulina* digesters (0.21 L Biogas.$L_{reactor}^{-1}$.day^{-1}) than that of the *Scenedesmus* digesters (0.15 L Biogas.$L_{reactor}^{-1}$.day^{-1}) was consistent with the greater change in VFA concentrations.

For both *Spirulina* and *Scenedesmus* digesters the initial DW loading was 20 $g.L^{-1}$, which corresponded to 16.8 g $VS.L^{-1}$ for *Spirulina* sp. and 16.3 g $VS.L^{-1}$ for *Scenedesmus* sp. The ultimate yield for the *Spirulina* digesters (0.11 L CH_4.g VS^{-1} loaded) was greater than that of the *Scenedesmus* digesters (0.06 L CH_4.g VS^{-1} loaded). Organic loading rates vary widely across studies (Table 3). The initial loading rate of the batch digesters in this study may have been too high for continuous operation. In addition, the high protein content of the Spirulina biomass could potentially lead to ammonia and sulphur inhibition in continuous operation. The biogas yields measured in this work are low compared to those found in other studies (Table 3).

Mechanical pre-treatment had little effect on the VFA profile of *Spirulina* digestion. *Spirulina* filaments appeared to degrade rapidly under the conditions in the AD reactors, both with and without mechanical pre-treatment. While milling increased the soluble COD of the *Spirulina* slurry by over 100%, this was not reflected in the VFA values, suggesting that the soluble intracellular components were primarily proteins, carbohydrates and long chain fatty acids. The data differ significantly from that presented by Samson and LeDuy (1983), who investigated the impact of ultrasonic pre-treatment (mechanical disintegration) of *Spirulina* on digestion efficiency. The semi-continuous AD system had an average VFA concentration of 8685 mg $CH_3OOH_{equivalent}.L^{-1}$ when digesting the ruptured substrate, compared to 3249 mg $CH_3OOH_{equivalent}.L^{-1}$ for the whole cell biomass. The difference may be accounted for by the lower retention time (20 days) and increased VS loading rate (2 kg $VS.m^{-3}.day^{-1}$).

The higher VFA concentrations in the digesters fed with disrupted *Scenedesmus* (Figure S4b, supplementary information) as opposed to whole cell *Scenedesmus* (Figure S1b, supplementary information) highlighted the benefit of disruption in liberating complex organic molecules that could be converted to VFAs. This immediately increased the BMP of the system. The higher initial VFA production resulted in an extended lag phase (18 days compared to 10 days) as more time was needed for the microbial consortia to acclimatise to the change in environment.

Mechanical disruption of *Spirulina* resulted in a methane yield 47% greater than without disruption. This can be attributed to increased microbial activity during the early stages of digestion, as well as to the extended period of methane production. Samson and LeDuy (1983) reported that the mechanical pre-treatment of *Spirulina maxima* positively influenced acidogenic bacteria, but did not increase methane production. The results shown in the current study indicate that an increase in VFA production results in a greater consumption of acetic acid by methanogens and so a greater methane yield. Unless the concentrations become inhibitory, this should always be the case. AD of mechanically disrupted *Scenedesmus* resulted in a methane yield 76% greater than without disruption. The substantial increase related to the greater amount of acetic acid produced and consumed. This agrees with studies by Gonzalez-Fernandez et al. (2012) who found a 2-fold increase in methane production from *Scenedesmus* biomass disrupted by ultrasound (2012a), and a 2-fold increase in the biodegradability of *Scenedesmus* biomass heat treated at 90°C (2012b), compared to untreated biomass.

The results in Table 2 confirm that *Spirulina* sp. degraded more easily than *Scenedesmus* sp.. The larger consumption of both total and solid COD as well as the total destruction of VS supported this result. The residual, apparently indigestible, biomass resulted in a solid COD value in the region of 4800 $mg.L^{-1}$, which remained relatively unchanged from day 30 for the whole cell digesters and day 34 for the digesters fed ruptured biomass. The destruction profiles for the *Scenedesmus* sp. whole cell and ruptured cell digesters revealed that mechanical disruption of the cells allowed more of the original solid COD to be consumed. The result differs from the *Spirulina* sp. digesters where the residual solid COD was not affected by mechanical pre-treatment. The difference is largely due to the extent of disruption. From the images taken during milling, partially disrupted cells were seen after bead mill operation had stopped, which contributed to solid COD. However these cells degrade more easily through digestion, increasing the concentration of soluble organics. This also led to a high net destruction of solid COD. The low destruction of solid COD in the whole cell digesters supported this result and highlighted the resistance of *Scenedesmus* sp. to degradation. This confirmed that breakdown of COD in the ruptured cell digesters was most likely not a result of cell fragment degradation. In an intact cell, the soluble intracellular components still count as solid COD because they are trapped in the cell.

In conclusion, the anaerobic digestion of the whole cell or mechanically disrupted biomass of the microalgae *Spirulina* sp. and *Scenedesmus* sp. was investigated through analysis of the rate of solubilisation of COD, production of VFAs and evolution of methane over time in batch anaerobic reactors. Anaerobic digestion of whole cell *Spirulina* resulted in a substantially higher methane productivity (0.18 L $CH_4.L_{reactor}^{-1}.day^{-1}$) and methane yield (0.113 L $CH_4.g^{-1}$ VS) compared to the digestion of whole cell *Scenedesmus* (0.12 L $CH_4.L_{reactor}^{-1}.day^{-1}$ and 0.054 L $CH_4.g\ VS^{-1}$). This can be attributed to a higher production of VFAs in the *Spirulina* digesters as a direct result of the greater degradability of the algal biomass. The higher resistance of *Scenedesmus* sp. to degradation resulted in reduced methane yields.

Mechanical pre-treatment improved the final methane yields in both substrates, from 0.113 to 0.166 L $CH_4.g\ VS^{-1}$ for *Spirulina* and 0.054 to 0.097 L $CH_4.g\ VS^{-1}$ for *Scenedesmus*. The effect of pre-treatment was greater for *Scenedesmus* due to the relatively inefficient digestion of the whole unruptured cells, attributable to the highly recalcitrant cell wall. This study

suggests that hydrolysis is the rate-limiting step in anaerobic digestion of microalgae. Without pre-treatment, methane yield is dependent on cell wall composition, which affects resistance to bacterial degradation. It was shown that mechanical cell disruption of recalcitrant microalgal species, such as *Scenedesmus*, can alleviate this limitation, resulting in improved degradation efficiencies and higher final methane yield, to a level comparable with that of less recalcitrant biomass such as cyanobacteria. Despite pre-treatment, the methane yields achieved here are substantially lower than the theoretical methane potential calculated from their average elemental composition: 0.319 L.g VS^{-1} for *Spirulina platensis* and 0.260 L.g VS^{-1} for *Scenedesmus dimorphus* (Ward et al., 2014), and lower than those found in comparable studies (Table 3). This is likely largely due to the fact that the AD process was not optimised in this study.

Different species of algae result in different COD, VFA and methane production profiles during AD. Of the two species tested here, *Spirulina* appears to be the more promising substrate for AD due to its higher digestibility. This species does not require pre-treatment, however, the effects of the greater salinity of the biomass and the potential of the high protein content to lead to ammonia and sulphur inhibition in continuous operation should be taken into account.

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582

583 **Tables**

584 **Table 1:** Properties and characteristics of *Scenedesmus* sp. and *Spirulina* sp. biomass

Constituent	*Scenedesmus* sp.	*Spirulina* sp.
	mg.g TS^{-1}	mg.g TS^{-1}
Volatile solids	850	860
C	460	410
H	70	61
O	430	450
N	40	77
P	Trace	Trace
S	Trace	2.6
Total COD	1540	1690
C/N ratio	5.33	11.5
Cell wall composition	cellulose, algaenan, glycoproteins	protein, polysaccharides, peptidoglycans
Cell morphology	rigid ellipsoidal	filamentous
Cell size (length x width, µm)	9 x 4	60-500 x 10

585

586 **Table 2:** Efficiency of AD in the destruction of COD, VFAs and VSs for whole cell *Spirulina*
587 sp. and *Scenedesmus* sp.

Substrate	Initial solid COD ($mg.L^{-1}$)	COD destruction		VFA destruction[c]	VS destruction[d]
		Solid[a] (%)	Total[b] (%)	(%)	(%)
Whole cell *Spirulina*	23800	88	85	89	23
Ruptured *Spirulina*	12200	68	81	92	27
Whole cell *Scenedesmus*	22000	53	56	98	13
Ruptured *Scenedesmus*	18600	75	81	80	24

588 [a] Calculated from the difference in final and initial solid COD concentrations

589 [b] Additive destruction of both solid and soluble COD

590 [c] Calculated by taking point of maximum VFA conc. as the initial conc

591 [d] Calculated using Varel's eq: VS destroyed (g) = (mol CO_2 + mol CH_4) x (12/(carbon content of biomass))
592 (Varel et al., 1988)

593

594 **Table 3:** Comparison of organic loading rates and biogas yields during anaerobic digestion of
595 *Spirulina* sp. and *Scenedesmus* sp.

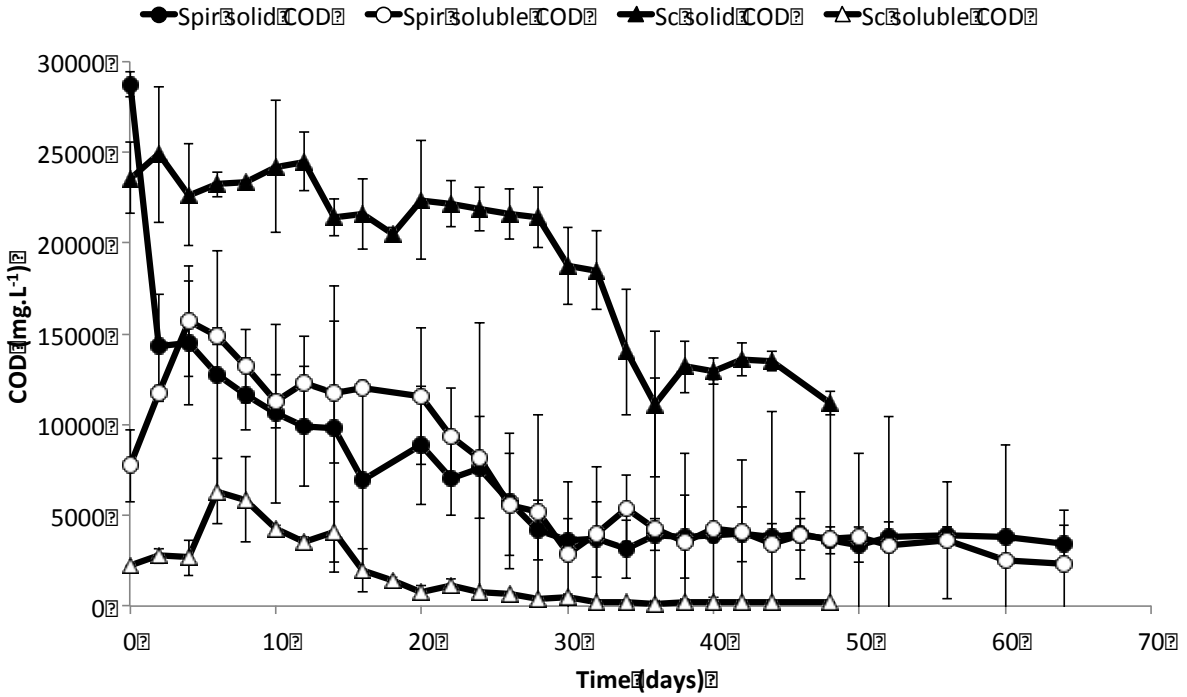
Reference	**Species**	**Organic loading rate**		**Biogas yield**
		g $VS.L^{-1}$	g $TS.L^{-1}$	L CH_4.g VS^{-1}
This study	*Spirulina platensis*	16.8	20	0.113
Inglesby & Fisher, 2012	*Spirulina maxima*		0.5	0.173
Chen, 1987	*Spirulina maxima*	0.91		0.32
Varel et al., 1988	*Spirulina maxima*	22.5		0.33
Samson & Leduy, 1986	*Spirulina maxima*	20		0.35

Mussgnug et al., 2012	*Spirulina platensis*		2	0.481
This study	*Scenedesmus* sp.	16.3		0.054
Tran et al., 2014	*Scenedesmus*	2-3.5		0.13-0.14
Yen et al., 2007	*Scenedesmus* sp.	4		0.143
Zamalloa et al., 2012	*Scenedesmus*	2		0.21
Mussgnug et al., 2012	*Scenedesmus obliquus*		2	0.287
Yang et al., 2011	*Scenedesmus* sp.	18		0.29

596

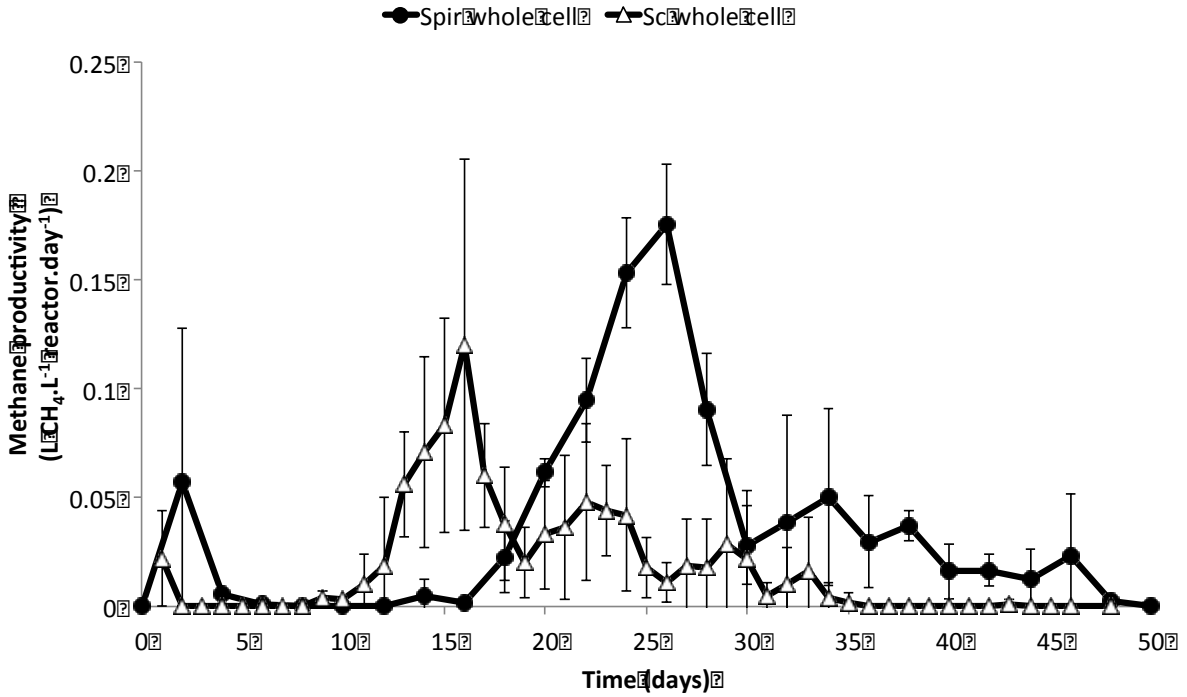
597

598 **Figure legends:**



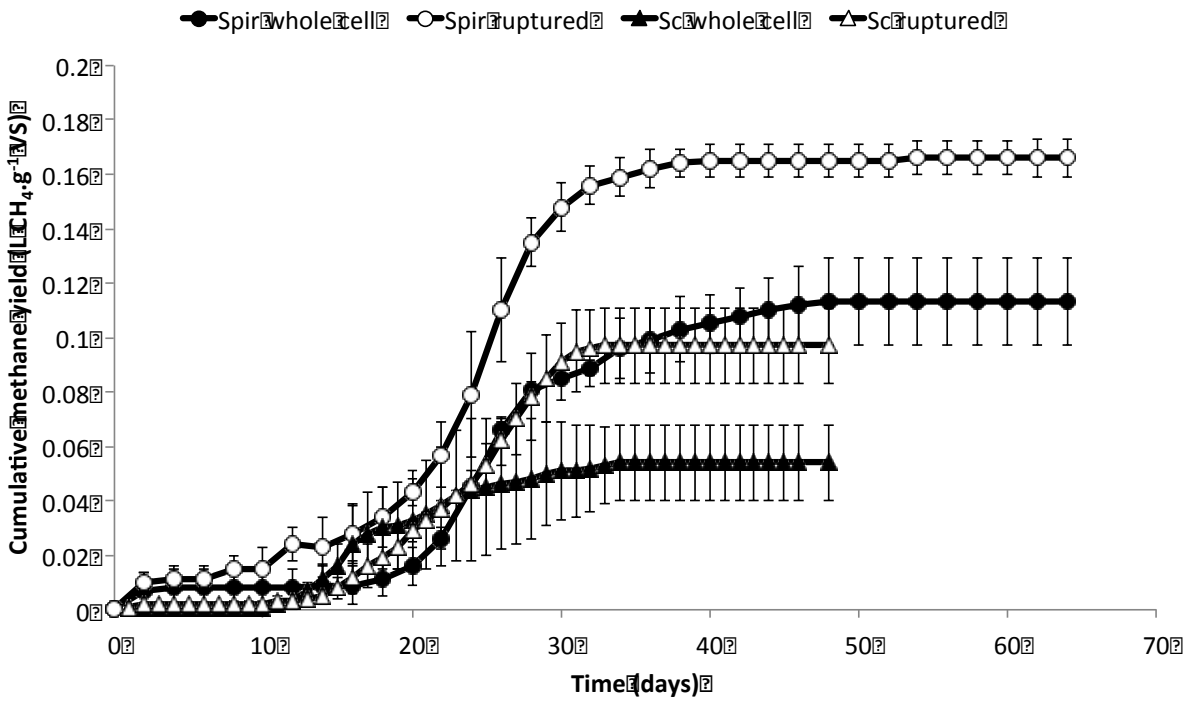
599

600 **Figure 1:** Solid (filled symbols) and soluble (open symbols) COD concentration profiles of
601 whole cell *Spirulina* sp. (Spir, circles) and *Scenedesmus* sp. (Sc, triangles) batch digestion
602 (n=3), error bars represent standard deviation



603

604 **Figure 2:** Methane productivity from the digestion of unruptured *Spirulina* sp. (Spir, circles)
605 and *Scenedesmus* sp. (Sc, triangles) biomass. Data represent mean values (n=3), error bars
606 represent standard deviation



607

608 **Figure 3:** Effect of mechanical pretreatment on methane yield in batch anaerobic digesters
609 fed *Spirulina* sp. (Spir, circles) and *Scenedesmus* sp. (Sc, triangles) biomass. Data represent
610 mean values (n=3), error bars represent standard deviation

611