

THE ANAEROBIC DIGESTION OF HALOPHYTIC
MICROALGAE

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THE UNIVERSITY
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**THE ANAEROBIC DIGESTION OF HALOPHYTIC
MICROALGAE**

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Thesis by Publication

This doctoral thesis is submitted as a portfolio of peer-reviewed publications according to the “PhD Rules & Specifications for thesis” of the University of Adelaide. The journals in which these papers were published or accepted are closely related to the research field of this work. The citation index is listed and the journals ranked in order of impact factor in reference to their scientific significance.

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Peer reviewed scientific publications

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- 2- Nguyen, L.T.A. Ward, A.J. Lewis D., (2014). Utilisation of turbidity as an indicator for biochemical and chemical oxygen demand. *Journal of Water Process Engineering*. 4:137-142
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- 4- Ward, A.J. Lewis, D.M., (2015). Pre-treatment of halophytic microalgae and associated methane production. *Bioresource Technology*. 177:410-413
- 5- Erkelens, M. Ward, A.J. Lewis, D.M., (2014). Microalgae digestate effluent as a growth media for *Tetraselmis sp.* in the production of biofuels. *Bioresource Technology*. 167:81-86

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- 3- Ward, A.J. Lewis, D. Ball, A. (2013). The anaerobic digestion of saline biomass under high salinity conditions. (Presented at the Bioenergy Australia Conference, Sydney, Australia, 2012)

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- 1- Ward, A.J. (2013). Molecular comparison of nested and non-nested PCR 16S rDNA polymerase chain reaction (PCR) amplicons. (Presented at the Bioenergy Australia conference, Sydney, Australia, 2013).

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ABSTRACT

The anaerobic digestion of microalgae is a potential environmentally feasible option for creating a renewable source of energy for industrial and domestic needs. Microalgae anaerobic digestion is a key unit process that integrates efficiency and beneficially into the production of microalgae derived biofuels. Anaerobic digestion culminating in methane fermentation improves the economic viability of microalgae liquid biofuel production and presents an opportunity for power generation from wastewater derived microalgae. However the anaerobic digestion of halophytic microalgae biomass is not straight forward due to several technical restraints including low concentration of digestible biodegradable substrate, recalcitrant substrate constituents, cell wall degradability and effects from salinity and associated metal ions.

To address the quantification of low biodegradable substrate associated with microalgae cultures, development of a high throughput methodology to determine the quantification of suspended microalgae biomass content and other water quality parameters via turbidity measurements was determined. The development of the new management tool allows faster operational control from a simple turbidity analysis, reducing time delays to fewer than 5 minutes and avoids expensive laboratory testing. Further development of this management tool will support the operational control for biofuel pond management and wastewater treatment plants. This management tool provides a rapid quantification of biomass and allows harvesting volumes to be calculated to allow consistent volatile solid and chemical oxygen demand loading to anaerobic digesters.

The anaerobic digestion of halophytic microalgae biomass however, has a significant challenge to be mitigated before this technology can be beneficial for the burgeoning microalgae industry. The halophytic microalgae biomass as a potential substrate feedstock for anaerobic digestion will have salinities > 35 ppt. To address this issue the first section of my PhD research focussed on the changes undertaken in the bacterial community associated with the anaerobic digestion of piggery effluent under increasing saline conditions with the aim of establishing a saline tolerant anaerobic digestion inoculum capable of digesting feedstock's under high salinity conditions.

Favourable results from this inoculum development study allowed the second part of the PhD research to be investigated where the anaerobic digestion of halophytic biomass was investigated utilising the inoculum established from the initial component of the reported study. Results of the later study demonstrated that a hyper saline inoculum was achieved and subsequent DGGE fingerprinting of the bacterial community detected several high salinity methanogens at a salinity of 7% and validated the establishment of a halo-tolerant anaerobic digestion community. Establishment of a halo tolerant anaerobic digestion community was further validated by significant methane production at the high 7% salinities. This inoculum was then used for all other reported studies.

Another major difficulty associated with the anaerobic digestion of microalgae is the need to disrupt the cell wall allowing the cell contents to be processed by the bacterial community. In this study I compared the methane production from lipid extracted, pre-treated disrupted and non-pretreated *Tetraselmis* sp. microalgae respectively. Results demonstrate that a methane production of 122 mL per g VS for the lipid extracted *Tetraselmis* sp. biomass. This result demonstrates that after the

extraction of lipid for use in biofuel production residual lipid extracted microalgae biomass is a viable feedstock for methane production. A methane production of 252 mL per g VS and 248 mL per g VS was reported for the non-disrupted algae and pre-treated disrupted *Tetraselmis* sp. respectively. This study also identified the ability of the anaerobic digestion microbial community to undertake cell lyses via microbial degradation of the *Tetraselmis* sp. microalgae. Cell lyses by the anaerobic digestion microbial community can offer a direct conversion pathway for energy production were whole biomass can be harvested and concentrated and directly fed to the anaerobic digester without energy intensive pre-treatment or processing being required.

Investigation was also undertaken to quantify the suitability of anaerobically digested halophytic *Tetraselmis* sp. microalgae digestate as a nutrient feed stock to form a closed loop nutrient system. To determine microalgae digestate suitability I established that the following factors needed to be observed: growth, lipid content, and the bacterial community diversity. Microalgae digestate was diluted according to the concentration of NH_4^+ content (20, 40, 60, 80 mg/L) and compared against a standard medium for *Tetraselmis* sp.. The growth rate on the microalgae digestate media was not as rapid as the F/2 standard medium and the high microalgae digestate media concentrations correlated with lower total lipid contents, additionally acyl carrier proteins (ACP) gene expression rates displayed lower lipid gene expression within high microalgae digestate treatments. Lastly, higher concentrations of microalgae digestate were correlated with a higher bacterial diversity in the bacterial community throughout the investigation. No significant difference in lipid production and satisfactory growth was recorded for the lower microalgae digestate treatments. These results confirmed the suitability of

microalgae digestate as a suitable nutrient source for use in the production of *Tetraselmis* sp. biomass for lipid and biofuel production.

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Chapter One

Chapter 1: Introduction

1.1 Overview.

The major aim of my PhD research was to anaerobically digest halophytic microalgae biomass into valuable products. These include methane bio-gas that can be utilised for on-site energy production, and the remineralisation of valuable nutrients that can be used in a closed loop system for the continuous production of halophytic microalgae biomass.

The first stage of my PhD was to undertake a comprehensive literature review to investigate what experimental work that had been reported on the anaerobic digestion (AD) of microalgae biomass. This literature review identified several key restraints to the AD of microalgae. These key restraints included inhibition from salinity, cell wall permeability, concentrating and dewatering of biomass and ammonia inhibition. The final literature review is presented in Chapter 2 and was published in the Journal of Algal research.

One problem identified in the literature review was the requirement for a consistent and concentrated biomass feeding rate for the digester. This was achieved by dewatering and concentrating microalgae biomass. Work was undertaken on quantifying algae biomass content within cultures to allow correct harvest rates to be utilised to deliver sufficient biomass after the concentrating and dewatering steps. Microalgae culture biomass concentrations are highly variable and can change daily depending on growth conditions and environmental factors. Daily harvesting of the same volume of pond culture would result in different quantities of microalgae biomass depending on the cell concentration at the time of harvest. Therefore multiple

regression correlation equations were developed to model the amount of microalgal biomass within the culture solution. These correlations allowed the quantification of the dependant variables: total solids, volatile solids, chemical oxygen demand and culture cell density, which were correlated with turbidity as the predictor variable. Turbidity can be readily measured utilising a handheld turbidity meter allowing simple operation by a pond operator, removing many hours of laboratory work to generate the equivalent data. This multiple regression model also has the potential to be utilised with the management of microalgae raceways at microalgae based wastewater treatment facilities. This work is presented in Chapter 3 and was published in the Journal of Water Process Engineering.

Another major challenge of this project was to undertake AD under high ionic conductivity that results from the high salinities that are associated with the culture of halophytic microalgae. To address this issue microbiological genetic fingerprinting methods were utilised to understand the response of the microbial community under the influence of high ionic conductivity. To determine the activity and response of the microbes associated with this three stage process: two separate primer sets were used; one to target the overall microbial population (Muyzer primers) and the second methanogenic specific primers were used to investigate the methanogen microbial population. Methanogen archea have been deemed the most sensitive microbes in the process of converting organic material to methane. Therefore the investigation of these methanogen archea will have an integral role identifying the effects ionic conductivity plays on this community and their response to increasing environmental ionic conductivity. Results of this investigation are contained in Chapter 4 and published in the Journal of Algal Research.

Due to the microbial investigation a halophytic AD inoculum was produced allowing further work to be undertaken on the AD of halophytic microalgae under high ionic conditions. With this inoculum an investigation of the cell wall permeability and pre-treatment methods was undertaken, which included quantifying the methane potential of lipid extracted microalgae biomass. This work is presented in Chapter 5 and published in the Journal Bioresource Technology.

Investigation of the digestate as a nutrient source was necessary as utilisation of the digestate produced from anaerobically digested microalgae biomass was required. This work also investigated the associated microbial community with the digestate and its effect on the microalgae culture and growth. Lipid production from microalgae biomass was also investigated utilising microalgae digestate during this study. This work is presented in Chapter 6 and published in the Journal of Bioresource Technology.

1.2 Background

Anaerobic digestion (AD) processes utilise microbial activity in the absence of oxygen for the stabilisation of organic materials and liquefaction of solids, by conversion to methane and inorganic end products including carbon dioxide and ammonia nitrogen (McCarty, 1964a). The primary objective of the anaerobic process is to stabilise organic matter with a concurrent reduction in odours, pathogen concentration, and the amount of solid organic material that requires further processing, making AD highly suitable for the processing of residual biofuel biomass. AD of organic biological matter is a complex process involving many types of microbes that work in an assembly line fashion (Metcalf and Eddy, 2006, McCarty, 1964a). Older

research and literature identified a three stage assembly line process is involved in the AD process. The first of three stages are hydrolysis and liquefaction of feedstock to a size and form that can pass through the microbial cell walls for use as energy (Parkin and Owen, 1986). The second fermentation step produces volatile fatty acids (VFA) and hydrogen by hydrolytic and acidogenic bacteria. The third methanogenesis stage deals with methane biogas production by methanogenic archaea (Guerrero et al., 1999, Parkin and Owen, 1986).

More current literature identifies this process as a four stage process (Speece, 2008). The first stage is the liquefaction and hydrolysis of the feedstock. The second stage involves the acidogenesis where volatile fatty acids are produced. The third stage is the acetogenesis stage where longer chain VFAs are further reduced to acetate. The fourth methanogenesis stage involves the conversion of this acetate to methane by methanogen archaea (Speece, 2008).

1.3 History of AD

Historically AD has been used for the stabilisation of raw, domestic sewage sludge typically removed from primary sedimentation (McCarty, 1964a). As early as the eighteenth century the formation of methane from anaerobic decomposition of organic deposits was known (Hughes et al., 1982). France is credited with having made one of the first significant contributions towards the anaerobic treatment of wastewater suspended solids. In December 1881 and January 1882 there appeared in the French journal *Cosmos* a description of an airtight chamber developed by M. Louis Mouras called the "Mouras automatic scavenger" in which suspended solid

organic materials in wastewater were liquefied (Monigo, 1882) cited in (Hughes et al., 1982).

Other studies on liquefaction of wastewater solids in the absence of oxygen were conducted by several authors these included W.D. Scott-Moncrieff in 1890/91, A.C. Houston in 1892/93, the Massachusetts State Board of Health in 1894, Donald Cameron of Exeter in England in 1895, A.N. Talbot in Illinois in 1894 and Champaign, Illinois in 1887 (Metcalf and Eddy, 1915) cited in (Hughes et al., 1982). Their discoveries were utilised in the treatment of wastewater suspended solids and were the beginnings of the first anaerobic digester now commonly known as the septic tank (Hughes et al., 1982).

The value of methane production during sludge decomposition and solids liquefaction was first recognised in 1895 by Donald Cameron, and gas produced in septic tanks at Exeter was used for heating and lighting at the Exeter sewage works (Hughes et al., 1982). In 1897 waste disposal tanks at a leper colony in Matunga, Bombay, are reported to have also been equipped with gas collectors and used to drive gas engines (Buswell and Hatfield, 1928-1929).

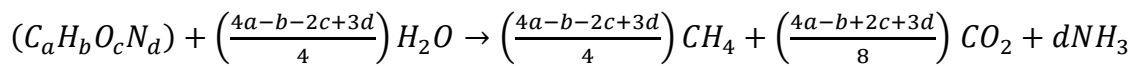
Research continued on the development of septic tanks and AD by engineers such as Clark, H.W. 1899, William O Travis, 1904, and Imhoff, K., 1905 (Metcalf and Eddy, 1915) cited in (Hughes et al., 1982). Collection of gas and heating experiments were conducted in 1914 in Germany and in 1923 the gas was collected on a large scale and delivered to the municipal gas system at the Essen-Rellinghausen plant Germany (Hughes et al., 1982).

Numerous studies up to the 1930's have led to a better understanding of the importance and the operation of anaerobic systems. Hence by the end of the 1930's

sufficient understanding of the process had developed to allow practical application for the treatment of municipal waste sludge's (Hughes et al., 1982).

Further development in AD continued and a better understanding of the process was developed. Buswell et al. (1932) developed several stoichiometric equations to quantify the end products produced from the AD process.

EQ-1



Where a = carbon content, b = hydrogen content, c = oxygen content and d is the nitrogen content contained in the substrate.

EQ-2

$$\text{Methane yield (litres/g (VS) destroyed)} = \left(\frac{4a+b-2c-3d}{12a+b+16c+14d}\right) * V_m$$

Where V_m is the molar volume of methane = 22.14 l at 0 °C and 1 atm.

When the composition of the wastewater or substrate is known, Equations 1 and 2 allow the theoretical gas composition to be calculated on a percentage basis and also the volume of methane gas to be calculated. The volume of gas is dependent on the quantity of volatile solids available or the Chemical Oxygen Demand (COD) of the substrate being digested.

Equations 1 and 2 are now formally accepted and a large quantity of literature and wastewater engineering manuals utilise these equations to calculate the theoretical gas production and quantities of end products produced from different substrates (Metcalf and Eddy, 2006, Sialve et al., 2009). The percentage of methane gas and other products produced can be calculated from Equation 1. In Equation 1,

the organic matter is stoichiometrically converted to methane, carbon dioxide and ammonia. The adaption of Equation 1 (Buswell and Boruff, 1932) to Equation 2 allows the specific methane yield expressed in litres of CH₄ per gram of volatile solids (VS) to be calculated (Sialve et al., 2009). The use of Equation 1 and 2 allows the theoretical stoichiometric gas potential to be compared to actual gas volumes produced during AD operation and experimental work. They have become very useful to determine the efficiency of the digester setup and operational conditions used in digesters.

1.4 Development of AD

The development of AD has become quite detailed over the years since its first discovery back in 1882. A large amount of experimental research has been done on different aspects of AD including hydraulic retention times, loading rates, carbon nitrogen ratios, temperature and pH (Parkin and Owen, 1986, McCarty, 1964a, McCarty, 1964b, McCarty, 1964c, McCarty, 1964d). Currently digesters are operated under two different modes, the batch mode and the continuous mode: batch digesters are the simplest, the process involves loading the feedstock into the digester vessel and starting the digestion process. Once the digestion is complete, the residue is removed and another batch digestion started. The second mode is the continuous digestion mode; continuously run digesters involve regular feeding of waste into the digester vessel to continuously produce biogas (Metcalf and Eddy, 2006). This type of digester is suitable for large-scale operations processing large amounts of continuous waste and is highly suited to the processing of residual biofuel biomass (Metcalf and Eddy, 2006).

Since 1882 a large amount of research has been done on the design and engineering of the actual digester configuration. Different designs have become available and include covered pond digesters (Heubeck and Craggs, 2010), plug flow digesters (Lansing et al., 2010), membrane filtration digesters (Anderson et al., 1986, Nagano et al., 1992), up-flow anaerobic sludge blanket digesters (USAB) (Goodwin et al., 2001, Gao et al., 2007, Ramasamy et al., 2004), two stage digesters (Ward and Kumar, 2010, Dinsdale et al., 1996), anaerobic sequencing batch reactors (ASBR) (Dugba and Zhang, 1999, Shao et al., 2008), anaerobic film expanded bed digesters (AFEB) (Kelly and Switzenbaum 1984), buoyant filter bed reactors (BFBR) (Haridas et al., 2005) and submerged in pond fermentation pits (Green et al., 1995). These anaerobic digesters have been developed to digest different substrates with different characteristics for example the USAB reactor is ideal for wastewaters with a high suspended solid loading rates (Goodwin et al., 2001), whereas the BFBR reactors are ideal for wastewaters with a high lipid contents (Haridas et al., 2005). Most research into anaerobic digester technology has predominately evolved and changed to suit the treatment of wastewater where energy maximisation has not been the first priority (Shilton and Guieysse, 2010).

Recent research on AD has utilised molecular biology techniques to identify and monitor different microbes associated with the AD process (Patil et al., 2010a, Patil et al., 2010b, Skilman et al., 2009). These new genetic and molecular techniques are revolutionising the understanding of the AD process as they are directly quantifying the microbial responses that these microbial communities undergo when stress or environmental change is applied to the AD community (Patil et al., 2010a). One molecular method utilised to study the microbial community utilises a genetic fingerprinting technique called polymerase chain reaction denaturing gel gradient

electrophoresis (PCR DGGE) to separate the microbial community (Patil et al., 2010b, Patil et al., 2010a). The separation of the microbial community is important as it allows the diversity and identification of the methanogen and microbial community and allows the influence from high salinity conditions and the associated response and adaptation of these communities. The PCR DGGE technique is used in this PhD study to investigate the microbial community change when high ionic conductivities are applied to the AD microbial community.

The AD process has been adapted to deal with a large amount of different wastewaters from industry, food processing plants, intensive livestock system wastes and human effluent aspects (Speece, 2008). Nallathambi et al. (1997) provides an excellent review on various types of substrates that have been used for digestion. Although this publication was published in 1997 it still provides a good insight into the diversity of wastewaters and plant feed stocks that have been researched in scientific literature. Substrates covered in this paper include weeds, woods, grasses, leaves fruit and vegetable solid waste, organic fractions of municipal wastes and fresh and salt water derived biomasses. A large quantity of literature has been produced on the AD of microalgae. The fundamentals of this research is not discussed in this introduction section as a comprehensive literature review on the AD of microalgae is presented in chapter two of this thesis.

1.5 Biofuel and the AD perspective

The AD of biomass for bio-gas production received renewed attention when global oil crisis occurred, due to its viability as an alternative fuel source (Sialve et al., 2009). Interest in AD has greatly increased during these periods as bio-gas was seen

as a renewable and viable source of energy. In the 1970s oil crisis there was a strong interest in the cultivation of highly productive macroalgae for bio energy production (Briand and Morand, 1997). Development of feedstock for methane bio-fuel production from marine macro-algae biomass led to significant plans for extensive marine farms (North, 1980) but these failed as the economic climate became favourable once again for fossil fuels.

The “first and second generation of bio-fuels” have focused largely on the production of bio-fuels from food plant crops (Habig and Ryther, 1983). In these systems solar energy is used to drive the photosynthetic fixation of carbon dioxide to organic matter. The organic matter (biomass) is then harvested and then used directly as a combustible fuel or converted to another form such as ethanol hydrogen or methane (Keenan, 1976, Hansson, 1983, Chang et al., 2010). These “first and second generation bio-fuels” have been highly criticised for their use of valuable food crops as feed stock for fuel production. This negative criticism is due to use of valuable agricultural land and scarce water resources for their production (Fenton and Ohuallachain, 2012). Biofuel production from food crops places increased pressure on the price of food and the use of valuable land and water resources, and has been deemed unsustainable (Ras et al., 2010).

Recent research focus on bio-fuel production has transitioned towards what is known as “third generation of bio-fuels” (Mussnug et al., 2010). Third generation biofuels utilise macroalgae and microalgae as a feedstock for the production of alternative bio-fuel (Mussnug et al., 2010). Microalgae are highly productive and are generally able to produce large quantities of biomass more efficiently than terrestrial plants (Collet et al., 2010, Stephans et al., 2013). The photosynthetic efficiency of microalgae in engineered systems can reach 4-5% of the solar energy compared to

1-2% for terrestrial plants (Walker, 2009, Shilton and Guieysse, 2010). Some species of microalgae that have also been identified for their potential as feedstock for bio-fuels are grown in a marine environment (Fon Sing et al., 2014). The use of saline water for their production offers a great incentive due to their ability to use unproductive land and sea or brackish water, hence reducing pressure on current agricultural land and water resources. However ionic conductivity / salinity at moderate concentrations can inhibit microbes associated with AD and pose a significant problem to overcome (Parkin and Owen, 1986, McCarty, 1964b).

1.6 Ionic conductivity

Ionic conductivity is made up of multiple elements and can vary depending on the source of water and its associated environment. The sodium ion which makes up a large percentage of the ionic conductivity of seawater has been shown to cause inhibition in AD (Sialve et al., 2009, Speece, 2008). The AD under high ionic conductivities is a major issue addressed by my PhD research. The inhibition by sodium can vary depending on the source of inoculum and overall composition of the total ionic conductivity of water and substrate being digested (Metcalf and Eddy, 2006, Parkin and Owen, 1986, Mc Carty and Mc Kinney, 1961, Speece, 2008). The inhibition by sodium on AD is well documented and reported in scientific literature (Speece, 2008, Parkin and Owen, 1986, Chen et al., 2008, McCarty, 1964c, Mc Carty and Mc Kinney, 1961, Rinzema et al., 1988, Kugelman and McCarty, 1964, Zhang et al., 2012, Metcalf and Eddy, 2006).

Parkin et al. (1986) and McCarty (1964b) have recorded that a sodium concentration of 3500-5500 mg/l can have a moderate inhibitory effect on AD and at

sodium concentrations above 8000 mg/l can be extremely inhibitive. Halophytic microalgae used in the experiments reported in this thesis are grown at ionic conductivities that average 7% (Fon Sing et al., 2014). Approximately 21000 mg/L of this salinity or ionic conductivity is comprised of sodium (Segar, 1997).

This average microalgae production sodium concentration is approximately 2.5 times higher than the extremely inhibitory sodium concentrations reported in literature by (Parkin and Owen, 1986, McCarty, 1964b). This high sodium concentration of the culture environment for the *Tetraselmis* sp. microalgae utilised as feedstock in this thesis demonstrates the challenge to be overcome by this research. The development of a halophytic AD microbial community is essential for the implementation of this biotechnology into a high salinity biofuels production system.

1.7 PhD objectives

The main focus of my PhD will be to investigate the influence of high ionic conditions on the anaerobic digestion microbial community utilising *Tetraselmis* sp. microalgae as a feedstock. The development of a halophytic AD microbial community and the determination of the anaerobic digestibility of *Tetraselmis* sp. microalgae and quantification of AD end products are key objectives of my PhD study. These valuable end products include methane bio-gas that can be utilised for energy production on site, and the remineralisation of valuable nutrients that can be used in a closed loop system for the continuous production of halophytic microalgae biomass. It is envisaged that this high salinity AD community could reduce electrical consumption and offset the nutrient use within the microalgae biomass production systems greatly benefitting

the economics and environmental sustainability of the microalgae based biofuels industry.

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Chapter Two

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Review article

Anaerobic digestion of algae biomass: A review

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ABSTRACT

The anaerobic digestion of microalgae is a prospective environmentally feasible option for creating a renewable source of energy for industrial and domestic needs. Microalgae anaerobic digestion is a key unit process that integrates efficiency and beneficially into the production of microalgae derived biofuels. Anaerobic digestion culminating in methane fermentation improves the economic viability of microalgae liquid biofuel production and presents an opportunity for power generation from wastewater derived microalgae. However the anaerobic digestion of microalgae biomass is not straight forward due to several technical restraints including low concentration of digestible biodegradable substrate, recalcitrant substrate constituents, cell wall degradability, low carbon to nitrogen ratio, ammonia toxicity and effects from salinity and associated metal ions.

Current production methods for liquid biofuel production from microalgae produce approximately 60–70% residual biomass that is currently a byproduct. Anaerobic digestion provides biogas, but it can also provide essential nutrient recovery from lipid extracted microalgae biomass. The biogas produced from the anaerobic digestion process can be used to generate onsite electrical power or thermal heat to offset biomass processing and extraction processes. When both of these processes are integrated and operated simultaneously, the benefits to microalgae biofuel production and wastewater treatment derived energy production are increased significantly. To consider the integration of anaerobic digestion into a commercial-scale integrated microalgae production and biofuel refinery facility or wastewater treatment plant we present a review of the literature, the current state of the art and future directions for research.

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1. Introduction

1.1. Algae based biofuels

With the current increasing global population and the associated increase in fossil fuel use and demand, there has been an increased interest in renewable energy sources based on biomass transformation [1–3]. The use of agricultural derived biomass to produce biofuels has gained momentum [4,5]. This push for agricultural based biofuel production can lead to other less obvious problems for example eutrophication, resource depletion, reduced biodiversity due to current farming practices and the direct competition with current food crops [5,6].

Microalgae offer an interesting alternative feedstock for the production of biofuels. Microalgae have a high total yield and hence a lower land use footprint and can utilise land areas that are unsuitable for food production [1,7]. In addition microalgae production has the potential to utilise CO₂ emissions and offers the potential for a carbon neutral biofuel [8].

Biofuel production from microalgal feedstock has several challenges to overcome before it can become a mainstream industry capable of producing the quantity of biofuel required at a competitive price. Challenges faced by the industry include demand for fertiliser due to microalgae's significant utilisation of nutrients, high energy inputs required for harvesting and dewatering biomass and for the lipid extraction and conversion processes. Anaerobic digestion can offer a pathway to eliminate some of the overheads of the production cycle by producing biogas for utilisation in electricity production or thermal energy production. This benefit is highly dependent on the biofuel plant process and location, and the costs associated with natural gas prices and electricity prices will determine the efficiency improvement resulting from the biogas utilisation. In addition, the recovery of valuable nutrients from biomass via anaerobic digestion is essential for the sustainability of the algae biofuel industry. It is anticipated that the incorporation of anaerobic digestion in microalgae biofuel production and bio-refinery processes will increase the cost effectiveness of the production methods, helping it to become economically feasible and environmentally sustainable. Fig. 1 illustrates the conceptual implementation of anaerobic digestion into algal production processes. Three pathways have been defined: pathway 1 shows the direct anaerobic digestion after the biomass harvest and concentration step. Pathway 1 could be utilised in a wastewater process where the cell wall is degradable by bacterial activity within the digester. The second pathway

illustrates the anaerobic digestion of biomass after cell wall disruption prior to conversion. The third pathway is the traditional biodiesel practice where lipid is extracted and residual algal biomass is converted to biogas by anaerobic digestion and methane fermentation.

1.2. Historical perspective of anaerobic digestion

Historically anaerobic digestion has been exploited for the stabilisation of raw, domestic sewage sludge that is typically removed from primary sedimentation basins [9]. However anaerobic digestion for bio-methane production has received renewed attention due to its viability as an alternative and renewable fuel source [10,11].

Municipal and Industrial anaerobic digestion of organic waste streams is widely practiced and recognised as a mature technology for producing biogas [12]. Anaerobic digestion has been included in the “first generation of biofuels” that have been developed in recent years. The “first generation biofuels” have focused largely on the production of biofuel from terrestrial plant crops [13]. In these systems solar energy is used to drive the photosynthetic fixation of carbon dioxide to organic matter. The energy crop is harvested and then used directly as a combustible fuel or converted to another form such as ethanol, hydrogen or methane [14–16]. These “first generation biofuels” have been highly criticised for their use of valuable food crops as feedstocks for fuel production. This criticism is due to the utilisation of valuable agricultural land and scarce water resources for feedstock production. With the upward pressure exerted on food prices, biofuel production from food crops has been deemed unsustainable [6,16].

2. Macroalgae and anaerobic digestion

Interest in the cultivation of microalgae for the production of bio energy, as one form of solar energy, was born in the 1950s, but when the global supply of oil was interrupted twice during the 1970s, interest in the cultivation of highly productive macroalgae for bio energy production was accelerated [11]. Macroalgae received a large amount of attention as a biofuel feedstock due to its prolific growth in eutrophic coastal water fouling beaches and coastal waterways. Anaerobic digestion has been used to dispose and process this material for the production of biogas [11,17]. Development of feedstocks for methane biogas production from macroalgae biomass led to significant plans for extensive marine farms [18,19], but these failed as the economic and geopolitical climate became favourable once again for fossil fuels.

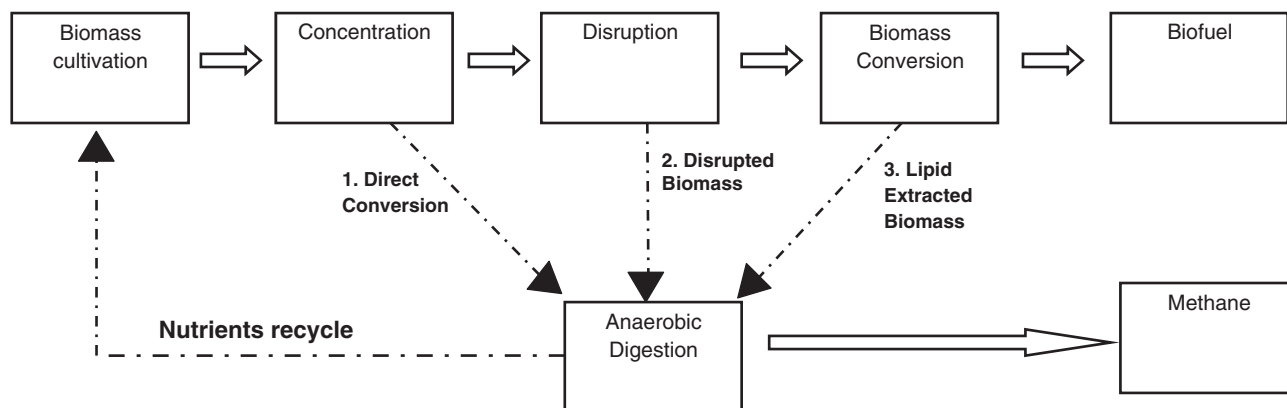


Fig. 1. Conceptual visualisation of anaerobic digestion incorporation into algal biofuel production.

Some of the problems that have been associated with anaerobic digestion of marine macroalgae include recalcitrant material such as polyphenols, cellulosic fibres and lignin type components resulting in the reduced biodegradability of the biomass by bacterial processes, hence limiting digestibility and gas production [20,11]. Also some high sulfide containing macroalgae species have been found to inhibit the anaerobic digestion process [17]. Other problems associated with the use of macroalgae for biofuel production include the seasonal growth associated with different types of macroalgae and hence variable feedstock for biogas production [11,17]. Macroalgae are again receiving attention as a substrate for anaerobic digestion, but macroalage are not discussed in the context of this review article.

3. Microalgae and anaerobic digestion

3.1. Historical and current perspectives

The focus on biofuel production is shifting towards what are known as second and third generation biofuels [10]. The second generation of biofuels utilise alternatives to food sourced biomass crops for feedstocks in biofuel production. The third generation or advanced biofuels are sourced from non-food crops, but the resulting fuel is indistinguishable from its petroleum counterparts [5]. A promising approach within the second and third generations of biofuels is the use of microalgae as a biofuel feedstock [10]. Microalgae are highly productive and are able to produce large quantities of biomass more efficiently than current

cultivation practices for terrestrial crops. The photosynthetic efficiency of microalgae in engineered systems can reach 4–5% of the solar energy compared to 1–2% for terrestrial plants [21].

The first authors to report on the anaerobic digestion of microalgae biomass were Golueke et al. [22]. They investigated the anaerobic digestion of *Chlorella vulgaris* and *Scenedesmus*, microalgae species grown as part of a wastewater treatment process. Oswald, the co-author from the Golueke et al. [22,23] papers, continued research on anaerobic digestion of microalgae that is reported in a series of scientific publications describing the role of microalgae in sewage treatment using “Advanced Integrated Wastewater Pond Systems” [24–33].

This early work by Oswald and co-authors identified several key factors that could hinder the digestion of microalgae biomass; these factors are discussed later in this review article. The data shown in Table 1 provides a summary of the research that has been undertaken on several different microalgae species to date and highlights the gas potential from microalgae as a viable process for the production of biogas.

The lowest gas production recorded from freshwater microalgae biomass was ~70 mL g⁻¹ VS (volatile solids) for untreated *Microcystis* sp. [34]. The gas production reported in this experiment was low due to the researcher's investigating inoculum start up volumes during the bio-methane potential assays rather than maximising gas productivity. The authors later recorded a gas production of 153 mL g⁻¹ VS for the same microalgae species utilising an optimised inoculum ratio. The authors Lakaniemi et al. [35] reported a low production rate of 24 mL g⁻¹ VS for the saline microalgae species *Dunaliella tertiolecta*. This low

Table 1
Methane production from the anaerobic digestion of microalgae biomass reported in scientific literature. (NR = Not reported).

Microalgae species	C/N Ratio	Methane yield	Loading rate	Reference
<i>Arthrospira maxima</i>	4.3–5.33	173 mL g ⁻¹ VS	500 mg/TS/L	[48]
<i>Arthrospira platensis</i>	N/R	481 mL g ⁻¹ VS	2000 mg/TS/L	[10]
Blue green algae	N/R	366 mL g ⁻¹ VS	281.96 mg/VS/L	[121]
<i>Chlamydomonas reinhardtii</i>	N/R	587 mL g ⁻¹ VS	2000 mg/TS/L	[10]
<i>Chlorella kessleri</i>	N/R	335 mL g ⁻¹ VS	2000 mg/TS/L	[10]
<i>Chlorella</i> sp., <i>Pseudokirchneriella</i> sp. and <i>Chlaqumydomas</i> sp.	N/R	0.28–0.60 m ³ /kg/VS	402 mg VS	[8]
<i>Chlorella</i> sp., <i>Scenedesmus</i> , <i>Euglena</i> and <i>Oscillatoria</i>	N/R	300–800 mL g ⁻¹ VS	N/R	[23]
<i>Chlorella</i> sp., <i>Scenedesmus</i>	N/R	170–320 mL g ⁻¹ VS	1.44–2.89 g/VS/L	[22]
<i>Chlorella sorokiniana</i>	N/R	212 mL g ⁻¹ VS	N/A	[122]
<i>Chlorella vulgaris</i>	N/R	403 mL g ⁻¹ VS	2 g/VS/L	[66]
<i>Chlorella vulgaris</i>	N/R	286 mL g ⁻¹ VS	5000 mg/VS/L	[35]
<i>Chlorella vulgaris</i>	6	240 mL g ⁻¹ VS	1000 mg/VS/L	[6]
<i>Chlorella vulgaris</i>	N/R	189 mL g ⁻¹ VS	N/R	[122]
<i>Chlorella vulgaris</i>	N/R	0.40–0.45 L	2677–6714 mg (COD)	[43]
<i>Dunaliella</i>	N/R	440 mL g ⁻¹ VS	910 mg/VS/L	[123]
<i>Dunaliella salina</i>	N/R	505 mL g ⁻¹ TS	2000 mg/TS/L	[10]
<i>Dunaliella tertiolecta</i>	N/R	24 mL g ⁻¹ VS	5000 mg/VS/L	[35]
<i>Durvillea Antarctica</i>	N/R	492 mL g ⁻¹ VS	3000 mg/dry/TS/d	[72]
<i>Euglena gracilis</i>	N/R	485 mL g ⁻¹ VS	2000 mg/TS/L	[10]
Lake Chaohu natural population consortium	N/R	295 mL g ⁻¹ VS	N/R	[77]
<i>Macrocystis pyrifera</i> and <i>Durvillea Antarctica</i> (50% blend)	N/R	540 mL g ⁻¹ VS	3000 mg/dry/TS/d	[72]
<i>Macrocystis pyrifera</i>	N/R	545 mL g ⁻¹ VS	3000 mg/dry/TS/day	[72]
<i>Microcystis</i> sp.	N/R	70.33–153.51 ml	1500–6000 mg/VS	[34]
<i>Nannochloropsis oculata</i>	N/R	204 mL g ⁻¹ VS	N/R	[110]
<i>Nannochloropsis salina</i> (lipid extracted biomass)	4.4	130 mL g ⁻¹ VS	2000 mg/l/VS	[80]
<i>Phaeodactylum tricornutum</i>	N/R	0.35 L g ⁻¹ COD	1.3 ± 0.4–5.8 ± 0.9	[47]
<i>Scenedesmus obliquus</i>	N/R	287 mL g ⁻¹ VS	2000 mg/TS/L	[10]
<i>Scenedesmus obliquus</i>	N/R	240 mL g ⁻¹ VS	2000 mg/VS/L	[47]
<i>Scenedesmus</i> sp.	N/R	170 mL g ⁻¹ COD	1000 mg/COD/L	[61]
<i>Scenedesmus</i> sp. (single stage)	N/R	290 mL g ⁻¹ VS	18,000 mg/VS/L	[107]
<i>Scenedesmus</i> sp. (two stage) Note: 46 mL/g/VS Hydrogen	N/R	354 mL g ⁻¹ VS	18,000 mg/VS/L	[107]
<i>Scenedesmus</i> sp. and <i>Chlorella</i> sp.	N/R	16.3–15.8 ft ³	7.8–9.2 ft ³ /lb (VS)	[22]
<i>Scenedesmus</i> sp. and <i>Chlorella</i> sp.	6.7	143 mL g ⁻¹ VS	4000 mg/VS/L	[67]
<i>Spirulina Leb 18</i>	N/R	0.79 g/L	72,000 mg/L/TS	[124]
<i>Spirulina maxima</i>	4.16	0.35–0.80 m ³	20–100 kg/m ³ (VS)	[125]
<i>Spirulina maxima</i>	N/R	320 mL g ⁻¹ VS	910 mg/VS/L	[123]
<i>Spirulina maxima</i>	N/R	330 mL g ⁻¹ VS	22,500 mg/VS/L	[126]
<i>Spirulina platensis</i> UTEX1926	N/R	0.40 m ³ kg	N/R	[12]
<i>Tetraselmis</i>	7.82	0.25–0.31 L g ⁻¹ VS	2000 mg/VS	[109]
C/N ratio-[127]				
Waste water grown community	N/R	497 mL g ⁻¹ TS	2.16 g/L/TS	[81]
<i>Zygonium</i> sp.	N/R	344 mL g ⁻¹ TS	N/R	[76]

production rate was attributed to the effects of salinity. The highest methane production recorded was by De Schampheleire and Verstraete [8] who recorded a gas production of $600 \text{ mL g}^{-1} \text{ VS}$ for a mixed undetermined freshwater microalgae consortium. The data shown in Table 1 also highlights the difference in units and terminology used to report gas production from microalgae. Units range from gas production per grams of chemical oxygen demand (COD) destroyed, gas produced per gram of volatile solids loaded and gas produced per gram of total solids loaded. The standardisation of terminology and standard units to report biogas productivities are essential for comparing microalgae and other digestible substrates.

The methods used to determine volatile solids are also used to determine the ash free dry weight (AFDW) of microalgae [36,37]. Ash free dry weight is used extensively by phycologists to report quantities of microalgae biomass. When reporting microalgae biomass, the ash free dry weight or the volatile solids (digestible component) of the microalgae biomass is a percentage of the total solids and varies between species. The data in Table 2 includes the AFDW or VS of some common microalgae species. The variation in AFDW and VS can vary by up to 50% between species and can significantly affect predicting the theoretical biogas production potential for the anaerobic digestion of microalgae.

4. Problems with anaerobic digestion of microalgae

4.1. Low concentration of digestible substrate

The majority of authors listed in Table 1 conclude that the concentrating or harvesting of microalgae biomass presents a fundamental challenge to the financial viability of an energy system using microalgae biomass as a substrate for anaerobic digestion or alternative biofuel production.

Gouleke et al. [22] identified the low volatile solids loading rate that is associated with microalgae when used as a digestible substrate. The low VS rate is due to the low concentration of microalgae biomass present in large volume of water. Significant research has focussed on engineering issues associated with the, harvesting, dewatering, and further concentrating of the microalgae biomass energy. Engineering issues common in microalgae production for biofuel are discussed by Benemann et al. [26], Chen et al. [38], and Molina et al. [39–42]. Regarding the data presented in Table 1, all experiments except for the work published by Sanchez-Hernandez and Trvieso [43] and De Schampheleire and Verstraete [8] were performed using concentrated microalgae. In the

Sanchez-Hernandez and Trvieso [43] paper no concentrating step was reported and the chlorophyll *a* ranged from 2.87 mg/L to 9.62 mg/L. This higher chlorophyll *a* content would indicate that the microalgae were at a higher density, and the problem of low volatile solids may have not been evident in the experiment.

In the De Schampheleire and Verstraete [8] experiment the authors came to the conclusion that a concentrating step would be required for optimal performance of the anaerobic digestion process. Results indicate that the digester completely failed once during the experimental period. The authors observed that the required volatile solids loading rate comprising of microalgae biomass was too dilute and contained excessive water, leading to the washout of the anaerobic bacteria community. Bacterial washout is due to a low digestible content of the wastewater or digestible feedstock. Hence when the subsequent hydraulic retention time within the digester is shortened to less than the bacterial generational time, the result is a decreased bacterial population [44,45].

McCarty [46] indicated that a settling tank could be utilised after the digester to allow bacteria and solids to settle via gravity. These solids could then be reintroduced to the digester for further processing. This step is essential to reduce bacterial washout when the hydraulic retention time (HRT) is lower than the solid retention time (SRT) of the substrate.

Bacterial washout can also be addressed by better anaerobic digester design. Zamalloa [47] used a laboratory scale membrane reactor to anaerobically digest *Phaeodactylum tricornutum*. The addition of the membrane to the reactor gave a hydraulic retention time of 2.5 days, while the solids retention time was increased to between 10 and 20 days depending on the solids loading rate. The decoupling of the hydraulic retention times and the solids retention time can also be achieved by utilising upflow anaerobic sludge blanket (USAB) reactors, anaerobic membrane reactors (AnMBR), anaerobic filters (AF) and anaerobic fluidised bed reactors (AFBR) [48] and by fermentation cells [24] or by in-pond digesters [27,28].

The paper by Collet et al. [1] reports a novel approach to concentrating microalgae. The authors first use a gravity settling-step to separate the microalgae before transferring it into a centrifuge for dewatering and concentrating to a higher percent biomass solid. The results indicated that by settling the culture for 1 h, 65% of the microalgae biomass was separated into slurry with a concentration 20 times higher than in the original culture stream. The authors then used centrifugation and reported a further concentration factor of five times. However this initial settling step was more effective with non-motile microalgae species.

Table 2

The volatile solids (VS) or ash free dry weights (AFDW) as a percentage of the total solids (TS) reported for different microalgae species.

Species	Fresh or saltwater	VS and AFDW as % of TS	Reference
<i>Arthrospira maxima</i>	Brackish	80–93%	[48]
<i>Blue green algae</i>	Fresh	94%	[121]
<i>Chorella vulgaris</i>	Fresh	93%	[128]
<i>Chorella vulgaris</i>	Fresh	90%	[6]
<i>Chlorogloeopsis fritschii</i>	Salt	92%	[128]
<i>Dunaliella sp.</i>	Saltwater	82%	[36]
<i>Isochrysis galbana</i>	Saltwater	86%	[36]
<i>Nannochloropsis oculata</i>	Saltwater	45%	[110]
<i>Nannochloropsis salina</i>	Fresh	77%	[80]
<i>Nannochloropsis sp.</i>	Saltwater	93%	[36]
<i>Nitzschia closterium</i>	Saltwater	78%	[36]
<i>Phaeodactylum tricornutum</i>	Fresh	82%	[47]
<i>Porphyridium cruentum</i>	Saltwater	91%	[36]
<i>Scenedesmus dimorphus</i>	Fresh	88%	[128]
<i>Scenedesmus obliquus</i>	Fresh	72%	[47]
<i>Scenedesmus sp.</i>	Fresh	60%	[61]
<i>Spirulina maxima</i>	Saltwater	86%	[58]
<i>Spirulina platensis</i>	Salt	92%	[128]
<i>Spirulina platensis</i>	Fresh	93%	[47]
<i>Wastewater consortium</i>	Fresh	88%	[91]
<i>Wastewater consortium (lipid extracted)</i>	Fresh	86%	[91]

The publications by Golueke et al. [29], Benamann et al. [26] and Harun et al. [49] investigated the use of chemical coagulation, flocculation and centrifugation as a means of harvesting, concentrating and dewatering microalgae. All three papers discuss the high energy costs associated with the use of centrifugation and flocculation harvesting techniques. Golueke and Oswald [33] identified that digester performance is unaffected by the centrifugation or by alum addition as a flocculant. Their work has shown that concentrations in sludge up to 4% aluminium have no effect on digester stability or gas production. Many new commercially formulated coagulants exist and are comprised of cationic and anionic poly-electrolytes, synthetic polyacrylamide polymers and starch-based polymer flocculants [50–52]. Most of these flocculants are currently utilised in the wastewater treatment industry, and their use has shown very few detrimental effects to digester stability or gas production [50,51].

The authors Kalyuzhnyi et al. [53] and Callander [54] have reported improved anaerobic digester performance when commercially available chemical coagulants have been utilised. The increased performance is due to better solid retention times of particulate matter, allowing more complete digestion of solids and resulting in higher conversions to biogas. Barford et al. [52] also noted that the use of the chemical flocculants resulted in an increased biomass concentration in the digester compared to the control that did not utilise a flocculant. The author noted that this higher concentration of particulate matter enabled considerably higher solids loadings per unit volume to be applied to the digester. However the authors also noted that the higher concentration of biomass could induce ammonia inhibition due to the much higher loading rates that could be applied to a digester with a flocculated biomass. Zhang et al. [55] reported that a high pH by chemical adjustment associated with struvite formation had no negative effect to the performance of anaerobic digestion.

With the high cost associated with these harvesting and dewatering steps many new cost efficient laboratory and pilot scale technologies are under development. Many of these technologies are still to be proven in full commercial scale settings, and their impact to anaerobic digestion is yet to be established.

4.2. Cell wall degradability and pre-treatment of microalgae biomass

Golueke et al. [22] demonstrated the ability of microalgae to pass through an anaerobic digester intact and remain undigested. The authors noted that microalgal cells are known to be able to effectively resist bacterial attack and found intact microalgae cells in digestate leaving a digester after a 30-day hydraulic retention time. Sanchez-Hernandez and Trvieso-Cordoba [43] observed that when *C. vulgaris* was added to a digester the chlorophyll *a* concentrations increased within the digester for the first two weeks of the experimental period but was still detectable 64 days after the start of the experiment. Zhou et al. [56] also found intact cells in digestate from a digester after 45 days. The longest duration reported for intact microalgae cells surviving within an anaerobic digester was reported by Mussgnung et al. [10]. They identified viable *Scenedesmus* cells after 6 months that had switched to mixotrophic growth.

Work by Mussgnung et al. [10] highlighted the role of the cell wall in the digestion process. Their results indicated that the higher gas production reported was due to the microalgae species that had either no cell wall or a cell wall made from protein. Gas production was observed to decrease for microalgal species that had a carbohydrate-based cell wall containing hemicellulose. The lowest gas production came from the species *S. obliquus* that has a particular rigid cell wall containing sporopollenin like biopolymers. Little or no cell wall degradation was detected in *S. obliquus* and very little gas was produced by the substrate. The authors concluded that the degradation of the cell wall was strongly correlated to the amount of gas produced during anaerobic digestion [10,57]. The results reported by Mussgnung et al. [10] also correlate to findings by Ras et al. [6] who noted changes in cell wall chemistry and its influence

on substrate degradability. Their results indicate the need for a pre-treatment step to disrupt the cell wall and increase bacterial hydrolysis before addition to the anaerobic digester [10,23,25,47,57–60]. Cell lysis is also essential for solvent extraction of the lipid fraction in microalgae biomass [42], allowing solvents to react with internal cell lipids. Hence microalgal cell wall disruption processes are essential for both lipid-based biofuel applications and for optimal microalgae anaerobic digestion or co-digestion processes.

Golueke and Oswald [23] investigated a thermal pre-treatment step of microalgal biomass wherein the temperature was raised above the thermal limit of the microalgal species, resulting in cell disruption. Chen and Oswald [25] undertook experiments that investigated thermal pre-treatment combined with chemical pre-treatment using sodium hydroxide and variable exposure times. Their results demonstrated that all pre-treatments tested produced better results than untreated control comparisons. It was demonstrated that the most efficient pre-treatment for microalgal biomass required heating to 100 °C for 8 h without an increase in pH using the addition of sodium hydroxide. This treatment increased gas productivity by 33% as compared to untreated microalgae biomass. This study also indicated that up to 60% of the untreated microalgae biomass added to the anaerobic digester will remain undigested due to the cell wall remaining intact throughout the digestion process [25]. More recent studies by Gonzalez-Fernandez et al. [61] reported that thermal pre-treatment of *Scenedesmus* sp. increased methane potential. At 70 °C, a 9% increase in methane production was reported, which increased to 57% at 90 °C when compared to untreated microalgae biomass. Further work by Gonzalez-Fernandez et al. [62] investigated the effect of the organic loading rates and the thermal pre-treatment of biomass at 90 °C for 1 h. Results indicated that a 2.9 and 3.4 fold increase in methane production for organic loading rates of 1 and 2.5 kg COD m⁻³ day respectively. Research reported by Alzate et al. [63] showed an increase of 46% to 62% in methane productivity utilising thermal hydrolysis. However results from De Schampelaire and Verstraete [8] reported no benefit when pre-treating a mixture of *Chlorella*, *Pseudokirchneriella* and *Chlamydomonas* microalgae species at 80 °C for 2.5 h.

Samson and Leduy [58] investigated thermo-chemical (heat and sodium hydroxide addition), mechanical and ultrasonic disintegration pre-treatment methods. The authors reported that at a temperature of 50 °C there was a 20% increase in substrate solubilisation and at 150 °C there was a 43% increase in substrate solubilisation. Their experiments indicated that the ultrasonic treatment gave similar results as the 150 °C heat treatment. The time taken for the ultrasonic treatment was relatively short and only took 10 min compared to 1 h for the thermal pre-treatment. Paris and Oswald [25] and Samson and Leduy [58] both indicated that the simple addition of sodium hydroxide was inefficient as a pre-treatment step for the anaerobic digestion of microalgae biomass or biosolids.

Gonzalez-Fernandez et al. [60] investigated sonic disruption pre-treatment of microalgae biomass. They utilised a frequency of 20 Hz but at varying power levels. All sonicated biomass exhibited higher methane production during the first days of digestion compared to untreated biomass. Overall the highest microalgae biodegradability of 44% was recorded for the longest sonication treatment as compared to 23% for un-sonicated biomass.

Samson and Leduy [58] reported a 26% increase in the solubilisation of microalgal substrates by freezing the biomass. This was due to the disruption of the microalgae cell wall by ice crystals. Keymer et al. [64] adapted high pressure thermal hydrolysis (HPTH), a commercially available technology used for the disruption of waste activated sludge biosolids for the purpose of pre-treating microalgae biomass. HPTH processes heat substrate to approximately 160 °C at a pressure of approximately 6 bars. After these conditions have been maintained for 20–30 min the contents are then reduced in pressure via a flash drum where the pressure change causes the cells to rupture and release the cell contents. Keymer et al. [64] reported that the process substantially

increased methane potential for lipid extracted and non-lipid extracted algae. The authors also reported an extraction method using a soxhlet apparatus with hexane to extract the lipid that increased the biogas potential of the microalgae biomass. When both lipid extraction and HPTL were combined an increase in the digestibility of the lipid extracted and HPTL microalgae biomass of 110% was recorded compared to untreated microalgae biomass. However this process is energy intensive but energy balances demonstrate that HPTL coupled with anaerobic digestion can be energy positive due to the increased methane potential from the substrate [65].

The various mechanical, physical, thermal and chemical methods used to improve microalgae methane potential can have a high energy requirement. Several authors have found that the energy consumption for the pre-treatment of microalgae biomass is equal to or higher than the energy gained from the microalgal cell [42,59,66–69]. Due to this high energy demand alternative methods including enzymatic and bacterial methods have also been investigated. Lu et al. [66] cited results by Sander and Murthy [70] wherein they reported the cell walls of a mixed microalgae culture to be susceptible to degradation by lipase and cellulase. Results reported by Ehimen et al. [71] showed an increase in methane production by treating *Rhizoclonium* biomass with the addition of an enzymatic mixture. The greatest increase in gas production resulted from the addition of the single enzyme cellulase. Bacterial cell disruption has also been shown to increase methane production [66]. The authors Lu et al. [66] demonstrated an increase of 17–24% in biogas production by adding the bacterium *Clostridium thermocellum* to *C. vulgaris* biomass.

4.3. The carbon/nitrogen ratio associated with microalgae biomass

Vergara-Fernandez et al. [72], Sialve et al. [59] and Yen and Brune [67] identified further difficulties with the anaerobic digestion of microalgae biomass, due to the low carbon to nitrogen ratio present in microalgal species. Data reported in Table 1 shows that the carbon/nitrogen (C/N) ratio varies from 4.16 to 7.82 for microalgal species that have been investigated for anaerobic digestion. When the C/N ratio is below 20 there is an imbalance between carbon and nitrogen requirements for the anaerobic bacterial community or consortia [59]. This imbalance leads to nitrogen release in the form of ammonia during digestion, which can become inhibitory to methanogenic bacteria and result in volatile fatty acids accumulating within a digester [59]. Ammonia-nitrogen and volatile fatty acids (VFA) are important intermediates in anaerobic digestion processes but can also be potential inhibitors when allowed to accumulate [44].

To overcome problematically low C/N ratios, several researchers have investigated co-digestion, where microalgae has been co-digested with other waste streams or biomass to increase the C/N ratio. These studies include Gonzalez-Fernandez et al. [73] and Shouquan et al. [74] who investigated the addition of microalgae to pig manure prior to digestion. Saxena et al. [75] recorded increased methane production for the anaerobic co-digestion of green filamentous microalgae and water hyacinth supplemented with cow manure. Ramamoorthy and Sulochana [76] also investigated the addition of *Zygonium* sp. with various quantities of cow manure. Shuchuan et al. [77] reported a significant increase in methane potential when blue green algae was co-digested with corn stalks. Samson and Leduy [78] undertook a co-digestion experiment wherein they blended sewage sludge with *Spirulina maxima* and observed a 2-fold increase in gas production when a mixture of 50% by weight of sewage sludge to microalgae ratio was used. Yaun et al. [79] reported increased methane potential in the co-digestion of municipal wastewater solids with *Chlorella* sp. and *Spirulina platensis* microalgae species respectively. Park and Li [80] investigated co-digestion using *Nannochloropsis salina* and lipid-rich fats, oil and grease. They observed higher methane production and were able to use an increased organic loading rate due to a more balanced C/N ratio with less inhibition and digester imbalance. An increase in gas

production was also reported by Salerno et al. [81] by co-digesting domestic municipal wastewater grown microalgae and soybean oil.

Glycerol is a carbon-rich by-product of the trans-esterification conversion of lipids to biodiesel, and it can be used as a carbon source to maximise gas production in anaerobic co-digestion [82]. Ehimen et al. [83] investigated using glycerol produced from transesterified microalgae lipid. A slight increase in gas production rate was noted in this study; however a low application rate of glycerol was utilised. An increase in gas production was also observed by Salerno et al. [81] when co-digesting glycerol and domestic municipal wastewater derived microalgae biomass.

Yen and Brune [67] considered the addition of paper waste to improve the C/N ratio of the microalgae combination comprised of *Scenedesmus* sp. and *Chlorella* sp. Yen and Brune [67] illustrated that with the addition of waste paper there was an increase in the C/N ratio from 6.7 to 36.4. Results from this experiment showed that the best co-digestion ratio was 50% paper and 50% microalgae. The final C/N ratio of this combination was 18.0 with 1170 ± 75 mL/day biogas produced. In comparison the microalgae-only anaerobic digestion produced 573 ± 28 mL/day of biogas, which was about a 50% reduction in gas production compared to the more favourable C/N ratio treatment. Yen and Brune [67] concluded that the best C/N ratio for anaerobic digestion is in-between 20:1 and 25:1. This conclusion is similar to the C/N ratio discussed by the authors Parkin and Owen [44] wherein they indicated an optimum C/N ratio range of between 20:1 and 30:1.

Yen and Brune [67] indicated that as the C/N ratio increased the amount of total ammonia-nitrogen decreased as the C/N ratio became more favourable thus reducing ammonia-nitrogen inhibition effect. Ehimen et al. [83] suggested that a C/N ratio of 15 or below can result in a build up of free ammonia-nitrogen, which can be detrimental to anaerobic digestion processes. The high C/N ratio treatment of 36.4/1 used in the experiment was on the upper extreme for anaerobic digestion as high volatile fatty acid (VFA) concentrations can also become inhibitory to anaerobic digestion. At a high C/N ratio, the amount of total ammonia-nitrogen can be too low for the cellular needs of the anaerobic microorganisms. It has been shown that a minimum concentration of 50 to 200 mg/L of nitrogen as ammonia is essential for the requirements of the bacterial community associated with anaerobic digestion [44,84]. The co-digestion of two substrates may improve C/N ratio and VFA/alkalinity ratios and attenuate unfavourable ratios in a single substrate.

One problem that must be considered with the co-digestion of a second waste stream or biomass is the seasonal availability of the feedstock and location of production. This problem was highlighted previously where seasonal growth of macroalgae limited anaerobic digestion to only six months of the year [11,17].

4.4. Lipids and microalgae

Lipids are an attractive substrate for anaerobic digestion and have a higher theoretical methane potential compared to proteins and carbohydrates [85]. However due to their low alkalinity and buffering capacity, lipids can cause inhibition due to their intermediate products such as long chain fatty acids (LCFAs) and VFAs [80]. It has been suggested that the conversion of microalgal biomass to methane rich biogas is energetically more favourable than lipids removal from microalgae biomass with the total lipid content is lower than 40% [59]. However, the removal of lipids from microalgae biomass for liquid biofuel production prior to anaerobic digestion of the residual microalgae biomass can be beneficial to anaerobic digestion processes, as high lipid concentrations can be inhibitory [59,80,86].

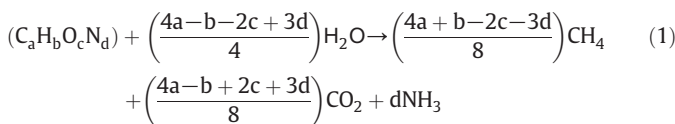
Crine et al. [86] reported that there was no inhibition for lipid concentrations of 5, 10 and 18% respectively. However inhibition was observed for lipid concentrations of 31, 40 and 47%, where inhibition increased due to higher lipid fractions. It is anticipated that the lipid concentration for economically viable lipid-based biofuel microalgae

species generally exceeds 30%, which could have negative consequences for anaerobic digestion if the lipids are not extracted.

Lipid extraction methods used on microalgal biomass can affect the digestibility of residual microalgal biomass. Ehimen et al. [83] and Thiel [87] reported a significant decrease in gas production due to residual chloroform from the Bligh and Dyer extraction process even though it had been heat treated to remove residual entrained solvents after the extraction process [88]. Butanol, hexane and methanol have been shown to have no detrimental effects on anaerobic digestion when residual solvents are removed by heating [83].

5. Theoretical methane production

When the C, H, O and N composition of a wastewater or substrate is known, the stoichiometric relationship reported by Buswell and Boruff [89] can be used to estimate the theoretical gas composition on a percentage molar basis.



where a, b, c and d equal the carbon content, hydrogen content, oxygen content and nitrogen molar composition respectively [59,89,90].

$$\text{Methane yield (litres/g (VS) destroyed)} = \left(\frac{4a + b - 2c - 3d}{12a + b + 16c + 14d}\right) * V_m \quad (2)$$

where V_m is the molar volume of methane or 22.14 L at 0 °C and 1 atm [59]. Eq. 2 is used to calculate the volume of methane gas depending on the amount of volatile solids (VS) available in the substrate being digested.

The data shown in Table 3 illustrates the theoretical methane potential for several microalgae species utilising Eq. 2 and values from literature. However Eq. 2 overestimates the gas production as it assumes 100% conversion of the volatile solids to biogas and also does not consider the needs for bacterial cell maintenance and anabolism [59,90]. When the theoretical methane potential was calculated for lipid extracted and non lipid extracted wastewater microalgae consortium reported in Chinnasamy et al. [91], a 13% decrease in the theoretical methane potential was reported highlighting the residual gas potential of lipid-extracted microalgae biomass.

6. Inhibition of anaerobic digestion

6.1. Ammonia-nitrogen toxicity

Ammonia-nitrogen is produced from the biological breakdown of nitrogenous matter, mostly in the form of proteins and urea [84]. The high

nitrogen and protein levels found in microalgae can lead to significant release of ammonia-nitrogen during anaerobic digestion [59]. The equilibrium established between un-ionised ammonia- (NH_3-N) and ammonium- (NH_4-N) nitrogen can be affected by a change in pH or temperature within the anaerobic digester. An increase in temperature or pH can be very detrimental to the bacterial community as the equilibrium shifts to the more toxic un-ionised form of ammonia-nitrogen NH_3-N [44,59,92].

McCarty [93] indicated that ammonia gas within the digester is inhibitory at a much lower concentration than the aqueous ionised form of ammonium-nitrogen. Ammonia toxicity has been shown to affect methanogenic bacteria in two ways: (1) the ammonium ion may inhibit the methane synthesising enzyme directly, and (2) the hydrophobic ammonia-nitrogen molecule may diffuse passively into the cell, causing proton imbalance and/or potassium deficiency [59,94]. Ammonia-nitrogen is toxic at high levels and has a moderately inhibitive effect from 1500–3000 mg/L. Above 3000 mg/L there is a strong inhibitive effect associated with ammonium-nitrogen [44], which can lead to a drop in gas production. Inhibition of the methanogenic or acidogenic groups of anaerobic microbes was not quantified in this study.

There is a large amount of conflicting information in the literature relating to the ammonia-nitrogen tolerance of anaerobic microbes. Research based on methane production and growth rate comparisons indicate that inhibitory effects are greater for the acidogenic bacteria compared to the methanogenic bacteria [84,95–100]. It has been observed that acetate consuming methanogens have relatively high resistance to high ammonia-nitrogen concentrations [84,94,100–102].

This difference in opinion demonstrates the distinctive responses that can be associated with bacterial consortiums that are involved with anaerobic digestion. Among the methanogenic strains commonly found in digesters (for example: *Methanospirillum hungatei*, *Methanosarcina barkeri*, *Methanobacterium thermoautotrophicum*, and *Methanobacterium formicicum*) the species *M. hungatei* was found to be the most sensitive microbe to ammonia-nitrogen, with inhibition observed at 4200 mg/L compared to the other three strains where inhibition did not occur until ammonia-nitrogen levels were above 10,000 mg/L [103]. The inhibition of *M. hungatei* at 4200 mg/L is the only methanogenic strain out of the five methanogenic strains tested that corresponds to the findings of Parkin and Owen [44].

The utilisation of volatile fatty acids by methanogens must balance the production of volatile fatty acids by hydrolytic and acetogenic bacteria in order to maintain digestion stability. Efficient digester performance is therefore dependent upon maintaining the ammonia-nitrogen concentration below the inhibitory limits for all of the associated digestion bacteria [100].

A solution to the problem of digester stability and balancing the bacterial populations and end products is to separate the bacterial communities. This can be done by utilising a two-stage anaerobic digestion process [72]. The metabolic pathways of the two-stage anaerobic digestion processes are the same as those of single-stage anaerobic digestion. However the stages are physically separated with the hydrolytic and

Table 3

Biochemical analysis of microalgae species and their theoretical methane potentials calculated from the mean carbon, nitrogen, hydrogen and oxygen values.

Species	Carbon	Hydrogen	Nitrogen	Oxygen	Calculated methane potential (ml/g/VS)	Reference
<i>Chlorogloeopsis fritschii</i>	54.4 ± 2.1	6.9 ± 0.5	7.3 ± 0.3	31.4	309	[128]
<i>Spirulina platensis</i>	55.7 ± 0.4	6.8 ± 0.1	11.2 ± 0.1	26.4	319	[128]
<i>Chlorella vulgaris</i>	52.6 ± 0.8	7.1 ± 0.1	8.2 ± 0.2	32.2	283	[128]
<i>Scenedesmus dimorphus</i>	53.4 ± 0.6	7.8 ± 0.2	7.9 ± 0.1	31.0	260	[128]
Wastewater consortium	49.4 ± 0.1	6.7 ± 0.1	9.3 ± 0.2	21.6	347	[91]
Wastewater consortium (lipid extracted)	45.9 ± 1.9	6.2 ± 0.1	9.3 ± 0.9	23.6	303	[91]
<i>Nannochloropsis</i> sp. (lipid extracted)	49.7	7.1	5.8	26.7	340	[3]
<i>Nannochloropsis</i> sp.	47.6	6.6	5.5	21.7	383	[3]
<i>Nannochloropsis</i> sp. (low lipid)	52.0	7.5	4.8	22.4	414	[3]
<i>Nannochloropsis</i> sp. (high lipid)	51.5	7.3	4.5	22.4	414	[3]

acetogenic bacteria in the first stage and the methanogenic bacteria in the second phase [72]. Several authors have indicated that by using two-stage digestion there is increased degradation of organic matter, improved biogas production and better control over the conditions inside the digester limiting inhibition of the microbial populations [72,104–106].

Yang et al. [107] demonstrated that by utilising a two-stage digestion process an increased methane yield of 22% was achieved when anaerobically digesting *Scenedesmus* sp. The authors also recorded 46 ml g⁻¹ VS of hydrogen production in the first stage of the two-stage digestion process. The hydrogen production was in addition to the methane production recorded for the second stage. A soluble COD reduction of 75% was recorded for the single stage digestion, whereas a soluble COD reduction of 81.8% was recorded for the two stage process.

Recent work by Inglesby and Fisher [48] has shown that the integration of microbial fuel cells can be beneficial in decreasing ammonia-nitrogen inhibition during anaerobic digestion. Improved performance is achieved by allowing the ammonium ion to migrate across the cation exchange membrane from the anode to the cathode. The use of the microbial fuel cells decreases the chance of free ammonia-nitrogen inhibition of methanogenic bacteria improving the stability of the anaerobic digestion process [48].

6.2. Saline microalgae and the effect of salinity

Some species of microalgae that have been identified for their potential as feedstock for liquid biofuels are grown in a saline environment. The use of saltwater for their production offers a sustainable alternative due to the ability to use non-arable land and seawater, hence reducing pressure on current agricultural land and scarce freshwater resources. Several scientific publications cited in Table 1 have dealt with saline species of microalgae. The marine species *Macrocystis pyrifera* and *Tetraselmis* sp. have been used as a substrate for anaerobic digestion. The cyanobacteria *Spirulina* sp. has also been identified for having the potential to be grown in saline waters [108].

Alkaline earth metal salts are needed in very low concentrations for cellular metabolism in bacteria, and higher concentrations can be extremely toxic to methanogenic bacteria [93]. Salinity and more specifically sodium divalent cations do pose a problem to bacteria associated with anaerobic digestion [44,93]. Vergara-Fernandez et al. [72] demonstrated that the digestion of marine microalgae is possible. They recorded a total biogas production of between 95 and 260 mL g⁻¹ TS microalgae loaded using a two-stage digestion process to obtain these results. However this work did not quantify the salinity of the concentrated paste that was used to feed the digester, and the actual salinity of the substrate was less than that of seawater salinities.

Asinari Di San Marzano et al. [109] showed that a gas production of 310 ml and 440 mL g⁻¹ VS for dry anaerobically digested *Tetraselmis* sp. of saline microalgae. Both methane production rates are for *Tetraselmis* sp. microalgae biomass that has a salinity of <1 g/L. However they also recorded 450 mL g⁻¹ VS added with a substrate salinity of 35 g/L. The authors did not indicate whether this is for dry or wet *Tetraselmis* sp., and they did not indicate the source of inoculum used for this experiment. Buxy et al. [110] reported a methane potential of 204 mL g⁻¹ VS for the marine microalgae *Nannochloropsis oculata*. This microalga was grown under seawater salinity conditions, harvested, concentrated to a paste and then used for the bio-methane potential experiments. Due to the use of concentrated paste and its addition to a freshwater anaerobic digester, the final salinities of the digestion vessel are not reported.

High salinity levels have been shown to be inhibitory as it can cause bacterial cells to dehydrate due to increased osmotic pressure [84]. Salinity is made up of multiple elements and can vary depending on the source of water and its associated environment [111]. The light metal ions including sodium, magnesium, calcium and aluminium can all be toxic at high levels [84,93].

The sodium ion is the most inhibitory to anaerobic digestion of these metal ions, and it makes up a larger percentage of the light metal ions found in seawater [59]. However inhibition due to sodium varies depending on the source of inoculum and overall elemental composition of the saline water and substrate being digested [111]. Rinzeema [111] demonstrated that sodium concentrations of 5, 10 and 14 g/L caused 10, 50 and 100% inhibition in acetoclastic methanogenic bacteria respectively. These measured sodium inhibition rates were also observed by Parkin and Owen [44] who reported a moderate inhibitory effect on anaerobic digestion at sodium concentrations ranging from 3.5 to 5.5 g/L. Above 8.0 g/L of sodium can be extremely inhibitive.

However, Parkin and Owen [44] noted that amelioration of sodium ions and potassium ions was possible. When this amelioration occurred it could reduce the toxicity caused by light metal ions during anaerobic digestion. In addition, the presence of sodium ions was found to be antagonistic to ammonia-nitrogen inhibition. Experiments by Kugelman [112] demonstrated that at an ammonia-nitrogen concentration of 0.15 mol/L, the methane production from acetic acid was reduced by 20%. The addition of 0.002–0.05 mol of sodium (Na⁺) produced 5% more methane compared to that of the control. This research indicated a further increase of 10% in methane production was achieved by using a combination of sodium and potassium or sodium and magnesium cations [112].

Zhang et al. [113] identified that sodium induced build up of propionate to be problematic in their digester system, which became inhibitory and caused digester pH imbalance and digester failure. Zhang et al. [113] offered a solution to overcome the inhibition from sodium in syntrophic acetogenic bacteria. To help address high sodium inhibition, Zhang et al. [113] utilised an electrical current delivered to the digester via an iron anode and graphite cathode. They found by adding an electrical current to the digester enough free energy was produced to convert propionate acid to acetic acid allowing methanogens to further transform the acetic acid to methane [113].

6.3. Sulfur and its role in anaerobic digestion

Freshwater microalgal biomass contains low levels of sulfated amino acids and their digestion releases lower amounts of hydrogen sulfide than other types of substrates [59]. However oxidised sulfur compounds can be present in saline waters and saline substrates. These sulfur compounds can act as electron acceptors for sulfate-reducing bacteria that convert organic compounds in an anaerobic reactor and produce hydrogen sulfide gas. Hydrogen sulfide when present in gas is corrosive and can cause damage to machinery, such as gas-engine power generators, and piping [90]. Except for sulfide, sulfur compounds are not harmful to anaerobic bacteria unless at high concentrations.

Sulfide is needed for cellular metabolism in low concentrations by bacteria, but concentrations higher than 200 mg/L become extremely toxic to methanogenic bacteria [44,114]. Also like un-ionised ammonia-nitrogen, un-ionised sulfide is much more toxic than ionised sulfide. The speciation between the two compounds is also dependent on temperature and pH [44].

Sulfate reducing bacteria compete with methanogenic bacteria for acetate and hydrogen. The sulfate reducing bacteria have a higher affinity for acetate than methanogens, outcompeting them under low acetate concentrations [114]. This competitive inhibition results in the shunting of electrons from methane generation to sulfate reduction. Sulfate reducers and methanogens are very competitive at COD/SO₄ ratios of 1.7 to 2.7. An increase of this ratio is favourable to methanogens, whereas a decrease is favourable to sulfate reducers [90,114].

The conclusion on the optimum COD/SO₄ ratio is supported by the paper by Aspe et al. [115] wherein they found methane fermentation inhibition at a COD/SO₄ ratio lower than 0.5. Aspe et al. [115] highlighted the difference between inoculums used for seeding anaerobic digesters. Their research focused on two inoculums: one was sourced from

piggery effluent and the other was sourced from a marine sediment inoculum. The authors indicated that the sulfate reducing bacteria did not grow as well in the marine sediment sourced inoculum as compared to the piggery effluent sourced inoculum.

7. Bacterial consortium and its role in anaerobic digestion

Aspe et al. [115] highlighted the different results that can be obtained from the source of the bacterial inoculum. Little work has been done on bacterial communities within the microalgal anaerobic digester, and there is a lack of information in the scientific literature on this topic.

The authors Zhang et al. [113] and Patil et al. [116] used polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to profile the microbial communities associated with anaerobic digestion. This analysis revealed the presence of unknown and as yet uncultured microorganisms associated with the digestion process. Unknown organisms and differences within anaerobic communities are problematic when comparing the variety of results from similar substrates for anaerobic digestion. The inoculum source and the associated bacterial species can change over geographical and environmental locations.

The ability of bacteria to utilise environmental plasticity to change and adapt to different substrates and environmental conditions over time is reported by Buxy et al. [110], who reported an adaptation of bacteria to saline conditions when undertaking bio-methane potential experiments shown by an increase in gas potential and a decrease in the initial lag phase of the digestion process. Further investigation of the microbial community's adaptation and environmental plasticity could potentially offer improvements in the bacterial metabolism and the anaerobic digestion process under adverse conditions.

8. Anaerobic digestion and nutrient recycling

Nutrients are a large and expensive input into the mass production of microalgal biomass. Large amounts of nitrogen and phosphorous are needed for algae biomass production. With the proposed expansion of the commercial algal biomass production industry, the competition with current agricultural industries for organic fertilisers is expected to increase [5], which could increase fertiliser prices. Historically fertiliser prices are closely related to the cost of fossil fuel, and as fossil fuels become more expensive fertiliser prices will also increase [5,117]. This increase in oil prices combined with greater fertiliser and energy demand from the agricultural sector could result in increased oil prices making inorganic fertiliser un-competitive for algal biofuel production [5]. Due to these compounding factors, nutrient recovery from processed biomass via anaerobic digestion is highly desirable for the sustainable growth of the algal biomass.

Lyovo et al. [118] indicated that 45 kg of nitrogen is needed for one ton of algal biomass based on a composition of $\text{CO}_{0.48}\text{H}_{1.83}\text{N}_{0.11}\text{P}_{0.01}$ [118]. When analysing this stoichiometric relationship, an 11:1 ratio of nitrogen to phosphorus can be determined. This nitrogen to phosphorus ratio indicates that approximately 4 kg of phosphorous is required for every one tonne of algal biomass grown. This ratio further highlights the role that anaerobic digestion provides in the recycling of nutrient that is vital to the sustainability and economic feasibility of commercial-scale algal biofuel industries [1,59,64,80].

Phosphorus as a nutrient has had very little research attention when compared to other nutrients such as carbon and nitrogen [119]. Vaccari [119] stated, "the on-going supply of phosphorus has become one of the most significant sustainability issues facing our future" [119].

Several authors have highlighted that there are finite reserves of rock phosphorus available and human civilisation is heading towards a similar scenario as peak oil except with dwindling phosphorus reserves. However the exact quantity of reserves and timing of the shortage is still highly disputed [117,119,120]. This future decline in available phosphorus reserves must be addressed for the sustainable growth of algae for commodity products such as biofuel [2].

Anaerobic digestion of algal biomass produces a nutrient-rich digestate containing both nitrogen and phosphorus nutrients. Digestate nutrient values of 2940 mg/L ammonia-nitrogen, 390 mg/L of total phosphorous, and 320 mg/L of potassium have been reported by Collet et al. [1]. However, these values are in the moderate to high inhibition ranges recorded for ammonia-nitrogen by Parkin and Owen [44]. In contrast, studies conducted by Zamalloa [47] indicated a clear liquid digestate was produced with a total ammonia-nitrogen (TAN) concentration of 546 ± 48 mg/L and a phosphate concentration of 141 ± 41 mg/L from anaerobically digested *P. tricornutum*. These results demonstrate the high strength nutrient-rich digestate that can be produced from the anaerobic digestion of microalgae biomass. Results from De Schampelaire and Verstraete [8] also indicate that the high strength, nutrient-rich digestate nitrogen to phosphorous ratio from several digestion experiments was in the range of 10 to 17:1 that is ideal for the cultivation of algal species.

The gross chemical composition of microalgae can be highly dependent upon environmental conditions such as light intensity, temperature and nutrient availability. Generally microalgae contain varying proportions of proteins, lipids, carbohydrates, nucleic acids, pigments and vitamins [85]. The mineral composition of microalgae also meets the nutrient and mineral requirements for the microbial micro-flora that are associated with the anaerobic digestion process [59].

The use of digestate from digested microalgae biomass is highlighted in the research by Asinari Di San Marzano et al. [109], Benemann et al. [26], De Schampelaire and Verstraete [8], Gonzalez-Fernandez [60] but first by Golueke and Oswald [23]. Golueke and Oswald [23] and Ras et al. [6] both setup a closed looped system where microalgae was grown and then harvested and immediately digested to produce biogas. The digestate from the anaerobic digester was then fed back into the high rate pond or photo-bioreactor and used as a nutrient source for further microalgae growth.

A further synergistic benefit of integrating anaerobic digestion with a algal biofuel program is the ability to utilise the microalgae cultures for purifying the methane content of the biogas [12,59,85]. The concentration of carbon dioxide derived biogas from anaerobically digested microalgae is in the range of 30 to 50% [59]. From an energy recovery perspective the biogas CH_4/CO_2 ratio needs to be above 1 [72] indicating that a gas purification step is required for microalgae derived biogas. Due to the low solubility of methane and high solubility of carbon dioxide, uptake of carbon dioxide is high leaving high concentrations of methane after the purification step [1]. Also during this process other impurities such as hydrogen sulfide are removed from the biogas [1]. Biogas CO_2 bio-fixation by microalgae is discussed by Green et al. [28].

Research undertaken by Converti et al. [12] also highlighted an increased methane percentage in the biogas produced by utilising the microalgae culture to strip carbon dioxide gas from the biogas as it is produced. This is similar to the high methane gas composition recorded with the advanced integrated wastewater pond system utilising in pond fermentation pits [24]. Methane has been shown to be non-detrimental to microalgae growth and the upgrading of biogas via high density microalgae cultures would be beneficial due to the supply of carbon dioxide to microalgae cultures. The carbon dioxide that is stripped from the biogas would be utilised as a nutrient source by the microalgae [59].

9. Conclusions

Early and more recent research have provided greater understanding of the complexity of individual algal species as a substrate for anaerobic digestion or co-digestion. This knowledge will be extremely beneficial to the anaerobic digestion of algae and will allow the methane production rates from individual algae species to be increased and optimised. Each individual species of algae must be treated differently and processed specifically to optimise biogas yields. Anaerobic digestion of algal biomass is a key unit process that integrates efficiently and beneficially into the production of algae-based biofuels and algae-based

wastewater treatment. The integration of algae-based biofuel production with the anaerobic digestion of algae residuals is the most applicable scenario for the maximisation of methane-rich biogas.

Several technical issues including the low concentration of digestible (biodegradable) algal substrates and cell wall disruption can be overcome by the pre-treatment methods used to process algae for liquid or gaseous biofuels. The integration of anaerobic digestion into proposed algae-derived biodiesel operations has the benefit of being able to utilise glycerol, a by-product, in a co-digestion with microalgae improving the carbon to nitrogen ratio and thus increasing gas production. Gas production can also be utilised for electrical or thermal energy production, while algal cultures can also be utilised for biogas upgrading.

The resulting digestate can improve efficiency as it has been shown to be an ideal nutrient source for the production of algal biomass. The utilisation of this digestate for regrowth of additional algal biomass will help to close the nutrient loop associated with large scale algal biomass production and to achieve more widespread environmental sustainability.

With a greater understanding of algal species and their growth and biological characteristics, the anaerobic digestion of macroalgae and microalgae, and their residues, can be optimised to play a promising role in the sustainable future of clean energy derived from algal biomass.

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2.12. Supplementary literature review

This supplementary literature review addresses further experimental work on the anaerobic digestion (AD) of microalgae that has been completed since the publication of “Anaerobic digestion of algal biomass: A review” (Ward et al., 2014) (chapter 2). Additional publications investigating the pre-treatment of microalgae biomass have been published. The pre-treatment methods that have been reported since publication of Ward et al. (2014) include ultrasonic pre-treatment (Passos et al., 2014, Ometto et al., 2014), auto-hydrolysis and alkaline pre-treatment (Mahdy et al., 2014c), thermal pre-treatment (Kinnunen et al., 2014, Ometto et al., 2014, Passos and Ferrer, 2015, Zielinski et al., 2015, Mendez et al., 2014) and enzymatic pre-treatment (Mahdy et al., 2014a, Munoz et al., 2014, Mahdy et al., 2014b, Ometto et al., 2014).

Ultrasonic pre-treatment experimental findings were consistent with previous reported literature, where ultrasonic pre-treatment of biomass increases the methane yield from anaerobically digested microalgae biomass (Ometto et al., 2014, Passos et al., 2014). However Passos (2014) concluded that the energy requirements to ultrasonically pre-treat microalgae biomass was greater than the additional methane recovered from the increased cell wall disruption. This finding was also noted by Ometto (2014) where results indicated that the energy input and energy output ratio was negative for all ultrasonic treatments tested.

Results on the auto-hydrolysis pre-treatment method reported in Mahdy et al. (2014c) also identified an increased methane yield utilising auto-hydrolysis pre-treatment methods as previously reported in Ward et al. (2014).

Table 1: Methane biogas production from anaerobic digestion of different species of microalgae biomass.

Microalgae species	C/N Ratio	Methane Yield	Loading Rate	Reference
Waste water grown community	N/R	196.4 (ml/Ch ₄ /g VS)	5 g COD/L	(Passos et al., 2014)
<i>Scenedesmus obliquus</i>	N/R	411 (ml/Ch ₄ /g VS)	N/R	(Ometto et al., 2014)
<i>Chlorella sorokiniana</i>	N/R	318 (ml/Ch ₄ /g VS)	N/R	(Ometto et al., 2014)
<i>Arthrospira maxima</i>	N/R	141 (ml/Ch ₄ /g VS)	N/R	(Ometto et al., 2014)
<i>Chlorella vulgaris</i>	N/R	160 (ml/Ch ₄ /g VS)	N/R	(Mahdy et al., 2014c)
' <i>Scenedesmus</i> ' sp.	N/R	165 (ml/Ch ₄ /G VS)	N/R	(Mahdy et al., 2014c)
Waste water grown community	N/R	225 (ml/Ch ₄ /g VS)	1.7 to 0.3 g VS/L ⁻¹ D ⁻¹	(Kinnunen et al., 2014)
<i>Oocystis</i> sp.	N/R	170 (ml/Ch ₄ /g VS)	0.7 g VS/L ⁻¹ D ⁻¹	(Passos and Ferrer, 2015)
<i>Chlorella vulgaris</i> (68%) & <i>Scenedesmus</i> sp. (28%)	N/R	347 (ml/Ch ₄ /g VS)	1 g VS/L ⁻¹	(Zielinski et al., 2015)
<i>Scenedesmus</i> sp.	N/R	194 (ml/Ch ₄ /g COD)	3.2% TS/L ⁻¹	(Mendez et al., 2014)
<i>Chlorella vulgaris</i>	N/R	225 (ml/Ch ₄ /g COD)	1.6% TS/L ⁻¹	(Mendez et al., 2014)
<i>Chlorella vulgaris</i>	N/R	299 (ml/Ch ₄ /g COD)	N/R	(Mahdy et al., 2014a)
<i>Chlamydomonas reinhardtii</i>	N/R	289 (ml/Ch ₄ /g COD)	N/R	(Mahdy et al., 2014a)
<i>Chlorella vulgaris</i>	N/R	255 (ml/Ch ₄ /g COD)	N/R	(Mahdy et al., 2014b)
<i>Nanochloropsis gaditana</i>	N/R	282 (ml/CH ₄ /g VS)	2 g TS/L ⁻¹	(Munoz et al., 2014)
<i>Scenedesmus</i> sp.	6.0	140 (ml/CH ₄ /g VS)	N/R	(Ramos-Suarez et al., 2014)
<i>Chlorella</i> sp.	N/R	210 (ml/Ch ₄ /g VS)	6.7 g VS/L ⁻¹	(Li et al., 2014)
<i>Chlorella</i> sp.	6.80	337 (ml/Ch ₄ /g VS)	10 g VS/L ⁻¹	(Zhao et al., 2014)
<i>Chlorella</i> sp. (LEB)	5.51	314 (ml/Ch ₄ /g VS)	10 g VS/L ⁻¹	(Zhao et al., 2014)
<i>Nanochloropsis</i> sp.	7.55	357 (ml/Ch ₄ /g VS)	10 g VS/L ⁻¹	(Zhao et al., 2014)
<i>Nanochloropsis</i> sp. (LEB)	6.36	399 (ml/Ch ₄ /g VS)	10 g VS/L ⁻¹	(Zhao et al., 2014)
<i>Nanochloropsis salina</i>	14.87	557 (ml/Ch ₄ /g VS)	10 g VS/L ⁻¹	(Zhao et al., 2014)
<i>Nanochloropsis salina</i> (LEB)	8.46	383 (ml/Ch ₄ /g VS)	10 g VS/L ⁻¹	(Zhao et al., 2014)

(N/R = not reported and (LEB) = lipid extracted biomass

Table 1 Continued: Methane biogas production from anaerobic digestion of different species of microalgae biomass.

Microalgae species	C/N Ratio	Methane Yield	Loading Rate	Reference
<i>Nanofrustulum</i> sp.	9.47	507 (ml/Ch ₄ /g VS)	10 g VS/L ⁻¹	(Zhao et al., 2014)
<i>Nanofrustulum</i> sp. (LEB)	6.89	304 (ml/Ch ₄ /g VS)	10 g VS/L ⁻¹	(Zhao et al., 2014)
<i>Phaeodactylum tricornutum</i>	6.86	337 (ml/Ch ₄ /g VS)	10 g VS/L ⁻¹	(Zhao et al., 2014)
<i>Phaeodactylum tricornutum</i> (LEB)	5.68	339 (ml/Ch ₄ /g VS)	10 g VS/L ⁻¹	(Zhao et al., 2014)
<i>Nanochloropsis</i> sp.	N/R	399 (ml/Ch ₄ /g VS)	1 g VS/L ⁻¹	(Alzate et al., 2014)
<i>Nanochloropsis</i> sp. (LEB)	N/R	381 (ml/Ch ₄ /g VS)	1 g VS/L ⁻¹	(Alzate et al., 2014)
<i>Nanochloropsis gaditana</i> (LEB)	N/R	236 (ml/Ch ₄ /g VS)	N/R	(Hernandez et al. 2014)
<i>Tetraselmis</i> sp. (LEB)	N/R	203 (ml/Ch ₄ /g VS)	N/R	

(N/R = not reported and (LEB) = lipid extracted biomass

However the authors also report that the use of sodium hydroxide with *Chlorella vulgaris* resulted in the formation of precipitating by- products from the addition of the chemical catalysts. They conclude that these precipitates may have contributed to the resultant lower methane production that was reported (Mahdy et al., 2014c).

Thermal pre-treatment experimental results reported by Kinnunen et al. (2014), Ometto et al (2014), Mendez et al. (2014), Zielinski et al. (2015) and Passos et al. (2014) also correlated with reported findings in Ward et al. (2014). All experimental results found increased methane potential when microalgae biomass was thermally pre-treated prior to AD. Ometto (2014) found one treatment combination for *Scenedesmus obliquus* that had a favourable energy return when thermally pre-treated. In contrast to this result, Passos (2015) and Ometto (2014) concluded that all the microalgae thermal pre-treatments they tested all utilised more energy than what was recoverable from the pre-treated biomass. Passos (2015) went further and calculated a theoretical total solids AD loading rate that would give a positive energy

balance if utilised. The author stipulated that a centrifugation step would be needed to reach the required total solids content needed for a positive energy balance. When the required theoretical loading rate was calculated and compared to scientific literature it indicated that this theoretical loading rate would be too high. The literature indicated that the calculated loading rate would be detrimental to the AD process due to high ammonia levels resulting from the breakdown of protein, hence causing AD inhibition. Passos (2015) also noted that a higher organic loading rate may not achieve a higher methane potential as current literature was inconclusive on gas potential at higher loading rates. The work by Menedez et al. (2014) adds additional experimental evidence to this. Menedez (2014) investigated higher loading rates utilising pre-treated substrate at loading rates ranging from 1.6% to 13% TS (W/V) basis. Results demonstrated that the increased loading rate did not yield additional methane production. Additionally results showed that a concentration above 3.2% TS (W/V) was detrimental and decreased methane production for the *Scenedesmus* sp. of microalgae biomass was recorded.

The author Ramos-Suarez et al. (2014) also investigated methane production from increased loading rates ranging from 2 to 6g VS L⁻¹. When *Scenedesmus* sp. was co-digested with *Opuntia maxima* sp. at a loading rate of 2g VS L⁻¹ the lowest methane production was reported. This increased to its maximum reported production at 4g VS L⁻¹ however this again decreased considerably at 6g VS L⁻¹.

The inoculum to substrate ratio, which also relates to loading rate was investigated by Li et al. (2014). Li et al. (2014) found a maximum methane production when a 1:3 inoculum to substrate ratio was utilised. Li et al. (2014) also reported if a ratio of 1 or below was used the methane was significantly reduced due to ammonia inhibition. This finding could have a significance implications to continuously run

anaerobic digesters where undigested biomass may continuously accumulate if high loading rates are utilised.

Pre-treatments utilising enzymatic activity also reported increased methane production as reported in Ward et al. (2014). The enzymes Viscozyme (carbohydrase) and Alcalase (protease) were tested by Mahdy et al. (2014a), (2014b) and both these enzymes were found to increase methane production from *Chlorella vulgaris* and *Chlamydomonas reinhardtii* microalgae species. However Mahdy (2014a) noted that the enzymatic hydrolysis of *Chlamydomonas reinhardtii* was not favourable due to the already high degradability of *Chlamydomonas reinhardtii*. The increased methane production from enzyme pre-treatment reported by Mahdy (2014) was insufficient to be a viable option. Alternatively the enzymatic pre-treatment of *Chlorella vulgaris* was seen as a promising pre-treatment to decrease the energetic input required for cell wall disruption of this species (Mahdy et al., 2014a).

Ometto et al. (2014) utilised the commercially available enzymes Depol™ 40L, Lipomod™ 957, Depol™ 220L, pectinase P2611 and Lipimod™ 166P to successfully pre-treat *Scenedesmus obliquus* and *Chlorella vulgaris* microalgae biomass (Ometto et al., 2014).

Munoz (2014) identified a new approach for the enzymatic pre-treatment of microalgae biomass. Munoz (2014) utilised live marine bacteria with cellulolytic activity as a whole-cell enzymatic pre-treatment for microalgal biomass (Munoz et al., 2014). This bacterial enzymatic pre-treatment had the advantage of being more energy efficient than current commercial enzymes. It also had the advantage of being able to be cultured on the same site as the microalgae reducing financial costs when compared to other commercial enzymatic pre-treatments (Munoz (2014). This method

also demonstrated positive gas production when microalgae biomass was pre-treated by this method (Munoz et al., 2014).

Further work investigating methane potential was undertaken on lipid extracted biomass (Zhao et al., 2014, Alzate et al., 2014, Hernandez et al., 2014). Positive gas potentials were reported for all lipid extracted biomass. However only Zhao et al. (2014) reported lower biogas production from extracted biomass. The extraction method used by Zhao et al. (2014) utilised a soxhlet with hexane and isopropanol as the solvent in the extraction method. Alzate et al. (2014) used ethanol but also used a thermal pre-treatment step for the disruption and the extraction process. Hernandez et al. (2014) used Supercritical carbon dioxide extraction and also utilised microwave pre-treatment. Both authors conclude that the extraction process constitutes a pre-treatment step and the more rigorous extraction methods also increase the solubilisation of cell contents allowing better conversion to methane by the AD microbial community (Hernandez et al., 2014, Alzate et al., 2014). The lower methane reported in Zhao et al. (2014) may have resulted from a less vigorous extraction technique than that used by Alzate et al. (2014 and Hernandez et al. (2014) resulting in less solubilisation of cell contents reducing the digestibility of the substrate.

The author Zhao et al. (2014) investigated the Bligh and Dyer extraction method (Bligh and Dyer, 1959) which uses chloroform mixed with solvent to extract the lipid. Chloroform has been identified as a major inhibitor in the AD process (Zhao et al., 2014). Chloroform disrupts the microbial cell membrane and compromises the cell's viability, resulting in inactivation of essential membrane functions and denaturation of essential enzymes (Yun et al., 2014). This inhibition was demonstrated during the experimental work undertaken by Zhao et al (2014) where extensive biological inhibition was reported when the Bligh and Dyer method was utilised.

Further work on the effect of chloroform on the AD process was carried out by Yun et al. (2014). Yun et al. (2014) investigated the effect of chloroform on both the acetogenic bacterial community and the methanogen archaea community. Chloroform inhibition 30, 60 and 90 concentrations for acetogenic microbial community were 138, 319, and 622 mg CHCl₃/L⁻¹, and 15, 37, 86 mg CHCl₃/L⁻¹ for the methanogen community respectively (Yun et al., 2014). Inhibition from chloroform was highlighted in Ward et al. (2014) however these more recent publications have now quantified the inhibitory concentration of chloroform on the AD microbial community. Also highlighted by this work is the higher sensitivity of the methanogen community when compared to acetogenic community this was also reported in Ward et al. (2014).

Further scientific literature on the AD of microalgae include the AD of halophytic microalgae and halophytic methanogen and acetogenic microbial communities (see chapter 4), Pre-treatment of microalgae biomass and lipid extracted biomass (see chapter 5) and AD digestate as a nutrient source (chapter 6).

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Chapter Three

STATEMENT OF AUTHORSHIP

Title of paper: Utilisation of turbidity as an indicator for biochemical oxygen and chemical oxygen demand.

Journal: Journal of Water Process Engineering

Le Anh Tu Nguyen (Equal first Author)

Performed the laboratory analysis, interpreted data and wrote manuscript and acted as the corresponding author

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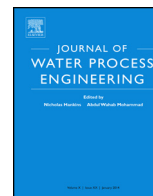
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Advanced integrated wastewater ponds

ABSTRACT

The objective for this paper was to develop a high throughput methodology to determine chemical oxygen demand (COD), five-day biochemical oxygen demand (BOD₅) and other water quality parameters via turbidity measurements of the treated effluent that has residual suspended microalgae. This methodology has twin applicability and can be utilised in wastewater management scenarios as well as pond management for the aquaculture and biofuel microalgae pond management applications. The final results indicated high correlations of turbidity with most of the studied standard parameters with the exception of BOD₅. This paper also suggested that COD is a more reliable method than BOD₅ in measuring the effluent from AIWPS®.

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1. Introduction

Frequent monitoring of effluent from wastewater treatment plants is essential for public safety. Advanced Integrated Wastewater Pond Systems (AIWPS®), which exploit microalgae for nutrient removal in municipal wastewater with the added benefit of carbon dioxide absorption and mitigation, are not exceptions. Monitoring, however, creates burdens on these systems as they are often designed for small operating flows and monitoring adds further delays in time and management of the wastewater plant. A management tool is, thus, required for rapid estimation and detection of desired monitoring parameters. A fast and reliable parameter is needed as an input measurement. While some significant parameters such as 5-day Biochemical Oxygen demand (BOD₅) need a period of six working days or 3 h for Chemical Oxygen Demand (COD) testing, the turbidity parameter is a highly probable candidate as less than 5 min is required for its analysis. Turbidity reflects the high recorded residual suspended microalgae conditions in the effluent in which AIWPS® experience.

The focus of this paper is, therefore, to initiate the development of a management tool by utilising turbidity of the treated effluent with residual suspended microalgae. This simple indicator can

be then correlated with a number of different parameters which are implicated under the current US EPA and South Australian EPA guidelines. Number of cells, total solids and volatile solids, 5-day BOD₅ and COD are the five parameters which were investigated for correlation with turbidity in this study.

1.1. AIWPS® system

A natural wastewater treatment system namely AIWPS® would provide answers to current industry challenges. The AIWPS® in which microalgae plays a key role in nutrient removal, carbon dioxide uptake and extra dissolved oxygen production is a simple upgrade from the Waste Stabilisation Pond (WSP). The system is a series of four ponds in order of Advanced Facultative Pond (AFP), High Rate Pond (HRP), Algal Setting Pond (ASP), and the final Maturation Pond (MP) [1]. While the AIWPS® has three times lower operating and maintenance cost than conventional wastewater treatment systems, only high rate ponds are currently applied in the regional Australian wastewater treatment facility (WWTF) [2].

In AIWPS®, ponds are highly integrated with one another in order to remove chemical oxygen demand, nutrients, pathogens and toxicants. The AFP with additional in-pond digesters promotes methane fermentation, disinfection, nutrient removal and odour control. The consumption of carbon dioxide from the microalgal photosynthesis in the facultative ponds upgrades methane from in pond digesters to 84% methane in the captured biogas [3]. Thus,

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electrical generation from captured Biogas is feasible. Unlike the AFP, in the HRP, the interaction between microalgae and aerobic bacteria is more profound [3]. The purpose of the algae is to provide extra dissolved oxygen for bacteria to breakdown BOD. Meanwhile, bacteria supply carbon dioxide and nutrient to promote the growth of those algae [2]. ASPs, however, have a primary focus on algae removal under gravity as the main driving force in order to meet required effluent quality management [4].

1.2. Microalgae involvement in AIWPS[®] system

The implication of microalgae in AIWPS[®] starts from the need to provide a system with sustainable energy consumption in order to compete in pollutant removal of conventional wastewater treatment [4] and later, Abdel-Raouf et al. [5] have pointed out that the photosynthetic capability of microalgae and the adsorption of nutrients namely nitrogen and phosphorus are of particular interest. In fact, the growth of microalgae increases dissolved oxygen which in turn encourages bacterial growth in order to maintain BOD removal as well as removing nutrients at the same time.

In general, the growth and species of microalgae that are present in the pond depend on the environment and location of the pond. Most microalgae species are classified under either Chlorophytes (green algae) or Cyanobacteria (blue-green algae). A study [5] found that eight most common species in natural microalgae populations were *Chlorella*, *Ankistrodesmus*, *Euglena*, *Chlamydomonas*, *Oscillatoria*, *Micractinium* and *Golenkinia*. In particular, in high rate ponds, under the mixing influence of paddle wheels, *Scenedesmus* and *Micractinium* are the two most common colonial microalgal species [2].

Microalgae also contribute to the removal of carbon dioxide which indirectly removes *E. coli*. In term of carbon dioxide, Singh and Ahluwalia [6] insist microalgae can both collect carbon dioxide (CO₂) from the atmosphere and water soluble carbon sources, in wastewater. While standard atmospheric conditions exist, which contains CO₂ at a level of 0.0387% (v/v) could not support the entire microalgae population. Thus, the main sources of carbon and specifically carbon dioxide for microalgae to undergo photosynthesis depend on bacteria during BOD breakdown processes in wastewater treatment ponds [4]. Furthermore, under rapid demand of dissolved CO₂, microalgae indirectly increase the pH value of the wastewater present in the pond to greater than 10. Under influence of microalgae, *E. coli* was efficiently removed with the recorded effluent from maturation pond at 10 MPN/100 mL, which met the US EPA guidelines for water reuse.

1.3. Limitation of AIWPS[®]

Even though, AIWPS[®] have benefits in WWT, management concerns are needed to be addressed. The first concern is the removal of total suspended solid (TSS). The primary reason is the addition of microalgae in suspension over the entire system and HRP in particular. While the impact of microalgae on nutrient removal and their tolerances with wastewater composition have been well investigated [5,7], there is limited data in regard to the effect of the high concentration of suspended microalgae in effluent.

1.4. Effectiveness of turbidity as an indicator

While, turbidity is a measure of water clarity, this parameter is an integrated measurement of suspended and dissolved particulates in the water. The methodology of measuring turbidity utilises the reflection of light that passes through the water without being scattered and absorbed. The measurements of light scattering are usually quantified and reported in nephelometric turbidity units (NTU) [8]. Under the requirement of US EPA and Australian and

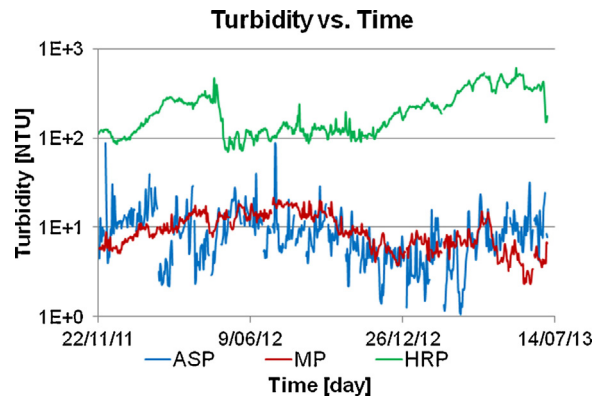


Fig. 1. Turbidity NTU measured in HRP, ASP and MP in the Delhi AIWPS[®] WWTF.

New Zealand Guidelines of Fresh and Marine Water Quality, the average discharge turbidity is to be lower than 2 NTU over a 24-h period. In addition, the instantaneous measurement should never exceed 5 NTU. The recommended turbidity should be met prior to disinfection. Currently, based on United State Environmental Protection Agency (2012) [9] use of turbidity as an indicator for total suspended solids is used in the State of Florida, US, Turbidity metres are also employed as a measure of system performance.

2. Materials and methods

2.1. Test samples

Due to the unavailability of colonial microalgae samples from an actual Advanced Integrated Pond System, water samples with a single suspended marine microalgae species (*Tetraselmis sp.*) were utilised as a sample proxy. *Tetraselmis sp.* was chosen as it is a conventional aquaculture feed utilised by the aquaculture industry [10,11] and is also utilised in the production of biofuel [12] and illustrates the twin applicability of these techniques use in other algae pond management scenarios. Under the unique growth condition in the laboratory, salt was added to the mixture at a concentration of 7.00% which was twice the concentration of natural seawater. High salinity was used for culture of this species as outlined in Fong Sing et al. [12] which details the commercial culture of this species.

The tests commenced after the *Tetraselmis sp.* growth medium provided a turbidity reading of higher than 418 NTU utilising the Hach 2100N Turbidimeter, which correspond to the average reading from high rate ponds in the Delhi AIWPS[®] WWTF during the highest recorded period between 20/03/13 and 20/06/13 (Fig. 1). Since this study purpose is to establish correlation in a wide range of turbidity which observed in a AIWPS[®]. The choice of average turbidity during highest recorded period is therefore necessary to limit the highest end value with lowest end recorded at 0 NTU. Tests were conducted in triplicate under minimum of five different turbidity values in order to establish regression models for the number of cells, total solids, volatile solid, COD and BOD₅ water quality parameters.

2.2. Cell counts

Since *Tetraselmis sp.* is considered as unicellular, direct counts are considered the best technique in examining the number of algal cells. From the previous five sets of test samples, 5 mL aliquots of well mixed sample were extracted for analysis. In order to limit microalgal cells mobility, three drops of lugol's solution were added to sample vials to kill and immobilise the cells. The sample vials containing the sample were shaken vigorously to homogenise

microalgae cells and keep microalgae in suspension. 1 mL of sample was then extracted using fine-tipped pipette. The extracted sample was then transferred to a hemocytometer with a coverslip already placed on top. The cells were then counted on the hemocytometer as described in the methods reported by [13].

The reading was conducted utilising an Olympus IX 50 microscope. Two microalgae counts were taken for each sample. For additional accuracy, equation one was utilised where the average number was multiplied by 0.98 to reduce any error from the sample counts. This method is described in more detail in [13].

$$\text{Number of cells [cells/L]} = A \times \frac{104}{0.98} \quad (1)$$

where A = average number of cell counted in the number of cells.

2.3. Total suspended solids and volatile solids

Total suspended solids (TSS) analysis was required in order to determine the quantity and soluble solids in the water samples containing the suspended microalgae (*Tetraselmis sp.*). Crucibles were initially fired at 550 °C to remove any residual volatiles. The crucible weight was then recorded before 5 mL of each sample was added. The sample filled crucibles were then placed in a Qualtex Solid-stat oven at a temperature of 104 °C until the final weight utilising four decimal place scales had stabilised with no significant change. The crucibles were then again placed in the drier. The process was repeated until no change in dry weight was observed. A volatile solid (VS) analysis was then carried out utilising the same samples. The crucibles were fired using a muffle furnace at 550 °C over a 16 h period ensuring complete combustion of all volatile matter. The same steady weight procedure was carried out in order to record the final ash weights of samples [14,15].

Since samples contained salt, a standard salt solution of 7.00% was prepared and placed under the same test conditions. The average weight of salt was then obtained in order to subtract salt content for each of five sample sets. Due to the influence of the additional salt the equations for total suspended solid and volatile solid [15] were modified as follow:

$$\text{TSS [mg/L]} = [(A - B) - (C - D)] \times 1000/V \quad (3)$$

$$\text{VS [mg/L]} = [(E - A)] \times 1000/V \quad (4)$$

where:

- A = weight of crucible + dried residue in mg,
- B = weight of crucible in mg,
- C = weight of crucible + dried salt in mg,
- D = weight of crucible in mg,
- E = weight of crucible + residue after ignition in mg, and
- V = sample volume in mL.

2.4. Five-day biochemical oxygen demand (BOD_5)

The BOD test procedure was developed under the 5-day BOD test methods reported in [14]. No seed was added as this method only determines the actual oxygen demand of living *Tetraselmis sp.* microalgae. After five days in a dark incubator, the determination of the final DO was undertaken. Standard laboratory conditions were maintained within the laboratory to ensure the correct initial and final readings from the DO probe [14].

According to the standard method, to produce meaningful results only bottles with a 2 mg/L minimum DO depletion and at least 1 mg/L residual DO is applicable for the calculation of BOD_5 under Eq. (5) [14]:

$$\text{BOD}_5 \text{ [mg/L]} = \frac{D1 - D2}{P} \quad (5)$$

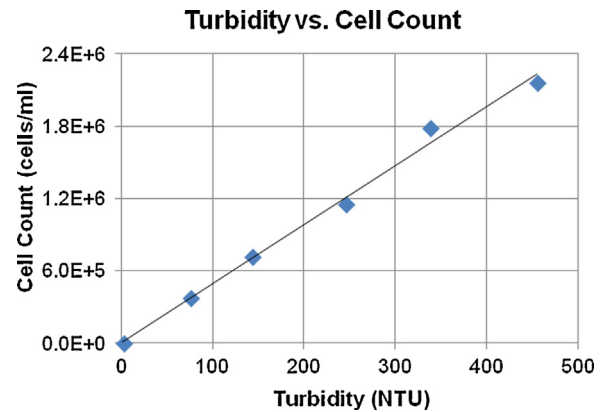


Fig. 2. Volatile solids under turbidity variation.

where:

D_1 [mg/L] = DO of diluted sample immediately after preparation

D_2 [mg/L] = DO of diluted sample after 5-day incubation period

P = decimal volumetric fraction of sample used

2.5. Chemical oxygen demand (COD)

The closed reflux potassium dichromate colorimetric method, which is outlined in [14], was determined to be the best method under the equipment availability within the laboratory. A serial dilution of 10% was applied to samples to bring them into a suitable range for the analysis. A wave length of 600 nm was set in the Shimadzu UV-vis spectrometer for the absorbance determination. The absorbance reading from the spectrometer is a direct correlation between the colour intensity which increases as the chemical oxygen demand of the sample increases. This relationship between chemical oxygen demand and absorbent has been shown to be linear, and the following regression equation was determined from running known concentration standards to form a calibration curve to describe this relationship mathematically. Thus, the change in absorbance and its correlation to COD is given by Eq. (6).

$$\text{COD [mg/L]} = 41020 \times X - 148.89 \quad (6)$$

where X = absorbent.

3. Results

3.1. Cell count, total suspended solids and volatile solids

The obtained results of turbidity in relation to cell counts suggest a linear regression with high correlation of $R^2 = 0.993$ as shown in Fig. 2. Similar trends were recognised for total suspended solids and volatile solids (Figs. 3 and 4) and with R^2 equal to 0.964, 0.978 respectively. The result is expected since turbidity is a measure of water clarity, and an integrated measurement of suspended and dissolved particulates in the water. With higher density of *Tetraselmis sp.* more light would be absorbed or scattered. Thus higher turbidity value would be observed. The use of the parameters in the model is acceptable since R^2 indicate a value greater than 0.95. The use of these regression equations is acceptable for use within the ranges analysed, however, additional testing of correlations for predictions are required for extrapolated values using univariate regression model [16]. The following equations were then established for the above parameters.

$$\text{Cell count [cells/mL]} = 4896.6 \times X \quad (7)$$

$$\text{TSS [mg/L]} = 29.959 \times X \quad (8)$$

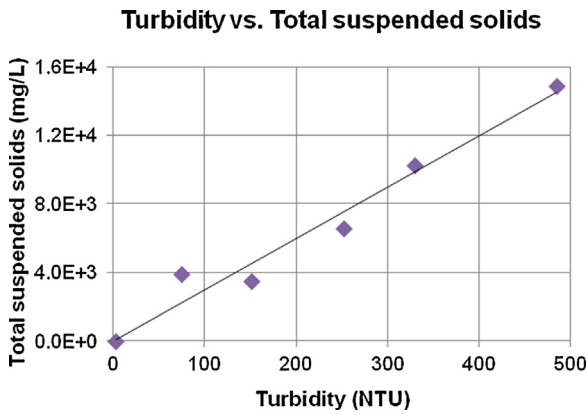


Fig. 3. Total suspended solids under turbidity variation.

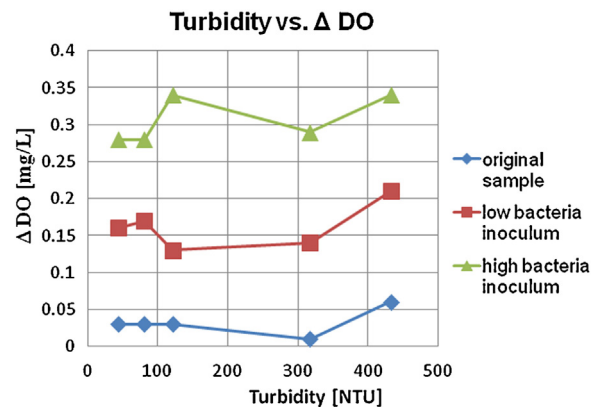


Fig. 6. The change in DO under turbidity variation.

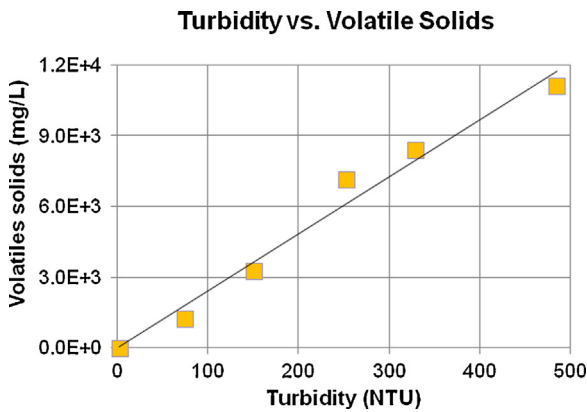


Fig. 4. Volatile solids under turbidity variation.

the results did not reach the minimum required depletion in DO of 2 mg/L under the standard condition, the calculation of BOD₅ would not produce meaningful results. From the outcome of BOD₅ experiments, with no light to support photosynthesis, the insignificant DO reading prove conclusively that there is no BOD₅ from live *Tetraselmis sp.* even with additional bacterial inoculums under the five day test regime (Fig. 6). The analysis of live microalgae in BOD₅ experiments is a concern as the microalgae may not breakdown fast enough during the period of five-day incubation. Further investigation would require the quantification of the ultimate BOD after 20 days. However, the quantification of 20 day BOD may not fully represent the oxygen demand of living algae as microalgae cells are able to resist bacterial attack [17]. Zhou et al. [18] reported live microalgae cells were detected in digestate from an anaerobic digester after 45 days. In fact, Chen and Oswald [19] found that up to 60% of untreated microalgae biomass would survive throughout the anaerobic digestion process as a result of their resistant cell wall. *Scenedesmus spp.* is another example of cell resistance to bacterial attacks, *Scenedesmus spp.* was still detected after 6 months in an anaerobic digester. During this 6 months period, the authors determined that *Scenedesmus spp.* cells had switched to mixotrophic growth and were still viable after the 6-month duration [20]. This literature highlights that the microalgae cells are very resistant to bacterial attack even in an extremely harsh bacterial environment such as an anaerobic digester.

In addition, the presence of microalgae in effluent does not grant additional adverse impacts. The presence of microalgae, in fact, indicates a negative oxygen demand because of their continuity in photosynthesis during daylight hours. The addition, microalgae is also considered to be beneficial for irrigation purposes, but could be detrimental for aquifer recharge schemes. Microalgae have been shown to be beneficial soil conditioners which promote microbial activity and improve water retention in soils [21]. In comparison to non-algae effluent, the addition of microalgae does not have a detrimental effect upon freshwater aquatic life. This, however, has the added benefit, as a carbonaceous source from biomass would be immediately consumed by fish, and other phytoplankton consuming aquatic life, upon its release into the natural water body [2].

3.3. COD experiment

Unlike the BOD₅ results, the results from COD experiment suggest a rise in the demand of oxygen as the turbidity increase (Fig. 7). A linear regression model is recognised with a correlation value of R² equal to 0.967. This correlation is similar to previous results reported for cell count, total solids and volatile solids, as COD is the measure of chemical decomposition of organic and inorganic

$$VS [mg/L] = 24.199 \times X \tag{9}$$

where X = turbidity value in NTU.

3.2. BOD₅ experiment

Under standard laboratory conditions, without the addition of seed, BOD₅ tests indicate no insignificant decline in DO (Fig. 5) with R² value is equivalent to 0.0087. Further addition of wastewater from Bolivar wastewater treatment facility was added to provide low bacterial inoculums (additional 1.5 mL of wastewater) and high bacterial inoculums (additional 4 mL of wastewater). The addition of seed was undertaken to determine if a rise in DO offset with the increase of additional bacteria in the samples. Since

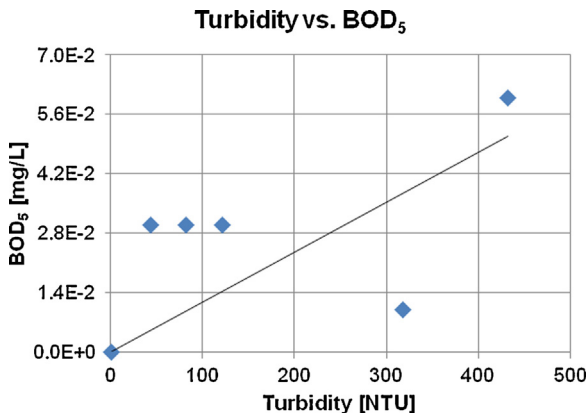


Fig. 5. BOD₅ under turbidity variation.

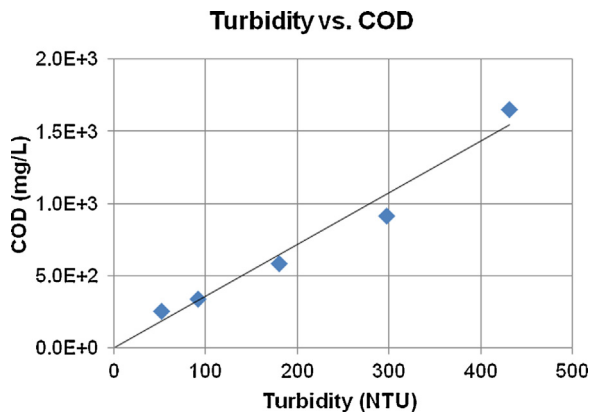


Fig. 7. COD under turbidity variation.

solids in the tested sample. Thus, as the volume of suspended solid increase, the light is absorbed or scatter and results in higher turbidity value. This outcome suggested a significant reduction can be achieved in estimating COD monitoring as the time delay could be further reduced by use of the regression model. An equation was later developed to model this correlation using the line of best fit.

$$\text{COD [mg/L]} = 3.5827 \times X \quad (10)$$

where X = turbidity value in NTU

Furthermore, there are limitations which may in turn pose influences on BOD₅ results. BOD₅ analysis requires high concentration of active, acclimated seed bacteria [14]. Pre-treatments are needed to minimise the effect of toxic waste and pH value and nitrifying organism in the reading of dissolved oxygen reading. Finally, the lack of stoichiometric validity reduces the usefulness of the obtained results [15].

On the other hand, COD in theory is corresponding to the ultimate BOD value. In reality, the COD method has a further advantage by quantifying the total demand in oxygen as some oxygen demands in wastewater are not readily oxidised under the ultimate BOD method of quantification [15]. In addition, from an operational and management point of view, COD is an efficient monitoring parameter because of its quick determination of 3.0 h [15]. Thus, COD analysis would be a more effective parameter than BOD₅ in determining the efficiency of the AIWPS[®]. However as specific laboratory equipment and hazardous chemicals are required the use of turbidity as a proxy to COD is much more desirable. The regression model for COD from the correlation with turbidity will allow rapid determination of COD and would reduce the time, expense and hazards involved with COD determination.

While both US EPA and Australian guidelines favour the use of BOD₅, COD is also adopted in the Australian guidelines as an important parameter. In fact, Tasmanian emission limit guidelines state that COD may be used in lieu of BOD testing [22]. However, the actual figure for COD is based on the type of industry for the area and the size and sensitivity of the receiving water body. According to the Australian and New Zealand Guidelines of Fresh and Marine Water Quality (2000) [23], in most cases the standard level for COD which is often set at the maximum value of 40 mg/L is considered on a case by case basis under the local EPA. The results of both the BOD and COD experiments clearly indicate that BOD does not capture the residual microalgae in effluent over a five-day period and may be overlooked by the USA EPA. Australian guidelines, on the other hand, are more rigorous and robust, and will account for this residual algae biomass as COD is also used to asset water discharge quality.

4. Conclusion

The results from laboratory experiments indicate a high correlation between turbidity and COD readings. Similar correlation regression models were recognised for number of cells, total suspended solids and volatile solids. Meanwhile, BOD₅ analysis does not fully represent the reality of the continuity in photosynthesis of living microalgae during daylight hours as well as its cell wall toughness and its resistance to bacterial attack and bacterial assisted breakdown.

The reduction of expensive laboratory tests and the minimum time delay is achievable under the management tool that is developed from the regression models utilising turbidity as an input parameter. Further development of this management tool will support the operational control for wastewater treatment plants in regional Australia and overseas operators.

Nevertheless, due to the choice of *Tetraselmis sp.* of microalgae and use of saline cultural medium as a sample proxy, the experimental outcomes are relevant to the *Tetraselmis sp.* and culture medium. Further studies and site specific ground proofing would be required for each specific site where this model is utilised. Individual site validation and calibration of regression models would be required for the various species of microorganisms located at each site. On-site calibration is also required to account for the nature of the wastewater and effluent variation at each site, and particular plant location.

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Chapter Four

STATEMENT OF AUTHORSHIP

Title of paper: Halophytic microalgae as a feedstock for anaerobic digestion.

Journal: Journal of Algal Research

Andrew Ward (First Author)

Provided the analysis used in this study, undertook culture work and microbiology analysis, collected gas and digester data, digester setup and operation drafted and editing of manuscript.

Signed..... Date 17/2/2015

Andrew S Ball (Co- author)

Supervised the study, supervised microbiology work and helped interpret data and drafting of the manuscript

Signed..... Date 17/2/15

David Lewis (Co-author)

Supervised project and drafted manuscript and help develop scientific approach

Signed..... Date 20/2/15



Halophytic microalgae as a feedstock for anaerobic digestion



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ABSTRACT

Anaerobic digestion can be employed to produce methane biogas from residual microalgae biomass derived from either a lipid based biofuel process or wastewater treatment. There is interest in using halophytic microalgae for biofuel production due to their potential robustness in large-scale open pond production. The anaerobic digestion of halophytic microalgae biomass would however be challenging due to the high salinities not typically experienced in anaerobic digestion scenarios. Halophytic microalgae biomass as a potential substrate feedstock for anaerobic digestion would have salinities in excess of 3.5%, which is typically found in marine environments. To investigate the anaerobic digestion of halophytic microalgae issue the first stage of the reported study focuses on the changes undertaken in the bacterial community associated with the anaerobic digestion of piggery effluent under increasing saline conditions, with the aim of establishing a saline tolerant anaerobic digestion inoculum capable of digesting feedstocks under high salinity conditions. Favourable results from this inoculum development study allowed the investigation of anaerobic digestion of halophytic microalgae. The reported results demonstrate that a saline tolerant inoculum was maintained. Subsequent denaturing gradient gel electrophoresis (DGGE) fingerprinting of the resulting halophytic bacterial community showed several halophytic methanogens. The inoculum was used to digest the halophytic microalgae. The resulting gas data showed that biogas production of 358 ± 53 mL/g of volatile solids (VS) with a methane content of $54 \pm 4.3\%$ methane was achieved at 7% salinity. The volume of biogas produced on a wet weight microalgae biomass basis was 122 ± 26 and 175 ± 25 mL/g of halophytic microalgae biomass respectively (74 ± 2.8 wt.% moisture content). The conversion of carbon in the feedstock to methane achieved an efficiency of 26.4% and 46.6% at 3.4% and 7% salinity respectively. A halo-tolerant anaerobic digestion microbial community could be further optimized to complete the loop with nutrient recycle required with the production of halophytic microalgae based biofuels and potentially, hypersaline wastewater treatment applications.

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1. Introduction

The anaerobic digestion of halophytic microalgae biomass offers a valuable opportunity to produce methane biogas from waste microalgae biomass derived from a lipid based biofuel process or saline wastewater treatment. This resultant methane biogas can be utilised to run a co-generation plant to produce renewable electricity and thermal energy. The production of electricity from extracted biomass could substantially reduce the requirements of electricity sourced from an electrical supply grid. The potential reduction in electrical requirements would greatly improve the commercial viability of the microalgae biofuel production operation as well as reducing the greenhouse gas emissions associated with the plant [32].

Anaerobic digestion of the residual 60–70% extracted microalgae biomass will also have the added benefit of recovering valuable nutrients, hence closing the nutrient loop associated with its production

[27,28]. Nutrients are biologically locked up within this residual microalgal biomass and anaerobic digestion has the potential to unlock these nutrients making them bio-available and suitable for regrowing microalgae biomass [24]. The use of anaerobic digestion under saline conditions is an appropriate process for integration into microalgae biofuel production and/or saline wastewater treatment, and is of utmost importance for increasing the feasibility of the microalgae biofuel industries [31,32].

The anaerobic digestion of halophytic microalgae biomass has several challenges to be addressed before the commercialisation of this technology can be beneficial for the microalgae industry. Two distinct challenges are faced when anaerobically digesting saline microalgae biomass: one being the associated high salinity from halophytic microalgae culture conditions and the second aspect is the physical characteristics of microalgae biomass as a substrate feedstock for anaerobic digestion.

Existing literature indicates that methane production decreases significantly as salinity concentrations increase [25,31]. The effects of salinity on anaerobic digestion are well documented [4,10,17,29], where it is

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suggested that anaerobic digestion can be significantly inhibited at sodium concentrations of 5745 mg L^{-1} [15]. However this inhibitory concentration was later reviewed by McCarty and was increased to 8000 mg L^{-1} [17]. This concentration is problematic to the anaerobic digestion of seawater derived biomass as surface sea water typically contains approximately $10,000\text{--}11,000 \text{ mg L}^{-1}$ of sodium [30]. Work by Lefebvre et al. [11] identified several methanogen species that are detectable at higher salinities using 16S rDNA techniques; however the specific methanogenic activity and methane production decreased as the salinity concentration increased. The authors also noted that the feedstock substrate influenced the reduction of methane by sodium inhibition [11]. Lefebvre et al. [11] indicated that when operating a reactor with distillery vinasse feedstock the reaction appeared to be inhibited at a lower salt concentration of 10 g L^{-1} compared to a second reactor operating with ethanol feedstock where inhibition was not detected until 60 g L^{-1} . This result demonstrates how the digestion feedstock substrate can influence the inhibition of the methanogenic bacteria. It is evident that independent analysis of each substrate should be undertaken to characterise its effect on the bacterial community response.

Due to the inhibitory effect of salinity on anaerobic digestion an initial investigation of a functioning piggery effluent anaerobic digestion community was undertaken utilising polymerase chain reaction denaturing gradient gel electrophoresis (PCR DGGE) analysis. This DGGE study utilised microbial community samples taken from an operating piggery effluent anaerobic digester subjected to increased salinity to investigate the response and adaptation of the microbial community to increasing saline conditions.

During the second phase of the reported study a halophytic alga, *Tetraselmis* sp., was utilised as a feedstock for bio-methane production utilising anaerobic digestion under saline conditions. Change in the microbial community during anaerobic digestion was investigated utilising PCR DGGE to profile the response and adaptation of the bacterial community. Methanogen bacteria have been shown to be highly influenced by environmental conditions when compared to the acidogenesis and hydrolysis stages of anaerobic digestion [18,25,31]. Therefore to specifically understand the activity of methanogen bacteria responsible for methane production, *Archaea* specific primers were also utilised in conjunction with universal bacterial primers. The *Archaea* specific primers target methanogen bacteria and provide a more complete understanding on the influence that increased salinity has on the methanogen microbial population.

Methane production was also recorded over a 100-day period during the second stage of the experiment allowing proper evaluation of *Tetraselmis* sp. to be evaluated for its gas potential under increased saline conditions.

2. Methods

2.1. Saline inoculum development

2.1.1. Piggery effluent feedstock source

Piggery effluent was collected at the University of Adelaide Roseworthy Campus piggery research facility, from the central piggery sump and transferred in 20 L containers. The collected piggery effluent was kept refrigerated at 4°C to inhibit any pre-digestion during storage. The effluent was used to establish a stable anaerobic digestion community for the inoculum development section used in the reported study.

2.1.2. Initial inoculum development digester conditions

A 5 L conical flask with an initial working volume of 2 L was used for the experiment. The conical flask was fitted with a stopper and an airtight tube from the stopper was connected to an inverted measuring cylinder and water displacement was used to monitor gas production. Piggery effluent was added to the inoculum digester at a rate of 500 mL per week until a total working volume of 4 L was achieved. The digester was then run for approximately 4 weeks under these

conditions until steady-state was achieved within the reactor. An aliquot (500 mL) of piggery effluent was fed to the digester over a five-day period in 100 mL aliquots. The digester was not fed on days six and seven to allow the digester time to process any undigested material. The anaerobic digester vessel was purged with nitrogen gas after each feeding to displace any oxygen that may have entered the vessel during the feeding period, and digestate was removed once the final volume was reached. The inoculum digester was placed on a magnetic stirrer hotplate and a constant temperature of 37°C was maintained during the entire experimental period. The magnetic stirrer hotplate was set at 90 rpm for the duration of the experimental period. The initial salinity of the digester was 1.6%. Gas was produced over the initial inoculum digester setup period, although the quantity and methane content are not reported. This initial procedure was undertaken to ensure a viable anaerobic digester bacterial inoculum. This stable functioning inoculum was then treated with increasing salinity to investigate the influence on the anaerobic microbial community.

2.1.3. Salinity increase of inoculum digester and sampling

Digester salinity was adjusted by adding Red Sea Aquarium Salt® to the piggery effluent feedstock. Piggery effluent feedstock was adjusted to the final treatment salinity concentrations required weekly at the start of the experimental period. Adjusted piggery effluent feedstock was stored at 4°C to reduce any pre-digestion during the experimental period. The initial increase in feedstock salinity ensured that the bacterial community present in the piggery effluent had been exposed to the higher salinities for the maximum period of time before being fed to the digester. To adjust the remaining digester volume each week, Red Sea salt was added to the remaining digester volume to give the final treatment salinities. The digester was fed (200 mL) once per week over a ten-week period. The feeding rate corresponded to a total solids (TS) loading rate of 1.42 g per week, and a volatile solid (VS) loading rate of 0.28 g per week, which is equivalent to $0.071 \text{ g VS L}^{-1}$ of the digester working volume. The adjusted lower feeding rate was utilised to reduce operational pressure upon the bacterial communities. The lower operating pressure was essential to allow bacterial communities to adjust to saline conditions without exerting pressure due to higher feeding rates. The lower feeding rates facilitate lower volatile fatty acid (VFA) production which balances the reduced metabolism and gas production rates, due to the influence of increased salinity.

The salinity adjusted digester was placed on a magnetic stirrer hotplate at a constant temperature of 37°C , which was maintained during the experimental period. The magnetic stirrer hotplate was set at 90 rpm for the duration of the experimental period. Prior to periodically increasing digester salinity, weekly samples (50 mL) were collected in Sardstedt® sample tubes and stored at -20°C for later use in bacterial DNA extractions. This sampling method was utilised for the duration of the experiment, and ensured that the bacterial communities had been exposed to the higher salinity environment for a minimum of seven days before being sampled for DNA extraction and PCR DGGE analysis.

2.1.4. DNA extraction

DNA extractions were undertaken on samples collected from the piggery effluent and the halophytic microalgae anaerobic digesters. DNA extractions were undertaken using a Fast DNA® spin kit for soil (MP Biomedicals, USA). An aliquot, (25 mL) of sample was used to conduct the DNA extractions that had an additional centrifugation step of 5 min at 1400 rpm, where the resultant concentrated pellet was then used for extraction as per manufacturer's instructions. A yield of 40 μL of DNA was obtained and stored at -20°C .

The V3 region of the 16S rDNA gene from the members of the domain Bacteria was amplified using the 314F primer with incorporation of a 40-bp GC clamp at the 5' primer end and a 518 R primer. PCR reagents and thermo-cycler conditions utilised are outlined in Muzyer et al. [21]. For members of the methanogenic *Archaea* bacterial species a nested PCR was used utilising the Met 357F/Met 1340R primers and

357F-GC/691R primer sets. Initial amplification using the Met 357F and Met 1340R primers used a touchdown PCR cycle which included a 3 min initial denaturation at 94 °C followed by 94 °C for 30 s and 68 °C for 30 s. This was followed by 72 °C for 90 s; this process was repeated ten times until the annealing temperature of 68 °C reached 58 °C. This was followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s of denaturation, annealing and extension followed by 10 min of final extension at 72 °C. The final reaction mixture (50 µL) contained 10 × PCR buffer (Sigma Aldrich Red Taq® Genomic DNA polymerase without MgCl₂) (5 µL), MgCl₂ (25 mM, 5 µL), Red Taq polymerase (1 U/µL, (2.5 µL), dNTP (10 mM, 1 µL) (Promega, Madison, WI, USA), 10 pmol (2 µL) each of forward and reverse primers and 2 µL DNA template. The second stage of the nested PCR utilised the primers 357F and 691R and was run utilising conditions outlined in Watanabe et al. [37] and 40 cycles were utilised for amplification. An aliquot (2 µL) of DNA template was used to provide a yield of 40 µL of DNA amplicon products, which were verified on a 1.2% agarose gel with SYBR® Safe then stored at –20 °C for later use (Invitrogen Life Technologies, USA).

2.1.5. PCR-DGGE of the bacterial community

Changes in the bacterial and methanogenic *Archaea* community were examined through the utilisation of PCR-DGGE, which identified diversity and dominance. Selective bacterial primers 341F GC & 518R [21] and methanogen specific primers Met 86F and Met 1340R in conjunction with 357F-GC and 691R were used to observe the microbial communities. DGGE analysis was conducted on a Universal Mutation Detection system Dcode system using a 6% polyacrylamide gel (BIORAD, USA). The denaturing gradient used for bacterial PCR amplicons was 40–60% and for the methanogen amplicons 40–55%. DGGE run conditions were 120 V at 60 °C for 10 h.

2.1.6. Statistical analysis of DGGE gels

DGGE gels were digitised and analysed using Phoretix 1D advanced gel analysis package. This software undertakes several procedures including: defining the different lanes, background subtraction, marker assisted normalisation, which includes compensating for intensity differences between lanes, and assigning the different bands in each lane. Each band was considered to be an operational taxonomic unit (OTU) and two bands in separate lanes were considered to be related if they migrated the same distance on the DGGE gel [22]. Phoretix analysis provided Gaussian values from gels and these were then used for further analysis. Dendrograms were produced from Dice–Sorensen's similarity matching index generated by Phoretix 1D software. Moving Windows Analysis (MWA) [33,34] consisted of plotting the evolution in time of the correlation values between two consecutive analysing dates. Consequently the rate of change (Δt) value can be calculated as the average of the respective window curve data points. The higher the changes between the DGGE profiles of two consecutive sampling points, the higher the corresponding moving window data point will be, and hence the higher the Δt values [14,33,34].

2.1.7. Feedstock change to microalgae

2.1.7.1. Source of biomass. The halophytic *Tetraselmis* sp. was grown in outdoor open raceway ponds located at Karratha, Western Australia, Australia (20S 45°47.72", 116E 44°9.88"). Microalgae culture conditions are described in Fon Sing et al. [7]. The biomass was harvested and concentrated to 20% solids by a T10 Evodos centrifuge and transported frozen to the laboratory. Biomass was then defrosted and resuspended to 10% TS concentration in synthetic seawater for experimental use. Prior to use the microalgae was sonicated using a Branson sonicator at a resonance of 10 kHz for 10 min to disrupt the microalgae cell wall to allow the bacteria to access the internal cell contents. Treatment salinity was also adjusted to the required treatment salinities for all treatments.

2.1.7.2. Experimental saline microalgae digester setup. Three 10 L Schott bottles with an initial working volume of 4.7 L were used for the experiment. The Schott bottles were fitted with a stopper and an air tight tube that was connected to a gas counting fluid displacement manometer fitted with a solenoid switch to release gas when a specified volume had been reached. Gas was then evacuated to a gas bag for storage and analysis. The halophytic *Tetraselmis* sp. was fed to the digester at an initial loading rate of 0.70 g VS L⁻¹ and decreased to 0.52 g VS L⁻¹ at day 50 due to the increase in overall volume and no removal of digestate. The microalgae were disrupted by sonication prior to feeding the digesters. Microalgae feedstock was stored at 4 °C to reduce any pre-digestion during the experimental period. No digestate was removed from the digester over the 50-day experimental period, hence eliminating any problems associated with bacterial washout due to short retention times. The initial digester working volume was 4.7 L and increased to 6.4 L at the end of the 50-day experimental period. The digesters were placed on magnetic stirrer hotplates and a constant temperature of 37 °C was maintained during the experimental period. The magnetic stirrer hotplate was set at 90 rpm for the duration of the experimental period.

The salinity of the 3 treatment digesters was 1.6, 3.4 and 7% respectively. Gas production and methane percentage were recorded for the 3.4 and 7% salinity treatments. Gas production for the 1.6% salinity digester was not recorded due to lower feeding rates being utilised. These lower feeding rates resulted from high dilutions, which were employed to reduce the associated salinity of the feedstock biomass. Methane was checked by an LMSxi multifunction gas analyser (Gas Data Ltd. UK). Digester pH, temperature and salinity were also recorded for the experimental period. On day 50 of the experimental period digesters were sampled for the microbial PCR DGGE investigations. The day 50 digestate samples were transferred to Sarstedt® 50 mL sample tubes and stored at –20 °C for later use in bacterial DNA extractions. The associated methods utilised in DNA extractions, PCR and DGGE protocols utilised are the same as appear in the inoculum development section of this study.

2.1.7.3. PCR identification of methanogenic Archaea. Identification of the methanogenic *Archaea* was undertaken on samples collected from the microalgae digesters on day 50 of the experimental period. Identification was conducted using the 357F and 691R methanogenic *Archaea* primers that target the conserved region on the 16S DNA. PCR amplicons underwent a cleanup stage using a Promega Wizard SV Gel and PCR Clean-Up System (Promega, USA). Products were sent to the Australian Genomic Research Facility for sequencing. PCR was conducted using a Red Taq® Genomic DNA polymerase and dNTP mix on a BIORAD T100 Thermocycler (Sigma Aldrich; Promega, USA; BIORAD, USA). Excised bands were identified using BLAST nucleotide sequences and were analysed using Sequencher software (Sequencher version 4.1.4). Homology searches were carried out with the BLAST server of the National Centre for Biotechnology Information (NCBI) using a blast algorithm (<http://www.ncbi.nlm.gov/libary.vu.edu/BLAST/>) for the comparison of a nucleotide query sequence database (blastn).

3. Results

3.1. Inoculum development (stage 1)

Gas was produced over the entire ten-week experimental period; however a much lower volume was noted for weeks 6, 7 and 8 although gas production did increase in the final two weeks. As this was the initial inoculum development investigation, the exact quantification of gas was desirable; however it was not essential due to the focus of this study being on the profiling of the microbial community by PCR DGGE. Table 1 shows the average weekly parameters of pH and salinity (%) and equivalent ionic conductivity (ms cm⁻¹) value over the experimental duration.

Table 1
Sample salinities, pH and the number of bacterial and methanogen OTUs detected by DGGE in piggery digester samples.

Week	pH	Salinity (%)	Salinity (ms cm ⁻¹)	Number of bacterial species detected	Number of Methanogen species detected
1	7.87 ± 0.01	1.6	19,428	28	12
2	7.77 ± 0.09	2.2	26,714	28	12
3	7.69 ± 0.02	2.8	233,999	29	11
4	7.70 ± 0.04	3.4	41,285	30	11
5	7.74 ± 0.02	4.0	48,571	30	11
6	7.78 ± 0.04	4.6	55,857	27	10
7	7.87 ± 0.10	5.2	63,142	29	10
8	7.87 ± 0.06	5.8	70,428	31	7
9	7.86 ± 0.04	6.4	77,714	28	9
10	7.65 ± 0.04	7.0	85,000	27	8

The microbial communities present in digester samples were assessed by DGGE using PCR-amplified V3 regions of the 16S rDNA fragment. Bands of the DGGE profile correspond to different PCR-amplified 16S rDNA fragments obtained from the different species. Duplicate PCR amplicons also resulted in identical DGGE patterns for all DGGE gels produced. DGGE results indicate a total of 31 different dominant operational taxonomic units (OTUs), or species of bacteria, detected in the piggery effluent digester over the ten-week period. Table 1 illustrates the number of bacterial OTUs that were detected at a particular salinity. Only the 5.8% salinity had all 31 OTUs of bacteria detected. The other salinities ranged from 27 to 30 bacterial OTUs detected within the anaerobic digester community.

The DGGE profiles generated from the bacterial community sampling were also analysed utilising MWA [33]. The MWA was used to quantify the change in the bacterial community over time as a result of the increasing salinity gradient applied to the bacterial community. The rate of change per week calculated for the piggery digester bacterial samples was $9.89 \pm 8.58\%$ per week over the experimental period. The MWA scores for the bacterial and methanogen populations are shown in Fig. 2.

A total of 14 different bands, indicative of dominant methanogen specific OTUs were detected in the piggery effluent digester over the ten-week period. Table 1 lists the number of OTUs that were detected at a particular salinity. The maximum number of methanogen OTUs detected was 12 in the 1.6 and 2.2% salinity treatments. After the 2.2% salinity treatment the number of methanogen OTUs detected decreased as the salt concentration increased. This decreasing trend in methanogen OTUs detected continued until a salinity of 5.5%, where only seven bands representing methanogen bacteria OTUs were detected. This trend however reversed and the number of OTUs detected began to increase after 5.8% salinity with 9 OTUs detected. This dropped to 8 OTUs detected at the highest 7.0% salinity treatment.

The dendrogram analysis of the DGGE gel utilising the methanogen primer sets clearly detects six different salinity groups of methanogens over the salinity gradient (Fig. 1, A). The dendrogram analysis groups the 1.6 and 2.2% salinity treatments as one group. The 2.8, 3.4 and 4.0% salinity treatments were found to be 95% similar to that group comprised of the 1.6 and 2.2% salinity treatments. The third group comprising of the 4.6 and 5.2% salinity treatments was found to be 93% similar to that of the 2 lower salinity treatment groups. The largest difference of 30% was found between the lower salinity groups under 5.2% salinity and the higher salinity groups. The higher salinity treatments were further grouped into the 5.8% salinity treatment where it was found to be 84% similar to the 6.4 and 7% salinity treatments. The 6.4 and 7.0% salinity treatments were found to be 92% similar to each other.

The results from the MWA showed that the methanogen population changed at a rate of $7.11\% \pm 10.07\%$ per week over the experimental duration and the bacterial species changed at a rate of $9.89 \pm 8.58\%$ per

week. The MWA score for both the bacterial population change and the methanogen population change over the experimental period is shown in Fig. 2.

3.2. Anaerobic digestion of saline microalgae biomass

3.2.1. Gas production

Gas production data including mean bio-gas production and methane percentage are shown in Table 2. All treatments produced biogas; however, no gas production and methane data were kept for the 1.4% salinity treatment. This was due to the need for constant dilution of feedstock due to increases in salinity due to the residual salt within the halophytic *Tetraselmis* sp. even though it had been washed and re-suspended in Milli Q water.

Biogas production over the 50 day period averaged 249 ± 54 mL and 358 ± 53 mL/g of VS fed to the 3.4% and 7% salinity digesters respectively. This equates to approximately 111 and 179 mL of methane when applied to the gas volume. The volume of biogas produced on a wet weight microalgae biomass basis was 122 ± 26 and 175 ± 25 mL/g of microalgae biomass respectively (74 ± 2.8 wt.% moisture content). This equates to 54 mL and 94 mL of methane per gramme of wet weight microalgae biomass. On a carbon conversion basis the conversion of carbon content in feedstock to methane a conversion efficiency of 26.4% and 46.6% was recorded for the 3.4 and 7% salinity treatments respectively. Carbon conversion in the feedstock to carbon dioxide was 12% and 21% for the 3.4 and 7% salinity treatments. This left a residual carbon content of 61.6% and 32.4% within the digestate. The pH of all digesters remained within the 6.5–7.6 range reported for efficient anaerobic digestion [25], indicating that there were no deleterious effects within the digesters.

The microbial communities present in the microalgae feedstock digester were assessed by DGGE using PCR-amplified V3 region of the 16S rDNA fragment. Samples were collected from digesters 50 days after the commencement of the study. A total of 20 different OTUs of bacteria were detected by DGGE fingerprinting in the piggery effluent digester at day 50. The number of bacterial OTUs that were detected for each of the experimental salinities tested is shown in Table 2. No treatments had the total number of OTUs detected and approximately 12 OTUs were detected at any one time from the 20 total bacterial OTUs detected. However the bacterial species detection rate was found to increase by 1 to 13 bacterial OTUs detected for the high salinity treatment.

A total of 13 different dominant methanogen OTUs were detected in the microalgae digester samples taken at day 50 of the experimental period. The number of methanogen OTUs that were detected at the three different treatment salinities is shown in Table 3. The maximum number of methanogen OTUs detected was 10 in the 7% salinity treatment, this then decreased to 9 OTUs in the 3.5% and further decreased to 7 methanogen OTUs in the 1.6% salinity treatment.

The dendrogram analysis of the DGGE gel utilising the methanogen primer sets clearly detects three different groups of methanogens over the salinity gradient, the 1.6 and 3.5% salinity treatments were grouped as one group and indicated that they have 75% similarity to each other (Fig. 1, B). The third group detected by Phoretix was the high salinity 7% treatment. This treatment has been determined to be 60% similar to the 1.6 and 3% salinity anaerobic digestion methanogen populations. This is the largest difference detected between the methanogen populations within the microalgae anaerobic digesters. The methanogen dendrogram produced for the day 50 samples, taken from the three different salinity microalgae anaerobic digesters, is shown in Fig. 1.

3.2.2. DGGE analysis of PCR amplicons sequencing

Excised bands were identified using BLAST nucleotide sequences and were analysed using Sequencher software (Sequencher version 4.1.4) (Fig. 1, B). Homology searches were carried out with the BLAST server of the National Centre for Biotechnology Information (NCBI) using a

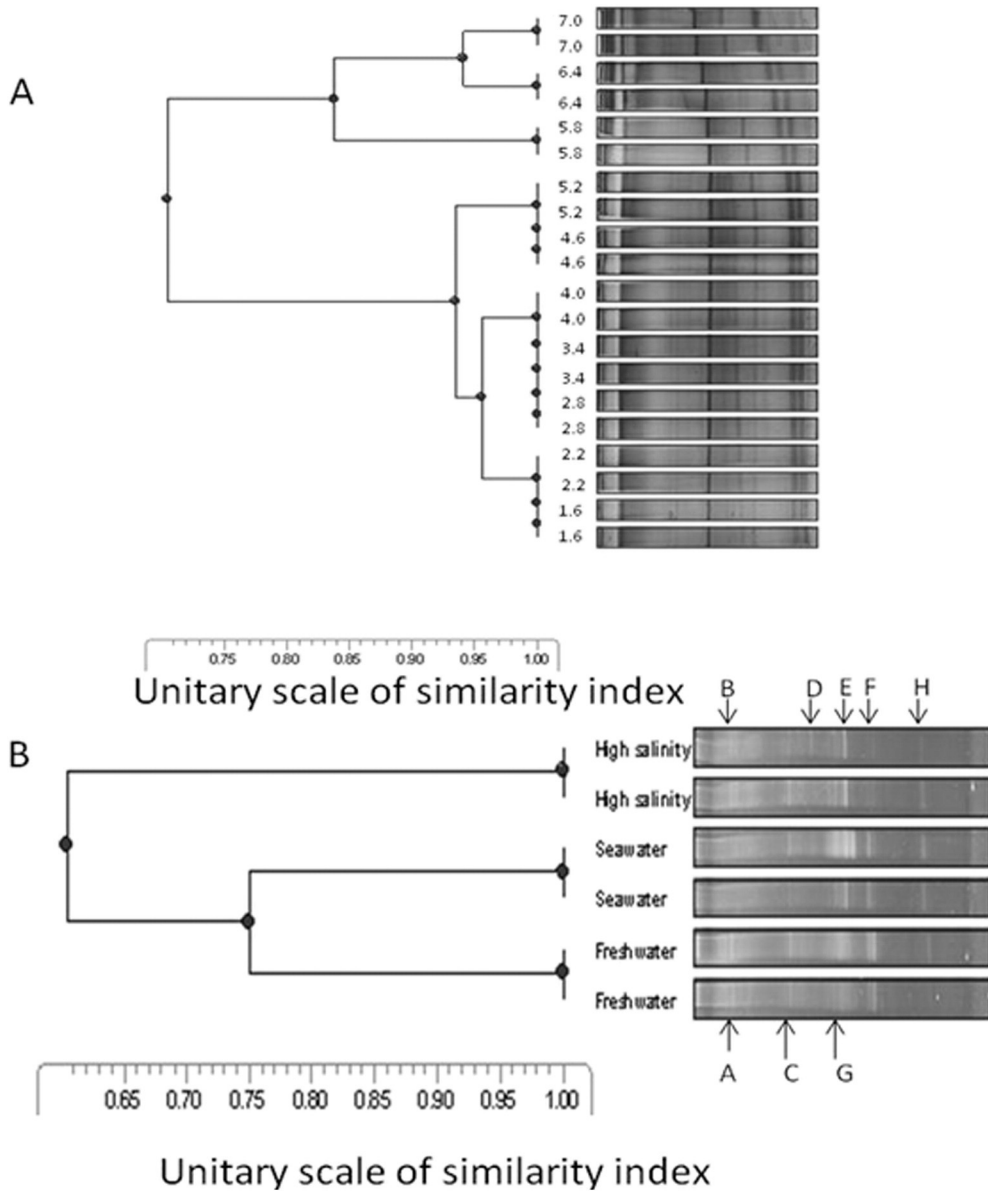


Fig. 1. UPGMA dendrogram constructed from Dice–Sorensen's similarity matching index produced from DGGE profiles generated using Phoretix advanced analysis package for: A) piggy effluent methanogen archaeal community for salinities 1.6 through to 7% salinity and B) microalgae methanogen community for the 1.6, 3.5 and 7% salinity treatments. The scale bar represents a unitary scale of similarity, and arrows indicate positions on the profile where bands particular to a specific lane or group of lanes were analysed.

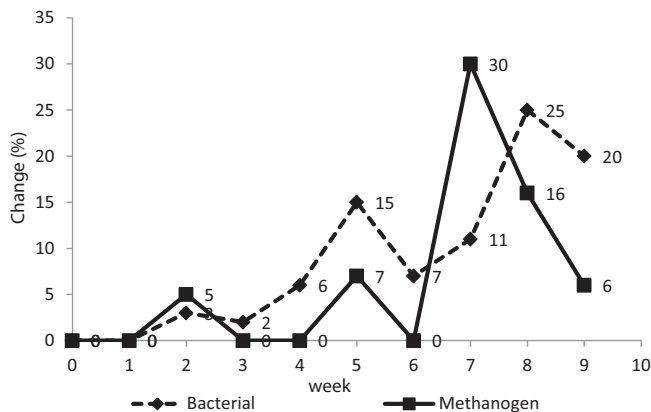


Fig. 2. MWA correlation for the piggy effluent bacterial inoculum and the piggy methanogen inoculum over the weekly experimental salinity treatment sampling periods.

blast algorithm (<http://www.ncbi.nlm.gov.library.vu.edu/BLAST/>) for the comparison of a nucleotide query sequence database (Blastn) (accessed on 18/07/2014). All blast sequences and their corresponding matches are shown in Table 4.

4. Discussion

The initial experimental anaerobic digester was originally inoculated and allowed to run at steady-state for a four week period to ensure a viable bacterial community existed. Stable conditions within the digester during the initial four weeks and commencement of the salinity adjustment were confirmed by the MWA results. Analysis of DGGE data by MWA confirmed that the bacterial and Archaeal communities were stable, as MWA analyses indicated a score of zero for both communities (Fig. 2). Wittebolle et al. [33] established that a low MWA score indicated stable bacterial communities.

Several methanogen OTUs were no longer detectable by DGGE analysis at the higher salinity treatments. This non-detection appears to be at a higher concentration than that presented in the scientific literature

Table 2

Biogas production, methane content, carbon dioxide content and pH for microalgae anaerobic digesters, where appropriate the results are the means of three measurements with standard errors given.

Treatment	Biogas production (ml g VS)	Mean methane content (%)	CO ₂ content (%)	pH
1.6% salinity	N/A	N/A	N/A	7.44 ± 0.07
3.4% salinity	249 ± 54	44 ± 8.99	21 ± 3.16	7.50 ± 0.13
7% salinity	358 ± 53	54 ± 4.3	27 ± 1.54	7.40 ± 0.05

[17,25,32]. The detection of 6 separate methanogen OTUs across the entire salinity gradient and the two additional methanogen OTUs detected in the 6.4 and 7% salinity treatments indicate a positive outlook for the anaerobic digestion process at higher salinities.

The initial digester feeding rate was reduced at the start of the experimental increase in salinity adjustment to the digesters. The initial start-up feeding rate of 1.42 g/TS per week was reduced to 0.46 g/TS per week for the experimental duration. The lower feeding rate of 0.46 g/TS was implemented to accommodate lower gas production from methanogen bacteria due to the possible detrimental effects from higher salinities, hence reducing the chances of souring or overloading digesters. McCarty [16] stated that methanogen bacteria are the slowest and most vulnerable step to environmental pressures in the anaerobic digestion process. The methanogens utilise volatile fatty acids produced from the breakdown of organic matter by acidogenic bacteria to produce methane [18,25]. This lower feeding rate would have allowed a decrease in methanogen bacterial activity without a build-up of volatile fatty acids due to the reduced conversion of VFAs to methane. No souring or crashing of the digester was observed (as confirmed by pH data). The pH remained relatively stable over the experimental period indicating no acidification or souring of the digesters by a build-up of VFAs and was within the optimal range for digestion reported by Parkin and Owen [25].

DGGE offers a powerful tool for the investigation of the dominant community structure and to analyse the structural variation of the dynamics of the system when influenced in this case by an increasing salinity gradient [21]. For the analysis of the DGGE profiles it was assumed that each band represented one bacterial species or OTUs. However Muyzer and Smalla [20] indicate that bands within a DGGE gel are not necessarily derived from the same species. To address this issue preliminarily DGGE gels were undertaken and from this a gradient that was very narrow and gave good separation of bands was utilised. The bacterial primer set 341F and 518R indicated a total of 47 OTUs. However the number of OTUs in a single salinity treatment ranged from 27 to 31 and only differed by 4 OTUs over the salinity gradient. The rate of change recorded by MWA indicated a rate of $9.89 \pm 8.58\%$ per week and the methanogen population recorded a $7.11 \pm 10.07\%$ rate of change per week as the salinity increased over the experimental period.

Due to the detection of high salinity methanogen OTUs and positive gas production at 7% salinity, further anaerobic digestion utilising halophytic microalgae as a feedstock with this anaerobic digester as a seed source for inoculum was considered a viable option for further investigation.

Positive biogas production from saline *Tetraselmis* sp. microalgae was recorded in the 3.4 and 7% salinity treatments during the

Table 3

Number of OTUs detected in the microalgae anaerobic digesters after 50 days utilising bacterial and methanogen primers.

Treatment	Salinity (ms cm ⁻¹)	Number of bacterial OTUs detected	Number of methanogen OTUs detected
1.6% salinity	21,250	12	7
3.4% salinity	42,500	12	9
7.0% salinity	85,000	13	10
Total number of OTUs detected		20	13

experimental period. Increased biogas results for the 7% salinity treatments compared to the 3.5% salinity treatment further validate that a halo-tolerant anaerobic digestion bacterial community had been established. Gas data is shown for the 3.4 and 7% salinity treatments in Table 3. Methane biogas production was approximately 44% higher in the high salinity (7% salinity) treatment when compared to the 3.4% salinity (seawater salinity) treatment.

Biogas production reported in the 7% salinity treatment was 193 mL g VS for *Tetraselmis* sp., representing increased biogas production at a salinity of 7% when compared to lower salinity treatments. Asinari Di San Marzano et al. [1] reported a methane production of between 250 and 310 mL of methane per g/VS. However this gas production was recorded in freshwater conditions, as the microalgae had been re-suspended in freshwater. Mottet et al. [19] reported methane yield from halophilic methanogens at 35 g L⁻¹ of salinity (3.5% salinity) and this was close to their defined reference value of methane production, however methanogenesis was considerably reduced at the higher salinities tested. Methane biogas production under high salinity conditions has been reported by Zhang et al. [36], where anaerobic digestion of sucrose at 5% salinity was shown. However this gas was produced when electrical anodes and cathodes were added to the digester. The addition of electrodes to the digester was to overcome the accumulation of VFA in the form of propionate. No build-up of propionate or VFA (volatile fatty acid) was observed in this study, which was demonstrated by the stable pH of the 7% and 3.4% salinity treatments (Table 3).

Results of the microalgae anaerobic digestion bacterial primer DGGE fingerprinting identified a total of 20 different bacteria OTUs present in the microalgae anaerobic digester samples. Between 12 and 13 of these bacteria OTUs were dominant at one of the three treatment salinities sampled. Results indicate that no specific trend was associated with the increase in salinity and this result was similar to the previous bacterial DGGE fingerprinting of the piggery effluent inoculum digesters in the inoculum development stage of this study.

However the initial piggery effluent inoculum digester recorded approximately 30% more OTUs than that detected in the microalgae anaerobic digesters. There was a lower number of bacterial OTUs detected in all three salinity treatments for the halophytic microalgae digesters. No specific trend indicating a decrease in bacterial OTUs was detected in both the halophytic microalgae and piggery effluent digesters, indicating no adverse effects from the increased salinity. The highest salinity treatment in the piggery effluent inoculum development work recorded 27 different bacterial OTUs at 7% salinity compared to only 13 OTUs in the 7% microalgae anaerobic digesters. It is therefore suggested that the lower number of bacterial OTUs detected during the operation of the digesters using halophytic microalgae feedstock was due to the elimination of piggery effluent and its associated bacterial load as a feed stock.

Analysis of the methanogen DGGE gels for the halophytic microalgae anaerobic digesters gave a differing result when compared to the results presented for the methanogen piggery effluent inoculum development digesters. A total of 13 different methanogen OTUs were detected over the three experimental salinities tested. From these 13 OTUs only 10 of the OTUs were detectable in any one of the three treatment salinities tested.

Although methanogen OTUs were detected at all three treatment salinities tested, the halophytic microalgae day 50 samples indicated that the highest number of methanogen OTUs detected was in the 7% salinity

Table 4
Similarities of sequences obtained from excised DGGE bands to sequences in the NCBI database (Blastn) and taxonomic position of the OTUs (database queries run on the 18/07/2014).

Excised DGGE bands	Accession no.	Closest relative (NCBI database)	Taxonomic group	E-value	Sequence number	Max identity	Reference
A	JF980398	Uncultured archaeon clone ADP20 16S ribosomal RNA gene, partial sequence	Archaea	1e–63	1	95%	Xia et al. [35]
B	NR_074094.1	<i>Methanococcus maripaludis</i> S2 strain S2 16S ribosomal RNA, complete sequence.	Archaea	0.31	2	86%	Hendrickson et al. [8]
C	NR_117705.1	<i>Methanospirillum stamsii</i> strain ps 16S ribosomal RNA gene, partial sequence.	Archaea	3e–35	3	95%	Parshina et al. [26]
D	NR_112981.1	<i>Methanospirillum lacunae</i> strain Ki8-1 16S ribosomal RNA gene, partial sequence.	Archaea	6e–32	4	93%	Lino et al. [12]
E	NR_102902.1	<i>Methanoplanus petrolearius</i> strain DSM 11571 16S ribosomal RNA gene, complete sequence.	Archaea	2e–76	5	93%	Brambilla et al. [2]
F	NR_028243.1	<i>Natrialba magadii</i> strain ATCC 43099 16S ribosomal RNA gene, complete sequence.	Archaea	3e–15	6	83%	Lodwick et al. [13]
G	NR_028225.1	<i>Methanogenium marinum</i> strain AK-1 16S ribosomal RNA gene, partial sequence.	Archaea	3e–105	7	95%	Chong et al. [5]
H	AB656041.1	Uncultured archaeon gene for 16S rRNA, partial sequence, Clone: A0610D001_101	Archaea	3e–35	8	88%	Itoh et al. [9]

treatment. DGGE fingerprinting undertaken on day 50 of the microalgae digester experiments indicates that there was a total of 13 methanogen OTUs detected and an increase in the number of methanogen OTUs in all treatments except for the 1.6% salinity treatment where only 7 methanogen OTUs were detected.

Lefebvre et al. [11], Omil et al. [23], and Feijoo et al. [6] suggested that the adaptation by the methanogenic communities to tolerate higher salinities and other detrimental environmental and inhibitory chemicals is made possible by the bacterial communities. This point was also raised by Buxy et al. [3] where they reported higher gas production under saline conditions after running an experiment for the second time. The ability of the anaerobic digestion bacterial communities to adapt to environmental change via their entrained environmental plasticity could offer a promising method to adapt a bacterial community to work at higher salinities; however few positive outcomes are reported in the scientific literature at 7% salinity as reported in this study. The results reported for this study validate findings by Lefebvre et al. [11], Omil et al. [23] and Feijoo et al. [6] and suggest that the inoculum development experiments influenced the methanogens into an adaptation period where they have become more tolerant to saline conditions and may now favour higher salinities than at the initial piggery effluent experimental salinity of 1.6%. Dendrograms generated from the DGGE methanogen microalgae gel by Phoretix software indicate that the 1.6% salinity treatment and the 3.4% treatment were 74% similar that the two methanogen populations are highly related. This similarity decreases to 60% for the high salinity treatment indicating that the high salinity 7% treatment methanogen community is 40% different to the 1.6% and the 3.4% salinity treatment. This result also suggests that a halo-tolerant community of methanogens are present within the 7% salinity treatment.

DNA sequences that were obtained from DGGE methanogen analysis were compared to sequences in the NCBI database and identified. Nucleotide sequence data (Table 4) illustrate that *Archaea* methanogens are present within the digesters and were detected by the DGGE fingerprinting technique. From the identification analysis all detected OTUs were matched to *Archaea*. From this identification excised bands A and G were identified as uncultured *Archaea* OTUs, samples C and G have a high (95%) similar to the *Methanospirillum stamsii* and *Methanogenium marinum* species of methanogen. Lower similarities (93%) were reported for excised bands D and E that were found similar to *Methanospirillum lacunae* and *Methanoplanus petrolearius* species of methanogen. Sample B was (86%) similar to *Methanococcus maripaludis* and sample F was (83%) similar to the *Natrialba magadii* strain of methanogen species. From the identified *Archaea* samples, bands B and E were found in samples associated with offshore drilling oil wells [2,8]. Bands A and C have been detected and reported in functioning anaerobic digesters [26,35]. Bands D and H have been detected in

environmental soil samples [9,12]. While samples G has been detected in a marine sourced sample [5] and sample F was found to be a Haloalkaliphilic *Archaea* methanogen [13].

The positive gas production and the DGGE fingerprinting analysis clearly identify the establishment of a halo-tolerant anaerobic digestion community. The reduction in biogas and reduction in detected methanogen OTUs identified in the 3.4% compared to the 7% salinity treatment during this investigation illustrates the operation and performance of a halo-tolerant anaerobic digestion community operating at conditions of 7% salinity.

5. Conclusion

A molecular investigation of the influence of salinity to a working piggery effluent digester was undertaken during this study. Results indicated biogas production at 7% salinity and a transformation in the methanogen community. This methanogen community was then used to inoculate digesters in the halophytic microalgae anaerobic digestion study. The halophytic microalgae anaerobic digestion study further confirmed the transformation to a halo-tolerant anaerobic digestion community. Positive gas production at 7% salinity indicated that the halophytic *Tetraselmis* sp. was suitable as a substrate for anaerobic digestion resulting in methane production. It is envisaged that this halo-tolerant anaerobic digestion microbial community could be further developed and may play a crucial role in closing the loop in the nutrient cycle associated with the production of halophytic microalgae based biofuel production systems. Further development of this halo-tolerant anaerobic community could also be utilised to anaerobically digest other hyper saline waste streams providing additional pathways for waste treatment.

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Chapter Five

STATEMENT OF AUTHORSHIP

Title of paper: Pre-treatment options for halophytic microalgae and associated methane production.

Journal: Bioresource Technology

Andrew Ward (First Author)

Performed laboratory analysis supervised study and developed scientific approach and methods utilised. Prepared manuscript

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15/02/2015

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Supervised project, experimental development and drafted manuscript

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Short Communication

Pre-treatment options for halophytic microalgae and associated methane production

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HIGHLIGHTS

- All anaerobic digestion successful at 7% salinity (2× seawater salinity).
- Methane production rate of 122 mL per g VS for lipid extracted *Tetraselmis* sp.
- Methane production rate of 252 mL per g VS for non-disrupted *Tetraselmis* sp.
- Methane production rate of 248 mL per g VS for pretreated disrupted *Tetraselmis* sp.
- Microbial degradation of *Tetraselmis* sp. demonstrated during anaerobic digestion.

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ABSTRACT

Methane production from lipid extracted, pre-treated disrupted and non-pretreated *Tetraselmis* spp. microalgae was investigated. The results demonstrated that 122 mL per g VS methane was produced for the lipid extracted *Tetraselmis* spp., demonstrating that lipid free *Tetraselmis* can be effectively digested in an anaerobic environment. A total of 252 mL per g VS and 248 mL per g VS of methane was reported for non-disrupted and pre-treated disrupted *Tetraselmis* sp. respectively. It was also observed that the microbial community caused cell lysis of *Tetraselmis* spp. during the anaerobic digestion process. Cell lyses can offer a direct conversion pathway of intact *Tetraselmis* spp. for energy production, thus negating the need for pre-treatment.

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1. Introduction

Anaerobic digestion of residual microalgae biomass after lipid extraction for lipid based biofuel production provides the ability to recover nutrients whilst producing methane for conversion to electrical and thermal energy (Ward et al., 2014). The anaerobic digestion of intact microalgal biomass offers great potential for biogas production from microalgae based wastewater treatment systems (Golueke et al., 1964).

One of the major problems associated with the anaerobic digestion of microalgae is the need to break the cell wall allowing the cell contents to be processed by the bacterial community to form precursor chemicals for the formation of methane biogas (Chen and Oswald, 1998; Gonzalez-Fernandez et al., 2012b; Mussgnug

et al., 2010; Samson and Leduy, 1983; Sialve et al., 2009; Ward et al., 2014).

Golueke et al. (1957) demonstrated the ability of *Scenedesmus* spp. and *Chlorella* spp. of microalgae to pass through an anaerobic digester intact and remain undigested. The authors noted that *Scenedesmus* spp. and *Chlorella* spp. microalgal cells are known to effectively resist bacterial attack, and the authors detected intact microalgae cells in the digestate after a 30-day hydraulic retention time.

Research undertaken by Mussgnug et al. (2010) highlighted the role of the cell wall in the digestion process. Mussgnug et al. (2010) results indicate that the highest gas production reported was due to microalgae species that had either no cell wall or a protein based cell wall. Gas production was observed to decrease for microalgal species that had a carbohydrate-based cell wall containing hemicellulose. The lowest gas production reported came from the species *Scenedesmus obliquus* that has a particular rigid cell wall containing a large proportion of sporopollenin like biopolymers.

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Little or no cell wall degradation was detected in *S. obliquus* and very low methane volumes were produced by the microalgae substrate when anaerobically digested. The authors [Musgnug et al. \(2010\)](#) and [Gonzalez-Fernandez et al. \(2012a\)](#) both concluded that the degradation of the cell wall was strongly correlated to the amount of biogas produced during anaerobic digestion.

Multiple authors indicated the need for a pre-treatment step to disrupt the cell wall increasing bacterial hydrolysis during anaerobic digestion ([Golueke et al., 1957](#); [Musgnug et al., 2010](#); [Ward et al., 2014](#)). The various mechanical, physical, thermal and chemical methods used to improve microalgae methane potential have a high energy investment ([Lee et al., 2013](#)). [Lee et al. \(2013\)](#) have shown that the disruption or cell lyses of *Tetraselmis* spp. of microalgae is higher than the energy content of the *Tetraselmis* spp. cell. Therefore the method of cell disruption and biomass treatment plays a critical role in energy utilisation and overall commercial feasibility of a microalgae based biofuels production and microalgae wastewater treatment systems.

This reported study investigates the role of cell disruption and biomass lipid extraction by comparing methane gas potentials from anaerobically digested *Tetraselmis* sp. with 3 different pre-treatment scenarios. The first treatment comprises microalgae that have been disrupted and the lipid content extracted with hexane, as is the standard procedure for lipid based biofuel production ([Pragya et al., 2013](#)). The second treatment evaluates the gas potential of disrupted *Tetraselmis* sp. biomass, and the third treatment investigates un-disrupted, un-treated, direct digestion of intact *Tetraselmis* sp. biomass for methane potential.

2. Methods

2.1. Source of microalgae

The microalgae *Tetraselmis* sp. (MUR 233) was grown in outdoor open raceway ponds located in Karratha, Western Australia, Australia (20S 45°47.72", 116E 44°9.88"). Microalgae biomass was first harvested utilising electroflocculation and then further concentrated to 20% dry weight solids by centrifugation utilising a T10 Evodos centrifuge and transported frozen to the laboratory. Biomass was then defrosted and resuspended to 10% solids w/w content for experimental use. Prior to use, the disrupted and lipid extracted microalgae biomass treatments were sonicated using a Branson sonifier at a resonance of 10 kHz for 10 min to disrupt the microalgae cell wall. The lipid extracted biomass was prepared from dried *Tetraselmis* spp. that was solvent extracted (Hexane) using a soxhlet apparatus. After lipid extraction the residual biomass was dried to remove any entrained solvent and resuspended in saline water (7%) at 10% solids w/w concentration for experimental use. For the third treatment, intact *Tetraselmis* spp. was used with no pre-treatment.

2.2. Digester setup

Twelve 500 mL Schott bottles with an initial working volume of 450 mL were used for the experiment, allowing four separate treatments in triplicate. The four treatments consisted of non-disrupted *Tetraselmis* spp., sonicated disrupted *Tetraselmis* spp., extracted *Tetraselmis* spp. and a control inoculum treatment. This inoculum was sourced from a anaerobic digester operating at 7% salinity ([Ward et al., 2015](#)). The control treatment contained only the inoculum volume as used in all treatments, and biogas produced from the control treatment was deducted from the biogas volumes produced from the three experimental treatments. The Schott bottles were fitted with a stopper and an air tight tube, which was connected to an inverted measuring cylinder. The displacement of

water within the measuring cylinder was used to quantify the gas volume produced, which was recorded and reset daily. The methane content was determined using a SRI 8610C gas chromatograph (SRI Instruments, Torrance, CA) fitted with a thermal conductivity detector (TCD), using helium as a carrier gas in a 0.3-m HaySep-D packed Teflon column with a capillary 6' silica gel column and a capillary molecular sieve column (6' MS13X) ([Labatut et al., 2011](#)). A total of 2.43 g of the *Tetraselmis* spp. was fed to the digester at the start of the experimental period, which equated to a loading rate of 5.4 g VS L⁻¹ per replicate. The *Tetraselmis* spp. feedstock was stored at 4 °C to prevent pre-digestion prior to use during the experimental period. The treatment digesters were placed in a water bath held at 37 °C for the duration of the experiment. The Schott bottles were shaken daily to resuspend settled material. Gas production and the corresponding methane percentage were recorded for all treatments over the experiment. The *Tetraselmis* spp. feedstock was characterised for total solids (TS) and volatile solids (VS) content. The TS content of the *Tetraselmis* spp. feedstock was determined by drying the samples at 50 °C until a constant weight was recorded. The VS content was determined using standard wastewater methods ([Clesceri et al., 1998](#)), where the oven dried samples were placed in pre weighted crucibles and then ignited for 2 h at 550 °C in a muffle furnace. The data was analysed for significant differences utilising a One-way ANOVA of variance with least significant difference test utilising the software package SPSS version 21. All data was checked for population normality and homogeneity of variance prior to analysis.

3. Results and discussion

All treatments produced biogas. The pH ranged between 7.22 and 7.88 for the control treatment. This was significantly different to the non-disrupted, disrupted and extracted treatments ($P = 0.00$), although no significant difference was found between the non-disrupted, disrupted and extracted treatments ($P = 1.00$). The pH for the non-disrupted, disrupted and extracted *Tetraselmis* spp. treatments ranged from 6.79 to 7.11 for the non-disrupted, 6.80 to 7.29 for the disrupted and 6.78 to 7.41 for the extracted biomass treatment respectively. The reduced pH within the non-disrupted, disrupted and extracted *Tetraselmis* spp. treatments was within the optimum anaerobic digestion operational pH range of 6.5–7.6 reported by [Parkin and Owen \(1986\)](#). This initial decrease in pH is believed to be due to the solubilisation of organic material and production of volatile fatty acids (VFA's) produced from the degradation of *Tetraselmis* spp. by the acetogenic bacteria. Increasing pH was noted in the non-disrupted, disrupted and extracted *Tetraselmis* spp. treatments during later stages of the experimental period. This indicates that the methanogenic bacteria were utilising the precursor VFA's and converting them to methane, which indicated that the acetogenic and methanogenic bacterial communities were balanced and essential for stable digester performance ([McCarty, 1964](#); [Ward et al., 2014](#)).

A significant difference ($P = 0.00$) between treatments was found for the total biogas produced for both the disrupted and non-disrupted *Tetraselmis* spp. biomass treatments. However no significant difference ($P = 0.607$) was found between the non-disrupted and disrupted *Tetraselmis* spp. treatments. Daily biogas production over the experimental period followed a similar trend for the non-disrupted and disrupted *Tetraselmis* spp. treatments ([Fig. 1](#)). Gas production increased rapidly and remained elevated until day 15 where upon, gas production reduced to the end of the experimental period. The extracted *Tetraselmis* spp. treatment also followed a similar trend with gas productivity until day 10 before a reduction occurred. This trend was repeated in the

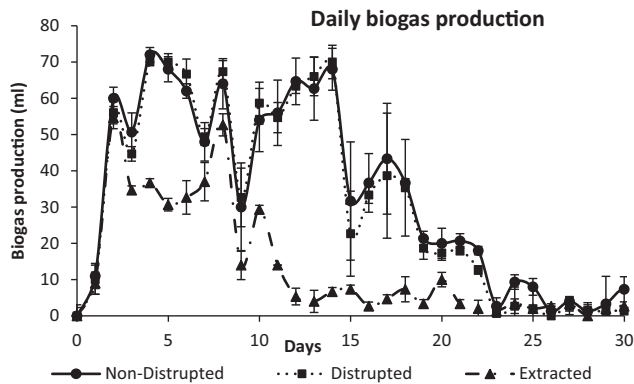


Fig. 1. The mean daily gas production (mL) and standard deviation for the non-distrupted, disrupted and extracted biomass over the experimental duration.

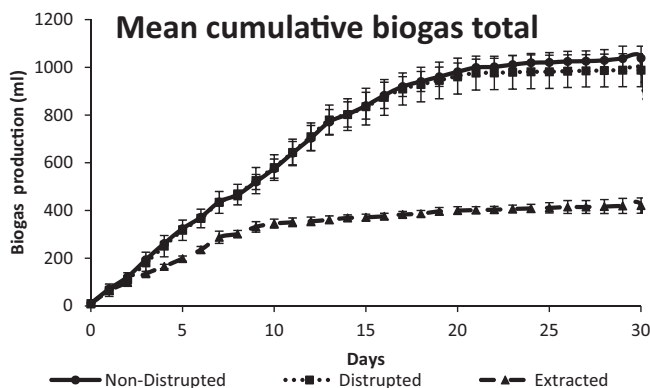


Fig. 2. The cumulative mean biogas production for the non-distrupted, disrupted and extracted biomass over the experimental duration.

cumulative gas production data (Fig. 2) where the gas production increased for the non-distrupted and disrupted *Tetraselmis* spp. treatment however gas production continued to increase until approximately day 20 and after this the cumulative gas production decreased with little additional biogas recorded over the final experimental period. The extracted *Tetraselmis* spp. gas production also increased daily until day 10 without any further increase for the final experimental period.

Biogas volumes of 23 ± 2 , 1060 ± 50 , 1009 ± 15 and 422 ± 41 mL were recorded for the control, non-distrupted, disrupted and extracted *Tetraselmis* spp. treatments respectively. The methane percentage recorded for the non-distrupted, disrupted and extracted *Tetraselmis* spp. biomasses were 58 ± 3.66 , 60 ± 2.55 and 34 ± 1.26 mL, respectively. This resulted in a methane production rate of 252 mL per g VS and 248 mL per g VS for the non-distrupted and disrupted treatments respectively. The recorded methane production rate was within the range of gas production volumes reported by Asinari Di San Marzano et al. (1983) for *Tetraselmis* spp., of between 250 and 310 mL per g VS. The lowest gas production rate of 122 mL per g VS was recorded for the extracted *Tetraselmis* spp. treatment. Lower gas production from the

extracted *Tetraselmis* spp. biomass was expected, as the removed lipid portion of the biomass has a higher theoretical methane potential when compared to protein or carbohydrate fraction of biomass as reported by Zamalloa et al. (2012). Ehimen et al. (2009) presented an additional contributing factor for lower gas production when using extracted biomass, which is affected by the method used for lipid extraction. In this experiment lipid was extracted from dry biomass with a soxhlet apparatus using hexane as a solvent as reported by (Pragya et al., 2013). Hexane, butanol and methanol extraction has been shown to be non-detrimental to anaerobic digestion if entrained solvents are vaporised by drying within a hot oven (Ehimen et al., 2009). However as *Tetraselmis* spp. was dried for the extraction process and redried after the extraction to remove entrained hexane, it is considered that this excessive drying of biomass could have significantly influenced the bio-methane potential of the substrate, further reducing gas potential. Kinnunen et al. (2014) reported a significant decrease in methane potential between dry and wet extracted *Nannochloropsis*, where wet biomass produced 482 mL per g VS compared to 194 mL per g VS for dry biomass. This reduction in methane clearly illustrates that the processing methods utilised for lipid extraction in microalgae needs to be considered if the residual biomass is targeted for anaerobic digestion. The conversion of carbon in the feedstock to methane in this experiment, achieved an efficiency of 29%, 60% and 59% for the extracted, non-distrupted and disrupted *Tetraselmis* spp. biomass respectively. TS conversion for the non-distrupted and disrupted *Tetraselmis* spp. treatments was 35% compared to 39% for the extracted *Tetraselmis* spp. biomass. Extracted *Tetraselmis* biomass in this study showed an 11% increase in TS conversion compared to the disrupted and non-distrupted *Tetraselmis* spp. treatments, however the VS conversion for the non-distrupted, disrupted and extracted *Tetraselmis* spp. biomass demonstrated much less variation at 66%, 65% and 66%, respectively. This indicates that the bacterial degradation rate of biomass was comparable for all treatments in this study. The TS and VS conversion rates for the disrupted and non-distrupted *Tetraselmis* spp. were also revealed in the biogas production over the experimental period. The initial and final TS and VS conversion over the experimental period are shown in Table 1.

The activity of this bacterial degradation has resulted in comparable gas production as well as similar VS and TS conversion rates for both non-distrupted and disrupted *Tetraselmis* spp. treatments.

Samson and Leduy (1983) reported a 26% increase in solubilisation of biomass when biomass was frozen, due to the disruption of the microalgae cell wall by ice crystal formation. Although *Tetraselmis* spp. biomass used in this experiment was subjected to freezing during transportation and storage, the 26% increase reported from freezing would not result in the similar gas production reported for the non-distrupted *Tetraselmis* spp. treatment when compared to the sonicated *Tetraselmis* spp. treatment as higher rates of TS and VS conversion were reported in this study.

The ability of the microbial community to degrade the *Tetraselmis* spp. allowing conversion of cell internal contents to methane biogas is an important finding from this study. Lee et al. (2013) has reported that the calculated theoretical energy requirement and the actual measured energy requirement to physically disrupt *Tetraselmis* sp. required more energy than what is contained and

Table 1
Extracted, disrupted and non-distrupted treatments initial TS and VS loading rate and the final TS and VS concentrations and the TS and VS destruction over the experimental period.

Treatment	Initial (%) TS	Final (%) TS	TS (%) destruction	Initial (%) VS	Final (%) VS	VS (%) destruction
Non-distrupted algae	10.63 ± 1.34	6.95 ± 0.09	35%	45.82 ± 0.41	15.70 ± 0.65	66%
Disrupted algae	10.63 ± 1.34	6.95 ± 0.17	35%	45.82 ± 0.41	15.79 ± 0.66	65%
Extracted algae	10.41 ± 0.66	6.40 ± 0.19	39%	45.69 ± 4.96	15.59 ± 1.12	66%

recoverable from the *Tetraselmis* spp. cells. The use of microbial degradation and associated enzymatic activity for cell lyses offers a promising outlook for future work and may play an important role in future biofuel operations. Cell lyses via an anaerobic digestion microbial community offers a direct conversion pathway for energy production where whole biomass can be harvested and concentrated, and directly fed to an anaerobic digester without energy intensive pre-treatment or processing being required.

4. Conclusion

This study demonstrated that the major problems associated with the disruption and lyses of microalgal cell walls are readily overcome by anaerobic digestion processes. No significant difference in methane production was found between pre-treated disrupted *Tetraselmis* sp. and non-disrupted non-treated *Tetraselmis* spp. Cell lyses by the anaerobic digestion microbial community provides a direct conversion pathway for energy production where whole biomass can anaerobically digest. Anaerobic digestion of residual lipid extracted biomass has been shown to be a potentially energetically viable process that also facilitates the recovery of valuable nutrients whilst producing methane for use as biogas.

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Chapter Six

STATEMENT OF AUTHORSHIP

Title of paper: Microalgae digestate effluent as a growth medium for Tetraselmis sp. in the production of biofuels

Journal: Bioresource Technology

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Provided the analysis used in this study, undertook training and supervision of initial culture work, contributed to drafting and editing of manuscript.

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Microalgae digestate effluent as a growth medium for *Tetraselmis* sp. in the production of biofuels



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HIGHLIGHTS

- *Tetraselmis* sp. is capable to utilise MDE media to form a closed loop system.
- Low diluted MDE media caused lower total lipid content and growth.
- Higher bacterial diversity observed within lower dilutions of MDE media.
- ACP lipid gene regulation was low in all MDE treatments.

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ABSTRACT

This study investigated an alternative nutrient source arising from anaerobically digested *Tetraselmis* sp. effluent (MDE) as a nutrient feed stock to form a closed loop nutrient system. To determine MDE suitability the following factors were observed: growth, lipid content, and the bacterial diversity. MDE was diluted according to the concentration of NH_4^+ content (20, 40, 60, 80 mg/L) and compared against F/2 medium a standard medium for *Tetraselmis* sp. The growth rate on the MDE medium was not as rapid as the F/2 medium and the less diluted MDE correlated (R^2) with lower total lipid contents (R^2 , 0.927), additionally acyl carrier proteins (ACP) gene expression rates displayed lower gene expression within MDE treatments. Lastly, higher concentrations of MDE were correlated with a higher bacterial diversity throughout the investigation. The suitability of MDE as a nutrient supplement for the production of *Tetraselmis* sp. biomass and lipid is feasible.

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1. Introduction

Microalgae biofuel production has recently become a viable option as a feedstock source for fuel (Fon Sing et al., 2014; Nashawi et al., 2010). The production of alternative sources of fuel such as the oil from microalgae has been shown to be effective at the laboratory and pilot scale (Fon Sing et al., 2014). Biomass and lipid produced by microalgae can be converted into various fuels with processes such as transesterification, pyrolysis, gasification, and hydrothermal liquefaction (Lane et al., 2013; Yang et al., 2011). Therefore optimising the performance of growth and lipid production of microalgae can be advantageous for those processes. However this potential shift to alternative fuel sources could lead to increasing pressure on the requirement for nutrients between the microalgal biofuel sector and the agriculture sector. Due to

the competition for nutrients, alternative nutrient sources are now being explored such as digestate effluent (Uggetti et al., 2014). The use of microalga digestate effluent (MDE), following anaerobic digestion of biomass or waste from the production of methane could offer a potential source of future nutrients and essentially form a closed loop system (Prajapati et al., 2014; Ward et al., 2014).

However the use of anaerobic digestate as a nutrient source for microalgae may affect growth rates, lipid production, and the growth of undesired organisms when compared to levels achieved with expensive defined media (Gao and Li, 2011; Vasseur et al., 2012; Xin et al., 2010). A major source of nitrogen within the anaerobic digestate is ammonia; studies have observed microalgal growth inhibition with the use of anaerobic digestate that is high in ammonia (Cho et al., 2013; Källqvist and Svenson, 2003). Growth inhibition occurs due to the ability of ammonia to negatively affect the photosynthesis process by restricting the electrons available for photosynthesis with the cell (Konig et al., 1987). With the use of an optimal concentration of anaerobic digestate, growth

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inhibition can be avoided and the growth of microalgae can be supported (Uggetti et al., 2014). Yang et al. (2011) observed the combination of seawater and wastewater can eliminate the need from all nutrient sources besides phosphate to support microalgal growth (Yang et al., 2011). Alternatively growth trials are required to determine the suitability of the microalgae species to grow on anaerobic digestate effluent. Studies have shown that not all microalgae species are suitable to grow on high concentrations of ammonia associated with digestate effluent. Microalgae species isolated from waste water locations have had better success with growth on substrates derived from waste (Abou-Shanab et al., 2013; Xin et al., 2010). Xin et al. (2010) screened 11 species of microalgae on wastewater to identify the most suitable species; only four of the species showed effective growth on the undefined medium (Xin et al., 2010). Recently Uggetti et al. (2014) investigated the use of anaerobic digestate as a substrate for a microalgae culture; it was found that the growth was satisfactory and the increase of digestate and microalgae may of reduced the initial growth (Uggetti et al., 2014).

The effects of innovative nutrient feeding regimes have to be understood to ensure that adequate lipid productivity can be achieved. Microalgae lipid production with MDE has shown to be directly affect by the concentration of ammonia in the digestate (Li et al., 2011; Uggetti et al., 2014; Wang et al., 2010). Lipid gene expression is a valuable tool when monitoring lipid production in microalgae due to its ability to show lipid activity throughout the different growth phases (Lei et al., 2012). This study will observe how lipid gene expression rates are affected by the use of MDE. Acyl carrier protein (ACP) plays a crucial role in fatty acid synthesis, its responsibility is to transfer acyl intermediates during fatty acid synthesis (Byers and Gong, 2007). Previous research has shown a positive correlation between ACP gene expression and lipid production (Lei et al., 2012). Monitoring lipid gene regulators like ACP represents a novel and simple method which can be used to evaluate the performance of a new nutrient source such as the use of digestate for lipid production. Additionally a traditional approach to monitor the final total lipid content will help determine how effective MDE can support lipid production. The combination of lipid gene expression and final total lipid content is a novel aspect of this study that will contribute to the understanding and the suitability of MDE for microalgae lipid production.

Large scale sterilisation of an open pond system is not feasible therefore the presence of bacteria has to be accepted and controlled (Erkelens et al., 2014). Studies have shown the growth of bacteria can compete for nutrient resources with microalgae (Li et al., 2011; Vasseur et al., 2012). Studies that have investigated the bacteria present with microalgae have shown that they are greatly affected by the use of digestate effluent (Vasseur et al., 2012). The presence of bacteria with microalgae can be beneficial as they are a natural symbiotic partner; studies have shown bacteria are capable of providing nutrients and vitamins to microalgae (Croft et al., 2005). There has been minimal reported research on the how the bacteria are influenced by the use of MDE for microalgal growth. A unique aspect of this work is the use of PCR-DGGE to observed changes within the bacterial diversity and how it is influenced by MDE.

The suitability of MDE for the growth of *Tetraselmis* sp. was analysed to determine how effective it was as a growth medium for biofuels production before large-scale applications. The suitability of MDE was determined by observing the effects of the growth, final total lipid content, and the effects to the bacterial diversity.

The aims of this research were:

1. To evaluate the potential suitability of anaerobic digestate as a potential feed stock for microalgae at large scale.

2. To assess how effective ACP gene regulation is for the assessment of lipid production.

2. Methods

2.1. Set up and experimental design

The reported investigation was conducted at laboratory scale. Plastic bag photobioreactors (5 L) were used with a light intensity of 210 $\mu\text{m photon/m}^2/\text{s}$ with continuous illumination. The temperature was maintained at room temperature (23–25 °C) throughout the experiment period. Each photobioreactor was allocated its own air intake for the purpose of mixing the microalgae. The flow rate of air was regulated at 4 L per min for all treatment replicates. Salt concentrations were achieved with the addition of Red Sea Salt to the desired salinity concentration of 7% (w/v). Sampling from photobioreactors was conducted daily; an aliquot (50 mL) was taken from each photobioreactor and stored at –40 °C for later analysis. Inoculation of the photobioreactor was conducted using a 10% (v/v) inoculum sourced from a stationary phase culture. F/2 medium was made according to Keller et al. (1987). Microalgae digestate was obtained from anaerobically digested *Tetraselmis* sp. Nutrient analysis of the digestate produced from the plug flow bioreactor was found to contain 833.2 NH_3 mg/L, 0.005 NO_3^- mg/L, 8.16 NO_2^- mg/L, 7.40 total phosphate mg/L. Other studies have shown that ammonia concentrations may be toxic to microalgae at high concentrations (Abeliovic and Azov, 1976; El-Shafai et al., 2004). Therefore in this study MDE was diluted according to the level of ammonia, the dilutions used within this study where 20, 40, 60, 80 mg/L of ammonia.

2.2. Anaerobic digestion plug flow reactor conditions

A 3 L plug flow anaerobic digester with a working volume of 2 L was utilised as the source of digestate for this experiment. The 3 L plug flow anaerobic digester utilised *Tetraselmis* sp. as a feedstock. The microalgae was disrupted by sonication pretreatment at 10 KHz for 15 min by a Branson sonifier prior to being fed into the digester. Disrupted microalgae feedstock was stored at 4 °C to reduce any pre-digestion prior to digester feeding. A hydraulic retention time was used and a feeding rate of 1.0 g of ash free dry weight of *Tetraselmis* sp. biomass was applied to the reactor. The equivalent volume of digestate was removed from the digester prior to feeding, thus maintaining a constant volume within the digester. Removed digestate was stored in a glass bottle and allowed to accumulate until sufficient volume was collected.

2.3. DNA and RNA extraction

DNA extractions were conducted on samples collected from the photo-bioreactors. DNA extractions were undertaken using a MO Bio DNA extraction kit (MO Bio, USA). An aliquot, (1.2 mL) of sample was used to conduct the DNA extraction which had a homogenisation stage for 10 min as per the manufacturer's instructions. A yield of 100 μL of DNA was obtained and stored at –20 °C.

RNA was extracted using a MO Bio Power Plant RNA extraction kit (MO Bio, USA). An initial amount (1.2 mL) of sample was used as directed by the supplier's instructions. The total yield of RNA from the kit was 50 μL . RNA, which was then stored at –80 °C for later use if not used immediately. The MO Bio Power Plant RNA extraction kit included a DNAase stage.

PCR was conducted with universal microalgal primers (Sherwood and Presting, 2007), with the isolated RNA used as template to identify the presence of DNA. If no PCR amplicons were identified, the production of RNA to cDNA was conducted using a Turbo RNA to cDNA kit (Invitrogen Life Technologies, USA). An

aliquot (2 μ L) of RNA template was used to provide a yield of 20 μ L of cDNA. cDNA products were verified on a 1.2% agarose gel with SYBR[®] Safe then stored at -20°C for later use (Invitrogen Life Technologies, USA).

2.4. Real-time PCR for the quantification of specific genes of interest

Real time PCR assays were performed using a Corbett Roto-Gene 6000 (QIAGEN, USA) with a KAPA Master Mix 1X SYBR green (KAPA, USA) with a final volume of 20 μ L per reaction. Template DNA (1 μ L) was used for each reaction. Each run included a standard curve which was formed by serial dilutions of the target gene. At the end of each run a melt curve was undertaken to determine specific PCR products where present.

2.5. PCR identification of *Tetraselmis* sp. inoculums

Identification of the *Tetraselmis* sp. was undertaken before the initial inoculation of photo-bioreactors. Identification was conducted using universal microalgal primers which targets the conserved region on the 23S chloroplast DNA (Sherwood and Presting, 2007). PCR amplicons underwent a cleanup stage using a Promega Wizard SV Gel and PCR Clean-Up System (Promega, USA). Products were sent to the Australian Genomic Research Facility for sequencing (AGRF). PCR was conducted using a KAPA master mix on a BIORAD T100 Thermocycler (KAPA, USA; BIORAD, USA). Retrieved sequences were analysed using the BLASTN algorithm of the National Center of Biotechnology Information (NCIB) database to determine their identities.

2.6. Microscopy of *Tetraselmis* sp.

The reported investigation was conducted under non sterile conditions to simulate open pond conditions. The possibility of contamination of other microalgae represented an important consideration. Morphological identification of *Tetraselmis* sp. was used to differentiate it from possible contamination by other microalgae. An Olympus IX50 Microscope at 40 \times magnification and haemocytometer (0.2 mm deep) were used to determine total cell volume per millilitre. Cells were allowed to settle for 5 min before cell counting was undertaken. Triplicate cell counts were undertaken for each sample.

2.7. Growth of bacteria via real-time PCR

The final concentration of bacteria was analysed at the end point to determine how it would affect harvested biomass rates. The growth of bacteria was not selective to any individual bacteria. The use of universal bacterial primers 314F and 581R (Muyzer et al., 1993) with RT-PCR was used to determine final bacterial cell concentration. Day 10 was selected due to it coinciding with the harvesting day. Samples were conducted in duplicate, a mean value was used to determine the concentration and form a standard error. Mean Ct values were calculated from duplicates and compared to a standard curve to determine total cell number per mL.

2.8. Total lipid content of *Tetraselmis* sp.

The total lipid production of the microalgae was investigated to calculate the total lipid yield expected from treatments. The final volumes of each day 10 culture were centrifuged (3257g \times 5 min at 21 $^{\circ}\text{C}$). Samples were dried at 35 $^{\circ}\text{C}$ for 12 h before lipid extraction. Dried microalgae (700 mg) was used for the lipid extraction stage. Samples underwent chloroform/methanol extraction and was quantified gravimetrically (Bligh and Dyer, 1959).

2.9. Lipid gene expression rate via real time PCR

ACP lipid gene regulation was investigated throughout the growth phase of this study. Real time PCR was conducted on cDNA libraries, with 1 μ L cDNA used as template. Each sample was run in duplicates. Lipid gene expression primers were taken from Lei et al. (2012) which were designed for *Haematococcus pluvialis* (Lei et al., 2012). *Tetraselmis* sp. has the ability to produce lipid during the exponential phase. Subsequently samples were selected over the exponential phase from days 2, 4, 6 and 8 (Guzmán et al., 2010). Gene expression was compared using normalisation to 23S rRNA (BIORAD, USA). The amplification efficiencies of the standard curves of both 23S rRNA and ACP genes were close (97–99%). The ACP target gene was normalised to the 23S rRNA target of the same sample using the formula; $\Delta C(t)_{\text{sample}} = \text{average } C(t)_{\text{ACP}} - \text{average } C(t)_{\text{23S RNA}}$. For the $2^{-\Delta\Delta C(t)}$ analysis (Livak and Schmittgen, 2001).

2.10. PCR-DGGE of the bacterial community

Changes in the bacterial community were examined through the use of PCR-DGGE which identified diversity and dominance. Selective bacterial primers 341F GC & 518R (Muyzer et al., 1993) were used to observed the bacterial community. DGGE analysis was conducted on a Universal Mutation Detection system Dcode system using a 6% polyacrylamide gel (BIORAD, USA). The denaturing gradient used for bacterial PCR amplicons were 40–60%. DGGE run conditions were 60 V at 60 $^{\circ}\text{C}$ for 20 h.

2.11. Statistical analysis

DGGE gels were digitalized and then analysed using TotalLab 20.1 to determine the band intensity. The band intensities were then analysed using the Shannon Weaver diversity index ($H = -\sum (\frac{n_i}{N}) \log(\frac{n_i}{N})$) for each sample over time (Shannon and Weaver, 1963).

3. Results and discussion

The use of digestate was investigated as a potential replacement of F/2 medium. Its suitability as a replacement nutrient source was quantified on its ability to grow *Tetraselmis* sp. and in terms of the microalgae's lipid production.

3.1. Genetic identification of *Tetraselmis* MUR233

Initially the *Tetraselmis* sp. strain used in this study was assessed in terms of the similarity to other isolates. The PCR amplicons which were produced were analysed on BLAST, and a high similarity was observed to other *Tetraselmis* species. Due to the high similarity of our sample to other *Tetraselmis* sp., it would be considered to be a *Tetraselmis* sp.

3.2. Effect of microalgae digestate effluent on growth for *Tetraselmis* sp.

The results show that *Tetraselmis* sp. was capable of growth in MDE medium (Fig. 1). Higher rates of biomass production are highly desirable when assessing the commercial potential of a new feeding nutrient source such as MDE. The growth of microalgae was monitored to determine the growth rate of *Tetraselmis* sp. on MDE medium (Fig. 1). F/2 medium achieved the highest growth rate of 1.8×10^6 cells per/mL over a 10 d period. For MDE medium, there was little difference between each of the MDE dilutions though the highest growth was observed in the 80 mg/L dilution

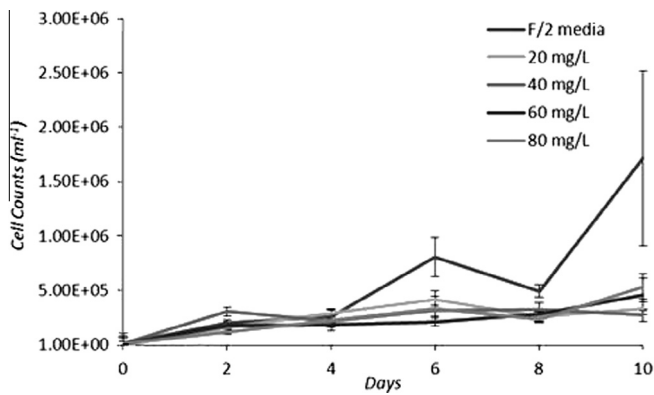


Fig. 1. Cell count of *Tetraselmis* sp. in F/2 medium and MDE medium over 10 days.

(0.5×10^6 cells per/mL). This indicates that the use of microalgae digestate was not as effective as F/2 medium for the growth of *Tetraselmis* sp. and at large scale may be detrimental to the production of biomass. Various studies have shown similar results when growing microalgae on digestate, low growth was attributed to poor nutrient ratios, shading and ammonia (Abou-Shanab et al., 2013; Levine et al., 2011; Li et al., 2011; Arumugam et al., 2011; Nielsen et al., 2012; Uggetti et al., 2014). An example of a poor nutrient ratio was shown when Li et al. (2011) compared TAP medium to municipal waste water concentrate. TAP medium achieved a growth rate of $0.4614 \text{ g/L d}^{-1}$ while non-autoclaved raw municipal wastewater concentrate achieved $0.1677 \text{ g/L d}^{-1}$ (Li et al., 2011). Nielsen et al. (2012) grew *Ulva lactuca* on digestate and found that lowering the concentrations of NH_4^+ had a positive effect on growth rates (Nielsen et al., 2012). In the reported study there was no significant difference between growth rates of *Tetraselmis* sp. on the various dilution of digestate effluent, indicating that 20 mg/L NH_4^+ may have been too high for *Tetraselmis* sp. Uggetti et al. (2014) observed a similar trend with a growth trial of microalgae on anaerobic digestate; it was observed that an increase of both digestate and the microalgae initial concentrations may have reduced the initial growth rate of the microalgae. Their study concluded that anaerobic digestate showed satisfactory growth rates and biomass production (Uggetti et al., 2014). In addition, the low growth that occurred could be due to the potential unsuitability of MDE for the growth of *Tetraselmis* sp. Xin et al. (2010) grew 11 various microalgal species on secondary effluent and found only four were capable of growth on the secondary effluent (Xin et al., 2010). Abou-Shanab et al. (2013) determined the best microalgae to be grown on waste water was commonly found at the source of the wastewater (Abou-Shanab et al., 2013). Other studies have reported success in growing *Chlorella vulgaris* on piggy digestate with a growth of 11.6×10^6 cells per/mL over 6 days (Kumar et al., 2010). It is hypothesised that the microalgal species used in the reported study may have not been optimal in terms of microalgal productivity though the MDE medium was still capable to support growth. The *Tetraselmis* sp. used within this study was isolated from hyper-saline ponds, and would not be expected to be found around wastewater locations and may not be the best suited microalgae for the growth on MDE medium. The results confirm that MDE medium is capable of growing *Tetraselmis* sp. but not as effectively as F/2 medium.

3.3. Total lipid and ACP gene regulation

The *Tetraselmis* sp. was chosen due to its production of lipid in the exponential phase (Fon Sing et al., 2014; Guzmán et al., 2010). The total lipid content was determined at the end of the

exponential phase (day 10). Total lipid was found to be at its highest in the F/2 medium (49%). The lipid content grown on the digestate varied from 48% total lipid in 20 mg/L NH_4^+ to 27% total lipid in 80 mg/L NH_4^+ and found to be negatively correlated with ammonia concentrations ($R^2 = 0.927$) (Fig. 3). The gene regulation of ACP was used to quantify how alternative nutrient regimes would affect lipid productivity. ACP was monitored over the exponential phase as it was the main stage in the life cycle of *Tetraselmis* sp. where lipid production was undertaken (Fig. 2). Day 0 was normalised using $2^{-\Delta\Delta C(t)}$ to determine changes in the expression rate over the exponential phase. The inoculants used came from a stationary phase culture where lipid production does not commonly occur (Costa et al., 2004). F/2 medium displayed the highest level of gene expression rates with a 4.75 fold increase on day 4, slowly returning to normal at day 8 which is close to the end of the exponential phase. ACP gene expression rates in the microalgal digestate mostly decreased after day 0, although an ammonia concentration of 60 mg/L gene expression increased slightly for samples taken on days 4 and 6 (Fig. 2). The overall performance of ACP gene expression in microalgal digestate generally showed a negative impact. The results suggested that ACP gene regulation was not up-regulated when compared with that in ACP gene regulation in F/2 medium. However, other pathways may have been used for the production of lipid. Studies have shown other genes such as FATA, KASIII, acetyl CoA, and pyruvate decarboxylases are implicated in the production of lipid (Lei et al., 2012; Li et al., 2012). Nevertheless the overall lipid production was not significantly different when compared to what was observed in the F/2 medium, suggesting the potential of MDE medium as a nutrient source.

The lower lipid production and low ACP gene regulation in *Tetraselmis* sp. grown on MDE medium may have been due to ammonia and its ability to change internal cell pH and cause a lack of electron donors for photosynthesis (Abeliovic and Azov, 1976; Källqvist and Svenson, 2003). The reported results are similar to those reported in other studies that investigated the use of digestate effluent for lipid production. Wang et al. (2010) investigated the use of dairy digestate for lipid production in *Chlorella* sp.; it was observed that the growth of *Chlorella* sp. on higher dilutions of digestate resulted in a higher percentage of total fatty acids (Wang et al., 2010). A 10% dilution of digestate had a total lipid content of 9%, while microalgae grown on digestate which was diluted 20% and 25% had a higher total lipid content of 13.6% and 13.7% (Wang et al., 2010). Levine et al. (2011) investigated the growth of *Neochloris oleoabundans* in the presence of ammonia and its ability to grow on anaerobically digested dairy manure. *N. oleoabundans* grown on modified Bold's Basal Medium with $50 \text{ mg L}^{-1} \text{ NH}_4^+$ produced no lipid while growth in the $10 \text{ mg L}^{-1} \text{ NH}_4^+$ digestate achieved $3.6 \pm 0.6 \text{ mg L}^{-1} \text{ d}^{-1}$ of lipid. *N. oleoabundans* grown on

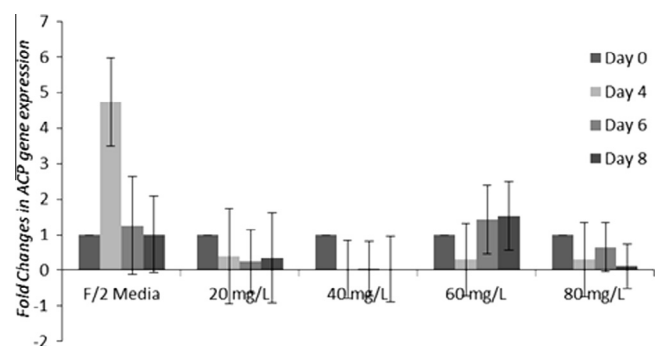


Fig. 2. ACP gene regulation for F/2 medium and digestate medium over the exponential phase.

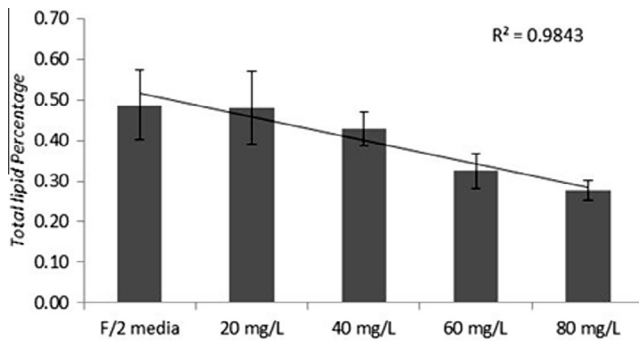


Fig. 3. Total Lipid content of *Tetraselmis* sp. at day 10.

1:50 dilution of digestate achieved a $2.57 \text{ mg L}^{-1} \text{ d}^{-1}$. A 1:200 dilution had a lipid productivity of $4.7 \text{ mg L}^{-1} \text{ d}^{-1}$ (Levine et al., 2011). The reduced level of lipid production reported may have been caused by the increasing presence of ammonia in the medium (Levine et al., 2011). Therefore the trend seen within the results was similar to that observed within other studies, the less diluted the digestate the lower the total lipid content.

3.4. Bacterial community dynamics

PCR-DGGE was used to determine the bacterial community dynamics with the use of microalgal digestate effluent. The growth of bacteria with the microalgae is expected to occur, and it is essential that there is a positive symbiosis between bacteria and microalgae (Croft et al., 2005). There was a significant increase in the bacterial diversity in the MDE medium treatments, which indicated that the MDE medium may have influenced the bacterial community. MDE medium was also observed to influence the initial bacterial diversity on day 0 for all the MDE treatments; as the concentration of MDE increased the bacterial community diversity also increased on day 0 ($R^2 = 0.852$).

The bacterial content on the harvest day (day 10) were investigated to determine if the MDE medium may have influenced the bacterial cell content during the harvest stage. The final bacterial cell counts on the harvest day were the following: F/2 $9.19 \times 10^2 \pm 5.60 \times 10^1$; 20 mg/L NH_4^+ $1.19 \times 10^{10} \pm 3.32 \times 10^9$; 40 mg/L NH_4^+ $3.14 \times 10^5 \pm 8.60 \times 10^4$; 60 mg/L NH_4^+ $1.04 \times 10^7 \pm 8.27 \times 10^5$; 80 mg/L NH_4^+ $3.55 \times 10^{10} \pm 7.80 \times 10^9$. The final bacterial growth in the MDE medium treatments were observed to be higher in comparison to F/2 medium, this indicates that the use of MDE medium enhanced the bacterial content on the harvest day. The enhanced bacterial growth can be associated to the high diversity of bacteria on day 0 within the MDE treatments (Fig. 4), the bacteria within the hypersaline MDE would have already undergone natural selection for the best suited bacteria to grow within a hypersaline environment. The bacterial diversity of the F/2 medium treatment on day 0 was very low and remained low throughout the investigation (Fig. 4), this indicating the bacterial community within the F/2 medium was not well adjusted to the hypersaline conditions and had little effect on the bacterial content during the harvesting stage.

The presence of bacteria within this study was similar to that found with other studies that have utilised digestate effluent as a nutrient source for microalgae. Li et al. (2011) grew *Chlorella* sp. on non-sterile municipal wastewater and found that 10% of the final biomass was bacteria. Levine et al. (2011) also detected bacterial growth with the growth of *N. oleoabundans* on anaerobically digested dairy manure (Levine et al., 2011). Vasseur et al. (2012) investigated the relationship between microalgal and bacterial growth on digestate. They observed that with the presence of

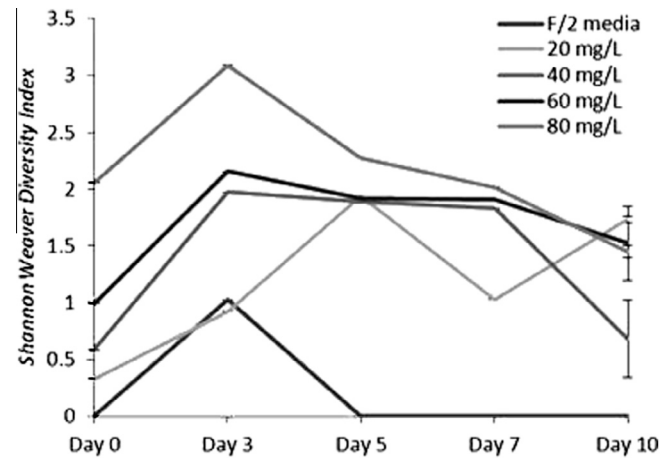


Fig. 4. Bacterial diversity in photobioreactors for F/2 medium and microalgal digestate effluent.

bacteria increased the carbon conversion efficiency, bacteria from the anaerobic digesters were capable of recycling the carbon lost during photosynthesis (Vasseur et al., 2012). Therefore the use of anaerobic digestate at large-scale should be monitored closely as the reported data indicates that non-sterile digestate effluent can influence the bacterial community, though as shown in other studies the presence of bacteria can be beneficial.

4. Conclusion

The overall performance of MDE for biofuel production is feasible but needs further development to optimise performance with regard to providing a commercially viable medium. The final evaluation of MDE for *Tetraselmis* sp. production was; (1) lower microalgal growth in low diluted MDE medium when compared to F/2 medium, (2) higher lipid production rates at lower MDE concentrations, (3) low lipid ACP gene expression found in MDE medium in comparison to F/2 medium, (4) high bacterial diversity was found to be parallel to low dilution of MDE, (5) *Tetraselmis* sp. growth and lipid production can be supported by MDE.

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Chapter Seven

7.0 Summary of conclusions

7.1 Introduction

All the objectives of my PhD were achieved. The principal outcome was the characterisation of the response of a methanogenic anaerobic digestion community, when influenced under high saline conditions that are associated with high salinity microalgae used in a biofuels production system. This work demonstrated that as the salinity increased the species abundance of the methanogen community decreased. However, when high salinities were investigated several species of methanogens were detected and only appeared at these higher salinities. Further work utilising these detected high salinity methanogens allowed the anaerobic digestion of halophytic microalgae resulting in methane production and nutrient recovery.

7.2 Biomass quantification and feedstock management

The development of a simplified and cost effective protocol for the quantification of biomass and resulting chemical parameters that is essential for maintaining optimum anaerobic digestion was achieved. Utilising turbidity as an indicator for Chemical oxygen demand (COD) and volatile solids content allows improved management of pond system and permits the harvesting of a precise volume of microalgal culture to deliver a standardised quantity of volatile solids or COD loading to an anaerobic digester. This is essential and has the added advantage of being able to be utilised within microalgae based wastewater systems and also biofuel pond scenarios.

A strong correlation between COD and volatile solids and turbidity as the indicator variable was found. Another finding from this work was the failure of this relationship

when it was applied to biological oxygen demand (BOD). Experimental results indicated that there was a relationship between BOD and turbidity and this may have ramifications to wastewater quality, as microalgae are not adequately accounted for with BOD testing. This methodology has the potential to reduce labour and laboratory costs, hence reducing the cost of pond management and digester management.

7.3 Inoculum development

This work highlighted the difference between utilising a standard PCR procedure utilising single primers when compared to utilising several sets of primers in a nested PCR method. The nested PCR resulted in a much higher detection rate when compared to the single PCR and primer set. This additional resolution allowed a greater understanding of the bacterial community due to better detection rates. The profiling of the genetic diversity of the anaerobic community highlighted the decrease in methanogen species as a result of increased salinity. However the detection of several methanogen species at the higher salinities was a novel finding. Further work with this inoculum and the introduction of microalgae as a feedstock further enhanced the microbial diversity and an increase in the number of species at 7% salinity was noted utilising the DGGE molecular fingerprinting technique. Quantification of gas production also confirmed the transformation of this microbial community to halophytic conditions due to a reduced detection rate of methanogenic activity when they were reintroduced to lower salinity waters. This result confirmed the formation of a truly halophytic anaerobic digestion community able to digest microalgae biomass at 7% salinity.

7.4 Pre-treatment

Results from this work indicated that lipid extracted biomass was suitable for anaerobic digestion and highlighted the residual energy that is recoverable from the extracted biomass. It was demonstrated that the microbial breakdown of *Tetraselmis* sp. cells occurred due to microbial attack. No significant difference was found between the gas production from sonicated and non-sonicated *Tetraselmis* sp. cells. This result indicated that the conversion of *Tetraselmis* sp. to methane can exclude the energy intensive cell disruption process. This offers a direct pathway to methane production where cells can be directly fed into an anaerobic digester. This direct pathway can potentially eliminate cost and energy required for pre-treatment of microalgae biomass and again increases the cost effectiveness of microalgae biofuel production.

7.5 Digestate as a nutrient source

The overall performance of microalgae digestate for biofuel production is feasible but may need future development to optimize its performance with regards to microbial community. The final evaluation of microalgae digestate for *Tetraselmis* sp. production demonstrated no significant difference in lipid productivity when compared to commercial F/2 media. However as the concentration of digestate increased and associated nitrogen levels became higher than commercial F/2 media, lipid production decreased. The growth and lipid production of *Tetraselmis* sp. can be supported by anaerobic digestate, however proper management of the microbial community must be undertaken to limit competition within the microalgal community. The use of

anaerobic digestate as a nutrient source has the potential to reduce production costs significantly. There is also the added benefit of a reduced carbon footprint as the need for commercial nutrients are reduced decreasing the associated CO₂ emissions of microalgae biofuel production.

7.6 Future work

Future work resulting from this study would involve the full commercialisation of the halophytic inoculum. This would require the implementation of a pilot scale methane digester into a microalgae biofuel production plant. Further work into the optimisation of digestate as a commercial nutrient replacement could then be undertaken at pilot scale to maximise the benefits of its use. Future laboratory scale work to be undertaken would include the investigation of the halotolerant digestion inoculum for higher salinities and to evaluate the ionic conductivity limits of the methanogen community. The investigation of the acetogenic community for hydrogen production under high ionic conditions would also be beneficial. The investigation into the suitability of this inoculum to treat other high ionic conductivity wastewaters would also be beneficial. This research could lead to other opportunities for bio-methane production incorporated into the treatment of other high ionic conductivity wastewaters, leading to greater sustainability and environmental outcomes of these operations.

Chapter Eight

Appendix

Appendix 8.1

Molecular comparison of nested and non nested 16S rDNA polymerase chain reaction (PCR) amplicons.

Andrew Ward Supervisors: David Lewis and Andy Ball

Introduction

To investigate the microbial diversity of methanogenic Archaeal bacteria under increasing saline conditions. A molecular genetic study was carried out on marine microalgae anaerobic digestion microbial communities. To validate the PCR methods employed in this investigation, three digesters at 1.6, 3.4 and 7% salinity were sampled for methanogenic populations. Methanogenic communities were investigated using a denaturing gel gradient electrophoresis (DGGE) using the V3 region of the 16S rDNA gene. Two PCR methods were compared for the best detection of methanogen bacteria.

Table 1: Biogas production and conditions recorded for anaerobic digesters

Treatment	Biogas production (ml/g/VS)	Methane content (%)	Temp (C°)	Mixing (RPM)	Ph
1.6% Salinity	N/A	N/A	37 ± 0.5	90	7.44 ± 0.07
3.4% Salinity	165 ± 54	44 ± 9	37 ± 0.5	90	7.50 ± 0.13
7% Salinity	290 ± 53	54 ± 4	37 ± 0.5	90	7.40 ± 0.05

Methods

The two PCR methods were compared for the best detection of methanogen bacteria. For DGGE analysis the variable V3 regions of the 16S rDNA gene were amplified using the methanogen specific primers 0357F-GC and 0691R. The second PCR primer set consisted of a nested PCR using the methanogen specific primers Met86F and Met 1340R and 0357F-GC and 0691R primers sets. To validate data via gas production three anaerobic digesters were set up and operated for a 50 day period. The digester treatment salinities were 1.6, 3.4 and 7.0% salinity respectively.

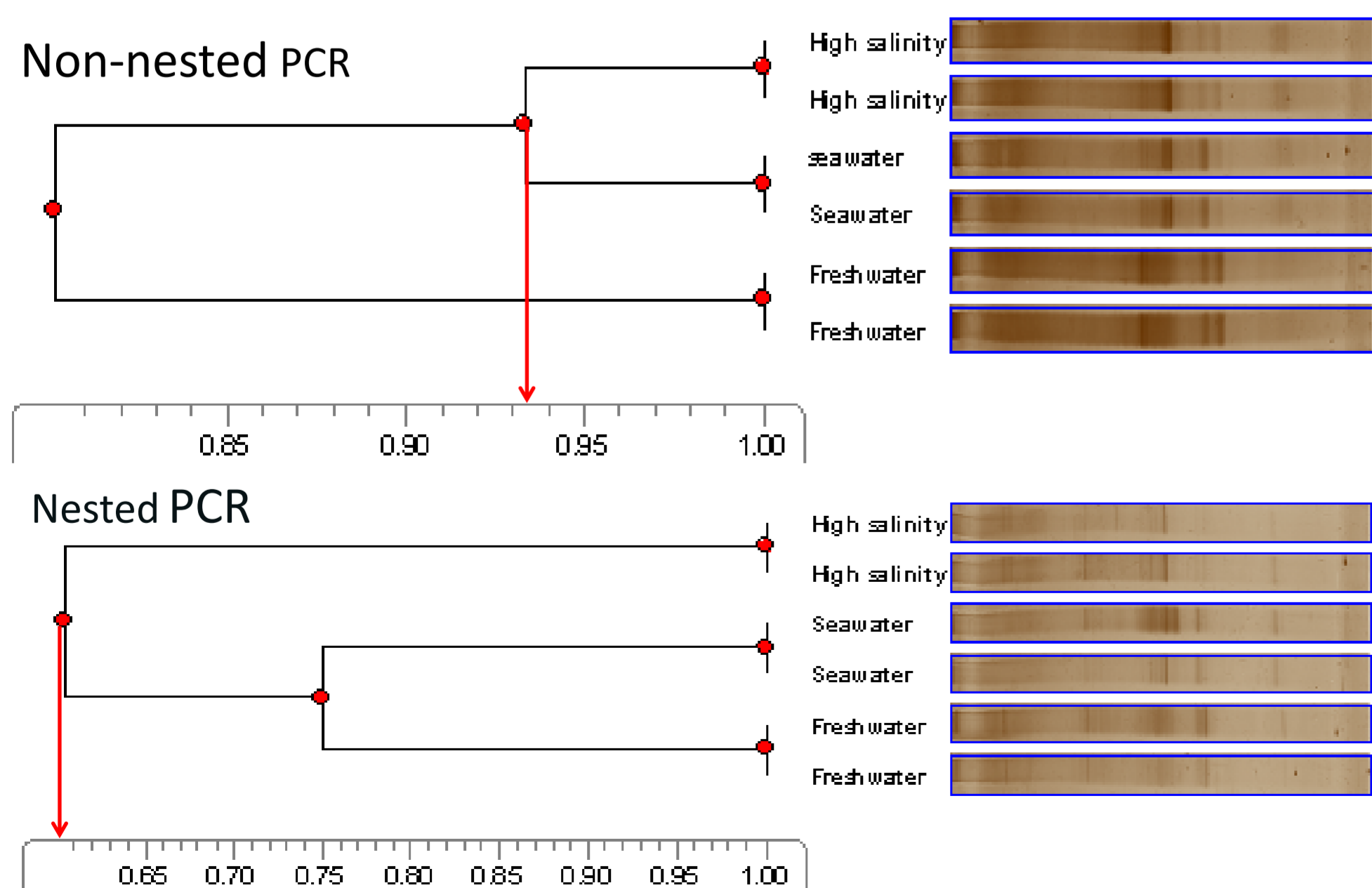
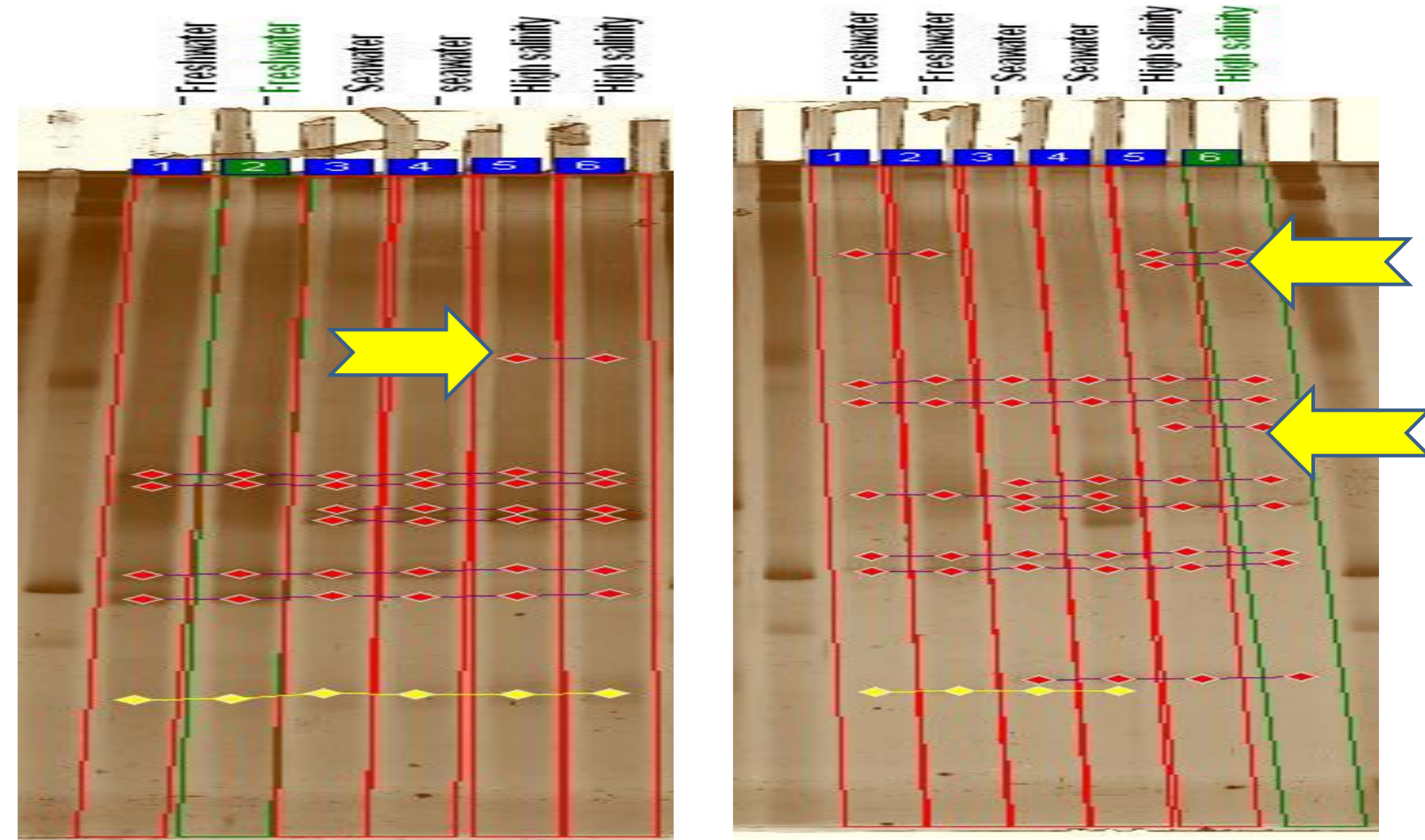


Figure 1: Dendograms from Phoretix analysis of DGGE gels (arrows indicates the similarity between high salinity and seawater communities).



Non-nested DGGE results

Nested PCR results

Figure 2: DGGE gels for the nested and non-nested PCR amplicons. Arrows indicate high salinity methanogen bacteria species.

Results

Good separation and quality of band patterns were achieved in DGGE gels by both primer sets at all three digester salinities (Figure 2). The data shown in Table 1 lists the gas production and digester conditions recorded during the experimental period. Table 2 indicates higher detection rates and sensitivity by the nested PCR when compared to the non-nested PCR method. Gas data closely correlates to the nested PCR dendograms shown in Figure 1. Nested dendograms indicate a significant difference between the seawater and high salinity communities which corresponds to the large difference in gas data between the high salinity and seawater salinity communities respectively. This difference in gas production does not correlate with the non-nested dendograms where little difference between communities is recorded. Both methods indicate the presence of high salinity methanogen bacteria in the 7% treatments (arrows in Figure 2)

Table 2: Number of methanogen bacterial species detected

PCR Treatment	1.4% Salinity	3.4% Salinity	7% Salinity
Nested	7	9	10
Non-Nested	5	7	8

Conclusion

Both PCR methods tested detected methanogen bacteria species. The nested PCR method had a much higher sensitivity and detection rate than the non-nested PCR method. The gas data clearly correlated with the difference in the bacteria communities indicated in the dendograms for the nested PCR method tested, Hence making the nested PCR the better method. Also gas data and detection of high salinity methanogen bacteria at 7% salinity indicates anaerobic digestion of saline microalgae at 7% salinity is achievable.

(This work has been submitted to The Journal of Phycology for publication)

Appendix 8.2

Ward, A.J. (2015). The Anaerobic Digestion of Microalgae Feedstock, “Life-Cycle Environmental Impacts of Biofuels and Co-products” In N.R. Moheimani, M.P. McHenry, de Boer, K. and P.A. Bahri Eds.) *Biomass and Biofuels from Microalgae. Biofuel and Biorefinery Technologies*, 2, 331-345.

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