Investigation of the closed loop processing systems developed for microalgal biofuels.

Mason Erkelens

This thesis is submitted for the degree of Doctoral of Philosophy

In

The School of Chemical Engineering

At

The University of Adelaide

February 2015.

Panel of Supervisors

Principal supervisor

A/Prof David M Lewis

PhD University of Adelaide

School of Chemical Engineering

The University of Adelaide

Email: david.Lewis@adelaide.edu.au

Phone: +61 8 83135503

Fax: +61 8 83134373

Co supervisors

Professor Peter Ashman

PhD University of Sydney

School of Chemical Engineering

The University of Adelaide

Email peter.ashman@adelaide.edu.au

Phone: +61 8 83135072

Fax: +61 8 83134373

Professor Andy Ball

PhD University of Liverpool

School of Applied Science

Royal Melbourne Institute of Technology

Email: andy.ball@rmit.edu.au

Phone: +61 3 99256594

Fax: +61 3 9925 7110

Declaration for a thesis that contains publications

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due references has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applications, any partner institution responsible for the joint-award of this degree.

I give consent for this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published work contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Signature ……………………………………………………………. Date …………………..

Thesis by publication

This doctoral thesis is submitted as a portfolio of peer-reviewed publication according to the "*PhD Rules & Specification 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 information is listed and the journals ranked in the order of impact factor in reference to their scientific significance.

	Journal Title	Impact Factor	Citations
1	Bioresource Technology	5.039	5
2	Bioresource Technology	5.039	2
3	Bioresource Technology	5.039	-
4	Bioresource Technology	5.039	-
5	Bioprocess and Biosystems Engineering	1.823	-

The thesis is composed of the following papers:

1. Mason Erkelens, Andrew S. Ball, David M. Lewis (2014), *The influences of the recycle process on the bacterial community in a pilot scale microalgae raceway pond.*, Journal of Bioresource Technology.
2. Mason Erkelens, Andrew S. Ball, Andrew Ward, David M. Lewis (2014), *Microalgae digestate effluent as a growth media for Tetraselmis sp. in the production of biofuels*, Journal of Bioresource Technology.
3. Mason Erkelens, Andrew S. Ball, Andrew Ward, David M. Lewis (2014), *The influence of protozoa with a filtered and non-filtered seawater culture of Tetraselmis sp.*, Journal of Bioresource Technology.
4. Mason Erkelens, Andrew S. Ball, David M. Lewis (2015), *The application of activated carbon for the treatment and reuse of the aqueous phase derived from the hydrothermal liquefaction of a halophytic Tetraselmis sp.*, Journal of Bioresource Technology,
5. Mason. Erkelens, Xiaoyu Ma, Wynand Van Den Berg, Andrew. S. Ball, Steven. Amos, David. M. Lewis (2015), *Reducing microalgae biofuels water footprint by recycling water and its effects on the bacteria and microalgal communities*, Bioprocess and Biosystems Engineering, Submitted

The following additional outcomes resulted from PhD work associated with the research carried out:

Sanaz Orandi*, Mason Erkelens*, Andrew S Ball, Amini, David M Lewis (2014), *An Investigation of the bacterial community dynamics of a microbial biofilm, exploited for the biotreatment of acid mine drainage*, joint lead author, submitted to the Journal of Extremophiles.

Mason Erkelens, Peter Forward, Martin Lambert, *The bacterial community within a 28km bore system with iron bacteria."* Submitted.

Mason Erkelens*, Peter Forward, Martin Lambert, *Characterisation of Bacterial Iron Nanowires from biofilm: towards large scale production of nanomaterias from the environmental waste*, Poster presentation, ICONN conference, Adelaide, 2014.

Mason Erkelens, Krishna Kant, Peter Forward, Martin Lambert, *Large scale production of iron oxide nanowires*, Oral presentation, RACI conference, Adelaide, 2014.

Mason Erkelens, Andy Ball, David Lewis, *The influence of the recycle process on the bacterial community in a pilot scale microalgae raceway pond*, Poster presentation, ISAP conference, Sydney, 2014.

Mason Erkelens, Mohamad Taha, Akin Adututu, Laureta, John Aniborg, Andy Ball, *Sustainable remediation – The application of bioremediated soil for the use in the degradation of TNT chips,* Journal of Environmental Management, Impact factor 3.55.

Mason Erkelens, Andy Ball, David Lewis, *The influence of microalgae digestate on microalgae biofuel production*, Poster presentation, EMBL symposium, 2012.

Mason Erkelens, Andy Ball, *Sustainable remediation – The application of bioremediated soil for the use in the degradation of TNT chips,* Oral presentation, Australian Energetics Symposium, 2012.

Awards

2012 Awarded Travel research scholarship

2012 Joint Research Engineer scholarship

2012 EMBL PhD travel symposium scholarship

2012 Awarded EMBL PhD research travel scholarship

If I want to summarise my thesis in one sentence, I would say:

"*Looks like pea soup, but it's actually the future of crude oil production.*"

Mason Erkelens

December 2014.

Acknowledgements

I would like to thank everyone who has helped me get to where I am today.

Firstly I would like to thank my family for supporting me throughout my whole educational journey, they have supported me and pushed me to exceed.

I would like to thank Kerri Wester my partner for being there with me from my honours, she has been great support during my studies.

I would like to especially thank Prof Andy Ball for accepting me into honours, and then taking me on for a PhD. Without Prof Andy Ball I would not be here to this day, he took me on as a 3rd year student, and then accepted me for honours. The training in the Ball Lab was the best I have ever received, from that training I have been able to be competitive within the scientific community. Without him I would have not be where I am today and I thank him for that.

I would also like to thank A/Prof David Lewis, he accepted to take me on as a PhD student and as my Primary supervisor. He has been a great teacher and also a great mentor! A/Prof David Lewis has made it possible for me to finish the PhD.

I would also like to thank Prof Peter Ashman for accepting me as his PhD student, he has been very good to have a supervisor.

I would also like to thank Prof Martin Lambert for support me through this PhD by continuous encouragement, this encouragement has helped me finish my thesis for submission.

I would like to thank all my co-authors in all my papers, Steven Amos, Andrew Ward, Sanaz Orandi, Ivy Ma, Wynand van den Berd, Daniel Lane, Anne Philcox. I will remember all of you and hope that someday we will cross paths in the same research group again.

I would also like to thank Prof Alan Cooper and his research group for accepting me to join their weekly meetings, it has opened my eyes to the future of the metagenomics field.

Abstract

The algal biofuels industry is under development and being investigated at large scale all around the world. To improve the viability of algal biofuels the ability to use closed loop systems that recycle waste and water thereby decreasing the overall waste produced while increasing profitability is being investigated. The aim of this work was to investigate closed loop systems associated with the algal biofuel production, focusing on its effects on the production of algal biomass and lipid and on the natural microbial community. The key areas of algal production that have been the focus of the research are the introduction of water, and the recycling of water and the recycling of waste produced during the biomass to biofuel stage of the microalgal biofuel process. Water is a key part of microalgal biofuel production; the source of water can contain many different microorganisms that can affect microalgal growth.

Recycling waste streams back into the culture as a nutrient stream is an effective way to reduce the cost of production. Within this thesis I investigated two waste streams as a potential nutrient stream, microalgae digestate and the hydrothermal liquefaction aqueous phase (Chapter 3 and 4). I observed that high concentrations of either of the waste streams resulted in reduced growth in comparison to F/2 media. Negative growth was associated with high concentrations of ammonia, and the effect of the use of waste streams was species dependent.

There is currently little known about the changes in the bacterial and algal communities during the harvesting/recycle process. Within chapter 5 and 7 I investigated the bacterial and algal diversity present during these processes. It was observed that while the electroflocculation stage had little impact on the bacterial community, the centrifuge stage was shown to have a much higher impact on the bacterial community. The recycling process also increased the dominance of *Tetraselmis MUR233* over various recycle stages. A benefit of recycling is the

prevention of undesired microorganisms entering into the culture. One microorganisms that is of interest is Protozoa, due to the potential damages to microalgae biomass production. Within chapter 6 I observed the effects of protozoa within the culture; it was observed that was no significant difference between the final total lipid or final total dry weight produced in the presence and absence of protozoa. This study shows the ability of *Tetraselmis MUR233* to outgrow any potential damage caused by the presence of the protozoa.

Developing further understanding of these processes can help improve potential outcomes when these processes are undertaken.

Contents

Chapter 1, Introduction 14

Chapter 2, Literature Review 18

1 Overview 18
2 Recycling water 20
3 Recycling nutrients 21
3.1.1 Carbon 22
3.1.2 Phosphorus 24
3.1.3 Nitrogen 25
3.1.4 Trace metals 27
4 Conclusion 28

Chapter 3, Microalgae digestate effluent as a growth medium for *Tetraselmis sp.* in the production of biofuels. 32

Abstract 34
1 Introduction 34
2 Methods 35
2.1 Set up and experimental design 35
2.2 Anaerobic digestion plug flow reactor conditions 35
2.3 DNA and RNA extraction 35
2.4 Real-time PCR for the quantification of specific genes of interest 36
2.5 PCR identification of *Tetraselmis sp.* Inoculums 36
2.6 Microscopy of *Tetraselmis sp.* 36
2.7 Growth of bacteria via real-time PCR 36
2.8 Total Lipid content of *Tetraselmis sp.* 36
2.9 Lipid gene expression rate via real time PCR 36
2.10 PCR-DGGE of the bacterial community 36
2.11 Statistical analysis 36
3 Results and Discussion 36
3.1 Genetic identification of *Tetraselmis MUR233* 36
3.2 Effect of microalgae digestate effluent on growth for *Tetraselmis sp.* 36
3.3 Total lipid and ACP gene regulation 37
3.4 Bacterial communitydynamics 38
4 Conclusion 38
5 Acknowledgments 38
6 References 38

Chapter 4, The application of activated carbon for the treatment and reuse of the aqueous phase derived from the hydrothermal liquefaction of a halophytic *Tetraselmis sp.*. 40

Abstract
1 Introduction 42

2 Methods 43
2.1 HTL aqueous phase 43
2.2 Chemical composition 43
2.3 Experimental design 43
2.4 Growth analysis of *Tetraselmis MUR2* 43
3 Results and discussion 44
3.1 Chemical composition of the HTL AP and effect of activated carbon 44
3.2 Growth analysis of *Tetraselmis MUR233* on the HTL aqueous phase 44
4 Conclusion 45
5 Acknowledgements 45
6 References 46

Chapter 5, The influences of the recycle process on the bacterial community in a pilot scale microalgae raceway pond. 47

Abstract 48
1 Introduction 49
2 Method 50
2.1 Process description and plant design 51
2.2 Experimental design and sampling plan 51
2.3 Molecular analysis 51
2.4 Identification of bands of interest 51
2.5 Quantification of bacteria with real-time PCR 51
2.6 Statistical analysis 51
2.7 Results and discussion 51
2.8 Bacterial cell counts with real time PCR 51
2.9 Bacteria community dynamics via PCR-DGGE 51
2.10 Conclusion 52
3 Acknowledgments 52
4 References 52

Chapter 6, The influence of protozoa with a filtered and non-filtered seawater culture of *Tetraselmis sp.,* and effects to the bacterial and algal communities over 10 days. 54

Abstract 55
1 Introduction 55
2 Methods 56
2.1 Experimental design 56
2.2 DNA extraction 56
2.3 PCR-DGGE 56
2.4 Growth of *Tetraselmis sp.* and total lipid & dry weight content 56
3 Results and discussion 57
3.1 Microscope observation of cultures 57
3.2 Final total lipid and dry weight 57
3.3 Bacteria community analysis 57

3.4 Algal community analysis 58
4 Conclusion 60
5 Acknowledgments 60
6 References 60

Chapter 7, Reducing microalgae biofuels water footprint by recycling water and its effects to the bacteria and microalgal communities. 61

Abstract 63
1 Introduction 64
2 Materials and methods 65
3 Experimental design 65
3.1 Cell counts 66
3.2 DNA extraction & polymerase chain reaction (PCR) 66
3.3 Denaturing Gradient Gel Electrophoresis (DGGE) and identification of bands of interest. 67
3.4 Statistical analysis 67
4 Results and Discussion 68
4.1 Growth of *Tetraselmis sp.* in FT and RT treatments 68
4.2 Bacteria community dynamics between the use of recycled media and fresh seawater 69
4.3 Algal community dynamics between the use of recycled media and fresh seawater 71
4.4 Water footprint 73
5 Conclusion 74
6 Acknowledgments 74
7 References 74

Chapter 8 Conclusion 79

Chapter One.

Chapter 1: Introduction

Objectives and achievements

The main aim of this research was to develop a deeper understanding of the closed loop systems that has been highlighted as a crucial component of any future algal biofuel technology.

Large scale microalgal biofuel production is widely regarded as a significant potential future fuel. The first goal of large scale microalgal biofuel production is to continuously grow sufficient amounts of algal biomass to feed downstream processes. After production algal biomass has to undergo harvesting and dewatering. Various methods in combination can be used within this stage, including electroflocculation, settling tanks, centrifugation and filter press.

The concentrated microalgae biomass is then converted into fuel; some conversion methods include lipid extraction of followed by transesterification, digestion of biomass to methane and hydrothermal liquefaction.

These processes produce various waste streams that can be reincorporated within the microalgal biofuel system. With the development of large scale biofuel, various closed loop systems have been introduced to reduce the cost of operations and overall economic feasibility of the process. Closed loop systems can involve the reuse of water and nutrients, for the growth of microalgae biomass to re-feed the downstream processes. Within this thesis I examined closed looped systems used within the microalgal biofuels field and determined its impact to the microalgae, the natural bacterial community, algal biomass and lipid production. As there are many different microalgae used within the biofuel industry various closed loop systems will have varying effects on the microalgae of interest, therefore this thesis will examine the potential of applying these closed loop systems as a method of improvement.

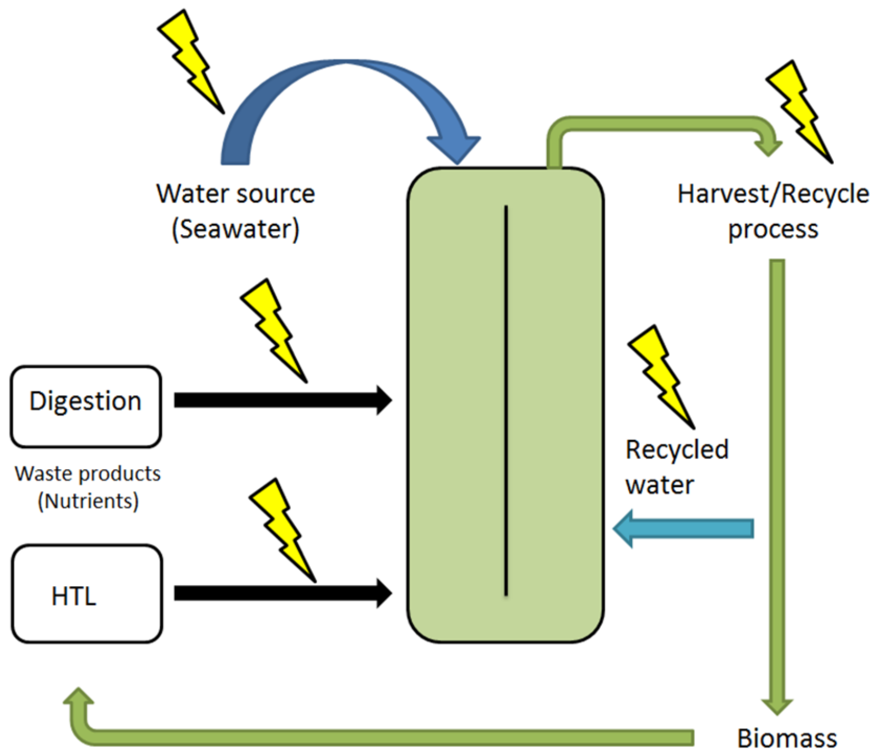

Figure 1. A summary of some of the processes used for the production of *Tetraselmis sp.* biomass for large-scale biofuel production. Blue arrows represent water sources for the open pond, green arrows represents the biomass processes, and black arrows represents potential nutrients sources from waste production; HTL: Hydrothermal liquefaction). The lightning bolts indicate sections that were focused on within this thesis, and are investigated in various chapters.

The specific objectives were:

1) Evaluate the use of microalgal digestate as a nutrient source to from a closed loop nutrient system.

The use of digestate as a biofertilizer for microalgae growth is a popular method of increasing the economic and environmental sustainability of microalgae biofuels. The digestion of microalgal biomass is used to produce methane; this process is commonly applied to lifecycle analyses that produce a biomass waste stream commonly seen with the transesterification process. Within this study I assessed the use of microalgal digestate, a by-product of the digestion for methane production that utilised microalgal

biomass. As the digestate effluent has been determined as a potential source of ammonia I examined its potential use as a nutrient source for *Tetraselmis sp.* and how it may be incorporated into a closed loop nutrient system.

2) Evaluate the use of the hydrothermal liquefaction aqueous phase as a nutrient source to grow microalgae.

Hydrothermal liquefaction is a method used to convert biomass into type II kerogen; this method has recently become increasingly popular within the future development of microalgae biofuels. During the hydrothermal liquefaction process four phases are produced, the oil, liquid, gas, and aqueous phase. Within this study I assessed the potential for the use of the hydrothermal liquefaction aqueous phase as a nutrient source for microalgal biofuels.

3) Analyse the effects of multiple recycle stages on the microalgal and bacterial communities.

Water reuse is a process that is applied to ensure the low consumption of water and increase the economic stability of a large scale process. Little is known about how the bacterial and microalgae community are affected by multiple water recycling. Within this study I examined the effects the *Tetraselmis MUR233* over various recycle cycles with non-sterile seawater sourced from Whyalla SA, together with an investigation into the impact on the bacterial and algal populations.

4) Analyse the impact of protozoa within a microalgae culture, by comparing a filtered and non-filtered seawater culture.

Protozoa have been identified as a potential problem for large scale microalgae growth, although there have also been various reports stating that microalgae and protozoa can be successfully co-cultured. I assessed the potential problems that may be caused by sourcing seawater containing protozoa and directly culturing microalgae with no filtration. This study will also assess what could potentially happen if water is not recycled and fresh seawater is used to replenish the water lost during the harvest.

5) Analyse the bacterial community throughout the harvesting process within a large scale open pond raceway.

Recycling water has been practiced at large scale at Karratha Western Australia to harvest the water and concentrate the biomass. I investigated the recycling and harvesting cycle to determine its overall effects on the bacterial community. Bacteria are an important symbiotic partner with microalgae, therefore understanding how they are affected within a large scale microalgae open raceway pond is important and novel. Within this study I investigated how the bacterial community was affected by a two stage harvesting recycle process which involved firstly electroflocculation and then centrifugation. The first stage, electroflocculation had little effect on the size and diversity of the bacterial community. The second stage, centrifugation resulted in a significant reduction in both the size and diversity of the bacterial community. Each stage re-introduced the water back into the raceway pond; however after one cycle there was little difference in terms of the bacterial community within the raceway pond.

Chapter 2.

Literature review: recycling of water and nutrients to form a self-sustained microalgal biofuels process.

1 Overview

The concept of algal biofuels has been evolving over many years; one area in particular is the way to increase the self-sustainability of the process by recycling waste streams. The unique aspect of algal biofuel research is the involvement of many different research fields from engineering, chemistry, microbiology and biotechnology. With the input from all the different research fields the ability to practice large-scale algal biofuel production has become a reality at various locations around the world. Large scale biofuel production however still has many different aspects that can further improve the economic viability of algal biofuels production. Recycling waste streams to produce algal biomass is an effective method of reducing production costs; two main waste streams within the algal biofuel production process are water and nutrient rich effluent (Du et al., 2012; Yang et al., 2011). The first waste stream produced by algal biofuel production is the water that is discarded during the harvesting and recycling process; the second main waste stream is the nutrient rich effluent produced during the conversion of biomass into biofuels. Utilising these waste streams to produce microalgal biomass is an effective way to form a self-sustained closed looped process. Reusing these waste streams and forming closed loops systems can increase the economic viability of the algal biofuel process and reduce the overall nutrient and water footprint of algal biofuel plants. Monitoring how these closed loop systems affect biomass production and evaluating their effects on the algae of interest is essential to prevent any negative impacts on biomass and biofuel production.

Applying waste streams within the algal biofuels process is desirable, though the effects on the algae of interest are not always similar to other what was observed in other studies. Research has shown that the impact of using waste streams can be beneficial for some algal species while negative for other algae species (Du et al., 2012). Understanding the potential problems at large scale and the compatibility of the species of interest to function with different waste streams is important; additionally, understanding how other non-desired organisms function with waste streams is important to monitor and determine any potential change in the dominance within the culture.

2 Recycling water

Water is a key part of the production of algal biofuels; recycling of water has been shown to be effective at reducing the water footprint of the microalgal biofuel process along with capturing nutrients that would otherwise have been lost within the harvested water. Water has always been a topic of priority within the algal biofuel sector, this is due to competition for water with agriculture and human consumption. The main argument is that the use of water for microalga production will be in direct competition with other uses (e.g. crop production) and will eventually lead to increased water prices. The selection of microalgae has to be tailored to the water environment surrounding the location of interest for potential large scale production. Within Australia, freshwater is not as abundant as seawater, therefore marine algae are a more desirable option for large scale due to their reduced cost. Additionally, sourcing water from a natural source that is unrestricted and not purchased can greatly reduce costs in comparison to sourcing water from a water utilities company.

Recycling water during the harvesting stage has been shown to have an improved effect on microalgal growth (Singh & Olsen, 2011; Yang et al., 2011). The benefit of recycling the water back into the open raceway is the minimization of introducing new microorganisms from external water sources (Day et al., 2012), the recovery of nutrients that have not been utilised (Fon Sing et al., 2014), and the reduction of the water footprint (Singh & Olsen, 2011). There has been little research that has investigated the changes in the microalgal and a bacterial community during the harvesting/recycle processes of water. Understanding how these processes affect the microalgal and bacterial communities can help develop an understanding of what may be occurring when harvesting and recycling is undertaken. As this water has to be reused it is important to determine if there is any potential negative impact to the microalgae of interest, for example understanding if some species are unaffected by the harvesting recycle system may increase competition with the microalgal species of interest. Bacteria are important to microalgae due to the close symbiotic relationship shared between the two microorganisms; bacteria utilise microalgae and their metabolites as a source of carbon, while the microalgae utilise the bacteria for as a source of vitamins (Cole, 1982; Kazamia et al., 2012).

The recycle and harvesting process will be used multiple times over a year as harvesting of biomass is conducted; how this affects the species of interest and the microbial community is

important to monitor. Fon Sing (2014) investigated the effects of a large scale recycling process over multiple stages. They observed an increase growth of *Tetraselmis MUR233* within the recycled pond in comparison to a pond that received fresh media after each harvest (Fon Sing et al., 2014).

If the water during the harvesting and recycle process is not recycled and new water is sourced each time the potential of introducing new microorganisms into the culture increase. Seawater has all different types of microbes presence such as bacteria, algae, and protozoa (Cole, 1982; Day et al., 2012). The presence of other microorganisms besides the microalgae of interests has to be accepted as the complete removal of every other micro-organisms is not feasible and not economically wise, therefore evaluating the performance of *Tetraselmis MUR233* in the presence of other microorganisms is important. Protozoa have been identified as a major concern due to their ability to consume microalgae; there have been varied results in the terms of effects on algal biomass production with the presence of protozoa within a culture. Some studies have reported the complete clarification of an algae culture in the presence of protozoa (Day et al., 2012), while others have reported that the presence of protozoa has little impact on the growth of microalgae. For example, Sananurak (2009) observed that in a large scale open pond, *Tetraselmis sp.* was not greatly affected by the presence of protozoa, while the rotifers growth was observed to be unstable (Sananurak et al., 2008).

The reuse of water has often been discussed by proponents of algal biofuel due to its beneficial effects on both the economical and water foot print; however there are a number of additional benefits and they are summarised below:

- It is more economical to reuse the water generated from the harvesting/recycle process.
- The water footprint is reduced.
- The species of interest will become more dominant over various recycle cycles.
- The bacterial that have the strongest symbiotic relationship will be maintained and thrive.
- The mitigation of new other microorganisms entering the microalgae pond such as protozoa.
- The harvesting system can be used to clean the water of all microbes if needed.

3 Recycling nutrients

Nutrients are one of the most important aspects of large scale microalgae production; nitrogen, phosphorus, carbon and trace elements are essential to the successful production of microalgae (Chen et al., 2011; Yang et al., 2011). There are many variations to concentration and forms of nutrients that can be used to culture microalgae, this is often due to the ability of the species of interest to utilise different nutrient sources and the most economical viable source.

3.1 Carbon

Carbon is a highly important nutrient for the production of lipid and biomass as it has the highest requirement for algae growth according to the Redfield ratio (Tett et al., 1985). Algae can utilise carbon as a form of chemical energy such as sugars or as CO_2 which is converted to carbonic acid (Tsuzuki et al., 1990). Different waste streams have been utilised as a carbon source such as flue gas, dairy waste and industrial effluents (Iyovo et al., 2010a; Iyovo et al., 2010b). Carbon dioxide is naturally present within the atmosphere and has been the cause of much concern due to its negative environmental impact with its increase atmospheric concentration (reference). The ability of algae's to use CO_2 as a source of carbon from the atmosphere is essential when forming a future sustainable source of algae.

Various studies have investigated the effects of using CO_2 from waste gaseous streams for the production of algal biomass. Chiu etal.(2009) investigated the difference in lipid accumulation under different CO_2 concentrations for *Nannochloropsos oculata*. The study tested various concentrations of CO_2, 2%, 5%, 10% and 15% CO_2. It was observed that growth under 2% CO_2 resulted in the highest lipid accumulation in comparison to the other CO_2 concentrations used (Chiu et al., 2009). Jiang 2011 cultivated *Nannochloropsis sp.* under 15% CO_2 at a continuous flow rate of 0.1 L min^{-1}, and under high light and nitrogen deprivation conditions they achieved 2.23 g L^{-1} cell density with a total lipid content of 59.9% (Jiang et al., 2011). Another study compared lipid and growth production of *Dunaliella viridis* under various CO_2 concentrations (0.035% and 1% CO_2) and under different nitrogen and light conditions. A 1% CO_2 enriched-air with sufficient O_2 and nitrogen resulted in the highest growth rates of *Dunaliella viridis*; triglycerides were also increased by 22% when *D. viridis* was grown in 1% CO_2 enriched-air under N deprived conditions (Gordillo et al., 2001).

The effects of adding either 10% CO_2 or a real flue gas containing 5.5% CO_2 on the growth and lipid production of *B. braunii* and *Scenedesmus sp.* were compared. While there was no significant difference between the yields obtained using the two carbon sources (Yoo et al.,

2010). Zeiler (1995) also investigated the assimilation and utilization of CO_2 from flue gas. At laboratory scale they showed efficient utilisation of simulated CO_2, as well sulphur oxide and nitrogen oxide when used as feedstock to produce biomass. Additionally at their large scale facility (Two 0.1 hectare ponds) in New Mexico they demonstrated there was no significant engineering barrier to prevent moving from small scale to large scale (Zeiler et al., 1995). Other studies have also shown direct utilisation of actual flue gas at demonstration-scale ponds with seawater (Negoro et al., 1993). Currently in Hawaii flue gas produced by power plants is being used as a CO_2 source for the production of algal biomass (Pedroni P, 2001). Doucha (2009) investigated the use of flue gas as a source of CO_2 in pilot plant photobioreactors at large scale. The investigation observed that 38.7% of the CO_2 in flue gas was utilized for the biosynthesis of biomass with 4.4 kg of CO_2 in flue gas required for the production of 1 kg of dry algal mass (Doucha et al., 2005). Douskova (2009) investigated the bioremediation of flue gas as a means of reducing microalgal biomass production costs. Their investigation compared flue gas to different concentrations of CO_2 and O_2. The highest growth of *C. vulgaris* strain P12 was observed under cooled flue gas which contained 13-20% (v/v) of CO_2. Higher growth was observed in flue gas in comparison to a controlled mixture of clean CO_2 and O_2; this was due to lower partial pressure of O_2 in flue gas (Douskova et al., 2009). Oxygen concentrations can influence the phenomena of photorespiration and photoinhibition which may have caused the lower growth in the clean CO_2 and O_2 mixture (Foyer & Noctor, 2003). Another large scale study was conducted for a 100 L air-lift photobioreactor for the cultivation of *S. obliquus WUST4*. Flue gas from a combustion chamber was captured and utilized as a carbon source. Under optimal conditions a CO_2 removal ratio of 67% (Li et al., 2011) was observed. However, the use of flue gas has also been shown to have an inhibitory effect on algae production due to presence of NO_x (Hauck et al., 1996; Matsumoto et al., 1997), though other studies have shown no inhibitory effects of flue gas on algae productions due to the presence of NO_x (Doucha et al., 2005). Overall the use of flue gas appears highly beneficial in comparison to the use of clean CO_2 due to the higher growth rates and environmental benefits for using flue gas.

Industrial effluents that are rich in carbon have also been investigated for their potential for use in the production of microalgae biomass. Yeh (2012) investigated the growth of *C. vulgaris ESP-31* under heterotrophic growth conditions. His investigation showed that *C. vulgaris ESP-31* did not grow well under heterotrophic conditions but did grow well under photoheterotrophic conditions with a 15 fold increase in biomass. The highest carbohydrate

concentration was obtained when carbon was added to the media, although the highest lipid content (19%-53%) was obtained when using MBL media due to its low nitrogen concentration (Yeh & Chang, 2012). Organic carbon from industrial dairy waste has been used for the mixotrophic cultivation of *C. vulgaris*. A comparison was formed between phototrophic and mixotrophic growth with dairy waste as an organic carbon source showed that lipid productivity was shown to be at the highest with mixotrophic growth (253 mg/L d) while phototrophic growth only achieved 42 mg/L d. In terms of biomass, once again mixotrophic growth led to greater biomass production (3.58 +- 0.12 g/L d compared with 1.22 +_ 0.12 g/L d) (Abreu et al., 2012). The use of carbon waste streams is certainly effective and can be incorporated in the production of algal biomass, though as discussed each species will vary in the production rates that can be achieved with different waste streams.

3.2 Phosphorus

Phosphorus is an essential nutrient due to its high use in cell synthesis e.g. for phospholipids and DNA production. Wu et al. (2012) investigated the limitations of phosphorus and the effects on *C. raciborskii*. They concluded that inorganic phosphorus played an important role in regulating growth, photosynthesis and metabolism. It was observed the synthesis of *Chl-a* by *Microcystis aeroginosa* was inhibited by low P conditions (Wu et al., 2012). The composition and concentration of stored chemical energy has been shown to change under different phosphorus nutrient levels. Sigee et.al. (2007investigated the effects of different phosphorus levels on algal growth and observed that t only at very low phosphorus levels was an increase in carbohydrate synthesis demonstrated (Sigee et al., 2007). Cade-Menun (2010) investigated the effects of phosphorus stress on algae and showed that in low phosphorus conditions the algae had significantly higher C:P and N:P ratios (Cade-Menun & Paytan, 2010). Vanucci et al. (2012) demonstrated that over the whole growth cycle there was an increase in cell volume under phosphorus limited conditions (Vanucci et al., 2012). *B. braunii KMITL2* was shown to produce higher biomass at high concentration of phosphorus (444mg L^{-1}). Ruangsomboon (2012) found there was a 7.3 fold increase in biomass when the phosphorus concentration increased from 22- 444 mg L^{-1}, although lower lipid content was also observed. Within the study they observed maximum lipid production at a phosphorus concentration of 222 mg L^{-1} (Ruangsomboon, 2012).

Algae have the ability to store phosphorus internally and utilise these internal source of phosphorus when the medium is depleted. Anu Ruiz (2014) investigated the effects when

microalgae are deprived of phosphorus within a continuous culture that is fed with nitrogen. It was found that the microalgae were still capable of growth, though when phosphorus was supplemented back into the culture over time the microalgae increased growth (Ruiz-Martinez et al., 2014). The ability of microalgae to tolerate phosphorus deprivation and continuously grow is a good indication of how well microalgae can survive within harsh environments.

A potential source of phosphorus that can be used within the closed loop algal biofuels process is the ash produced during the combustion and gasification, Lane et al (2014) observed that the ash produced by the combustion of microalgae consists of a high content of trace metals and phosphorus that can potentially be utilised for the growth of microalgae. To date there has been little research within this area as the need for phosphorus is much lower in comparison to the carbon and nitrogen (Lane et al., 2013). Utilising ash as a source of phosphorus for the production of microalgal biomass can potentially help reduce the overall costs of microalgae biomass production, though determining the effectiveness of this ash as a phosphorus source still needed to be investigated.

3.3 Nitrogen

Nitrogen is one of the most important nutrients for microalgae production. Its influence on lipid and biomass production by microalgae has been well researched. The application these waste sources of nitrogen for microalgal growth have been investigated due to the fact that nitrogen availability generally leads to increases in lipid and biomass production. Different waste sources can potentially be used as a nitrogen nutrient source; each form may have a different effect on the algae in terms of growth and lipid production. The source and concentrations of nitrogen in the media will also have an effect on the target species. Nitrogen is commonly added for increasing the growth of algae; the Redfield ratio identified the optimal molar ratio to be 16:1 (Tett et al., 1985). Under nitrogen deficient conditions algae will increase the production of lipids and carbohydrates rather than proteins. The introduction of a nitrogen rich stage for growth followed by a nitrogen limited stage for carbon energy production is the common cycle used within the microalgae biofuel process (Richardson et al., 1969). Therefore monitoring the ratio of nitrogen to carbon within a culture is important to determine to potential products that are being produced.

As there are many waste streams that contain nitrogen, the waste streams can be utilised to form closed loop nutrient systems. Closed loops systems can include the use of by-products

produced during the biomass to biocrude conversion within the algal biofuel process. Biomass can be converted into biofuels, with the digestion of biomass or the conversion of biomass into biocrude with hydrothermal liquefaction.

After the extraction of lipid from microalgae a large amount of biomass is leftover, this biomass can be used for digestion. Digestion works anaerobically with methanogens which are capable of breaking down the microalgae biomass into methane (Rösch et al., 2012). After digestion has taken place and methane production has ceased the digestate effluent can be collected and reused as a source of nitrogen due to its high ammonia content. Studies have shown that digestate can be used for the growth of microalgae (Iyovo et al., 2010a). This is important since it displays the use of waste streams as a nutrient source. The benefit of using microalgae digestate for microalgae growth is due to its self-sustaining cycle. The reuse of digestate has been shown to be an economical advantage for microalgae fuel production.

A problem with some of these waste streams is the high concentration of ammonia. Ammonia is a common source of nitrogen for algal growth; the most common source of ammonia is through manure, digestate and farming fertilizer (Uggetti et al., 2014). Studies have shown that when the concentration of digestate is increased, lipid and growth production if negatively affected. Xin et.al (2010) examined the growth of 14 different algae on digestate effluent; only 4 of the 11 species were able to grow on the digestate (Xin et al., 2010).

Hydrothermal liquefaction has been shown to be the new focus of biomass to biocrude conversion. This process has been shown to be an effective method for dealing with biomass in comparison to the traditional solvent extraction and lipid conversion with transesterfication (Biller et al., 2012; Brown et al., 2010). During hydrothermal liquefaction a waste product in the form of the aqueous phase is produced which is rich in nitrogen. Current research has shown that microalgae can utilise this waste stream for microalgae growth, though heavy dilution, up to 500-fold is required (Biller et al., 2012). Studies have identified that the hydrothermal liquefaction aqueous phase (HTL AP) is highly toxic to some microalgae, due to its high concentration of ammonia, phenols, nickel and total organic carbon (Biller et al., 2012; Jena et al., 2011). The concentration of ammonia within the HTL AP is much higher in comparison to digestate effluent, although the composition is highly variable due to the presence of hydrocarbons, high levels of organic nitrogen and other heavy metals not commonly seen in digestate effluent.

Table 1, A comparison of different types of hydrothermal liquefaction aqueous phases observed.

	U Jena et.al 2011	L. Garcia Alba et.al 2013	P. Biller 2012					Du. et.al 2011
	S. platensis	*Desmodesmus sp.*	*Chlorogloeopsis*	*Spirulina*	*Chlorella 300oc*	*Chlorella*	*S. dimorphus*	*Chlorella vulgaris*
TKN	16200	–	5636	8136	6636	6888	3139	9650 ± 1582
Ammonia	12700	1964	4748	6295	5673	5920	5280	1343 ± 75
Nitrate	26.76	70	508	194	329	237	192	211 ± 20
Phosphate	795	159	280	2159	3109	1121	1470	343 ± 43
Phenols	50.9	58	178	98	108	158	80	
Potassium	–	–	303	1506	1460	1419	1150	775.45
Nickel	–	–	3.8	0	0.1	0.4	0.8	0.005
TOC	–	–	9060	15,123	11,373	13,764	11,119	45700 ± 1513
Total dry weight	*C. minutissima* , 500x, 0.52g/L, 12 days	20x, 0.21g/L, 96 hours	100x 0.498g/L, 12 days	400x 0.66g/L, 12 days	100x, 0.88g/L, 12 days	200x, 0.09 g/L, 12 days	400x, 0.05g/L, 12 days	50x, ~0.6g/L, 5 days

The overall the growth that is achieved using HTL AP is very low even when high dilutions are used to reduce the toxicity (Table 1). The use of the HTL AP as a potential nutrient source may not be suitable for algae. As shown in table 1 various different microalgae have undergone growth trials using the HTL AP, the highest growth is 0.6g/L by Du et.al 2011 which is very low for *Chorella Vulgaruis* (Du et al., 2012).

Many of these nitrogen waste streams contain of high concentrations of ammonia. Ammonia can cause inhibitory effects at high pH levels (pH 8.5-10) Ammonia is capable to penetrate the cell and elevate the internal pH, preventing the production of electron acceptors for photosynthesis (Abeliovich & Azov, 1976). Exposure of microalgal cells to ammonia for several hours at a high pH (pH 9-10) results in a long-term negative effect. At large scale *C. vulgaris* has been shown to be effective at ammonia removal. Kim (2010) utilised *C. vulgaris* to remove ammonia at a large scale waste water facility. A reduction of 7.6 mg/L to 3.4 mg/L of ammonia occurred during the rapid growth phase (24 to 96 h) at a constant pH of 7 (Kim et al., 2010). With effective control of pH the use of ammonia has been shown to be an effective means of nitrogen at large-scale.

3.4 Trace metals

Trace metals are an important part of the production of algae for biofuels. There has been little research in the alteration of trace metals concentration at large scale, but small scale studies have been conducted to investigate the effects of trace metals on organisms. Understanding what effects trace metals have at small scale will give a better understanding of large scale production problems.

The function of iron has been well documental in the chloroplast structure, function and development (Pushnik et al., 1984; Terry and Abadía, 1986; Guerinot, 2010). Iron has also been shown to play a key part in regulating phytoplankton and microalgal biomass (Behrenfeld et al., 2006). Hu (2004) reported that when there is an iron deficiency present it

reduces the growth and overall biomass of the algae (Hu, 2007). Borowitzka (1990) identified Fe as one of the major nutrients which was added to ponds for *Dunaliella salina* production at large scale (Borowitzka and Borowitzka, 1990). High iron concentrations can help increase total lipid and biomass concentration (Liu et al., 2008). Iron can also prolong the exponential phase and increase final cell density (Liu et al., 2008). Chen (2011) investigated the changes in concentration of metals during the growth of *D. tertiolect*. The investigation monitored the concentrations of iron, cobalt, zinc, manganese, molybdenum, silicon, calcium and sulphur over time. Only iron and molybdenum dropped statistically significantly in concentration, iron (8 % decrease) and molybdenum (49 % decrease) (Chen et al., 2011).

Copper concentrations have been shown to have varying effects on different types of microalgal growth. Bentley-Mowat and Reid (1977) found concentrations of lead and copper as high as 5×10^{-4} M were required to inhibit the growth of *Phaeodactylum tricornutum, Tetraselmis sp., Dunaliella tertiolecta,* and *Cricosphaera elongata* in s88 medium (Bentley-Mowat & Reid, 1977).

As trace metals are found in natural water sources it is essential to understand the concentrations present to maximise lipid and biomass production. Iron has been shown to play a major role in biomass and lipid production at both small and large scale and represents a key element to monitor due to its impact on productivity. There has to date been little research on the waste streams that can be potentially utilised as a source of trace metals, although preliminary analysis suggests that they are commonly found within most waste sources.

4 Conclusion

Forming closed loop systems to recycle the waste products to produce microalgal biomass is an effective way of increasing the economic viability and maximising the benefits of producing fuel using microalgal biomass. There are many different waste streams that can be used to produce biomass; however their suitability needs to be assessed before implementation at large scale. As discussed previously different waste streams will have different effects on various species. Additionally not only should the impact on the algal species of interest be determined but also the impact on the natural bacterial and microalgae communities should also be assessed to prevent any potential shift in the culture dynamics

5 References

Abeliovich, A., Azov, Y. 1976. Toxicity of ammonia to algae in sewage oxidation ponds. *Applied and Environmental Microbiology*, **31**(6), 801-806.

Abreu, A.P., Fernandes, B., Vicente, A.A., Teixeira, J., Dragone, G. 2012. Mixotrophic cultivation of< i> Chlorella vulgaris</i> using industrial dairy waste as organic carbon source. *Bioresource Technology*, **118**, 61-66.

Bentley-Mowat, J., Reid, S. 1977. Survival of marine phytoplankton in high concentrations of heavy metals, and uptake of copper. *Journal of Experimental Marine Biology and Ecology*, **26**(3), 249-264.

Biller, P., Ross, A.B., Skill, S., Lea-Langton, A., Balasundaram, B., Hall, C., Riley, R., Llewellyn, C. 2012. Nutrient recycling of aqueous phase for microalgae cultivation from the hydrothermal liquefaction process. *Algal Research*, **1**(1), 70-76.

Brown, T.M., Duan, P., Savage, P.E. 2010. Hydrothermal liquefaction and gasification of Nannochloropsis sp. *Energy & Fuels*, **24**(6), 3639-3646.

Cade-Menun, B.J., Paytan, A. 2010. Nutrient temperature and light stress alter phosphorus and carbon forms in culture-grown algae. *Marine Chemistry*, **121**(1), 27-36.

Chen, M., Tang, H., Ma, H., Holland, T.C., Ng, K., Salley, S.O. 2011. Effect of nutrients on growth and lipid accumulation in the green algae< i> Dunaliella tertiolecta</i>. *Bioresource Technology*, **102**(2), 1649-1655.

Chiu, S.-Y., Kao, C.-Y., Tsai, M.-T., Ong, S.-C., Chen, C.-H., Lin, C.-S. 2009. Lipid accumulation and CO< sub> 2</sub> utilization of< i> Nannochloropsis oculata</i> in response to CO< sub> 2</sub> aeration. *Bioresource Technology*, **100**(2), 833-838.

Cole, J.J. 1982. Interactions between bacteria and algae in aquatic ecosystems. *Annual Review of Ecology and Systematics*, 291-314.

Day, J.G., Thomas, N.J., Achilles-Day, U.E., Leakey, R.J. 2012. Early detection of protozoan grazers in algal biofuel cultures. *Bioresource Technology*, **114**, 715-719.

Doucha, J., Straka, F., Lívanský, K. 2005. Utilization of flue gas for cultivation of microalgae <i>Chlorella sp.) in an outdoor open thin-layer photobioreactor. *Journal of Applied Phycology*, **17**(5), 403-412.

Douskova, I., Doucha, J., Livansky, K., Machat, J., Novak, P., Umysova, D., Zachleder, V., Vitova, M. 2009. Simultaneous flue gas bioremediation and reduction of microalgal biomass production costs. *Applied Microbiology and Biotechnology*, **82**(1), 179-185.

Du, Z., Hu, B., Shi, A., Ma, X., Cheng, Y., Chen, P., Liu, Y., Lin, X., Ruan, R. 2012. Cultivation of a microalga *Chlorella vulgaris* using recycled aqueous phase nutrients from hydrothermal carbonization process. *Bioresource Technology*, **126**, 354-357.

Fon Sing, S., Isdepsky, A., Borowitzka, M., Lewis, D. 2014. Pilot-scale continuous recycling of growth medium for the mass culture of a halotolerant< i> Tetraselmis</i> sp. in raceway ponds under increasing salinity: A novel protocol for commercial microalgal biomass production. *Bioresource Technology*, **161**, 47-54.

Foyer, C.H., Noctor, G. 2003. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiologia Plantarum*, **119**(3), 355-364.

Gordillo, F.J., Niell, F.X., Figueroa, F.L. 2001. Non-photosynthetic enhancement of growth by high CO2 level in the nitrophilic seaweed Ulva rigida C. Agardh (Chlorophyta). *Planta*, **213**(1), 64-70.

Iyovo, G.D., Du, G., Chen, J. 2010a. Poultry manure digestate enhancement of Chlorella Vulgaris biomass under mixotrophic condition for biofuel production. *J Microbial Biochem Technol*, **2**, 51-57.

Iyovo, G.D., Du, G., Chen, J. 2010b. Sustainable Bioenergy Bioprocessing: Biomethane Production, Digestate as Biofertilizer and as Supplemental Fee d in Algae Cultivation to Promote Algae Biofuel Commercialization. *J Microbia l Biochem Technol*, **2**, 100-106.

Jena, U., Das, K., Kastner, J. 2011. Effect of operating conditions of thermochemical liquefaction on biocrude production from< i> Spirulina platensis</i>. *Bioresource Technology*, **102**(10), 6221-6229.

Jiang, L., Luo, S., Fan, X., Yang, Z., Guo, R. 2011. Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO2. *Applied Energy*, **88**(10), 3336-3341.

Kazamia, E., Czesnick, H., Nguyen, T.T.V., Croft, M.T., Sherwood, E., Sasso, S., Hodson, S.J., Warren, M.J., Smith, A.G. 2012. Mutualistic interactions between vitamin B12-dependent algae and heterotrophic bacteria exhibit regulation. *Environmental Microbiology*, **14**(6), 1466-1476.

Kim, J., Lingaraju, B.P., Rheaume, R., Lee, J.-Y., Siddiqui, K.F. 2010. Removal of Ammonia from Wastewater Effluent by< i> Chlorella Vulgaris</i>. *Tsinghua Science & Technology*, **15**(4), 391-396.

Lane, D.J., Ashman, P.J., Zevenhoven, M., Hupa, M., van Eyk, P.J., de Nys, R., Karlström, O., Lewis, D.M. 2013. Combustion Behavior of Algal Biomass: Carbon Release, Nitrogen Release, and Char Reactivity. *Energy & Fuels*, **28**(1), 41-51.

Lardon, L., Hélias, A., Sialve, B., Steyer, J.-P., Bernard, O. 2009. Life-cycle assessment of biodiesel production from microalgae. *Environmental science & technology*, **43**(17), 6475-6481.

Li, F.-F., Yang, Z.-H., Zeng, R., Yang, G., Chang, X., Yan, J.-B., Hou, Y.-L. 2011. Microalgae Capture of CO2 from Actual Flue Gas Discharged from a Combustion Chamber. *Industrial & Engineering Chemistry Research*, **50**(10), 6496-6502.

Negoro, M., Hamasaki, A., Ikuta, Y., Makita, T., Hirayama, K., Suzuki, S. 1993. Carbon dioxide fixation by microalgae photosynthesis using actual flue gas discharged from a boiler. *Applied Biochemistry and Biotechnology*, **39-40**(1), 643-653.

Pedroni P, D.J., Beckert H, Bergman P, Benemann J. 2001. A proposal to establish an international network on biofixation of CO2 and greenhouse gas abatement with microalgae. Workshop held in January 2001 at the EniTechnologie Research Facility in Monterotondo, Italy.

Richardson, B., Orcutt, D., Schwertner, H., Martinez, C.L., Wickline, H.E. 1969. Effects of nitrogen limitation on the growth and composition of unicellular algae in continuous culture. *Applied microbiology*, **18**(2), 245-250.

Rösch, C., Skarka, J., Wegerer, N. 2012. Materials flow modeling of nutrient recycling in biodiesel production from microalgae. *Bioresource Technology*, **107**, 191-199.

Ruangsomboon, S. 2012. Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, Botryococcus braunii KMITL 2. *Bioresource Technology*, **109**(0), 261-265.

Ruiz-Martinez, A., Serralta, J., Pachés, M., Seco, A., Ferrer, J. 2014. Mixed microalgae culture for ammonium removal in the absence of phosphorus: Effect of phosphorus supplementation and process modeling. *Process Biochemistry*.

Sananurak, C., Lirdwitayaprasit, T., Menasveta, P. 2008. Development of a closed-recirculating, continuous culture system for microalga (Tetraselmis suecica) and rotifer (Brachionus plicatilis) production.

Sigee, D.C., Bahrami, F., Estrada, B., Webster, R.E., Dean, A.P. 2007. The influence of phosphorus availability on carbon allocation and P quota in Scenedesmus subspicatus: a synchrotron-based FTIR analysis. *Phycologia*, **46**(5), 583-592.

Singh, A., Olsen, S.I. 2011. A critical review of biochemical conversion, sustainability and life cycle assessment of algal biofuels. *Applied Energy*, **88**(10), 3548-3555.

Tett, P., Droop, M., Heaney, S. 1985. The Redfield ratio and phytoplankton growth rate. *Journal of the Marine Biological Association of the United Kingdom*, **65**(02), 487-504.

Tsuzuki, M., Ohnuma, E., Sato, N., Takaku, T., Kawaguchi, A. 1990. Effects of CO_2 concentration during growth on fatty acid composition in microalgae. *Plant Physiology*, **93**(3), 851-856.

Uggetti, E., Sialve, B., Latrille, E., Steyer, J.-P. 2014. Anaerobic digestate as substrate for microalgae culture: The role of ammonium concentration on the microalgae productivity. *Bioresource Technology*, **152**, 437-443.

Vanucci, S., Pezzolesi, L., Pistocchi, R., Ciminiello, P., Dell'Aversano, C., Iacovo, E.D., Fattorusso, E., Tartaglione, L., Guerrini, F. 2012. Nitrogen and phosphorus limitation effects on cell growth, biovolume, and toxin production in< i> Ostreopsis</i> cf.< i> ovata</i>. *Harmful Algae*, **15**, 78-90.

Xin, L., Hong-ying, H., Jia, Y. 2010. Lipid accumulation and nutrient removal properties of a newly isolated freshwater microalga,< i> Scenedesmus</i> sp. LX1, growing in secondary effluent. *New biotechnology*, **27**(1), 59-63.

Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M., Chen, Y. 2011. Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance. *Bioresource Technology*, **102**(1), 159-165.

Yeh, K.-L., Chang, J.-S. 2012. Effects of cultivation conditions and media composition on cell growth and lipid productivity of indigenous microalga< i> Chlorella vulgaris</i> ESP-31. *Bioresource Technology*, **105**, 120-127.

Yoo, C., Jun, S.-Y., Lee, J.-Y., Ahn, C.-Y., Oh, H.-M. 2010. Selection of microalgae for lipid production under high levels carbon dioxide. *Bioresource Technology*, **101**(1), S71-S74.

Zeiler, K.G., Heacox, D.A., Toon, S.T., Kadam, K.L., Brown, L.M. 1995. The use of microalgae for assimilation and utilization of carbon dioxide from fossil fuel-fired power plant flue gas. *Energy Conversion and Management*, **36**(6–9), 707-712.

Chapter 3

STATEMENT OF AUTHORSHIP

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

Journal: *Bioresource Technology*

Mason Erkelens (First Author)

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

Signed Date......5/11/14

Andrew Ward (Co- author)

Provided the digestate used for this study, drafted the manuscript.

Signed Date..5/11/14

Andew S Ball (Co- author)

Supervised the study, helped interpret the data, and drafted the manuscript.

Signed Date...11/02/15

David M Lewis (Co- author)

Supervised the study and developed the scientific approach, helped interpret the data, and drafted the manuscript.

Signed ..... Date..19/12/14

Bioresource Technology 167 (2014) 81–86

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

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

Mason Erkelens [a,b,*], Andrew J. Ward [a], Andrew S. Ball [b], David M. Lewis [a]

[a] School of Chemical Engineering, University of Adelaide, Adelaide 5005, Australia
[b] School of Applied Sciences, RMIT, Bundoora 3083, Australia

HIGHLIGHTS

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

ARTICLE INFO

Article history:
Received 3 April 2014
Received in revised form 30 May 2014
Accepted 31 May 2014
Available online 9 June 2014

Keywords:
Digestate effluent
Tetraselmis sp.
Biofuels
Microalgae
PCR-DGGE

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.

© 2014 Elsevier Ltd. All rights reserved.

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

* Corresponding author at: School of Chemical Engineering, University of Adelaide, Adelaide 5005, Australia. Tel.: +61 883133959.
E-mail address: mason.erkelens@adelaide.edu.au (M. Erkelens).

http://dx.doi.org/10.1016/j.biortech.2014.05.126
0960-8524/© 2014 Elsevier Ltd. All rights reserved.

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 μm photon/m^2/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 μL) of RNA template was used to provide a yield of 20 μL 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 where 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 μL per reaction. Template DNA (1 μL) 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 Themocycler (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× 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 (3257*g* × 5 min at 21 °C). Samples were dried at 35 °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 μL 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; $\Delta C(t)$sample = average C(t)ACP − average C(t)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 °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 ($H = -\sum(\frac{n}{N})\log(\frac{n}{N})$) 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

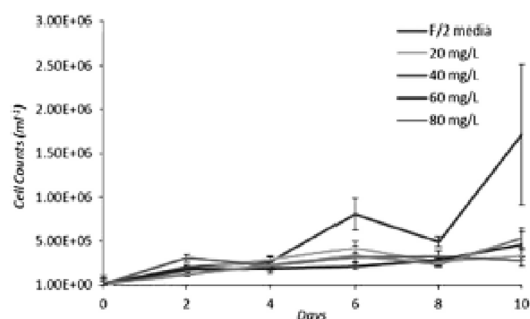

Fig. 1. Cell count of *Tetraselmis* sp. in F/2 medium and MDE medium over 10 days.

(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 g/L d^{-1} while non-autoclaved raw municipal wastewater concentrate achieved 0.1677 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 piggery 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 mg L^{-1} NH_4^+ produced no lipid while growth in the 10 mg L^{-1} NH_4^+ digestate achieved 3.6 ± 0.6 mg L^{-1} d^{-1} of lipid. *N. oleoabundans* grown on

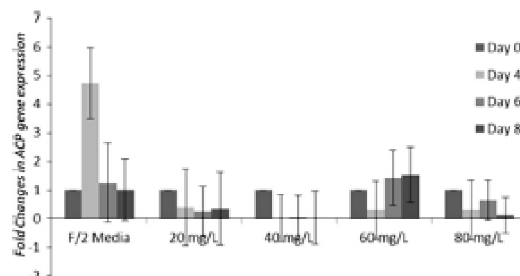

Fig. 2. ACP gene regulation for F/2 medium and digestate medium over the exponential phase.

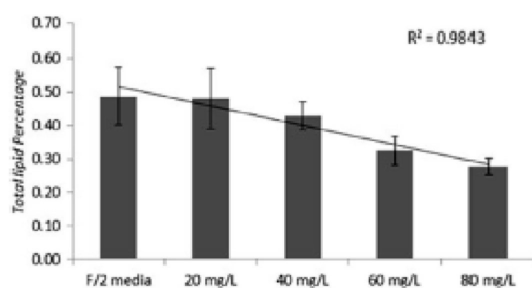

Fig. 3. Total Lipid content of *Tetraselmis* sp. at day 10.

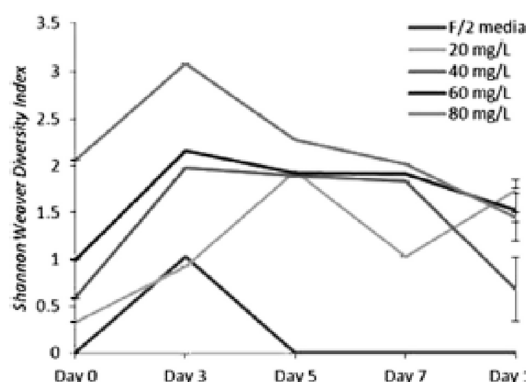

Fig. 4. Bacterial diversity in photobioreactors for F/2 medium and microalgal digestate effluent.

1:50 dilution of digestate achieved a 2.57 $mg\,L^{-1}\,d^{-1}$. A 1:200 dilution had a lipid productivity of 4.7 $mg\,L^{-1}\,d^{-1}$(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 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.

Acknowledgements

We would like to thank the South Australian Museum, South Australian Regional Facility for Molecular Evolution and Ecology for allowing us to conduct work within their facilities. This research was supported under Australian Research Council's Linkage Projects funding scheme (Project LP100200616) with industry partner SQC Pty Ltd, and the Australian Renewable Energy Agency advanced biofuels investment readiness program funding agreement number Q00150.

References

Abeliovic, A., Azov, Y., 1976. Toxicity of ammonia to algae in sewage oxidation ponds. Appl. Environ. Microbiol. 31, 801–806.

Abou-Shanab, R.A.I., Ji, M.-K., Kim, H.-C., Paeng, K.-J., Jeon, B.-H., 2013. Microalgal species growing on piggery wastewater as a valuable candidate for nutrient removal and biodiesel production. J. Environ. Manage. 115, 257–264.

Arumugam, M., Agarwal, A., Arya, M.C., Ahmed, Z., 2011. Influence of organic waste and inorganic nitrogen source on biomass productivity of Scenedesmus and Chlorococcum sp.. Int. J. Energy Environ. 2, 1125–1132.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Byers, D.M., Gong, H., 2007. Acyl carrier protein: structure–function relationships in a conserved multifunctional protein family. Biochem. Cell Biol. 85, 649–662.

Cho, S., Lee, N., Park, S., Yu, J., Luong, T.T., Oh, Y.-K., Lee, T., 2013. Microalgae cultivation for bioenergy production using wastewaters from a municipal WWTP as nutritional sources. Bioresour. Technol. 131, 515–520.

Costa, R.A.A.M.D., Koening, M.L., Macedo, S.J.D., 2004. Urban secondary sewage: an alternative medium for the culture of Tetraselmis chuii (Prasinophyceae) and Dunaliella viridis (Chlorophyceae). Braz. Arch. Biol. Technol. 47, 451–459.

Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G., 2005. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438, 90–93.

El-Shafai, S.A., El-Gohary, F.A., Nasr, F.A., van der Steen, N.P., Gijzen, H.J., 2004. Chronic ammonia toxicity to duckweed-fed tilapia (Oreochromis niloticus). Aquaculture 232, 117–127.

Erkelens, M., Ball, A.S., Lewis, D., 2014. The influences of the recycle process on the bacterial community in a pilot scale microalgae raceway pond. Bioresour. Technol. 157.

Fon Sing, S., Isdepsky, A., Borowitzka, M., Lewis, D., 2014. Pilot-scale continuous recycling of growth medium for the mass culture of a halotolerant *Tetraselmis* sp. in raceway ponds under increasing salinity: a novel protocol for commercial microalgal biomass production. Bioresour. Technol. 161, 47–54.

Gao, T., Li, X., 2011. Using thermophilic anaerobic digestate effluent to replace freshwater for bioethanol production. Bioresour. Technol. 102, 2126–2129.

Guzmán, H., Jara Valido, A., Duarte, L., Presmanes, K., 2010. Estimate by means of flow cytometry of variation in composition of fatty acids from Tetraselmis suecica in response to culture conditions. Aquacult. Int. 18, 189–199.

Källqvist, T., Svenson, A., 2003. Assessment of ammonia toxicity in tests with the microalga, Nephroselmis pyriformis, Chlorophyta. Water Res. 37, 477–484.

Keller, M.D., Selvin, R.C., Claus, W., Guillard, R.R.L., 1987. Media for the culture of oceanic ultraphytoplankton 1,2. J. Phycol. 23, 633–638.

Konig, A., Pearson, H., Silva, S.A., 1987. Ammonia toxicity to algal growth in waste stabilization ponds. Water Sci. Technol. 19, 115–122.

Kumar, M.S., Miao, Z.H., Wyatt, S.K., 2010. Influence of nutrient loads, feeding frequency and inoculum source on growth of Chlorella vulgaris in digested piggery effluent culture medium. Bioresour. Technol. 101, 6012–6018.

Lane, D.J., Ashman, P.J., Zevenhoven, M., Hupa, M., van Eyk, P.J., de Nys, R., Karlström, O., Lewis, D.M., 2013. Combustion behavior of algal biomass: carbon release, nitrogen release, and char reactivity. Energy Fuels 28, 41–51.

Lei, A., Chen, H., Shen, G., Hu, Z., Chen, L., Wang, J., 2012. Expression of fatty acid synthesis genes and fatty acid accumulation in haematococcus pluvialis under different stressors. Biotechnol. Biofuels 5, 18.

Levine, R.B., Costanza-Robinson, M.S., Spatafora, G.A., 2011. Neochloris oleoabundans grown on anaerobically digested dairy manure for concomitant nutrient removal and biodiesel feedstock production. Biomass Bioenergy 35, 40–49.

Li, Y., Chen, Y.-F., Chen, P., Min, M., Zhou, W., Martinez, B., Zhu, J., Ruan, R., 2011. Characterization of a microalga Chlorella sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresour. Technol. 102, 5138–5144.

Li, Y., Fei, X., Deng, X., 2012. Novel molecular insights into nitrogen starvation-induced triacylglycerols accumulation revealed by differential gene expression analysis in green algae Micractinium pusillum. Biomass Bioenergy 42, 199–211.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

Nashawi, I.S., Malallah, A., Al-Bisharah, M., 2010. Forecasting world crude oil production using multicyclic hubbert model. Energy Fuels 24, 1788–1800.

Nielsen, M., Bruhn, A., Rasmussen, M., Olesen, B., Larsen, M., Møller, H., 2012. Cultivation of Ulva lactuca with manure for simultaneous bioremediation and biomass production. J. Appl. Phycol. 24, 449–458.

Prajapati, S.K., Kumar, P., Malik, A., Vijay, V.K., 2014. Bioconversion of algae to methane and subsequent utilization of digestate for algae cultivation: a closed loop bioenergy generation process. Bioresour. Technol. 158, 174–180.

Shannon, C., Weaver, W., 1963. The Mathematical Theory of Communication. University of Illinos Press, Urbana, IL.

Sherwood, A.R., Presting, G.G., 2007. Universial primers amplify a 23s rDNA plastid marker in eukayotic algae and cyanobacteria. J. Phycol. 43, 605–608.

Uggetti, E., Sialve, B., Latrille, E., Steyer, J.-P., 2014. Anaerobic digestate as substrate for microalgae culture: the role of ammonium concentration on the microalgae productivity. Bioresour. Technol. 152, 437–443.

Vasseur, C., Bougaran, G., Garnier, M., Hamelin, J., Leboulanger, C., Chevanton, M.L., Mostajir, B., Sialve, B., Steyer, J.-P., Fouilland, E., 2012. Carbon conversion efficiency and population dynamics of a marine algae–bacteria consortium growing on simplified synthetic digestate: first step in a bioprocess coupling algal production and anaerobic digestion. Bioresour. Technol. 119, 79–87.

Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., Ruan, R.R., 2010. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae Chlorella sp.. Bioresour. Technol. 101, 2623–2628.

Ward, A., Lewis, D., Green, F., 2014. Anaerobic digestion of algae biomass: a review. Algal Res..

Xin, L., Hong-ying, H., Jia, Y., 2010. Lipid accumulation and nutrient removal properties of a newly isolated freshwater microalga, Scenedesmus sp. LX1, growing in secondary effluent. New Biotechnol. 27, 59–63.

Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M., Chen, Y., 2011. Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance. Bioresour. Technol. 102, 159–165.

Chapter 4

STATEMENT OF AUTHORSHIP

Title of paper: The application of activated carbon for the treatment and reuse of the aqueous phase derived from the hydrothermal liquefaction of a halophytic Tetraselmis sp..

Journal: *Bioresource Technology*

Mason Erkelens (First Author)

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

Signed Date...19/02/295

Andew S Ball (Co- author)

Supervised the study, helped interpret the data, and drafted the manuscript.

Signed Date...11/02/15

David M Lewis (Co- author)

Supervised the study and developed the scientific approach, helped interpret the data, and drafted the manuscript.

Signed ...... Date...19/12/14

Bioresource Technology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

The application of activated carbon for the treatment and reuse of the aqueous phase derived from the hydrothermal liquefaction of a halophytic *Tetraselmis* sp.

Mason Erkelens [a,*], Andrew S. Ball [b], David M. Lewis [a,c]

[a] School of Chemical Engineering, University of Adelaide, 5005, Australia
[b] School of Applied Sciences, RMIT University, 3083, Australia
[c] Muradel Pty Ltd, Maylands, 5069, Australia

HIGHLIGHTS

- Active carbon treatment greatly affected the organic nitrogen content.
- 1000× dilution of the treated HTL AP generated 0.41 g/L of biomass.
- Heavy dilutions required for microalgae growth.

ARTICLE INFO

Article history:
Received 4 November 2014
Received in revised form 27 January 2015
Accepted 29 January 2015
Available online xxxx

Keywords:
Active carbon
Hydrothermal liquefaction
Tetraselmis sp.

ABSTRACT

The aim of this study was to determine how the treatment of the HTL AP with activated carbon would affect both growth and chemical composition of the microalgae. *Tetraselmis MUR233* was grown in HTL AP (filtered and unfiltered) at 500×, 1000×, and 2000× dilutions in hyper saline conditions. The organic nitrogen and carbon component of the HTL AP was greatly reduced with the activated carbon treatment (TKN 52,000 ± 520 mg/L to 5900 ± 59 mg/L; TOC 19,000 ± 190 mg/L to 13,000 ± 130 mg/L). Growth of *Tetraselmis MUR233* was achieved on all dilutions of HTL AP, with a maximum growth observed in the AP filtered 1000× dilution treatment (0.41 ± 0.09 g/L), this compares to a yield of 0.49 ± 0.10 g/L when grown in traditional culture media.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

The production of green crude via hydrothermal liquefaction (HTL) is a now a popular method proposed to be implemented into design plans for large scale microalgal biofuels production (Orfield et al., 2014). HTL is capable of converting biomass into renewable crude oil (known as green crude) under sub-critical water conditions and produces solid/organic, aqueous and gas phases (Anastasakis and Ross, 2011). The organic phase produced from HTL is predominately hydrocarbon and the solid, gaseous and aqueous phases generated are by-products. Current research activities are focussing on potential applications of the AP by hydrothermal gasification and the utilisation of the AP for nutrient recycling (Biller and Ross, 2011; Garcia Alba et al., 2013; Orfield et al., 2014).

The AP has been shown to be capable of supporting the growth of some microalgae with varying success, though the success was only achievable with significant dilution (Biller and Ross, 2011; Garcia Alba et al., 2013; Jena et al., 2011). Previous studies have investigated the use of growing microalgae on the AP with the same species used as a biomass source for HTL. Garcia Alba et al. (2013) reported the generation of 0.21 g/L of *Desmodesmus* sp. with a 20× dilution of the AP. They also showed that increased biomass can be generated by combining the AP with commercial medium (Garcia Alba et al., 2013). Jena et al. (2011) also investigated the ability of *Chlorella minutissima* to grow on the AP; the highest total dry weight achieved within their study was 0.52 g/L with a 500× dilution. Du et al. (2012) observed the highest total microalgal biomass produced on the AP with a total dry weight of 0.5 g/L at a 500× dilution (Du et al., 2012). High dilutions of AP is required due to the high concentrations of ammonia, phenols and nickel compounds that are all known to have inhibitory effects on microalgae growth (Azov and Goldman, 1982; Fiorentino et al., 2003; Garcia Alba et al., 2013).

* Corresponding author at: Gate 5, Frome Road, Adelaide, 5005 South Australia, Australia. Tel.: +61 8 83133959.
E-mail address: mason.erkelens@adelaide.edu.au (M. Erkelens).

http://dx.doi.org/10.1016/j.biortech.2015.01.129
0960-8524/© 2015 Elsevier Ltd. All rights reserved.

Please cite this article in press as: Erkelens, M., et al. The application of activated carbon for the treatment and reuse of the aqueous phase derived from the hydrothermal liquefaction of a halophytic *Tetraselmis* sp.. Bioresour. Technol. (2015), http://dx.doi.org/10.1016/j.biortech.2015.01.129

The use of the HTL AP has been focused on reducing the concentration of toxic compounds by heavy dilutions, though little research has been conducted on the active carbon pre-treatment on the HTL AP before utilising it for microalgal growth. The application of using pretreatments for the HTL AP before utilising it for microalgae growth is a novel step forward to ensuring its potential future success as a nutrient source. Removing some of the compounds within the AP could potentially increase the growth of microalgae on the AP. Activated carbon has been used within various industries such as water purification, wastewater treatment, air filters, purification of gases and medical uses (Apul et al., 2013; Kovalova et al., 2013). One application that is relevant to the AP is the use of activated carbon to remove organic contaminants (Apul et al., 2013; Petrova et al., 2011).

Within this study we demonstrate the first step towards the application of activated carbon upon the AP and determine its effects on composition and the overall effects on the growth of *Tetraselmis MUR233*. The aim is to observe the effects of the use of active carbon on the HTL AP and its effects to microalgae growth.

2. Methods

2.1. HTL aqueous phase

The HTL aqueous phase was obtained from a proprietary continuous sub-critical water reactor (SCWR) (www.muradel.com.au). A 20% slurry was reacted with the continuously operated SCWR at 350 °C with a 10 min reaction time. At the end of the process the HTL product was collected, cooled, and the AP was collected stored at 4 °C for future use.

2.2. Chemical composition

The chemical composition of the HTL aqueous phase was analysed by Eurofins (Eurofins, South Australia, NATA accredited) to determine the composition in comparison to other studies that have utilised the HTL aqueous phase as a growth medium. Analysis consisted of total recoverable hydrocarbons (TRH; 2013 NEPM Fraction), phenol (USEPA 8270 Phenols), ammonia (Method: APHA 4500-NH_3 Ammonia Nitrogen by FIA), total nitrogen (nitrate and nitrite) (Method: APHA 4500-NO_3/NO_2 Nitrate–Nitrite Nitrogen by FIA), total organic carbon (Method: APHA 5310B Total Organic Carbon), total nitrogen (Method: APHA 4500 TKN), total phosphate (Method: APHA 4500-P E. Phosphate), nickel (Method: USEPA 6010/6020 Heavy Metals), and potassium (Method: USEPA 6010/6020 Heavy Metals).

2.3. Experimental design

Within this investigation we observed how the growth of *Tetraselmis MUR233* was affected using the HTL AP. Powdered activated carbon (20 mg/L) (AJAX chemicals) was used to treat the HTL aqueous phase for 10 min; the concentration of active carbon used within this study is similar to the concentration used within other waste water studies (e.g. Margot et al., 2013; Namasivayam and Kadirvelu, 1999). After the HTP AP was exposed to the active carbon it was centrifuged at 3270*g* for 3 min to separate the activated carbon from the treated HTL AP. As the HTL AP is highly concentrated a series of dilutions were required using MilliQ water; the dilutions selected within this study were 500×, 1000×, and 2000× for both the filtered and non-filtered AP. The HTL treatment was compared against a standard microalgal growth media, F/2 media (Guillard and Ryther, 1962) to give an indication of the competiveness of the HTL AP for the growth of *Tetraselmis MUR233*. Each treatment was carried out in triplicate using conical flasks (500 mL) on a shaker table at 210 μm photon/m^2/s continuous illumination and a temperature of 21 °C. All treatments were supplemented with salt to hypersaline conditions (70 g Red Sea Salt per L) as described previously (Erkelens et al., 2014).

2.4. Growth analysis of Tetraselmis MUR233

The growth rate of *Tetraselmis MUR233* using HTL AP was analysed by monitoring absorbance over time. An appropriate wavelength was determined by placing isolated species of *Tetraselmis MUR233* within a full spectrum to determine the peak absorbance (680 nm). Each treatment received a 10% inoculum of *Tetraselmis MUR233* from a stationary phase culture. On the final day of the investigation the dry weight (g/L) was assessed. Samples (50 mL, in duplicate) were taken from each treatment to determine the final total dry weight. The samples then underwent a wash cycle where the samples were centrifuged at 3000*g* then washed with MilliQ water. This wash step was repeated four times to completely remove salts that would affect the final weight. The pellets were then stored at 40 °C for 24 h. The pH of the culture medium was measured every second day over the whole investigation to determine the uptake of carbonic acid of the algae.

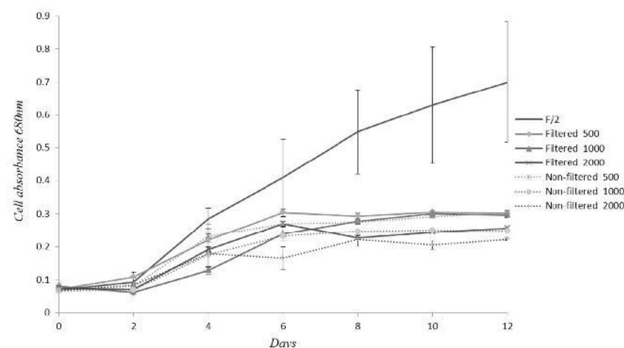

Fig. 1. Cell absorbance at 680 nm over the investigation for each treatment.

Please cite this article in press as: Erkelens, M., et al. The application of activated carbon for the treatment and reuse of the aqueous phase derived from the hydrothermal liquefaction of a halophytic *Tetraselmis* sp.. Bioresour. Technol. (2015), http://dx.doi.org/10.1016/j.biortech.2015.01.129

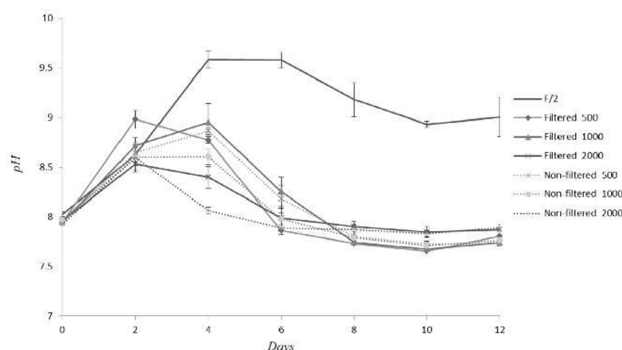

Fig. 2. Changes of pH over the investigation between each treatment.

3. Results and discussion

Within this investigation the effects of activated carbon on the composition of HTL AP and the subsequent effect upon the growth of *Tetraselmis MUR233* was assessed. The successful growth of the microalgae using the HTL AP would represent a key step in the formation of a closed nutrient loop for this emerging environmental biotechnology.

3.1. Chemical composition of the HTL AP and effect of activated carbon

The chemical composition of the HTL AP was investigated before and after treatment using activated carbon. Other studies have identified that compounds such as phenols, nickel and ammonia found within the AP can all cause growth inhibition of algae (Garcia Alba et al., 2013).

The active carbon had the following effects on the HTL AP; $NH4^+$ before 2800 ± 28.00 mg/L, after 3100 ± 310 mg/L; Total nitrate and nitrite before 0.2 ± 0.00 mg/L, after 1.7 ± 0.51 mg/L; TKN before 52,000 ± 520 mg/L, after 5900 ± 59 mg/L; Total phosphate before 1.25 ± 0.01 mg/L, after 0.49 ± 0.1 mg/L; Potassium before 2400 ± 336 mg/L, after 3500 ± 35 mg/L; Nickel before 0.1 ± 0.00 mg/L and after 0.067 ± 0.02 mg/L; Total organic carbon before 19,000 ± 190 mg/L, after 13,000 ± 130 mg/L; TRH (Total C10–36) before 500 ± 5 mg/L, after 300 ± 3 mg/L; Total phenols before 1 ± 0.01 mg/L, after 0.1 ± 0.00 mg/L.

The concentration of ammonia in the AP was not significantly affected by the activated carbon treatment. The concentration of ammonia found within this investigation was very high (2800 mg/L ammonia) in comparison to other waste streams that have been utilised to grow microalgae. Erkelens et al. (2014) utilised anaerobic digestate to grow *Tetraselmis* sp. The anaerobic digestate had a concentration of 823 mg/L and the study reported that the less dilute the anaerobic digestate the less the growth (Erkelens et al., 2014). Therefore the high concentrations of ammonia present in the AP causes concern due to the heavy dilutions that are required to prevent growth inhibition. The total Kjeldahl nitrogen (TKN) was shown to be the largest component of the AP at 52,000 ± 520 mg/L, though after the activated carbon treatment the concentration was shown to reduce to 5900 ± 59 mg/L. Activated carbon therefore significantly reduces the nitrogen component of the AP by absorption (Sabio et al., 2004).

The total recoverable hydrocarbon (TRH; C10 to C36) concentration within the non-treated AP was 500 ± 5 mg/L; following activated carbon treatment of the AP the TRH was reduced to 300 ± 3 mg/L. The TRH present within the AP was very low compared to other forms of hydrocarbon contamination (Simons et al., 2013). The total organic carbon was also observed to drop from 19,000 ± 190 mg/L to 13,000 ± 130 mg/L with the use of activated carbon. This suggests that the overall absorption of the organic carbon with the activated carbon within the AP was not as significant as the nitrogen component implying a stronger absorption relationship between the active carbon and TKN.

Toxic compounds identified within the AP such as phenol and nickel were found to be at low concentrations. Phenol was found to be present at 1 ± 0.01 mg/L initially and 0.1 ± 0.00 mg/L after the activated carbon treatment. Nickel was present at a concentration of 0.1 ± 0.00 mg/L initially and 0.067 ± 0.02 mg/L after activated carbon treatment. As the AP was heavily diluted the dilution of the nickel and phenol would potentially have minimal effect on microalgal growth. The increase in concentration of some of the HTL components following active carbon treatment may be due to the removal of other components of the HTL AP by the active carbon, thereby increasing the concentration of non-effected compounds within the HTL AP. Additionally active carbon is known to leach potassium, this may have also contributed to the increase in potassium seen after the active carbon treatment (Altland and Locke, 2012).

Overall, the application of activated carbon to the AP resulted in some beneficial changes in the composition of the AP, with the removal of selective compounds including organic nitrogen and organic carbon; this result is consistent with previous studies (Apul et al., 2013; Kovalova et al., 2013; Petrova et al., 2011). The active carbon used within this study could potentially be recharged by combustion and reused, this type of process has been displayed to be effective (Sabio et al., 2004).

3.2. Growth analysis of Tetraselmis MUR233 on the HTL aqueous phase

Growth of *Tetraselmis* MUR 233 was assessed on various dilutions of the HTL AP and was compared between activated carbon filtered, non-filtered treatments and F/2 medium. *Tetraselmis MUR233* was capable of growth on the HTL AP on all dilutions (Fig 1). Unsurprisingly the F/2 medium was found to have the highest absorbance (OD_{680}) overall (0.70); the next highest growth was observed by the filtered 500× dilution (0.30), followed by the filtered 1000× dilution (0.30) and the non-filtered 500× dilution (0.30) (Fig 1). These results suggest that the activated carbon filter treatment can increase the growth of the *Tetraselmis MUR233* when compared to a non-filtered culture.

Please cite this article in press as: Erkelens, M., et al. The application of activated carbon for the treatment and reuse of the aqueous phase derived from the hydrothermal liquefaction of a halophytic *Tetraselmis* sp.. Bioresour. Technol. (2015), http://dx.doi.org/10.1016/j.biortech.2015.01.129

Table 1
Comparison between studies that have utilised HTL AP to produce microalgae biomass.

	Jena et al. (2011)	Garcia Alba et al. (2013)	Biller and Ross (2011)					Du et al. (2012)	This study	
	S. platensis	*Desmodesmus* sp.	*Chlorogloeopsis*	*Spirulina*	*Chlorella* 300oc	*Chlorella*	*S. dimorphus*	*Chlorella vulgaris*	*Tetraselmis* sp. (Before)	*Tetraselmis* sp. (After)
TKN	16,200	–	5636	8136	6636	6888	3139	9650 ± 1582	52,000 ± 520	5900 ± 59
Ammonia	12,700	1964	4748	6295	5673	5920	5280	1343 ± 75	2800 ± 28.00	3100 ± 310
Nitrate	26.76	70	508	194	329	237	192	211 ± 20	0.2 ± 0.00	1.7 ± 0.51
Phosphate	795	159	280	2159	3109	1121	1470	343 ± 43	1.25 ± 0.01	0.49 ± 0.15
Phenols	50.9	58	178	98	108	158	80		1 ± 0.01	0.1 ± 0.00
Potassium	–	–	303	1506	1460	1419	1150	775.45	2400 ± 336	3500 ± 35
Nickel	–	–	3.8	0	0.1	0.4	0.8	0.005	0.1 ± 0.00	0.067 ± 0.02
TOC	–	–	9060	15,123	11,373	13,764	11,119	45,700 ± 1513	19,000 ± 190	13,000 ± 130
Total dry weight	*C. minutissima*, 500×, 0.52 g/L, 12 days	20×, 0.21 g/L, 96 h	100×, 0.498 g/L, 12 days	400×, 0.66 g/L, 12 days	100×, 0.88 g/L, 12 days	200×, 0.09 g/L, 12 days	400×, 0.05 g/L, 12 days	50×, ~0.6 g/L, 5 days	500×, 0.23 g/L, 12 days	1000×, 0.41 g/L, 12 days

Examination of the changes in pH during growth of the microalgae in HTL AP is shown in Fig. 2. The pH was found to be significantly higher within the F/2 medium in comparison to all the HTL AP treatments (Fig 2). This may have occurred due to the higher growth of *Tetraselmis MUR233* observed within the F/2 medium in comparison to the HTL AP treatments; the growth of *Tetraselmis MUR233* would increase the amount of carbonic acid removed from the water leading to an overall increase in pH. As the absorbance was much lower within the HTL treatments the uptake of the carbonic acid was lower. This is a concern as if the pH rises above 8.5, ammonia is capable of passing through the cell membrane and inhibiting photosynthesis by reducing the electron donors (Azov and Goldman, 1982; Uggetti et al., 2014). All HTL AP treatments reached a pH of 8.5 on day 2; pH then slowly decreases back to 7.5 by day 8. The presence of ammonia may therefore be responsible for the poor growth of *Tetraselmis MUR233* within these cultures.

Examination of the final dry weight of microalgae at the end of the experiment were observed, the following dry weights were achieved; F/2 0.49 ± 0.10 g/L; Non filtered 500 0.23 ± 0.01 g/L; Non Filtered 1000 0.11 ± 0.07 g/L; Non filtered 2000 0.17 ± 0.01 g/L; Filtered 500 0.24 ± 0.04; Filtered 1000 0.41 ± 0.09 g/L; Filtered 2000 0.21 ± 0.01 g/L. Growth using F/2 media resulted in the highest biomass (0.49 ± 0.10 g/L). The highest biomass produced with the HTL AP treatments were observed in the 1000× dilution of the filtered AP (0.41 ± 0.09 g/L). The lowest dry weight was found in the non-filtered 2000× dilution AP (0.11 ± 0.07 g/L). Generally within the HTL AP treatments, the total dry weights varied with dilutions and no trend was observed. Similar observations were made by Biller and Ross (2011) suggesting a high sensitivity to the various inhibitors found within the AP and/or the balance of nutrient required for growth vary throughout the each dilution.

Overall the growth within this study was similar to what has been found within other studies that utilise the HTL AP as a nutrient source (Table 1). These results confirm that AP is highly variable in content due to the HTL conditions and the biomass that is used as feed. The AP used in this current study was found to have the highest nitrogen and TOC content together with the lowest ammonia concentration. Phenol content was also low in studies (Table 1). The growth achieved within this study is also similar to that observed within other studies, with most studies ranging in the less than 1 g/L range. This suggests that with the application of activated carbon, growth can be improved although growth trials need to be undertaken to determine the right dilution to use for the growth of microalgae.

4. Conclusion

Overall the growth of microalgae on the HTL AP fraction is an option, though within a large scale operation where large amounts of the AP are produced this may because a significant build-up due the high dilutions that are required for microalgal growth. With the application of activated carbon upon the AP growth of *Tetraselmis MUR233* was possible. The optimal condition to grow *Tetraselmis MUR233* on AP was obtained by treating the AP with activated carbon and a dilution of 1000×.

Acknowledgements

This research was supported under Australian Research Council's Linkage Projects funding scheme (Project LP100200616) with industry partner SQC Pty Ltd, and the Australian Renewable Energy Agency advanced biofuels investment readiness program funding agreement number Q00150.

Please cite this article in press as: Erkelens, M., et al. The application of activated carbon for the treatment and reuse of the aqueous phase derived from the hydrothermal liquefaction of a halophytic *Tetraselmis* sp.. Bioresour. Technol. (2015), http://dx.doi.org/10.1016/j.biortech.2015.01.129

References

Altland, J.E., Locke, J.C., 2012. Biochar affects macronutrient leaching from a soilless substrate. HortScience 47, 1136–1140.

Anastasakis, K., Ross, A.B., 2011. Hydrothermal liquefaction of the brown macro-alga *Laminaria saccharina*: effect of reaction conditions on product distribution and composition. Bioresour. Technol. 102, 4876–4883.

Apul, O.G., Wang, Q., Zhou, Y., Karanfil, T., 2013. Adsorption of aromatic organic contaminants by graphene nanosheets: comparison with carbon nanotubes and activated carbon. Water Res. 47, 1648–1654.

Azov, Y., Goldman, J.C., 1982. Free ammonia inhibition of algal photosynthesis in intensive cultures. Appl. Environ. Microbiol. 43, 735–739.

Biller, P., Ross, A.B., 2011. Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content. Bioresour. Technol. 102, 215–225.

Du, Z., Hu, B., Shi, A., Ma, X., Cheng, Y., Chen, P., Liu, Y., Lin, X., Ruan, R., 2012. Cultivation of a microalgae *Chlorella vulgaris* using recycled aqueous phase nutrients from hydrothermal carbonization process. Bioresour. Technol. 126, 354–357.

Erkelens, M., Ward, A.J., Ball, A.S., Lewis, D.M., 2014. Microalgae digestate effluent as a growth medium for *Tetraselmis* sp. in the production of biofuels. Bioresour. Technol.

Fiorentino, A., Gentili, A., Isidori, M., Monaco, P., Nardelli, A., Parrella, A., Temussi, F., 2003. Environmental effects caused by olive mill wastewaters: toxicity comparison of low-molecular-weight phenol components. J. Agric. Food Chem. 51, 1005–1009.

Garcia Alba, L., Torri, C., Fabbri, D., Kersten, S.R., Brilman, D.W., 2013. Microalgae growth on the aqueous phase from hydrothermal liquefaction of the same microalgae. Chem. Eng. J. 228, 214–223.

Guillard, R.R., Ryther, J.H., 1962. Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (cleve) Gran. Can. J. Microbiol./Rev. Can. Microbiol. 8, 229–239.

Jena, U., Das, K.C., Kastner, J.R., 2011. Effect of operating conditions of thermochemical liquefaction on biocrude production from Spirulina platensis. Bioresour. Technol. 102, 6221–6229.

Kovalova, L., Siegrist, H., von Gunten, U., Eugster, J., Hagenbuch, M., Wittmer, A., Moser, R., McArdell, C.S., 2013. Elimination of micropollutants during post-treatment of hospital wastewater with powdered activated carbon, ozone, and UV. Environ. Sci. Technol. 47, 7899–7908.

Margot, J., Kienle, C., Magnet, A., Weil, M., Rossi, L., De Alencastro, L.F., Abegglen, C., Thonney, D., Chèvre, N., Schärer, M., 2013. Treatment of micropollutants in municipal wastewater: ozone or powdered activated carbon? Sci. Total Environ. 461, 480–498.

Namasivayam, C., Kadirvelu, K., 1999. Uptake of mercury (II) from wastewater by activated carbon from an unwanted agricultural solid by-product: coirpith. Carbon 37, 79–84.

Orfield, N.D., Fang, A.J., Valdez, P.J., Nelson, M.C., Savage, P.E., Lin, X.N., Keoleian, G.A., 2014. Life cycle design of an algal biorefinery featuring hydrothermal liquefaction: effect of reaction conditions and an alternative pathway including microbial regrowth. ACS sustain. Chem. Eng. 2, 867–874.

Petrova, B., Tsyntsarski, B., Budinova, T., Petrov, N., Velasco, L.F., Ania, C.O., 2011. Activated carbon from coal tar pitch and furfural for the removal of *p*-nitrophenol and *m*-aminophenol. Chem. Eng. J. 172, 102–108.

Sabio, E., Gonzalez, E., Gonzalez, J., González-Garcıa, C., Ramiro, A., Ganan, J., 2004. Thermal regeneration of activated carbon saturated with *p*-nitrophenol. Carbon 42, 2285–2293.

Simons, K.L., Sheppard, P.J., Adetutu, E.M., Kadali, K., Juhasz, A.L., Manefield, M., Sarma, P.M., Lal, B., Ball, A.S., 2013. Carrier mounted bacterial consortium facilitates oil remediation in the marine environment. Bioresour. Technol. 134, 107–116.

Uggetti, E., Sialve, B., Latrille, E., Steyer, J.-P., 2014. Anaerobic digestate as substrate for microalgae culture: the role of ammonium concentration on the microalgae productivity. Bioresour. Technol. 152, 437–443.

Please cite this article in press as: Erkelens, M., et al. The application of activated carbon for the treatment and reuse of the aqueous phase derived from the hydrothermal liquefaction of a halophytic *Tetraselmis* sp.. Bioresour. Technol. (2015), http://dx.doi.org/10.1016/j.biortech.2015.01.129

Chapter 5

STATEMENT OF AUTHORSHIP

Title of paper: The influences of the recycle process on the bacterial community in a pilot scale microalgae raceway pond.

Journal: Bioresource Technology

Mason Erkelens (First Author)

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

Signed Date 19/02/2015

Andew S Ball (Co- author)

Supervised the study, helped interpret the data, and drafted the manuscript.

Signed Date 11/03/15

David M Lewis (Co- author)

Supervised the study and developed the scientific approach, helped interpret the data, and drafted the manuscript.

Signed ...... Date 19/12/14

Bioresource Technology 157 (2014) 364–367

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

The influences of the recycle process on the bacterial community in a pilot scale microalgae raceway pond

Mason Erkelens [a,b,*], Andrew S. Ball [b], David M. Lewis [a]

[a] School of Chemical Engineering, University of Adelaide, 5005, Australia
[b] School of Applied Sciences, RMIT University, 3083, Australia

HIGHLIGHTS

- Bacteria were present over the whole harvest/recycle process at large scale.
- Electroflocculation had little effect over the bacterial community.
- Bacteria found within this study are a symbiotic partner to *Tetraselmis* sp.

ARTICLE INFO

Article history:
Received 20 December 2013
Received in revised form 10 February 2014
Accepted 14 February 2014
Available online 24 February 2014

Keywords:
Pilot scale
Tetraselmis
Recycle
Microalgae biofuels
Electroflocculation

ABSTRACT

The use of recycled media has been shown to be a necessary step within the lifecycle of microalgal biofuels for economic sustainability and reducing the water footprint. However the impact of the harvesting of microalgae on the bacterial load of the recycled water has yet to be investigated. Within this study PCR–DGGE and real-time PCR was used to evaluate the bacterial community dynamics within the recycled water following harvest and concentration steps for a pilot scale open pond system (120,000 L), which was developed for the production of green crude oil from *Tetraselmis* sp. in hyper saline water. Two stages were used in the harvesting; Stage 1 electroflocculation, and Stage 2 centrifugation. Electroflocculation was shown to have little effect on the bacterial cell concentration. In contrast bacterial diversity and cell concentration within the centrifugation step was greatly reduced.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Improvements in the flocculation methods to increase the efficiencies of harvesting microalgae have been well researched and has been demonstrated with dissolved air flocculation (DAF)(Chu et al., 2011; Haarhoff and Edzwald, 2013), electroflocculation (Lee et al., 2013) and centrifugal force (Pahl et al., 2013). Combinations of these technologies has been shown to shorten the time required for recovering microalgae; for example by combining electroflocculation with dissolved air flotation, the flocculation time was reduced from 30 min to 14 min (Xu et al., 2010). Flocculation methods have been shown to be both effective for low concentrations of microalgae and inexpensive, this makes it suitable for the initial concentration of the biomass (Molina Grima et al., 2003). The wastewater industry has long made use of centrifugation for the dewatering of solids. Similarly, the recovery of microalgal cells via centrifugation results in the rapid harvesting of up to 94% of the algal biomass (Molina Grima et al., 2003).

Reduction and reuse of waste is a key part of the environmental and economic sustainability for the production of microalgal biofuels (Cho et al., 2011). One aspect of this is the use of recycled water from the harvest process. The large volumes of water which are found in open systems together with the low density of microalgae provide challenges in efficiencies and cost effectiveness; therefore it is essential to reclaim the water from the harvest process. An example of why recycling water is essential was shown by Yang et al. (2011); their life cycle analysis of biodiesel production from microalgae showed that the recycling of water from harvest reduces the water and nutrient usage by 84% and 55% respectively. These authors also showed that by reclaiming the water following microalgal harvest no further additions of potassium, magnesium and sulphur to the open pond system were required (Yang et al., 2011). Furthermore the use of recycled water collected from harvesting stages prevents new input of water from external sources (which may also contain undesired organisms), significantly reducing the costs associated with the acquisition of water.

* Corresponding author at: Frome Road, Gate 5, Adelaide 5005, South Australia, Australia. Tel.: +61 8 83133959.
E-mail address: mason.erkelens@adelaide.edu.au (M. Erkelens).

http://dx.doi.org/10.1016/j.biortech.2014.02.056
0960-8524/© 2014 Elsevier Ltd. All rights reserved.

Previous research has focused on the ability to use recycled water, with a main focus on nutrient recycling. To date minimal research has been conducted on the bacterial community dynamics within the recycled water (Cho et al., 2011). When developing a harvesting system it is important to influence the growth towards the desired organism; previous studies have shown that recycled water can enhance the growth of unwanted microorganisms during the flocculation process, which is not desired when growing organisms of interest (Guo et al., 2011). Bacteria have shown to have a varying effect on microalgal growth: the symbiosis between microalgae and bacteria has shown to be beneficial due to bacterial ability to produce B12, an essential vitamin for microalgae (Goecke et al., 2013; Kazamia et al., 2012); particular negative aspects of enhanced bacterial growth are the introduction of competition for nutrients and loss of nitrogen through denitrification processes (Christenson and Sims, 2011). To maintain a large scale open pond in a sterile condition is impractical as it is exposed to the environment; however ensuring that the conditions are selective towards desired microorganisms is achievable. Monitoring the bacterial population in terms of biomass and diversity within the recycled water from a harvesting system is essential to prevent an increase in the bacterial load with each harvest cycle.

The aim of this investigation was to determine the effects on the bacterial community dynamics during the harvest of *Tetraselmis* sp. This study also assessed the effectiveness of using molecular biological methods such as polymerase chain reaction (PCR) combined with denaturing gradient gel electrophoresis (DGGE) and real time PCR (RT-PCR) as tools to effectively determine the overall efficiency of a pilot scale microalgal biofuels harvest system. These tools have been commonly used to evaluate microbial communities and effects of treatment in other systems (Erkelens et al., 2012).

2. Methods

2.1. Process description and plant design

Harvesting of *Tetraselmis* sp. in hyper saline water was conducted on a daily basis from an open pond system (120,000 L) (Muradel, Australia). The harvest of the microalgae was conducted over a two stage system involving firstly electroflocculation and secondly continuous centrifugation. The electroflocculation unit consisted of aluminium sheets with a separation of 0.15 m between each electrode and a DC power supply. The electroflocculator unit was 2.4 m × 1.2 m × 0.15 m with a linear flow velocity 0.075–0.08 m s^{-1} with an electroflocculation duration of 30 s. A voltage between 10 and 20 V was used. This electroflocculation unit has been previously described (Lee et al., 2013). Water recycled from this process was returned to the open pond system. The harvested *Tetraselmis* sp. would then undergo stage 2 of the harvesting process. The centrifuge system which was used in this harvesting process was an Evodos type 10 (Evodos, Netherlands). Harvested microalgae from stage 2 would then undergo downstream processes for bioconversion to green crude oil, while the supernatant from the centrifuge was discharged.

2.2. Experimental design and sampling plan

Samples were collected from the 120,000 L open pond system developed for the continuous production of microalgae biomass. An initial water sample of 5 L was taken from the ponds and a final water sample was taken from the open pond system after five days as a comparison over time to observe bacterial community changes. Throughout the five days the harvesting process was continuous with recycled water reintroduced into the open ponds. In addition, samples from the electroflocculation (100 mL) and centrifugation (100 mL) stages were taken.

2.3. Molecular analysis

The extraction of DNA was conducted using a Mo Bio Ultra Clean DNA extraction kit (Mo bio USA) with 1.2 mL of sample used. Extracted DNA was stored at −20 °C for future use if not used immediately. The presence of DNA was confirmed on a 0.8% agarose gel with SybrSAFE. The eubacterial PCR primers set 341FGC and 518R were used to determine changes in community dynamics (Erkelens et al., 2012). DNA template (2 µL) was used together with a KAPA master mix. PCR–DGGE was performed using a D-Code system (BioRad) with a 6% polyacrylamide gel (35.5:1 acrylamide/bis-acrylamide) with a denaturing gradient of 40–60%. The DGGE run conditions were 60 V at 60 °C for 20 h. Following electrophoresis the gel was stained with silver (Girvan et al., 2003).

2.4. Identification of bands of interest

Bands of interest were excised from the PCR–DGGE gel; excised bands were left overnight at 55°Cc in 50 µL molecular grade water. The bands where then reamplified using 2 µL of template using primers 341F 518R (Rölleke et al., 1996). Reamplified PCR amplicons underwent the MoBio PCR clean-up kit (MoBio, USA). Clean PCR amplicons were quantified using a Nano-drop lite (Thermofisher Scientific Australia) and then sent to the Australian Genomics Research Facility (AGRF, Australia) according to submission requirements. Sequences were analysed using National Centre for Biotechnology Information using a BLAST algorithm (<http://www.ncbi.nlm.gov.library.vu.edu.au/BLAST/>), and a comparison was formed against a nucleotide sequence database for identification.

2.5. Quantification of bacteria with Real-Time PCR

For real-time PCR analysis for bacterial quantification, DNA template (1 µL) was used together with a 20 µL reaction of KAPA Sybrgreen Master Mix on a Qiagen Rotor Gene 6000. Bacterial selective primers 314F and 518R were used to quantify the number of gene copies (Rölleke et al., 1996). After the run was completed a melt curve was conducted to ensure the product of interest was produced, the melt curve conditions were: 50–95 °C with a 0.5 °C increase every 5 s. Two negative controls were added within the samples where added within the run to screen for contamination.

2.6. Statistical analysis

PCR–DGGE gels were digitalised and quantified to determine the changes within the bacterial community. Digitalised gels were analysed using Total Lab Quant to determine the bacterial community dynamics occurring at each stage (Total Lab, UK). A Shannon Weaver index was applied to the PCR–DGGE gels to determine the diversity within each bacterial community sample (Marzorati et al., 2008). Equitability index was also assessed to determine the evenness of the bacterial community (Marzorati et al., 2008). The results from the real time PCR analysis of samples were statistically analysed using a *T*-TEST to determine significant difference between bacterial cell counts.

3. Results and discussion

3.1. Bacterial cell counts with real time PCR

The results of the QPCR analyses showed that the microalgal harvesting stage had a significant impact on the number of bacteria

present in the recycled water (Table. 1). The use of real time PCR was shown to be a highly sensitive tool for the detection of organisms. A standard was formed with a known bacterial cell count; the standard used gave an R^2 value of 0.98. The overall bacterial cell concentration was higher in the final pond sample (34,983 ± 8,798 gene copies/mL) than in the initial pond sample (21,102 ± 1286). There was no significant increase in the bacterial cell concentration following the reintroduction of recycled water to the open pond system ($P = 0.43$). The most significant observation was that following centrifugation, cell concentrations in the recycled water was significantly reduced ($P = 0.014$) (Table. 1). In contrast electroflocculation had no impact on the bacterial population, as it was optimised for pre-concentration of microalgae biomass prior to centrifugation.

3.2. Bacteria community dynamics via PCR–DGGE

Having established that the harvesting process had a significant effect on bacterial numbers, PCR–DGGE was used to assess the impact of harvesting and the subsequent reuse of recycled waters on the diversity of the bacterial community (Fig. 1).

The presence of bacteria in a large scale open pond system is inevitable as it is impractical to maintain selective growth solely towards the desired organisms. Monitoring and controlling the bacteria is essential to prevent competition with the target microalgal species. The initial bacterial diversity in the pond was low with only a few bands evident from the PCR–DGGE profile (Fig. 1). The bacterial diversity in the recycled water from the electroflocculation stage was much higher, as evidenced by the large number of bands compared to the initial pond sample; this indicated that the electroflocculation unit concentrated the bacterial community greatly in comparison to the open pond samples. This also indicates that the bacteria may have been present within the open pond system but in low numbers; due to the concentration of bacteria in the electroflocculation stage they appeared on the PCR–DGGE gel (Fig. 1). In contrast no bands were detectable within the recycled water from the centrifuge stage, which indicates that the centrifuge had a significant effect in reducing the bacterial diversity. However real-time PCR results confirmed their presence, albeit at low concentrations (1,216 cells ± 602 mL^{-1}). Real-time PCR displayed a higher sensitivity in comparison to PCR–DGGE which indicated no dominant bacteria were present in the centrifuge samples. The bacterial community within the recycled water from the centrifuge was effective at removing the bacteria suggesting that the use of a centrifuge may be an effective means of controlling undesired microorganisms in the water. These observations were confirmed following further analysis of the PCR–DGGE. The Shannon Weaver Diversity Index between the samples confirmed the high bacterial diversity associated with the electroflocculation process (Fig. 2).

Further analysis of the data was carried out to assess whether the different treatments resulted in changes in dominance of members of the bacterial community. The dominance was determined by the Equitability index (Fig. 3). In the initial open pond sample an even spread of dominance was observed. Throughout the harvesting process there was no increase in the dominance in the bacterial community, suggesting the bacterial community remained stable throughout the process (Fig. 3). The recycled water from the centrifuge stage had no bands present within the sample so an Equitability index analysis was shown as zero. Studies have shown bacteria present in recycled water have the ability to remain and recover after a water treatment process; for example Jjemba et al. (2010) found that bacteria were able to regrow in a recycled water distribution system after various filtration stages (Jjemba et al., 2010).

Bacteria within the PCR–DGGE gel which were of interest were identified to determine their role within the open pond system (Table. 2). Band one was identified as *Cyanobacterium*, suggesting the presence of a competitor within the open pond though it should be pointed out that the cell density of this organism was relatively low, so it was assumed to have little effect on the total biomass yield (Table. 1). The other bacteria found within this study

Table 1
Enumeration of bacteria (expressed as gene copies per mL) with real-time PCR at different stages of the harvest process.

Sample Location	Cell count (gene copies/mL)
Initial pond sample	21,102 ± 1286
Electroflocculation recycled water	18,270 ± 997
Centrifuge recycled water	1216 ± 602
Final pond sample	34,983 ± 8798

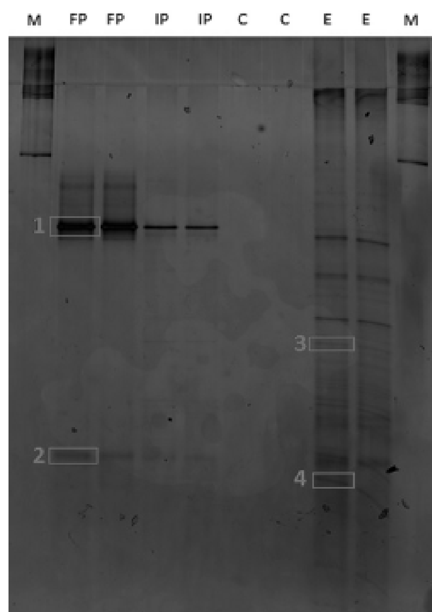

Fig. 1. PCR–DGGE of the bacterial community present in the open pond system and recycled water. (M, Marker; FP, Final pond sample; IP, Initial pond sample; C, Centrifuge recycled water; E, Electroflocculation recycled water.).

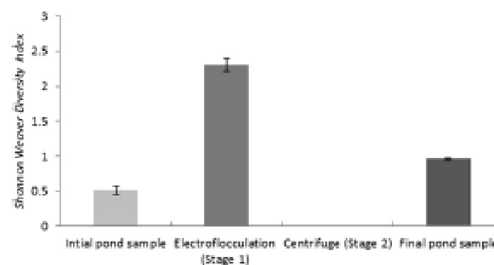

Fig. 2. Shannon Weaver diversity of the bacterial diversity at different stages in the pond.

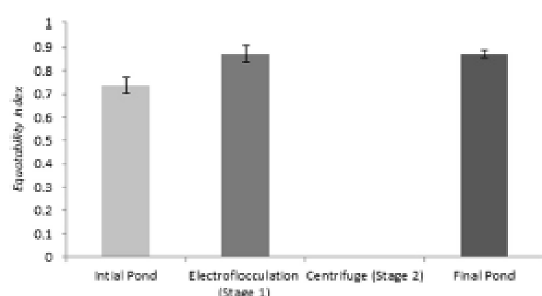

Fig. 3. Equitability index of the bacterial community present in the harvest system.

Table 2
Identification of bacteria from the PCR–DGGE.

Band	Species	Identity%	Accession
1	Unicellular *Cynobacterium*	92	GU594033.2
	Prochlorales *Cynobacterium*	91	DQ059300.1
2	Uncultured bacteria	98	HQ436829.1
	Uncultured bacteria	98	EU443037.1
3	*Phaeobacter* sp.	88	HG423273.1
	Phaeobacter sp.	88	KF009759.1
4	*Ruegeria* sp.	87	AB793108.1
	Ruegeria sp.	87	JX861574.1

where *Phaeobacter* sp. and *Ruegeria* sp. (Table. 2). These bacteria are known for their production of antibacterial compounds, their main use is as a probiotic for the aquaculture industry (Porsby et al., 2008; Rao et al., 2007). Additionally studies have observed that *Ruegeria* sp. is capable of utilising *Tetraselmis indica* as a carbohydrate source, degrading the cell wall for carbohydrates and additionally prolonging the life of *T. indica* (Arora et al., 2012). PCR–DGGE has been shown to be an effective tool for observing the bacterial community, especially as this is one of the first studies to investigate the bacterial community within the recycle process of a 120,000 L raceway pond designed for microalgae biofuel.

4. Conclusion

It was observed the bacterial community from the water recycled at the electroflocculation stage still consisted of bacteria, while the centrifuge stage greatly reduced the bacterial community. It is recommended that total removal of the bacteria is not essential but can be controlled much better with the centrifuge process, though the removal of organisms which are not of interest from the recycled water may not be beneficial as the removal of symbiotic partners with the microalgae may occur. In addition it was observed real-time PCR was more sensitive than PCR–DGGE in terms of the detection of low numbers of bacteria.

Acknowledgements

The authors would like to thank Marissa Miller for obtaining the samples. The authors would also like to thank the South Australian Regional Facility for Molecular Evolution and Ecology for the use of their facilities. This research was supported under Australian Research Council's Linkage Projects funding scheme (Project LP100200616) with industry partner SQC Pty Ltd. The views expressed here are those of the authors and are not necessarily those of the Australian Research Council.

References

Arora, M., Anil, A.C., Delany, J., Rajarajan, N., Emami, K., Mesbahi, E., 2012. Carbohydrate-degrading bacteria closely associated with *Tetraselmis indica*: influence on algal growth. Aquatic Biol. 15, 61–71.
Cho, S., Luong, T.T., Lee, D., Oh, Y.-K., Lee, T., 2011. Reuse of effluent water from a municipal wastewater treatment plant in microalgae cultivation for biofuel production. Bioresour. Technol. 102, 8639–8645.
Christenson, L., Sims, R., 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotechnol. Adv. 29, 686–702.
Chu, W.-H., Gao, N.-Y., Templeton, M.R., Yin, D.-Q., 2011. Comparison of inclined plate sedimentation and dissolved air flotation for the minimisation of subsequent nitrogenous disinfection by-product formation. Chemosphere 83, 647–651.
Erkelens, M., Adetutu, E.M., Taha, M., Tudararo-Aherobo, L., Antiabong, J., Provatas, A., Ball, A.S., 2012. Sustainable remediation – the application of bioremediated soil for use in the degradation of TNT chips. J. Environ. Manage. 110, 69–76.
Girvan, M.S., Bullimore, J., Pretty, J.N., Osborn, A.M., Ball, A.S., 2003. Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Appl. Environ. Microbiol. 69, 1800–1809.
Goecke, F., Thiel, V., Wiese, J., Labes, A., Imhoff, J.F., 2013. Algae as an important environment for bacteria – phylogenetic relationships among new bacterial species isolated from algae. Phycologia 52, 14–24.
Guo, M., Huang, J., Hu, H., Liu, W., 2011. Growth and repair potential of three species of bacteria in reclaimed wastewater after uv disinfection. Biomed. Environ. Sci. 24, 400–407.
Haarhoff, J., Edzwald, J.K., 2013. Adapting dissolved air flotation for the clarification of seawater. Desalination 311, 90–94.
Jjemba, P.K., Weinrich, L.A., Cheng, W., Giraldo, E., LeChevallier, M.W., 2010. Regrowth of potential opportunistic pathogens and algae in reclaimed-water distribution systems. Appl. Environ. Microbiol. 76, 4169–4178.
Kazamia, E., Czesnick, H., Nguyen, T.T.V., Croft, M.T., Sherwood, E., Sasso, S., Hodson, S.J., Warren, M.J., Smith, A.G., 2012. Mutualistic interactions between vitamin B12-dependent algae and heterotrophic bacteria exhibit regulation. Environ. Microbiol. 14, 1466–1476.
Lee, A.K., Lewis, D.M., Ashman, P.J., 2013. Harvesting of marine microalgae by electroflocculation: the energetics, plant design, and economics. Appl. Energy 108, 45–53.
Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W., 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. Environ. Microbiol. 10, 1571–1581.
Molina Grima, E., Belarbi, E.H., Acién Fernández, F.G., Robles Medina, A., Chisti, Y., 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol. Adv. 20, 491–515.
Pahl, S., Lee, A., Kalaitzidis, T., Ashman, P., Sathe, S., Lewis, D., 2013. Harvesting, thickening and dewatering microalgae biomass. In: Borowitzka, M.A., Moheimani, N.R. (Eds.), Algae for Biofuels and Energy, vol. 5. Springer, Netherlands, pp. 165–185.
Porsby, C.H., Nielsen, K.F., Gram, L., 2008. *Phaeobacter* and *ruegeria* species of the *roseobacter* clade colonize separate niches in a Danish turbot (*Scophthalmus maximus*)-rearing farm and antagonize *Vibrio anguillarum* under different growth conditions. Appl. Environ. Microbiol. 74, 7356–7364.
Rao, D., Webb, J.S., Holmström, C., Case, R., Low, A., Steinberg, P., Kjelleberg, S., 2007. Low densities of epiphytic bacteria from the marine alga *Ulva australis* inhibit settlement of fouling organisms. Appl. Environ. Microbiol. 73, 7844–7852.
Rölleke, S., Muyzer, G., Wawer, C., Wanner, G., Lubitz, W., 1996. Identification of bacteria in a biodegraded wall painting by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl. Environ. Microbiol. 62, 2059–2065.
Xu, L., Wang, F., Li, H.-Z., Hu, Z.-M., Guo, C., Liu, C.-Z., 2010. Development of an efficient electroflocculation technology integrated with dispersed-air flotation for harvesting microalgae. J. Chem. Tech. Biot. 85, 1504–1507.
Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M., Chen, Y., 2011. Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance. Bioresour. Technol. 102, 159–165.

Chapter 6

STATEMENT OF AUTHORSHIP

Title of paper: The influence of protozoa with a filtered and non-filtered seawater culture of Tetraselmis sp., and effects to the bacterial and algal communities over 10 days.

Journal: *Bioresource Technology*

Mason Erkelens (First Author)

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

Signed Date 19/02/2015

Andew S Ball (Co- author)

Supervised the study, helped interpret the data, and drafted the manuscript.

Signed Date 11/02/15

David M Lewis (Co- author)

Supervised the study and developed the scientific approach, helped interpret the data, and drafted the manuscript.

Signed ...... Date 19/12/14

Bioresource Technology 173 (2014) 361–366

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

The influence of protozoa with a filtered and non-filtered seawater culture of *Tetraselmis* sp., and effects to the bacterial and algal communities over 10 days

Mason Erkelens [a,b,*], Andrew S. Ball [b], David M. Lewis [a]

[a] School of Chemical Engineering, University of Adelaide, 5005, Australia
[b] School of Applied Sciences, RMIT University, 3083, Australia

HIGHLIGHTS

- An 11 μm filter prevented protozoa entering into a culture.
- *Tetraselmis* sp. out grew the potential damage of protozoa.
- *Tetraselmis* sp. remained dominant within a non-filtered culture.
- Protozoa did not cause any significant effect in 10 days.

ARTICLE INFO

Article history:
Received 15 June 2014
Received in revised form 19 September 2014
Accepted 23 September 2014
Available online 5 October 2014

Keywords:
Protozoa
Tetraselmis sp.
Biofuels
PCR-DGGE
Filters

ABSTRACT

In this study a filter was used to remove protozoa and its effects on a *Tetraselmis* sp. culture were evaluated in terms of final total lipid, final total dry weight, cell counts, and both the bacterial and algal communities. The protozoa species observed within this study was identified as *Cohnilembus reniformis*. It was observed that on the final day no *C. reniformis* were present in filtered cultures compared to the non-filtered culture which contained 40 ± 3 *C. reniformis*/mL. The presence of *C. reniformis* within the culture did not affect the total lipid or the total dry weight recovered, suggesting that *Tetraselmis* sp. was capable of surviving and growing in the presence of *C. reniformis*. Overall it is suggested that an 11 μm filter was effective at removing protozoa, though growing a microalgae culture without filtration did not show any significant effect.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

The concept of microalgal biofuels is now highly advanced and practiced at large scale all over the world (Fon Sing et al., 2014; Stephens et al., 2010). Microalgal biofuels are highly desirable due to their potential to be carbon neutral (Fon Sing et al., 2014; Sharif Hossain et al., 2008). Microalgae can be processed into biofuels by transesterification, where lipids are extracted and converted to biodiesel (Sostaric et al., 2012), or the use of hydrothermal liquefaction that converts biomass into type 2 kerogen (Eboibi et al., 2014). These processes require a constant feedstock of microalgae that has been economically produced with minimum amount of losses due to grazing microorganisms. There are many methods that are used to produce microalgal biomass; however the production of microalgal biomass requires water, nutrients and sunlight. The most important aspect of microalgal biofuels is the source of water for the culture. In Australia due to the drought conditions, seawater is more favourable as it is highly abundant and does not interfere with agricultural industries.

A problem with the introduction of any water source for the culturing a target microalgae is the control of other microorganisms within the source water. The prevention of non-desired microorganisms is essential to ensure that the desired microorganism is dominant within the culture (Day et al., 2012; Erkelens et al., 2014). In open raceway ponds it is impractical to maintain the culture in a sterile condition due to its constant exposure to the environment and also from an economic stand point, therefore control methods targeting non desired organisms should be implemented. The species of interest in this study is *Tetraselmis* sp. which recently was used in a study that reported this microalga's ability

* Corresponding author at: Frome Road, Gate 5, Adelaide 5005, South Australia, Australia. Tel.: +61 8 83133959.
E-mail address: mason.erkelens@adelaide.edu.au (M. Erkelens).

http://dx.doi.org/10.1016/j.biortech.2014.09.115
0960-8524/© 2014 Elsevier Ltd. All rights reserved.

to produce biofuels at large scale (Fon Sing et al., 2014). However, many organisms are known to graze on *Tetraselmis* sp. which causes concerns for large scale culturing of *Tetraselmis* sp. (Day et al., 2012).

One method of control is the use of filters to prevent the entry of these non-desired microorganisms into open raceway ponds. Ideally selecting a filter that can prevent the entry of selected undesired organisms can be advantageous. Most protozoa range between 16 and 70 μm in size; therefore a filter that can prevent the entry of these large microorganisms may have a positive effect on biomass production (Day et al., 2012). It is essential to assess how filters are able to prevent non desired microorganisms entering the microalgae cultures, as well the effects on the microalgae culture with and without the filter in place. Moreno-Garrido and Canavate (2001) observed the clarification of a dense culture of *Dunaliella salina* within 2 days due to the introduction of grazers. To prevent grazer damage they introduced formaldehyde, metronidazole, ammonia, hydrogen peroxide and quinine sulphate. The most successful treatment within the study was quinine sulphate which only affected the grazers and not the microalgae (Moreno-Garrido and Canavate, 2001). Another method to combat the damage of rotifer was investigated by Richmond (1986); he observed that maintaining the optimal values of pH, temperature and light can increase the growth of microalgae and minimise the damage caused by grazers (Richmond, 1986). Sananurak et al. (2009) was able to create a large scale closed-recirculating system, continuous culture of *Tetraselmis suecica* and rotifers (*Brachionus plicatilis*) for larval fish culture. Within their study they showed that the microalgal density was very stable throughout the 28 days even with rotifers present (Sananurak et al., 2009).

Within pilot scale open ponds the presence of bacteria is normal; it has been shown however that the bacterial dynamics of a pond can be easily controlled within a harvesting system (Erkelens et al., 2014). Bacteria are an important symbiotic partner with microalgae; recent research has shown that bacteria are capable of producing vitamins that are important for the growth of microalgae (Croft et al., 2005). Studies have also observed that the presence of protozoa within a culture can cause shifts in algal dominance. Grobbelaar et al. (1981) observed a culture of *Chlorella* sp. that was infected by *Stylonychia* sp., the dominant algae then changed from a *Chlorella* sp. dominant culture to a *Scenedesmus* sp. culture. This change in dominant algae was associated with the large size of *Scenedesmus* sp., as it was too large to be consumed (Grobbelaar et al., 1981). Monitoring the effects on the bacterial and algal community is therefore important to ensure there are no negative impacts on the beneficial bacteria and the algae of interest. Introducing a selective method such as a filter to allow beneficial bacteria to enter an open pond system and excluding microorganisms such as protozoa could be more beneficial.

This study selected a 11 μm filter to selectively remove protozoa that are known to commonly be 16–70 μm in size (Day et al., 2012). This pore size will still allow for other microorganisms to pass through and ensure less clogging for large volumes of water. Currently there is little known about how effective an 11 μm filter can be to prevent the introduction of theses larger protozoa that may destroy the microalgae culture. The aim of this study is to compare a filtered seawater culture against a non-filtered seawater culture to evaluate the effects on microalgal biomass and total lipid production together with changes in the bacterial and algal population. To monitor effects to the bacterial and algal communities we used polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE); this has been shown to be an effective tool for evaluating microalgae processes (Erkelens et al., 2014).

2. Methods

2.1. Experimental design

Seawater (40 L) was obtained from the Spencer Gulf at Whyalla, South Australia. Two vessels (each 2 L) were prepared to compare between filtered and non-filtered seawater. The filtered sea water was vacuumed filtered through an 11 μm sterile glass filter. Filtered water was collected in a sterile container and placed directly into the culture vessel. Both cultures were nutrient enhanced with F/2 media; this was conducted by treating the cultures volume as the total water and enhancing it with F/2 nutrients (Guillard, 1975). A constant flow of air (4 L/min) was used with the culture; the light source used within this study was a 100 W fluorescent light globe with continuous illumination. The microalgal cultures were incubated in a 21 °C constant temperature room. A culture of *Tetraselmis* sp. was obtained and used as an inoculum. The inoculum was in a stationary phase and was added to achieve a 10% (v/v) inoculum of the initial volume. An aliquot (50 mL) of water was sampled every second day over 10 days from each treatment. On the final day the cultures were centrifuged and the pellet harvested for further analysis such as dry weight and total lipid content. All samples were stored at −20 °C if not used immediately.

2.2. DNA extractions

An aliquot (5 mL) of sample was taken from the initial 50 mL samples and was centrifuged and resuspended with 300 μL of sterile PBS. The resuspended pellets underwent DNA extraction using a MoBio PowerPlant DNA extraction (MoBio, USA). An aliquot (50 μL) of solution six was used to elute the DNA at the final step and stored at −20 °C if not used immediately. To confirm the presence of DNA, samples were run on a 0.8% agarose gel with SybrSafe.

2.3. PCR-DGGE

To determine the influence of an 11 μm filter on the bacterial and algal communities PCR-DGGE was conducted. PCR was conducted on the extracted DNA using a KAPA PCR mixture (KAPA, South Africa). Primers used to amplify the microalgal community targeted the 18s rRNA region; these primers have previously been shown to have been used successfully for analysing microalgal communities (Diez et al., 2001). The bacterial community was amplified by targeting the 16s rRNA region; the primers were developed for 314FGC and 518R which is known as a marker for bacterial communities (Erkelens et al., 2014; Muyzer et al., 1993). PCR amplicons were analysed on a 1% agarose gel with SybrSafe. PCR amplicons from both the bacteria and microalgal communities were then loaded onto a 6% polyacrylamide gel with a denaturing gradient of 40–60%. The run conditions for the 6% polyacrylamide gel were on a BioRad D-Code mutation over 20 h, 60 °C at 60 v. After the run was completed the DGGE gels were developed via silver staining (Erkelens et al., 2012; Radojkovic and Kusic, 2000). The developed gels where then digitalized with a scanner for further analysis with Total Lab.

2.4. Growth of *Tetraselmis* sp. and total lipid & dry weight content

After day 10 culture aliquots were prepared into separate 50 mL tubes and centrifuged at 3000g for 10 min. The samples were washed three times with milliQ water and centrifuged at each step for 10 min at 3000g. After the final wash the samples were dried at 37 °C for 2 d and the initial and final weights were taken to determine the total dry weight content. The total lipid content was determined by a chloroform–methanol extraction (Bligh and

Dyer, 1959). Dried microalgae (500 mg) was used for each sample ($n = 2$). Following lipid extraction the change in mass was taken to calculate the total percentage of lipid present within the pellet. A t-test was conducted to determine significant differences between the total lipid and total dry weight content between the filtered and non-filtered samples. An Olympus IX50 Microscope at 20× magnification and haemocytometer (0.2 mm deep) was used to determine total cell count of *Tetraselmis* sp. per a millilitre. Cells were allowed to settle for 5 min before cell counting was undertaken. Triplicate cell counts were undertaken for each sample.

3. Results and discussion

3.1. Microscope observation of cultures

The cultures were observed with a microscope to visually inspect differences within the microalgal cultures. It was immediately observed within the non-filtered culture that *Cohnilembus reniformis* was present; it was compared to other studies to determine the species (Day, 2013). The final counts of *C. reniformis* were taken from both cultures; the non-filtered culture was observed to have 40 ± 3 *C. reniformis*/mL present at day 10. In contrast no *C. reniformis* were present within the filtered culture. Therefore the use of an 11 µm filter can prevent protozoa entering into a culture. Protozoa are known to consume microalgae, in particular *Tetraselmis* sp. (Lubzens, 1987; Luna-Andrade et al., 2002).

Tetraselmis sp. growth was determined by observing the cell counts every second day throughout the investigation (Fig. 1). It was clearly seen that the growth of *Tetraselmis* sp. was steady in both the filtered and non-filtered cultures. The stationary phase was achieved in days 8 and 10 respectively. There was no significant loss of cell numbers within the non-filtered culture in comparison to the filtered culture.

3.2. Final total lipid and dry weight

The final total lipid and the final biomass dry weight were observed within both cultures. There was no significance difference between the filtered and non-filtered seawater (Total lipid P-value: 0.23; Total dry weight P-value: 0.38) (Fig. 2). The presence of protozoa in the non-filtered seawater over 10 days did not result in a reduction of total dry weight. It would be expected that the presence of protozoa within the microalgal culture would add to the total biomass on day 10 (Day et al., 2012; Moreno-Garrido and Canavate, 2001). There was also no significant difference between the total lipid content of the filtered and non-filtered seawater. The total lipid achieved after day 10 was also similar to that

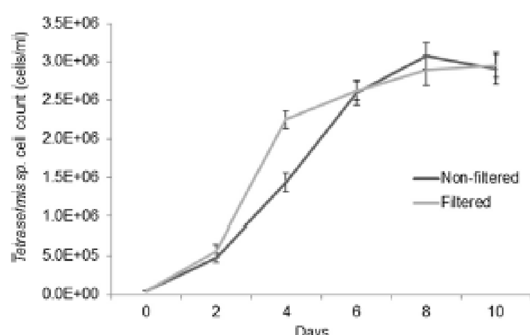

Fig. 1. Cell count of *Tetraselmis* sp. for the filtered and non-filtered cultures over the 10 days.

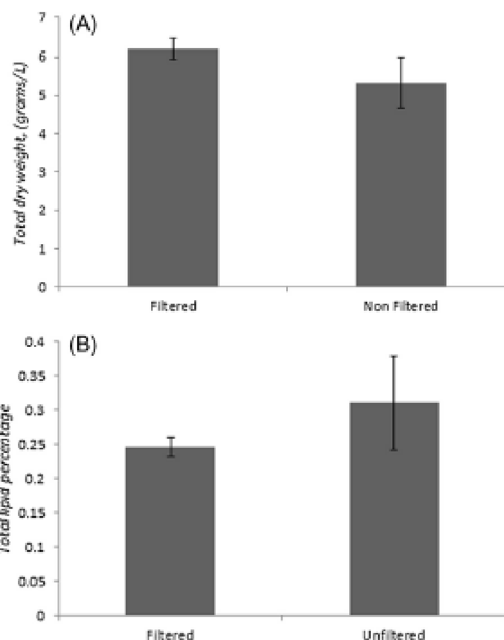

Fig. 2. The final total dry weight (A) and total lipid (B) content found on day 10 comparing non-filtered and filtered sea water. In panel B change to state non-filtered.

reported for other studies (Huang et al., 2013). Protozoa are known to consist of lipid and must have also contributed to the final total lipid content.

While some studies have reported that the presence of protozoa can cause complete loss of cultures, other studies have shown that this is not always the case. Within this study the *Tetraselmis* sp. was grown at the optimal growth conditions (i.e. nutrient, light and temperature) suggesting that *Tetraselmis* sp. may have out grown any significant damage that the protozoa may have caused to the cultures (Richmond, 1986). Sananurak et al. (2009) investigated the growth of *Tetraselmis* sp. and rotifers (*B. plicatilis*) within a continuous culture for 28 days at large scale. The microalgal density was found to be much more stable in comparison to the rotifer cell count; this was attributed to the optimal photosynthetic conditions (Sananurak et al., 2009). Therefore the presence of protozoa within a culture does not necessarily always lead to complete loss of a microalgae culture; instead if optimal growth conditions are maintained the microalgae culture is capable to outgrow most of the damages caused by protozoa. Other studies have also observed the relationship between the algal and protozoa. Additionally some microalgae maybe too large to be consumed by the protozoa, preventing losses (Grobbelaar et al., 1981). This maybe the case for this study as well.

3.3. Bacteria community analysis

Bacteria are known to be a symbiotic partner with microalgae; this symbiosis has been shown to be essential for microalgae to grow (Croft et al., 2005). The bacterial community present in the filtered and non-filtered seawater was compared to determine the influence on the *Tetraselmis* sp. cultures (Fig. 3). It was observed the bacterial community was influenced by the filtration

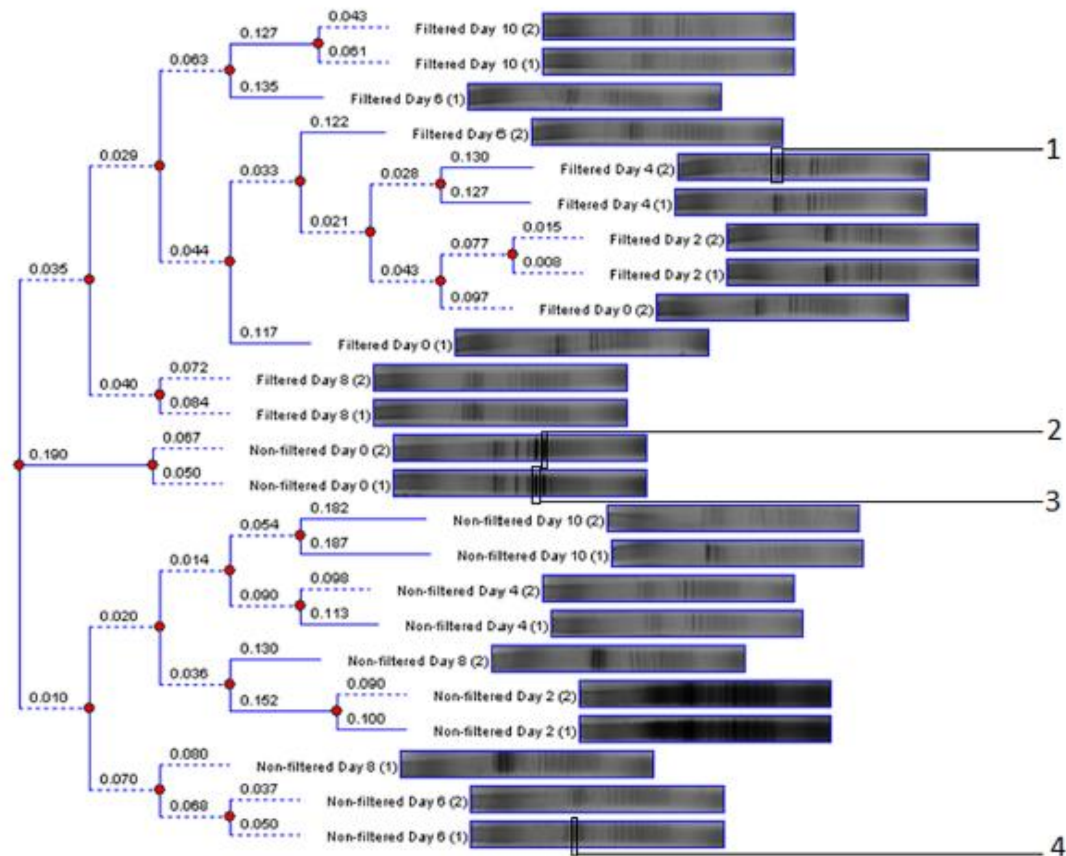

Fig. 3. Dendogram comparing the bacterial communities found in the filtered and unfiltered microbial communities.

step. There was a difference between the filtered and non-filtered bacterial community since two clusters where formed within the dendrogram, a cluster for filtered and non-filtered (Fig. 3). The impact of protozoa on the bacterial community was not clearly observed within this study. This may have occurred due to bacterial colonisation; for example bacteria that would have grown as colonies larger than the filter size would have been excluded. Berdjeb et al. (2011) investigated the effects of grazers on the bacterial community; they observed that there was no clear influence on the bacterial community within the presence of grazers (Berdjeb et al., 2011). Therefore the filter may have had a larger impact on the bacterial community rather than the presence of protozoa.

Bands that were deemed dominant within the bacterial PCR-DGGE gel were excised and sequenced to identify key bacteria present within the samples (Table 1). The bacteria identified as dominant within this study are all known to be naturally present within marine environments (Table 1). Some of the bacteria within this study are known to have a relationship with microalgae and plants. For example *Rhizobiales* sp. is known for its role in plant defence mechanisms; these defence mechanisms were associated with carbohydrate metabolism (Kutuzov and Andreeva, 2012). *Tetraselmis* sp. is known for its production of carbohydrates which may assist in the growth of *Rhizobiales* sp. (Ho et al., 2012). *Rhizobiales* sp. may have been utilising *Tetraselmis* sp. as a carbon source and in return providing nitrogen fixation (Kutuzov and Andreeva, 2012). Additionally the presence of *Methylobacterium* sp. was observed within the culture as a dominant bacterium. *Methylobacterium* sp. is a consumer of methanol compounds and is capable of producing methane. Their role within the cultures may have been with the degradation of dead microalgae under anaerobic conditions (Skovran et al., 2011). *Rhodospirillaceae* sp. was also observed to be a dominant bacterium within the filtered and non-filtered cultures. *Rhodospirillaceae* sp. is also known to have the ability to fix nitrogen within the environment (Madigan et al., 1984; Madigan, 1995). *Phaeospirillum* sp. is a phototropic non sulphur bacteria, there has been little research regarding its relationship with microalgae (Takaichi et al., 2011). The presence of bacteria within the seawater suggested that it may be acting a positive symbiotic relationship for the growth of microalgae within the cultures. The use of filtrations has caused an overall change in the bacterial community, though beneficial bacteria were still able to pass through the filter.

3.4. Algal community analysis

Maintaining a monoculture within the large-scale raceway ponds is important to ensure the microalgae of interest with the desired properties remains dominant. One concern is the ability of small non-valuable microalgae to move through the filter and

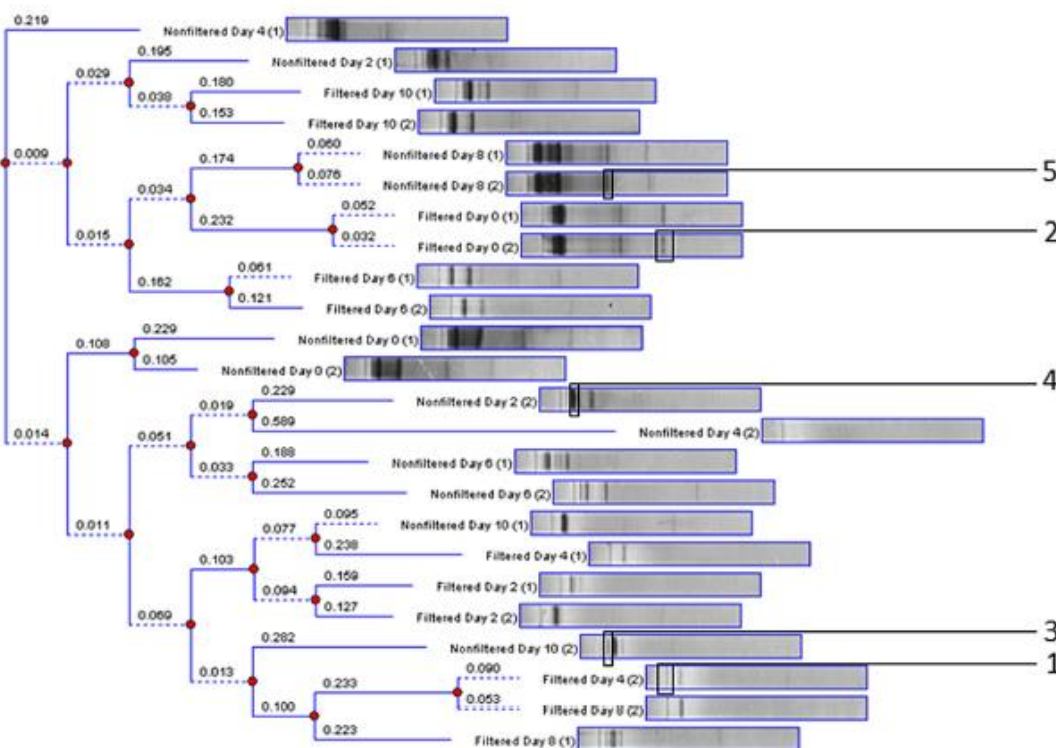

Fig. 4. Dendogram comparing the algal communities within filtered and non-filtered cultures.

Table 1
Dominant bacteria found within the bacterial communities in both the filtered and unfiltered microalgae cultures according to fig. 3.

Band	Species	Identity	Accession number
1	*Phaeospirillum* sp.	92	HF559004.1
	Phaeospirillum sp.	92	NR_108482.1
2	*Uncultured Rhizobiales*	94	JQ403003.1
	Uncultured Rhizobiales	94	JQ401838.1
3	*Rhodospirillaceae bacterium*	89	KC545306.1
	Rhodospirillaceae bacterium	89	FR693291.1
4	*Methylobacterium* sp.	94	D32231.1
	Alphaproteobacterium GMD37D2	94	AY162081.1

flourish. It was observed there was no significant difference between the filtered and unfiltered algal communities. The effects of the filter was observed to have an impact on day 0, there was a clear difference seen between the two treatments (Fig. 4). Algae that may grow larger than 11 µm would not have the ability to pass through the filter; therefore the introduction of a filter did prevent some algae entering into the culture causing a difference between the day 0 cultures. The influence of the filter was only temporary as the algal cultures throughout the investigation displayed little dissimilarity between each treatment.

It was observed within this study the most dominant band found within the algal PCR-DGGE gel was *Tetraselmis* sp.. The fact that *Tetraselmis* sp. was the dominant microalgae confirmed that the initial inoculum of *Tetraselmis* sp. was strong enough to remain dominant within both the filtered and non-filtered cultures.

Both macro and microalgae were identified within the study. Interestingly macroalgae (Band 2,5, *Porphyra* sp.) was found within both cultures indicating their ability to travel through the small filter (Table 2). The presence of the other algae within the filtered cultures was not unexpected as there are many species of algae smaller than 11 µm. Even with the presence of other algae within the culture *Tetraselmis* sp. was still able to dominate in both filtered and non-filtered cultures. This investigation confirms *Tetraselmis* sp. as a key microalga for mass production with seawater due to its ability to dominate cultures and compete against the other microalgae present in the seawater. The presence of grazers within the non-filtered culture did not appear to cause any large change in the algal community. It is suspected that the algae

Table 2
Algae identified within both filtered and non-filtered cultures according to Fig. 4.

Band	Species	Identity	Accession number
1	*Dictyochloropsis splendida*	91	GU017665.1
	Dictyochloropsis splendida	91	GU017660.1
2	*Porphyra pendula*	87	DQ084430.1
	Porphyra drewiana	87	AY766362.1
3	*Uncultured Chlorodendraceae*	85	EF024582.1
	Uncultured Dunaliellaceae	85	EF024336.1
4	*Tetraselmis chuii*	99	AJ437327.1
	Tetraselmis chuii	99	DQ207405.1
5	*Porphyra pseudolinearis*	86	GU319858.1
	Porphyra perforata	86	GU319856.1

within the non-filtered culture out grew the grazers and their ability to cause damage within the culture in the 10 days.

4. Conclusion

This study showed that the presence of protozoa did not significantly affect the final total lipid or final dry weight of the study. The 11 μm filter did affect the bacterial community; the algal community was only initially impacted by large change in the algal community on day 0. Overall this study confirmed that (1) an 11 μm filter is effective at removing protozoa, (2) and the presence of protozoa had little observed impact on the *Tetraselmis* sp. cultures within the 10 days.

Acknowledgements

This research was supported under Australian Research Council's Linkage Projects funding scheme (project LP100200616) with industry partner SQC Pty Ltd, and the Australian Renewable Energy Agency advanced biofuels investment readiness program funding agreement number Q00150. The views expressed herein are those of the authors and are not necessarily those of the Australian Research Council.

References

Berdjeb, L., Ghiglione, J.F., Jacquet, S., 2011. Bottom-up versus top-down control of hypo- and epilimnion free-living bacterial community structures in two neighboring freshwater lakes. Appl. Environ. Microbiol. 77, 3591–3599.
Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.
Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G., 2005. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438, 90–93.
Day, J.G., 2013. Grazers: the overlooked threat to the sustained production of future algal biofuels. Biofuels 4, 459–461.
Day, J.G., Thomas, N.J., Achilles-Day, U.E., Leakey, R.J., 2012. Early detection of protozoan grazers in algal biofuel cultures. Bioresour. Technol. 114, 715–719.
Diez, B., Pedros-Alio, C., Marsh, T.L., Massana, R., 2001. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl. Environ. Microbiol. 67, 2942–2951.
Eboibi, B.E., Lewis, D.M., Ashman, P.J., Chinnasamy, S., 2014. Effect of operating conditions on yield and quality of biocrude during hydrothermal liquefaction of halophytic microalga *Tetraselmis* sp.. Bioresour. Technol. 170C, 20–29.
Erkelens, M., Adetutu, E.M., Taha, M., Tudararo-Aherobo, L., Antiabong, J., Provatas, A., Ball, A.S., 2012. Sustainable remediation – the application of bioremediated soil for use in the degradation of TNT chips. J. Environ. Manage. 110, 69–76.
Erkelens, M., Ball, A.S., Lewis, D.M., 2014. The influences of the recycle process on the bacterial community in a pilot scale microalgae raceway pond. Bioresour. Technol. 157, 364–367.
Fon Sing, S., Isdepsky, A., Borowitzka, M.A., Lewis, D.M., 2014. Pilot-scale continuous recycling of growth medium for the mass culture of a halotolerant *Tetraselmis* sp. in raceway ponds under increasing salinity: a novel protocol for commercial microalgal biomass production. Bioresour. Technol. 161, 47–54.
Grobbelaar, J.U.S., Soeder, C.J., Toerien, D.R., 1981. Open semi-defined systems for outdoor mass culture of algae. Wastewater for Aquaculture, 3, p. 24.
Guillard, R.R., 1975. Culture of phytoplankton for feeding marine invertebrates. Culture of Marine Invertebrate Animals. Springer, pp. 29–60.
Ho, S.H., Chen, C.Y., Chang, J.S., 2012. Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus CNW-N*. Bioresour. Technol. 113, 244–252.
Huang, X., Huang, Z., Wen, W., Yan, J., 2013. Effects of nitrogen supplementation of the culture medium on the growth, total lipid content and fatty acid profiles of three microalgae (*Tetraselmis subcordiformis, Nannochloropsis oculata* and *Pavlova viridis*). J. Appl. Phycol. 25, 129–137.
Kutuzov, M.A., Andreeva, A.V., 2012. Rhizobiales-like protein phosphatases (Rhilphs): a role in plant defence responses? Plant Omics 5, 590.
Lubzens, E., 1987. Raising rotifers for use in aquaculture. Rotifer Symposium IV. Springer, pp. 245–255.
Luna-Andrade, A., Aguilar-Duran, R., Nandini, S., Sarma, S., 2002. Combined effects of copper and microalgal (*Tetraselmis suecica*) concentrations on the population growth of *Brachionus plicatilis* Müller (Rotifera). Water Air Soil Pollut. 141, 143–153.
Madigan, M., Cox, S.S., Stegeman, R.A., 1984. Nitrogen fixation and nitrogenase activities in members of the family Rhodospirillaceae. J. Bacteriol. 157, 73–78.
Madigan, M.T., 1995. Microbiology of nitrogen fixation by anoxygenic photosynthetic bacteria. Anoxygenic Photosynthetic Bacteria. Springer, pp. 915–928.
Moreno-Garrido, I., Canavate, J., 2001. Assessing chemical compounds for controlling predator ciliates in outdoor mass cultures of the green algae *Dunaliella salina*. Aquacult. Eng. 24, 107–114.
Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.
Radojkovic, D., Kusic, J., 2000. Silver staining of denaturing gradient gel electrophoresis gels. Clin. Chem. 46, 883–884.
Richmond, A.E., 1986. Microalgaculture. Crit. Rev. Biotechnol. 4, 369–438.
Sananurak, C., Lirdwitayaprasit, T., Menasveta, P., 2009. Development of a closed-recirculating, continuous culture system for microalga (*Tetraselmis suecica*) and rotifer (*Brachionus plicatilis*) production. Sci. Asia 35, 118–124.
Sharif Hossain, A., Salleh, A., Boyce, A.N., Chowdhury, P., Naqiuddin, M., 2008. Biodiesel fuel production from algae as renewable energy. Am. J. Biochem. Biotechnol. 4.
Skovran, E., Palmer, A.D., Rountree, A.M., Good, N.M., Lidstrom, M.E., 2011. XoxF is required for expression of methanol dehydrogenase in *Methylobacterium extorquens* AM1. J. Bacteriol. 193, 6032–6038.
Sostaric, M., Klinar, D., Bricelj, M., Golob, J., Berovic, M., Likozar, B., 2012. Growth, lipid extraction and thermal degradation of the microalga *Chlorella vulgaris*. New Biotechnol. 29, 325–331.
Stephens, E., Ross, I.L., Mussgnug, J.H., Wagner, L.D., Borowitzka, M.A., Posten, C., Kruse, O., Hankamer, B., 2010. Future prospects of microalgal biofuel production systems. Trends Plant Sci. 15, 554–564.
Takaichi, S., Maoka, T., Sasikala, C., Ramana, Ch.V., Shimada, K., 2011. Genus specific unusual carotenoids in purple bacteria, Phaeospirillum and Roseospira: structures and biosyntheses. Curr. Microbiol. 63, 75–80.

Chapter 7

STATEMENT OF AUTHORSHIP

Title of paper: Reducing microalgae biofuels water footprint by recycling water and its effects to the bacteria and microalgal communities.

Journal: *The Journal of Applied Phycology.*

Mason Erkelens (Joint first author)

Performed the analysis, interpreted the data, wrote the final manuscript and manuscript evaluation, and acted as the corresponding author.

Signed Date..19/02/2015

Xiaoyu Ma (Joint first author)

Conducted the analysis of the culture, and wrote the initial manuscript.

Signed Date.....8/10/2014

Wynand Van Den Berg (Co- author)

Helped with the interpretation and drafting of the manuscript

Signed Date..4/2/2015

Steven. Amos (Co- author)

Helped with the flocculation of the culture.

Signed Date..19/12/14

Andew S Ball (Co- author)

Supervised the study, helped interpret the data, and drafted the manuscript.

Signed Date..11/02/15

David M Lewis (Co- author)

Supervised the study and developed the scientific approach, helped interpret the data, and drafted the manuscript

Signed ..... Date..19/12/14

Title: Reducing microalgae biofuels water footprint by recycling water and its effects to the bacteria and microalgal communities.

Authors

Mason. Erkelens[1*+], Xiaoyu Ma[1+], Wynand Van Den Berg[1], Andrew. S. Ball[2], Steven. Amos[1], David. M. Lewis[1]

+ Both authors contributed equally to this investigation.

Affiliation:

1) School of Chemical Engineering, University of Adelaide, South Australia, 5005.

2) Applied Sciences, RMIT, Victoria, Australia, 3083.

Corresponding Author*: Mason Erkelens

Email: mason.erkelens@adelaide.edu.au

Phone: 08 8313 3959 Fax: 08 8313 4373

Abstract

In order to enable microalgae derived biocrude competitive with fossil fuels, it is essential to increase microalgal production rates coupled with reduced operating costs. One method is to recycle the water used in production. However the effects of this water recycle process on both the microalgal and bacterial communities is unknown. The aim of this study was to compare the use of fresh seawater and the reuse of the harvested water to replenish the microalgae cultures, and how that effected the bacterial and algal communities. PCR-DGGE was used to evaluate the bacterial and algal communities. The dominating bacteria were identified as members of *Vibrio sp., Cyanobacterium sp*., and *Rhizobium sp..* It was also

observed that the algae culture was more monoculture when reusing recycled media in comparison to using fresh seawater.

Keywords: *Tetraselmis sp.;* Bacteria; Recycle Media; Microbial community; PCR-DGGE

1 Introduction.

Biofuels produced from microalgae has raised significant attention as a potentially sustainable alternative to fossil fuels (Chisti 2007; Singh and Gu 2010; Stephens et al. 2013). The popularity of microalgal biofuels is due to the carbon neutral process that can be utilised to produce a renewable energy resource. Furthermore, microalgal biomass requires less land and production energy than traditional biofuel crops such as palm oil (Biller et al. 2012; Tilman et al. 2009). Tilman et al. (2009) determined that the use of land for microalgal biofuels does not compete for fields that are used for agriculture, or the freshwater that additionally would be used for food crops (Tilman et al. 2009).

Tetraselmis sp. has generated a lot of attention recently as a suitable microalga for biofuel production due to its fast growth rate, high biomass content and presence of lipid during most stages of growth (Fon Sing et al. 2014; Griffiths and Harrison 2009; Huerlimann et al. 2010; Montero et al. 2010). Studies have shown *Tetraselmis sp.* to have an average lipid production of 3.9 to 32 mg/L/day (Griffiths and Harrison 2009; Huerlimann et al. 2010; Montero et al. 2010). However, technical challenges still exist for commercialising microalgae to produce biocrude economically. Large scale production of microalgal biocrude encounters difficulties such as inconsistent microalgal growth rates due to environmental influences (Mata et al. 2010), the requirement of nutrients, and the need to develop more economical harvesting techniques (Lee et al. 2010; Pienkos and Darzins 2009; Scott et al. 2010). To help reduce operating cost and the water footprint, the harvested water containing some nutrients

that otherwise would have been disposed of can be recycled back to the microalgal culture (Biller et al. 2012; Yang et al. 2011).

It is well known that complex interactions between microalgae and bacteria exist in natural habitats (Mouget et al. 1995). Some studies have shown a coherent growth of bacteria attached to phytoplankton cells (Sapp et al. 2007). Bacterial symbiosis with microalgae has been observed to be due to their production of vitamin B12 (Kazamia et al. 2012) and nitrogenous compounds by bacteria (Ceh et al. 2013), in exchange for the microalgal supply carbon sources such as lipid and starch, both of which are critical elements for bacterial growth (Goecke 2013). Erkelens et.al (2014) observed that the bacteria community was present throughout a harvesting/recycle cycle process during continuous production of microalgae; the bacteria observed within were *Cyanobacteria sp, Phaeobacter sp,* and *Ruegeria sp.* (Erkelens et.al 2014). Therefore, maintaining such beneficial bacteria within the microalgae cultures may be crucial for maintaining high yields of microalgal biomass.

The aim of this study was to observe the growth of *Tetraselmis subcordiformis* and the bacterial and algal community over multiple harvesting and recycle cycles with non-sterile seawater adjusted to hypersaline conditions. Achieving this aim will give a novel insight on the effects of harvesting and recycle of both the bacterial and algal communities over multiple harvesting/recycle cycles, and the effects on the growth rate of the *Tetraselmis subcordiformis.*

2 Materials and methods

2.1 Experimental design.

Tetraselmis subcordiformis was grown in 5L photo-bioreactors for a total period of 17 d at 25ºC under 24 h cool-white fluorescent lamps. The medium was initially made from seawater obtained from the Spencer Gulf near Whyalla, South Australia, and enhanced with F/2

media; additionally the salt content was also increased to hyper saline conditions (7%) using RedSea Salt (Red Sea USA). Duplicate photo bioreactors (PBRs) were created to cultivate *Tetraselmis subcodiformis*. The investigation was carried out over four recycle periods with a harvesting threshold of 500,000 cells/ml, after the fourth time the threshold of 500,000 cell/ml is reached we will finalise the investigation. Depending on the treatment the water, reclaimed recycled media was returned back to the PBR for the recycled treatment (RT), or fresh hypersaline seawater was used for make up for the water discarded after harvesting (FT). Recycled media was obtained by collecting supernatant from chemical flocculation of 2.5L microalgae medium (20 g/L aluminium sulphate solution, with 35 mL added for each harvest). When either the RT or FT was replaced back into the PBR it was enhanced with F/2 medium. Samples (40 mL) were taken from the middle of the PBRs on day 0, the final day, and prior to harvest. Samples were stored at -20oC for future analysis.

2.2 Cell counts.

Cell counts were conducted daily using an Olympus IX50 microscope with a Neubauer improved haemocytometer (Precicolor, HBG, Germany). Cell counts where undertaken daily with triplicate samples.

2.3 DNA extraction & polymerase chain reaction (PCR).

Duplicate aliquots (12 ml) of each sample were defrosted at room temperature and centrifuged at 3270 g for 10 min. 0.2 g of wet sample was placed in PowerBead Tubes, and Solutions C1 to C6 was added according to the MoBio PowerSoil DNA extraction Kit instructions (MoBio, USA). DNA amplification of the bacterial community was performed using PCR in a 48 µl solution with 16S rDNA primers pairs (Muyzer et al. 1993). The PCR solution consisted of 38 µl dH2O, 5 µl 10×buffer, 5 µl dNTP, 2 µl 314F GC, 2 µl 907R, 0.2 µl Taq. The PCR cycle conditions were: 94ºC for 10 min, then 33 cycles of 94ºC 1min, 55ºC for 1 min, 72ºC for 1

min, then 72ºC for 10 min and 8ºC for the final hold stage. PCR amplicons where then stored at -20 ºC for future use. Microalgae DNA was amplified using 18S rRNA primers pairs (Diez et al. 2001). The PCR solution for the algal community was 38 µl dH2O, 5 µl 10×buffer A, 5 µl dNTP, 2 µl R0580, 2 µl P0579, 0.2µl Taq. PCR cycle conditions were: 94ºC for 2:10 min, 34 times cycles of 94ºC 0.5 min, 56ºC for 45 s, 72ºC for 2:10 mins, then 72ºC for 7 min, and 8ºC for the final hold stage. PCR amplicons were validated on a 0.8% agarose gel using SYBRsafe.

2.4 Denaturing Gradient Gel Electrophoresis (DGGE) and identification of bands of interest.

PCR amplicons were analysed on a DGGE gel with a 40% -60% gradient. An aliquot (15 µl) of each sample was loaded into a well with 2µl of loading dye. The PCR-DGGE gels were then run for 60ºC at 60V for 20 h. After a successful run, the PCR-DGGE gels were developed by silver staining (Girvan et al. 2003). Bands that were of interest were excised from the PCR-DGGE gel with a sterilised razor blade and transferred in molecular grade water (100 µl), the bands were left at 55ºC overnight. Re-amplification was then carried out using PCR with the isolated band as the DNA template and the addition of the primer sets described above, though this time without a GC clamp present on the forward primer for both 18S and 16S bands. PCR amplicons were then adjusted to the submission requirements of the Australian Genomics Research Facility (AGRF). The sequences where compared to a nucleotide database (National Centre for Biotechnology Information) via a BLAST algorithm to determine the band of interests identification (http://www.ncbi.nlm.gov.library.vu.edu.au/BLAST).

2.5 Statistical analysis

Gels obtained from PCR-DGGE were digitalised and Total Lab Quant 1.02, software was used to quantify the changes in the bacterial and algal communities at each harvest stage. Shannon

Weaver and Equitability indices were calculated using the Gaussians volumes obtained from Total Lab Quant 1.02. This allowed the determination of the diversity and evenness of bacterial and algal community distributions respectively (Girvan et al. 2003).

3 Results and Discussion.

3.1 Growth of *Tetraselmis sp.* in FT and RT treatments.

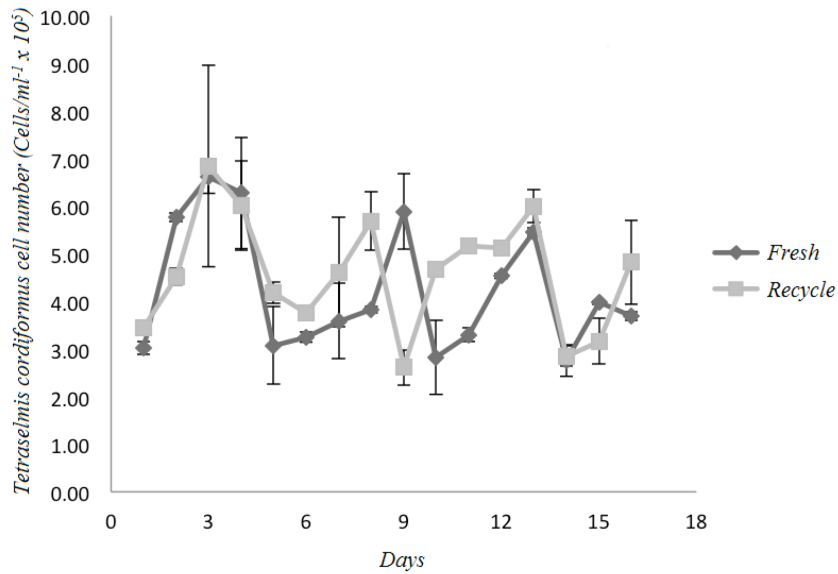

Figure 1. *Tetraselmis subcordiformus* growth curves in recycled and fresh medium the four harvests.

Over the harvest and recycling stages cell counts were performed daily (Figure 1). There was no significant difference between the growth rates of the FT and RT, though a slight enhancement of the growth rate was seen in the RT on day 7.

3.2 Bacteria community dynamics between the use of recycled media and fresh seawater.

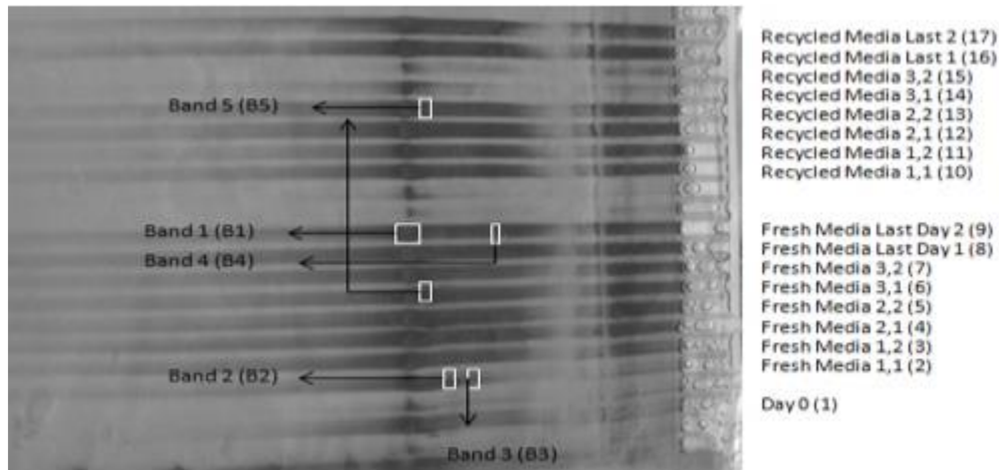

Figure 2. Bacteria selected for identification within the bacteria PCR-DGGE gel.

Table 1. Bacteria identified within the RT and FT treatments.

Band	Species	Similarity	Accession number
Band 1	*Vibrio sp.*	86%	JQ068793.1
	Vibrio corallitlyticus	86%	JQ307097.1
Band 2	*Cylindrospermum stagnale*	79%	NR_102462.1
	Cylindrospermum stagnale	79%	NR_117352.1
Band 3	*Rhizobium sp.*	96%	KF647254.1
	Rhizobium sp.	96%	HG518324.1
Band 4	*Uncultured organism*	85%	JN447524.1
	Uncultured bacterium clone	85%	KF817477.1
Band 5	*Uncultured marine bacterium*	91%	KF185572.1
	Uncultured marine bacterium	91%	KF185463.1

The bacterial community for the RT and FT treatments were investigated using PCR-DGGE. It is important to investigate the bacteria communities present within a microalgae culture since bacteria can play a significant role in microalgal growth, as bacteria has been found to either stimulate or prohibit microalgal growth (Cole 1982). Therefore understanding how the harvesting and recycling process effects the bacterial community is essential (Goecke 2013). Some bacteria are able to provide essential growth element such as vitamins and nitrogen

sources to microalgae (Kazamia et al. 2012; Mouget et al. 1995). Promoting such bacteria growth could contribute to a higher *Tetraselmis subcordiformis* production rates.

It was observed that the bacterial community through the whole investigation was stable and there was little differences seen within the bacterial communities (Figure 2). A dominating bacterium found within both the FT and RT treatments was Vibrio sp. (Figure 2), studies have observed that Vibrio sp. has the ability to fix nitrogen within aquatic environments (Chimetto et al. 2008; Criminger et al. 2007). The other bacteria found within this study were uncultured *Cyanobacterium sp.* (band 2), and *Rhizobium sp.* (band3), these bacteria where found both in RT and FT treatments. *Cyanobacterium sp.* are a group of phototropic bacteria that have the ability to fix nitrogen and photosynthesize (Meeks and Elhai 2002). Many *Rhizobium sp.* bacteria also specialise in nitrogen fixation (Masson-Boivin et al. 2009), though studies have recently found that *Rhizobium sp.* also aid in plant defence (Soto et al. 2006; Yang et al. 2009). *Cyanobacteria sp.* does not require specific environmental partners to survive (Meeks and Elhai 2002), however *Rhizobium sp.* requires a host plant, normally legumes, to generate nitrogen. There might be a link between *Rhizobium sp.* and *Tetraselmis subcordiformis* though further research will be required to show this symbiosis.

Figure 3. Bacterial communities analysis with recycled media and fresh media: (a) Shannon Weaver Index (b) Equitability Index (c) Bacteria dendogram ("F" represents Fresh Media, and "R" represents Recycled Media. Also F4 (1), F4 (2), and R4 (1), R4 (2) are samples from the last date of growth period.)

The dendogram indicated further distinctions between the FT and RT treatments. For the RT bacteria community there is a gradual shift between the first harvest and last harvest samples, while the bacterial community within the FT treatments is more randomly distributed throughout the dendogram (Figure 3c). The gradual change of bacterial community within the

RT treatment may indicate that the bacteria present within the RT culture were under a higher selective environment; therefore bacteria more suited to the hypersaline conditions and are able to grow with *Tetraselmis subcordiformis* and become more dominant.

To further estimate the diversity and distribution of the bacterial community within the RT and FT treatments, Shannon Weaver index and Equitability indices were calculated (Figure 3a and 3b). The diversity of RT bacterial community tended to increase before the first harvest, and remained stable during the other harvest cycles, and finally decreased at the last harvest cycle of the investigation. The diversity of the bacterial community from FT did not show a clear trend in this study, exhibiting increases and decreases in bacterial diversity throughout the experimental period (Figure 3a). The RT bacterial diversity would have had fewer introductions of new bacteria during each harvest cycle stage in comparison to the FT treatment which used fresh seawater to replenish the water loss during the harvesting stage, this may explain why the RT treatment bacterial diversity was much more stable in comparison to the FT treatment.

The Equitability index shows how evenly the bacterial communities are distributed (Figure 3b). The Equitability index of the RT treatment declined gradually from the first harvest to last harvest (EI: 0.7 – 0.5), this may indicate that a few bacterial strains such as *Vibrio sp.* where becoming dominant over the investigation. Again, the Equitability index of the FT treatment bacteria community did not follow a trend, this could be a result of the harvesting and reintroducing new bacteria during each harvest cycle.

3.3 Algal community dynamics between the use of recycled media and fresh seawater.

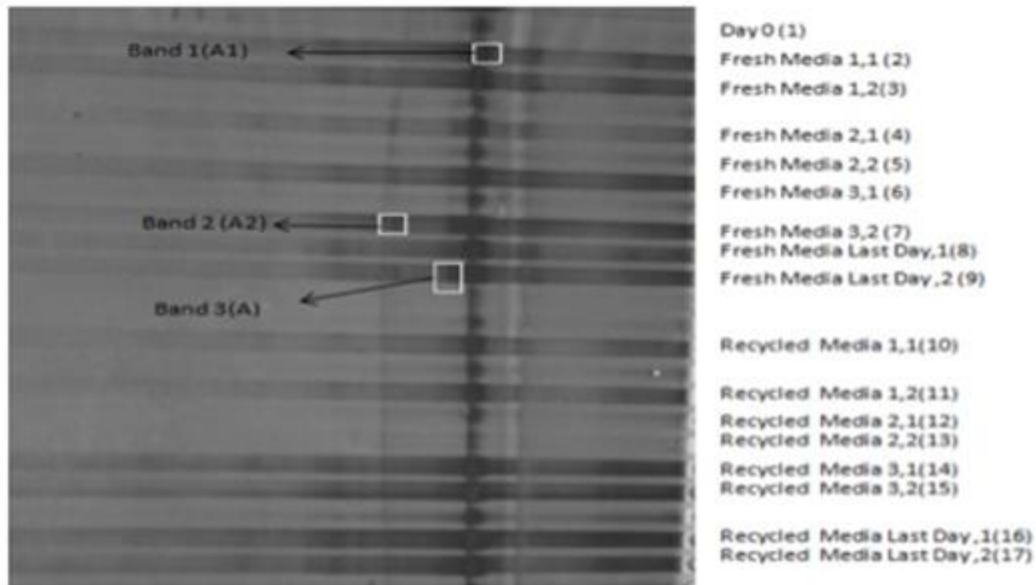

Figure 4. Microalgae selected for identification within the algal PCR- DGGE gel.

Table 2.Algae identified within the both the recycled and fresh media.

Band	Species	Similarity	Accession number
Band 1	*Tetraselmis subcordiformis*	99%	FJ559380.1
	Tetraselmis cordiformis	97%	HE610165.1
Band 2	*Tetraselmis striata*	98%	FN563077.1
	Tetraselmis carteriiformis	98%	L42992.1
Band 3	*Chlamydomonas pitschmannii*	99%	Z15152.1
	Chlamydomonas Moewusii	97%	X68916.1

The algal community was investigated to observe how competitive *Tetraselmis subcordiformis* was in comparison to other algae in unsterile conditions. It was observed that there were two *Tetraselmis sp.* present throughout all of the samples; they were identified as band 1 and band 2 (Figure 4). Additionally it was observed that there were more microalgae present within the FT culture in comparison to the RT culture (Figure 4), this suggests that the environmental pressure in hyper saline conditions help *Tetraselmis subcordiformis* (Band 1) to become dominant over time. The FT would have introduced new algae as fresh seawater was added to replace the lost water during the harvest cycles, as a result of the continuous

introduction of the new algae into these hyper saline conditions caused constant changes in the algal diversity.

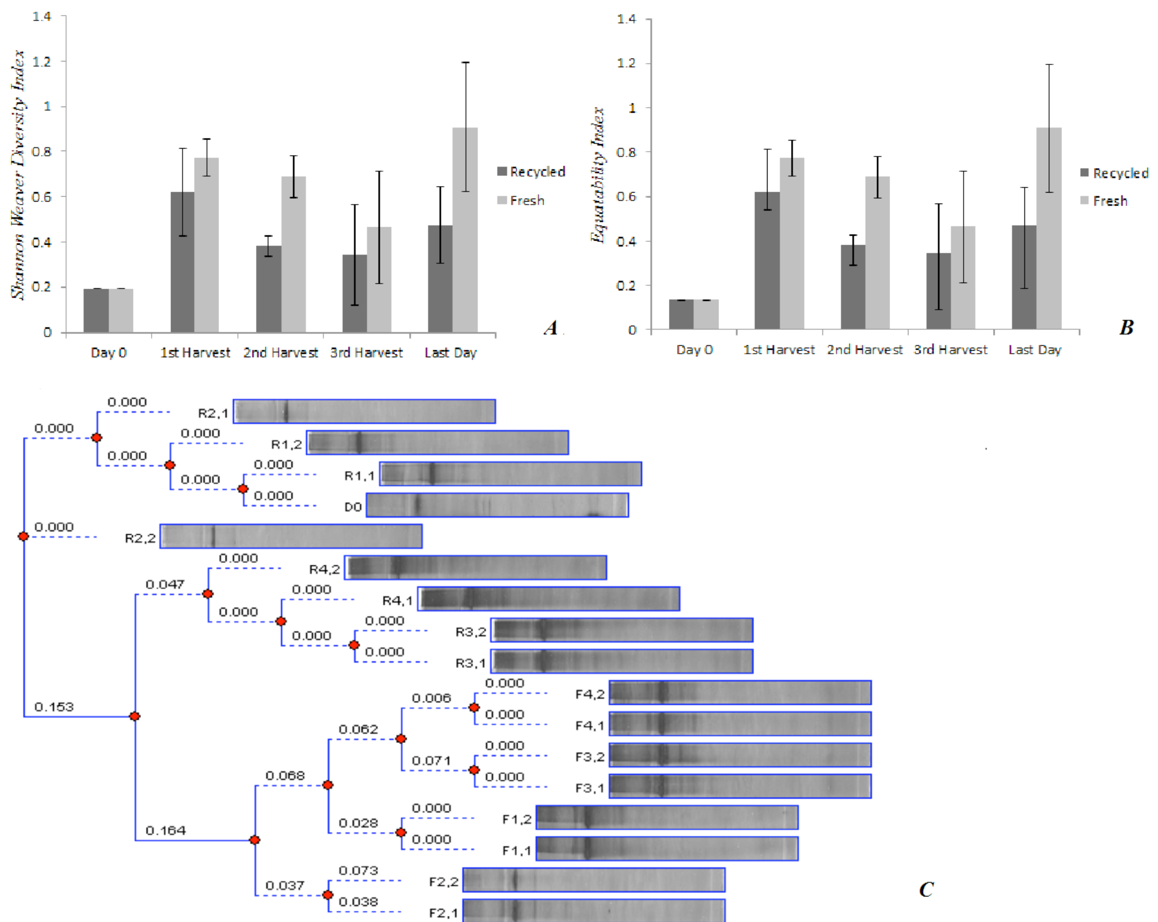

Figure 5. Algal communities analysis for recycled and fresh seawater media: (a) Shannon Weaver Index (b) Equitability Index (c) Dendogram (F: Fresh seawater medium, R: Recycled medium, and F4,1; F4,2; R4,1;R4,2 represent the last day of growth period.).

The FT and the RT treatments were separated into two groups according to the algal dendogram (Figure 5c). The FT was found to have similar algal communities over the whole investigation. The RT algal communities were separated into two clusters in the dendogram (Figure 5c). The Shannon Weaver index illustrates that the FT had a higher diversity over the whole investigation in comparison to the RT, additionally a similar trend was observed with the algal Equitability Index, where a more dominant microalgae where observed in the RT

treatment in comparison to the FT treatment. This indicates that the use of a RT can maintain a microalgal monoculture better than if adding fresh seawater to replace lost water during harvesting.

3.4 Water footprint.

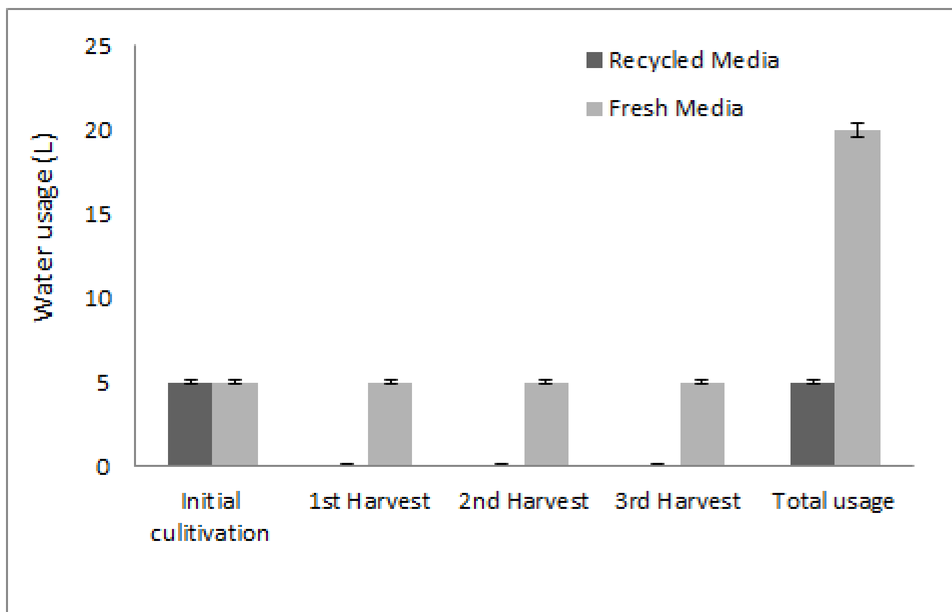

Figure 6. RT and FT treatments seawater usage comparison in litres.

In order to compare the water footprint of RT and FT treatment, the seawater usage was compared for recycled and fresh media treatments. It was obvious that RT used the same amount of seawater throughout the period, while FT required continuous addition of seawater. For three repetitive harvests, eliminating the minor loss of water from evaporation, a total of 15 litres of seawater was saved. Therefore a large sum of seawater usage could be reduced for larger scale production.

4 Conclusion.

It was observed within this investigation that the use of recycled media helped control non-desired bacteria and was slightly selective towards *Tetraselmis subcordiformis*. The

dominating bacteria found within both the RT and FT treatments where *Vibrio sp., Cyanobacterium sp.* and *Rhizobium sp.*. The water footprint was significantly reduced using recycled media. Hence it is suggested for large-scale production to use recycled media not only to be selective towards certain beneficial bacteria, but also to obtain higher microalgae growth rate with reduced water footprint for the bio crude oil production.

5 Acknowledgement.

This research was supported under Australian Research Council's Linkage Projects funding scheme (project LP100200616) with industry partner SQC Pty Ltd, and the Australian Renewable Energy Agency advanced biofuels investment readiness program funding agreement number Q00150. The views expressed herein are those of the authors and are not necessarily those of the Australian Research Council.

6 References.

Biller P et al. (2012) Nutrient recycling of aqueous phase for microalgae cultivation from the hydrothermal liquefaction process Algal Research 1:70-76

Ceh J, Kilburn MR, Cliff JB, Raina JB, Keulen M, Bourne DG (2013) Nutrient cycling in early coral life stages: Pocillopora damicornis larvae provide their algal symbiont (Symbiodinium) with nitrogen acquired from bacterial associates Ecology and Evolution 3:2393-2400

Chimetto LA, Brocchi M, Thompson CC, Martins RC, Ramos HR, Thompson FL (2008) Vibrios dominate as culturable nitrogen-fixing bacteria of the Brazilian coral Mussismilia hispida Systematic and applied microbiology 31:312-319 doi:10.1016/j.syapm.2008.06.001

Chisti Y (2007) Biodiesel from microalgae Biotechnology advances 25:294-306 doi:10.1016/j.biotechadv.2007.02.001

Cole J (1982) Interactions Between Bacteria and Algae in Aquatic Ecosystems Annual Review of Ecology and Systematics 13:291-314

Criminger JD, Hazen TH, Sobecky PA, Lovell CR (2007) Nitrogen fixation by Vibrio parahaemolyticus and its implications for a new ecological niche Applied and environmental microbiology 73:5959-5961 doi:10.1128/AEM.00981-07

Diez B, Pedros-Alio C, Marsh TL, Massana R (2001) Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques Applied and environmental microbiology 67:2942-2951 doi:10.1128/AEM.67.7.2942-2951.2001

Fon Sing S, Isdepsky A, Borowitzka M, Lewis D (2014) Pilot-scale continuous recycling of growth medium for the mass culture of a halotolerant Tetraselmis sp. in raceway ponds under increasing salinity: A novel protocol for commercial microalgal biomass production Bioresource technology

Girvan MS, Bullimore J, Pretty JN, Osborn AM, Ball AS (2003) Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils Applied and environmental microbiology 69:1800-1809

Goecke F, Thiel, V, Wiese, J, Labes, & Imhoff, JF (2013) Algae as an important environment for bacteria – phylogenetic relationships among new bacterial species isolated from algae Phycologia 52:14-24

Griffiths MJ, Harrison STL (2009) Lipid productivity as a key characteristic for choosing algal species for biodiesel production Journal of Applied Phycology 21:493-507 doi:10.1007/s10811-008-9392-7

Huerlimann R, de Nys R, Heimann K (2010) Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production Biotechnology and bioengineering 107:245-257 doi:10.1002/bit.22809

Kazamia E et al. (2012) Mutualistic interactions between vitamin B12-dependent algae and heterotrophic bacteria exhibit regulation Environmental microbiology 14:1466-1476

Lee AK, Lewis DM, Ashman PJ (2010) Energy requirements and economic analysis of a full-scale microbial flocculation system for microalgal harvesting Chemical Engineering Research and Design 88:988-996

Masson-Boivin C, Giraud E, Perret X, Batut J (2009) Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? Trends in microbiology 17:458-466 doi:10.1016/j.tim.2009.07.004

Mata TM, Martins AA, Caetano NS (2010) Microalgae for biodiesel production and other applications: A review Renewable and Sustainable Energy Reviews 14:217-232 doi:10.1016/j.rser.2009.07.020

Meeks JC, Elhai J (2002) Regulation of Cellular Differentiation in Filamentous Cyanobacteria in Free-Living and Plant-Associated Symbiotic Growth States Microbiology and Molecular Biology Reviews 66:94-121 doi:10.1128/mmbr.66.1.94-121.2002

Montero MF, Aristizábal M, García Reina G (2010) Isolation of high-lipid content strains of the marine microalga Tetraselmis suecica for biodiesel production by flow cytometry and single-cell sorting Journal of Applied Phycology 23:1053-1057 doi:10.1007/s10811-010-9623-6

Mouget JL, Dakhama A, Lavoie MC, Noüe J (1995) Algal growth enhancement by bacteria: Is consumption of photosynthetic oxygen involved? FEMS Microbiology Ecology 18:35-43

Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA Applied and environmental microbiology 59:695-700

Pienkos PT, Darzins A (2009) The promise and challenges of microalgal-derived biofuels Biofuels, Bioproducts and Biorefining 3:431-440 doi:10.1002/bbb.159

Sapp M, Schwaderer AS, Wiltshire KH, Hoppe HG, Gerdts G, Wichels A (2007) Species-specific bacterial communities in the phycosphere of microalgae? Microbial ecology 53:683-699 doi:10.1007/s00248-006-9162-5

Scott SA, Davey MP, Dennis JS, Horst I, Howe CJ, Lea-Smith DJ, Smith AG (2010) Biodiesel from algae: challenges and prospects Current Opinion in Biotechnology 21:277-286

Singh J, Gu S (2010) Commercialization potential of microalgae for biofuels production Renewable and Sustainable Energy Reviews 14:2596-2610

Soto MJ, Sanjuán J, Olivares J (2006) Rhizobia and plant-pathogenic bacteria: common infection weapons Microbiology 152:3167-3174

Stephens E, de Nys R, Ross IL, Hankamer B (2013) Algae Fuels as an Alternative to Petroleum J Pet Environ Biotechnol 4:2

Tilman D et al. (2009) Beneficial biofuels—the food, energy, and environment trilemma Science 325:270

Yang J, Kloepper JW, Ryu C-M (2009) Rhizosphere bacteria help plants tolerate abiotic stress Trends in plant science 14:1-4

Yang J, Xu M, Zhang X, Hu Q, Sommerfeld M, Chen Y (2011) Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance Bioresour Technol 102:159-165 doi:10.1016/j.biortech.2010.07.017

Chapter 8

Conclusion

This thesis presented the different closed loop systems that are used within the microalgae biofuels sector. Previously there was little known about how these closed loop systems affected the algae of interest and the microbial community present within the culture. Developing a greater understanding of closed looped systems provide insight into the future research goals within the algal biofuel industry.

Nutrient closed loops

Nutrient recycle loops are an important research area within the algal biofuel industry due to the potential economic benefits. Digestate in particular was identified as a key nutrient, its benefits are associated with a high nitrogen content and potential methane production. Digestate may be an alternative nutrient source although it may only be suitable for some microalgae as studies have also shown negative growth on digestate. With this consideration the application of digestate as a nutrient source is feasible but is highly species selective.

With the current trends in algal biofuels, HTL is becoming more popular. The use of the HTL AP is now seen as a potential nutrient source. HTL AP has a much higher nitrogen content of in comparison to algal digestate effluent. As discussed in chapter 4 the use of the HTL AP has it draw backs due to its negative impact on microalgal growth and high levels of dilution required for the growth of *Tetraselmis sp.*. However it was shown that it was possible to introduce the use of active carbon filtration to improve the growth of microalgae digestate. I believe that future research within the algal biofuels field will be more focused on the HTL AP and utilising it to produce biomass or energy. There is plenty of scope with the various microalgae that it can be applied too. However further research is required to firmly seed the HTP AP as a nutrient source.

Nutrient recycling has been identified as a process that would be applied at large scale. Initial testing of the suitability of the microalgae of interest is suggested to determine its potential performance on waste streams.

Water closed loops

The recycling of water is an essential step within the algal biofuels sector to ensure that the water footprint is reduced and that the technology is economically viable. Within this thesis I have displayed that there are many additional benefits of recycling water; the culture tended more

towards a monoculture of *Tetraselmis sp.* with each recycle period, whilst the beneficial bacteria associated with the growth of the *Tetraselmis sp.* were preserved.

Additionally it was shown that the introduction of new water into a culture can introduce undesired microorganisms such as other microalgae, bacteria, and protozoa. Interestingly the presence of protozoa was found to have no impact on our culture over 10 days. This was an interesting outcome as the perception of large-scale microalgal growth was if protozoa are present, there exists a high potential for biomass losses. The rotifers were more varied in terms of their growth patterns in comparison to *Tetraselmis sp.* which exhibited highly stable growth patterns throughout the experiment. This indicates that the impact of protozoa may be may be species and culture dependent, with optimisation of the algal culture preventing culture damage. Further research within this area may lead to enhanced understanding of why some different studies report very different outcomes in terms of microalgal growth in the presence of protozoa. I believe that future research on closed loop water systems will be focused on tracing nutrients through large scale open ponds, and on how symbiotic organisms grow and improve microalgal growth within these closed loop water systems. There is little known about the nutrients loads within each section, the changes to the chemical composition, trace metals and vitamins over each recycling stages, and how the harvesting systems after electroflocculation effects the chemical composition of the water. In addition, following algal death and lysis during the water recycle process, and how the microorganisms consume the dead algae and the products will also lead to significant increased knowledge.

Overall this thesis expanded the understanding of closed loop systems used within the algal biofuels sector.