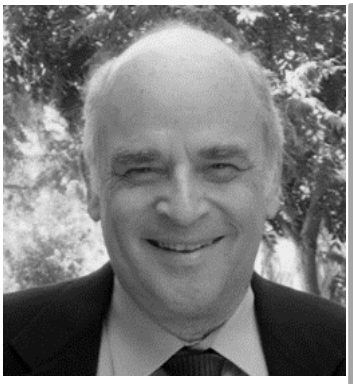

Dr John Benemann

"There is an enormous amount of ongoing research in which the facile assumption is made that algal wastewater treatment provides an easy route to algal biofuels. ***This thesis is a reality check*** *for such research-the route to algal biofuels, even with wastewater treatment carrying most of the financial load, will certainly not be easy nor straightforward."*

Wastewater treatment high rate algal pond (WWT HRAP) biomass for low-cost liquid biofuel production

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

in Chemical and Materials Engineering, The University of Auckland

by Abbas Mehrabadi

Jan. 2017

Abstract

Currently despite intense efforts algal biofuel production is still not economically competitive with fossil fuel. To lower algal biofuel production costs, replacement of pure algal biomass with free gravity-harvested algal-bacterial biomass produced in wastewater treatment high rate algal ponds (WWT HRAPs) has been suggested as a niche opportunity. While most WWT HRAP studies have focused on optimization of treatment performance, the biomass energy productivity and its suitability for quality biofuel production have not been previously investigated in detail. Hence, the objectives of this study are to:

- Examine the biomass energy yield potential of WWT HRAP,
- Evaluate different strategies to improve biomass energy yield and quality of without impacting pond treatment performance, and
- Examine the suitability of HRAP biomass for conversion to biodiesel, pyrolytic bio-oil and bio-crude production.

The average biomass production, energy content and energy yield of two identical pilot-scale WWT HRAP (monitored weekly over one year) were 21.5 ton VSS/ha/year, 19.2 GJ/ton and 413 GJ/ha/year respectively. Biomass energy yield is dependent on several factors increasing with warmer climate, lower grazing pressure, higher biomass algal proportion and higher lipid content. Since at full-scale climate conditions are not controllable, and there is little opportunity to increase biomass lipid content without impacting on pond treatment performance, biomass energy yield can only be increased by controlling zooplankton grazing or improving algal biomass productivity.

HRAP tend to select of colonial algal species due to the pond mixing that maintains them in suspension. Colonial algae are therefore more easily harvested by simple gravity settling compared to unicellular algal species and are due to their larger size are unable to be grazed by the majority of zooplankton. Hence, an investigation was made of the treatment performance and biomass energy production of the most dominant WWT HRAP colonial algal species. Of the colonial species tested, *Mucidosphaerium pulchellum* and *Micractinium pusillum* cultures had the highest nutrient removal and the highest biomass energy yield under simulated New Zealand summer and winter conditions. However, due to much better settleability, *Micractinium pusillum,* had the greatest potential for both wastewater treatment and biomass energy yield.

Two outdoor mesocosm-scale (HRAM) experiments using different air:CO_2 mixtures (up to 10% CO_2) conducted in summer and winter showed that the biomass energy yield could be

improved with CO_2 addition. In the summer experiment, compared to the aerated cultures (the control), the highest improvements of the biomass energy yield and its gravity harvestable proportion (43.8% and 102%, respectively) were achieved in the 5% CO_2 cultures (pH 6-7). While in the winter experiment, the greatest improvements (~ 14% for the biomass energy yield and~ 33% for the harvestable fraction) occurred in the 0.5% CO_2 cultures (pH 7-8). These experiments indicate maintaining a pond pH of 7-8 in winter and 6-7 in summer with CO_2 addition would be most beneficial.

To assess the quality of WWT HRAP biomass for biodiesel production, the biomass lipids were extracted and profiled during both the annual monitoring of the pilot-scale HRAP and the two CO_2 addition HRAM experiments. The biomass lipid profiles were highly complex which led to production of low-quality biodiesel. CO_2 addition did not affect biodiesel quality and only enhanced biodiesel productivity by up to 20% due to increased biomass productivity. Overall, less than 30% of the biomass energy yield (413 GJ/ha/year) was recovered in the form of low-quality biodiesel.

The low lipid content and high lipid complexity of the WWT HRAP biomass together with the technical limitations of lipid extraction such as drying, cell disruption and solvent extraction make energy recovery from the whole biomass more attractive. Thus, biomass energy recovery via conversion of the whole biomass through pyrolysis and hydrothermal liquefaction (HTL) was investigated at different temperatures. Overall, temperature had a positive effect on the yields of the target products but negatively affected its quality so that the maximum yield (7 wt% pyrolytic bio-oil and 24.9 wt% bio-crude) had the lowest quality (highest complexity and nitrogen content) and was produced at the highest temperatures (500 ºC in pyrolysis and 300 ºC in HTL). The maximum % of the energy content of the biomass recovered in the biofuel products, were 15% and 47.4% for the pyrolytic bio-oil and bio-crude respectively. While, HTL is a more favourable conversion process to maximise energy recovery from such a complex biomass, it was not feasible from an energy yield point of view at the tested conditions. Further investigations on the use of the by-products are required to improve biomass energy recovery and consequently the economic viability.

Overall based on the results WWT HRAP biomass is not a promising feedstock for low-cost quality liquid biofuel production due to its high complexity which leads to low-quality biofuel production.

Acknowledgements

First, I would like to express my deep gratitude and appreciation to my supervisors, Prof. Mohammed Farid and Dr Rupert Craggs for their kindly and continuous support, advice and enthusiasm during my journey towards completing this research. Their insightful and critical comments on my research work and particularly on my journal papers are greatly appreciated. It has been an honour for me to be part of their world class research teams.

I also wish to acknowledge Mr Valerio Montemezzani, Dr Jason Park and Dr Donna Sutherland for their help, valuable discussion and technical advice during the experiments. I would like also to thank technical staff at the National Institute of Water and Atmospheric Research Ltd. (NIWA) and at the University of Auckland, especially Mr Peter Martin and Mr Raymond Hoffmann for their expertise, dedication and technical assistance.

I gratefully acknowledge the financial support for this PhD research which was rendered by National Institute of Water and Atmospheric Research Ltd. (NIWA) through NIWA's MBIE funded project, *Wastewater Algal Biofuel Programme*.

Finally, special thanks to my lovely wife, Azadeh, and my parents for their patience and supporting me spiritually throughout this three years research and my life in general.

Thesis structure

The thesis consists of nine chapters which have been structured as a series of scientific papers that are either published or submitted for publication in international journals. These chapters may have some overlap particularly in the Introduction and Method sections. Design of all experiments, experimental work and analysis of the results were undertaken by myself, and the manuscripts were completed with the assistance of my supervisors, Prof. Mohammed Farid and Dr Rupert Craggs. All references are listed at the end of thesis.

Chapter 1: **Wastewater treatment high rate algal ponds (WWT HRAP) for low-cost biofuel production**

This chapter provides an overview of the benefits of WWT HRAPs relative to open raceway ponds typically used for algal cultivation. In addition, parameters influencing algal energy content and biomass energy yield in WWT HRAP are discussed. Recovering algal biomass energy through different biofuel conversion pathways is also reviewed.

Chapter 2: **Variation of biomass energy yield in wastewater treatment high rate algal ponds**

This chapter examines the biomass energy yield potential of WWT HRAP by operating and monitoring of two identical pilot-scale WWT HRAPs (8 m^3 volume, 0.3 m depth and 31.8 m^2 surface area) for one year. The effects of different parameters including algal species dynamics, zooplankton grazing, environmental conditions, biomass chemical composition and biomass algal proportion on overall biomass energy yield are addressed.

Chapter 3: **Beneficial colonial algal species for wastewater treatment and biomass production in high rate algal ponds (HRAPs)**

The results of pond monitoring (chapter 2) indicated that there was no difference in both the biomass energy yield and nutrient removal of the ponds when one was populated by unicellular species and the other with colonial species. However, the settleability of the colonial species was much better than that of the unicellular species, which would lower the cost of algal harvest. Hence, the focus of this chapter is on identification of the most beneficial wastewater colonial species for both wastewater treatment and biomass energy production. The performance of five dominant HRAP colonial species (*Mucidosphaerium pulchellum*, *Micractinium pusillum*, *Coleastrum* sp., *Desmodesmus* sp. and *Pediastrum*

boryanum) are compared based on the batch monoculture experiments of each species conducted on pre-frozen and pre-filtered primary settled sewage over 10 days under simulated New Zealand summer and winter conditions.

Chapter 4: **Effect of CO_2 addition on biomass energy yield in wastewater treatment high rate algal mesocosms (WWT HRAM)**

It has been suggested that algal production in WWT HRAPs is limited by the dissolved CO_2 concentration, particularly, in warm months. Therefore, the biomass energy yield is also limited by CO_2 availability. This chapter investigates if CO_2 addition improves the biomass energy yield (and gravity harvestable fraction) of algal-based wastewater treatment without negatively impacting treatment performance. Two outdoor experiments are conducted using 15 replicate mesocosms supplemented with different CO_2 concentrations during New Zealand summer and winter conditions. The cultures are compared in terms of productivity, chlorophyll a (chl-a) content, chemical composition, energy content, algal dynamics and settleability of the biomass.

Chapter 5: **Biodiesel production potential of wastewater treatment high rate algal pond biomass**

In this chapter the quantity and quality of biodiesel produced from WWT HRAP biomass are examined by profiling the biomass lipids extracted during year-long monitoring. Moreover, effect of CO_2 addition on the quality and quantity of the biodiesel is assessed by profiling the biomass lipids extracted during the CO_2 experiments. The overall energy balance on biodiesel production from WWT HRAP biomass is, also, discussed.

Chapter 6: **Pyrolysis of wastewater treatment high rate algal pond (WWT HRAP) biomass**

This chapter includes the description of pyrolytic bio-oil production from WWT HRAP biomass at different temperatures (300-500 ºC) and heating regimes (stepwise and non-stepwise) in terms of yield, chemical and elemental composition, and energy content. In addition, thermal decomposition behaviour of the WWT HRAP biomass is investigated using TGA under simulated pyrolysis conditions. Moreover, potential uses of process by-products, i.e. bio-char and aqueous phase, are proposed based on their composition and characteristics. The energy balance on pyrolysis of WWT HRAP biomass is, also, discussed in detail.

Chapter 7: **Wastewater treatment high rate algal pond biomass for bio-crude production**

In this chapter the potential of bio-crude oil production by HTL of wet WWT HRAP biomass is examined at different temperatures (150-300 ºC) by measuring yield, elemental/chemical composition and energy content of the products. The benefits and opportunities which may be provided by using WWT HRAP biomass as HTL feedstock are outlined. In addition, an overall lumped reaction kinetic model for degradation of biomass volatile organic fraction is developed. The solid residue and aqueous phase are also analysed and their potential applications are proposed. Finally, the energy balance for HTL of WWT HRAP biomass is, also, discussed in detail.

Chapter 8: **Conclusions**

In this chapter the main findings of this study are summarized.

Chapter 9: **Future research needs**

Suggestions for further investigations in the study of bioenergy production from WWT HRAP biomass are provided in this chapter.

The structure of this thesis complies with the University of Auckland guidelines given in the Doctorial Handbook, 2011.

Table of Contents

List of Tables

List of Figures

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Nature of contribution by PhD candidate	The literature search and critical review were undertaken by myself. I also undertook the manuscript writing for this paper.
Extent of contribution by PhD candidate (%)	90%

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Dr Ruppert Craggs	Advise and manuscript revision

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Nature of contribution by PhD candidate	The literature review, the design of the experiment, the experimental work and analysis of results were undertaken by myself. I also undertook the manuscript writing for this paper.
Extent of contribution by PhD candidate (%)	80%

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Dr Ruppert Craggs	Providing experimental set-up and chemicals, advising on the experimental study and manuscript revision

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Nature of contribution by PhD candidate	The literature review, the design of the experiment, the experimental work and analysis of results were undertaken by myself. I also undertook the manuscript writing for this paper.
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CHAPTER 1

Introduction

This chapter is based on the following publication:

Mehrabadi, A., Craggs, R., Farid, M. M., 2015. Wastewater treatment high rate algal ponds (WWT HRAP) for low-cost biofuel production. *Bioresource technology*, 184, 202-214.

Chapter preface

Growing energy demand and water consumption have increased concerns about energy security and efficient wastewater treatment and reuse. Wastewater treatment high rate algal ponds (WWT HRAPs) are a promising technology that could help solve these challenges concurrently where climate is favorable. WWT HRAPs have great potential for biofuel production as a by-product of WWT, since the costs of algal cultivation and harvest for biofuel production are covered by the wastewater treatment function. Generally, 800-1400 GJ/ha/year energy (average biomass energy content: 20 GJ/ton; HRAP biomass productivity: 40-70 tons/ha/year) can be produced in the form of harvestable biomass from WWT HRAP which can be used to provide community-level energy supply. In this chapter the benefits of WWT HRAPs are compared with conventional mass algal culture systems. Moreover, parameters to effectively increase algal energy content and overall energy production from WWT HRAP are discussed including selection of appropriate algal biomass biofuel conversion pathways.

1.1 Introduction

Of all biomass sources, microalgae have received considerable renewed attention to become a feedstock for large-scale biofuel production. They are an interesting feedstock for biofuel production in comparison with the other biomass sources as they have higher photosynthetic efficiency and biomass productivity than terrestrial crops, and the ability to grow on wastewater and marginal land [1]. Despite recent intense efforts to make algal-based biofuel economically viable with fossil fuels, there are still several obstacles to overcome. To improve the performance of algal biofuel production systems, each step from algal production to biofuel conversion must be considered. The major costs for algal production include fertilizer and chemical expenses; water pumping; while biomass harvest and dewatering can have a high energy demand; and algal biomass biofuel conversion pathways have particular technological limitations [2, 3]. A promising niche opportunity to avoid/overcome these obstacles is to use microalgae that are grown as a by-product of wastewater treatment in high rate algal ponds (HRAP).

Wastewater treatment high rate algal pond (WWT HRAP) are shallow (30-40 cm deep), paddlewheel-mixed open raceway ponds which are designed to optimise natural biological treatment processes [4]. Their performance is based on a symbiotic relationship between bacteria and microalgae and they provide low-energy wastewater treatment, while recovering

dissolved nutrients as harvested algal biomass that could be used as a biofuel feedstock [4]. Compared with conventional mechanical wastewater treatment technologies typically used for wastewater treatment in large cities, WWT HRAP has lower capital and operating costs [5]. In fact, algal-based treatment systems (i.e. WWT HRAP) replace electrical energy consumed in conventional mechanical treatment technologies by capturing solar energy through algal photosynthesis [4]. Thus, more than 50% of the energy used by mechanical systems could be saved by applying WWT HRAPs [6]. However, HRAP require a large land area (1.7-2.7 ha/ML/day; [6]) and are being natural systems have some variability in treatment performance. On the other hand, WWT HRAPs are much more efficient than the conventional waste stabilization ponds (WSP) used to treat the wastewater of small to medium-sized communities world-over. WWT HRAPs can achieve much more efficient nutrient removal than WSPs in shorter time, 4-8 days compared with 30 – 60 days [7, 8]. In addition, since WSPs are unmixed, only unicellular, motile or buoyant algal genera including *Chlorella, Chlamydomonas* and *Anabaena,* tend to grow in them Pearson [9]. Chemical flocculation or other expensive processes are often required to remove the algae to improve effluent quality prior to discharge. The gentle mechanical mixing provided by the paddlewheel in WWT HRAPs, promotes non-motile colonial algal genera including *Micractinium, Pediastrum* and *Scenedesmus* that can grow and form large microbial flocs [10] with a diameter greater than 100 μm [11] which can be passively harvested by gravity with no energy use [12].

Although, the primary goals of such a system are wastewater treatment and nutrient recovery, the easily harvestable biomass produced in WWT HRAP has a heating value of 18-22 kJ/g and therefore has potential for conversion to various biofuels [8, 12]. The costs of microalgal cultivation and harvesting are covered by the tertiary-level wastewater treatment function. In particular, no extra nutrients are needed for microalgal cultivation, and algal biomass (algal/bacterial flocs) is easily harvested by simple gravity sedimentation [6]. Therefore, there is a niche opportunity for use microalgae grown in WWT HRAPs for low-cost community-level biofuel production [8, 12].

Energy production in WWT HRAP is a function of several factors that can be classified in three categories: environmental, operational and biological. To enhance low-cost biomass energy production in WWT HRAP all possibilities to increase biomass productivity, biomass energy content and harvestability should be considered.

Various biofuels can be produced by conversion of the whole or part of WWT HRAP biomass including [2, 6]:

- Conversion of the whole biomass to biogas by anaerobic digestion
- Conversion of the whole biomass to bio-oil by thermochemical reactions.
- Extraction of the lipid fraction and transesterification to biodiesel
- Fermentation of the carbohydrate fraction and distillation of bioethanol

Although biofuel production from freshwater and marine microalgae has recently been the subject of intensive interest [3, 13], there have been few reviews on biofuel production from algal-bacterial community grown as a by-product of wastewater treatment in HRAP [6, 10]. The purpose of this chapter is to outline the benefits and opportunities provided by using WWT HRAP algae for biofuel production by reviewing current literature. In addition, the influence of different parameters on energy production in these systems is discussed. Moreover, the parameters that effectively increase algal energy content and overall energy production from WWT HRAP are discussed including the advantages and limitations of different biomass conversion pathways.

1.2 Low-cost algal biomass production in WWT HRAPs

Microalgal biomass was first suggested as a feedstock for biofuel production in the 1960s [14]. High value microalgae and products are commercially cultivated today in both closed photobioreactors and open raceway ponds although over 90% of current production is from open raceways. Moreover, biofuel cannot be economically converted from algae grown in photobioreactor systems due to their high capital and operation costs [3]. Consequently, open raceway ponds are increasingly being chosen for commercial-scale microalgal cultivation for biofuel production.

Commercial cultivation of freshwater algae in HRAP requires a water source and nutrients (N, P and micronutrients) which can be costly. The annual make up water volume for microalgal production is in the range of 11–13 ML/ha/year, while production of one ton of algal biomass requires approximately 40–100 kg of N and 3-12 kg of P [15]. Moreover, world phosphorus resources are limited with 30-50 years supply left at the current rate of consumption [16]. Therefore, nutrient recovery and water reuse within algal biofuel production processes would be particularly beneficial. CO_2 addition is an important operational parameter for microalgal production but requires a high investment (5940 $/ha) [15].

In contrast, WWT HRAPs are essentially a low cost algal cultivation system since costs of water and nutrients are provided by the wastewater, CO_2 is partially provided via respiration of wastewater bacterial community and other operation costs as well as algal harvest are covered by the wastewater treatment function [8]. Approximately 2500 m^3 of wastewater (with an average 30 $g/m^3$of ammonia) can be treated by production of 1 ton of algal biomass with average 7% nitrogen content [15]. Moreover, the microalgal species cultivated in HRAPs for biofuel production are usually small (< 30 μm) and costly to harvest [17]. Whereas growth of colonial algal species and formation of algal-bacterial floc with the large size of 50-200 μmin WWT HRAPs enable gravity harvesting, minimising harvest costs [11, 18]. Due to these factors, coupling wastewater treatment with algal cultivation for biofuel production majorly reduces the energy requirement for algal biomass production to ~4 MJ/kg [19] and consequently reduces the overall production cost of the biofuel.

A potential drawback for the use of WWT HRAP as an algal cultivation system for biofuel production is the low lipid content of the biomass. Similar biomass production (40-70 tons/year/ha) can be achieved in both algal cultivation HRAP and WWT HRAP [12, 20], however, the lipid content of WWT HRAP biomass is low. Algal cultivation HRAP biomass can have a lipid content in the range of 20-40% (by dry weight) [13, 20], while the lipid content of WWT HRAP biomass is in the range of 10-30% [8, 18, 21]. A lower lipid content results in lower biomass energy content because 30-50 % of biomass energy content comes from lipids [22]. The typical energy content of algal cultivation HRAP biomass is 18-24 kJ/g [20, 23] compared with17-22 kJ/g for WWT HRAP biomass [12]. The main reason for this difference is the composition of the biomass since WWT HRAP biomass contains a mixture of algae and bacteria. As the lipid content of bacteria is typically <10% [24], this reduces the overall lipid content and hence energy content of WWT HRAP biomass.

1.3 The effect of microalgae chemical composition on biomass energy content

Algal cells are composed of carbohydrates ($C_1H_{1.67}O_{0.83}$, ~17 kJ/g), lipids ($C_1H_{1.83}O_{0.17}N_{0.0031}P_{0.006}S_{0.0014}$, ~37 kJ/g) and proteins ($C_1H_{1.56}O_{0.3}N_{0.26}S_{0.006}$, ~24 kJ/g) [22], and their energy content is typically in the range of 18-24 kJ/g. The biochemical composition of microalgae and consequently microalgal energy content vary between species and are affected by several parameters.

Microalgal biomass synthesis begins with the photosynthesis reactions: the light-dependent reaction (a set of photochemical and redox reactions taking femtoseconds to

milliseconds) and temperature-dependent (or light-independent) reaction (a series of enzymatic reactions, which take seconds to hours) [1, 22]:

$$Light dependent\ reaction: 2H_2O + light \rightarrow 4H^+ + O_2 + energy$$

$$Temperature dependent\ reaction: 4H^+ + CO_2 \rightarrow CH_2O + H_2O$$

Cell proteins catalyse cell metabolism reactions, provide a physical support for chlorophyll, and play a critical role in the selective permeability of the cell membrane [22]. Cell lipids and carbohydrates function as energy reserves and form the structural components of the cell. They are, also, responsible for responding to the variation of environmental factors [25]. For example, the percentage of intracellular waxes increases in response to low temperature due to isolate cell and prevent heat lost [25].

When photosynthetic energy supply equals algal energy demand optimal growth rates occur, but when there is an imbalance between energy supply and demand as a result of environmental variation, the photosynthetic apparatus and algal growth are affected [26]. For instance, under conditions of low temperature and high light intensity, more light will be absorbed and surplus energy will be produced by the cell since the activity of the carboxylase enzyme will be low. Consequently, the microalgal cells will reduce their chlorophyll content to prevent cell damage [26] and protein synthesis and cell division will decline while production of storage lipids will increase.

Microalgal lipids can generally be grouped into two categories: structural lipids or polar lipids (33-35 kJ/g) and storage lipids or non-polar lipids (39-43 kJ/g). Structural lipids typically have a high content of polyunsaturated fatty acids (PUFAs).Whilst storage lipids are mainly in the form of triacylglycerol (TAGs) made of predominately saturated and monounsaturated fatty acids which can be easily catabolized to provide metabolic energy [1, 22, 25].

Microalgae are capable of producing a wide range of fatty acids from medium (C12) to long (+C20) carbon chain length. The most common fatty acids in freshwater microalgae are C16 and C18 [1, 13, 22, 25]. Thompson [25] reported that during logarithmic growth phase, green algae produce a large amount of polar lipids and polyunsaturated C_{16} and C_{18} fatty acids. As algae approach stationary phase the lipid profile often changes with higher amounts of neutral lipids1ncluding 18:1 and 16:0 fatty acids [1, 25], which increase the overall energy content of the algae [23].

1.4 Parameters affecting biomass energy production in WWT HRAP

The main characteristics of algal species that are beneficial for wastewater treatment include high nutrient removal capacity, high growth rate at different nutrient loads, an ability to grow under variable temperature and light conditions, and easy harvesting by gravity settling [11, 18].

For biofuel production, the critical characteristics to maximise biomass energy production in WWT HRAP are culturing algal species that have high productivity and high energy content without impacting the wastewater treatment function of the ponds. Therefore, an understanding the factors that affect the dynamics and growth of naturally occurring algal species in WWT HRAPs is essential. Furthermore, although high productivity is most important for biofuel production, high lipid content and particularly high content of specific lipids (i.e., saturated and mono-saturated fatty acids with medium chain length, C16 to C18, these don't need costly upgrading processes) could be beneficial with the exception of bioethanol production.

Biomass energy yield in WWT HRAPs, (the combination of algal biomass productivity, dominant algal species and their composition) is affected by factors which can be divided into three main categories: environmental (light and temperature), operational (CO_2 concentration, nutrient concentration, cultivation mode, hydraulic retention time, mixing, and algal recycling) and biological (algal species contamination and grazers) [6, 18].

1.4.1 Environmental factors

1.4.1.1 Light

Light of frequency 400 to 700 nm (photosynthetically active radiation) provides the energy for algal photosynthesis and production [22]. Only 12.8-14.4% of solar energy can theoretically be converted into algal biomass however, actual photosynthetic efficiency is much lower than this, typically only 1-2.5% [18]. In temperate climates algal biomass productivity varies with seasonal variation in solar radiation. For example, Sutherland et al. [27] found a 250% increase in biomass concentration (measured as volatile suspended solids, VSS), in summer when there was three times more solar radiation than in winter.

Biomass productivity may be improved by increasing the availability of light within the pond water column. Pond depth and hydraulic retention time can be adjusted to vary the algal culture density to prevent self-shading and improve light availability within the pond [18].

Moreover, light intensity and photoperiod affect microalgal composition and lipid profile in terms of polarity and degree of saturation [1, 13]. Generally, at high light intensity the fatty acid synthesis pathway produces more saturated and mono-unsaturated fatty acids and less total polar lipids [1]. However, high light intensities can cause photoinhibition where the amount of photons being absorbed the photosynthetic apparatus is higher (50 to 80%) than the consumption of photons by the photosynthesis reaction [28]. Excess electrons accumulate in the photosynthetic electron transport pathway and can cause an over-production of reactive oxygen, which in turn, inhibits photosynthesis and damages cell membrane lipids (PUFA), proteins and other macromolecules. As a result, biomass productivity and nutrient removal efficiency decrease [1]. Under these conditions, algal cells synthesise more TAG instead of carbohydrate or protein as a safety measure since TAG synthesis consumes double the amount of the reductant NADPH (from the electron transport pathway) which is used for CO_2 assimilation [1].

1.4.1.2 Temperature

Temperature is a crucial parameter affecting algal growth. Many microalgal species are able to photosynthesise and grow over a wide temperature range between 5 and 40 °C [18, 26]. However, their optimal temperature is often between 28 and 35 °C [18]. Below the optimum temperature, temperature increases will enhance cell chlorophyll content, intracellular enzymatic activity and biomass productivity. Biomass productivity will typically double for each 10 °C increase in temperature until unfavourable temperatures are reached [26].

Temperature also has a major influence on microalgal biochemical composition, especially the lipid profile. In many species, the ratio of unsaturated to saturated fatty acids decreases with increasing temperature [1, 28]. This not only means that the algal cell energy content increases but also that the proportion of lipids that are beneficial for biodiesel production and quality increases. For example, Wu et al. [29] showed that the saturated fatty acid content of *Monoraphidium sp.* increased from 30% to 34% when temperature was increased from 25 to 35 ^{o}C. Moreover, the fraction of fluid lipids decreases in all lipid classes when algal cells are grown at higher temperature. Therefore, temperature has considerable influence on the energy content of algal biomass and the fraction of high quality lipids.

1.4.2 Operational factors

1.4.2.1 CO_2 availability

Microalgae, like other plants, are able to capture CO_2 and convert it to valuable organic molecules including lipids, the basic component for biofuel production, through photosynthesis.

The performance of WWT HRAP (in terms of nutrient removal, algal productivity, lipid content and profile) is enhanced with CO_2 addition [11]. With WWT HRAP the CO_2 for CO_2 addition can be sourced from the biogas produced by digestion of the wastewater solids or the flue gas following combustion of the biogas.

CO_2 addition is used to increase the C: N ratio of the wastewater (often 2.5-4:1) up to 6:1 which is more typical of algal biomass [12]. Park and Craggs [30] found that WWT HRAP biomass productivity was increased by 30% with CO_2 addition under summer conditions. Park et al. [11] further showed that CO_2 addition also increased the plant availability of N by 17% by reducing pond water pH and shifting the equilibrium of ammonia towards ammonium. Therefore CO_2 addition to WWT HRAP promotes both nutrient recovery and algal productivity, and consequently energy production [11, 28, 30].

Furthermore, CO_2 addition has been found to increase algal cell fatty acid content. Muradyan et al., [31] found > 30% increase in algal cell fatty acid content when the CO_2 concentration was increased from 2% to 12 %. Within this range, increased CO_2 concentration enhanced the synthesis of de novo fatty acids [31].

Moreover, CO_2 addition has been shown to increase algal energy content by up to 10% via changing lipid composition. For example, growth on CO_2 enriched (2-10% CO_2) culture media changed the algal biomass lipid profile and increased the degree of fatty acid saturation in several green algae [32, 33].

1.4.2.2 Hydraulic retention time (HRT)

HRT is an important operational factor which affects culture concentration, dominant species, algal/bacterial ratio and WWT HRAP performance in terms of treatment and energy production [10, 34]. Algal/bacterial ratio often decreases with increasing HRT while nutrient removal improves [11, 34]. Park et al. [11] reported that the % of algae in HRAP biomass increased from 56% when grown at an 8 day HRT to 80.5% at a 4 day HRT. These results suggest that longer HRAP HRTs decrease the proportion of algae in the biomass which will

affect biomass composition. Furthermore, the biomass ash content will increase as the ash content of bacteria (10-20%) is higher than that of microalgae (<10 %) [24]. Hydraulic retention time also influences algal population dynamics due to differences in the specific growth rate of algal species [10] which would further alter the overall biochemical composition and energy content of the algal biomass.

1.4.2.3 Mixing

Algal production and nutrient removal are limited by the uptake of nutrients into the algal cells from the culture media. Mixing reduces both the boundary layer around individual algal cells as well as thermal stratification within the pond, both of which can reduce the efficiency of nutrient assimilation and gas exchange [10]. Moreover, mechanical mixing increases cell light exposure frequency, promoting algal photosynthesis by increasing the light climate of the cell when more is needed or reducing the light climate when light is in surplus at the pond surface and photoinhibition is likely.

Unmixed wastewater treatment ponds tend to grow algae that are either have a similar density to water, are buoyant or are motile, and often all available light is absorbed in the top 300 mm of the pond [28, 35]. As a result, the algal biomass does not settle and contains few colonial species or large algal/bacterial flocs [8] requiring expensive high energy use mechanical harvest.

1.4.2.4 Cultivation mode

Cultivation mode also effects on microalgal dynamics and biomass productivity in the WWT HRAP. For example, Johnson [10] operated two WWT HRAPs (500 m^2, 150 m^3) in both continues and batch modes. The HRT of continues mode was 4 days and WWT HRAP didn't feed for two month in batch mode. He found that batch culture tends to favour small microalgae such as *Chlorella* sp. which have smaller size and need lower nutrient to grow, while *Micractinium* sp. outcompeted *Chlorella* sp. in continuous culture as they have higher growth rate [10]. Since each algal species has a different biochemical composition, the overall composition and energy content of WWT HRAP biomass would vary with cultivation mode.

1.4.2.5 Algal recycling

Another parameter that enhances the final amount of biomass energy is controlling the dominant microalgae in the WWT HRAP for species that have high productivity, are easily settleable and have a high energy content. Park et al. [12] showed that maintaining the

dominance of *Pediastrum boryanum* in a WWT HRAP increased biomass productivity, harvestability, energy content, and consequently the net biomass energy yield by 66%. The dominance of *Pediastrum boryanum* (which is a potential species for both wastewater treatment and energy production) was maintained simply by recycling 10% of the harvested biomass back into the WWT HRAP [12].

1.4.2.6 Nutrients

The availability of the nutrients is extremely important for species dominance, biomass productivity and biomass chemical composition. Green algae have a typical biochemical composition of $C_{106}H_{181}O_{45}N_{16}P$ [28] and require sufficient amounts of carbon, nitrogen, and phosphorus to grow optimally. Municipal primary wastewater typically contains 30 g/m^3 NH_4^+ - N and 5 g/m^3 PO_4^{3-} - P, so P is in excess for the N that is available.

Generally, algal cell internal nutrient concentrations are a function of the nutrient concentration in their environment [36] which can favour particular algal species. Fulton [36] showed that algal species that had N:P ratios similar to the culture media N:P ratio often dominated the culture [36]. Klok et al. [37] found that under nitrogen limiting conditions, the algal intracellular N concentration decreased by half. Furthermore, nutrients have a significant influence on lipid synthesis in microalgae. When nitrogen is deficient, protein synthesis reduces or even stops and cell growth declines, while lipid and carbohydrate synthesis increases [13, 22]. Algal lipid content can double or triple under nitrogen limiting conditions [23, 25], which, in turn, increases microalgal energy content from 18 kJ/g (low lipid content) up to 29 kJ/g (high lipid content) [22, 23]. However, nitrogen limitation also reduces algal growth, often resulting in lower overall lipids production. Phosphorus limitation (though less likely in WWT HRAP) induces the replacement of membrane phospholipids with non-phosphorus lipids which are more useful for biofuel production. As phospholipids known as gum, the raw material with high phosphorous content has problem during storage due to precipitate formation [1, 38].

Therefore, cultivation of microalgae under near-nutrient limiting conditions can increase microalgal lipid content, quality and overall energy content but comes at the expense of reduced productivity. Extending the HRAP HRT is a potential strategy to create nutrient limiting condition. However, further research is required to find optimum HRTs for WWT HRAP in different seasons to produce more beneficial biomass for biofuel production with

minimum negative effects on wastewater treatment rate, biomass production and algal/bacterial ratio.

1.4.3 Biological factor

1.4.3.1 Invertebrates

The biomass concentration in WWT HRAP can be rapidly reduced to less than 10% within 2-3 days by herbivorous zooplankton grazers (mainly cladocerans and rotifers) [6, 18]. Moreno-Garrido and Canavate, [39] observed the complete elimination of a *Dunaliella salina* from an algal production HRAP by a ciliate in less than 5 days. Grazer control in WWT HRAP could potentially be used to select for beneficial algae by maintaining zooplankton that graze on poorly settleable, unicellular algae, thus promoting the dominance of larger, settleable algae.

1.5 Algal biofuel options

Algal biomass biofuel conversion processes can be classified into chemical, biochemical and thermochemical processes. Chemical conversion includes transesterification of the lipid fraction to biodiesel. Biochemical conversion consists of fermentation of the carbohydrate fraction to bioethanol and anaerobic digestion of the whole biomass to produce biogas. Thermochemical conversion involves the thermal decomposition of algal organic components into liquid or gaseous fuels and can be divided into hydrothermal liquefaction, pyrolysis and gasification [2, 40]. Selection of the most appropriate conversion process is a function of: the biomass biochemical composition and water content; the desired type of biofuel; economic and technical considerations; and environmental standards [2, 6, 40]. The efficiency and limitations of each biofuel conversion process are discussed in detail in the next section.

1.5.1 Biodiesel production through transesterification of the lipid fraction

Biodiesel is a mixture of mono-alkyl esters of long chain fatty acids derived from a renewable lipid feedstock such as algal lipid and has a heating value in range of 39-41 kJ/g [2, 40]. The lipids are converted to biodiesel by the transesterification reaction, in which the carbonyl group R1 of an ester is exchanged with the organic group R2 of an alcohol and is catalysed by either a strong acid donating a proton to the carbonyl group (electrophilic), a strong base removing a proton from the alcohol (nucleophilic), an enzyme, or a heterogeneous catalyst [2]. The enzymatic process is still costly compared to other catalysts,

but it does not need neutralization; requires less alcohol, and can convert feedstocks with a high free fatty acid content [40].

Parameters that effect on the overall yield of biodiesel are: reaction temperature, reaction time, alcohol/lipid ratio, catalyst type and dose, mixing intensity, and lipid profile [2, 38]. The transesterification reaction occurs at 35-90 °C and takes 1 to 6 h to complete (Table 1.1). Reaction efficiency can be improved by using alcohol in excess of the stoichiometric ratio of alcohol in reaction which is 3. Conversion efficiency and the quality of the biodiesel are affected by the lipid profile. Algal lipid extract not only contains triacylglycerols (TAGs), but undesirable lipids such as phospholipids, chlorophyll and polyunsaturated fatty acids which cannot be converted to biodiesel [2, 40]. Therefore, the overall yield of the reaction is between 80-90%, however, the conversion efficiency of TAGs is more than 90%. Undesired lipids produce an unstable and viscous biodiesel that may polymerize over time into waxy solids [6]. Typically only 1/3-1/4 of total algal lipid can be converted to biodiesel, which is only 10 wt% of the algal biomass [41]. Further benefits from this conversion process may be made by also utilizing the by-products. The main by-product of this reaction is glycerine which is produced in the range of 8-12% of the volume of the biodiesel [2].

There are many obstacles to economical production of biodiesel from microalgae including: the low lipid content of algal biomass, dewatering and drying of algal biomass which normally contains more than 99% water and subsequent solvent recovery after lipid extraction and biodiesel production.

Table 1.1. Example of published results on biodiesel production from microalgae lipid fraction

Species	**Lipid content** (%)	**Reaction temperature** (°C)	**Time** (min)	**Catalyst**	**Lipid: solvent ratio**	**Conversion efficiency** (%)	**Yield** (g biodiesel/ g dry biomass)	**Ref.**
Dunaliella tertiolecta	19	340	0.5	Titainia	NG[a]	82.3	NG	[42]
Nannochloropsis oculata	18					11.4		
Chlorella vulgaris	38.9	60	120	KOH	NG	NG	0.13	[43]
Nannochloropsis oculata	24	60	1140	H_2SO_4	1:600	14	NG	[44]
				NaOH		1		
				Sodium methoxide		8		
Chlorella sp.	12			H_2SO_4		92		
				NaOH		79		
				Sodium methoxide		90		
***Chlorella vulgaris* ESP-31**	13.52 – 63.17	40 - 45	2880	*Burkholderia* lipase	1:66-426	91.15 – 95.74	0.1268 – 0.5821	[45]
Chlorella pyrenoidosa	NG	60	30	H_2SO_4	NG	94	0.0834	[46]
Nannochloropsis oceanic	24.8	60	30	H_2SO_4	NG	100	0.2963	[38]

[a]Not given

As was mentioned previously, growing microalgae under stress conditions can increase lipid content, but overall lipid productivity may even decline due to the reduction in biomass productivity [13]. However, the quality of algal lipids that are produced under stress conditions may be improved. Moreover, dewatering, drying, lipid extraction, and solvent recovery are all energy intensive steps [47]. Despite other more cost-effective extraction methods (including using pulsed electric field and high pressure homogenising) having been developed, further research is required to develop a scalable, efficient wet extraction method. Another issue with the use of algal lipids is the high free fatty acid content that occurs during the algal growth phase, which when transesterified in presence of a base catalyst can result in soap production. Soap production can be avoided by using an acid catalyst, however, this requires more time and uses more excess alcohol to achieve the same efficiency [40].

Since the main goal of WWT HRAP is wastewater treatment and there is little opportunity within this context to substantially alter the lipid content of the algal biomass (typically <30 %), it is unlikely, given that only 1/3-1/4 of total algal lipid could be converted, that more than 10 wt % of the algal biomass is converted to biodiesel (Table 1.1).

1.5.2 Bio-methane production through anaerobic digestion of the whole biomass

Anaerobic digestion (AD) is the anaerobic bacterial conversion of organic material directly into biogas at temperatures ranging between ambient to 55 °C [48]. It is commercially developed and can be used to treat biomass with a high moisture content (90–99%) [40]. Anaerobic digestion occurs in three consecutive stages of hydrolysis, fermentation and methanogenesis [40]. In the hydrolysis step complex polymers are broken down into soluble sugars. Then, fermentative bacteria convert the sugars into alcohols, acetic acid, volatile fatty acids, and a gas containing H_2 and CO_2 [40]. Subsequently, this mixture is metabolised into methane (60-70%) and CO_2 (30-40%) through methanogenesis (Table 1.2). The hydrolysis step is often the rate limiting step of AD [49, 50].

Harvested biomass, which contains more than 97% moisture can be digested to biogas with an average yield ranging from 0.20 to 0.4 m^3 CH_4 /kg biomass (Table 1.2) which is enough to generate 1-1.25 kWh electricity [6, 49].

Theoretically the yield of methane from the different components of algal biomass is highest for lipid, followed by protein and then carbohydrate [50]. This means that more biogas is could be produced from biomass with a higher lipid content. However, lipid

hydrolysis takes much longer (3.2 days) than protein hydrolysis (0.43 day) and carbohydrate hydrolysis (0.18 day) [49, 50].

AD of algal biomass is affected by several factors including biomass biochemical composition, HRT, loading rate, pH, temperature, substrate to inoculum ratio, and co-digestion [49-51]. Typically, AD is conducted in heated mixed digesters with a HRT of between 14 and 28 days and a loading rate of between 2-10 kg VS/m^3. (Table 1.2) [49-51]. An important factor affecting the CH_4 content of biogas is the liquor pH, which controls the speciation of the carbonate and ammonia equilibria. If the pH goes up (> 8.5), more free ammonia is formed which is toxic. However, CO_2 from the biogas will dissolve in the liquor and decrease the pH, which increases the biogas CH_4 content [50].

The main issues with AD are the low biodegradability of the microalgae cell wall and potentially the low C:N ratio of microalgal biomass (5:1-10:1, depending on microalgal cell protein content) which may limit methane production. A low C:N ratio releases more ammonia into the digester liquor which can be toxic to the bacteria and inhibit methanogen activity unless they are adapted. This results in the accumulation of more fatty acids (as an intermediate component).

The low biodegradability of microalgae can be solved to some extent by pre-treatment by various methods. Chen and Oswald [52] found that pre-treating algal biomass at 100 °C for 8 h increased biogas production yield by 33% for digestion at 38 °C over 28 days. Alternatively, adding cellulosic materials such as agriculture wastes, wastepaper (C:N from 170:1 to 1000:1) or sewage sludge (C:N from 6:1 to 16:1) may improve digestion performance by increasing cellulase activity [21, 51]. For instance, Yen and Brune [51] showed that the optimum C:N ratio is in the range of 20:1-25:1 and increased the methane yield by 50%.

Table 1.2. Example of published results on biogas production from algal biomass

Species	Temperature (°C)	HRT (days)	Process mode	Pretreatment	Substrate: Inoculum ratio	Loading rate (g VS/ L/d)	Yield (L CH_4 g^{-1} VS)	Biogas production Rate (L L^{-1} d^{-1})	CH_4 in biogas (%)	Conversion efficiency (%)	Ref.
Scenedesmus spp. and Chlorella spp.	35	10	Semi-continuous	No	NG[a]	2 - 6	0.09 – 0.14	0.18 -0.818	68 -72	NG	[51]
50% (Scenedesmus spp. and Chlorella spp.) + 50% waste paper						4	0.29	1.17	0.61		
Scenedesmus obliquus	33	30	Batch	No	0.25	2	0.21	NG	NG	NG	[48]
	33	2.2	Continuous			2.7	NG	0.4	74.3	26 – 31	
	54		Continuous			2.8	NG	0.6	77.1		
Phaeodactylum tricornutum	33	30	Batch		0.25	2	0.35	NG	NG	NG	
	33	2.2	Continuous			1.9	NG	0.8	75.1	50	
	54		Continuous			2	NG	0.8	78.6		
40%Chlamydomonas, 20% Scenedesmus, 40% of an unknown microalgae	35	NG	Batch	NG	0.5	10	0.06 – 0.32	NG	NG	60 – 70	[53]
				110, 140, 170 °C for 15 min, Ultrasound, Biological	1	3, 10, 20	0.25 – 0.39			40 – 60	
				NG	3	10	0.1 – 0.32			50 - 60	
Chlorella sp. (70%) + Scenedesmus sp. (30%)	37	23	Batch	No	1	10	0.336	NG	56 - 60	25 - 30	[54]
				50, 80, 120 °C for 30 min		10	0.351,0.384, 0.405				
				Ultrasound 130 W for 30, 90, 180 s		10	0.356,0.368, 0.385				
				Alkali pH 9, 11, 13		10	0.363,0.327, 0.213				
Mix culture	35	15	Continues	No	NG	1.0	0.13	0.12	68.5	NG	[21]
				Microwave 900 W for 3 min			0.17	0.16	69.3		
		20		No		0.75	0.17	0.14	68.1		
				Microwave 900 W for 3 min			0.27	0.20	68.5		
				120 °C for 2 h			0.27	0.6 – 1.25			

[a]Not given

1.5.3 Bioethanol production through fermentation of the carbohydrate fraction

Bioethanol can be produced through the fermentation of microalgae carbohydrate [55] at 30-40 °C over 24-96 h (Table 1.3). Compared with transesterification, the yield of fermentation is likely to be higher since algal cells grown under normal conditions tend to contain more carbohydrate than lipid. Algae typically accumulate lipids under stress conditions, while carbohydrate is the first product of photosynthesis [1, 56].

The fermentation process involves several steps; firstly microalgal carbohydrates should be released from the cell by disrupting the cell wall which is not readily fermentable. Then the polysaccharides should be hydrolyzed to fermentable sugars by microorganisms such as *Saccharomyces cerevisiae* [57]. Then a suitable yeast is added to commence fermentation to ethanol (31.1 MJ/kg heating value). The bioethanol must be purified by distillation (which is an energy intensive step) and leaving a solid residue of all other cell components including the lipid and protein which can be used as a cattle-feed [40] or used for methane production through AD.

The overall yield of bioethanol from microalgae feedstock is in the range of 50-70 % of the fermentable organic matter (Table 1.3) [6, 40, 56]. Thus, based on the carbohydrate portion of growth phase algal biomass0.1-0.35 kg bioethanol/kg biomass is achievable (Table 1.3) [6]. The yield of bioethanol could be improved by increasing reaction time, temperature, pre-treatment of algal biomass and increasing the yeast concentration [2, 55]. Pretreatment could increase the fermentation efficiency by more than 30% compared to untreated microalgae [55]. Harun et al. [55] obtained up to 60% higher ethanol concentration from lipid extracted microalgal biomass compared with dried intact biomass.

Table 1.3. Example of published results on bioethanol production from carbohydrate fraction of microalgae

Species	**Carbohydrate content** (%)	**Pre-treatment**	**Reaction time** (h)	**temperature** (°C)	**Yeast**	**Yield** (g bioethanol/ g biomass)	**Ref.**
Chlorococum sp.	NG[a]	No	60	30	*Saccharomyces bayanus*	0.23	[55]
				40		0.37	
		Supercritical Lipid extraction		30		0.65	
				40		0.37	
Chlorococcum humicola	32.52	Cell lysis (by 3-10% H_2SO_4 at 100 – 200 °C for 5 to 60 min)	50	30	*Saccharomyces cerevisiae*	0.0267 –0.52	[58]
Scenedesmus obliquus	29	H2SO4 2 N for 30 min at 120 °C	NG	30	*Kluyveromyces marxianus IGC 2671*	0.023	[59]
Chlorella vulgaris FSP-E	50.39	Cellulases + Amylases	NG	30	*Zymomonas mobilis ATCC*	0.178 –0. 214	[57]

		H_2SO_4			*29191*	0.233	
Spirulina platensis	58	0.5N H_2SO_4	24	30	*Saccharomyces cerevisiae MV 92081*	0.16	[60]
		0.5 N HNO_3				0.16	
		0.5 N HCl				0.13	
Chlorella vulgaris	55	Cellulases + amylases + endoglucanases	26	33	*Saccharomyces cerevisiae*	0.167	[61]

[a]Not given

1.5.4 Bio-crude oil production by hydrothermal liquefaction (HTL) of wet biomass

Hydrothermal liquefaction (HTL) is a process to convert wet algal biomass (75-98% moisture) into bio-crude oil at sub-critical temperatures (200-350 ^oC), high pressure (5-20 MPa) and in presence or absence of a catalyst for 5-120 minutes (Table 1.4) [47, 62-64]. In HTL, biomass is broken down in hot compressed water to shorter carbon chains that have a higher energy density [40, 63]. The product of microalgal liquefaction is a heavy oil or tarry material, called bio-crude oil, which contains C17-C18 n-alkanes and polyaromatic hydrocarbons [47, 62]. The bio-crude oil yield of HTL is in the range of 30–50 wt% and it has a heating value of between 30 and 40 kJ/g (Table 1.4). The by-products of HTL are a mixture of gases (CO_2, H_2, CH_4, C_2H_4, C_2H_6, N_2) with 20% yield, the residual solids with a yield of less than 10 wt%, and the aqueous phase (20-30 wt% yield) which contains a high portion of nutrients and could be recycled back to the WWT HRAP [63]. The residual solids are composed of >20% carbon and have an energy content in range of 8-10 kJ/g [65].

In most cases, the yield of bio-crude oil is 5–15% higher than the lipid content of the microalgae (Table 1.4). This means that bio-crude oil is also derived from the algal carbohydrate and protein, however, the yield of HTL is affected by microalgal composition, being higher from lipid than protein and lower for carbohydrates [47, 62].

The yield of HTL is also affected by reaction time, temperature, water content, heating rate, catalyst type, and separation method [63]. Biller and Ross (2011) liquefied *Chlorella vulgaris*, *Nannochloropsis oculata*, *Porphyridium cruentum*, and *Spirullina* sp.at 350 ^0C for 1 hr in presence of Na_2CO_3, HCOOH and water. Both *C.vulgaris* and *N.oculata* had the highest bio-crude oil yield because they had the highest lipid content. Brown et al. [47] found that HTL of *Nannochloropsis* sp. gave the highest bio-crude oil yield (43 wt%) at 350 °C which was higher than initial microalgal lipid content (28 wt%). Yu et al. [64] found that HTL of *Chlorella pyrenoidosa*at 20% solids over a temperature range of 240-300 ^oC for 30-120 minutes gave an average bio-crude oil yield of 34.4 wt%. Low conversion temperature produced a bio-crude oil that was like asphalt whereas a high conversion temperature produced a pourable bio-crude oil. The yield of bio-oil can be increased in the presence of

catalysts. For example, bio-crude oil production from biomass with a high carbohydrate content, can be improved by adding the catalyst Na_2CO_3 [62].

An issue with algal HTL bio-crude oil quality is the high nitrogen and oxygen content which is derived from protein and makes the oil unstable unless upgraded. The nitrogen content can reach 5 wt% of the oil and increases with conversion temperature. When the oil is combusted this nitrogen will be converted to NO_x which can react with rainwater and form acidic rain [62, 63].

Table 1.4. Example of published results on bio-crude oil production from algal biomass

Species	**Temperature** (°C)	**Holding time** (min)	**Catalyst**	**Water content** (%)	**Algal composition** (%)			**Major bio-oil components**	**N:C ratio in product**	**Yield** (g Bio-oil/ g biomass)	**Heating value** (kJ/g)/**%Energy recovery**	**Ref.**
					L	P	C					
Nannochloropsis sp.	350	60	5 - 80% wt Pd/C	79	28	52	12	Phenol, long-chain fatty acids, alkanes and alkenes	0.049	0.43	39	[47]
Chlorella vulgaris	350	60	No	90	25	55	9	Phenols, Indole, Heptadecane	0.083	0.38	54.2	[62]
			1 M Na_2CO_3						0.066	0.28	44.2	
			1 M HCOOH						0.074	0.28	31.7	
Nannochloropsisocculata			No		32	57	8		0.060	0.36	66.1	
			1 M Na_2CO_3						0.054	0.26	50.0	
			1 M HCOOH						0.057	0.28	41.1	
Porphyridium cruentum			No		8	43	40		0.074	0.20	51.6	
			1 M Na_2CO_3						0.069	0.28	42.1	
			1 M HCOOH						0.078	0.18	35.2	
Spirulina			No		5	65	20		0.095	0.25	50.7	
			1 M Na_2CO_3						0.061	0.15	21.0	
			1 M HCOOH						0.078	0.18	19.3	
Chlorella pyrenoidosa	280	120	No	80	0.1	71.3	NG[a]	NG	0.087	0.359	65.4	[64]
Scenedesmus obliquus	250	5	No	93 - 95	16.8	28	NG	NG	0.06	0.176	33.8	[66]
	375								0.07	0.506	35.6	
Phaeodactylum tricornutum	250				21.9	37.5			0.06	0.408	30.3	
	375								0.07	0.543	35.9	
Nannochloropsis gaditana	250				25.1	43.9			0.04	0.344	35.4	
	375								0.06	0.543	37.2	
Scenedesmus almeriensis	250				21.8	51.7			0.05	0.357	35.3	
	375								0.07	0.581	36.2	
Tetraselmis suecica	250				19.5	43.6			0.07	0.294	29.3	
	375								0.07	0.456	36.0	
Chlorella vulgaris	250				20.4	41.2			0.07	0.330	34.4	
	375								0.08	0.553	35.0	
Porphyridium purpureum	250				12.1	45.6			0.06	0.247	32.7	
	375								0.08	0.471	35.0	
Dunaliella tertiolecta	250				23.4	50.8			0.06	0.448	34.6	
	375								0.07	0.553	34.9	
Mix-culture	350	60	No	94	14	NG	NG	aliphatics, phenols, fatty acids, ketones, indoles	0.056	0.445	39	[65]
Nannochloropsis oceanica	300	30	No	80	24.8	19.1	22.7	fatty acids, amides, pyrroles, indoles	0.07	0.40	36.35	[38]

[a] Not given

1.5.5 Bio-oil production through pyrolysis of dry biomass

Pyrolysis is the thermal cracking of dry biomass in the absence of oxygen at around 500 ^{o}C for about 30-120 minutes to biofuels including: bio-oil, gases and biochar (Table 1.5) [63]. This process can be divided into fast or slow pyrolysis but both involve three steps: (i) dehydration (vaporization of intracellular water content at 80 °C to 190 °C); (ii) volatilization (volatile components are volatized at 190 °C to 600 °C and after condensation, form stable liquid and gases products); and (iii) decomposition (which occurs at temperatures equal or higher than 600 °C and result in the formation of solid biochar) [63, 67].

Pyrolysus bio-oil is usually composed of aliphatic hydrocarbons, aromatic hydrocarbons, phenols, long-chain fatty acids and acids [67, 68]. The exact composition and conversion yield of bio-oil varies with several parameters including: microalgal biochemical composition, pre-treatment, conversion temperature, heating rate, residence time, catalyst type and condensation process [63, 67, 68]. The overall conversion efficiency and energy content of the bio-oil are 20-45 wt% and 25-35 kJ/kg respectively (Table 1.5) [2, 63, 67, 68]. Babich et al. [68] showed that the 60 wt% of *Chlorella* (containing 34% protein, 7% lipid and 15.5% carbohydrate) decreased via volatilization during pyrolytic conversion at temperatures ranging between 300-450 ^{o}C and 20-50 % of the volatized mass was converted to bio-oil. Moreover, pyrolysis with a Na_2CO_3 catalyst at 450 ^{o}C decreased the bio-oil yield and increased the gas yield from 15% to 30%. Bio-oil produced via catalytic pyrolysis was less acidic (pH 3.7) compared to that produced by non-catalytic pyrolysis (pH 2.5), making it more easy to transport and store [68]. Porphy and Farid [67] pyrolysed *Nannochloropsis* biomass (16 wt% lipid) at 200 ^{o}C, 300 ^{o}C and 400 ^{o}C before and after lipid extraction, giving bio-oil yields of 10-30% and 5-20%, respectively. Bio-oil yield at 300 ^{o}C was higher than that at 200 ^{o}C (where volatilization was not complete) and at 400 ^{o}C (where most of the volatile components were decomposed to gas). The composition of the bio-oil from the lipid extracted biomass residue included: acetone, methyl ethyl ketone and aromatics such as pyrrole, pyridine compounds [67].

The main issues with pyrolysis and subsequently the use of bio-oil are the high energy demand for biomass drying, poor thermal stability of bio-oil and its corrosiveness (acidity) which still need to be overcome [68]. Improvement in the stability of bio-oil, decrease of acidity and increase of the energy density achieve by decrease of oxygen content and bio-oil may be upgraded by hydrogenation of alkalis at 200-300 ^{o}C and 150-200 bar for 1 hour followed by catalytic cracking of the oil using Ru/C as a catalyst [68].

Table 1.5. Example of published results on pyrolysis bio-oil production from algal biomass

Species	Heating rate (°C/min)	Experimental temperature (°C)	Reaction time (min)	Catalyst	Lipid content (%)	Yield (wt%)	Heating value (kJ/g)	Bio-gas content (%)	Heating value (kJ/g)	Bio-char content (%)	Heating value (kJ/g)	Ref.
Chllorella protothecoides	Fast pyrolysis	500	NG[a]	No	14.57	17.5	30	30	NG	52.5	NG	[69]
Microcystis aeruginosa					12.5	23.7	29	55		21.3		
Tetraselmis chui	10	500	NG	No	NG	43	NG	20	3.4	37	NG	[70]
Chlorella like						41		22	1.8	37		
Chlorella vulgaris						41		25	4.8	34		
Chaetocerous muelleri						53		14	1.2	33		
Dunaliella tertiolecta						63		13	2.4	24		
Synechococcus						44		18	1.4	38		
chlorella	10	450	30	No	NG	57	27	15	NG	28	NG	[68]
				Na_2CO_3		42	33	30		28		
Nannochloropsis sp.	30	300 - 350	30	No	16	30	32	20	5	50	7	[67]
Scenedesmus sp.	Fast pyrolysis	480	120	No	11.5	55	18.4	NG	NG	14.6	4.6	[71]

[a] Not given

1.5.6 Gasification of algal biomass

Gasification is the thermochemical partial oxidation of hydrocarbons in the biomass at high temperature (800-1000 °C) to a combustible gas mixture (typically containing H_2, CH_4, CO_2, and C_2H_4) [63]. Gasification is energy intensive and the gas mixturehas a relatively low calorific value (only 4–6 MJ/ m^3), although the co-product of gasification, bio-char, has a heat value similar to black coal (30 MJ/kg) [72]. The low heating value of the gas mixture does make it suitable to be either burned directly, used as a gas engine fuel, or used as a feedstock (syngas) for the production of chemicals (e.g. methanol) [2]. Hirano et al. [73] found that a slight positive energy balance (1.1:1) was achievable by gasification of Spirulina, however, this is too low to be economically profitable.

1.6 Which conversion route is more beneficial?

WWT HRAP can produce algal/bacterial biomass with an energy value of 800-1400 GJ/ha/year. However, the cost of the energy required for algal biomass production (~4 MJ/kg) is covered by the wastewater treatment function of the pond. Moreover, concentrating the algal biomass from 0.02% in the WWT HRAP effluent to 2% can be done by gravity sedimentation in simple in-ground harvester. Therefore the only energy requirement for WWT HRAP biomass biofuel production is for the biofuel conversion process. Hence, the

main question is which algal biofuel conversion process has the largest positive energy balance?

Since there are three main steps during the conversion of biomass to biofuel (1) dewatering biomass to the required concentration, 2) pretreatment and operation at the required temperature/pressure, and 3) product upgrading) the energy consumed by each biofuel conversion process should be compared.

The required moisture content of feedstock for each conversion process is different. While wet biomass with only 2% solids is suitable for pond-based anaerobic digestion and fermentation, HTL requires biomass with at least 20% solids, and the biomass feedstock for transesterification, pyrolysis and gasification must be at least 90% solids. As the solid fraction increases, the energy demand for dewatering increases considerably. Recent studies confirmed that to concentrate biomass slurry from 2% (harvester outflow) to 30% by centrifugation, 1-2 MJ energy per kg biomass is needed [19, 74]. If it is assumed that dewatering from 30% to 90% occurs by heating and the heat capacity of algal slurry is 4.3 kJ/kg, the energy requirement would be 4.5 MJ/kg biomass [19]. Although the energy used in dewatering is considerable, it can be partly recovered using heat exchangers that use the heat of the steam to warm the new biomass.

For biogas production, the 2% solids outflow of the algal harvest pond is suitable for ambient temperature, unmixed pond-based digesters but further concentration to 5-8% solids is required for heated mixed digesters. Collet et al. [74] showed that 3 MJ/kg biomass, energy is needed to operate a heated mixed digester to recover 35% of the potential energy (0.3 m^3, 7 MJ). Similar energy yields can be achieved on an annual average basis in ambient temperature pond based digesters, although there may be substantial seasonal variation. Since biogas is a mixture of methane (~65%) and CO_2 (~35%), upgrading is required for use as a vehicle fuel or as a natural gas supplement, part of which can be done by scrubbing the biogas in the WWT HRAP [6].

HTL bio-crude oil production requires a biomass concentration of 20% (Table 1.4). Therefore, harvested biomass with 2% solids needs an additional dewatering step compared to AD. Concentration can be done by centrifugation of the algal slurry which uses 0.5-1 MJ/kg biomass to obtain a final biomass concentration of 15%. In comparison with the biodiesel production route, HTL avoids the need for high lipid biomass, the energy and capital cost of cell disruption, and solvent recovery, but it requires significant energy inputs

to establish the process conditions (350 ^{o}C, 20 MPa). For example, for processing of 6.7 kg biomass paste which contains 1 kg dry biomass (15% solid content), 5.7 kg water would be heated which means 9.5 MJ energy/kg dry biomass is required. If the yield of the HTL process is considered to be 40% of the biomass, 15.5 MJ/kg biomass or 75% of potential energy can be captured. As the nitrogen and oxygen content of HTL products are high, the products are unstable and further upgrading is required. During the upgrading process, oxygen is converted to CO_2 and ammonia is produced from nitrogen. Part of the energy required for the HTL process can be recovered by using the HTL reactor outflow to heat the inflow while the aqueous phase which contains the ammonia and other nutrients may be able to be recycled for use as fertiliser.

Biodiesel production, is limited firstly by the low lipid content of algal biomass and secondly by only one-third of the biomass lipid content being suitable for transesterification. Furthermore, the algal cells must be disrupted and the lipids extracted and purified to maximize conversion yield. Cell disruption and lipid extraction can be done by either wet or dry methods. Wet biomass (<20% solid content) can be disrupted by passing it through a pressure homogenizer which has an energy consumption of 0.7 MJ/kg biomass [75]. In contrast, dry extraction requires a solid content of >90% and the cells can then be ruptured by milling which has an energy consumption of 1.2 MJ/kg biomass [19]. Assuming that the lipid extraction procedure for dried ruptured algae is similar to that of dried disrupted soybean, this requires 0.76 MJ/kg to extract the lipids and recover solvent [19, 75]. Transesterification of the extracted lipids has an energy requirement of1-3 MJ/kg biomass. While only 20% of the total available energy (4 MJ/kg biomass) in the algal biomass can be converted to biodiesel [19].

Pyrolysis of dry biomass (>90% solids) at 550 ^{o}C has an energy consumption in the range of 1.1 - 2.5 MJ/kg biomass [19, 67]. Assuming, on average, 35% conversion efficiency and a 30 MJ/kg heat value of the oil phase, the yield of energy recovery is 52%. However, beneficial use of co-products of process, including the gas phase (30% of final products, 2.8 MJ/kg) and char phase (35% of final products, 6 MJ/kg) can increase the yield of energy recovery up to 67%.

No actual data has been reported in the literature on the energy consumption of bioethanol production from microalgae. Moncada et al. [61] simulated the energy requirement for fermentation of lipid extracted *Chlorella vulgaris* biomass with 132 g bioethanol (99.7%

purity) produced from 1 kg lipid extracted biomass requiring 5.3 MJ. However, the energy value of the bioethanol that was produced was only 4.1 MJ which equates to ~80% of input energy. This means that using the established fermentation process with ethanol distillation, algae bioethanol production is not even energetically viable. The required energy input could be reduced from 5.3 MJ to 4 MJ by replacing the distillation step with a molecular sieve column, but the fermentation process would still not be energetically viable.

The total energy input, total energy output and net energy ratio (NER), defined as the total energy input divided by the total energy output, are presented in Table 1.6. Although, the energy demand for pumping and product upgrading are not included and it is assumed that all energy requirements for cultivation are covered by the HRAP wastewater treatment function. Of the conversion pathways that were compared, AD had lowest NER, at only 35% of all potential energy (which is equal to 280-500 GJ/year/ha of biofuel energy. So, even AD, the simplest algal biofuel conversion pathway is far from optimal in terms of biofuel energy conversion. To improve biofuel energy conversion from WWT HRAP biomass, several technical limitations need to be solved and a combination of conversion pathways may need to be used. For example, biomass remaining from wet lipid extraction for biodiesel production can be used as a substrate for AD, since the disruption of algal cells before the lipid extraction step will also improve the digestability of the biomass and consequently the yield of biogas. Alternatively, if dry extraction is used, the biomass residue could then be used as a substrate for bio-oil production by pyrolysis.

Table 1.6. Comparison of different conversion routes

Process		Total energy input (MJ/kg biomass)	Total energy output (MJ/kg biomass)	NER	Yield of recovery (%)
Biogas production		3	7	0.42	35
Biodiesel production	Wet route	3.4	4	0.85	20
	Dry route	8	4	2	20
Bioethanol production		5.3	4.1	1.29	20
Bio-crude oil production		10.5	15.5	0.67	75
Pyrolytic bio-oil production		8	10.5	0.76	50

1.7 Summary and Research needs

To produce sustainable and commercially viable algal biofuel, several major challenges in the pathway of microalgal biofuel production, from cultivation to conversion, need to be solved. Of all the scenarios to produce low-cost algal biomass, WWT HRAPs seem to be the most promising since they combine cost-effective wastewater treatment with algal biomass

production (a by-product) at no additional cost as nutrients are assimilated from the wastewater.

The potential amount of low-cost energy produced in WWT HRAP is a function of the productivity, chemical composition, and harvestability of the biomass. However, all these parameters are limited by environmental, operational and biological factors. Studies have shown that low-cost energy production in WWT HRAP can be improved by optimizing HRT (in terms of increasing biomass energy yield), CO_2 addition (in terms of increasing biomass productivity and improving the lipid profile), biomass recycling (in terms of increasing biomass productivity and promoting desired species), and controlling grazers (in terms of minimizing productivity losses and increasing harvestable biomass productivity). Therefore, further research of WWT HRAP is required to address the following questions: (i) how can invertebrates be controlled in large-scale WWT HRAP to prevent productivity losses and promote the dominance of settleable algal species?; (ii) which of the typical WWT HRAP algal species are more beneficial for both wastewater treatment and low-cost energy production (specify the desired species)?; and (iii) which of the HRAP operation factors (extending HRT or CO_2 addition) is the most practical option to improve WWT HRAP performance at large-scale? Answering these questions will provide more understanding of how to produce sustainable low-cost biomass for biofuel production.

Moreover, various conversion processes may be used to convert WWT HRAP biomass to biofuel. AD, HTL, pyrolysis and gasification are based on conversion of whole biomass while biodiesel and bioethanol only convert the lipid and carbohydrate fractions through transesterification and fermentation respectively. Although the efficiency of conversion methods is affected by several parameters, biomass biochemical composition is more important for selection of the most appropriate conversion process. Since, WWT HRAP biomass contains microalgae and bacteria, more research is required to determine the chemical composition and lipid profile of the biomass to help select the most beneficial conversion and upgrading processes.

The HTL process, compared with other conversion pathways has the highest conversion efficiency and can recover >70% of energy available in WWT HRAP harvested biomass. However the energy demand of HTL is high and bio-oil upgrading is required. Thus, further research is required to improve recovery yield, reduce HTL energy demand by operating at lower temperature and develop upgrading processes. To improve energy recovery from

WWT HRAP biomass further study is needed on the possibility of using the combination of conversion processes and finding the best integrated option.

1.8 Conclusions

WWT HRAP bring algal biofuel production closer to economic reality by producing and harvesting biomass at little or no additional cost to the WWT plant. Of the range of operational and biological factors, CO_2 addition shows great promise on enhancement quantity and quality of biomass produced in WWT HRAP. It not only increases biomass and lipid productivity, but also improves lipid quality. The current limitations on biodiesel production from microalgae, together with their low lipid content, make energy recovery from the whole algal biomass most attractive. However, a combination of conversion processes will be needed to maximise total energy recovery.

CHAPTER 2

Variation of biomass energy yield in wastewater treatment high rate algal ponds

This chapter is based on the following publication:

Mehrabadi, A., Farid, M. M., Craggs, R., 2016. Variation of biomass energy yield in wastewater treatment high rate algal ponds. *Algal Research*, 15, 143-151.

Chapter preface

This chapter investigates the biomass energy yield potential of WWT HRAP (calculated by multiplying biomass productivity and biomass energy content) by weekly monitoring of two parallel identical pilot-scale HRAPs. We address experimentally, for the first time, the influence of algal species dynamics, zooplankton grazing, environmental conditions, biomass chemical composition and biomass algal proportion on overall biomass energy yield. The algal species composition and algal proportion in the HRAP effluent varied with season and grazing pressure. The highest biomass lipid content was achieved when effluent ammonia concentration was lowest. Biomass productivity depended on season and zooplankton grazing pressure and biomass energy content increased algal proportion and lipid content of the HRAP biomass. The average biomass energy yield in the HRAPs was 113.3 $kJ/m^2/d$ and it was significantly higher in summer compared to winter. Results suggest improving algal proportion and productivity would promote biomass energy yield in WWT HRAP by enhancing biomass energy content and productivity concurrently.

2.1 Introduction

There is renewed interest worldwide in replacing fossil transportation fuels with algal-based biofuels. Microalgal biomass was first suggested as a feedstock for biofuel production in the 1960s [14]. It can be converted to various kinds of biofuel including: biogas by anaerobic digestion of the whole biomass, bio-oil through thermochemical conversion of the whole biomass, biodiesel by transesterification of the lipid fraction, and bioethanol via fermentation of the carbohydrate fraction [2, 6, 76].

Although intensive research has been conducted to try to make algal-based biofuel production an economic reality, there are still many obstacles across the entire process (from cultivation to conversion) [3, 12, 77-80]. The major costs of algae cultivation for biofuel production are: capital cost of algal production system, fertilizer and chemicals, and pumping of water for cultivation; biomass harvest and dewatering (which have high energy demands as algal species are small (<30 μm) and pond medium is >99% water); and algal biomass biofuel conversion pathways for which there are specific technological limitations [3, 11, 13, 15, 17, 76]. Even low-cost algal production system (open raceway ponds) are not yet economical for biofuel production alone and combining algal biofuel production with wastewater treatment is considered to be the most promising option.

Wastewater treatment high rate algal ponds (WWT HRAP), as part of an advanced treatment pond system, offer a niche opportunity for low-cost algal biomass production since the algal cultivation and harvest costs are included in the wastewater treatment operation. In particular, addition of nutrient fertiliser is not required for microalgal cultivation on human and animal wastewater, and the biomass (algal/bacterial flocs) is relatively easily harvested and may be concentrated to 2 wt% solids by simple gravity sedimentation [6]. Therefore, WWT HRAP could make community-level low-cost algal biofuel production feasible by producing and harvesting biomass as a by-product of the WWT plant.

Efficient wastewater treatment with nutrient recovery and low-cost biofuel production both rely on maximizing algal productivity, which in the context of biofuel production means maximizing energy production. Therefore, any practical strategies that improve the algal yield from wastewater HRAPs should also benefit energy production. The biomass energy yield in WWT HRAP is a function of the biomass productivity and its energy content. Both are affected by the dominant algal species, the proportion of algae in the biomass and chemical composition of the biomass. However, these factors are limited by environmental (light and temperature), operational (CO_2 concentration, nutrient concentration, cultivation mode, hydraulic retention time, mixing, and algal recycling) and biological conditions (algal species contamination and grazer occurrence) [81]. Therefore, to produce sustainable low-cost energy in the form of biomass in WWT HRAP, a greater understanding of the factors which affect productivity and energy content of biomass are essential.

Several studies have reported the biomass productivity potential of the WWT HRAP [12, 82, 83] and suggested a number of strategies such as CO_2 addition, biomass recycling and controlling zooplankton grazers to promote HRAP biomass productivity and culturing under nutrient-limiting conditions that might improve microalgae energy content [13, 23, 77, 84, 85]. However, these have either been short-term studies in outdoor pilot-scale systems or been carried out under controlled laboratory conditions with little focus on biomass energy yield from WWT HRAP and how it is influenced by different factors. In this paper, the biomass productivity and energy content of two identical WWT HRAPs operated in parallel was measured over a whole year. The variation of biomass energy yield potential of WWT HRAP was related to factors including: biomass chemical composition; algal proportion; algal species dynamics; environmental conditions, and; zooplankton grazing.

2.2 Materials and methods

2.2.1 Environmental variables and HRAPs operational parameters

The current study involved operating and sampling two identical pilot-scale WWT HRAPs in parallel (West (WHRAP) and East (EHRAP)) to assess HRAP biomass energy yield potential. The ponds were located at the Ruakura Research Centre, Hamilton, New Zealand (37º47′S, 175º19′E). Each HRAP was a single-loop raceway with sloped embankments, separated by a central baffle with depth of 30 cm, surface area of 31.8 m^2 and total volume of 8 m^3. The pond water was circulated with a mean surface velocity of 0.15 m/s using a paddlewheel. The HRAPs received 0.5-1 m^3/d of primary settled domestic wastewater at hourly intervals that was pumped from the Ruakura sewer. Although technically WWT HRAP is used for tertiary-level treatment and receives secondary treated wastewater, in this study primary settled wastewater was used which had the similar BOD and COD concentration to secondary treatment. The pond hydraulic retention time (HRT) was varied with season from 8 days in winter (Jun.-Aug.) by diluting the influent with tap water (to simulate recycling of treated effluent). During spring (Sep.-Nov.) and autumn (Mar.-May) the HRT was maintained at 6-6.5 days while in summer (Dec.-Feb.) the HRT was maintained at 5 days.

To avoid free ammonia inhibition and carbon limitation, the maximum pH of the HRAPs was maintained below 8 by CO_2 addition. CO_2 was automatically injected into the pond water when the pH exceeded 8 and stopped when pH was less than 7.8. The HRAP effluent flowed by gravity from the pond bottom into 250 L settling tanks, from which the settled biomass was harvested daily using a peristaltic pump (Masterflex, Cole-Parmer, HV-07523-60). The HRAPs were run with no control of the dominant algal species or of the zooplankton population. Further details of the HRAP construction and operation were previously described in Park et al. [11] and Park and Craggs [84].

The pH, dissolved oxygen (DO) and temperature of the HRAP water were continually measured using a DataSond 4a (Hydrolab, HACH Environment, USA). The data were logged at 15 min intervals using a data logger (CR10X, Campbell Scientific Inc, UT, USA) and downloaded weekly. Over the course of study, daily climate data (solar radiation, evaporation and rainfall) were downloaded from NIWA's National Climate Database (http://cliflo-niwa.niwa.co.nz/).

2.2.2 Measurement of water quality

Concentrations of water quality variables in the pond influent were periodically analyzed according to standard methods [86].The pond water ammoniacal-N concentration was sampled weekly as this was the main form of nitrogen in the primary settled sewage [30]. Since the N:P ratio of the domestic wastewater is usually 6:1, which is lower than N:P ratio of the algal biomass (often 16:1) [12, 28], the effect of phosphorus was not considered in this study. Pond influent and water samples were filtered through Whatman GF/F filters (with 0.7 µm pore size) and the concentration of ammonium (NH_4^+-N) was determined colorimetrically [86] using a spectrophotometer (HACH RD2008, Germany).

2.2.3 Algae assessment and relative abundance

HRAP algal species and their relative abundance were determined weekly during the experimental period using the methodology developed by Park et al. [11]. A well-mixed sub-sample of pond water was settled in an Utermöhl chamber (diameter: 25 mm -volume: 10 ml). Three random pictures were taken using a microscope Leica DM 2500, equipped with a Leica DFC 420 digital camera (Leica Microsystem, Switzerland). The procedure was repeated three times and a total of nine pictures were taken for each HRAP. The numbers of cells of each algal species were counted and multiplied by the mean cell biovolume to obtain their relative abundance. The mean biovolume (μm^3) of each algal species were assessed according to Vadrucci et al. [87] equations by measuring the size of 150 cells/colonies using the freeware software "ImageJ" V 1.43u.

2.2.4 Measurement of Chlorophyll-a

The biomass chl-a content (which can be used as an indicator of the proportion of algae in the HRAP biomass) was determined spectrophotometrically using the monochromatic equations for methanol of Ritchie [88]. A known volume of pond water was filtered through a 25 mm Whatman GF/F filter (with 0.7 µm pore size), and the filter was placed in a centrifuge tube with 10 ml of pure methanol and placed in a water bath and boiled at 65 ºC for 5 min. The tubes were cooled and then refrigerated at 4 ºC in the dark for 12 hours (h) for full chlorophyll extraction. The tubes were then centrifuged at 2000 relative centrifugal force (rcf) for 15 minute (min) and the absorbance of the supernatant was measured using a Shimadzu UV 1601 spectrophotometer.

2.2.5 Measurement of biomass lipid, carbohydrate and protein composition

Samples of HRAP effluent were taken at weekly intervals and the biomass concentrated by centrifugation (2000 rcf, 10 min). The biomass was frozen until the lipid, carbohydrate and protein content was analyzed.

Total lipids were extracted based on a modified procedure adopted from Bligh and Dyer method [89] and measured gravimetrically. A 20-30 mg sample of the centrifuged frozen biomass was placed in a centrifuge tube with a 20 ml mixture of distilled water, methanol and chloroform (1:2:1). The centrifuge tube was placed horizontally on a shaking table overnight (~ 6-cm oscillation at ~2 cycles per second). An additional 5 mL of chloroform and 4 mL of distilled water were then added to the tube to give a final ratio of water: methanol: chloroform of 0.9:1:1. The tube was then vortex mixed for 30 seconds and centrifuged at 3500 rcf for 10 minutes. Most of lipids are soluble in the chloroform and form a dense layer at the bottom of the centrifuge tube. The remaining cell debris creates the middle layer, while the methanol and water create the top layer. The lipid-chloroform layer was removed using a pipette and then filtered through a GFF filter and transferred into a pre-weighed glass tube. A second and occasionally third re-extraction was conducted by adding another 5 mL of chloroform to the remaining biomass in the centrifuge tube, vortex mixing for 30 s and then centrifuging at 3500 rcf for 5 min. The lipid-chloroform layer was removed from the tube, filtered and then combined with the first extract in the glass tube which was subsequently placed into an oven (at 60 ºC overnight) and sparged with nitrogen gas to allow the chloroform to evaporate and drive off any water. The tubes were cooled down under a nitrogen stream in a desiccator and then weighed. The lipid weight fraction was obtained by dividing lipid weight by the biomass weight. The pond inflow was analysed occasionally and was found to have no fat content to interfere with the results.

Total carbohydrate was measured using a modified procedure of the phenol sulphuric acid method [90]. A 0.5 mg sample of centrifuged frozen biomass was placed into a glass tube with 2 ml of distilled water, followed by 1 ml of 5% aqueous phenol (wt/vol) and 5 ml of concentrated sulphuric acid. After vortex mixing, a golden brown color (resulting from acid digestion of the biomass) developed and samples were cooled down for 30 min at room temperature. Subsequently, the absorbance was read at 485 nm and the mass of carbohydrate was determined by interpolation from a standard curve for D-glucose.

The protein fraction was measured by a procedure adapted from Lowry et al. [91] by Moheimani et al. [90]. A 0.5 mg sample of centrifuged frozen biomass was placed in a glass centrifuge tube along with 5 ml of Biuret reagent, vortex mixed for 30 s, followed by heating at 100 ºC for 1h in water bath. Subsequently, the tube was removed and immediately 0.5 ml Folin-phenol reagent was added to the tube while being vortex mixed. The tube was then placed in a 10-15 ºC water bath for 20 min to cool down before centrifuging at 1400 rcf for 10 minutes and measuring the absorbance of the blue supernatant at 660 nm for comparison with protein standards.

2.2.6 Measurement of biomass productivity

The biomass productivity was calculated based on the volatile suspended solids (VSS) concentration following Equation (2-1) [12]:

$$P = \frac{X \times Q_c}{A} \qquad (2-1)$$

$$Q_c = Q_{inf} + ((rainfall - evaporation) \times surface\ area)$$

where P is the areal biomass productivity (g VSS/m^2/d^{-1}), X is the WWT HRAP biomass concentration (g VSS/m^3) , A is WWT HRAP surface area (m^2), Q_{inf} is WWT HRAP daily inflow ($m^3.d^{-1}$), and Q_c is daily outflow ($m^3.d^{-1}$) corrected for net evaporation. To measure VSS, a known volume of pond water was filtered onto a pre-rinsed, pre-combusted and pre-weighed Whatman GF/F filter (with 0.7 µm pore size), dried (at 80 ºC overnight), cooled in desiccator and weighed to determine the dry weight and used to calculate the total suspended solids (TSS) concentration. The dried filter was then ashed at 550 °C for 1 h in muffle furnace (F.E.KILN, RTC1000, Bartlett Instrument Company, UK) and cooled in a desiccator before weighing to determine the ash weight. The ash weight was subtracted from the dry weight and used to calculate the VSS concentration.

2.2.7 Measurement of biomass energy content

To assess the potential of the WWT HRAP for low-cost energy production, the calorific value (kJ/g) of the biomass produced in the pilot-scale HRAPs was measured using a bomb calorimeter (Parr 1341, Parr Instrument Company, Moline, IL 61265 USA) at weekly intervals over a year. Biomass samples from both HRAPs were collected, centrifuged and dried at 60 ºC overnight. A 20-50 mg sub-sample of the dried biomass was combusted in the bomb calorimeter in presence of pure oxygen. The biomass energy content was calculated by measuring the increase in temperature of the water surrounding the bomb calorimeter using a

digital thermometer (Parr 6775, Parr Instrument Company, USA). Results were compared to those measured when combusting a Benzoic acid standard**.**

2.2.8 Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) in Excel software (Excel, Microsoft office 2010).

2.3 Results and discussion

2.3.1 Environmental variables and HRAP operational conditions

Solar radiation was continuously monitored during the experimental period (Aug. 2013-Jul. 2014). Water temperature, DO and pH of the HRAPs were also monitored continually using DataSondew 4a (Hydrolab, HACH Environment, USA). Mean daily pond water temperature and solar radiation are shown in Fig. 2.1. In winter (Jun.-Jul. 2014), the average daily pond temperature and light intensity (ranging from 6.2 to 14 ºC, and 0.9 to 11.9 $MJ/m^2/d$, respectively) were at a minimum. While maximum mean daily values (24.8 ºC and 32.3 $MJ/m^2/d$, respectively) occurred in summer (Dec. 2014-Feb. 2015). The average daily summer-time water temperature and solar radiation were 21.7 ºC and 21.2 $MJ/m^2/d$, respectively. Both the mean water temperature and average solar radiation were lower in spring (Sep.-Nov.) (17.5 ºC and 17.5 $MJ/m^2/d$) and autumn (Mar.-May) (16.5 ºC and 12.8 $MJ/m^2/d$).

As CO_2 addition was used to control maximum daytime pH to below 8, there was little seasonal variation in pH which ranged between 6.1 (the minimum just before sunrise, due to algal and bacterial respiration) and 8 (the controlled daytime maximum). The maximum day-time DO due to algal photosynthesis in both HRAPs, reached as high as 300% saturation around midday during summer months, while the minimum DO (0-20% saturation, just before sunrise, due to algal and bacterial respiration) did not differ with season.

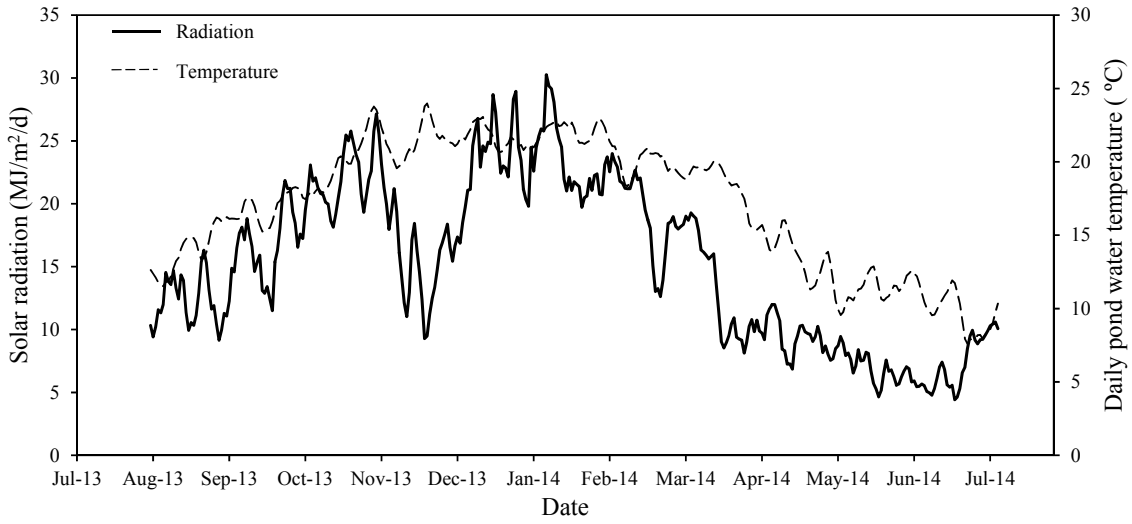


Figure 2.1. Average daily solar radiation and HRAP water temperature during monitoring period (Aug. 13- Jul. 14)

2.3.2 Water quality and nitrogen assimilation

The annual average concentrations of water variables in the primary settled sewage were 110 ± 15 g/m^3 of COD, 60 ± 10 g/m^3 of BOD_5, 25 ± 3 g/m^3 of TSS, 15.1 ± 7.1 g/d of NH_4^+-N, 1.0 ± 0.8 g/d of NO_3^--N and 2.4 ± 1.1 g/d of PO_4^{3-}-P. Since nitrogen significantly affects biomass productivity and energy content by altering algal chemical composition [13, 23, 25], both the wastewater influent and the pond water were sampled weekly for ammoniacal-N concentration. Nitrogen removal efficiencies and biomass organic nitrogen in both HRAPs are summarized in Table 2.1.

Table 2.1. Seasonal variation in nitrogen removal and biomass organic nitrogen (BON) during the pond monitoring

	Influent Load (g/day)	**Effluent Load** (g/day)	**Removal** (g/d)	**BON*** (g/day)	**Effluent** Load (g/day)	**Removal** (g/d)	**BON** (g/day)
		WHRAP			**EHRAP**		
Spring	13.4 ± 6.0	4.4 ± 2.1	9.0	8.6 ± 4.3	2.7± 1.9	10.7	10.4 ± 6.3
Summer	12.1 ± 7.0	1.7 ± 1.0	10.4	10.2 ± 3.7	1.2 ± 1	10.9	10.5 ± 4.8
Autumn	14.7 ± 6.5	2.8 ± 2.0	11.9	11.3 ± 2.1	4.4 ± 3.5	10.3	6.5 ± 3.6
Winter	20.3 ± 13.2	10.3 ± 1.0	10.0	7.2 ± 1.6	9.8 ± 2.8	9.5	7.5 ±1.3

*Nitrogen assimilated by biomass was calculated by multiplying biomass productivity and biomass nitrogen content. Assuming a 7 wt% nitrogen content of biomass [30].

Over the course of the study, on average 70% of the wastewater nitrogen was removed in both ponds, although this varied seasonally with 50% in winter and >80% in summer. As can be seen in Table 2.1. >75% of removed nitrogen was assimilated into the biomass.

2.3.3 Algal relative abundance

The relative abundance of algal species in the pilot-scale WWT HRAPs at monthly intervals over the year-long study is shown in Fig. 2.2. Microscopic monitoring showed considerable variation in the algal community during the monitoring period with the most abundant species including *Pediastrum* sp., *Micractinium* sp., *Ankistrodesmus* sp., *Monoraphydium* sp., *Desmodesmus* sp., *Coelastrum* sp. and *Mucidosphaerium* sp. Changes in dominant algal species were related to season and blooms of invertebrate grazers. For example, *Ankistrodesmus* sp. (a poorly-settleable unicellular species) was dominant in both pilot-scale HRAPs during winter and early spring 2013 comprising >75% of the algal community. In mid-spring (Oct. 2013) the *Ankistrodesmus* sp. population in both ponds declined rapidly during an invertebrate grazer bloom and was replaced by a combination of *Micractinium* sp. and *Mucidosphaerium* sp. In summer, when the solar radiation and pond water temperature were more favourable for both algae and invertebrates, the zooplankton population increased and as a result the poorly-settleable unicellular species and colonial species with no defence mechanism against grazing e.g. *Mucidosphaerium* sp. were eaten and their abundance decreased. Therefore, the relative abundance of *Mucidosphaerium* sp. in the WHRAP and *Micractinium* sp. in the EHRAP declined due to another invertebrate bloom in each pond and both HRAPs were populated with species such as *Pediastrum* sp., *Micractinium* sp. and *Coelastrum* sp. which due to their size or spines, are less able to be grazed by zooplankton [27, 77, 92, 93]. Later in autumn and early winter months, when solar radiation and pond water temperature decreased some zooplankton grazers were still alive in the EHRAP. It was populated by *Pediastrum* sp. While in the same period of time in WHRAP, as no invertebrates grew *Micractinium* sp which had higher growth rate was dominant. Later in winter, as the weather conditions were not favourable for zooplankton proliferation, both HRAPs were populated by fast growing poorly-settleable algal species like

Mucidosphaerium sp., *Micractinium* sp. and *Monoraphydium* sp.

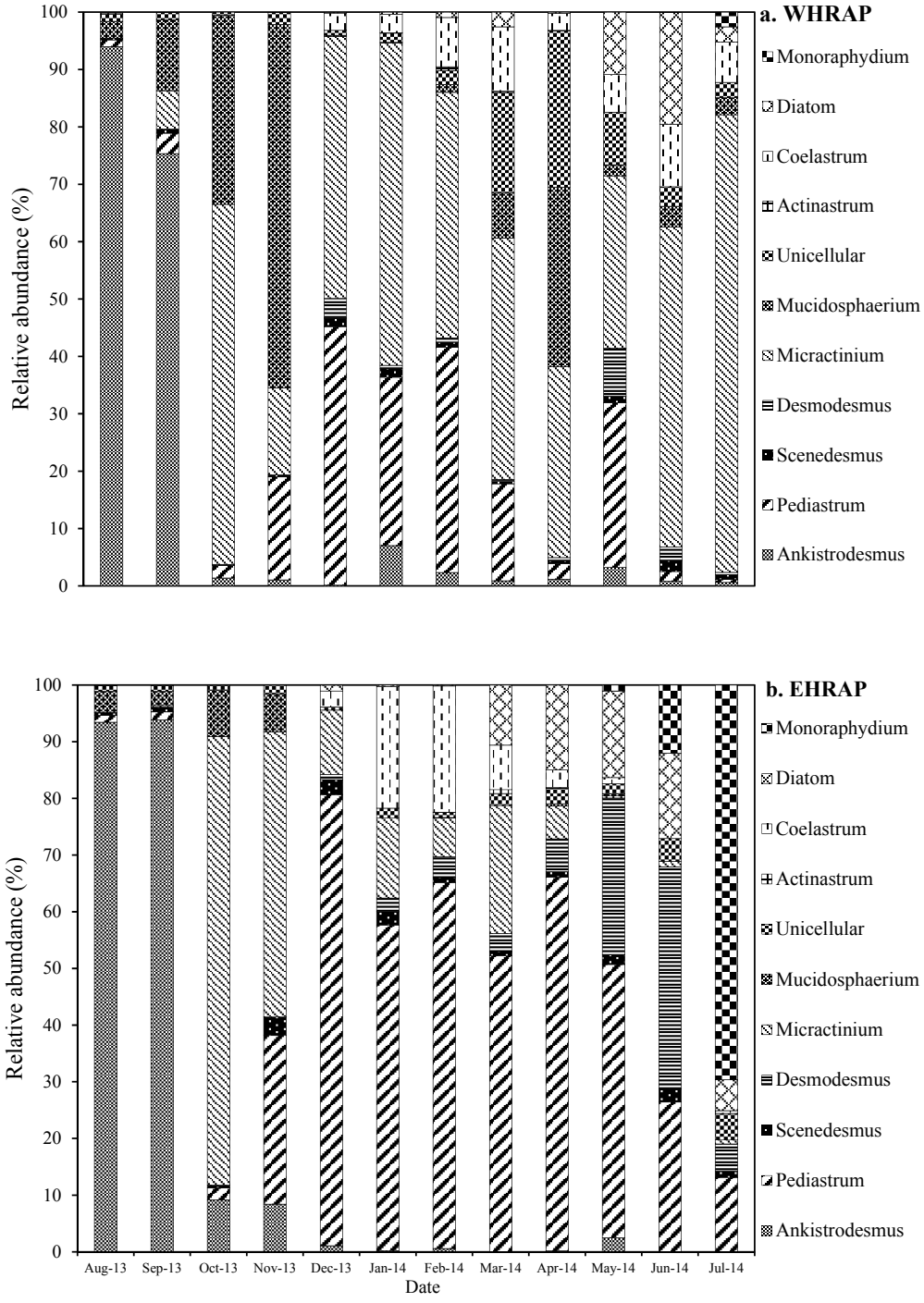


Figure 2.2. Relative abundance of algae in both WHRAP and EHRAP over one year (Aug. 13- Jul. 14)

2.3.4 Biomass productivity

The WWT HRAP biomass concentration and productivity are important parameters for both wastewater treatment and sustainable biofuel production. The average weekly areal biomass productivity (including both algae and bacteria) of the HRAPs, calculated using

Equation (1) is shown in Fig. 2.3. Despite the different algal species composition of the WHRAP and EHRAP, the mean annual biomass productivities of both ponds were similar, 5.8 ± 3.2 g VSS/m^2/d and 6.0 ± 3.2 g VSS/m^2/d, respectively. These values are in line with literature values for WWT HRAPs [7, 15-16, 18, 30].

Algal productivity changed seasonally with changes in water temperature and light intensity (Fig. 2.1 and 2.3), and was greatly reduced by grazing during zooplankton blooms. The influence of seasonal changes and zooplankton grazing on the HRAP biomass productivity has been previously documented [12, 27, 82].

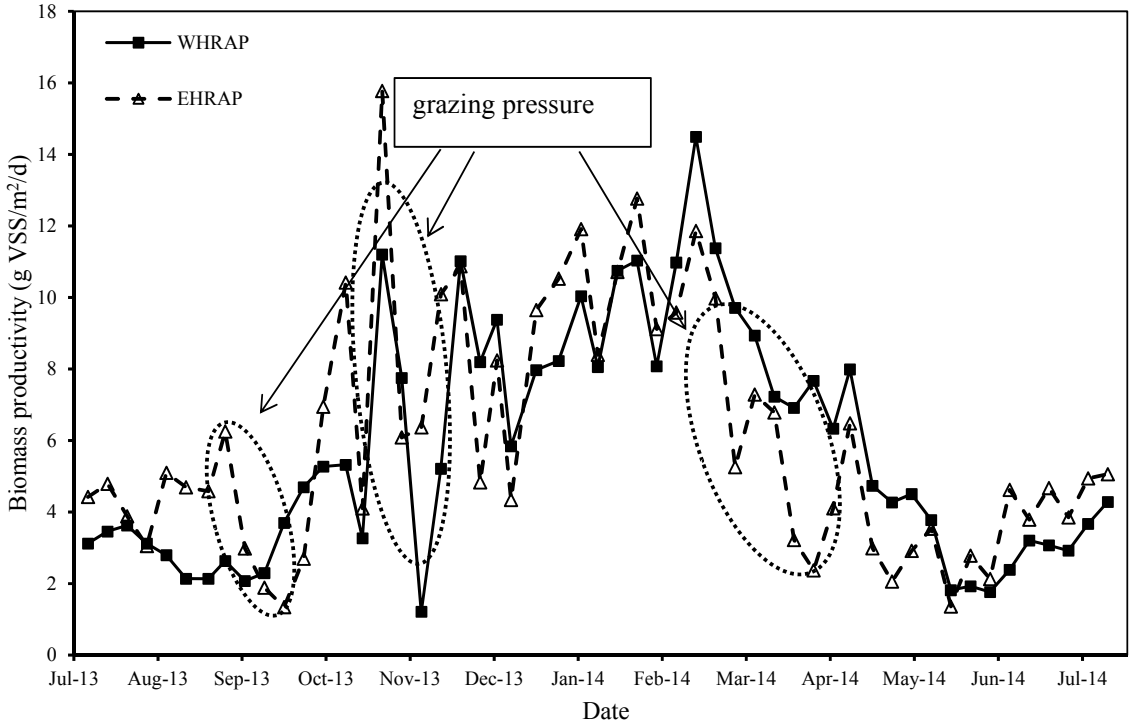


Figure 2.3. Mean areal biomass productivity (g VSS/m^2/d) in the HRAPs during the year (Aug. 13- Jul. 14)

2.3.5 Biomass composition

The composition of WWT HRAP biomass is dependent on microbial composition, and environmental and operational conditions [76], and is important for low-cost energy production in terms of high biomass yield and lipid content. The average annual lipid content of the biomass in the west and east WWT HRAPs was similar (24 ± 7 wt% and 23.4 ± 8 wt% respectively) despite the variation in the microalgal community of the ponds over the year (Fig. 2.2). These lipid content values are in line with literature values for wastewater HRAP grown biomass [8, 10, 94]. There was some variation in the biomass average lipid content over the year between spring, summer, autumn and winter (24 ± 0.5 wt%, 26 ± 1 wt%, 21.5 ±

0.5 wt%, and 23 ± 1 wt%, respectively). This slight variation in the biomass lipid content between seasons suggests that there was little effect of environmental factors (temperature and solar radiation) on the biomass chemical composition.

The highest biomass lipid content (45 wt%,) and the lowest biomass protein content (26 wt%) occurred in summer (late December to early January) and coincided with the lowest ammonia concentration (<1 mg/L) in the pond water (Fig. 2.4). There was a positive relationship between the proportion of algae in the biomass and the pond water ammonia concentration, while the biomass lipid content was inversely related to the ammonia concentration. In particular, there was a significant ($p<0.05$) inverse relationship between biomass lipid content and ammonia concentration at low HRAP ammonia concentrations (<5 mg/L). These results are in good agreement with a number of studies that have shown cultivation of microalgae under nitrogen-limiting conditions to enhance lipid content, but reduce microalgae production [1, 13, 23, 25].

For example, when the outflow ammonia concentration decreased from 3 mg/L to 0.2 mg/L, the biomass lipid content increased from 20 wt% to 40 wt%, while the chl-a/VSS ratio declined by >30%. These results are in agreement with work by Park and Craggs [8] in which biomass lipid content decreased and biomass productivity increased with an increase in the pond water ammonia concentration. Protein synthesis declines with nitrogen limitation, consequently cell growth decreases and the proportion of algae in the biomass is reduced. As a safety mechanism during nitrogen limitation, the algae's carbon fixation processes divert to lipid synthesis resulting in higher biomass lipid content. At outflow ammonia concentrations > 5 mg/L the biomass algal and lipid content did not increase further and even declined at times due to zooplankton grazing.

The major constituent of the biomass in both WWT HRAPs was protein (annual average 43.4 ± 7.5 wt%) (Fig. 2.4). This high percentage of protein may be explained by the high and similar protein content of both algal and bacterial biomass. WWT microalgae are typically composed of only 15-30 wt% lipid, but 10-30 wt% carbohydrate and 30-60 wt% protein, [22, 95, 96]. While, bacteria are typically composed of <10 wt% lipid, 20-40 wt% carbohydrate and 25-50 wt% protein [24]. WWT HRAP biomass typically consists of microalgae (60-80 wt%), bacteria (20-30 wt%) and other organic matter (5-10 wt%) such as zooplankton and detrital material [11, 27, 97]. Therefore one explanation for the periods of lower biomass lipid content may be an increase in the proportion of bacteria (with lower lipid content than

algae) and detrital organic matter (typically with no lipid content) in the biomass. Lower lipid content biomass would convert to a lower yield and quality of liquid biofuels.

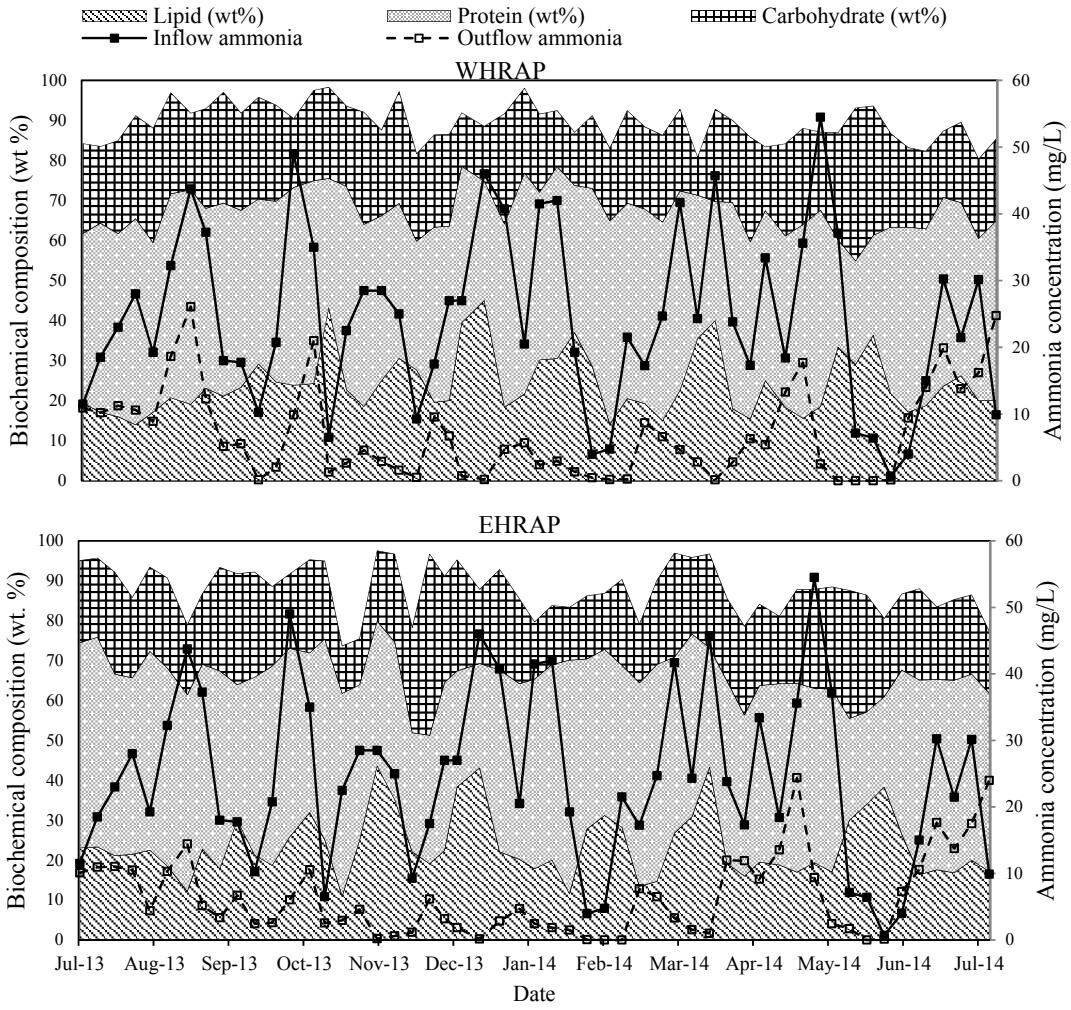


Figure 2.4. Mean weekly chemical composition of biomass produced in the ponds and the pond inflow and outflow ammonia concentration

2.3.6 Biomass energy content

Although, the primary goals of using microalgae in WWT HRAP are wastewater treatment and nutrient recovery, the easily harvestable biomass produced in such a system has an energy content of 18-22 kJ/g and can be considered as a low-cost feedstock for biofuel production [8, 12]. The WWT HRAP biomass consists of microalgae (18-24 kJ/g), bacteria (17-22 kJ/g) and other organic matter which have probably lower energy content [23, 98, 99]. This implies that changes in the pond microbial community and chemical composition of the biomass affect the biomass energy content. The energy content of the WWT HRAP biomass

is a function of the ratios of algae, bacteria and other organic matter and the biomass chemical composition (the ratios of proteins (24 kJ/g), carbohydrates (17 kJ/g), and lipids (37 kJ/g)). Hence, an increase in the lipid content and the proportion of algae in the biomass should result in the enhancement of overall biomass energy content.

Weekly data on the biomass energy content and the proportion of algae in the biomass (indicated Chl-a/VSS) are shown in Fig. 2.5. The HRAPs biomass energy content and the Chl-a.VSS^{-1} varied between 13-25 kJ/g, and 0.37-3.4 wt% respectively. The average annual biomass energy content was 19.2 ± 2.6 kJ/g which was in line with literature values for WWT HRAP biomass [12, 100]. The lowest energy content was measured in summer during an invertebrate grazer bloom when Chl-a.VSS^{-1} reduced to <0.5 wt%.

The biomass energy content depended on the proportion of algae in the biomass which was affected by environmental, operational and biological parameters. The biomass energy content did not vary significantly with season, although it positively correlated with Chl-a/VSS ($p<0.05$) (Fig. 2.5) with the highest values of both variables occurring in winter (1.75 ± 0.05 wt%; 21.7 ± 0.1 kJ/g). The lower algal content in summer compared to winter may be explained by the reduction of algal growth resulting from the lower ammonia concentration in the pond (Fig. 2.4), the change in dominant algal species, grazing pressure and an increase in bacterial growth due to the higher pond water temperature. In addition, the 5 day hydraulic retention time used in summer was probably too long for optimal operation of WWT HRAP under New Zealand summer conditions.

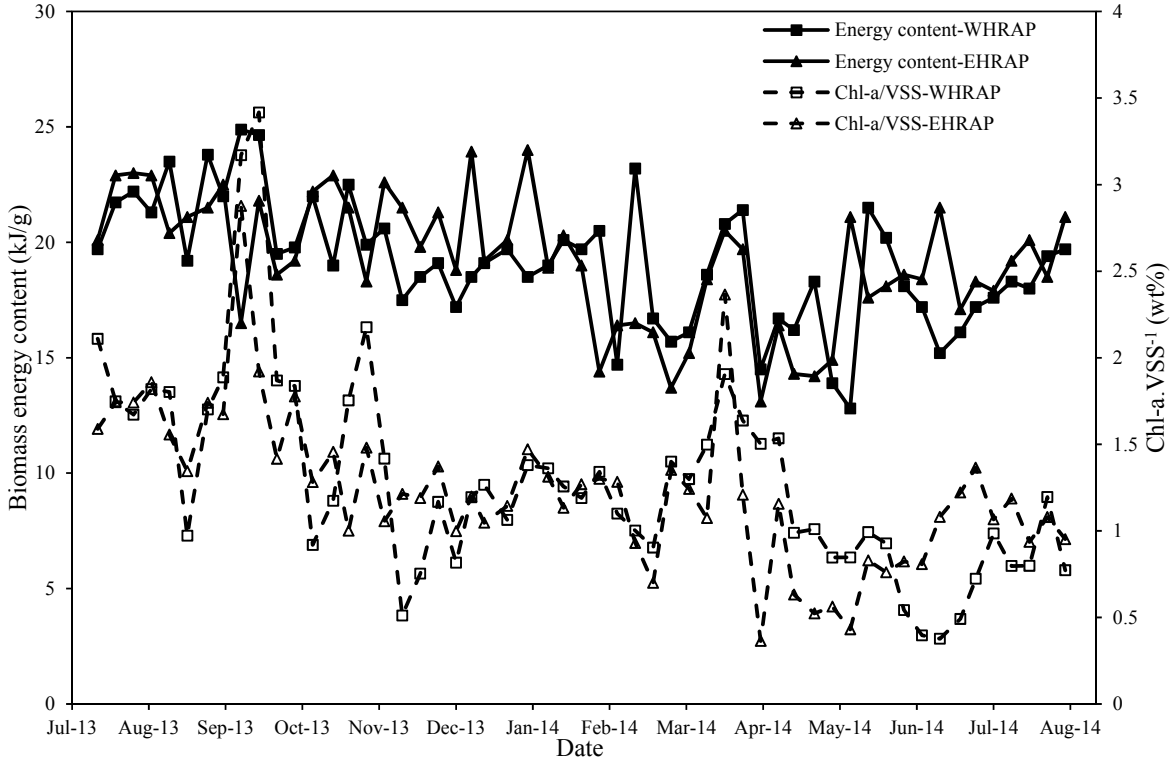


Figure 2.5. Energy content and proportion of algae in the pond biomass measured over the one year study

Previous studies have reported that the proportion of algae in pond biomass often decreases with increasing HRT [8, 34]. Park and Craggs [8] showed that under summer conditions the percentage of algae in HRAP biomass increased from 56% when grown at an 8 day HRT to 80.5% when grown at a 4 day HRT. A comparison between the summer-time 5 d HRT TSS (206 mg/L) and biomass energy content (19.2 kJ/g) results of this study and those of previous study [12] conducted in the same HRAP operated with a 4 day HRT (201 mg/L and 20.5 kJ/g) further illustrates the effect of HRT on the HRAP. The higher pond TSS concentrations measured at the longer HRT, could have limited algal growth and production due to shading.

Moreover, as there was no control of zooplankton, the fast growing algal species such as *Ankistrodesmus* sp. (which was dominant in the HRAPs in winter 2013) was grazed and replaced by a combination of the slower growing larger species (*Micractinium* sp. and *Pediastrum* sp.) (Fig. 2.2) which probably resulted in the lower algal proportion in the HRAP biomass. In addition, Sutherland et al. [27] found that the chl-a content of algae grown in wastewater treatment HRAP did not decline during summer compared to winter and the algal proportion reduction in the HRAP biomass produced under warm weather conditions was due

to an increase in the development of bacteria flocs, and the higher zooplankton growth rate in the warmer pond water.

The direct positive relationship between microalgal biomass lipid content and energy content is well documented [22, 23, 95], because 30-50 % of the microalgal energy content comes from lipids. However, the relationship between WWT HRAP biomass (comprised of algae, bacteria, zooplankton and detrital material) lipid content and energy content has not been previously considered. The lipid and the energy content of the biomass produced in the HRAPs in this study also had a direct positive relationship (Fig. 2.6), however, it was not highly significant ($p<0.15$). For example, autumn biomass lipid and energy content (21.5 ± 0.5 wt%; 16.8 ± 0.2 kJ/g) were both lower than summer values (26 ± 1 wt%; 19.2 ± 0.2 kJ/g) while the chl-a.$VSS^{-1}$of biomass (indicator of algal proportion) in both seasons did not significantly differ, 1.13 ± 0.12 wt% in autumn and 1.17 ± 0.03 wt% in summer. Moreover, the biomass energy content and algal proportion were lower in summer than in winter 2013, while the WWT HRAP biomass lipid content was higher in summer. While it was expected to have a significant higher energy content when the lipid content increased, the protein reduction, carbohydrate and ash content would increase (Fig. 2.4) lowering the effect of lipid content on biomass energy content.

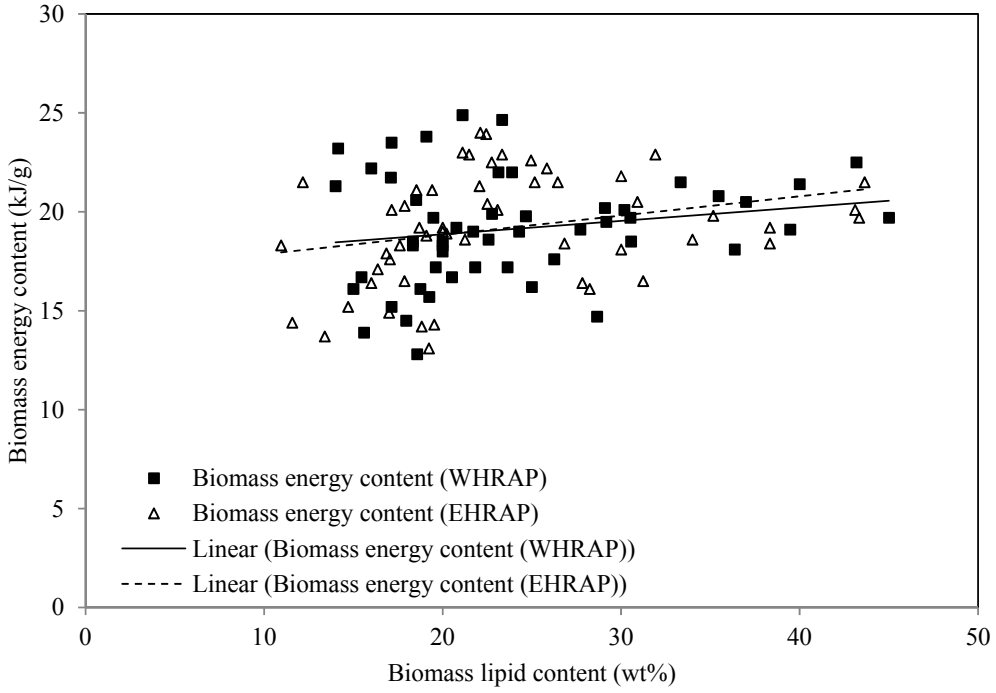


Figure 2.6. The relationship between energy content and lipid content of the HRAP biomass

2.3.7 Biomass energy yield potential of WWT HRAP

The biomass energy yield potential of the WWT HRAP over the year-long study is shown in Fig. 2.7 and was calculated by multiplying the energy content and the productivity of the biomass. Results showed that in a worst case scenario, i.e. operating WWT HRAP with no control of the zooplankton population and dominant species, and sub-optimal HRT, the annual biomass energy yield potential of such a system under New Zealand climate conditions was 113.3 kJ/m^2/d (with average biomass productivity: 5.9 g VSS/m^2/d and mean energy content of 19.2 kJ/g). Much higher values (220-360 kJ/m^2/d) have been previously reported for the same experimental system and elsewhere [12, 101] where the HRAP biomass productivity was enhanced by controlling invertebrate grazers and the dominant algal species.

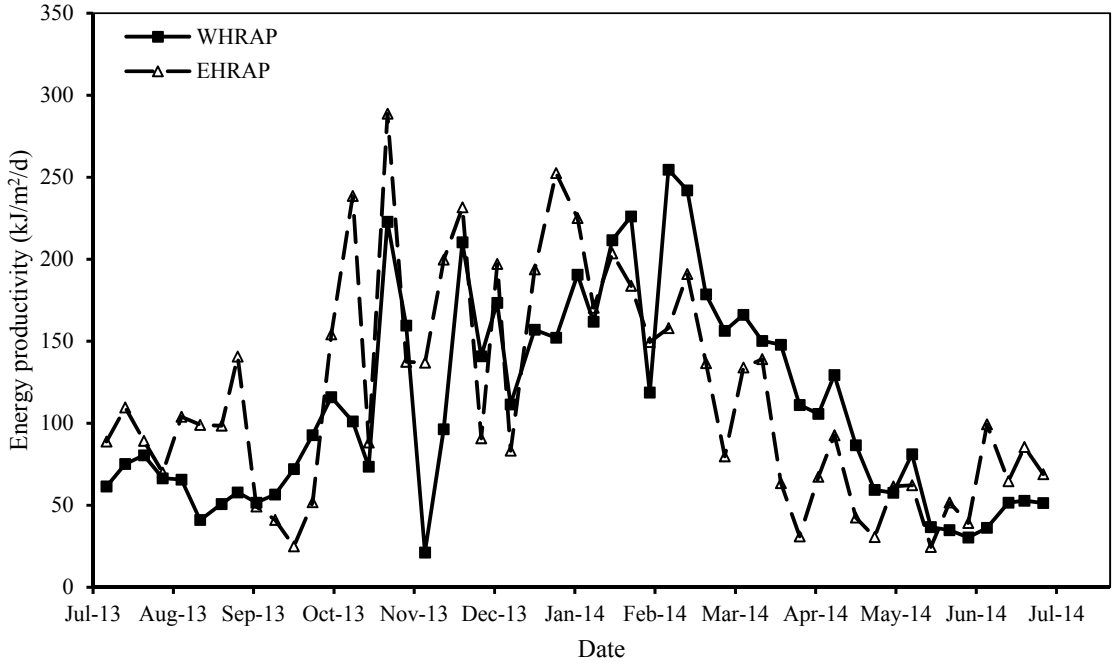


Figure 2.7. Daily areal energy productivity (kJ/m^2/d) in the HRAPs over the year

The summer average daily biomass energy yield (175 ± 5 kJ/m^2/d) was ~2.5x higher than that in winter (68 ± 18 kJ/m^2/d). The energy content of the biomass grown in the WWT HRAPs under winter conditions was slightly higher than that in summer, although, the mean biomass productivity in summer was triple that in winter. Therefore, changes in biomass productivity had a greater influence than the biomass energy content on the WWT HRAP biomass energy yield. The highest biomass energy yield (288 kJ/m^2/d) was achieved when the biomass productivity was maximum (15.8 g/m^2/d). Park et al. [12] had a similar result, finding that biomass productivity had more influence on HRAP biomass energy yield than

the biomass energy content. This research indicates that HRAP biomass (particularly algal biomass) productivity is the most important parameter for integrated algal-based tertiary-level wastewater treatment and low-cost biofuel production. Hence, to maximize the biomass energy yield from WWT HRAP, strategies to enhance algal biomass productivity such as zooplankton grazer control, algal recycling and optimization of operational parameters (such as HRT and CO_2 addition) need to be employed.

It has previously been found that WWT HRAP biomass productivity was enhanced by 30-50% by CO_2 addition which was controlled by maintaining the daytime maximum pond water pH below 8 [30, 84]. Moreover, Sutherland et al. [102] recently showed further improvements in HRAP productivity with higher levels of CO_2 addition, by maintaining the daytime maximum pond water pH below 7. They found that the biomass concentration and the algal biovolume were increased by 22%-45%, and 100-560%, respectively when pH was maintained at 6.5 compared with pond mesocosms without CO_2 addition. The proportion of algae in pond mesocosm biomass was also increased at higher CO_2 addition rates (lower maximum pH). However, the influence of lower maximum pH on gravity harvestable biomass productivity which is important for low-cost biofuel production was not investigated. Therefore, further research is required to fully understand the effect of lower maximum pH on both the productivity and quality of gravity harvestable biomass.

In addition, algal production is improved with higher mixing which reduces both the boundary layer around individual algal cells as well as thermal stratification within the pond, and increases the cell light exposure frequency which promotes algal photosynthesis [10, 103]. Sutherland et al. [103] showed that biomass productivity, the algal/bacterial ratio and the proportion of settable colonial species were all enhanced by increasing the mixing frequency to reduce laminar flow and dead zones in the pond.

WWT HRAP biomass productivity and potential for low-cost energy production in the form of biomass could be further enhanced by zooplankton population control. Moreno-Garrido and Canavate [39] observed the complete elimination of *Dunaliella salina* from an algal production HRAP by a ciliate in less than 5 days. Park and Craggs [84] showed under New Zealand summer conditions, the high biomass productivity (on average, 20.7 g VSS/m^2/d, more than double the summer biomass productivity in this study) was achieved in WWT HRAP by controlling the population of zooplankton grazers. Alternatively, Park et al. [12] found that the biomass productivity in WWT HRAP could be enhanced by up to 19%

even in presence of invertebrate grazers by recycling a small portion of the harvested biomass which was dominated by *Pediastrum boryanum* (which is harder to graze due to its large colony size).

The results of this study indicate that changes in biomass productivity (especially algal productivity) are more dependent than biomass energy content on environmental, biological and operational conditions and therefore have a greater influence on WWT HRAP biomass energy yield. The effect of environmental conditions on biomass productivity have been well documented [1, 26, 104] and it has been shown that biomass productivity increases under more favourable solar radiation and temperature conditions [e.g. [105]]. Therefore higher biomass energy yields would be expected under more favourable climates than New Zealand's temperate climate especially with the application of the aforementioned enhancement strategies.

2.4 Conclusions

This study has measured the biomass energy yield in WWT HRAP in terms of biomass productivity and biomass energy content. The two identical pilot-scale WWT HRAPs that were operated in parallel and monitored for a year without control of either dominant algal species or zooplankton grazers had an average daily biomass energy yield of 113.3 kJ/m^2/d. This biomass energy yield was much lower than literature values (220-360 kJ/m^2/d) due to the reduction of algal biomass by zooplankton grazing, not controlling algal species and operation at a longer than optimal HRT. WWT HRAP biomass energy content correlated strongly with seasonal changes in the HRAP effluent chl-a/VSS ratio and to less extent the biomass lipid content. Biomass energy yield from WWT HRAP may be enhanced by employing operational strategies to increase algal productivity such as: optimizing pond HRT, biomass recycling to control algal species, and control of zooplankton grazers. Increasing WWT HRAP algal productivity has the combined benefit of raising both the biomass productivity and the biomass energy content.

CHAPTER 3

Beneficial colonial algal species for wastewater treatment and biomass energy production in high rate algal ponds (HRAP)

This chapter is based on the following publication:

Mehrabadi, A., Farid, M. M., Craggs, R. 2017. Potential of five different isolated colonial algal species for wastewater treatment and biomass energy production. *Algal Research*, 21, 1-8.

Chapter preface

While several unicellular algal species have been evaluated extensively for their wastewater treatment and biofuel production potential, there has been little focus on the colonial species that typically predominate in high rate algal ponds and which have the benefit of being easily harvested by cost-effective, simple gravity settling. This chapter investigates the wastewater treatment performance of five wastewater colonial algal species that are common in high rate algal ponds: *Mucidosphaerium pulchellum*, *Micractinium pusillum*, *Coleastrum* sp., *Desmodesmus* sp. and *Pediastrum boryanum* and their potential value for biofuel production under simulated New Zealand summer and winter conditions. The results showed *Mucidosphaerium pulchellum* and *Micractinium pusillum* were the most beneficial colonial species for both treatment and biofuel production.

3.1 Introduction

The techno-economic feasibility of biofuel production from algal biomass has been questioned for several years and it has been highlighted that even the use of conventional raceway ponds for biofuel production alone is not currently financially viable [3, 15, 106-109]. One opportunity to lower algal-based biofuel production costs is where the algal biomass is produced as an essentially free by-product of tertiary-level wastewater treatment in High Rate Algal Ponds (HRAP) [6, 76]. Wastewater treatment HRAPs are a component of enhanced wastewater treatment pond systems which have received much attention worldwide as an upgrade option for traditional wastewater treatment ponds, due to their higher nutrient removal rates and the ability to recover resources in the form of algal biomass [103, 110].

To effectively combine low-cost tertiary-level wastewater treatment and low-cost algal production in WWT HRAPs for biofuel, the WWT HRAP must be mainly dominated by algal species that have both high treatment and energy production potential. The main characteristics of beneficial algal species for wastewater treatment are: 1) high nutrient removal capacity at typical wastewater nutrient loads, 2) ability to grow under seasonally variable environmental conditions, and 3) easy harvest by simple gravity settling [11, 18]. The important characteristics of algal species for low-cost biomass energy yield in WWT HRAP without impacting their wastewater treatment function include: 1) high year-round productivity, 2) high energy content (resulting from beneficial biomass chemical composition), and 3) high settleability to achieve the highest settleable algal biomass yields [76].

Over recent years, many studies have been conducted to investigate beneficial algal species for biofuel production using wastewater (municipal/industrial/animal manure) as a nutrient source [100, 111-115]. The majority of these studies have found high biofuel production and wastewater treatment potential of the tested algae. However, only a limited number of algal species including *Chlorella* sp., *Scenedesmus* sp., *Ankistrodesmus* sp., and Monoraphidium sp. have been assessed [100, 111-115] and these are typically motile or poorly-settleable unicellular algae that require chemical flocculation / energy consumption for efficient removal. Moreover, most studies have been carried out under simulated moderate conditions without taking into account natural seasonal variation in performance. In addition nutrient and operational stress conditions (such as cultivation under N starvation or high light intensity) have been suggested to improve algal biomass quality for biofuel production while they may negatively affect wastewater treatment performance [100, 111, 114-117].

While poorly-settleable unicellular species have been cultivated on wastewater under controlled conditions for biofuel production, research on algal-based wastewater treatment ponds has shown that WWT HRAPs are often populated by colonial species which have similar wastewater treatment performance to unicellular species but can be cost-effectively harvested using gravity settling [11, 118]. Park et al. [12] conducted an experiment in outdoor pilot-scale WWT HRAPs populated by >80% *Pediastrum boryanum* (a readily settleable colonial species) and showed that >80% NH_4^+-N and 50-75% PO_4^{3-}-P were removed year-round. They found that not only high nutrient removal was achievable by *Pediastrum boryanum* dominance but also high harvest efficiency was achieved, which would improve the economic viability of WWT HRAP for combined wastewater treatment and low-cost energy production. Our previous study has also shown >70% year-round nutrient removal in outdoor pilot-scale WWT HRAPs dominated by colonial species such as *Micractinium* sp., *Mucidosphaerium pulchellum*, *Coleastrum* sp., *Desmodesmus* sp., and *Pediastrum* sp. [119]. In a comparative lab-scale experiment, Sutherland et al. [103] found that under continuous mixing the nutrient removal capacity of colonial species (*Mucidosphaerium pulchellum* and *Pediastrum boryanum*) and poorly settleable unicellular species (*Chlorella* sp.) were similar. However, compared with the two other species, *Pediastrum boryanum* was highly settleable so that >55% of culture biomass settled at 10 min. While a number of colonial microalgal species have been assessed for wastewater treatment potential [11, 12, 85, 93, 103] there has been little focus on algal biomass quality (i.e. biochemical composition and biomass energy content) for biofuel production. It has been found that colonial species can be maintained in

WWT HRAP by recycling a small (<20%) portion of the harvested biomass back to the pond [11, 12, 85]. This could provide an opportunity to maintain the most beneficial species for both wastewater treatment and production of algal biomass for biofuel in WWT HRAP. Therefore, the aim of this study was to investigate the performance of typical WWT HRAP colonial algal species for efficient wastewater treatment (in terms of nutrient removal) as well as biomass energy yield (in terms of growth rate, biochemical composition and energy content) for further use as biofuel feedstock.

3.2 Materials and methods

Laboratory-scale batch experiments were conducted to determine the wastewater treatment and energy production potential of five common colonial algal species isolated from pilot-scale WWT HRAPs at the Ruakura Research Centre, Hamilton, New Zealand (37º47´S, 175º19´E). Performance was compared under New Zealand summer and winter simulated conditions (as used previously in Park et al. [93]) using pre-frozen pre-filtered primary settled sewage.

3.2.1 Microalgal species isolation

Colonial algal species which were predominantly present in the pilot-scale WWT HRAP at the Ruakura Research Centre (see Mehrabadi et al. [119] for more details) were isolated, identified and grown in pure culture under both New Zealand summer and winter simulated conditions.

The isolation procedure involved a combination of serial dilution and selection of a single healthy colony of each species using a microscope (Leica DM 2500). The single colonies were placed into autoclaved flasks with 100 ml sterile growth medium (Bold 3N growth medium modified by replacing $NaNO_3$ with $(NH_4)_2SO_4$).

Five species were isolated including *Mucidosphaerium pulchellum*, *Micractinium pusillum*, *Coleastrum* sp., *Desmodesmus* sp., *Pediastrum boryanum* (Fig. 3.1). *Mucidosphaerium pulchellum* (HC Wood) C. Bock, Proschold & Krienitz is a colonial species with 4-64 spherical cells connected by mucilaginous stalks and forms colonies with a diameter of up to 80 µm [120]. *Micractinium pusillum* has small spherical cells (diameter: 3-7 µm) which develop cell wall spines (length: 20-35 µm) and grow as colonies (diameter: up to 150 µm) in the presence of zooplankton grazers (e.g. *Brachionus calyciflorus*) [121]. *Coelastrum sphaericum* Nägeli grow as hollow spherical colonies of closely-packed cells (8-

128 cells per colony) with a diameter of up to 100 µm [122]. *Desmodesmus abundans* (Kirchner) E. Hegewald has flat ellipsoidal cells (length: 10-15 µm, width: 5-8 µm) that grow as either unicells or 4-celled colonies and develop cell wall spines in presence of zooplankton grazers (e.g *Daphnia*) [123]. *Pediastrum boryanum* is a star-shaped flat colonial species typically with 8 to 32 and sometimes 64 cells. The number of cells is fixed from when the juvenile colony emerges from the parent cell and the colony size increases as the cells grow (colony diameter: 4-80 µm). A silica skeleton with horn-like projections on the outer cells makes *Pediastrum* colonies denser than the other colonial species [93, 103].

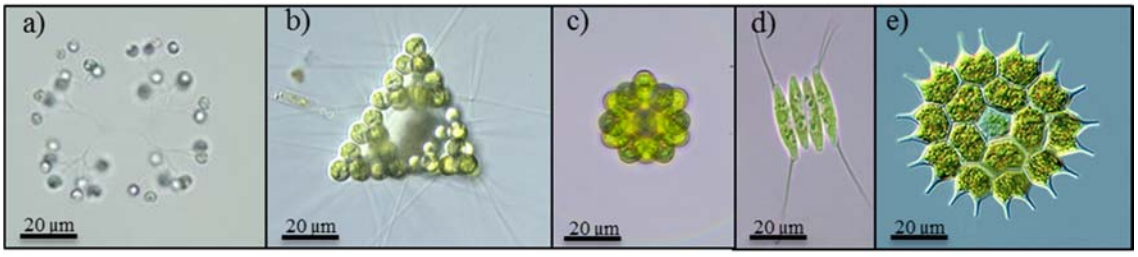


Figure 3.1. Colonial algal species: a) *Mucidosphaerium pulchellum*, b) *Micractinium pusillum*, c) *Coleastrum* sp., d) *Desmodesmus* sp., e) *Pediastrum boryanum*

3.2.2 Culture conditions

The isolated species were grown under both New Zealand summer and winter simulated conditions in growth chambers as used by Park et al. [93] (6150CP-6400CP, Contherm Scientific Ltd.) to grow sufficient biomass for use as inoculum in the experiment. Day/night temperature, light/dark cycle and light intensity for simulated summer conditions were: 25/19 ºC, 14/10 h, and 250 µ mol photon/m^2/s, and for simulated winter conditions were: 13/9 ºC, 10/14 h, and 120 µ mol photon/m^2/s.

The purity of cultures was regularly monitored and just prior to the experiment, the cultures were subsampled and grown up over 5 days (summer) and 10 days (winter) to provide and exponential phase inoculum.

3.2.3 Wastewater collection, storage and characterization

A 100 L volume of primary settled sewage was collected from the Ruakura sewer for use as the growth medium in the experiment. To ensure consistency of the growth medium the wastewater was initially placed in a freezer at -18 ºC for 72 h and then stored in a cold room maintained at 4 ºC. Physicochemical parameters of the fresh wastewater including COD, BOD_5, total nitrogen, total phosphorous and total Kjeldahl nitrogen (TKN) were measured using standard methods for wastewater [86].

3.2.4 Experimental set-up

A 100 ml volume of pure algal culture (that had been diluted with deionized water to have a biomass concentration of 12 mg/L) was pipetted into an autoclaved Erlenmeyer flask with 900 ml of pre-frozen filtered primary settled sewage. To avoid interference between wastewater solids and the algal culture biomass, pre-frozen wastewater was filtered through a 0.7 µm glass fibre filter (LabServ, LBS0GFF.047) before use. Triplicate flasks for each algal species were placed in each of the summer and winter simulated condition growth chambers (6150CP-6400CP, Contherm Scientific Ltd.). The algal cultures were bubbled with pre-filtered 1% CO_2-air mixture (0.2 l/min) to avoid carbon limitation, provide mixing and maintain pH<8 to prevent ammonia volatilisation and phosphate precipitation. The batch experiments were conducted over 10 days and samples (15 ml) of the cultures were removed from the flasks every two days to check culture purity and measure biomass (TSS) and nutrient (NH_4^+-N and DRP) concentrations.

3.2.5 Measurement of nutrient concentrations and removal efficiency

Ammonia and dissolved reactive phosphorous (DRP) concentrations were measured spectrophotometerically using standard methods [86]. The percent nutrient removal was calculated using Equation (3-1):

$$\%Nutrient\ removal = \frac{(Initial\ concentration - Final\ concentration)}{Initial\ concentration} \times 100 \quad (3-1)$$

3.2.6 Measurement of biomass concentration, growth rate and productivity

A known volume of algal culture was filtered onto a pre-rinsed and pre-weighed Whatman GF/F filter, dried (at 80 ºC overnight), cooled in desiccator and weighed to measure the dry weight and used to calculate the biomass concentration in terms of mg TSS/L. The specific growth rate and productivity of each algal species were determined using Equation (3-2) and Equation (3-3) [124]:

$$\mu = \frac{\mathrm{Ln}(X_{t2}) - \mathrm{Ln}(X_{t1})}{(t2 - t1)} \quad (3-2)$$

$$P = \frac{X_{t2} - X_{t1}}{t2 - t1} \quad (2-3)$$

where µ is specific growth rate (d^{-1}), P is productivity (mg/L/day), X_{t2} and X_{t1} are the biomass concentration (mg TSS/L) at time t_2 and t_1 (days), respectively. The specific growth

rates were calculated for the time interval of the highest increase in culture biomass concentration.

3.2.7 Measurement of biochemical composition and energy content of species

The protein, carbohydrate, lipid and energy content of each algal biomass were determined from culture samples taken on day 5 and day 10. A 100 ml volume of culture was centrifuged (Sigma centrifuge 4K15) at 4000 rpm for 5 min and frozen until analysis. The biochemical composition and energy content were measured as described previously [119].

3.2.8 Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) in Excel software (Microsoft office 2010).

3.3 Results and discussion

3.3.1 Wastewater characterization

The physicochemical properties of primary settled sewage are summarized in Table 3.1. The majority of nitrogen and phosphorus were present in dissolved inorganic forms, mainly ammonia (NH_4^+-N) and DRP (PO_4^{3-}-P) which is typical for this wastewater [8, 30]. As the nutrients concentration is important for algal growth, their concentration did not change before and after filtration.

Table 3.1. Initial physicochemical properties of primary settled sewage

Chemical Oxygen Demand (COD)	g O_2/m^3	70
Carbonaceous Biochemical Oxygen Demand ($cBOD_5$)	g O_2/m^3	15
Total Alkalinity	g/m^3 as $CaCO_3$	200
Total Solids (TS)	g/m^3	270
Volatile Total Solids	g/m^3	103
Total Nitrogen	g/m^3	41
Total Kjeldahl Nitrogen (TKN)	g/m^3	41
Nitrate-N + Nitrite-N	g/m^3	0.005
Ammonia	g/m^3	40
Total Phosphorus	g/m^3	4.7
Dissolved Reactive Phosphorus	g/m^3	3.7

3.3.2 Biomass concentration and species growth rate

The biomass concentration and exponential phase specific growth rate of each algal species are shown in Fig. 3.2 and summarized in Table 3.2. The day 10 biomass yields under summer conditions ranged from 991 mg/L to 1700 mg/L, but were more than 65%, on average, lower under winter conditions. *Mucidosphaerium pulchellum* had the highest growth under both conditions achieving significantly ($p<0.05$) higher biomass concentrations of 1700 ± 74 mg/L and 584 ± 49 mg/L under summer and winter conditions respectively. *Micractinium pusillum* had the second highest growth under both conditions achieving biomass concentrations of 1395 ± 67 mg/L and 363 ± 10 mg/L under summer and winter conditions respectively. *Coelastrum* sp. had the lowest biomass concentration (991.5 ± 29 mg/L) under summer conditions while *Pediastrum boryanum* had the lowest biomass concentration (158.7 ± 21.9 mg/L) under winter conditions.

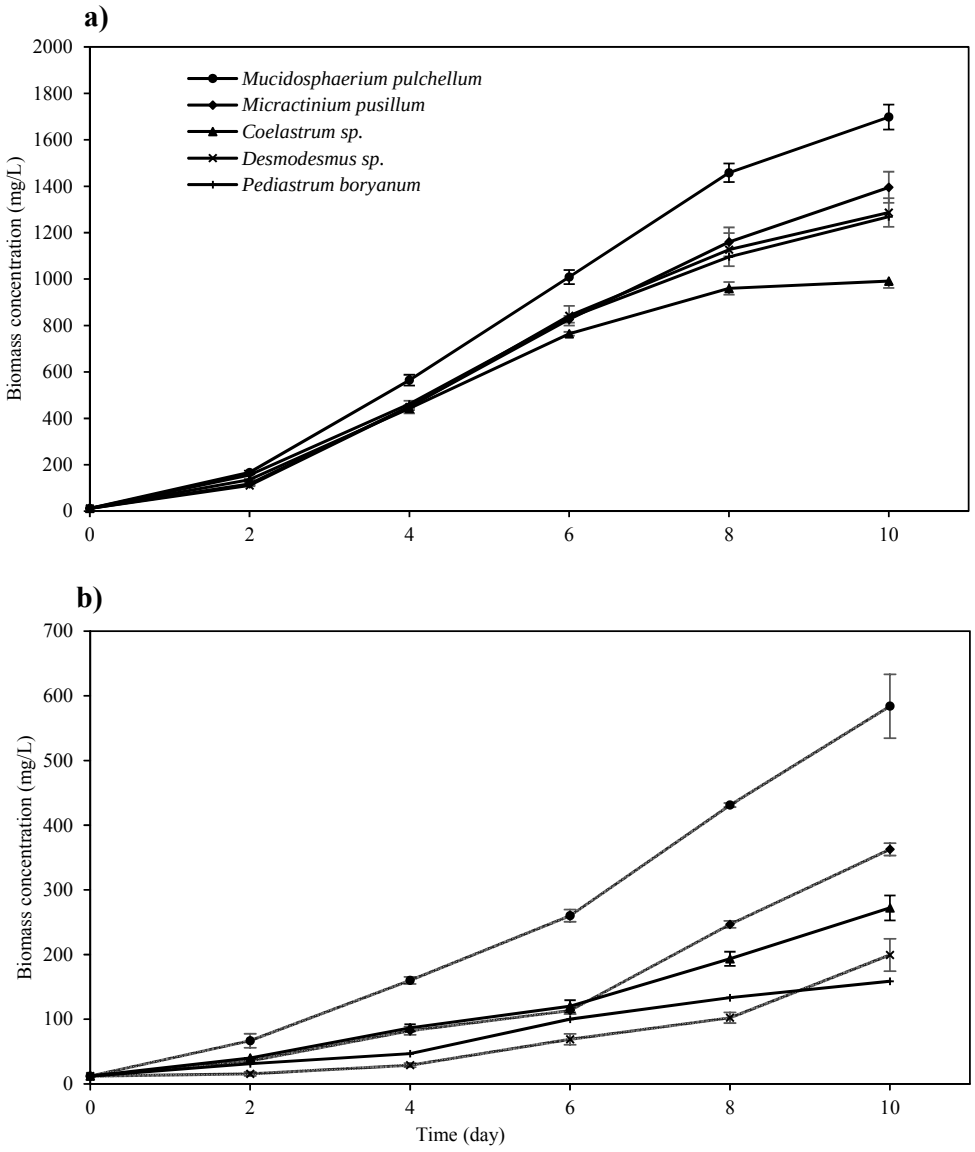


Figure 3.2. Growth curves and biomass concentration of species monoculture batch experiment under similar a) summer and b) winter conditions

Mucidosphaerium pulchellum and *Micractinium pusillum* had the highest growth rates (summer: 0.59 ± 0.01 d^{-1} and 0.57 ± 0.02 d^{-1}; respectively; and winter: 0.38 ± 0.03 d^{-1} and 0.29 ± 0.01 d^{-1} respectively) of all the algal species while the growth rate of the other species did not differ significantly (Table 3.2). Both *Mucidosphaerium pulchellum* and *Micractinium pusillum* had the smallest cell size of the algal species studied and their higher growth rate may be related to their smaller size. Previous studies [103, 125, 126] have indicated that the higher growth rate of species with small cells is due to faster nutrient uptake due to their larger surface area/volume ratio. The growth rate of *Micractinium pusillum*, *Desmodesmus*

sp., *Pediastrum boryanum* and *Coelastrum* sp. were quite similar during exponential phase under summer conditions (Fig. 3.2; Table 3.2). To ensure that the maximum growth rates were measured in the 10 day duration of the winter simulated condition experiments, the experiments for the species *Mucidosphaerium pulchellum* and *Pediastrum boryanum* were extended to 45 days and found that those calculated within first 10 days were maximum.

Table 3.2. Average specific growth rate of colonial species

Species	Specific growth rate (d^{-1}) under summer conditions	Specific growth rate (d^{-1}) under winter conditions
Mucidosphaerium pulchellum	0.59 ± 0.01	0.38 ± 0.03
Micractinium pusillum	0.57 ± 0.02	0.29 ± 0.01
Coealstrum sp.	0.55 ± 0.01	0.24 ± 0.05
Desmodesmus sp.	0.56 ± 0.03	0.26 ± 0.04
Pediastrum boryanum	0.56 ± 0.02	0.20 ± 0.02

The differences in growth rates found between the algal species in this study showed a similar trend to those found by Park et al. [85] for isolated HRAP colonial algal species grown on artificial wastewater under New Zealand summer simulated conditions where *Micractinium* sp. had a higher growth rate (0.27 d^{-1}) than *Scenedesmus* sp. (0.24 d^{-1}) and *Pediastrum boryanum* (0.22 d^{-1}) (Park et al., 2013a). Moreover, Johnson [10] also reported a higher growth rate for *Micractinium* sp. (0.25 d^{-1}) than *Pediastrum boryanum* (0.22 d^{-1}) when cultured in modified Bold medium under constant laboratory conditions (27 ± 1 °C; continuous illumination at 103 ± 11 μmol/m^2/s; aeration). The higher growth rates in this study may result from differences in the culture conditions, particularly the CO_2 addition which ensured that the algal cells were not CO_2 limited and controlled culture pH, minimizing any pH inhibition or potential ammonia toxicity. Moreover, algae use less energy to assimilate ammonia than the nitrate nitrogen source typically used in Bold growth medium [127], so may have grown better on the ammonia-rich wastewater.

All algal species continued to grow even when the culture nutrients had been removed, however nutrient limitation appeared to reduce the size of the algal colonies in some species. For example, the colony size of *Pediastrum boryanum* reduced over the course of study (Fig. 3.3) and this may have been a consequence of nutrient limitation. Alternatively reduced colony size may have been due to release of many juvenile colonies from the large

(reproductive) colonies (reported in Park et al. [93]) that were present in the cultures on day 0 of the experiment.

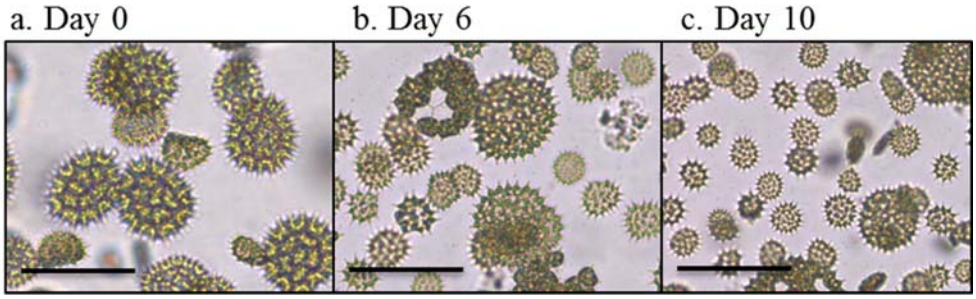


Figure 3.3. Example of reduced cell/colony size of *Pediastrum boryanum* species grown under simulated summer conditions. Scale bars equal to 100 μm.

3.3.3 Dissolved nutrient concentrations and nutrient removal efficiency

Since, low-cost tertiary-level wastewater treatment with nutrient recovery is the primary benefit of HRAP utilization, it was essential to evaluate the nutrient removal capability of the algal species in this study. Ammonia and DRP concentrations in the algal cultures are shown in Fig. 3.4 and nutrient removal efficiencies calculated on day 4 (summer conditions) and day 10 (winter conditions) are given in Table 3.3. *Mucidosphaerium pulchellum* cultures had significantly higher ($p<0.05$) nutrient removal, for example 73% for ammonia and 57% for DRP after only 2 days culture under summer conditions. While during the same time period, ammonia and DRP removal efficiencies of *Micractinium*, *Coelastrum,* and *Pediastrum* ranged between 51-61% and 29-32%, respectively and did not differ significantly relative to each other however their removal efficiencies were significantly higher ($p<0.05$) than those of *Desmodesmus* sp.. By day 4 under summer conditions, all species tested demonstrated a high nutrient removal capacity with >95% of ammonia and >85% DRP removed. These results are similar to those observed with cultures unicellular algal species [28, 94, 114, 115, 128, 129] which implies that colonial species are as beneficial as unicellular species for wastewater treatment and nutrient resource recovery.

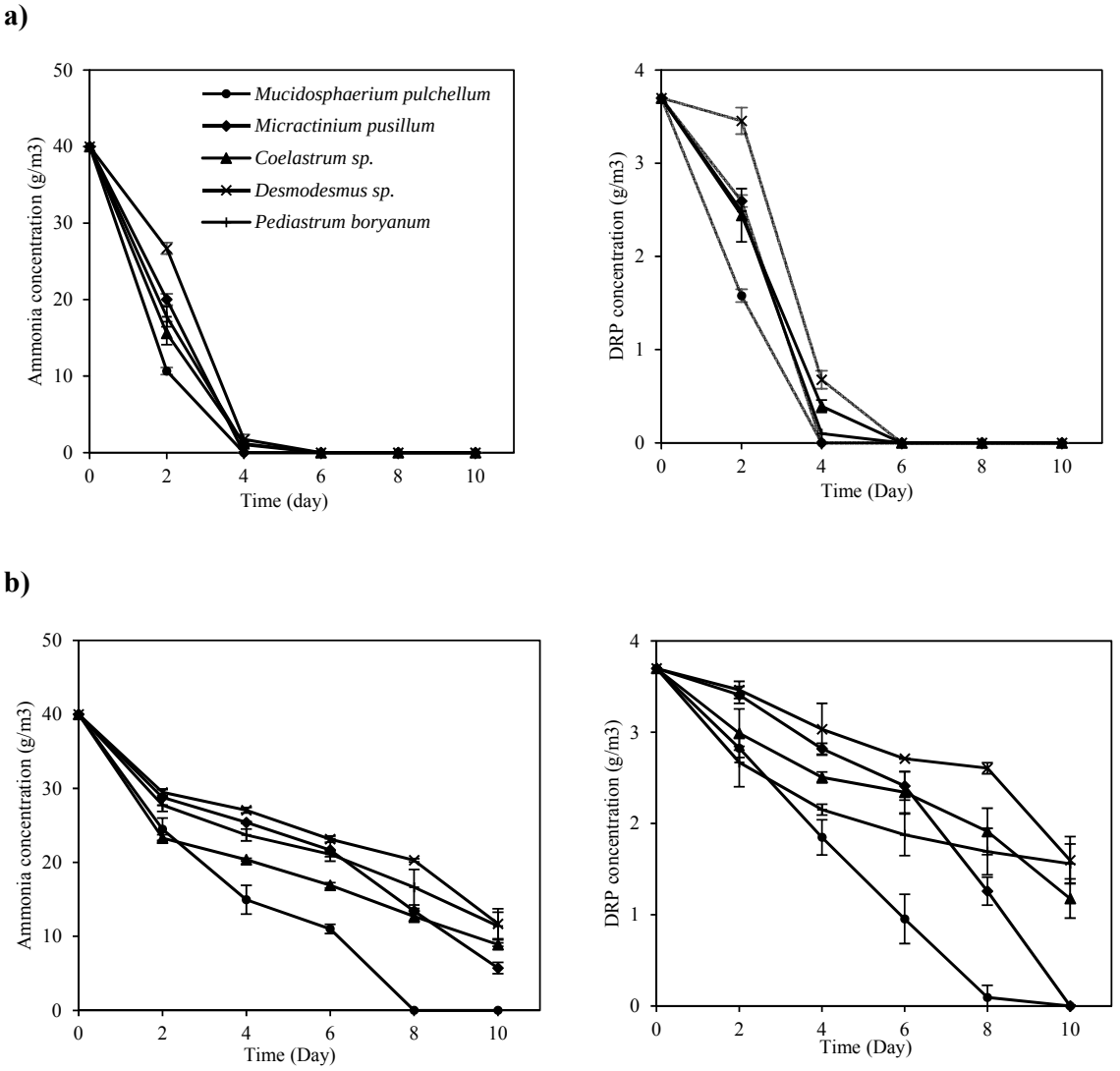


Figure 3.4. Nutrient concentrations in batch cultures of colonial algal species grown under simulated a) summer and b) winter conditions

Under winter conditions the rate of nutrient removal by all algal cultures was lower than under summer conditions, *Mucidosphaerium pulchellum* and *Micractinium pusillum* cultures were most efficient at removing nutrients but only *Mucidosphaerium pulchellum* had completely removed both nutrients by day 10. Nutrients are assimilated by algae through intracellular mechanisms hence the rate of nutrient removal was reduced in winter due to the reduction of the rates of photosynthetic reactions and enzymatic activities in response to decrease in light availability and water temperature [26, 104].

Table 3.3. Nutrient removal efficiencies on day 4 in summer and day 10 in winter

	Mucidosphaerium pulchellum	*Micractinium* sp.	*Coelasteum* sp.	*Desmodesmus* sp.	*Pediastrum boryanum*
Initial NH_4^+-N (g/m^3)	40				
Initial DRP (g/m^3)	4.7				
Summer conditions (Day 4)					
NH_4^+-N (g/m^3)	0	0	1.2 ± 0.3	1.7 ± 0.6	1.0 ± 0.4
%NH_4^+-N removal	100	100	97	96	97
DRP (g/m^3)	0	0	0.4 ± 0.06	0.7 ± 0.1	0.1 ± 0.02
%DRP removal	100	100	92	85	98
Winter conditions (Day 10)					
NH_4^+-N (g/m^3)	0	5.7 ± 0.7	8.9 ± 0.2	11.7 ± 2.0	11.4 ± 1.9
%NH_4^+-N removal	100	85	78	71	71.5
DRP (g/m^3)	0	0	1.5 ± 0.2	2.0 ± 0.2	2.0 ± 0.1
%DRP removal	100	100	68	57	57

3.3.4 Biochemical composition and energy content of algal biomass

The quality of algal biomass and beneficial biochemical composition for biofuel use is highly dependent on cultivation conditions [76]. Although several studies have investigated the biochemical composition of algal species grown on wastewater for biofuel production, there has been little evaluation of the composition and potential energy value of species grown on wastewater treatment HRAP. With this regards, the biochemical composition and energy content of the biomass of each colonial algal species are summarized in Table 3.4. The average total lipid content of the algal cultures varied between 14.4 ± 1.2 wt% and 48.2 ± 1.8 wt% depending on algal species, culture length and culture conditions. These lipid contents are in agreement with literature values for algal species grown on wastewater [111, 113, 115, 130].

Under summer conditions (by day 5) *Mucidosphaerium pulchellum* and *Micractinium pusillum* cultures had the highest lipid content (32.3 ± 3.0 wt% and 24.4 ± 2.4 wt% respectively) and the lowest protein content (37.2 ± 6.2 wt% and 40.3 ± 1.3 wt% respectively) of the algal species which were related to the complete removal of nutrients in these cultures. Both ammonia and DRP were totally removed from the *Mucidosphaerium pulchellum* and *Micractinium pusillum* cultures by day 4 under summer conditions and did not occur in the other algal cultures until day 6 (Fig. 3.4a). The nutrient limiting conditions experienced in the *Mucidosphaerium pulchellum* and *Micractinium pusillum* cultures from day 4 would have affected their biochemical composition. Nitrogen is one of the most

important macro elements contributing to algal biomass production and composition. Microalgae biomass comprises 1-10 wt% of nitrogen, and nitrogen plays an important role in regulating the protein and lipid content of algal cells [28, 116, 131]. Over the remainder of the 10 day summer cultivation period, the production of nutrient-based macromolecules such as proteins was reduced, and biomass production continued at slower rate using intracellular nutrients. This resulted in a >30% reduction in algal protein content from day 10 compared to day 5. The reduced protein content would have contributed to the decline in growth rate and algae approached stationary phase (Fig. 3.2a). Since, apart from N and P, other growth requirements such as light and CO_2 were available, the alga cells shifted their metabolism to produce non- nutrient-limited macromolecules such as lipids (except phospholipids) which can act as energy sink to prevent cell damage by photoinhibition [13, 26]. Therefore, the reduction in protein measured from day 5 to day 10 for all species, coincided with an increase in the lipid content by >30%. This value is in good agreement with literature values for various algal species cultivated under nitrogen-limiting conditions. Illman [23] showed that the protein content of five *Chlorella* strains cultivated under nitrogen limiting condition decreased whereas their lipid content increased by >70%. Rodolfi et al. [13] reported similar results for *Nannochloropsis* sp. and showed that the lipid content of species increased from 13 wt% to 50 wt% after cultivation under nitrogen deprivation for 18 days.

Under summer condition, the carbohydrate content of all species, except for *Pediastrum boryanum*, decreased which could have resulted from the complete removal of phosphorus from the cultures. Apart from nitrogen, phosphorus is also, an essential nutrient for cell wall construction and many other cellular processes including energy supply [132]. It has been shown that under phosphorus limiting conditions, carbohydrates are used to supply the energy for the intracellular processes of algae [22].

Table 3.4. Biochemical composition and energy content of colonial algal species

	Mucidosphaerium pulchellum		*Micractinium pusillum*		*Coelasteum* sp.		*Desmodesmus* sp.		*Pediastrum boryanum*	
Summer conditions										
	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10
Protein (wt%)	37.2 ± 6.2	13.5 ± 1.5	40.3 ± 1.3	16.8 ± 0.4	46.0 ± 5.5	28.1 ± 1.4	45.1 ± 7.5	27.1 ± 3.0	47.4 ± 4.6	30.2 ± 7.4
Carbohydrate (wt%)	29.3 ± 2.1	27.8 ± 6.9	34.5 ± 1.5	29.2 ± 5.7	38.2 ± 3.5	30.0 ± 3.4	28.2 ± 3.0	23.0 ± 1.0	28.5 ± 2.1	33.9 ± 2.1
Lipid (wt%)	32.3 ± 3.0	48.2 ± 1.8	24.4 ± 2.4	46.3 ± 3.6	14.4 ± 1.2	30.4 ± 0.9	21.6 ± 3.1	40.2 ± 0.9	22.1 ± 0.4	31.5 ± 5.8
Energy content	20	24.5	22	25	19.1	22	19.5	23.7	19.8	24.5

(kJ/g)										
Winter conditions										
Protein (wt%)	46.4 ± 2.7	37.0 ± 3.0	38.1 ± 3.5	39.6 ± 1.3	45.4 ± 0.4	44.1 ± 6.6	44.8 ± 4.6	43.7 ± 3.2	46.6 ± 1.4	40.3 ± 1.1
Carbohydrate (wt%)	25.8 ± 5.2	29.9 ± 4.7	37.4 ± 0.8	26.5 ± 0.8	28.6 ± 2.7	30.4 ± 1.6	23.2 ± 3.5	31.2 ± 0.4	28.1 ± 5.3	29.1 ± 7.1
Lipid (wt%)	27.1 ± 1.5	31.0 ± 3.3	21.8 ± 4.1	30.9 ± 0.7	23.2 ± 1.3	15.1 ± 3.2	31.4 ± 3.2	25.3 ± 2.6	24.1 ± 5.2	23.9 ± 3.7
Energy content (kJ/g)		21.1		19.9		18.1		17.7		19

Although several studies have investigated the biochemical composition of algal species, there has been a little evaluation of species biochemical composition grown under cold weather conditions would help select the most beneficial species for year-round wastewater treatment and biofuel production. Under winter conditions, *Mucidosphaerium pulchellum* had the highest lipid content (27.1 ± 1.5 wt%) on day 5 while both *Mucidosphaerium pulchellum* and *Micractinium pusillum* had the highest lipid content (31.0 ± 3.3 wt% and 30.9 ± 0.7 wt%, respectively) on day 10. The increase in lipid content was probably due to the reduction in the ammonia concentration in the cultures. In contrast, the lipid content of *Desmodesmus*, *Coelastrum* and *Pediastrum* reduced from day 5 to day 10. This may have been due to these cultures being in a more active growth stage on day 10 than on day 5 when they were only just coming out of lag phase.

The energy content of the algal species ranged from 17.7 kJ/g to 25 kJ/g (Table 3.4) with higher values for *Mucidosphaerium pulchellum* and *Micractinium pusillum* under both summer and winter conditions. These values are in a good agreement with literature values of 18-24 kJ/g [22, 23]. Algal energy content is a function of cell protein (24 kJ/g), carbohydrate (17 kJ/g), and lipid (37 kJ/g) ratios. Typically 30-50% of algal energy content comes from lipids and there is a positive relationship between lipid and energy content of algal biomass [22, 23, 119]. The higher biomass energy content of *Mucidosphaerium pulchellum* and *Micractinium pusillum* cultures under both summer and winter conditions is due to the higher lipid:protein:carbohydrate ratio in these cultures.

3.4 Which species is more beneficial for combined wastewater treatment and biomass energy production?

Since the primary objective of using algae in WWT HRAP is efficient wastewater treatment with nutrient recovery, the species chosen to be cultivated in WWT HRAP for biofuel production should have these qualities as well as high productivity and lipid content.

Moreover, species that are easily harvested (ideally by low-cost gravity settling) are necessary for both effective treatment and economical biofuel production.

Of all species investigated in this study, *Mucidosphaerium pulchellum* and *Micractinium pusillum* appear to be the most promising species for an integrated wastewater treatment and algal biomass production for biofuel due to their high nutrient removal capacity, high growth rate, and high lipid and energy content under both New Zealand summer and winter culture conditions. These characteristics enable treatment of more wastewater, and production of more energy-rich biomass than the other species tested. *Mucidosphaerium pulchellum* and *Micractinium pusillum* had the highest exponential phase volumetric productivity under both summer and winter simulated conditions (188.9 ± 10 mg/L/day under summer conditions and 57.2 ± 3 mg/L/day under winter conditions for *Mucidosphaerium pulchellum*, and 177.2 ± 11 mg/L/day under summer conditions and 40.9 ± 4 mg/L/day under winter conditions for *Micractinium pusillum*) (Table 3.5). Although, achieving such high volumetric biomass and energy productivities in actual outdoor conditions is not possible due to environmental, operational and biological factors that limit the biomass productivity [12, 119].

Of the two promising species identified in this study, it has been shown that *Mucidosphaerium pulchellum* is poorly settleable 9, 22]. Sutherland et al. [103] showed that <5% and <45% of a *Mucidosphaerium pulchellum* culture settled within 10 min and after 90 min, respectively, while >55% of *Pediastrum boryanum* culture biomass settled after only 10 min. This implies that more chemicals and energy would be required to harvest *Mucidosphaerium pulchellum* from the treated wastewater to enable the biomass to be used as biofuel feedstock. So without further research to find a cost-effective harvest method *Mucidosphaerium pulchellum* does not seem to be a promising candidate for low-cost wastewater treatment and biofuel production.

Previous studies [11, 133-135] and our observations from monitoring pilot-scale WWT HRAPs [119], have shown that unlike *Mucidosphaerium pulchellum*, *Micractinium pusillum* can be easily harvested by sedimentation in a similar way to *Pediastrum boryanum*. However, when grown in laboratory pure culture, *Micractinium pusillum* grows as either single cells or small colonies which have low harvestability. When cultured under outside conditions with the inevitable presence of zooplanktons the cells develop spines and form flocs, which greatly enhance biomass settleability [81, 121, 135]. Therefore, further research is required to understand how the HRAP invertebrate population can be controlled to minimize the productivity loss from grazing but improve the dominance and harvestability of

Micractinium pusillum. Since the growth rates, productivity and energy content of *Pediastrum boryanum* and *Micractinium pusillum* under New Zealand summer conditions were quite similar and *Pediastrum boryanum* is less vulnerable to being grazed [93], another possible strategy is to promote the dominance of *Pediastrum boryanum* in WWT HRAP in warmer months and *Micractinium pusillum* in colder months.

Table 3.5. Volumetric biomass, lipid and biomass energy yield calculated based on specific growth rate, lipid and energy content at day 5 in summer and day 10 in winter

	Mucidosphaerium pulchellum		*Micractinium pussilum*		*Coelastrum* sp.		*Desmodesmus* sp.		*Pediastrum boryanum*	
	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Volumetric biomass productivity (mg/L/day)	188.9 ± 10	57.2 ± 3	177.2 ± 11	40.9 ± 4	157.2 ± 8	29 ± 10	169.3 ± 17	14.4 ± 7	156.6 ± 20	18.7 ± 5
Volumetric lipid productivity (mg/L/day)	61.0 ± 2.3	17.7 ± 1.2	43.2 ± 3.1	12.6 ± 1.0	22.6 ± 1.5	4.8 ± 0.5	36.6 ± 1.6	3.6 ± 1.1	34.6 ± 2.2	4.5 ± 0.2
Volumetric energy productivity (kJ/L/day)	3.8 ± 0.6	1.2 ± 0.2	3.9 ± 0.3	0.8 ± 0.1	3.0 ± 0.4	0.5 ± 0.1	3.3 ± 1.2	0.2 ± 0.1	3.1 ± 0.2	0.3 ± 0.1

3.5 Conclusions

The wastewater treatment and the biomass energy yield potential of five wastewater colonial algal species typically found in the wastewater treatment high rate algal ponds were investigated under New Zealand summer and winter conditions. In summer, all species grew well and removed nutrients completely by day 6, while under winter conditions, only *Mucidosphaerium pulchellum* and *Micractinium pusillum* showed efficient nutrient removal. *Mucidosphaerium pulchellum* and *Micractinium pusillum* also had the highest growth rates and showed the highest biomass productivities under both conditions (*Mucidosphaerium pulchellum:* 0.59 ± 0.01 d^{-1} and 188.9 ± 10 mg/L/day in summer, and 0.38 ± 0.03 d^{-1} and 57.2 ± 3 mg/L/day in winter; *Micractinium pusillum:* 0.57 ± 0.02 d^{-1} and 177.2 ± 11 mg/L/day in summer, and 0.29 ± 0.01 d^{-1} and 40.9 ± 4 mg/L/day in winter). Moreover, biochemical composition and energy analysis indicated that *Mucidosphaerium pulchellum* and *Micractinium pusillum* had the highest lipid and energy content. However, previous research

has shown that *Mucidosphaerium pulchellum* has poor harvesability compared with *Micractinium pusillum*, indicating that *Micractinium pusillum* is the most promising species for both wastewater treatment and biofuel feedstock production.

CHAPTER 4

Effect of CO_2 addition on biomass energy yield in wastewater treatment high rate algal mesocosms (WWT HRAM)

This chapter is based on the following publication:

Mehrabadi, A., Farid, M. M., Craggs, R. Effect of CO_2 addition on biomass energy yield in wastewater treatment high rate algal mesocosms. *Algal Research*, 22, 93-103.

Chapter preface

This chapter investigates the effect of CO_2 addition on algal biomass energy yield in WWT HRAM. Two experiments were conducted using HRAMs under outdoor conditions during summer and winter. The effects of CO_2 augmentation were evaluated by operating HRAMs with continuous sparging of different CO_2:air mixtures including air (control mesocoms), 0.5%, 2%, 5% and 10% CO_2. Performance was compared by determining the productivity, chlorophyll a content, chemical composition, energy content, algal species composition and settleability of the biomass. The total biomass energy yield and its gravity harvestable proportion (calculated by multiplying biomass concentration, energy content and harvest efficiency) were highest for the 5% CO_2-HRAMs in summer and for the 0.5% CO_2-HRAMs in winter. These results show that CO_2 addition (indicated by maintaining a culture pH of 6-7 in summer and 7-8 in winter) not only improves biomass productivity and energy content but selects for easily harvestable colonial algal species which are less susceptible to zooplankton grazing.

4.1 Introduction

Microalgal biomass cultivated as a by-product of wastewater treatment in high rate algal ponds (WWT HRAPs) has been highlighted as a promising feedstock to reduce production costs for community-level algal based biofuel production [14, 76]. WWT HRAPs offer a niche opportunity by producing harvested algal biomass during near tertiary-level treatment of wastewater [6] without the addition of nutrient fertiliser and using simple gravity sedimentation to harvest and concentrate the biomass to 1- 2 wt% solids [6].

The biomass energy yield potential of WWT HRAP is a function of productivity, algal dominance, chemical composition, and harvestability of the biomass [12, 119]. However, all these parameters become limited by environmental, operational and biological factors. Our previous study of a pilot-scale HRAP showed that the biomass energy yield potential of WWT HRAP was highly dependent on climate conditions (decreasing by >250% from summer to winter) and zooplankton grazing pressure (decreasing by >50% within few days during a summertime zooplankton bloom) [119].

To enhance the biomass energy yield of WWT HRAP different practical strategies can be employed such as: CO_2 addition which can improve biomass productivity, algal and lipid content, and lipid profile; optimizing hydraulic retention time (HRT) which can increase biomass energy content through increasing biomass lipid content; biomass recycling which

can increase biomass productivity, energy content and harvestability by promoting the dominance of readily harvestable algae; and zooplankton control which can prevent productivity loss due to grazing and increase the settleability of the biomass (since some of the algal species develop spines as a defence mechanisms in presence of grazers which could further improve the algal-bacterial flocs formation) [12, 76, 81]. However, further research is needed to identify the most practical strategy/strategies to improve WWT HRAP performance both in terms of sustainable treatment and production of energy-rich biomass.

Of these strategies, CO_2 addition has been recommended for several reasons including: 1) to avoid high pH (>8.5) inhibition and free ammonia toxicity of algal and aerobic bacterial growth, 2) to increase the availability of ammonium and dissolved reactive orthophosphate for algal uptake, 3) to increase the C/N ratio of the wastewater (typically 3:1) in the pond to overcome carbon limitation for algal assimilation of all wastewater nutrients, 4) to enhance the proportion of algae in the pond biomass (typically 2.5-4:1), and 5) to improve the lipid content and profile of the algal biomass in terms of lowering polyunsaturated fatty acids [8, 15, 32, 102, 136-139]. Although pH reduction and increasing C/N ratio of the wastewater can be done by adding organic acids, as conversion of organic carbon to CO_2, before assimilation, is an energy intensive pathway the algal growth rate would not be as high as using CO_2 directly. Park and Craggs [8] found that WWT HRAP biomass productivity was improved by 30-50 % by CO_2 addition and maintaining the day-time maximum pond water pH below 8 during summer conditions. Sutherland et al. [102]investigated the effects of CO_2 addition on wastewater microalgae performance showing that, in summer, the biomass concentration and the algal biovolume of CO_2 enriched HRAMs (pH 6.5) were enhanced by 22-45% and 100-560%, respectively compared with HRAM without CO_2 addition. They also found similar results under winter conditions where, the maximum photosynthetic rate, biomass concentration and algal biovolume increased by up to 172%, 20% and 181%, respectively in 2% and 5% CO_2 HRAMs [138]. As stated earlier, the culture CO_2 concentration also affects algal lipid content. Sun et al. [140] reported >100% enhancement of the lipid content of batch cultures of *Chlorella sorokiniana* sparged with a 10% CO_2-air mixture compared to control cultures which were sparged with air.

Despite there being several studies on the positive effects of CO_2 addition on the performance of wastewater microalgae in HRAPs (in terms of nutrient removal, and quantity and quality of the biomass), there have been no publications that have investigated the effects of CO_2 addition on biomass energy yield in WWT HRAP. This study investigates, for the first time, the effects of different CO_2 addition concentrations on parameters that influence

total and gravity harvestable biomass energy yield including: productivity, harvest efficiency, biochemical composition and energy content.

4.2 Materials and methods

4.2.1 Experimental set-up

To study the effect of different CO_2 addition concentrations on the biomass energy yield of HRAMs, two outdoor experiments were conducted using fifteen replicate foil-wrapped plastic mesocosms (water depth: 0.3 m; volume: 16 L; surface area: 0.06 m^2) at the Ruakura Research Centre, Hamilton, New Zealand ($37^0$47′S, $175^0$19′E). The mesocosms were wrapped to ensure sunlight only entered through the water surface. Experiment 1 was carried out in January 2014 (NZ summer) over 21 days and Experiment 2 was conducted in July and August 2014 (NZ winter) over 30 days. The HRAMs were inoculated from an adjacent pilot-scale HRAP and they were mixed continuously using magnetic stirrers. During the summer experiment the mesocosms were operated with a 4 day HRT by replacing 4 litres of culture with primary settled sewage every morning (at ~ 9 am). During the winter experiment the mesocosms were operated with an 8 day HRT by daily replacement of 2 litres of culture with primary settled sewage. The experiments were conducted without control of zooplankton grazers or dominant algal species.

Supplementary carbon was supplied to the cultures in the form of CO_2:air mixtures. The CO_2 addition system consisted of four CO_2 gas cylinders, a gas regulator, a gas flow meter (0–12 L·min− 1 range), an air pump and gas diffusers (Fig. 4.1). The CO_2 gas was blended with air (via the air pump) to provide different CO_2 concentrations including air (control mesocosm), 0.5%, 2%, 5% and 10%. The CO_2 addition system was similar to the system used by Sutherland et al. [102]. The sparged CO_2 concentration (in gas phase) and culture pH (in liquid phase) were measured regularly during the daytime using a portable gas analyser (Biogas 5000, Geotech) and pH meter (TPS WP-91, TPS Pty. Ltd., Springwood Australia) respectively. The gas blends were continuously bubbled into the cultures at 10 L·min− 1 through a gas diffuser placed on the bottom of the HRAMs. Daily air temperature, solar radiation, evaporation and rainfall data were downloaded from NIWA's National Climate Database (http://cliflo-niwa.niwa.co.nz.ezproxy.auckland.ac.nz/).

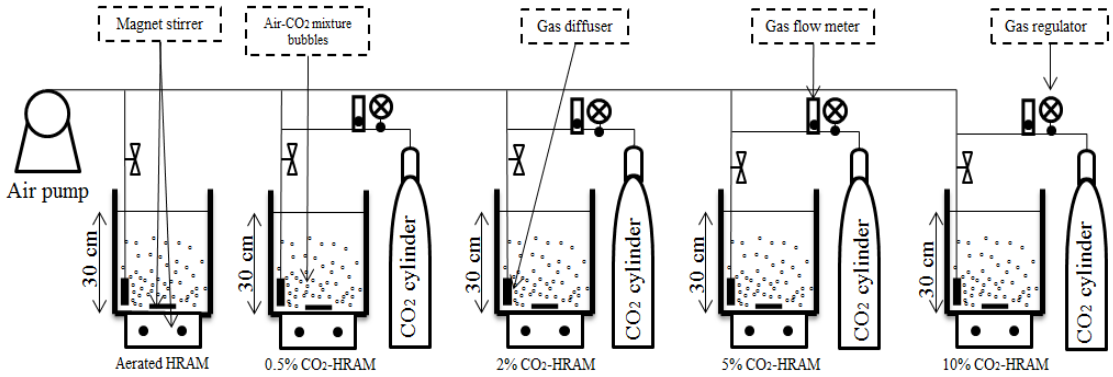


Figure 4.1. Schematic diagram of high rate algal mesocosms (HRAMs) supplemented by different CO_2:air mixtures

4.2.2 Measurement of nutrient concentration

Concentrations of dissolved nutrients (Ammonium (NH_4^+-N), nitrate and dissolved reactive orthophosphate (PO_4^{3-}-P, DRP)) in the HRAM influent and effluents were analyzed twice a week. Samples were filtered through Whatman GF/F filters and concentrations of ammonium (NH_4^+-N), nitrate (NO_3^--N) and DRP were determined colorimetrically according to standard methods (APHA 2008) using a spectrophotometer (HACH RD2008, Germany).

4.2.3 Algal relative abundance

The relative abundance (%) of algal species present in the HRAM was determined by comparing their biovolume as described previously [119]. Biovolume was calculated by counting the numbers of cells of each algal species and multiplying by the mean cell biovolume for that species, assessed according to the equations of Vadrucci et al. [87].

4.2.4 Measurement of biomass chlorophyll a (Chl-a) content

The biomass HRAM effluent chl-a content (as a proxy for the proportion of algae in the biomass) was determined spectrophotometrically using a Shimadzu UV 1601 spectrophotometer as described previously [119]. The chl-a content of biomass filtered from a known volume of HRAM effluent was extracted by boiling in methanol at 65 ºC for 5 min and then refrigerating at 4 ºC in the dark for 12 h. Chl-a concentrations were calculated from the absorbance of the supernatant after centrifuging (at 3000 rpm for 15 min) using the trichromatic equations for methanol [88].

4.2.5 Measurement of biomass productivity and 1h-harvest efficiency

The areal biomass productivity (g VSS/m^2/day) was calculated using the following Equation (4-1) [12]:

$$P = \frac{X \times Q_c}{A} \qquad (4-1)$$

$$Q_c = Q_{inf} + ((rainfall - evaporation) \times surface\ area)$$

where P is the areal biomass productivity, X is the WWT HRAM biomass concentration (g VSS/m^3, that is also used to calculate biomass energy yield), A is the WWT HRAM surface area (m^2), Q_{inf} is the WWT HRAP daily inflow (m^3/d) and Q_c is the daily outflow (m^3/d) corrected for net evaporation. A known volume of HRAM effluent was filtered onto a pre-rinsed, pre-combusted and pre-weighed Whatman GF/F filter, dried (at 80 ºC overnight), cooled in desiccator and weighed to determine the total suspended solids (TSS) concentration. The samples were then ashed at 550 °C for 1 h in a muffle furnace (F.E. KILN, RTC1000, Bartlett Instrument Company, UK) and cooled in a desiccator before weighing. The VSS was calculated as the difference between the TSS and residue on the filter after combustion.

The harvest efficiency of the biomass in the HRAM effluents was measured using Imhoff cones twice a week throughout the study. A known volume (1L) of mesocosm effluent was placed into each Imhoff cone and after one hour and the gravity settled biomass was collected and dried at 80 ºC overnight and weighted after cooling in a desiccator. The 1h-harvest efficiency of the biomass was calculated by comparing of the weight of the settled biomass to the total suspension solids (TSS).

4.2.6 Measurement of biomass biochemical composition and energy content

The biomass chemical composition (protein, carbohydrate and lipid fraction) was measured twice a week according to modified standard methods described previously [119]. The energy content of the biomass was measured three times during the experiment using an oxygen bomb calorimeter (Parr 1341, Parr Instrument Company, Moline, IL 61265 USA).

4.2.7 Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) in Excel software (Excel, Microsoft office 2010).

4.3 Results and discussion

4.3.1 Environmental variables and HRAM operational conditions

Mean daily ambient temperature and irradiance of both experiments are shown in Fig. 4.2. The average daily ambient temperature and irradiance were 19.6 ± 2.8 °C and 24.7 ± 7.7

MJ/m^2, respectively in Experiment 1 (summer) and 9.5 ± 1.3 °C and 8.5 ± 2.6 MJ/m^2, respectively in Experiment 2 (winter). Minimum and maximum values of environmental parameters (air temperature and light intensity) occurred in the early morning and mid-afternoon (3 pm), respectively. Mesocosm water pH varied with changing CO_2 concentration so that the HRAMs supplemented with higher concentrations of CO_2 had lower day-time pH. In the summer experiment the mesocosm water pH ranged between 8.1 and 10.1 in the aerated HRAMs, 6.8 and 7.9 in the 0.5% CO_2-HRAMs, 6.2 and 7.3 in the 2% CO_2-HRAMs, 6 and 6.8 in the 5% CO_2-HRAMs, and 5.9 and 6.3 in the 10% CO_2-HRAMs. The pH ranges of the HRAMs in the winter experiment were similar to those in summer experiment, except that the pH of the control HRAMs only ranged between 7.5 and 9.1 due to reduced algal biomass and photosynthesis in response to the lower winter sunlight levels. Minimum and maximum pH occurred in the early morning and mid-afternoon (3 pm), respectively. The largest diurnal variation of the pH values typically occurred in control cultures, while the lowest variation occurred in the 10% CO_2-HRAMs.

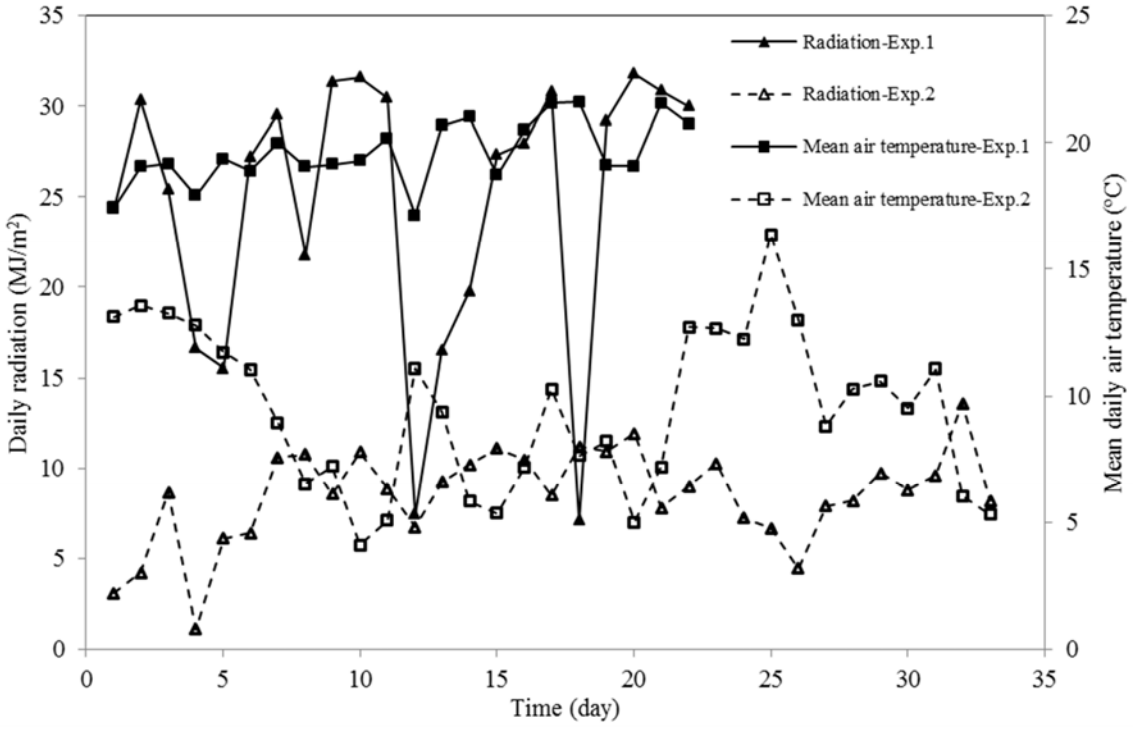


Figure 4.2. The average daily air temperature and diurnal radiation during the CO_2 experiments

4.3.2 Nutrient concentration and removal efficiency

The average influent concentrations of NH_4^+-N and DRP were 36.1 ± 10.3 mg/L and 4.1 ± 0.9 mg/L, respectively in the summer experiment; 32.3 ± 8.2 mg/L and 4.3 ± 1.1 mg/L, respectively in the winter experiment. The nitrate concentration was negligible compared to

ammonium concentration. During the summer experiment 60-87% of ammonia and 42.9-61.2% of DRP were removed by microalgae in HRAMs, while removal efficiency of both nutrients was ~50% lower in the winter experiment (Table 4.1). During the summer experiment the control HRAMs had the highest nutrient removal and this was likely due to ammonia volatalization and phosphate precipitation at the high pH (>8.5) [8]. While nutrient removal efficiencies in other mesocosms increased with higher CO_2 concentration (Table 4.1). In winter, CO_2 addition did not affect significantly the nutrient removal due to acidification of algae.

Table 4.1. Average dissolved nutrients concentration and nutrient removal in the different HRAMs during the CO_2 addition experiments

Parameter	**Influent**	**Effluent**				
		Air	0.5% CO_2	2% CO_2	5% CO_2	10% CO_2
The summer experiment						
NH_4^+-N (mg/L)	36.1 ± 10.3	5.0 ± 0.6	14.5 ± 1.0	14.1 ± 0.4	13.3 ± 0.2	12.6 ± 0.3
% NH_4^+-N removed		86.7 ± 7.5	60.7 ± 8.2	60.4 ± 6.1	63.7 ± 4.8	64.3 ± 5.7
DRP (mg/L)	4.1 ± 0.9	2.5 ± 0. 4	2.1 ± 0.2	1.9 ± 0.3	1.6 ± 0.1	1.7 ± 0.3
% DRP removed		61.2 ± 8.9	42.9 ± 5.3	51.6 ± 6.5	55.4± 3.7	58.5 ± 7.1
The winter experiment						
NH_4^+-N (mg/L)	32.3 ± 8.2	20.0 ± 2.5	22.2 ± 1.3	21.9 ± 2.2	22.8 ± 1.8	23.6 ± 0.9
% NH_4^+-N removed		38.2 ± 7.2	30.2 ± 5.1	31.5 ± 6.2	29.2 ± 5.6	26.8 ± 4.2
DRP (mg/L)	4.3 ± 1.1	3.0 ± 0.3	2.9 ± 0.4	2.8 ± 0.2	3.0 ± 0.1	3.3 ± 0.4
% DRP removed		30.2 ± 6.1	30.4 ± 8.2	32.6 ± 5.1	29.6 ± 3.2	22.2 ± 7.8

4.3.3 Concentration, productivity and algal proportion of the biomass

Increasing biomass productivity (especially algal productivity) is a critical issue in the concept of using WWT HRAP for algal biofuel production. The average biomass concentration (organic matter, g VSS/m^3) and the biomass chl-a content (as a proxy for the proportion of algae) of the HRAMs are summarized in Table 4.2 and the average biomass productivity (g VSS/m^2/day) of the HRAMs is shown in Fig. 4.3. In the summer experiment increasing the CO_2 concentration resulted in enhancement of both the biomass concentration and the proportion of algae. The biomass concentration and the biomass chl-a content in 2%, 5% and 10% CO_2-HRAMs were significantly higher than those of the control ($p<0.05$) which were in agreement with the literature [102]. The increase in the chl-a/biomass concentration ratio during the summer experiment implies that the biomass productivity enhancement was

likely due to algal proliferation. In the winter experiment, the biomass and chl-a concentrations either did not increase significantly, or even decreased relative to the control in response to increased CO_2 concentration. This was probably due to inhibition of the growth of microbial community and algal photosynthesis at low pH and suboptimal environmental conditions. It has been shown that the optimum pH range for bacterial growth is 8 [138]. At high levels of dissolved inorganic carbon (DIC) in the culture and under low temperature-low light conditions, the key enzymes that catalyse entry of CO_2 into the Calvin cycle (the process that plants and algae use to convert CO_2 to essential cell components) are inactivated. Acidification of chloroplast stroma results in inhibition of photosynthesis and reduced algal growth [141, 142].

Table 4.2. The biomass and chl-a concentrations of the different HRAMs during the CO_2 addition experiments

	Air	0.5% CO_2	2% CO_2	5% CO_2	10% CO_2
The summer experiment					
Biomass concentration (g VSS/m^3)	215.6 ± 31.2	234.9 ± 45.2	293.8 ± 54.3	309.3 ± 46.9	333.2 ± 41
Chl-a (mg/m^3)	2925 ± 601	3151 ± 746	4014 ± 713	5091 ± 1004	5541 ± 1524
The winter experiment					
Biomass concentration (g VSS/m^3)	151.5 ± 44.5	163.4 ± 48.1	161.7 ± 40.3	150 ± 36.7	129.9 ± 33.6
Chl-a (mg/m^3)	1680 ± 677	1733 ± 748	1588 ± 752	1303 ± 691	954 ± 499

The biomass productivity ranged between 11.2 to 23.5 g VSS/m^2/day in summer; and 3.4 to 7.4 g VSS/m^2/day in winter (Fig. 4.3). The biomass productivity of the HRAMs, in the summer experiment, increased whenever zooplankton grazers did not bloom (Fig. 4.3a). The biomass productivities of mesocosms supplemented by 2%, 5% and 10% CO_2 were significantly higher ($P<0.05$) relative to the biomass productivity of the aerated and 0.5% CO_2-HRAMs. The highest mean biomass productivity (20.9 g VSS/m^2/day) occurred in 10% CO_2-HRAMs while the lowest mean biomass yield was occurred in aerated cultures (13.5 g VSS/m^2/day). Inhibition of algae and bacteria growth by a high free ammonia concentration at high pH (>8.5) during the entire culture period as well as grazing by a bloom of zooplankton between day 11 and day 18 resulted in low overall productivity of the aerated (control) cultures. At high pH (>8.5) ammonium (NH_4^+-N) converts to ammonia (NH_3) which is toxic and inhibits the activity of wastewater algal and bacterial community [102, 137]. Increases in the concentration, productivity and algal proportion of the biomass in response to increased CO_2 concentration resulted from several potential pathways:

1) Elevated CO_2 concentration and reduced pH increase the availability of nutrients and decrease ammonia toxicity, since ammonium converts to ammonia gas and DRP precipitates at pH> 8.5;

2) Increased DIC concentration increases light utilisation and the photosynthetic rate of algae [102, 138];

3) The activity of carbon-concentrating mechanisms (CCMs) declines in response to increased CO_2 availability. Based on the alkalinity at lower pH inorganic carbon will bemore in the form of CO_2. As CO_2 is the preferential form of inorganic carbon it will reduce considerable amount of energy involved in CCMs,, lowering CCM activity means that more energy is available for growth [141, 143];

4) Changes in the dominant algal species and decreased zooplankton population at high culture CO_2 concentration (lower pH). Zooplankton invertebrates did not bloom in the mesocosms with high CO_2 concentration (low pH) and therefore, the high CO_2 HRAMs were dominated by fast growing species. For example, *Pediastrum* sp. was dominant in aerated HRAM while, in the 10% CO_2-HRAM *Micractinium* sp. was dominant (explained by details in section 3.4). It has been shown by Park et al. [85] that the growth rate of *Micractinium* sp. is higher than that of both *Pediastrum* sp. and *Scenedesmus* sp. (the most abundant species in these experiments) in zooplankton grazer-free cultures. Sutherland et al. [102], also, found that in zooplankton-free cultures operated at low pH (pH 6.5) *Micractinium* biovolume doubled compared with *Pediastrum* biovolume. Hence, as *Micractinium* sp. became dominant, the biomass concentration and consequently productivity increased due to its high growth rate.

Biomass productivities of all HRAMs in the winter experiment were a third to a quarter lower than those in the summer experiment due to less favourable environmental conditions. Productivities in all treatments gradually increased over the course of the winter experiment as the daily solar radiation and mean daily temperature increased (Fig. 4.2). The average biomass productivity in the winter experiment ranged between 4.5 and 5.6 g VSS/m^2/day. In contrast to the summer experiment, the biomass productivity did not differ significantly between HRAMs supplemented by different CO_2-air mixtures or even reduced in response to CO_2 addition. The highest productivity occurred in the 0.5% CO_2-HRAMs, while the lowest productivity occurred in the 10% CO_2-HRAMs due to inhibition of algal and bacterial growth at the low pH achieved under winter conditions (low solar radiation and water temperature, Fig. 4.2). This inhibition may result from the reduction of the activity of the microalgae carbon concentrator enzyme at such low pH [144]. Moreover, the pH of the control rarely

exceeded 8.5, hence, the biomass productivity of the control was similar to the other mesocosms.

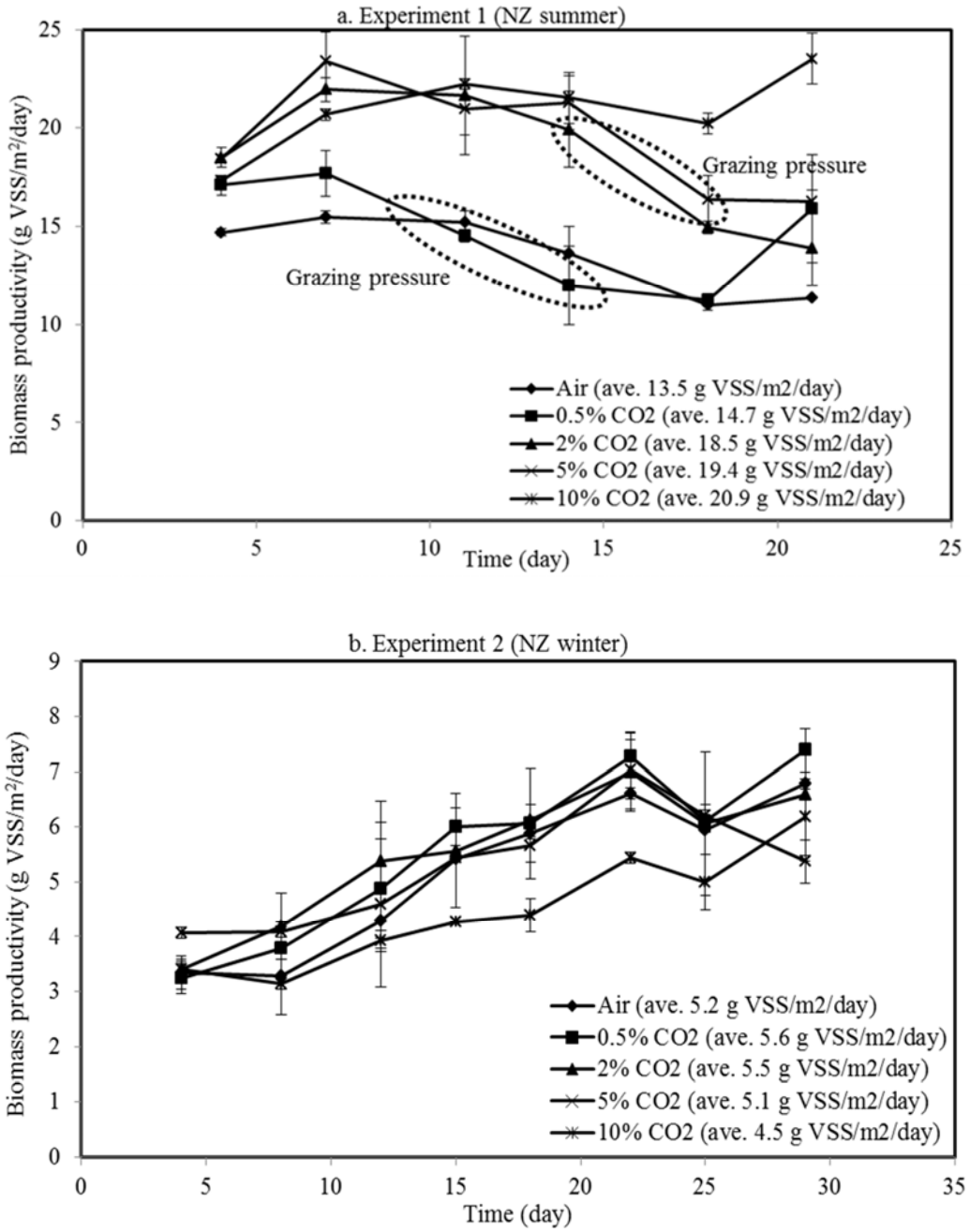


Figure 4.3. Biomass productivity of the HRAMs during the CO_2 experiments

4.3.4 Chemical composition and energy content of the biomass

The biochemical composition and the energy content of the biomass produced in HRAMs supplemented by different CO_2-air mixtures are summarized in Table 4.3. The average biomass lipid fraction and biomass energy content ranged 18 – 26.1 wt% and 19.3 – 21.6

kJ/g, respectively in the summer experiment which were higher than those (16.8 – 17.7 wt% and 20.8 - 22.8 kJ/g, respectively) in the winter experiment. The biomass lipid content, decreased gradually with increased CO_2 concentration in both experiments, which is in contrast to previous studies which has shown a positive relationship between algal lipid content and CO_2 concentration in algal monoculture experiments [31, 140], This was probably due to the changes in algal species observed in this study at the different CO_2 concentrations and culture pH levels. In the summer experiment, the biomass lipid content of the aerated HRAMs was significantly ($p<0.05$) higher than that of the other mesocsosms which did not differ significantly from each other. As day-time pH of the aerated mesocosms was usually high (>8.5), much of the ammonium may have been converted to ammonia gas and volatilized, leading to nitrogen-limiting conditions, reduced protein synthesis and increased biomass lipid content. Park and Craggs [8], also, found that the lipid content of biomass harvested from HRAP maintained at pH <8 by CO_2 addition decreased by 20% compared with the biomass harvested from HRAP without CO_2 addition. The slight changes in the biomass chemical composition of the other treatments, (which had pHs lower than 8 and no nitrogen limitation), were probably due to changes in algal composition.

In both experiments, there was little difference in the biomass energy content between treatments. HRAP biomass energy content has been previously reported as a function of the microbial and chemical composition of the biomass and therefore enhanced by increasing the algal and lipid proportion of the biomass [119]. The slight changes in biomass energy content were probably due to changes in the proportion of algae in the biomass, algal population dynamics and the biomass lipid profile.

Table 4.3. The biochemical composition and energy content of the HRAMs biomass during the CO_2 addition experiments

	Protein (wt%)	Carbohydrate (wt%)	Lipid (wt%)	Energy content (kJ/g)
The summer experiment				
Air	46.7 ± 7.0	19.4 ± 1.7	26.1 ± 4.0	21.6 ± 1.0
0.5% CO_2	50.4 ± 5.9	18.6 ± 1.7	20.5 ± 3.5	20.0 ± 2.2
2% CO_2	48.9 ± 5.4	17.6 ± 1.4	19.1 ± 3.2	19.3 ± 1.0
5% CO_2	54.7 ± 5.9	16.2 ± 2.6	18.0 ± 3.7	21.2 ± 0.5
10% CO_2	48.9 ± 4.8	19.5 ± 2.8	18.1 ± 2.4	19.8 ± 1.5
The winter experiment				
Air	45.0 ± 4.0	22.1 ± 1.5	17.6 ± 2.1	21.0 ± 0.5
0.5% CO_2	48.2 ± 4.1	22.5 ± 2.1	17.7 ± 1.9	20.8 ± 0.6
2% CO_2	45.7 ± 3.3	23.4 ± 2.2	16.8 ± 2.4	21.2 ± 2.7
5% CO_2	46.0 ± 5.7	24.2 ± 3.3	17.3 ± 2.0	22.8 ± 0.6
10% CO_2	45.8 ± 6.7	23.7 ± 1.9	17.3 ± 1.7	22.2± 1.0

4.3.5 Biomass harvestability

Efficient biomass harvest is one of the main challenges to overcome to produce economical algal biofuel. It has been suggested that harvest costs could account for up to 50% of total algal cultivation costs [17]. Therefore, developing a cost-effective biomass harvest method is crucial, and the effect of any strategy chosen to improve WWT HRAP performance, on biomass settleability should be considered. Although it has been reported that biomass productivity should increase in response to increased DIC concentration, the effect of CO_2 concentration on biomass settleability which is highly dependent on algal species [11, 12, 17] has not been investigated previously.

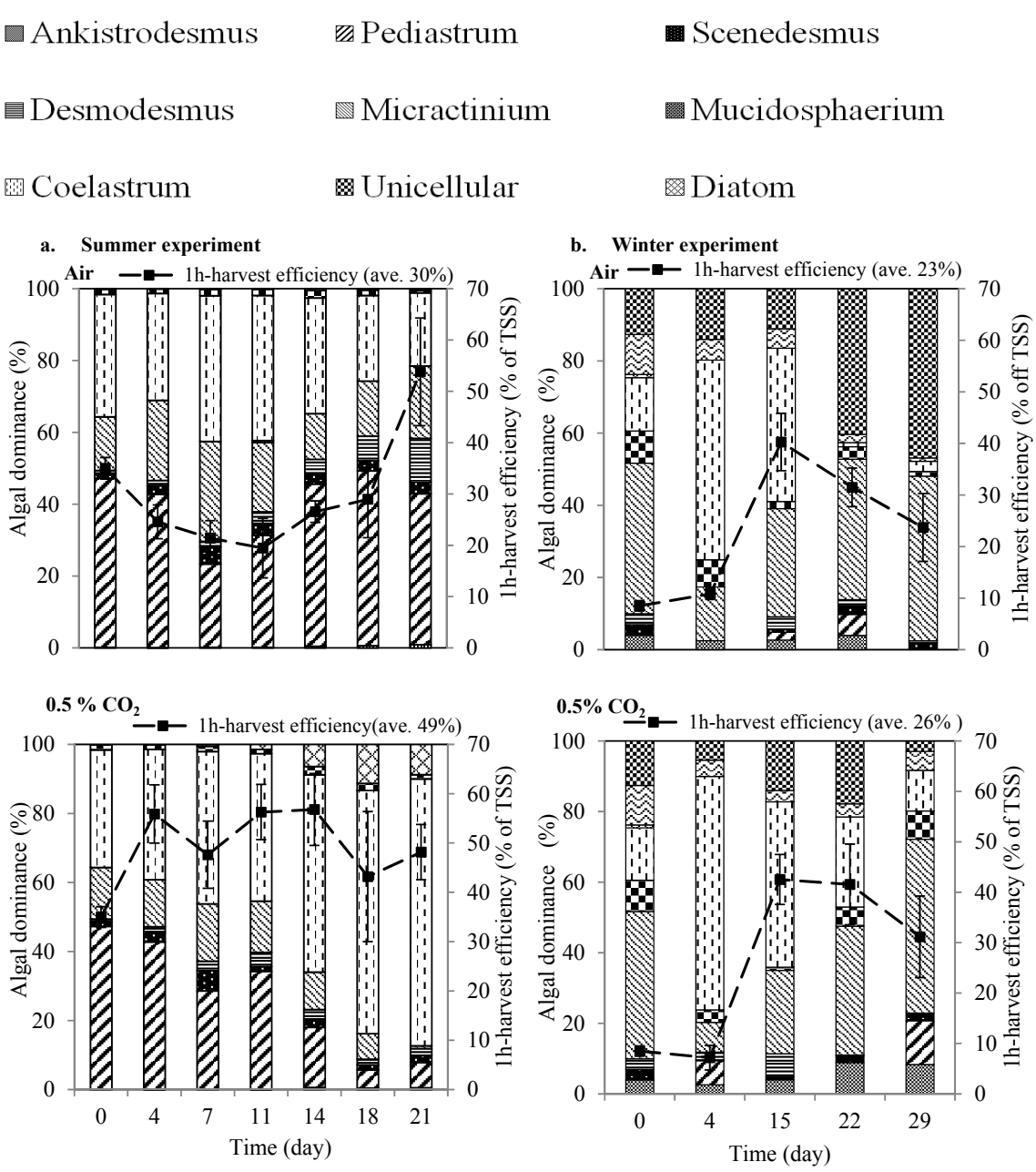
The efficiency of gravity-based settleability of the biomass produced in the HRAMs over the course of this study and the biovolume-based relative abundance of the algae species found in the HRAMs are illustrated in Fig. 4.4. The 1h-harvest efficiency changed in response to the mesocosm algal species composition, improving when larger species were present and when larger algal flocs formed. During the summer experiment the average 1h-harvest efficiency of the biomass in the 0.5% CO_2-HRAMs was significantly higher than those of the control, 2% CO_2 and 10% CO_2-HRAMs ($p<0.05$). The dominance of *Pediastrum* sp. (a large and easily harvestable algal species) was highest (40 ± 9%) in the control. The lower biomass harvestability in the control in spite of the dominance of *Pediastrum* sp. was likely due to either the lack of algal/bacterial aggregation and floc formation because of the inhibition of wastewater bacterial proliferation (occurred typically at pH >8.5) or the lower effect of autoflocculation (occurred typically at culture pH >8.5) on harvesatbility compared to algal/bacterial floc formation at pH 7.5-8 [102, 145, 146]. The mean 1h-harvest efficiency of the 2% CO_2-HRAM biomass in the summer experiment was near one-fifth lower than that of the 0.5% CO_2-HRAM biomass while their algal communities were similar during the experiment. Microscopic observation indicated that the colony size of *Coelastrum* decreased in response to reducing pH (i.e. increasing DIC concentration) (Fig. 4.5) could have contributed to the reduced biomass harvestable efficiency. The results are in-line with other studies which have reported a similar reduction in cell/colony size of algal species with decreasing culture pH from 9 to 6.5 (corresponded to DIC of 135 mg/L to 965 mg/L) [102, 147].

The variation of algal composition between the HRAM may be explained by the pH preference of algal species and the effects of invertebrate grazing. In the summer experiment, the control HRAMs were populated mainly by *Pediastrum* sp. (41.1% dominance) while it

was replaced by *Coelastrum* sp. (33.4% dominance) in the 2% CO_2-HRAMs. Both *Pediastrum* and *Coelastrum* are not preferred food species for microzooplanktons [81, 92] due to their large size and colony structure [93, 103, 122]. Hence, changes in algal dominance were likely due to pH preference, which has been previously reported for both fresh/seawater algal species in CO_2 enrichment studies [102, 138, 148, 149].

In the summer experiment *Micractinium* sp. became dominant in both the 5% CO_2 and 10% CO_2-HRAMs, but the biomass 1h-harvest efficiency of the 5% CO_2–HRAMs was almost twice that of the 10% CO_2-HRAMs. One possible explanation could be lower zooplankton grazing at low pH and high DIC level of the 10% CO_2-HRAMs [81, 150, 151]. No live grazers were found during microscopic observation of the 10% CO_2-HRAMs. Laboratory studies have shown that in the absence of invertebrate grazers, *Micractinium* sp. has a higher growth rate than *Pediastrum* sp., *Scenedesmus* sp. and *Coelastrum* sp [85, 119]. However in zooplankton-free culture *Micractinium* grows as individual cells (rather than as a colony), and do not develop spines resulting (Fig. 4.5, [102]), resulting in lower biomass harvest efficiency. This implies that although CO_2 addition increases biomass productivity and the proportion of algal biomass, it can reduce the gravity settling harvestable biomass productivity. Reduced gravity harvest would increase costs for complete harvest and negatively impact the potential for low-cost biomass production in WWT HRAP for biofuel.

In the winter experiment, water temperature and HRT were lower and shorter than the optimal conditions for zooplankton growth in the HRAMs [81, 119]. Therefore, the HRAMs were dominated by poorly settleable algae (either colonial algae such as *Micractinium, Mucidosphaerium pulchellum* and *Coelastrum* that grew individually or with small colony size) or single cell species such as *Monoraphidium* (Fig. 4.3 and 4.4). Hence, the 1h-biomass harvest efficiencies of the HRAMs except for 10% CO_2-HRAMs were lower compared to the summer experiment while they did not differ significantly from each other.


Ankistrodesmus
Pediastrum
Scenedesmus
Desmodesmus
Micractinium
Mucidosphaerium
Coelastrum
Unicellular
Diatom
a. Summer experiment
Air
1h-harvest efficiency (ave. 30%)
Algal dominance (%)
1h-harvest efficiency (% of TSS)
0.5 % CO2
1h-harvest efficiency(ave. 49%)
Time (day)
b. Winter experiment
Air
1h-harvest efficiency (ave. 23%)
1h-harvest efficiency (% off TSS)
0.5% CO2
1h-harvest efficiency (ave. 26%)

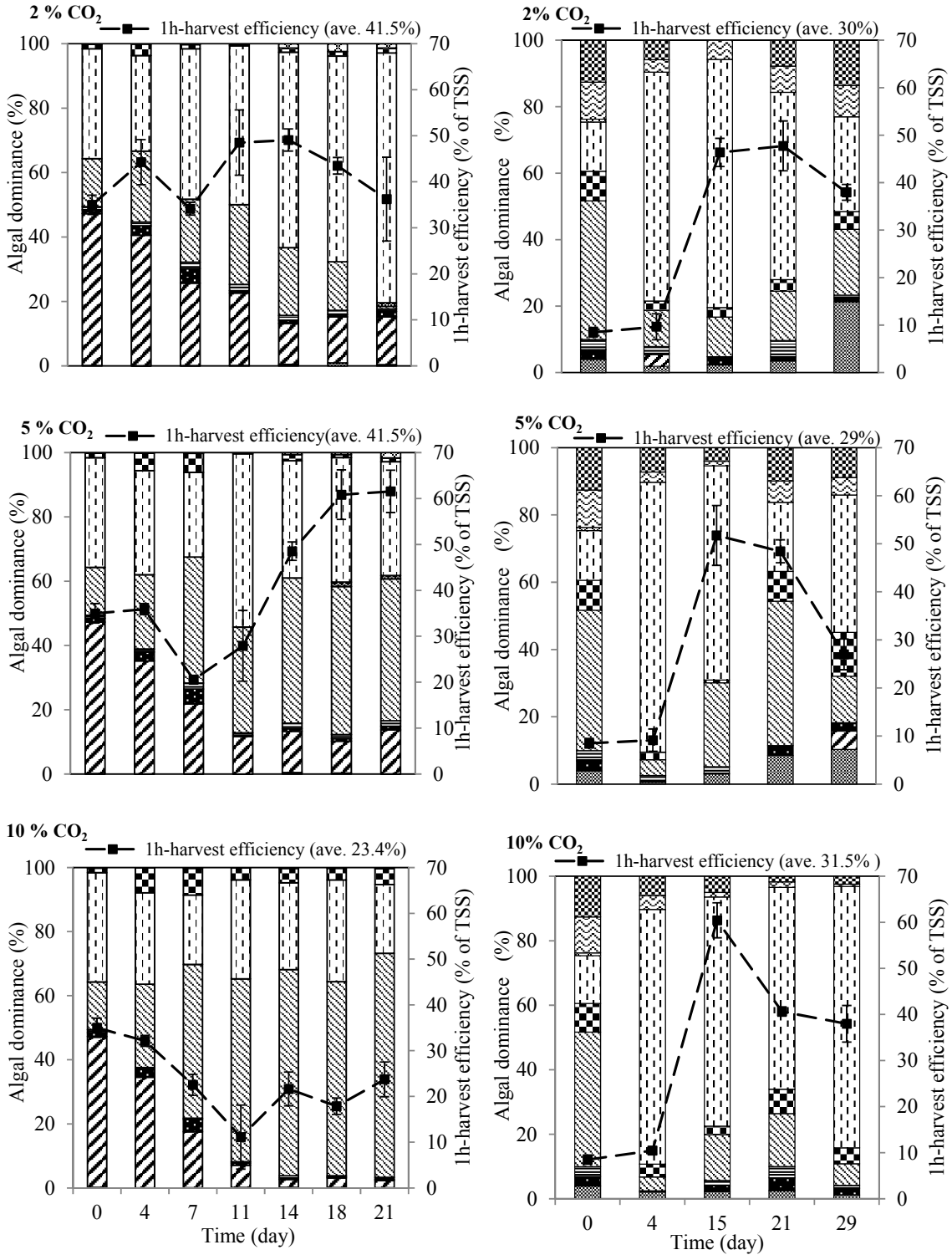
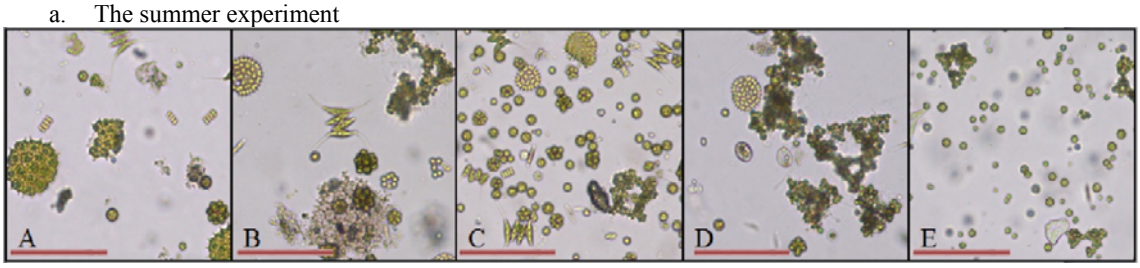


Figure 4.4. The biovolume-based algal abundance and the 1h-harvest efficiencies of HRAMs during the CO_2 experiments. HRT was 4 days in summer and 8 days in winter.

a. The summer experiment



b. The winter experiment

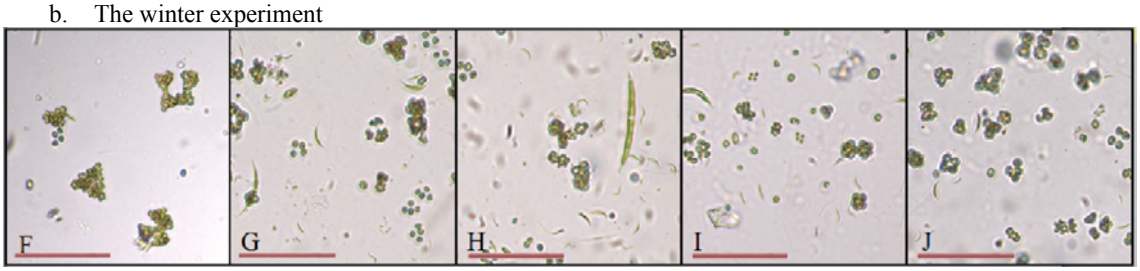


Figure 4.5. Microscopic pictures of the HRAM biomass (at day 18 in the summer experiment and at day 15 in the winter experiment): A and F) aerated HRAMs, B and G) 0.5% CO_2-HRAMs, C and H) 2% CO_2-HRAMs, D and I) 5% CO_2-HRAMs, E and J) 10% CO_2-HRAMs. Scale bars equal to 100 µm.

4.3.6 Effect of CO_2 concentration on the potential for biomass energy yield in HRAMs

The total daily biomass energy yield and the gravity harvestable energy yield (calculated by multiplying the productivity (g TSS/m^2/day), energy content (kJ/g TSS) and 1-h harvest efficiency (% of TSS) of the HRAM biomass are shown in Fig. 4.6. During the summer experiment CO_2 augmentation significantly improved the biomass energy yield in the 2%, 5% and 10% CO_2-HRAMs compared with the control and 0.5% CO_2-HRAMs ($p<0.05$) (Fig. 4.6a). In the winter experiment the biomass energy yield increased compared to the control, in HRAMs with low levels (<2%) of CO_2 augmentation, while it decreased in HRAPs with higher levels (>5%) of CO_2 augmentation. For example the biomass energy yield in the 0.5% CO_2-HRAMs increased by >1/10^{th} relative to the control while it decreased in the 10% CO_2 – HRAMs by 1/20^{th}.

CO_2 augmentation also enhanced the gravity harvestable biomass energy yield (Fig. 4.6) whenever the culture conditions (as explained before) were favourable for easy-harvest algal proliferation. According to the results, more gravity harvestable energy could be produced by operating an integrated wastewater treatment-algal cultivation system with dynamic pH control, i.e. at pH 6-7 (in summer and at pH 7-8 in winter. In winter, the gravity harvestable biomass energy yield of the 10% CO_2-HRAMs was only slightly higher than that of the 0.5%

CO_2-HRAMs, and not sufficient to be more economically viable. These results imply that CO_2 addition may offer a mechanism to assist with controlling microalgal community structure as well as zooplankton grazers in wastewater HRAPs. Low pH appeared to not only control the population of zooplankton grazers but promoted high growth rate species like *Micractinium* sp. which also form big flocs and are easily settleable (increasing both total daily biomass energy yield and the gravity harvestable energy yield). Sutherland et al. [102] obtained similar results and found that more gravity settleable biomass could be produced by operating WWT HRAM under suggested conditions (6<pH<7) in summer.

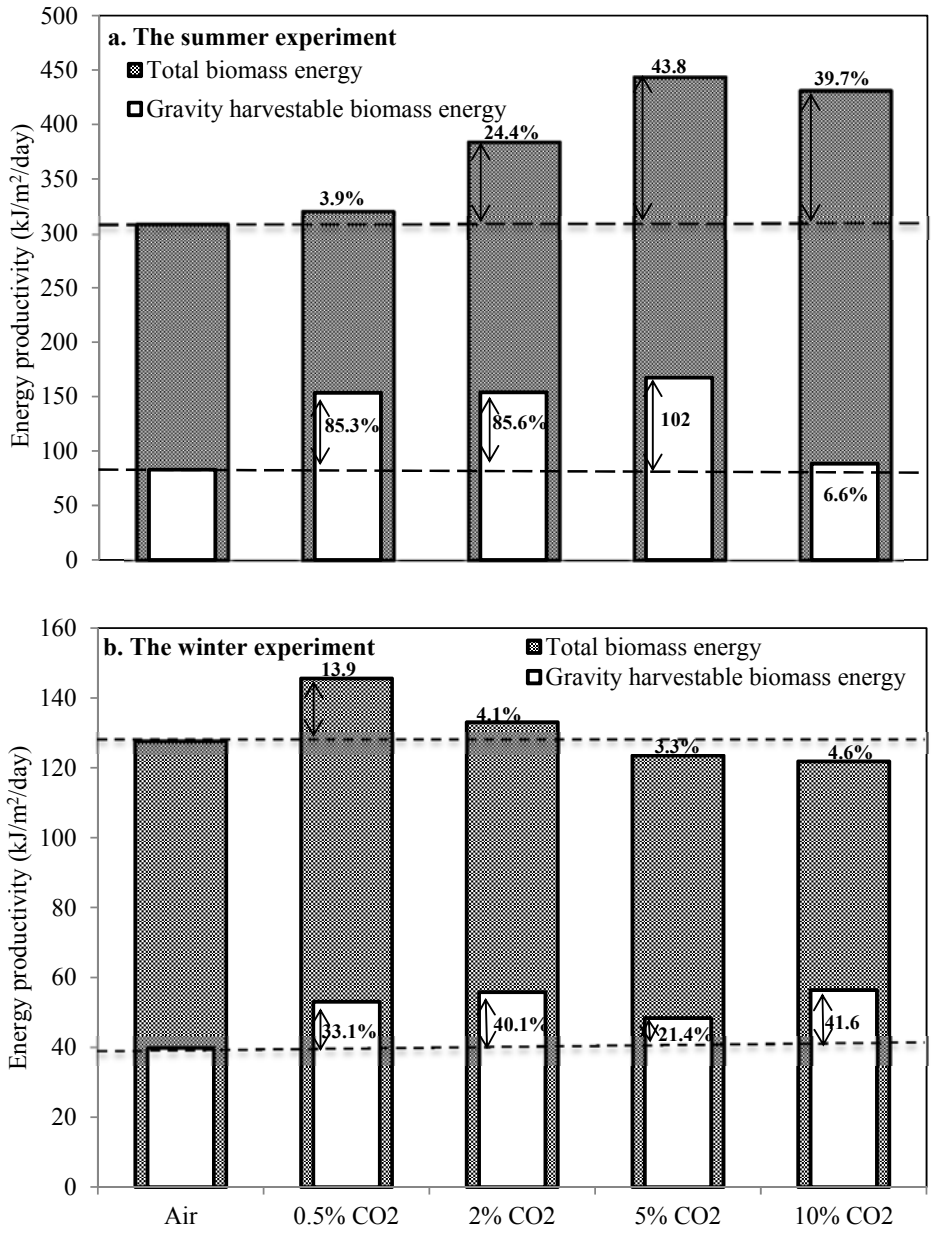


Figure 4.6 The average daily total and gravity harvestable biomass energy yield in the HRAMs during the CO_2 experiments. HRT was 4 days in summer and 8 days in winter.

A previous pilot-scale WWT HRAP study [119] showed that only 400-450 GJ/ha/year of biomass energy could be produced when operated without control of zooplankton grazers and pH was maintained <8 by CO_2 addition. The present study has demonstrated that the biomass energy yield of such a system could be more than doubled by operating at suggested pH ranges (6-7 in summer and 7-8 in winter), which also provide some zooplankton grazer control and increase the gravity harvestable biomass energy yield by more than 1/3rd, both at no extra cost. Further research is required to further improve the gravity harvestable biomass energy yield. One possible potential strategy is to operate WWT HRAPs with both CO_2 addition and recycling of a small portion of the harvested biomass back to the HRAP. Park et al. [12, 85] showed that recycling a proportion of gravity harvested biomass back into the pilot-scale HRAP contributed to a 2/3^{rds} increase in gravity harvestable biomass energy yield (from 117 to 195 kJ/m^2/day) compared to a control pond without recycling.

To maintain low pH (6-7) in summer will require a large amount of CO_2 and an efficient CO_2 delivery system. The most promising option to supply large amounts of CO_2 is to use flue gas (10-15% CO_2) or biogas (with 30-40% CO_2) as part of a biogas scrubbing system. Moreover, the capital cost of a CO_2 addition system for large-scale HRAPs is high (~25% of the total capital cost of the HRAP) [102, 110]. Further research is therefore required on cost-effective and efficient CO_2 addition for large-scale integrated wastewater treatment and algal cultivation for biofuel production.

4.4 Conclusions

This study has shown that CO_2 addition is a promising strategy to enhance gravity harvestable biomass energy yield of algal wastewater treatment and cultivation systems. CO_2 augmentation promoted HRAM performance through combined improvements of the biomass and algal productivity (by controlling invertebrate grazer populations, and overcoming CO_2 limitation to algae growth), the biomass energy content (by changing both the microbial species and chemical composition of the biomass) and the biomass harvestability (by promoting growth of colonial algal species, formation of algal-bacterial flocs and controlling invertebrate grazer populations). Increasing algal culture DIC concentrations in the present study has demonstrated that the total and harvestable biomass energy yield of such a system could be enhanced by nearly 1.5 times and over 2 times respectively in summer and over 1.1 times and 1.4 times, respectively in winter relative to the control. In summer, the greatest biomass energy yield (443.3 kJ/m^2/day) and the highest

gravity harvestable biomass energy yield (179.9 kJ/m^2/day) were measured in the 5% CO_2-HRAMs (pH 6-7). While in winter the highest biomass energy yield and its gravity harvestable proportion occurred in the 0.5% CO_2-HRAMs (pH 7-8). These results imply that to maximize gravity harvestable biomass energy yield in WWT HRAP, pond water pH should be varied with season and climate.

CHAPTER 5

Biodiesel production potential of wastewater treatment high rate algal pond biomass

This chapter is based on the following publication:

Mehrabadi, A., Craggs, R., Farid, M. 2016. Biodiesel production potential of wastewater treatment high rate algal pond biomass. *Bioresource Technology*, 221, 222-233.

Chapter preface

This chapter investigates the production potential and quality of biodiesel from wastewater treatment high rate algal pond (WWT HRAP) biomass and how it is affected by CO_2 addition to the pond. The investigation was conducted by profiling the lipid samples extracted during the monitoring of two parallel identical pilot-scale HRAPs (chapter 2) and CO_2 experiments (chapter 4). Gas chromatography (GC) analysis showed that all the lipid samples were dominated by palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acid methyl esters. According to the results, the potential annual biodiesel production that could be produced from WWT HRAP was 0.9 ± 0.1 g/m^2/d (3.2 ± 0.5 ton/ha/year) which could be increased to 1.1 ± 0.1 g/m^2/d (4.0 ton/ha/year) by lowering culture pH to 6-7 during warm summer months. However, the fatty acid methyl ester profile of the biodiesel, indicated that was of low quality and could not meet the standards in terms of cetane number, saponification value, cloud point and energy content and therefore, it cannot be used directly as a transportation fuel.

5.1 Introduction

Increasing fossil fuel consumption has accelerated global warming and caused several environmental problems such as air pollution and changing marine ecosystem[152]. To reduce the environmental problems associated with use of transportation fuels, replacement of petro-diesel by premium quality biodiesel derived from a renewable feedstock such as algal has been suggested for several decades [3, 76, 152]. Of all renewable biofuel resources, microalgae have been highlighted due to: 1) high biomass and lipid productivities (up to 100x greater than oil seeds), 2) temperature tolerance, 3) ability to grow on wasteland and wastewater, 4) ability to bio-fix high amounts of CO_2 (1.7-2.4 tons of CO_2 per one ton of microalgae biomass), and 5) relatively simple life cycle [3, 153-155]. It has been predicted, based on small-scale controlled experiments, that 7-60 ton/ha/year biodiesel could potentially be produced from pure fresh/seawater algae [156-159]. These values are much higher than the potential values for oil seed-based biodiesel: ~0.4 ton/ha/year from soybean,~ 0.7 ton/h/year from canola,~2 ton/ha/year from jatropha,~4.5 ton/ha/year from palm [160-162].

Biodiesel production from algae involves six sequential steps [157, 163] including: 1) species selection (in terms of biomass and lipid productivities as well as suitability of lipids for biodiesel production), 2) algal cultivation, 3) biomass harvesting, concentrating and dewatering (optional), 4) lipid extraction (optional) and purification, 5) biodiesel production through esterification of the lipid fraction, and 6) biodiesel purification.

To date, despite intensive efforts, there are still several obstacles to efficient production of algal biodiesel, which have prevented it from being economic and competitive with petro-diesel. The low lipid content (typically < 30 wt%) of algal biomass, the high cost of nutrient fertilisers and high energy demands for biomass harvest and dewatering are the main obstacles [19, 164-166]. In addition, there are several technological limitations for lipid extraction, purification and esterification [19, 164, 167-169]. However, to lower production costs, different strategies including genetic engineering of algal lipid synthesis pathway to improve lipid content and profile, using wastewater as a nutrient source for cultivation of pure species, cultivation of easily settleable species, applying wet extraction methods, and enzymatic esterification to improve biodiesel yield have been employed [12, 100, 170-173].

A number of studies have shown that 30-50% of the total energy demand for algal biodiesel production is from the cultivation and harvesting of biomass [19, 107, 174-176]. While biodiesel production from pure algal biomass is still uneconomic, one opportunity to lower biodiesel production costs is where the algal-bacterial biomass is produced as an essentially free by-product of wastewater treatment in high rate algal ponds [6, 76]. In fact, in such system cultivation and harvest costs are covered by treatment function. Hence, it is of interest to see whether the lipid content of such free feedstock is higher or lower than pure algal cultures, how it varies seasonally, and based on the fatty acid profile what the quality of the biodiesel produced from it would be. In addition, since WWT HRAP biomass is a consortium of different algal and bacterial species [119, 177], the biomass lipids may be varied which would result in higher downstream processing costs and consequently further diminish the potential for low-cost, high quality biodiesel production. Although, to reduce the algal lipid variability, culturing under nutrient starvation conditions has been suggested [116], as the main goal of WWT HRAP is wastewater treatment, there is little opportunity within this context to substantially alter the lipid content of such biomass. Therefore, to improve the quality biodiesel production potential in WWT HRAP, there is a need to find strategies to improve productivity, content and profile of the WWT HRAP biomass lipid without impacting pond treatment performance. Hence, the aims of this study are first to investigate the production potential of biodiesel from WWT HRAP biomass by measuring productivity, lipid content and lipid profile of biomass produced in two identical pilot-scale WWT HRAPs over one year, and second to assess the potential to improve biodiesel production and quality by CO_2 addition to the algal mix culture.

5.2 Materials and methods

5.2.1 Experimental set-up

To assess potential of WWT HRAP for biodiesel production three sets of experiment were conducted at the Ruakura Research Centre, Hamilton, New Zealand (37°47′S, 175°19′E). In experiment 1 two identical pilot-scale WWT HRAPs (West (WHRAP) and East (EHRAP)) were operated in parallel and sampled weekly over a year (July 2013-August 2014). The ponds culture depth, surface area and mean surface water velocity were 30 cm, 31.8 m^2 and 0.15 m/s, respectively. The ponds were fed with 0.5-1.0 m^3/day of primary settled domestic wastewater at hourly intervals from the Ruakura sewer. The pond hydraulic retention time (HRT) was changed with season from 8 days in winter to 5 days in summer. It is noteworthy that based on the literatures [12, 30] optimal HRT, in summer, is 4 days and therefore, either longer or shorter HRT may reduce biomass productivity in WWT HRAP. To avoid free ammonia inhibition and carbon limitation, the maximum pH of the HRAPs was kept below 8 during the daytime by CO_2 addition, if necessary. The ponds were operated with no control of the dominant algal species or of the zooplankton population.

Exp.2&3 were conducted to investigate the effect of CO_2 addition on quality and quantity of the biodiesel produced from WWT HRAP biomass. Both experiments were conducted under outdoor conditions in summer (Exp. 2, January 2014) lasted for 21 days and winter (Exp. 3, July-August 2014) lasted for 30 days. Fifteen replicate foil-wrapped plastic mesocosms (water depth of 0.3 m; volume of 16 L; surface area of 0.06 m^2) were used in each experiment and the cultures were sampled twice a week. The mesocosms were inoculated from adjacent pilot-scale WWT HRAP dominated by *Pediastrum* sp., *Micractinium* sp. and *Coelastrum* sp. in summer and by *Micractinium* sp., *Ankistrodesmus falcatus*, *Mucidosphaerium* sp. and *Monoraphodium* sp. in winter. The biomass concentration at start time was 174 g VSS/m^3 in summer experiment and 79 g VSS/m^3 in winter experiment. The cultures were fed semi-continuously (at 9 am) with primary settled domestic wastewater on 4 and 8 days hydraulic retention time in summer and winter, respectively. The mesocosms were supplemented by a mixture of air and CO_2 (with mole fractions of 0.04% CO_2, 0.5% CO_2 (control), 2% CO_2, 5% CO_2, 10% CO_2) using a gas diffuser placed on the bottom of the buckets while cultures were mixed continuously by individual magnetic stirrer. Over the course of study, daily climate data (temperature, solar radiation, evaporation and rainfall) were downloaded from NIWA's National Climate Database (http://cliflo-niwa.niwa.co.nz/).

5.2.2 Nutrient concentrations

During the sampling period, the influent and effluent of WWT HRAPs/HRAMs were filtered through Whatman GF/F filters (with 0.7 μm pore size) and then the concentrations of ammonium (NH_4^+-N) and dissolved reactive phosphorous (PO_3^--P) were determined colorimetrically [86] using a spectrophotometer (HACH RD2008, Germany).

5.2.3 Algal species composition

During the sampling period, a well-mixed sub-sample of HRAP/HRAM effluents was settled in an Utermöhl chamber (diameter: 25 mm -volume: 10 ml) and viewed on a microscope Leica DM 2500, equipped with a Leica DFC 420 digital camera (Leica Microsystem, Switzerland). Microalgae species were identified to species level, where possible, based on the taxonomic descriptions of John et al. [178].

5.2.4 Biomass productivity

The biomass productivity was calculated based on the volatile suspended solids (VSS) concentration following Equation (5-1) [12]:

$$P = \frac{X \times Q_c}{A} \qquad (5-1)$$

$$Q_c = Q_{inf} + ((rainfall - evaporation) \times surface\ area)$$

where P is the areal biomass productivity (g VSS/m^2/d), X is the HRAP/HRAM biomass concentration (g VSS/m^3) , A is surface area (m^2) of the HRAP/HRAM, Q_{inf} is HRAP/HRAM daily inflow (m^3/d), and Q_c is daily outflow (m^3/d) corrected for net evaporation. To determine the VSS a known volume (50 ml) of the pond/mesocosm effluent was filtered onto a pre-rinsed, pre-combusted and pre-weighed Whatman GF/F filter (with 0.7 μm pore size) and then dried in an oven (at 80 ºC (to prevent lipid damage and weight lost) overnight). The sample was then combusted at 550 ºC for 1 hour in muffle furnace (F.E.KILN, RTC1000, Bartlett Instrument Company, UK). The weight loss was recorded as VSS concentration.

5.2.5 Biomass lipid content and productivity

During the sampling period, lipid fraction of the HRAP/HRAM biomass were extracted and measured gravimetrically according to a modified procedure adopted from Bligh and Dyer method [89] as described previously [119]. A known amount (20-30 mg) of centrifuged frozen HRAP/HRAM biomass was placed in a centrifuge tube with a 20 ml mixture of

distilled water, methanol and chloroform (1:2:1). The centrifuge tube was mixed using a shaking table overnight (~ 6-cm oscillation at ~2 cycles per second). The day after, an additional 5 mL of chloroform and 4 mL of distilled water were added to the tube. The tube was then vortex mixed for 30 s and centrifuged at 3500 relative centrifugal force (rcf) for 10 min. The lipid-chloroform layer placed at the bottom of the tube was removed and filtered through a GFF filter and transferred into a pre-weighed glass tube. A second and occasionally third re-extraction was conducted by adding another 5 mL of chloroform to the remaining biomass in the centrifuge tube followed by vortex mixing for 30 s and centrifuging at 3500 rcf for 5 min. The lipid-chloroform layer was then removed, filtered and combined with the lipid-chloroform layer obtained from the first extraction. The chloroform was evaporated in an oven (at 60 ºC overnight) while the oven was purged continuously by nitrogen gas. The tube was cooled down under a nitrogen stream in a desiccator, weighed and stored for further analysis. The ash-free mass fraction of lipids was obtained by dividing lipid weight by the VSS. The ash-free biomass lipid productivity was then calculated by multiplying the productivity with ash-free lipid mass fraction of the biomass. The WWT HRAP/HRAM influent was analysed occasionally and was found to have no fat content to interfere with the results.

5.2.6 Esterification and characterization of extracted lipids

To assess the potential of biodiesel production from wastewater algae and its quality, the lipids extracted from the WWT HRAP/HRAMs biomass were profiled after esterification using gas chromatography (GC). To esterify the extracted lipids, a known amount (2-5 mg) of lipids (without pre-purification i.e. degumming and chlorophyll removal) was placed in a 20 ml screw-top Pyrex-glass vial with 5 ml of freshly prepared 5 v/v% BF_3-methanol and mixed vigorously for 30 s. 100 µL of methanol containing heptadecanoic acid (2 mg/ml) was added to the vial as an internal standard and the mixture was heated at 70 ºC by a heat block (DBH20D, Ratek, Australia) for 20 min. Subsequently 1 ml of distilled water and 5 ml of dichloromethane: hexane mixture (1:4, v/v) were added to the vial. The vial was vortex mixed for 30 s, centrifuged at 2500 rcf for 10 min and the upper layer containing mixture of fatty acids methyl esters (FAMEs) and hexane was then transferred to a new vial. A second and third re-extraction was conducted by adding another 5 ml of dichloromethane: hexane mixture (1:4, v/v) to the left over. To remove the water which might present in the FAMEs and hexane mixture, a known amount (0.5-1 g) of anhydrous sodium sulphate was then added to the mixture, followed by transferring FAMEs:hexane mixture to a new vial to evaporate

hexane under N_2 stream at 50 ºC. Subsequently, the total dry FAMEs were dissolved in dichloromethane (DCM) and separated in a GC-2010 Shimadzu instrument equipped with a flame ionization detector using a 0.32 mm (I.D.) × 30 m HP-INNOWax capillary column (Agilent Technologies, USA). 2.5 µL of sample was injected at split ratio of 20:1 while helium was used as carrier gas at 2.2 ml/min. The injector and detector temperatures were set at 250 ºC and 275 ºC, respectively. The column was programmed as follows: oven temperature held at 50 ºC for 1 min, ramped to 200 ºC at 15 ºC/min, maintained at 200 ºC for 12 min, heated to 250 ºC at 2 ºC/min, and kept at 250 ºC for 2 min. Individual peaks were identified by comparison of their retention time with those of standard compounds. FAME mix (CRM18918, Sigma-Aldrich Co., USA) comprised of C8-C24; methyl palmitate (C16:0), methyl palmitoleate (C16:1), methyl stearate (C18:0), methyl oleate (C18:1), methyl linoleate (C18:2), methyl linolenate (C18:3), methyl $^{\gamma}$-linolenate (C18:3n6) and methyl architate (C20:0) were used as standards. In addition, the quantification of individual FAMEs (mg/g of the initial lipid sample) was performed according to standard method (AOCS Official Method Ce 1-62) and using standard curves.

5.2.7 Estimation of biodiesel properties based on FAME profile

It has been shown that quality of biodiesel (as a mixture of FAMEs) is a strong function of amount of individual FAME [179, 180]. In addition a number of studies have shown that the biodiesel properties such as cetane number could be calculated based on biodiesel FAME profile using empirical equations [181, 182]. To assess the quality of the WWT HRAP biomass-based biodiesel, five of important biodiesel properties including saponification value (SV, mg KOH/g biodiesel), iodin value (IV, g I_2/100 g biodiesel), cetane number (CN), cloud point (CP, ºC), and higher heating value (HHV, kJ/g biodiesel) were calculated according to FAME profile following Equations (5.2-5.6) [181, 182]:

$$SV = 268 - (0.418 \times P + 1.3 \times S + 0.695 \times O + 0.77 \times L + 0.847 \times LL) \quad (5-2)$$

$$IV = \sum \frac{254 \times D_i N_i}{M_i} \quad (5-3)$$

$$CN = 46.3 + \frac{5.458}{SV} - 0.225 \times IV \quad (5-4)$$

$$CP = (0.526 \times P) - 4.992 \quad (5-5)$$

$$HHV = 49.43 - 0.015 \times IV - 0.041 \times SV \quad (5-6)$$

where P, S, O, L, LL are the weight fractions of C16:0, C18:0, C18:1, C18:2, C18:3, respectively in total FAMEs and Di, Ni and Mi denote the number of double bound, the wt% and the molecular mass of fatty acid component (i).

5.2.8 Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) in Excel software (Excel, Microsoft office 2010).

5.3 Result and discussion

5.3.1 Biomass algal composition

Microscopic monitoring of the WWT HRAPs/HRAMs effluent showed that, over the course of study, the WWT HRAPs/HRAMs were populated by more than 10 algal species with the most abundant species being *Pediastrm* sp., *Micractinium* sp., *Ankistrodesmus falcatus*, *Monoraphodium* sp., *Desmodesmus* sp., *Coleastrum* sp. and *Mucidosphaerium* sp. (Table 5.1). During the pond monitoring, algal dynamics was affected by environmental conditions and blooms of zooplankton grazers, while in the CO_2 addition experiments, due to the similar environmental conditions for all cultures, the HRAM pH level was the main influencing factor. The ponds were dominated mainly by colonial species such as *Pediastrum* sp., *Coelastrum* sp. and *Micractinium* sp. in summer when the weather conditions were favourable for invertebrate proliferation and unicellular species such as *Ankistrodesmus falcatus* were grazed. While fast-growing unicellular species such as *Ankistrodesmus falcatus* and *Monoraphodium* sp. dominated the ponds in winter where the zooplankton invertebrates could not grow and grazed them (see Mehrabadi et al., [119] for more details). Similar to the ponds, the WWT HRAMs were populated by colonial species in summer. However, since the culture pH changed with CO_2 concentration, different cultures were populated by different colonial species. *Pediastrum* sp. and *Coelastrum* sp. were the most abundant species in the aerated HRAMs and in 0.5 and 2% CO_2-HRAMs, respectively while *Micractinium* sp. dominated in 5% and 10% CO_2-HRAMs. In the winter experiment, all the WWT HRAMs were populated by mixture of fast growing colonial and unicellular species such as *Micractinium sp.*, *Mucidosphaerium sp., Coelastrum* sp., *Ankistrodesmus falcatus* and *Monoraphodium sp*. Although the colonial species grew individually or with reduced colony size due to the low abundance of zooplankton grazers [135].

Table 5.1 Dominant algal species during the pond monitoring and the CO_2 experiments

		The pond monitoring experiment (WHRAP)	**The pond monitoring experiment (EHRAP)**
Winter	Aug. 2013	*Ankistrodesmus falcatus* (>90%)	*Ankistrodesmus falcatus* (>90%)
Spring	Sep. 2013	*Ankistrodesmus falcatus* (>75%)	*Ankistrodesmus falcatus* (>90%)
	Oct. 2013	*Micractinium* sp. (>40%)	*Micractinium* sp. (>70%)
	Nov. 2013	*Mucidosphaerium* sp. (>70%)	*Micractinium* sp. (>40%)
Summer	Dec. 2013	*Micractinium* sp. (>50%) and *Pediastrum* sp. (>39%)	*Pediastrum* sp. (>70%)
	Jan. 2014	*Micractinium* sp. (>55%)	*Pediastrum* sp. (>55%)
	Feb. 2014	*Micractinium* sp. (>40%) and *Pediastrum* sp. (>30%)	*Pediastrum* sp. (>65%)
Autumn	Mar. 2014	*Micractinium* sp. (>40%)	*Micractinium* sp. (>25%) and *Pediastrum* sp. (>45%)
	Apr. 2014	*Mucidosphaerium* sp. (>50%)	*Pediastrum* sp. (>55%)
	May. 2014	*Micractinium* sp. (>29%) and *Pediastrum* sp. (>22%)	*Pediastrum* sp. (>36%) and *Desmodesmus* sp. (>34%)
Winter	Jun. 2014	*Micractinium* sp. (>53%)	*Pediastrum* sp. (>39%) and *Desmodesmus* sp. (>30%)
	Jul. 2014	*Micractinium* sp. (>72%)	*Monoraphydium* sp. (>82%)
		The summer CO_2 addition experiment	**The winter CO_2 addition experiment**
Aerated HRAM		*Pediastrum* sp. (>35%) and *Coelastrum* sp. (>30%)	*Micractinium* sp. (>40%) and *Mucidosphaerium* sp. (>20%)
0.5% CO_2-HRAM		*Coelastrum* sp. (>50%)	*Micractinium* sp. (>30%) and *Coelastrum* sp. (>30%)
2% CO_2-HRAM		*Coelastrum* sp. (>50%)	*Coelastrum* sp. (>50%)
5% CO_2-HRAM		*Micractinium* sp. (>30%) and *Coelastrum* sp. (>35%)	*Micractinium* sp. (>20%) *Coelastrum* sp. (>40%)
10% CO_2-HRAM		*Micractinium* sp. (>50%)	*Coelastrum* sp. (>60%) and *Micractinium* sp. (>15%)

5.3.2 Environmental variables and nutrient concentrations

Mean values of temperature, solar radiation, influent nutrient concentrations and percentages of nutrient removed during the experiments are summarized in Table 5.2. Over the course of the one year HRAP experiment (Exp. 1), mean daily temperature and solar radiation varied considerably. The average daily temperature and light intensity ranged between 9.7 ± 1.9 and 21.9 ± 1.4 °C, and between 6.1 ± 2.5 and 25 ± 7.3 MJ/m^2/day, respectively where the minimums were recorded in the winter and the maximums occurred in the summer. In the summer CO_2 addition mesocosm experiment, the average daily temperature and irradiance were 19.6 ± 2.8 °C and 24.7 ± 7.7 MJ/m^2/day, respectively while

in the winter CO_2 addition experiment they were much lower, 9.5 ± 1.3 °C and 8.5 ± 2.6 $MJ/m^2/day$, respectively. The day-time pond pH was kept ≤ 8 via CO_2 addition, if necessary, while the day-time mesocosm pH was different corresponding to the CO_2 concentration so that the HRAMs sparged with higher concentration of the CO_2:air mixture had a lower day-time pH. In both CO_2 addition experiments, the highest day-time pH (ranging from 8.1 to 10.1) occurred in aerated HRAMs while the lowest day-time pH (ranging from 5.9 to 6.3) occurred in 10% CO_2-HRAMs (Table 5.2).

The ammoniacal-N (the dominant form of N in domestic wastewater) and DRP concentrations in the HRAPs/HRAMs influent and effluent were measured and the average percentage removals were calculated during sampling periods. As the nitrate concentration was negligible compared to the ammonium concentration, it is not reported. The mean monthly ammoniacal-N and DRP pond influent concentrations ranged from 12.5 ± 13.3 to 34.1 ± 11.2 mg/L, and from 1.9 ± 2.0 to 5.0 ± 0.9 mg/L, respectively. Overall, 31.5-92.1% of ammoniacal-N and 31.2-86.5% of DRP were removed in the ponds, with highest nutrient removal in summer where the environmental conditions were favourable for algal proliferation. In the summer CO_2 addition experiment, the average influent concentrations of NH_4^+-N and DRP were 36 ± 10 mg/L and 4.1 ± 0.9 mg/L, respectively; and in the winter CO_2 addition experiment, they were 32 ± 8 mg/L and 4.3 ± 1 mg/L, respectively. Over the course of the CO_2 addition experiments, % nutrient removal ranged between 59-86% for ammonia and 39-61% for DRP in summer, while removal efficiency of both nutrients was much lower (50% of the summer CO_2 addition experiment) in winter. In the both CO_2 addition experiments the highest nutrient removal ($p<0.05$) occurred in the aerated HRAMs most likely due to ammonia volatilization and phosphorus precipitation at high pH (>8.5) [30]. Except for the aerated HRAMs, the higher CO_2 concentration resulted in the higher nutrient removal efficiency in summer. While, in winter, CO_2 addition negatively affected nutrient removal, which was probably due to inhibition of the growth of microbial community at low pH and suboptimal environmental conditions. It has been shown that nitrifying bacteria grow optimally in the range of pH 7.5-8.5 and their growth are inhibited at 10<pH< 6.5. In addition, at high levels of dissolved inorganic carbon (DIC) in the culture and under low temperature and low light conditions, the key enzymes that catalyse CO_2 entry into the Calvin cycle (the process that plants and algae use to convert CO_2 to essential cell components) are inactivated. Acidification of chloroplast stroma results in inhibition of

photosynthesis and reduced algal growth and consequently reduces the nutrient removal efficiencies [138].

Table 5.2 Environmental conditions, culture pH and nutrient concentrations and percentage removal during the pond monitoring and the CO_2 experiments. The temperature column represents water temperature in the pond experiment and ambient temperature in the CO_2 addition experiments.

		HRT (day)	Day-time pH	Temperature (ºC)	Sunlight intensity ($MJ/m^2/d$)	Primary NH_4^+-N (mg/L)	% NH_4^+-N removed	Primary PO_4^{3-}-P (mg/L)	% PO_4^{3-}-P removed
The pond monitoring experiment									
Aug. 2013	WHRAP	8	≤8	12.1 ± 0.8	10.3 ± 4.1	22.2 ± 4.3	53.0	3.3 ± 0.8	32.2
	EHRAP						58.1		35.2
Sep. 2013	WHRAP	7	≤8	14.7 ± 1.6	12.7 ± 4.8	32.8 ± 10.9	55.3	4.7 ± 1.4	31.2
	EHRAP						75.6		46.9
Oct. 2013	WHRAP	7	≤8	17.2 ± 1.3	18.0 ± 5.1	28.6 ± 17.3	78.1	4.7 ± 3.2	37.1
	EHRAP						78.0		45.6
Nov. 2013	WHRAP	6	≤8	20.8 ± 1.8	21.8 ± 4.6	22.2 ± 9.1	84.9	3.3 ± 1.4	65.2
	EHRAP						82.9		66.5
Dec. 2013	WHRAP	5	≤8	21.5 ± 1.5	16.0 ± 6.5	27.9 ± 13.8	82.1	3.7 ± 1.8	59.0
	EHRAP						88.3		69.0
Jan. 2014	WHRAP	5	≤8	21.7 ± 1.0	25.0 ± 7.3	30.8 ± 12.6	90.4	3.5 ± 2.2	76.4
	EHRAP						92.1		72.9
Feb. 2014	WHRAP	5	≤8	21.9 ± 1.4	22.4 ± 3.7	23.5 ± 8.9	85.5	3.6 ± 1.9	68.2
	EHRAP						89.9		73.2
Mar. 2014	WHRAP	6	≤8	19.7 ± 1.1	18.5 ± 3.8	34.1 ± 11.2	85.2	5.0 ± 0.9	78.0
	EHRAP						86.9		85.6
Apr. 2014	WHRAP	6	≤8	17.3 ± 2.3	11.1 ± 3.8	25.7 ± 8.4	85.9	2.6 ± 1.3	73.8
	EHRAP						72.3		62.1
May. 2014	WHRAP	8	≤8	12.6 ± 2.4	8.7 ± 2.6	26.3 ± 23.6	73.1	3.2 ± 2.7	55.2
	EHRAP						59.7		41.1
Jun. 2014	WHRAP	8	≤8	11.4 ± 1.4	6.1 ± 2.5	12.5 ± 13.3	92.0	1.9 ± 2.0	51.3
	EHRAP						86.7		57.4
Jul. 2014	WHRAP	8	≤8	9.7 ± 1.9	8.0 ± 2.7	24.0 ± 11.7	31.5	3.4 ± 1.6	31.8
	EHRAP						37.1		45.4
The summer CO_2 addition experiment									
Air		4	9.1 ± 1.0	19.6 ± 1.3	24.7 ± 7.7	36.0 ± 10.0	84.0 ± 3.5	4.1 ± 0.9	67.2 ± 10.2
0.5% CO_2		4	7.4 ± 0.5				53.3 ± 4.1		45.4 ± 13.2
2% CO_2		4	6.7 ± 0.3				54.7 ± 3.6		54.6 ± 4.5
5% CO_2		4	6.4 ± 0.2				59.4 ± 5.1		62.3 ± 15.4
10% CO_2		4	6.1 ± 0.2				57.2 ± 4.7		61.2 ± 10.0
The winter CO_2 addition experiment									
Air		8	8.5 ± 0.5	9.5 ± 3.1	8.5 ± 2.6	32.2 ± 8.2	37.9 ± 5.0	4.3±1.0	35.5 ± 3.2
0.5% CO_2		8	7.4 ± 0.5				31.0 ± 6.1		30.2 ± 5.0
2% CO_2		8	6.7 ± 0.3				28.6 ± 5.4		34.9 ± 1.2
5% CO_2		8	6.4 ± 0.2				25.5 ± 3.2		30.2 ± 2.8
10% CO_2		8	6.1 ± 0.2				24.0 ± 6.4		19.5± 2.1

5.3.3 Biomass and lipid productivity

The biomass and lipid productivities are crucial measurements since higher biomass and lipid productivities would result in higher potential for biodiesel production. The average biomass concentration and productivity as well as lipid content and productivity of all three sets of experiments are summarised in Table 5.3. The average monthly biomass concentration and productivity, over the one year pond monitoring, ranged from 56.3 ± 14.6 to 237.2 ± 31.7 g VSS/m^3, and from 2.0 ± 0.3 to 11.1 ± 2.5 g VSS/m^2/day, respectively with the higher values in the warmer months (Table 5.2). However, the differences were observed between the biomass concentrations and productivities of the two ponds in warm months when only one of those was under zooplankton grazing pressure (see Mehrabadi et al., [119] and Montemezzani et al., [135] for more details). These pond annual biomass productivities are comparable with values (2.3-12.1 g/m^2/day) reported in the literature for similar systems [12, 27, 82, 177, 183].

It has been well documented that the CO_2 augmentation in microalgae cultures significantly enhances biomass productivity [84, 102, 138, 155]. In good agreement with the literature, CO_2 addition improved the biomass concentration and productivity where the environmental conditions were favourable for algal proliferation. Compared with the control HRAMs, the biomass productivity increased significantly ($p<0.05$) by 26-40% in the summer experiment with the higher CO_2-supplemented cultures having higher productivity. The increase in biomass productivity may have resulted from: 1) higher ammonium availability at lower pH, 2) increased light utilization by algae at higher CO_2 concentration, 3) reduction of activity of carbon-concentrating mechanisms at higher CO_2 concentration, 4) changing algal dynamics due to at higher CO_2 concentrations controlling the zooplankton grazer population [102, 135, 138]. Overall, in the winter mesocosm CO_2 addition experiment, the biomass concentrations and productivities were one-third to one-fourth of those in the summer CO_2 addition experiment which was due to sub-optimal environmental conditions, lower sunlight and lower temperature. Compared to the control HRAMs, the biomass productivity declined with increasing CO_2 concentration so that the highest productivity occurred in the control cultures (0.5% CO_2-HRAMs). It was probably resulted from inhibition of algal and bacterial growth at the low pH under winter conditions, i.e. sub-optimal solar radiation and water temperature [138]. In fact, at low pH under sub-optimal conditions, due to high concentration of dissolved inorganic carbon in the culture, the activity of the extracellular carbonic anhydrase enzyme of microalgae reduces and therefore cells may have to expend more

energy to maintain the intracellular pH within the range necessary for cell function, which could reduce growth rates [144, 184].

Table 5.3 Biomass concentrations and productivities, and ash free lipid weight fraction and productivities during the pond monitoring and the CO2 experiments

The pond monitoring experiment (WHRAP)												
	Winter	**Spring**			**Summer**			**Autumn**			**Winter**	
	Aug. 2013	Sep. 2013	Oct. 2013	Nov. 2013	Dec. 2013	Jan. 2014	Feb. 2014	Mar. 2014	Apr. 2014	May 2014	Jun. 2014	Jul. 2014
VSS (g/m^3)	105.6 ± 15.1	68 ± 5.9	142 ± 48.2	135 ± 97.3	142.6 ± 46.1	183.2 ± 22.9	223.3 ± 31.7	237.2 ± 54.7	140.7 ± 15.8	134.3 ± 15.1	56.3± 14.6	103.7 ± 26.3
Biomass productivity ($g/m^2/day$)	3.2 ± 0.3	2.2 ± 0.3	4.3 ± 1.3	5.9 ± 4.5	7.9 ± 2.4	8.6 ± 1.0	10.2 ± 1.4	11.1 ± 2.5	7.2 ± 0.6	4.3 ± 0.4	2.0 ± 0.3	3.4 ± 0.6
Ash free lipid content (wt%)	19.4 ± 2.8	22.2 ± 1.8	26.7 ± 2.2	29.2 ± 10.4	30.8 ± 8.4	31.5 ± 14.1	31 ± 10.5	21.4 ± 2.9	31 ± 12.3	24.8 ± 9.2	29.0 ± 7.9	25.5 ± 2.7
Lipid productivity ($g/m^2/day$)	0.6 ± 0.1	0.5 ± 0.1	1.1 ± 0.3	1.5 ± 1.0	2.4 ± 0.8	2.6 ± 1.0	3.2 ± 1.2	2.4 ± 0.6	2.2 ± 0.9	1.0 ± 0.3	0.6 ± 0.1	0.9 ± 0.1
The pond monitoring experiment (EHRAP)												
VSS (g/m^3)	138.3 ± 25.0	140.6 ± 37.9	159.6 ± 142.3	178.9 ± 114.3	135.2 ± 45.8	216.2 ± 32.7	230.7 ± 35.3	183.5 ± 64	89.9 ± 40.9	89.5 ± 19.9	78.8± 46.2	134.8 ± 30.0
Biomass productivity ($g/m^2/day$)	4.2 ± 0.8	4.6 ± 1.3	4.7 ± 3.9	8.1 ± 5.2	7.7 ± 3.0	10.1 ± 1.5	10.5 ± 1.6	8.6 ± 2.9	4.6 ± 2.0	2.9 ± 0.6	2.7 ± 1.4	4.5 ± 0.6
Lipid content (wt%)	24.1 ± 0.8	20.3 ± 4.5	27.7 ± 5.6	29.8 ± 12.5	29.8 ± 8.4	29.7 ± 12.9	26.5 ± 9.8	23.0 ± 7.6	28.6 ± 10.1	21.0 ± 2.0	37.9 ± 7.4	20.9 ± 1.8
Lipid productivity ($g/m^2/day$)	1.0 ± 0.2	0.8 ± 0.4	1.3 ± 1.3	1.8 ± 0.7	2.1 ± 1.0	2.6 ± 1.1	2.3 ± 0.6	1.9 ± 1.1	1.2 ± 0.7	0.5 ± 0.1	0.8± 0.3	0.8 ± 0.2

CO_2 addition experiments											
	The summer experiment						**The winter experiment**				
	Air	**0.5% CO_2**	**2% CO_2**	**5% CO_2**	**10% CO_2**		**Air**	**0.5% CO_2**	**2% CO_2**	**5% CO_2**	**10% CO_2**
VSS (g/m^3)	215.6 ± 31.2	234.9 ± 45.2	293.8 ± 54.3	309.3 ± 46.9	333.2 ± 41.0		151.5 ± 44.5	163.4 ± 48.1	161.7 ± 40.3	150.0 ± 36.7	129.9 ± 33.6
Biomass productivity ($g/m^2/day$)	13.5 ± 1.9	14.7 ± 2.6	18.5 ± 3.4	19.4 ± 2.9	20.9 ± 2.1		5.2 ± 1.4	5.6 ± 1.5	5.5 ± 1.2	5.1 ± 1.1	4.7 ± 1.0
Ash free lipid content (wt%)	28.3 ± 6.3	22.9 ± 8.4	22.3 ± 4.8	20.2 ± 6.5	21.2 ± 6.9		20.8 ± 1.6	20.2± 2.4	19.5 ± 1.7	19.8 ± 2.1	20.0 ± 1.6
Lipid productivity ($g/m^2/day$)	3.7 ± 0.5	3.4 ± 1.5	4.0 ± 0.5	3.9 ± 1.1	4.5 ± 1.8		1.1 ± 0.3	1.1 ± 0.4	1.1 ± 0.3	1.0 ± 0.3	0.9 ± 0.2

To evaluate the potential value of the WWT HRAP biomass for biodiesel production the lipid productivity of such a biomass should be assessed. The average monthly lipid content and productivity of the biomass produced in the ponds were 19.4 ± 2.8 to 37.9 ± 7.4 wt% of

ash-free biomass, and 0.6 ± 0.1 to 3.2 ± 1.2 g/m^2/day, respectively. Overall, the higher values occurred in warm seasons when the nutrient concentration was low due to faster assimilation by microbial community. However, the highest (37.9 ± 7.4 wt%) average monthly biomass lipid content which occurred in June 2014 (NZ winter) in the EHRAP coincided with the lowest mean monthly ammonia concentration (2 ± 1 mg/L) in the pond (Table 5.2). It has been shown that the lipid content of algae/WWT HRAP biomass is highly dependent on algal species, temperature, sunlight, growth phase and nutrient concentration in culture medium [1, 13, 113, 116, 119]. The highest biomass lipid content in winter and when the ammonia concentration was at the lowest average level (in both influent and effluent) implies that nutrient concentration, particularly ammonia concentration, is the main influencing factor which was consistent with the literature [10, 84, 185, 186]. As lipid productivity is a function of total lipid content and biomass productivity, it was significantly higher ($p<0.05$) in warmer months compared to colder months due to higher mean values of both lipid content and biomass productivity.

In contrast with the literature, in both CO_2 addition experiments, the CO_2 addition insignificantly affected the total biomass lipid content which was probably resulted from changes in algal dynamics. While it has been shown that supplementation of the pure algal culture with higher CO_2 concentration (ranging from 0.04% (air) to 5% CO_2) can improve the algal lipid content up to 30% [32, 155, 187]. In fact, in pure culture and under high CO_2 concentration, photorespiration is reduced and the activity of CO_2 fixation enzyme (Ribulose-1,5-bisphosphate carboxylase/oxygenase) is increased towards the production of more 3-phosphoglycerate which are further catalysed for synthesis of fatty acids [144, 187]. While in the WWT HRAP, as an algal mix culture, at different pH different species will dominate and therefore, no CO_2 stress would occur. The results could illustrate the difference between the effect of CO_2 addition on lipid content of pure culture experiments and real scale wastewater treatment-based algal mix culture. The highest lipid content, in the summer CO_2 addition experiment, occurred in the aerated HRAMs and coincided with lower ammonium concentration resulting from ammonia volatilization at high pH. Although the biomass lipid content did not differ significantly between the HRAMs, by increasing CO_2 concentration the biomass productivity was considerably enhanced ($p<0.05$) in summer which resulted in increase of the lipid productivity up to 40% compared to the control HRAMs. This implies more lipid would be available year-round for biodiesel production if operate the pond at lower pH via CO_2 addition in warm months.

5.3.4 Biomass lipid profile

To produce high amount of quality biodiesel not only feedstock lipid content and productivity are important but also obtaining appropriate lipid profile is equally important. Quality biodiesel is comprised of fatty acid methyl esters of C16:0, C18:1 and C18:3 that are derived from esterification of the triacylglyceride and hydrocarbon fractions of renewable lipid sources [133, 180, 182, 188, 189]. It has been shown that the optimum ratio of C16:0, C18:1 and C18:3 for production of quality biodiesel which could meet different standardsis 0-1:4-5:0-1 [180, 189].

In addition, feedstock lipid profile has a high impact on operational factors and yield of esterification [179, 180, 190]. If free fatty acids content in feedstock was >5wt% (normally occurred during the algal growth phase) more excess alcohol must be supplied and alkaline catalyst should be substituted by acid catalyst which increase reaction time up to five folds [40, 190]. Moreover, the feedstock lipid complexity and impurities such as chlorophyll reduces the yield of esterification to <90% [76]. Furthermore, increasing chain length and reducing unsaturation degree of lipids increase cetane number, heat of combustion, melting point, and viscosity of biodiesel while decrease oxidative stability and cause lipid polymerization into waxy solids [179, 180, 182]. Therefore, complete profiling of WWT HRAP biomass lipids is essential before using such a free biomass for quality biodiesel production.

The weight fraction of the individual fatty acid methyl esters (FAMEs) in the WWT HRAP/HRAMs biomass lipids are summarized in Table 5.4 and Table 5.5. According to the conversion of internal standard, the conversion of esterification reaction was >90%. Fig. 5.1 shows an example of gas chromatogram of esterified extracted lipids. As can be seen (Fig. 5.1 and Tables 5.4 & 5.5), the FAMEs profile of the WWT HRAP/HTRAMs biomass was complex so that ~50 wt% of the FAMEs could not be identified. The complexity of the WWT HRAP lipid profile resulted from the complexity of microbial community comprised of different algal and bacteria species with different lipid profiles [24, 119, 177].

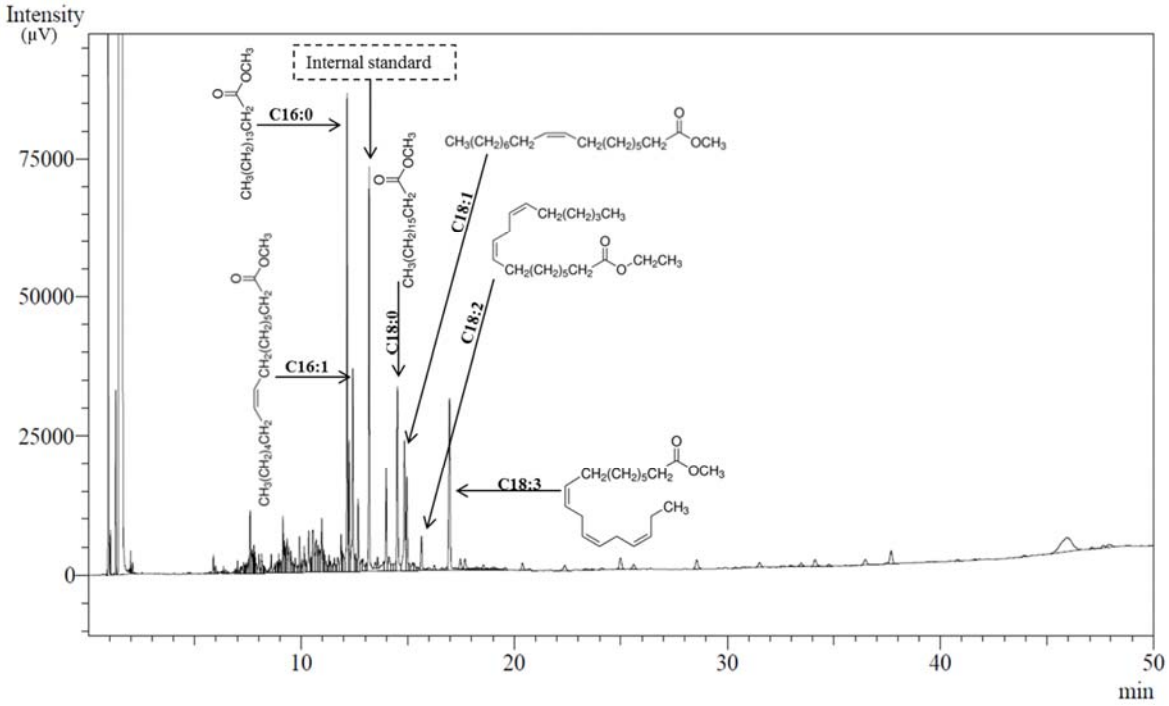


Figure 5.1 An example of gas chromatogram of the WWT HRAP/HRAM biomass FAMEs profile

Methyl esters of palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1) and linolenic (C18:3) acids were the most abundant FAMEs species in the pond biomass lipids (Table 5.4). The composition was in agreement with what has been reported for wastewater microalgae/biomass lipids [10, 100, 111, 177, 189]. The biomass methyl esters during the pond monitoring were dominated by the saturated FAMEs (23-32.2 wt% of the total FAMEs) while the monounsaturated and the polyunsaturated FAMEs ranged 10.8-19.7 wt% and 4.8-10.7 wt% of the total FAMEs, respectively. Doma et al. [177] found a similar trend in lipid profile of WWT HRAP biomass when the biomass was dominated by *Micractinium* sp. and *Scenedesmus* sp. as algae and *Gomphonema oilvecum* as bacteria. They reported 59.8 wt%, 13.7 wt% and 12.4 wt% for saturated, monounsaturated and polyunsaturated FAMEs, respectively. In good agreement with the literature the average saturated and monounsaturated FAMEs were higher in warm months compared to cold months [1, 13]. However, comparing the biomass FAMEs profile over the seasons and between both ponds indicated that the fluctuation was insignificant corresponding to changes in environmental conditions and algal dynamic.

Table 5.4 Biomass fatty acid methyl esters composition (wt% of total FAMEs) at monthly intervals during the ponds monitoring experiment

		Winter	Spring			Summer			Autumn			Winter	
		Aug. 2013	Sep. 2013	Oct. 2013	Nov. 2013	Dec. 2013	Jan. 2014	Feb. 2014	Mar. 2014	Apr. 2014	May 2014	Jun. 2014	Jul. 2014
C10:0	**WHRAP**	0	0.5 ± 0.1	0.1 ± 0.1	1.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0	0.1 ± 0.1	00	0.3 ± 0.1	0.4 ± 0.2
	EHRAP	0.2 ± 0.1	0.1 ± 0.1	0.6 ± 0.2	0.1 ± 0.1	0.3 ± 0.1	1.3 ± 0.2	0	0	0	1.1 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
C12:0	**WHRAP**	0.1 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	1.2 ± 0.3	0.3 ± 0.2	0.7 ± 0.3	0.1 ± 0.1	0	1.1 ± 0.3	0.1 ± 0.1	0.7 ± 0.3	0.3 ± 0.1
	EHRAP	0.3 ± 0.1	0.1 ± 0.1	0.6 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.8 ± 0.2	0	0.6 ± 0.3	1.1 ± 0.2	0.9 ± 0.2	0.2 ± 0.1	0.1 ± 0.1
C14:0	**WHRAP**	3.0 ± 0.4	4.5 ± 0.6	4.8 ± 1.0	4.8 ± 0.4	2.2 ± 0.4	3.1 ± 0.5	5.1 ± 0.4	3.7 ± 0.7	4.8 ± 0.8	2.7 ± 0.4	4.8 ± 0.5	1.9 ± 0.4
	EHRAP	4.0 ± 0.3	2.2 ± 0.1	3.3 ± 0.3	3.7 ± 0.3	2.9 ± 0.3	2.2 ± 0.2	2.2 ± 0.2	2.8± 0.3	5.6 ± 1.1	2.9 ± 0.3	2.4 ± 0.3	1.8 ± 0.2
C16:0	**WHRAP**	13.1 ± 3.1	12.7 ± 0.3	14.7 ± 2.1	14.1 ± 2.1	15.2 ± 1.3	16.1 ± 2.2	17.5 ± 3.4	17.0 ± 4.1	13.1 ± 2.4	15.8 ± 3.1	15.8 ± 3.1	12.6 ± 1.3
	EHRAP	12.0 ± 1.5	13.2 ± 0.2	15.4 ± 0.9	19.5 ± 1.2	13.4 ± 1.4	15.9 ± 1.9	16.8 ± 4.1	16.1 ± 2.3	14.5 ± 1.6	17.3 ± 2.9	16.2 ± 2.1	12.4 ± 2.3
C18:0	**WHRAP**	6.9 ± 0.5	7.7 ± 0.6	7.6 ± 0.2	6.2 ± 0.5	8.4 ± 2.1	8.1 ± 0.7	5.9 ± 0.4	6.3 ± 1.3	7.3 ± 0.7	5.6 ± 0.4	7.4 ± 0.7	6.6 ± 0.6
	EHRAP	7.5 ± 0.3	6.4 ± 0.2	7.9 ± 0.5	8.6 ± 0.6	7.9 ± 1.1	6.9 ± 0.3	3.2 ± 0.2	5.5 ± 0.5	8.0 ± 1.5	4.8 ± 0.7	6.8 ± 0.6	6.4 ± 0.2
C20:0	**WHRAP**	1.0 ± 0.2	0.7 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	2.4 ± 0.2	0.2 ± 0.1	0.4 ± 0.3	0.6 ± 0.5
	EHRAP	0.7 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.1 ± 0.1	0.3 ± 0.2	0.4 ± 0.2	0.2 ± 0.1	1.0 ± 0.3	1.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
C22:0	**WHRAP**	0.5 ± 0.1	0.3 ± 0.1	0	0	0	0	0.2 ± 0.1	0	0	0	0.1 ± 0.1	0.3 ± 0.1
	EHRAP	0.3 ± 0.2	0.6 ± 0.2	0	0	0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.4 ± 0.2
C24:0	**WHRAP**	0	0	0	0	0	0	0.4 ± 0.2	0	0	0.2 ± 0.2	0.1 ± 0.1	0.3 ± 0.1
	EHRAP	0	0.1 ± 0.1	0	0.1 ± 0.1	0	0	0.3 ± 0.1	0.1 ± 0.1	0	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
Total	WHRAP	24.6 ± 2.1	26.7 ± 0.9	27.9 ± 1.3	28.2 ± 3.1	26.9 ± 0.8	28.6 ± 0.7	29.6 ± 3.1	27.2 ± 1.6	28.8 ± 0.9	24.6 ± 0.3	29.6 ± 0.7	23.0 ± 3.1
	EHRAP	25.0 ± 3.2	23.0 ± 2.0	28.2 ± 2.3	32.2 ± 1.4	25.0 ± 2.3	27.6 ± 1.2	22.8 ± 0.7	26.2 ± 0.9	30.7 ± 2.1	27.6 ± 0.7	26.3 ± 1.3	21.9 ± 2.3
C16:1	**WHRAP**	5.1 ± 0.4	5.3 ± 0.2	6.3 ± 0.2	5.9 ± 0.3	6.4 ± 0.4	6.6 ± 0.2	9.0 ± 2.3	10.5 ± 0.5	7.5 ± 0.4	10.6 ± 1.7	6.7 ± 1.3	7.6 ± 2.1
	EHRAP	5.6 ± 0.2	5.6 ± 0.1	5.2 ± 0.4	6.7 ± 1.1	5.7 ± 1.2	5.6 ± 0.6	12.5 ± 1.4	12.7 ± 0.3	6.3 ± 0.8	14.8 ± 3.1	8.2 ± 0.3	8.1 ± 0.9
C18:1	**WHRAP**	5.8 ± 1.1	5.8 ± 0.4	4.5 ± 1.1	5.1 ± 0.2	6.5 ± 0.9	6.3 ± 2.1	5.3 ± 0.8	3.7 ± 0.2	4.1 ± 1.1	4.4 ± 0.3	4.3 ± 0.6	4.2 ± 0.2
	EHRAP	5.3 ± 0.1	5.4 ± 0.2	3.4 ± 0.3	4.9 ± 0.6	5.5 ± 0.3	6.2 ± 0.8	5.1 ± 0.3	4.8 ± 0.9	5.0 ± 0.2	4.9 ± 0.6	4.3 ± 0.2	2.6 ± 0.1
Total	WHRAP	10.9 ± 2.1	11.1 ± 1.1	10.8 ± 0.9	11.0 ± 0.8	12.9 ± 0.6	12.9 ± 1.3	14.3 ± 1.2	14.2 ± 0.9	11.6 ± 2.1	15.0 ± 0.8	11.0 ± 0.7	11.8 ± 0.7
	EHRAP	10.9 ±	11.0	8.6 ±	11.6	11.2	11.8 ±	17.6 ±	17.5 ±	11.3 ±	19.7	12.5	10.7 ±

		1.3	± 2.0	0.6	± 0.5	± 0.3	1.3	3.1	3.1	1.3	± 1.4	± 2.1	0.3
C18:2	**WHRAP**	1.9 ± 0.1	3.2 ± 0.2	4.5 ± 0.4	4.6 ± 0.3	4.3 ± 0.2	3.7 ± 0.4	3.5 ± 0.5	3.0 ± 1.3	3.2 ± 0.3	2.9 ± 0.9	5.5 ± 0.5	1.7 ± 0.4
	EHRAP	3.1 ± 0.1	1.9 ± 0.1	3.0 ± 1.1	3.5 ± 0.5	3.7 ± 0.7	3.9 ± 0.3	2.6 ± 0.2	3.0 ± 0.8	2.7 ± 0.5	2.5 ± 0.3	3.8 ± 0.7	1.7 ± 0.6
C18:3	**WHRAP**	3.9 ± 0.3	3.2 ± 0.4	5.5 ± 2.3	4.7 ± 0.7	3.4 ± 0.3	3.7 ± 0.7	5.0 ± 1.0	2.1 ± 0.3	1.8 ± 0.6	4.1 ± 0.7	2.8 ± 0.3	4.5 ± 0.5
	EHRAP	5.2 ± 0.5	4.7 ± 0.7	3.6 ± 2.1	4.2 ± 0.3	1.0 ± 0.2	6.7 ± 0.6	5.0 ± 0.5	3.8 ± 0.2	2.4 ± 0.3	4.4 ± 0.2	4.1 ± 1.1	5.9 ± 1.3
C18:3 n6	**WHRAP**	0	0	0	0.1 ± 0.1	0	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0
	EHRAP	0.1 ± 0.1	0.2 ± 0.1	0	0	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0	0
Total	WHRAP	5.8 ± 0.2	6.4 ± 0.3	10.0 ± 0.3	9.4 ± 0.6	7.8 ± 0.3	7.5 ± 0.2	8.7 ± 2.1	5.2 ± 0.9	5.2 ± 0.7	7.2 ± 2.1	8.4 ± 0.3	6.2 ± 0.2
	EHRAP	8.4 ± 0.6	6.8 ± 0.8	6.6 ± 0.4	7.7 ± 0.7	4.8 ± 0.8	10.7 ± 1.3	7.8 ± 2.1	7.0 ± 2.1	5.3 ± 0.4	7.1 ± 0.7	7.9 ± 0.6	7.6 ± 0.5
Total known FAMEs	WHRAP	41.3 ± 5.1	44.2 ± 3.1	48.7 ± 4.1	48.6 ±3.2	47.6 ±6.3	49.0 ± 3.2	52.6 ± 2.1	46.6 ± 5.1	45.6 ± 3.2	46.8 ± 2.2	49.0 ± 0.15	41.0 ± 4.1
	EHRAP	44.3 ± 3.2	40.8 ± 1.5	43.4 ± 3.5	51.5 ± 6.2	41.0 ± 2.3	50.1 ± 3.2	48.2 ± 2.1	50.7 ± 6.7	47.3 ± 4.4	54.4 ± 5.1	46.7 ± 3.2	40.2 ± 3.7
Unknown FAMEs	**WHRAP**	58.7	55.8	51.3	51.4	52.4	51.0	47.4	53.4	54.4	53.2	51.0	59.0
	EHRAP	55.7	59.2	56.6	48.5	59.0	49.9	51.8	49.3	52.7	45.6	53.3	59.8

According to the biomass lipid profile of HRAMs during the last week of CO_2 addition experiments, similar to the pond biomass lipid profile, saturated fatty acids formed the majority of the known FAMEs followed by monounsaturated FAMEs and polyunsaturated FAMEs (Table 5.5). Palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1) and linolenic (C18:3) acids were predominant fatty acids in all cultures in the both CO_2 addition experiments. Despite the considerable impacts of CO_2 addition on pure culture algal lipid profile which have been reported in the literature [32, 155, 191], slight changes in the biomass lipid profile of the WWT HRAP/HRAM biomass, illustrated ineffectiveness of CO_2 addition for improving the mixed culture lipid profile. This might results from the dominance of different species with most likely similar lipid profile in different CO_2-supplemented cultures.

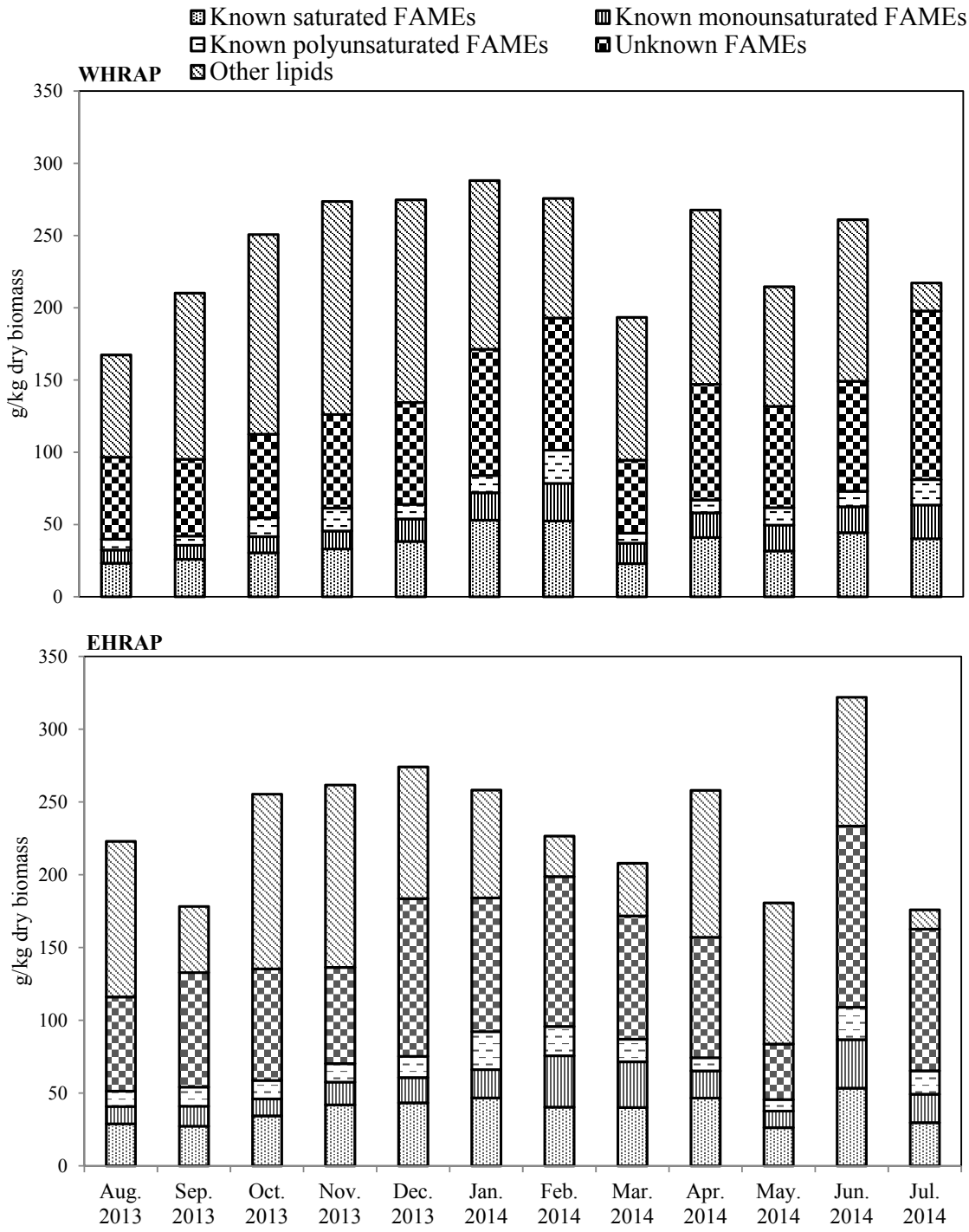
Table 5.5 WWT HRAM biomass fatty acid methyl esters composition (wt% of total FAMEs) over the last week of summer and winter CO_2 addition experiments

The summer CO_2 addition experiment					
	Air	**0.5% CO_2**	**2% CO_2**	**5% CO_2**	**10% CO_2**
C10:0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
C12:0	0.3 ± 0.5	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
C14:0	1.2 ± 0.2	1.4 ± 0.1	1.8 ± 0.4	1.7 ± 0.2	1.2 ± 0.1
C16:0	13.8 ± 0.2	10.5 ± 1.0	13.6 ± 0.1	13.9 ± 1.6	16.1 ± 1.0
C18:0	4.7 ± 1.0	3.0 ± 0.5	4.2 ± 0.5	3.1 ± 0.3	3.3 ± 0.7
C20:0	0.8 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
C22:0	0.3 ± 0.1	0.1 ± 0.1	0.0	0.0	0.0
C24:0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Total	21.7 ± 0.2	16.1 ± 0.8	20.9 ± 1.3	19.7 ± 2.0	21.7 ± 0.2
C16:1	4.9 ± 0.3	7.3 ± 0.2	6.2 ± 0.5	5.2 ± 1.0	5.1 ± 1.5
C18:1	6.8 ± 1.0	4.2 ± 0.1	5.0 ± 0.1	5.5 ± 1.0	5.9 ± 0.3
Total	11.7 ± 0.5	11.5 ± 0.2	11.2 ± 0.3	10.7 ± 1.0	11.0 ± 1.0
C18:2	4.4 ± 0.2	4.2 ± 0.2.0	3.3 ± 0.4	6.1 ± 0.3	5.5 ± 0.7
C18:3	6.7 ± 0.5	5.5 ± 2.0	4.4 ± 0.1	6.9 ± 1.1	7.1 ± 1.0
C18:3n6	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Total	11.4 ± 0.4	9.9 ± 1.0	7.9 ± 0.3	13.2 ± 0.6	12.8 ± 0.7
Total known FAMEs	44.8 ± 3.5	37.5 ± 4.1	40.0 ± 3.2	43.6 ± 5.1	45.5 ± 4.1
Others	51.2	62.5	60	56.4	54.5
The winter CO_2 addition experiment					
C10:0	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
C12:0	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
C14:0	1.4 ± 0.2	1.9 ± 0.1	4.8 ± 0.5	2.1 ± 0.5	2.2 ± 0.4
C16:0	14.0 ± 0.1	13.7 ± 2.0	15.1 ± 3.0	16.1 ± 2.1	13.5 ± 3.1
C18:0	8.2 ± 0.4	7.4 ± 0.6	7.4 ± 0.1	6.3 ± 0.9	6.1 ± 0.8
C20:0	0.7 ± 0.1	0.6 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	1.0 ± 0.2
C22:0	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
C24:0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.2
Total	25.9 ± 2.1	25.2 ± 1.5	29.7 ± 1.3	26.8 ± 0.5	24.5 ± 1.2
C16:1	5.4 ± 0.6	6.0 ± 0.3	5.7 ± 0.1	3.5 ± 0.5	3.7 ± 0.8
C18:1	3.4 ± 1.0	4.3 ± 0.7	4.9 ± 1.0	5.9 ± 0.3	4.2 ± 1.5
Total	8.8 ± 0.5	10.3 ± 0.4	10.6 ± 0.2	9.4 ± 0.3	7.9 ± 0.9
C18:2	1.5 ± 0.1	2.6 ± 0.6	2.8 ± 0.4	2.0 ± 0.2	1.9 ± 0.1
C18:3	3.3 ± 1.0	4.5 ± 1.5	4.5 ± 1.0	3.1 ± 0.1	2.6 ± 0.1
C18:3n6	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1

Total	5.1 ± 1.0	7.4 ± 0.6	7.6 ± 0.5	5.4 ± 0.1	4.8 ± 0.1
Total known FAMEs	39.8 ± 2.1	42.9 ± 1.4	47.9 ± 1.6	41.6 ± 2.1	37.2
Others	60.2	57.1	52.1	58.4	62.8

5.4 Potential of biodiesel production from WWT HRAP biomass

To evaluate the potential of biodiesel production from WWT HRAP/HRAMs biomass the mean mass fraction of total known FAMEs per unit biomass was calculated as shown in Fig. 5.2. In a good agreement with the literature [83, 191, 192], all experiments showed that 60 ± 15 wt% of total lipids (equal to 14.8 ± 4.0 wt% of ash-free biomass) was convertible to the FAMEs (raw biodiesel, i.e. mixture of desired and undesired FAMEs). With regards to the biomass productivity (Table 5.3), 0.9 ± 0.1 g/m^2/d (equals to 3.2 ± 0.5 tons/ha/year) raw biodiesel could be produced year-round from WWT HRAP biomass when the ponds were operated with no control of dominant species and zooplankton population. The results were in line with what have been reported for biodiesel production from algal biomass cultivated in municipal wastewater sources in literature [189, 193]. Assemany et al. [193] estimated that, theoretically, 3.6 tons/ha/year biodiesel can be produced from algal consortium grown on pre-UV disinfected domestic wastewater while the biomass productivity was estimated to be 40 ton/ha/year based on a 5 month outdoor experiment when the average temperature was 24 ºC. The results of the CO_2 addition experiments indicated that the potential value of WWT HRAP for raw biodiesel production could be increased up to 1.1 g/m^2/d (equals to 4.0 ton/ha/year) (by assuming average 20% biomass productivity increase in spring and autumn) by lowering the WWT HRAP pH via CO_2 addition during the warm months.


Known saturated FAMEs
Known monounsaturated FAMEs
Known polyunsaturated FAMEs
Unknown FAMEs
Other lipids
WHRAP
EHRAP
g/kg dry biomass
0
50
100
150
200
250
300
350
Aug. 2013
Sep. 2013
Oct. 2013
Nov. 2013
Dec. 2013
Jan. 2014
Feb. 2014
Mar. 2014
Apr. 2014
May. 2014
Jun. 2014
Jul. 2014

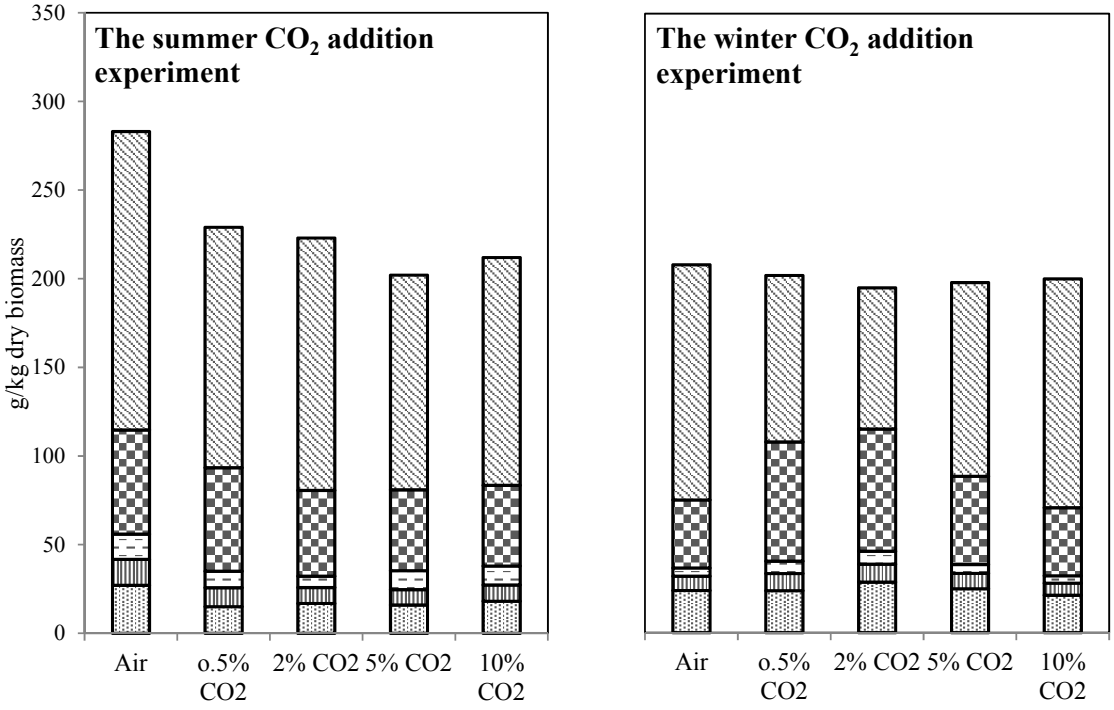


Figure 5.2 The amount of individual FAME categories (g/kg dry biomass)

5.5 Quality of the WWT HRAP biomass-based biodiesel

As the esterification reaction does not change the feedstock fatty acids composition [180](Ramos et al., 2009), it can be concluded that the biodiesel properties are function of the feedstock lipid profile. This means that the overall properties of biodiesel including cetane number (CN), iodine value (IV), saponification value (SV), cloud point (CP) and energy content (HHV) can be predicted according to the structural properties of fatty acids [179, 180, 182].

CN, IV, SV, CP and HHV of the WWT HRAP biomass-based biodiesel defined according to Eq. 5.2-5.6 are summarised in Table 5.6. Over the course of study, in all experiments, CN, as a proxy for ignition quality of the fuel and a prime indicator of biodiesel quality, insignificantly fluctuated between 37.7 and 42.5 while the minimum CN for quality biodiesel according to UNE-EN 14214 is 51. The higher CN values were obtained where the ratio of saturated: unsaturated FAMEs was higher. The CN values were lower than what have been reported (44-95) for biodiesel produced from pure algae in the literature [115, 182]. This resulted from the complexity of the lipid feedstock in present study. The oxidation instability of biodiesel is function of the total amount of unsaturated FAMEs measured as IV. Higher IV

results in the higher polymerisation of glycerides which can lead to formation of deposits and deterioration of the lubricant [115, 179, 182]. The oxidation rate of polyunsaturated FAMEs is much (up to 100 times) higher than monounsaturated FAMEs [180](Ramos et al., 2009). Hence, according to the EN-14214 standard, the level of polyunsaturated FAMEs should be <12wt% of the final product. The WWT HRAP/HRAM biomass-based biodiesel had much lower IV values (17.1-38.1 g I_2/100 g biodiesel) compared with the maximum standard value of IV (120 g I_2/100 g biodiesel) and values reported for biodiesel produced from pure algal biomass (40-140 g I_2/100 g biodiesel) [155, 182]. This could result to the least degree of biodiesel oxidation at higher temperature and during storage time. The higher SV results in the higher percentage of free fatty acids which may lead to soap formation and lower yield of biodiesel [194]. The average SV was >239 mg KOH/g biodiesel which was much higher than values (170-217 mg KOH/g biodiesel) have been reported for pure algal biodiesel [115, 182, 195]. CP relates to biodiesel cold properties and defines the onset crystallisation temperature of the biodiesel. The higher degree of unsaturation results in the lower CP which means the biodiesel could be used in colder regions. In fact, as the melting point of saturated fatty acids is high, the unsaturated fatty acids act as a solvent and prevent clogging the injector at cold weather. According to the CP values (Table 5.6), the biodiesel produced from the WWT HRAP biomass, can be used, in warm regions [196, 197]. In addition, the HHV values insignificantly fluctuated between 38.7 and 39.2 kJ/g in all experiments which were close but lower than what have been reported for pure algal biodiesel, 39-41 kJ/g [2, 40, 169].

Table 5.6 The estimated properties of the raw biodiesel which could be produced from WWT HRAP/HRAM biomass

The pond monitoring experiment													
		Winter	**Spring**			**Summer**			**Autumn**			**Winter**	
		Aug. 2013	**Sep. 2013**	**Oct. 2013**	**Nov. 2013**	**Dec. 2013**	**Jan. 2014**	**Feb. 2014**	**Mar. 2014**	**Apr. 2014**	**May 2014**	**Jun. 2014**	**Jul. 2014**
SV[a]	WHRAP	244.8	243.5	240.7	243.0	240.0	240.4	242.4	246.0	246.2	245.4	242.2	246.1
	EHRAP	242.8	245.0	243.6	239.0	244.6	239.4	247.0	245.3	244.0	245.5	243.0	246.4
IV[b]	WHRAP	23.2	23.8	31.9	30.1	27.9	27.6	32.1	23.7	20.8	29.4	26.8	25.4
	EHRAP	28.7	25.4	22.4	27.5	19.1	34.8	33.6	31.1	21.1	33.9	28.6	28.1
CN	WHRAP	41.1	41.0	39.2	39.6	40.1	40.1	39.1	41.0	41.6	39.7	40.3	40.6
	EHRAP	39.9	40.6	41.3	40.1	42.0	38.5	38.8	39.3	41.6	38.7	39.9	40.0
CP[c]	WHRAP	1.9	1.7	2.7	2.4	3.0	3.5	4.2	4.0	1.9	3.3	3.3	1.6
	EHRAP	1.3	2.0	3.1	5.3	2.1	3.4	3.8	3.5	2.6	4.1	3.5	1.5
HHV[d]	WHRAP	39.0	39.1	39.1	39.0	39.2	39.2	39.0	39.0	39.0	38.9	39.1	39.0
	EHRAP	39.0	39.0	39.1	39.2	39.1	39.1	38.8	38.9	39.1	38.9	39.0	38.9

	The summer CO_2 addition experiment						The winter CO_2 addition experiment				
	Air	**0.5% CO_2**	**2% CO_2**	**5% CO_2**	**10% CO_2**		**Air**	**0.5% CO_2**	**2% CO_2**	**5% CO_2**	**10% CO_2**
SV	242.3	248.9	247.1	243.8	242.6		245.2	243.9	242.7	244.8	247.8
IV	35.5	32.0	27.3	38.1	37.8		19.2	25.5	26.1	19.9	17.1
CN	38.3	39.1	40.2	37.7	37.8		42.0	40.6	40.4	41.9	42.5
CP	2.3	0.5	2.2	2.3	3.5		2.4	2.2	3.0	3.5	2.1
HHV	39.0	38.7	38.9	38.9	38.9		39.1	39.0	39.1	39.1	39.0

[a] The unit of saponification value (SV) is mg KOH/g biodiesel

[b] The unit of iodine value (IV) is g I_2/100 g biodiesel

[c] The unit of cloud point (CP) is ºC

[d] The unite of energy content (HHV) is kJ/g biodiesel

5.6 Is WWT HRAP biomass a promising feedstock for biodiesel production?

Biodiesel is a lipid-based fuel and therefore its quantity and quality are function of feedstock lipid content and profile. The lower lipid content and the higher complexity of lipids result in higher processing costs. In terms of quantity, the potential of raw biodiesel production from WWT HRAP biomass is higher than what have been reported for majority of traditional oil seed such as soybean, canola and jatropha [160, 198, 199]. While in terms of quality, the WWT HRAP/HRAM biomass-based biodiesel has lower quality compared to oil seed-based biodiesel since the lipid profile of oil seeds are simple and mainly predominated by biodiesel fatty acids which leads to lower processing costs [169, 199-201]. Therefore, less purification and upgrading processes are required for production of oil seed-based quality biodiesel rather than production of quality biodiesel from WWT HRAP biomass. Although, according to the results, it can be concluded that the WWT HRAP biomass is not a promising feedstock for low-cost quality biodiesel production, its quality can be improved by blending with oil seeds-based biodiesel [100].

From energy standpoint of view, it has been shown that, the average annual energy content of the WWT HRAP biomass is 19.2 MJ/kg biomass [119] and as predicted the energy content of the raw biodiesel produced from the WWT HRAP biomass would be ~ 39 MJ/kg biodiesel. Considering WWT HRAP biomass as feedstock for biodiesel production, based on the results (Fig. 5.2), on average, 148 ± 40 g raw biodiesel/kg biomass could be produced which is ~ 30% of the biomass energy (<6 MJ/kg biomass). Comparing recoverable energy with the total energy (3.5-8 MJ/kg biomass) required for dewatering, drying (optional), lipid extraction and esterification without considering purification of product shows unsuitability of this route [19, 75, 76, 172, 174, 175]. Therefore, it may better to recover the WWT HRAP

biomass energy through the processing of the whole biomass via processes suchs as hydrothermal liquefaction (HTL), pyrolysis or anaerobic digestion (AD) [76]. It has been reported that, ~75%, ~50% and ~35% of the algal biomass energy could be recovered through HTL, pyrolysis and AD processes, respectively [76]. The higher energy recovery through suggested conversion routes may improve the net energy balance. However, further research is still needed on processing of the WWT HRAP biomass via other conversion routes to define the most promising conversion pathway for commercial scale energy recovery from such a free biomass.

5.7 Conclusions

The year-round biodiesel production potential from WWT HRAP biomass and the effect of CO_2 addition on this potential were investigated in outdoor experiments using WWT HRAPs/HRAMs. The mean monthly HRAP biomass and lipid productivities varied between 2.0 ± 0.3 - 11.1 ± 2.5 g VSS/m^2/d, and between 0.5 ± 0.1 - 2.6 ± 1.1 g/m^2/d, respectively, with higher values in the warmer summer months. Only <15 wt% of the HRAP ash-free biomass could be converted to raw biodiesel (a mixture of desirable (<30 wt%) and non-desirable (>70 wt%) FAMEs) through esterification of the biomass lipid fraction. Annual average raw biodiesel production was 0.9 ± 0.1 g/m^2/d. CO_2 addition enhanced biodiesel production during warm summer months without negatively affecting pond treatment performance.

CO_2 addition (by lowering culture pH to 6-7) during warm summer months increased biomass productivity to 1.1 ± 0.1 g/m^2/d, but had little effect on both the biomass lipid content and profile. Consequently CO_2 addition did not change the quality of biodiesel. The quality of the WWT HRAP biomass-based biodiesel (in terms of cetane number, saponification value, cloud point and energy content) was low so that it cannot be used directly as a transportation fuel and would need to be blended with quality biodiesel before use.

CHAPTER 6

Pyrolysis of wastewater treatment high rate algal pond (WWT HRAP) biomass

This chapter is based on the following publication:

Mehrabadi, A., Craggs, R., Farid, M. 2016. Pyrolysis of wastewater treatment high rate algal pond (WWT HRAP) biomass. *Algal Research*. In press.

Chapter preface

This chapter investigates the potential of pyrolitic bio-oil production from wastewater treatment high rate algal pond biomass. The pyrolitic bio-oil produced at different temperatures (300 ºC, 400 ºC and 500 ºC) was assessed in terms of yield, chemical and elemental composition, and energy content. Thermal decomposition behaviour of the biomass was confirmed using thermogravimetric analysis (TGA). Stepwise pyrolysis and TGA were employed to determine the bio-oil conversion at different temperature intervals. TGA results indicated that a maximum of 50 ± 2 wt% of the initial biomass was pyrolysed at 500 ºC which was in agreement with the product conversion results in pyrolysis. The highest yield of the liquid fraction was obtained at 500 ºC. At <400 ºC, the liquid fraction was mainly dominated by an aqueous phase, while the bio-oil phase was mainly produced at 400-500 ºC. Elemental analysis indicated that the bio-oil contained >65 wt% carbon, 6-9 wt% nitrogen, 8-10.2 wt% hydrogen and had an energy content of 34.4-37 kJ/g, all with the higher values at higher temperature except for nitrogen. GC-MS analysis showed high complexity of the liquid fraction in which aromatics and acids were dominant in the bio-oil and aqueous phases, respectively. Energy balance on system indicated that using the non-condensable gases and bio-char as fuel to supply the process energy demand could make algal-based bio-oil feasible from energy point of view. However, further research is required to make bio-oil production economical.

6.1 Introduction

Microalgae biomass have been highlighted as a promising feedstock for production of different kinds of biofuels for several years [3, 14, 202] owing to high productivity, zero CO2 emission and ability to grow on wasteland and wastewater [1, 22]. Algal-based biofuels including biogas, bioethanol, biodiesel and bio-oil could be produced through conversion of the whole or part of algal biomass [76]. However, due to high biomass production costs and technological limitations of conversion routes, algal-based biofuel production is not yet economically competitive with the fossil fuels [17, 77]. Therefore, to reduce the cost of algal biofuel, the use of algae that is a by-product of wastewater treatment in high rate algal ponds (WWT HRAP) has been suggested [14]. Several studies have shown WWT HRAP biomass productivities of 20-60 tonnes/ha/year, consisting of 60-80 wt% algae, 20-30 wt% bacteria, and 5-10wt% other organic matter [11, 27, 119]. Similar to algal biomass, WWT HRAP biomass is typically composed of 40-55 wt% protein, 15-30 wt% lipid and 15-25 wt%

carbohydrate [119]. A high lipid fraction is desirable for biofuel production, although, both the production of high lipid content algal biomass, and current methods of lipid extraction are costly [19, 203]. Therefore, for algal-based biofuel to be economical, conversion all biomass components to fuel must be maximized. Thermochemical conversion (pyrolysis) of biomass in the absence of oxygen has been suggested as a pathway to enhance the energy conversion of either low-lipid content microalgae or algal residues from lipid extraction [67, 68, 204].

Pyrolysis is a thermal cracking technique that can be applied to dry algal biomass/residue in the absence of oxygen at around 500 ºC to produce renewable high energy content oil (20-33 kJ/g), gas (2-5 kJ/g) and char (4-8 kJ/g) [68, 71, 76]. In general, the algal pyrolysis process involves three steps: (i) dehydration (vaporization of intracellular and loosely bonded water at <200 ºC); (ii) volatilization and decomposition of volatile organic components (at <200-500 ºC) which forms stable liquids and gases after condensation; and (iii) char formation (which occurs at temperatures >500 ºC) [67, 76, 203, 205].

In comparison with conventional pyrolitic bio-oil feedstock such as woody biomass, algal biomass is more suitable for high quality energy rich bio-oil production owing to its high lipid content, which has a low oxygen and high carbon and hydrogen content (H and C are key elements in determination of energy content and quality of products) [69, 203]. Compared with WWT HRAP biomass, lignocellulosic biomass is mainly composed of hemicellulose, cellulose and/or lignin components which have low tendency to volatilise at low temperatures [70, 71]. In addition, the lignocellulose-based pyrolitic oil is acidic (pH<3), unstable and has higher viscosity with relatively low energy content (19 kJ/g) compared to algal-based pyrolitic bio-oil, therefore, further upgrading is required [68, 71]. Moreover, algal-based pyrolitic bio-oil contains linear hydrocarbons and more aromatic compounds, which contribute to the higher energy content and quality [71, 203, 206].

The thermal decomposition behaviour of algal-based biomass depends highly on the biomass composition, since microalgae are composed of many different types of lipid, carbohydrate and protein, which all have different thermal decomposition characteristics [207-209]. Grierson et al. [70] conducted slow pyrolysis on biomass of six different algal species (*Tetraselmis chui*, *Chlorella* sp., *Chlorella vulgaris*, *Chaetocerous muelleri*, *Dunaliella tertiolecta* and *Synechococcus* sp.) at 500 ºC to examine effect of their composition on pyrolysis products and found, for all species that the greatest weight loss occurred during the second step of algal pyrolysis (250–350 ºC). However, yields of liquid, gas and char phases, and average molecular weight of the liquid phase (which is a proxy for product properties) significantly varied between algal species. The maximum liquid and gas

yields were for both *Chlorella* species (41 wt% and 22-25 wt%, respectively) and the minimum was for *D. tertiolecta* (24 wt% and 13 wt%, respectively) at 500 ºC. In contrast the highest char yield was observed for *D. tertiolecta* (63 wt%), while the lowest bio-char (30 wt%) was produced through pyrolysis of *Chlorella* species. Although they did not report the biochemical composition of tested algal species, the differences in yield of products indicates the effect of algal composition on the process yield.

Gong et al. [210] investigated thermal decomposition behaviour and pyrolysis products distribution of *Chlorella vulgaris* and *Dunaliella salina* at different temperatures (300-700ºC) while the holding time was 20 min. TGA results indicated that *D. salina* biomass decomposition began at lower temperature (150 ºC) relatives to *C. vulgaris* (160 ºC) and occurring at higher rate. This was probably due to the cell wall structure and higher protein content of *D. salina* since proteins need less activation energy for decomposition compared with the lipids and carbohydrates [210]. For both species the maximum bio-oil yields (55.4 wt% for *D.salina* and 49.2 wt% for *C. vulgaris*) were obtained at 500 ºC where the lowest bio-char yields occurred. Reduction of the bio-oil yields at temperatures >500 ºC was more likely due to occurring secondary reactions resulted in increased gas and char yields. A direct relationship between temperature and gas yield in was found, showing an increase from~0.5 wt% at 300 ºC to maximum 17.7 wt% at 700 ºC [210]. While the temperature has been highlighted as the main parameter influencing the yield of algal pyrolytic bio-oil production, temperature effects on the quality of algal bio-oil have been not addressed in detail.

Although, pyrolysis of algal biomass has been widely investigated, there has been little focus on the potential of pyrolitic bio-oil production from WWT HRAP biomass. This study investigates the potential of bio-oil production from WWT HRAP biomass as well as its thermal decomposition behaviour. The bio-oil produced from pyrolysis of WWT HRAP biomass at different temperatures and heating regimes was assessed in terms of yield, chemical and elemental composition, and energy content. In addition, the characteristics of bio-char (produced as a by-product of pyrolysis) were determined in terms of elemental composition, heavy metal concentration, surface area and energy content.

6.2 Materials and methods

6.2.1 Feedstock characterization

WWT HRAP biomass was collected from two identical outdoor pilot-scale WWT HRAPs (8 m^3 volume, 0.3 m depth and 31.8 m^2 surface area) located at the Ruakura Research

Centre, Hamilton, New Zealand (37°47′S, 175°19′E). Microscopic analysis showed that the biomass including the following algal species: *Coleastrum* sp., *Actinustrum* sp., *Diatom* sp. and *Mucidosphaerium pulchellum.* The biomass was oven dried at 80 ºC (to prevent weight lost) overnight prior to analysis and grinded to a particle size of <5 mm before use in pyrolysis experiments. Replicate total protein, lipid and carbohydrate content measurement were performed according to the Lowry method [90], the Bligh and Dyer [89] and the phenol sulphuric acid [202] methods respectively, as described in our previous study [119]. Duplicate ultimate analysis and energy content of the biomass were measured using Carlo-Ebra EA 1108. The concentrations of exchangeable cations and heavy metals in the biomass were determined using Inductively Coupled Plasma Mass Spectroscopy (ICP-MS).

6.2.2 Thermal study

Thermal decomposition behaviour of the biomass during pyrolysis under stepwise and non-stepwise heating was investigated using a Shimadzu TGA-50 apparatus. A known weight (5-10 mg) of dried biomass was heated from room temperature to 800 ºC at 20 ºC/min, using either stepwise or non-stepwise heating at atmospheric pressure while the instrument was purged with argon at 75 ml/min. A heating rate of 20 ºC/min was chosen to have better understanding and comparison between TGA and pyrolysis results. The weight loss of sample was recorded and used to plot the TGA and derivative thermogravimetry (DTG) graphs. TGA analysis was also conducted on biomass residue after lipid extraction (Bligh and Dyer method [89]). All experiments were performed in duplicate. To ensure that the results were not sensitive to the biomass particle size and there was no heat resistance in the biomass under pyrolysis conditions due to its particle size, in a primary experiment, the thermal decomposition behaviour of feedstock was assessed at two different heating rates (5 and 20 ºC/min). No significant differences were found between the decomposition behaviour of the biomass at different heating rates which implied that there was no heat resistance in tested particle size range and the products yields would not change with changing the heating rate up to 20 ºC/min.

6.2.3 Pyrolysis of WWT HRAP biomass

The triplicate pyrolysis experiments were conducted on dry WWT HRAP biomass at three different temperatures (300 ºC, 400 ºC and 500 ºC). The maximum pyrolysis temperature was chosen based on safe temperature range (≤500 ºC) of the pyrolysis unit used in this study. In each run, a known amount (50-100 g) of dry biomass (<4wt% water content) was loaded into a semi-batch pyrolysis reactor (Fig. 6.1). Both stepwise and non-stepwise

heating were applied at 20 ºC/min heating rate for 30 minutes at atmospheric pressure while the reactor was purged continuously with N_2 (40 ml/min) to prevent oxidation reactions. A pyrolysis time of 30 minutes was chosen after conducting stepwise TGA. The TGA results indicated that the biomass decomposition was terminated within <30 minutes. Throughout the pyrolysis, temperature was monitored with four thermocouples installed inside the reactor. The condensable gases were condensed using two condensers (80 ºC and 0 ºC) installed in series.

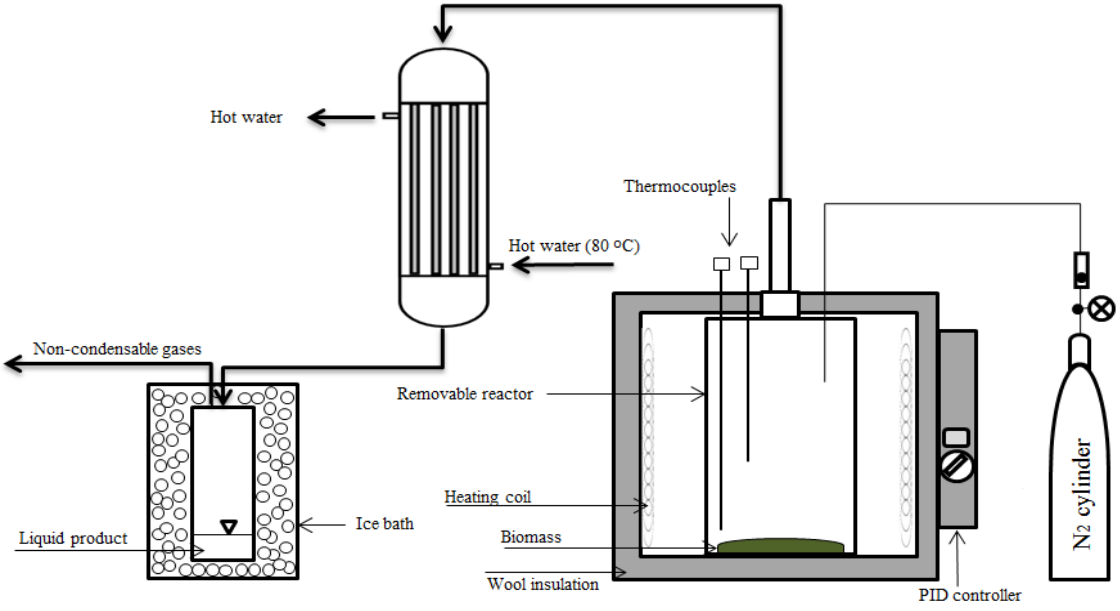


Figure 6.1 Schematic of semi-batch pyrolysis set-up

In stepwise experiments, the biomass/solid residue (bio-char produced in previous step) was heated to desired temperature and held for 30 min. The condensable gases and 1g of solid residue were then collected and stored for further analysis, and the yields were determined. Whereas in non-stepwise experiments the fresh dry biomass was loaded into the reactor and pyrolysed at 300 ºC, 400 ºC and 500 ºC (Fig. 6.2). Yield of liquid and solid phases were determined according to the mass of each fraction, while the gas phase yield was determined by difference.

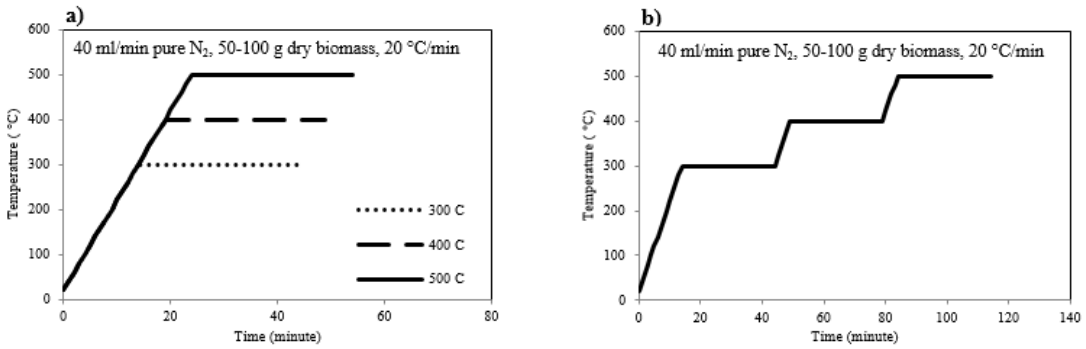


Figure 6.2. Different heating regime employed during the pyrolysis of WWT HRAP biomass

The energy needed for pyrolysis of the biomass at three different temperatures (300 ºC, 400 ºC and 500 ºC) was measured using a DSC instrument (DSC 404 F3 Pegasus, NETZSCH). A known weight (5-10 mg) of dry biomass was placed in an aluminium pan caped using an aluminium lid with a tiny hole on it. The sample was then heated from room temperature to a target temperature at 5 ºC/min and maintained at this temperature for 30 min while the reactor was purged continuously with pure argon (100 ml/min). The difference between the area under the curves of sample pan and reference pan (empty pan) represents the energy required for pyrolysis. Measuring of required energy for pyrolysis was conducted in duplicate.

6.2.4 Characterization of liquid fraction

The condensable gases obtained from pyrolysis of WWT HRAP biomass were condensed and allowed to separate gravimetrically into distinct aqueous and oil phases (Fig. 6.3) and were stored in a fridge for further analysis.

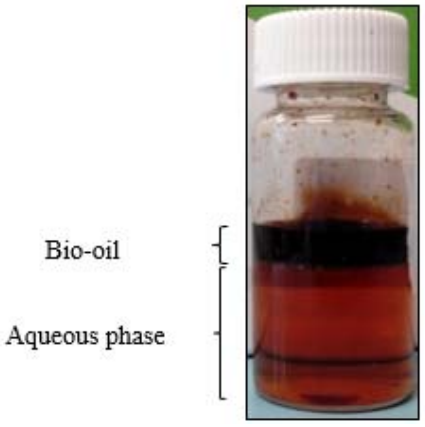


Figure 6.3. Example of pyrolysis liquid fraction after separation

The densities of both liquid phases were determined using a digital density meter (DMA 500, Anton Paar) and the water content of the aqueous phase was determined using Karl-

Fischer titration (ASTM D1744). The cold properties (melting point and melting latent heat) of the aqueous phase were determined using DSC Shimadzu-60 apparatus. A known weight (5-10 mg) of aqueous phase was placed in an aluminium pan covered with a lid. Cold properties of the sample were determined by comparing the heat flow through the sample pan with that through a reference pan (without any sample). The thermal behaviour of the aqueous phase was evaluated by cooling the sample to -60 ºC using liquid nitrogen and then heating it to 30 ºC at 3 ºC/min.

The chemical composition of both liquid phases was determined by GC-MS using a fused silica ZB-1701 column (30 m × 0.25 mm × 0.15 μm, 86% dimethylpolysiloxane, 14% cyanopropylphenyl, Phenomenex). A one microliter sample of each liquid phase was filtered using a Simplepure syringe filter (0.45 μm) and injected into the column. The bio-oil phase sample was diluted with dichloromethane (DCM) in a 1:4 v/v ratio before injection into the GC-MS instrument, while the aqueous phase sample was tested without dilution. The injector temperature was set at 250 ºC and the GC oven temperature programming started isothermally at 40 ºC for 2 min, ramped to 270 ºC at 5 ºC/min, then increased to 280 ºC at 10 ºC/min and held at this temperature for 20 min. Helium (99.99%) was used as carrier gas at 1.0 mL/min and the MS source was set to 70 eV. Identification of compounds was carried out using mass spectra acquired in scan mode from 15 to 450 atomic mass units based on mass-spectra library data base provided by NIST.

The duplicate ultimate analysis (C, H, N and S concentration) and energy content measurement of oil and aqueous phases were performed using Carlo-Ebra EA 1108 at the Campell micro analytical laboratory at the University of Otago in Dunedin, New Zealand while oxygen concentration was determined by difference. The analytical method was based on the complete and quick oxidation of the sample by "flash combustion" at 1020 ºC while helium (99.99%) was used as a carrier gas. The combustion gas mixture was directed to a chromatographic column where the components (carbon dioxide, water, sulphur dioxide and nitrogen) were separated and their concentrations determined based on the weight of the sample. The energy contents were calculated automatically by an elemental analyser based on elemental concentration.

6.2.5 Bio-char (solid fraction) analysis

The duplicate ultimate analysis and energy content of the bio-char (solid fraction) were also determined following similar procedures to those used for liquid fraction. The concentrations of exchangeable cations and heavy metals in the bio-char produced through

pyrolysis of the WWT HRAP biomass at different process conditions were determined using ICP–MS. In triplicate; ~100 mg of dry sample was digested with 10 mL of concentrated (65%) HNO_3 in a 80 mL maxi-44 Teflon tube, diluted with 40 mL distilled water, it was then allowed to settle and the supernatant of the digested sample was analysed using the ICP-MS. The specific surface area (SSA) of the bio-char was measured based on the BET nitrogen adsorption isotherm method using a Micromeritics 3Flex instrument.

6.2.6 Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) in Excel software (Excel, Microsoft office 2010).

6.3 Results and discussion

6.3.1 WWT HRAP biomass characteristics

The characteristics of the WWT HRAP biomass used in this study are summarised in Table 6.1. The biomass had a high protein content (42 ± 2 wt%) and relatively low lipid content (20 ± 2 wt%). This implies that biodiesel production from this biomass may be as uneconomical as that from cultured algal biomass due to the low lipid level, high cost of lipid extraction and purification, and complex lipid profile [163, 167, 211]. The biomass might be a promising feedstock for pyrolytic bio-oil production due to its relatively low oxygen content (34.2 wt%) compared to most woody biomass (35-45%) [69, 212]; relatively high content of carbon (36.7±1.2 wt%) and hydrogen (5.7 ± 0.3 wt%) and relatively high energy content (19.7 ± 0.5 kJ/g). However, due to the high protein content, the nitrogen content (5.4 ± 1 wt%) of the biomass was higher than for other pyrolytic bio-oil feedstock such as woody biomass (<1 wt%) [69]. This would reduce bio-oil quality through co-production of nitrogenous compounds. In addition, the biomass ash content was high which may result in lower liquid as well as higher bio-char fractions compared with the pure algal biomass. The high ash content was probably due to the presence of *Diatom* sp. and impurities such as detrital materials and salts coming into WWT HRAP with wastewater. *Diatom* sp. typically has up to 50 wt% ash content [213-215] and wastewater typically contains 5-10 wt% detrital materials [119]. Moreover, the biomass contained a high level of exchangeable cations, while heavy metal levels were below those for the safe application of biosolids to land [216]. This implies that solid phase (bio-char) produced by pyrolysis may have great potential for land applications as a soil amendment.

Table 6.1. Characteristics of WWT HRAP biomass used in pyrolysis experiment

Biomass composition (wt%)		**Elemental composition (wt%)**		**Exchangeable cations (cmole/kg)**		**Heavy metals (mg/kg)**	
Proteins	42 ± 2	C	36.7 ± 1.2	Mg	12.2 ± 2.1	Cr	21.3 ± 1.6
						Cu	447.2 ± 10
Carbohydrates	17 ± 4	H	5.7 ± 0.3	K	18.8 ± 1.1	Pb	58.4 ± 3
Lipids	20 ± 2	N	5.4 ± 1	Ca	32.6 ± 3	Hg	52.2 ± 1.3
Ash	17 ± 3	S	1 ± 0.1	Na	5.5 ± 0.5	Zn	10607.7 ± 23
Moisture	4	O	34.2			As	25.7 ± 2.3
Energy content (kJ/g)	19.7 ± 0.5					Cd	9.1 ± 0.4

6.3.2 Thermal studies and pyrolysis of WWT HRAP biomass

TGA thermal degradation of WWT HRAP biomass can be divided into three steps (Fig. 6.4a): below <200 ºC water is evaporated (4 wt%); above 200 ºC biomass degradation occurs with the rate of weight loss increasing up to temperatures of 300 ºC above which it slowly declined. The main pyrolysis reactions occurred over a broad temperature range (200-500 ºC) where the majority of organic materials were decomposed with the greatest (46±3 wt%) weight loss and maximum rate (1±0.1 mg/min) (Fig. 6.4); above 500 ºC decomposition of carbonaceous matter in the solid residue occurred at a very slow degradation rate (0.09±0.01 mg/min) corresponding to char formation [203-205].

Thermal decomposition of the biomass can be divided into three temperature intervals including 200-300 ºC, 300-400 ºC and 400-500 ºC. Under stepwise pyrolysis (Fig. 6.4b) the significant weight loss fraction (23 ± 2 wt%) occurred between 200-300 ºC ($p<0.05$). The weight loss then decreased to 14 ± 1.2 wt% and 9 ± 2 wt% of the initial weight of the biomass at 300-400 ºC and 400-500 ºC, respectively.

The higher weight loss at lower temperatures supports the hypothesis that energy rich bio-oil can be produced with lower consumption of energy. However, further analysis is required to determine the quality of liquid phase produced by the pyrolysis of WWT HRAP biomass at each temperature interval.

Stepwise TGA analysis was also conducted on biomass residue after lipid extraction and showed a lower total weight loss (25 ± 1 wt%) at <400 ºC compared with 37 ± 2 wt% for biomass before lipid extraction. Since the boiling points (240-350 ºC) of most algal lipids (C8-C18) are below this temperature [179], this result indicates that lipid conversion occurs below this temperature. Babich et al. [68] found similar results (10% reduction of weight loss

at 230 ºC) with TGA analysis of hexane pre-extracted *Chlorella* sp. biomass. They concluded that the main contribution to decomposition at 230 ºC was related to lipids and other hexane extractives. However, further studies with different model compounds such as pure lipids, proteins and carbohydrates are required to identify the contribution of each fraction in the thermal decomposition of WWT HRAP biomass.

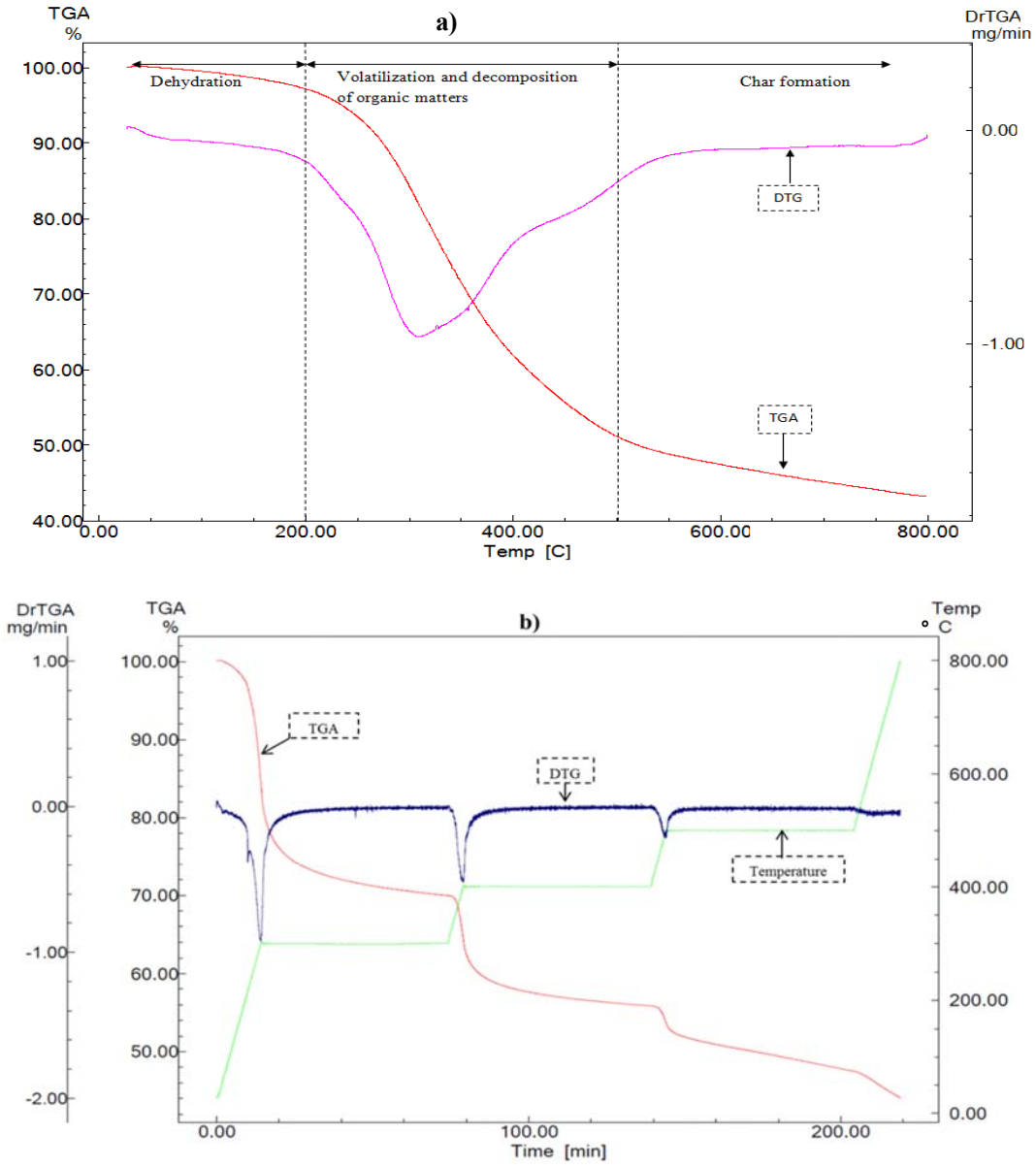


Figure 6.4. TGA and DTG curves for WWT HRAP biomass conversion under different heating regimes

The yields of solid, liquid and gas fractions at different temperatures and heating regimes are illustrated in Fig. 6.5 and were in good agreement with the TGA results. For the non-

stepwise heating (Fig. 6.5a), the highest yield of the liquid fraction at tested conditions (30 ± 3 wt% of initial biomass) occurred at 500 ºC and 50 ± 2% of it was produced at <300 ºC. While stepwise pyrolysis (Fig. 6.5b) showed that the remaining 30 ± 1% and 20 ± 1% of the liquid fraction were produced at 300-400 ºC and 400-500 ºC, respectively. The yield of the bio-oil phase of the liquid fraction increased with temperature with the highest (4.7 wt% of initial biomass) amount produced in the 400-500 ºC temperature range, which was significantly higher (more than 3 times more) than that produced at both <300 ºC and 300-400 ºC temperatures ($p<0.05$). The liquid fraction produced at <400 ºC under the both heating regimes was mainly dominated by an aqueous phase (>70%) (Fig. 6.5) which means that most dehydration reactions occurred at low temperatures since free moisture was already removed prior to pyrolysis.

Interestingly, a high amount of bio-char (>50 wt% of the initial biomass) was produced under both heating regimes which was much higher than that reported for pyrolysis of pure algae biomass (14-38 wt%) [15, 18, 21]. This was probably due to the high non-volatile solid content of WWT HRAP biomass. It has been reported that the ash content of WWT HRAP biomass is typically around 20 wt% of the biomass [12].

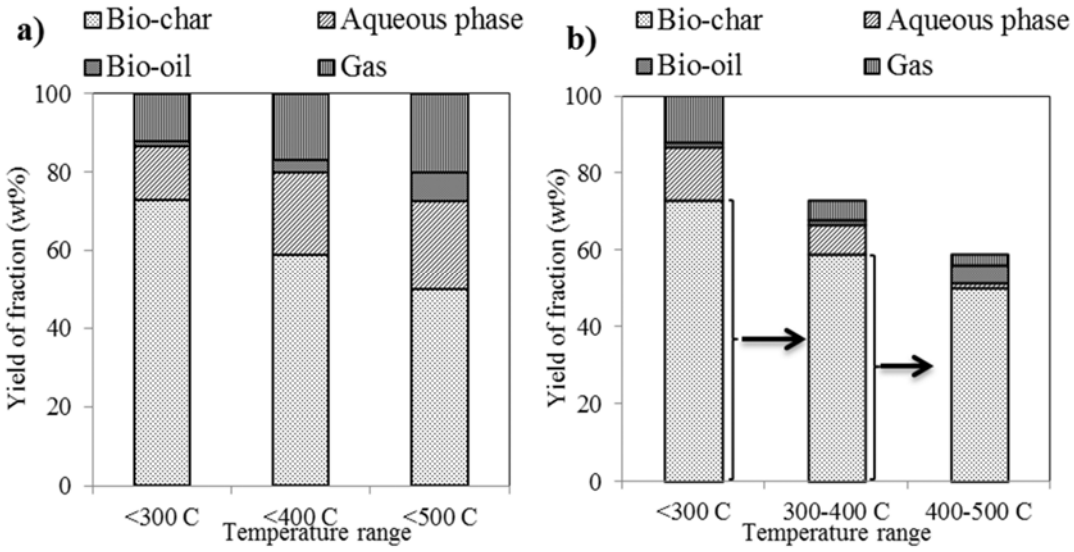


Figure 6.5. Yield of different pyrolytic products produced at different temperatures and different heating regimes

6.3.3 Analysis of bio-oil and aqueous phases of the liquid fraction

The bio-oil and aqueous phases were separated gravimetrically and their average densities were 1.08 ± 0.03 kg/L and 1.13 ± 0.05 kg/L, respectively. The energy content of bio-oil produced under different process conditions varied in a narrow range between 34.7 ± 0.2 kJ/g and 37 ± 0.8 kJ/g (Table 6.2). There was a positive relationship between process

temperature and the energy content of the bio-oil as the bio-oil with the highest energy content (37 ± 0.8 kJ/g) was obtained at 400-500 ºC (stepwise heating). This corresponded to higher carbon (73.9 ± 1.5 wt%) and hydrogen (9.5 ± 0.5 wt%) content, but lower oxygen (8.9 wt%) content. The energy content of the bio-oil was higher than that previously reported for algal-based pyrolytic bio-oil (18-30 kJ/g) and more comparable with that of fossil fuel (30-42 kJ/g) [68, 69, 71, 206]. However, the quality of the bio-oil was lower than fossil fuel oil due to higher content of oxygen (10.6-15.5 wt%) and nitrogen (6.4-8.9 wt%) (Table 6.2) compared to fossil oil (<1.5 wt% oxygen and <0.7 wt% nitrogen) [69]. The high oxygen content may increase the reactivity of the bio-oil which may change its composition during storage. In addition, the high nitrogen content may reduce the fuel value of the bio-oil from an environmental point of view due to increased NOx emissions, and would increase refinery costs. The acid catalyst used in Pyrolysis would be neutralised by the ammonia that is produced, while recovery of ammonia requires a hydrogen source [203, 217]. Since the nitrogen mainly originates from protein, the inverse relationship observed between bio-oil nitrogen content and process temperature indicates that proteins were decomposed at lower temperature.

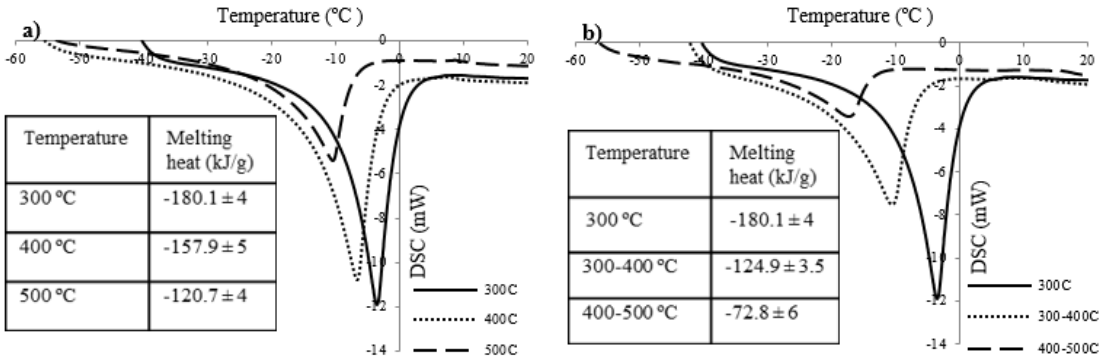
Table 6.2 Ultimate analysis, energy and water contents of the pyrolytic aqueous and bio-oil phases

Non-stepwise	C (wt%)	H (wt%)	N (wt%)	S (wt%)	O (wt%)	Water content (wt%)	Energy content (kJ/g)
Non-stepwise							
Aqueous phase							
300 ºC	7.7 ± 0.3	11.0 ± 1.3	3.6 ± 0.3	<0.4	77.3	78 ± 5	16.1 ± 1
400 ºC	7.7 ± 1.2	9.6 ± 2.1	3.7 ± 0.2	0.7 ± 0.1	78.3	79 ± 2	14.5 ± 0.7
500 ºC	14.5 ± 0.4	10.8 ± 0.5	5.5 ± 0.6	0.5 ± 0.1	68.7	70 ± 6	18.3 ± 0.5
Oil phase							
300 ºC	69.2 ± 0.4	8.7 ± 1.1	8.9 ± 2	2.6 ± 0.2	10.6	---------	34.7 ± 0.2
400 ºC	70.5 ± 0.7	9.7 ± 0.3	7.4 ± 0.4	1.1 ± 0.5	11.3	---------	35.6 ± 2.5
500 ºC	66.6 ± 1.3	10.2 ± 0.6	6.4 ± 0.9	1.3 ± 0.1	15.5	---------	35.5 ± 0.5
Stepwise							
Aqueous phase							
300-400 ºC	9.6 ± 0.4	10.6 ± 0.3	7.9 ± 0.3	<0.4	71.5	73 ± 5	16.3 ± 2.1
400-500 ºC	5.1 ± 0.8	11.2 ± 1.2	2.8 ± 0.1	<0.4	80.5	71 ± 3	15.5 ± 0.3
Oil phase							
300-400 ºC	69.8 ± 1.2	9.1 ± 0.3	7.4 ± 0.3	1.3 ± 0.2	12.4	---------	35.3 ± 0.5
400-500 ºC	73.9 ± 1.5	9.5 ± 0.5	7 ± 0.1	0.7 ± 0.1	8.9	---------	37.0 ± 0.8

Karl-Ficher analysis indicated that the water content of the liquid fraction aqueous phase was high >70% (Table 6.2) particularly at temperatures below <400 ºC (Fig. 6.5). This production of a significant amount of water at <400 ºC was probably due to dehydration reactions between amines and carboxylic acids derived from the decomposition of proteins and lipids respectively (Reaction 6-1) [71].

$$Amines\ (RHN\dot{R}) + Carboxylic\ acids\ (\ddot{R}\mathrm{COOH}) \rightarrow \mathrm{Amides\ (R\dot{R}NC}\ddot{\mathrm{O}}) + \mathrm{Water\ (H2O)} \quad (6\text{-}1)$$

DSC analysis results indicated cold properties that were comparable with antifreeze (Fig. 6.6) (from -12 ºC to -22 ºC) [218, 219] which may be due to the presence of compounds including acids and linear amides (Table 6.4).



Temperature	Melting heat (kJ/g)
300 ºC	-180.1 ± 4
400 ºC	-157.9 ± 5
500 ºC	-120.7 ± 4

Temperature	Melting heat (kJ/g)
300 ºC	-180.1 ± 4
300-400 ºC	-124.9 ± 3.5
400-500 ºC	-72.8 ± 6

Figure 6.6 DSC curves of the aqueous phase produced at different temperature and heating regimes

The most abundant compounds in the liquid fraction (bio-oil and aqueous phases) determined using GC-MS analysis are summarized in Tables 6.3 and 6.4. Although more than 500 peaks were found on the chromatograms, less than 100 compounds (representing <60% of total area of chromatogram) could be identified. The high complexity of the liquid fraction implies that refinery costs would be very high as the bio-oil is not distillable. The GC-MS analysis indicated that both liquid phases contained nitrogenous and oxygenated compounds. The nitrogenous compounds such as pyrroles, indoles and amides were probably derived from the Millard reaction between decomposed proteins and carbohydrates, while the oxygenated compounds such as acids and alcohols resulted from decomposition of carbohydrates and lipids [71, 203, 220].

All identified compounds were categorised in three different groups (acids, aromatics, and hydrocarbons) based on their chemical structure. The bio-oil phase was dominated by hydrocarbons and aromatics while the aqueous phase was dominated mainly by acids. There was no relationship for production of specific compounds at different temperatures which indicates the complexity of pyrolysis reactions. The aromatic compound content of the bio-oil

increased with increasing temperature up to 35.7% of the total chromatograph area at 500 ºC, while the hydrocarbon content had a reverse trend. These results were similar for the composition of the bio-oil produced through both stepwise and non-stepwise pyrolysis of the biomass. The GC-MS analysis of the bio-oil produced in stepwise pyrolysis indicated that the aromatic compound content increased from 21% at 300-400 ºC to 33.8% at 400-500 ºC while the hydrocarbon content declined from 32.4% to 26.2%. This implies that more aromatic compounds, which could be important industrial chemicals, could be produced at higher temperatures. The high complexity of this bio-oil would it costly to refine, however, it could possibly be used directly as an additive to increase gasoline octane number [206].

The aqueous phase was mainly dominated by acids such as propanoic acid, butanoic acid (due to pyrolysis of proteins and lipids), and nitrogenous compounds such as acetamide which is formed from the combination of acetic acid with an amide (Table 6.4). The high organic acid content would likely limit initial market acceptance as it could cause corrosion [68, 203]. Although, the pH of the aqueous phase produced in this study (5.5<pH<6.5) was higher than that reported in the literature (pH<3.5) due to the presence of nitrogen-based alkaline compounds such as the amides [68, 206]. Moreover, a catalyst could be used during pyrolysis to reduce acidity of the aqueous phase. For example, Babich et al. [68] found that use of Na_2CO_3 as a catalyst during pyrolysis of *Chlorella* sp increased the aqueous phase liquid pH from 2.5 (without the catalyst) to 3.7.

Table 6.3. The most abundant compounds (as % of total chromatogram area) in pyrolytic bio-oil produced at different temperatures and heating regimes

<300 ºC		**300-400 ºC**		**<400 ºC**		**400-500 ºC**		**<500 ºC**	
Oil phase									
Component	Area (%)	Component	Area (%)	Component	Area (%)	Component	Area (%)	Component	Area (%)
Hydrocarbons									
Acetamide, N,N-dimethyl-	0.8	Acetamide	6.1	1-Nonene	1.5	1-Hexadecanol	1.0	Hexadecanamide	1.0
Dodecanamide	1.0	Heptadecanenitrile	1.5	Decane, 2-methyl-	1.3	Heptadecanenitrile	2.4	Hexadecane	1.3
Pentadecane	1.0	Hexadecanamide	1.1	Dodecane	1.3	Octadecanenitrile	1.3	Hexadecanenitrile	7.9
		Hexadecanenitrile	2.3	Hexadecanamide	1.4	Pentadecane	1.4	Nonadecanenitrile	1.5
		n-Hexadecanoic acid	1.7	Hexadecanenitrile	7.1	Pentadecanenitrile	3.9	Nonane, 5-butyl-	1.0
		Tetradecane	1.1	Tridecane	1.2	Tridecane	1.4		
Σ	**5.8**	**Σ**	**32.4**	**Σ**	**24.0**	**Σ**	**26.2**	**Σ**	**19.7**
Aromatics									

1H-Indole, 2-methyl-	0.7	1H-Indole, 2,3-dimethyl-	0.4	Benzene, 1,3-dimethyl-	1.6	5H-1-Pyrindine	1.1	1H-Benzimidazole, 2-methyl-	0.9
2,5-Pyrrolidinedione, 1-methyl-	1.4	Benzenepropanenitrile	0.6	Benzenepropanenitrile	0.9	Benzenepropanenitrile	0.9	Cholest-4-ene	1.0
2-Piperidinone	2.7	Benzyl nitrile	0.5	Cyclohexadecane	1.1	Cyclododecane	1.3	Cyclododecane	1.6
Benzyl nitrile	1.0	Cyclododecane	0.6	Ethylbenzene	2.3	Cyclopropane, nonyl-	1.1	Cyclotetradecane	1.0
Cyclotridecane	1.1	Indole	0.7	Indole	1.1	Cyclotetradecane	1.1	Indole	1.2
Phenol	5.0	Naphthalene, 2-methyl-	0.8	Phenol	3.2	Ethylbenzene	1.2	Naphthalene, 1-methyl-	1.4
Phenol, 2,5-dimethyl-	1.1	Phenol	2.0	Phenol, 4-methyl-	3.6	Phenol	3.2	Phenol	3.5
Phenol, 2-methyl-	1.8	Phenol, 4-methyl-	2.6	p-Xylene	1.6	Phenol, 4-methyl-	4.0	Phenol, 2-methyl-	1.4
Phenol, 4-methyl-	5.4	Styrene	0.6	Pyridine, 2-methyl-	1.6	p-Xylene	0.9	Phenol, 3,5-dimethyl-	1.5
Undecanenitrile	2.6			Pyrrole	1.6	Pyridine, 2-methyl-	0.9	Phenol, 4-methyl-	4.5
				Styrene	1.5	Pyrrole	0.8	Pyrrole	1.0
						Styrene	0.8		
Σ	**33.0**	**Σ**	**21.0**	**Σ**	**31.8**	**Σ**	**33.8**	**Σ**	**35.7**

Table 6.4. The most abundant compounds (in % of total area of chromatogram) in pyrolytic aqueous phase produced at different temperatures and heating regimes

300 ºC		**300-400 ºC**		**400 ºC**		**400-500 ºC**		**500 ºC**	
Aqueous phase									
Component	Area (%)	Component	Area (%)	Component	Area (%)	Component	Area (%)	Component	Area (%)
Acids									
2-Pentenoic acid	4.5	2-Pentenoic acid	2.2	Acetamide	22.5	Acetamide	6.6	2-Pentenoic acid	5.9
Acetamide	16.7	Acetamide	21.8	Butanoic acid	7.6	Acetamide, N-methyl-	2.7	Acetamide	16.6
Butanoic acid	7.4	Butanoic acid	2.9	Propanoic acid	19.4	Butanoic acid	8.6	Butanoic acid	7.1
Crotonic acid	4.7	Butanoic acid, 3-methyl-	7.2			Butanoic acid, 2-methyl-	2.4	Crotonic acid	2.6
Propanoic acid	10.5	Propanamide	2.3			Pentanoic acid	2.4	Isocrotonic acid	5.3
		Propanoic acid, 2-methyl-	2.3			Propanoic acid	10.6	Propanoic acid	7.4
						Propanoic acid, 2-methyl-	5.8		
Σ	**47.0**	**Σ**	**41.6**	**Σ**	**58.1**	**Σ**	**47.3**	**Σ**	**46.4**
Aromatics									
2-Pyrrolidinone	3.1	1-Imidazolidinemethanol, 4,4-dimethyl-2,5-dioxo-	18.8	1,2,4-Triazine-3,5(2H,4H)-dione	5.7	2,4-Imidazolidinedione, 5,5-dimethyl-	11.6	2,4,5-Trioxoimidazolidine	1.8
5-Isopropyl-2,4-imidazolidinedione	1.9	2,4-Imidazolidinedione, 5-ethyl-5-methyl-	8.0	2,4-Imidazolidinedione, 5-methyl-	1.2	dl-5-Ethyl-5-methyl-2,4-imidazolidinedione	4.2	2,4-Imidazolidinedione, 5,5-dimethyl-	4.9
dl-5-Ethyl-5-	1.4	2-Pyrrolidinone	2.5	5-Isopropyl-2,4-	2.0	Phenol	1.7		

methyl-2,4-imidazolidinedione				imidazolidinedione					
		5-Isopropyl-2,4-imidazolidinedione	2.8	dl-5-Ethyl-5-methyl-2,4-imidazolidinedione	2.8	Phenol, 4-methyl-	1.0		
Σ	**7.5**	**Σ**	**37.5**	**Σ**	**12.4**	**Σ**	**20.1**	**Σ**	**10.0**

6.3.4 Analysis of bio-char

Results from measurements of ultimate analysis, energy content, exchangeable cation and heavy metal concentrations as well as the specific surface area of the solid (bio-char) fraction of pyrolysed WWT HRAP biomass are reported in Table 6.5. An inverse relationship was observed between pyrolysis temperature and the concentration of volatile elements such as C, H and N and energy content of the bio-char. The bio-char energy content (14.7-21 kJ/g) was much higher than that previously reported for algal bio-char (4-7 kJ/g) [17-18] which was probably due to higher C and H content of the tested biomass in this study. Hence, the bio-char has potential to also be used as fuel source. BET analysis indicated that the bio-char had a very low specific surface area (<3.2 ± 0.3 m^2/g) compared with pure carbon (up to 1200 m^2/g) [221] implying that this kind of bio-char is unsuitable for use as an adsorbent.

Table 6.5. Characteristics of bio-char produced during the pyrolysis at different temperatures and heating regimes

Temperature	C (wt%)	H (wt%)	N (wt%)	S (wt%)	Energy content (kJ/g)	Exchangeable cations (cmole/kg)				Surface area (m^2/g)
						Mg	K	Ca	Na	
Non-stepwise pyrolysis										
<300 ºC	43.9 ± 0.4	4.7 ± 1	6.2 ± 1.5	0.9 ± 0.1	21.0 ± 0.5	32.0 ± 2.3	41.9 ± 3	67.7 ± 5	16.2 ± 3	NQ*
<400 ºC	39.0 ± 1.3	3.3 ± 0.2	4.9 ± 0.6	0.7 ± 0.1	17.4 ± 1	46.2 ± 7.1	60.3 ± 2.1	65.9 ± 4	22.9 ± 5	<2
<500 ºC	36.7 ± 0.9	1.7 ± 0.1	5.2 ± 1.1	0.8 ± 0.2	14.7 ± 0.8	56.1 ± 5.3	68.0 ± 8	158.9 ± 11	31.3 ± 2	3.2 ± 0.3
Stepwise pyrolysis										
300-400 ºC	44.1 ± 0.3	3.9 ± 0.4	6.0 ± 1.3	0.8 ± 0.1	19.9 ± 0.2	--------	--------	--------	--------	---------
400-500 ºC	37.1 ± 0.2	1.8 ± 0.2	5.0 ± 0.7	0.7 ± 0.1	14.9 ± 0.3	--------	--------	--------	--------	--------

*NQ: not quantifiable

The concentrations of exchangeable cations and heavy metals (except mercury) in the bio-char increased with pyrolysis temperature (Tables 6.5 & 6.6). The mercury content declined with increasing pyrolysis temperature as it evaporates above 356.7ºC (its boiling point) (Table 6.6). The high concentrations of exchangeable cations together with the high C and N content, indicate the potential of using this bio-char as a soil amendment, particularly as the content (determined using ICP-MS) of most metals (Table 6.6) were low enough to

meet the NZ sewage sludge land application guideline (grade A (1–600 mg/kg dry wt) or grade B (7.5–1500 mg/kg dry wt)) [213]. The high Zn content of the bio-char could restrict its use, however this may be mitigated since pyrolysis is known to reduce both the bio-availability and phytotoxicity of Zn [222].

Table 6.6. Heavy metal concentrations (mg/kg) in bio-char produced during the pyrolysis at different temperatures and heating regimes

	<300 ºC	**<400 ºC**	**<500 ºC**
Cr	50.4 ± 12	69.0 ± 8	80.7 ± 11
Cu	443.6 ± 10	555.2 ± 32	796.4 ± 54
Zn	14285.6 ± 89	16351.6 ± 110	20551.2 ± 132
As	33.4 ± 3	63.6 ± 2.2	64.0 ± 6
Cd	21.7 ± 2.5	26.2 ± 3.1	36.7 ± 3
Hg	40.3 ± 4	0.4 ± 0.1	0.2 ± 0.1
Pb	180.7 ± 7	300.9 ± 25	255.4 ± 6

6.4 Is WWT HRAP biomass a promising feedstock for pyrolytic bio-oil production?

Maximizing the bio-oil fraction and the energy recovered as bio-oil are crucial for the economic viability of pyrolysis. The proportions of energy recovered in each fraction of pyrolysed WWT HRAP biomass were calculated (Equation 6-1) and are illustrated in Fig. 6.7.

$$Energy\ recovery = \frac{energy\ content\ of\ the\ fraction \times yield\ of\ the\ fraction}{energy\ content\ of\ the\ biomass} \times 100 \qquad (6\text{-}1)$$

There was a positive relationship between pyrolysis temperature and the energy recovered in the bio-oil and gas fractions (Fig. 6.7), while energy recovered in the bio-char decreased with temperature. Maximum bio-oil energy recovery (2.5 MJ/kg biomass) occurred at 500 ºC, which was >2-times and >5-times higher than the bio-oil energy recovery at 400 ºC and 300ºC respectively ($p<0.05$). As can be seen in Fig. 6.7, 10-28% and 38-78% of the total biomass energy (19.7 ± 0.5 MJ/kg) were recovered in the non-condensable gas and bio-char respectively.

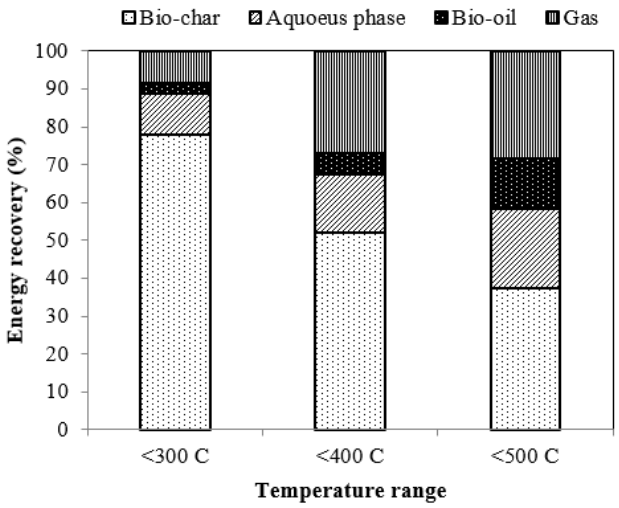


Figure 6.7. The percentage of energy recovery in different pyrolysis fractions for different temperatures

To assess the feasibility of pyrolitic bio-oil production from WWT HRAP biomass, an energy balance needs to be made on whole the process, from cultivation to bio-oil production. The energy required for culturing and harvest of the biomass is essentially covered by wastewater treatment function of the pond [6]. It has been estimated in our previous study (Mehrabadi et al., 2015) that ~ 6 MJ/kg biomass energy is needed to concentrate, mechanically dewater and thermally dry the biomass. The energy needed for the pyrolysis of the biomass to produce bio-oil was calculated using the DSC measurements. DSC results indicated that the energy required for pyrolysis of dry WWT HRAP biomass (<4wt% moisture) under similar process conditions was 0.8 ± 0.1 MJ/kg biomass at 300 ºC, 1.2 ± 0.2 MJ/kg biomass at 400 ºC, and 2.9 ± 0.1 MJ/kg biomass at 500 ºC which are in line with literature values (e.g. Grierson et al., 2009). The energy demand to produce maximum bio-oil by pyrolysis of 1 kg of dry WWT HRAP biomass (energy content: 19.7 ± 0.5 MJ/kg) at 500 ºC along with the energy value of the co-products of the process are listed below and summarized in Fig. 6.8:

Energy demands

- Energy required for culturing and harvesting is negligible as it is a co-product.
- Energy required for concentrating, dewatering and drying of the biomass:~6 MJ/kg
- Energy required for pyrolysis of dry biomass at 500 ºC:~2.9 MJ/kg

Available energy in product and co-products

- Energy recovered in bio-oil: 2.5 MJ (7 ± 1 wt% yield, 35.5 ± 0.5 MJ/kg)

- Energy recovered in non-condensable gases: 5.6 MJ (calculated by difference, 20 ± 2 wt% yield)
- Energy recovered in bio-char: 7.4 MJ (50 ± 3 wt% yield, 14.7 ± 0.8 MJ/kg)
- Energy recovered in aqueous phase: 4.2 MJ (23 ± 2 wt% yield, 18.3 ± 0.5 MJ/kg)

This energy balance shows that the total energy needed for pyrolitic bio-oil production from WWT HRAP biomass was~45% of the total energy of the WWT HRAP biomass, and only <15% of the WWT HRAP biomass energy was recovered in the pyrolysis bio-oil. The energy in both the bio-char and non-condensable gas stream which contains combustible gasses such as methane (Grierson et al., 2009; Maddi et al., 2011) could be used as fuel sources to supply the process energy demand (Fig. 6.8). Further exergy analysis and research are required to assess the energy needed to remove condensable gases and upgrade bio-oil, and other beneficial uses of pyrolysis co-products that might make WWT HRAP biomass-based bio-oil production economically feasible.

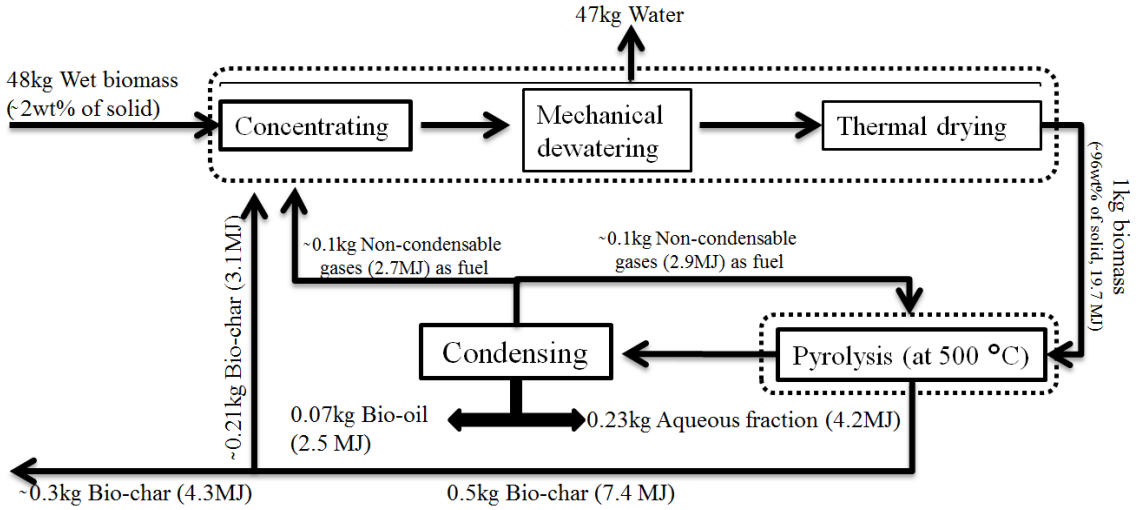


Figure 6.8 Total mass and energy balance for pyrolysis of 1 kg dry WWT HRAP biomass at 500 °C

6.5 Conclusions

The thermal behaviour of WWT HRAP biomass and its potential to be converted to pyrolytic bio-oil at different temperatures (300 ºC, 400 ºC and 500 ºC) were investigated in terms of yield, chemical composition, and energy content of products. Stepwise pyrolysis was employed to determine the proportion of products produced at different temperature intervals and the most suitable temperature range for high quality bio-oil production.

- At <400ºC the liquid fraction was mainly dominated by water, while the bio-oil phase was mainly produced at 400-500 ºC. Elemental and chemical composition analyses illustrated that higher quality bio-oil with higher aromatic and energy content as well as lower nitrogen content was produced at higher temperature (400-500 ºC). However, the GC-MS analysis showed that the bio-oil was highly complex (>500 compounds) even at low temperatures which could lead to high refinery costs.
- Overall, <15% of the WWT HRAP biomass energy (19.7 MJ/kg) was recovered in the pyrolysis bio-oil at 500 ºC. This was very low compared to the energy required to pyrolyse the WWT HRAP biomass (40-50% of biomass energy).

Use of the non-condensable gases and bio-char as fuel could help offset the pyrolysis energy demand. However, to make bio-oil production economically feasible, further research is required to maximize value of other co-products such as utilization of bio-char as a soil amendment or use of the aqueous phase for antifreeze/chemical production.

CHAPTER 7

Wastewater treatment high rate algal pond biomass for bio-crude oil production

This chapter is based on the following publication:

Mehrabadi, A., Craggs, R., Farid, M. 2017. Wastewater treatment high rate algal pond biomass for bio-crude oil production. *Bioresource Technology*, 224, 255-264.

Chapter preface

Hydrothermal liquefaction (HTL) has been highlighted as a promising conversion method to produce petroleum-like bio-crude from wet algal biomass. While several studies have investigated the potential of bio-crude production from pure algal biomass at different operational conditions, there has been little detailed focus on HTL of wastewater treatment high rate algal pond (WWT HRAP) biomass. This chapter investigates the production potential of bio-crude from WWT HRAP biomass as an essentially free feedstock, in terms of yield, elemental/chemical composition and higher heating value (HHV). The biomass slurry (2.2 wt% solid content, 19.7 kJ/g HHV) collected from a full-scale WWT HRAP, was hydrothermally liquefied at a range of temperatures (150-300 °C) for one hour. The bio-crude yield varied between 3.1 and 24.9 wt%. Both the yield and nitrogen content of the bio-crudes increased with increasing HTL temperature while the oxygen content decreased. GC-MS analysis indicated that pyrroles, indoles, amides and fatty acids were the most abundant bio-crude compounds. In addition to the bio-crude, HTL of WWT HRAP biomass resulted in production of 10.5-26 wt% water-soluble compounds (containing up to 293 mg/L ammonia), 1.0-9.3 wt% gaseous phase and 44.8-85.5 wt% solid residue with a HHV of 12.2-18.1 kJ/g. The maximum biomass energy recovery (47.4%, 9.3 kJ/(g biomass)) occurred at 300 °C however, it was lower than the energy requirement of the HTL process at tested conditions.

7.1 Introduction

Among the different kinds of biofuel feedstock, microalgae have been highlighted due to their high productivity, ability to grow on wastewater and wasteland, ability to fix high amounts of CO_2 and simple life cycle [3, 154, 202]. Algal biomass could be converted to different liquid biofuels via conversion of the whole or a fraction of biomass. Biodiesel and bioethanol could be produced via esterification and fermentation of lipid and carbohydrate fractions, respectively. On the other hand, whole biomass could be thermochemically converted to pyrolitic bio-oil and bio-crude via pyrolysis and hydrothermal liquefaction (HTL), respectively [76]. Of all algal conversion routes, HTL has received intensive interest owing to: 1) ability to process wet biomass, 2) no need to lipid-rich algae since all the biomass components would be converted to bio-crude, 3) no need to use catalyst for conversion of biomass, 4) self-separating majority of products from the water , and 5) high potential of HTL aqueous phase to be used as fertiliser or recycled into the pond as nutrient source for algal cultivation [223-226]. However, this process has some disadvantages

including using feedstock with lower lipid content results in production of lower quality bio-crude and requiring further solvent extraction of bio-crude from solid fraction [224, 226].

HTL is a thermochemical process in which biomass organic compounds are converted to bio-crude (main product), solid residue, gaseous and water-soluble products at high temperature (200-374 ºC) and pressure (5-22 MPa) and presence/absence of a catalyst [76, 227]. Under HTL conditions, biomass constituents including lipids, proteins and carbohydrates are hydrolysed into their monomers such as fatty acids, amino acids and glucose at lower temperatures. The majority of the monomers are soluble in water [228, 229]. When the temperature increase, complex reactions including re-polymerization, dehydration, decarboxylation, deamination, condensation and cyclization occur which convert monomers into bio-crude [230-232]. Although by increasing temperature all monomers are converted to bio-crude, conversion of the protein-based monomers occurs at lower temperature relatives to lipid-based monomers followed by carbohydrate-based monomers [233, 234].

Bio-crude is an energy dense (30-40 kJ/g) product which could potentially be used as a substitute for petroleum crudes [223, 230, 235]. Yield of bio-crude, under HTL conditions, is function of different parameters including feedstock composition and concentration, reaction time, reaction temperature and catalyst type [63, 223, 230, 231, 236-238]. To investigate the effect of algal cell structure and composition, Barreiro et al. [66] liquefied eight different species (*Scenedesmus obliquus, Scenedesmus almeriensis, Phaeodactylum tricornutum, Nannocholoropsis gaditana, Tetraselmis suecica, Chlorella vulgaris, Porphyridium purpureum, Dunaliella tertiolecta*) at 250 ºC or 375 ºC for 5 min. A broad range of bio-crude yields (17.6-44.8 wt%) was obtained at 250 ºC while, the yields changed in a narrow range (45.6-58.1 wt% of initial biomass) at 375 ºC. At 250 ºC, the higher conversion was obtained from the species which had higher lipid content as well as no or reduced cell wall such as *Dunaliella tertiolecta* which indicated the direct effects of species features on yield of HTL process at mild temperatures. Although at 375 ºC the effect of species features on the yields of the bio-crude reduced, the lower yields were obtained from the species which had higher carbohydrate content. It has been reported that the carbohydrate fraction convert in less extend to the bio-crude [62].

Chen et al. [230] investigated the effect of temperature and time on yields of HTL products. They conducted HTL experiments on wastewater treatment pond biomass comprised of microalgae, macroalgae, bacteria (47.5 wt% ash, 12.9 kJ/g energy content) at different temperatures (260, 280, 300 and 320 ºC) and different reaction time (0 (heat up time was not counted), 0.5, 1 and 1.5h). The highest yield of bio-crude (26 wt% of initial biomass)

occurred at 300 ºC after 1h processing. The results illustrated positive relationship between temperature and bio-crude yields at temperatures ≤300 ºC while the yield of bio-crude decreased at temperature beyond 300 ºC due to conversion of a fraction of bio-crude to gaseous products. The effect of reaction time on bio-crude yield is highly dependent on temperature. It plays a critical role at relatively low temperatures (<240 ºC) so that longer reaction time results into more bio-crude [224]. While Chen et al. [230] showed that at mild temperatures (240-320 ºC) the bio-crude yield insignificantly and inconsistently fluctuated with increased reaction time. This implies that long retention time is not an essential factor for bio-crude formation at mild temperatures. However, longer reaction time at mild temperatures may change the bio-crude chemical composition, particularly resulting in a higher concentration of cyclic compounds [230] which produce a higher quality bio-crude. At temperatures above the critical point of water (374 ºC), increase reaction time results in lower bio-crude yield due to further decomposition of bio-crude into the gaseous products [229, 239].

It has been shown that increasing feedstock concentration results into higher bio-crude yield [229, 237]. Valdez et al. [229] investigated the effect of feedstock solid content on HTL product composition using a slurry of *Nannochloropsis* sp. with 5-35 wt% solid content processed at 350 °C for 60 min. The bio-crude yields increased from 36 to 46 wt% with feedstock concentration. Although higher loadings result in higher bio-crude yields, the recommended feedstock solid concentration for economical hydrothermal process of algal biomass is 15-20 wt% [231, 240]. At lower concentrations, the associated capital costs of pumping and heating of large quantity of water can create economic barriers. While at higher concentration, high energy demands as well as technological limitations for dewatering, pumping and mixing of biomass are considered as economic barriers.

The effect of catalysts on yield and quality of bio-crude has been evaluated by several researchers [236, 238]. It has been shown that use of heterogeneous catalysts such as Ce/HZSM-5, Pt/Al_2O_3, $Co/Mo/Al_2O_3$ and Ni/Al_2O_3 could improve the energy content and quality of bio-crude by lowering its O and N content via in-situ de-oxygenation and de-nitrogenation of bio-crude, however, catalysts may have negative effect on bio-crude yield [236, 238]. For example, Biller et al. [236] found that the use of Ni/Al_2O_3 resulted in a lower bio-crude yield and a higher yield of gaseous products in liquefaction of *Chlorella vulgaris* and *Nannochloropsis occulta* at 350 ºC for 1h.

While several lab-scale batch studies have shown the effectiveness of hydrothermal liquefaction route for conversion of the whole biomass of different algae species, a few

number of studies have shown the positive energy balance on pilot-scale continues HLT plant [227, 241] which means the process is not yet economically profitable [235, 242]. Most of studies have been performed under experimental conditions which would never be achieved at true industrial conditions such as the use of pure algal biomass or feedstock preparation via mixing washed and dried biomass with de-ionized water. To lower the high bio-crude production costs and achieve a positive energy balance for the whole process from cultivation to conversion, a niche opportunity may exist where the algal biomass is produced as an essentially free by-product of near tertiary-level wastewater treatment in high rate algal ponds [6, 76]. In fact, using WWTHRAP biomass could offset the water, nutrient and harvest costs [6, 107]. 20-40 tonnes/ha/year free biomass (algal-bacterial consortium) with chemical composition of 40-55 wt% protein, 15-30 wt% lipid and 15-25 wt% carbohydrate and energy content of 13-25 kJ/g could be produced in WWT HRAP [12, 27, 119, 227]. These properties are comparable to those reported for pure algal biomass, 20-60 tonnes/ha/year, 20-60 wt% protein, 20-40 wt% lipids, 10-50 wt% carbohydrates and 18-24 kJ/g [20, 22]. However, WWT HRAP biomass a have higher ash content (15-30 wt%) [12, 65, 83] which would reduce the bio-crude yield.

While several studies have investigated the potential of bio-crude production from pure algal biomass at different operational conditions, there has been little detailed focus on HTL of wastewater treatment high rate algal pond (WWT HRAP) biomass. Hence, this study aims to examine the potential of such free biomass directly collected from a full scale WWT HRAP for bio-crude production to outline the benefits and opportunities which may be provided by using WWT HRAP biomass as HTL feedstock. In addition, a lumped reaction kinetic model was developed for WWT HRAP biomass decomposition and the effects of processing temperature on yields of products were assessed. The chemical and elemental compositions as well as energy content of the bio-oil and the solid residue were measured to examine the possible reaction pathways for bio-crude formation. Furthermore, to evaluate the possibility of using the HTL aqueous phase as a fertiliser, the ammonia concentration and chemical composition of aqueous phase were measured.

7.2 Materials and methods

7.2.1 Feedstock characterization

The biomass slurry was directly collected from the harvest pond of a full-scale WWT HRAPs (2850 m^3 volume, 0.3 m depth and 9650 m^2 surface area) located in Cambridge, New Zealand (North Island, New Zealand (lat. 37° 53′ 54.63″ S, long. 175° 26′ 17.15″ E)) [110].

Microscopic analysis showed that the biomass included the following dominant algal species: *Pediastrum* sp., *Micractinium* sp., and *Desmodesmus* sp. To determine total and volatile suspended solids (TSS & VSS) of the collected biomass, a known volume (5-10 ml) of biomass was filtered onto a pre-washed, pre-combusted and pre-weighed Whatman GF/F filter (with 0.7 µm pore size). The filter was then oven dried (at 80 ºC overnight), weighted, ashed (at 550 ºC for one hour) and weighted again. The TSS was determined by subtracting the weight of filter before filtration from the weight of filter after drying. The VSS was measured as weight loss of oven dried sample before and after ashing.

The ammonium (NH_4^+-N) concentration of filtrate was determined colorimetrically [86] using a spectrophotometer (HACH RD2008, Germany). The protein, lipid and carbohydrate content of centrifuged biomass (3000 rcf for 5 min) were measured according to the Lowry method [90], the Bligh and Dyer [89] and the phenol sulphuric acid methods [90], respectively as described in our previous study [119]. Ultimate analysis and higher heating value (HHV) measurement of the biomass were performed using Carlo-Ebra EA 1108 elemental analyser. All analysis was performed in duplicate.

7.2.2 Hydrothermal liquefaction (HTL) of WWT HRAP biomass

HTL experiments were conducted loading 400 ml of the biomass slurry with the initial solid content of 2.2 wt% into a 1L Parr reactor (Parr Instrument Company, USA, Model 4540) (Fig.7.1). The reactor feedstock solid content was limited 2.5 wt%. The reactor was sealed and the headspace was purged with nitrogen to remove any air. The reactor was then pressurized up to 30 bar with nitrogen to keep water in liquid phase at elevated temperature. Under HTL conditions water acts as both solvent and reactant to hydrolytically decompose the proteins, lipids, and carbohydrates in the biomass [233]. The experiments were conducted at seven different temperature including 150 °C, 200 °C, 220 °C, 240 °C, 260 °C, 280 °C, and 300 °C for one hour. The maximum temperature was chosen based on the reactor temperature limited to 300 °C. The reactor was heated up to the desired conversion temperature using an electric resistance heater while the mixture was continuously mixed using an internal mixer at 500 rpm. It should be mentioned that the retention time did not include the heat-up times (20-35 min).

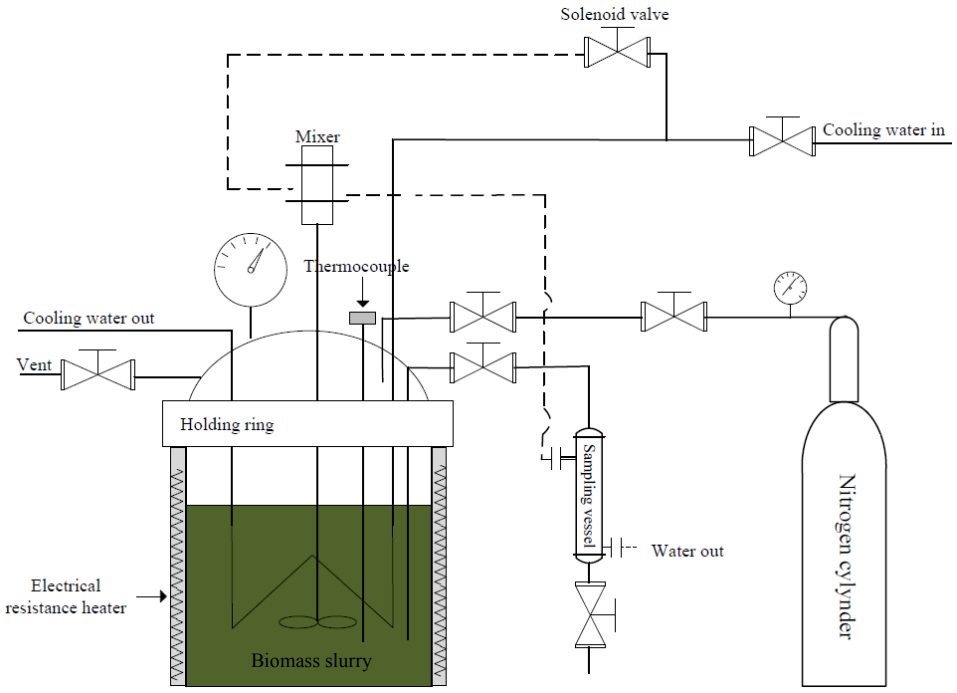


Figure 7.1 Schematic diagram of hydrothermal liquefaction set-up

After one hour, the heater was removed and the reactor was rapidly cooled down to room temperature by flowing tap water through the internal cooling coil. Subsequently the reactor was depressurised venting the headspace gases and the product mixture was transferred into a glass bottle. The reactor was then rinsed with known volume of dichloromethane (DCM) to ensure that all the products were collected and the rinsings were then added to the product glass bottle. 40 ml of product mixture, after vortex mixing, was then placed in a centrifuge tube with 10 ml of DCM, vortex mixed for 1 min and centrifuged at 7000 rcf for 10 min. Most of the HTL bio-crude components were extracted by the DCM which formed a dense layer at the bottom of the centrifuge tube. The remaining cell debris created the middle layer, while the aqueous phase created the top layer. The bio-crude-DCM layer was transferred into a pre-weighed glass tube. A second and third re-extraction was conducted by adding another 10 ml DCM to the left over in the centrifuge tube followed by vortex mixing for 1 min and centrifuging at 7000 rcf for 10 min. The bio-crude-DCM layer was removed from the tube and combined with the first extract in the glass tube. The glass tube was subsequently placed in a vacuum evaporator (Speedvac Thermo high-capacity) while it was purged with nitrogen gas to allow the DCM to evaporate. The tube was then weighed and stored in a fridge for further analysis. The left over (aqueous phase and solid residue) was then filtered onto a pre-rinsed, pre-combusted and pre-weighed Whatman GF/F filter (with 0.7 µm pore size). Weight

of solid residue was determined after oven drying of the filter at 80 ºC overnight. The water of aqueous phase was also dried off following similar procedures to those used for bio-crude fraction. The tube was then weighed and stored in a fridge for further analysis. Yields of the bio-crude, aqueous and solid phases were determined dividing the mass of each fraction by the mass of dry feedstock initially loaded into the reactor, while the gas phase yields were determined by difference. The yields of gas phase might be overestimated due to lose of a fraction of some compounds such as ammonia during the drying of aqueous and bio-crude fractions. All HTL experiments were conducted in duplicate.

To study the degradation kinetics of WWT HRAP biomass under HTL conditions, the reactor was sampled (10 ml) at different time (5, 10, 20, 30, 40, 50, 60 min) after the reactor reached the conversion temperature. The pressure drop due to sampling was negligible. The bio-crude compounds were extracted using DCM and the VSS were determined as described before.

7.2.3 Characterization of bio-crude

Chemical composition of the bio-crudes was determined by GC-MS using a fused silica ZB-1701 column (30 m × 0.25 mm × 0.15 μm, 86% dimethylpolysiloxane, 14% cyanopropylphenyl, Phenomenex). A known amount (5-10 mg) of dried bio-crude was dissolved in 2 ml DCM and one microliter sample was injected into the column. The injector temperature was set at 250 ºC and the GC oven temperature programming started isothermally at 40 ºC for 2 min, ramped to 280 ºC at 7 ºC/min, and then held at this temperature for 5 min. Helium (99.99%) was used as carrier gas at 1.0 mL/min and the MS source was set to 70 eV. Scan range of mass spectrum was in m/z of 15-500. A DCM blank was run before and after each sample to monitor instrument carryover. Identification of compounds was performed comparing mass spectra acquired in scan mode with mass spectra provided by NIST library data base (NIST, Shimadzu Company).

The ultimate analysis (C, H, N and S concentration) and HHV of the bio-crudes were measured using Carlo-Ebra EA 1108 at the Campell micro analytical laboratory at the University of Otago in Dunedin, New Zealand while the oxygen weight fraction was determined by difference. The analytical method was based on the complete and quick oxidation of the sample by "flash combustion" at 1020 ºC while helium (99.99%) was used as a carrier gas. The combustion gas mixture was directed to a chromatographic column where the components (carbon dioxide, water, sulphur dioxide and nitrogen) were separated and their concentrations were determined based on the weight of the sample. The bio-crude HHV

was calculated by the elemental analyser based on the elemental composition using Dulong's formula [229].

7.2.4 Characterization of aqueous phase

The aqueous phase ammonia concentrations of the samples withdrawn from the reactor were determined as described in section 2.1 after extraction of oily compounds by DCM. In addition, chemical composition of aqueous phase collected at 60 min was determined by GC-MS using a DB-WAX column (30 m × 0.25 mm × 0.25 μm). A known amount (5-10 mg) of dry sample was dissolved in 5 ml water and filtered using a Simplepure syringe filter (0.45 μm). One microliter sample was injected into the column at a split ratio of 20:1 while helium (99.99%) was used as carrier gas at 1.0 ml/min. The injector temperature was set at 250 ºC and the GC oven temperature programming started isothermally at 50 ºC for 5 min, ramped to 250 ºC at 10 ºC/min, and held at this temperature for 20 min and the MS source was set to 70 eV. Scan range of mass spectrum was in m/z of 15-500. Identification of compounds was carried out by comparing mass spectra acquired in scan mode with mass-spectra provided by NIST library data base (NIST, Shimadzu Company).

7.2.5 Bio-char (solid fraction) analysis

The ultimate analysis and HHV of the solid residues were also determined following similar procedures to those used for the bio-crude fractions.

7.2.6 Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) in Excel software (Excel, Microsoft office 2010).

7.3 Results and discussion

7.3.1 WWT HRAP biomass characteristics

The characteristics of the feedstock used in this study are summarised in Table 7.1. The biomass lipid content was as low as 15 ± 3 wt% which implies the unsuitability of the biomass for biodiesel production. While due to conversion of whole biomass through HTL process the biomass might be a promising feedstock for bio-crude production due to its relatively high level of carbon (34.1 ± 0.5 wt%), hydrogen (5.6 ± 0.1 wt%), and HHV (19.7 ± 0.2 kJ/g). The biomass composition and chemical composition was comparable what were obtained for the pond biomass and the biomass used in pyrolysis experiment. The biomass had high ash content (29.5 ± 1 wt%) compared with pure freshwater algal biomass (<10 wt% ash content) [66, 223, 233, 243]. The main components of the non-combustible material were

probably silica and calcium which have usually high level in municipal wastewater [65]. The presence of high level of silica could be confirmed by presence of *Pediastrum* sp. which has a silica skeleton [93]. In addition to the high ash content, similar to pure algal biomass, the protein content (40 ± 1.5 wt%) and consequently the nitrogen content (4.9 ± 0.1 wt%) of the biomass were high which may reduce the quality of bio-crude due to co-production of nitrogenous compounds under HTL conditions.

Table 7.1 Characteristics of WWT HRAP biomass used in HTL experiment

Biomass composition (wt%)		**Elemental composition (wt%)**	
Proteins	40 ± 1.5	C	34.1 ± 0.5
Carbohydrates	16 ± 2	H	5.6 ± 0.1
Lipids	15 ± 3	N	4.9 ± 0.1
Ash	29.5 ± 1	S	<0.3
VSS (g/L)	15.4 ± 1.5	O	25.7 ± 0.3
HHV (kJ/g)	19.7 ± 0.2		

7.3.2 Kinetic of volatile suspended solids degradation

According to the literatures, temperature is the most important operating variable in algal HTL process which could highly affect organic conversion pathways [230, 233, 244]. Influence of temperature on degradation and solubilisation of total volatile suspended solids (VSS) during the experiments is illustrated in Fig. 7.2. The percentages of VSS degradation were calculated according to Eq. (7-1).

$$VSS\ degradation\ (\%) = \left(\frac{VSS_0 - VSS_t}{VSS_0}\right) \times 100 \qquad (7-1)$$

Overall, the degradation of VSS increased sharply within a short time and then reached equilibrium (Fig. 7.2). Occurring different equilibriums at different temperatures resulted from the complexity of the biomass comprised of different lipids, proteins and carbohydrates with different degradation temperatures. As the temperature increased the activation energy of the decomposition of more organic compounds is provided and therefore, the VSS degradation rate increased. The percentages of VSS degradation at equilibrium varied within a broad range, 22.3-75.5%. The lowest degradation occurred at the lowest temperature. In fact liquefaction, at low temperatures, acts more likely similar to hot extraction of certain components rather than conversion [66, 224]. The results illustrated that at the temperatures ≤220 ºC, increase in temperature remarkably improved the VSS degradation. For example, the equilibrium degradation of VSS was doubled when the temperature was increased from 150 ºC to 200 ºC. While at the conversion temperatures >220 ºC, increase in temperature

affected VSS degradation but to less extent. The results were comparable with those which have been reported for pure algal biomass degradation in the literatures [224, 229, 244]. Yu et al. [224] hydrothermally liquefied *Chlorella pyrenoidosa* at 100-300 ºC for 30 min. They showed that an increase in temperature from 160 ºC to 200 ºC reduced solid residue carbon content (as a proxy of VSS degradation) from 90% to 35% of initial carbon content of feedstock. While the temperature changes from 220 ºC to 240 ºC resulted in reduction of solid residue carbon content from 22% to 12% of initial one.

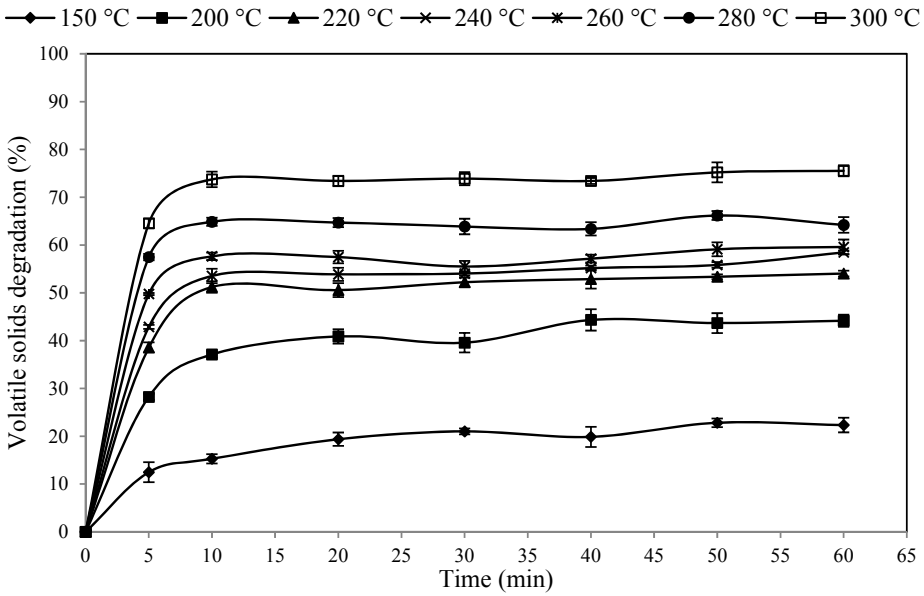


Figure 7.2 The influence of temperature on degradation of the volatile suspended solids during the HTL experiment

With respect to the isotherm conversion time, overall, the degradation reaction could be divided in two distinct steps. Step one occurred within first 10 min in which the VSS is degraded fast and linearly and step two occurred within 10-60 min in which the VSS remained unchanged and no significant degradation occurred. The results suggest that to liquefy the WWT HRAP biomass treatment time does not need to be more than 10 min. This was in a good agreement with the recent literatures which shows a shift from conventional HTL (long heat up and processing time) toward fast HTL (rapid heat up and processing time <10 min) at mild conversion temperatures [244-246]. However, the optimum time for fast HTL should be further investigated since it has been shown that, at mild temperatures, water-soluble compounds are typically dominant in short conversion time (<20 min) while they are converted to bio-crude at longer time [230, 247].

To simplify the kinetic of the process a lump kinetic model based on VSS degradation is developed. To calculate the overall biomass degradation rates first-order kinetics was used (Eq. 7-2).

$$Ln\left(\frac{VSS}{VSS_0}\right) = -Kt \qquad (7-2)$$

where VSS_0 and VSS are the volatile suspended solids concentration (g/L) at time 0 (loaded into the reactor) and t, respectively and K is the overall kinetic rate coefficient (1/min) of biomass degradation reaction. It was assumed that the degradation of volatile suspended solids during the heat up period was negligible. K is related to the reaction temperature according to Arrhenius equation (Eq. 7-3).

$$K = K_0 \exp\left(\frac{-E}{RT}\right) \qquad (7-3)$$

in which K_0 is the Arrhenius pre-exponential factor constant, E is the overall activation energy (kJ/mole) for degradation of biomass organic fraction, R is the gas constant and T is the conversion temperature (K). Since at conversion time >10 min no sensible VSS degradation occurred, the first-order kinetic model of the degradation results is shown for only first 10 min (Fig. 7.3a) where the kinetic rate coefficient was determined using Eq. (7-2). Although, no sensible VSS degradation occurred at >10 min, it is noteworthy that the interactions between the water-soluble compounds, the bio-crude and gaseous fraction are continuously occurring which were not the focus of this study.

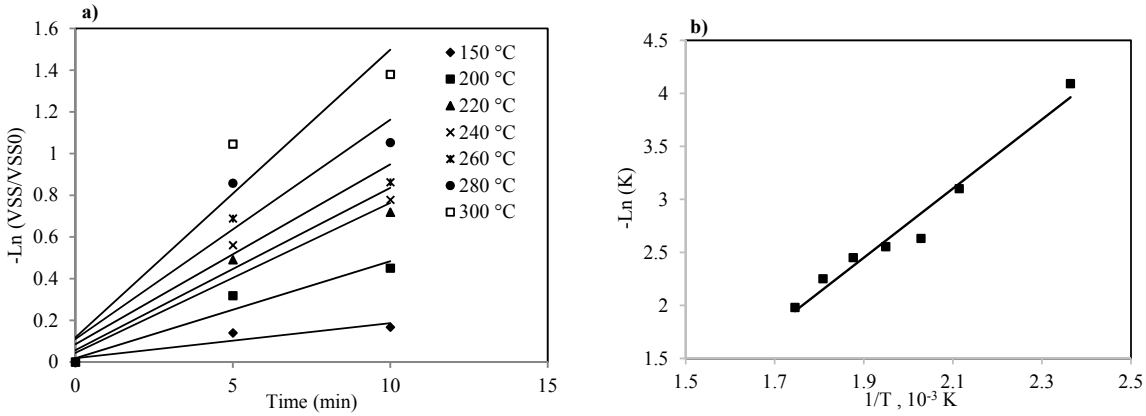


Figure 7.3 HTL kinetic model: a) First-order kinetic model of the volatile solid degradation results, b) Arrhenius plot for the first-order conversion rate coefficient presented in Fig. 3a

As shown in Fig. 7.3a, the reaction rate constant was highly temperature dependent. To determine K_0 and activation energy, the conversion rate constant was plotted versus temperatures according to Eq.7-3 (Fig. 7.3b). K_0 and overall activation energy were 42.2 and 27.1 kJ/mole. The activation energy was comparable with what has been reported (26

kJ/mole) for degradation and conversion of *Nannochloropsis* sp. to water soluble products and bio-crude compounds at 250-400 °C [247].

7.3.3 Product yields

The effect of temperature on products distribution is shown in Fig. 7.4. The changes of products yields over a broad range of temperature indicated the critical role of temperature. Overall, increase of temperature increased yields of the bio-crude while decreased yields of the solid residue.

The yields of solid residue changed between 44.8 ± 1.1 wt% and 85.5 ± 1.7 wt% with the highest yield occurred at the lowest temperature. It is noteworthy that the feedstock ash content (~ 30 wt%) was counted as solid residue. At the lowest temperature (150 ºC) the biomass was converted less to liquid/gaseous products. While when the temperature increased more degradation occurred and therefore more biomass was converted to the water-soluble products and the bio-crude.

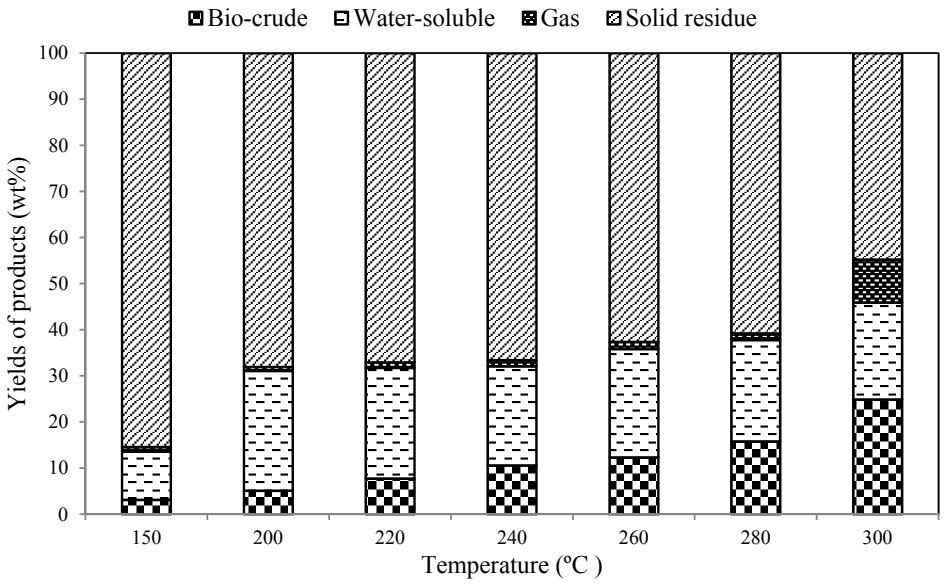


Figure 7.4 Yields of different products produced during the HTL of the biomass at different temperatures for 1h

The bio-crude yields ranged from 3.1 ± 0.1 wt% to 24.9 ± 0.1 wt% with the highest yield obtained at the highest temperature (300 ºC). Notably, the bio-crude yields were lower compared with what have been reported for HTL of pure algal biomass (20-45 wt% of initial biomass) at similar temperature range [62, 66, 223, 232, 233, 239]. While the bio-crude yields were comparable with those (≤ 30 wt% of initial biomass) reported for HTL of mixed culture high ash content biomass [65, 227, 230, 248]. The lower bio-crude yields might result

from either the high non-volatile solid content of the WWT HRAP biomass or the high resistant and thick cell wall of the *Pediastrum* and *Desmodesmus* species [11, 239]. Increase in the bio-crude yield with temperature was more significant at higher temperatures (at ≥280 ºC) coincided with the reduction of the yields of water-soluble compounds. This suggests that, at such temperature range, the water-soluble compounds may be further converted to the bio-crude. This observation was consistent with what have been suggested for formation of the bio-crude from the water-soluble products at mild temperatures and relatively long reaction times (>20 min) [230, 233, 244, 247].

Moreover, temperature not only affects the bio-crude yield but also affect its appearance. The bio-crude produced at 150 ºC was brownish-green solids in which the green colour was probably due to extraction of chlorophyll by DCM. By increasing the conversion temperature from 200 ºC to 260 ºC black solid bitumen-like bio-crude was produced and at >260 ºC the bio-crude viscosity reduced and became oilier (Fig. 7.5). This suggests that the ratio of the high viscosity bio-crude compounds (known as heavy bio-crude) to low viscosity bio-crude compounds (known as light bio-crude) reduces at the higher conversion temperatures. This implies that the bio-crudes produced at higher temperatures would have lower boiling points which would be more suitable for use as transportation fuels.

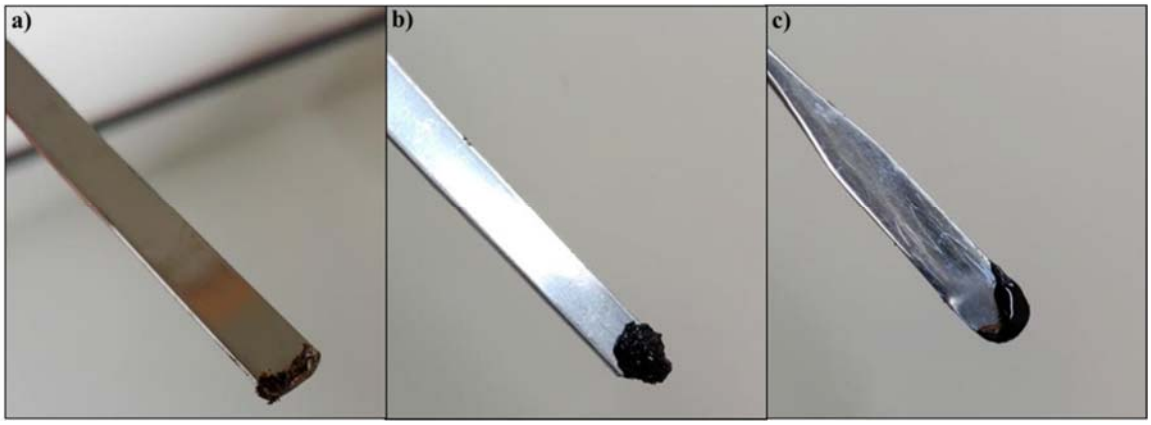


Figure 7.5 The HTL bio-crude appearance at: a) 150 ºC, b) 260 ºC and c) 300 ºC

The yields of water soluble products were between 10.5 ± 0.5 wt% and 26.0 ± 1.0 wt%. An increase in the temperature from 150 ºC to 200 ºC significantly improved the water soluble components formation which was probably due to decomposition and conversion of majority of the proteins into water-soluble compounds at this low temperature range [233]. While at the higher temperatures the yields of water-soluble compounds reduced inconsistently. The yields of gaseous products were ranged from 1.0 ± 0.2 wt% to 9.3 ± 1.5 wt% and were relatively unchanged at temperatures ≤280 ºC. The yields of gas phase

increased considerably at 300 ºC which suggests that part of the water-soluble compounds or the solid residue was converted to the gaseous products. The results were in line with the literatures [229, 244]. In addition, it has been reported that at temperatures ≥350 ºC, both water soluble compounds and bio-crude are converted to gaseous products and its yield considerably increases [229, 239]. It is noteworthy that the HTL gas phase produced at <300 ºC, is typically dominated by CO_2 (>95%) while at the higher temperatures where the decomposition of bio-crude occurs CH_4 and H_2 are also produced [65, 244].

7.3.4 Analysis of bio-crude

Ultimate analysis and HHV of the bio-crudes collected at different temperatures after 60 min are summarised in Table 7.2. The bio-crudes comprised of 71-72.4 wt% C, 8.7-9.8 wt% H, 0.9-4.8 wt% N, 1.7-3.5 wt% S and 12-15.7 wt% O. By raising temperature, the bio-crude carbon and hydrogen contents insignificantly fluctuated while its nitrogen content consistently increased. It was probably due to the production of the bio-crude mainly from protein-based monomers at <350 ºC [233]. However, it should be noted that the increase of the bio-crude nitrogen content was significant ($p<0.05$) at the lower temperatures which might be due to the higher impact of the temperature on conversion rate of the protein-based monomers into the bio-crude at the lower temperatures [233, 246].

In contrast with the nitrogen trend, the oxygen content of the bio-crudes continuously reduced as temperature increased. Under HTL conditions oxygen is removed continuously in the form of CO_2 from water-soluble and bio-crude organic compounds through decarboxylation and depolymerisation reactions [224, 230]. As the lower content of oxygen usually result in the higher bio-crude HHV, reduction of O_2 at the higher temperature is favourable. The bio-crude HHV ranged between 37.5 ± 0.1 kJ/g and 38.9 ± 0.0 kJ/g (Table 7.2) which were comparable with what have been reported for algal-based bio-crude (30-40 kJ/g) [65, 83, 249, 250]. Overall, no significant relationship was found between the reaction temperature and the bio-crude HHV. The highest (38.9 kJ/g) bio-crude HHV occurred at 200 ºC where the bio-crude hydrogen content was at the highest level (9.8 wt%).

Oxygen, nitrogen and energy content of bio-crude are the main indicators of bio-crude quality. Compared with fossil fuel oil with <1.5 wt% oxygen, <0.7 wt% nitrogen and 42 kJ/g energy content [69, 224], it can be concluded that the bio-crudes had lower quality. However, the bio-crude sulphur content was comparable with that of fossil fuel oil (0.05-5 wt%). The high oxygen content of the bio-crudes not only resulted in lower HHV but also may reduce the stability of the bio-oil experienced by change its composition during storage [230].

Moreover, the high nitrogen content may reduce the fuel value of the bio-crude from an environmental point of view. Since when the bio-crude is combusted this nitrogen will be converted to NOx which can react with rainwater and form acidic rain [76]. The results indicated that the bio-crude could not be used directly as transportation fuel and needs further upgrading to reduce its oxygen and nitrogen contents before use which would consequently increase refinery costs. Although, by conducting catalytic HTL part of nitrogen could be removed to lower refinery costs [19, 236].

Table 7.2 Ultimate analysis and HHV of the HTL bio-crude produced at different temperatures

Temperature (°C)	C (wt%)	H (wt%)	N (wt%)	S (wt%)	O (wt%)	HHV (kJ/g)
150	71 ± 0.1	8.9 ± 0.05	0.9 ± 0.01	3.5 ± 0.5	15.7	37.6 ± 0.1
200	71.8 ± 0.05	9.8 ± 0.0	2.1 ± 0.05	1.9 ± 0.0	14.4	38.9 ± 0.0
220	71.5 ± 0.1	9.5 ± 0.02	3.4 ± 0.0	1.8 ± 0.02	13.8	38.5 ± 0.1
240	71.1 ± 0.1	9.1 ± 0.0	4.3 ± 0.05	1.75 ± 0.05	13.7	37.6 ± 0.0
260	71.6 ± 0.3	9.2 ± 0.05	4.5 ± 0.02	1.7 ± 0.05	13	38.0 ± 0.0
280	72.4 ± 0.1	9.0 ± 0.02	4.7 ± 0.01	1.9 ± 0.03	12	38.0 ± 0.0
300	72.2 ± 0.05	8.7 ± 0.0	4.8 ± 0.02	1.8 ± 0.01	12.5	37.5 ± 0.1

The most abundant bio-crude organic compounds identified using GC-MS are listed in Table 7.3. The bio-crude comprised of a complex organic mixture, especially at the higher temperature, which made it difficult to identify all the peaks on the chromatograms. This implies that although the yield of the bio-crude increased at the higher temperatures, the bio-crude may require more upgrading due to higher complexity.

The identified compounds were categorised into two different groups based on their chemical structure including linear and cyclic compounds. Both groups consisted of nitrogenous and oxygenate compounds such as indole, phytol, dodecanamide and cresol produced from interactions of biomass constituents under HTL conditions. The content of linear and cyclic compounds were 33.4-62.2% and 15.4-31.4% of the total chromatograph area, respectively. The content of the linear compounds decreased with temperature while the fraction of cyclic compounds increased with temperature, except at 150 ºC. Under HTL conditions at higher temperatures (>250 ºC) and relatively long reaction time re-polymerization and cyclization become more intense, which is may be due to involving water-soluble compounds in the reactions at such temperatures [230]. Similar trends have

been reported by Chen et al. [230] in isothermally liquefaction of mixed algal feedstock at 260-300 ºC for 0-90 min.

Pyrrols, indoles, amides and fatty acids were the most abundant compounds however, there was no relationship for their production with the temperature. Pyrrols and indoles are produced from side chain of amino acids through condensation, cyclization and dehydration reactions [234]. Fatty acids are produced through decomposition of lipids and amides are formed from reaction of fatty acids with amino acids/ammonia (originated from proteins) [230, 237]. The bio-crude fatty acid contents increased with temperature since the rate of lipid decomposition increases with temperature [233].

Table 7.3 The most abundant compounds (as % of total chromatogram area) in HTL bio-crude produced at different temperatures and 60 min

	Cyclic compounds		**Linear compounds**	
	Component	**Area ratio (%)**	**Component**	**Area ratio (%)**
150 ºC	Indole	10.7	Phytol	12.7
	p-Cresol	5.5	Eicosane	8.2
	Phenol	4.6	n-Hexadecanoic acid	6.8
	Caprolactam	2.3	Z-5-Nonadecene	4.9
	Phenol,2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl-	2.0	9,12-Octadecadienoic acid (Z,Z)-	4.6
	Σ	**31.4**	**Σ**	**62.2**
200 ºC	Phenol	3.2	Heneicosane	6.1
	Indolo[2,3-a]quinolizine, 1,2,3,4,6,7,12,12b-octahydro-	2.5	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	4.8
	Quinoline, 4-methyl-	2.1	Octacosyl acetate	3.6
	Pyrrolidine, 1-acetyl-	1.3	9-Tricosene, (Z)-	3.3
	Phenol, 4-ethyl-	1	Pentadecanoic acid	2.4
	Σ	**15.4**	**Σ**	**52.1**
220 ºC	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	4.7	n-Hexadecanoic acid	16.1
	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	1.6	1-Docosene	2.9
	p-Cresol	1.2	1-Docosene	2.0
	17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol	1.1	Isophytol	1.8
	2-Piperidinone	1.0	Pentadecanoic acid	1.3
	Σ	**16.2**	**Σ**	**35.6**
	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	5.0	Oleic Acid	8.3
	cis-1-Chloro-9-octadecene	3.6	n-Hexadecanoic acid	8.5
	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	1.5	1-Docosene	1.9

240 ºC	3-(phenylmethyl)-			
	Cholestan-3-ol, 2-methylene-, (3.beta.,5.alpha.)-	1.1	Dodecanamide	1.9
	p-Cresol	1.1		
	Σ	**17.5**	**Σ**	**35.1**
260 ºC	p-Cresol	1.47	Oleic Acid	5.6
	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	1.4	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	4.9
	5.alpha.-Cardanolide, 3.beta.,14,19-trihydroxy-	1.1	9-Tricosene, (Z)-	2.3
	4-pyridinecarboxamide, 2-chloro-	1.0	Dodecanamide	1.8
	Octacosyl acetate	0.9	n-Hexadecanoic acid	1.6
	Σ	**21.8**	**Σ**	**36.7**
280 ºC	9H-Pyrido[3,4-b]indole	2.0	n-Hexadecanoic acid	3.6
	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	1.9	Dodecanamide	2.0
	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester	1.4	1-Docosene	1.9
	Cyclotetracosane	1.0	Heneicosane	1.5
	Cholest-3-ene, (5.beta.)-	1.0		
	Σ	**22.2**	**Σ**	**34.1**
300 ºC	9H-Pyrido[3,4-b]indole	1.7	n-Hexadecanoic acid	3.1
	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	1.4	1-Docosene	2.3
	2,5-Piperazinedione, 3-benzyl-6-isopropyl-	1.3	cis-Vaccenic acid	2.1
	p-Cresol	1.3	Tetradecanamide	1.9
	Cyclopentadecane	1.2	1-Tetracosene	1.3
	Σ	**28.3**	**Σ**	**33.4**

7.3.5 Analysis of HTL by-products

While the aim of early HTL studies was maximizing yield of the bio-crude, recent studies have focused on beneficial use of HTL by-products to improve algal HTL economically. In this section the potential application of aqueous phase and solid residue would be proposed with regards to their properties.

7.3.5.1 Analysis of aqueous phase

In general, recovering nitrogenous compounds such as ammonia in aqueous phase (AP) rather than in bio-crude fraction is preferable since less upgrading processes are required to remove them and improve the quality of bio-crude. In addition, N-rich AP could be recycled and used as nitrogen source for algal biomass production [225, 228, 232]. Ammonia is one of

the main nitrogenous compounds usually presents in HTL AP which could be easily assimilated by microalgae. It is derived from proteins under hydrothermal liquefaction conditions. Proteins are hydrolysed and degraded into amino acids, amine and carboxyl groups. Subsequently N and H are removed from amino acids in the form of ammonia through deamination reaction most likely at <240 ºC within a short period of time [231, 249, 251, 252].

To examine suitability of the AP produced at different temperatures for use as nitrogen source for algal cultivation, the AP ammonia concentrations (as a proxy for suitability of AP) were measured over the isothermal processing time (Fig. 7.6). Overall, the ammonia concentrations increased with the temperature and time so that the maximum ammonia concentration (292.9 mg/L) occurred at 300 °C after 60 min. However, the ammonia concentration increased at lower rate at both the higher temperatures and the longer time. For example, at 10 min, an increase in reaction temperature from 150 ºC to 200 ºC increased significantly ammonia concentration from 84.3 mg/L to 146.0 mg/L. While the ammonia concentration increased from 262.5 mg/L at 260 ºC to only 277.5 mg/L at 280 ºC and 10 min. Regardless of the reaction temperature, the ammonia concentration increased continuously with time at the lower temperatures while it increased sharply at higher temperatures and reached to an equilibrium within <20 min. This was probably due to the production of majority of the ammonia during the heat up time which was not measured. With respect to the ammonia concentration (≥239.1 mg/L) at the temperatures ≥240 ºC after 10 min and initial feedstock nitrogen concentration (440 mg/L), >54 wt% of feedstock nitrogen was recovered in the form of ammonia in a short processing time.

The results suggest that in order to reduce the bio-crude nitrogen content (originated from conversion of water-soluble compounds) the WWT HRAP biomass could be pre-treated to <240 ºC for a short time. The N-rich AP could be recycled back into the culture system and the biomass could be then subjected to HTL at higher temperatures. In this case more quality bio-crude can be produced, however, the overall yield of the bio-crude may reduce. Costanzo et al. [249] showed that up to 45% of initial nitrogen content of mixed-culture algal feedstock dominated by *Chlorella sorokiniana*, *Chlorella minutissima*, and *Scenedesmus bijuga* was recovered in AP by conducting HTL at 225 ºC for 15 min. While the yield of bio-crude produced from liquefaction of treated biomass at 350 ºC for 60 min is reduced by 34% compared with what was usually produced from untreated biomass.

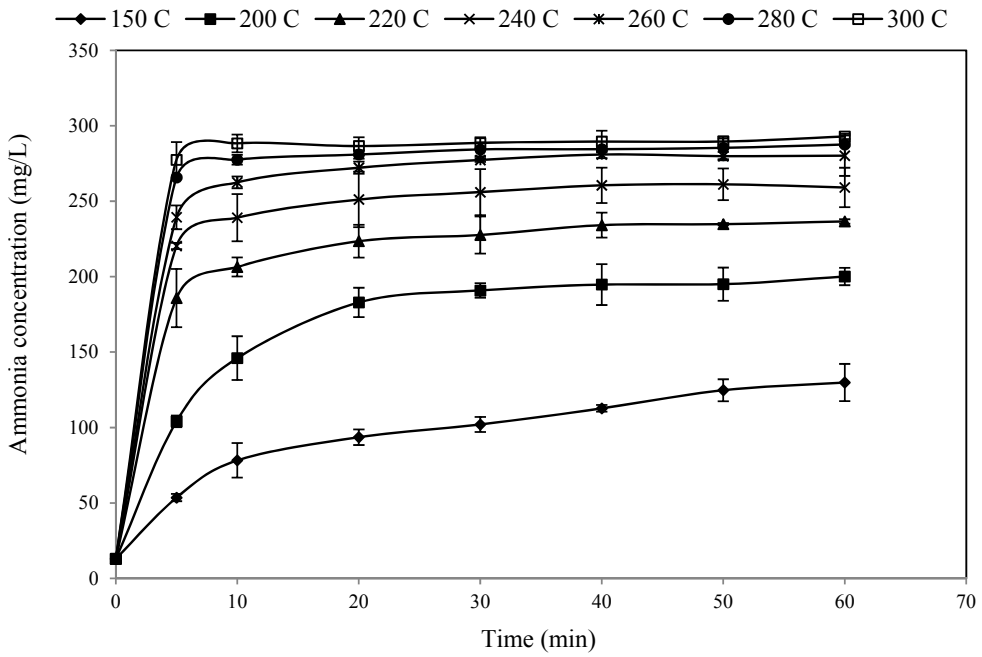


Figure 7.6 Ammonia concentrations of HTL aqueous phase produced at different temperatures and different sampling times

Suitability of the AP for use as an ammonia source not only depends on its ammonia concentration but also on the presence of other water-soluble compound such as phenolic compounds, which may inhibit the algal growth [253]. Hence, GC-MS analysis of the AP was performed and the most abundant compounds in the AP are listed in Table 7.4. The aqueous phase was mainly dominated by organic acids such as acetic acid, and oxygenate and nitrogenous compounds such as diethyl phthalate, 2-piperidinone, 2-pyrrolidinone and acetamide. Similar compounds have been reported in the AP produced from HTL of different algae [230, 254, 255]. These compounds are produced through carboxylation of amino acids and reaction between amine and carboxylic acids [230, 231]. Of dominant compounds, acetic acid is a useful compounds which could be utilized by microalgae as carbon source [225, 232, 256] while the other compounds may inhibit the algal growth. Adams et al. (1995) showed that diethyl phthalate inhibits the growth of *Selenastrum capricornutum* (green algae) at concentration >16 mg/L. Pham et al. [255] also found that the growth of *Spirulina* sp. was reduced in presence of 2-piperidinone and 2-pyrrolidinone. This meant that recycling of the HTL AP may not be a promising option. However, further research is required to find: 1) the toxicity level of individual compound on wastewater algae, 2) effect of HTL operational conditions on production rate of toxic compounds, and 3) promising strategies for removing toxic compounds before recycling HTL AP into the pond.

Table 7.4 The most abundant water-soluble compounds (as % of total chromatogram area) produced during the HTL experiment at different temperatures and 60 min

	Component	Area ratio (%)
150 ºC	Diethyl Phthalate	68.2
200 ºC	2-Piperidinone	34.6
	Acetamide	16.3
220 ºC	Acetic acid	50.2
	2-Piperidinone	21.7
	2-Pyrrolidinone	16.7
240 ºC	Acetic acid	33.9
	1,4,7,10,13,16-Hexaoxacyclooctadecane	28.1
	2-[2-[2-[2-[2-[2-[2-[2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethanol	15.6
	2H-1,3-benzoxazine-7-carboxamide, 3,4-dihydro-6-hydroxy-3-methyl-N-octadecyl-5,8-dipropyl-	11.5
260 ºC	1,4,7,10,13,16-Hexaoxacyclooctadecane	23.6
	2-Piperidinone	15.6
	2-Pyrrolidinone	8.3
	Acetic acid	6.1
280 ºC	Acetic acid	58.9
	2-Piperidinone	15.9
300 ºC	Acetic acid	36.0
	Diethyl Phthalate	17.3
	2-Piperidinone	7.7

7.3.5.2 Analysis of solid residue

The ultimate analysis and HHV measurement of the solid residue produced through hydrothermal liquefaction of WWT HRAP biomass were measured and are summarised in Table 7.5. The solid residue comprised of 21.9-32.2 wt% C, 3.2-4.9 wt% H and 1.4-4 wt% N and its HHV ranged 12.2-18.1 kJ/g. Overall, an inverse relationship was observed between the conversion temperature and the concentrations of the C, H and N. The HHV of the solid residues followed similar trend. The elemental composition and HVV of the solid residue produced at 150 ºC was similar to that of feedstock (Table 7.1 and 7.5). While there was a significant difference between the carbon content and HHV of the solid residue produced at 150 ºC and those produced at the higher temperatures. This could confirm again the hypothesis that HTL at low temperature is a hot extraction route and the major biomass decomposition reactions occur at higher temperatures. Similar results have been reported by Yu et al. [224] in which *Chlorella pyrenoidosa* was liquefied at a wide range of temperatures (100-300 °C) for 30 min. They found that an increase in conversion temperature from 160 °C

to 300 °C reduced initial feedstock carbon content (51.4 wt%) from 46.2wt% to 5.1 wt% in solid residue, respectively. The HVV of solid residue in the current study was higher than what has been reported (8-10 kJ/g) by Roberts et al. [65] for the solid residue produced through liquefaction of WWT HRAP biomass at 350 ºC for 1h. This was probably due to the higher C and H contents of the tested biomass and the lower conversion temperature in current study. Lower the conversion temperature resulted in less decomposition and consequently production of energy-rich solid residue. The results suggest that the solid residue produced from HTL of WWT HRAP biomass could be potentially used as fuel source.

Table 7.5 Ultimate analysis and HHV of the HTL solid residue produced at different temperatures

Temperature (°C)	C (wt%)	H (wt%)	N (wt%)	S (wt%)	HHV (kJ/g)
150	32.2 ± 0.01	4.9 ± 0.1	4.0 ± 0.02	<0.3	18.1 ± 0.1
200	26.4 ± 0.02	3.7 ± 0.02	3.1 ± 0.01	<0.3	14.3 ± 0.1
220	24.5 ± 0.1	3.6 ± 0.05	2.2 ± 0.01	<0.3	13.6 ± 0.0
240	24.9 ± 0.02	3.6 ± 0.01	2.1 ± 0.1	<0.3	13.7 ± 0.1
260	25.0 ± 0.1	3.6 ± 0.03	1.8 ± 0.02	<0.3	13.7 ± 0.0
280	23.0 ± 0.1	3.3 ± 0.05	1.6 ± 0.05	<0.3	12.7 ± 0.1
300	21.9 ± 0.01	3.2 ± 0.05	1.4 ± 0.01	<0.3	12.2 ± 0.0

7.4 Is WWT HRAP biomass a promising feedstock for bio-crude production?

From economic point of view, maximising energy recovery in the form of bio-crude is crucial. Energy recovery is a function of yield and energy content of each fraction produced which could be calculated based on Equation (7-4). The energy recovered in the bio-crude and the solid residue was calculated from the experimental results while for the combined aqueous and gaseous phases it was calculated by difference. The results are illustrated in Fig. 7.7.

$$Energy\ recovery\ (\%) = \frac{HHV\ of\ the\ fraction \times yield\ of\ the\ fraction}{HHV of\ the\ biomass} \times 100 \qquad (7\text{-}4)$$

Overall, there was a positive relationship between the temperature and the proportion of energy recovered in the bio-crude so that the maximum energy recovery (47.4% of feedstock biomass energy content, i.e. 19.7 kJ/g) occurred at the highest temperature (300 ºC). The

results were lower compared with what have been reported (50-80%) for HTL of pure algal biomass at similar temperature range [62, 66, 224, 229]. Over the temperatures the energy recovered in solid residue consistently reduced. Similar trend, except at 150 ºC, was found for the aqueous and gaseous phases. Reduction of energy recovered in the aqueous and gaseous fractions reinforced the hypothesis of conversion of the water-soluble compounds to the bio-crude at the higher temperatures. Significant increase ($p<0.05$) in proportion of the energy recovered in the aqueous and gaseous phases from 150 ºC to 200 ºC, was probably due to the decomposition of majority of the protein fraction into the water-soluble compounds [230].

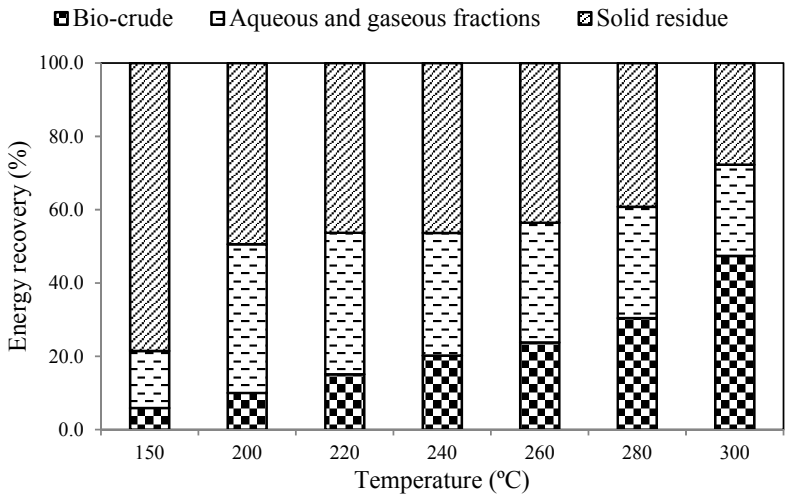


Figure 7.7 The percentage of energy recovered in different HTL fractions at different temperatures

To examine the feasibility of the bio-crude production from the WWT HRAP biomass, an energy balance needs to be made on the whole process, from cultivation to bio-crude production. As feedstock comes from wastewater treatment ponds, the energy required for cultivation and harvest of the biomass would be essentially covered by wastewater treatment function of the pond [6]. Assuming the harvested biomass (~2 wt% solid content) is directly subjected to HTL reactor (similar to this study) then no energy is required for concentrating the biomass. Assuming algal slurry had similar enthalpy to that of water, hence the energy required (according to steam tables) to liquefy the biomass at 300 ºC is 1.3 MJ/kg slurry. This is similar to those (1.25 MJ/kg slurry) which has been reported for HTL of algal feedstock with different solid concentration (up to 20 wt% solids) at 300 ºC [257]. On the other hand, the results indicated that the maximum energy of 186.7 kJ/kg slurry (based on 47.4% energy recovery from a slurry with ~2wt% solid content and 19.7 kJ/(g dry biomass) energy content) could be recovered in the bio-crude from HTL of the WWT HRAP biomass. The energy balance illustrates that the energy return on energy invested (EROEI) of the HTL of the

WWT HRAP biomass at 300 ºC is <15%. It is noteworthy that the results are specific to the tested conditions and the energy required for solvent extraction, solvent recovery and upgrading of the bio-crude was not counted in the energy balance. Similar negative energy balance has been reported for HTL of algal biomass with solid content <10 wt% [258]. The results were considerably lower than those (EROEI >1) reported by Biller and Ross [62] where they liquefied a 10 wt% slurry of *Chlorella, Nannochloropsis, Porphyridium* and *Spirulina* at 350 ºC for 1h. It was probably due to the higher reaction temperature, lower ash content (7-26 wt%) and higher feedstock concentration in their study resulted in higher bio-crude yields (25-40 wt%) and energy recovery [62].

According to the results if the bio-crude is considered as the only HTL product the process seems not feasible under tested conditions (using batch reactor with low feedstock concentration and without recovering waste heats) and hence modification is required. As stated earlier, 1.3 MJ/(kg slurry) energy is required to heat up the slurry containing less than 20 wt% solid concentration to 300 °C. In this case one possible modification is to process the concentrated biomass, i.e. feedstock with 15-20 wt% solid content instead of ~2 wt% solid content. Assuming energy recovery through HTL (47.4% of biomass energy) does not change by concentrating biomass, thereby the minimum feedstock concentration to supply the concentrating and processing energy demands (i. e., EROEI = one) is 14 wt%. This means that the economy of the process is highly dependent on feedstock concentration which needs further investigations. Vardon et al. [258] conducted HTL experiment on *Scenedesmus* sp. and *Spirulina* sp. at 300 °C for 30 min and showed that if the solid content of feedstock increases to >10 wt% the EROEI would be higher than one.

In addition, compared to the lab-scale batch HTL studies, in an optimized continues industrial-scaled process the heat lost is minimized using efficient heat exchanger for preheating of feedstock by hot product stream which leads to positive energy balance. For example, Muradel Pty Ltd [241] reported an EROEI of 2.67 where a slurry of *Tetraselmis* sp. was converted to the bio-crude in a continues pilot-scale HTL reactor and must of the heat was recovered. However, they did not considered the required energy for solvent extraction of the bio-crude from solid fraction, solvent recovery and the bio-crude upgrading.

Moreover, in an industrial scale, the solid residue (>12 MJ/kg) could also be used to supply the HTL energy demands. Furthermore, the aqueous phase which contains high level of ammonia could be used as ammonia source for further algal cultivation or sold as fertiliser which in turn improves the economy of the process.

7.5 Conclusions

Hydrothermally liquefaction of the WWT HRAP wet biomass (2.2wt% solid content) for bio-crude production was investigated at various temperatures (150-300 ºC). The bio-crude yields ranged from 3.1 wt% to 24.9 wt% with the higher yields at the higher temperatures. The bio-crudes consisted of 0.9-4.8 wt% nitrogen and 12-15.7 wt% oxygen, had a HHV of 37.5-38.9 kJ/g, and were dominated by pyrroles, indoles, amides and fatty acids. The bio-crude nitrogen content increased with increasing HTL temperature while the oxygen content declined. The results were comparable to those reported for HTL of pure algal biomass, however, the bio-crudes had lower quality than fossil fuel oil and needed further upgrading prior to use. Overall, up to 47.4% of the biomass energy (19.7 kJ/g) was recovered in the bio-crudes. To improve the economics of the process the solid residue having >12 kJ/g energy content could be used as a fuel source for the process and aqueous phase containing 30-67% of the initial biomass nitrogen in the form of ammonia could be used for further algal cultivation or as fertilizer. However, further research on the toxicity level of the AP compounds is required. Since literature on HTL of pure algal biomass indicates a positive energy balance at higher feedstock concentration, it would be valuable to determine the effects of biomass concentration on yield of HTL of WWT HRAP biomass.

CHAPTER 8

Conclusions

It has been proposed that replacement of pure algal biomass with free gravity-harvested algal-bacterial biomass produced in WWT HRAP could bring algal-based liquid biofuel production closer to economic reality. In such a system, the production and harvest costs of the biomass are covered by the WWT plant. While several studies have investigated different strategies to improve pond treatment function, there has been little evaluation of the quantity, quality and suitability of such biomass for high quality liquid biofuel production. Therefore, the aim of this study was to test the hypothesis: "*WWT HRAP biomass is a promising feedstock for low-cost production of algal-based liquid biofuel*". A series of experiments were conducted that investigated both biomass energy production and biomass energy recovery as different liquid biofuels. First, the quantity and quality of the WWT HRAP biomass energy and the possibilities for their improvement were investigated. Then, the suitability of WWT HRAP biomass energy for conversion to the quality biodiesel, pyrolytic bio-oil and bio-crude was assessed.

Over the course of one year monitoring two parallel identical pilot-scale WWT HRAPs, the mean annual biomass productivity was 21.5 ton VSS/ha/year and was highly dependent on seasonal changes in temperature and solar radiation, as well as zooplankton grazing pressure. The mean annual biomass energy content was 19.2 GJ/ton biomass and it was function of biomass algal proportion and to lesser extent the biomass lipid content. Biomass energy of 413 GJ/ha/year was produced in the outdoor wastewater treatment ponds which was much lower than literature values (800-1200 GJ/ha/year) estimated for either WWT HRAP or pure algal cultivation system based on controlled experiments. This considerable difference was most likely due to lower biomass algal content and productivity resulted from zooplankton grazer blooms, no control of algal species and operating the ponds at sub-optimal HRT, particularly in the warm months. As the both biomass productivity and energy content were a function of the algal productivity, to further improve the biomass energy yield, the operational, biological and environmental conditions need to be modified. Environmental parameters (temperature and solar radiation) are not controllable at full-scale, but operational and biological parameters can be, therefore two strategies were investigated.

To lower zooplankton grazing pressure and improve algal productivity, the pond could be dominated by large colonial species which are not preferable food for majority of the micro-zooplankton invertebrates. Among five different dominant colonial species, *Mucidosphaerium pulchellum* and *Micractinium pusillum* showed the highest nutrient removal capacity as well as the highest biomass energy yield under both summer and winter conditions. Both species grew faster and showed the highest biomass productivities as well as

the highest lipid and energy content (*Mucidosphaerium pulchellum*: 0.59 ± 0.01 d-1, 188.9 ± 10 mg/L/day, 32.3 ± 3.0 wt% and 20 kJ/g in summer, and 0.38 ± 0.03 d-1, 57.2 ± 3 mg/L/day, 31.0 ± 3.3 wt% and 21.1 kJ/g in winter; *Micractinium pusillum*: 0.57 ± 0.02 d-1, 177.2 ± 11 mg/L/day, 24.4 ± 2.4 wt% and 22 kJ/g in summer, and 0.29 ± 0.01 d-1, 40.9 ± 4 mg/L/day, 30.9 ± 0.7 wt% and 19.9 kJ/g in winter).While both species were equally beneficial, *Micractinium pusillum* is the promising species due to its much higher settleability which leads to lower additional costs for complete biomass harvesting.

To further improve the biomass energy yield without impacting pond treatment function, effect of CO_2 addition was investigated conducting two mesocosms-scale outdoor experiments in summer and winter. The total and harvestable biomass energy yield enhanced by up to nearly 1.5 times and over 2 times respectively in summer and over 1.1 times and 1.4 times, respectively in winter relative to the aerated cultures. The maximums, in summer, occurred in the cultures supplemented with 5% CO_2 (pH 6-7), while in winter occurred in the 0.5% CO_2 cultures (pH 7-8). These results indicated that the wastewater algal growth is limited by CO_2 concentration year-round and therefore CO_2 addition is essential to vary culture pH with seasons and climate.

Although maximising biomass energy yield in WWT HRAP is crucial, the suitability and recoverability of such biomass energy for economical algal liquid biofuel production is equally important. Hence, the second part of this study was focussed on assessing the suitability of such free biomass for quality biodiesel, pyrolytic bio-oil and bio-crude production.

In contrast with the literature values for the pure algal lipid profile, the biomass FAME profile was highly complex so that less than 30 wt% of the FAMEs profile belonged to biodiesel desired lipids. Overall, the results illustrated that 0.9 ± 0.1 $g/m^2/d$ low quality biodiesel (3.2 ± 0.5 ton/ha/year) could be produced from the WWT HRAP at the tested conditions. Although, the production potential of the low-quality biodiesel could be further improved by 20% via CO_2 addition, it was much lower than estimated values (>7 ton/ha/year) for the quality biodiesel production from pure algal biomass.

The low quantity and quality of the biomass lipids, together with the current limitations of lipid extraction and purification make energy recovery from the whole biomass more attractive. Hence, the recoverability of the WWT HRAP biomass energy through pyrolysis and hydrothermal liquefaction (HTL) were investigated. In the both experiments, effect of temperature as the main influencing operational parameters on yield and quality of the products and consequently on energy recovery was evaluated. However, the highest yields of

the pyrolytic bio-oil (7 wt%) occurred at the maximum temperature (500 ºC), the majority of that was mainly produced at 400-500 ºC and interestingly at <400 ºC water was the dominant product. The bio-oils had low quality so that they comprised of 6.4-8.9 wt% N and was highly complex (>500 compounds) even at low temperatures which could lead to high refinery costs. Overall, less than 15% of the WWT HRAP biomass energy was recovered in the form of the low-quality bio-oil which was much lower than what (50% energy recovery) have been reported for the pyrolysis of pure algal biomass. Similar to the pyrolysis results, increased temperature in HTL experiment lowered the bio-crude quality (by increasing N content and complexity of the bio-crude) while enhanced its yield. The highest (24.9 wt%) yield of the bio-crude occurred at the highest temperature (300 ºC). Overall, maximum 47.4% of initial biomass energy (19.7 MJ/kg) was recovered in the bio-crude at the highest temperature which was lower than literature values (50-80 % energy recovery) for HTL of pure algal biomass. In terms of quality (N, O, energy content and chemical composition complexity), both bio-oil and bio-crude had comparable quality to those reported for pyrolysis and HTL of pure algal biomass while in terms of quantity, the yields of the main products were much lower.

This study has demonstrated that (at tested conditions) although the use of the WWT HRAP biomass would offset algal cultivation and harvest costs, the production costs of quality liquid biofuel would be higher than for production of liquid biofuel from pure algal biomass due to the low quality and quantity of the WWT HRAP biomass energy.

CHAPTER 9

Future work

9.1 Biomass energy production in WWT HRAP

This research has shown that the production potential of low-cost and quality biomass energy in WWT HRAP is a function of algal productivity which is limited by operational, biological and environmental parameters. In addition, maintaining the dominance of the most beneficial colonial algal species in the pond is preferable for enhancement of biomass energy yield all year-round and maximising the biomass energy recovery as well as lowering the upgrading costs via lowering the complexity of the biomass composition. Although the results of lab-scale batch monoculture experiment determined *Micractinium* sp. as the most beneficial colonial species for both treatment and biomass energy production, further investigation is required to address: "Which species is more beneficial from conversion point of view?" Detail analysis of the chemical composition of the species and their cell wall resistance (influencing on cell rupture during the conversion process and consequently on yield of biofuel) would help to select the promising wastewater algae for effective and low-cost treatment as well as biomass energy and liquid biofuel production. Afterwards, there is a need to answer: "How to maintain selected species dominant in the pond all year-round? Can recycling a fraction of its gravity-harvested biomass back into the pond improves its dominance as has been proven for Pediastrum sp.?". CO_2 addition showed great potential to improve biomass energy yield and its harvestable fraction in mesocosms-scale experiment in both summer and winter. While the transferability of the results from mesocosms-scale to pilot-scale and from pilot-scale to industrial scale should be investigated.

In addition, it has been shown that recycling a fraction of gravity-harvested biomass of the *Pediastrum* sp. into the pond not only improves the dominance of this species but also enhances the WWT HRAP biomass energy yield. Similarly, in the current study, CO_2 addition improved the biomass energy yield. The question which is raised is: "Can the biomass energy yield be further improved by a combination of both strategies?".

9.2 WWT HRAP biomass conversion

In pyrolysis experiment, under the tested conditions, >60% of the pyrolytic liquid phase was water mainly produced through dehydration reactions occurred in the hot zone of the reactor. The interaction between pyrolysis gases and water production rate depends on residence time of the volatile in the reactor hot zone controlled by inert gas flow rate. Therefore, to reduce the water production rate and improve the yield of bio-oil there is a need to address the following question: "What is the optimum inert gas flow rate required to

minimise the dehydration reactions and water production?". In addition as the bio-oil comprises of high amount of aromatics, especially at higher temperatures, the suitability of bio-oil for use as gasoline additive for improving its octane number needs to be investigated.

According to the results, HTL was the most promising route for maximising biomass energy recovery in liquid biofuels while its energy return on energy invested (EROEI) was <1 at tested conditions which means it was not an economical process and therefore, the process needs further modifications. According to the literature published on pure algal biomass, using higher feedstock concentration (10-20 wt% solid content) as well as catalysts would improve the EROEI and quality of the bio-crude. Therefore, it would be valuable to determine the effects of biomass concentration and catalyst on yield, composition and energy density of the HTL products of WWT HRAP biomass.

In addition, in the current study all energy analysis was performed based on the lab-scale batch experiments, resulted in the negative EROEI. While at the industrial scale the conversion processes are typically continuous in which cold streams could be pre-heated by hot streams which could improve EROEI. Hence, further experiment and exergy analysis are required on continuous process. Moreover, to further improve economy of the liquid biofuel from WWT HRAP biomass, the beneficial applications of the conversion by-products need to be investigated.

Of course, answering the raised questions would provide more understanding of how to produce low-cost liquid biofuels from WWT HRAP biomass sustainable and economically profitable.

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