Macroalgae as feedstock for the production of liquid biofuels

Thesis submitted by
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Statement on the contribution of others

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Abstract

Policies focused on reducing dependence on foreign oil and on the mitigation of greenhouse gas emissions are driving the development of liquid biofuels. Currently, commercial liquid biofuels are produced from edible crops. However, this method of production represents a major issue for the food supply chain and the extensive use of arable land. Alternative feedstocks such as macroalgae have the potential to increase the production of biomass for biofuels in a sustainable way, without impacting on the food supply chain or the use of arable land. This thesis investigates the potential of this alternative feedstock for the production of high-energy liquid fuels such as biocrude and biodiesel.

In chapter 2, the biomass production and biochemical properties of six marine and freshwater species of green macroalgae cultivated in outdoor conditions were evaluated to assess the potential conversion to high-energy liquid biofuels, specifically biocrude and biodiesel, and the value of these products. Biomass productivities were typically 2-times higher for marine macroalgae (8.5 - 11.9 $g/m^2/d$) than for freshwater macroalgae (3.4 - 5.1 $g/m^2/d$) based on dry weight. The biochemical compositions of the species were also distinct, with higher ash content (25.5 - 36.6%) in marine macroalgae and higher calorific value (15.8 - 16.4 MJ/kg) in freshwater macroalgae. Lipid content was highest for freshwater *Oedogonium* and marine *Derbesia*. Lipids are a critical organic component for biocrude production by hydrothermal liquefaction and the theoretical biocrude yield was

therefore highest for *Oedogonium* (17.7%) and *Derbesia* (16.2%) based on dry weight. Theoretical biocrude yields were also higher than biodiesel yields for all species due to the conversion of the whole organic component of biomass, including the predominant carbohydrate fraction. However, all marine species had higher biomass productivities and therefore had higher projected biocrude productivities than freshwater species, up to 7.1 t/ha/yr of biocrude for *Derbesia*. The projected value of the six macroalgae was increased by 45 - 77% (up to US$7,700/ha/yr) through the sequential extraction of protein and the subsequent conversion of the residual biomass to biocrude. This chapter highlights the importance of optimising biomass productivities for high-energy fuels and targeting additional co-products to increase value.

In Chapter 3, the six species of marine and freshwater green macroalgae were converted to biocrude through hydrothermal liquefaction in a batch reactor. The influence of the biochemical composition of biomass on biocrude yield and composition was assessed. The freshwater macroalgae *Oedogonium* afforded the highest biocrude yield of all six species at 26.2%, based on dry weight. *Derbesia* (19.7%) produced the highest biocrude yield for the marine species followed by *Ulva* (18.7%). In contrast to significantly different yields across species, the elemental profiles of the biocrudes were remarkably similar with higher heating values of 33 - 34 MJ/kg. Biocrude productivity was highest for marine *Derbesia* (2.4 g/m^2/d) and *Ulva* (2.1 g/m^2/d), and for freshwater *Oedogonium* (1.3 g/m^2/d). These species were therefore identified as suitable feedstocks for scale-up and further HTL studies based on biocrude productivity, as a function of biomass productivity and the yield of biomass conversion to biocrude.

In Chapter 4, the three species of macroalgae selected in Chapter 3 were treated with the aim of reducing nitrogen, sulfur and ash within the biomass prior to hydrothermal liquefaction, as high ash, nitrogen and sulfur contents in biomass were identified in the previous chapter as major issues to both the processing of macroalgae and obtaining a biocrude of high quality. The treatments were the nutrient starvation of cultures and post-harvest washing of biomass in freshwater. Subsequently, hydrothermal liquefaction of macroalgae was carried out in a batch reactor heated for 8 minutes with a maximum temperature of 345 ^{o}C. Nutrient starvation effectively reduced nitrogen and sulfur levels within the biomass, which led to a reduction in nitrogen by 51 - 59% and sulfur by 64 - 88% within the biocrude, based on dry weight. The yield of biocrude was highest for *Derbesia* at 38.6 - 41.7% ash-free dry weight and *Oedogonium* at 35.6 - 38.8% ash-free dry-weight when not starved, but was reduced by up to 19% when the biomass was starved. The washing of biomass consistently reduced the ash content for all species by 7 - 83%. The removal of ash affected neither the quality nor the quantity of biocrude produced. The two treatments demonstrate that macroalgal biomass can be effectively manipulated in the production process to modify the composition of the feedstock and, consequently, improve the quality of biocrude. Additionally, reducing the ash content of biomass minimises its potential impact on HTL processing equipment.

Finally, Chapter 5 assessed the suitability of three municipal wastewater sources at various exchange rates for the cultivation of the selected freshwater macroalga *Oedogonium* sp., demonstrating that the delivery of high dissolved inorganic nutrient loads with low

exchange rates of primary treated effluent (5%/d) supported high biomass productivity. A continuous culture of *Oedogonium* in a pilot-scale pond system yielded biomass productivities of 7 - 10 g/m^2/d based on dry weight and nutrient removal rates of 0.50 g N/m^2/d and 0.11 g P/m^2/d. Chemical oxygen demand, microbes and metals were also reduced in the treated water. The biomass produced had a relatively consistent biochemical composition that would yield 26 - 27% of the dry weight as biocrude through hydrothermal liquefaction. The results demonstrate that freshwater macroalgae can be used to treat multiple components of municipal wastewater and simultaneously deliver biomass that can be converted to biocrude for the production of drop-in fuels.

In summary, the research presented throughout this thesis describes the potential of macroalgae as an alternative feedstock for the production of liquid biofuels such as biocrude. Additionally, this thesis demonstrates the suitability of wastewater as a source of water and nutrients for the mass production of freshwater macroalgae.

Abbreviations

A− = washed

A+ = not washed

AA = amino acids

AAC = Advanced Analytical Centre

AD = anaerobic digestion

afdw = ash-free dry weight

AMCRC = Advanced Manufacturing Cooperative Research Centre

ANOVA = analysis of variance

APAF = Australian Proteome Analysis Facility

Aq. = aqueous

ARENA = Australian Renewable Energy Agency

atm = atmosphere

ATS = algal turf scrubber

BC = biocrude

BOD = biochemical oxygen demand

CARB = carbohydrate

Chaet. = Chaetomorpha linum

CHG = catalytic hydrothermal gasification

Clad. (freshwater) = *Cladophora vagabunda*

Clad. (marine) = *Cladophora coelothrix*

COD = chemical oxygen demand

DAF = effluent from the dissolved air flotation unit

DCM = dichloromethane

Derb. = Derbesia tenuissima

DIN = dissolved inorganic nitrogen

DIP = dissolved inorganic phosphorus

DMSP = dimethylsulfopropionate

dw = dry weight

E.coli = *Escherichia coli*

EIA = Energy Information Administration

Eq. = equation

ER = energy recovery

FA = fatty acids

FAME = fatty acid methyl esters

FAO = Food and Agriculture Organization

FRP = filterable reactive phosphorus

fw = fresh weight

FW = freshwater

GHG = greenhouse gas

HHV = higher heating value

HPC = heterotrophic plate count

HRAP = high rate algal pond

HSD = honestly significant different

HTL = hydrothermal liquefaction

HV = hand valve

IEA = International Energy Agency

JCU = James Cook University

LIP = lipid

M = marine

MARFU = Marine Aquaculture Research Facility Unit

met:lys = methionine:lysine ratio

MUFA = monounsaturated fatty acids

N− = starved

N+ = not starved

NOx = nitrogen oxides

NR = nutrient removal

NRV = non-return valve

OEA = Organic Elemental Analysis

Oedog. = Oedogonium sp.

P = productivity

P.aeruginosa = Pseudomonas aeruginosa

PAR = photosynthetically active radiation

PE = protein extract

PI = pressure indicator

PR = pressure regulator

PRIM = primary treated effluent

PROT = protein

PRV = pressure relief valve

PUFA = polyunsaturated fatty acids

S = surface

SE = standard error

SEC = secondary treated effluent

SFA = saturated fatty acids

t = time

TI = temperature indicator

TIC = total inorganic carbon

TN = total nitrogen

TOC = total organic carbon

TP = total phosphorus

TSS = total suspended solids

Ulva = Ulva ohnoi

V = value

W = weight

W_f = final weight

W_i = initial weight

WTI = Western Texas Intermediate

Y = yield

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Chapter 1

General introduction

1.1. The demand for energy and biofuels

Energy is a fundamental resource that is directly linked to well-being and prosperity across the globe. With a continuously growing population and improvements in the quality of life, the global demand for energy is projected to increase at a steady pace in the next decades. In 2013 the global consumption of energy increased by 2.3% with fossil fuels representing more than 80% of total energy consumption (IEA Report, 2013; BP Statistical Review, 2014). Of these fossil fuels, oil products remain the most important final energy commodity comprising 31.5% of total energy in 2011 driven by their use in transport (IEA Report, 2013). Given improvements in oil and gas extraction technologies, potential reserves, and the increased exploitation of unconventional reserves such as shale oil, it is probable that fossils fuels will continue to be available at reasonably low cost for the next ten to twenty years (Brennan & Owende, 2010; EIA Report, 2012). However, fuel reserves are unevenly distributed around the globe and repeated fuel crises arising from geopolitical conflicts are compelling governments to develop independent sources of energy to improve domestic fuel security. Additionally, burning large quantities of fossil fuels has driven unprecedented greenhouse gas (GHG) emissions on a global scale and, as a consequence, compatible mitigation strategies are required to neutralize the excess carbon dioxide (CO_2). In 1992, member countries of the United Nations Framework Convention on Climate Change agreed on a common objective of “stabilising greenhouse gas concentrations in the

atmosphere at a level that would stop dangerous anthropogenic interference with the climate system". It is increasingly recognized that there is not a single solution to this complex problem and that combined actions are needed, including the more efficient use of fossil fuels, changes in vehicle technologies, expansion of public transport and the introduction of innovative fuels and technologies (The Royal Society, 2008).

In the last two decades, the use of liquid biofuels in the global transport sector has grown rapidly, driven mostly by policies focused on the reduction of dependence on foreign oil and the mitigation of GHG emissions. Bioenergy crops can reduce or offset GHG emissions by directly removing CO_2 from the atmosphere as they grow and store carbon in biomolecules. Fuels from bioenergy crops are thus considered emission-neutral when burnt. First generation biofuels obtained from biomass that are generally food crops have reached commercial levels of production and consist mainly of fuel ethanol produced from corn-starch in the United States and sugarcane in Brazil (Lee & Lavoie, 2013). The remainder of current liquid biofuel production is mostly biodiesel produced from vegetable oils from rapeseed and soybean in Europe and the United States. The global production of biofuels reached 10% of the world's total primary energy supply in 2011 and it is projected that the share of biofuel will continue to grow (IEA Report, 2013). Despite these potential benefits, further development of first generation biofuels will remain limited due to the competition for arable land with food and fibre crops, increased land requirements and the related destruction of natural habitats, the security and quality of the food chain, and high water and fertiliser requirements (FAO Report, 2008). Therefore, depending on the method used to produce the feedstock and process the fuel, some crops can generate more GHG

than fossil fuels (BioGrace Report, 2012). For example nitrous oxide, a GHG with a global warming potential around 300 times greater than that of CO_2, is released from nitrogen fertilisers (Ravishankara et al., 2009). Greenhouse gases can also be emitted by direct or indirect land-use changes related to increased biofuel production, for example, when carbon stored in forests or grasslands is released from the soil during land conversion to crop production (Fargione et al., 2008; Searchinger et al., 2008). In contrast, second generation biofuels that are produced from non-food materials (such as lignocellulosic biomass), animal or plant wastes and organic residues are regarded as more sustainable alternatives to fossil fuels and conventional – first generation – biofuels (Cherubini, 2010). Although significant progress continues to be made to overcome technical and economic challenges, second generation biofuels face major constraints to their commercial deployment such as relatively immature processing technologies and competition for land use (IEA Report, 2009). To produce a sustainable biofuel resource, feedstocks must comply with a number of conditions including (1) competitiveness with the cost of petroleum fuels, (2) mitigation of GHG emissions, (3) nil or low usage of arable land and (4) minimal use of water and fertilisers (McKendry, 2002a; Brennan & Owende, 2010). Third generation biofuels produced from algal biomass, including microalgae, cyanobacteria and macroalgae, could meet these conditions and therefore make a contribution to meeting primary energy demands, while simultaneously providing environmental benefits (Wang et al., 2008). The use of algae as a bioenergy feedstock has many advantages as they have high yields of biomass per hectare and are a homogenous feedstock that can be harvested year-round, providing a reliable and continuous supply of biomass. Furthermore, the production of algae can utilise wastewater streams thereby

greatly reducing the requirements for water and nutrients, and can be coupled with intensive carbon emitting industries such as power generation from fossil fuels for the direct sequestration of CO_2 emissions (Schenk et al., 2008).

However, despite all of these advantages, algae are not yet viably cultivated at scale for the production of biofuel as the costs of production are not competitive with fossil fuels. Consequently, there has been an unprecedented interest in the research field of algal biofuels to close the gap between technical challenges and commercialisation (Foley et al., 2011; Coelho et al., 2014). Research has focused on all aspects of algae-to-biofuel processes including the search for highly productive species of algae, innovative and cost-effective culture systems, integration with wastewater streams, harvesting methods and conversion pathways to liquid biofuels. The immense taxonomic diversity of algae that have evolved over billions of years provides a genetic pool that is orders of magnitude larger than that of land plants (Georgianna & Mayfield, 2012). This potential pool is reflected in the diversity of algal species being explored for fuel production, ranging from cyanobacteria and microalgae to macroalgae, from both freshwater and marine environments. To date, the selection of species of algae for the production of biofuels has for the most part focused on microscopic unicellular algae (~5 - 100 μm in diameter, i.e. microalgae and cyanobacteria) due to the high yield and lipid content of a number of species within these groups (Chisti, 2007; Brennan & Owende, 2010; Borowitzka, 2013; Liu et al., 2013a). However, it is recognized that there are major hurdles in the development of microalgal biofuels due to significant technological challenges in the cultivation, harvesting and dewatering of microalgal biomass (Georgianna & Mayfield,

2012). An alternative biomass resource for the production of biofuels and bioproducts that has to a large part been overlooked is macroalgae. Macroscopic multicellular algae, or macroalgae, are larger in size (> 100 μm in length) and represent an abundant resource that remains to be developed for biomass production. Importantly, macroalgae have a number of key advantages as a feedstock, providing the potential to increase the viability of algal biofuels.

1.2. Macroalgae as feedstock for liquid biofuels: resources assessment

1.2.1. Overview of macroalgae

Macroalgae are a very large group of eukaryotic, multicellular organisms that can be found in virtually all aquatic environments. The distribution of macroalgae is worldwide with more than 10,000 identified species (AlgaeBase; Canadian Museum of Nature). Although most species of macroalgae live in marine environments – commonly known as seaweeds – there are also large numbers originating from brackish and freshwater environments (i.e. not all macroalgae are seaweeds). Macroalgae are generally classified as Phaeophyceae or brown algae, Rhodophyta or red algae, and Chlorophyta or green algae, based on their composition of photosynthetic pigments. Brown macroalgae belong to the monophyletic group of heterokonts (or stramenopiles) and share numerous hereditary traits with the unicellular microalgae diatoms. Red macroalgae are more closely related to green macroalgae and are differentiated by simple chloroplasts and floridean starch as the primary carbohydrate reserve (Adl et al., 2005). In contrast, green macroalgae use starch as the primary carbohydrate reserve and have evolutionary and biochemical similarities with higher plants. The life cycles of macroalgae are complex and diverse, with variations in

annual and perennial life histories, combinations of sexual and asexual reproductive strategies, and alternation of generations (Roesijadi et al., 2010). This diversity within macroalgae offers the potential to select highly productive and robust species for the production of biomass as a feedstock for biofuels. At present, there is no commercial exploitation of macroalgae for the production of biofuels, however, the possibility of harvesting wild stocks, or of utilising the existing mariculture industry and the development of intensive land-based cultivation systems, provides a range of options for the sustainable mass production of macroalgae.

1.2.2. Harvesting wild stocks

Wild stocks of freshwater and marine macroalgae represent a significant natural resource for the capture and conversion of solar energy into biomass. However, the exploitation of benthic macroalgae such as kelps is considered to be unsustainable due to the consequences of continuous harvesting on biodiversity and ecosystem services (Benson et al., 2014). In contrast, the exploitation of free-floating blooms of macroalgae is considered to have a low impact on aquatic ecosystems (Titlyanov & Titlyanova, 2010). Temporary free-floating algal blooms (green, red or brown tides) result from a build-up of nutrients in natural waters, which rapidly leads to large quantities of macroalgae that can be harvested and converted to bioenergy (Savage, 2011). Macroalgal blooms occur in freshwater and in marine environments and are typically a result of anthropogenic activity (Smetacek & Zingone, 2013). The most dramatic occurrence of macroalgal blooms is in the Yellow Sea, China, where the phenomenon has become an annual occurrence along the coast of Qingdao. For example, more than a million tonnes of the green macroalga *Ulva prolifera*

(formerly *Enteromorpha prolifera*, Hayden et al., 2003) were removed from the beach in the cleanup of the green tide prior to the Olympics in 2008 (Keesing et al., 2011; Liu et al., 2013b). Analysis of satellite images of the green tide show that a mosaic of macroalgae was spread over 84,000 km^2 (Liu et al., 2013b) highlighting both its abundance and the complexity of harvesting it as a resource.

1.2.3. Mariculture

Mariculture involves the cultivation and harvest of marine organisms and is currently the major source of macroalgae providing more than 90% of production, compared to 10% from the harvest of wild stocks (Roesijadi et al., 2010; Paul & Tseng, 2012). The majority of the aquaculture production of macroalgae utilises brown and red seaweeds from the genera *Saccharina* (formerly *Laminaria*, Lane et al., 2006), *Undaria* and *Pyropia* (formerly *Porphyra*, Sutherland et al., 2011) for human food products. Other mass-produced species of macroalgae are brown and red seaweeds from the genera *Macrocystis* and *Saccharina* (alginate), *Gelidium* and *Gracilaria* (agar), and *Kappaphycus* and *Eucheuma* (carrageenans) for hydrocolloids. The global production of farmed seaweeds reached approximately 20 million tons in 2012 with a value exceeding 6 billion US dollars, and the sector continues to grow (Paul & Tseng, 2012; FAO Report, 2014). Commercial production is mainly carried out on the seabed, on ropes or on nets in the open sea, near-shore coastal sites, and closed bays or lagoons. The biomass is traditionally harvested by hand at low tide (Crawford, 2002). Fragments of adult plants, juvenile plants or spores are seeded onto ropes or nets and the seaweeds grow to maturity in the sea. Knowledge of the life history is critical for most species and on-land cultivation of specific life history phases

is often necessary for seeding. The productivity of farmed seaweeds is reportedly high, up to 120 - 150 t/ha/yr fresh weight (Gao & McKinley, 1994; Lüning & Pang, 2003; Titlyanov & Titlyanova, 2010), however, mariculture is limited to the production of high-value commodities due to the economics of production. Given the scale of production, however, large quantities of biomass wastes are produced after the extraction of high-value chemicals from brown and red seaweeds. These wastes are typically rich in carbohydrates and can be converted to biofuels (Adams et al., 2009; Rothe et al., 2012; Wei et al., 2013). For example, the waste products from the extraction of rhizoidal filaments from the red seaweed *Gelidium* for the paper industry (Seo et al., 2010), or from the extraction of ulvans from the green seaweed *Ulva* (Lahaye & Ray, 1996), have been used for the research-scale production of ethanol and methane. Farmed brown seaweeds from the genus *Saccharina* are also regarded as a possible feedstock for enzymatic conversion into ethanol after the extraction of high-value chemicals (i.e. mannitol, fucoidans) due to their high growth rate and high carbohydrate content (Kraan, 2013). Currently, mariculture of seaweeds in Asia is a relatively low-technology business where attached plants are placed in the sea and there is a high labour component for these operations (Crawford, 2002). New approaches for the mass culture of brown seaweeds in particular were developed in the United States in the 1980s for the production of biogas, but the program did not achieve any significant results in either near-shore or off-shore ocean cultivation (Huesemann et al., 2010). However, given the development of macroalgal biomass as a food, and as a renewable resource for the production of bio-chemicals (Jung et al., 2013; van Hal et al., 2014) and biofuels (Kraan et al., 2013; Coelho et al., 2014), there is a surge in new research programs for the development of the high-technology off-shore mass production of seaweeds (Kelly &

Dworjanyn, 2008; Seafarm Project in Sweden; Algae for Biogas Project in Denmark; SeaBioGha Project in Ghana).

1.2.4. Land-based intensive aquaculture

The mass production of macroalgae can also occur in intensive land-based systems, which provide a high degree of control over culture parameters to maximise biomass productivity while minimising land requirements. Factors to be considered for the selection of culture systems include climate, the efficiency of light utilisation, the ability to regulate temperature and pH, energy and water requirements, the hydrodynamic stress placed on the algae and the ease of construction on a large scale at a low cost (Borowitzka, 1999). The final choice of system is almost always a compromise between all of these considerations to achieve an economically acceptable outcome. Additionally, the possibility of integrating land-based culture of macroalgae with wastewater streams provides a lower-cost source of water and nutrients in addition to the benefits of bioremediation that can increase the viability of the process. The main types of intensive culture systems with the potential to achieve the large-scale production of macroalgal biomass and biofuel are open tanks and ponds, high-rate algal ponds (HRAP) and algal turf scrubbers (ATS).

1.2.4.1. Open tanks and ponds

Cultivation in open tanks and ponds is considered a high-technology method of production, however, the fundamental principles of these systems are simple. Both systems work on the same principle, with the exception that tanks are generally positioned above ground and open ponds dug into the ground. These systems consist of a reservoir of water, typically

with an influent, an effluent, and a mixing device. Light attenuation increases with depth, therefore the use of an efficient mixing device that alternately exposes individual fragments of macroalgae to sunlight allow for deeper cultures, reducing the surface area of cultivation and land requirements. In addition, mixing provides additional CO_2 to the algae through the partial dissolution of gases into water, with the positive effect of reducing water pH. Macroalgae can be cultured all year-round in intensive land-based systems providing for high yields of biomass up to 40 $g/m^2/d$ dry weight (dw) for the red seaweed *Gracilaria ferox* over a period of 32 months (Capo et al., 1999) and over 70 $g/m^2/d$ (dw – summer values) for the red seaweed *Asparagopsis armata* and green seaweed *Ulva rigida* (Mata et al., 2010a). However, the cost of construction of open tanks and ponds combined with the energy required for mixing can be prohibitive for cost efficient scale-up of this technology (Lundquist, 2010; Benemann, 2013).

1.2.4.2. High rate algal ponds

High rate algal ponds (HRAPs) have been developed as the most productive and cost-effective method for the production of microalgae (Benemann, 2013) but with limited application to the production of marine and freshwater macroalgae. HRAPs are relatively shallow, mechanically mixed, raceway-type open ponds that were initially developed for the treatment of wastewater (Oswald & Golueke, 1960) and have become the major commercial scale system for the production of microalgae for pigments and biofuels (e.g. Sapphire Energy Inc., USA; Cyanotech Corp., USA; Cellana, USA). HRAPs can be well over one hectare in size (Weissman et al., 1998) and water depth varies between 0.25 and 0.6 m to allow efficient light utilisation and reduce water turbidity, depending on the water

source (Craggs et al., 2012a). Water mixing is performed by paddlewheels to reach typical velocities of 0.15 to 0.30 m/s. Although HRAPs have been almost exclusively used for the culture of microalgae, they have been used successfully for the commercial production of the marine seaweed *Ulva* in South Africa (Bolton et al. 2009, Nobre et al. 2010) and Australia (MBD Energy Ltd.). In contrast to tank and conventional open pond systems, HRAPs can be operated at relatively low cost and scaled-up with relatively high productivities (Benemann, 2013). However, the control of water temperature and pH (by CO_2 addition) is difficult with this technology and the selection of suitable macroalgal species will be critical in achieving desirable productivities (Lundquist et al., 2010).

1.2.4.3. Algal turf scrubbers

Algal turf scrubbers (ATS) are intensive land-based culture systems originally developed as a low-cost, environmentally-compatible alternative to conventional technologies for water quality management and for the production of algal biomass (Adey & Hackney, 1989; Adey & Loveland, 2007). In ATS, water is periodically added to a shallow raceway (typically 1 - 3 cm deep) creating a wave surge that prevents the development of boundary layers which limit nutrient and metabolite exchange, and prevents light-shielding of the interior portions of the algal turf (Adey & Loveland, 2007). The wave surge flows down the raceway providing nutrients to the bed of benthic algae attached on nylon mesh (Mulbry & Wilkie, 2001). Nutrients are removed by the algae for growth and the water is collected at the end of the raceway with a lower concentration of nutrients. Algae, generally filamentous green macroalgae from the genera *Rhizoclonium*, *Microspora*, *Ulothrix*, and *Oedogonium* (freshwater), or *Ulva* (marine), are harvested partially and

frequently to stimulate rejuvenation, which leads to high sustainable production. Biomass production rates of up to 25 g/m^2/d dw in pilot-scale ATS treating wastewater are among the highest recorded values for low-cost managed ecosystems (Mulbry et al., 2008).

In addition, the development of intensive land-based culture systems such as open tanks, ponds, HRAP and ATS provides the potential to integrate the production of algal biomass with waste streams where algae provide bioremediation benefits, in particular the removal of CO_2, nitrogen, phosphorous and other contaminants. This integrated approach provides water and nutrients at lower cost for the production of algal biomass.

1.2.5. Integration with waste streams

The mass cultivation of algae requires large quantities of nutrients to maintain high productivity, of which carbon, nitrogen and phosphorus are often the first elements that become limiting (Christenson & Sims, 2011). Carbon dioxide and bicarbonate are the two forms of dissolved inorganic carbon that can be assimilated by autotrophic algae. High rates of photosynthesis, indicated by high water pH (> 10), typically decrease the availability of carbon to the algae, which in turn decreases biomass productivity (Craggs et al., 2012a). The addition of CO_2 to the cultures increases carbon availability and enables pH to be maintained at an optimum of 7.5 - 8.5. Different waste streams can be used to add CO_2 to the cultures, the most likely being flue gas from electricity generation, for example, collected from anaerobic digesters (Benemann, 2003) or coal-fired power stations (Cole et al., 2013; Roberts et al., 2015). Other important elements for algal metabolism such as nitrogen and phosphorus are also limited resources. For example, the process of converting

nitrogen gas into ammonia fertiliser, the Haber process, is energy intensive and currently consumes 1% of the world's energy supply (Sode et al., 2013). The extraction of phosphorus from phosphate rocks is also energy intensive and efforts are now focusing on recycling these resources rather than discharging them to the environment. Nitrogen and phosphorus as well as trace elements essential to algal growth are often present in significant quantities in wastewater streams. Therefore, wastewaters derived from municipal, agricultural, and industrial activities represent cost-effective and sustainable sources of nutrients and water for the mass production of macroalgae (Pittman et al., 2011). The concept of growing algae directly in wastewater was first suggested in the context of treating wastewater for nutrient and metal removal (Oswald & Golueke, 1960). The concentration of nitrogen can reach 100 mg/L in municipal wastewater and over 1000 mg/L in agricultural wastewater (Pittman et al., 2011), while industrial wastewater typically accumulates very high concentrations of heavy metals (Roberts et al., 2013a). However, the ability of some algal species to grow in these extreme environments makes them an efficient means to recover nutrients and contaminants from wastewater, while providing biomass for the production of biofuels.

A wide variety of wastewater sources have been used to grow macroalgae including effluents from municipal treatment facilities (Sode et al., 2013), agriculture and aquaculture (Bolton et al., 2009; Cole et al., 2014), and industrial energy generation (Saunders et al., 2012; Roberts et al., 2013a; Roberts et al., 2015). However, the selection of reliable species of algae and the land required to treat the totality of wastewater generated by these facilities are often limiting factors for the integration of algal treatment.

The selection of reliable species of algae depends on factors such as productivity, the biology of the alga, nutrient requirements, the ability to remain the dominant species in culture, the tolerance to environmental fluctuations, and biochemical properties suited to the production of biofuels. This necessitates the careful study and selection of robust macroalgae species with high growth rates, but more importantly, adapted to these artificial culture conditions. To make a substantial contribution to biofuels production however, considerable scale-up from current activities is necessary, which involves research into the selection of robust species, including genetic improvement, as well as the development of cultivation methods, harvesting techniques, pre-treatment of biomass and conversion processes to biofuels (Friedlander, 2008).

1.3. Macroalgae as feedstock for liquid biofuels: conversion processes

Macroalgal biomass can be converted into biofuels using a number a processes. The choice for the type of conversion process is generally based on a combination of factors including the type, biochemical properties and quantity of source biomass, the desired form of fuel (i.e. end-use requirements), and economic and environmental considerations (McKendry, 2002b). Conversion processes can be categorised as: (1) biological – for fermentation and anaerobic digestion processes; (2) physico-chemical – including press, solvent and supercritical CO_2 extraction of lipids for subsequent transesterification; and (3) thermochemical – including direct combustion, pyrolysis, hydrothermal liquefaction and gasification. Among all these processes, fermentation, transesterification, and

hydrothermal liquefaction yield primarily liquid fuel products (Fig. 1.1) which are typically easy to store and can be used in most modes of transport.

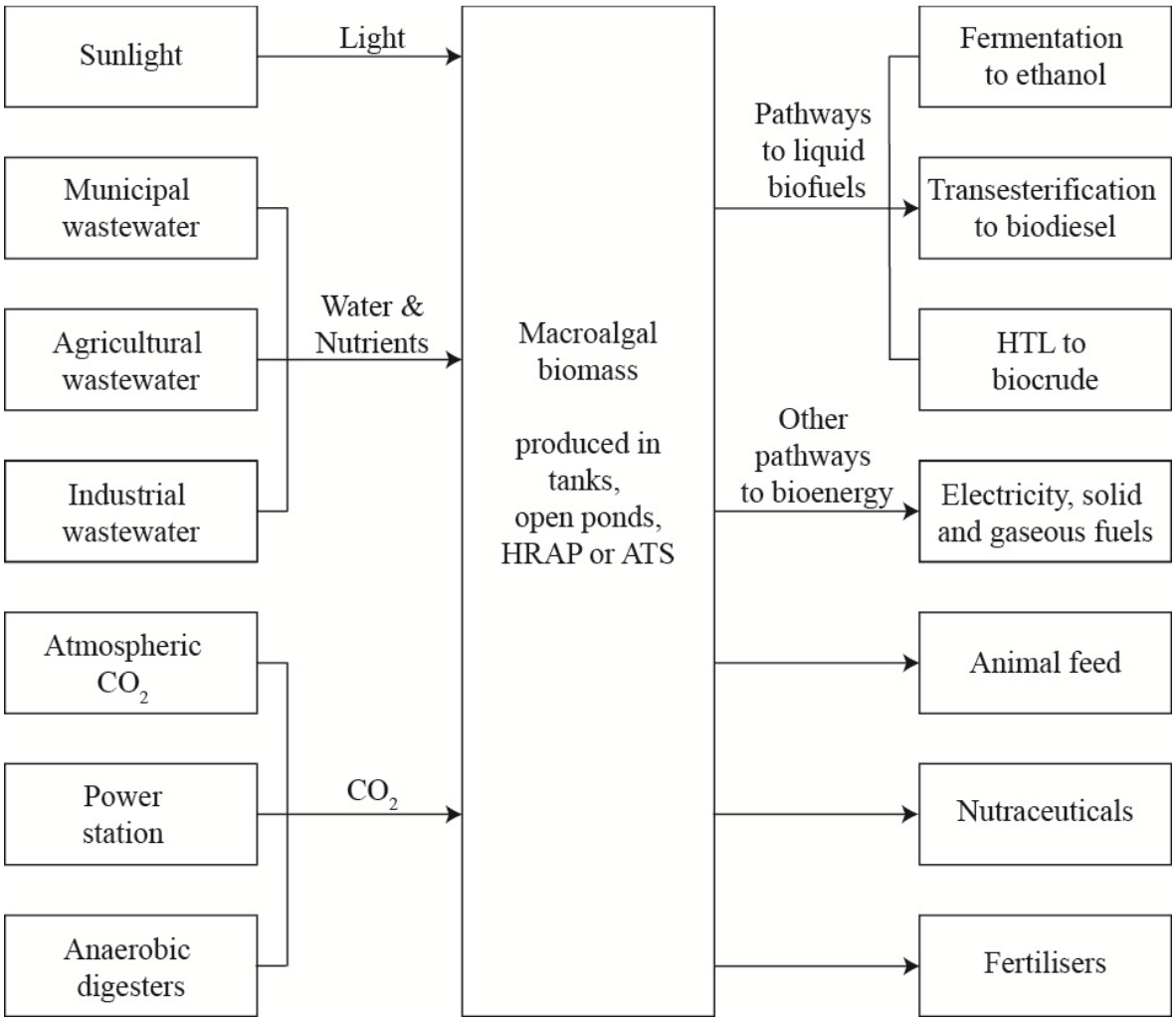

Figure 1.1. Process schematic of the integration of the culture of macroalgae with waste streams for the production of liquid biofuels and other potential applications.

1.3.1. Fermentation to ethanol

Complex structural and storage carbohydrates (sugars) such as cellulose and starch in macroalgae can be converted to ethanol through fermentation (John et al., 2011). These complex sugars are typically broken down to fermentable sugars using acid or enzymatic

hydrolysis and then fermented in the presence of yeast (e.g. *Saccharomyces cerevisiae*) to produce ethanol (Demirbas, 2005). Distillation of the diluted alcohol products (10 - 15% ethanol) is necessary to obtain a concentrated ethanol fuel (typically 95% by volume after one distillation) that can be used pure or in blend with gasoline in cars (Brennan & Owende, 2010). The use of ethanol in blends with gasoline (e.g. E10 - E95) has the benefit of decreasing the consumption of petroleum fuel, increasing the octane of gasoline to which it is added (thereby improving the performance of the fuel blend), and providing oxygen for more complete combustion results (Wyman, 1994). Additionally, the co-products of the fermentation process can be used for animal-feed or gasification to increase the viability of the process (McKendry, 2002b).

Macroalgae are an attractive feedstock for the production of ethanol via fermentation as they do not have complex structural biopolymers such as hemicellulose or lignin, which eliminates some of the mechanical, chemical or enzymatic pre-treatment steps required to break down these biopolymers into fermentable sugars (John et al., 2011). Macroalgae generally have a high carbohydrate content in the form of laminarin and mannitol for brown macroalgae, glucan and galactan for red macroalgae and cellulose and starch for green macroalgae. Brown macroalgae from the genus *Saccharina* can accumulate over 50% of the dry weight as the sugars laminarin and mannitol (Adams et al., 2009), which have been converted to ethanol with a yield of 0.38 g ethanol/g sugars after acid hydrolysis (Kim et al., 2011). This yield is 75% of that of ethanol produced from simple sugars like glucose, fructose, sucrose or xylose in corn and sugarcane (0.51 g ethanol/g of sugars based on the stoichiometric biochemistry of yeast) (Kim & Day, 2011). Similarly, sulfated galactans from the red macroalga *Kappaphycus alvarezii* have been converted to ethanol

with a yield of 0.27 g ethanol/g sugars after pre-treatment by methanolysis (Li et al., 2014). The wild-harvested green macroalga *Ulva lactuca* has also been identified as a suitable feedstock for the production of ethanol, butanol, and acetone through fermentation with yields of 0.35 g products/g sugars consumed (van der Wal et al., 2013). However, further development is needed to close the technical gaps in the cultivation of macroalgae and the conversion of this unique feedstock into ethanol, including the search for new enzymes and yeasts, and improvements in the dark fermentation process (John et al., 2011; Daroch et al., 2013).

1.3.2. Transesterification to biodiesel

The lipid fraction of macroalgae can be extracted as oil and converted to biodiesel through transesterification, which involves the reaction of the triglycerides with an alcohol (methanol) to produce fatty acid methyl esters – or biodiesel – and glycerol (Chisti, 2007). In industrial processes, alcohol is added in excess to ensure methyl esters are formed with a high yield of 98% and acid and alkali catalysts are used to speed up the reaction (Fukuda et al., 2001). While microalgal feedstocks have been attracting attention for the production of biodiesel due to their high lipid content (Mata et al., 2010b), few macroalgae have been considered as feedstock. However, the lipid and fatty acid contents of macroalgae can reach values of 15% and 6% of the dry weight, respectively, which makes macroalgal biomass a technically suitable feedstock for the production of biodiesel (Gosch et al., 2012). Brown and green marine macroalgae including *Fucus*, *Ascophyllum*, *Ulva* and *Pelvetia* collected on Galician beaches in Spain (Maceiras et al., 2011), and freshwater macroalgae from the genera *Oedogonium* and *Spirogyra* (Hossain et al., 2008), have been

successfully converted to biodiesel, although with yields below 10% based on dry weight. However, biodiesel production requires the use of dried biomass to prevent undesirable reactions during transesterification. This drying process and the effective extraction of oils prior to transesterification are significant limitations as they are energy intensive and increase the cost of production of biodiesel.

1.3.3. Hydrothermal liquefaction to biocrude

Hydrothermal liquefaction or HTL is a thermochemical process that involves the chemical and physical transformation of biomass into bio-crude oil (or biocrude) in high-temperature and high-pressure liquid water (Peterson et al., 2008). HTL simulates the natural geological processes involved in the formation of fossil fuel, but in the time scale of hours or even minutes (Patil et al., 2008). Compared to the fermentation or transesterification of biomass that use only fermentable sugars or triglycerides, respectively, HTL has the advantage of using the whole organic component of biomass, which generally results in higher yields of biofuel. Additionally, HTL utilises wet biomass – in slurries with typical concentrations of dry solids of 5 to 30% – to feed the reactor, thereby offering a significant advantage in the case of algae as no drying of biomass is required (Toor et al., 2011; Biller & Ross, 2011). During HTL, algal biomass is degraded in hot compressed water at conditions approaching the critical point of water (374^{o}C and 22.1 MPa). The thermochemical decomposition of biomass relies on the unique properties of water at these subcritical conditions, where it acts simultaneously as a solvent, reactant, and both acid and base catalyst, due to its increased auto-ionisation. Elevated temperatures and pressures reduce the density, polarity and dielectric constant of water, resulting in the hydrolysis and dissolution of solid biomass

(Peterson et al., 2008). A complex network of cascading reactions, involving the newly liberated low molecular weight hydrocarbons, leads to the formation of a synthetic biocrude, gases (principally CO_2), water-soluble chemicals and insoluble residues (biochar). Biocrude produced through the HTL of algae has a high energy density (30 - 40 MJ/kg) that is 70 - 95% of that of petroleum (fossil) crude (López Barreiro et al., 2013). The difference in energy density is due to the presence of residual heteroatoms (principally O, N, S) that are typically removed from the biocrude product by hydrotreatment to deliver a range of drop-in fuels and chemicals.

Recent work on the HTL of macroalgal biomass suggests that this feedstock is suitable for the production of biocrude (Zhou et al., 2010; Anastasakis & Ross, 2011; Elliott et al., 2013a; this thesis as published in Neveux et al., 2014a, b, c). The marine macroalga *Ulva prolifera*, an invasive and predominant species during green tides in China, has been successfully converted to biocrude through HTL in a batch reactor (Zhou et al., 2010; Xu et al., 2015). A maximum yield of 35% based on ash-free dry weight was obtained at 370^{o}C with 20% K_2CO_3 catalyst and a reaction time of 60 minutes. Similarly, the brown seaweeds *Saccharina latissima* and *Sargassum patens* harvested at sea have been converted to biocrude with yields of 19% and 32% respectively using comparable operating conditions (340 - 350^{o}C; 15 min; ~10% dry solids) in a batch reactor without catalyst (Anastasakis & Ross, 2011; Li et al., 2012). More recently, researchers at the Pacific Northwest National Laboratory have investigated the conversion of wild-harvested *Saccharina* spp. in a continuous-flow HTL reactor, which has provided data for the scaling-up of this process (Elliott et al., 2013b). Following the reaction at 350^{o}C, nearly

60% of the carbon contained in the biomass feedstock was recovered in the biocrude product. The separation of biocrude and aqueous products was achieved by gravity, which considerably lowers the cost of the recovery of products compared with the use of solvents. Additionally, catalytic hydrothermal gasification (345 - 348^{o}C) of the process water (aqueous product) produced clean water and fuel gas with a conversion efficiency of 99% of the remaining carbon. These results demonstrate the potential of HTL to convert wet macroalgal biomass to biocrude and the importance of recovering all the products of the reaction through simple and cost-efficient methods to improve the viability of macroalgal biofuels.

1.4. Aims and outline

The overarching aim of this thesis is to investigate the potential of macroalgae as a new source of biomass for the production of liquid biofuels. As highlighted, the field of macroalgal biofuels has received little attention to date, and the limited amount of data available describes for the most part the conversion of biomass harvested from the wild (Coelho et al., 2014). However, much of the value proposition of macroalgae lies in the ability to successfully integrate production into land-based bioremediation with the concomitant production of liquid biofuels. Therefore, the objective of this thesis is to provide an initial assessment of the benefits and limitations associated with the cost-effective and sustainable production of macroalgae in intensive land-based culture systems with an emphasis on the integration of the culture of macroalgae with municipal wastewater. Although this work focuses mainly on the biological part of the process, the objective is also to demonstrate the production of biodiesel and biocrude as high-energy

liquid fuels suited for heavy transportation vehicles and aviation. These two high-energy liquid fuels with an energy value > 30 MJ/kg, as opposed to ethanol with a value < 30 MJ/kg, were chosen on the basis of a recent assessment by the Australian Renewable Energy Agency (ARENA Report, 2012). This report highlights the need to develop advanced drop-in renewable heavy-transport, marine and aviation fuels based on sustainable biomass supplies for military and commercial transport. Key issues related to the development of this technology using macroalgae as a biomass feedstock are addressed in the following chapters including the selection of reliable species of macroalgae, the method of culture, the conversion to high-energy liquid fuels, and the integration of the culture of macroalgae with wastewater streams.

In **Chapter 2**, the aim was to determine the best pathway to convert macroalgae into high-energy liquid fuels, based on the projected quantity and value of the biofuel produced. Six species of marine and freshwater green macroalgae were selected for this study from forty species (green, red and brown macroalgae) tested under intensive cultivation outdoors in tanks. Productivities quantified on a unit area basis ($g/m^2/d$, dw) and the biochemical profiles of the macroalgae provided the basis to calculate the potential yield of biodiesel and biocrude from each biomass, and the projected productivity and value of these biofuels. Two conversion pathways were compared in this chapter, the esterification of fatty acids to biodiesel and the hydrothermal liquefaction of the organic component of biomass to biocrude. The potential of extracting protein prior to converting the residual biomass to biocrude was also examined as an option to add value to the process. Finally, a

sensitivity analysis was used to evaluate the relative influence of each production parameter on the potential value of feedstocks.

In **Chapter 3**, the aim was to further investigate the conversion of the six macroalgae to biocrude using hydrothermal liquefaction based on the conversion process selected in the previous chapter (Chapter 2). The objective was to provide a direct comparison of the yield, elemental composition, and productivity of biocrudes, in order to select the best species among the six macroalgae tested. Feedstocks were converted through hydrothermal liquefaction in a bench-scale batch reactor. The operating conditions to process the feedstock slurries – specifically 330 - 341^{o}C for 5 minutes and a concentration of dry solids of 6.6% – were selected based on the literature and preliminary trials carried out in the laboratory. The condensed products of the reaction – biocrude, aqueous product and biochar – were quantified after separation and the yield of biocrude was multiplied with biomass productivity to determine biocrude productivity per unit area per unit of time (g of biocrude produced/m^2/d) for each species of macroalgae.

HTL was identified as an efficient conversion pathway in the previous chapters, however, high ash, nitrogen and sulfur contents in biomass were identified as major issues to both the processing of macroalgae and obtaining a biocrude of high quality.

Therefore, the aim of **Chapter 4** was to assess if the manipulation of freshwater and marine macroalgae through nutrient starvation and the post-harvest washing of biomass could reduce the content of ash, nitrogen and sulfur prior to HTL processing, and whether

these changes could be carried through the conversion process affording a desirable biocrude product. This chapter evaluated the effects of starvation and washing on the composition of the three species of macroalgae selected in Chapter 3 (*Derbesia tenuissima*, *Ulva ohnoi* and *Oedogonium* sp.). Subsequently, the yield and elemental composition of biocrude and HTL co-products produced from macroalgae subjected to combinations of starvation and washing were assessed. Finally, the variation in the content of carbon in each of the treated algal feedstocks was correlated with the yield of biocrude.

Important aspects of the production process were resolved in the previous chapters (Chapters 2 - 4) including the selection of the most promising conversion pathway, the most reliable species, and the manipulation of biomass in culture and post-harvest to improve the quality of feedstocks and the conversion to biocrude. However, it is increasingly recognised that the cost-efficient and sustainable large-scale cultivation of macroalgae for the production of biofuel will rely on the integration of biomass production in wastewater streams, as a source of water and nutrients.

Therefore, the aim of **Chapter 5** was to investigate the possibility of growing the freshwater macroalga *Oedogonium* sp. in municipal wastewater for simultaneous nutrient removal and biomass production. This chapter evaluated the suitability of three wastewater sources – effluents from the primary and secondary clarifiers and underflow effluent from the dissolved air flotation unit – at different exchange rates to support macroalgal growth in small-scale culture trials, and analysed how the difference in water quality influences the biochemical composition of the algae. The most effective wastewater source and exchange

rate were subsequently investigated in pilot-scale cultures of *Oedogonium* in open ponds. Finally, the nutrient removal rates and the potential yield and scale of biocrude production using macroalgae and municipal wastewater were calculated for the open pond system.

In **Chapter 6** the results of the previous chapters are synthesised and discussed to provide a holistic overview of the potential of macroalgae for the production of liquid biofuels and biocrude in particular. The main knowledge gaps relating to the development of macroalgae cultivation for the simultaneous bioremediation of wastewater and biomass production for biofuels are addressed, with an emphasis on the work conducted in this thesis and the data available in the recent literature. Finally, a model of the overall process is presented to provide a framework of the critical developments required for the future of liquid biofuels from macroalgae.

Chapter 2

Comparing the potential production and value of high-energy liquid fuels and protein from marine and freshwater macroalgae[1]

2.1. Introduction

Biomass represents a carbon-neutral renewable resource for the production of biofuels and biomaterials (Perlack et al., 2005; Ragauskas et al., 2006; Farine et al., 2012). However, the expansion of biofuel production requires the development of fast-growing crops that can provide continuous and affordable biomass with a minimal impact on the environment (Fargione et al., 2008; Brennan & Owende, 2010; Frank et al., 2013). Algae, and more specifically both marine and freshwater macroalgae, are now recognised as targets for low-cost feedstocks for biofuels (Rowbotham et al., 2012) and in particular high-energy liquid biofuels (> 30 MJ/kg) for aviation and heavy vehicle transport (ARENA Report, 2012). Marine macroalgae (seaweeds) are already cultivated at scale (> 20 million tonnes per annum) in a well-established and valuable industry for food and phycocolloids production (Chopin & Sawhney, 2009; Paul & Tseng, 2012). More recently, new technologies have been investigated for the conversion of macroalgal biomass to bioenergy (Ross et al., 2008;

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[1] **Chapter 2** is adapted from Neveux N, Magnusson M, Maschmeyer T, de Nys R, Paul NA, 2014a. Comparing the potential production and value of high-energy liquid fuels and protein from marine and freshwater macroalgae. *Global Change Biology Bioenergy*, **7**, 673-689.

Rowbotham et al., 2012) and, at the same time, macroalgal proteins are now considered a suitable source for human and animal nutrition (Holdt & Kraan, 2011; Boland et al., 2013).

There are numerous pathways to bioenergy from macroalgae that depend on the biochemical composition of the target species. The key biochemical components of lipid, protein, carbohydrate and ash contents vary substantially between the taxonomic grouping of species, and between marine or freshwater origin (Holdt & Kraan, 2011; Gosch et al., 2012; Jung et al., 2013). There are also effects of seasonal, environmental and culture conditions on the biochemical compositions of species (Fleurence, 1999; Taylor et al., 2005; Adams et al., 2011; Angell et al., 2014). Importantly, the options for the conversion of macroalgal biomass to liquid biofuels vary from the traditional fermentation of carbohydrates to ethanol (Kraan, 2013) and the esterification of fatty acids for biodiesel production (Gosch et al., 2012), to the more recent use of thermochemical conversion, such as pyrolysis and hydrothermal liquefaction (HTL), that yield a liquid biocrude (Rowbotham et al., 2012). Of these, the extraction and esterification of fatty acids to biodiesel and the HTL of whole biomass to biocrude, with subsequent refining, represent two promising pathways for the production of high-energy liquid fuels from algae for the aviation industry (Aresta et al., 2005; Brennan & Owende, 2010; Biller & Ross, 2012; Rowbotham et al., 2012, Frank et al., 2013). These pathways focus primarily on the lipid and carbohydrate components of the biomass due to the high conversion efficiency of lipids and the high proportion of carbohydrate in macroalgal biomass, respectively (Biller & Ross, 2012; Rowbotham et al., 2012). Consequently, the pre-extraction of the protein

component of the biomass represents an attractive option to add value to biomass *in-toto* in a biorefinery concept (Lammens et al., 2012).

Regardless of the technology and processing opportunities, the development of liquid biofuels from macroalgae inextricably relies on high biomass productivities and the integration of production systems with marine (de Paula Silva et al., 2008; Bolton et al., 2009; Nobre et al., 2010) and freshwater (Mulbry et al., 2008) wastewater streams. Productivities for land-based cultivated macroalgae (Capo et al., 1999; Bolton et al., 2008; Mulbry et al., 2008; Mata et al., 2010a) are higher than for many land crops (Kraan, 2013) and are also higher than that of macroalgae cultivated at sea, due to the ability to control both the supply of dissolved carbon and nutrients, and limit the action of epiphytes and grazers (Capo et al., 1999; Lüning & Pang, 2003). Furthermore, macroalgae in land-based systems can deliver simultaneous biomass production, CO_2 capture, and the removal of aquatic contaminants including nutrients (Gao & McKinley, 1994; Israel et al., 2005; Mata et al., 2010a) and more intractable industrial contaminants (Saunders et al., 2012; Roberts et al., 2013a). Given that industrial and agricultural waste streams, including land-based aquaculture, represent the primary resource for intensive macroalgal biomass production, the focus must be on macroalgae that are robust and highly productive in land-based systems within these environments (de Paula Silva et al., 2008; Paul & de Nys, 2008; Lawton et al., 2013a).

In this chapter, the biochemical features of six selected marine and freshwater green macroalgae were quantified and compared to identify the most promising species for the

production of high-energy liquid fuels. These species were selected as they have relatively simple morphologies, are suited to intensive land-based production in nutrient-rich water (Bolton et al., 2008; Mulbry et al., 2008; Mata et al., 2010a) and are resistant to contamination with a high tolerance to environmental fluctuations (de Paula Silva et al., 2008; Lawton et al., 2013a). Biomass productivities were quantified per unit area ($g/m^2/d$, dw) and the biochemical profiles of each species analysed. These biochemical data provided the basis to firstly calculate the potential yield of high-energy liquid biofuel from each biomass, using either esterification of fatty acids to obtain biodiesel or HTL of the organic fraction to obtain biocrude, and secondly to calculate the projected productivity and value of these biofuels. Subsequently, I evaluated the potential of extracting protein prior to converting the residual biomass to biocrude, as an option to add value to the production of biocrude. Finally, I used sensitivity analyses for the highest value marine and freshwater species to evaluate the influence of the production parameters on the potential value of feedstocks.

2.2. Materials and methods

2.2.1. Study organisms

Six species of green macroalgae were selected from the culture collections at the Marine & Aquaculture Research Facilities Unit at James Cook University (Townsville, $19^o33'S$; $146^o76'E$). These included four species of marine green macroalgae (seaweed), *Chaetomorpha linum* (Kutzing), *Cladophora coelothrix* (Kutzing), *Derbesia tenuissima* (Crouan) and *Ulva ohnoi* (Hiraoka and Shimada), hereafter referred to by genus and origin. *Chaetomorpha*, *Cladophora* and *Ulva* were originally collected from the bioremediation

pond at Good Fortune Bay Fisheries Ltd. (20°02'S; 148°22'E) in May 2010. *Derbesia* was collected from a shallow coastal rock platform at Rowes Bay, Townsville (19°29'S; 146°83'E) in August 2010. For the two freshwater species, *Cladophora vagabunda* (Hoek) was originally collected from the freshwater ponds at the Townsville Barramundi Fish farm, Kelso (19°36'S; 146°70'E) in March 2011 and *Oedogonium* sp. (Lawton et al., 2013a) was collected from an irrigation channel in the Brandon sugar cane region (19°55'S; 146°35'E) in April 2011, hereafter also referred to by genus and origin. All macroalgae were maintained in stock cultures in outdoor tanks at James Cook University for at least 3 months prior to the experimental period in August 2011.

2.2.2. Culture experiments

Macroalgae were cultured in an outdoor tank-based system with the same regime of nutrient addition and water exchange. This enabled the biomass productivities of marine and freshwater species to be compared simultaneously. Each species was cultured in triplicate in 50 L batch culture cylindrical tanks (Blyth Enterprise Pty. Ltd., Australia) stocked at 2 g/L fresh weight (fw) with a water exchange rate of 0.25 vol/d (12.5 L/d). Each tank had a footprint of 0.16 m^2 and a water depth of 0.36 m. Nutrients and trace elements were provided with 60 mg/L of f/2 medium (Guillard & Ryther, 1962) with each water exchange. Water motion in batch cultures was provided through an aeration ring around the base of the tank bottom, ensuring the biomass had an even exposure to sunlight in the water column (Fig. 2.1).

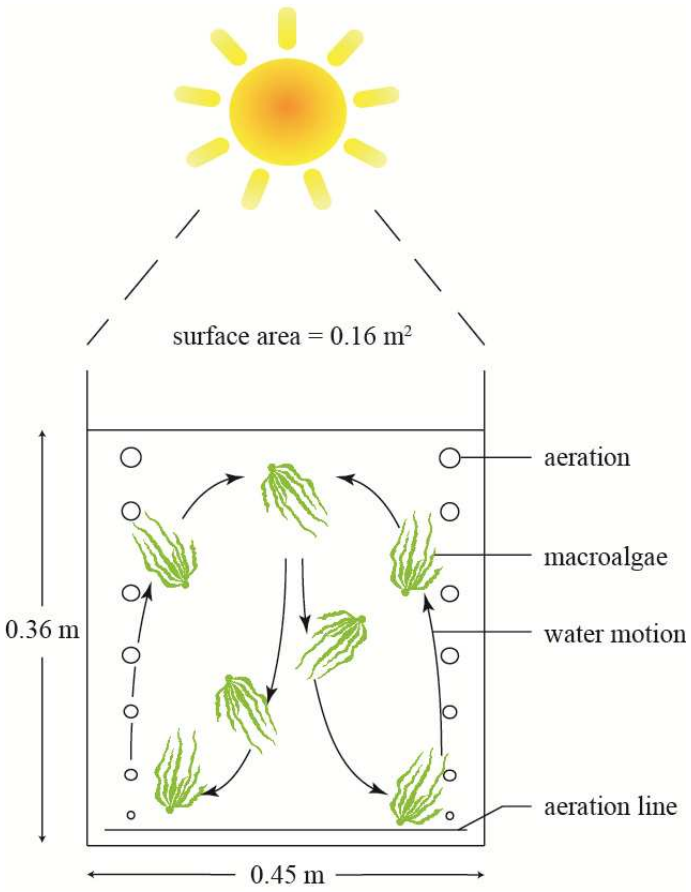

Figure 2.1. Schematic of a batch culture tank. Macroalgae moves freely within the water column driven by aeration from the base of the tank.

The experimental conditions for all cultures were maintained for three culture cycles of 6 days with biomass productivities being measured at day 6, 12 and 18. Culture tanks were randomly repositioned every 2 days in the holding tank. The entire biomass within each culture tank was harvested every 6 days using an aquarium fish net (2 mm screen), placed in a mesh bag (200 µm), spun to constant fresh weight in a domestic centrifuge (MW512; Fisher & Paykel, Australia), weighed and subsequently restocked at 2 g/L. After 18 days, all biomass in each tank was harvested using a fish net. A sub-sample of each replicate (n = 3 tanks) for each of the six species was weighed and oven-dried at 60^{o}C (Binder, Germany)

to a constant weight to determine the fresh to dry weight ratio (fw:dw). Remaining biomass was freeze-dried at -55^{o}C and 120 µbars for 48 hours (VirTis BTK Manifold; Quantum Scientific, Australia). Dried samples were then ground to a mean particle size of < 500 µm and placed in a desiccator for 30 minutes to reach a stable moisture content (defined as dry weight or dw). Powdered macroalgae were stored in air-tight vials under refrigeration and used for all subsequent biochemical analyses.

Environmental culture conditions were monitored and adjusted accordingly. Salinity and pH were recorded daily (YSI 63; YSI, USA). Salinity for marine species was adjusted daily to 35 g/L (of dissolved salts) using dechlorinated freshwater. Salinity of freshwater cultures was stable at 0 - 1 g/L for the duration of the experiment. The pH in batch cultures varied from 8.2 (sunrise) to 9.4 (sunset) for marine species and from 8.4 (sunrise) to 10.5 (sunset) for freshwater species. The culture tanks were placed within a larger holding tank which acted as a water bath to maintain the batch cultures at 25^{o}C. All cultures were held outdoor under full ambient sunlight. Light (photosynthetically active radiation) was monitored hourly using a data logger (Li-1400; LI-COR Inc., USA) adjacent to the tanks for the duration of the experiment. Total photons received for the final 6-day culture cycle was 260 mol photons/m^2 with a peak daily irradiance of 1870 µmol photons/m^2/s.

2.2.3. Biomass productivity

Macroalgae productivity was determined for each culture cycle using the following equation:

$$P = (W_f - W_i) / (t * (fw:dw) * S) \quad \textbf{Eq. 2.1}$$

where P is the biomass productivity ($g/m^2/d$, dw), W_f is the final weight and W_i is the initial weight of algae (g, fw), t is the number of days in culture, *fw:dw* the fresh to dry weight ratio and S is the surface area of the culture (m^2). Mean biomass productivities for each species were analysed by 1-factor Analysis of Variance (ANOVA, see Quinn & Keough 2002 for details) followed by a pairwise comparison for each species combination using Tukey's Honestly Significant Different (HSD) multiple comparisons (significant differences at $p < 0.05$ are reported) using the SPSS Statistics software (v20; IBM, USA). Biomass productivities of the species were analysed for the final 6-day culture cycle (n = 3 replicate tanks per species) as this was the source of the biomass for all biochemical analyses.

2.2.4. Proximate analysis

Ash (dry inorganic) content was determined after combustion of the macroalgal sample (~100 mg) in a muffle furnace (SEM Ltd., Australia) at 550^{o}C until constant weight was reached. Moisture content was determined by drying the sample (~1.5 g) at 110^{o}C in a moisture balance (MS70; A&D Company Ltd., Japan). Total lipids of macroalgal samples were extracted using a mixture of chloroform: methanol (2:1, v/v) and quantified by weight (Folch et al., 1957), as described in Gosch et al., (2012). Proteinogenic amino acids (protein content) were quantified using the Water AccQTag method at the Australian Proteome Analysis Facility (APAF Ltd., Australia). Total carbohydrates were determined by difference, by subtracting ash, moisture, total lipid and protein contents from 100%. Mean values of ash, moisture, lipid, protein and carbohydrate were analysed separately using 1-factor ANOVAs and Tukey's HSD multiple comparisons.

2.2.5. Ultimate analysis

Carbon, hydrogen, oxygen, nitrogen and sulfur contents of macroalgal samples were analysed externally (OEA Laboratory Ltd., UK) using an elemental analyser. Higher heating values (HHV) were calculated from the ultimate analysis of samples, incorporating the ash content (Channiwala & Parikh, 2002). HHV were analysed using a 1-factor ANOVA and Tukey's HSD multiple comparisons.

2.2.6. Biodiesel yield

Biodiesel yield was determined through the conversion of biomass fatty acids (FA) to fatty acid methyl esters (FAME), the components of crude biodiesel (Chisti et al., 2007), following the relationship:

$$Y_{BIODIESEL} = W_{FAME} \qquad \textbf{Eq. 2.2}$$

where $Y_{BIODIESEL}$ is the crude biodiesel yield (wt%), corresponding to W_{FAME}, the FAME content (wt%) extracted from the macroalgae.

FA were converted to FAME using a direct esterification method adapted for macroalgae (Gosch et al., 2012). This method simultaneously extracts and esterifies FA to FAME for subsequent separation and quantification by gas chromatography – mass spectrometry (Agilent 7890 GC with FID – Agilent 5975C EI/TurboMS; Agilent, Australia). The FAME profile of macroalgae was used to analyse the quality of biodiesel, through the calculation of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) concentrations.

2.2.7. Theoretical biocrude yield

Although a complex reaction cascade occurs in the production of biocrude through HTL, it has been demonstrated that the conversion of lipids, proteins and carbohydrates is additive and that the yield of biocrude can be estimated based on the feedstock biochemical content (Biller & Ross, 2011) using the following equation:

$$Y_{BIOCRUDE} = (Y_{LIP} * W_{LIP}) + (Y_{PROT} * W_{PROT}) + (Y_{CARB} * W_{CARB}) \quad \textbf{Eq. 2.3}$$

where $Y_{BIOCRUDE}$, Y_{LIP}, Y_{PROT} and Y_{CARB} are biocrude, lipid, protein and carbohydrate HTL yields (wt%), and W_{LIP}, W_{PROT} and W_{CARB} are lipid, protein and carbohydrate contents (wt%) of macroalgae. The theoretical biocrude yields were calculated as a range with an upper and a lower limit for each species. The upper limit used the biochemical yield conversion factors of 0.80, 0.18, 0.15 for lipids, proteins and carbohydrates, respectively, and the lower limit used conversion factors of 0.55, 0.11, 0.06 for the same components (Biller & Ross, 2011). These conversion factors are based on the yields of a range of model compounds obtained through HTL performed at 350^{o}C for 1 h and 10% solids.

2.2.8. Theoretical protein yield

The theoretical protein yield (wt%) was calculated from the sum of all amino acids (AA). The essential amino acids were calculated from the sum of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine.

2.2.9. Projected areal productivities

The projected productivities of biodiesel, biocrude and protein were determined by multiplying individual yields by the biomass productivity for each species, using the following equation:

$$P_{BIOPRODUCT} = P * Y_{BIOPRODUCT} /100\% \quad \textbf{Eq. 2.4}$$

where $P_{BIOPRODUCT}$ is biodiesel, biocrude or protein productivity ($g/m^2/d$), P is the biomass productivity ($g/m^2/d$, dw) and $Y_{BIOPRODUCT}$ is the biodiesel, biocrude or protein yield (wt%).

2.2.10. Projected production values – at scale with sequential extraction

In order to evaluate the potential value of macroalgal feedstock at scale, $P_{BIOPRODUCT}$ was converted into t/ha/yr and the values of comparable commodities were used to estimate the value per hectare per year of each species in US$. The value of crude diesel ($3.1/gal) was converted to $975.0/t according to the specific gravity of 0.84 at 15^{o}C for crude diesel (Tat & Van Gerpen, 2000) and assuming that one US gallon contains 3.785 L. Then, the price of biodiesel ($941.4/t) was derived from crude diesel price after adjustment for volume with a biodiesel specific gravity of 0.87 (Miao & Wu, 2006), using the conversion factor of 0.9655 (= 0.84/0.87) to account for this difference in quality. Similarly, the value of WTI (West Texas Intermediate) crude oil ($105.3/bbl) was converted to $798.1/t according to the specific gravity of 0.83 at 15^{o}C for WTI crude oil (Weaver, 2004) and assuming that one barrel contains 158.987 L. Then, the price of biocrude ($682.5/t) was derived from WTI crude oil price after adjustment for volume with biocrude specific gravity of 0.97 (Jena & Das, 2011), using the conversion factor of 0.8550 (= 0.83/0.97) to account for this

difference in quality. Soybean meal ($431.9/t) was used to estimate the value of the protein in a conservative way, acknowledging that soybean meal is composed of about 50% amino acids (Lywood et al., 2009) whereas the protein extract would theoretically be close to 100% amino acids.

The values of crude diesel, WTI crude oil and soybean meal were based on the 2012-2013 average price index sourced from Indexmundi (http://www.indexmundi.com/australia/). Projected values of biodiesel, biocrude and protein were calculated for each product singularly and then sequentially for the extraction of protein prior to conversion of the residual biomass to biocrude. The sequential extraction of lipids (value estimated from soy oil price at $1169.7/t, Indexmundi) or fatty acids for biodiesel production (see above for value), prior to the conversion of the residual biomass to biocrude, was also calculated for comparison (Annex 1, Table S2.1).

2.2.11. Projected production values – sensitivity analysis

Sensitivity analysis was used as a tool to visualise the relative importance of production parameters under a range of different cases. This tool has recently been used for algal biofuels as it is particularly useful where there are knowledge gaps or uncertainty for the parameters of different systems. For example, sensitivity analysis provides a mechanism to synthesise laboratory, pilot and commercial scale information into a single package whilst acknowledging the limitations and uncertainties of each parameter to define unfavourable, standard and favourable cases (Yang et al., 2011; Ong et al., 2012; Liu et al., 2013c). Sensitivity analyses were used in the present study to provide context for the outcomes of protein extraction prior to biocrude production from the residual biomass for the most

valuable marine species (*Derbesia* and *Ulva*) and the most valuable freshwater species (*Oedogonium*), given that this sequential process yielded the highest projected values (see Results and Annex1, Table S2.2). It also served to provide additional context for projections, for example, while there is no commercial scale production of *Derbesia* and *Oedogonium*, there are analogous culture systems in place for both marine (*Ulva* – Bolton et al., 2009) and freshwater algae (Park et al., 2011a). Similarly, while there are no reported yields from HTL of macroalgae for large-scale continuous flow reactors, there are laboratory data (batch reactor) yields for the green macroalga *Ulva* (Zhou et al., 2010) and a range of microalgae (Annex 1, Table S2.3) that can be used for projections. Full calculations and references for the sensitivity analyses are provided in the supporting information (Annex 1).

Values for biomass productivity were defined as standard (centre, average of the current study), favourable (right of centre, 24.0 $g/m^2/d$ for *Derbesia* from Magnusson et al., 2014, 26.1 $g/m^2/d$ for *Ulva* from Bolton et al., 2009 and 16.0 $g/m^2/d$ for *Oedogonium* from Cole et al., 2014) and unfavourable (left of centre, one standard deviation below the average of the current study). Values for theoretical biocrude conversion yield were defined as standard (centre, upper limit of the current study) with favourable (right of centre, 50% increase from the upper yield) and unfavourable (left of centre, lower limit of the current study). Values for protein content were defined as standard (centre, average of the current study), favourable (right of centre, one standard deviation above the average of the current study) and unfavourable (left of centre, one standard deviation below the average of the current study). Values for biocrude and protein extract, adjusted from the values of WTI

crude oil and soybean meal (see above section – Projected production values), were defined as standard (centre, average price for 2012-2013 from Indexmundi), favourable (right of centre, maximum price for 2012-2013 from Indexmundi) and unfavourable (left of centre, minimum price for 2012-2013 from Indexmundi).

Projected values for the sequential extraction of protein and the conversion of the residual biomass for *Derbesia*, *Ulva* and *Oedogonium* (US$/ha/yr) were calculated separately for each species according to the following equation:

$$Value = 3.65 * P * [(Y_{BIOCRUDE\text{-}AA} * Price\text{-}_{BC}) + (W_{PROTEIN} * Price\text{-}_{PE})] / 100\% \quad \textbf{Eq. 2.5}$$

where the multiplier of "3.65" is derived from the conversion of productivity in g/m^2/d to productivity in t/ha/y, P is the biomass productivity (g/m^2/d, dw), $Y_{BIOCRUDE\text{-}AA}$ is the biocrude yield (wt%) after protein extraction, $Price\text{-}_{BC}$ is the 2-year average price (US$/t) of biocrude derived from WTI crude oil price, $W_{PROTEIN}$ is the protein (AA) content (wt%) of macroalgae and $Price\text{-}_{PE}$ is the 2-year average price (US$/t) of the protein extract derived from soybean meal price.

2.3. Results

2.3.1. Biomass productivity

Biomass productivity (g/m^2/d, dw) for outdoor batch cultures was up to two times higher for marine macroalgae than for freshwater macroalgae (Fig. 2.2; ANOVA, $F_{5,12} = 63.09$, $P < 0.001$). *Derbesia* (11.9 g/m^2/d) and *Ulva* (11.4 g/m^2/d) were the most productive species. *Oedogonium* (5.1 g/m^2/d) had the highest productivity of the two freshwater species, and freshwater *Cladophora* (3.4 g/m^2/d) the lowest productivity of all species. These biomass

productivities are for the final 6-day cycle and were consistent with the previous two cycles, for example, ranging from 11.5 to 12.7 g/m^2/d for *Derbesia*, 10.8 to 11.9 g/m^2/d for *Ulva* and 4.9 to 5.5 g/m^2/d for *Oedogonium*.

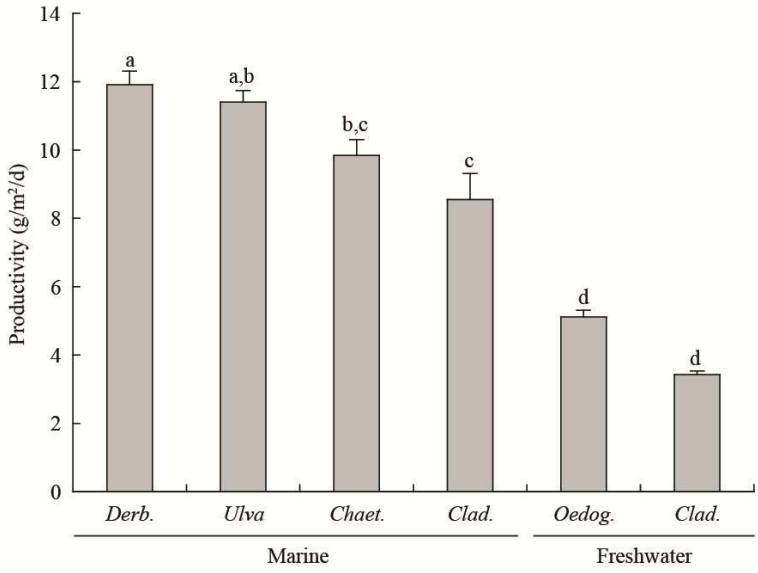

Figure 2.2. Biomass productivity of macroalgae.
Data show means (n = 3 ± SE) of productivity dry weight of marine (M) and freshwater (FW) macroalgae. Species sharing the same letter above the bars are not significantly different (Tukey's HSD, $p < 0.05$).

2.3.2. Proximate analysis

The proximate and biochemical composition of macroalgae, expressed as the percentage of the dry weight of samples, varied substantially between species (Table 2.1). Ash content ranged from 17.8 to 36.6% and freshwater macroalgae typically had lower ash contents than marine macroalgae (ANOVA, $F_{5,12} = 15.43$, $P < 0.001$). Marine *Chaetomorpha* (36.6%) and *Derbesia* (34.7%) had the highest ash content, and the freshwater *Cladophora* (17.8%) the lowest. The organic component varied widely across species as well, in many

cases by a factor of two. Lipid content ranged from 1.9 to 10.4% and varied independently from macroalgae marine or freshwater origin. Marine *Derbesia* (10.4%) and freshwater *Oedogonium* (9.4%) had the highest lipid content and marine *Ulva* (1.9%) had the lowest (ANOVA, $F_{5,12} = 276.58$, $P < 0.001$). Variation in protein content was primarily driven by the difference between marine and freshwater species, ranging from 11.1 to 26.8% (ANOVA, $F_{5,12} = 97.70$, $P < 0.001$). Protein contents were above 20% for three species and highest for freshwater *Cladophora* (26.8%) and *Oedogonium* (22.5%). *Derbesia* (21.6%) had the third highest protein content, which was the highest of all marine species and was double that of *Chaetomorpha* (11.1%), which had the overall lowest protein content. Carbohydrates were the main organic component of all species, ranging from 26.9 to 45.4% (ANOVA, $F_{5,12} = 14.11$, $P < 0.001$). Marine *Cladophora* (45.4%) and freshwater *Oedogonium* (44.4%) had the highest carbohydrate contents, ~75% higher than *Derbesia* (26.9%), which had the lowest content.

Table 2.1. Proximate and biochemical analysis of macroalgae.

Data show means (n = 3 ± SE) of content dry weight of marine (M) and freshwater (FW) macroalgae. Species sharing the same letter in superscript are not significantly different (ANOVA, Tukey's HSD, $p < 0.05$).

Species	*Derb.*	*Ulva*	*Chaet.*	*Clad.*	*Oedog.*	*Clad.*
Source	M	M	M	M	FW	FW
Proximate (wt%)						
Ash	$34.7^{a} \pm 0.4$	$30.7^{a,b} \pm 0.5$	$36.6^{a} \pm 1.0$	$25.5^{b,c} \pm 1.0$	$20.6^{c} \pm 4.2$	$17.8^{c} \pm 1.5$
Moisture	$6.4^{a,b} \pm 0.5$	$7.2^{a} \pm 0.4$	$5.1^{b} \pm 0.4$	$6.7^{a,b} \pm 0.3$	$6.5^{a,b} \pm 0.4$	$5.7^{a,b} \pm 0.3$
Biochemical (wt%)						
Lipid	$10.4^{a} \pm 0.1$	$1.9^{d} \pm 0.1$	$3.3^{c} \pm 0.1$	$4.6^{b} \pm 0.2$	$9.4^{a} \pm 0.3$	$5.3^{b} \pm 0.3$
Protein*	$21.6^{b} \pm 0.2$	$16.3^{c} \pm 0.2$	$11.1^{d} \pm 0.4$	$17.8^{c} \pm 1.1$	$22.5^{b} \pm 0.3$	$26.8^{a} \pm 0.4$
Carbohydrate**	$26.9^{b} \pm 0.6$	$43.9^{a} \pm 0.8$	$43.9^{a} \pm 0.8$	$45.4^{a} \pm 1.7$	$41.0^{a} \pm 4.0$	$44.4^{a} \pm 0.5$

*sum of amino acids; **determined by difference.

2.3.3. Ultimate analysis

The carbon content of macroalgae ranged from 26.5 to 37.5% on a dry weight basis (Table 2.2). Freshwater *Cladophora* (37.5%) and *Oedogonium* (36.6%) had the highest carbon content of all species. Marine *Cladophora* (30.9%) and *Derbesia* (29.2%) had the highest carbon content of the marine species, whereas marine *Chaetomorpha* (26.5%) had the lowest. Carbon content correlated with the HHV that ranged from 10.3 to 16.4 MJ/kg (ANOVA, $F_{5,12} = 39.88$, $P < 0.001$). Freshwater *Cladophora* (16.4 MJ/kg) and *Oedogonium* (15.8 MJ/kg) had the highest HHV of all species. Marine *Cladophora* (12.7 MJ/kg) and *Derbesia* (12.4 MJ/kg) had the highest HHV of the marine species and marine *Chaetomorpha* (10.3 MJ/kg) had the lowest. Nitrogen content was species dependent and ranged from 3.4 to 6.5%. Both freshwater *Cladophora* (6.5%) and marine *Cladophora* (5.2%) had the highest nitrogen content and marine *Chaetomorpha* (3.4%) had the lowest.

Table 2.2. Ultimate analysis of macroalgae.

Data show means (n = 3 ± SE) of C, H, O, N, S (wt%) and HHV (MJ/kg) of marine (M) and freshwater (FW) macroalgae. Species sharing the same letter in superscript are not significantly different (Tukey's HSD, $p < 0.05$).

Species	*Derb.*	*Ulva*	*Chaet.*	*Clad.*	*Oedog.*	*Clad.*
Source	M	M	M	M	FW	FW
C	29.2 ± 0.3	27.7 ± 0.3	26.5 ± 0.6	30.9 ± 0.3	36.6 ± 1.9	37.5 ± 1.2
H	4.8 ± 0.1	5.5 ± 0.1	4.1 ± 0.1	5.0 ± 0.1	5.7 ± 0.2	5.9 ± 0.1
O	27.4 ± 0.3	41.1 ± 0.4	31.0 ± 1.0	34.9 ± 0.8	30.9 ± 1.9	32.9 ± 0.5
N	4.5 ± 0.0	3.5 ± 0.1	3.4 ± 0.1	5.2 ± 0.1	4.8 ± 0.2	6.5 ± 0.1
S	2.8 ± 0.1	5.0 ± 0.1	2.1 ± 0.1	2.3 ± 0.1	0.4 ± 0.0	1.8 ± 0.1
HHV*	12.4 ± 0.2^{b}	$11.7 \pm 0.2^{b,c}$	10.3 ± 0.3^{c}	12.7 ± 0.1^{b}	15.8 ± 0.8^{a}	16.4 ± 0.6^{a}

*calculated from Channiwala & Parikh (2002).

2.3.4. Biodiesel yield

Yields of crude biodiesel ranged from 1.6 to 4.9% on a dry weight basis (Table 2.3). Freshwater *Cladophora* (4.9%) and *Oedogonium* (4.7%) had the highest biodiesel yields of all species (ANOVA, $F_{5,12} = 119.23$, $P < 0.001$). The third highest biodiesel yield was obtained from marine *Derbesia* (4.2%), which was more than 2.5 times higher than the lowest biodiesel yield of marine *Ulva* (1.6%).

Table 2.3. Theoretical biodiesel, biocrude and protein yields**.**

Data show means (n = 3 ± SE) of yield from marine (M) and freshwater (FW) macroalgae. Species sharing the same letter in superscript are not significantly different (Tukey's HSD, $p < 0.05$).

Species	*Derb.*	*Ulva*	*Chaet.*	*Clad.*	*Oedog.*	*Clad.*
Source	M	M	M	M	FW	FW
Biodiesel (wt%)						
Total	4.2 ± 0.2^{b}	1.6 ± 0.1^{d}	$2.1 \pm 0.1^{c,d}$	2.6 ± 0.1^{c}	$4.7 \pm 0.1^{a,b}$	4.9 ± 0.2^{a}
SFA	1.5 ± 0.0^{a}	0.7 ± 0.0^{c}	0.7 ± 0.0^{c}	1.0 ± 0.1^{b}	1.1 ± 0.0^{b}	1.5 ± 0.0^{a}
MUFA	0.5 ± 0.0^{b}	$0.4 \pm 0.0^{b,c}$	0.3 ± 0.0^{c}	0.6 ± 0.0^{b}	0.5 ± 0.0^{b}	1.1 ± 0.1^{a}
PUFA	2.2 ± 0.2^{c}	0.5 ± 0.1^{d}	1.1 ± 0.0^{d}	0.9 ± 0.0^{d}	3.1 ± 0.1^{a}	2.3 ± 0.1^{b}
Biocrude (wt%)						
Upper	$16.2 \pm 0.0^{a,b}$	11.1 ± 0.1^{d}	11.2 ± 0.2^{d}	13.7 ± 0.1^{c}	17.7 ± 0.6^{a}	15.7 ± 0.4^{b}
Lower	9.7 ± 0.0^{a}	5.5 ± 0.1^{d}	5.7 ± 0.1^{d}	7.2 ± 0.1^{c}	10.1 ± 0.3^{a}	8.5 ± 0.2^{b}
Amino acids (wt%)						
Total	21.6 ± 0.2^{b}	16.3 ± 0.2^{c}	11.1 ± 0.4^{d}	17.8 ± 1.1^{c}	22.5 ± 0.3^{b}	26.8 ± 0.4^{a}
Essential	9.1 ± 0.1^{b}	6.4 ± 0.1^{c}	4.4 ± 0.1^{d}	7.1 ± 0.1^{c}	$9.7 \pm 0.3^{a,b}$	10.1 ± 0.1^{a}

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

The quality of biodiesel (FA concentrations, measured as FAME) also differed between species (Table 2.4). The quantity of saturated fatty acids (SFA) in all species was primarily driven by palmitic acid (C16:0) content. The proportion of SFA was highest in marine *Ulva* (43.0%) and marine *Cladophora* (38.7%), and lowest in freshwater *Oedogonium* (23.5%). The same species, *Ulva* (25.2%) and marine *Cladophora* (25.0%), had the highest monounsaturated fatty acid (MUFA) content. This was driven primarily by high concentrations of oleic acid (C18:1) for *Ulva* (1.6 mg/g) and for marine *Cladophora* (3.4 mg/g) relative to their total FA content. The two species with the highest proportion of PUFA were *Oedogonium* (66.4%) and *Derbesia* (53.2%), for which the concentrations of

hexadecatrienoic acid (C16:3) and α-linolenic acid (C18:3) were particularly high, with 6.1 mg/g and 12.8 mg/g respectively for *Oedogonium*, and 4.9 mg/g and 9.5 mg/g respectively for *Derbesia*. However, the FA content of macroalgae differed from the total lipid content and the lipid:FA ratio ranged from 1.1 to 2.5 across all species, and was highest for marine *Derbesia* (2.5) and freshwater *Oedogonium* (2.0) and lowest for freshwater *Cladophora* (1.1). This high ratio shows that *Derbesia* and *Oedogonium* had the highest proportions of non-FA lipids.

Table 2.4. Biodiesel profiles of macroalgae.

Data show means (n = 3 ± SE) of fatty acid methyl esters (FAME, mg/g) of marine (M) and freshwater (FW) macroalgae. Chemical properties of biodiesel are expressed as a proportion [wt%] of total fatty acid content.

Species	*Derb.*	*Ulva*	*Chaet.*	*Clad.*	*Oedog.*	*Clad.*
Source	M	M	M	M	FW	FW
C14:0	1.02 ± 0.04	0.30 ± 0.01	1.66 ± 0.00	2.18 ± 0.22	0.76 ± 0.20	3.27 ± 0.14
C14:1 (n-5)	0.40 ± 0.01	0.29 ± 0.01	0.27 ± 0.00	0.29 ± 0.00	0.43 ± 0.01	0.37 ± 0.01
C15:0	0.46 ± 0.02	0.32 ± 0.01	0.30 ± 0.01	0.32 ± 0.00	0.50 ± 0.01	0.45 ± 0.01
C15:1 (n-5)	0.73 ± 0.03	0.43 ± 0.02	0.40 ± 0.01	0.46 ± 0.01	0.83 ± 0.02	0.68 ± 0.02
C16:0	9.84 ± 0.15	5.08 ± 0.10	4.09 ± 0.23	6.51 ± 0.36	8.59 ± 0.17	10.19 ± 0.3
C16:1 (n-9)	0.28 ± 0.01	0.21 ± 0.01	0.24 ± 0.00	0.55 ± 0.10	0.30 ± 0.01	0.58 ± 0.05
C16:1 (n-7)	1.74 ± 0.05	1.26 ± 0.03	1.03 ± 0.11	1.59 ± 0.14	1.68 ± 0.42	2.02 ± 0.09
C16:2 (n-6)	0.41 ± 0.03	0.22 ± 0.01	0.30 ± 0.01	0.60 ± 0.02	0.93 ± 0.02	0.44 ± 0.05
C16:2 (n-4)	0.22 ± 0.01	0.24 ± 0.01	1.46 ± 0.05	0.36 ± 0.02	0.66 ± 0.25	1.62 ± 0.15
C17:0	0.24 ± 0.01	0.22 ± 0.01	0.22 ± 0.00	0.20 ± 0.00	0.27 ± 0.02	0.31 ± 0.07
C16:3 (n-6)	0.26 ± 0.02	0.21 ± 0.01	0.21 ± 0.01	0.22 ± 0.00	0.84 ± 0.38	0.46 ± 0.11
C16:3 (n-3)	4.92 ± 0.52	0.32 ± 0.02	0.22 ± 0.01	0.24 ± 0.00	6.05 ± 0.85	0.24 ± 0.01
C16:4 (n-3)	0.40 ± 0.02	0.77 ± 0.21	1.33 ± 0.04	1.25 ± 0.17	1.43 ± 0.16	3.70 ± 0.12
C18:0	0.53 ± 0.00	0.28 ± 0.01	0.25 ± 0.00	0.27 ± 0.02	0.36 ± 0.00	0.33 ± 0.01
C18:1 (n-9)	1.76± 0.06	1.61 ± 0.05	1.36 ± 0.04	3.39 ± 0.25	1.24 ± 0.09	6.64 ± 0.66
C18:2 (n-6)	1.93 ± 0.09	0.39 ± 0.03	4.35 ± 0.06	1.99 ± 0.06	2.17 ± 0.12	7.45 ± 0.57
C18:3 (n-6)	0.87 ± 0.04	0.27 ± 0.01	0.29 ± 0.01	0.25 ± 0.01	1.39 ± 0.06	0.59 ± 0.04
C18:3 (n-3)	9.46 ± 0.62	0.97 ± 0.18	0.63 ± 0.18	2.64 ± 0.17	12.84±1.21	3.98 ± 0.15
C18:4 (n-3)	0.96 ± 0.10	1.18 ± 0.36	0.35 ± 0.08	0.41 ± 0.05	2.58 ± 0.04	0.28 ± 0.02
C20:0	0.24 ± 0.01				0.21 ± 0.01	
C20:1 (n-9)	0.22 ± 0.00			0.21 ± 0.00	0.21 ± 0.00	0.46 ± 0.02
C20:2 (n-6)			0.23 ± 0.00	0.21 ± 0.00	0.30 ± 0.01	0.29 ± 0.01
C20:4 (n-6)	0.38 ± 0.01		0.23 ± 0.00		0.32 ± 0.03	0.30 ± 0.01
C20:3 (n-6)	1.46 ± 0.06	0.24 ± 0.01	0.60 ± 0.02	0.50 ± 0.01	0.43 ± 0.13	1.15 ± 0.05
C20:5 (n-3)	1.15 ± 0.10	0.30 ± 0.02	0.32 ± 0.04	0.79 ± 0.05	1.13 ± 0.52	1.84 ± 0.03
C22:0	0.91 ± 0.03	0.49 ± 0.01		0.24 ± 0.01		
C24:0	1.38 ± 0.01	0.22 ± 0.00	0.38 ± 0.05	0.33 ± 0.01	0.31 ± 0.09	0.51 ± 0.04
C22:6 (n-3)		0.25 ± 0.01	0.26 ± 0.02			0.48 ± 0.03
Total FAME	42.2 ± 1.7	16.1 ± 1.1	21.0 ± 0.7	26.0 ± 0.9	46.8 ± 1.0	48.6 ± 1.9
Biodiesel chemical profile [wt%]						
SFA	34.6	43.0	32.9	38.7	23.5	31.0
MUFA	12.2	25.2	17.0	25.0	10.0	23.1
PUFA	53.2	31.8	50.2	36.4	66.4	45.9
Ratio						
lipid:FA	2.5	1.2	1.6	1.8	2.0	1.1

2.3.5. Theoretical biocrude yield

The theoretical yields of biocrude from macroalgae through HTL yielded 2 to 7 times more biocrude than the esterification of fatty acids (FA) yielded biodiesel (Table 2.3, ANOVA, $F_{5,12} = 75.27$, $P < 0.001$). Overall, theoretical biocrude yields ranged from 5.5 to 17.7% on a dry weight basis. For each species, the theoretical biocrude yields calculated as a range with lower and upper limits, were highest for freshwater *Oedogonium* (10.1 - 17.7%) and marine *Derbesia* (9.7 - 16.2%), which were ~75% higher than the lowest yields for marine *Ulva* (5.5 - 11.1%).

2.3.6. Theoretical protein yield

The theoretical protein yield (sum of individual amino acids) ranged from 11.1 to 26.8% dw and was highest for freshwater *Cladophora* and *Oedogonium* and marine *Derbesia* (Table 2.3, ANOVA, $F_{5,12} = 97.70$, $P < 0.001$). The quality of the protein also differed between species (Table 2.5). Both aspartic and glutamic acids – and their respective amides – were the main amino acids in all species and were highest in freshwater *Cladophora* (37.9 and 41.3 mg/g, respectively) and lowest in marine *Chaetomorpha* (17.7 and 15.7 mg/g, respectively). The essential amino acid content, expressed as a proportion of total amino acids, was highest for freshwater *Oedogonium* (43%) and marine *Derbesia* (42%), and lowest for freshwater *Cladophora* (38%). The quantity of the essential amino acid methionine, expressed as a relative amount of total amino acids, and the ratio of methionine to lysine were highest in marine *Derbesia* (2.1% and 0.31%, respectively), *Ulva* (1.6% and 0.30%, respectively) and freshwater *Oedogonium* (1.9% and 0.28 respectively), and lowest in marine *Chaetomorpha* (1.0% and 0.12%, respectively). The

protein:N ratio for green macroalgae ranged from 3.3 to 4.8, highest for marine *Derbesia* (4.8) and freshwater *Oedogonium* (4.7) and lowest for marine *Chaetomorpha* (3.3).

Table 2.5. Amino acids profiles of macroalgae.

Data show means (n = 3 ± SE) of α-amino acids (mg/g, tryptophan and cysteine not included) of marine (M) and freshwater (FW) macroalgae. Chemical properties of proteins are expressed as a proportion [wt%] of total amino acid content.

Species	*Derb.*	*Ulva*	*Chaet.*	*Clad.*	*Oedog.*	*Clad.*
Source	M	M	M	M	FW	FW
aspartic acid/ asparagine	23.0 ± 0.4	22.7 ± 0.3	17.7 ± 0.8	26.6 ± 3.5	25.3 ± 0.5	37.9 ± 0.8
glutamic acid/ glutamine	33.0 ± 0.4	20.0 ± 0.4	15.7 ± 0.6	26.9 ± 1.6	29.4 ± 0.6	41.3 ± 1.4
histidine*	4.7 ± 0.1	2.8 ± 0.0	1.6 ± 0.1	2.8 ± 0.1	4.6 ± 0.1	3.7 ± 0.1
serine	11.2 ± 0.2	9.4 ± 0.1	5.1 ± 0.3	8.4 ± 0.9	11.4 ± 0.1	14.3 ± 0.1
arginine	12.6 ± 0.2	10.3 ± 0.1	6.0 ± 0.1	10.3 ± 0.5	13.2 ± 0.2	21.1 ± 1.4
glycine	12.4 ± 0.2	9.5 ± 0.1	6.5 ± 0.1	10.7 ± 0.5	12.4 ± 0.0	14.9 ± 0.1
threonine*	11.2 ± 0.2	9.1 ± 0.1	4.2 ± 0.3	8.0 ± 1.3	12.3 ± 0.1	14.1 ± 0.1
alanine	14.7 ± 0.2	13.7 ± 0.3	6.3 ± 0.4	11.6 ± 0.4	16.2 ± 0.3	13.9 ± 0.2
proline	10.0 ± 0.1	8.5 ± 0.1	7.6 ± 0.2	9.4 ± 0.3	11.5 ± 0.2	14.3 ± 0.3
lysine*	14.8 ± 0.2	8.8 ± 0.1	9.8 ± 0.3	10.8 ± 0.3	15.2 ± 0.5	21.1 ± 0.4
tyrosine	8.4 ± 0.1	5.7 ± 0.0	2.8 ± 0.2	3.7 ± 0.8	8.0 ± 0.1	8.9 ± 0.3
methionine*	4.6 ± 0.0	2.6 ± 0.1	1.2 ± 0.1	1.8 ± 0.4	4.3 ± 0.1	3.7 ± 0.2
valine*	14.3 ± 0.2	10.7 ± 0.1	6.8 ± 0.2	12.5 ± 0.3	14.6 ± 0.2	15.7 ± 0.0
isoleucine*	10.2 ± 0.1	7.4 ± 0.1	5.1 ± 0.2	8.7 ± 0.2	10.7 ± 0.0	10.5 ± 0.1
leucine*	18.1 ± 0.2	12.0 ± 0.1	8.5 ± 0.4	15.7 ± 0.3	21.8 ± 0.3	19.6 ± 0.2
phenylalanine*	13.1 ± 0.2	10.2 ± 0.1	6.4 ± 0.2	10.5 ± 0.3	14.0 ± 0.1	12.8 ± 0.2
Total AA**	216.2 ± 2.3	163.2 ± 2.0	111.3 ± 4.1	178.5±11.4	224.8 ± 2.9	267.9 ± 4.4
Protein chemical properties [wt%]						
essential	42.1	38.9	39.2	39.7	43.4	37.8
non-essential	57.9	61.1	60.8	60.3	56.6	62.2
lysine	6.8	5.4	8.8	6.1	6.7	7.9
methionine	2.1	1.6	1.0	1.0	1.9	1.4
Ratio						
met:lys	0.31	0.3	0.12	0.16	0.28	0.18
protein:N	4.8	4.6	3.3	3.4	4.7	4.1

* Essential amino acids; ** Total α-amino acids (tryptophan and cysteine not included).

2.3.7. Projected areal productivities

The projected areal productivities of biodiesel, biocrude and protein, calculated by integrating biomass productivity and biochemical composition (Eq. 2.4), demonstrated that biocrude productivity was consistently higher (by 40 - 80%) than biodiesel productivity across all species on a dry weight basis (Fig. 2.3a). Marine species had a higher productivity of biocrude than freshwater species due to their higher growth rates, for which *Derbesia* (1.15 - 1.93 $g/m^2/d$) and *Ulva* (0.63 - 1.26 $g/m^2/d$) had the maximum projected biocrude productivity of the marine species, and *Oedogonium* (0.52 - 0.90 $g/m^2/d$) the highest of the freshwater species. Freshwater *Cladophora* (0.29 - 0.54 $g/m^2/d$) had the lowest overall biocrude productivity even though it had the third highest theoretical biocrude yield. The most productive species in terms of protein were marine *Derbesia* (2.57 $g/m^2/d$) and *Ulva* (1.86 $g/m^2/d$), and the least productive species was freshwater *Cladophora* (0.92 $g/m^2/d$) (Fig. 2.3b).

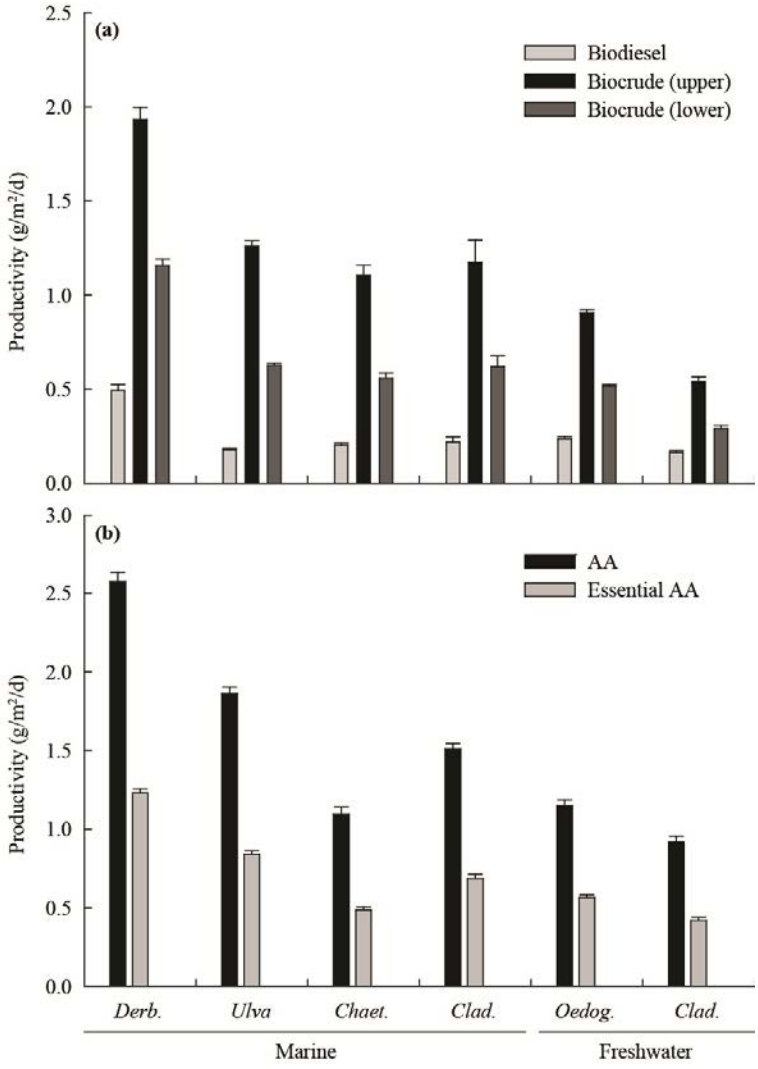

Figure 2.3. Projected areal productivities of biofuels and bioproducts from macroalgae. Data show means (n = 3 ± SE) of theoretical productivities dry weight of biodiesel and biocrude – upper and lower limits (a); amino acids (AA) and essential AA (b) of marine (M) and freshwater (FW) macroalgae.

2.3.8. Projected production values – at scale with sequential extraction

To assess the potential value of macroalgae at scale, the projected value of biodiesel, biocrude and protein was calculated per unit hectare of production (Eq. 2.5) by scaling biomass productivities and bioproduct yields (Table 2.6; values rounded to the nearest $100). With a starting point of a single product use for the entire biomass, the conversion

to biocrude was the most valuable option for five of the six species. Marine *Derbesia* had the highest projected productivity of biocrude at 7.1 t biocrude/ha/yr. Notably, *Derbesia* was the most valuable biomass in each scenario of biodiesel ($1,700/ha/yr), biocrude ($4,800/ha/yr) and protein ($4,100/ha/yr) production. Marine *Ulva* was the second most valuable species for biocrude ($3,100/ha/yr) and protein ($2,900/ha/yr) production. *Oedogonium* was the most valuable of the freshwater species, however, biomass productivities were half that of *Derbesia* and correspondingly the projected value per ha was also proportionally lower for biodiesel ($800/ha/yr), biocrude ($2,300/ha/yr), and protein ($1,800/ha/yr). Freshwater *Cladophora* was an anomaly in that it had a higher projected value per unit hectare for protein ($1,400/ha/yr) compared to biocrude ($1,300/ha/yr).

In the scenario where protein is extracted prior to HTL of residual biomass to biocrude, the projected value of the feedstock increased by 45 to 77% (Table 2.6, scenario 5). The pre-extraction of protein followed by the production of biocrude was the most valuable option for all species and was highest for marine *Derbesia* ($7,700/ha/yr) and *Ulva* ($5,200/ha/yr), and *Oedogonium* was the highest of the freshwater species ($3,500/ha/yr). In this instance each product generated by *Derbesia, Ulva* and *Oedogonium* – protein ($4,100/ha/yr, $2,900/ha/yr and $1,800/ha/yr, respectively) and biocrude ($3,700/ha/yr, $2,300/ha/yr and $1,700/ha/yr, respectively) – accounted for approximately half of the projected value of the feedstock. *Derbesia* had the highest protein productivity (9.4 t/ha/yr) of all species, and *Oedogonium* had the highest protein productivity (4.2 t/ha/yr) of the freshwater species. *Derbesia* and *Ulva* had the highest projected biocrude productivity post-extraction of protein (5.4 t/ha/yr and 3.4 t/ha/yr, respectively), again corresponding to

the highest value ($3,700/ha/yr and $2,300/ha/yr, respectively), while *Oedogonium* had a projected biocrude productivity post-extraction of 2.5 t/ha/yr corresponding to a value of $1,700/ha/yr. Given the highest projected values for *Derbesia and Ulva* for marine species and *Oedogonium* for freshwater species, these species were further considered using sensitivity analysis.

Table 2.6. Projected productivity and value of commodities produced by macroalgae.
Data show macroalgae projected productivities (P, in metric t/ha/yr) and values (V, in US$/ha/yr) of commodities generated by marine (M) and freshwater (FW) macroalgae through different scenarios including conversion to biodiesel (1), to biocrude (2), extraction of protein (3), and HTL conversion of residual biomass to biocrude after protein extraction (4). Theoretical values of protein extract plus biocrude from residual biomass (5) is also presented. Products prices are derived from equivalent commodities prices (see Methods section 2.2.10). Note that theoretical values (V) are rounded to the nearest $100 for each scenario.

Scenario			1	2	3	4	5
Commodity			Biodiesel	Biocrude	Protein	Biocrude - Protein	3 + 4
Price (US$/t)			941	682	432	682	
Species	Source						
Derb.	M	P	1.8	7.1	9.4	5.4	
		V	$1,700	$4,800	$4,100	$3,700	$7,700
Ulva	M	P	0.6	4.6	6.8	3.4	
		V	$600	$3,100	$2,900	$2,300	$5,200
Chaet.	M	P	0.7	4.0	4.0	3.3	
		V	$700	$2,700	$1,700	$2,300	$4,000
Clad.	M	P	0.8	4.3	5.5	3.3	
		V	$700	$2,900	$2,400	$2,200	$4,600
Oedog.	FW	P	0.8	3.3	4.2	2.5	
		V	$800	$2,300	$1,800	$1,700	$3,500
Clad.	FW	P	0.6	2.0	3.4	1.4	
		V	$600	$1,300	$1,400	$900	$2,400

2.3.9. Projected production values - Sensitivity analysis

Sensitivity analyses were used to predict the relative influence of different parameters on the value of the feedstock (US$/ha/yr) for the most valuable marine species, *Derbesia* and *Ulva,* (Fig. 2.4a,b) and the most valuable freshwater species, *Oedogonium* (Fig. 2.4c). The most valuable processing scenario, the sequential pre-extraction of proteins and subsequent HTL of residual biomass to biocrude (scenario 5 in Table 2.6), was used for each species. Therefore, the parameters for each sensitivity analysis were biomass productivity, protein content of the biomass, theoretical biocrude yield, and the commodity prices for biocrude and protein (Annex 1, Table S2.2).

Under standard conditions (centre lines, Fig. 2.4), *Derbesia* had a higher projected value ($7,700/ha/yr) than *Ulva* ($5,200/ha/yr) and *Oedogonium* ($3,500/ha/yr). The influence of each parameter was also assessed in both favourable and unfavourable conditions to assess the potential range of the feedstock value relative to the empirical values in the literature or potential fluctuations in market prices. Biomass productivity was the most influential parameter that could potentially double the value of *Derbesia* and *Ulva*, and triple the value of *Oedogonium* when higher biomass productivities of > 15 $g/m^2/d$ (dw) are achieved at larger scale (Annex 1, Table S2.2). Theoretical biocrude yield was the second most influential parameter that could increase the value of *Derbesia* by 24%, of *Ulva* by 22% and of *Oedogonium* by 25%, assuming that HTL optimisation translates to maximum yields of 12.2 to 20.6% using the residual biomass after protein extraction. The other parameters – protein content, and biocrude and protein prices – had a lesser impact on the projected feedstock value.

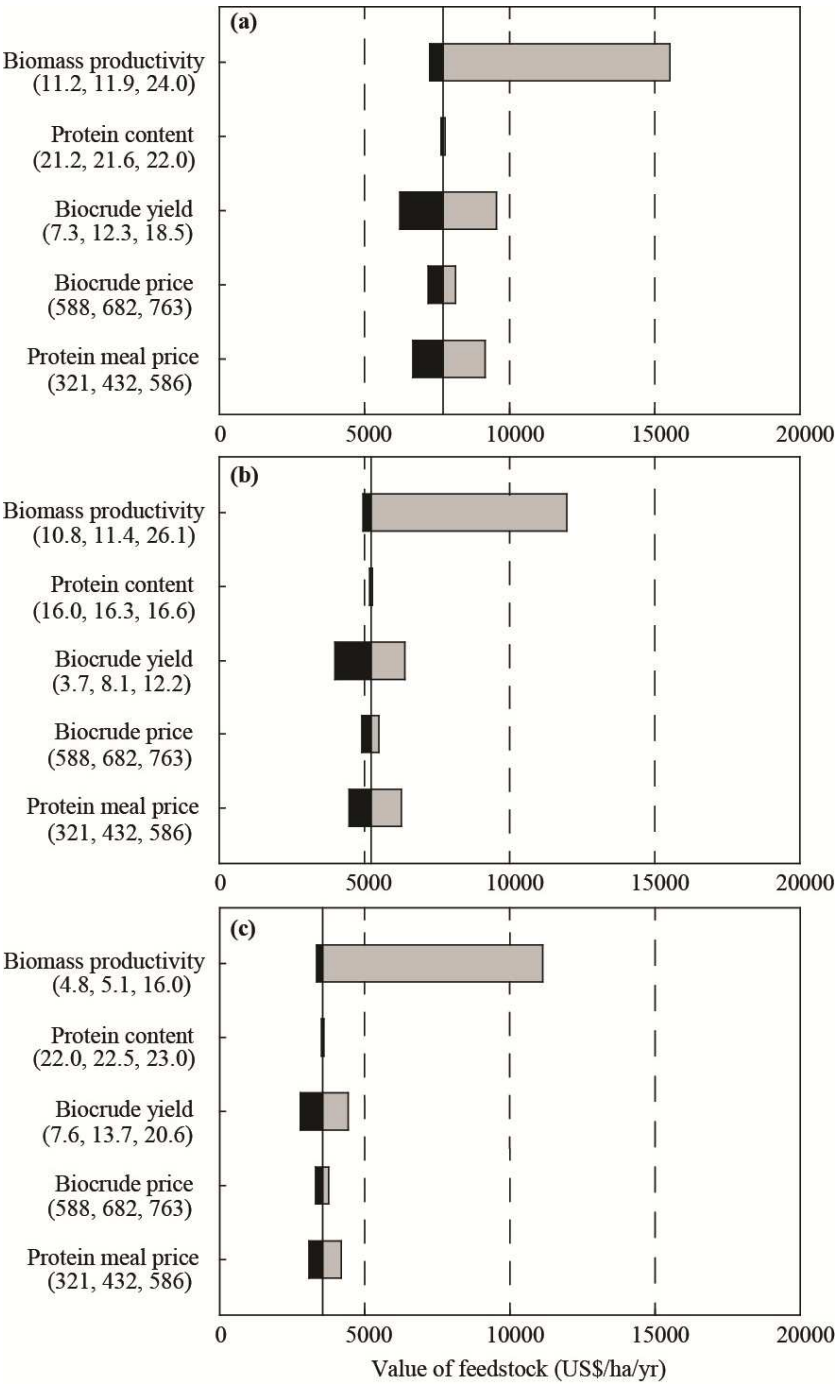

Figure 2.4. Sensitivity analysis. Sequential protein extraction followed by conversion of residual biomass to biocrude for marine *Derbesia* (a), marine *Ulva* (b) and freshwater *Oedogonium* (c). Variation in the value of selected feedstock (US$/ha/yr) is associated with the variation of each parameter while the other parameters remain the same. Values for each parameter are indicated in brackets (unfavourable, standard, favourable).

Notably, if all favourable conditions were summed for each parameter, the projected ceiling value per ha per year of *Derbesia* would reach $23,600/ha/yr, *Ulva* would reach $18,100/ha/yr and *Oedogonium* would reach $17,100/ha/yr.

2.4. Discussion

Of the two theoretical pathways considered in the present study to convert biomass to high-energy biofuel, the HTL of biomass to biocrude was more attractive than the extraction and esterification of fatty acids to biodiesel. Higher theoretical yields were achieved through HTL as the whole organic fraction of biomass is used in the conversion, including proteins, carbohydrates, and the entire lipid component (Frank et al., 2013). Importantly, the sequential extraction of proteins and subsequent conversion of the residual biomass by HTL could add significant value to the feedstock. This multiple or sequential product approach is considered to be critical for the viability of biofuel applications for microalgae (Vardon et al., 2011; Chakraborty et al., 2012; Miao et al., 2012) but to date there have been no empirical analyses of co-products from macroalgae and, more specifically, no analysis of the sequential extraction of protein followed by conversion to biocrude. However, this option needs to be considered on a species by species basis as protein content generally varies substantially between species (Lourenço et al., 2002) as exemplified by the significant differences between related green macroalgae in this study. Although freshwater macroalgae had a higher theoretical yield of biocrude and higher protein content, marine macroalgae had higher projected productivities of both biocrude and protein per unit area of production. The importance of this "areal" metric is highlighted in the sensitivity analyses for marine *Derbesia* and *Ulva* and freshwater *Oedogonium*, in

which biomass productivity is the single most influential parameter for feedstock value for macroalgal cultivation at scale.

2.4.1. Biomass productivity

Of the six species of green macroalgae considered in the present study, marine macroalgae had higher biomass productivities than freshwater macroalgae under identical culture conditions. The biomass productivity of marine *Derbesia* (43 t/ha/yr, dw) was similar to sugar beet (Renouf et al., 2008) and twice that of the promising industrial biomass crop *Miscanthus* (20 t/ha/yr, McKendry, 2002a). Furthermore, the carbon productivity of *Derbesia* equated to 13 t C/ha/yr, which is similar to or higher than most land crops (Stephens et al., 2013), irrespective of the higher ash content in macroalgae. In contrast, freshwater macroalgae had lower biomass productivities (12 - 18 t/ha/yr), yet were typically twice the average annual biomass productivity of soybean (6 - 8 t/ha/yr) (Salvagiotti et al., 2008). Most importantly, however, marine *Derbesia* and *Ulva* cultured at scale have biomass productivities that exceed 20 $g/m^2/d$ (dw), or effectively > 73 t/ha/yr (Bolton et al., 2009; Magnusson et al., 2014), while freshwater *Oedogonium* at scale has values twice that of the present study exceeding 16 $g/m^2/d$ (dw), or effectively > 55 t/ha/yr (Cole et al., 2014). These high biomass productivities at scale highlight the conservative nature of the data presented in this study, and justify the use of higher favourable values in the sensitivity analyses. Biomass productivities contrast with terrestrial crops due, in part, to the filamentous or leaf-like structure of green macroalgae that provides a uniform morphology with no differentiation of tissues and, therefore, all cells within the biomass are photosynthetic. Furthermore, this homogeneity of cells within marine and freshwater

filamentous green macroalgae translates into a homogenous feedstock for biomass applications.

2.4.2. High-energy liquid fuels

Notably, the potential applications for macroalgal biomass are a direct function of the biomass productivities and their biochemical profiles. As an outcome, the species with the highest lipid content, specifically the marine *Derbesia* and freshwater *Oedogonium*, had the highest theoretical yields of biocrude (16 - 18%, dw). The biochemical profiles of the selected macroalgae were similar in composition to the model compounds used by Biller and Ross (2011) for determining the individual conversion factors of lipid, protein and carbohydrate. In particular, carbohydrates as the major biochemical component in green macroalgae correspond with the model compounds of starch and glucose used in the equation (Biller & Ross, 2011). This supports the use of these factors to calculate the theoretical biocrude yields. Additionally, these theoretical yields were comparable to the yields obtained from the HTL of green and brown macroalgae (Zhou et al., 2010; Anastasakis & Ross, 2011), but noticeably lower than the yields obtained from a range of microalgae (26 - 57%; Annex 1, Table S2.3). The projected biodiesel yields were less attractive than for biocrude due to the generally lower fatty acid contents of green macroalgae compared to other seaweeds (Gosch et al., 2012). Although HTL represents a more efficient utilisation of all organic components of the biomass, a number of hurdles remain for the commercialisation of this technology including a reduction in the energy requirements to operate at high temperature, a reduction of the hydrogen demand for biocrude upgrading, and an efficient method for nitrogen recycling (Frank et al., 2013). In

contrast, while biodiesel production is a less effective process for deriving high-energy fuels from macroalgae, this technology is commercial and can be integrated with alternative bioenergy production including, for example, anaerobic digestion of residual biomass after fatty acid extraction (Chisti, 2007; Krohn et al., 2011). However, biodiesel derived from green macroalgae will likely contain higher oxygen contents than biocrude, further increasing the hydrogen demand required for upgrading (Frank et al., 2013). It also appears that the high proportions of PUFA, that are detrimental to the quality of biodiesel due to increased rates of oxidation during storage (Chisti, 2007), represent a major hurdle to the production of biodiesel from green macroalgae. In a similar way, biocrude from algae, while consistent in quality (see typical elemental composition in Annex 1, Table S2.3), contains high amounts of nitrogen compared to conventional crude oil, which represents an issue for refining (Jazrawi et al., 2013). However, the pre-extraction of protein from biomass would facilitate the removal of the majority of nitrogenous organic compounds that would otherwise influence the nitrogen content of the resulting crude (Peterson et al., 2008; Toor et al., 2011). Therefore, the sequential extraction of protein followed by HTL conversion of the residual biomass could ensure the highest quality of the respective products in a way that would not otherwise be achieved through the single use of the biomass for either biofuel or protein meal. In this scenario, the higher proportion of carbohydrates and lipids compared to the original feedstock could also enable fine-tuning of the HTL settings, for example, through the use of catalysts such as Na_2CO_3 that could double the yield of biocrude (Biller & Ross, 2011). Furthermore, the HTL co-products of this process (biochar, aqueous and gas products) may offer additional

opportunities to increase the value of macroalgal feedstock in commercial production (Biller & Ross, 2012).

2.4.3. Protein

The development of efficient separation technology for multiple product streams will be critical for algae (Chakraborty et al., 2012) but could potentially be achieved in the same facility, for example, using mild HTL conditions to extract proteins and then altering conditions to process the remaining organic material to biocrude (Yoshida et al., 1999; Biller & Ross, 2012). The protein extracts of green macroalgae could potentially complement terrestrial plant protein (soybean) meal in food and animal feed industries (Lammens et al., 2012). All six species of green macroalgae had a high proportion of the two most limiting amino acids in livestock diet, methionine and lysine (Boland et al., 2013). The protein extract of *Derbesia*, *Ulva* and *Oedogonium* contained 2.1%, 1.6% and 1.9% of methionine and 6.8%, 5.4%, 6.7% of lysine, respectively (Table 2.5). This is comparable to soybean meal at 0.9% methionine and 2.8% lysine (Glencross et al., 2007), assuming that soybean meal contains ~50% crude protein (Glencross et al., 2007; Lywood et al., 2009). Furthermore, the relative amount of methionine to lysine for *Derbesia* (0.31), *Ulva* (0.30) and *Oedogonium* (0.28) is within the range of 0.27 to 0.38 and is therefore suitable for humans, pigs and poultry (Boland et al., 2013).

2.4.4. Alternative bio-products for bio-refinery

The strategy of sequential treatment of biomass to derive multiple co-products (the bio-refinery concept) is arguably the most important aspect for the development of biofuels

more broadly, including from microalgae and terrestrial biomass crops (Fatih Demirbas, 2009; Foley et al., 2011). It is also notable that thermochemical conversion such as HTL could yield additional "niche" products rather than just commodities that would enable higher returns for the same biomass, for example, by targeting valuable polysaccharides (Chakraborty et al., 2012). Green macroalgae have high proportions of carbohydrates, mostly in the form of glucose-based cellulose and starch that are involved in cell wall formation and energy storage, respectively (Lobban & Harrison, 1996). However, there are also high-value polysaccharides unique in form and function that could be recovered from the biomass prior to HTL, the most prominent examples being sulfated polysaccharides such as fucoidan in brown seaweeds (kelps) and ulvan in *Ulva* (Lahaye & Robic, 2009). Similarly, non-free fatty acid lipids such as pigments, phospholipids, glycolipids, and other neutral lipids such as residual triglycerides, sterols and free alcohols could be recovered from biodiesel production and be used as feedstock for further HTL processing or targeted specifically for high-value nutraceuticals (Krohn et al., 2011; see also Annex 1, Table S2.1). These niche-market nutraceutical products offer the opportunity to bridge the technology gap for biomass production by justifying the development of larger culture systems and fast-tracking the expected economies of scale to compete with commodity biomass (ARENA Report, 2012).

2.4.5. Limitations and perspectives

There are considerable limitations for the development of algae-based biofuels, including the technical developments for efficiencies in conversion and refining (Biller & Ross, 2012; Rowbotham et al., 2012). However, the present and recent studies highlight that

biomass production is a key limiting step, which includes the selection of robust species and the scale-up of operations on non-arable land (Lawton et al., 2013a; Stephens et al., 2013). There are both benefits and problems associated with land-based production of marine and freshwater macroalgae. Marine macroalgae are typically larger than freshwater macroalgae and therefore simpler to handle (see images of *Derbesia*, *Ulva* and *Oedogonium* in Fig. 2.5) but may require the removal of salts through freshwater rinsing, which is an additional cost. In contrast, freshwater macroalgae are relatively low in salt and higher in carbon than marine macroalgae, and can be cultured on marginal land or in aquatic waste streams (Mulbry et al., 2008; Pittman et al., 2011; Saunders et al., 2012; Lawton et al., 2013a; Cole et a., 2014). However, freshwater macroalgae have consistently lower biomass productivities than marine macroalgae. Notably, strain selection and selective breeding offer clear opportunities to deliver tailored crops, with the added benefit that the macroalgae production is a continuous process in comparison to the fixed cycles of terrestrial crops.

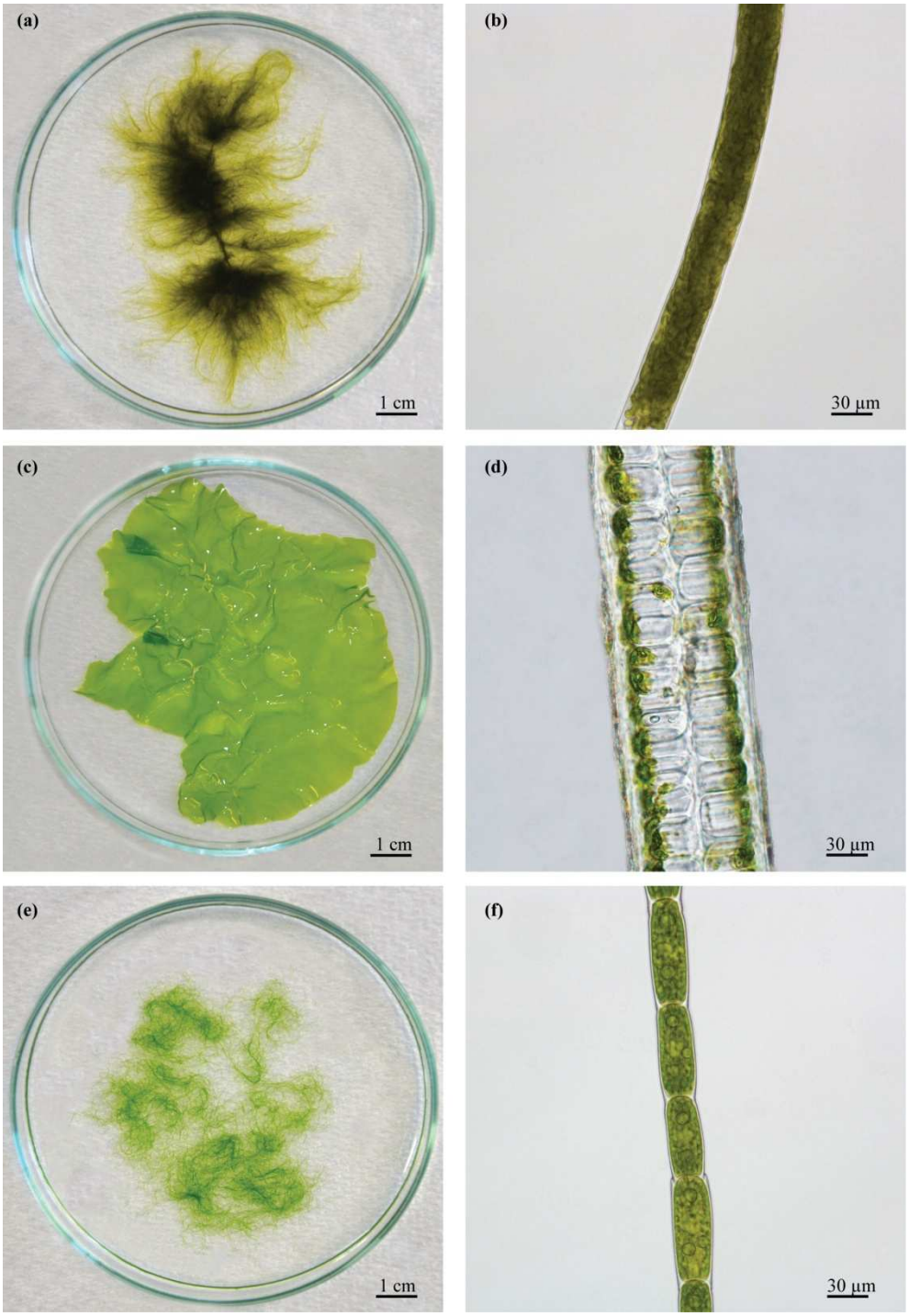

Figure 2.5. Specimen photos of *Derbesia tenuissima* (a,b), *Ulva ohnoi* (c,d), and *Oedogonium* sp.(e,f) showing growth habit in culture (Nikon D7000) (a,c,e) and cellular detail at 400x magnification (Olympus DP73 camera connected to Olympus BX53 microscope) (b,d,f; note that *Ulva* is a transverse section).

2.5. Conclusions

A major outcome of this chapter is the identification of two novel species of filamentous macroalgae, marine *Derbesia* and freshwater *Oedogonium*, alongside the well-established marine *Ulva* (Fig. 2.5), for the production of biocrude. While I highlight the sequential production of protein and biocrude as an important driver to increase feedstock value, it is clear from the sensitivity analyses that key drivers to deliver high value per unit area are biomass productivity and HTL technology optimisation.

Chapter 3

Biocrude yield and productivity from the hydrothermal liquefaction of marine and freshwater green macroalgae[2]

3.1. Introduction

The decline in fossil fuel reserves and increasing greenhouse gas emissions necessitate the development of alternative and sustainable energy sources. Phototrophic biomass has been identified as a primary feedstock for energy capture and the production of renewable liquid fuels (Perlack et al., 2005). Critical factors in the selection of a biofuel feedstock are productivity, scalability and a continuous supply of biomass. Given these criteria, macroalgae are particularly suitable as they are highly productive, are produced at scale and can be delivered as a continuous feedstock supply (Chopin & Sawhney, 2009). Furthermore, macroalgae can be grown within a broad range of environments, from the open ocean through to land-based tanks and ponds production systems, and do not require arable land for cultivation thereby avoiding the highly contentious food *versus* fuel debate (Pimentel et al., 2009). Notably, production systems can also be integrated into an industrial ecology framework where the culture of algae in wastewater provides bioremediation applications in agriculture (Mulbry et al., 2007), mineral processing

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[2] **Chapter 3** is adapted from Neveux N, Yuen AKL, Jazrawi C, Magnusson M, Haynes BS, Masters AF, Montoya A, Paul NA, Maschmeyer T, de Nys R, 2014b. Biocrude yield and productivity from the hydrothermal liquefaction of marine and freshwater green macroalgae. *Bioresource Technology*, **155**, 334-341.

(Saunders et al., 2012), and aquaculture (de Paula Silva et al., 2008; Mata et al., 2010a). The combination of biomass production with these established industries enables macroalgae to become a bioresource for the production of renewable fuels and co-products (Ragauskas et al., 2006). However, the application of algae for renewable fuels is also dependent on the biochemical composition of the biomass, as this directly affects the quantity and quality of the fuel (Rowbotham et al., 2012). The culture medium and environmental conditions affect the biochemical properties of macroalgae significantly and interactively (Saunders et al., 2012; Angell et al., 2014), and hence the subsequent conversion of algal biomass to energy (Bruhn et al., 2011) and renewable fuels (Rowbotham et al., 2012).

Renewable fuels can be produced through the refining of a liquid biocrude produced through the thermochemical conversion of biomass, either through hydrothermal liquefaction (HTL) or pyrolysis (Rowbotham et al., 2012). HTL is a medium temperature process (200 - 374^{o}C), carried out under sufficient pressure to maintain water in a liquid state. Under subcritical conditions, water participates in a series of complex reaction cascades with macromolecules including hydrolysis, fragmentation, aromatisation, dehydration and deoxygenation to produce lower molecular weight compounds resulting in liquid biocrude, solid biochar, aqueous and gaseous fractions (Toor et al., 2011). For algal biomass, HTL offers several advantages over pyrolysis through the use of wet biomass thereby avoiding energy losses associated with drying, and also through enhanced reaction rates and an efficient separation of products (Peterson et al., 2008). In addition, HTL delivers a high-energy biocrude (30 - 40 MJ/kg) that is lower in oxygen and moisture content compared to pyrolysis biocrude and, therefore, provides a more stable product

(Peterson et al., 2008; Bridgwater, 2012). This biocrude can subsequently be refined to deliver a diversity of 'drop-in' renewable fuels ranging from petroleum to aviation fuel (Larson, 2006).

The combination of high biomass productivities for macroalgae, comparable to highly productive terrestrial crops (Larson, 2006; Kraan, 2013), and efficient conversion processes such as HTL (Aresta et al., 2005), provides a new focus on high-energy fuels derived from algal biomass. The first critical step in assessing and selecting species of macroalgae for the production of biomass and subsequent conversion to advanced high energy biofuels is the quantitative comparison of biomass productivity and biochemical composition (Chapter 2). The second step is to quantify the conversion of this biomass to biocrude through the HTL process and assess the quality of this biocrude. The third and final step is to quantify and compare biocrude productivities, specifically the mass of biocrude produced per unit area of culture per unit of time.

Therefore, the aim of this chapter was to provide a direct comparison of the yield and elemental composition of biocrude and co-products resulting from HTL of marine and freshwater macroalgae, and then compare their biocrude productivities. To do this, four marine and two freshwater green macroalgae were cultivated in outdoor tanks simultaneously (Chapter 2). These species were selected based on a combination of their ability to be cultured in land-based systems, high productivities, resistance to contamination and a high tolerance to environmental fluctuations (de Paula Silva et al., 2008; Lawton et al., 2013a; Angell et al., 2014). This approach differs substantially from previous studies, where macroalgal biomass was collected from natural environments or

cultivated under laboratory conditions and enables a true comparative assessment of macroalgae originating from different environments in terms of biocrude production.

3.2. Materials and methods

3.2.1. Algae collection

Six species of green macroalgae (Chlorophyta) were selected for this study including four species of marine macroalgae, *Derbesia tenuissima* (Crouan), *Ulva ohnoi* (Hiraoka and Shimada), *Chaetomorpha linum* (Kutzing), *Cladophora coelothrix* (Kutzing) and two species of freshwater macroalgae, *Cladophora vagabunda* (Hoek), *Oedogonium* sp. (Lawton *et al.*, 2013a), hereafter referred to by origin and genus, e.g. marine *Cladophora*. Refer to Chapter 2 for details. All species were maintained as stock cultures in outdoor tanks at the Marine & Aquaculture Research Facilities Unit at James Cook University (Townsville). In these tanks, macroalgae species underwent fundamental changes in terms of composition and morphology compared to their natural environment (data non-reported), due to the changes of the environmental conditions. For this reason, macroalgae were maintained in tanks for a period of at least 3 months for acclimation, prior to the start of the experiment.

3.2.2. Algae culture

The experimental culturing of algae was conducted in an outdoor tank-based recirculation system, where productivity of marine and freshwater species was compared on a dry weight basis. Each species was cultured in triplicates at 2 g/L fw in 50 L tanks, with a 0.25 vol/d water exchange rate. Experimental culturing of algae was carried on for three cycles

of 6 days and biomass was restocked at 2 g/L for each new cycle while the excess biomass was discarded. After 18 days, all biomass in each tank was harvested using a fish net (2 mm screen) and the biomass productivity was calculated on a dry basis. This biomass was freeze-dried, ground to a mean particle size of < 500 µm and stored in air-tight vials. Proximate, biochemical and ultimate analysis were performed on the dry biomass and the results are listed in Table 3.1. Refer to Chapter 2 for details on macroalgae culture and analyses.

Table 3.1. Proximate, biochemical and ultimate analysis of marine (M) and freshwater (FW) macroalgae.

Data show means (n = 3) of content dry weight of marine (M) and freshwater (FW) macroalgae.

Species	*Derb.*	*Ulva*	*Chaet.*	*Clad.*	*Oedog.*	*Clad.*
Source	M	M	M	M	FW	FW
Proximate (wt%)						
Ash	34.7	30.7	36.6	25.5	20.6	17.8
Moisture	6.4	7.2	5.1	6.7	6.5	5.7
Biochemical (wt%)						
Lipid	10.4	1.9	3.3	4.6	9.4	5.3
Protein*	21.6	16.3	11.1	17.8	22.5	26.8
Carbohydrate**	26.9	43.9	43.9	45.4	41.0	44.4
Ultimate (wt%)						
C	29.2	27.7	26.5	30.9	36.6	37.5
H	4.8	5.5	4.1	5.0	5.7	5.9
O	27.4	41.1	31.0	34.9	30.9	32.9
N	4.5	3.5	3.4	5.2	4.8	6.5
S	2.8	5.0	2.1	2.3	0.4	1.8
HHV (MJ/kg)	12.4	11.7	10.3	12.7	15.8	16.4

*sum of amino acids; **determined by difference; HHV = higher heating value.

3.2.3. Hydrothermal processing

Hydrothermal liquefaction of macroalgae was performed on each of the three replicates of the six species, for a total of 18 runs. HTL was performed using a custom-built stainless steel reactor system, assembled from commercially available components (Swagelok Company, Australia) and the setup is illustrated in Fig. 3.1. A slurry (6.6% solids) composed of 1 g of dry, powdered algae and 14 mL of distilled water was loaded in the 20 mL stainless steel tube reactor for each run. The reactor was subsequently fitted with a gasket and attached to the pressure-head, specifically engineered to handle pressures up to 25 MPa at 350^{o}C. The head-space of the system was purged with nitrogen 3 times, then pressurised with nitrogen to 9 MPa at ambient temperature, to ensure that the aqueous phase would remain liquid at high temperatures and to minimise the transport of vapour from the reactor into the connecting tubing, after the reactor was heated and began to generate steam pressure. A vapour reducer (50 mm length of tubing, 1.75 mm internal diameter; clearance between tube and thermocouple of 0.165 mm) served to dampen pressure spikes and inhibit diffusive interchange between the reactor and the cold tubing. The reactor was subsequently immersed in a pre-heated fluidised sand bath (model SBL-2D; Techne, UK) set to 350^{o}C to initiate the HTL process. Typically, the internal temperature (determined by an internal thermocouple) rose on average to 262^{o}C (11.9 MPa) within 1 minute, 310^{o}C (13.1 MPa) within 2 minutes and 325^{o}C (13.8 MPa) within 3 minutes of reaction time. Internal reaction temperatures between 330^{o}C and 341^{o}C (maximum temperature) (14 - 17 MPa) were maintained for a further 5 minutes (total of 8 minutes reaction time) before the reactor was quenched in an ice/water bath for 1 minute to cool the reactor to room temperature.

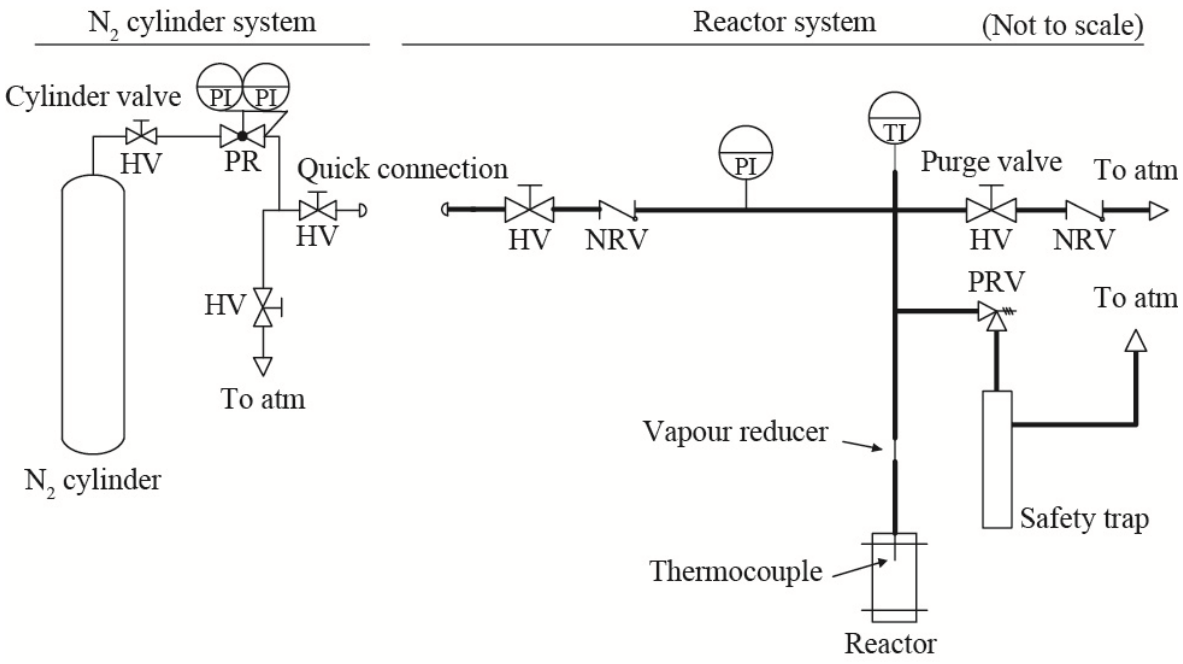

Figure 3.1. Schematic representation of the HTL system.

HV = hand valve, PR = pressure regulator, PI = pressure indicator, TI = temperature indicator, NRV = non-return valve, PRV = pressure relief valve, atm = atmosphere.

3.2.4. Products separation and analysis

Use of the batch reactor system described above did not permit analysis of the gas produced by the reaction as this phase was vented inside a fume hood immediately following the reaction quench and prior to disassembly. In contrast, the condensed phases were separated and analysed. The reaction mixture (minus gas product) was diluted with dichloromethane (DCM) and distilled water (25 mL each), suction filtered over Whatman grade 2 paper and the residue was further washed with DCM and water, followed by drying at 80^{o}C for 12 hours to afford a dry solid fraction (biochar). The biphasic filtrate was transferred to a separation funnel to isolate the aqueous phase and the biocrude phase (dissolved in DCM). The aqueous phase was further washed twice with 25 mL DCM. The

DCM phase and washings were subsequently combined, concentrated under reduced pressure at 40^{o}C and 451 mbar and vacuum dried at 50^{o}C and 23 mbar in a rotary evaporator to give a dark brown oil (biocrude). Biocrude and biochar yields were calculated separately using the following equation:

$$Y_{PRODUCT} = W_{PRODUCT} / W_{FEEDSTOCK} * 100\% \quad \textbf{Eq. 3.1}$$

where $Y_{PRODUCT}$ is biocrude or biochar yield (wt%) on a dry weight basis, $W_{PRODUCT}$ is the mass of product (g) and $W_{FEEDSTOCK}$ is the mass of macroalgal feedstock used (g). In addition, biocrude yield was calculated on an ash-free dry weight basis where $W_{FEEDSTOCK}$ was replaced by $W_{ORGANIC\ BIOMASS}$ ($W_{ORGANIC\ BIOMASS} = W_{FEEDSTOCK}$ – ash content – moisture content) in Eq. 3.1 (see also Eq. 4.1). The elemental composition of biocrude and biochar was analysed externally (OEA Laboratory Ltd., UK) to determine the carbon, hydrogen, oxygen, nitrogen and sulfur contents on a dry weight basis. The HHV of biocrude and biochar was calculated from their ultimate analysis using the unified correlation proposed by Channiwala and Parikh (2002). The extracted aqueous phase was diluted to 100 mL volumetrically for subsequent quantification of total organic carbon (TOC) and total nitrogen (TN) (Trop-Eco Laboratory, JCU, Australia).

3.2.5. Energy recovery and mass balance

The chemical energy recovery (ER) was calculated for the biocrude and biochar phases according to the following equation:

$$ER = (HHV_{PRODUCT} * W_{PRODUCT}) / (HHV_{FEEDSTOCK} * W_{FEEDSTOCK}) * 100\% \quad \textbf{Eq. 3.2}$$

where ER is the energy recovery of HTL products (%), $HHV_{PRODUCT}$ is HTL products higher heating value (MJ/kg), $W_{PRODUCT}$ is the mass of HTL products (g), $HHV_{FEEDSTOCK}$ is

the macroalgae higher heating value (MJ/kg) and $W_{FEEDSTOCK}$ is the mass of macroalgae used (g). A similar equation was used to determine the carbon and nitrogen recoveries in product streams, specifically the mass balances of carbon and nitrogen for each isolated condensed phase, by substituting HTL products and feedstock HHV with carbon or nitrogen contents. The remaining carbon and nitrogen fractions allowed an estimation of the mass partitioned to the combined gas phase and losses.

Biocrude productivity was determined for each species by multiplying biocrude yield with biomass productivity (from Chapter 2), using Eq. 2.4.

3.3. Results and discussion

3.3.1. Yields of HTL products

Biocrude yields from HTL of freshwater and marine macroalgae are presented on both dry weight and ash-free dry weight (afdw) basis in Table 3.2. The freshwater species *Oedogonium* (26.2% dw) and *Cladophora* (19.7% dw) had the highest biocrude yield based on dry weight in accordance with their lower ash content, and therefore higher organic content. Marine *Derbesia* (19.7% dw) had the highest yield of the marine species, identical to that of freshwater *Cladophora* despite having twice the ash content, followed closely by *Ulva* (18.7% dw). In contrast, marine *Chaetomorpha* (9.7% dw) had the lowest biocrude yield, partly due to its high ash content. The proportion of inorganic content (ash + moisture) is a detrimental factor for the liquefaction of macroalgae as it constitutes a fraction with no calorific value, and therefore reduces the biocrude yield for the same quantity of feedstock processed. Moreover, ash causes slagging and fouling issues in large-

scale continuous flow reactors due to the presence of alkali metals, earth alkaline metals or halides (Peterson et al., 2008; Jazrawi et al., 2013).

Table 3.2. Products yield and biocrude productivity of marine (M) and freshwater (FW) macroalgae.

Data show means (n = 3 ± SE) of yield of marine (M) and freshwater (FW) macroalgae.

Species	*Derb.*	*Ulva*	*Chaet.*	*Clad.*	*Oedog.*	*Clad.*
Source	M	M	M	M	FW	FW
Yield (wt%, dw)						
Biocrude	19.7 ± 1.6	18.7 ± 0.8	9.7 ± 0.4	13.5 ± 1.0	26.2 ± 2.6	19.7 ± 1.8
Biochar	8.1 ± 1.4	12.1 ± 1.1	8.4 ± 1.6	10.4 ± 0.8	10.2 ± 2.5	18.7 ± 2.9
Aqueous + Gas*	72.2 ± 2.2	69.2 ± 0.3	82.0 ± 1.6	76.1 ± 0.4	63.6 ± 2.6	61.7 ± 3.3
Biocrude *(afdw)*	33.4 ± 2.7	30.1 ± 1.3	16.6 ± 0.7	20.0 ± 1.5	35.9 ± 3.6	25.7 ± 2.3
Productivity ($g/m^2/d$, dw)						
Biocrude	2.4 ± 0.3	2.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	0.7 ± 0.1

*determined by difference; dw = dry weight; afdw = ash-free dry weight.

The species of macroalgae tested here also had a wide range of lipid, protein and carbohydrate contents within their organic fraction, regardless of whether they originated from a freshwater or marine environment. Generally, a higher organic carbon content delivered a higher yield of biocrude. Freshwater *Oedogonium* (35.9% afdw) and marine *Derbesia* (33.4% afdw) had the highest biocrude yields of all species based on ash-free dry weight, as they had the highest proportion of lipids that are efficiently converted to biocrude through the rapid hydrolysis of triglycerides at temperatures of 330 to 340^{o}C (King et al., 1999; Wang et al., 2013). The choice of operating HTL at temperatures of 330 - 340^{o}C with fast heating and cooling rates also ensured not only the decomposition of proteins and carbohydrates, but also favoured the formation of liquid hydrocarbons rather than solid or gaseous compounds due to overprocessing (Peterson et al., 2008; Torri et al.,

2013). In addition, there are specific reaction mechanisms that influence the conversion of individual species. For example, at temperatures above 300^{o}C, the low lipid content of freshwater *Cladophora* was compensated for by a high amount of protein, which contributes to the formation of biocrude when it degrades into low molecular weight compounds (Torri et al., 2013). Notably, among marine species, *Ulva* had a lower lipid and protein content than marine *Cladophora*, but still produced more biocrude (30.1% afdw) than did *Cladophora* (20.0% afdw) suggesting interlinked reaction cascades between specific biochemical compounds, for example Maillard-type or condensation reactions. These reactions result in the formation of hydrophobic compounds which contribute to the overall yield of the dichloromethane soluble fraction as biocrude (Torri et al., 2013).

Biocrude yields obtained in this study were similar to those produced from HTL of the green marine macroalga *Ulva prolifera* (23.0% dw, Zhou et al., 2010) and the brown marine macroalga *Saccharina latissima* (19.3% afdw, Anastasakis & Ross, 2010) and were somewhat lower than yields obtained for a range of microalgae (26-57% dw, reviewed in López Barreiro et al., 2013). This difference in biocrude yield between microalgae/cyanobacteria and macroalgae has been highlighted by the co-liquefaction of *Spirulina platensis* and *Ulva prolifera*, where the yield of biocrude increases with the ratio of microalgae to macroalgae (Jin et al., 2013). The higher yields achieved by microalgae are generally attributed to a higher lipid content, compared to macroalgae that have commonly higher carbohydrate content. Importantly, there are mechanisms to increase biocrude yield through the use of catalysts, such as Na_2CO_3 (Zhou et al., 2010), that are particularly suited to the conversion of macroalgae due to the specific enhanced conversion of carbohydrates (Ross et al., 2010; Biller & Ross, 2011). However, a catalyst such as

Na_2CO_3 is detrimental to the conversion of lipids and should therefore be considered on a species by species basis (Biller & Ross, 2011; Wang et al., 2011). In addition, a reduction of ash in biomass through either selected culture or post-harvest processing prior to HTL will enhance biocrude yields per unit biomass.

Generally, the biocrude yield obtained from HTL of macroalgae in this study did not fit the additive conversion model of lipid, protein and carbohydrate compounds proposed by Biller and Ross (2011) for microalgae (Chapter 2). However, underestimates were also found for some microalgae (e.g. *Nannochloropsis* sp.) and a cyanobacterium (*Spirulina platensis*) processed under identical conditions (López Barreiro et al., 2013). Maximum theoretical biocrude yields calculated from the biochemical composition of the algae used in Chapter 2 fitted the model for marine *Chaetomorpha*, within the fit of the theoretical model, and with an acceptable margin of error (< 5%). However, maximum theoretical yields were underestimated by 22 - 56% for the remaining species. The most notable difference is the high biocrude yield obtained for *Ulva* (18.7%), which contained low lipids (< 2%), compared to the maximum theoretical yield (11.1%). This discrepancy is again most likely due to the model not accounting for biocrude produced through the interactions between biochemical compounds, on top of individual additive conversion yields.

Biochar yields were also quantified following the separation of products and were found to vary between species, with no correlation with origin (freshwater or marine). Yields ranged from 8.1% dw for marine *Derbesia* to 18.7% dw for freshwater *Cladophora* (Table 3.2). These values are comparable to biochar yields reported for the HTL of other species of

macroalgae processed at comparable operating conditions (5 - 25%, Zhou et al., 2010; Anastasakis & Ross, 2011), and somewhat higher than values found for a range of microalgae (Frank et al., 2013). This suggests that the greater proportions of carbohydrates in macroalgae have a positive impact on char formation, as shown by Biller and Ross (2011), using the liquefaction of the model compounds glucose and starch.

The remaining aqueous and gas yields, and eventual losses during products separation, were combined and calculated by difference. These ranged from 61.7% for freshwater *Cladophora* to 82.0% for marine *Chaetomorpha*. This is in accordance with previous results for the HTL of algae where most of the mass balance is recovered in the aqueous phase, while gaseous products generally account for 5 - 25% for the same operating temperature (Biller & Ross, 2011; Garcia Alba et al., 2012).

3.3.2. Elemental composition of HTL products

The elemental composition of biocrude, biochar and aqueous products obtained from the liquefaction of macroalgae was characterised through the ultimate analysis of biocrude and biochar on a dry basis, and through TOC and TN analysis of the aqueous phase, and the elemental compositions of these are presented in Table 3.3. Although biocrudes varied significantly between species in terms of yield, their elemental compositions were very consistent regardless of the feedstock. All species generated a biocrude composed of 71 - 73% carbon, 7 - 8% hydrogen, 10 - 11% oxygen, 6 - 7% nitrogen and 0 - 1% sulfur, which is comparable to the range of values reported in the literature for the liquefaction of macro- (Zhou et al., 2010; Anastasakis & Ross, 2011) and microalgae (Frank et al., 2013). There was only a minor variation (< 2%) in the carbon content of the biocrude between species,

with the highest carbon content in marine *Derbesia* (73.0%), *Ulva* (72.6%) and freshwater *Oedogonium* (72.1%), and the lowest content in marine *Chaetomorpha* (70.9%). The energy value of biocrudes was therefore also consistent across species, corresponding to a HHV of 33 - 34 MJ/kg. Among all six species investigated, the largest relative differences in the elemental composition of the biocrude were for the content of nitrogen and sulfur. The nitrogen content of biocrude varied from 5.8% for *Ulva* to 7.1% for marine *Cladophora*. The accumulation of nitrogen in the biocrude fraction was proportionally higher for those species that contained lower levels of nitrogen in their biomass. Values for the nitrogen content of biocrude reported in this study are consistent with a range of values obtained for the liquefaction of other macro- and microalgae species, where nitrogen varies between 5% and 8%, with the exception of *Nannochloropsis* that yields a biocrude particularly low in nitrogen (4 - 4.5%) in relation to its protein content (Biller & Ross, 2011; Duan & Savage, 2011a). The presence of nitrogen remains one of the main concerns for the quality of biocrude produced through HTL as high nitrogen levels in biocrude increase the energy demand for refining to comply with legislation on regulated NOx emissions (Larson, 2006) and can poison the current commercial hydrotreatment catalysts used in conventional crude oil refining (Jazrawi et al., 2013). Similarly, low sulfur biocrude is preferable, as sulfur must otherwise be removed through a hydro-desulfurisation process (Peterson et al., 2008). Importantly, sulfur was markedly lower in the biocrudes than in the originating biomass. This is best illustrated for *Ulva* with 5.0% sulfur in the biomass and 0.4% sulfur in the biocrude (also see section 3.3.3 for further discussion).

Table 3.3. Ultimate analysis and energy recovery of biocrude and biochar, and total organic carbon and total nitrogen concentrations of aqueous phase following HTL of marine (M) and freshwater (FW) macroalgae.

Species	*Derb.*	*Ulva*	*Chaet.*	*Clad.*	*Oedog.*	*Clad.*
Source	M	M	M	M	FW	FW
Biocrude (wt%)						
C	73.0	72.6	70.9	71.6	72.1	71.1
H	7.5	8.2	7.7	8.0	8.1	8.3
O	10.6	11.0	11.4	10.6	10.4	10.6
N	6.5	5.8	6.8	7.1	6.3	6.8
S	0.7	0.4	0.1	0.9	0.8	1.3
HHV (MJ/kg)	33.2	33.8	32.5	33.3	33.7	33.5
ER (%)	52.5	54.0	30.6	35.3	55.7	40.3
Biochar (wt%)						
C	19.9	9.6	48.1	44.2	12.2	36.1
H	3.0	2.3	5.3	4.1	1.4	3.9
O	22.1	35.1	25.5	20.1	6.6	20.6
N	1.3	0.9	2.7	3.7	1.2	2.6
S	9.3	16.3	0.8	1.7	0.0	0.3
HHV (MJ/kg)	9.1	4.0	20.5	18.3	5.2	15.1
ER (%)	5.3	3.4	16.4	14.6	2.3	16.5
Aqueous phase (mg/L)						
TOC	7064	4629	3357	4950	10643	5319
TN	2093	1629	993	2371	1857	2373

HHV = higher heating value; ER = energy recovery; TOC = total organic carbon; TN = total nitrogen.

In contrast to the consistency of the elemental composition of the biocrudes, biochar composition varied markedly between species resulting in two distinct groupings (Table 3.3). The first grouping of marine *Derbesia* and *Ulva*, and freshwater *Oedogonium*, formed an organic-poor biochar with a low carbon content ranging from 9.6% for marine *Ulva* to 19.9% for marine *Derbesia*. Consequently, these biochars had a low HHV ranging from 4.0 MJ/kg for *Ulva* to 9.1 MJ/kg for *Derbesia*. The second grouping of filamentous

species, including marine *Chaetomorpha* and both marine and freshwater *Cladophora*, formed an organic-rich biochar with a high carbon content ranging from 36.1% for freshwater *Cladophora* to 48.1% for marine *Chaetomorpha*. Consequently, these biochars had a high HHV ranging from 15.1 MJ/kg for freshwater *Cladophora* to 20.5 MJ/kg for *Chaetomorpha*. Notably, biochars generated by HTL of marine *Chaetomorpha* and both marine and freshwater *Cladophora*, that are relatively high in energy, carbon, and nitrogen, may be suitable as slow release fertilisers for use in agriculture (Bird et al., 2011) or as feedstock for subsequent thermochemical processes such as pyrolysis (López Barreiro et al., 2013) and could represent a valuable by-product of HTL.

The total organic carbon (TOC) and total nitrogen (TN) contents of the aqueous product also varied markedly between species. TOC dissolved in aqueous products ranged from 3.4 g/L for marine *Chaetomorpha* to 10.6 g/L for freshwater *Oedogonium* and was generally higher for carbon-rich species. TN in aqueous products ranged from 1.0 g/L for *Chaetomorpha* to 2.4 g/L for both freshwater and marine *Cladophora*. In this study, values for the TOC and TN of aqueous products were lower than values reported in the literature for microalgae (Biller et al., 2012) due to lower organics, particularly lower protein content in the biomass. Inorganic nitrogen in the aqueous product is generally in the form of NH_4^+ as a result of the breakdown of protein (Garcia Alba et al., 2012) and NH_4^+ is an important nutrient that can be recycled in macroalgal culture (Mata et al., 2010a). In addition, the aqueous phase contains beneficial macronutrients such as phosphorus and potassium, and mineral elements such as sodium, that could be recycled as nutrient inputs into algal culture (Biller et al., 2012). While this result remains to be tested for macroalgae, the

recovery of all valuable components from the HTL process will be critical to long-term process sustainability.

3.3.3. Energy recovery and mass balance

The chemical energy recovered in both biocrude and biochar varied across species according to the HTL yields and the feedstock HHV (Eq. 3.2), and therefore corresponded to the previous grouping of species (Table 3.3). Marine *Derbesia* and *Ulva*, and freshwater *Oedogonium*, had the highest energy recovery in their biocrude, ranging from 52.5% for *Derbesia* to 55.7% for *Oedogonium*, and the lowest energy recovery in biochar ranging from 2.3% for *Oedogonium* to 5.3% for *Derbesia*. Among the six species studied, the filamentous species marine *Chaetomorpha,* and marine and freshwater *Cladophora,* had the lowest energy recovery in biocrude, ranging from 30.6% for *Chaetomorpha* to 40.3% freshwater *Cladophora,* and the highest energy recovery in biochar, ranging from 14.6% for marine *Cladophora* to 16.5% freshwater *Cladophora*. For both groupings however, a significant proportion of biomass energy partitioned to the combined aqueous and gas products (including eventual losses), ranging from 42% for marine *Derbesia* and freshwater *Oedogonium,* to 53% for marine *Chaetomorpha*.

The distribution of carbon and nitrogen in product streams was determined from the ultimate analysis of biocrude and biochar, and from TOC and TN analysis of the aqueous phase. The remaining carbon and nitrogen fractions partitioned to the gas phase and eventual losses were determined by difference. The carbon and nitrogen mass balances resulting from HTL of macroalgae are presented in Fig. 3.2a and 3.2b. The proportion of carbon recovered in HTL products follows the same pattern as energy recovery with the

same groupings within the six species (Fig. 3.2a). Marine *Derbesia* and *Ulva,* and freshwater *Oedogonium,* had the highest carbon recovery in their biocrude, ranging from 48.9% for *Ulva* to 51.6% for *Oedogonium.* A high proportion of carbon was also recovered in their aqueous phase, ranging from 23.4% for *Ulva* to 40.9% for *Oedogonium*. Consequently, a relatively low proportion of biomass carbon was recovered in biochar (3.5 - 5.5%) and gas products (3.9 - 23.6%). In contrast to the first grouping of species, marine *Chaetomorpha,* and marine and freshwater *Cladophora*, had a lower carbon recovery in their biocrude, ranging from 26.0% for *Chaetomorpha* to 37.3% for freshwater *Cladophora*, and similarly a lower recovery of carbon in their aqueous phase, ranging from 17.8% for *Chaetomorpha* to 22.4% for marine *Cladophora.* Consequently, a large proportion of biomass carbon was recovered in their biochars (14.9 - 18.2%) and associated gas phases (24.7 - 40.9%).

The distribution of nitrogen within the product streams for the six species mirrored that of carbon (Fig. 3.2b). In relative terms, the highest recovery of biomass nitrogen into biocrude occurred for marine *Derbesia* and *Ulva,* and for freshwater *Oedogonium,* ranging from 28.6% to 34.6%. In absolute terms however, *Derbesia*, *Ulva* and *Oedogonium* had a combination of low biomass nitrogen levels and high biocrude yield. Consequently, based on Eq. 3.2 (specifically, $N_{BIOCRUDE} = N_{RECOVERY} * N_{FEEDSTOCK} / Y_{BIOCRUDE}$), these species had the lowest nitrogen content in their biocrude, even though they had the highest biomass to biocrude nitrogen conversion ratio. For *Derbesia, Ulva* and *Oedogonium*, the mass of nitrogen recovered in the aqueous phase was also high, ranging from 54.8 - 65.4%. As a result, these same species had the lowest nitrogen recovery in their biochar (2.3 - 3.1%) and gas (1.1 - 8.0%) product streams. For *Chaetomorpha*, and both marine and freshwater

Cladophora, the opposite trend occurred with only 18.4% to 20.6% nitrogen recovered in their biocrude fractions. The recovery of nitrogen to their aqueous phase was lower, ranging from 41.2% for *Chaetomorpha* to 51.3% for freshwater *Cladophora*, resulting in higher recovery of nitrogen in biochar (6.8 - 7.4%) and gas (10.8 - 32.6%) fractions.

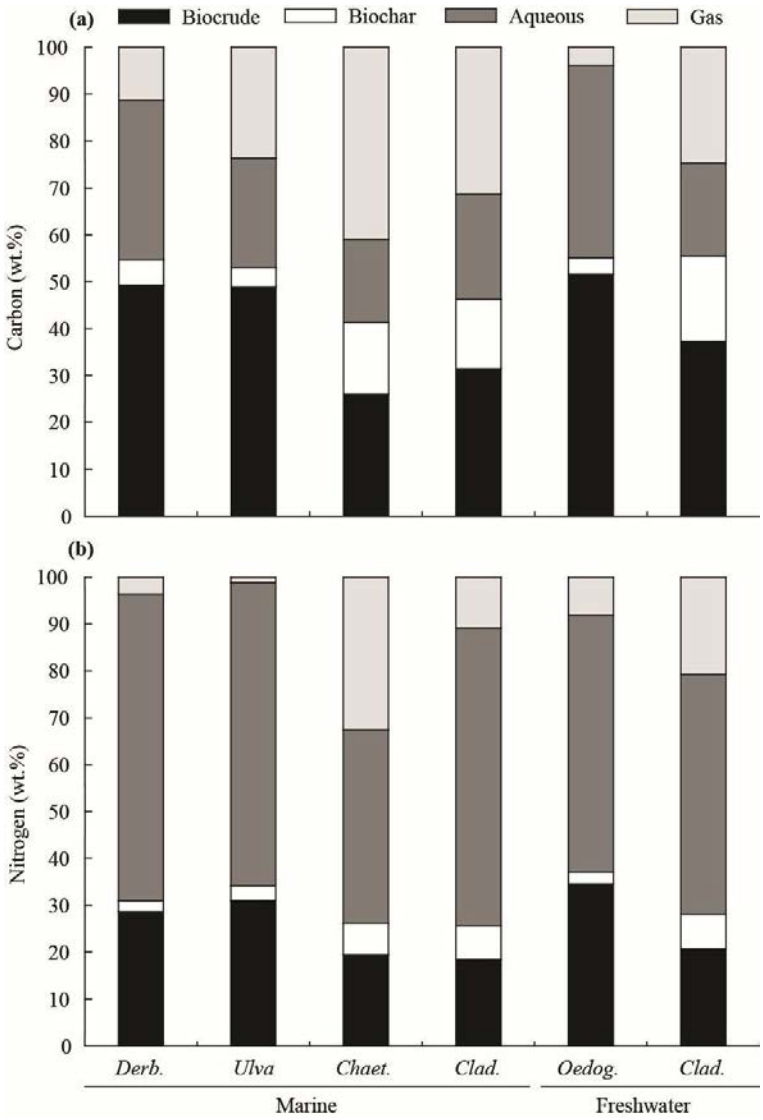

Figure 3.2. Distribution of carbon and nitrogen in HTL product streams.
Data show means (n = 3) of carbon and nitrogen conversion ratios (wt%) from macroalgal biomass to HTL products.

For all six species, the proportion of total organic constituents partitioned to the combined aqueous and gas phases was generally higher than to biocrude and biochar, with the combined aqueous and gas products (as well as eventual losses) accounting for 45 - 60% carbon, 60 - 70% hydrogen, 80 - 90% oxygen and 60 - 75% nitrogen of the original biomass. Notably, there was a marked decrease in the oxygen content of the biocrude compared to the original feedstock for all species, corresponding to a minimum recovery of 3.6% for marine *Chaetomorpha*, to a maximum of 8.8% for freshwater *Oedogonium*. This major reduction of the oxygen content in biocrude highlights the efficiency of HTL over pyrolysis, where the oxygen content is generally higher and therefore leads to a biocrude lower in energy (Bridgwater, 2012; Rowbotham et al., 2012).

Sulfur, both organic and inorganic, was also effectively excluded from the biocrude fraction, despite the conversion ratio varying widely between species. For example, only 0.3 - 5.2% of the original sulfur in the biomass was transferred to the biocrude for the marine species, while the ratio increased to 14.3 - 53.5% for freshwater species. This suggests that the organic sulfur content of macroalgae found in some amino acids and sulfolipids is transferred to a higher degree to biocrude, while the inorganic sulfur content that is higher in marine species is transferred to the other HTL products rather than the biocrude.

3.3.4. Biocrude productivity

Biocrude productivity was calculated for all species by multiplying the biomass productivities determined in Chapter 2 with biocrude yield (Eq. 2.4), and the results are presented in Table 3.2. Biocrude productivity on an areal basis was primarily influenced by

biomass productivity (dw), with the positive impact of higher biomass productivities overriding the negative impact of higher ash content for marine species. Therefore, biocrude productivities were highest for marine *Derbesia* (2.4 g/m^2/d) and *Ulva* (2.1 g/m^2/d), followed by freshwater *Oedogonium* (1.3 g/m^2/d) where lower biomass productivity was compensated for by a higher biocrude yield. This highlights the importance of comparing biocrude productivities rather than biocrude yields for species selection, as *Derbesia* had 46% higher biocrude productivity than *Oedogonium*, despite a higher biocrude yield achieved with the liquefaction of *Oedogonium* (Table 3.2). Notably, higher biomass productivities than those achieved in this study for the same species will result in significant overall increases in biocrude productivities, for example by 100% for marine species *Derbesia* and *Ulva*, and by 200% for freshwater *Oedogonium* (sensitivity analysis in Chapter 2). In addition, the biocrude productivity of *Derbesia*, *Ulva* and *Oedogonium* accounted for 30% (*Ulva*) to 36% (*Oedogonium*) of their biomass productivity demonstrating a relatively efficient conversion of biomass organic content that correlates with both energy recovery and elements conversion ratio.

3.4. Conclusions

HTL of macroalgae demonstrated the influence of biomass ash and organic carbon contents on biocrude yield. Carbon, hydrogen, and therefore the HHV of biocrude, were consistent across all six species investigated. Marine *Derbesia* and *Ulva*, and freshwater *Oedogonium*, had the highest biocrude yields based on ash-free dry weight, and the highest biocrude productivities. Their biocrude was also lowest in nitrogen, and recovered the highest proportions of the biomass energy. The selection of these species demonstrates the

efficacy of the simultaneous assessment of biocrude productivities, as a function of biomass productivity and biocrude yield, to identify suitable feedstock for HTL applications.

Chapter 4

Pre- and post-harvest treatment of macroalgae to improve the quality of feedstock for hydrothermal liquefaction[3]

4.1. Introduction

Macroalgal biomass is a diverse and abundant resource for the innovative production of renewable liquid fuels and chemicals (Ross et al., 2008; Rowbotham et al., 2012; Chapter 2; Chapter 3). Macroalgae are often highly productive on an areal basis, are simple to harvest and process, and can be produced on non-arable land, as well as in freshwater and in the sea. These advantages combine ideally with the efficiencies of hydrothermal liquefaction (HTL), a thermochemical process using hot compressed water at conditions approaching the critical point of water (374^{o}C; 22.1 MPa) to decompose wet biomass to a liquid biocrude (Toor et al., 2011; Biller & Ross, 2012). The thermochemical decomposition of biomass relies on the unique properties of water at these subcritical conditions, where it acts simultaneously as a solvent (similar to acetone), reactant, and both acid and base catalyst, due to its increased auto-ionisation. Elevated temperatures and pressures reduce the density, polarity and relative permittivity/dielectric constant of water, resulting in the hydrolysis and dissolution of solid biomass (Peterson et al., 2008).

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[3] **Chapter 4** is adapted from Neveux N, Yuen AKL, Jazrawi C, He Y, Magnusson M, Haynes BS, Masters AF, Montoya A, Paul NA, Maschmeyer T, de Nys R, 2014c. Pre- and post-harvest treatment of macroalgae to improve the quality of feedstock for hydrothermal liquefaction. *Algal Research*, **6**, 22-31.

A complex network of cascading reactions involving the newly liberated low molecular weight hydrocarbons leads to the formation of an oily biocrude, gases (principally CO_2), water-soluble chemicals and insoluble residues (biochar).

Biocrude produced through the HTL of algae has a high energy density that is 70 - 95% of that of petroleum crude (López Barreiro et al., 2013). The difference in energy is due to the presence of heteroatoms (O, N, S), derived mainly from the protein and carbohydrate fractions of the biomass, accounting for 10 - 20% of the mass of algal biocrude (Zhou et al., 2010; Frank et al., 2013; Chapter 3). The reduction or removal of these heteroatoms within the biocrude prior to upgrading into liquid hydrocarbon 'drop-in' fuel or into feedstock for the production of industrial chemicals would be highly beneficial (Li & Savage, 2013). Previous studies have demonstrated that it is possible to reduce the content of O, N, and S within the biocrude through catalytic hydrotreating, although this treatment requires substantial H_2 and energy inputs (Duan & Savage, 2011b; Duan & Savage, 2011c; Elliott et al., 2013b). Other studies have demonstrated that oxygen and trace metals can be efficiently reduced within the biocrude through thermal treatment, but the nitrogen content is not improved by such treatment (Duan & Savage, 2011a; Roussis et al., 2012). The presence of nitrogenous and sulfurous compounds in the biocrude is particularly detrimental as nitrogen can poison the active sites of catalysts used in conventional refining and both elements can participate in the formation of harmful nitrogen and sulfur oxides emissions during combustion (Jazrawi et al., 2013). Another issue for HTL processing of macroalgae – and specifically marine macroalgae – is the presence of inorganic compounds (ash as silicates, hydroxides, metal oxides, halides, carbonates, and sulfates

with alkali-metal counterions) which can precipitate and deposit on reactor walls, thereby blocking reactors, and in the case of halides in particular, cause corrosion and the degradation of the stainless steel reaction vessels (Peterson et al., 2008; Jazrawi et al., 2013). Trace amounts of metals (i.e. Fe, Mg, Zn, Ni) can also be transferred to the biocrude and become a significant challenge for upgrading in conventional refinery units (Roussis et al., 2012).

One route to circumvent both the extensive treatment of the biocrude and the HTL processing issues arising from nitrogen, sulfur and ash in macroalgae, would be to reduce these components in the feedstock prior to hydrothermal upgrading. Nitrogen is a key element in algal metabolism that is essential for the formation of proteins and chlorophyll, and therefore photosynthesis (Merzlyak et al., 2007). However, the content of nitrogen is highly variable in macroalgae and can be reduced through a starvation process, where the biomass continues to grow in a low nitrogen environment, thereby diluting the internal nitrogen pool to a minimum (Angell et al., 2014). Sulfur also has a pivotal role in algal cell physiology and homeostasis through its role in the formation of dimethylsulfopropionate (DMSP), sulfolipids and various amino acids (Giordano et al., 2008). The treatment of macroalgae through nutrient starvation and washing may result in a decreased content of sulfur within the biomass. Consequently, the HTL processing of this biomass may improve the quality of biocrude. Similarly, the minimisation of the ash content of the biomass through the removal of salts can be expected to reduce the mechanical demands on HTL processing equipment. To my knowledge this is the first report on the combined effects that metabolic manipulation of the content of nitrogen, sulfur and ash in the biomass have

on the yield and quality of biocrude. This approach, which focuses on tailoring the algal feedstock for a specific purpose, is a critical first step in the delivery of an improved biocrude that minimises hydrotreating requirements (particularly hydrodenitrogenation and hydrodesulfurisation) for the production of a fully fungible biofuel.

Therefore, the aim of this chapter was to assess if the manipulation of freshwater and marine macroalgae through nutrient starvation, and the post-harvest washing of biomass, would reduce the content of nitrogen, sulfur and ash prior to the HTL processing, and whether these changes would be carried through the HTL process, affording a desirable biocrude product. Firstly the effects of starvation and washing on the composition of biomass were evaluated. Subsequently, the yield and elemental composition of biocrude and HTL co-products produced from algae subject to combinations of starvation and washing were assessed. Finally, the variation in the content of carbon in each of the treated algal feedstocks was correlated with the yield of biocrude.

4.2. Materials and methods

4.2.1. Culturing of macroalgae

Three species of green macroalgae (Chlorophyta) were selected based on their high productivity in land-based culture and high conversion yield to biocrude (Chapter 2; Chapter 3). Samples were harvested in November 2012 from stock cultures held in outdoor tanks at James Cook University (Townsville). Species were the marine macroalgae *Derbesia tenuissima* (Magnusson et al., 2014) and *Ulva ohnoi* (Lawton et al., 2013b) and the freshwater macroalga *Oedogonium* sp. (Lawton et al., 2013a). Macroalgae were placed

in 50 L cylindrical tanks in an outdoor system to be cultured for 36 days. Biomass was initially stocked at 2 g/L fw for marine species and 0.5 g/L fw for the freshwater species based on individual stocking density trials (Lawton et al., 2013a; Angell et al., 2014; Magnusson et al., 2014). Macroalgae were cultivated in a batch culture system, described in detail in Chapter 2. Biomass was harvested every 6 days (six cycles of 6 days each in total) using a net (2 mm screen), spun to a constant fresh weight, weighed and subsequently re-stocked at initial stocking densities for a new cycle. Excess biomass was discarded. Water in the batch tanks was entirely renewed every 6 days using saltwater (35 g/L of dissolved salts) for marine species and dechlorinated freshwater (0 - 1 g/L of dissolved salts) for the freshwater species. Environmental conditions were monitored daily and adjusted accordingly. Salinity for marine species was adjusted daily by adding dechlorinated freshwater to compensate for evaporation. Salinity in freshwater cultures was stable for the duration of the experiment. The pH in batch cultures varied naturally between 8.3 (sunrise) to 9.4 (sunset) for marine species and between 8.4 (sunrise) to 10.3 (sunset) for the freshwater species. Culture tanks were placed inside a larger holding tank for temperature control at 25^{o}C with a continuous flow of water. Light was monitored hourly using a photosynthetically active radiation data logger (Li-1400; LI-COR Inc., USA) adjacent to the tanks for the duration of the experiment. Total photons received over each 6-day culture cycle ranged from 301 to 349 mol photons/m^{2}.

4.2.2. Nutrient starvation and washing treatments

A schematic diagram of the culture method and treatments is shown in Fig. 4.1. The initial growth phase (18 days, three 6-day culture cycles) provided nutrients in excess until stable

productivity was reached, using f/2 medium (Guillard & Ryther, 1962) for marine species and f/4 medium for freshwater species. During this phase, eight replicates of each of the three species were cultured (N+; n = 8). The second phase, or starvation phase (also 18 days, three 6-day culture cycles), consisted of removing the nutrients supply from half of the culture replicates (N−; n = 4), while the other half remained supplied with the same nutrients as in the growth phase (N+; n = 4). After a total of 36 days of culture, all biomass in each tank was harvested, spun and weighed.

Then, macroalgae were further treated to quantify the effect of washing on the ash (dry inorganic) content of biomass. The biomass from each replicate of each species both not starved (N+; n = 4) and starved (N−; n =4) was divided in equal amounts. Half of the biomass then remained not washed (A+; n = 4), while the other half of the biomass was washed (A−; n = 4) three times for 1 minute by immersing the biomass in town water (~3 L/100 g of algae), stirring and draining the water at each wash. As a result of the starvation and washing procedures, four treatment combinations existed for each species denominated N+/A+, N+/A−, N−/A+, N−/A−.

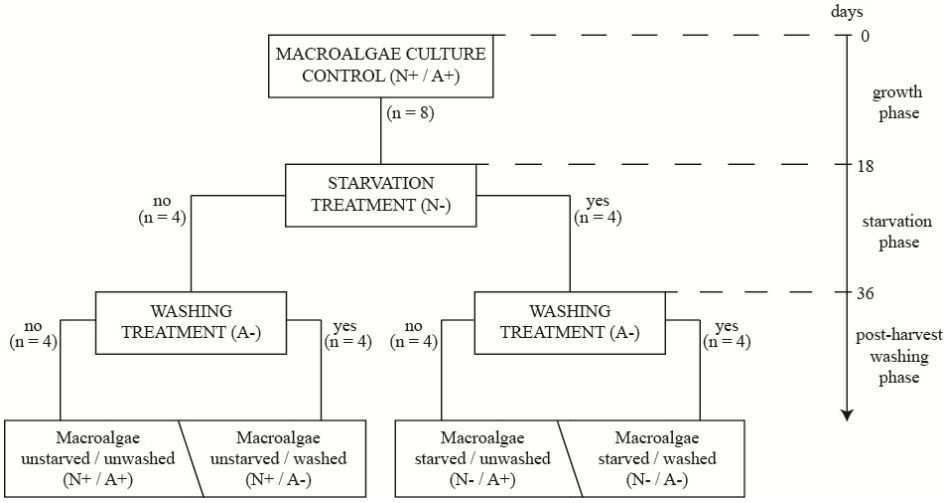

Figure 4.1. Schematic diagram of the experimental set up.

4.2.3. Biomass characterisation

A sub-sample of each replicate of each of the treatments was weighed (fw) and oven-dried for 12 hours at 60^{o}C, placed in a desiccator for 30 minutes at room temperature to reach stable moisture content, and weighed again (dw) to determine the fresh to dry weight ratio (fw: dw). The remaining biomass was freeze-dried, ground to a mean particle size < 500 µm, placed in a desiccator for 30 minutes and then stored in air-tight vials under refrigeration until further analyses. Powdered macroalgae (dw) was used for ash, moisture, lipid and ultimate analyses (see Chapter 2 for details). Protein content was determined using the nitrogen to protein conversion factors of 4.8 for *Derbesia*, 4.6 for *Ulva* and 4.7 for *Oedogonium* (Chapter 2). Carbohydrates were determined by difference by subtracting the sum of ash, moisture, lipid and protein weight percentages from 100%.

4.2.4. Hydrothermal liquefaction

Hydrothermal liquefaction (HTL) of the three macroalgae was performed on each replicate (n = 4) of the four treatments, for a total of 48 runs. HTL was performed using a custom-built stainless steel batch reactor system as described in detail in Chapter 3. A slurry (6.6% solids) composed of 2 g of algae powder and 28 mL of distilled water was loaded in the 35 mL (internal volume) reactor tube for each run. The reactor was subsequently fitted with a gasket and attached to a pressure-head, able to handle pressure up to 25 MPa at 350^{o}C. The reactor was purged three times at room temperature with N_2 to remove excess oxygen, after which it was pressurised to 7 MPa with N_2. The reactor was then immersed in a fluidised sand bath set to 350^{o}C to initiate the reaction. The temperature in the reactor was

monitored via a thermocouple located inside the reactor above the slurry and the pressure was monitored externally. Typically, the internal temperature rose on average to 252^{o}C (9.6 MPa) within 1 minute, 309^{o}C (11.5 MPa) within 2 minutes and 328^{o}C (12.9 MPa) within 3 minutes of reaction time. Internal reaction temperatures of between 330^{o}C and 345^{o}C (maximum temperatures and pressures, 14 - 17 MPa) were maintained for a further 5 minutes (total of 8 minutes reaction time) before the reactor was quenched in an ice/water bath for 1 minute to cool the reactor and contents to room temperature.

4.2.5. Product separation and analysis

The separation of HTL products was performed as described in Chapter 3 (section 3.2.4). Biocrude and biochar yields were calculated separately on an ash-free dry weight basis (afdw) using the following equation:

$$Y_{PRODUCT} = W_{PRODUCT} / W_{ORGANIC\ BIOMASS} \times 100\% \qquad \textbf{Eq. 4.1}$$

where $Y_{PRODUCT}$ is the yield of biocrude or biochar (% afdw) and $W_{PRODUCT}$ is the mass of biocrude or biochar (g). $W_{ORGANIC\ BIOMASS}$ is the organic mass of algae processed (g) and was calculated by subtraction of the sum of ash (g) and moisture (g) from the total mass of the algae.

The ultimate analysis of biocrude and biochar was performed externally (OEA Laboratory Ltd., UK). The aqueous phase (post-separation) was transferred to a volumetric flask and made up to 100 mL using distilled water for subsequent quantification of total organic carbon, inorganic carbon and total nitrogen (Trop-Eco Laboratory, JCU, Australia).

4.2.6. Chemical energy recovery and mass balance

The chemical energy recovery (ER) was calculated for the biocrude and biochar products according to Eq. 3.2. The HHV of biomass, biocrude and biochar was calculated with the unified correlation proposed by Channiwala and Parikh (2002).

Eq. 3.2 was also used to determine the mass balance in product streams, specifically the mass of elements C, H, O, N, and S recovered in biocrude and biochar, by substituting HTL products and feedstock HHV with elemental contents. The remaining elemental fractions allowed an estimation of the energy and mass partitioned to the combined aqueous and gas phases, and losses.

4.2.7. Statistical analysis

Factorial analyses of variance (two- and three-way ANOVAs) were performed to assess the main effects and interactions between starvation and washing treatments on the composition and productivity of biomass, and on the yield of biocrude, using STATISTICA 10 software (StatSoft Inc., USA). Residual plots and normality tests were used to ensure ANOVA assumptions were met. Significant differences between the treatments are reported at $\alpha = 0.05$ level of significance. As there were significant interactions in each ANOVA (see section 4.3), no formal post-hoc comparisons were made between treatments for each main effect. The productivity of the biomass was only formally analysed for the last culture cycle (cycle 6, 3rd cycle of starvation), since this was the biomass used for all subsequent biochemical analyses and HTL. The elemental composition of HTL products was not analysed formally as the individual replicates of

biocrude, biochar and aqueous phases were combined for each treatment prior to elemental analysis. All results are reported on a dry weight basis unless stated otherwise.

4.3. Results

4.3.1. Biomass productivity

The productivity of macroalgae cultured for six cycles of 6 days is presented in Fig. 4.2 with the nutrient starvation treatment starting after three cycles. All cultures that were continuously provided with nutrients (N+, cycle 1 to 6) had a stable productivity over the entire culture period. *Ulva* had the highest productivity with an average (± standard error) of 21.6 ± 0.9 g/m^2/d dw over the six culture cycles, followed by *Derbesia* at 12.7 ± 0.5 g/m^2/d dw and *Oedogonium* at 9.7 ± 0.2 g/m^2/d dw. Predictably, the nutrient starvation phase (N−, cycle 4 to 6) resulted in a consistent and in some cases dramatic decrease in productivity (cycle 6, ANOVA, $F_{1,18} = 476.7$, $P < 0.05$). After one cycle of starvation (cycle 4), the productivity of the marine species decreased by more than 50% to 7.0 g/m^2/d dw for *Ulva*, and 5.7 g/m^2/d dw for *Derbesia*. The subsequent cycles of starvation resulted in further decreases in productivity for *Ulva* and *Derbesia* to 0.5 and 0.9 g/m^2/d dw respectively. In the third cycle of starvation (cycle 6) there was no further increase in biomass, and therefore no further dilution of the internal nitrogen pool. Consequently, the cultivation phase was completed at this stage. Interestingly, the productivity of freshwater *Oedogonium* remained stable in the first cycle of starvation (cycle 4), maintaining growth at 10.6 g/m^2/d dw without the addition of nutrients, with a subsequent decrease in productivity to 3.7 g/m^2/d dw in the final culture cycle (cycle 6). A significant interaction effect between species and the starvation treatment (ANOVA, $F_{1,18} = 46.4$, $P < 0.05$) was

the result of the marine species having higher growth rates under nutrient supply and the freshwater species being less affected by nutrient starvation.

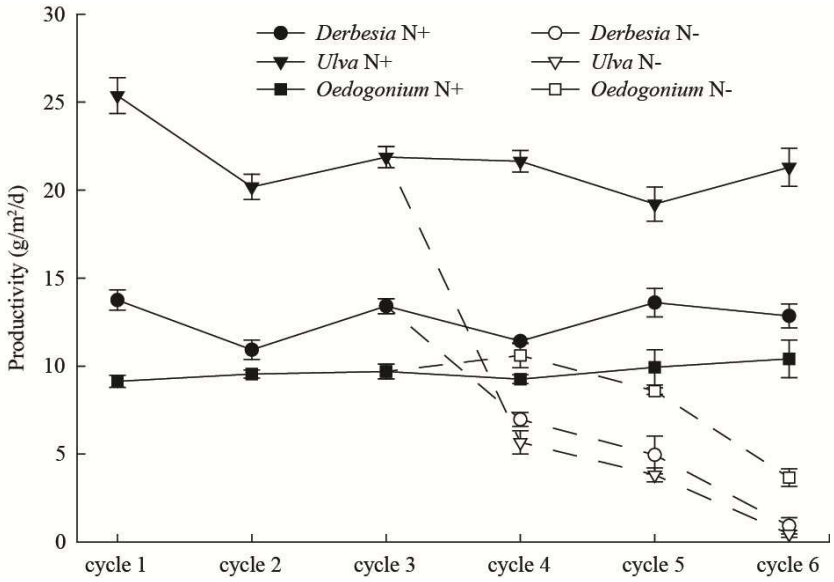

Figure 4.2. Biomass productivity of macroalgae.
Data show means (n = 4 ± SE) of biomass productivity dry weight of not starved (N+) and starved (N−) macroalgae over six culture cycles of 6 days. The nutrient starvation treatment starts after cycle 3.

4.3.2. Feedstock characterisation

Table 4.1 shows the proximate, biochemical, ultimate and elemental analyses for each of the macroalgae species subjected to the various nutrient (N+, N−) and washing (A+, A−) treatments. As hypothesized, nutrient starvation (N−) had a significant effect on the organic profile of the biomass, primarily the protein content, with an average reduction in protein of 73 ± 3% for *Derbesia*, 75 ± 2% for *Ulva* and 71 ± 4% for *Oedogonium*, compared with biomass that was not starved of nutrients (N+) (ANOVA, $F_{1,36} = 589.4$, $P < 0.05$).

Table 4.1. Proximate, biochemical, ultimate and elemental analysis of macroalgae.
Data show means (n = 4) of content dry weight of macroalgae not starved (N+), starved (N−), not washed (A+) and washed (A−).

Species	*Derbesia*				*Ulva*				*Oedogonium*			
Treatment	N+	N+	N−	N−	N+	N+	N−	N−	N+	N+	N−	N−
	A+	A−	A+	A−	A+	A−	A+	A−	A+	A−	A+	A−
Proximate (wt%)												
Ash	27.4	5.0	22.8	3.4	27.6	15.4	26.0	14.9	7.0	6.7	6.7	6.1
Moisture	8.8	8.0	8.7	8.3	11.9	9.5	12.2	10.6	7.0	7.2	6.9	7.8
Biochemical (wt%)												
Lipid	11.1	11.2	4.9	5.5	2.1	2.1	1.2	1.4	7.4	8.5	3.1	3.2
Protein	25.0	33.5	7.9	7.2	18.2	21.0	4.8	4.8	19.7	19.8	6.7	4.7
Carb.*	27.7	42.4	55.8	75.5	40.1	52.0	55.8	68.3	58.9	57.8	76.6	78.2
Ultimate (wt%)												
C	36.1	48.0	34.0	42.9	31.1	35.9	30.5	33.1	44.1	44.4	41.2	41.0
H	5.8	7.3	5.7	6.8	5.5	6.1	5.5	5.9	6.7	6.7	6.4	6.4
O	29.4	33.3	38.9	45.5	42.1	45.1	49.2	53.3	38.8	39.2	46.6	46.9
N	5.2	7.0	1.6	1.5	4.0	4.6	1.0	1.1	4.2	4.2	1.4	1.0
S	1.9	1.0	1.6	0.4	4.9	4.7	5.2	5.4	0.2	0.2	0.2	0.1
HHV**	16.5	21.9	14.7	18.2	13.5	15.5	12.6	13.6	19.2	19.3	17.1	17.0
C:N	6.9	6.9	20.7	28.5	7.9	7.9	29.4	31.5	10.5	10.5	28.8	40.6
Elemental (g/kg)												
Cl	95.9	0.5	81.9	0.6	56.1	8.0	35.2	1.6	3.2	3.5	4.5	4.7
Na	55.8	0.8	49.0	0.8	28.3	0.7	27.6	3.0	3.3	3.1	0.7	0.8
K	19.0	0.6	16.0	0.6	24.6	4.6	17.9	8.1	10.8	12.7	19.1	18.8
Mg	12.4	4.5	8.5	2.2	33.8	33.6	29.3	32.3	3.2	3.2	3.2	3.1
Ca	5.7	6.2	5.4	6.8	4.1	8.6	4.7	11.4	3.9	4.4	3.0	3.1
Fe	0.8	1.2	0.3	0.4	0.5	0.6	0.2	0.2	1.0	1.0	0.4	0.4
P	4.2	3.0	1.3	0.9	2.4	2.3	0.7	0.7	3.1	3.6	0.6	0.6

*carbohydrate content determined by difference; **HHV (MJ/kg) = higher heating value.

This reduction is highlighted in Fig. 4.3 with a substantial decrease of the nitrogen content of biomass resulting from nutrient starvation. This treatment also had a significant effect on lipid content with an average reduction in lipid of 53 ± 2% for *Derbesia*, 36 ± 10% for

Ulva and 58 ± 4% for *Oedogonium* (ANOVA, $F_{1,36} = 220.3$, $P < 0.05$). Consequently, there was an increase in carbohydrate content of 91 ± 8% for *Derbesia*, 36 ± 4% for *Ulva* and 34 ± 5% for *Oedogonium* (ANOVA, $F_{1,36} = 539.6$, $P < 0.05$). This modification of the organic profile was also manifested at the elemental level, with an average reduction in the carbon and energy content of 7 ± 1% and 12 ± 1% respectively, across all species. Finally, nutrient starvation (N−) had a small but significant effect on the ash (dry inorganic) content of macroalgae (ANOVA, $F_{1,36} = 28.5$, $P < 0.05$), and in contrast to the other effects, this was not species-dependent, there being no interaction between species and the starvation treatment (ANOVA, $P = 0.11$).

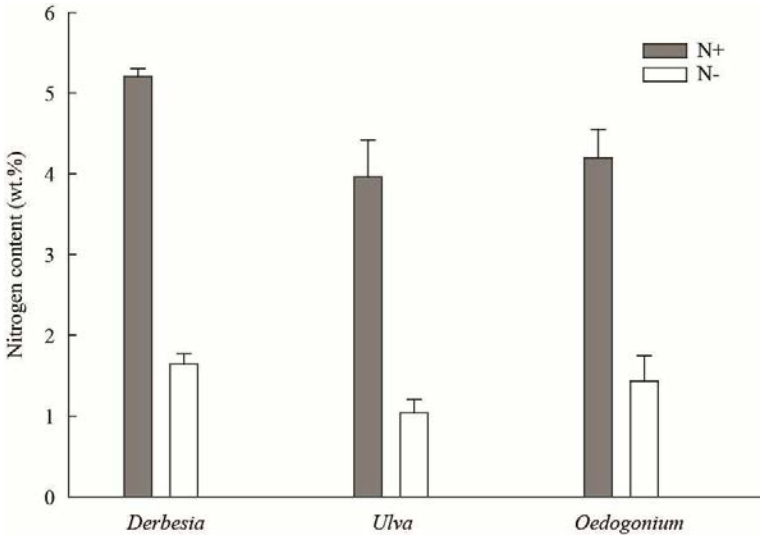

Figure 4.3. Effect of starvation treatment on macroalgae nitrogen content.
Data show means (n = 4 ± SE) of biomass nitrogen content dry weight of not starved (N+) and starved (N−) macroalgae.

There was a significant effect of the washing treatment on the ash content of all macroalgae (ANOVA, $F_{1,36} = 425.8$, $P < 0.05$) as predicted, with the largest effect on marine species (Fig. 4.4). Washing had the most significant effect on *Derbesia* (A−),

reducing the ash content by 83 ± 2% on average after three washing cycles. This was followed by *Ulva* (A−) with a reduction in the ash content of 43 ± 3% on average after three washing cycles. For freshwater *Oedogonium*, which initially had a low ash content, the washing treatment was less effective but still reduced the ash content by 7 ± 2%. These changes in ash content related to changes in specific elements, including key metals and halides in the marine species. For example, sodium decreased markedly by 98 ± 0% for *Derbesia* (A−) and 94 ± 1% for *Ulva* (A−), while chlorine decreased by 99 ± 0% for *Derbesia* (A−) and 90 ± 2% for *Ulva* (A−). Similarly, washing significantly reduced potassium and magnesium in *Derbesia* (A−) by 97 ± 0% and 69 ± 2% respectively. Potassium was reduced by 65 ± 6% in *Ulva* (A−). Consequently, the washing treatment led to a concomitant increase in the organic content of marine macroalgae. The content of carbon and therefore HHV of washed biomass increased by 29 ± 2% for *Derbesia* (A−) and 12 ± 2% for *Ulva* (A−).

Of the three species investigated, freshwater *Oedogonium* generally had the highest content of carbon and energy, whereas *Derbesia* had the highest content of protein and lipid. The combination of starvation and washing (N−/A−) was effective in producing biomass with a low protein content, reaching a minimum of 4.7% for *Oedogonium*, 4.8% for *Ulva* and 7.2% for *Derbesia*, corresponding to the lowest nitrogen content of 1.0% for *Oedogonium*, 1.1% for *Ulva* and 1.5% for *Derbesia* (Table 4.1). This combination of treatments (N−/A−) also produced the biomass with the lowest ash content of 3.4% for *Derbesia*, 6.1% for *Oedogonium* and 14.9% for *Ulva*.

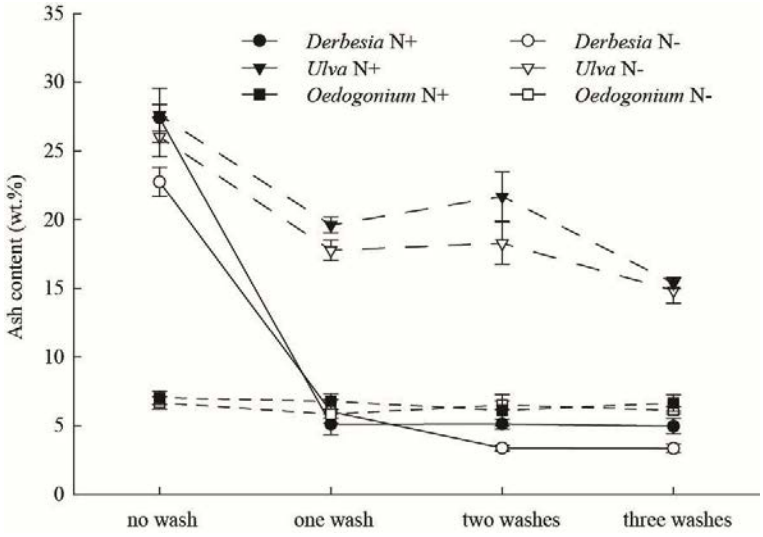

Figure 4.4. Effect of washing treatment on macroalgae ash content.
Data show means (n = 4 ± SE) of biomass ash content dry weight of not starved (N+) and starved (N−) macroalgae, washed three times with freshwater.

4.3.3. HTL products yield

Fig. 4.5 shows the effect of the starvation and washing treatments on the yield of biocrude produced during the HTL processing of the three macroalgae species, on an ash-free dry weight basis. Of the two treatments, only nutrient starvation of the biomass had a significant effect on the yield of biocrude for *Derbesia* (ANOVA, $F_{1,12} = 20.4$, $P < 0.05$) and *Oedogonium* (ANOVA, $F_{1,12} = 9.3$, $P < 0.05$). Washing increased the yield of biocrude on a dry weight basis, but this was only the result of processing biomass with higher organic content, which compensated for the loss of inorganic material through washing. On an ash-free dry weight basis, washing had no significant effect on the yield of biocrude (ANOVA $F_{1,36} = 0.7$, $P = 0.42$).

When not starved of nutrients (N+), *Derbesia* had the highest yield of biocrude in the range of 38.6 - 41.7% afdw, compared to a yield in the range of 35.6 - 38.8% afdw for *Oedogonium* and 32.3 - 32.6% afdw for *Ulva*. The starved biomass (N−) that was

inherently lower in carbon and energy, generally yielded less biocrude than the biomass that was not starved (N+). However, the response to starvation ultimately varied among species with an interaction effect between species and the starvation treatment (ANOVA, $F_{1,36} = 5.1$, $P < 0.05$). The reduction was highest for *Derbesia*, where the yield decreased by 19 ± 3% on average, compared to *Oedogonium* and *Ulva*, where the yields decreased by 13 ± 4% and 0 ± 6% respectively. These reductions led to yields in the range of 31.4 - 33.4% afdw for starved *Derbesia*, 32.2 - 32.6% afdw for starved *Oedogonium* and 30.6 - 34.0% afdw for starved *Ulva*.

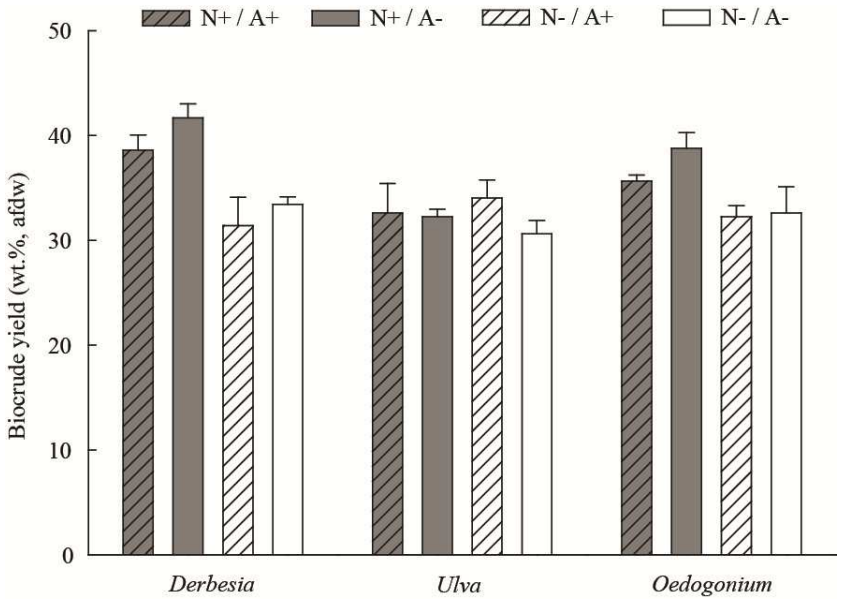

Figure 4.5. Effect of starvation and washing treatments on biocrude yield.
Data show means (n = 4 ± SE) of biocrude yields ash-free dry weight following HTL of macroalgae not starved (N+), starved (N−), not washed (A+) and washed (A−).

Fig. 4.6 shows that the yield of biochar varied from 4% to 20% on an ash-free dry weight basis across all species and treatments. The starvation of biomass (N−) led to a decreased yield of biochar by 37 ± 7% on average across species, compared with biomass that was

not starved (N+) (ANOVA, $F_{1,36} = 32.5$, $P < 0.05$). This decrease was largest for *Ulva* (50 ± 4%) and *Oedogonium* (43 ± 5%). In contrast, the post-harvest washing of biomass had no significant effect on the yield of biochar (ANOVA, $F_{1,36} = 0.0$, $P = 0.88$).

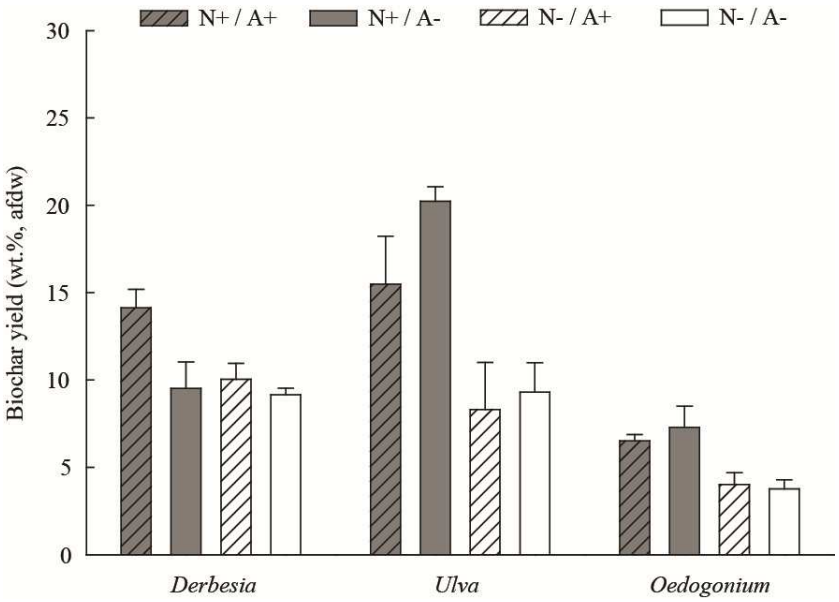

Figure 4.6. Effect of starvation and washing treatments on biochar yield.
Data show means (n = 4 ± SE) of biochar yields ash-free dry weight following HTL of macroalgae not starved (N+), starved (N−), not washed (A+) and washed (A−).

4.3.4. HTL products characterisation

As hypothesized, the quality of biocrude was improved by the starvation treatment, which was manifested through important changes in the key quality parameters of nitrogen and sulfur content, as shown in Table 4.2. Starved biomass (N−) produced a biocrude that was lower in nitrogen compared to biomass that was not starved (N+), with an average decrease in the content of nitrogen in the biocrude of 51 ± 1% for *Derbesia*, 53 ± 2% for *Ulva*, and 59 ± 0% for *Oedogonium*. Similarly, the content of sulfur in the biocrude decreased markedly by 66 ± 2% for starved *Derbesia*, 64 ± 4% for starved *Ulva* and 88 ± 0% for

starved *Oedogonium*. In contrast, nutrient starvation had no effect on the content of carbon and hydrogen in the biocrude, with consistent values ranging from 72 - 74% for carbon, 7 - 8% for hydrogen and 31 - 34 MJ/kg for the HHV across all species and treatments. The decrease of the nitrogen and sulfur content of biocrude, as a result of starvation, was consequently compensated for by an increase in the oxygen content to absolute values ranging from 14.8% to 17.2% for all starved biomass. The washing of biomass had no effect on the elemental composition of biocrude.

Of the three species, starved *Oedogonium* produced the biocrude with the lowest nitrogen content of 2.1% and 2.2%, whether washed or not (N−/A− and N−/A+), followed by *Ulva* at 3.0% (N−/A−) and 2.7% (N−/A+) and *Derbesia* at 3.0% (N−/A−) and 3.2% (N−/A+). These biocrudes were also the lowest in sulfur, with concentrations at the ppm level for *Oedogonium* (below the limit of detection) and ranging between 0.2 - 0.3% for *Derbesia and Ulva*.

The quality of biochar produced by the HTL of macroalgae was also only influenced by the starvation treatment (Table 4.2). The biochars produced from starved biomass (N−) generally had a higher content of carbon and hydrogen than biochars from the untreated biomass (N+), with higher energy values ranging between 18 - 20 MJ/kg for starved *Derbesia*, 13 - 16 MJ/kg for starved *Ulva* and 25 MJ/kg for starved *Oedogonium*. Interestingly, the biochar produced from starved *Oedogonium* contained up to 60% carbon with a relatively low inorganic content in the range of 26 - 28% (calculated by subtraction of the sum of C, H, O and N percentages from 100%).

Table 4.2. Ultimate analysis of biocrude, biochar and aqueous products.

Data show means (n = 4) of content dry weight of HTL products following conversion of macroalgae not starved (N+), starved (N−), not washed (A+) and washed (A−).

Species	*Derbesia*				*Ulva*				*Oedogonium*			
Treatment	N+ A+	N+ A−	N− A+	N− A−	N+ A+	N+ A−	N− A+	N− A−	N+ A+	N+ A−	N− A+	N− A−
Biocrude (wt%)												
C	71.9	72.2	73.5	73.5	71.9	73.2	73.1	72.0	71.7	71.7	71.7	72.3
H	7.7	7.9	7.6	7.5	7.2	8.1	6.9	6.9	7.4	7.3	6.6	7.3
O	11.7	11.3	14.8	14.8	12.0	11.9	16.2	15.9	13.8	13.8	17.2	17.0
N	6.1	6.7	3.2	3.0	6.4	5.7	2.7	3.0	5.3	5.3	2.2	2.1
S	0.8	0.7	0.3	0.2	0.7	0.5	0.2	0.2	0.4	0.3	0.0	0.0
HHV*	33.0	33.3	33.0	32.9	32.3	33.8	32.0	31.6	32.2	32.2	31.1	32.1
Biochar (wt%)												
C	28.1	29.7	40.1	45.6	16.3	8.0	34.5	28.5	38.9	41.5	60.0	59.7
H	3.5	2.9	3.0	3.3	2.0	1.6	2.7	2.1	3.2	3.3	4.3	4.2
O	2.7	2.8	3.2	3.7	2.8	1.8	4.0	3.2	4.2	4.3	6.0	4.6
N	2.0	3.1	2.4	2.7	1.4	0.7	1.6	1.3	3.6	3.9	2.6	2.7
S	7.0	5.6	6.6	4.3	14.9	18.1	10.4	13.0	0.6	0.9	1.1	0.7
HHV*	14.3	14.0	17.9	19.9	9.3	6.3	15.8	13.3	16.9	18.0	25.5	25.3
Aqueous phase (mg/L)												
TOC	5750	6214	3807	5150	4750	5343	4064	4071	6921	5843	6121	5743
TIC	557	650	14	29	414	414	29	29	93	100	21	43
TN	1636	1421	465	398	1243	1286	317	272	1043	1021	272	274

*HHV (MJ/kg) = higher heating value; TOC = total organic carbon; TIC = total inorganic carbon; TN = total nitrogen.

The effect of treatments on the composition of the aqueous phase was assessed by measuring the concentration of total organic (TOC) and inorganic carbon (TIC) and total nitrogen (TN). For all three species, the aqueous phase produced from the starved biomass (N−) had a concentration of TOC that was reduced by 17 ± 6% compared to biomass that was not starved (N+). There were similar reductions in TIC of 86 ± 9%, and TN of 74 ± 1% (Table 4.2). The concentration of TOC ranged from 3807 mg/L for starved biomass to

6921 mg/L for biomass that was not starved, and was noticeably higher than the concentration of TIC at ≤ 650 mg/L across all species and treatments. The concentration of TN in the aqueous phase was relatively consistent across all species, and was lower for starved biomass (272 - 465 mg/L) that had initially less nitrogen, compared with biomass that was not starved (1021 - 1636 mg/L).

4.3.5. Chemical energy recovery and mass balance

While the heteroelements nitrogen and sulfur decreased with the various treatments, it is instructive to examine how these lower values partition across the product streams. Thus, the elemental and energy recoveries of the HTL products presented in Table 4.3 were determined from the ultimate analysis of biocrude and biochar, and calculated by difference for the remaining combined aqueous and gas products. Importantly, the two treatments of nutrient starvation and washing had a substantial effect on the distribution of nitrogen and sulfur. The mass balance shows that most of the nitrogen did not report to the char, but was partitioned between the biocrude and the combined aqueous and gas phases. The relative recovery of nitrogen in the biocrude increased with the treatments, following the trend: untreated < washed < starved < starved and washed biomass. In terms of nitrogen partitioning, HTL of starved and washed biomass (N−/A−) had the effect of pushing a higher proportion of the biomass nitrogen into the biocrude fraction. This differed from untreated algal samples (N+/A+), in which the combined aqueous and gas phases ended up with proportionally more nitrogen than the biocrude. For example, N−/A− *Ulva* retained 64.4% of its nitrogen in the biocrude fraction, whereas the biocrude generated from N+/A+ *Ulva* only retained 31.8% of the biomass nitrogen.

Table 4.3. Element conversion ratio and energy recovery (ER) in HTL product streams. Data show means (n = 4) of element conversion ratio and energy recovery based on dry weight (wt%) in HTL products following conversion of macroalgae not starved (N+), starved (N−), not washed (A+) and washed (A−).

Species		*Derbesia*				*Ulva*				*Oedogonium*			
Treatment		N+ A+	N+ A−	N− A+	N− A−	N+ A+	N+ A−	N− A+	N− A−	N+ A+	N+ A−	N− A+	N− A−
C	Biocrude	49.0	54.5	46.6	50.6	45.1	49.3	50.1	49.6	49.8	53.9	48.5	49.5
	Biochar	7.0	5.1	8.1	8.6	4.9	3.4	5.7	5.9	4.9	5.9	5.1	4.7
	Aq.+ Gas	44.0	40.4	45.3	40.8	50.0	47.3	44.2	44.5	45.2	40.3	46.4	45.7
H	Biocrude	32.7	39.5	28.6	32.7	25.5	32.0	26.4	26.6	33.7	36.3	29.0	32.3
	Biochar	5.4	3.3	3.7	3.9	3.4	3.9	2.4	2.4	2.7	3.1	2.3	2.1
	Aq.+ Gas	61.9	57.1	67.8	63.4	71.1	64.1	71.2	71.0	63.7	60.6	68.7	65.6
O	Biocrude	9.7	12.3	8.2	9.6	5.6	6.4	6.9	6.8	10.9	11.7	10.3	10.2
	Biochar	0.8	0.7	0.6	0.7	0.6	0.6	0.4	0.4	0.6	0.7	0.4	0.3
	Aq.+ Gas	89.4	87.0	91.3	89.7	93.8	93.0	92.7	92.8	88.5	87.6	89.3	89.5
N	Biocrude	28.8	34.7	41.8	58.7	31.8	30.0	55.1	64.4	38.7	42.2	43.5	58.8
	Biochar	3.5	3.7	9.8	14.4	3.3	2.3	7.7	8.3	4.9	5.8	6.3	8.6
	Aq.+ Gas	67.7	61.6	48.4	26.9	64.9	67.7	37.2	27.3	56.5	52.0	50.2	32.6
S	Biocrude	10.3	23.8	4.3	12.2	2.8	2.5	0.7	1.0	58.2	50.7	8.0	19.1
	Biochar	33.3	46.4	29.1	77.5	28.2	57.9	10.2	16.5	16.2	27.7	24.2	40.8
	Aq.+ Gas	56.4	29.8	66.6	10.3	69.0	39.6	89.1	82.5	25.6	21.6	67.7	40.2
ER	Biocrude	49.1	55.3	48.5	53.2	46.9	52.8	53.3	53.1	51.4	55.6	50.6	53.1
	Biochar	7.8	5.3	8.4	8.8	6.4	6.1	6.4	6.8	4.9	5.8	5.2	4.8
	Aq.+ Gas	43.0	39.4	43.2	37.9	46.7	41.1	40.3	40.1	43.7	38.6	44.2	42.0

Aq. + Gas = aqueous and gas products are combined and determined by difference.

The effect of both treatments on the sulfur content of macroalgae and consequently its distribution in HTL product streams was variable (Table 4.3). The starvation treatment (N−) led to a 65 ± 6% reduction in the recovery of sulfur in biocrude on average across all

species, while the washing treatment had the opposite effect with a slight increase of the recovery of sulfur in the biocrude. For marine species and particularly for *Ulva*, the majority of the sulfur (76 - 99%) contained in the biomass was effectively excluded from the biocrude phase. For freshwater *Oedogonium* that has an inherently low sulfur content, the same effect occurred with most of the sulfur excluded from the biocrude phase, particularly after starvation (81 - 92%), resulting in biocrudes with a sulfur content at the ppm level. In a similar way, most of the oxygen did not report to the biocrude but to the combined aqueous and gas phases (87 - 94%), with a relatively high consistency in the distribution of oxygen in HTL product streams across treatments. As a result, the starved biomass (N−) that initially had a higher content of oxygen produced a biocrude that was also higher in oxygen, compared with biomass that was not starved (N+).

Despite a high variability in the elemental composition of the macroalgal feedstocks, there was little variation in the recovery of carbon and hydrogen in the biocrude (Table 4.3). This manifested through a slight decrease in the recovery of both elements after the starvation treatment (< 5%, compared with untreated biomass), and a slight increase in their recovery following the washing treatment (< 6%, compared with untreated biomass). This relatively consistent recovery of carbon in biocrude across treatments was best illustrated by a plot of biomass carbon content and biocrude yield in Fig. 4.7, which showed that the two variables were strongly correlated ($R^2 = 0.96$). The line shown in this figure represents a linear correlation of the data, within a carbon content of biomass in the range of 29.7 - 48.2%:

$$Y_{BIOCRUDE} = 0.885 \times C_{BIOMASS} - 7.455 \quad \textbf{Eq. 4.2}$$

where $Y_{BIOCRUDE}$ is the yield (dw) of biocrude and $C_{BIOMASS}$ is the carbon content (dw) of the biomass. *Ulva* and *Oedogonium* had a relatively narrow range of carbon values across treatments, however, the biocrude yield varied linearly across a wide range of biomass carbon values for *Derbesia*.

Approximately half of the biomass energy was transferred to the biocrude with most of the remainder transferred to the combined aqueous and gas phases.

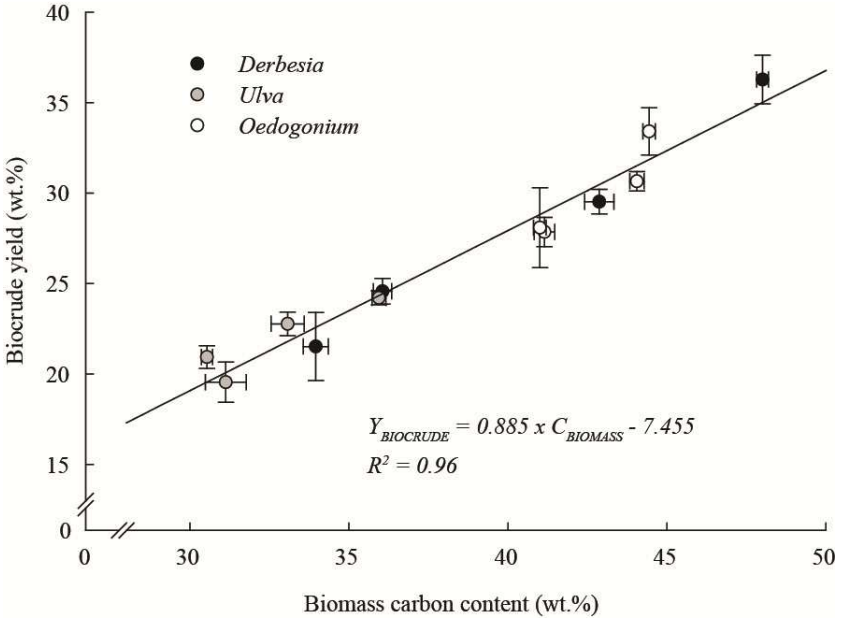

Figure 4.7. Effect of biomass carbon content on biocrude yield.
Data show means (n = 4 per treatment ± SE) of the effect of biomass carbon content on biocrude yield (% dw) for the three species of macroalgae.

4.4. Discussion

The results demonstrate that it is indeed possible to effectively manipulate the composition of macroalgal biomass through pre- and post-harvest treatments. The nutrient starvation of the cultures and washing of the biomass, individually or combined, significantly affected the quality of macroalgal feedstocks. Restricting the supply of nutrients to macroalgal cultures for 18 days resulted in an effective reduction of the content of nitrogen and sulfur in biomass, and consequently an effective reduction in the content of nitrogen and sulfur in biocrude. However, starved biomass was also lower in carbon per unit of mass and this led to a decreased yield of biocrude. The decrease in carbon was due to the modification of the organic profile of the biomass, with a lower proportion of proteins and lipids, and a higher proportion of carbohydrates that are less dense in carbon. Most importantly, starvation resulted in a major decrease in biomass productivities that is due to the essential metabolic roles of nutrients, particularly nitrogen, in photosynthesis, the synthesis of proteins, and the catalytic capacity of enzymes (Hein et al., 1995). Therefore, the efficiency of starvation in enhancing the quality of biocrude is offset by a simultaneous decrease of the overall yield and productivity of biocrude, as highlighted in Table 4.4. Furthermore, the efficiency of starvation in reducing the content of nitrogen and sulfur in biocrude is balanced by an increase of the oxygen content of the biocrude, which counteracts some of the potential benefits made in terms of the requirements for the upgrading of biocrude to a blendable fuel.

Table 4.4. Summary table of the productivity and yield of biomass, biocrude and biochar. Data show means (n = 4) of productivity and yield for macroalgae not starved (N+), starved (N−), not washed (A+) and washed (A−). Values for biomass productivity are an average of the three last weeks of culture (starvation phase).

Species	*Derbesia*				*Ulva*				*Oedogonium*			
Treatment	N+	N+	N−	N−	N+	N+	N−	N−	N+	N+	N−	N−
	A+	A−	A+	A−	A+	A−	A+	A−	A+	A−	A+	A−
Biomass												
P (g/m^2/d dw)	12.6	9.3	4.3	3.3	20.7	16.7	3.3	2.7	9.9	9.8	7.6	7.6
P (g/m^2/d afdw)	8.1	8.1	2.9	2.9	12.5	12.5	2.0	2.0	8.5	8.5	6.6	6.6
Biocrude												
Y (% afdw)	38.6	41.7	31.4	33.4	32.6	32.3	34.0	30.6	35.6	38.8	32.2	32.6
P (g/m^2/d afdw)	3.1	3.4	0.9	1.0	4.1	4.0	0.7	0.6	3.0	3.3	2.1	2.1
Heteroatoms (wt%)												
O	11.7	11.3	14.8	14.8	12.0	11.9	16.2	15.9	13.8	13.8	17.2	17.0
N	6.1	6.7	3.2	3.0	6.4	5.7	2.7	3.0	5.3	5.3	2.2	2.1
S	0.8	0.7	0.3	0.2	0.7	0.5	0.2	0.2	0.4	0.3	0.0	0.0
Biochar												
Y (% afdw)	14.1	9.5	10.0	9.2	15.5	20.2	8.3	9.3	6.5	7.3	4.0	3.8
P (g/m^2/d afdw)	1.1	0.8	0.3	0.3	1.9	2.5	0.2	0.2	0.6	0.6	0.3	0.2

P = productivity; Y = yield.

Washing macroalgal biomass with freshwater to reduce the ash content (i.e. inorganic salts, alkali metals and halides) was globally beneficial to the biocrude production process. For all species, washing was effective in removing the external salts (trapped between algae blades or filaments) following harvesting and dewatering. In addition, I believe that a second mechanism of osmosis caused variation in the response of the species to the treatment. The exceptional decrease of ash content in *Derbesia* (> 80%) was most likely due to the coenocytic siphonous structure of the alga, composed of a single giant cell (Lobban & Harrison, 1996), enabling direct contact and passive diffusion of osmolytes between the entire internal cytoplasm and the external medium (freshwater). The osmotic

effect was less effective at removing the internal salts of *Ulva* which is two cells thick, or of the freshwater *Oedogonium* that already has a low internal ash content. Washing did not affect biocrude production, where the loss of (inorganic) biomass harvested per unit area was compensated for by higher biocrude yields per unit of biomass processed (on a dry basis). Importantly, a major benefit of using washed biomass, especially for processing of marine species, will be a reduced load of ash in the continuous flow reactor reducing the effects of corrosion (Peterson et al., 2008; Jazrawi et al., 2013). It is also important to consider the effect of washing in terms of life cycle analysis (Grierson et al., 2013), where the organic fraction is concentrated within the biomass (higher energy content), therefore increasing the efficiency of biomass transportation and processing. The disposal of the water used to wash the biomass, containing dissolved inorganic salts (1 - 5 g/L, when using 3 L/100 g of algae), could be achieved through its recycling in marine macroalgal cultures to compensate for evaporation.

In this study, the whole nutrient supply to the cultures (not only nitrogen) was restricted to evaluate the effect of the starvation treatment in a scaled-up algal cultivation concept. In this concept, wastewater is used as a cost-effective nutrient source to grow algae at optimal conditions (Roberts et al., 2013b), before transferring the biomass to a nutrient-free environment (polishing tank) in order to reduce the nitrogen content. Of the species of macroalgae investigated, the freshwater *Oedogonium* showed promising results, with the ability to be starved of nutrient for a period of 6 - 8 days without a significant impact on productivity. After an extended period of starvation of 18 days, the liquefaction of starved *Oedogonium* produced a biocrude of high quality with a low content of nitrogen (2%) and

sulfur (< 0.1%), reducing the hydrogen requirement for biocrude upgrading into a blendable fuel. The nitrogen content of the biocrudes reported in this study for starved macroalgae, particularly *Oedogonium*, is noticeably lower than the nitrogen content of biocrudes reported to date in other HTL studies of algae (listed in Frank et al., 2013), which demonstrates the potential benefits of the starvation treatment. For other algal species, and especially for marine species for which nutrient starvation had a major impact on biomass productivity, the assessment of profitability in terms of biocrude productivity and refining costs (hydrogen demand for the hydrotreatment) will determine if the starvation treatment of the cultures is beneficial. For this assessment, it will be critical to determine the length and intensity (nutrient concentration) of the starvation treatment, if at all, on a species by species basis.

Furthermore, the relationship between the nitrogen content of biomass and biocrude showed that a higher proportion of biomass nitrogen recovered in the biocrude phase for (starved) biomass that contained a lower proportion of nitrogen. Given that nutrient starvation could be detrimental to the aim of recycling nitrogen and the overall productivity of biocrude production, the possibilities of selectively extracting nitrogen from biomass as protein, prior to HTL of the residual biomass, is a key area of future research.

In terms of conversion efficiency, *Oedogonium* showed promising results with a high yield of biocrude (36 - 39% afdw), even after starvation (32 - 33% afdw). It is also important to note that *Oedogonium* had the lowest productivity of the three species investigated, however, higher productivities of 15 - 20 g/m^2/d dw at large-scale have been demonstrated

by Cole et al. (2014). *Derbesia* that was not starved of nutrients afforded the highest yield of biocrude (39 - 42% afdw) of the three species, and this was higher than the yield previously reported (Chapter 3). It was also higher than yields reported in the literature for green and brown seaweeds (Zhou et al., 2010; Anastasakis & Ross, 2011). These higher yields are most likely due to a lower ash content and a higher lipid content than the marine species that have been the focus of research to date (Zhou et al., 2010; Anastasakis & Ross, 2011), and therefore a higher proportion of organic carbon. Furthermore, these yields are comparable to the middle range of yields obtained for several microalgae and cyanobacteria species processed under similar conditions, including *Dunaliella tertiolecta*, *Chlorella vulgaris* and *Spirulina platensis* (Minowa et al., 1995; Biller & Ross, 2011; Jena et al., 2011; Frank et al., 2013). The data presented here confirm that yields of > 35% afdw biocrude can be achieved through the HTL of low-lipid feedstocks such as micro- (Yu et al., 2011a) and macroalgae.

Notably, *Ulva* had the lowest biocrude yield of all three species, mainly due to a low carbon content, and this yield was not affected by the starvation treatment for the same reason. However, *Ulva* had the highest productivity of the three species, and consequently the highest productivity of biocrude in untreated conditions (4.1 g biocrude/m^2/d, compared to 3.0 - 3.1 g biocrude/m^2/d for *Derbesia* and *Oedogonium*), highlighting that selecting species with a high biocrude yield is not systematically the preferred option (Table 4.4), unless a high biomass productivity can also be achieved (Chapter 2; Garcia Alba et al., 2012). The optimisation of biomass productivities is therefore central to improving efficiencies in the production of biocrude. Similarly, several studies showed that

the operating conditions of HTL including temperature, solids loading, residence time, and the use of heterogeneous catalysts greatly influence biocrude yield and composition, and the optimisation of operating parameters will also be critical in achieving maximum recovery of biomass energy (Biller & Ross, 2011; Garcia Alba et al., 2012; Torri et al., 2012).

Finally, a significant portion of biomass energy was also recovered in the biochar, aqueous and gaseous co-products. The starvation of biomass resulted in a lower yield and higher quality of biochar, with higher carbon and energy and a lower inorganic fraction than biochar produced from biomass that was not starved. This high carbon and low ash biochar is suitable for agriculture as a soil ameliorant and fertiliser and could add value to the overall production process while providing benefits for long term sequestration of carbon (Bird et al., 2012). In contrast to biochar, a high portion of the biomass energy was recovered in the combined aqueous and gas phases. The aqueous phase has been the focus of studies investigating the recycling of nutrients (N, P, K) back into algal cultures (Biller et al., 2012; Elliott et al., 2013b; Garcia Alba et al., 2013; Pham et al., 2013) or the gasification of the organics to recover some energy as hydrogen (Elliott et al., 2013a), to add value to the overall process. Similarly, the carbon dioxide that forms most of the gas phase could be recycled back into algal cultures to enhance growth (Yu et al., 2011b; Cole et al., 2013). The recovery of all co-products from HTL will be critical to increase the value of the algal biocrude production process.

4.5. Conclusions

In conclusion, this study has demonstrated that macroalgae can be manipulated in culture, and in post-harvest processing, to specifically improve the composition of feedstock for the production of biocrude. The treatments of nutrient starvation and the washing of biomass were effective in reducing the content of nitrogen, sulfur and ash in biomass, which resulted in an improved quality of biocrude. While further optimisation of the HTL process will improve the recovery of biomass energy to biocrude, I demonstrate that the optimisation of culture protocols and post-harvest processing is a powerful tool to add viability to the algae-to-biofuel concept.

Chapter 5

Cultivation of freshwater macroalgae in municipal wastewater for nutrient removal and biofuel production

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5.1. Introduction

Population growth and socio-economic development have rapidly increased the demand for water, resulting in an increasing need to reclaim or re-use abundant sources of wastewater such as municipal wastewater (FAO Report, 2012). In industrialised countries, the daily production rate of municipal wastewater reaches approximately 265 litres per capita, a rate that is likely to increase to 375 litres per capita in areas with significant industrial contributions (Ellis, 2004; FAO AQUASTAT database). Most of the sewage produced is directed to wastewater treatment plants where contaminants are separated from the water to produce a wastewater stream and a solid waste (sludge) suitable for discharge or re-use. Conventional municipal wastewater treatment consists of a primary treatment where solid material and large particles are partitioned, a secondary treatment in which suspended and soluble organic substances are degraded by microorganisms and a facultative tertiary treatment in which the quality of the effluent is further improved before discharge to the environment.

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[3] **Chapter 5** is adapted from Neveux N, Magnusson M, Mata L, Whelan A, de Nys R, Paul NA, 2015. The treatment of municipal wastewater by the macroalga *Oedogonium* sp. and its potential for the production of biocrude. *Algal Research*, accepted for publication.

If not treated appropriately, wastewater represents a risk for eutrophication of aquatic ecosystems combined with a significant socio-economic impact on coastal activities such as tourism and fisheries (Asano & Cotruvo, 2004). However, wastewater also represents large quantities of freshwater and nutrients, and efforts are focused on putting this potentially valuable resource to beneficial use rather than discharging it to the environment. For instance, the reclamation of treated wastewater to irrigate parks and agricultural land and the reuse of the sludge to produce biogas and agricultural fertilisers are now common practices (FAO Report, 2012). Nonetheless, the nutrients, heavy metals and pathogenic microorganisms that may still remain in treated wastewater and sludge can adversely impact soil and water quality, hence limiting their reuse (Cheng, 2003; Asano & Cotruvo, 2004).

A complementary method that allows efficient recovery of nutrients and energy from wastewater is the cultivation of algae. The concept of growing algae directly in wastewater ponds for simultaneous wastewater treatment and biomass production was first suggested in the 1960s and has been regularly promoted since (Oswald & Golueke, 1960; Craggs et al., 2012a). Algae are fast growing photosynthetic organisms that are potentially able to produce large quantities of biomass on land that is unsuitable for conventional agriculture. Additionally, the ability of algae to grow in nutrient-rich water and to efficiently remove nutrients and metals from wastewater make them an attractive option to explore for integrating into wastewater treatment plants (Pittman et al., 2011). Different types of wastewaters collected at various stages of the purification process have been successfully tested for their ability to support algal growth, including primary effluent (Craggs et al.,

1994; Wang et al., 2010), secondary effluent (Ruiz-Marin et al., 2010; Sturm and Lamer, 2011) and centrate wastewater, which is the water from the activated sludge thickening process (Wang et al., 2010; Sode et al., 2013). Until recently, the use of algae has been restricted to microalgae, with the accepted paradigm being that freshwater microalgae are the most suitable target for the bioremediation of wastewater streams (Rawat et al., 2011). However, freshwater macroalgae also have demonstrated applications in the bioremediation of wastewater streams due to their high productivity and competitive dominance which enables monocultures to be maintained in open systems (Lawton et al., 2013a; Roberts et al., 2013a). More specifically, filamentous green macroalgae from the genus *Oedogonium* are particularly effective at remediating waste nutrients from intensive aquaculture production with biomass productivities of 16 - 36 g/m^2/d dw and nutrient removal rates of ~1.0 g N/m^2/d and ~0.1 g P/m^2/d (Cole et al., 2014; Cole et al., 2015).

The potential of freshwater macroalgae for cultivation in municipal wastewater is yet to be quantified but hinges on two considerations. Firstly, the biomass production must be sufficiently high in the wastewater source to support the extraction of nutrients. This is because treatment is correlated to biomass productivity (Lundquist et al., 2010) and wastewater sources at different stages of the treatment process have different water qualities (Wang et al., 2010). This variation in water quality is compounded by changes in the quality of the influent sewage water throughout the year (Craggs et al., 2012a). Secondly, the resulting biomass composition must be of suitable quality for the generation of co-products such as biofuels (Chapter 2). In many cases it is expected that large-scale algal cultivation for the production of fuel will have to rely on waste nutrients to be

sustainable (Park et al., 2011a). Consequently both the biomass productivity and biomass composition are critical for an initial assessment of the suitability of freshwater macroalgae as an effective and sustainable component of a wastewater treatment plant. The aim of this chapter was therefore to assess the potential of the freshwater macroalga *Oedogonium* sp. for simultaneous nutrient removal and biomass production. Firstly, I evaluated the suitability of three wastewater sources, the effluents from the primary and secondary clarifiers and underflow effluent from the dissolved air flotation unit, at various exchange rates to support algal growth in small-scale culture trials and analysed how the water quality influences the biochemical composition of the algae. Secondly, the most effective wastewater source and exchange rate were investigated in pilot-scale cultures of *Oedogonium* in open ponds. Finally, the nutrient removal rates and the potential yield and scale of biofuel production using macroalgae and municipal wastewater were calculated for the open pond system.

5.2. Materials and methods

5.2.1. Source biomass

The filamentous freshwater green macroalga *Oedogonium* sp. (GenBank accession number: EKC701473, Lawton et al., 2014) was selected for this study due to its high productivity in land-based systems, resistance to contamination, tolerance to environmental fluctuations and biochemical profile suited to the development of bio-products (Lawton et al., 2013a; Cole et al., 2014; Chapter 2; Chapter 3). *Oedogonium* biomass is maintained as stock cultures held in outdoor tanks at James Cook University aquaculture facility (Townsville, 19^{o}33’S; 146^{o}76’E).

5.2.2. Small-scale culture trials

Three wastewater sources were collected from the Cleveland Bay Municipal Wastewater Purification Plant (Townsville) and transported to James Cook University to be tested as a nutrient source for the culture of *Oedogonium*, from March to April 2014. The wastewaters were the primary treated effluent (PRIM), the secondary treated effluent (SEC) and the underflow effluent from the dissolved air flotation unit (DAF) (Fig. 5.1). The three wastewaters were held in separate 1,000 L sumps with the water quality measured at days 0, 21 and 42 (end) of the experiment (Table 5.1 shows the properties of the three wastewater sources).

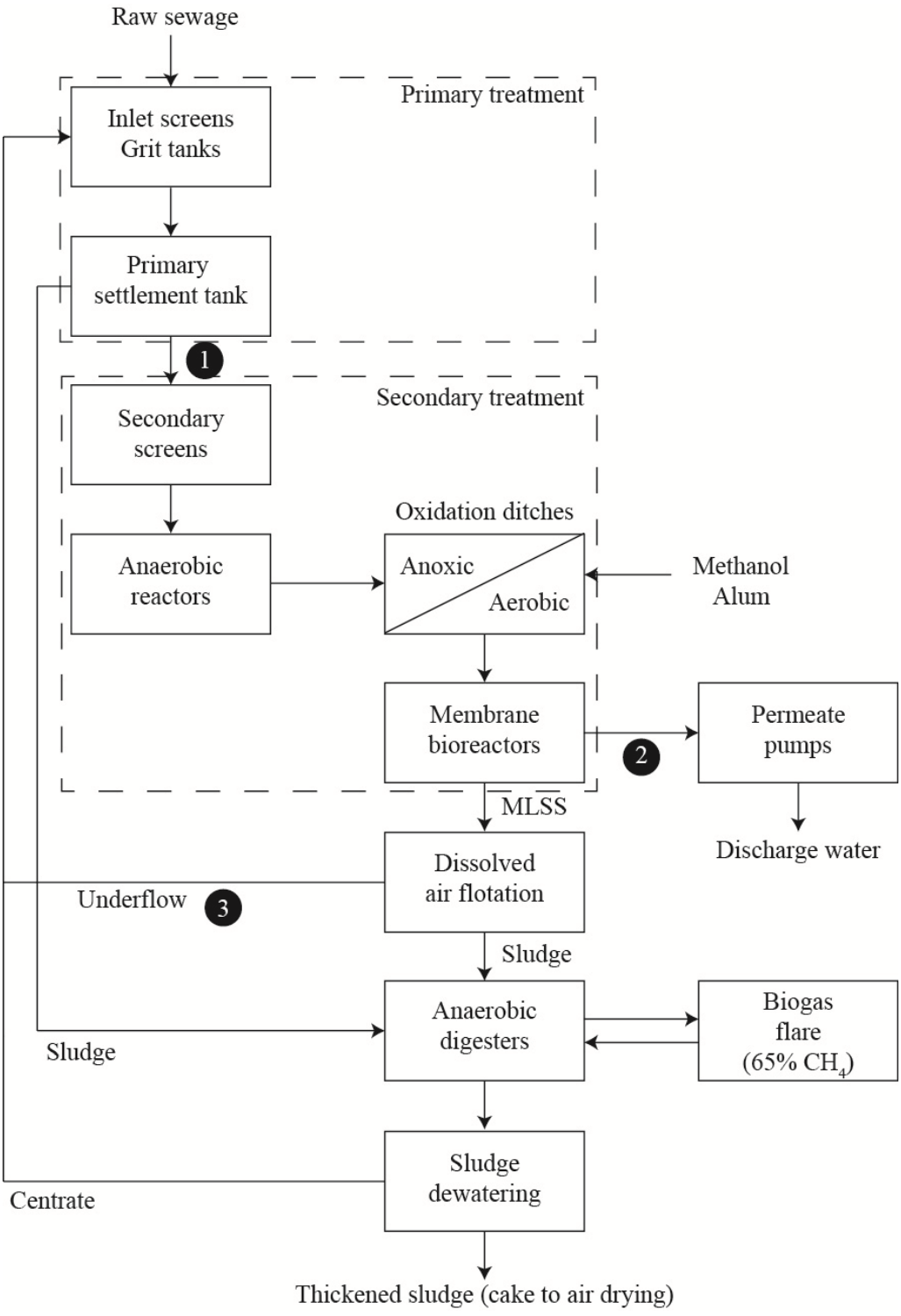

Figure 5.1. Simplified schematic diagram of Cleveland Bay Municipal Wastewater Treatment Plant. 1 = PRIM ; 2 = SEC ; 3 = DAF; MLSS = mixed liquor suspended solids.

Table 5.1. Properties of the three wastewater sources.

Data show means (n = 3) of properties of wastewaters taken at day 0, 21 and 42.

Water properties	Unit	PRIM	SEC	DAF
Alkalinity (total CO_3)	mg CO_3/L	272	196	193
pH		8.1	7.6	7.8
Salinity	g/L	1.03	0.99	1.00
Conductivity	mS/cm	2.29	2.16	2.20
TSS	mg/L	50	2	22
TOC	mg/L	9	5	5
COD	mg/L	31	12	24
Nitrogen (total N)	mg N/L	27.20	1.12	2.79
Dissolved	mg N/L	23.81	0.89	2.05
Inorganic	mg N/L	23.11	0.66	1.70
Ammonia	mg N/L	17.15	0.02	0.10
Nitrite	mg N/L	5.19	0.01	0.02
Nitrate	mg N/L	0.77	0.64	1.58
NO_x	mg N/L	5.97	0.64	1.59
Organic	mg N/L	0.70	0.22	0.36
Particulate	mg N/L	3.38	0.23	0.74
Urea N	mg N/L	0.31	0.04	0.04
Phosphorus (total P)	mg P/L	5.04	0.23	1.11
Dissolved	mg P/L	4.30	0.20	0.93
Inorganic (FRP)	mg P/L	4.13	0.20	0.83
Organic	mg P/L	0.17	0.01	0.10
Particulate	mg P/L	0.75	0.03	0.18
DIN:DIP molar ratio		12.7	7.5	5.0
Microbes				
E.coli	cfu/100mL	$5.4.E10^6$	50	$8.8.E10^3$
P.aeruginosa	cfu/100mL	$0.2.E10^6$	1	$0.7.E10^3$
Faecal coliforms	cfu/100mL	$6.2.E10^6$	50	$8.8.E10^3$
Total coliforms	cfu/100mL	$75.0.E10^6$	75	$11.6.E10^3$
Metals				
Ca	mg/L	29	28	28
K	mg/L	20	19	20
Al	µg/L	35	225	370
Fe	µg/L	115	380	240
Mn	µg/L	88	5	22
Zn	µg/L	9	13	21

PRIM = primary effluent; SEC = secondary effluent; DAF = effluent from DAF unit; cfu = colony-forming unit.

Small-scale trials were conducted in 20 L cylindrical tanks in an outdoor system, described in Chapter 2. Each tank was stocked with 5 g fw of biomass (equivalent to 0.25 g/L) and filled with nutrient-free freshwater. PRIM, SEC and DAF were subsequently added to the cultures at various exchange rates to acclimate the algae progressively to the wastewater. The exchange rates tested were 5, 10 and 20% (v/v) renewal per day, which represent reasonable exchange rates that can be used at large scale. To perform the water exchange, the appropriate volume of water was drained from the tank through a sieve (200 μm) to retain the algae in the tank, and replaced with the same volume of wastewater. After 2 weeks of culture, the biomass in each tank was harvested using a filter bag (200 μm), drained to constant fresh weight in a domestic centrifuge (washing machine Fisher & Paykel, Australia - spin mode) and weighed to determine biomass productivity. The biomass was subsequently dried and stored in vacuum-sealed bags at room temperature. Only the biomass productivity from the second week of culture at each time was analysed as this biomass was then acclimated to the water source and the biochemical composition was analysed for these same samples. This 2-week culture experiment was run three times (a total of 6 weeks of culture) to show consistency in biomass productivity. Biomass productivity was analysed using a factorial analysis of variance (3-way ANOVA - STATISTICA 10; StatSoft Inc., USA), with water source and exchange rate as a fixed factors, and culture time as a random factor.

Environmental conditions throughout the experimental period were monitored daily. The culture tanks were placed in a larger holding tank acting as a water bath with continuously

flowing water at 25 - 27^{o}C for temperature control. The pH of the cultures varied naturally between 8.5 (sunrise) and 10.8 (sunset), and ambient photosynthetically active radiation (PAR) ranged from 250 to 423 mol photon/m^2/week, which corresponds to the season of high-photon irradiance (summer) in Townsville.

5.2.3. Production of biomass in pilot-scale open ponds

The continuous culture of *Oedogonium* was conducted outdoors for 8 weeks, from June to August 2014. The pilot-scale system consisted of parabolic cultivation ponds, each with a surface area of 16 m^2 (depth 89 cm; length 721 cm; width 221 cm) and a total volume of culture of 10 m^3. A single aeration line running along the base of the pond ensured constant water motion in the ponds. In an analogous manner to the small scale experiment, each pond (n = 3) was initially stocked with 2.5 kg fw of *Oedogonium* (0.25 g/L) and filled with nutrient-free freshwater. Primary treated effluent (PRIM) was added to the ponds at a rate of 5% per day (v/v) (or a hydraulic residence time of 20 days) to acclimate the algae progressively to the wastewater. These effluent (PRIM) and water exchange rate (5%) were chosen as they resulted in the highest biomass productivity in the small-scale culture trials, acknowledging that the PRIM sampled in June-August (dry season) for this experiment was more concentrated in nutrients than the PRIM sampled in March-April (rainy season), effectively delivering the equivalent of 10% per day from the culture trials. Every morning, 0.5 m^3 of water were drained from each pond and replaced by 0.5 m^3 of PRIM after it was filtered at 150 μm through a sand filter. The biomass was harvested on a weekly basis through a mesh filter bag (200 μm), drained and spun to constant fresh weight and weighed to determine biomass productivity. This biomass was used to restock the ponds for the

subsequent week of culture at the same initial stocking density each week (0.25 g/L), while the excess biomass was sun-dried on trays for 4 days until constant dry weight was achieved. Dried biomass was packed in sealed bags and stored in the dark at room temperature.

Water quality was measured at the start of the experiment and then on each day of harvest, for both the influent (PRIM) and effluent water drained from the ponds. Water temperature was recorded hourly using a temperature logger (see Fig. 5.3). The pH in the ponds increased from 9.3 (sunrise) to 9.9 (sunset) on average, due to the photosynthetic uptake of CO_2 through the course of the day. Ambient PAR ranged from 123 to 228 mol photon/m^2/week for the duration of the experiment, which corresponds to the season of low-photon irradiance (winter) in Townsville.

5.2.4. Biomass characterisation

Approximately 15 g fw from each sample were weighed immediately after harvest and centrifugation, oven-dried for 12 hours at 60^{o}C, placed in a desiccator for 30 minutes at room temperature to reach stable moisture content, and weighed again (dw) to determine the fresh to dry weight ratio (fw:dw). The biochemical composition of the biomass was analysed at the end of the 2-week period for the three small-scale culture trials and at the end of each of the 8 weeks for the pilot-scale production of biomass, for each replicate (n = 3 for all times). Moisture content of the algae was determined using a moisture balance (MS70; A&D Company Ltd., Japan) set at 110^{o}C, and ash (dry inorganic) content was determined by combustion (550^{o}C, 6h) in a muffle furnace (SEM Ltd., Australia).

Subsamples of dried biomass were sent to OEA Laboratories Ltd. (UK) to determine the internal carbon, hydrogen and nitrogen contents, while other subsamples were analysed by the Advanced Analytical Centre (JCU, Australia) for metal content.

Biomass productivity was calculated according to Eq. 2.1 and nutrient removal rate (NR) was determined as follow:

$$NR = P \times E_{BIOMASS} / 100 \quad \textbf{Eq. 5.1}$$

where P is biomass productivity ($g/m^2/d$, dw) and $E_{BIOMASS}$ is the content of an element (e.g. N, P, K, S) in the dried biomass.

5.2.5. Biomass application

The carbon content of biomass was used to assess the potential of this feedstock for biofuel production. The Equation 4.2 was used to estimate the theoretical yield of biocrude produced at 330 - 345^{o}C for 5 minutes with a solid to water loading ratio of 6.6% (Chapter 4). For *Oedogonium* biomass with a carbon content within the range of 29.7 - 48.2%, it was shown that the yield of biocrude could be calculated accurately ($R^2 = 0.96$).

5.3. Results

5.3.1. Small-scale culture trials

The physicochemical properties of the effluents from the primary (PRIM) and secondary clarifiers (SEC) and from the dissolved air flotation unit (DAF) differed substantially for all variables (Table 5.1). PRIM had the highest concentration of nitrogen, phosphorus, carbon, chemical oxygen demand (COD), suspended solids and microbes, while SEC had the lowest.

Most of the nitrogen and phosphorus were present in dissolved inorganic forms (DIN and DIP), which are readily available for algal uptake. PRIM was comparatively rich in nitrogen with a molar DIN:DIP ratio of 12.7 while the other two wastewaters had proportionally more phosphorus with ratios of 7.5 for SEC and 5.0 for DAF. The mineral content of the three wastewaters was comparable with a few exceptions (Table 5.1; Annex 2, Table S5.1); noticeably, the aluminium content in SEC and DAF was markedly higher than in PRIM due to the addition of flocculating agent (aluminium sulphate) in the activated sludge process, iron was typically higher in SEC, and manganese was higher in PRIM.

The biomass productivity varied considerably between the treatments, demonstrating that PRIM was the most suitable wastewater source for culture (ANOVA, $F_{2,18} = 191.1$, $P < 0.001$) (Fig. 5.2). *Oedogonium* had the highest productivity of 12.7 - 13.8 g/m^2/d dw with PRIM at the exchange rates of 5, 10 and 20% per day. There was no statistical difference in productivity between these three exchange rates (ANOVA, $F_{2,6} = 2.9$, $P = 0.13$) as nutrients were provided in excess. However, the average (± standard error) biomass productivity was slightly higher for the 5% and 10% treatments at 13.6 ± 0.4 and 13.8 ± 0.4 g/m^2/d dw respectively compared with the 20% treatment at 12.7 ± 0.1 g/m^2/d dw. The two other wastewaters (SEC and DAF) did not have sufficient nutrients at the exchange rates investigated for the algae to maintain high productivity over 2 weeks of culture, resulting in nutrient-starved biomass (see N and P content in subsequent paragraph). The highest productivity using SEC and DAF was obtained with the 20% exchange per day (6.3 and 9.2 g/m^2/d dw respectively).

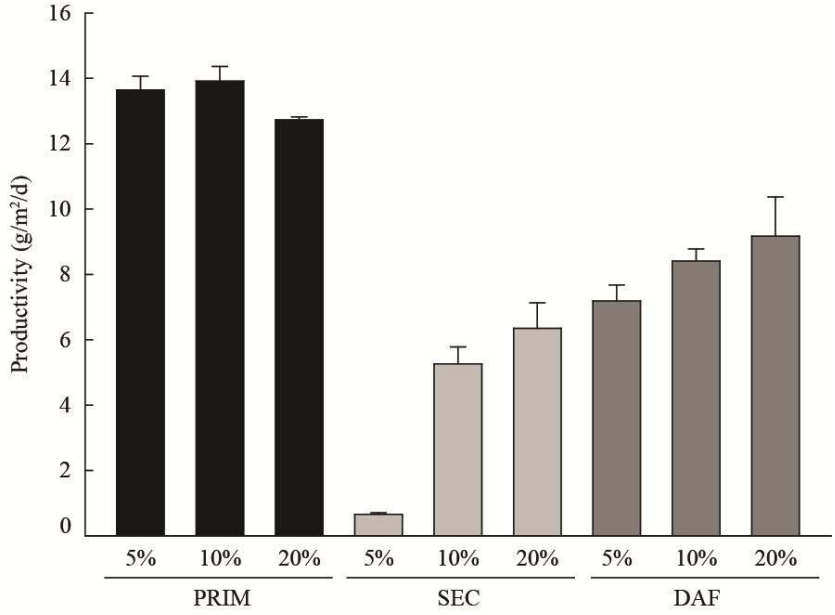

Figure 5.2. Biomass productivity for the small-scale culture trials.
Data show means (n = 3 ± SE) of productivity dry weight of biomass cultured in primary effluent = PRIM; secondary effluent = SEC and effluent from DAF unit = DAF at various exchange rates.

The elemental analyses presented in Table 5.2 also show that the biomass cultured with PRIM was not limited by nutrient availability as it had the highest content of carbon (45.2 - 45.6%), nitrogen (4.7 - 5.7%) and phosphorus (6.0 - 8.5 g/kg). In contrast, biomass cultured in SEC and DAF presented the characteristics of starved biomass with low contents of carbon (38.5 - 41.3%), nitrogen (1.0 - 2.8%) and phosphorus (0.2 - 5.2 g/kg). These results confirm that the supply of nutrients in these two effluents was not sufficient to sustain algal growth.

PRIM was the most effective nutrient source to grow *Oedogonium* at the water exchange rates of 5 - 20% per day, resulting in the highest nitrogen uptake rates of 0.6 - 0.7 g $N/m^2/d$

and phosphorus uptake rates of 0.08 - 0.09 g $P/m^2/d$. Therefore these parameters were further investigated in a continuous culture of *Oedogonium* in pilot-scale ponds.

Table 5.2. Biochemical composition of the biomass for the small-scale culture trials. Data show means (n = 3) of content of biomass cultured in wastewaters at various exchange rates.

Properties	PRIM			SEC			DAF		
	5%	10%	20%	5%	10%	20%	5%	10%	20%
Proximate (wt%)									
Ash	7.7	6.9	6.2	6.7	6.6	6.4	7.1	7.0	6.8
Moisture	5.3	4.8	4.1	4.9	4.3	4.2	4.4	4.7	4.5
Ultimate (wt%)									
C	45.2	45.3	45.6	39.9	38.5	39.1	41.0	41.3	40.9
H	7.0	7.0	7.1	6.6	6.3	6.4	6.7	6.8	6.7
O*	30.1	30.7	31.3	40.8	42.9	42.8	39.8	38.4	38.2
N	4.7	5.3	5.7	1.0	1.5	1.1	1.1	1.8	2.8
Metals (g/kg)									
Ca	4.6	2.1	2.1	3.5	2.7	2.9	2.3	2.3	2.3
K	17.8	19.6	17.6	22.1	14.4	21.0	10.6	13.6	12.0
P	8.5	6.0	6.4	0.2	0.2	0.9	5.2	2.3	3.4
S	2.2	1.8	2.1	0.3	0.4	0.5	0.9	0.5	0.7
Al	0.13	0.03	0.09	0.71	3.17	6.12	0.79	0.34	0.51
Fe	0.60	0.17	0.30	0.27	1.23	2.13	0.38	0.22	0.29
Mn	0.50	0.10	0.17	0.02	0.08	0.11	0.04	0.04	0.05
Zn	0.03	0.04	0.05	0.07	0.15	0.35	0.08	0.09	0.11

* determined by difference. PRIM = primary effluent; SEC = secondary effluent; DAF = effluent from DAF unit.

5.3.2. Production of biomass in pilot-scale open ponds

Oedogonium was successfully cultivated for 8 weeks in ponds using primary treated effluent (PRIM) as the nutrient source, and simultaneously delivered an improved quality of effluent wastewater. Table 5.3 shows the properties of the influent wastewater and the

evolution of the water quality in the ponds throughout the cultivation period. The influent (PRIM) had an average total nitrogen concentration of 41.2 ± 8.1 mg/L, predominantly in the form of DIN (29.7 ± 9.9 mg/L), an average total phosphorus concentration of 6.7 ± 1.0 mg/L, predominantly in the form of orthophosphate (5.8 ± 1.3 mg/L), and an average COD of 53 ± 18 mg/L. The ponds were initially filled with nutrient-free freshwater and the concentration of nutrients increased gradually in the ponds with the addition of wastewater at 5% per day. The proportion of wastewater to freshwater reached 50% within 14 days and 90% (v/v) within 45 days of culture (Annex 2, Fig. S5.1). The algae adapted well to the increase in nutrients and the concentration of nitrogen in the ponds reached a plateau at 16 mg/L after 7 weeks, whereas the concentration of phosphorus and COD reached a peak after 5 weeks of culture at 2 mg/L and 39 mg/L respectively before decreasing to 1.7 mg/L and 23 mg/L during the final 3 weeks (Table 5.3). This decrease indicates that following a period of acclimation and nutrient build-up in the ponds, the removal rates of phosphorus and COD were higher than the mass of each supplied with a 5% exchange per day. After 8 weeks of culture, the effluent had lower concentrations of all forms of nitrogen and phosphorus compared with the influent wastewater, including dissolved organic and particulate forms (Table 5.3). Similarly the concentration of COD and microbes was greatly reduced in the ponds, which indicates that the organic material present in the wastewater feed was degraded in the ponds and subsequently converted to inorganic forms of nitrogen, phosphorus and carbon. Noticeably, ammonia-N was rapidly converted to oxygenated forms in the ponds. Metal elements that were present far in excess in the influent wastewater (i.e. Na, Ca, B, S) reached similar concentration in the pond effluents after 5 to 6 weeks (Annex 2, Table S5.3). Other metal elements that were partially (i.e. K,

Ba, Sr) or more efficiently assimilated by the algae (i.e. Al, Mn, Zn) had lower concentration in the pond effluents than in influent wastewater after 8 weeks of culture.

Table 5.3. Properties of the influent and effluent waters in the ponds.

Data show means (n = 9) of properties of influent wastewater and means (n = 3) of properties of pond effluents over 8 weeks.

Water properties		Influent	Pond effluents								
		PRIM	w_0	w_1	w_2	w_3	w_4	w_5	w_6	w_7	w_8
Alkalinity (total CO_3)	mg CO_3/L	216	60	107	156	130	152	172	180	190	195
TOC	mg/L	13	6	6	8	8	9	10	10	11	11
COD	mg/L	53	10	14	23	32	39	37	35	33	23
Nitrogen (total N)	mg N/L	41.2	0.7	3.5	5.2	9.9	11.1	11.6	13.8	15.6	15.8
Dissolved	mg N/L	37.7	0.7	3.0	4.4	9.3	10.5	11.5	12.4	15.2	15.0
Inorganic	mg N/L	29.7	0.3	2.1	3.3	9.1	9.7	10.5	11.6	14.3	14.7
Ammonia	mg N/L	7.9	0.2	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Nitrite	mg N/L	10.8	0.0	2.0	3.3	8.0	7.5	8.7	8.2	6.9	4.0
Nitrate	mg N/L	11.0	0.1	0.1	0.0	1.1	2.2	1.7	3.4	7.4	10.7
NO_x	mg N/L	21.7	0.1	2.1	3.3	9.1	9.7	10.4	11.6	14.3	14.7
Organic	mg N/L	8.0	0.3	0.9	1.0	0.2	0.8	1.0	0.8	0.8	0.2
Particulate	mg N/L	3.5	0.1	0.5	0.9	0.6	0.6	0.1	1.4	0.4	0.8
Phosphorus (total P)	mg P/L	6.7	0.1	0.7	1.3	1.5	1.7	2.0	1.8	1.6	1.7
Dissolved	mg P/L	6.2	0.1	0.7	1.1	1.3	1.5	1.9	1.6	1.5	1.4
Inorganic (FRP)	mg P/L	5.8	0.1	0.6	1.0	1.3	1.3	1.8	1.6	1.4	1.4
Organic	mg P/L	0.4	0.0	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1
Particulate	mg P/L	0.4	0.0	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.2
Microbes (total HPC)	org/1mL	$1.4.E+10^6$	151	3000	3000	50000	2800	26200	3000	3000	3000
E.coli	cfu/100mL	$3.5.E+10^3$	2	6	34	2	2	800	6	34	2
P.aeruginosa	cfu/100mL	1200	1201	4	24	4800	2	7800	1	2	2
Faecal coliforms	cfu/100mL	$3.6.E+10^3$	3	18	34	4	200	800	6	34	2
Total coliforms	cfu/100mL	$6.4.E+10^3$	101	48	26	10	2	800	6	2	2
Metals											
Ca	mg/L	33	-	17	21	23	26	28	28	29	29
K	mg/L	24	-	7	8	10	12	14	14	16	16
S	mg/L	26	-	10	14	17	19	21	22	24	24
Al	µg/L	6	-	1	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Fe	µg/L	< 100	-	<100	<100	<100	<100	<100	<100	<100	<100
Mn	µg/L	74	-	3	10	5	7	7	6	3	1
Zn	µg/L	7	-	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5

PRIM = primary effluent; FRP = filterable reactive phosphorus; HPC = heterotrophic plate count.

The productivity of the algae ranged from 6.8 to 9.9 g/m^2/d dw with the water temperature in the ponds oscillating between 17.4 - 24.5^{o}C over the course of the experiment (Fig. 5.3).

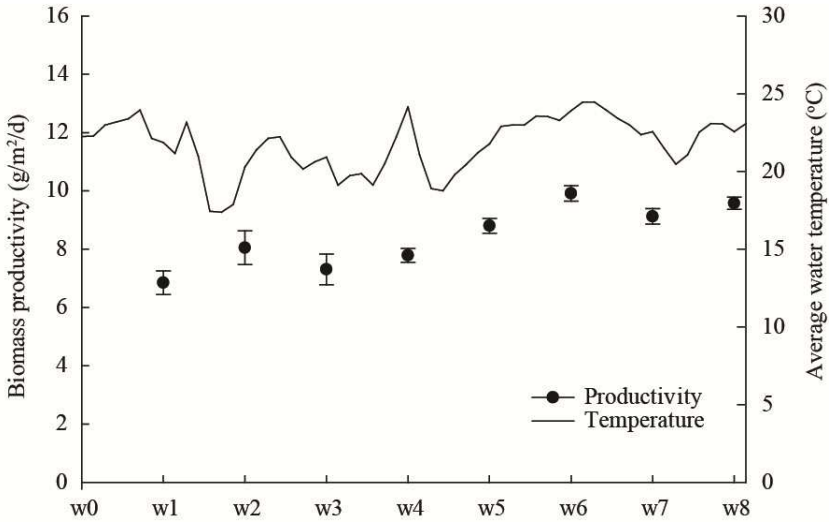

Figure 5.3. Biomass productivity and water temperature in the ponds.
Data show means (n = 3 ± SE) of productivity of biomass and average daily water temperature in the ponds over 8 weeks.

The lag phase observed in the first week corresponded to the initial lack and then progressive build-up of nutrients in the ponds. The biomass produced varied in composition with a decrease in the carbon content from 44 ± 1% to 39 ± 1% during the first six weeks followed by stabilisation at 38 - 39% (Table 5.4). This decrease in carbon content corresponded with an increase in the ash content of the biomass from 10 ± 2% to 19 ± 1% after 6 weeks. The nitrogen content remained relatively constant at 5.5 ± 0.4% on average whereas potassium stabilised around 2.0 ± 0.2% after 4 weeks of acclimation (Table 5.4). In contrast, phosphorus increased progressively from 0.6 ± 0.1% to 1.1 - 1.3%. Algal biomass also accumulated high concentrations of minerals such as calcium, magnesium, aluminium, boron, manganese and sulfur (Table 5.4; Annex 2, Table S5.4).

Table 5.4. Biochemical composition of the biomass produced in pilot-scale ponds. Data show means (n = 3) of content of biomass over 8 weeks.

Properties	w_0	w_1	w_2	w_3	w_4	w_5	w_6	w_7	w_8
Proximate (wt%)									
Ash	11.0	9.0	12.9	15.1	16.1	16.3	19.5	19.8	19.5
Moisture	4.5	3.5	3.6	4.1	4.2	4.4	5.2	4.9	4.9
Ultimate (wt%)									
C	43.5	43.7	42.1	40.5	39.5	39.8	38.8	38.2	38.5
H	6.7	6.8	6.5	6.4	6.2	6.3	6.1	6.1	6.2
O*	28.6	31.0	29.1	29.3	28.3	27.4	25.4	25.6	25.7
N	5.7	6.0	5.8	4.7	5.8	5.9	5.1	5.5	5.1
Metals (g/kg)									
Ca	1.4	1.4	1.8	7.6	6.3	5.3	10.1	16.1	11.9
K	25.2	17.1	28.6	23.7	20.0	18.4	18.7	18.1	20.8
P	5.9	6.6	6.6	8.3	9.5	8.4	9.1	13.0	11.4
S	2.7	2.1	2.5	1.8	2.3	2.6	1.9	2.7	3.0
Al	0.11	0.25	0.26	0.25	0.44	0.62	0.38	0.49	0.51
Fe	0.30	0.29	0.35	0.27	0.32	0.40	0.25	0.33	0.28
Mn	0.26	0.32	0.25	0.22	0.28	0.27	0.19	0.28	0.20
Zn	0.06	0.06	0.06	0.05	0.07	0.06	0.05	0.06	0.06

*determined by difference.

5.3.3. Nutrient removal

All forms of nitrogen and phosphorus in the influent PRIM were considerably reduced by the algae. Nitrogen and phosphorus uptake rates reached a stable state after approximately 4 weeks of culture at 0.50 ± 0.01 g N/m^2/d and 0.11 ± 0.02 g P/m^2/d respectively (Fig. 5.4a). The difference in nutrient concentration between the influent and effluent water at week 8 corresponded to a removal efficiency of 62% for nitrogen, 75% for phosphorus and 57% for COD, with the addition of PRIM of 5% per day. Particularly, the algal treatment removed the majority of ammonia-N, with consistently low or no total ammonia-N in the

pond effluents. Potassium and sulfur that are also key elements for algal metabolism were efficiently assimilated. The removal rate of potassium and sulfur were consistent over the course of the experiment at 0.17 ± 0.03 g K/m^2/d and 0.20 ± 0.05 g S/m^2/d on average (Fig. 5.4b). In contrast, the removal rate of calcium increased drastically over time, reaching a plateau at 0.50 ± 0.04 g Ca/m^2/d from week 3 to week 5 and then increasing to 1.20 ± 0.24 g Ca/m^2/d in the last 3 weeks of culture. The increase of calcium content in biomass was the main driver of the increase in ash content, with magnesium and sodium participating to a lesser extent (Annex 2, Table S5.4). The uptake of other elements of interest in water treatment, such as aluminium, iron, and manganese, were similar over time with a higher removal rate for aluminium at 46 ± 7 mg Al/m^2/d, followed by iron at 29 ± 5 mg Fe/m^2/d and manganese at 22 ± 4 mg Mn/m^2/d on average during the last 4 weeks of culture (Fig. 5.4c).

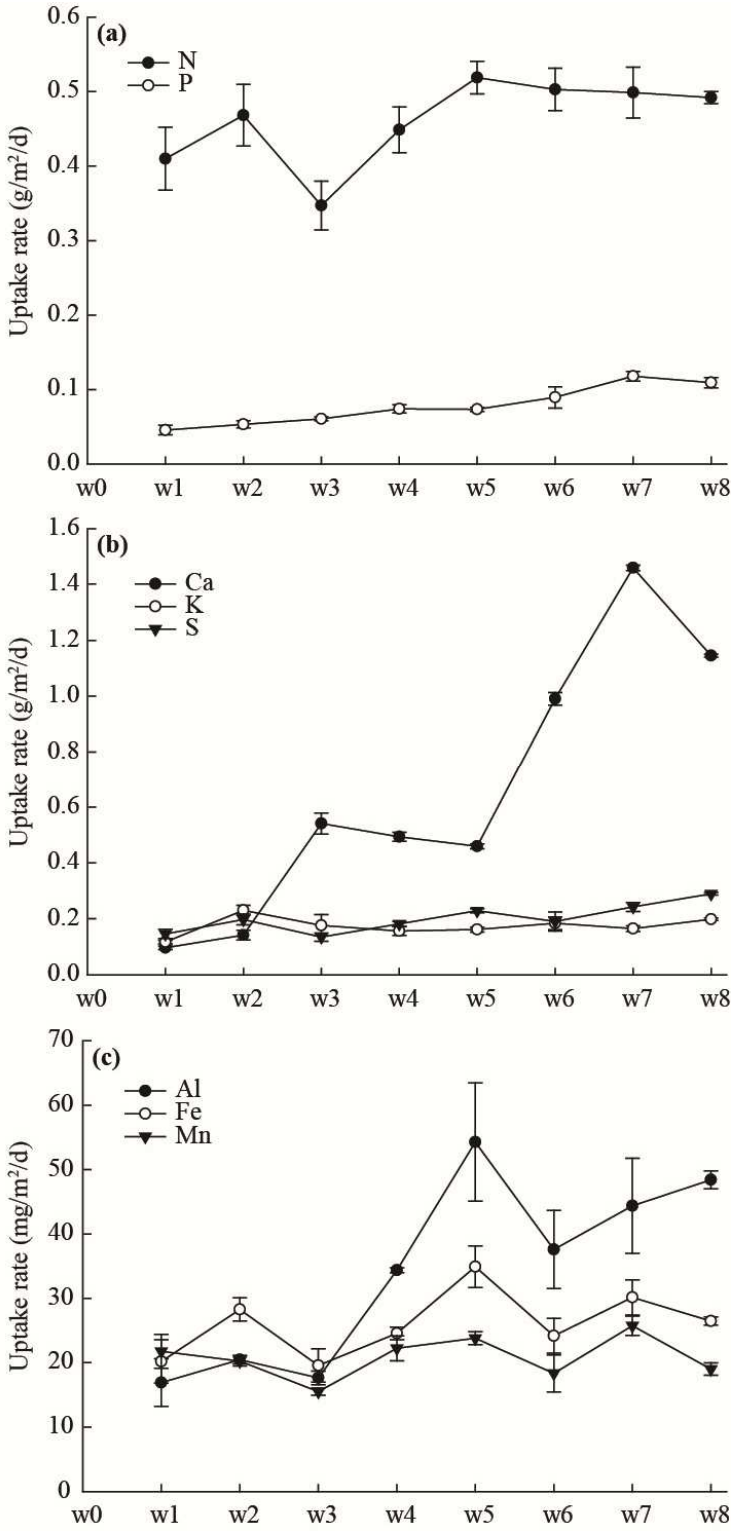

Figure 5.4. Nutrient and metal removal rates for (a) nitrogen and phosphorus, (b) calcium, potassium and sulfur and (c) aluminium, iron and manganese.

Data show means (n = 3 ± SE) of removal rates of nutrients by the algae over 8 weeks.

5.3.4. Biomass applications

The biomass produced in the ponds represented a suitable feedstock for the production of biocrude through hydrothermal liquefaction (HTL). The elemental composition of the biomass was used to estimate the theoretical yield and quality of biocrude. With a carbon content of 38.5% at week 8, it was estimated that biomass would yield 26 - 27% biocrude on a dry weight basis (Eq. 4.2) (35% on ash-free dry weight basis). It was determined previously that the elemental composition of biocrude produced by HTL is relatively consistent among species of macroalgae, with typically 72 - 73% carbon and 7 - 8% hydrogen (Chapter 4). Furthermore, *Oedogonium* biomass had a high nitrogen content of 5.1% at week 8, which would result in a biocrude with relatively high nitrogen (5.0 - 5.5%). The remainder of the content of heteroatoms in biocrude is expected to range between 10 - 14% for oxygen and 0.0 - 0.4% for sulfur.

5.4. Discussion

5.4.1. Small-scale culture trials

The results demonstrate that freshwater macroalgae can be maintained as a monoculture and cultured continuously to treat both nutrient and metal components of municipal wastewater with the best results for growth and treatment using the high-nutrient primary treated effluent (PRIM). Small-scale trials showed that the nutrients supplied with a low volume of PRIM (5% to 10% exchange per day) were sufficient to maximise biomass productivity, but that higher exchange rates may increase water turbidity and reduce productivity likely by limiting the photosynthetic activity of the algae. For the same reason,

using raw sewage as a nutrient source remains challenging as it contains higher suspended solids and COD than primary effluent. In contrast, secondary treated effluent had a much lower nutrient concentration that was not sufficient to sustain algal growth with the water exchange rates in this study (5% to 20% exchange per day). An estimated 100 - 200% volume exchange per day would be the minimum required here to provide the same quantity of nitrogen that of primary effluent at 5% per day. Therefore, secondary effluent will only be useful if the objective of the algal culture is to further improve the quality of wastewater prior to discharge. This process would treat the remaining nutrients in secondary effluent and also a significant quantity of dissolved metals, in particular the aluminium added in the activated sludge process. Effluent water from the DAF unit was also an interesting source of nutrients as these nutrients are normally returned from the sludge thickening process back to the primary settlement tank (Fig. 5.1). Although the volume of DAF effluent is generally low (1 - 2% of the total volume treated), the water has suitable properties to grow *Oedogonium* efficiently such as low suspended solids and a relatively high concentration of phosphorus. This effluent had similar properties to that of centrate water from the sludge thickening process, which was successfully tested for the culture of species of microalgae (Wang et al., 2010) and macroalgae (Sode et al., 2013).

5.4.2. Production of biomass in pilot-scale open ponds

The continuous production of a monoculture of *Oedogonium* in pilot-scale wastewater treatment ponds demonstrated the potential to reliably treat wastewater and produce biomass over time. This demonstration was conducted during winter and resulted in productivities ranging between 7 - 10 $g/m^2/d$ dw. These productivities are equivalent to a

scaled 25 - 36 t/ha/yr which is comparable to the productivity of microalgae cultivated in high-rate algal ponds treating domestic wastewater in temperate climates (Craggs et al., 2012a). In addition, this productivity is comparable to the productivity of *Oedogonium* cultivated in aquaculture waste streams at the same time of the year (Cole et al., 2013). Daily solar irradiance and temperature are the main environmental factors driving algal productivity and consequently winter values determine the surface area of culture required for effective year-round wastewater treatment (Craggs et al., 2012a). Based on a recent culture trial of *Oedogonium* in an aquaculture waste stream at scale (Cole et al., 2015), the average productivity of *Oedogonium* cultured in primary effluent could reach as high as 87 - 130 t/ha/yr (or 23.9 - 35.7 $g/m^2/d$ dw). Higher biomass productivities are always the primary objective for algal culture as this results in a decreased area of culture required for similar nutrient removal.

Oedogonium also displayed other important attributes during the continuous wastewater treatment in open ponds including that it remained uncontaminated through the course of the experiment, was resilient to fluctuations in water quality and weather, and – because it is a macroalga – it was easily harvested by overflow and concentrated on mesh to 14 - 28% dry solids. Finally, *Oedogonium* was able to grow at a pH above 10, which demonstrates its ability to use bicarbonate as a carbon source as well as CO_2 (Cole et al., 2013).

5.4.3. Nutrient removal and bioremediation potential

The biomass productivity of *Oedogonium* equated to a high efficiency in removing nutrients and metals from the incoming primary treated effluent. The removal rate of

nitrogen was higher in the small-scale culture trials (0.6 - 0.7 g $N/m^2/d$ - summer values) than in the continuous culture of *Oedogonium* in pilot-scale ponds (0.4 - 0.5 g $N/m^2/d$ - winter values). Given that productivity decreases during winter, lower water exchange rates (< 5%/d) would be necessary to increase the nutrient removal efficiency. The nutrient removal rates achieved here (0.50 g $N/m^2/d$ and 0.11 g $P/m^2/d$) were lower than those obtained in aquaculture wastewater streams (1.09 g $N/m^2/d$ and 0.13 g $P/m^2/d$, Cole et al., 2014). However, *Oedogonium* was particularly efficient at removing phosphorus from the primary effluent of the municipal wastewater stream. This reflects the higher phosphorus value in this wastewater stream and the flexibility of the alga to adapt to different water sources.

In addition to nitrogen and phosphorus, the organic compounds in the primary effluent were also degraded in the ponds, with a > 50% reduction in COD. The oxygen released through algal photosynthesis tends to promote the growth of aerobic bacteria and the degradation of organic compounds, which in turn provides additional nutrients and CO_2 to the algae (Oswald, 1991). Overall the abundance of unwanted microbes decreased between the influent and effluent water with, for example, a 3-log removal of *Escherichia coli*. The UV irradiance and oxygen provided through the aeration in the ponds for mixing algae through the water column may have played a significant part in the degradation of microbes and organic compounds in the wastewater (Craggs et al., 2012a; Prieto-Rodriguez et al., 2013). Finally, there were also positive changes in the remaining inorganic components of the wastewater as *Oedogonium* biomass accumulated large quantities of metals (ash content of 19 - 20%) including heavy metals and contaminants

such as arsenic, cadmium, lead and zinc. This represents a major advantage of the treatment by algae since conventional secondary treatment does not cope with the removal of metal contaminants and these are ultimately released to the environment (Cheng, 2003).

Irrespective of the substantial reductions of nutrients and both organic and inorganic components, the pond effluents still had nutrient and COD concentrations exceeding the allowable level for discharge, and managing the load of nutrients supplied to the cultures is a critical step in meeting standards for direct release. Open pond systems designed to fulfill the same purpose to that of mechanical secondary treatment is likely to be limited by land availability (Fortier & Sturm, 2012; Craggs et al., 2012b). For example, it is estimated that the wastewater source at Cleveland Bay treatment plant would require an area of 94 ha of algal culture to hold the volume of wastewater received daily (29 ML) using the operating pond system and water exchange rate described in the pilot-scale experiment. This area of culture could increase by a factor of 1 to 5 to treat the primary effluent with the same efficiency to that of mechanical secondary treatment, depending on variation in the amounts of nitrogen and phosphorus to be treated and the removal rate of these elements, which in turn relies on the productivity of the algae. Furthermore, an additional area of approximately 15% of the surface area of culture would be required for operational infrastructure (Fortier & Sturm, 2012).

5.4.4. Biomass applications

The viability of integrating algal pond systems into wastewater treatment plants can be increased through the valorisation of secondary products generated by wastewater

treatment. Specifically, anaerobic digestion of primary sludge and hydrothermal liquefaction of algal biomass represent attractive conversion pathways to bioenergy as no drying of biomass is needed (Fig. 5.5). In addition, the moisture content of the algae (14 to 28% dry solids) harvested from the ponds is suitable for direct HTL processing, although a pre-treatment such as mechanical grinding may be required, as particle size affects pumpability and pressure control in continuous flow reactors (Jazrawi et al., 2013). It was calculated that the algae produced in the pilot-scale system would have a biocrude yield of approximately 26 - 27% dw (or 35% afdw), which is in the range of yields obtained with the HTL of low-lipid biomass (López Barreiro et al., 2013). This equates to a theoretical biocrude production equivalent to 52 - 75 barrels/ha/yr for a potential production area of 94 ha, based on a conservative biomass productivity of 25.5 - 36.5 t/ha/yr. The composition of the biomass from the primary effluent is characterised by relatively high contents of ash and nitrogen which effectively decrease the yield and quality of the biocrude. However, there are simple pre-treatments that can reduce these components significantly, thereby producing a biocrude of higher quality (Chapter 4). Finally, by-products of HTL such as process water and CO_2 could ultimately be returned to the algal ponds for nutrient and carbon recycling, assuming the proximity of infrastructure, which would contribute to the sustainability of this technology.

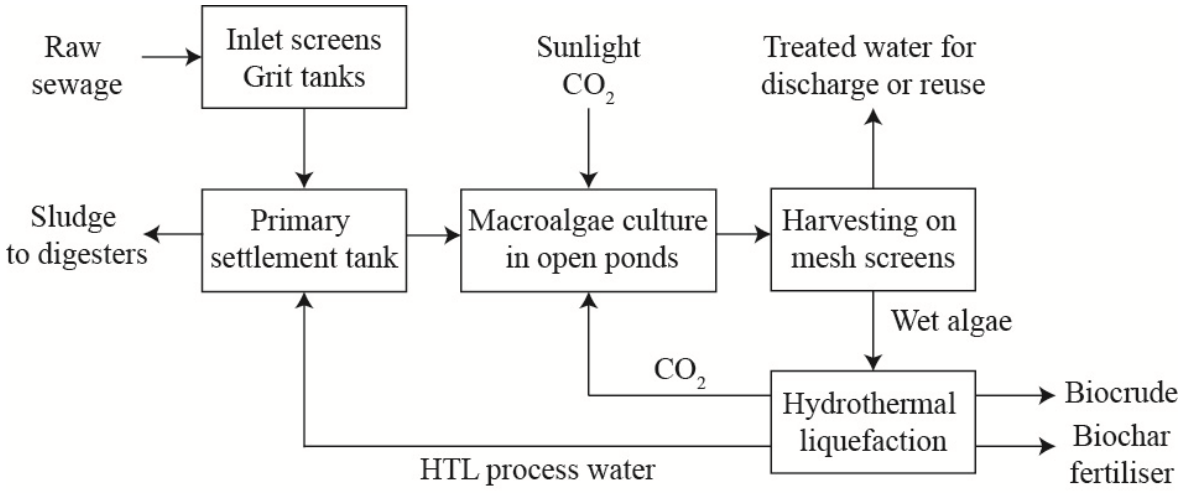

Figure 5.5. Simplified schematic diagram of the integration of algae ponds and hydrothermal liquefaction of biomass to wastewater treatment.

5.5. Conclusions

The results demonstrate the potential of integrating the culture of macroalgae with wastewater treatment operations. Wastewater effluent from the early treatment stages provide sufficient nutrients to maximise biomass productivity at low water exchange rates, thus reducing energy demands for pumping higher volumes of water with lower nutrient loads. In return, the culture of macroalgae represents an efficient tool for reducing nutrients, COD, microbes and also some of the more intractable metals in wastewater that could in turn influence the microbial processes in secondary treatment (Principi et al., 2006). The algal treatment alone may not be sufficient in consistently meeting the standards of discharge, which impacts directly on the need for mechanical secondary treatment or higher surface area of culture and land for efficient nutrient removal. These results emphasize the dynamic nature of any biological treatment process based on algae and this would need to be managed through feedback mechanisms to ensure that the operational parameters are aligned with the primary goals of the culture, whether it be

nutrient removal or biomass production, as the two may be mutually exclusive at certain times of the year. Macroalgal biomass produced from waste nutrients can be conveniently harvested and converted to liquid biofuels, such as biocrude, with the reliability of a monoculture feedstock with the ecologically dominant freshwater macroalgae from the genus *Oedogonium*.

Chapter 6

General discussion

This thesis successfully establishes the suitability of macroalgal biomass produced in land-based systems as a feedstock for the production of liquid biocrude. The screening of more than forty indigenous species of macroalgae from tropical Northeast Australia (green, red and brown), tested for cultivation in land-based systems, resulted in the selection of the six most productive and reliable species for the production of macroalgal biomass. The biomass productivity and biochemical composition of the selected species were quantified and provided the basis to compare the theoretical production and value of the feedstocks for the production of biocrude and biodiesel. The hydrothermal liquefaction of macroalgal feedstocks for the production of biocrude was identified as the most promising conversion pathway to high-energy liquid biofuels. The six species of macroalgae selected were subsequently converted to biocrude through HTL in a batch reactor and the three species with the highest biocrude productivity (expressed as g biocrude produced/m^2/d) were selected for further improvement. The treatments selected to specifically improve the biochemical composition of macroalgae for the production of biocrude were the nutrient starvation of cultures and post-harvest washing of biomass in freshwater. These two treatments were effective in improving the biochemical composition of the three macroalgae by reducing the contents of nitrogen, sulfur and ash within the biomass, which resulted in the production of biocrudes of higher quality, with lower contents of nitrogen and sulfur. Finally, among the three species selected, the freshwater macroalga

Oedogonium sp. was cultured in municipal wastewater for simultaneous nutrient removal and biomass production. Different combinations of wastewaters and water exchange rates investigated for the culture of *Oedogonium* sp. in small-scale trials resulted in the selection of the most effective combination (primary effluent at an exchange rate of 5%) for production in pilot-scale ponds. In this pilot-scale experiment conducted over a period of 8 weeks, *Oedogonium* was efficient at removing nutrients and contaminants from primary treated effluent for simultaneous nutrient removal and biomass production. This pilot-scale pond system produced a desirable feedstock for hydrothermal conversion to biocrude.

A major outcome of this thesis is that the sustainable production of macroalgal biofuels requires the continuous and cost-effective production of macroalgal biomass. Therefore, the development of cost-effective and reliable systems of production is critical to close the gap between the promising concept of macroalgal biofuels and commercialisation, particularly in the context of today's price for fossil crude oil (approximately $US 50 for WTI in the beginning of 2015).

The development of any successful biofuel operation relies on a reduction of the system inputs (e.g. capital and operating costs) and an increase of the system outputs (e.g. biomass and biofuel yield and quality). It is therefore important for research to focus on all aspects of the production process, with the aim of reducing the costs of production and maximising the value of the macroalgal biomass and biofuel. The key factors in the production of the macroalgal biofuels identified in this thesis include (1) the co-location of production facilities with a source of wastewater, (2) the selection of an efficient cultivation system, (3) the selection of highly productive and reliable species of macroalgae, (4) the

optimisation of culture protocols, (5) the selection and optimisation of the conversion process, and (6) the possibility of producing multiple products from a single macroalgal feedstock with a focus on recycling the co-products produced during the process in an industrial ecology framework. These factors are summarised and discussed in the following sections and an integrated model is proposed for the sustainable development of macroalgal biofuels.

6.1. Co-location of production facilities

The mass production of macroalgae for conversion to biofuel requires large quantities of water and nutrients, therefore the access to a low-cost and abundant source of water and nutrients such as wastewater is imperative (Lundquist et al., 2010; Craggs et al., 2012a; Benemann, 2013). The co-location of production facilities with a wastewater source or wastewater treatment facility should consequently be the primary objective of any realistic and sustainable operation aimed at producing biofuels or any other low-cost commodity such as animal feed or fertilisers (Benemann, 2013).

In Chapter 5 of this thesis, wastewater collected from a municipal treatment plant was tested as a low-cost and abundant source of water and nutrients for the culture of the freshwater macroalga *Oedogonium* sp. in an intensive land-based system. The two main factors that can be manipulated when integrating the culture of algae into the treatment of wastewater, the quality of wastewater (nutrient concentration) and the rate at which nutrient-rich wastewater is added to the cultures (nutrient flux), were evaluated in this chapter. The three wastewaters (effluent from the primary and secondary clarifiers and underflow effluent from the dissolved air flotation unit) and the three exchange rates (5%,

10% and 20% volume exchange per day) all proved to be suitable for the survival and growth of *Oedogonium* over 2 weeks. Among all the treatment combinations tested, the use of primary treated effluent from the primary clarifier at a low volume exchange rate of 5%/d was the best option to maximise biomass productivity. Further culture experiments in pilot-scale ponds (with a volume of 10 m^3) demonstrated that primary treated effluent is an effective source of water and nutrients to maintain high productivity of *Oedogonium* (7 - 10 $g/m^2/d$ dw) for at least 8 weeks. The results of the pilot-scale experiment conducted in winter combined with the productivity values obtained with the cultivation of *Oedogonium* in wastewater from an aquaculture facility (Cole et al., 2015) suggest a potential for higher annual productivity values (15 - 20 $g/m^2/d$ dw).

The integration of mass cultivation of algae with municipal treatment plants is promising for the cost-effective and sustainable removal of nutrients and the production of biomass. However, as highlighted in Chapter 5, the area of land required to integrate algal treatment with a municipal treatment plant combined with the cost of land near urban areas – where most large municipal wastewater plants are located – can be prohibitive for the development of this technology (Park et al., 2011b; Fortier & Sturm, 2012). Nonetheless, it was recently demonstrated in Christchurch, the third largest city in New Zealand, that the primary treated effluent from the city's wastewater treatment plant could be used successfully to grow microalgae in a 5-ha demonstration HRAP system over a period of 15 months (Craggs et al., 2012b). This system was able to remove approximately 50% biochemical oxygen demand (BOD), 65% NH_4^+-N, 19% filterable reactive phosphorus (mostly PO_4^{3+}) and 2-log removal of *E.coli* with a biomass productivity of 8 $g/m^2/d$ afdw. Although the nutrient removal efficiency of this system was not optimal, the results

obtained provide additional data for the development of this technology at scale. A system of this size was designed so that it could be replicated in smaller communities in New Zealand and worldwide (Craggs et al., 2012b), where the price of land may be lower and where the proximity to agriculture or aquaculture operations that produce nutrient-rich wastewater potentially provides an additional source of water and nutrients (Fortier & Sturm, 2012).

The possibility of using the primary effluent from municipal wastewater treatment plants or the effluent from agriculture and aquaculture facilities is attractive as these effluents are widely available in large quantities and at low-cost. Additionally, these effluents are generally highly concentrated in nutrients which reduces the energy required to pump sufficient nutrients to the cultures of macroalgae, and they do not require extensive pre-treatment, usually only the removal of large solids and organic particles.

The selection of the location near a source of wastewater is therefore crucial for the development of intensive cultivation of algae at scale for the production of biofuels. Other important aspects of the production process may be influenced by the location of the algal treatment facilities. On a broader scale, this includes the amount of sunlight and seasonal variations of temperature and weather that directly affect the productivity of the algae. Therefore, tropical latitudes should be favoured to maximise biomass productivity. The selection of the location also includes regulatory considerations and the cost of land and staff, therefore countries or regions where the density of population and wages are lower would reduce capital and operating costs. Finally, this aspect includes the possible proximity to a waste source of CO_2 to provide additional carbon to the cultures at low cost. Meeting all these conditions is certainly difficult for a single operation. However, the

selection of the location is arguably the first factor to be considered to determine the viability of an algae-to-biofuel operation (Lundquist et al., 2010; Meyer et al., 2010; Fortier & Sturm, 2012).

6.2. Cultivation system

The mass production of macroalgae relies on an efficient cultivation system that can be scaled-up and operated at low-cost, while delivering high biomass productivity. After the location (i.e. price of land), the highest capital cost for the production of algal biofuels is in most cases related to the cultivation system (Lundquist et al., 2010).

The cultivation systems used for experimental work in this thesis consisted of tanks with a capacity of 20 L and 60 L. Tanks were chosen to grow macroalgae due to their ability to simulate intensive land-based culture conditions and their flexibility in the design of culture experiments. In Chapter 5, pilot-scale ponds with a capacity of 10 m^3 (footprint of 16 m^2) were used to grow the freshwater macroalga *Oedogonium* sp. in wastewater to provide better estimates of the biomass productivity on a larger scale (Fig 6.1a).

These culture systems are well-suited to conduct research, however, two important criteria must be considered to select systems for large scale operations. Firstly, the cost of construction should be as low as possible and a single unit of the cultivation system should be as large as possible (several hectares) and most likely dug in the ground with plastic liners or compacted clay as a base to minimise capital costs (Benemann, 2013). Secondly, the operating costs should be as low as possible and this can be achieved with a reduction of the energy consumed for mixing, as well as a reduction of the maintenance requirements (Lundquist et al., 2010).

For these two reasons, the use of open tanks and ponds (built above ground) or closed photobioreactors for the production of algal biomass for fuels is simply not viable on a large scale (Lundquist et al., 2010; Craggs et al., 2012a; Benemann, 2013). In contrast, the use of high-rate algal ponds (HRAP) or algal turf scrubbers (ATS) could meet these requirements while maintaining high biomass productivity (Adey et al., 2011; Park et al., 2011b; Craggs et al., 2012a).

Moreover, HRAP and ATS have previously been used in the treatment of wastewater from municipal (Adey et al., 2011; Park et al., 2013), agriculture (de Godos et al., 2009; Kangas & Mulbry, 2014) and aquaculture facilities (Pagand et al., 2000; Valeta & Verdegem, 2013). Although the use of HRAPs have been extensively described for the low-cost production of microalgae in wastewater (Craggs et al., 2011; Craggs et al., 2012b; Park et al., 2014; Sutherland et al., 2014), only limited studies have demonstrated the suitability of this system for the cultivation of macroalgae (Bolton et al., 2009; Nobre et al., 2010). In contrast, ATS systems can only use attached filamentous macroalgae and studies with this system have been described for the treatment of water from marine (Adey & Hackney, 1989; Adey et al., 2013) and freshwater waste streams (Craggs et al., 1996; Mulbry & Wilkie, 2001; Mulbry et al., 2008; Adey et al., 2011). However, recent estimates assessing the efficiency of ATS for the treatment of agricultural drainage water suggest that the cost of nutrient removal is still high (Kangas & Mulbry, 2014) and further work using alternative species of macroalgae and operating conditions are required to fully demonstrate the viability of this technology.

Figure 6.1. Cultivation of *Oedogonium* sp. in open ponds at the Marine Aquaculture Research Facilities at James Cook University Townsville (a, left) and at Good Fortune Bay Fisheries Ltd. Townsville (b, right).

6.3. Species selection

Following the confirmation of the location and cultivation system, the selection of reliable species of macroalgae is the next most important factor to be considered to ensure maximum biomass productivity. This involves the screening of indigenous species adapted to local climatic conditions. Selecting indigenous species of macroalgae also avoids the introduction of non-endemic species that represent a biosecurity hazard for local ecosystems. The selection of the most reliable species should be then based on the ability of the algae to maintain high productivity and quality (i.e. biochemical composition) overtime. This relies on high growth rate, high resistance to contamination and high tolerance to environmental fluctuation, including variations in light intensity, temperature, pH, salinity, and nutrient availability.

Preliminary work in this thesis involved the screening of more than forty species of indigenous macroalgae from both freshwater and marine environments. Freshwater species

from the genera *Oedogonium*, *Cladophora*, *Rhizoclonium*, *Spirogyra* and *Hydrodictyon* were collected from freshwater ponds, creeks, irrigation channels and other nutrient-rich freshwater bodies. Marine species from the genera *Derbesia*, *Ulva*, *Chaetomorpha*, *Cladophora*, *Bryopsis*, *Caulerpa* (green), *Asparagopsis*, *Ceramium*, *Gracilaria*, *Halymenia*, *Hypnea*, *Champia* (red), *Sargassum*, *Dictyota*, *Padina*, *Cystoseira* and *Turbinaria* (brown) were collected from saltwater ponds, local aquaria, fish tanks, coastal rock platforms, intertidal zones and coral reefs. The testing of these species for intensive culture in tanks in batch conditions provided the basis to select the most productive and reliable species for the long-term production of biomass. The six most productive and reliable species selected for this thesis were all green macroalgae adapted to nutrient-rich and fast-changing environments such as eutrophic ponds, irrigation channels and intertidal zones. Collecting species from these environments ensures their ability to assimilate large quantities of nutrients, be competitively dominant and resistant to variations of temperature, pH, and salinity throughout the year. The six species selected have a simple homogenous structure of thin filaments or thin blades (Fig. 2.5) that maximises the surface area of exchange with the surrounding water, thereby increasing the potential for nutrient uptake (Hein et al., 1995). Additionally, the absence of complex reproductive structures gives them the ability to grow at higher rates and be competitively dominant particularly in nutrient-rich environments (Camp et al., 2014). For three of the six species however, *Chaetomorpha linum*, *Cladophora coelothrix* and *Cladophora vagabunda*, the ability to remain in suspension in the water column was low as the algae accumulated on the surface, forming a mat that prevented light reaching deeper parts of the water column. Therefore, these species had a lower light utilisation efficiency and consequently a lower growth rate

than the three species selected for the production of biomass, *Ulva ohnoi*, *Oedogonium* sp., and *Derbesia tenuissima*.

Ulva is the most studied genus of macroalgae for the production of biomass and biofuels (Coelho et al., 2014). However, the use of *Oedogonium* sp. (GenBank accession number: EKC701473, Lawton et al., 2014) and *Derbesia tenuissima* for the production of biomass and biofuels was described in this thesis for the first time. These three species of macroalgae are promising as they are highly productive (15 - 20 $g/m^2/d$ dw), reliable in that they can maintain high productivity year-round (in a tropical climate) and are relatively high in energy (16 - 19 MJ/kg). *Ulva* has been successfully cultivated in HRAP (Bolton et al., 2009) and ATS (Adey et al., 2013) at scale over extended periods, demonstrating high biomass productivity and dominance over other species. Similarly, *Oedogonium* has been successfully cultivated in municipal wastewater (Chapter 5 of this thesis), aquaculture wastewater (Cole et al., 2015) and heavily contaminated industrial wastewater (Roberts et al., 2013a; Roberts et al., 2015). However, the possibility of growing *Oedogonium* sp. and *Derbesia tenuissima* in HRAP or ATS remains to be tested.

6.4. Optimisation of culture protocols

The optimisation of culture protocols is a key factor and a powerful tool to increase the viability of the production process. The primary objective of the culture is to maximise the productivity and tailor the biochemical composition of the biomass for a specific purpose such as the production of liquid biocrude. Macroalgae require nutrients, water, light and CO_2 for growth (Fig. 1.1) and the supply of each of these elements without limitation is a condition to achieve maximum theoretical biomass productivity (Lundquist et al., 2010).

The two main culture parameters that can be managed to maintain high productivity are the flux to maintain optimal delivery of nutrients and water, and the density of macroalgae to ensure the optimal use of light and CO_2.

The nutrient flux (g nutrients/m^2/d) can be managed in line with the concentration of nutrients in wastewater and the ability of macroalgae to assimilate these nutrients. Providing sufficient nutrients to the algae ensures maximum growth while limiting the excessive addition of nutrients ensures the highest quality of wastewater treatment. In Chapter 5, a nutrient flux of 0.80 g N/m^2/d and 0.15 g P/m^2/d was provided through the addition of primary effluent at 5% volume exchange per day. This nutrient flux was sufficient to maintain high productivity of *Oedogonium* for 8 weeks, but was slightly overestimated to ensure the acceptable removal of nutrients with the macroalgal treatment. This resulted in the removal of 62% N and 75% P (compared to > 85% N and P removal in conventional wastewater treatment).

The second parameter requiring continuous management to optimise biomass productivity is the density of macroalgae in culture. The optimal stocking density varies on a species by species basis. For example, marine species have a higher productivity at relatively higher stocking densities (1 to 4 g/L fw for *Ulva* and *Derbesia*) (Chapter 2; Chapter 4; Angell et al., 2014; Magnusson et al., 2014). In contrast, freshwater species have higher productivity at lower stocking densities (0.25 to 0.50 g/L fw for *Oedogonium*)(Chapter 4; Lawton et al., 2013b; Cole et al., 2014). The optimal stocking density can be maintained through frequent or continuous harvesting of macroalgae using simple and effective methods such as continuous overflow on mesh screens (Fig 6.1b).

The management of nutrient flux and stocking density is central to maintaining high biomass productivity, although these culture parameters offer little control on the quality (i.e. biochemical composition) of the biomass produced. However, the quality of biomass can be improved using temporary treatments. In Chapter 4, the nutrient starvation and washing treatments demonstrated that macroalgal biomass can be manipulated in culture and post-harvest to reduce the contents of nitrogen, sulfur and ash before HTL processing. The nutrient starvation treatment was effective at producing biomass with a lower content of nitrogen and sulfur (70 - 75% reduction for both elements for the three macroalgae species), which resulted in the production of biocrude of higher quality, with low contents of nitrogen (2 - 3%) and sulfur (0.0 - 0.3%). This type of biocrude requires a less intensive hydrotreatment, which would reduce the cost of refining. The washing treatment of biomass in freshwater was effective at producing a feedstock with a low ash content (up to 90% reduction of ash for marine species), which reduces the mechanical demand on HTL processing equipment. In addition, the removal of ash from macroalgal biomass early in the production process represent a significant reduction of transport and processing requirements, particularly for a fraction of the biomass with no calorific value. Temporary treatments can however have a 'cost', with for example a decrease in biocrude productivity following the nutrient starvation treatment (Chapter 4). Importantly, these treatments need to be managed on a species by species basis to achieve a positive outcome.

In summary, the judicious management of the culture parameters and temporary treatments has the potential to increase the productivity of a system and therefore increase the viability of macroalgal biofuels.

6.5. Conversion process

The selection and optimisation of the conversion process is central to the sustainable production of biofuels from macroalgal feedstocks. Among the two conversion processes investigated in this thesis, HTL and transesterification, HTL was identified as the most effective process to produce a high-energy liquid fuel from macroalgae as it allows the conversion of wet feedstocks and converts the whole organic component of biomass.

In Chapters 3 and 4, macroalgal feedstocks were converted to biocrude with a batch HTL reactor and a set of operating conditions (6.6% solids; 330 - 340^{o}C; 14 - 17 MPa; 8 minutes) selected from preliminary laboratory work and the data published in the literature. The yield of biocrude produced at these conditions (17 - 42% afdw) was comparable to the mid-range of values reported in the literature for the conversion of micro- and macroalgal feedstocks (López Barreiro et al., 2013). The chemical composition of biocrude produced from the six species of macroalgae (untreated) was consistent, with typically a HHV of 32 - 34 MJ/kg (following the formula of Channiwala & Parikh, 2002), a carbon content of 71 - 74% and a nitrogen content of 5 - 7%. These values were also comparable to the mid-range of values obtained with the processing of various micro- and macroalgae species (Biller & Ross, 2011; Garcia Alba et al., 2012; Elliott et al., 2013a; Frank et al., 2013). All these results highlight the relatively high yield and quality of biocrude produced from the conversion of low-lipid macroalgal feedstocks (from 2 to 11% lipids).

Species of algae with high lipid content generally yield more biocrude, and consequently, these species are often the focus of HTL studies. However, recent results – including in this thesis – demonstrated that due to the conversion of all the organic macromolecules of biomass (carbohydrates, proteins and lipids), HTL eliminates the need to promote lipid

accumulation and provides focus on species with high growth rates (Jones et al., 2014; Neveux et al., 2014b). Therefore, the selection of species of algae (micro- and macroalgae) for the production of biocrude should be based on the comparison of biocrude productivities (g biocrude/m^2/d) rather than biocrude yields (wt%). It was highlighted in Chapters 2, 3 and 4 that higher biomass productivities often offset the lower yields of biocrude, the most explicit example being for the highly productive marine macroalga *Ulva ohnoi*, with a lipid content of 2%, a biocrude yield of up to 33% afdw and a biocrude productivity of up to 4 g/m^2/d, the highest productivity of all the species investigated.

In addition, the yield and chemical composition of biocrude can be improved by the selection of suitable HTL operating conditions (Anastasakis & Ross, 2011; Biller & Ross, 2011; Garcia Alba et al., 2012). As a general rule, higher temperatures (up to 350^{o}C) produce a higher yield of biocrude with a higher content of carbon and nitrogen, and a lower content of oxygen (Peterson et al., 2008; Brown et al., 2010; Garcia Alba et al., 2012). Higher temperatures also lead to higher reaction rates and consequently, a reduction in the size and cost of the reactor (Elliott et al., 2015). Similarly, high solid loadings (e.g. 15 - 30% solids) are suitable for HTL of algal feedstocks as this increases the yield of biocrude, reduces carbon losses within the HTL system and allows for smaller systems to process the same quantity of biomass, thereby decreasing capital costs (Elliott et al., 2015). In contrast, it has been demonstrated that longer reaction times (> 15 minutes) have little effect on the yield and quality of biocrude at subcritical temperatures of 300^{o}C and above (Garcia Alba et al., 2012). Therefore, short reaction times should be promoted to reduce the energy involved in the reaction (Faeth et al., 2013; Bach et al., 2014).

In summary, the use of high temperatures of approximately 350^{o}C, high solid loadings > 15% solids and short residence of < 15 minutes provide the best conditions for HTL of algal feedstocks to increase the yield and quality of biocrude while decreasing capital costs and energy inputs (Biller & Ross, 2011; Elliott et al., 2013b; Jazrawi et al., 2013). However, higher temperatures also increase the content of nitrogen in biocrude, which represents a major issue for refining as nitrogen (and sulfur) can poison the active sites of the catalysts used in refinery. The use of pre-treatments such as the starvation treatment (discussed in section 6.4) or the extraction of proteins using sequential HTL (Jazrawi et al., 2015) or solvents, or the use of catalysts provide multiple options to reduce the content of nitrogen and sulfur in biomass or biocrude prior to refining. The use of catalysts (homogenous and heterogeneous) in HTL is not limited to the reduction of nitrogen and sulfur in biocrude, and numerous catalysts have been shown to increase the yield and quality of biocrude (short chain hydrocarbons), with for example the use of alkali increasing the formation of aromatic hydrocarbons (Biller, 2013; Elliott et al., 2015).

Most research in the field of algal HTL to date (including in this thesis) describe the use of batch reactors. These laboratory scale batch-type reactors are useful for research purposes, however, the development of this technology at scale requires the use of continuous-flow reactors (Jones et al., 2014; Elliott et al., 2015). To date, two major studies have described the use of continuous-flow reactors for the conversion of microalgal (Jazrawi et al., 2013) and macroalgal feedstocks (Elliott et al., 2013a). These studies highlight two main engineering issues that need to be addressed for further development of this technology at scale.

Firstly, maintaining high pressure (15 to 25 MPa) in the continuous-flow system represents a technical challenge, particularly at high temperatures around 350^{o}C. Therefore, the choice of metallurgy is critical for the construction of the reactor at reasonably low-cost, while providing a high level of safety.

Secondly, pumping concentrated algal slurries of > 15% solids (particularly for macroalgae) is a major issue in continuous-flow systems as viscous slurries often tend to obstruct the valves and pipes of the reactor. Therefore, particle size is an important parameter to be monitored and feedstocks may require wet grinding or maceration before processing. However, it is hypothesised that larger continuous-flow reactors for commercial production would offer better pumping control and reduce blockages in the system (Jazrawi et al., 2013).

The use of continuous-flow reactors for the production of biocrude at scale is at the early stage of research and development (Elliott et al., 2013b; Jazrawi et al., 2013; Jones et al., 2014; Elliott et al., 2015; Licella, Australia). Processing algal feedstocks using different temperatures, solids loadings, residence times and catalysts would provide the data needed to define a range of suitable operating conditions and the optimisation of the design of continuous-flow reactors (Jones et al., 2014). Finally, the possibility of recovering and recycling the co-products of the reaction (solid, gas and aqueous products) provides an array of options to increase the viability of algal HTL.

6.6. Industrial ecology

The concept of industrial ecology involves the integration of all the components of the production process, where the primary products are recovered efficiently and where the co-products are recycled within the process to increase the overall productivity of the system. Assuming the proximity of each component of the system, the wastewater treatment, cultivation of macroalgae and HTL processing, most of the co-products could theoretically be recycled.

As described earlier in this discussion, wastewater originating from municipal or agricultural treatment facilities is the main starting material of the system, providing water and nutrients. The pre-treatment of wastewater (raw sewage) involves the removal of large solids and most of the organic particles (sludge) in grit tanks and primary settlement tanks respectively. While the large solids are typically sent to landfill in conventional wastewater treatment, the sludge can be further processed in anaerobic digesters to reduce the quantity of sludge produced (Appels et al., 2008). The fermentation process in anaerobic digesters produces biogas that can be converted to heat and electricity for re-use on site. This effective method is already in use in wastewater treatment plants the world over (Grady et al., 2012). The pre-treatment of raw wastewater provides large quantities of primary effluent for the cultivation of macroalgae in intensive land-based systems, such as HRAPs.

Following the optimisation of the cultivation system, macroalgae can treat the primary effluent efficiently, producing treated water and biomass during the process. While some of the treated water could be re-used in the HRAPs to compensate for evaporation, the remaining treated water is suitable for discharge in the environment, for irrigation of

agricultural fields or for further treatment, depending on the quality of wastewater and environmental regulations.

The wet macroalgal biomass produced in wastewater is a high-quality feedstock for the production of biocrude, with the possibility of removing most of the nitrogen from the biomass prior to HTL processing in order to improve the quality of biocrude. To our knowledge, two methods have been described to reduce nitrogen in the biomass before conversion to biocrude, through nutrient starvation of the cultures (Chapter 4) and through soft HTL in a two-stage HTL approach (Jazrawi et al., 2015). Although these methods demonstrated promising results, they were not entirely satisfactory due to a reduction in the productivity of biocrude. Therefore, alternative methods for the extraction of proteins from biomass require development. The advantages of this integrated process include growing macroalgae at a high rate without nutrient deprivation, the production of a protein extract suitable for human or animal food (after purification), and the production of a residual biomass primarily composed of carbohydrates and lipids for HTL processing.

The use of protein-extracted biomass would potentially produce a biocrude of high quality with low content of nitrogen and sulfur, as well as co-products such as a solid phase (biochar), a gas phase and an aqueous phase (process water). Although the separation of these products has been achieved with the use of organic solvents in most HTL studies, commercial production of biocrude will rely on the separation of these products by gravity (Elliott et al., 2013b). The biochar that is commonly rich in carbon, nitrogen and phosphorus can be precipitated and isolated from the other phases for further treatment, and be recycled as a soil ameliorant in agriculture (Bird et al., 2012). The gas phase that is mainly composed of CO_2 could be recycled directly or after further purification in the

culture of macroalgae. Finally, the recycling of the HTL process water has been described in several studies (Biller et al., 2012; Elliott et al., 2013b; Garcia Alba et al., 2013; Pham et al., 2013). One of the most promising options is the use of the process water for the production of hydrogen through catalytic hydrothermal gasification (CHG) (Duan & Savage, 2011d; Elliott et al., 2013b; Cherad et al., 2014). In addition, it was demonstrated that the hydrogen produced in the CHG process could be used for the hydrotreatment of the biocrude through the removal of oxygen, nitrogen and sulfur for the production of drop-in liquid fuels (Elliott et al., 2015). Following CHG, the process water could subsequently be returned to the primary settlement tank of the wastewater treatment to recycle the nutrients (e.g. nitrogen and phosphorus) back into the system.

6.7. General conclusions

The results presented in this thesis and the synthesis of this work with the most recent literature have been used for a proposed model that describes the integration of wastewater treatment, cultivation of macroalgae and HTL processing in the following process diagram (Fig. 6.2).

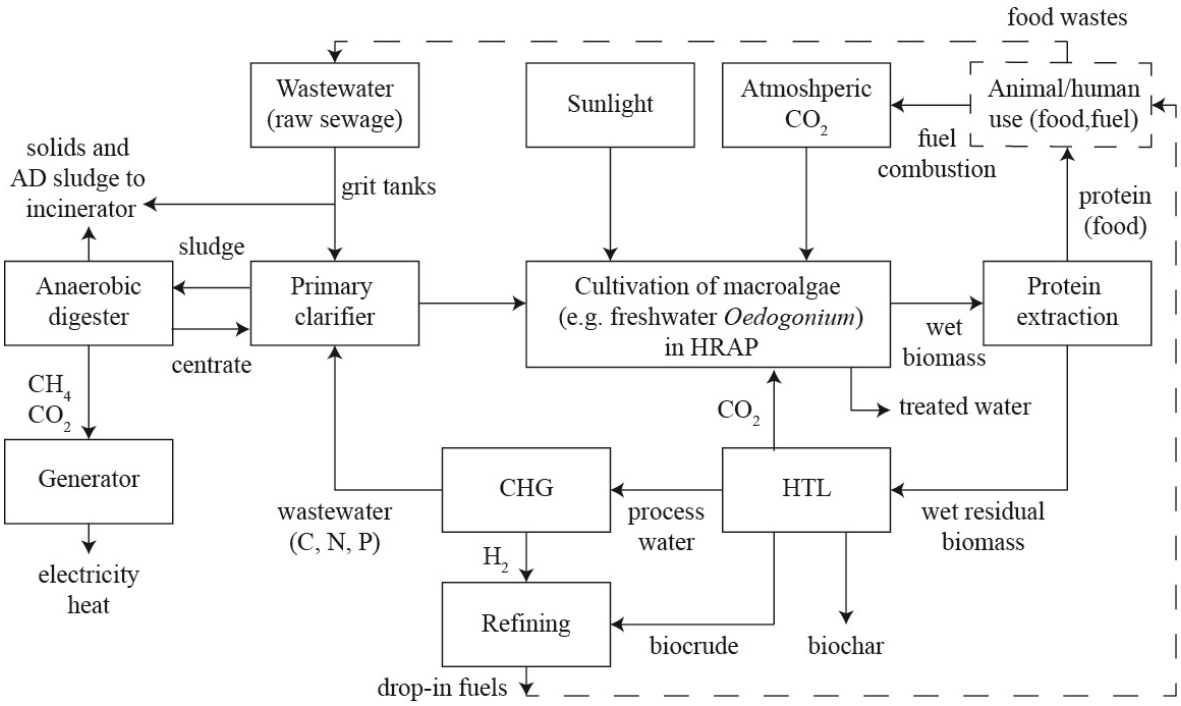

Figure 6.2. The proposed model describes the integration of wastewater treatment, cultivation of macroalgae and HTL processing in an industrial ecology framework. AD = anaerobic digestion; CHG = catalytic hydrothermal gasification.

The proposed model describes an optimistic view of the sustainable production of biofuels from wastewater through the cultivation of macroalgae. While the cultivation of terrestrial crops have been developed and improved for thousands of years, we only begin to understand how to cultivate algae intensively (both micro- and macroalgae). Algae have a tremendous potential for the capture of solar energy and carbon dioxide. Converting this biomass to bio-crude oil using hydrothermal liquefaction is simply a method to reproduce one of the most efficient natural processes to concentrate this energy. It is crucial to continue developing alternative energies such as biofuels to anticipate the grand economic, social and environmental challenges that face the world. The work presented in this thesis aims to contribute to the sustainable and realistic development of algal biofuels. The model

proposed is optimistic and acknowledges that to make this concept a reality will require a progressive change in the way we manage and recycle fundamental and finite resources, using macroalgae as a key resource in that process.

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Annex 1

Annex to Chapter 2

Table S2.1. Projected productivity and value of commodities produced by macroalgae.

Data show macroalgae projected productivities (P, in metric t/ha/yr) and values (V, in US$/ha/yr) of commodities generated by marine (M) and freshwater (FW) macroalgae through different scenarios Theoretical values of protein extract plus biocrude from residual biomass (5) is also presented. Note that theoretical values (V) are rounded to the nearest $100 for each scenario. FA = fatty acids.

Scenario			1	2	3	4	5	6	7	8	9	10
Commodity			Biodiesel	Biocrude	Protein	Biocrude - Protein	3+4	Lipid	Biocrude - Lipid	6+7	Biocrude - FA	1+9
Price (US$/t)			941	682	432	682		1170	682		682	
Species	Source											
Derb.	M	P	1.8	7.1	9.4	5.4		4.5	3.5		5.6	
		V	$1,700	$4,800	$4,100	$3,700	$7,800	$5,300	$2,400	$7,600	$3,800	$5,500
Ulva	M	P	0.6	4.6	6.8	3.4		0.8	4.0		4.1	
		V	$600	$3,100	$2,900	$2,300	$5,200	$900	$2,700	$3,600	$2,800	$3,400
Chaet.	M	P	0.7	4.0	4.0	3.3		1.2	3.1		3.4	
		V	$700	$2,700	$1,700	$2,300	$4,000	$1,400	$2,100	$3,500	$2,300	$3,000
Clad.	M	P	0.8	4.3	5.5	3.3		1.4	3.1		3.6	
		V	$700	$2,900	$2,400	$2,200	$4,600	$1,700	$2,100	$3,800	$2,500	$3,200
Oedog.	FW	P	0.8	3.3	4.2	2.5		1.8	1.9		2.6	
		V	$800	$2,300	$1,800	$1,700	$3,500	$2,000	$1,300	$3,300	$1,800	$2,600
Clad.	FW	P	0.6	2.0	3.4	1.4		0.7	1.4		1.5	
		V	$600	$1,300	$1,400	$900	$2,300	$800	$1,000	$1,800	$1,000	$1,600

Table S2.2. Sensitivity analyses of marine *Derbesia* and *Ulva* and freshwater *Oedogonium* for parameters influencing the value of feedstock (US$/ha/yr) for sequential extraction of protein from biomass and hydrothermal liquefaction of the residual biomass to biocrude. Values (A); Parameters (B); References (C); "Best Case" scenarios (D).

A) Sensitivity Analysis Values

	Parameter	Biomass productivity	Protein content	Biocrude yield	WTI crude oil price	Soybean meal price
	Unit	US$/ha/yr	US$/ha/yr	US$/ha/yr	US$/ha/yr	US$/ha/yr
Species	Case					
Derbesia	Favourable	15,526	7,780	9,544	8,137	9,149
	Standard	7,705	7,705	7,705	7,705	7,705
	Unfavourable	7,245	7,630	6,221	7,199	6,660
Ulva	Favourable	11,973	5,283	6,394	5,502	6,273
	Standard	5,230	5,230	5,230	5,230	5,230
	Unfavourable	4,954	5,176	3,980	4,911	4,475
Oedogonium	Favourable	11,135	3,590	4,426	3,755	4,194
	Standard	3,549	3,549	3,549	3,549	3,549
	Unfavourable	3,341	3,509	2,774	3,308	3,084

B) Sensitivity Analysis Parameters

	Parameter	Biomass productivity	Protein content	Biocrude yield	WTI crude oil price	Soybean meal price
	(P)	(P1)	(P2)	(P3)	(P4)	(P5)
	Unit	$g/m^2/d$	wt%	wt%	US$/t	US$/t
Species	Case					
Derbesia	Favourable	24	22	18.5	763.3	585.8
	Standard	11.9	21.6	12.3	682.5	431.9
	Unfavourable	11.2	21.2	7.3	587.9	320.7
Ulva	Favourable	26.1	16.6	12.2	763.3	585.8
	Standard	11.4	16.3	8.1	682.5	431.9
	Unfavourable	10.8	16	3.7	587.9	320.7
Oedogonium	Favourable	16	23	20.6	763.3	585.8
	Standard	5.1	22.5	13.7	682.5	431.9
	Unfavourable	4.8	22	7.6	587.9	320.7

C) Sensitivity Analysis References

	Parameter	Biomass productivity	Protein content	Biocrude yield[a]	WTI crude oil price[b]	Soybean meal price[b]
	(P)	(P1)	(P2)	(P3)	(P4)	(P5)
Case	Species					
Favourable	*Derbesia* *Ulva* *Oedogonium*	Magnusson[c] Bolton[d] Cole[e]	This Study, +1 SD	(P3) = standard + (standard * 0.5)	Maximum price within the last 2 years	Maximum price within the last 2 years
Standard	All species	This Study, average	This Study, average	(P3) = $0.80*W_{LIP} + 0.15*W_{CARB}$	Average price within the last 2 years	Average price within the last 2 years
Unfavourable	All species	This Study, -1 SD	This Study, -1 SD	(P3) = $0.55*W_{LIP} + 0.06*W_{CARB}$	Minimum price within the last 2 years	Minimum price within the last 2 years

[a] W_{LIP} and W_{CARB} are lipid and carbohydrate contents (wt%) of macroalgae respectively; conversion factors from Biller & Ross, 2011

[b] commodity prices (average 2012-2013) from Indexmundi, http://www.indexmundi.com/australia/

[c] Magnusson et al., *2014*

[d] Bolton et al., 2009

[e] Cole et al., 2014

D) Sensitivity Analysis "Best Case"

All parameters are considered favourable	
Value of feedstock (US$/ha/yr) = 3.65[c] * (P1) * [((P2) * (P5)) + ((P3) * (P4))] / 100%	
Derbesia	US$23,660/ha/yr = 3.65 * 24.0 * [(22.0 * 585.8) + (18.5 * 763.3)] / 100
Ulva	US$18,135/ha/yr = 3.65 * 26.1 * [(16.6 * 585.8) + (12.2 * 763.3)] / 100
Oedogonium	US$17,051/ha/yr = 3.65 * 16.0 * [(23.0 * 585.8) + (20.6 * 763.3)] / 100

[c] multiplier derived from the conversion of productivity in $g/m^2/d$ to productivity in t/ha/yr

Table S2.3. Biocrude yield from several studies on hydrothermal liquefaction of macroalgae and microalgae. M = marine origin, FW = freshwater origin, dw = dry weight, afdw = ash-free dry weight.

Reference	Species	Origin	T	Time	Biocrude	Equation[a]	
			(°C)	(min)	yield (wt%)	Mass of feed	Algae basis
Macroalgae							
Zhou *et al*.	*Enteromorpha prolifera*	M	300	30	23.0	algae + catalyst	dw
Anastasakis & Ross	*Laminaria saccharina*	M	350	15	19.3	algae + catalyst	afdw
Microalgae[b]							
Dote *et al.*	*Botryococcus braunii*	FW	300	60	64.0	algae	afdw
Minowa *et al*.	*Dunaliella terciolecta*	M	300	5	43.8	algae	afdw
Yang *et al*.	*Microcystis viridis*	FW	340	30	33.0	cyanobacteria	afdw
Biller & Ross	*Chlorella vulgaris*	FW	350	60	36.0	algae	afdw
	Nannochloropsis occulata	M	350	60	35.0	algae	afdw
	Porphyridium cruentum	M	350	60	27.1	algae + catalyst	afdw
	Spirulina	FW	350	60	29.0	cyanobacteria	afdw
Garcia Alba *et al*.	*Desmodesmus* sp.	FW	375	5	49.0	algae	afdw
Vardon *et al*.	*Spirulina*	FW	300	30	32.6	cyanobacteria	afdw
Ross *et al*.	*Chlorella vulgaris*	FW	350	60	27.3	algae + catalyst	afdw
	Spirulina	FW	350	60	20.0	cyanob. + catalyst	afdw
Biller *et al*.	*Nannochloropsis occulata*	M	350	60	34.3	algae + catalyst	afdw
	Chlorella vulgaris	FW	350	60	38.9	algae + catalyst	afdw
Zou *et al*. (a)	*Dunaliella terciolecta*	M	360	50	25.8	algae	dw
Zou *et al*. (b)	*Dunaliella terciolecta*	M	360	30	36.9	algae	dw
Brown *et al*.	*Nannochloropsis* sp.	M	350	60	43.0	algae	dw
Duan & Savage	*Nannochloropsis* sp.	M	350	60	57.0	algae	dw
Jena *et al*.	*Spirulina platensis*	FW	350	60	39.9	cyanobacteria	dw

Table S2.3. *continued*

Reference	Species	Origin	T	Time	Biocrude	Equation[a]	
			(^{o}C)	(min)	yield (wt%)	Mass of feed	Algae basis
Valdez *et al*.	*Nannochloropsis* sp.	M	350	60	39.0	algae	dw
Yu *et al*.	*Chlorella pyrenoidosa*	FW	280	120	39.4	algae	dw
These biocrudes are composed of 68-75% carbon, 8-10% hydrogen, 9-19% oxygen and 4-8% nitrogen (dry weight)[c]							

[a] Biocrude yield (wt%) = Mass of biocrude (g) / Mass of feed (g) *100%

[b] reproduced and modified from Lopez Barreiro *et al.,* 2013

[c] Frank *et al.,* 2013

Annex 2

Annex to Chapter 5

Table S5.1. Metal content of the wastewaters.

Data show means (n = 3) of metal content of wastewaters taken at day 0, 21 and 42.

Element	Unit	PRIM	SEC	DAF
Ca	mg/L	29	28	28
K	mg/L	20	19	20
Mg	mg/L	31	29	32
Na	mg/L	343	357	376
Al	µg/L	35	225	370
As	µg/L	2	< 1	< 1
Cd	µg/L	< 0.1	< 0.1	< 0.1
Cu	µg/L	7	11	17
Fe	µg/L	115	380	240
Hg	µg/L	< 0.1	< 0.1	< 0.1
Mn	µg/L	88	5	22
Mo	µg/L	5	3	3
Ni	µg/L	2	1	1
Pb	µg/L	1	2	2
Se	µg/L	< 10	< 10	< 10
Sr	µg/L	260	256	256
V	µg/L	< 10	< 10	< 10
Zn	µg/L	9	13	21

PRIM = primary effluent; SEC = secondary effluent; DAF = effluent from DAF unit.

Table S5.2. Metal content of the biomass for the small-scale culture trials.

Data show means (n = 3) of metal content of biomass cultured in wastewaters at various exchange rates.

	PRIM			SEC			DAF		
mg/kg	5%	10%	20%	5%	10%	20%	5%	10%	20%
Al	131	30	86	714	3170	6120	791	338	509
As	0.7	1.8	1.3	3.9	4.5	5.2	2.2	2.8	2.6
B	1.5	<0.1	<0.1	<0.1	<0.1	0.4	0.9	<0.1	0.6
Ba	65	86	81	114	150	184	73	111	100
Ca	4560	2140	2110	3510	2680	2940	2340	2300	2290
Cd	<0.1	<0.1	<0.1	0.11	0.11	0.16	0.30	0.16	0.25
Co	0.5	0.2	0.3	0.2	1.0	1.3	0.4	0.4	0.5
Cr	1.1	0.8	0.8	1.2	2.3	3.7	1.2	1.0	1.1
Cu	29	25	23	20	161	306	36	73	71
Fe	598	174	302	274	1230	2130	378	216	288
Hg	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
K	17800	19600	17600	22100	14400	21000	10600	13600	12000
Mg	4210	3930	4290	3080	2650	3630	2820	2800	2980
Mn	497	100	174	18	75	105	41	36	46
Mo	0.16	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Na	<250	<250	<250	478	<250	330	1860	<250	1140
Ni	3.0	0.8	1.1	0.8	1.8	3.1	1.1	0.8	1.2
P	8450	6020	6380	219	<240	885	5150	2330	3440
Pb	2.7	0.9	2.7	0.9	8.3	18.4	2.6	1.8	2.4
S	2240	1780	2140	314	362	477	855	487	673
Se	<1	<1	<1	<1	<1	<1	<1	<1	<1
Sr	141	100	101	169	107	127	111	84	90
V	0.2	0.2	0.2	1.6	4.7	7.1	1.1	0.6	0.8
Zn	30	40	46	74	146	347	82	91	106

PRIM = primary effluent; SEC = secondary effluent; DAF = effluent from DAF unit.

Table S5.3. Metal content of the influent and effluent waters in the ponds.

Data show means (n = 9) of metal content of influent wastewater and means (n = 3) of properties of pond effluents over 8 weeks. PRIM = primary effluent.

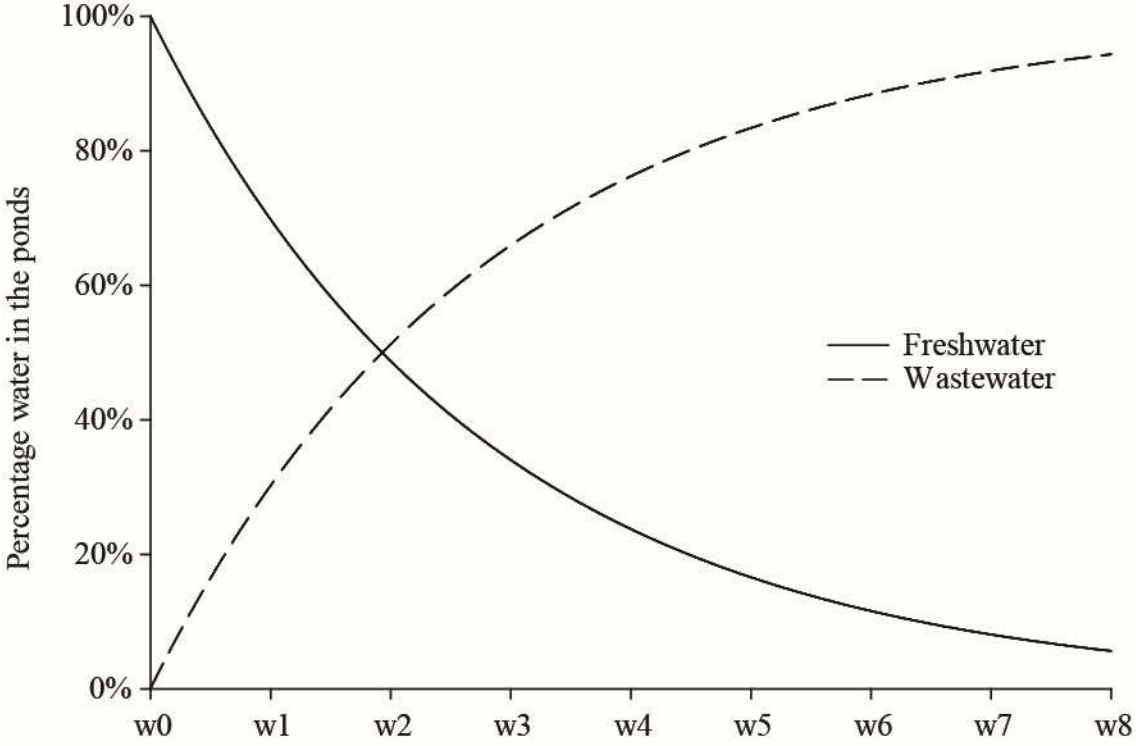
Element		Influent	Pond effluents							
		PRIM	w_1	w_2	w_3	w_4	w_5	w_6	w_7	w_8
Ca	mg/L	33	17	21	23	26	28	28	29	29
K	mg/L	24	7	8	10	12	14	14	16	16
Mg	mg/L	33	16	21	25	27	27	29	29	30
Na	mg/L	400	190	261	301	327	346	352	372	371
S	mg/L	26	10	14	17	19	21	22	24	24
Al	µg/L	6	1	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
As	µg/L	8	4	5	5	6	7	7	7	7
B	µg/L	220	106	144	175	199	223	237	259	266
Ba	µg/L	29	25	23	18	16	14	9	6	7
Cd	µg/L	< 0.05	0.3	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Co	µg/L	0.7	0.3	0.5	0.6	0.7	0.8	0.8	0.8	0.9
Cu	µg/L	4	4	4	3	3	3	3	3	3
Cr	µg/L	7	3	4	5	5	6	6	7	7
Fe	µg/L	< 100	< 100	< 100	< 100	< 100	< 100	< 100	< 100	< 100
Hg	µg/L	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Mn	µg/L	74	3	10	5	7	7	6	3	1
Mo	µg/L	3	2	2	2	3	3	3	3	3
Ni	µg/L	1.8	0.7	0.8	0.9	1.0	1.2	1.2	1.2	1.2
Pb	µg/L	0.1	< 0.05	0.1	0.2	< 0.05	0.1	0.1	< 0.05	< 0.05
Se	µg/L	5	1	2	3	4	4	5	5	5
Sr	µg/L	356	181	226	248	278	291	283	278	282
V	µg/L	10	5	7	8	9	9	9	9	9
Zn	µg/L	7	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5

Table S5.4. Metal content of the biomass produced in pilot-scale ponds.

Data show means (n = 3) of metal content of biomass over 8 weeks.

mg/kg	w_0	w_1	w_2	w_3	w_4	w_5	w_6	w_7	w_8
Al	113	247	256	245	442	619	383	492	506
As	1.2	1.3	1.1	1.3	1.5	1.4	1.3	2.0	2.0
B	1.8	2.4	3.7	9.4	13.2	11.4	12.1	16.4	19.8
Ba	91	65	63	85	73	61	68	86	69
Ca	1433	1417	1777	7600	6290	5277	10110	16133	11930
Cd	0.06	0.07	<0.1	0.06	0.08	0.07	0.07	0.13	0.15
Co	1.4	1.2	1.0	0.9	0.9	0.8	0.7	1.2	1.3
Cr	1.7	1.5	2.0	2.0	2.5	2.5	2.0	2.4	2.6
Cu	31	30	25	18	24	21	14	18	18
Fe	296	291	352	273	316	398	246	333	277
Hg	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
K	25167	17133	28600	23733	20033	18400	18700	18067	20767
Mg	3083	4360	4197	5293	4493	4157	4267	5587	4877
Mn	259	316	253	215	285	271	187	284	199
Mo	0.2	0.2	0.4	0.3	0.2	0.2	0.2	0.2	0.4
Na	668	472	694	1142	1197	1097	1116	1443	1843
Ni	1.8	2.2	2.4	2.7	2.9	2.2	2.0	3.2	3.9
P	5947	6600	6607	8333	9477	8360	9110	12967	11400
Pb	0.6	1.3	1.0	0.8	1.2	1.2	1.1	2.3	1.9
S	2.7	2.1	2.5	1.8	2.3	2.6	1.9	2.7	3.0
Se	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Sr	79	44	57	134	116	110	175	262	202
V	0.6	0.6	0.6	0.6	0.9	1.0	0.7	0.9	1.1
Zn	55	60	59	46	67	62	49	61	55

Figure S5.1. Evolution of the proportions of freshwater and wastewater in the cultivation ponds over 8 weeks.



Annex 3

List of publications from this thesis

Neveux N, Magnusson M, Maschmeyer T, de Nys R, Paul NA, 2014a. Comparing the potential production and value of high-energy liquid fuels and protein from marine and freshwater macroalgae. *Global Change Biology Bioenergy*, **7**, 673-689.

Neveux N, Yuen AKL, Jazrawi C, Magnusson M, Haynes BS, Masters AF, Montoya A, Paul NA, Maschmeyer T, de Nys R, 2014b. Biocrude yield and productivity from the hydrothermal liquefaction of marine and freshwater green macroalgae. *Bioresource Technology*, **155**, 334-341.

Neveux N, Yuen AKL, Jazrawi C, He Y, Magnusson M, Haynes BS, Masters AF, Montoya A, Paul NA, Maschmeyer T, de Nys R, 2014c. Pre- and post-harvest treatment of macroalgae to improve the quality of feedstock for hydrothermal liquefaction. *Algal Research*, **6**, 22-31.

Neveux N, Magnusson M, Mata L, Whelan A, de Nys R, Paul NA, 2015. The treatment of municipal wastewater by the macroalga *Oedogonium* sp. and its potential for the production of biocrude. *Algal Research*, accepted for publication.

Contributed to

Paul NA, Neveux N, Magnusson M, de Nys R, 2014. Comparative production and nutritional value of "sea grapes" – the tropical green seaweeds *Caulerpa lentillifera* and *C. racemosa*. *Journal of Applied Phycology*, **26**, 1833-1844.