Green tide algae integrated aquaculture for nitrogen bioremediation

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Abstract

Green tide algae bloom in eutrophic environments with fast growth rates, efficient nutrient uptake and broad environmental resilience. These same characteristics are sought after for algae in integrated aquaculture systems. To evaluate the potential use of naturally occurring green tide algae in tropical integrated aquaculture the effects of key variables on resilience, biomass productivity and bioremediation potential were quantified. Three locally abundant green tide algal species (*Cladophora coelothrix, Chaetomorpha indica* and *Ulva* sp.) demonstrated a high tolerance across the extremes of salinity (15 to 45‰) and total ammonia nitrogen (TAN from 7 up to 700 µmols l^{-1}) characteristic of tropical aquaculture bioremediation ponds. In a preliminary *in situ* experiment *Cladophora coelothrix* demonstrated high growth and survival rates, a high predicted productivity (4 t ha^{-1} for a 7-day harvest cycles) and corresponding nitrogen removal (23 kg N $harvest^{-1}$). Subsequently, it was determined that large scale, *in situ* productivity of *Cladophora coelothrix* during four winter months correlated primarily with nitrogen concentration, position in the pond and stocking density, with a lesser influence of salinity, temperature and the ratio of nitrate-nitrogen and total ammonium nitrogen. Growth and resilience were then determined in factorial laboratory and mesocosm experiments, according to environmental fluctuations of an entire growing season. *Cladophora coelothrix* growth was high irrespective of seasonal fluctuations. Temperature was the key variable for seasonal growth, followed by nitrogen concentration and salinity, and interestingly growth was limited by ~20% when nitrate-N and TAN were available simultaneously. In addition to adequate nitrogen levels, seaweeds under intensive culture require a high flux of dissolved inorganic carbon (Ci) to support productivity. In this study, higher levels of inorganic carbon (Ci), in particular CO_2 significantly enhanced the productivity of *Cladophora coelothrix* (26%), *Chaetomorpha*

linum (24%) and *Cladophora patentiramea* (11%), despite the demonstrated HCO_3^- utilization through high pH compensation points (9.7-9.9) and growth in pH levels up to 9. Finally, classification and regression tree (CART) analysis in combination with logistic equations, using relevant laboratory, mesocosm and field data from all previous data chapters, were used to develop annual model simulations for large-scale algal culture for two locally abundant green tide algae. The logistic model simulations demonstrate that *Cladophora coelothrix* had a high environmental tolerance and the highest predicted annual productivity of 24.3 T dry weight ha^{-1} $year^{-1}$, equating to a nitrogen removal of 140 kg ha^{-1} $year^{-1}$, whereas the lower environmental tolerance and overall growth of *Cladophora patentiramea* resulted in a lower annual productivity of 10.8 T dry weight ha^{-1} $year^{-1}$. In conclusion, this study has demonstrated that green tide algae can be cultured in tropical land-based ponds with high productivity and nitrogen bioremediation. *Cladophora coelothrix*, in particular, demonstrated a broad environmental tolerance, high biomass productivity and nitrogen bioremediation, and these results support the use of this green tide algal species in integrated aquaculture.

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Chapter 1: General Introduction

1.1 Intensive aquaculture production and the environment

The demand of aquatic protein for human consumption has increased substantially in the past decade. At the same time, natural fisheries stocks have declined to alarmingly unsustainable levels due to overfishing (Froese & Proel 2010, Garcia & Rosenberg 2010, Thurstan et al. 2010). This phenomenon has driven the rapid development of aquaculture (Naylor & Burke 2005, Neori et al. 2007, Jiang 2010). In the past decade, aquaculture production has had a steady eleven percent growth per annum to reach production of over fifty million tons of cultured finfish, crustaceans, molluscs and other aquatic animals in 2008 (FAO 2010). As the world's population expands to over six billion the dependence on aquaculture as a primary protein source will increase, and it is predicted that in 2012 more than fifty percent of the world's aquatic protein will be supplied through aquaculture (FAO 2010).

Most aquaculture production takes place in freshwater, where herbivorous and filter-feeding species are cultured extensively (Bostock et al. 2010). In contrast, marine and brackish aquaculture is dominated by the intensive production of high value finfish and crustaceans (Bostock et al. 2010). As marine and brackish aquaculture production continues to intensify there is an increase in the use of artificially compounded feeds, particularly in large-scale intensive production of carnivorous finfish and crustaceans (Tacon & Metian 2008). Much of this intensive production is carried out in land-based ponds and raceways, all of which rely on high water exchanges to manage waste products produced through the metabolism of high protein compounded feeds, or from uneaten waste feed (Hall et al. 2011). These processes produce water that is high in dissolved nutrients, in particular inorganic nitrogen and phosphorus, and the discharge of untreated wastewater is an environmental concern (Lin &

Fong 2008). This potential for environmental damage has led to regulations for intensive aquaculture wastewater discharge (DERM 2011).

1.2 The environmental footprint of intensive aquaculture on the marine environment

One of the major impacts of intensive aquaculture on the natural environment arises from the discharge of nutrient-rich wastewater in coastal areas (Sara 2007, Lin & Fong 2008). High value carnivorous species of crustaceans and finfish require feeds containing elevated protein levels to sustain the high growth rates in intensive aquaculture systems (Tacon et al. 2009, Olsen 2011). However, a high percentage of the nutrients within feeds are un-utilized and remain as waste in the water (Gutierrez-Wing & Malone 2006), which consequently contains organic and inorganic nutrients as ammonium and nitrates, phosphorus, dissolved organic carbon (Herbeck et al. 2012). Therefore, the discharge of untreated wastewater has the potential to cause eutrophication of receiving environments (Thomas et al. 2010), and these impacts have been associated with high-input intensive systems (Diana 2009).

Nevertheless, nutrient rich wastewater from intensive aquaculture operations can be treated prior to discharge to the environment. Constructed wetlands are efficient buffer areas that can mitigate some negative effects from excess nutrients (Sindilariu et al. 2009, Su et al. 2011). Settling basins or ponds are also efficient in reducing concentrations of potential pollutants, in particular the suspended solids fraction of the wastewater (Prapaiwong & Boyd 2012). In these systems biological transformation (denitrification) through microbial activity transforms more toxic dissolved nitrogen forms (ammonium) to less toxic compounds (nitrates) (Erler et al. 2008, 2010). However, the most effective method to remove dissolved nitrogen from aquatic environments is through the integrated culture of macroalgal biomass (Cahill et al. 2010). Integrated algal culture can remove large amounts of dissolved nutrients, in particular nitrogen (Al-Hafedh et al. 2012).

1.3 Seaweed-based integrated aquaculture

The underpinning concept of modern integrated aquaculture has been practiced in Asian countries for centuries. Polyculture uses the waste from one system as a valuable resource for a subsequent system to optimize nutrients usage. Integration of marine finfish and prawns aquaculture with seaweed culture can produce sustainable and cost-effective operations to reduce environmental impacts (Barrington et al. 2009, Troell 2009). Seaweeds are photosynthetic organisms that incorporate inorganic nutrients (particularly nitrogen and phosphorus) dissolved in the wastewater into organic molecules as proteins, pigments and carbohydrates in the presence of sunlight (Figure 1.1). The seaweed biomass produced can be supplied to a large commodity market to manufacture and extract a variety of valuable products and compounds, including food, animal feeds, phycocolloids and fertilizers (Chopin and Sawhney 2009) (Figure 1.1). Therefore, seaweed-based integrated aquaculture has great potential for the bioremediation of nitrogen and phosphorous from intensive aquaculture.

Targeting algal species for bioremediation systems, however, cannot be based solely on high value. Wastewater bioremediation depends on the physiological capacity of the targeted species to thrive in sub-optimal and sometimes harsh culture conditions with dynamic environmental fluctuations, particularly in tropical land-based pond systems (de Paula Silva et al. 2012). These conditions include unnaturally high nitrogen and severe fluctuations of the key limiting environmental variables of temperature, salinity and light intensity. Therefore, to develop integrated systems it is essential to utilise robust species that deliver efficient and reliable removal of excess dissolved nutrients with high biomass productivity.

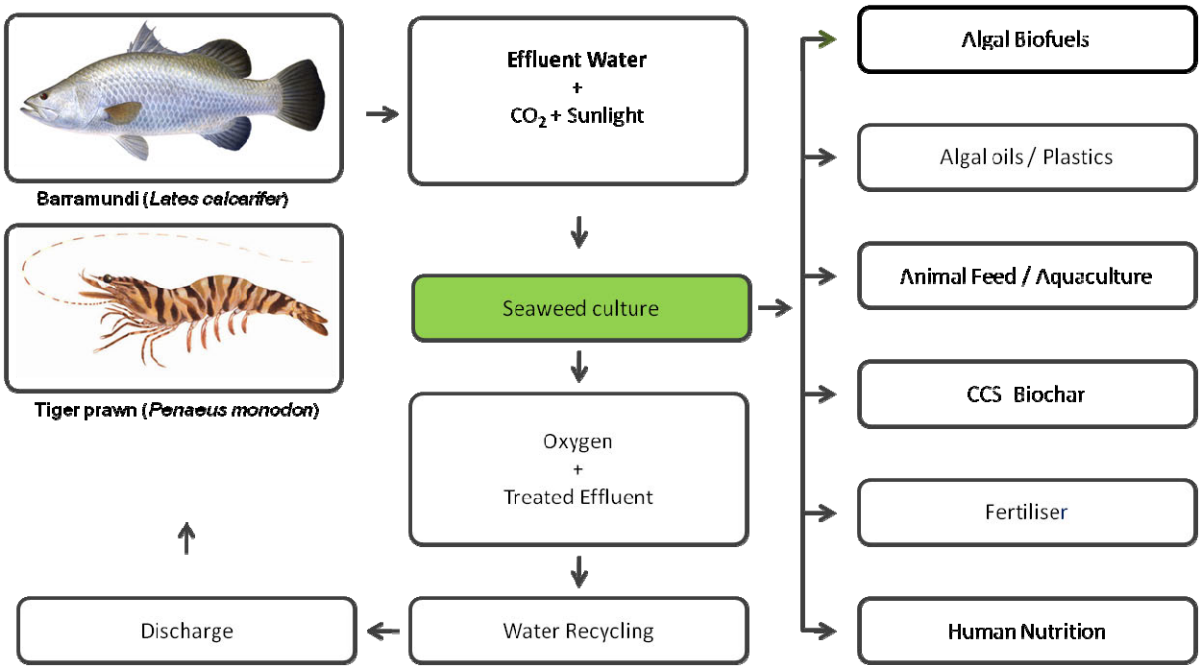

Figure 1.1 Diagram representing the bioremediation of aquaculture wastewater through integrated seaweed culture systems and end products.

In this context, green tide algal species have excellent potential for bioremediation systems due to their effective uptake and storage of inorganic nutrients, high biomass productivity and broad environmental tolerance (Taylor et al. 2001, de Paula Silva et al. 2008, Pérez-Mayorga et al. 2011). Green tide algae have been considered fouling organisms in the targeted culture of high value seaweeds (Paul & de Nys 2008). However, the new focus on seaweed biomass production for novel and diverse applications provides a clear impetus to develop green tide algae aquaculture with a focus on bioenergy. For example, the production of biochar from green tide algae through pyrolisis results in energy and a high-value soil ameliorant (Bird et al. 2011, Bird et al. 2012). The production of biofuels from green tide algae through hydrothermal liquefaction (Zhou et al. 2010, Zhou et al. 2012) has potential as a supply of renewable energy and liquid fuels.

1.4 Green tide algae characterization

1.4.1 Species diversity and biology

The grouping of seaweeds as green tide algae is comprised of species that inhabit the marine-terrestrial interface, including coastal and estuarine areas. In particular, green tide algae are within the class Ulvophyceae, and are fast-growing and ephemeral species with foliose or filamentous morphology from the genera *Ulva* (including *Enteromorpha*, see (Hayden et al. 2003), *Cladophora* and *Chaetomorpha* (Morand & Merceron 2005). They form large accumulations of biomass under suitable environmental conditions, and are amongst the most common fouling organisms in many coastal areas worldwide (Merceron et al. 2007, Ye et al. 2011). Staggering quantities of algal biomass can accumulate in shallow water, constituting a major nuisance in sheltered bays around the world (Liu et al. 2010). The most recent and largest green tide algal bloom was reported in the 2008 Olympic games, covering an area of more than 1200 km^2 (Pang et al. 2010). These algal blooms are generally explained by increased nutrient loads and favourable environmental conditions of light, temperature and salinity (Liu et al. 2009).

Green tide algal species are characterized by high rates of nutrient uptake and growth (Cohen & Fong 2004a, Fong et al. 2004, Kennison et al. 2011). These characteristics are linked with their thalli morphology, where the thin filaments or blades facilitate the exchange of gases and nutrients with the environment (de los Santos et al. 2009). Moreover, the high tolerance of opportunistic algal species to changes in salinity, temperature and light levels enhances their success in eutrophic estuaries and lagoons, which are often characterized by highly fluctuating abiotic conditions (Choi et al. 2010, Kim et al. 2011, Luo et al. 2012).

1.4.2 Environmental factors limiting seaweed growth

Seaweed growth is regulated by environmental parameters, in particular temperature, salinity and irradiance (Lobban & Harrison 1994). The overall stress tolerance and physiologically

hardy nature of bloom-forming species of the genera *Ulva*, *Chaetomorpha* and *Cladophora* means that they are tolerant of a wide range of these environmental factors (Taylor et al. 2001). Consequently, green tide algae are encountered from the cold Antarctic waters to shallow warm tropical areas (Smith et al. 2005, McKamey & Amsler 2006). They also thrive across a broad salinity gradient ranging from freshwater to hypersaline environments (Thomas et al. 1990, Dodds & Gudder 1992), and can effectively grow in light levels as low as 2 µmols photons m^{-2} s^{-1} (Taylor et al. 2001) while tolerating light levels over 1000 µmols photons m^{-2} s^{-1} (Bischof et al. 2006).

However, stress from one abiotic factor may reduce tolerance to another, even if species has a broad tolerance to any single environmental factor (Lotze & Worm 2002). For example, the interaction of key environmental factors, such as fluctuating temperature and light may have a detrimental effect on growth (Hernandez et al. 2008). Therefore, understanding the interactions between limiting factors important to growth is essential in implementing seaweed-based integrated aquaculture, particularly in open systems subject to seasonal changes in conditions (Abreu et al. 2011).

1.4.3 Green tide algae nutrient physiology

The availability of nutrients plays a major role in regulating the growth and development of seaweeds. The nutrient physiology of seaweeds is based on nutrient uptake, assimilation and storage (Harrison & Hurd 2001, Kennison et al. 2011). Nutrient supply is often limited in natural settings, and bloom forming species (i.e. green tide algae) are dependent on nutrient pulses from anthropogenic activities to sustain growth (Cohen & Fong 2006, Lin & Fong 2008). In contrast, integrated culture systems provide saturating levels of nutrients which fluctuate in relation to the aquaculture production (Abreu et al. 2011). Seaweeds require a wide variety of nutrients for growth, including micro (Fe, Zn, Cu, Mn, Mo) and macro

(nitrogen, phosphorus and carbon) nutrients (Harrison & Hurd 2001). Nitrogen and phosphorus are the two nutrients that most commonly limit seaweed growth, and therefore productivity, in most natural environments, and seaweeds have developed mechanisms that enable them to uptake these nutrients in various sources in a broad range of concentrations (Cohen & Fong 2004a).

Green tide algae have a high surface to volume ratio which promotes a higher nutrient uptake rate (an increased membrane surface area for uptake) (Raven & Taylor 2003). Inorganic nitrogen is present in two common sources ammonium and nitrate. Growth with ammonium as a nitrogen source is generally higher than with nitrate, because ammonium ions diffuse through the cellular membrane, whereas nitrate uptake incurs a cost through enzymatic reactions (Raven et al. 1992). However, when ammonium and nitrate are supplied in combination this does not always translate directly to increases in growth as competition for uptake can occur between sources, for example, ammonium can inhibit nitrate uptake by inactivating or slowing the synthesis of nitrate reductase (Young et al. 2005).

Adequate nitrogen supply is essential in sustaining high biomass productivity in seaweed-based integrated culture systems. However, the oversupply of nitrogen may not be beneficial as uptake is limited by nitrogen assimilation into organic molecules (Barr & Rees 2003). In fact, very high nitrogen levels, in particular ammonium, can have a detrimental effect on growth of certain green tide species (de Paula Silva et al. 2008). Nevertheless, a high nitrogen supply promotes luxury uptake which is stored as organic molecules and inorganic nitrogen in vacuoles (Harrison & Hurd 2001). Nitrogen stored intracellularly can be used to sustain growth when the exogenous supply of nitrogen is limited, but may also provide energy for regulatory processes to overcome detrimental effects of environmental factors on growth (Hernandez et al. 2008). The negative effects of low salinity and the interactive effects of temperature and salinity on growth are ameliorated in the presence of high nitrogen levels

(Kamer & Fong 2001). Furthermore, dense algal cultures are usually limited by dissolved carbon availability, and therefore carbon needs to be supplied to sustain high growth (Demetropoulos & Langdon 2004a).

1.5 Integrated culture systems

1.5.1 Integrated finfish – seaweed aquaculture for nitrogen bioremediation

Integrated finfish - seaweed aquaculture systems have been recognized as one of the most promising forms of sustainable aquaculture (Chopin et al. 2001, Neori et al. 2004). The primary role of seaweed in these systems is wastewater treatment through the uptake and conversion of nitrogenous compounds. In land-based integrated aquaculture, also known as partitioned aquaculture, fish and seaweeds are each cultured in individual ponds or tanks.

The choice of seaweed species for integrated aquaculture system depends on high growth and nitrogen concentration, environmental tolerance, ease of cultivation, control of life cycle and resistance to epiphytes. In addition, the seaweed species also requires a market value (Neori et al. 2004). The most common seaweed genera in integrated aquaculture are *Gracilaria and Porphyra* (red algae) and *Laminaria*, *Fucus* (brown algae), for the high value phycocolloids and human food (Chopin & Sawhney 2009). Green tide algal species from the genera *Ulva* have also been extensively studied, and perhaps are the most efficient and more widely used seaweed species in integrated aquaculture (Neori et al. 2003, Msuya & Neori 2008, Bolton et al. 2009, Copertino et al. 2009, Mata et al. 2010).

The green tide algae genera, including *Ulva*, have the greatest potential for integrated aquaculture with fast growth, efficient nitrogen accumulation and high environmental tolerance (Choi et al. 2010, Deng et al. 2012, Kim et al. 2011, Pérez-Mayorga et al. 2011, Luo et al. 2012). This is in part because green tide algal species can be cultured in a variety of

systems, are easily propagated by fragmentation, and are resistant to fouling. Furthermore, new applications for seaweed biomass, in particular through biomass energy, are increasing the economic viability of these systems (Zhou et al. 2010). Nevertheless, seaweed species for integrated aquaculture have to be thoroughly investigated for their bioremediation potential as a primary goal. The quantification of productivity on nitrogen sources and concentrations is also essential for bioremediation systems. Nitrogen and phosphorus are often supplied at saturation through the wastewater from fish culture, and this then results in carbon becoming a limiting factor in seaweed growth in bioremediation systems (Mata et al. 2007).

1.5.2 Enhancing productivity of green tide algae in integrated aquaculture through carbon supplementation

The additional carbon supply in the form of carbon dioxide (CO_2) has been used to overcome dissolved inorganic carbon limitation, enhancing the biomass productivity of some seaweed species in intensive culture (Demetropoulos & Langdon 2004a). Furthermore, carbon dioxide from anthropogenic emissions has been successfully used to enhance seaweed productivity and consequently the bioremediation potential, increasing the environmental and economic benefits of algal integrated systems (Israel et al. 2005).

Despite productivity enhancements for seaweed species following CO_2 additions, not all seaweed species benefit from additional supply of CO_2 (Israel & Hophy 2002). This is because although all seaweeds use CO_2 for photosynthesis, which is fixed by Rubisco within chloroplasts, some species have also developed carbon concentrating mechanisms (CCM) which allows them to utilize HCO_3^- as a carbon source for photosynthesis (Giordano et al. 2005, Raven et al. 2008). Productivity increases in seaweed biomass through CO_2 enrichment are therefore dependent on the ability of a species to uptake and utilise bicarbonate (HCO_3^-) and carbon dioxide (CO_2). Green tide algae have evolved diverse and efficient mechanisms to

use the HCO_3^- in water (Choo et al. 2002) and consequently their biomass productivity may not be enhanced by CO_2 enrichment (Israel & Hophy 2002). In conclusion, the optimisation of biomass productivity is critical to remediating nutrients, in particular nitrogen, in integrated aquaculture. The interaction between temperature, nutrients, salinity, carbon source and light all impact algal productivities and nitrogen uptake. Determining the interactions between these factors and understanding their impacts will deliver tangible benefits in the development of integrated seaweed aquaculture systems for bioremediation through optimised species selection and environmental management. Therefore, the overall aim of this thesis is to optimise the integrated aquaculture of green tide algae for nitrogen bioremediation within the tropics.

1.6 – Aims and chapter summaries

This thesis aimed to develop models for the bioremediation of intensive aquaculture wastewater through green tide algae integrated culture. The importance of key environmental factors, including temperature, salinity and nutrients (nitrogen and carbon), were quantified for different scenarios in intensive aquaculture in the tropics. Bioremediation potential and biomass productivity were assessed at multiple scales, from controlled environment experiments to *in situ* operational aquaculture conditions. Green tide algal species from the genera *Cladophora*, *Chaetomorpha* and *Ulva* were targeted as candidates for the integrated culture systems.

In Chapter 2, the initial model for the bioremediation of finfish aquaculture wastewater was developed using a combination of field surveys, controlled environment and *in situ* experiments. Natural algal biomass carrying capacity was first determined for an operational bioremediation pond receiving nutrient-rich wastewater. The tolerance to variable salinity and total ammonia nitrogen (TAN) correspondent to conditions relevant to tropical pond-

based was assessed. Subsequently, productivity and bioremediation were quantified *in situ*. These data was then used to develop a growth/harvest model for seaweed-based integrated aquaculture.

In Chapter 3 a more complex model using field survey values representing the annual variation of the key environmental variables from an operational bioremediation pond was developed. Biomass productivity and bioremediation potential were assessed in an operational bioremediation pond receiving effluents from intensive finfish aquaculture. Subsequently, the interactive effects of temperature, salinity, nitrogen concentrations and sources on growth were determined for the green tide alga *Cladophora coelothrix*.

In Chapter 4 the processes of carbon utilisation by three green tide algae, *Cladophora coelothrix*, *Cladophora patentiramea* and *Chaetomorpha linum* were quantified. This is essential to understand how carbon dioxide (CO_2) enrichment affects biomass productivity. First, in a series of laboratory experiments carbon concentration mechanisms (CCM) and affinity for bicarbonate (HCO_3^-) as a carbon source for photosynthesis were quantified. Two techniques were used in the laboratory experiments; pH drift in closed vessel and growth response on different CO_2/HCO_3^- ratios determined by variable pH levels. Thereafter, CO_2 was used to overcome dissolved carbon (DIC) limitation, and the growth response was related to the specific HCO_3^- affinity.

In Chapter 5 an annual productivity model for integrated green tide algae aquaculture was developed using data from Chapters 2, 3 and 4. I used classification and regression tree analysis to partition growth into specific combinations of environmental factors determined through a year-long survey of an operational aquaculture settlement pond. The growth rates for each month were then used as input data for a logistic regression model. The resilience and biomass productivity of *Cladophora coelothrix* in relation to fluctuating environmental

conditions encountered in tropical aquaculture was compared with another promising green tide algae; *Cladophora patentiramea*.

The results of this study were discussed in Chapter 6.

Chapter 2: Integrating filamentous green tide algae into tropical pond-based aquaculture[1]

2.1 Introduction

Integrated aquaculture, the sequential use of species to optimize nutrient use, can produce sustainable and cost-effective operations that reduce the environmental impacts of aquaculture effluents (Chopin et al. 2001, Neori et al. 2004, Crab et al. 2007). The incorporation of algae in these systems to remove dissolved nutrients is highly efficient with model bioremediation genera such as *Ulva* (Chlorophyta, Ulvaceae) removing up to 80% of dissolved ammonium in aquaculture wastewaters (Neori et al. 2000). A range of algal species have also been targeted, often based on their ability to value-add an additional product (Matos et al. 2006, Pereira et al. 2009). However, the diversity of species trialed in bioremediation systems to date is limited and mostly focused on temperate aquaculture systems. Consequently the focus of bioremediation has also been on temperate species of algae (Chopin et al. 2001, Neori et al. 2004). The potential use of integrated systems in tropical aquaculture is enormous, particularly as the majority of production is pond-based with a resultant point-source discharge (Troell 2009, Barrington et al. 2009).

While the scale and structure of tropical aquaculture provides exceptional opportunity for integrated development to minimize nutrient discharge, the operational parameters affecting the selection and implementation of algae are significantly different from those affecting temperate systems. Pond-based systems also provide different operational considerations to cage and tank-based systems that have been the focus of integrated systems. Tropical pond-based aquaculture systems are typically characterized by large fluctuations in salinity and

[1] **de Paula Silva PH, McBride S, de Nys R, Paul NA (2008) Integrating filamentous green tide algae into tropical pond-based aquaculture. Aquaculture 284:74-80.**

nitrogen availability, while in contrast to temperate systems, light and temperature are relatively stable. Seasonal (monsoonal wet season) rainfall and high levels of evaporation (dry season) characteristic of tropical Northern Queensland, Australia result in salinity changes from 5‰ to 45‰ while management practices such as feeding rate, water flux and exchange rates (also significantly affected by seasonal rainfall) result in variations of nutrient levels in effluent, in particular total ammonia nitrogen (TAN) (Boyd 2003, Metaxa et al. 2006). Ultimately any algal species to be used in tropical integrated aquaculture systems must have a broad tolerance to fluctuating environmental conditions. Furthermore, species with promise need to be assessed *in situ* in bioremediation ponds, in the early stages of the evaluation process, as the transfer from controlled conditions to ponds can provide contrasting outcomes (Paul and de Nys 2008).

One mechanism to select suitable species for integration into tropical pond-based aquaculture is to capitalize on the process of "natural" selection in the ponds themselves, especially as flow-through facilities deliver unfiltered water, with associated biota from adjacent coasts, to the bioremediation ponds. Abundant endemic species present in pond-based mesocosms offer a wide pool of suitable species to select and develop for bioremediation that may not be obvious in local nutrient-poor environments. Two of these species, *Cladophora coelothrix* and *Chaetomorpha indica*, are often referred to as green tide algae because of their excessive nuisance growth under eutrophic conditions (Taylor et al. 2001, Raven and Taylor 2003). They have fast growth and efficient nutrient uptake, as well as a tolerance of low dissolved oxygen, low pH and high levels of nutrients in the water (Morand and Merceron 2005), all of which are essential for pond-based integrated aquaculture. However, the implementation of such species into integrated aquaculture systems remains dependent on their success (growth) under *in-situ* environmental parameters (Chopin et al. 2001, Troell et al. 2003).

Given the need to identify algal bioremediation species that tolerate and grow well across the broad range of salinity and nutrients characteristic of tropical pond-based aquaculture we quantified the efficacy of green tide algae in the bioremediation of aquaculture effluent. Growth rates of three species of filamentous green algae, *Cladophora coelothrix*, *Chaetomorpha indica* and *Ulva* sp. were measured in response to a broad salinity gradient, and subsequently in response to the interaction between salinity and nitrogen (TAN) in laboratory studies. Growth and productivity were then quantified *n-situ* under operational conditions. Data from controlled environment and field were used to calculate potential seaweed productivity and nitrogen extraction from aquaculture effluent under operational conditions.

2.2 Materials and methods

2.2.1 Study organisms: Green tide algae

Three species of green tide filamentous algae (all Ulvophyceae) were used in experiments. *Cladophora coelothrix* Kützing and *Chaetomorpha indica* (Kützing) Kützing (hereafter in this chapter *C. coelothrix* and *C. indica*) are related algae in the family Cladophoraceae. *C. coelothrix* species form dense mats composed of entangled branched filaments on the water surface, and ascending filaments from the water column that are loosely attached to the benthos. *C. indica* species have unbranched filaments as masses of long strands, or are entangled with other seaweed species. These two algae co-exist in floating mats on-site at Good Fortune Bay Fisheries Ltd. (Figure 2.1a). Both species have uniseriate cell growth patterns (Womersley 1984). *Ulva* sp. (hereafter in this chapter *Ulva*) (Ulvaceae) is part of a broad species complex with the genus *Enteromorpha* (Hayden et al. 2003) and has diffuse cell growth forming foliose and tubular green filaments, which are attached or free floating (Womersley 1984).

2.2.2 Sample collection

Algal samples were collected from a sedimentation pond of Good Fortune Bay Fisheries Ltd., a land-based, intensive salt water barramundi (*Lates calcarifer* Bloch) farm, Bowen, Australia (Latitude: 20.02°S Longitude: 148.22°E) from February to May of 2007 (Figure 2.1a). The site has a mean annual temperature of 30°C. *C. coelothrix* and *C. indica* co-exist and were collected from extensive floating mats within the pond. *Ulva* was collected from the same pond attached to the bottom and rocks. Algae used for experiments were transported in plastic bags filled with seawater and kept in the outdoor seawater recirculation system tanks with constant aeration at James Cook University – Marine and Aquaculture Research Facilities Unit. Isolated cultures of each species were maintained during the period of the experiments.

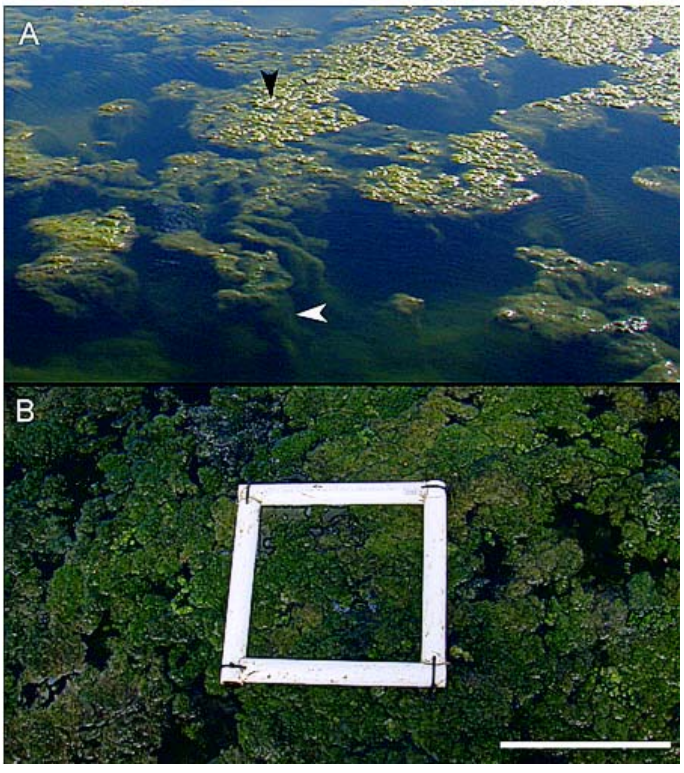

Figure 2.1 Images of green tide algae and bioremediation pond. (A) Floating mats (black arrow) at the pond surface. Mats can be thick (white arrow) indicating the large biomass that can be contained per unit area. (B) Quadrate used to sample biomass per unit area for growth calculations in the logistic model (Section 3.4). Density of the green tide mats was up to 3000 g FW m^{-2}. Scale bar, 25 cm.

2.2.3 General culture conditions and methods for growth experiments in controlled environment

Filaments of each species were rinsed with filtered seawater to remove any visible epiphytes. Small pieces of algae were cultured in Petri dishes in a controlled temperature room (30 ± 3°C) and mean light intensity of 100 µmol photons m^{-2} s^{-1}. The dishes were placed under light and were repositioned daily to reduce any potential effect of light variation. Growth was measured using images taken on a dissecting microscope (Leica MZ125) at the beginning and end of each experiment. Due to morphological characteristics (refer to section 2.1), length (mm) was used to quantify the growth of the uniseriate filaments of *C. coelothrix* and *C. indica*, whereas area (mm^2) was used to quantify the growth of the diffuse thallus of *Ulva*. Both length and area were measured using the software Leica Image Manager 5.0. Specific growth rates (SGR) were calculated from initial length or area (*Li*) and final length or area (*Lf*) using the following equation:

$$SGR = 100 [\ln (Lf / Li)/t] \quad (1)$$

Culture experiments were run for a minimum of 5 days. This timeframe is suitable given the ability of filamentous algae to grow rapidly under a broad range of environmental conditions (Cohen & Fong 2004b) and provides for large effects (see results).

2.2.4 Effect of salinity on growth

As green tide algae can tolerate environments ranging from fresh to marine water (Taylor et al. 2001), growth rates of the three species were examined across a range of salinities, from 0‰ to 45‰ with increments of 5‰. Similar sized pieces of algae with a mean length of 5 mm for *C. coelothrix* and *C. indica*, and a mean area of 1.5 mm^2 for *Ulva*, were excised from the seaweed samples. Filtered sterile seawater (37‰) was used as basal culture medium.

Salinity treatments less than 37‰ were prepared by diluting this medium with distilled water, while higher salinity treatments were made through the addition of artificial seawater salt (Aquasonic). The medium for the 0‰ treatment was distilled water.

All algae were cultured in plastic culture dishes containing 5 ml of medium (n = 5 replicates of each species per salinity treatment). Nutrients (nitrogen as NH_4Cl and phosphate as $NaH_2PO_4.H_2O$) were added to the culture dishes daily to ensure that growth was not limited. Distilled water (0.4 ml) containing 370 µmol l^{-1} of TAN (NH^-_3/NH_4^+) and 22 µmol l^{-1} of PO_4^- was added daily as a source of nutrients, and this also served to adjust for increases in salinity resulting from calculated evaporation rates. The photoperiod used was 14 L:10 D, which is the annual average *in situ*. Growth (SGR) was measured after 6 days of culture.

2.2.5 Interactive effects between nitrogen (TAN) and salinity on growth

High variations of TAN in tropical aquaculture systems occur due changes in feed supplied to the main cultured species and water exchange. Due to the potential interactive effects of nitrogen and salinity on the growth *in-situ*, a broad range of nitrogen concentrations were examined 0, 7, 35, 70 and 700 µmols l^{-1} (equating 0, 0.1, 0.5, 1, 10 mg l^{-1} respectively) under three levels of salinity. These were 15‰ (the level at which *C. indica* and *Ulva* performed well and also the lower limit for *C. coelothrix*), 36‰ (seawater), and 45‰ (the highest salinity level tested in Section 2.4).

Filaments of a similar size (mean length of 5 mm for *C. coelothrix* and *C. indica* and a mean area of 1.5 mm^2 for *Ulva*) were cultured in 75 ml culture vessels containing 30 ml of seawater media (n = 6 replicate vessels for each species*salinity*nutrient treatment). Larger containers were used in this experiment to limit the effect of evaporation on the salinity of the culture medium which occurred previously. A photoperiod of 14 L:10 D was used. Growth (SGR) was measured over 6 days.

2.2.6 Growth and survival in aquaculture effluent under a controlled environment

As an intermediate step between controlled culture conditions and *in situ* trials we determined the survival and growth of *C. coelothrix*, *C. indica* and *Ulva* in water (effluent) sampled directly from an operational bioremediation pond (36‰, 130 µmol l^{-1} ~1.8 mg l^{-1} TAN level) and returned to controlled culture conditions. Fragments of *C. coelothrix*, *C. indica* and *Ulva* were generated (5 mm and 1.5 mm^2, as described above) and cultured in filtered (0.45 µm) aquaculture effluent for 5 days. Survival (alive/dead) and growth (SGR of surviving fragments) were quantified. These data permitted us to make direct comparisons with laboratory cultures in modified seawater, and to compare growth in aquaculture effluent data from in situ experiment (Section 2.7).

2.2.7 Growth experiments *in situ*

To assess bioremediation efficiency, growth rates of the green tide species (*C. coelothrix* and *C. indica*) were quantified under standard farm operating conditions (36 ‰, 130 µmol l^{-1} ~1.8 mg l^{-1} TAN level). *Ulva* was not used in this experiment because it requires substratum to attach and grow. Mesh floating cages (7 cm diameter), containing either 0.1 and 0.3 g of each species, were attached to floating frames constructed from PVC pipes. These weights were used as they either sparsely (0.1 g), or completely (0.3 g), cover the water surface in the cage. Ten floats (experimental blocks), each containing two cylinders with 0.1 or 0.3 g of each species (eight cylinders per float), were positioned across the operational bioremediation pond. Specific growth rates (SGR) were calculated after 10 days.

2.2.8 Biomass measurements *in situ*

To calculate the potential removal of nitrogen from the effluent in the settlement pond using a growth and harvest scenario the ambient density of algal biomass was assessed to determine

natural biomass carrying capacities. Samples were randomly selected from existing algal mats from around the pond. Samples were taken during the wet (monsoonal summer, n = 20 in February) and dry seasons (n = 8 in August) from the pond using a quadrate (0.067 m²) to estimate the maximum standing biomass per hectare at each time (Figure 2.1b). The samples were only obtained from the floating mats as a conservative estimate, and to provide information on the harvestable (floating) portion of the algae.

The relative abundance of *C. coelothrix* and *C. indica* were assessed in each pond sample by viewing sub-samples (n = 4) under the microscope. This was necessary as even though these species co-exist in the floating mats they had different growth properties in experiments, which would affect the input into any model. Fresh weights for samples were recorded at the collection site after drying using a salad spinner, and were subsequently oven dried (65°C for 24 h) to acquire the dry weight for each sample. Percentage nitrogen of the dry mass for each pond sample (n = 20) was quantified by isotope analysis using a Carlo-Erba elemental autoanalyser (Environmental Biology Group, Australian National University, Canberra). This allowed for the calculation of the amount of nitrogen per unit of fresh weight, and also the amounts of nitrogen accumulated as with algal growth.

2.2.9 Data analysis

Statistical analyses were performed using the software SYSTAT 10. A 2-way ANOVA was used to compare the effect of different salinity treatments on the growth of the three species (Section 2.4). The interactive effect of salinity and nitrogen concentrations on the growth rates of the 3 species was analyzed using a 3-factor fixed-effect ANOVA (Section 2.5). The effects of initial size on the growth rates of 2 species at 10 experimental blocks within the pond were analyzed using a 3-factor mixed model ANOVA (Section 2.7). For the ANOVA the assumptions of homogeneity of variance and normality were assessed by scatter plots of

residuals and normal curves of residuals, respectively (Quinn and Keough, 2002).

Calculations for the bioremediation potential (nitrogen removal) were made by logistic models using the average seaweed biomass per unit area (carrying capacity) for the more abundant species (*C. coelothrix*) (Section 2.7), approximated field experiment growth rates (Section 3.3), and the average percentage of nitrogen in the tissue of the samples. Calculations are outlined in detail in Results, including suggested harvesting regimes based on relevant variables for the monsoonal summer, and the dry season (Section 3.4).

2.3 Results

2.3.1 Effect of salinity on growth

The growth responses of the three species followed different trends across the range of salinities (Figure 2.2), as demonstrated by a significant interaction between the species and salinity (species*salinity, $P < 0.001$; Table 2.1). Optimum growth rates (SGR) occurred at different salinities for each of the species. Growth rates of *C. coelothrix* were optimum at 30‰ with a specific growth rate greater than 20% day $^{-1}$. Above this salinity growth decreased as salinity increased, to a minimum at 45‰ (Figure 2.2). *C. indica* had the highest specific growth rate of more than 30% day^{-1} from 15 to 20‰, while *Ulva* had an optimum growth rate of 25% day^{-1} at 15 ‰ (Figure 2.2). In general, all the species had a broad range of salinity tolerance. *C. coelothrix* grew from 15 to 45‰, and *C. indica* and *Ulva* had an even broader tolerance growing from 5 to 45‰ (Figure 2.2). Freshwater (0‰) represented the lower limit, with no species growing at this salinity (Figure 2.2).

Table 2.1 Output for 2-factor ANOVA testing the response of the three species of green tide algae to changes in salinity.

Source	**df**	**MS**	**F**	***P***
Salinity	8	1052.77	13.97	< **0.001**
Species	2	2947.46	39.11	< **0.001**
Salinity * Species	16	908.04	12.04	< **0.001**
Error	108	75.36		

2.3.2 Interactive effects of salinity and nitrogen (TAN) on growth

There was a significant interaction between salinity and nitrogen on growth for the different species of green tide algae (species*salinity* TAN, $P < 0.001$; Table 2.2). This was again (similar to 3.1) driven by different optimum growth rates of each species, this time in a specific combination of salinity and TAN level. General growth responses showed that all algae had a broad tolerance across all salinity*TAN treatments, but with clear differences in the growth response curves (Figure 2.3a-c). In general, the growth rates of *C. coelothrix* peaked at median TAN levels (35-70 µmol l^{-1}) but decreased for all salinities at the highest TAN (700 µmol l^{-1}) (Figure 2.3a). In contrast, *C. indica* had a uniform positive growth trend with increasing TAN for all salinities, although at 15‰ the peak was at 35 µmol l^{-1} TAN (Figure 2.3b). The growth response of *Ulva* was most variable, increasing almost exponentially with increasing TAN up to 70 µmol l^{-1} at higher salinities, yet the reverse was true at the lower (15‰) salinity (Figure 2.3c).

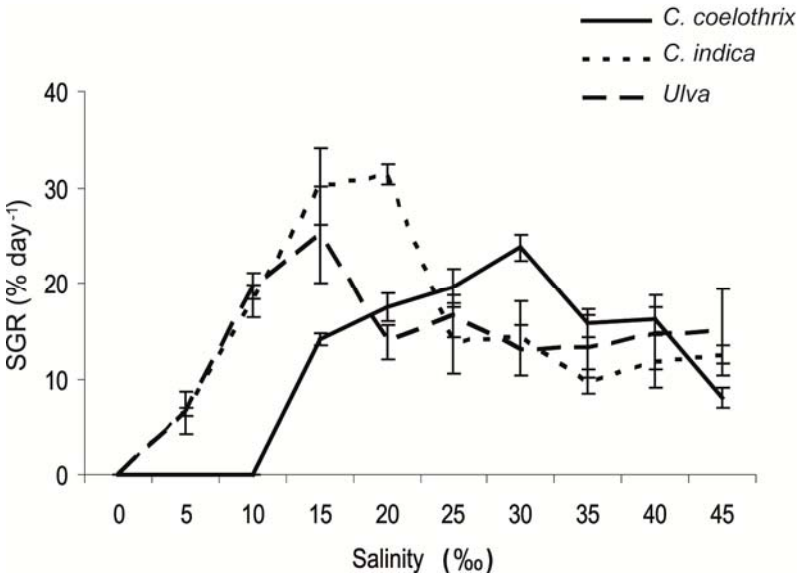

Figure 2.2 Mean specific growth rates (SGR) (+ 1SE) of *C. coelothrix*, *C. indica* and *Ulva* sp. in a range of salinities. (n = 5 for each salinity treatment*species).

The individual combination growth optima of each species also differed. *C. coelothrix* had the highest growth rate (~14% day $^{-1}$) at 36‰ salinity and higher levels of TAN (35 and 70 µmol l^{-1}) (Figure 2.3a). The highest growth rates for *C. indica* (15% day $^{-1}$) were associated with the highest levels of nitrogen (700 µmol l^{-1}) and were indistinguishable between 36 and 45‰ (Figure 2.3b). *Ulva* clearly grew fastest (~25% day $^{-1}$) at high salinity (45‰) and high TAN (70 and 700 µmol l^{-1}) (Figure 2.3c).

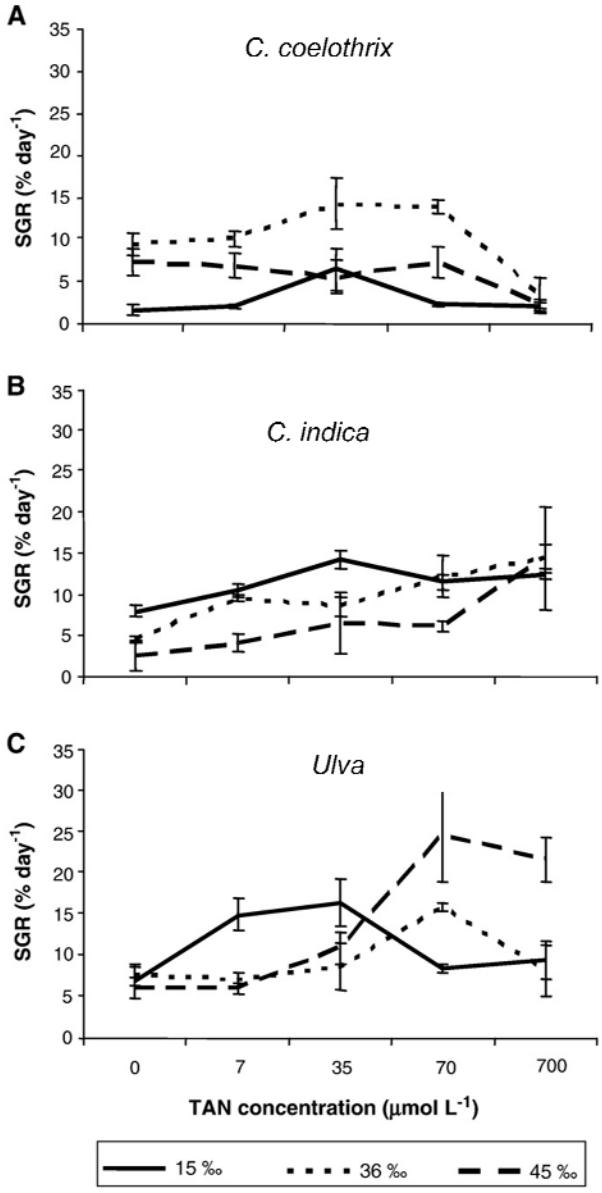

Figure 2.3 Mean specific growth rates (SGR) (+ 1SE) of *C. coelothrix* (A), *C. indica* (B) and *Ulva* sp. (C) cultured in different combinations of salinity and total ammonia nitrogen (TAN) concentrations. (n = 6 for each salinity*TAN*species).

2.3.3 Growth and survival in aquaculture effluent and *in situ*

Growth rates of *C. coelothrix* and *C. indica* were very high when cultured directly in aquaculture effluent (~44% day^{-1}) under controlled conditions (Figure 2.4a). Both *C. coelothrix* and *C. indica* grew significantly faster than *Ulva* (23% day^{-1}) (Tukey's HSD, ANOVA, $F_{2,47}$ = 71.191, P < 0.001). However, survival of generated fragments varied

markedly between all three green tide species, from 100% for *C. coelothrix*, to 40% for *Ulva*, and to a low 26.6% for *C. indica* (χ^2 = 39.3, DF = 2, P < 0.001) (Figure 2.4b).

Growth was substantially lower for the *in situ* trial than any of the controlled condition experiments. *In situ* growth rates of *C. coelothrix* (6.6 ± 2.1% day $^{-1}$ for 0.1 g, 4.9 ± 1.9% day $^{-1}$ for 0.3 g) were much higher than *C. indica* (-2.4 ± 2.5% day $^{-1}$ for 0.1g, -6.7 ± 2.7% day $^{-1}$ for 0.3 g) (Figure 2.5). In fact, positive growth rates for *C. indica* were only recorded in 3 of the 40 samples (a maximum individual growth rate of 7.54% day $^{-1}$), as most of the samples had visibly senesced. Because of the loss of *C. indica* replicates in the majority of experimental blocks (creating an unbalanced design), we could not analyse growth rates using the desired 3-factor mixed model ANOVA. However, the growth of *C. coelothrix* could still be analysed separately, testing the effect of initial size on growth in the 10 blocks around the pond in a 2-factor mixed model ANOVA. There was no effect of initial size on growth of *C. coelothrix* ($F_{1,8}$ = 0.10, P = 0.766; Table 2.3), however, there was a significant effect of block ($F_{8,15}$ = 5.11, P < 0.001). Within-pond (block) variation in growth of *C. coelothrix* resulted in individual growth rates ranging from -8.5 to 13.6 day $^{-1}$.

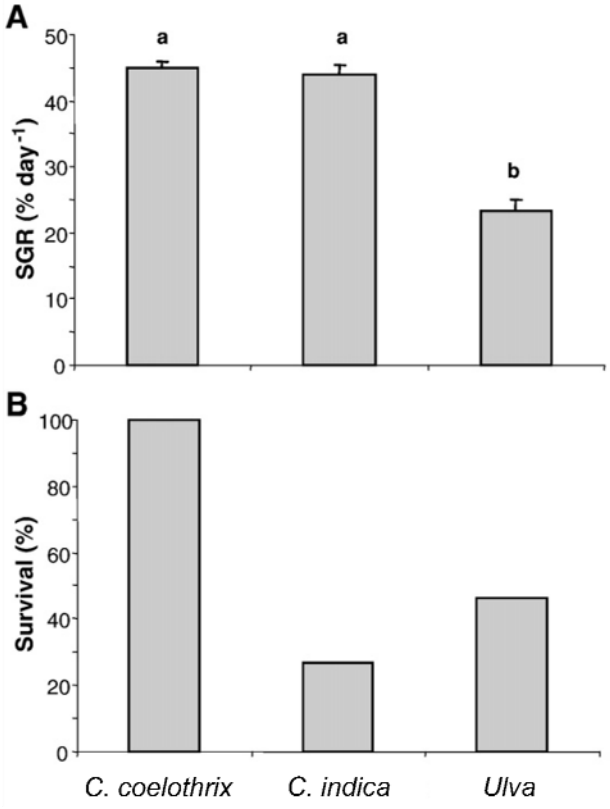

Figure 2.4 Mean specific growth rates (SGR) (+ 1SE) (A) and survival (B) of green tide algae cultured in aquaculture effluent under laboratory conditions. (*C. coelothrix* n = 30; *C. indica* n = 8; *Ulva* sp. n = 14). Different letters denote significant difference α = 0.05.

2.3.4 Bioremediation potential

Estimates of natural carrying capacity based on the average density of floating mats of algae, measured as kilograms of fresh weight per square metre (kg FW m^{-2}), varied between the monsoonal wet season (1.5 ± 0.816 kg FW m^{-2} 1SD) and dry season (1 ± 0.786 kg FW m^{-2} 1SD). This equates to seasonal pond (1 ha) carrying capacities of 15000 kg (wet season) and 10000 kg (dry season), and represents a conservative value as some monsoonal samples contained more than 3 kg FW m^{-2} (giving a maximum potential capacity of 30000 kg). Because the majority of samples across both seasons (>90%) were solely *C. coelothrix*, growth rates for this species were used to calculate potential nitrogen removal rates. Data used for logistic regression (below) are the approximate mean (7% day^{-1}) and upper (15%

day^{-1}) growth rates of *C. coelothrix* from the *in situ* experiments (Section 3.3) and a mean nitrogen content of green tide algae sampled from the pond of 0.58% FW (± 0.139 1SD) calculated from 20 samples (mean ±SD: % N dry weight = 2.90 ± 0.97, C:N = 9.18 ± 3.17).

Table 2.2 Output for the 3-factor ANOVA testing the interactive effect of salinity and TAN (total ammonia nitrogen) on growth of the three species of green tide algae.

Source	**df**	**MS**	**F**	***P***
Species	2	430.06	22.71	**<0.001**
Salinity	2	31.85	1.68	0.189
TAN	4	154.51	8.16	**<0.001**
Species*Salinity	4	201.09	10.62	**<0.001**
Species*TAN	8	86.93	4.59	**<0.001**
Salinity*TAN	8	74.61	3.94	**<0.001**
Species*Salinity*TAN	16	51.19	2.70	**<0.001**
Error	152	18.93		

The calculation of nitrogen removal uses the basic logistic equation: Biomass (B) $_{t+1}$ = B_t + r * B_t (($K - B_t$)/K), and assumes a constant growth rate (r = 15 %, maximum for any block) and maximum carrying capacity (K = 15000 kg in monsoon). Growth is maximal over the linear portion of the harvestable biomass curve (5500 – 9500 kg) and harvest is calculated for a 7-day cycle (Figure 2.6a). These parameters were selected on the basis of maximal growth within the linear range of the curve and an appropriate management regime. This then equates to an approximate 4-tonne harvest from a standing stock (day 0) of 5500 kg in the pond after seven days (Figure 2.6a) with nitrogen removal of 23 kg N harvest^{-1} (equating to 3.3 kg N day $^{-1}$ ha $^{-1}$).

Changes in growth rates will alter the amount that can be harvested. For example, sub-optimal growth at 7% results in a 15 day 4-tonne harvesting cycle (Figure 2.6a). Furthermore, differences in the natural carrying capacity between seasons will also affect yield with decreased biomass and subsequent nitrogen removal (Figure 2.6b). Each model illustrates the best case scenario based on optimum standing stock relating to growth, and limiting the frequency of harvest (monsoonal summer = 5500 kg, dry season = 4500 kg). This model also allows the addition of data from relevant growth experiments under controlled conditions to assess potential variation in productivity and nitrogen removal.

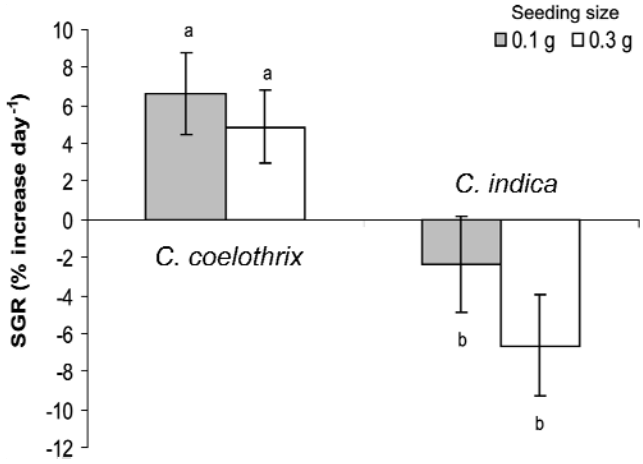

Figure 2.5 Mean specific growth rates (SGR) of *C. coelothrix* and *C. indica* at two different seeding sizes *in situ* in the bioremediation pond. (*C. coelothrix* 0.1 g samples n = 15 and 0.3 g samples n = 15; *C. indica* 0.1 g samples n = 9 and 0.3 g samples n = 6). Different letters denote significant difference $\alpha = 0.05$.

2.4 Discussion

The filamentous green tide algae trialled have excellent potential for implementation in tropical integrated aquaculture systems. This is based on their ability to survive, and grow, under the conditions that characterize tropical pond-based aquaculture. Growth of *C. coelothrix* in particular proved to be high *in situ*. Moreover, high growth rates and tolerance to variation in salinity and TAN in the controlled environment demonstrate its potential for

broader application. This study was one of the first to consider the potential role of what are essentially fouling organisms, or green tide species, in integrated pond-based aquaculture. These forms of algae have been largely overlooked in terms of their application in bioremediation, and this is understandable given that their economic value is unclear. However, there are economic drivers which can be applied to these species, in particular where legislative control of water quality affects the productivity of aquaculture farms within limited infrastructure. This is the case for the aquaculture industry in Northern Queensland, Australia, where aquaculture adjacent to the World Heritage listed Great Barrier Reef Marine National Park is regulated in terms of the nitrogen load within discharge waters (maximum total nitrogen of 3 mg l^{-1}, equating to 210 µmol l^{-1}), and the total amount of nitrogen discharged per day (1 kg ha^{-1} day^{-1}) (DERM 2011). Removal of nitrogen from aquaculture effluent prior to discharge provides the opportunity to increase feed inputs, and therefore productivity of fixed infrastructure. The ability to ameliorate nitrogen through a farm management plan incorporating algal removal through controlled harvest can thus have tangible economic benefits. Furthermore, the harvest algae have potential commercial uses ranging from fertilisers to aquaculture feeds.

Table 2.3 Output for the 2-factor mixed model ANOVA testing the effect of initial size on growth of *C. coelothrix* at different blocks (sites) around an operational bioremediation pond.

Source	**df**	**MS**	**F**	***P***
Initial Size	1	2.15	0.10	0.766
Block	8	111.59	5.11	< **0.001**
Size * Block	8	22.58	1.04	0.453
Error	15	21.82		

However, any algae that can be incorporated into a management plan for aquaculture must be suitably resilient to produce efficient and reliable removal of nitrogen waste. A broad environmental tolerance was observed for the studied green tide species, yet each had an optimum salinity (*C. coelothrix* 30‰, *C. indica* 20‰ and *Ulva* 15‰) that could serve to aid bioremediation in fluctuating environments, in particular if they are utilised as a species consortium. Notably, tropical aquaculture species (crustaceans and fish) are normally cultured between 25 and 40‰, where all three algal species had growth rates over 10% day^{-1}. Under climatic extremes the salinity of ponds can fluctuate from 5‰ to 45‰ in response to rainfall (wet season) or evaporation (dry season), and again these conditions are within the spectrum for growth, albeit sub-optimal. Similar high tolerance has been documented for *Cladophora*, *Chaetomorpha* and *Ulva* species in salinities up to 50‰ in other regions, supporting their broad utility (Birch et al. 1981, Lavery & McComb 1991).

Given the highly variable nature of tropical pond-based aquaculture systems the interaction between the key environmental parameters of nitrogen and salinity will affect productivity. The results presented in this study and those for other resilient species (Taylor et al. 2001) confirm that a diversity of factors affect growth. However, the transfer of these results into operational systems is the key to species selection. The performance of *C. indica* in this study highlights the difficulty of transferring controlled environment outcomes to operational farm systems. *C. indica* has excellent growth under controlled conditions approximating the temperature (28°C ± 2.0°C), nitrogen load (130 µmol l^{-1}), and salinity (36‰) of the operational pond, but failed to grow (essentially died) under short-term *in situ* trials. In contrast, *C. coelothrix* performed well under these same conditions and this may be due to its regenerative capabilities upon fragmentation. While there was variable growth of *C. coelothrix* across locations (-8.5 to 13.6% day^{-1}) within the pond due to variation in water quality, the survival of *C. coelothrix* upon fragmentation was high in all trials (100%). This is

in contrast to the *C. indica*, the other uniseriate filamentous algae, for which only 25% of fragments regenerated. Survival and growth were considered in tandem for the *in situ* growth trial, a factor that influenced the highly negative growth of *C. indica*. However, given the high growth rate of *C. indica* in all controlled experiments, further research to develop fragmentation methods to improve survival is warranted.

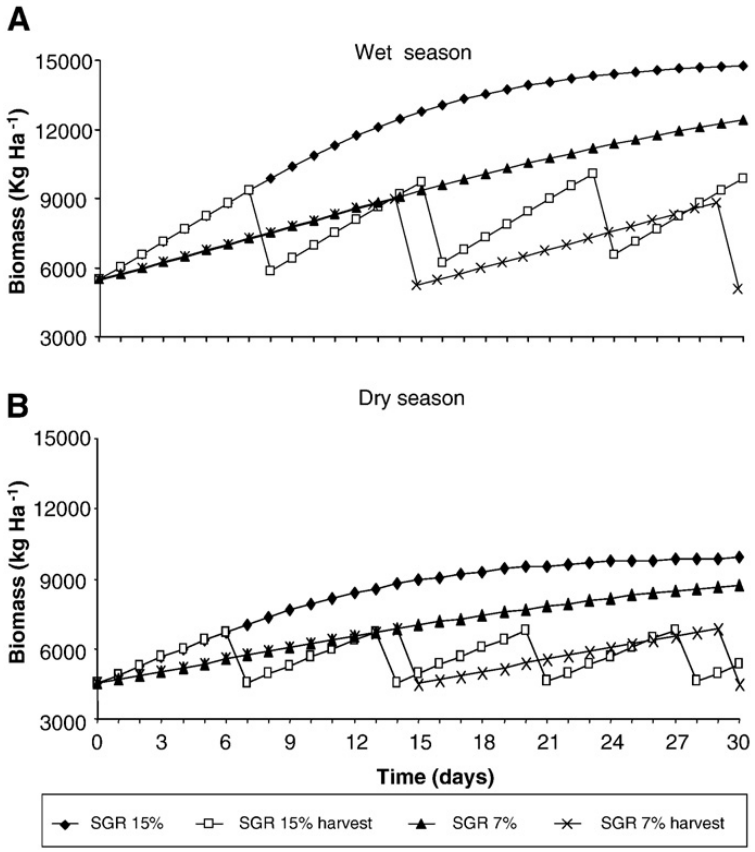

Figure 2.6 Logistic growth of green tide algae in monsoonal wet season (A) and dry season (B) at high (15% day^{-1}) and average (7% day^{-1}) growth rates. Models include a 1-tonne fresh weight (FW) harvesting component. Optimum stocking density (5500 kg FW wet season, 4500 kg dry season) uses the linear portion of the curves with the minimum number of harvests per unit time.

One of the traits that bioremediation species often have is high levels of internal nitrogen (this may be organic but also inorganic forms that are stored in vacuoles) (Harrison and Hurd 2001). Given that bioremediation potential is a function of growth and nitrogen assimilation,

C. coelothrix is an excellent candidate. The mean tissue nitrogen level of 2.9% (of dry weight) is similar to that of the bioremediation species *Ulva rotundata*, 2.9%, *Enteromorpha intestinalis*, 3.2%, and *Gracilaria gracilis*, 3.4% (Hernandez et al. 2002). However, *C. indica* also offers potential given its high growth rate at very high nitrogen concentrations. Under this scenario the potential also exists for higher (luxury) uptake similar to that of *Porphyra amplissima*, with high growth and nitrogen content when cultured in a high nitrogen environment (Carmona et al. 2006).

In summary we have demonstrated that green tide algae can be cultured in ponds with high productivity and nitrogen assimilation. The optimisation of growth under controlled conditions can be managed in an aquaculture context, and data can be used for the development of models for nitrogen stripping. These can be tailored to suit the activities of aquaculture facilities that typically work in cycles relating to maintenance and grow-out of stock. Providing functional growth-bioremediation models to aquaculture facilities will be critical in developing the integration of algal bioremediation in the tropics with mesocosm-like ponds, and these models can be developed to incorporate new components for more complex environmental scenarios.

The inability of traditionally high value genera such as *Gracilaria*, *Palmaria* and more recently targeted high yield species such as *Asparagopsis armata* (Matos et al. 2006, Schuenhoff et al. 2006) to survive the broad environmental fluctuations of tropical pond-based systems provides an impetus for the selection of alternative bioremediation species. The “natural” selection and development of algae endemic to tropical pond-based mesocosms is effective, in this instance, in delivering bioremediation species. The high productivity and nitrogen removal of *Cladophora coelothrix,* allied with a broad tolerance to the tropical pond-based environment, supports the integration of filamentous green tide algae into tropical pond-based aquaculture to promote sustainability.

Chapter 3: Seasonal growth dynamics and resilience of the green tide alga *Cladophora coelothrix* in high-nutrient tropical aquaculture[2]

3.1 Introduction

High levels of inorganic nutrients (nitrogen and phosphorus) are present in many anthropogenically impacted environments, including intensive aquaculture (Sarà 2007, Lin & Fong 2008, Abreu et al. 2009). Arguably the most effective method to remove dissolved inorganic nutrients from aquatic environments is through the integrated culture of algal biomass, in particular for point-source discharge from land-based aquaculture (Crab et al. 2007, reviewed by Troell 2009). However, a major constraint on integrated aquaculture is the selection of robust algal species which are effective across seasonal fluctuations in the environment and are also responsive to aquaculture operational cycles, that is, species that keep functioning through both peak and off-peak production (Neori et al. 2004).

Green tide algae are typically coastal or estuarine species which form opportunistic blooms under eutrophic conditions, sometimes at staggering scales, (Lavery & McComb 1991, Raven & Taylor 2003, Pang et al. 2010). In the past, green tide algae have been considered pest species in the targeted culture of high-value seaweeds (Fletcher 1995, Paul & de Nys 2008). More recently, green tide algae have instead been targeted to treat aquaculture effluents because of their high environmental tolerance (de Paula Silva et al. 2008). These are primarily rapid growth rates, an ability to use multiple sources of nitrogen and a broad environmental tolerance (Pedersen & Borum 1997, Taylor et al. 2001). This recent emphasis on high productivity biomass for integrated aquaculture is warranted given a new focus on

[2] **de Paula Silva PH, Paul NA, de Nys R (2012) Seasonal growth dynamics and resilience of the green tide alga *Cladophora coelothrix* in high-nutrient tropical aquaculture. Aquaculture Environmental Interactions 2:253-266.**

algal biomass for diverse and innovative bioenergy applications, such as biocrude (Zhou et al. 2010) and biochar (Bird et al. 2011, 2012). It is unclear, however, how characteristics unique to tropical land-based aquaculture, in particular monsoonal fluctuations in temperature and salinity with high nitrogen concentrations present in multiple sources (ammonium and nitrate), will influence year-round productivity of green tide algae. In natural settings, complex interactions exist between environmental factors and nitrogen, leading to dynamic growth responses of species which can explain patterns of abundance (Lotze & Worm 2002). For example, *Ulva* (*Enteromorpha) intestinalis* is more resilient in low salinity (15‰) when nitrogen concentration is high (>150 µM of nitrate-N), rather than low (<19 µM, Kamer & Fong 2001). Nitrogen source is also a driver of productivity, as ammonium can inhibit nitrate uptake even at low concentrations (Raven et al. 1992, Neori 1996). These examples demonstrate the need to understand species-specific responses to interactions between key factors under environmental conditions relevant to land-based aquaculture in order to optimise algal productivity and bioremediation and produce a reliable supply of biomass.

This study, therefore, quantified the productivity, resilience and, by extension, the bioremediation potential of *Cladophora coelothrix* in an operational settlement pond receiving effluents from intensive finfish aquaculture. I evaluated growth of this green tide alga at multiple scales, with a 4 month *in situ* experiment, a controlled environment experiment in the laboratory, and a flow-through outdoor mesocosm experiment. This combination of *in situ* and controlled environment data provided a comprehensive assessment of the factors which drives productivity of a green tide alga in an integrated aquaculture system. The *in situ* data provided a correlative assessment of algal productivity relative to marked changes in environmental conditions across a 4 month period. I subsequently simulated annual variation in environmental variables (temperature, salinity and nutrients) relevant to this tropical land-based aquaculture system in order to test the growth and

resilience of *Cladophora coelothrix* in two controlled experiments. Classification and regression tree analyses were used for interpretation of these complex data sets, providing comparisons of the relative importance of environmental variables for the growth of this green tide alga.

3.2 Materials and Methods

3.2.1 Environmental parameters monitoring *in situ*

This study was conducted at Good Fortune Bay Fisheries Ltd., an intensive saltwater barramundi (*Lates calcarifer* Bloch) farm located at Bowen, Northern Queensland, Australia (Latitude 20.02°S, Longitude 148.22°E). This site is located in the dry tropics and is characterized by a wet season (during austral summer) followed by a dry season (austral winter). The primary bioremediation area at the farm consists of a 5 ha settlement pond receiving the effluent water from intensive finfish cultivation. Seasonal and spatial variations in the physicochemical properties of the effluent water in the settlement pond were determined by water quality measurements taken fortnightly from May 2008 to April 2009 (Table 3.1). Temperature (°C) and salinity (‰) were measured using a multi-parameter probe (Oakton model PCD 650). Samples were consistently made at 8 fixed collection points around the settlement pond, ~3 m from the edge and at a depth of ~30 cm (Site 1 closest to the inlet and Site 8 closest to the outlet). Nitrogen was measured from water samples taken at the same 8 sites. These samples were filtered through cellulose membrane filters (Sartorious, Minisart 0.45 µm) and placed on ice for transportation to the laboratory, where they were stored frozen. Nitrogen analysis was done with a Hach colorimeter (model DR/890) following the prescribed methods: total ammonia nitrogen (TAN) was analyzed using the salicylate method; nitrate and nitrite (NO_x^-) were analyzed simultaneously using a cadmium reduction technique. However, since nitrite is rapidly oxidized to nitrate, the nitrogen

enrichments used in the controlled experiments were nitrate (NO_3^--N) and/or ammonium (TAN). The farm is located at a latitude 20.02°S in the tropics, and has a yearly average of light availability of 1000 µmols photons m^{-2} s^{-1} (calculated from the global solar exposure from the Australian Bureau of Meteorology).

3.2.2 Algal biomass collection

The green tide alga *Cladophora coelothrix* Kützing (and hereafter in this chapter *C. coelothrix*) has a broad geographic range, from temperate to tropical climates, predominantly in Europe and Australia (Guiry & Guiry 2012). It forms a large bloom in the settlement pond of Good Fortune Bay Fisheries Ltd. that is most pronounced in the second half of the year from June through to December. *C. coelothrix* was sampled in May 2009 from the dense floating mats in the pond. Samples were collected by hand and restocked in experimental plots (experiment 1, below) or placed in seawater with aeration for controlled environment experiments (experiments 2 and 3). Isolated cultures were maintained for two months before the experimental trials in outdoor aquaria on a recirculating system (~28ºC, 36‰) at the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University, Townsville, Australia.

Table 3.1 Mean values (± 1 SD, n = 8) of environmental variables (temperature and salinity) and concentrations of total inorganic nitrogen, total ammonia nitrogen (TAN) and NO_X^- (NO_2^- + NO_3^-) at the study site from May 2008 to April 2009. Note: settlement pond was dry throughout October 2008.

	Months / 2008							**Months / 2009**			
Variables	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**
Temperature (°C)	25.3 ± 0.3	23.5 ± 0.4	25.8 ± 0.3	24.6 ± 0.3	28.3 ± 0.2	33.9 ± 0.3	31.3 ± 0.6	28.5 ± 0.3	29.4 ± 0.5	29.2 ± 0.5	27.1 ± 0.2
Salinity (‰)	31.7 ± 0.2	32.1 ± 0.6	31.3 ± 0.3	33.5 ± 0.4	40.5 ± 0.3	40.2 ± 0.6	37.4 ± 0.4	30.4 ± 0.6	17.7 ± 0.6	20.2 ± 0.6	31.6 ± 0.4
Total Nitrogen (µM)	47.9 ± 12	37.1 ± 14	55.7 ± 15	70.7 ± 10	67.9 ± 15	87.1 ± 17	80.7 ± 10	95.7 ± 12	81.4 ± 15	82.1 ± 12	76.4 ± 10
NO_x^--N (µM)	32.1 ± 10	26.4 ± 07	36.4 ± 11	47.9 ± 12	39.3 ± 10	50.7 ± 10	43.6 ± 10	51.4 ± 10	53.4 ± 12	50.7 ± 13	43.6 ± 08
TAN (µM)	15.7 ± 05	10.7 ± 10	19.3 ± 08	22.9 ± 15	18.6 ± 18	36.4 ± 13	37.1 ± 12	43.5 ± 22	30.0 ± 15	31.4 ± 11	32.9 ± 12
NO_x^--N:TAN	2.0:1	2.5:1	1.9:1	2.1:1	2.1:1	1.4:1	1.2:1	1.2:1	1.8:1	1.6:1	1.3:1

3.2.3 Experiment 1: *In situ* biomass productivity

Productivity of *C. coelothrix* was measured for 4 months, from June to September 2008 over the winter season, at 8 positions around the settlement pond. This culture period provided marked variation in environmental conditions relative to annual variation (see Table 3.1). Three experimental plots containing different densities were located at each of the 8 sites. The densities used in this experiment (0.25, 0.5 and 1 kg m^{-2} fresh weight), were based on the growth/harvest model in de Paula Silva et al. (2008). The culture units (1 m^2, 15 cm deep) were made by attaching plastic oyster trays (XL6 Aquatray, Tooltech Pty LTD) to PVC pipe for flotation, which were then anchored by pickets to the periphery of the pond. Culture units were stocked with freshly collected samples, after excess water had been removed using a hand-operated salad spinner. No aeration or other agitation was provided to the cultures.

Productivity was analyzed for a 14 day growth cycle at the start of each month, from June to September 2009. Biomass was completely harvested from each culture unit, spun-dry and weighed using a digital scale (Adam APFL 4000). Sub-samples of each unit were oven dried for 48 h at 60°C to determine the fresh to dry weight ratio (on average FW:DW = 3.5). Productivity (g DW m^{-2} day^{-1}) was then calculated using equation 1:

$$P = \{[(Wf - Wi)/FW{:}DW]/A\}/T \quad (1)$$

where *Wi* is the initial biomass, *Wf* is the final biomass, FW:DW is the fresh to dry weight ratio (3.5), A is the area of culture unit (1 m^2) and T the number of days in culture (14 days).

3.2.4 Experiment 2: Interactive effects of temperature, salinity and nitrogen (concentration and source) on growth

The values for temperature, salinity and nitrogen (concentration and source) for the controlled environment experiments were derived from the *in situ* annual survey of the

settlement pond (see Table 3.1). The potential interactions between these factors were evaluated using a factorial design, where the nitrogen sources (NO_3^--N or TAN) were added as single source to the culture medium at 4 relevant nitrogen concentrations: low (35 µM), average (70 µM), high (105 µM) and very high (210 µM), with the latter included as the maximum permitted discharge concentration in Australia (equivalent to 3 mg l^{-1}, Department of Environment and Resource Management, Australia, 2011). The salinity levels selected represented the annual average of 32‰, as well as the extreme values in the wet season (17‰) and dry season (40‰) (Table 3.1). Three consecutive 10 day experiments (1 day apart) were run at 24, 28 and 33ºC using a controlled environment test chamber (Sanyo model MLR 351), reflecting the average (28ºC) and seasonal extremes in water temperature (see Table 3.1).

Culture media were prepared using a base of sterile seawater (32‰) with low inorganic nutrients concentration (NO_x^--N 3.5 µM; TAN 0.0 µM; PO_4^- 0.65 µM). Salinities lower than 32‰ were diluted with distilled water and the background nitrogen and phosphorus levels were restored to previous levels. Artificial sea salts without nitrogen (Aquasonic) were added to increase salinity. Culture media (f/2, Guillard & Ryther 1962) were prepared without nitrogen. Ammonium (NH_4Cl) or nitrate (KNO_3) was then added to respective treatments to give nitrogen concentrations of 35, 70, 105 and 210 µM (equating to approximately 0.5, 1, 1.5 and 3 mg l^{-1}).

Prior to experiments the algal samples were rinsed and cleaned to remove any surface contaminants and epiphytes, and maintained for 5 days at 28°C with 150 µmol photons m^{-2} s^{-1}, 12L:12D photoperiod, in seawater (36‰) with f/2 medium renewed daily. Algae were kept in batch culture to expose them to the same nutrient concentration prior to experiments. Samples of ~100 mg fresh weight (FW) were taken from the stock culture and placed into culture vessels (120 ml clear plastic, Techno Plas) containing 100 ml of growth media (n = 3

for each salinity*nitrogen concentration*nitrogen source). Culture vessels were randomly allocated a position within the environmental test chamber, with a controlled light intensity of 150 µmol photons m^{-2} s^{-1}, photoperiod of 14L:10D, and constant temperature (24, 28 or 33ºC, in consecutive experiments). Samples were repositioned daily to limit any potential artefacts from light variation within the chamber. Culture media in all vessels was replaced at day 5 with a total growth period of 10 days. Algal biomass was measured at the beginning and after 10 days, and growth was calculated as daily growth rates (% day^{-1}) using equation (2):

$$DGR = [(Wf / Wi)^{(1/T)}-1]*100 \quad (2)$$

where *Wi* = initial fresh weight, *Wf* = final fresh weight and T = days.

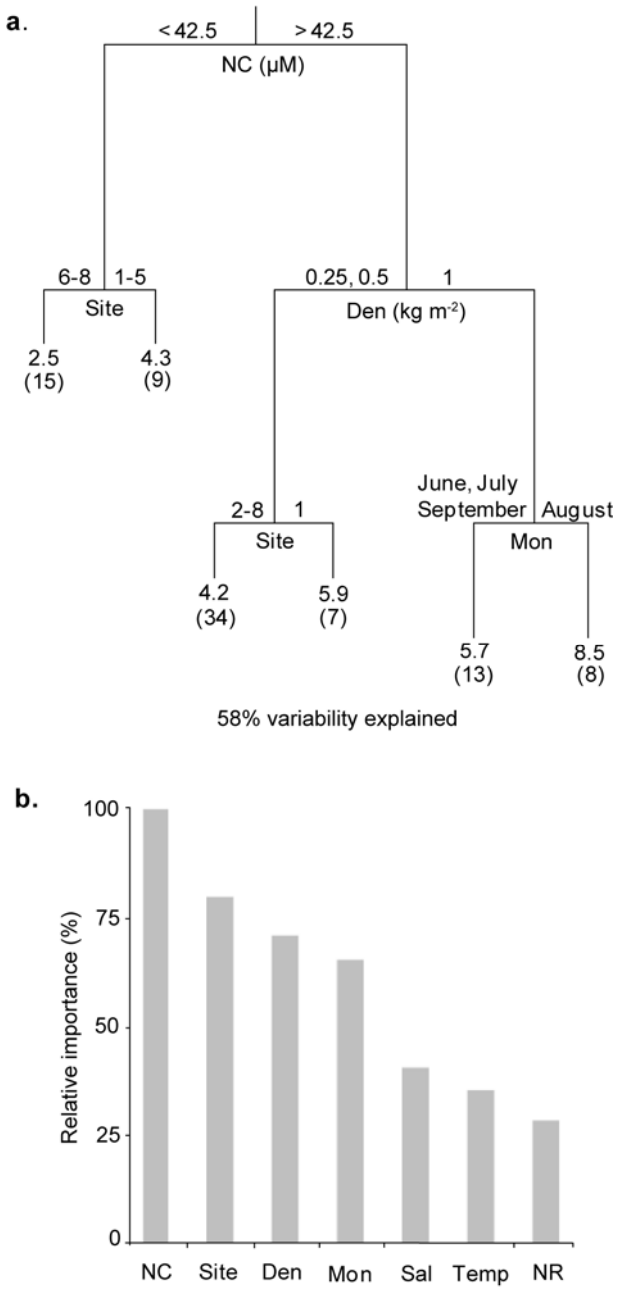

Figure 3.1 a) Univariate classification and regression tree (CART) explaining 58% of the variability in the model. The model is based on the productivity in different months (Mon), positions within the pond (Site) and stocking densities (Den), under different environmental conditions of temperature (Temp), salinity (Sal), nitrogen concentrations (NC) and nitrogen sources ratios (NR). Numbers below terminal nodes are the mean productivity for the specific interaction of explanatory factors, and numbers in brackets indicate the number of cases. b) Relative importance of all explanatory factors for *C. coelothrix* productivity *in situ*.

3.2.5 Experiment 3: Interactive effects of single and multiple nitrogen sources with salinity on growth

Both ammonium and nitrate were available to varying degrees within the pond and throughout the year (see Table 3.1). The effect of different ratios of nitrogen sources on growth is potentially important but could not be effectively evaluated at the laboratory scale. To test the effect of different nitrogen ratios (NO_3^--N:TAN) on growth I instead used an outdoor flow-through system to simulate the conditions in the settlement pond by maintaining specific nitrogen ratios throughout the experiment. The experimental design comprised of 2 single source nitrogen treatments, NO_3^--N or TAN (1:0 and 0:1), and 3 multiple nitrogen sources in different NO_3^--N:TAN ratios (3:1, 1:1 and 1:3). All treatments had nitrogen concentration over saturation levels (105 µM) according to experiment 2 (see Results). Two salinities (17 and 32‰) were used to represent the wet and dry seasons from the seasonal survey (n = 3 for each nitrogen ratio*salinity).

The flow-through system consisted of 10 raised 70 l sumps, supplying a constant gravity feed of 1 V (volume) h^{-1} to 220 ml culture vessels (clear plastic, Techno Plas) containing an initial mass of 100 mg FW. Sumps were refilled twice a day to maintain flow rates. Media for each treatment were prepared as for experiment 2. Dechlorinated tap water (NO_x^--N 7 µM; TAN 0.0 µM; PO_4^- 0.65 µM) was used to prepare 17‰ culture media, and background levels of phosphate and nitrate were restored. Stocks of NH_4Cl and KNO_3 were used to make both single and multiple nitrogen sources treatments and fresh nutrient media were prepared daily. Culture vessels were repositioned daily to avoid any potential effects of variable flow rates, temperature and light in the experiment. Stock biomass was acclimated in batch culture for 5 days prior to the experiment, as before. Daily growth rates (DGR) were calculated over 8 days of culture, according to equation (2).

Temperature and light intensity were measured daily. Temperature (minima and maxima) was measured using 4 thermometers spaced throughout the culture area. Light intensity was measured hourly from 0700 to 1800 on the first day (using a LiCor Li 250 meter), and then subsequently at 0700, 1200 and 1800 on remaining days. Average (1 ± SD) daily temperature and surface irradiances (PAR) over the course of the experiment were 26 ± 5ºC and 981 ± 192 µmol photons m^{-2} s^{-1}, respectively. Salinity was also monitored daily using an Oakton P650 multi-parameter probe.

3.2.6 Statistical analyses

Classification and regression trees (CART) were used to analyse the data from the *in situ* and controlled environment experiments (TreesPlus 2000). I ran univariate CART analyses to explain variation in a single response variable (productivity in experiment 1 and DGR in experiments 2 and 3) by multiple explanatory variables. Experiment 1 had a combination of variables that were categorical (site, month and density) or continuous (total nitrogen concentration, temperature, salinity and nitrogen ratio). Experiments 2 and 3 were comprised only of categorical variables (temperature, salinity, nitrogen concentration and source, and, salinity and nitrogen ratios, respectively). CARTs explain variation using a stepwise process which splits explanatory variables in a pairwise manner to create homogenous clusters of data (De'ath & Fabricius 2000). The branch splits minimize the sums of squares (SS) within groups, which is equivalent to a least-squares linear model. Pruning of the final tree was made by 10-fold cross-validation to develop a simple predictive model (De'ath & Fabricius 2000). In our analyses, fifty 10-fold cross validations were performed and the “best" tree (minimum estimated cross-validated (CV) error) was selected as the final model for each experiment (see Figures 3.1a, 3.3a & 3.5a). These analyses identified the relative importance of explanatory variables involved in complex interactions for each model (see Figures 3.1b, 3.3b & 3.5b).

Missing values where culture units were lost during experiment 1 were omitted from the CART analysis and the surrogate function in TreePlus was disabled. The data sets for each experiment were plotted in two ways, firstly as the tree from the CART analysis (mean values and number of replicates are listed below each of the terminal nodes) and, secondly, as the means for relevant main effects for each experiment (see Figures 3.2, 3.4 & 3.6).

3.3 Results

3.3.1 Environmental parameters monitoring *in situ*

There were large temporal variations in both temperature and salinity in the settlement pond (Table 3.1). Annual averages of temperature and salinity were 28ºC and 32‰, respectively. In the wet season (during austral summer, November to March) temperature was high and salinity low and the extremes of these variables were 33ºC and 17‰, respectively (Table 3.1). Conversely, in the dry season (austral winter, June to September) temperature was lower and salinity higher, with extremes values at 24ºC and 40‰, respectively (Table 3.1). Both nitrogen concentration and source varied throughout the year and between sites within the settlement pond. The annual average for total inorganic (NO_x^--N + TAN) nitrogen concentration was 70.3 µM (43.2 µM NO_x^--N and 27.1 µM TAN Table 3.1). The highest mean nitrogen concentration across the 8 sites sampled was recorded in January (austral summer, peak production) at 94.9µM (and 51.4 µM NO_x^--N and 43.5 µM TAN, Table 3.1), while the lowest level of nitrogen recorded was in June (austral winter, off-peak production) at 37.1 µM (26.4 µM NO_x^--N and 10.7 µM TAN, Table 3.1). NO_x^--N was always present at a higher concentration than TAN throughout the year, with NO_x^--N:TAN ratios varying, on average, from 1.2:1 to 2.5:1 (Table 3.1). Individual sites within the pond showed variation from a low of 1.3:1 at site 1 in June through to a high of 14.6:1 at site 8 in August.

3.3.2 Experiment 1: *In situ* biomass productivity

C. coelothrix had sustained productivities *in situ*. Stocking density was the most important split for higher nitrogen concentrations in the CART (>42.5 µM, Figure 3.1a, branches to the right). The stocking density of 1 kg m^{-2} yielded the highest productivities overall, particularly in the month of August (8.5 g DW m^{-2} d^{-1}), whereas lower stocking densities had lower productivities 4.2 and 5.9 g DW m^{-2} d^{-1} (0.25 and 0.5 kg m^{-2}, respectively) except for site 1, which was closest to the inlet point (Figure 3.1a). Productivity in the settlement pond correlated with all of the explanatory variables in the model, however, the relative importance of each factor varied (Figure 3.1b). The CART model (58% of the variability explained) revealed that total nitrogen concentration (NC 100%) was the main driver of productivity and was correspondingly the first branch in the tree, splitting replicates with >42.5 µM from those <42.5 µM (Figure 3.1a). The position within the pond (Site), the stocking density (Den) and the months (Mon) in which they were cultured also influenced productivity *in situ* (79.7, 70.6, 65.2% relative to the nitrogen concentration, respectively, Figure 3.1b). However, the influence of each of these variables depended on nitrogen concentration. For example, "Site" was the most important split for low nitrogen concentrations, grouping sites 1-5 and 6-8 with mean productivities of 4.3 and 2.5 g DW m^{-2} d^{-1}, respectively (Figure 3.1a, branches to the left). Moreover, even though salinity and temperature varied substantially over 4 months (32 to 40‰ and 24 to 28°C from June to September, respectively), these factors had the least influence on productivity (40.5 and 35.1%, respectively), while the ratio NO_x^--N:TAN had the lowest overall relative importance at 28.2% (Figure 3.1b). None of these three variables influenced the splits in the tree.

Nitrogen concentration at the sites followed a clear trend, from highest at the inlet through to lowest at the outlet for each of the months (Figure 3.2a). Overall, nitrogen concentration was lowest in June and increased steadily through to September, however, the largest differences

in nitrogen concentration between months were found at the inlet sites, in particular Site 1 adjacent to the inlet (Figure 3.2a). This differentiation between Site 1 and the remaining sites also corresponded to a split in the tree with a higher productivity at Site 1 (5.9 g DW m^{-2} d^{-1}) compared to Sites 2-8 (4.2 g DW m^{-2} d^{-1}) under high nitrogen with lower stocking densities of 0.25 and 0.5 kg m^{-2} (Figure 3.1a).

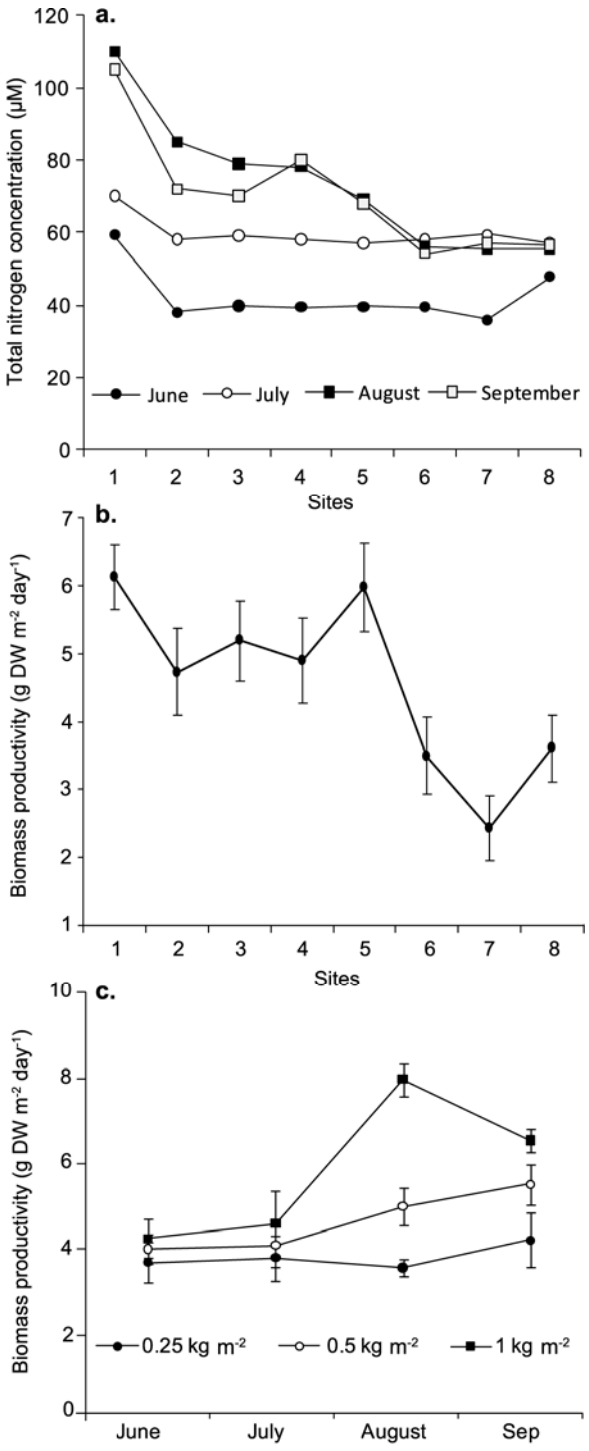

Figure 3.2 a) Total nitrogen concentration for each of the 8 culture sites around the settlement pond for 4 months, from June to September (n = 1 for each site*month). b) Mean productivity (± 1SE) of *C. coelothrix* across 3 stocking densities at the 8 different positions (site) within the settlement pond (n = 12 for each site). Data show mean value of the three stocking densities (0.25, 0.5 and 1 kg m^{-2}). c) Mean productivity (± 1SE) of *C. coelothrix* cultured at the 3 different densities across the months between June and September (n = 3 for each density*month).

In general, the site within the pond influenced productivity in a consistent way over the months. The productivity across all densities for the sites 1-5 was on average 40% higher than productivity at the sites 6-8 (~5.4 ± 0.28 and ~3.2 ± 0.37 g m^{-2} day^{-1}) (Figure 3.2b). Furthermore, average *in situ* productivity across all sites increased between the months of June to September, with a clear effect of stocking density in August and September (Figure 3.2c), corresponding to the same split in the tree above (Figure 3.1a, right side). This effect correlated with an increase in nitrogen concentration over months (Table 3.1). In June and July, when nitrogen was lower (37.1 and 55.7 µM, respectively), there was no obvious difference in productivity across the three densities tested (~3.8 ± 0.19 and ~4.0 ± 0.27 g m^{-2} day^{-1}, respectively, Figure 3.2c). For the months of August and September, in which the nitrogen concentration was higher (~70 µM), productivity was greater for the 2 higher stocking densities tested (Figure 3.2c). Productivity of algal cultures with stocking densities with 1 kg m^{-2} reach its peak productivity in August (8.5 g m^{-2} day^{-1}, see also this mean value in the CART diagram Figure 3.1a), while at lower stocking densities (0.5 kg m^{-2}) the peak productivity was reached in September (Figure 3.2c). No difference was found for cultures stocked with 0.25 kg m^{-2} in all months, indicating that there was no growth limiting factors for the low density treatment (Figure 3.2c).

3.3.3 Experiment 2: Interactive effects of temperature, salinity and nitrogen (concentration and source) on growth

C. coelothrix daily growth rates varied from a maximum of 8.3% day^{-1} at lower temperatures and nitrogen concentrations above 70 µM, irrespective of salinity (Figure 3.3a, right branches), to a minimum of 2.9% day^{-1}, at high temperature, low salinity and nitrogen concentrations ≤105 µM (Figure 3.3a, branches to the left). Overall the CART model explained 76.3% of the variability in the data. In this experiment, temperature was the most important explanatory variable (nominally 100%, Figure 3.3b) and was also the first split in

the tree (Figure 3.3a), splitting the annual average and high temperature treatments from the low temperature treatments. Nitrogen concentration and salinity were subsequently the most important variables for the 28 and 33°C split and the 24°C treatments, respectively (25 and 21%, Figure 3.3b). Nitrogen source had a negligible influence on growth, <1 % (Figure 3.3b).

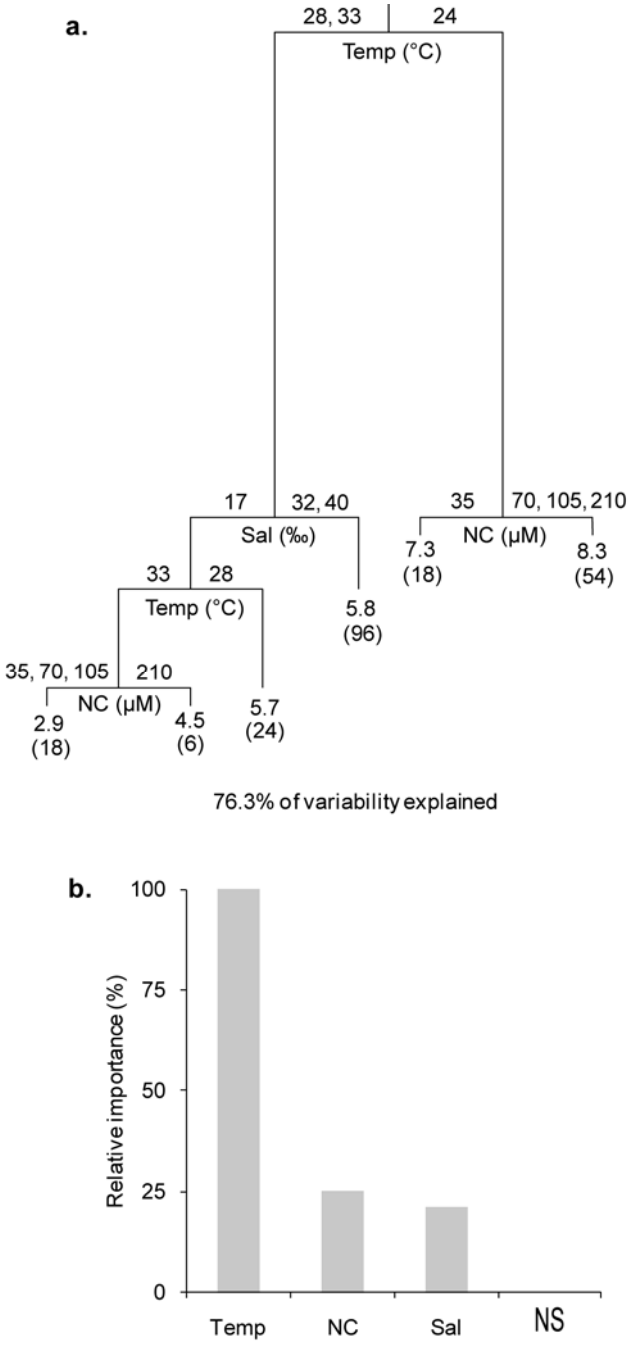

Figure 3.3 a) Univariate classification and regression trees (CART) based on environmental factors in Table 1, explaining 87% of the variation of DGR. Numbers below terminal nodes are the mean DGR for the specific interaction of explanatory factors, and numbers in brackets indicate the number of cases. Explanatory factors used were: temperature (Temp 24, 28 and 33ºC), salinity (Sal 17, 32 and 40‰), nitrogen concentration (NC 35, 70, 105 and 210 µM) and nitrogen source (NS, not represented in any terminal node). b) Relative importance of all explanatory factors for *C. coelothrix* in the laboratory model.

Nitrogen concentration was important for growth of *C. coelothrix* growth at low and mid temperatures (Figure 3.4a 24°C & b 28°C). Concentrations of 70 µM and above (up to 210 µM) promoted higher daily growth rates than a nitrogen concentration of 35 µM, regardless of the salinity or nitrogen source (Figure 3.4a & b). Moreover, growth appeared to saturate with nitrogen concentration at 70 µM (Figure 3.4a & b). There was a marginal interaction between nitrogen concentration and salinity at low temperature (24°C, Figure 3.4a), which corresponds to an analogous branch split in the tree (Figure 3.3a, to the right). This resulted in lower growth at low salinity and low nitrogen concentration (17‰ and 35 µM) compared to low salinity treatments with higher nitrogen concentrations (70, 105 and 210 µM, Figure 3.4a).

Under average to high temperatures (28 and 33°C), the average to high salinity (32 and 40‰) treatments grouped and were higher than any combination of low salinity treatments (branches to left, Figure 3.3a). The largest, distinct combination of explanatory variables in the model related to temperature at 33°C and salinity at 17‰ with a final split between high and low nitrogen concentrations (Figures 3.3a & 3.4c). The low nitrogen concentrations in this split yielded the lowest overall growth rate for any treatment combination at 2.9% day^{-1}. Interestingly for each "extreme" treatment there was a split which yielded reasonable growth rates, for example, low salinity combined with average temperature (28°C) yielded relatively high growth in comparison to high temperature (33°C) (Figure 3.3a). Similarly, where potentially negative combinations of low salinity (17‰) and high temperature (33^0C) existed, higher nitrogen concentrations (210 µM) yielded enhanced growth compared to low nitrogen; the lowest of all treatment combinations (Figure 3.3a, to the left).

At the highest temperature tested (33°C), growth rates were high (>5% day^{-1}) provided that salinity was high (>32‰). This result was consistent irrespective of nitrogen concentration and source (Figure 3.4c). The combination of extreme conditions of high temperature and

low salinity (33°C and 17‰), characteristic of the wet season, had the lowest overall growth rates (<3% day^{-1}, Figure 3.4c). However, these growth rates were comparable to higher salinities if cultured at the highest nitrogen concentration (e.g. 4.8% day^{-1}) with a trend of increasing growth with increasing nitrogen at 17‰ (Figure 3.4c).

3.3.4 Experiment 3: Interactive effects of single and multiple nitrogen sources with salinity on growth

Daily growth rates in the mesocosm experiment were always higher at 32‰ than 17‰, irrespective of the ratio of nitrogen source available (Figure 3.5a).The CART model with these two explanatory variables, salinity and nitrogen ratio, explained 86% of the variation in the data and both variables were important for growth in the CART model tested (100% vs. 85%, Figure 3.5b), with salinity dictating the primary branch split and nitrogen ratio driving all subsequent splits in the model (Figure 3.5a). The interactions between salinity and nitrogen ratio were distinct for each salinity treatment. Under low salinity (17‰), there was a negative influence of 1:3 NO_3^--N: TAN treatment that yielded the lowest overall average productivity. The remaining ratio treatments then partitioned between single source and combinations (1:0 and 0:1 vs. 1:1 and 1:3, Figures 3.5a & 3.6). For higher salinity (32%), there was a single split between single source and multiple nitrogen sources, i.e. irrespective of ratio (Figures 3.5a & 3.6). In general, the single source nitrogen provided the higher productivity for both salinity treatments (16% more in 32‰, and ~22% on average more in 17‰).

Because experiment 3 contained only 2 factors, the tree (Figure 3.5a) almost entirely reflects the trends of the mean values for each of the combination of treatments (Figure 3.6). Overall, daily growth rates were high for most treatments, other than low salinity with single source ammonium (>10% day^{-1}, Figure 3.6), indicating high resilience across salinities and nitrogen

sources. Growth rates were higher (by ~20%) with a single nitrogen source (1:0 and 0:1) than with any combination (3:1, 1:1 and 1:3) of NO_3^--N and TAN (Figure 3.6). High salinity typically yielded higher growth rates than low salinity (32 and 17‰, respectively), similar to experiment 2, regardless of the NO_3^--N:TAN (Figure 3.6). Single sources of nitrogen promoted the highest daily growth rates both at high salinity (15.5 and 14.2% day^{-1} respectively, Figure 3.6) and low salinity (11.9 and 11.5% day^{-1}, respectively). Growth rates were lower when nitrogen was supplied as multiple sources. At salinity 32‰, there was no clear difference between any combinations of nitrogen sources, whereas at the lower salinity (17‰) growth rates were lower in the treatments with NO_3^--N:TAN ratios of 3:1 (Figure 3.6). All of these interpretations are consistent with combinations of salinity and nitrogen ratio from the CART output (Figure 3.5a).

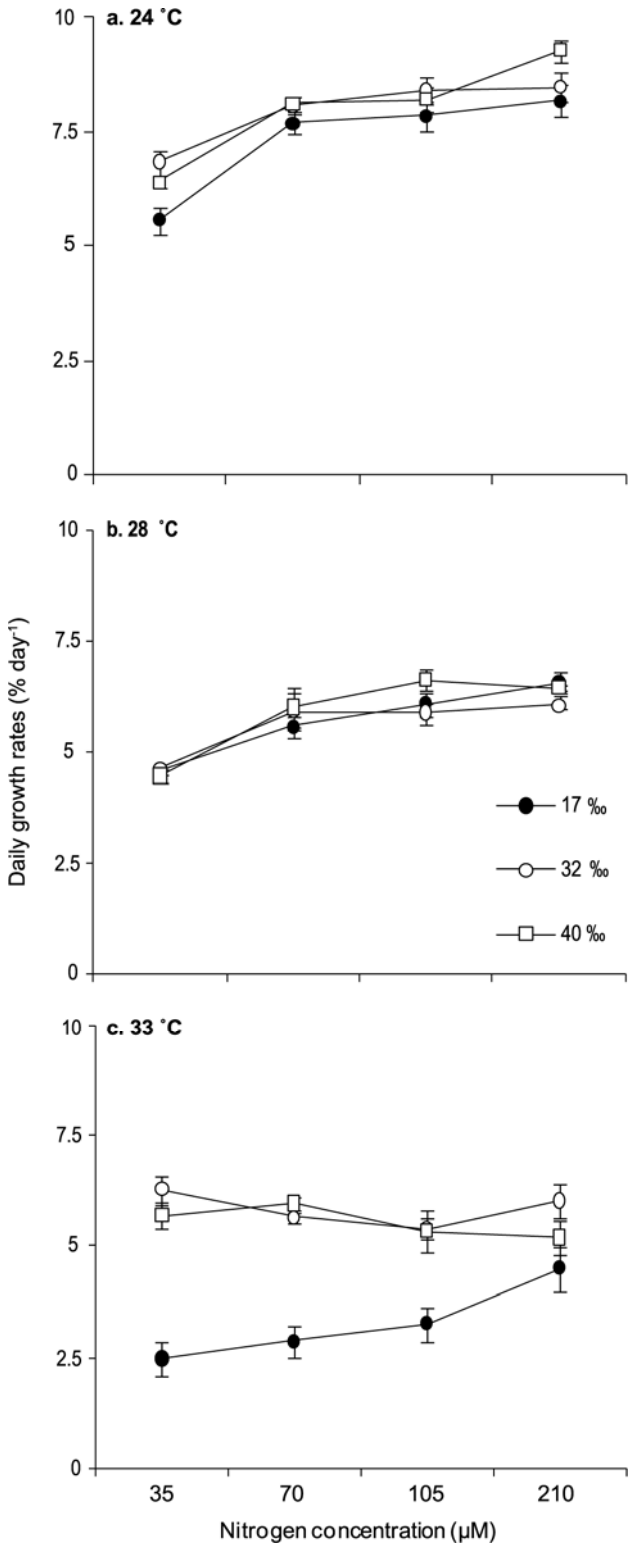

Figure 3.4 Growth of *C. coelothrix* in a combination of salinities (17, 32 and 40‰) and nitrogen concentrations (35, 70, 105 and 210 µM) at 3 different temperatures a) 24^0C, b) 28^0C and c) 33^0C. Data show mean daily growth rates of nitrate or ammonium (± 1SE, n = 6).

3.3.5 Comparison of growth and productivity between experiments

The CART models developed for experiments 1-3 constitute a valuable tool for quantitatively assessing the response of a species to dynamic environmental conditions. We used data derived from experiments at 3 different scales, from *in situ* cultures through to controlled environment experiments. Even though the *in situ* experiment only ran for 4 months, it captured marked variation in conditions in the settlement pond (temperature ~24 to 28^{0}C, salinity ~32 to 40‰ and nitrogen concentration ~35 to 70 µM), as well as highlighting management tools for the system (e.g. position within the pond and stocking densities). The controlled environment model from experiment 2, in contrast, represents the environmental variation of an entire growing season and provides an analysis of the resilience of *C. coelothrix* in conditions relevant to this integrated system.

Each experiment had different conditions and therefore there is no clear mechanism to correlate data across these experiments. For example, the water delivery systems was different (continuous and flow-through for experiment 1 and 3 respectively, and static for experiment 2), and the stocking densities used *in situ* (approximately 6.8, 3.4 and 1.7 g l^{-1}) were higher than that for the laboratory and mesocosm experiments (1 and 0.5 g l^{-1}, respectively). Nevertheless, it is notable that the relative importance of variables from the CART models followed the same trend across the different scales of investigation, with temperature, salinity and nitrogen concentration as the main drivers of growth, albeit with some minor variations. The laboratory model (experiment 2) demonstrated that temperature was clearly the main driver of changes in growth rates throughout the year, followed by nitrogen concentration and salinity. Given that the period of the *in situ* trial corresponded to the lowest temperature and highest salinity, i.e. close to the optimum conditions as predicted from experiment 2, it was therefore both predicted and demonstrated that nitrogen concentration was the major driver of growth in experiment 1. All of the models

demonstrated that the nitrogen source or the ratio between nitrogen sources had the least influence on growth, consistent with a strong relationship between the laboratory, mesocosm and *in situ* models.

3.4 Discussion

One pathway for the selection of algae for biomass applications in integrated aquaculture is to utilise species that already exist and opportunistically bloom in aquaculture facilities, including green tide algae (de Paula Silva et al. 2008, Hernandez et al. 2008). In order for these species to be truly integrated, and become a reliable and commercial source of biomass, it is essential to understand the influence of seasonal changes in environmental conditions on growth, biomass yield and bioremediation potential. Here I demonstrate that the green tide alga *C. coelothrix* is tolerant to the concurrent and extreme seasonal fluctuations in temperature, salinity, nitrogen concentrations and sources that are characteristic of tropical land-based aquaculture. Because these environmental factors typically co-vary, we used 3 different scales of investigation combined with classification and regression tree analyses to partition the complex data sets and determine the relative importance of different factors under different conditions. I found that growth of *C. coelothrix* was sustained in most combinations of environmental factors and nitrogen (concentrations and sources), demonstrating resilience in this high-nutrient system. Furthermore, the CART models revealed that all factors influenced growth but to different extents, with temperature as the main driver of seasonal variation in productivity. Salinity and nitrogen concentration (but not source) were also important, but only in conditions representative of the extremes of seasonal fluctuations. Although nitrogen was not a critical factor compared to large seasonal changes in temperature and salinity, it remained the most crucial factor for growth within a single season for the *in situ* experiment. This study defines growth and resilience of an alga for

integrated aquaculture across 3 different scales of experimentation and provides formal models of these data as a tool for managing biomass yields in changing environments.

Seasonal fluctuations of temperature and salinity are recognized constraints for biomass production and bioremediation through integrated algal culture (Yang et al. 2006, Marinho-Soriano et al. 2009), respectively. This problem has been resolved or managed in some instances using alternation of species with complementary tolerance ranges across seasons (Buschmann et al. 2008, Skriptsova & Miroshnikova 2011). Here I instead selected *C. coelothrix* as it was a pre-adapted and reoccurring bloom-forming species, and found that it had a high environmental tolerance to both extremes of temperature and salinity that corresponded to dry winter and wet summer seasons in the tropics. These tolerances were similar to those reported for *C. coelothrix* in the tropics where growth was maintained in temperatures up to 35ºC (Cambridge et al. 1987) and salinities ranging from 15 to 45‰ (de Paula Silva et al. 2008). However, even if a species has a broad tolerance to any single environmental factor, stress from an additional factor may reduce overall performance (Rivers & Peckol 1995b, Lotze & Worm 2002). Therefore understanding and predicting the interactions between environmental factors is critical for implementing seaweed-based integrated aquaculture, particularly in open systems subject to seasonal changes in conditions (Abreu et al. 2011).

Classification and regression tree (CART) analyses are a practical tool for determining the relative importance of factors that often co-vary in nature (De'ath & Fabricius 2000) and also for partitioning factors involved in complex interactions in manipulative experiments (this study). These results demonstrate that the interaction between environmental factors is the most critical aspect for determining species resilience in integrated aquaculture. Aquaculture in the tropics is characterized by two distinct seasons (wet vs. dry) which also correlate with fish production cycles (peak in the wet vs. off-peak in the dry). This leads to a simultaneous

seasonal extreme of temperature (33°C) and salinity (17‰) in the austral summer, an interaction that produced the lowest overall growth of *C. coelothrix,* similar to other *Cladophora* species (Thomas et al. 1988). However, the environmental stresses relating to temperature and salinity are buffered by high nitrogen concentration (this study, Hernandez et al. 2008), potentially by providing osmotic balance in the low salinities (Kamer & Fong 2001). Alternatively, the dry season extreme (winter) in the tropics yielded conditions that were optimal for growth (<28ºC, >32‰), even at relatively low nitrogen concentrations (35 µM). The CART model based on the controlled environment component predicted that the optimal conditions for *C. coelothrix* are temperature from ~24 to 28°C and salinities from ~32 to 40‰. This was confirmed *in situ* for 4 months (June to September, austral winter), where high productivity was achieved for biomass cultured in the settlement pond and highlights, in this case, the importance of nitrogen concentration as a driver of productivity when temperature and salinity are close to optimal.

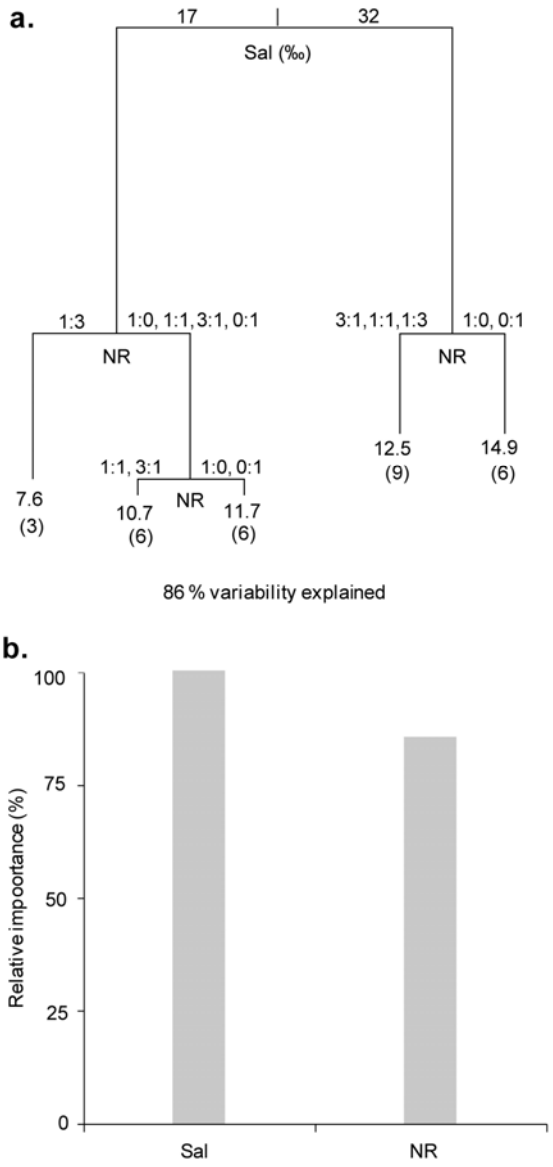

Figure 3.5 a) Univariate classification and regression tree (CART) for DGR in different salinities (Sal 17 and 32‰) and ratios of nitrate-N and TAN (NR 1:0, 3:1, 1:1, 1:3 and 0:1). Numbers below terminal nodes are the mean productivity for the specific interaction of explanatory factors, and numbers in brackets indicate the number of cases. The CART model explained 86% of the variability of *in situ* productivity. b) Relative importance of explanatory factors for *C. coelothrix* growth in the outdoor controlled environment model.

Adequate nitrogen supply is essential to sustain high biomass productivity in seaweed-based integrated culture systems (Neori et al. 1991, Cohen & Neori, 1991, Harrison & Hurd 2001). Nitrogen concentration was the most important factor in the *in situ* experiment, where it doubled in concentration over the 4 months (from June to September), and was also the factor

with the highest spatial variability in the settlement pond. Correspondingly, productivity was higher in August and September than in July and July, as nitrogen concentrations were low in the latter. Within the pond, higher productivity was achieved in sites receiving effluent with higher nitrogen concentration. Similar large effects of nitrogen concentration are seen for other integrated species (Matos et al. 2006, Schuenhoff et al. 2006, Paul & de Nys, 2008), indicating it can commonly be a limiting factor even for integrated systems. However, even when nitrogen is not limiting, it may still have a role in maintaining growth rates under extreme conditions (Dodds and Gudder 1992).

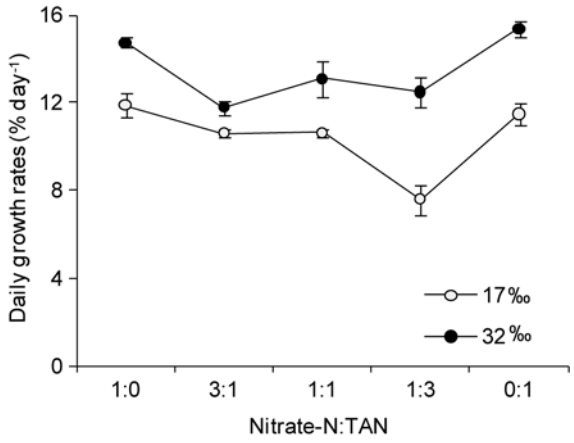

Figure 3.6 Daily growth rates of *C. coelothrix* across different nitrate-N and TAN ratios at 2 salinities (n = 3 for each species*salinity*N-ratios). Data show mean ± 1SE.

Clearly an ability to tolerate high and variable nitrogen concentrations is an advantage in integrated algal culture systems, and this includes tolerance of specific nitrogen sources such as the high ammonium concentrations from fish production (Phillips & Hurd 2003, Neori et al. 2004). However, the CART models show that nitrogen sources (nitrate or ammonium, single source or in combination) had less influence on productivity than total nitrogen concentration. The ratio of nitrate to ammonium *in situ* was highly variable, yet there were no clear preferences for either nitrogen source. In theory, growth with ammonium as a nitrogen

source should be higher than nitrate, because ammonium ions diffuse through the cellular membrane whereas nitrate uptake incurs a cost through enzymatic reactions (Raven et al. 1992). This lack of preference for a nitrogen source is a common feature across a broad taxonomic range of algae (Navarro-Angulo & Robledo 1999, Carmona et al. 2006), and it is likely that *C. coelothrix* can utilize nitrate and ammonium simultaneously (Phillips & Hurd 2003, Cohen & Fong 2004a) or sequentially (Neori 1996). Our manipulative experiments revealed that it was not the preference for nitrate or ammonium that was important, but the co-supply of multiple sources that reduced growth below potential. Therefore, full growth potential is unlikely to be achieved in this treatment pond, or any pond where ammonium has not been oxidised to nitrate. The explanation for this antagonistic result is that ammonium can inhibit nitrate uptake by inactivating or slowing the synthesis of nitrate reductase (Berges et al. 1995, Young et al. 2005), even at low concentrations (<5 μM) (Thomas & Harrison 1987). Enhancing the oxidation of ammonium to nitrate by denitrifying bacteria using biofilters could be an important strategy to complement existing conversions in order to increase the nitrate to total ammonium nitrogen ratio above 1:3, as even this ratio was detrimental in our study. However, this may infer further production costs and a comprehensive economic analysis is necessary. Furthermore, values *in situ* commonly reach 2:1 during times of the year where environmental factors are optimal and therefore algal productivities may be significantly enhanced.

Implementation of large scale effluent bioremediation must be supported by appropriate models suitable for up-scaling to annual productivity and bioremediation (Troell et al. 2003). Here, I used defined experimental designs to create predictive CART models that assess the relative importance of multiple, co-varying environmental variables for algal growth in an integrated system. The controlled environment model captured the seasonal trends in algal productivity over an entire annual cycle and, importantly, the relevant output of this model

was consistent with the *in situ* experiment in the operational settlement pond. The experimental design used in study is similar to studies used for extrapolation to commercial scenarios (Troll et al. 2003). Therefore at the maximum productivity of 8.5 g DW m^{-2} day^{-1}, the system would produce 31 T ha^{-1} $year^{-1}$, which is equivalent to 2.5 t ha^{-1} $month^{-1}$. At this site the integrated algal system using *C. coelothrix* could remove 72.5 kg ha^{-1} of nitrogen per month (at 2.9% nitrogen of dry weight, de Paula Silva et al. 2008). The maximum productivity and bioremediation that can be achieved using this integrated pond system is comparable or higher than others (Msuya et al. 2006, Marinho-Soriano et al. 2009) respectively), however, not as high as intensive algal culture using aeration and high water exchange (Abreu et al. 2011). Therefore, the addition of paddlewheels in the settlement pond for aeration and water flow could provide a means to enhance spatial productivity and could also enhance the conversion of ammonium to nitrate in the water column. However, these energy-intensive strategies would be better placed if there was an additional economic driver for the biomass, for example, the productivity and nitrogen content of the *C. coelothrix* biomass was high enough to promote use as biochar (Bird et al. 2011, 2012) and other green tide algae have demonstrated conversions to biofuels (Zhou et al. 2010). Given the formidable task of operational management of temperature and salinity in large, multiple hectare bioremediation areas, the selection of resilient algal species is fundamental to delivering effective and reliable biomass and bioremediation within integrated tropical land-based aquaculture.

Chapter 4: Enhanced production of green tide algal biomass through additional carbon supply[3]

4.1 Introduction

Macroalgal biomass is an emerging resource for sustainable bioenergy (Ross et al. 2008) and advanced biofuels (Zhou et al. 2010, Wargacki et al. 2012). Bioenergy applications rely on the production of a high volume/low value biomass opening opportunities to develop the culture of new commercial species. Green tide algae have the potential to meet these criteria as they are fast growing species (Raven & Taylor 2003) with a tolerance to a broad range of environmental conditions (Taylor et al. 2001). Furthermore, they are highly suitable as a bioenergy feedstock for ethanol (Yanagisawa et al. 2011), biogas (Bruhn et al. 2011, Migliore et al. 2012) and thermo-chemical conversion to biocrude (Zhou et al. 2010, Zhou et al. 2012). Green tide algae can be cultured extensively in open water culture (Pierri et al. 2006) or harvested from natural blooms (Bird et al. 2012). Alternatively, they can be cultured intensively in land-based ponds and tanks integrated into nutrient-rich aquaculture (de Paula Silva et al. 2008, Neori 2008, Robertson-Andersson et al. 2008) and municipal (Tsagkamilis et al. 2010) waste streams for bioremediation.

Dissolved inorganic carbon (Ci) is usually the limiting factor for growth in intensive cultivation with nutrient-rich systems, as the rate of Ci assimilation by the algae is greater than the rate of CO_2 diffusion from the air into the water, even when vigorously aeration is used (Bidwell & McLachlan 1985). Total Ci in the water is composed of carbon dioxide (CO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), which are part of a buffered system in equilibrium, according to the following equation:

[3]**de Paula Silva PH, Paul NA, de Nys R, Mata L (2013) Enhanced Production of Green Tide Algal Biomass through Additional Carbon Supply. Plos One, DOI: 10.1371/journal.pone.0081164.**

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO^{3-} \leftrightarrow 2H^+ + CO_3^{2-}$$

The relative amounts of each fraction co-vary according to pH levels, total alkalinity and to a lesser extent on salinity and temperature (Lobban & Harrison 1994). At pH 6, the molar fraction of the total Ci is divided equally between CO_2 and HCO_3^-, the only usable forms of carbon for most of algae. The concentration of CO_2 at pH 8.5 is negligible, as HCO_3^- is in equilibrium with CO_3^{-2}, which is not a direct source of inorganic carbon for algal photosynthesis (Maberly 1992). Above pH 9, the relative fraction of HCO_3^- continues to decrease relative to CO_3^{-2} leading to Ci limitation. Intensive algal cultivation suffers from large daily pH fluctuations due to the counter effects of photosynthesis and respiration, and in a carbon limited system pH levels can cross at least 2 units (Mata et al. 2007) providing a unique setting to evaluate the benefits of dosing Ci at commercial scales.

Increasing total dissolved CO_2 concentrations in land-based intensive seaweed cultivation can therefore significantly enhance biomass productivity (Gao et al. 1991, Gao et al. 1993, Demetropoulos & Langdon 2004a, Zou 2005). However, CO_2 enrichment can also have no effect or may even be detrimental for some species (Andria et al. 1999, Israel et al. 1999, Israel & Hophy 2002). The lack of widespread positive responses to CO_2 enrichment in algae has been attributed to the presence of carbon concentration mechanisms (CCMs). These mechanisms allow algae to utilize the HCO_3^- pool in seawater, which is the most common form of carbon (Giordano et al. 2005). However, the efficiency of HCO_3^- use is species specific, with some species relying on HCO_3^- to complement CO_2 as a carbon source, while others can efficiently saturate carbon requirements using HCO_3^- alone (Raven et al. 2008). Therefore, enhanced productivity through CO_2 enrichment is affected by the capability and efficiency with which species use HCO_3^-. Quantifying and understanding the response of algae to CO_2 enrichment is a critical first step in optimisation of growth under intensive culture.

The major objective of this study was to quantify the ability of three green tide algal species, *Cladophora coelothrix*, *Cladophora patentiramea* and *Chaetomorpha linum*, to utilise alternative carbon sources under intensive culture, and subsequently quantify their growth response to removing carbon limitations. These three species were selected as they are clearly identified targets for biomass production for bioremediation and bioenergy applications in tropical Australia (de Paula Silva et al. 2008, de Paula Silva et al. 2012), and worldwide (Aresta et al. 2005, Pierri et al. 2006, Migliore et al. 2012). Specifically, we quantified the affinity for HCO_3^- as a carbon source using the pH drift technique to determine the compensation points. We subsequently quantified growth under laboratory conditions at different pH levels with defined carbon sources. Finally, the three species were cultured for four weeks in an outdoor experiment testing the effects of CO_2 enrichment and HCO_3^- affinity on productivity and biomass composition (carbon, nitrogen and ash).

4.2 Materials and methods

4.2.1 Algae collection and stock cultures

Three green tide algal species were collected from aquaculture facilities in Queensland, Australia. *Cladophora coelothrix* Kützing and *Chaetomorpha linum* (O. F. Müller) Kützing (hereafter in this chapter *C. coelothrix* and *C. linum*) were collected from the settlement pond and intake channel, respectively, of an intensive fish farm (Latitude: 20.02^0S Longitude: 148.22^0E, barramundi *Lates calcarifer*). *Cladophora patentiramea* (Montagne) Kützing (hereafter in this chapter *C. patentiramea*) was collected from the intake dam of an intensive prawn farm (Latitude: 18.26^0S Longitude 146.03^0E, tiger prawns *Penaeus monodon*). Algal samples were hand collected and placed in aerated seawater for transportation to the James Cook University, Marine Aquaculture Research Facility Unit (MARFU). Stock cultures of each algal species were maintained in 70 l tanks within a recirculating system (~27ºC, 36‰).

4.2.2 Algal affinity for HCO_3^-

Two approaches were used to quantify the ability of the three algal species to utilize HCO_3^- as a source of Ci; pH drift technique (compensation point), and algal growth response to different pH levels.

4.2.2.1 pH drift in closed vessel

The pH drift technique is a reliable method to determine HCO_3^- utilization (Murru & Sandgren 2004). As the photosynthetic uptake of CO_2 and/or HCO_3^- results in a near stoichiometric production of hydroxyl ions, the pH of the culture media increases in response to photosynthesis. At pH 9, dissolved CO_2 is virtually absent and species without mechanisms of HCO_3^- utilization reach their limit of Ci extraction. Consequently, pH will not increase beyond this level, enabling the ability to utilise HCO_3^- to be evaluated (Maberly 1990).

The pH drift assays were carried out in a culture chamber (Sanyo model MLR-351) with constant temperature (28°C) and irradiance (150 µmol photons m^{-2} s^{-1}). Basal culture media was prepared using filtered sterile seawater (NO_3-N 0.06 mg l^{-1}, PO_4^--P 0.02 mg l^{-1}, Ci 1.9 mM and 32 ‰) enriched with f/2 growth media (Ryther & Guillard 1962). Algal samples were collected from the stock cultures, washed clean and pre-incubated for five days in the conditions described above. Approximately 100 mg fresh weight of filaments were incubated in closed airtight 120 ml graduated culture vessels filled with 130 ml of freshly prepared growth media (pH 7.9), leaving a minute air space. Culture vessels were repositioned and stirred hourly during the experiment to minimise any artefacts relating to light source or the formation of a boundary layer.

Thirty-six culture vessels were prepared for each species and, after one hour in culture, three random culture vessels for each species (n = 3) were destructively sampled for pH measurements (YSI 63 pH meter). This procedure was repeated every two hours until the maximum pH reached a stable level for at least two consecutive measurements (pH compensation point). This compensation point represents the pH at which the Ci taken up by the algae equals the CO_2 released by respiration and/or photorespiration into the medium.

4.2.2.2 Effects of pH on algal growth

The HCO_3^- affinity of the algae can be inferred from their growth response at different pH levels because the relative amount of CO_2 and HCO_3^- available for growth is pH dependent. Above pH 8.5, where CO_2 is virtually absent, species with no or little ability to use HCO_3^- experience a steep decrease in growth. In contrast, species with the ability to efficiently use HCO_3^- respond more slowly to the increase in pH as they utilize HCO_3^- for growth.

To test HCO_3^- affinity, algal biomass was transferred from the outdoor stock cultures to the laboratory and pre-cultured in f/2 enriched growth media for five days (in conditions described in the previous section 2.2.1). Samples of each algal species (~100 mg fresh weight) were incubated in 100 ml of seawater enriched with f/2 (Ryther & Guillard 1962) within 120 ml plastic culture vessels with the lid loosely placed on top. The culture media in each treatment was buffered to maintain constant pH (+ 0.1 units), and correspondingly Ci ratios, using biological Tris (Sigma) at a final concentration of 25 mM. The water pH was adjusted to the desired pH levels (7, 7.5, 8, 8.5 and 9) using freshly prepared 1 M NaOH or HCl solutions. The culture media was prepared and replaced every day to renew Ci and to maintain the original CO_2:HCO_3^- ratios for each treatment. Samples were again stirred and repositioned daily to a new position in the culture chamber. Treatments were weighted at the

beginning and end of a ten day experimental period. Daily growth rates (DGR; % day^{-1}) were then calculated using the following equation:

$$DGR = [(Wf / Wi)^{(1/T)}-1]*100 \quad (1)$$

where *Wi* is the initial fresh weight, *Wf* is the final fresh weight and T is the culture period in days.

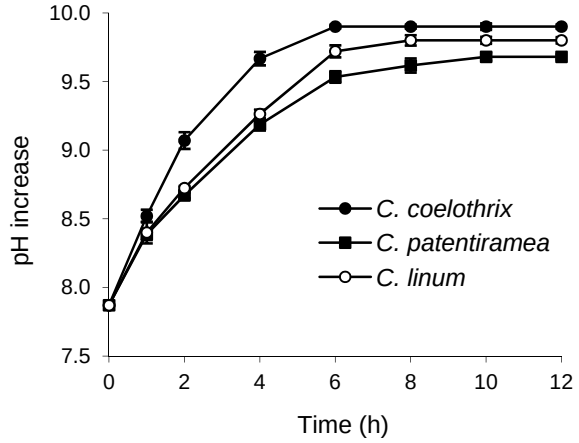

Figure 4.1 pH drift experiment for the three green tide algal species *C. coelothrix*, *C. patentiramea* and *C. linum*. Error bars represent standard deviation (n = 3).

4.2.3 Algal productivity under CO_2 enrichment

A CO_2 enrichment experiment was performed outdoor using recirculating cultivation systems at the Marine Research Facility Unit (MARFU) at James Cook University between August and September 2010. Two independent sumps were used, one was supplied directly with CO_2 gas stream (food grade 99.9% – BOC Australia) delivered through an air stone fixed at the bottom of the sump and the flow of CO_2 gas was manually adjusted throughout the day to maintain pH between 6.5 and 7, whereas the other acted as a control sump with no additional CO_2. These systems provided a constant water flow of 2 volumes (vol) h^{-1} to polyethylene white buckets with 5 l capacity, 0.035 m^2 surface area, containing a ring of aeration in the

bottom to maintain the algae in tumble culture. The buckets were stocked with 3 g fresh weight l^{-1} (n = 3 for each species*CO_2 treatment).

Cultures were acclimated for two weeks at these conditions and a formal growth experiment conducted over the subsequent four week period. Algal biomass of each tank was harvested weekly to determine productivity and subsequently restocked at the original density of 3 g fresh weight l^{-1}. The algae were collected in mesh bags (0.1 mm mesh) and the biomass drained to a constant fresh weight in a washing machine (spin cycle 1000 rpm). Productivity (g m^{-2} day^{-1} dry weight) was then calculated using equation (2):

$$P = \{ [(Bf - Bi) / FW:DW] / A \} / T \quad (2)$$

where *Bi* is the initial biomass, *Bf* is the final biomass, FW:DW is the fresh to dry weight ratio, A is area of culture vessels and T the number of days in culture. The dry weights were acquired individually for each week from excess centrifuged biomass oven dried at 65°C for 48 h. Resulting FW:DW ratios were on average 3.5:1 for *C. coelothrix*, 5:1 for *C. patentiramea* and 5.9:1 *C. linum*.

The water pH, temperature and salinity were measured daily in the inflow and outflow water of seaweed cultures at 08:00, 12:00 and 18:00 using an YSI 63 multi-parameter meter. Throughout the experiments water temperature and salinity averaged 28ºC (2 ± SD) and 35‰ (1 ± SD), respectively. Ambient surface photosynthetic active radiation (PAR) was measured continuously using a LI-192S (2p) sensor placed near the tanks. Daily average PAR recorded during light hours for the experimental period was 881 ± 152 µmol photons m^{-2} s^{-1}. Water samples were collected twice a week at 12:00 from the inflow and outflow of tumble cultures for alkalinity determination. The samples were fixed with 200 µM of saturated $HgCl_2$ solution, immediately taken to the lab and stored in the fridge until alkalinity analysis. Alkalinity was calculated using potentiometric titration by the Australian Centre for Tropical

Freshwater Research (ACTFR) at James Cook University. Ci concentration and sources were calculated using the pH, alkalinity, salinity, and temperature original values of collection time, using the software CO_2sys (Lewis & Wallace 1998). Nitrogen and phosphorus were measured from water samples collected from the inflow and immediately analysed by cadmium reduction and ascorbic acid techniques (HACH model DR/890), respectively. Average nitrogen and phosphorus concentrations during the experiment were ~2.4 and 0.16 mg l^{-1}, respectively.

4.2.4 Biomass analysis

The biomass of each tank was harvested at the completion of the four week growth period for nitrogen, carbon and ash analysis (n = 3 for each CO_2*species treatment). Biomass was spun dry and then oven dried at 60°C for 48 h, milled and stored in glass containers prior to analysis. Nitrogen and carbon were quantified for each sample using isotope analysis. Ash was quantified using a Carlo-Erba elemental autoanalyzer (Environmental Biology Group, Australian National University, Canberra).

4.2.4 Statistical analysis

Two-way fixed-effect analyses of variance (ANOVA) were used to compare the growth response of the three green tide algal species to different pH and correspondent CO_2 and HCO_3^- ratios (section 2.2), and the effects of CO_2 enrichment on biomass productivity (section 2.3) using SYSTAT 12. Post-hoc comparisons were made to assess the differences between treatments in both experiments (Tukey's HSD multiple comparisons). The ANOVA assumptions of homogeneity of variance and normality were assessed by scatter plots and normal curve of the residuals, respectively (Quinn & Keough 2002). To test whether the the biomass composition was influenced by CO2 enrichment, we used a two-factor permutational multivariate analysis of variance (PERMANOVA, PRIMER v.6). The two fixed-factors were

species and CO_2 enrichment, while the dependent variables were the % nitrogen, carbon and ash content of dried biomass. Biomass composition data was fourth-root transformed for the PERMANOVA.

Table 4.1 Summary output for significant interactions in the ANOVAs (Species*pH and Species*CO_2), analysing the effects of varying pH on growth and CO_2 enrichment on algal productivity, and PERMANOVA (Species*CO_2) testing effects of CO_2 enrichment on biomass composition.

Source	df	*MS*	*F*	*P*
ANOVA				
Species*pH	12	16.16	7.01	**<0.001**
Species*CO_2	2	5.91	3.73	**0.031**
PERMANOVA				
Species*CO_2	2	7.06	16.33	**<0.001**

4.3 Results

4.3.1 Algal affinity for HCO_3^-

4.3.1.1 pH drift in closed vessel

The pH drifted from 7.9 to over 9.7 for all three algal species (Figure 4.1). *C. coelothrix* had the highest pH compensation point of 9.9, which was reached after six h in culture. *C. linum* and *C. patentiramea* used the HCO_3^- in the water at a slower rate, taking eight and ten hours to achieve the slightly lower pH compensation points of 9.8 and 9.7, respectively (Figure 4.1).

4.3.1.2 Effects of pH on algal growth

All three species had decreasing growth rates with increasing pH above the optimum of pH 7.5. However, there was a significant interaction between the species and the pH levels in which they were cultured ($P < 0.001$, Table 4.1, Figure 4.2), driven by different optimal pH ranges for growth. In other terms, integrating the pH drift results of 3.1.1, different growth responses were reflective of different HCO_3^- affinities. Both *C. coelothrix* and *C. linum* had higher growth rates at pH levels between 7 and 8.5, whereas the optimum pH range for *C. patentiramea* was between 7 and 8 (Figure 4.2). There were no significant differences in growth rates within the optimal pH range for each species (Tukey's HSD, $P > 0.05$). The highest individual growth rates for all three species were measured at pH 7.5, with growth rates of 14.5, 8.8 and 8.2% day^{-1} for *C. linum*, *C. patentiramea* and *C. coelothrix*, respectively (Figure 4.2). Growth rates for *C. linum* and *C. coelothrix* decreased above the optimal pH range (from pH 8.5 to pH 9), by 48% and 35%, respectively. Growth rates for *C. patentiramea* decreased above the optimal pH range (from pH 8 to pH 8.5) by 30%, and subsequently by 47% to pH 9. The highest susceptibility of *C. patentiramea* growth to increasing pH levels (lower CO_2:HCO_3^- ratio) supports a relatively lower capability of using HCO_3^-. In accordance with the pH drift experiment, the lower sensitivity of *C. coelothrix* to changes in pH supports its more efficient use of HCO_3^-.

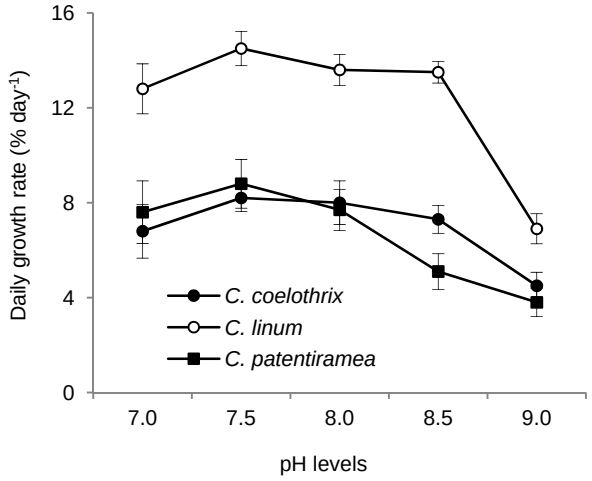

Figure 4.2 Mean daily growth rates (DGR) (±1 SE) of *C. coelothrix*, *C. patentiramea* and *C. linum* cultured in different pH levels (n = 3 for each pH levels*species).

4.3.2 Algal productivity under CO_2 enrichment

Based on the differences in HCO_3^- utilization efficiencies between the three species, the subsequent step was to quantify increases in productivity through the addition of CO_2 in controlled intensive cultures. The addition of CO_2 decreased the pH of the inflowing seawater from ~ pH 8 (control) to pH 6.7. Under these conditions the concentration of CO_2 was twenty five times higher in the CO_2 enriched cultures compared to the control (Table 4.2). The addition of CO_2 also increased the concentration of HCO_3^- in the CO_2 enriched cultures to 2 mM, compared to 1.4 mM in the control cultures, because the hydration of CO_2 produces carbonic acid, and its subsequent de-protonation leads to the formation of HCO_3^-. After passing through the seaweed tanks, at 2 vol h^{-1}, all CO_2 was depleted from water within the control cultures. In contrast, there was a continual supply of CO_2 in the CO_2 enriched cultures for photosynthesis. HCO_3^- concentration in the control and CO_2 enriched cultures was ~1 mM and 1.5 mM, respectively. *C. coelothrix* cultures had the lowest concentration of all carbon forms (Table 4.2), and therefore the highest carbon uptake rates of all three species. *C. patentiramea* cultures had the highest Ci concentration in the water, in particular in the

control treatment, and therefore carbon uptake rates were lower for *C. patentiramea* when only HCO_3^- was present. These results confirm the laboratory data indicating that *C. patentiramea* has the least effective HCO_3^- utilisation of the three species.

The three algal species had different productivity (growth) responses to CO_2 enrichment, with a significant interaction between CO_2 supply and the species tested ($P < 0.001$, Table 4.1). The productivity of *C. coelothrix* and *C. linum* were significantly enhanced (~26 and 24%, respectively) when supplied with additional CO_2 (Figure 4.3) The productivity of *C. coelothrix* increased from 12.5 to 16.8 g DW m^{-2} day^{-1}, and *C. linum* from 9.5 to 12 g DW m^{-2} day^{-1} (Figure 4.3). The productivity of *C. patentiramea* (5.2 to 6.2 g DW m^{-2} day^{-1}) to CO_2 enrichment was not significantly different to that of the control (Tukey's HSD, $P > 0.05$).

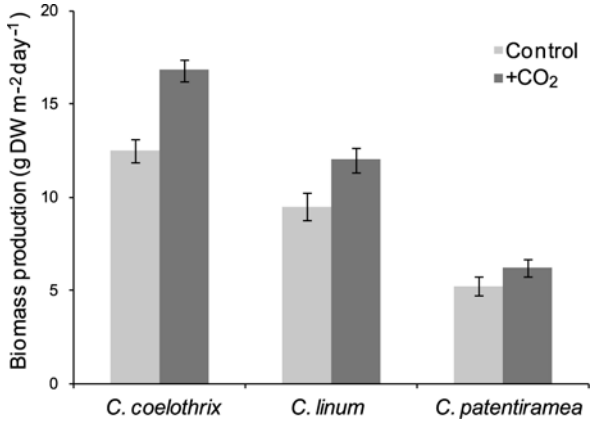

Figure 4.3 Biomass productivity of the three green tide algal species in response to CO_2 enrichment. Error bars represent standard error (n = 3 for each species).

4.3.3 Biomass analysis

In general, *C. coelothrix* and *C. linum* had higher carbon and nitrogen concentrations and lower ash contents that *C. patentiramea* (Table 4.3). However, CO_2 enrichment influenced the biomass composition of the algal species in different ways (PERMANOVA, Species*CO_2, $P < 0.001$, Table 4.1). This interaction was mainly driven by positive influence

of CO_2 enrichment on carbon and nitrogen content in *C. coelothrix* and *C. linum* compared to the negative influence in *C. patentiramea*, which corresponded with an increase in ash content in the latter (Table 4.3). *C. coelothrix* biomass increased in carbon and nitrogen content by ~2%, while ash decreased by ~1% (Table 4.3). *C. linum* biomass also increased in carbon and nitrogen content, but to different degrees (by ~4% and 2%, respectively). In contrast, the biomass of *C. patentiramea* cultured under CO_2 enrichment had a lower and carbon (4%) and nitrogen (1%) content and a much higher ash content (7%) compared with the control (Table 4.3).

Table 4.2 Mean values (and standard deviation) for pH, dissolved inorganic carbon (Ci), carbon dioxide (CO_2) and bicarbonate (HCO_3^-) from the inflow and outflow of the green tide algal cultures with and without additional CO_2.

	CO_2 enrichment	pH	Ci (mM)	CO_2 (µM)	HCO_3^- (µM)
Inflow	+CO_2	6.73 ± 0.26	2.33 ± 0.20	250 ± 60	1980 ± 160
	Control	7.98 ± 0.16	1.63 ± 0.12	10 ± 05	1400 ± 100
C. coelothrix	+CO_2	7.42 ± 0.20	1.60 ± 0.15	40 ± 10	1510 ± 140
	Control	8.52 ± 0.12	1.17 ± 0.13	0	930 ± 130
C. linum	+CO_2	7.38 ± 0.19	1.62 ± 0.14	50 ± 10	1520 ± 110
	Control	8.47 ± 0.10	1.20 ± 0.13	0	980 ± 120
C. patentiramea	+CO_2	7.35 ± 0.20	1.62 ± 0.11	50 ± 10	1525 ± 100
	Control	8.38 ± 0.13	1.26 ± 0.14	0	1100 ± 140

4.4 Discussion

This study demonstrates that three green tide algal species, *C. coelothrix*, *C. linum* and *C. patentiramea*, have the ability to use HCO_3^- as a complementary carbon source to CO_2 for photosynthesis. However, this ability is restricted to a narrower pH range than for many other

green algae belonging to the same genera. There is a lower comparative complexity or efficiency of the mechanisms involved in the uptake or conversion of HCO_3^- to CO_2 for these species. Consequently, this corresponds to a relatively higher dependence on CO_2 as a carbon source for photosynthesis and this is reflected in the significant enhancement of productivity of these species when enriched with CO_2 in intensive culture.

Table 4.3 Mean values (and standard deviation) for % carbon (C) dry weight, % nitrogen (N) dry weight and % ash of three green tide algal species cultured with and without CO_2 enrichment.

Species	CO_2 enrichment	% C	% N	% Ash
C. coelothrix	$+CO_2$	33.29 ± 0.28	6.05 ± 0.03	25.20 ± 0.55
	Control	30.97 ± 0.30	4.07 ± 0.03	26.22 ± 0.56
C. linum	$+CO_2$	31.67 ± 0.70	5.98 ± 0.10	29.20 ± 0.88
	Control	27.12 ± 0.13	4.12 ± 0.13	33.43 ± 0.47
C. patentiramea	$+CO_2$	18.05 ± 0.45	3.07 ± 0.08	56.97 ± 1.97
	Control	22.53 ± 1.06	4.27 ± 0.17	50.66 ± 1.53

4.4.1 Algal affinity for HCO_3^-

4.4.1.1 pH drift in closed vessel

Comparatively, green algae as a taxonomic group photosynthesise at the highest pH levels with compensation points up to pH 10.8 (Maberly 1990). At these pH levels, CO_2 is absent and HCO_3^- is the only functional form of inorganic carbon, representing less than a quarter of the total Ci. Active photosynthesis at these pH levels is only possible because of diverse and highly efficient mechanisms to overcome CO_2 constraints through the utilization of HCO_3^- (Beer & Bjork 1994). There are at least two mechanisms to utilize HCO_3^- in green

macroalgae (Axelsson et al. 1999). The first is the extracellular dehydration of HCO_3^- into CO_2 through the periplasmic carbonic anhydrase (CA) enzyme, followed by diffusion of CO_2 into the cell, and this is the most widely distributed mechanism. The second mechanism is the direct uptake of HCO_3^- through the plasma membrane, mediated by an anion exchange protein (Larsson & Axelsson 1999). Some species of *Cladophora* have a third mechanism with the uptake the Ci through a vanadate-sensitive P-type H^+-ATPase (proton pump) (Choo et al. 2002). Species with these mechanisms raise the pH up to 10.5 in a closed vessel. The three species in this study did not raise the pH above 9.9 and therefore have limited HCO_3^- transport. They almost certainly concentrate carbon using the dehydration of HCO_3^- by CA into CO_2, as this is the most common mechanism of HCO_3^- utilization in algae (Badger & Price 1994). This mechanism usually operates at ~ pH 8.3, when the proportion of CO_2 in the total Ci pool is below 1% and HCO_3^- is more than 90%. The capacity to utilize HCO_3^- through this mechanism decreases sharply with increased pH, and is ineffective at pH 9.8 (Axelsson et al. 1995). The direct transport of HCO_3^- through an anion exchange protein usually operates at higher pH (~9.3) (Choo et al. 2002), and is the most probable mechanism for compensation points above 9.5 in the three species. These two mechanisms operate separately in other species of green algae with periplasmic CA activity dominating at lower pH, and direct uptake of HCO_3^- by an anion exchanger at higher pH (Choo et al., 2002). The relatively faster rate of pH increase for *C. coelothrix* supports more efficient HCO_3^- use than either *C. linum* or *C. patentiramea*. Nevertheless, the incapacity of the species in this study to raise the pH above 9.7-9.9 suggests that there is no proton pump mechanism involved in HCO_3^- transport. Choo et al. 2005 inhibited the proton pump mechanism in *Ulva procera*, an alga capable of a pH compensation point of 10.5, and the pH remained below 9.9, demonstrating a reliance on this third mechanism to elevate the pH compensation to its highest level.

In a comparative context, the two *Cladophora* species in this study are less efficient in the use of HCO_3^- than other species from the same genera with pH compensation points of ~pH 10.5 (Maberly 1990, Choo et al. 2005). However, as in this study, some species of *Cladophora* maintain a preference for dissolved CO_2 as a carbon source (Rivers & Peckol 1995a). These different responses may be related to the environmental niche prior to experiments, as the ability of algae to utilise HCO_3^- is strongly related to habitat (Maberly 1990). Individuals of the same species can express alternate strategies for carbon acquisition when in different habitats, or the habitat itself might select for survival of genotypes with different carbon acquisition strategies (Murru & Sandgren 2004). This relatively limited ability to utilise HCO_3^- is reflected in the growth response of all three species at different pH environments in this study.

4.4.1.2 Effects of pH on algal growth

As pH increases from 7 to 8, the relative proportion of Ci present as CO_2 is reduced by over 70%, while the relative proportion of HCO_3^- decreases by only 10%. This drastic change in the CO_2:HCO_3^- ratio had no effect on the growth of algae in this study. The comparative ratio of CO_2:HCO_3^- was maintained at each pH throughout the experiment through the addition of a biological buffer. Consequently, CO_2 is always available between pH 7 and 8 at concentrations that meet the carbon requirement for algal photosynthesis and growth. This does not, however, exclude the activation of the CA mechanism at ~pH 8, which supplies additional CO_2 derived from HCO_3^- to compensate for its lower availability at increased pH (Mata et al. 2007).

From pH 8 to 8.5, CO_2 decreases markedly and photosynthesis and growth depends on the efficiency of HCO_3^- utilization mechanisms. The growth rates of *C. linum* and *C. coelothrix* within this pH range changed little. In contrast, a significant decrease in growth for *C.*

patentiramea confirms that it is the least adapted to grow in the absence of CO_2. Above pH 8.5, HCO_3^- is replaced by CO_3^{2-} and growth decreased significantly for all species. Steeper decreases in growth rates for *C. patentiramea* and *C. linum* between pH 8 and 9, compared to *C. coelothrix*, correspond with the slower rate of increasing pH for these two species in the pH drift experiment. These data, together with the highest pH compensation point, confirm that *C. coelothrix* has the most efficient mechanisms of HCO_3^- utilisation. However, in a broader comparative context, the relatively low pH compensation points and significant decreases in growth rates from pH 8 to 9, again demonstrate that the three species in this study are not as efficient in the use of HCO_3^- as a carbon source compared to many other green tide algal species.

4.4.2 Algal productivity under CO_2 enrichment

The productivity of two of the three species of green tide algae, *C. coelothrix* and *C. linum*, was enhanced through the addition of CO_2. Notably, the enrichment treatment had twenty five times more CO_2 available than the control. This maintained the pH of the enriched water below pH 7.5 (excess CO_2), whereas the pH of the control cultures averaged 8.5 (depleted CO_2). Considering the relatively limited ability of species to utilize the HCO_3^- pool, and that this process has an energetic cost (Raven et al. 2008), the constant presence of CO_2 at pH 7.5 disproportionately facilitated photosynthetic carbon fixation, and ultimately enhanced biomass productivity. Enhanced growth rates with CO_2 enrichment have also been reported for other species capable of using bicarbonate (Gao et al. 1991, Gao et al. 1993, Demetropoulos & Langdon 2004a, Zou 2005). However, the magnitude of the growth responses to CO_2 enrichment is to some extent dependent on the efficiency of carbon concentrating mechanisms for each species. For example, species depending almost exclusively on CO_2 for photosynthesis can increase their biomass productivity up to three times when cultured in enriched CO_2 culture media (Kubler et al. 1999, Mata et al. 2007,

Mata et al. 2012). Any differences in growth relative to enhanced CO_2 can also be due to the effect of CO_2 on the rate of nitrogen assimilation (Rivers & Peckol 1995b, Gordillo et al. 2001). High levels of CO_2 can increase the rate of nitrogen assimilation in some algae by up-regulating nitrate reductase, the main enzyme in the nitrate assimilatory pathway (Mercado et al. 1999, Gordillo et al. 2001). This may be the case for *C. coelothrix* and *C. linum* in this study where they have a higher nitrogen content under CO_2 enrichment. Higher nitrogen and carbon contents on top of increased productivities with CO_2 enrichment represents a clear advantage for integrated systems focused on biomass production for bioremediation of waste streams (Israel et al. 2005, de Paula Silva et al. 2008).

In contrast, the nitrogen content of *C. patentiramea* decreased under CO_2 enrichment, suggesting no affect on assimilation. The effect of high CO_2 on the assimilation of nitrogen in algae is not consistent with decreases in assimilation for other species of algae (García-Sánchez et al. 1994, Andria et al. 1999). This effect may contribute to the relatively lack of increase in productivity of *C. patentiramea* under CO_2 enrichment. Notably, laboratory experiments suggested that *C. patentiramea* should be the most sensitive species to CO_2 enrichment based on relative capabilities of HCO_3^- utilization, which indicates that controlled, static, laboratory experiments may not be efficient to predict responses in flow environments (e.g. similar to commercial scale), potentially because of boundary layer/water motion effects on Ci distribution (Hurd 2000). An alternative but related driver to water motion is the morphological differences between the green tide algae. The two rapidly growing species which had enhanced growth under CO_2 enrichment, *C. coelothrix* and *C. linum*, have a fine filamentous morphology suitable for tumble culture. In contrast, *C. patentiramea* has tightly interwoven filaments (e.g. ball-like structure) that restrict light to the inner filaments (auto-shading), thereby potentially limiting photosynthesis and growth. These physical factors may have influenced small density cultures in the laboratory in a different

way than the dense cultures in the outdoor experiment, where individual, larger clumps could become limited. Regardless, *C. patentiramea* is a less favourable option for intensive cultivation because in addition to the lack of response to additional CO_2, high CO_2 decreases the nitrogen and carbon content while increasing ash content, and therefore the amount of biomass that can be converted into bioenergy or bio-products decreases substantially. In conclusion, intensive cultures of *C. coelothrix* and *C. linum* enriched with CO_2 had significantly enhanced productivity, despite their ability to utilise HCO_3^-. This demonstrates the potential for enhanced production for these two green tide algal species using CO_2 enrichment.

Chapter 5: Developing a simple predictive model for large-scale biomass applications

5.1 Introduction

One of the major constraints for integrated aquaculture is the selection of species that are resilient to dynamic environments are productive year-round and consequently provide consistent biomass for valuable products or bioremediation services. Much of the research in this field has focused on integrating species with existing commercial applications (Neori et al. 2004, Chopin et al. 2006), however, a renewed focus on algal biomass for broader applications, including biomass derived bioenergy, means that the pool of potential species has widened substantially to include those previously thought of as pests, such as green tide algae growing naturally in land-based aquaculture systems (Pagand et al. 2000, Paul & de Nys 2008). Of these, the genus *Cladophora* is highly diverse (Guiry & Guiry 2012) spanning freshwater to marine habitats (Dodds & Gudder 1992). The majority of species within this genus have not been investigated for biomass applications and, for those that have samples have been opportunistic collected from sporadic algal blooms (Zhou et al. 2010, Bird et al. 2012). For a more reliable source of biomass, a critical first step is to understand seasonal productivity. This can be delivered through field-based data that is, however, a this is time-consuming exercise (de Paula Silva et al. 2012). Furthermore, field-based research is, in most instances, unviable or has insufficient replication to deliver reliable and comprehensive management strategies for annual large-scale biomass production (Troell et al. 2003). Finally, field-based data may not accurately reflect environmental gradients that can differ markedly from year to year, particular in tropic regions.

Therefore, I present an innovative method to develop predictive models for large scale biomass applications. Classification and regression tree (CART) analysis were used to determine species resilience; and logistic equations to simulate large-scale integrated algal

culture. CART analyses are typically used to evaluate interactions in large data sets (Sheaves & Johnston 2009, Rozas & Minello 2010, Ferraro et al. 2012). They permit simultaneous analysis of categorical and continuous variables, and also help to explore data and predict scenarios within complex species by environment interactions (De'ath 2002). CART analyses were used to compare the resilience of two abundant green tide algal species, *Cladophora coelothrix* and *Cladophora patentiramea*, across seasonal extremes in environmental gradients relevant to land-based, tropical aquaculture systems. To do this I used the previously determined growth rates across the interactions of environmental variables (temperature, salinity, and nitrogen concentrations and sources) using manipulated gradients (annual minima, averages, maxima relevant to the system) in a repeated measures design. The outputs of the CART analysis and a repeated measures ANOVA for the same data were compared and key environmental interactions relevant to the system were derived from the tree. These groupings were used to generate predictive annual models for biomass production of each species using logistic regressions.

5.2 Materials and Methods

5.2.1 Study organisms and sample collection

Two species of green tide filamentous algae were used in experiments. *Cladophora coelothrix* Kutzing and *Cladophora patentiramea* (Montagne) Kützing (hereafter in this chapter *C. coelothrix* and *C. patentiramea*) are related algae in the family Cladophoraceae. The two species are uniseriate, branching filamentous algae (Womersley 1984) that form dense mats composed of entangled filaments on the water surface. Both species are seasonally abundant species in land-based aquaculture facilities in tropical North Eastern Australia, and undergo ‘bloom-and-bust’ growth cycles throughout the year (de Paula Silva, personal observation). *C. coelothrix* was sampled in May 2009 from the settlement pond of

Good Fortune Bay Fisheries Ltd, an intensive saltwater barramundi (*Lates calcarifer* Bloch) farm located at Bowen (Latitude 20.02°S, Longitude 148.22°E). *C. patentiramea* was collected in May 2009 from the intake dam of Pacific Reef Fisheries Pty Ltd**,** an intensive tiger prawn (*Penaeus monodon*) farm located in Ayr (Latitude 19.62°S, Longitude: 147.38°E), ~80 km North-East of Good Fortune Bay Fisheries Ltd. Isolated cultures of each species were maintained in outdoor aquaria on a recirculating system (~28ºC and ~36‰) at the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University.

5.2.2 Interactive effects of water quality parameters on growth

The seasonal values used for temperature, salinity and nitrogen (concentrations and sources) were based on an annual *in situ* survey of an operational settlement pond (see Table 1 in de Paula Silva et al. 2012) and were quantified in a full factorial design. The seasonal fluctuation of temperature was assessed in three consecutive experiments (one day apart), run at constant 24, 28 and 33ºC using a controlled environment test chamber. For each temperature experiment, we tested the annual average of salinity fluctuations (32‰), as well as the seasonal extremes representing the wet (17‰) and dry (40‰) seasons. The nitrogen supply to the algal samples was either ammonium (NH_4Cl) or nitrate ($NaNO_3$) as a single nitrogen source, added to the culture medium at 4 nitrogen concentrations: low (35 µM), average (70 µM), high (105 µM) and very high (210 µM). These are all within the natural range of nitrogen in the pond environment with the 210 µM being the maximum permitted discharge concentration in Australia (Department of Environment and Resource Management 2011). Growth was measured firstly after 5 days, at which time the nutrient media was replaced for all culture vessels, and subsequently after another 5 days (in a repeated measures design, see statistical analyses below). Growth was calculated as daily growth rates (% day^{-1}) for the two culture periods, 0 to 5 and 5 to 10 days, using equation (1):

$$DGR = [(Wf / Wi)^{(1/T)}-1]*100 \quad (1)$$

where *Wi* = initial fresh weight, *Wf* = final fresh weight and T = days).

5.2.3 Statistical analyses

Statistical analyses were performed using two techniques, classification and regression tree analysis (CART) using TreesPlus 2000 and a 6-factor repeated measures analysis of variance (ANOVA) using SYSTAT 12. We used CART to partition growth data according to the interactions of explanatory variables; culture periods (CP), species (SP), temperature (TP), salinity (SAL), nitrogen concentration (NC) and source (NS), and the relative importance of each variable in the model. CART analysis provides several advantages over traditional parametric tests in terms of flexibility with variable type (continuous and categorical variables can be analysed) and also with respect to the lack of assumptions for independence and linearity assumptions (Steinberg 2009).

Table 5.1 Parameters and values for the simulations that describe the annual productivity model for *Cladophora coelothrix* and *Cladophora patentiramea*.

Symbol	Unit	Description	Species	
			C. coelothrix	*C. patentiramea*
$B(t_0)$	t ha^{-1}	Initial biomass	10	10
r	% day^{-1}	Daily growth rates	6.6 - 12.3	1.3 - 9.7
k	t ha^{-1}	Carrying capacity	15	15
t	days	Harvest cycle	5	5
Adj. -	%	Penalty for multiple N	0 and 16	0 and 30
Adj. +	%	Positive CO_2 enrichment	26	11

The CART analysis was fitted using 10-fold cross-validation based on minimizing the error sum of squares (De'ath 2002, Sheaves 2006). The sum of squares is equivalent to the least squares of linear models (De'ath & Fabricius 2000). The cross-validation (CV) process repeatedly divides the data set into 10 random models in order to optimize the predictive power of the constructed tree (Steinberg & Colla 1995). When this process is completed the error counts from each of the 10 random models are summed to obtain the cross-validated (CV) error estimate (i.e. the cases incorrectly classified in the tree). Since cross-validation is a random process, different trees sizes can be produced. Thus, the analysis was run repeatedly 100 times and the most consistent model was selected as the final tree, appearing 73% of the

time using the SE rule which uses the smallest tree within ± 1 standard deviation (± 1 SE rule) with the lowest CV error (De'ath & Fabricius 2000). These analyses identified the relative importance of each factor across all interactions in the analysis according to their contribution the 'best' tree analysis (Steinberg & Colla 1995).

I subsequently ran the equivalent 6-factor repeated measures ANOVA for the CART analysis, using culture period as the within subjects effect. Each temperature experiment was conducted consecutively, which means that within temperature effects were not independent. However, I use a traditional parametric test of significance to create the full factorial output of a repeated measures analysis for comparison with the CART output. For the ANOVA, the assumptions of homogeneity of variance and normality were assessed by scatter plots and normal curves of the residuals, respectively. Daily growth rate (DGR) measurements were arcsine square root transformed where required (Quinn & Keough 2002).

5.2.4 Predictive model of annual productivity

Predictive models are widely used to optimize biomass production of terrestrial (Faria et al. 2010) and algal crops (Lacerda et al. 2011, Yang et al. 2011, Kumar & Das 2012). Logistic equations, in particular, are appropriate to calculate the growth of organisms with simple morphology and life cycles, including bacteria and many algae. Logistic models are a function of growth rate and carrying capacity, however, these variables vary according to species and environmental conditions and to accommodate these variances in the model complex equations are necessary (Buonomo et al. 2005). For this reason, I used the mutually exclusive groups from the CART analysis (0-5 days culture period, represented by the terminal nodes of the tree, see Figure 5.2) as a mechanism to allocate growth rates of specific months based on environmental data from de (de Paula Silva et al. 2012). The carrying capacity of the pond (K) and initial stoking density (Bt_0) were based on *in situ* algal biomass

sampling (de Paula Silva et al. 2008) and large-scale algal cultures (de Paula Silva et al. 2012). The growth rates for each month, initial biomass and carrying capacity of the system were then used as input in the following logistic equation for the model simulations (Figure 5.3):

$$\text{Biomass } (B)_{t+1} = Bt_0 + r^* Bt\,((K - Bt) / K) \quad (2)$$

where t represents the harvest cycle in days, r is the growth rate as DGR, and K is the carrying capacity of the pond. The input values for the model simulations are presented in Table 5.1. The harvest component of the logistic model was determined on 5-day harvest cycles over the exponential portion of the curve, and productivity was presented on a monthly basis as T DW per hectare (t ha^{-1}).

Table 5.2 Output for a 6-factor repeated measures ANOVA testing culture and environmental factors on *Cladophora coelothrix* and *Cladophora patentiramea* growth. The explanatory factors were culture periods (0-5 and 5-10 days), temperature (TP - 24, 28 and 33 ºC), salinity (SAL - 17, 32 and 40 ‰), species (SP – *C. coelothrix* and *C. patentiramea*), nitrogen concentration (NC - 35, 70, 105 and 210 µM) and nitrogen source (NS - NH_4^+ or NO_3^-).

Source	df	MS	F	P
Between Subjects				
TP	2	492.35	490.89	**<0.001**
SP	1	321.21	320.26	**<0.001**
SAL	2	538.84	537.24	**<0.001**
NC	3	13.27	13.23	**<0.001**
NS	1	2.61	2.60	**0.108**
TP * SP * SAL * NC	12	2.05	2.05	**0.021**
SAL * SP * NS * NC	6	2.12	2.12	**0.042**
Within Subjects				
CP	1	4052.70	1851.24	**<0.001**
CP * TP * NS	2	12.66	5.78	**0.003**
CP * TP * NC	6	7.80	3.56	**0.002**
CP * TP * SP * SAL	4	13.26	6.06	**<0.001**
CP * SP * SAL * NC	6	4.29	1.96	0.072

Subsequently an adjustment was used to accommodate for the decrease in growth rate for each species when multiple nitrogen sources are present in a variety of ratios as they are in the field, compared to single nitrogen sources (ammonium or nitrate) in the laboratory experiments. This leads to a reduction in growth of 16% for *C. coelothrix* and 30% for *C. patentiramea* (de Paula Silva et al. 2012). Therefore, these penalties (Adj. -, Table 5.1) were added for all months according to the interaction of salinity and nitrate-N:TAN ratios from the pond, except February and March because at low salinities there was no difference for growth on single or multiple nitrogen sources. As a final step I also include the potential effect of carbon dioxide (CO_2) enrichment (Adj. +, Table 5.1) for each species as a separate model (Figure 5.3, dotted line). For this, growth of *C. coelothrix* was enhanced by 26% and *C. patentiramea* by 11% across the board (Chapter 4).

5.3 Results

5.3.1 Interactive effects of water quality factors on growth

The CART analysis explained 85% of the total sum of squares, providing a confident dissection of the complex interactions. The output revealed that the culture period was the most important variable (nominally 100%), and correspondently the first split of the tree (Figures 5.1 & 5.2). Temperature and salinity had similar relative importance in the analysis (40 and 37%, respectively, Figure 5.1), and the interaction of these variables dictated the subsequent splits on both side of the tree (Figure 5.2). Overall, nitrogen concentration had very low importance (3.7%), while nitrogen source had no influence on the tree construction (i.e. it is not a driver of any split and is not present in the terminal nodes, Figure 5.2). Furthermore, the growth response to the interaction of temperature, salinity and nitrogen concentration was different for each species (SP = 58%, Figure 5.1).

The first split of the tree separated the culture periods (0 to 5 and 5 to 10 days) into two homogenous groups, where growth rates were always higher in the first 5 days than in the second 5 days of culture (Figure 5.2). The subsequent split was based on the growth response of each species to the combinations of temperature, salinity and nitrogen concentration (Figure 5.2, left branches). Overall *C. coelothrix* performed as well as, or better than, *C. patentiramea* in any specific combination of environmental factors. Temperature was the driver for *C. coelothrix* growth, whereas salinity was a driver for *C. patentiramea.*

In the 0 to 5 days culture period, where growth rates are selected for use in the annual productivity model (see Methods), annual average conditions (32‰ and 28 °C) resulted in high and similar daily growth rates for both species (> 9.5% day^{-1}, Figure 5.2, arrows 1a & b), independent of nitrogen concentration or source. Under winter conditions (40‰ and 24°C), *C. coelothrix* had higher daily growth rates when nitrogen concentration was over 70 µM (up to 210 µM) compared to 35 µM (12.3 and 10.4% day^{-1}, respectively, Figure 5.2, arrow 2a). In contrast, *C. patentiramea* had higher growth when nitrogen concentration was 105 µM or over, compared to lower nitrogen levels (8.6 and 6.5% day^{-1}, respectively, Figure 5.2, arrow 2b). Under summer conditions of high temperature and low salinity, *C. coelothrix* had considerably higher growth than *C. patentiramea* (6.6 and 1.3% day^{-1}, respectively), irrespective of the nitrogen (Figure 5.2, arrows 3a & b).

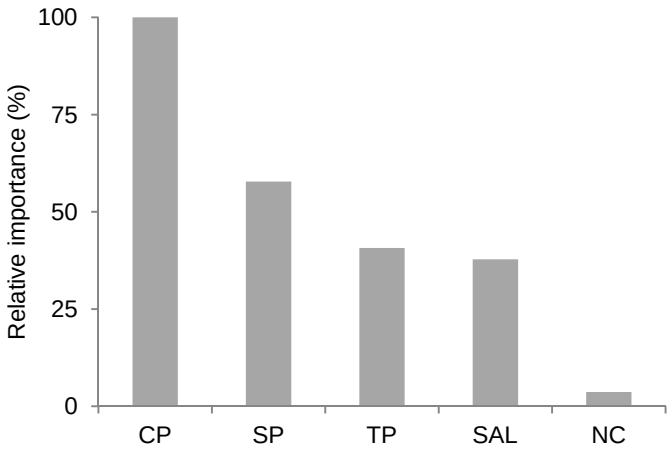

Figure 5.1 Relative importance of all environmental factors used in the laboratory CART analysis. The explanatory factors were culture periods (0-5 and 5-10 days), temperature (TP - 24, 28 and 33 ºC), salinity (SAL - 17, 32 and 40 ‰), species (SP – *C. coelothrix* and *C. patentiramea*), nitrogen concentration (NC - 35, 70, 105 and 210 µM) and nitrogen source (NS - NH_4^+ or NO_3^-).

In the subsequent 5 days of culture (5 to 10 days, right side, Figure 5.2) the interaction of temperature and salinity also affected growth of both species negatively (1.4% day^{-1}, right branches), characteristic of summer. In this culture period, *C. patentiramea* had higher growth than *C. coelothrix* in the combination of factors representative of annual average and winter (Figure 5.2, to right). *C. patentiramea* growth rates were high at low to mid temperatures (<28°C) provided that salinity and nitrogen were high (> 32‰ and 210 mM, respectively, Figure 5.2, right branches). *C. coelothrix* was more tolerant to the breadth of salinity and nitrogen concentrations and sources, again being influenced mainly by temperature (Figure 5.2, right)

The ANOVA outputs were consistent with the CART results, identifying three 4-factor interactions, each involving “species” (Table 5.2) High-order interactions are however difficult to interpret and explain (Tybout et al. 2001), and for this reason no figures are presented for the ANOVA results. All factors other than nitrogen source were significant

main effects ($P < 0.001$). The CART analysis, therefore, provides similar overarching results to the ANOVA but with a more thorough dissection of the relative influence of each environmental factor across all interactions in the tree analysis, which can be represented in a single graphic (Figure 5.2).

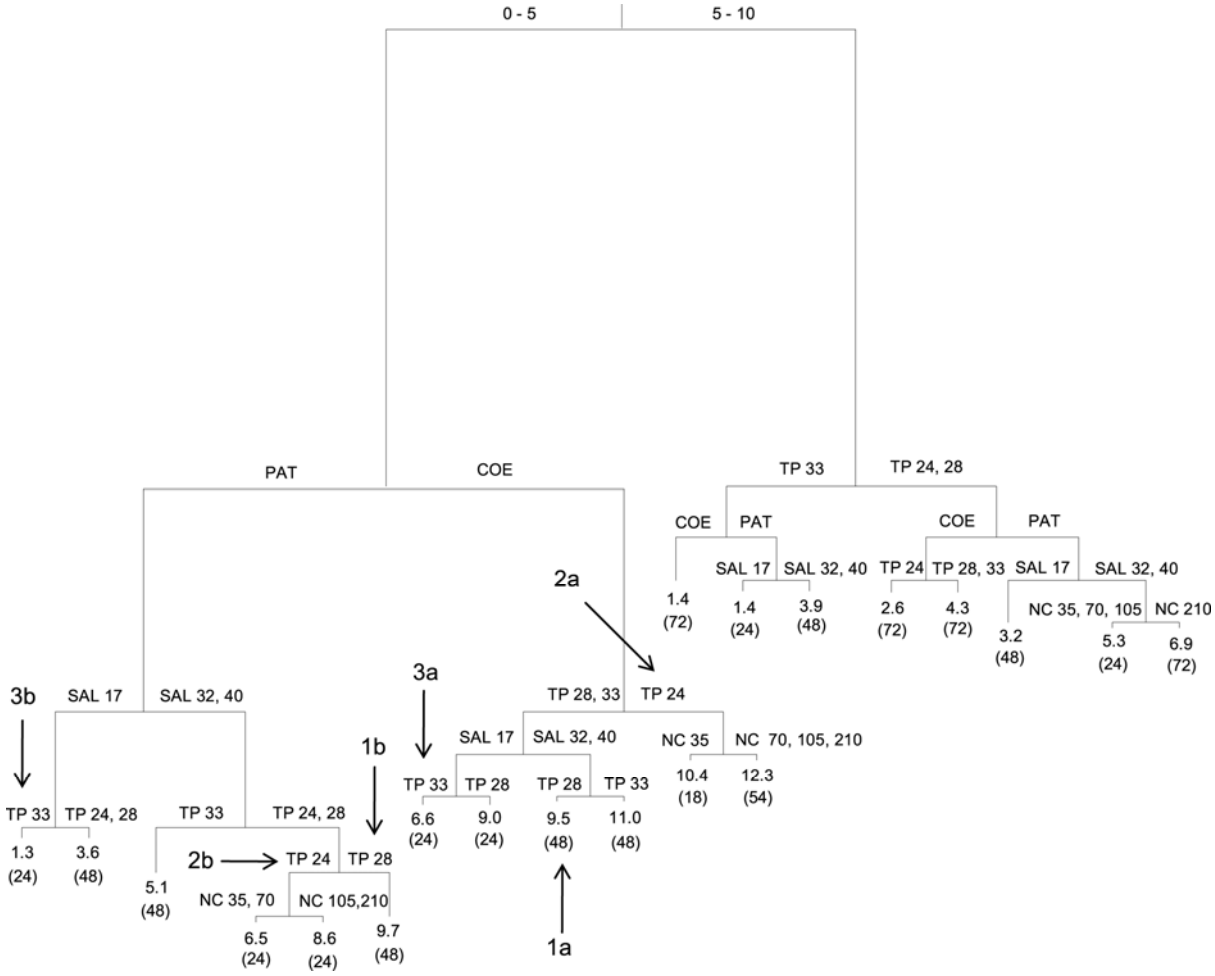

Figure 5.2 Univariate classification and regression trees (CART) based on environmental factors in Table 1, explaining 87 % of the variation of DGR between *C. coelothrix* and *C. patentiramea*. Numbers below terminal nodes are the mean DGR for the specific interaction of explanatory factors, and numbers in brackets indicate the number of cases. Explanatory factors used were: temperature (TP, °C), salinity (SL, ‰), nitrogen concentration (NC, µM), nitrogen source (NS, Nitrate-N or TAN, not present) and species (COE for *C. coelothrix* and PAT for *C. patentiramea*). Arrows 1, 2 and 3 represent the annual average and extremes of each season (winter and summer, respectively). Months represent the growth in specific combination of environmental variables used in the annual predictive model.

5.3.2 Predictive model of seasonal productivity

The predictive model showed that *C. coelothrix* annual productivity was >50% higher than *C. patentiramea* (Figure 5.3), estimated at 24.3 and 10.8 t (dry weight) ha^{-1}, respectively. Productivity varied across seasons with each species showing different trends (Figure 5.3). *C. coelothrix* had relatively stable productivity across the months, demonstrating high tolerance to the environmental fluctuations, with an average monthly productivity of 2.2 t ha^{-1} and a maximum productivity of 2.7 t ha^{-1} in August (Figure 5.3). In contrast, *C. patentiramea* had more variable productivity (Figure 5.3) with higher estimated productivity in January, and from April to September (average of 1.2 t ha^{-1}), when temperature was low to average (<28°C) and salinity mid to high (>32‰). Productivity of *C. patentiramea* decreased in the months with high temperature (~33°C, November and December) and low salinity (~17‰, February and March) to an average of 0.6 t ha^{-1}.

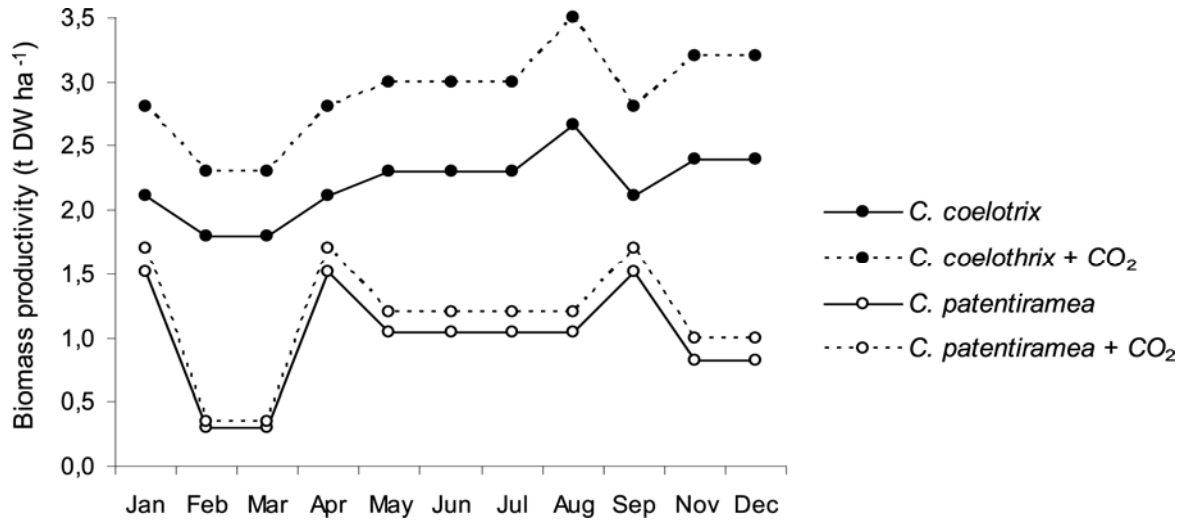

Figure 5.3 Predictive models for annual productivity, represented on a monthly basis, for *Cladophora coelothrix* and *Cladophora patentiramea* derived from the CART analysis and logistic regressions. Dotted line represents CO_2 enrichment.

The seasonal CART model was efficient in predicting the growth response of both targeted species to environmental and culture variables, providing an informative and simple summary

of complex interactions represented in a binary tree format. I then combined the simple predictive output for growth from the CART into a logistic growth curve to simulate productivity in the integrated system. The predictive model for annual productivity was cross-referenced to an extensive survey of relevant environmental data from the study site, the targeted species resilience to these specific conditions, and management factors. For example, annual productivity could potentially be enhanced through CO_2 enrichment to 31.3 and 12.6 t ha^{-1} respectively, for *C. coelothrix* and *C. patentiramea* (Figure 5.3, dotted line).

5.4 Discussion

This study demonstrates the effective use of classification and regression tree analysis to deliver predictive models for biomass productivity based on empirical data and logistic growth curves for two candidate algal species for integrated aquaculture, *C. coelothrix* and *C. patentiramea*. Predictive models formalise growth responses of the algae (Çelekli & Yavuzatmaca 2009), and can therefore be used in screening for resilient species using factorial laboratory experiments, and extrapolating this data to large-scale scenarios for biomass production. The CART output was used to extract the most important interactions in a factorial experimental design for each species and to determine seasonal changes in growth. Importantly, environmental interactions are recognized as critical for developing seasonal models (Acerenza & Fort 2005, Filotas et al. 2010, Heaven et al. 2012). The empirical component showed that all variables influence productivity to a certain degree and that both species were tolerant to concurrent and extreme seasonal fluctuations, sustaining high growth in many combinations of factors. Temperature and salinity were the main drivers of seasonal productivity in conditions characteristic of tropical pond-based aquaculture, demonstrating that these factors influence the boom and bust cycles with growth differing by up to 100%. Notably, the interaction of temperature and salinity affected each species differently, and

correspondingly the annual productivity model differed substantially. Estimated productivity for *C. coelothrix* was high across both seasons, and correlated with *in situ* productivity for overlapping environments with previous large-scale experiments (de Paula Silva et al. 2012). In contrast, *C. patentiramea* had substantially lower predicted productivity, particularly in months where conditions were close to the extremes, indicating that not all green tide species will be resilient in tropical aquaculture systems.

The cosmopolitan genus *Cladophora* is an excellent target for integrated aquaculture throughout the globe, as species inhabit marine, brackish and freshwater environments (Thomas et al. 1990, Higgins et al. 2008) from the cold Antarctic waters to tropical waters (Cambridge et al. 1991, McKamey & Amsler 2006). *Cladophora* species are also robust and have adapted to fluctuating regimes of environmental variables. However, there is strong interspecific variation in tolerance to temperature and salinity (Cambridge et al. 1990a, b, Thomas et al. 1990, Breeman et al. 2002), even between populations of a single species (Hayakawa et al. 2012). Similarly, in this study, *C. coelothrix* had a high environmental tolerance to both extremes of temperature and salinity characteristic of tropical systems (i.e. winter/dry and summer/wet seasons). In contrast, *Cladophora patentiramea* was less tolerant to concurrent high temperature (33 °C) and low salinity (17 ‰) in open tropical systems.

Open land-based pond systems are subjected to seasonal environmental fluctuations that are particularly pronounced in monsoonal regions. These environmental variables typically co-vary and complex interactions between extremes conditions may reduce overall tolerance to any single environmental variable (Rivers & Peckol 1995b, Lotze & Worm 2002). This means that tests of interactions between relevant factors to the culture site are critical to managing algal productivity in integrated aquaculture and predicting future responses to changing environments (Lenz-Wiedemann et al. 2010). Furthermore, aquaculture in the tropics is characterized by two distinct production cycles (peak in the wet vs. off peak in the

dry) leading to simultaneous variation in temperature, salinity and nitrogen (concentrations and sources). Dry season (winter) conditions (<28ºC, >32‰) provide the optimal environment for growth, even at the relatively low nitrogen concentrations (35 µM) that reflect off-peak fish production. In contrast, the interaction of high temperature and low salinity in the wet season (austral summer; >30ºC and down to 17‰) leads to reduced growth, even for *C. coelothrix*, which has a broad tolerance to each variable (Cambridge et al. 1987, de Paula Silva et al. 2008). The interactive effect of these variables was particularly marked for *C. patentiramea*, which is clearly reflected in the annual productivity model. This rapid identification of how tolerance may impact productivity is important for species selection, and can be done using laboratory scale experiments if they are combined with robust and flexible data analysis such as CART which can present complex operational scenarios in a simple binary output.

Logistic models are widely used for predicting microalgal growth but have not been broadly applied to integrated macroalgal culture (but see Hernandez et al. 2006, de Paula Silva et al. 2008). However, any model used must be able accommodate complex variances in growth relevant to open pond-based systems (Buonomo et al. 2005). I used CART to partition growth rates into exclusive groups based on *in situ* environmental data in order to develop an annual model for productivity, which enabled us to predict peaks and troughs of biomass production based on large environmental changes. The biomass productivity of *C. coelothrix* was estimated at ~2.2 t ha^{-1} $month^{-1}$ (>20 t ha^{-1} $year^{-1}$) showing strongly correlation with productivities determined *in situ* (reference for this statement) and demonstrating resilience to this particular pond system. Furthermore, the model provides flexibility to introduce negative and positive influences on the growth function, for example, targeting species that have enhanced productivity through inputs of CO_2 for biomass applications. This highlights the

opportunity to channel greenhouse gas emissions into algal production ponds as a source of biomass carbon and for CO_2 sequestration or abatement (Israel et al. 2005).

Biomass applications in integrated aquaculture have to date been focused on bioremediation efficiency and specific value-added products using species that are matched to the system (Chopin et al. 2006). However, many tropical aquaculture systems are extreme and it is not known whether there biomass options for year-round production. I have demonstrated that an annual predictive model can be used to select high-performing and resilient species that are naturally present in many circumstances, but rarely managed. The predictive power of the CART analysis, cross-referenced to *in situ* growth data, can be used to fast-track selection of species such as *C. coelothrix* from the multi-species communities that are present in these dynamic systems. This green tide alga has proved to be a highly resilient and productive species with a remarkable tolerance to distinct climatic season, sustaining reasonable growth rates under stress. Finally, if resilient species of green tide algae such as *C. coelothrix* are developed as a biomass source for bio-products (Bird et al. 2011) and bio-energy (Migliore et al. 2012; Zhou et al. 2010) this provides an additional driver for sustainable aquaculture and a shift towards algal biomass as a sustainable and valuable bioresource.

The world-wide demand for aquatic protein has increased substantially leading to the intensification of aquaculture. Intensively cultured aquaculture species, specifically high value carnivorous marine and brackish species such as barramundi (*Lates calcarifer*) and prawns (*Penaeus monodon*), requires large quantities of high protein (>50 %) formulated feeds (Tacon & Metian 2008). Consequently, nutrient-rich wastewater is a major issue in intensive culture systems which if discharged untreated can cause environmental damage to receiving environments (Sara 2007). Seaweed-based integrated aquaculture, the sequential use of complementary trophic levels species to optimize nutrient use, provides sustainable and efficient bioremediation of nutrient-rich wastewater, in particular dissolved nitrogen derived from high protein formulated feeds (Neori 2008). The majority of scientific research in integrated algal culture has focused on a few seaweed genera with consolidated markets such as human food and as a source of phycocolloids (Chopin and Sawnhey 2009, Pereira et al. 2009). However, many of these traditional high value algal species are not adapted to survive large environmental fluctuations characteristic of tropical pond-based systems, in particular open pond systems which have large fluctuations in temperature and salinity (Mata et al. 2006, Pereira et al. 2006, Abreu et al. 2011). This provides an impetus for the selection of alternative bioremediation species and in particular those species that are both resilient and have high biomass productivities.

Green tide algal species exhibit high growth, efficient nitrogen assimilation and a broad environmental tolerance (Taylor et al. 2001, Cohen & Fong 2004b, 2006, Nelson et al. 2008, Pérez-Mayorga et al. 2011), all of which are sought after characteristics of targeted species for integrated aquaculture. It was unclear however how characteristics unique to tropical land-based aquaculture, such as extremes in environmental parameters associated with

monsoonal activity and alternating operational cycles, will influence growth and nitrogen bioremediation efficiency. Therefore, the aim of this thesis was to provide an understanding of the key limiting factors characteristic of tropical pond-based aquaculture on the growth, and consequently the bioremediation potential, of four filamentous green tide algal species (*Cladophora coelothrix*, *Cladophora patentiramea*, *Chaetomorpha indica*, *Chaetomorpha linum*). These species were selected on their natural occurrence within intensive land-based aquaculture systems. The findings of this study demonstrate that the targeted green tide algae have a broad environmental tolerance, high productivity and bioremediation potential (Chapters 2 and 3). Furthermore, there is potential for the optimization of growth, biomass yield and bioremediation through intensification of culture methods and supplementation of a carbon source (Chapter 4). Finally, these results are used to develop novel predictive models for the selection of resilient and productive green tide algal species that are suitable for large-scale biomass applications in tropical pond-based aquaculture (Chapter 5).

The major findings of this study are:

<u>Chapter 2:</u> Integrating green tide algae into tropical pond-based aquaculture

***C. coelothrix* and *C. indica* have a broad tolerance to salinity and total ammonia nitrogen levels characteristic of tropical pond-based aquaculture. Growth of *C. coelothrix* in particular was high in short-term *in situ* trials, and the growth/harvest model predicted a high biomass productivity and nitrogen removal potential for this species. In contrast, *C. indica* performed well in controlled environment trials, but performed poorly under short-term *in situ* trials.**

Aquaculture in the tropics is characterized by two distinct seasons (wet and dry) which also correlate with aquaculture production cycles (peak in the wet and off-peak in the dry). This leads to a simultaneous fluctuation of two key limiting factors for integrated algal culture,

salinity and nitrogen (TAN), and any targeted algal species must be suitably resilient to the large and rapid changes in these factors. In general, green tide algae have a broad environmental tolerance (Dodds & Gudder 1992, Cohen & Fong 2004b) and correspondingly, *C. coelothrix and C. indica* demonstrated a broad tolerance to salinity and nitrogen levels that are within the spectrum for culture in tropical aquaculture pond-based systems. However, complex interactions between salinity and TAN for each species further demonstrated that responses vary significantly between algal species. These results and those for other green tide algal species confirm that the key factors salinity and nitrogen interact to affect growth (Fong et al. 1996, Kraemer et al. 2004), and these interactions will dictate the suitability of species for tropical pond systems more so than the tolerance to a single limiting factor.

Filamentous green tide algae have been overlooked in integrated aquaculture studies and their bioremediation potential in operational conditions is largely unknown. The high growth and resilience demonstrated in controlled environment experiments provided two species for further investigation on the bioremediation potential *in situ*, and the transfer of the results of small-scale experimental studies into operational systems is the key to species selection. *C. coelothrix* and *C. indica* were therefore cultured in an operational bioremediation pond to determine growth and nitrogen removal potential *in situ*. Although both species had high growth rates in a controlled environment, growth was significantly different in an operational bioremediation pond demonstrating that not all green tide algae are suitable for pond culture despite of their broad environmental tolerance. Transfer from controlled experiments to operational conditions is a recognized constraint in integrated aquaculture studies (Paul & de Nys 2008). *C. coelothrix* in particular had high growth *in situ* (up to 15 % day^{-1}) demonstrating comparable growth (Yokoyama & Ishihi 2010) and higher other green tide algae (Msuya et al. 2006). Bioremediation potential is a function of growth and nitrogen

assimilation and *C. coelothrix* also has a high nitrogen content (2.9%) making it an excellent candidate. A growth/harvest model predicted a maximum productivity of 4 T fresh biomass per hectare of algal culture for each 7-day cycle, with an estimated nitrogen removal of 23 kg N harvest^{-1}. High productivity and bioremediation potential allied with a high environmental tolerance warrant the integration of *C. coelothrix* into tropical pond-based aquaculture. In addition, the harvested algal biomass has potential commercial uses as fertilizers (Bird et al. 2011, Bird et al. 2012) and aquaculture feeds (Tolentino-Pablico et al. 2008), or in bioenergy production (Migliore et al. 2012, Zhou et al. 2012). In contrast, *C. indica* performed poorly in the bioremediation pond, essentially dying after short term *in situ* trials. This bioremediation pond is essentially a marine mesocosm in which bacteria, microalgae and macroalgae co-exist and compete for resources, and similarly there is competitive dominance by species over time (Lotze & Schramm 2000). This was further confirmed by sampling surveys of the floating algal mats within the bioremediation pond, where *C. coelothrix* was the dominant species (> 90 %) in this system.

The results of this chapter highlight the importance of interactions between multiple dynamic biotic and abiotic variables within tropical ponds with salinity and nitrogen being the most weighted in driving growth. Furthermore, the application of functional growth-bioremediation models based on operational aquaculture facilities is a useful tool in understanding large-scale algal bioremediation. Notably, these models can be further developed to incorporate new components for more complex environmental scenarios (Chapter 3).

<u>Chapter 3:</u> Seasonal growth dynamics and resilience of the green tide alga *Cladophora coelothrix* in high-nutrient tropical aquaculture

In a complex seasonal model, based on *in situ* environmental limits, temperature and salinity were the major drivers for algal productivity in land-based tropical ponds. Nitrogen (concentrations and sources) had limited influence on seasonal growth. In winter, where temperature and salinity are close to optimum levels, the *in situ* model determined that growth was primarily influenced by total nitrogen concentration. Notably, nitrogen concentration is ultimately correlated with management of culture within the pond and stocking density.

An annual survey of an operational bioremediation pond confirmed that the key factors of temperature, salinity and nitrogen that limit algal growth co-vary according to season and linked production cycles (nitrogen concentration and sources). This survey identifies the environmental limits to develop a seasonal model for green tide algae growth. This is the first study to develop predictive models for integrated aquaculture at different experimental scales (controlled environment and *in situ*), incorporating robust statistical analysis (CART), environmental and manage variables in levels and scale relevant to operational bioremediation ponds in the tropics. *C. coelothrix* demonstrated high resilience, sustaining growth in virtually all combinations of environmental variables (temperature and salinity) and nitrogen (concentrations and sources) relevant to tropical pond-based aquaculture. The seasonal model determined that temperature is the major driver of seasonal growth and importantly *C. coelothrix* tolerates temperatures up to 35 °C (Cambridge et al. 1987). Nitrogen concentration and salinity also influenced growth, and as determine in Chapter 2 this species is highly resilient to these variables.

More complex interactions were determined by the seasonal growth model, and the interaction between the extremes of temperature and salinity had the greatest effect on *C. coelothrix* growth. Interactions between limiting factors reduces the overal tolerance of resilient species (Xu & Lin 2008). However, in this instance, a high nitrogen concentration buffered the negative effect of high temperature and low salinity, perhaps providing the energy required for osmoregulation and antioxidative defence mechanisms (Kamer & Fong 2001, Luo & Liu 2011). In contrast, nitrogen either as nitrate or ammonium as a single source, was not a key variable for *C. coelothrix* growth in the seasonal model. The ability to take up, assimilate and grow in different forms of nitrogen is characteristic of green tide algae (Cohen & Fong 2004a, Fong et al. 2004, Copertino et al. 2009). The presence of multiple nitrogen sources was detrimental for growth compared to a single nitrogen source, and this contrasting result can be explained by the inhibition of ammonium on the nitrate reductase efficiency and synthesis (Young et al. 2005). However, nitrogen concentration remained more influential than any combination of nitrogen sources for a model developed *in situ*, showing strong correlation with the controlled environment model.

To implement large-scale integrated green tide algal culture it is essential to develop, and validate appropriate models *in situ* that are suitable for up-scaling to annual productivity and bioremediation (Troell et al. 2003). The controlled environment seasonal model was validated in a larger-scale *in situ* experiment over four months in the dry season (austral winter). *C. coelothrix* demonstrated high growth and resilience in this pond system, again in accordance with Chapter 2. Nitrogen concentration was the major variable controlling growth *in situ*, as predicted in the seasonal model given that temperature and salinity are optimum for growth in winter. Adequate nitrogen supply is essential to sustain high algal productivities and it can be a limiting factor even for integrated systems using nutrient-rich wastewater (Matos et al. 2006, Schuenhoff et al. 2006). Nitrogen concentration is a key management

factor for large-scale bioremediation, and therefore needs to be controlled where possible. The *in situ* model showed that providing a uniform nitrogen distribution within the pond is critical for large-scale bioremediation. Furthermore, appropriate stocking density is necessary to achieve high biomass productivities (Demetropoulos & Langdon 2004b), as dense cultures can be limited by low nitrogen concentrations, again in accordance with the *in situ* productivity model.

The results of this chapter clearly demonstrate that *C. coelothrix* is adapted to the seasonal fluctuations of environmental variables of an operational tropical bioremediation pond. The combination of *in situ* and controlled environment models identified the critical combinations of environmental and management variables appropriate for this system. These key variables can then be used to optimize species selection and manage large-scale culture. Furthermore, understanding the capabilities of this dynamic pond system provides a scientific basis to optimize growth through intensification of algal production (Chapter 4).

<u>Chapter 4:</u> Enhanced productivity of green tide algae through additional carbon supply in tank culture

All three green tide algae have an affinity for bicarbonate (HCO_3^-) as carbon source for photosynthesis, demonstrated through high pH compensation points (> 9.7) and a broad pH range for active growth (7 – 9.5). Carbon dioxide (CO_2) enrichment of *C. coelothrix* and *C. linum* cultures enhanced productivity and increased nitrogen and carbon bioremediation potential, despite the demonstrated affinity for bicarbonate (HCO_3^-) as a carbon source. C. *patenti*ramea had the lowest response to CO_2 enrichment in terms of productivity and nitrogen and carbon bioremediation.

Novel applications for algal biomass provide an impetus to develop more intensive culture methods, and this is also the case for green tide algae. Green tide algal biomass has recently

been targeted for the production of renewable energy sources (Ross et al. 2008) and advanced liquid fuels (Zhou et al. 2010, Zhou et al. 2012). The application of the growth model (Chapter 3) can be used on this basis to optimize green tide algae productivity. Intensive seaweed cultivation in integrated systems is normally not limited by nitrogen and phosphorus, but is limited by dissolved inorganic carbon (Mata et al. 2007). Carbon dioxide (CO_2) can be successfully used to supply dissolved inorganic carbon and thereby enhance algal productivity. However, seaweed species that are able to use bicarbonate (HCO_3^-) as a carbon source may not benefit from CO_2 enrichment (Israel & Hophy 2002). In general, green tide algae have evolved carbon concentrating mechanisms to utilize HCO_3^- as a carbon source (Choo et al. 2002). These mechanisms are however diverse and species-specific (Maberly 1990). Therefore the affinity for HCO_3^- was determined for three green tide algal species in this overall research study, *C. coelothrix*, *C. patentiramea* and *C. linum*. In addition, the growth response of these species to the enrichment of dissolved inorganic carbon through the controlled addition of CO_2 was quantified.

Green algal species have the highest affinity for HCO_3^- as carbon source amongst all algal groups, being able to photosynthesize in pH levels as high as 10.8 (Maberly 1990), where CO_2 is absent and HCO_3^- is the only functional carbon source. The green tide algae *C. coelothrix*, *C. patentiramea* and *C. linum* have affinity for bicarbonate (HCO_3^-) being able to raise the culture media pH (compensation point) up to 9.9. All three species also had a broad pH range for active growth (7-9.5). Active photosynthesis at high pH levels is dependent on mechanisms to overcome CO_2 constraints by utilizing the HCO_3^- (Giordano et al. 2005). Two HCO_3^- uptake mechanisms were determined for the green algae tested in this chapter: the extracellular dehydration of HCO_3^- into CO_2 through the periplasmic carbonic anhydrase (CA) enzyme, and the direct uptake of HCO_3^- through the plasma membrane, mediated by an anion exchange protein. The first mechanism is most commonly used and operates in

seawater with pH around 8.3, however, it is inefficient at pH above this level. The second mechanism, the direct transport of HCO_3^- through an anion exchange protein, operates at higher pH levels and was confirmed for all three green tide algae by pH compensation points well above 9. However, the inability of the three species to raise the pH above 9.7-9.9 demonstrates a comparative lower ability to utilize HCO_3^- than other green tide algae species (Choo et al. 2002). These differences in HCO_3^- affinity are strongly related to habitat (Maberly 1990). Algal species can alternate between HCO_3^- uptake mechanisms depending on carbon availability in fluctuating environments (e.g. intertidal) or particular habitats may select genotypes with different carbon acquisition strategies within the same population (Murru & Sandgren 2004).

Despite the affinity for HCO_3^- as carbon source, growth was significantly enhanced though the supply of carbon in the form of CO_2 for *C. coelothrix* and *C. linum*. CO_2 can diffuse through biological membranes providing a comparative energetic advantage over HCO_3^- which requires ATP facilitated process (Raven et al. 2008). Furthermore, these algal species had higher nitrogen content increasing not only productivity, but also bioremediation efficiency. Nitrate reductase production, the enzyme responsible for uptake and assimilation of nitrogen is stimulated in the presence of high CO_2 concentrations (Gordillo et al. 2001). *C. patentiramea* had lower productivity enhancements following CO_2 enrichment compared with the other two green tide algal species, but this restriction is probably more related to morphology than with affinity for HCO_3^- as a carbon source. Furthermore, all species were able to absorb over 90 % of the CO_2 supplied opening opportunity for broader bioremediation applications, for example, integrated algal culture for the bioremediation of greenhouse gases from industrial production (Israel et al. 2005).

In summary, this chapter demonstrated that the supply of carbon in the form of CO_2 can be successfully used to enhance the productivity of green tide algae with operating (CCMs). However, responses are species specific and biological factors such as morphology also plays a role in determining growth response to CO_2 enrichment. These results also provide the opportunity to integrated new optimizing variables, such as dissolved inorganic carbon supply, into large-scale annual bioremediation models (Chapter 5).

<u>Chapter 5:</u> Developing predictive models for optimizing large-scale biomass applications

The annual predictive model was efficient in determining species resilient and seasonal biomass productivity for a tropical pond system. *C. coelothrix* had a broad resilience and a high biomass productivity across the seasonal fluctuations of environmental variables, confirming its suitability for integrated culture in this particular system. In contrast, *C. patentiramea* was not resilient to the extremes of environmental fluctuations which significantly reduced its biomass productivity.

Predictive models for algal culture based on relevant information from operational system can be used to support management decisions and to optimize species selection through controlled experiments. Predictive models are well established for terrestrial (Faria et al. 2010) and algal biomass culture (Lacerda et al. 2011, Kumar & Das 2012). However, the use of predictive models as a functional tool has been established for pond-based integrated aquaculture. Open pond systems are complex environments and therefore difficult to adequately describe and predict interactions between co-varying limiting factors on algal growth. Therefore, the aim of this chapter was to develop simple predictive models for large-scale biomass production and bioremediation of two algal species from the genera *Cladophora*, based upon seasonal gradients of environmental factors from an operational

pond-based system in the tropics. Classification and regression tree analysis(CART) was used in combination with logistic equations to develop an annual predictive model.

The CART analysis partitioned the growth data of *C. coelothrix* and *C. patemtiramea*, in exclusive groups based upon seasonal gradients of environmental factors from an operational pond-based system in the tropics (Chapter 3). Subsequently, growth rates for each month were used as simulation values in logistic regression models. To increase the predictive power of the model the carrying capacity of the pond (Chapter 2) and optimum stocking density (Chapter 3) from field data were incorporated. Finally, to complete the model, the negative influence of multiple nitrogen sources (Chapter 3) and the positive influence of CO_2 (Chapter 4) were applied to the final biomass productivity.

The CART analysis revealed that temperature and salinity are the main environmental drivers for seasonal growth (40 and 37% relative importance), while nitrogen concentration had only a relatively modest influence (3.7%) and the source of nitrogen had no influence in the model. *C. coelothrix* was most resilient to environmental changes, whereas *C. patentiramea* had limited growth at high temperature (33°C) and low salinity (17‰). Based on these results, the logistic model simulations demonstrate that *C. coelothrix* had the highest predicted annual productivity of 24.3 T dry weight ha^{-1} $year^{-1}$, whereas the lower environmental tolerance and overall growth of *C. patentiramea* resulted in a lower annual productivity of 10.8 T dry weight ha^{-1} $year^{-1}$. Annual productivity could potentially be enhanced through CO_2 enrichment to >30 and 12 T dry weight ha^{-1} $year^{-1}$, respectively. Furthermore, the model predicted the peaks and troughs for the culture conditions of each month. *C. coelothrix* had relatively stable productivity across the months, with an estimated average monthly productivity of 2.1 T dry weight ha^{-1} $year^{-1}$ (24 T dry weight ha^{-1} $year^{-1}$) and a maximum productivity of 2.7 T dry weight ha^{-1} in August. These productivities were in agreement with large-scale experiments *in situ*, which determined a nitrogen removal of 23

kg ha^{-1} of cultured algae (140 kg of nitrogen removed annually). In contrast, *C. patentiramea* had more abrupt peaks and troughs, in which productivity was higher in January, April and September (1.7 T dry weight ha^{-1} $year^{-1}$), but decreased substantially in the months with high temperature (~33°C, November and December) and low salinity (~17‰, February and March) to an average of 0.6 T dry weight ha^{-1} $year^{-1}$.

The outcomes of this chapter demonstrate that predictive models developed through the combination of CART analysis and logistic equations are an effective tool for predicting species resilience, annual productivity and bioremediation for complex environmental settings such as tropical pond-based aquaculture. The models presented here were cross-referenced with detailed relevant environmental data from the study site, the resilience of the targeted species to these specific conditions and farm management strategies, and therefore had strong correlation with productivities and bioremediation determined in large-scale experiments *in situ*. Therefore, these models represent a simple approach to establishing effective integrated aquaculture systems based on high-performing and resilient algal species.

In conclusion, this study has provided rigorous scientific evidence that green tide algae can be cultured in tropical land-based ponds with high productivity and nitrogen bioremediation, however, species selection is a key factor for successful outcomes. Growth experiments ranging from controlled through to operational conditions can be used to develop efficient models to optimize species selection and manage large-scale integrated algal culture. These models can be tailored to suit a variety of aquaculture facilities with different effluent characteristics. *C. coelothrix*, in particular, has a broad environmental tolerance, high biomass productivity and nitrogen bioremediation. Furthermore, because harvested algal biomass can be converted into valuable bio-products, there are opportunities to deliver a valuable new integrated aquaculture process. Finally, the results of this thesis support the integration of

green tide algae to promote sustainability not only for the aquaculture industry, but for industrial production as whole.

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