Interactive effects of ocean acidification and declining water quality on tropical seagrass physiology

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Yan Xiang Ow (BSc Hons)

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STATEMENT OF CONTRIBUTION OF OTHERS

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ABSTRACT

Primary productivity is the conversion of inorganic carbon into structural and non-structural carbon (growth and storage) and is thereby the basis of the ecosystem services provided by seagrass meadows. These services include provision of habitat, carbon sequestration, stabilisation and trapping of sediments as well as food for invertebrates, fish, and mega-herbivores. Increased carbon dioxide (CO_2) dissolved in seawater (ocean acidification, OA), can enable seagrass productivity to increase even though they also use HCO_3^- as an alternate form of dissolved inorganic carbon (DIC). However, productivity responses to increasing pCO_2 might be affected by other environmental factors that influence metabolic processes, such as water temperature and local water quality. The lack of empirical evidence for interactive effects of OA and localized impacts (e.g. light, nutrients) hampers our ability to factor these into predictive models and into coastal management decision-making processes. Therefore, in this thesis I aimed to investigate the physiological responses of tropical Great Barrier Reef (GBR) seagrass species to increasing pCO_2 (simulating OA), temperature, and key water quality parameters.

In an initial experiment (Chapter 2), productivity and growth responses of common tropical seagrass species, *Cymodocea serrulata, Halodule uninervis* and *Thalassia hemprichii*, to CO_2 enrichment were quantified. The seagrasses were exposed for two weeks to pCO_2 levels (442 – 1204 μatm) approximating the range of end-of-century emission scenarios. Net productivity and carbon budgets (P_G:R) significantly increased with a rise in pCO_2 in all three species. The degree of productivity rise with pCO_2 was similar across species. While increased productivity in *H. uninervis* and *T. hemprichii* resulted in faster growth from CO_2 enrichment, this was not the case for *C. serrulata*. Varying carbon allocation strategies among species might have contributed to observed differences in growth responses and so internal carbon allocation was further explored in a later experiment (Chapter 5).

When light availability is reduced from declining water quality (e.g. land run-off), preference for utilisation of the DIC species (CO_2 vs HCO_3^-), and therefore response to increasing pCO_2, might be affected. To test this, *C. serrulata* and *H. uninervis* were exposed to two DIC concentrations (447 and 1077 μatm pCO_2), and three light treatments (35, 100 and 380 μmol m^{-2} s^{-1}) for two weeks (Chapter 3). DIC uptake mechanisms were separately examined by measuring net photosynthetic rates while subjecting *C. serrulata* and *H. uninervis* to changes in light and addition of bicarbonate (HCO_3^-) use inhibitor (carbonic anhydrase inhibitor, acetazolamide) and TRIS buffer (pH 8.0). DIC enrichment stimulated maximum photosynthetic rates (P_{max}) more in *C. serrulata* grown under lower light levels (36 – 60% increase) than for those in high light (4% increase) (DIC × light: $P = 0.049$). This was due to *C. serrulata*'s greater dependence on CO_2 in low light. However, this increase due to DIC did not compensate for low light, as net productivity of DIC-enriched plants at low light was 85 – 208 % lower than non-DIC enriched plants growing under high light. In contrast, photosynthetic responses in *H. uninervis* increased with higher light and were independent of the concentrations of the DIC substrates available. *H. uninervis* has more flexible HCO_3^- uptake pathways. Light availability strongly affected productivity and also influenced productivity responses to DIC enrichment, via both carbon fixation and acquisition processes.

Nitrogen availability can limit productivity responses to OA, since nitrogen-derived metabolites are required for carbon assimilation. In Chapter 4, the hypothesis that CO_2 and nitrate enrichment can have additive effects on seagrass productivity and biomass was tested. Nitrogen uptake and assimilation, photosynthesis, growth, and carbon allocation responses of *H. uninervis* and *T. hemprichii* to OA scenarios (428, 734 and 1213 μatm pCO_2) under two nutrient levels (0.3 and 1.9 μM NO_3^-, approximating average GBR flood plume levels) were measured. Net productivity (53 – 78 %) and growth (18 – 52 %) in *H. uninervis* increased with CO_2 enrichment ($P < 0.05$), but were not affected by nitrate enrichment. *T. hemprichii* did not show significant changes with pCO_2 or nitrate by the end of the experiment (24 days) in net productivity and growth. There was no evidence that nitrogen demand increased with pCO_2 enrichment in either species. Overall, nutrient increases to levels approximating flood plumes levels in the GBR only had small effects on seagrass metabolism, and high tissue nutrient

concentrations (2.53 – 2.75 %N) suggest that only small responses occurred because they were not nutrient limited.

Changes in ambient growth temperature can modulate seagrass response to OA by affecting assimilation and utilization of CO_2. Yet the combined effects of temperature and CO_2 enrichment on seagrass carbon metabolism are not known. In Chapter 5, *C. serrulata* and *H. uninervis* were exposed to three temperatures (20°C, 25°C and 30°C, spanning seasonal variation) and three target pCO_2 levels (present day 353 – 485 μatm; high 915 – 1102 μatm; extreme 1658 – 2297 μatm) for seven weeks. Net productivity, biomass allocation and enzyme activity as a proxy for carbon translocation (sucrose-phosphate synthase SPS and sucrose synthase SS) were measured. Net productivity in *C. serrulata* and *H. uninervis* increased by 109 % and 197 % ($P < 0.001$) over the 10ºC rise (20 – 30ºC), respectively. In addition, temperature rise stimulated the increase of above-ground biomass in *C. serrulata* (26 – 35 %; $P = 0.012$) and *H. uninervis* (42 – 88 %; $P = 0.006$). Differences in the allocation of fixed carbon in response to temperature were evident. At warmer temperatures (where net productivity was highest), *C. serrulata* exported more carbohydrates to its rhizomes, while *H. uninervis* increased shoot density. In comparison, responses to CO_2 enrichment were limited to *C. serrulata* increasing above- to- below-ground ratio ($P = 0.003$) and *H. uninervis* increasing net productivity the least at 30ºC (pCO_2 × temperature: $P = 0.047$). This study highlights that temperature exerts a much stronger control over carbon metabolism than CO_2 enrichment in tropical seagrasses.

In summary, the effects of OA on seagrass physiology varied with light availability and water temperature. Ocean acidification cannot fully compensate for productivity losses caused by reduced light. Nitrate fertilization did not enhance seagrass productivity responses to CO_2 enrichment, but it might have indirect impacts encouraging the growth of algae, which can thrive in nutrient and CO_2 enriched conditions. An overall analysis (Chapter 6) of results from all chapters suggests that nutrient status (leaf N content) might be a strong determinant of CO_2 responses, as *C. serrulata* and *H. uninervis* increased productivity at high pCO_2 when their tissue nutrient concentrations were elevated, but not at low tissue nutrient concentrations. Species-specific responses to OA and environmental parameters were consistently demonstrated, warning against the

generalisation of responses across seagrass species. Overall, seagrass productivity can increase under OA, which is likely to make them future "winners" (*sensu.* Fabricius et al 2011) among tropical marine habitats; however localised conditions will affect their response and many of these remain untested.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Seagrass biology

Seagrasses are distributed throughout the shallow (<50 m) coastal and island seas all around the world, except in polar regions (Hemminga and Duarte 2000). Globally, there are 72 seagrass species (Short et al. 2011), comprising a variety of growth forms, ranging from colonising, opportunistic through to persistent (Kilminster et al. 2015). Traits such as shoot turnover and response to disturbance, differ among seagrass species.

Seagrasses can form mono-specific meadows, or multi-species meadows, which may comprise a mix of growth forms (Green and Short 2003). Seagrasses grow through clonal expansion, spreading through shoot proliferation and rhizome extension with shoots emerging from meristems on the rhizome (Den Hartog 1970). They are aquatic angiosperms, which means that they produce flowering structures and either fruits or spathes that contain seeds. They have below-ground parts (rhizomes and roots) that anchor into the sediment (for most species) and which serve to propagate through habitat by clonal expansion. Seagrasses can absorb nutrients from sediment pore-water through their roots, and also access nutrients by direct uptake through leaves from the water column (Romero et al. 2006) (Fig. 1.1). Photosynthesis occurs in the leaves (source tissue), and the sucrose produced is translocated through the plant via a vascular network (described in section 1.6). The complex morphology means that there are multiple sink tissues for the photosynthates including leaves, rhizomes, roots, reproductive structures and storage as non-structural sugars and starch (Larkum et al. 2006).

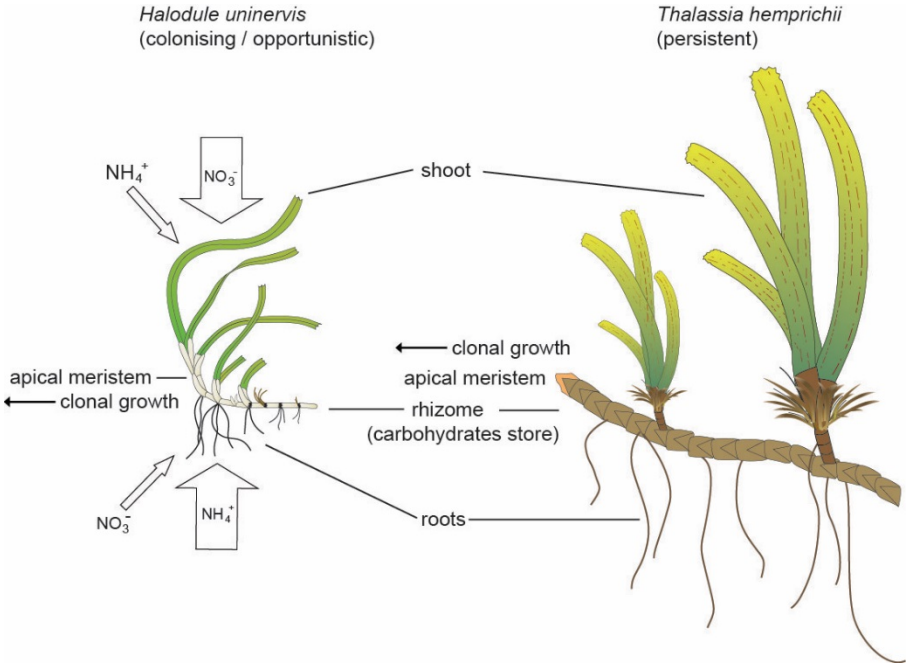

Fig. 1.1: Biology of seagrasses with contrasting growth traits – *Halodule uninervis* and *Thalassia hemprichii*. Clonal growth takes place in the direction of the apical meristem. Arrow size depicts the availability and uptake rates of each nutrient type (ammonium NH_4^+, or nitrate NO_3^-) from the sediment through roots or from the water column through leaves (Romero et al. 2006). Seagrass illustrations taken from Waycott et al. (2004).

1.2 Seagrasses in the Great Barrier Reef, Australia

One of the world's largest areas of seagrass (35,000 km^2) is in the Great Barrier Reef World Heritage Area (GBRWHA), spanning across tropical to sub-tropical regions (Coles et al. 2015) (Fig. 1.2). Fifteen seagrass species, around 20% of the world's total seagrass species, are found in the GBRWHA (Short et al. 2011). All growth forms, ranging from colonising, opportunistic through to persistent (Kilminster et al. 2015), are represented in the GBRWHA.

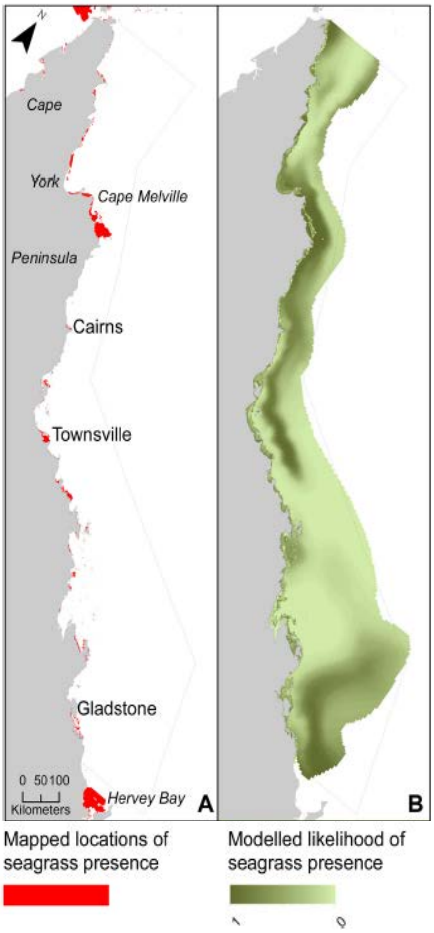

Fig. 1.2: Seagrass distribution in the Great Barrier Reef World Heritage Area. (A) Distribution of mapped seagrass locations in water less than 15 m (red areas). Data from McKenzie et al. (2014b). (B) Modelled likelihood of seagrass (green areas) being found at all depths (McKenzie et al. 2010).

1.3 Ecological importance of seagrasses

Seagrass meadows provide high value ecosystem services (Costanza et al. 1997). Primary production in tropical and sub-tropical seagrass meadows is one of the highest amongst plant communities in the world (Rasheed et al. 2008). An average of 24 – 44 % of this primary production is exported to the open ocean (Duarte and Cebrian 1996; Duarte et al. 2005), while up to 50 % is stored in below-ground tissue and buried within sediments (Duarte and Chiscano 1999). The latter represents 15% of carbon storage in the ocean

despite occupying only 1% of the total oceanic production (Duarte and Chiscano 1999; Duarte et al. 2005), making seagrass meadows hotspots for carbon sequestration (Fourqurean et al. 2012; Macreadie et al. 2014). In addition, seagrasses trap and stabilize the sediments they grow on, thereby buffering against sediment resuspension and erosion (Christiansen et al. 1981; de Boer 2007). Seagrass meadows support biodiversity by serving as foraging and nursery grounds to many species of finfish and shellfish, and marine megafauna such as green turtles and dugongs (Heck et al. 2003).

Ecosystem services provided by seagrass meadows are largely dependent on seagrass productivity. Seagrass productivity, defined in this thesis as the net photosynthetic rate (measured as oxygen evolution) (Silva et al. 2009), is affected by environmental conditions on both global and local scales. Flow-on responses of productivity, such as leaf growth and biomass production, are affected by environmental changes too. Research efforts are still describing seagrass responses to local water quality decline, mainly in terms of light and nutrients (Waycott et al. 2005; Orth et al. 2006; Lee et al. 2007). However, with growing concerns about ocean acidification (OA), which encompasses a global change in seawater chemistry, there is a need to not just understand OA, but how OA will affect other localised conditions (e.g. changes to light, nutrients).

1.4 Ocean acidification: a global change in seawater chemistry

Carbon dioxide (CO_2) emissions have drastically increased since the beginning of the industrial period in the late 18th century, largely due to fossil-fuel burning and large-scale changes in land-use practices (Sabine et al. 2004). To date, the ocean has absorbed about 30% of anthropogenic CO_2 (Sabine et al. 2004). This results in an overall decrease in seawater pH and increase in dissolved inorganic carbon (DIC) concentrations as CO_2 dissociates in water to give protons (H^+) and bicarbonate (HCO_3^-) ions - hence the term "ocean acidification" (OA) (Raven et al. 2005). On the current "business as usual" trajectory, present atmospheric CO_2 of 394 ppm is likely to approach 1000 ppm by the

year 2100 (RCP 8.5), with a projected drop in ocean pH of 0.3 – 0.4 units (Collins et al. 2013).

Dissolved inorganic carbon (DIC) concentration refers to the sum of dissolved carbonate species (CO_2, HCO_3^- and carbonate ions (CO_3^{2-})) in seawater. While OA increases the total DIC available, lowered pH also increases the relative availability of dissolved CO_2 (>250% with a 0.4 pH drop from ~8.2 to 7.8) while reducing the amount of dissolved CO_3^{2-} (>60%) (Gattuso and Hansson 2011; Koch et al. 2013). Changes in seawater carbon chemistry have various implications for different marine species.

Reduced solubility of CO_3^{2-} adversely affects many calcifying organisms (Hoegh-Guldberg et al. 2007; Fabricius et al. 2011). Calcifying organisms, such as foraminifera (Vogel and Uthicke 2012), corals (Albright et al. 2008; Marubini et al. 2008; de Putron et al. 2010) and coralline algae (Kuffner et al. 2007; Russell et al. 2011), demonstrated reduced calcification rates and growth under OA. Consequently, vital ecosystem functions performed by these calcifiers are threatened.

For marine macrophytes, the greater supply of CO_2 may benefit their primary production (Short and Neckles 1999; Koch et al. 2013). In ambient seawater (pH 8.1 – 8.3), dissolved CO_2 only exists as 0.5 – 1 % of the total DIC pool (Gattuso and Hansson 2011). In addition, the diffusion rate of CO_2 is slow in seawater, and is further subjected to resistance from the diffusion boundary layer surrounding photosynthetic surfaces (Maberly et al. 1992; Enriquez and Rodriguez-Roman 2006). A rise in seagrass productivity due to OA might in turn aid in carbon storage (Fourqurean et al. 2012) and modifying seawater pH (Manzello et al. 2012; Unsworth et al. 2012), indirectly modulating adverse OA conditions for coral reefs in close vicinity.

1.5 Declining local water quality

Global seagrass cover has declined at an alarming rate of 7% per year since the 1980s (Waycott et al. 2009). The main causes of the decline were thought to be increases in nutrient and sediment inputs to the coastal zones in both tropical and temperate regions

(Short and Wyllie-Echeverria 1996; Orth et al. 2006). Eutrophication and increased turbidity not only reduce seagrass productivity, they could also lower resilience and the ability to recover from natural destructive events such as cyclones (Orth et al. 2006; Unsworth et al. 2015).

Water quality describes the condition of the marine environment. When the delivery of pollutants, such as sediments, nutrient and organochlorine contaminants (e.g. herbicide and pesticide residues), to coastal areas reduces the "suitability" of the environment for marine organisms to survive in, a decline in water quality has occurred. In the GBRWHA, seagrass meadows are exposed to flood plumes each wet season, though the extent and intensity of water quality decline during these months are highly variable among years (Petus et al. 2014; Devlin et al. 2015) (Fig. 1.3). In the years 2008 to 2011, there was above average rainfall and run-off associated with widespread and unprecedented water quality decline also leading to record dugong and turtle mortality (McKenzie et al. 2015b). A wide variety of environmental parameters have contributed to declining water quality; salinity (Collier et al. 2014), herbicides (Negri et al. 2015), reduced light penetration (Petus et al. 2014) and elevated nutrients, which leads to phytoplankton and algal proliferation (Schaffelke et al. 2005), have been identified as having principal effects on seagrass primary production and biomass accumulation.

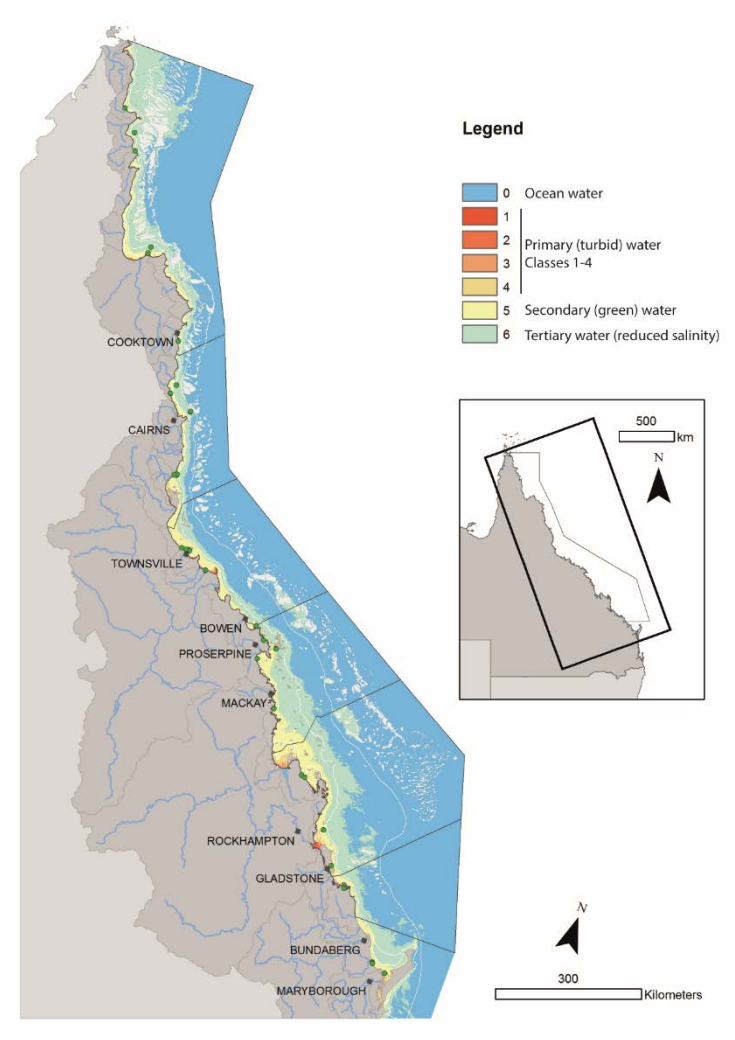

Fig. 1.3: Mean water type during the wet season (December – April from 2003 to 2015) showing that all the mapped seagrass, and much of the modelled deep-water seagrass is, on average, exposed to flood plume water at that time of year (Lønborg et al. 2015). Colour classes 1-4 are turbid primary water, 5 is green secondary water, and 6 has some freshwater influence. Colour classes 1-5 have high light attenuation properties (K_d) and elevated nutrients. Dots show long-term seagrass monitoring sites (McKenzie et al. 2015b).

1.6 Physiological controls of seagrass productivity

Seagrass productivity is affected by a number of environmental conditions. Environmental factors affect various pathways of carbon metabolism (fixation and assimilation) (Fig. 1.4), resulting in corresponding growth and biomass accumulation responses. In my thesis, the focus is on examining the effects of CO_2 enrichment (as a result of OA), temperature, and that of key water quality parameters on seagrass productivity. Figure 1.4 outlines the effect of these environmental factors on physiological processes, in particular, on carbon fixation. The following sections of this introduction will describe the processes outlined in Fig. 1.4 according to: OA, light, nutrients and temperature, which also corresponds to the order of the experimental chapters (2 – 5) of this thesis.

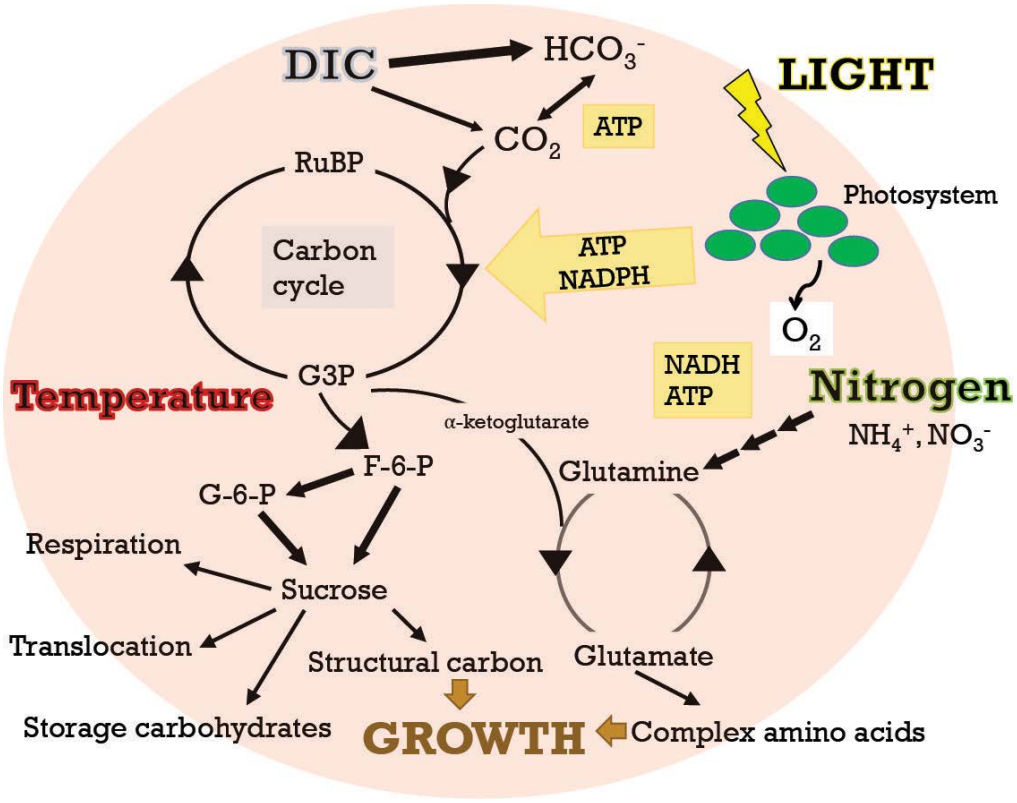

Fig. 1.4: Conceptual diagram of the physiological controls of CO_2 and key water quality parameters (light, nutrients and temperature) on carbon metabolism. CO_2 availability directly affects the carbon cycle, where carbon is fixed into glucose-3-phosphate (G3P). G3P is subsequently converted into sucrose, which is translocated to various organs for respiration, storage and growth. The carbon cycle is also affected by light availability, as the harvesting of light energy in the photosystems provides energy (ATP) and reducing equivalents (NADPH) for the carbon cycle and other downstream processes (Touchette and Burkholder 2000a). The assimilation of fixed carbon (G3P) into amino acids for growth requires nitrogen (Stitt and Krapp 1999). Nitrogen, in ammonium and nitrate forms, is taken up and converted into glutamine. G3P is converted to α-ketoglutarate for incorporation with glutamine to form glutamate, which is a pre-cursor for complex amino acids. Temperature plays an over-riding influence on carbon metabolism, as it regulates enzyme reaction rates of all processes, from light harvesting, carbon fixation to carbon assimilation (Sage and Kubien 2007).

1.6.1 Effects of OA (CO_2 enrichment)

Seagrass photosynthesis can be carbon-limited (Beer and Koch 1996; Thom 1996; Zimmerman et al. 1997; Jiang et al. 2010) due to the low availability and slow diffusion rates of dissolved CO_2 in ambient seawater (pH 8.1 – 8.3) (Gattuso and Hansson 2011). While many seagrasses are able to utilize HCO_3^- (~90 % of DIC pool in pH 8.1 – 8.3) in addition to CO_2, seagrasses generally exhibit greater preference for CO_2 than HCO_3^- (Durako 1993; Beer and Koch 1996; Invers et al. 2001). Acquisition of HCO_3^- involves the dehydration and transport of HCO_3^- into plant cells (Hellblom et al. 2001; Beer et al. 2002). Of the various HCO_3^- utilization pathways proposed, a number involves the active extrusion of protons (H^+) to drive the uptake of HCO_3^- (Bjork et al. 1997; Beer et al. 2002; Hellblom and Axelsson 2003). Active extrusion of H^+ is energetically costly compared to passive CO_2 uptake, and may be limited by low light availability (Burnell et al. 2014a).

Most seagrasses fix carbon via the C3 cycle (Koch et al. 2013). This means that elevated partial pressure of CO_2 (pCO_2) can help increase carboxylation and reduce oxygenation (photorespiration) of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), the initial carboxylating enzyme in C3 plants (Fig. 1.4) (Bowes and Ogren 1972; Koch et al. 2013). Hence, higher pCO_2 contributes to higher net photosynthesis (Long et al. 2004).

Short- to mid- term experiments have demonstrated CO_2 enrichment-induced responses in seagrasses such as increased photosynthesis, growth, leaf sugar contents, tissue C/N ratio, reproductive output and biomass ratio (Thom 1996; Zimmerman et al. 1997; Palacios and Zimmerman 2007; Jiang et al. 2010; Alexandre et al. 2012). Observations of long-term exposure around underwater CO_2 vents supported the experimental results, showing enhanced seagrass productivity and shoot density relative to adjacent control sites (Hall-Spencer et al. 2008; Fabricius et al. 2011; Apostolaki et al. 2014; Takahashi et al. 2015). Higher production could potentially improve the resilience of seagrasses, through expansion of areal cover, an increase in biomass (Palacios and Zimmerman 2007), and a reduction of light requirements (Zimmerman et al. 1997; Invers et al. 2001).

1.6.2 Effects of light

Light availability strongly influences photosynthesis and other processes in carbon metabolism. Essentially, light harvesting in photosystems provides energy in the form of adenosine triphosphate (ATP) and reducing equivalents (NADPH) for both the acquisition and fixation of carbon (Fig. 1.4) (Touchette and Burkholder 2000a).

Photosynthetic rates change with light availability in a manner that is described as a hyperbolic tangent (Jassby and Platt 1976). At low limiting irradiances, photosynthetic rates increase linearly with light availability. Beyond a certain light level, i.e. saturating irradiance (E_k), light-harvesting capacity is saturated and photosynthetic rates level off at the maximum photosynthetic rate (P_{max}).

Light intensity, when below saturating levels, influences the efficiency and rates of carbon fixation in seagrasses (Ralph et al. 2007). When photosynthesis is light-limited, the demand for DIC may be reduced (Durako and Hall 1992). Hence, limiting light levels should reduce seagrass response to OA. However, a study by Schwarz et al. (2000) revealed that the deep-water (10 – 12 m) seagrass species *Halophila ovalis* and *Cymodocea serrulata* exhibited a greater increase in relative electron transport rates (ETRs) than their intertidal conspecifics when exposed to increased DIC. The authors posited that low light adapted seagrasses may benefit more from CO_2 enrichment, since energetic requirements of HCO_3^- use meant that they have to be more dependent on CO_2.

Growth responses in seagrasses to CO_2 enrichment under low light conditions have been variable, ranging from no change in growth rates in *Zostera marina* (Palacios and Zimmerman 2007), to increased growth in *Amphibolis antarctica* (Burnell et al. 2014b) and *Thalassia hemprichii* (Liu et al. 2005). The interaction of light and CO_2 or DIC availability on seagrass productivity has only been explored in a few studies, and on a limited number of seagrass species (6 species) (Zimmerman et al. 1997; Schwarz et al. 2000; Mercado et al. 2003; Liu et al. 2005; Palacios and Zimmerman 2007; Burnell et al. 2014b).

1.6.3 Effects of nitrogen enrichment

Seagrass meadows can be subjected to fluctuations in water column nutrient levels (Romero et al. 2006). Moderate nutrient enrichment can benefit seagrass productivity and promote growth (Udy et al. 1999; Lee and Dunton 2000; Mellors 2003). Documented responses to nutrient fertilization included increased photosynthesis, plant biomass and tissue nitrogen content (Agawin et al. 1996; Udy and Dennison 1997; Lee and Dunton 2000). However, the increase in dissolved nutrients simultaneously enhanced the growth of other marine macrophytes, such as phytoplankton and seagrass epiphytes. These marine macrophytes compete for the same resources (light, nutrients, DIC) with seagrasses. Beyond a certain threshold, high nutrient enrichment may therefore lead to a decline in seagrass biomass and productivity due to the proliferation of algae and epiphytes (Burkholder et al. 2007).

Nitrogen availability can affect responses to elevated CO_2, as carbon and nitrogen metabolism are strongly coupled (Touchette and Burkholder 2007). Nitrate and nitrogen metabolites are needed to produce glutamine for the assimilation of fixed carbon into amino acids (Fig. 1.4). In doing so, end-product (sucrose) inhibition of photosynthesis can be avoided (Stitt and Krapp 1999). Carbon metabolic processes such as photosynthesis (Nielsen et al. 1998), organic acid synthesis and starch accumulation (Scheible et al. 1997) are also regulated by nitrogen metabolites. In turn, energy stored in carbon metabolites is essential for nitrogen uptake and incorporation (Touchette and Burkholder 2000b). In terrestrial plants, productivity initially increases with CO_2 enrichment, but is subsequently downregulated as nitrogen becomes limited (Stitt and Krapp 1999). In marine macroalgae, productivity responses to CO_2 enrichment were only enhanced with nutrient enrichment (Gordillo et al. 2003; Russell et al. 2009; Hofmann et al. 2014). Therefore, nitrogen enrichment could potentially augment CO_2 responses in seagrasses, bringing enhanced productivity while preventing nutrient imbalance in leaf tissue under OA conditions.

1.6.4 Effects of temperature

Water temperature affects both carbon fixation and carbon utilisation among various sinks in seagrasses (Fig. 1.4) (Touchette and Burkholder 2000a). In plants, net productivity responses to temperature follows an optimum curve pattern (Fig. 1.5) (Marsh et al. 1986; Perez and Romero 1992; Masini and Manning 1997). Below the thermal optima, photosynthetic rates increase with temperature due to higher Rubisco efficiency and capacity (Masini and Manning 1997; Sage and Kubien 2007). Enhanced Rubisco activity due to warmer temperatures also increases photorespiration in terrestrial C3 plants, but a greater increase in carboxylation could compensate for photo-respiratory losses, resulting in an overall increase in net photosynthesis (Sage and Kubien 2007). Beyond the thermal optima, reduced electron transport capacity in the photosystems might decrease photosynthesis (Sage and Kubien 2007). Furthermore, photo-respiratory losses (in the chloroplasts) and Krebs cycle activity (in the mitochondria) continue to rise with temperature. This results in respiratory demands increasingly exceeding that of photosynthetic production, thus reducing net productivity (Marsh et al. 1986; Perez and Romero 1992; Masini and Manning 1997). Reductions in growth and carbohydrate reserves could occur, as apparent in some seagrasses during summer months of seasonally high water temperatures (Marsh et al. 1986; Lee et al. 2007).

In Fig. 1.5, I have assembled all data on photosynthetic responses to increasing temperature (from ISI database prior to 2014 (Appendix 1)). These are presented relative to ambient growth temperature, which demonstrates that most seagrasses have thermal optima that are ~6ºC greater than their ambient growth temperature. Hence, seagrasses are relatively tolerant to small increases in water temperature. Optimal growth temperatures of tropical and subtropical species were 23 to 32 °C (Lee et al. 2007). However, there are few studies that have contributed information on responses to temperature at or below ambient growth temperature for tropical species (Fig 1.5). In addition, it is known that tropical terrestrial plants can acclimate to high temperatures by lowering their carbon losses to respiration as their environment warms up (Tjoelker et al. 2001). Thermal acclimation could result in metabolic responses deviating from classical Q_{10} relationships between increases in enzymatic and other metabolic processes with temperature rise (Tjoelker et al. 2001). However, such information on thermal acclimation in seagrasses is lacking in current literature.

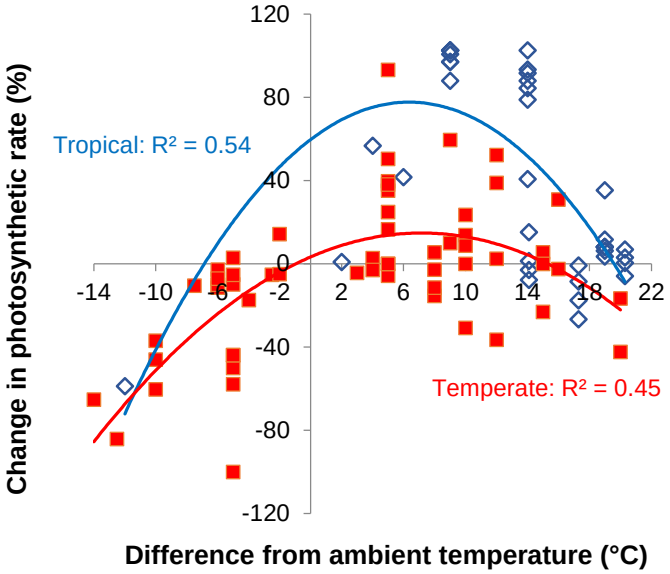

Fig. 1.5: The percentage change in photosynthetic rate as a function of temperature difference from ambient growth temperature. Data compiled from 15 studies, where ambient growth temperature was defined in each study (Appendix 1). Studies were published from 1986 to 2014, and covered both temperate (red squares) and tropical species (blue diamonds). R^2 of the fitted polynomial curves for tropical and temperate studies were given.

Seagrass responses to CO_2 enrichment could depend on the water temperature. Due to climate change, seawater temperature is projected to warm by 3 – 4ºC by end of the century (Koch et al. 2013). Seawater temperatures also vary with the seasons. Under cooler temperatures, where photosynthetic and growth rates are lower, CO_2 responses might be limited by the lowered carbon demand. In summer months that encompass the optimal growth temperature range, higher photosynthetic and growth demands could bolster responses to higher CO_2. Alternatively, enhanced photosynthetic rates (electron transport rates) and sucrose/starch synthesis with higher CO_2 availability might help support higher respiratory demands (Farrar and Williams 1991; Sage and Kubien 2007). Quantifying the interactive effects of temperature and OA on tropical seagrasses is essential to understanding what may happen in future more acidic seas.

1.7 Knowledge gaps

Seagrass productivity might benefit with OA in the future. However, local scale changes in temperature and water quality (e.g. turbidity, nutrients) could influence productivity responses to CO_2 enrichment. While effects of environmental factors on seagrasses have been documented in various separate single-factor studies, interactive effects of OA and local stressors have received little research attention, even though environmental stressors rarely occur in isolation. Possible interactive effects of OA (CO_2 enrichment) and key environmental parameters, such as temperature, light and nutrients, on seagrass productivity, need to be investigated to provide a more realistic prediction of seagrass response under future OA.

Around 60% of the studies that investigated OA effects on seagrasses were conducted on temperate species. Differences in CO_2 responses between temperate and tropical species are expected, mainly due to environmental differences between localities. Tropical regions tend to be warmer with a smaller seasonal temperature variation (Hobday and Lough 2011). Evolutionary and/or environmental history of tropical seagrasses could distinguish their CO_2 response from that of temperate seagrasses; however, this needs to be verified using empirical studies.

Among tropical seagrass species, there are differences in carbon uptake systems (Uku et al. 2005; Beer et al. 2006), growth rates and resource allocation strategies (Kilminster et al. 2015). Therefore, responses to OA may even differ between species in a multi-species seagrass meadow (Invers et al. 2001; Campbell and Fourqurean 2013b; Borum et al. 2015). To evaluate whether OA effects will vary among tropical species, the CO_2 responses of various tropical species need to be characterized.

1.8 Thesis aim and overview

The overarching aim of this PhD project is to investigate the responses of tropical seagrass species to OA in combination with key water quality parameters. A series of experiments, each documented in a chapter, were conducted to examine the interactive effects of water quality parameters with DIC / pCO_2 levels. DIC levels, as well as key water quality drivers (light, nutrients and temperature) were manipulated to simulate future predicted changes in climate and local environmental conditions.

In Chapter 2, the physiological responses of three tropical seagrass (*C. serrulata*, *H. uninervis* and *T. hemprichii*) to increased pCO_2 were quantified. I hypothesized that pCO_2 enrichment would increase photosynthetic and growth rates, but rate of responses may vary among species due to varying carbon uptake and allocation strategies (Campbell and Fourqurean 2013b).

Effects of light availability on carbon utilisation in *C. serrulata* and *H. uninervis* were investigated in Chapter 3. Photosynthetic (derived from photosynthesis-irradiance curves) and growth parameters, and productivity responses to carbon uptake inhibitors were used to evaluate relative changes in photosynthetic efficiency and light requirements in response to DIC levels. It was hypothesized that while DIC enrichment will increase photosynthesis and growth of seagrasses, the extent of increase will be greater under lower light levels. To further understand the influence of light availability on HCO_3^- use in tropical seagrasses, I tested the hypothesis that under limiting light availability, the ability to use HCO_3^- as a carbon substrate would be reduced (Spalding and Ogren 1982; Kubler and Raven 1995).

Chapter 4 documents the effects of nitrogen (NO_3^-) enrichment on CO_2 responses in *H. uninervis* and *T. hemprichii*. I hypothesized that firstly, pCO_2 and nitrate enrichment would have additive effects on seagrass productivity and biomass; secondly, pCO_2 enrichment would drive nitrogen demand. I examined species with different growth and storage strategies, the fast-growing species *Halodule uninervis* and the slow-growing species *Thalassia hemprichii* (Kilminster et al. 2015). Productivity, growth, nitrogen

uptake and assimilation were quantified to assess the effects of CO_2 and nitrate enrichment on seagrass.

In Chapter 5, the effects of seasonal temperature variation on carbon partitioning, under various CO_2 levels, were assessed. The hypothesis that CO_2 responses depend on ambient growth temperature was tested. Carbon metabolism (photosynthesis and respiration) and carbon partitioning processes (growth, biomass allocation etc.) in *C. serrulata* and *H. uninervis* were assessed and discussed in relation to growth strategies.

Finally in Chapter 6, the results of this study are synthesized and discussed. Experiments completed in this thesis are compared to each other, and with published studies to help identify key influential parameters and reconcile differing results among experiments. The chapter concludes with the implications of the findings on seagrasses and the ecological functions they provide under a changing climate.

This is the first study to measure productivity responses of the common tropical seagrass species *H. uninervis*. This is also the first study to explore interactive effects of OA and some water quality parameters on seagrasses in northern Australia. While the study was undertaken mainly on GBR seagrasses, methods and data developed in this thesis could be applied to seagrasses in the same bioregion (Indo-Pacific), which contains a high diversity of seagrasses (Short et al. 2011). Results could also be compared to studies beyond the region to elucidate locality-specific variation in responses. Overall, this research provides baseline data for undertaking further work on OA effects on tropical seagrasses. This is essential to develop a holistic framework to understand and manage future changes in seagrass meadows.

CHAPTER 2

RESPONSES OF THREE TROPICAL SEAGRASS SPECIES TO CO_2 ENRICHMENT [1]

Abstract

Increased atmospheric carbon dioxide leads to ocean acidification (OA) and carbon dioxide (CO_2) enrichment of seawater. Given the important ecological functions of seagrass meadows, understanding their responses to CO_2 will be critical for the management of coastal ecosystems. This study examined the physiological responses of three tropical seagrasses to a range of seawater pCO_2 levels in a laboratory. *Cymodocea serrulata*, *Halodule uninervis* and *Thalassia hemprichii* were exposed for two weeks to four different pCO_2 treatments, ranging from 442 – 1204 μatm, approximating the range of end-of-century emission scenarios. Photosynthetic responses were quantified using optode-based oxygen flux measurements. Across all three species, net productivity and energetic surplus (P_G:R) significantly increased with a rise in pCO_2 (linear models, $P < 0.05$). Photosynthetic-irradiance curve-derived photosynthetic parameters - maximum photosynthetic rates (P_{max}) and efficiency (α) - also increased as pCO_2 increased (linear models, $P < 0.05$). The response for productivity measures was similar across species, i.e. similar slopes in linear models. A decrease in compensation light requirement (E_c) with increasing pCO_2 was evident in *C. serrulata* and *H. uninervis*, but not in *T. hemprichii*. Despite higher productivity with pCO_2 enrichment, leaf growth rates in *C. serrulata* did not increase, while those in *H. uninervis* and *T. hemprichii* significantly increased with increasing pCO_2 levels. While seagrasses can be carbon limited and productivity can respond positively to CO_2 enrichment, varying carbon allocation strategies among

[1] Chapter 2 is adapted from Ow Y.X., Collier C.J. and Uthicke S. (2015) Responses of three tropical seagrass species to CO_2 enrichment. Marine Biology. 162: 1005-1017

species suggest differential species-specific growth responses. Thus, future increases in seawater CO_2 concentration may lead to an overall increase in seagrass biomass and productivity, as well as community changes in seagrass meadows.

2.1 Introduction

Anthropogenic carbon emissions have led to atmospheric carbon dioxide (CO_2) rising by 40% since pre-industrial times (Raven et al. 2005). By the end of this century, atmospheric CO_2 is predicted to double from current levels (Meehl et al. 2007; Collins et al. 2013). The rise in oceanic CO_2 concentration that follows is projected to decrease seawater pH by 0.3–0.4 units (Caldeira and Wickett 2003; Feely et al. 2004). This reduction can alter the carbonate chemistry of seawater in terms of the relative proportions of the dissolved inorganic carbon (DIC) species. Current concentrations of CO_2 and bicarbonate (HCO_3^-) in seawater are 8 and 1650 μmol kg^{-1} seawater respectively (Koch et al. 2013). Under the projected decrease in seawater pH, the proportion of CO_2 will have a greater proportional increase (>250%) than the other DIC constituents (HCO_3^-: 24% and carbonate CO_3^{2-}: -61%) (Koch et al. 2013). The higher concentration of utilisable carbon for photosynthesis (CO_2 and HCO_3^-) in acidified seawater may benefit marine macrophytes which are limited by the DIC concentration under current conditions (Beer et al. 2002).

Seagrasses can be carbon-limited at the seawater DIC composition under current CO_2 concentrations, given other conditions, such as light, nutrient availability and water temperature are non-limiting (Beer and Koch 1996; Thom 1996; Zimmerman et al. 1997; Invers et al. 2001). Most seagrasses utilise the C3 metabolism for carbon fixation (Koch et al. 2013). Elevated partial pressure of CO_2 (pCO_2) can increase carboxylation rates while reducing oxygenation rates of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), the initial carboxylating enzyme in C3 plants (Bowes and Ogren 1972; Koch et al. 2013). Furthermore, the predominant DIC species, HCO_3^-, appears to be less efficiently utilised in seagrasses – the increase in photosynthetic rates was much higher when seagrasses were enriched with CO_2 than with HCO_3^- (Sand-Jensen and Gordon

1984; Durako 1993; Beer and Koch 1996; Invers et al. 2001). Although seagrasses possess carbon concentrating mechanisms (CCMs) to more efficiently utilise HCO_3^-, whether these CCMs could effectively saturate the seagrasses to meet their DIC requirements under natural conditions is presently unknown (Beer et al. 2002; Koch et al. 2013). Overall, it is thought that higher pCO_2 not only increases passive diffusion of CO_2 for carbon fixation, but also lowers the loss of fixed carbon through photorespiration (Long et al. 2004). Laboratory and mesocosm experiments conducted over the short- and medium-term have shown an optimisation of photosynthetic performance, such as light requirements, photosynthetic efficiency and pigment content in response to CO_2 enrichment (Zimmerman et al. 1997; Jiang et al. 2010; Campbell and Fourqurean 2013b). This can result in higher rates of carbon fixation with flow-on effects to growth rate, carbohydrate content, biomass and reproductive output (Zimmerman et al. 1997; Jiang et al. 2010; Campbell and Fourqurean 2013b). In the field, higher seagrass productivity and biomass have been observed near natural CO_2 vents, suggesting that acidification of seawater may benefit seagrass meadow productivity in the long-term (Hall-Spencer et al. 2008; Fabricius et al. 2011; Russell et al. 2013).

Different seagrass species might vary in the manner and extent to which they respond to CO_2 enrichment. No previous studies have directly compared species responses to CO_2 enrichment, but responses to CO_2 depletion indicate that species are not affected uniformly by changing pCO_2 (Invers et al. 1997; Beer et al. 2006). This makes it difficult to determine whether findings are related to species or methodological differences. Most studies had focussed on temperate species, such as *Zostera marina* (Thom 1996; Zimmerman et al. 1997; Palacios and Zimmerman 2007), *Zostera noltii* (Alexandre et al. 2012), and *Posidonia oceanica* (Invers et al. 2002). Among temperate species, Invers et al (2001) demonstrated that pCO_2 enhancement of photosynthesis was higher in Pacific species (*Z. marina* and *Phyllospadix torreyi*) than in Mediterranean species (*P. oceanica* and *Cymodocea nodosa*). The few studies on tropical seagrasses yielded mixed results. For example, Jiang et al. (2010) showed increased growth and productivity in *T. hemprichii*, while *T. testudinum* showed little change in biomass and productivity to increased pCO_2 (Durako and Sackett 1993; Campbell and Fourqurean

2013a). Hence, differential response to CO_2 enrichment might exist between and within multi-species tropical seagrass meadows.

Differences in carbon utilisation and allocation strategies exist among tropical seagrass species (Hemminga and Duarte 2000; Uku et al. 2005). Species-specific differences in DIC uptake mechanisms would result in varying abilities among species to utilise the extra DIC (Invers et al. 2001; Uku et al. 2005; Campbell and Fourqurean 2013b). Species-specific carbon allocation strategies could affect how responses to CO_2 enrichment manifest at the plant scale. For example, in species that invest a greater proportion of biomass to below-ground tissue, such as *Halodule uninervis* and *Thalassia hemprichii*, there would be a higher metabolic demand on above-ground tissue for photosynthetic carbon fixation (Terrados et al. 1999; Hemminga and Duarte 2000; Tanaka and Nakaoka 2007). Increased availability of CO_2 in seawater could either allow for increasing photosynthetic capacity (e.g. more chlorophyll pigments, enhanced shoot growth), and/or increased storage of carbohydrates to support respiratory demands (Zimmerman et al. 1997; Jiang et al. 2010). In addition, small-bodied ephemeral species, such as *Halodule uninervis*, exhibit short turnover of leaves while bigger and more persistent species like *Cymodocea serrulata* and *Thalassia hemprichii* have longer shoot plastochrone intervals (Hemminga and Duarte 2000). Turnover rates of assimilated carbon could influence carbon demand (Arp 1991; Hemminga and Duarte 2000). Thus, various measures of productivity, such as tissue growth rates, carbohydrates storage or shoot production could vary among co-occurring species in response to CO_2 over different time scales.

Productivity of seagrass meadows is central to their ecological functions as a food source, including for megafauna such as dugongs and turtles, in bio-sequestration ("blue-carbon"), and substrate stabilisation (Duarte and Chiscano 1999; Gacia and Duarte 2001; Fourqurean et al. 2012; Vafeiadou et al. 2013). Understanding how productivity responses to CO_2 enrichment vary among species is vital for predicting future ecological change. In the present study, we quantified the photosynthetic and growth responses of three tropical seagrass species to increasing pCO_2 levels, bracketing the range of different end-of-century emission scenarios predicted (Collins et al. 2013). This allows for the quantification of the response to pCO_2 levels in seagrass productivity and growth. The three species examined, *Halodule uninervis*, *Cymodocea serrulata* and *Thalassia*

hemprichii, are common seagrasses found in the tropical Indo-Pacific region with contrasting growth strategies, ranging from rapid growth in *H. uninervis* to slow growth in *C. serrulata* and *T. hemprichii* (Hemminga and Duarte 2000). It was hypothesized that pCO_2 enrichment would increase photosynthetic and growth rates, but rate of responses may vary among species due to varying carbon uptake and allocation strategies (Campbell and Fourqurean 2013b).

2.2 Materials and Methods

2.2.1 Experimental species

Seagrasses were collected two to four weeks prior to the start of the experiment. Seagrass species *Cymodocea serrulata* and *Halodule uninervis* were collected from the intertidal meadow at Cockle Bay, Magnetic Island, Northern Great Barrier Reef (19°10.88'S, 146°50.63'E) in March 2013. Average daily and average maximum photosynthetically active radiation (PAR) at this site were 385 μmol m^{-2} s^{-1} and 961 μmol m^{-2} s^{-1}, respectively (Collier C, unpublished). Intact plugs of *H. uninervis* and sediment were collected with a trowel and placed into a plastic pot lined with a plastic bag. The bag was pulled up and secured over the seagrass to prevent moisture loss during transport. *C. serrulata* was collected by excavating intact shoots with connected horizontal rhizomes from the sediment before placing into seawater-filled containers for transport to aquaria. *Thalassia hemprichii* was collected from Green Island in the Northern Great Barrier Reef (16°45.37'S, 145°58.19'E), using a similar method to *C. serrulata*. At this site, average daily PAR was 344 μmol m^{-2} s^{-1} and average maximum PAR was 841 μmol m^{-2} s^{-1}, respectively (Collier C, unpubl. data). Average water temperatures at Cockle Bay (2005 – 2012) and Green Island (2003 – 2012) were 26.2ºC and 26.6ºC respectively (McKenzie et al. 2014a). Seagrasses were planted into orchid pots lined with a pool filter sock, in a mud and sand (roughly 20:80) mixture, within two days of collection. For acclimation, all species were kept in an outdoor flow-through aquarium prior to the experiment, under

average light levels of 350 μmol m^{-2} s^{-1}, average seawater temperature 25°C and salinity at 35 ppt.

2.2.2 Experimental setup

Seagrasses were exposed to four different seawater pCO_2 concentrations in a flow-through system for two weeks (Table 2.1). The experiment was conducted in an indoor flow-through aquarium system at the Australian Institute of Marine Sciences, Townsville. Sixteen glass aquaria with four replicates for each treatment (working volume 18 l) were supplied with fresh filtered seawater from four header tanks. Each aquarium contained all three species. Two sub-replicate pots of each species were placed in each aquarium. pH levels in the header tanks were monitored, as a proxy to control for CO_2 input, with eight potentiometric sensors (±0.01 pH unit) calibrated on the NBS scale. The sensors are connected to a feedback control system that regulates pH levels via a CO_2 gas injection system (AquaMedic, Germany). Pumps and diffusers installed in mixing tanks and experimental aquaria ensured thorough mixing of CO_2. Additional pH readings were taken regularly with a hand held pH probe (pH probe: Eutech, USA; console: Oakton, USA) and compared to Tris seawater standards (Batch 10, Supplied by A. Dixon, Scripps Institute of Oceanography). Water temperature remained constant throughout the experiment around 24°C (Table 2.1). Water samples, taken every five days, were analysed for dissolved inorganic carbon (DIC) and total alkalinity (AT) concentrations using a Vindta 3C analyser. Carbonate system parameters (Table 2.1) were calculated by measured values of AT, DIC, temperature and salinity using USGS CO2calc software (Robbins et al. 2010). Illumination was provided with LED lamps (Aqua Illumination) mounted about 40 cm above the aquaria, providing 400 μmol m^{-2} s^{-1} of light set on a 12 hour light:dark photoperiod. Duplicate water samples collected from each individual aquaria every five days were filtered (0.45 μm pore size) before being analysed for dissolved inorganic nitrogen and phosphorus concentration according to Ryle et al. (1981).

2.2.3 Photosynthetic response

Photosynthetic rates and respiration of the second youngest leaf (rank 2) of a haphazardly chosen shoot from each pot were measured using optical oxygen sensors ("optode", PreSens, Sensor spots-Pst3) and a PreSens Oxy 4 four-channel fiber-optic oxygen meter after two weeks. While the authors acknowledge that seagrasses could be sensitive to physical manipulations such as removing leaves (Schwarz et al. 2000), care was taken to reduce the impact on leaves such as using the whole leaf and gently rubbing epiphytes off with fingers instead of scrapping with a blade. Small transparent acrylic chambers (200 mL) were set in an array of four (i.e. four separate chambers allowing four parallel measures) and incubated at 25°C water temperature using a flow-through water system connected to a water bath (Lauda, Ecoline RE 106). Stirrer bars placed within the chambers provided even stirring. The leaves were held upright in the chamber to mimic natural orientation. Oxygen consumption (dark respiration) was measured over a 20-min period in the dark. Photosynthetic rates were then measured on the same leaf over a series of light steps (10, 30, 70, 110, 220, 400, 510 μmol m^{-2} s^{-1}) (Aqua Illumination LED), with each light step lasting 20 minutes. Illumination, measured with a PAR quantum sensor (Apogee, USA) was provided from the top of the incubation chambers. Seawater within the chambers was replaced with fresh media every two to three steps. Oxygen concentration data in the chambers were logged every 5s, and respiration and production rates were calculated by fitting a linear regression. Rates were normalised to the dry weight of the leaf. Leaves were dried at 60°C for 48 hours before weighing. Initial periods of incubation (~5 min) prior to stabilization of photosynthetic rates were omitted from regressions. Each optode was calibrated according to Collier et al. (2011).

Net productivity (NP) was taken to be the photosynthetic rate measured at 400 μmol m^{-2} s^{-1}, which was the experimental light level. Energetic surplus (P_G:R) was calculated as the ratio of gross productivity (sum of net photosynthetic rate and dark respiration rate) to dark respiration rate (Zimmerman et al. 1997). To determine photosynthetic parameters, photosynthesis versus irradiance (P-E) data plots were fitted to the adapted hyperbolic tangent model equation of (Jassby and Platt 1976):

$$P = P_{max} \times \tanh(\frac{\alpha P_{max}}{E})$$

where P_{max} is the maximal photosynthetic rate (mg O_2 g^{-1} DW h^{-1}), E is irradiance (μmol m^{-2} s^{-1}), and α described photosynthetic efficiency via the gradient of the curve at limiting irradiances (mg O_2 μmol^{-1}). Saturating irradiance (E_k) is the light level at which photosynthesis initially reaches the maximum rate, and compensation irradiance (E_c) is the light level when photosynthetic rate is equal to respiration rate.

2.2.4 Determination of growth rates

Growth was measured following Short and Duarte (2001). All shoots from each pot were marked at the top of the sheath with a needle at the start of the experiment. At the end of the experiment, the shoots were harvested. The length of new tissue growth was excised, dried at 60°C for 48 hours and weighed for determination of weight of new leaf growth. Leaf tissue growth was normalised to the above-ground biomass of its respective pot to derive relative leaf growth rates (RGR).

Specific leaf area (SLA) was calculated from biomass and areal measurements of leaves. Specific leaf area refers to the total leaf area normalised by the total biomass of the leaves and could be used to infer whole-plant changes in leaf biomass and area in response to pCO_2 enrichment (Chiariello et al. 2000). Leaves were separated from shoots and placed on a flat surface. Areal measurement of leaves were then carried out by capturing a clear image of all the leaves and analysing with CPCe software (version 3.6) (Kohler and Gill 2006). Finally, the leaves were dried at 60°C for 48 hours and weighed to obtain biomass measurements.

2.2.5 Chlorophyll content

A young mature leaf (rank 2) from each pot was collected and stored immediately at -20°C at the end of the experiment. To determine chlorophyll concentration, a 10-15 mm section of leaf was cut from the middle of a fully mature leaf and the width of the leaf segment was measured using a pair of callipers. The leaves were blotted dry and weighed before they were ground in a chilled mortar. Depending on the species and the weight of the leaf segment, 5 to 6 mL of cold (4°C) 90% acetone was added to extract chlorophyll from the sample. The solution was gently shaken, left in the dark to extract for 24 hours

at 4°C, and then centrifuged at 2680 g for 4 min to settle the pellet. The extract was measured for chlorophyll concentration according to Granger and Izumi (2002).

2.2.6 Non-structural carbohydrates (NSC) content

Roots and rhizomes were dried at 60°C for 48 hours, before being finely ground in a bead-beater (Daintree Scientific). Four replicate samples per treatment and species were sent to the Agriculture & Food Sciences lab in University of Queensland for non-structural carbohydrates content analysis. Briefly, soluble carbohydrates were extracted twice with 80% ethanol at 80°C for 10 min from 200 mg of ground plant material. Extracts were then passed through a de-colourising column to remove phenolic compounds. After acid hydrolysis, the amount of soluble carbohydrates was assayed with ferricyanide reagent and absorbance measured on a UV-Vis spectrophotometer at 420 nm (McCleary and Codd 1991).

Starch content was analysed according to Karkalas (1985). Residue from the soluble carbohydrate extraction was solubilised in boiling water. After cooling to room temperature, samples underwent enzyme digestion where amylase and amyloglucosidase was added. After incubation, the concentration of glucose is measured using a commercially available glucose oxidase/peroxidase (GOPOD) testing reagent (Megazyme)**.** Absorbance was then measured at 510 nm.

Total non-structural carbohydrates (NSC) content, which was the sum of the amount of soluble carbohydrates and starch content, were expressed as milligrams dry weight^{-1} of tissue.

2.2.7 Statistical analyses

All statistical analyses were carried out with R software (R Development Core Team 2014). Changes in photosynthetic and growth responses were tested using linear models with average pCO_2 levels for each treatment as explanatory variable. Data from sub-replicate pots from each tank were averaged for the analysis. Assumptions of homogeneity of variances and normality were checked using box plots and residual plots.

To satisfy the assumptions, photosynthetic efficiency (α) and compensation irradiance (E_c) for *T. hemprichii* were square-root transformed prior to analysis. One data point was identified as an outlier (> 2 SD from mean of remaining replicates) in each of the P_G:R and α datasets, and these outlier points were subsequently removed. To examine species differences in productivity and growth responses to increasing pCO_2, confidence intervals (CI) of the slopes (degree of response per 100 μatm rise in pCO_2) from linear models were calculated and compared.

2.3 Results

2.3.1 Experimental parameters

Water temperature (23.7 – 24.0°C) and salinity (35) in the experimental tanks were near-constant throughout the experiment (Table 2.1). Carbonate system parameters of the enriched pCO_2 treatments remained well within the target range (mean ± S.E.: control pCO_2 = 442 ± 6 μatm; low pCO_2 = 694 ± 20 μatm; intermediate pCO_2 = 884 ± 52 μatm; high pCO_2 = 1204 ± 59 μatm) (Table 2.1). Inorganic nutrient concentrations were similar among tanks, and averaged to an ammonium concentration of 0.22 ± 0.01 μM, nitrate concentration of 0.88 ± 0.3 μM and phosphate concentration of 0.19 ± 0.02 μM.

2.3.2 Photosynthetic performance

Carbon dioxide enrichment increased seagrass net productivity (NP). Under the chosen light level (400 μmol m^{-2} s^{-1}), NP significantly increased with increasing pCO_2 levels for all species (Fig. 2.1; Table 2.2). Across species, the increase in NP ranged from 0.757 to 1.040 mg O_2 g^{-1} DW h^{-1} for every 100 μatm increase in pCO_2; however no species difference in the slope was detected (based on overlapping confidence intervals) (Table 2.2).

Energetic surplus, or gross photosynthetic to respiration ratios (P_G:R), significantly increased with increasing pCO_2 for all three species (Fig. 2.1; Table 2.2). No distinct differences in the slopes (0.32 – 0.47 units as pCO_2 increased by 100 μatm, Table 2.2) indicated that P_G:R responses in the different species were similar.

Table 2.1: Measured and calculated parameters, and average nutrient concentrations for control and three enriched pCO_2 treatments. Water samples for DIC and nutrients (in duplicates) were taken every five days from each tank. Results were pooled and averaged over sampling times and tanks for each treatment (n = 4). Standard errors are given in brackets.

	Measured parameters				Calculated parameters					Nutrient concentrations		
pCO_2 treatment	pH range (NBS scale)	Temp (°C)	DIC (µmol kg^{-1} SW)	TA (µmol kg^{-1} SW)	pH (NBS scale)	pCO_2 (µatm)	HCO_3^- (µmol kg^{-1} SW)	CO_3^{2-} (µmol kg^{-1} SW)	CO_2 (µmol kg^{-1} SW)	NH_4^+ (µmol L^{-1})	PO_4^- (µmol L^{-1})	NO_3^- (µmol L^{-1})
High	7.69-7.85	23.9 (0.2)	2146 (7)	2296 (3)	7.76 (0.02)	1204 (59)	2024 (4)	86.2 (4.0)	35.3 (1.8)	0.217 (0.02)	0.206 (0.05)	0.968 (0.70)
Intermediate	7.81-7.98	24.0 (0.2)	2120 (10)	2290 (2)	7.89 (0.02)	884 (52)	1980 (7)	113.4 (6.2)	25.9 (1.5)	0.210 (0.03)	0.167 (0.04)	0.700 (0.50)
Low	7.91-8.01	23.7 (0.2)	2084 (7)	2289 (2)	7.98 (0.01)	694 (20)	1930 (7)	133.2 (3.4)	20.5 (0.6)	0.223 (0.03)	0.211 (0.06)	0.914 (0.61)
Control	8.12-8.16	23.7 (0.2)	2012 (8)	2281 (1)	8.14 (0.01)	442 (6)	1814 (6)	184.5 (2.6)	13.0 (0.2)	0.210 (0.03)	0.184 (0.03)	0.923 (0.64)

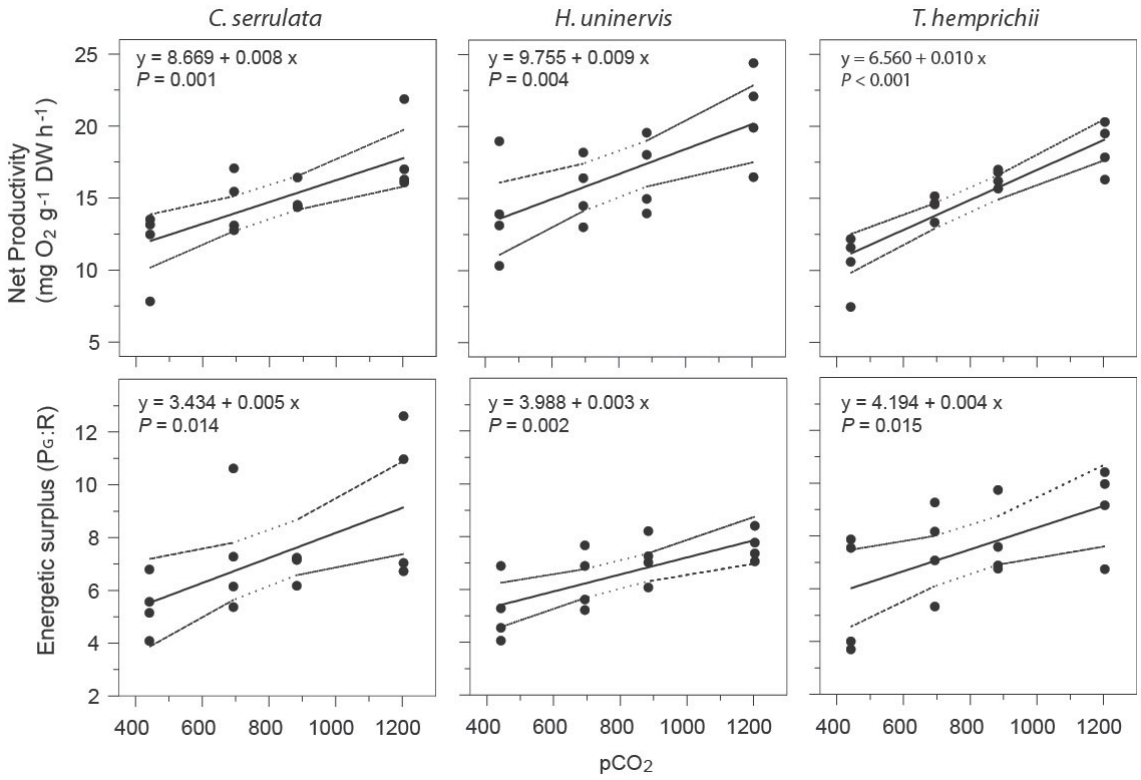

Fig. 2.1: Linear model fits (dotted lines indicates 95% confidence intervals) for net productivity and energetic surplus (P_G:R) of *C. serrulata*, *H. uninervis* and *T. hemprichii* in response to pCO_2 enrichment. n = 4.

Photosynthetic rates in all three species exhibited typical P-E (PAR) response curves. Photosynthetic rates increased linearly (initial slope, α) with light under limiting irradiances, before levelling off at the maximum photosynthetic rate (P_{max}) past saturating irradiance (E_k). Photosynthesis-irradiance (P-E) curves demonstrated a good fit ($R^2 > 0.85$; $P < 0.05$) to the adapted hyperbolic tangent model.

Increasing pCO_2 levels significantly increased maximum photosynthetic rates (P_{max}) for all three species (Table 2.2; Fig. 2.2). Maximal photosynthetic rates (P_{max}) increased by 0.677 to 0.929 mg O_2 g^{-1} DW h^{-1} for every 100 μatm rise in seawater pCO_2. Photosynthetic efficiency (α) significantly increased with pCO_2 levels across all species (Table 2.2; Fig. 2.2). Photosynthetic efficiency increased by 0.004-0.013 with every 100 μatm rise in pCO_2 level across all species.

Saturating irradiance (E_k) was not significantly altered by the pCO_2 treatments (Table 2.2; Fig. 2.2). Increasing pCO_2 enrichment reduced compensation irradiance (E_c) for *C. serrulata* and *H. uninervis* (Table 2.2; Fig. 2.2), however, in *T. hemprichii* E_c was not affected by pCO_2 enrichment (Table 2.2; Fig. 2.2).

Overall, most photosynthetic parameters responded significantly to pCO_2 increase. Although some variation exists in the slopes, overlapping CIs indicated that species differences were non-significant (Table 2.2).

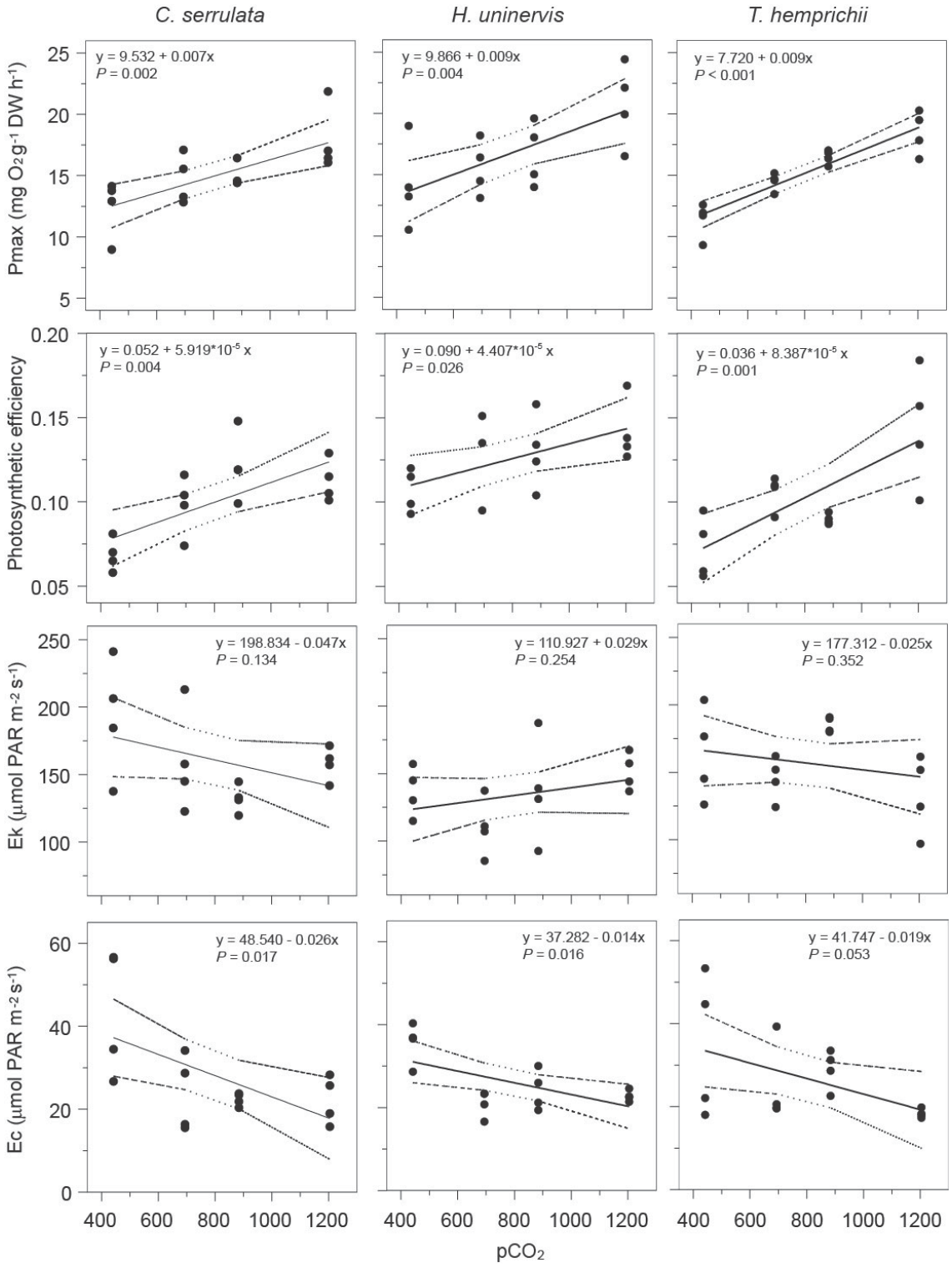

Fig. 2.2: Parameters derived from P-E curves. Top row - maximal photosynthetic rates (Pmax); second row – photosynthetic efficiency (α); third row – saturating irradiance (Ek); bottom row – compensation irradiance (Ec). Data were fitted with linear models (dotted lines 95% confidence intervals). n = 4.

2.3.3 Plant scale responses (leaf growth and rhizome carbohydrates)

Leaf growth responses to pCO_2 enrichment differed among species. *C. serrulata* did not show differences in growth rates with increasing pCO_2 levels (Fig. 2.3; Table 2.2). By contrast, growth rates increased with pCO_2 enrichment for *H. uninveris* (Fig. 2.3; Table 2.2) and *T. hemprichii* (Fig. 2.3; Table 2.2). Slopes for relative leaf growth rates (RGR) were about 0.001 units for every 100 μatm increase in pCO_2 for both species.

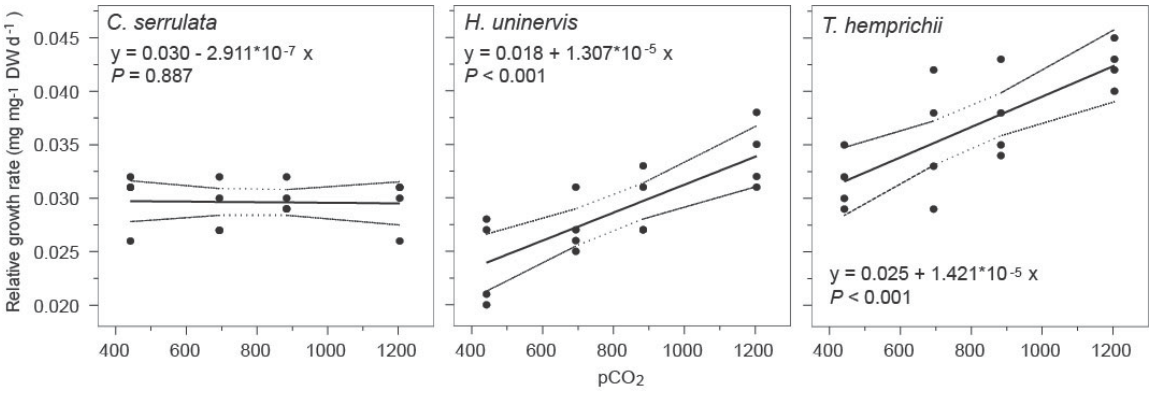

Fig. 2.3: Linear model fits (dotted lines indicates 95% confidence intervals) for relative growth rates of *C. serrulata*, *H. uninervis* and *T. hemprichii* in response to pCO_2 enrichment (mg DW mg^{-1} DW day^{-1}). n = 4.

For plant-scale response to pCO_2, amongst the three species only *T. hemprichii* displayed an increase in specific leaf area (SLA; leaf area per unit dry weight) with increasing pCO_2 (Table 2.2). No significant effects of pCO_2 on chlorophyll content were detected for all three species at the end of the experiment (Table 2.2). Starch content in *C. serrulata* rhizomes decreased as pCO_2 levels increased from 442 to 1204 μatm (Table 2.2). There were no significant changes for starch content in *H. uninervis* and *T. hemprichii* rhizomes with pCO_2 enrichment (Table 2.2). Neither NSC nor soluble carbohydrates content showed significant changes with pCO_2 enrichment for all three species (Table 2.2).

Table 2.2: Linear models for all response variables measured. All parameters were analysed with pCO_2 treatments as explanatory variable with tanks as replicates (n = 4). Slopes (pCO_2) and 95% confidence intervals (CI) are expressed per 100 μatm pCO_2. * denotes $P < 0.05$; ** denotes $P < 0.01$; *** denotes $P < 0.001$. NP- net productivity, P_G:R- gross photosynthesis to respiration ratio, P_{max}- maximum photosynthetic rate, α- photosynthetic efficiency, E_k- saturating irradiance, E_c- compensation irradiance, RGR- relative growth rate, SLA- specific leaf area, chl a- chlorophyll a, chl b- chlorophyll b, NSC- Total non-structural carbohydrates. α and E_c were square-root transformed for *T. hemprichii*.

Species	Parameter	Intercept	pCO_2	95% CI	R^2	$F_{(1,14)}$	*P*	
C. serrulata	NP	8.669	0.757	(0.352, 1.162)	0.502	16.094	0.001	**
	P_G:R	3.434	0.474	(0.111, 0.838)	0.313	7.845	0.014	*
	P_{max}	9.532	0.677	(0.291, 1.062)	0.468	14.179	0.002	**
	α	0.052	0.006	(0.002, 0.010)	0.427	12.190	0.004	**
	E_k	198.834	-4.748	(-11.141, 1.646)	0.093	2.537	0.134	
	E_c	48.540	-2.557	(-4.588, -0.525)	0.295	7.288	0.017	*
	RGR	0.030	-2.811×10^{-5}	$(-4.455 \times 10^{-4}, 3.893 \times 10^{-4})$	0.070	0.021	0.887	
	SLA	405.793	-0.346	(-5.883, 5.190)	0.070	0.018	0.895	
	chl a	0.795	-0.004	(-0.038, 0.031)	0.068	0.049	0.828	
	chl b	0.404	-0.004	(-0.020, 0.013)	0.055	0.216	0.649	
	NSC	10.450	0.026	(-0.844, 0.895)	0.071	0.004	0.951	
	sol carbs	10.020	0.049	(-0.823, 0.922)	0.070	0.015	0.906	
	starch	0.424	-0.023	(-0.041, -0.004)	0.277	6.741	0.021	*
H. uninervis	NP	9.755	0.867	(0.318, 1.416)	0.411	11.459	0.004	**
	P_G:R	3.988	0.322	(0.140, 0.505)	0.471	14.328	0.002	**
	P_{max}	9.866	0.857	(0.314, 1.401)	0.410	11.434	0.004	**
	α	0.090	0.004	(0.001, 0.008)	0.276	6.337	0.026	*
	E_k	110.927	2.866	(-2.296, 8.028)	0.027	1.418	0.254	
	E_c	37.282	-1.413	(-2.513, -0.312)	0.305	7.572	0.016	*
	RGR	0.018	0.001	$(7.240 \times 10^{-4}, 1.890 \times 10^{-3})$	0.596	23.128	0.000	***
	SLA	450.908	1.094	(-5.010, 7.199)	0.060	0.148	0.706	
	chl a	1.558	-0.009	(-0.081, 0.062)	0.065	0.080	0.782	
	chl b	0.776	-0.005	(-0.050, 0.041)	0.068	0.047	0.831	
	NSC	17.196	-0.077	(-0.796, 0.641)	0.067	0.054	0.820	
	sol carbs	2.469	0.050	(-0.122, 0.223)	0.042	0.390	0.542	
	starch	14.726	-0.128	(-0.751, 0.495)	0.057	0.194	0.667	

(Table 2.2 continued)

Species	Parameter	Intercept	pCO_2	95% CI	R^2	$F_{(1,14)}$	*P*	
T. hemprichii	NP	6.560	1.040	(0.746, 1.334)	0.791	57.586	0.000	***
	P_G:R	4.194	0.410	(0.094, 0.730)	0.309	7.712	0.015	*
	P_{max}	7.720	0.929	(0.691, 1.168)	0.822	70.109	0.000	***
	Sqrt α	0.215	0.013	(0.006, 0.019)	0.518	17.134	0.001	**
	E_k	177.312	-2.548	(-8.231, 3.135)	0.005	0.925	0.353	
	Sqrt E_c	6.67	-0.191	(-0.387, 0.004)	0.237	4.448	0.054	
	RGR	0.025	0.001	$(7.277\times10^{-4}, 2.114\times10^{-3})$	0.550	19.330	0.001	***
	SLA	333.021	6.169	(1.158, 11.182)	0.285	6.971	0.019	*
	chl a	0.934	-0.013	(-0.040, 0.014)	0.007	1.110	0.310	
	chl b	0.453	-0.008	(-0.022, 0.006)	0.028	1.430	0.252	
	NSC	7.477	0.624	(-0.230, 1.478)	0.089	2.457	0.139	
	sol carbs	1.894	0.227	(-0.241, 0.696)	0.005	1.082	0.316	
	starch	5.574	0.398	(-0.221, 1.016)	0.057	1.900	0.190	

2.4 Discussion

Under predicted future scenarios of ocean acidification, marine macrophytes on coral reefs could be amongst the "winners", because growth and survival will be enhanced by higher CO_2 availability (Fabricius et al, 2011; Koch et al, 2013). The present study supports this hypothesis as all three species benefitted from higher rates of photosynthesis (i.e. P_{max} increased) and adjusted photosynthetic kinetics in response to CO_2 enrichment of coastal seawater. Enhanced photosynthetic responses and growth rates were observed after two weeks of exposure to enriched pCO_2. Although photosynthetic responses were very similar among species, magnitude of plant-scale responses was species-specific.

2.4.1 Physiological responses to CO_2 enrichment

Carbon dioxide enrichment increased net productivity (NP) and energetic surplus (P_G:R) in all three species tested. The increases in NP and P_G:R were quantified as 0.757-1.040 and 0.322-0.474 units per 100 μatm pCO_2, respectively. This response is consistent with previous findings that photosynthetic rates increase with pCO_2 in seagrasses (Thom 1996;

Zimmerman et al. 1997; Invers et al. 2002; Alexandre et al. 2012). Having greater energetic surplus could indicate subsequent effects to plant scale responses, such as growth and shoot production (Invers et al. 2002; Palacios and Zimmerman 2007). Energetic status can affect growth, responses to physical disturbances such as grazing (Eklöf et al. 2009), abundance, and spatial distribution (Dennison et al. 1993; Zimmerman et al. 1997), and even reproductive output (Palacios and Zimmerman 2007).

Maximum photosynthetic rates (P_{max}) and efficiency (α) in all species were raised at higher pCO_2 levels although chlorophyll content was not affected. Photoacclimation has been reported for several temperate and tropical seagrass species (Invers et al. 1997; Zimmerman et al. 1997; Jiang et al. 2010; Alexandre et al. 2012), but this is the first study to compare short-term responses to CO_2 enrichment among three tropical species in one experiment. In general, P-E curves did not show differences among species in their photosynthetic responses to CO_2 enrichment. The responses over the range of pCO_2 in photosynthetic parameters were similar among species (similar slope, as shown by overlapping confidence intervals). Maximum relative electron transport rate ($rETR_{max}$), measured as a proxy for P_{max}, may increase with CO_2 enrichment (Jiang et al. 2010). While comparisons between quantum efficiency and O_2 production need to be viewed with caution (Beer et al. 2001), these findings indicate that there was a stronger response of P_{max} in the present study using respirometry per 100 μatm rise in pCO_2 (7.86% increase at 1204 μatm for 2 weeks) compared to $rETR_{max}$ (3.37% increase in $rETR_{max}$ at ~807 μatm (pH 7.75) for 3 weeks, Jiang et al 2010). Temperate species also increased P_{max} with CO_2 enrichment too, but showed much more variable response rates per 100 μatm pCO_2 (*Zostera marina* 0.59% increase per 100 μatm pCO_2 for 3 weeks, Zimmerman et al. 1997; *Zostera noltii* 9.64% increase per 100 μatm pCO_2 for 5 months, Alexandre et al. 2012).

Overall, our study concurs that seagrasses can raise productivity in response to pCO_2 enrichment, at least in the short-term (two weeks exposure). The responses of net productivity and P_G:R to increasing pCO_2 followed a linear trend, indicating that any future change in pCO_2 could effect seagrass productivity. In numerous studies on terrestrial plants, the initial stimulation of photosynthesis and growth in elevated CO_2 can decline over time, as Rubisco is down-regulated, carbohydrates accumulate and nitrogen

content decreases (Stitt and Krapp 1999). Observations of high seagrass abundance at CO_2 seep sites indicate seagrass productivity might continually benefit from CO_2 enrichment over the long term (decades) (Fabricius et al. 2011). However, interaction from other co-occurring influences, such as the lowered competition from photosynthetic calcifiers, or intrinsic genetic capacity to respond within the population, should be taken into account too. Whether such longer term acclimatory responses to CO_2 enrichment would manifest in tropical seagrasses remains unknown. Furthermore, the capacity of seagrasses to respond to increasing pCO_2 is likely to depend on other limiting factors such as nutrient or light availability (Invers et al. 1997).

Light availability is often the primary limiting factor for seagrass productivity. Exposure to low light conditions (such as high turbidity and high epiphyte loads) is a common factor causing seagrass loss (Waycott et al. 2009; Collier et al. 2012a). Here, a lowering in the light requirement to meet respiratory demands (E_c) and an increase in light efficiency (α) were observed with increasing pCO_2. This could imply that a lower amount of light energy would be required to meet metabolic balances (Schwarz et al. 2000; Long et al. 2004). Therefore, CO_2 enrichment could potentially increase the tolerance of seagrasses to conditions of low light, for example during flood plume events. In contrast, there was no change in the light level required to reach maximum photosynthetic rates (E_k). CO_2 enrichment increased E_k in *Z. marina* (Zimmerman et al. 1997), *Z. noltii* (Alexandre et al. 2012) and *T. hemprichii* (Jiang et al. 2010). An increase in P_{max} without a simultaneous rise in light requirement might be explained by the strong upregulation of photosynthetic efficiency (α). CO_2 enrichment can affect light requirements and this could be important for how seagrasses will respond to changing environmental conditions - including water quality - in the future.

Seagrasses can utilise the predominant HCO_3^- in seawater via carbon concentration mechanisms (CCMs), somewhat alleviating the problem of carbon limitation at higher pH (Durako 1993; Bjork et al. 1997; Uku et al. 2005; Campbell and Fourqurean 2013b). In favourable conditions where other factors are non-limiting, CCMs might cause some seagrasses to be carbon saturated (Schwarz et al. 2000; Beer et al. 2002). Such mechanisms were thought to be less efficient in the genus *Thalassia (T. hemprichii* and *T. testudinum*) rendering this genus less capable of utilising HCO_3^- than other species (Uku et al. 2005; Campbell and Fourqurean 2013b). Hence, an increase in

CO_2 availability would be important in raising productivity for *Thalassia*. *Cymodocea* and *Halodule* reportedly possess CCMs that allow them to utilise HCO_3^- under ambient conditions (Schwarz et al. 2000; Uku et al. 2005). Both species were able to increase photosynthetic rates under enriched pCO_2 conditions, where the relative increase in CO_2 was much greater than that in HCO_3^- (Koch et al. 2013). Both species have been observed to become more dominant and have increased biomass around highly enriched volcanic CO_2 seeps (Takahashi et al. 2015). All the three species tested responded at similar rates in terms of net productivity. It appears that regardless of whether they possess CCMs or not, CO_2 enrichment can increase photosynthetic rates for different species to a similar extent.

2.4.2 Sinks for carbon: plant-scale responses

As a result of increased photosynthetic rates and relatively stable dark respiration rates, energetic surplus (P_G:R) was increased at higher pCO_2 for all species. The rate of increase with pCO_2 levels in P_G:R was similar among the three species. There are a number of possible sinks for this additional fixed C. In this short-term study, we measured growth and storage carbohydrates in rhizomes, but other sinks, such as biomass or sexual reproduction exist.

Response in leaf growth rates to pCO_2 enrichment differed among species. Growth of *H. uninervis* and *T. hemprichii* responded strongly, but not in *C. serrulata*. Specifically, relative growth rate (RGR) increased significantly and in *T. hemprichii*, an increase in leaf area relative to leaf biomass (SLA) was also observed. Leaf growth responses vary among the limited number of studies on tropical seagrass. While Campbell and Fourqurean (2013a) observed no differences in leaf growth rates with pCO_2 enrichment in *T. testudinum*, Jiang et al. (2010) showed a 2.63% rate increase in leaf growth (per 100 μatm pCO_2) at pH 7.76 after 3 weeks of exposure in *T. hemprichii*. This is about half of the 5.62% rate of increase in leaf growth observed in *T. hemprichii* here. The effect of CO_2 enrichment on growth rate can be influenced by the tissue nutrient requirement of the species and other prevailing environmental conditions (Zimmerman et al. 1997; Palacios and Zimmerman 2007; Jiang et al. 2010; Alexandre et al. 2012;

Campbell and Fourqurean 2013a). Under nutrient limitation, seagrasses could direct the fixed carbon toward carbon-rich tissues such as below-ground tissues, instead of investing in nitrogen-rich tissue such as leaves (Poorter et al. 1996; Stitt and Krapp 1999). *C. serrulata*, which has a higher proportion of its biomass existing as shoots and leaves (Hemminga and Duarte 2000), might have required a simultaneous increase in nitrogen availability in order to assimilate the carbon into its leaves. Temperature strongly influences carbon and nitrogen metabolism (Touchette and Burkholder 2007) and could also affect the growth response of seagrasses to CO_2 (Atkin et al. 2005; Collier et al. 2011). Whether these, and other, environmental parameters affected the differences in growth response among species warrants further investigation.

Sink strength, or carbon demand, could modulate growth response in seagrasses to CO_2 enrichment, similar to that in terrestrial C3 species (Arp 1991; Poorter et al. 1996). Increased energetic surplus from CO_2 enrichment indicates extra assimilated carbon available for storage, growth and metabolism. While little change in NSC content was observed in the present study, seagrasses do possess a number of alternative "carbon sinks", with the size of carbon demand for each sink dependent on species-specific growth strategy (Doust 1981; Hemminga and Duarte 2000) (described further below). For example, the shorter time taken for shoot initiation for *H. uninervis* (average 7.9 days), compared to *C. serrulata* (average 21.2 days) and *T. hemprichii* (average 38.5 days) means a faster turnover of above-ground biomass for *H. uninervis* (Duarte 1991; Marba and Duarte 1998). Therefore *H. uninervis* might have a strong carbon demand in leaf growth. The relatively greater proportion of below-ground biomass in *H. uninervis* and *T. hemprichii* suggests higher storage potential and metabolic demand (Duarte 1991; Marba and Duarte 1998). In these species, more carbon could be directed to below-ground biomass, and/or leaf area could be expanded to increase photosynthetic rates. Extra carbon could also be directed to increased shoot production and flowering, as observed in *Z. marina* after one year of CO_2 enrichment (Palacios and Zimmerman 2007). Essentially, the extra carbon assimilated could be directed to a single "sink", such as the growth of new leaves, or it could be spread among various metabolic functions and storage organs. The latter makes distinguishing the fate of the extra carbon complicated, especially for short-term experiments such as this study.

In general, our results imply that the availability of higher pCO_2 might alter future interspecific competition among co-occuring species. With deteriorating water quality, i.e. low light and high nutrients, species that are able to readily assimilate and mobilise carbon resources with the extra CO_2 might outcompete other species. Under optimal growth conditions, species that are able to rapidly utilise the extra CO_2 to occupy more "space", i.e either upwards on vertical stems or via horizontal rhizomes, could potentially increase their abundance and distribution.

2.4.3 Seagrasses as "winners"?

The ability of marine macro-autotrophs to utilize the greater CO_2 availability suggests that they will thrive under future scenarios of climate change (Koch et al. 2013). This present study, has built evidence to support this, with increased growth, productivity and biomass from CO_2 enrichment (Zimmerman et al. 1997; Invers et al. 2002; Palacios and Zimmerman 2007; Jiang et al. 2010; Campbell and Fourqurean 2013a). This study has also quantified the change in physiological parameters with respect to CO_2 enrichment. Surveys at natural CO_2 seeps further attest to this, where greater seagrass cover, shoot density, root biomass and productivity were reported at low pH/ high CO_2 sites when compared to adjacent high pH/ low CO_2 sites (Hall-Spencer et al. 2008; Fabricius et al. 2011; Russell et al. 2013; Takahashi et al. 2015). For calcifying marine autotrophs, such as hard corals, foraminifera and coralline algae, ocean acidification lowers calcification and growth rates and increases rates of bioerosion (Kuffner et al. 2007; de Putron et al. 2010; Fabricius et al. 2011; Doo et al. 2014; James et al. 2014), and calcifying organisms might be outcompeted (Russell et al. 2011; Short et al. 2014). Thus, shifts in the ecological diversity and functions in coastal habitats could result from increased CO_2 levels.

This study demonstrated that tropical seagrasses can increase their photosynthetic rates, adjust photosynthetic performance and increase growth rates in response to CO_2 enrichment. Varying plant-scale responses to CO_2 enrichment among species might affect interspecies competition, especially in mixed species meadows (Takahashi et al. 2015). Under CO_2 enrichment scenarios, carbon utilisation and allocation traits among seagrass

species come into consideration, such as carbon uptake mechanisms, the ability to assimilate additional carbon and the response time of rhizome and shoot elongation to DIC enrichment (Hall-Spencer et al. 2008; Russell et al. 2013; Takahashi et al. 2015). Furthermore, environmental conditions such as reduced light and increased nutrients, which result from water quality changes, could limit species response to CO_2 enrichment in the long term. Changes in species composition and diversity in tropical seagrass meadows could potentially impact the functional diversity offered by these productive ecosystems. Inter-specific variation among seagrasses in response to ocean acidification, over different temporal scales, deserves further examination.

CHAPTER 3

LIGHT LEVELS AFFECT CARBON UTILISATION IN TROPICAL SEAGRASS UNDER OCEAN ACIDIFICATION [2]

Abstract

Under future ocean acidification (OA), increased availability of dissolved inorganic carbon (DIC) in seawater may enhance seagrass productivity. However, the ability to utilise additional DIC could be regulated by light availability, often reduced through land runoff. To test this, two tropical seagrass species, *Cymodocea serrulata* and *Halodule uninervis* were exposed to two DIC concentrations (447 μatm and 1077 μatm pCO_2), and three light treatments (35, 100, 380 μmol m^{-2} s^{-1}) for two weeks. DIC uptake mechanisms were separately examined by measuring net photosynthetic rates while subjecting *C. serrulata* and *H. uninervis* to changes in light and addition of bicarbonate (HCO_3^-) use inhibitors (carbonic anhydrase inhibitor, acetazolamide) and TRIS buffer (pH 8.0). We observed a strong dependence on energy driven H^+-HCO_3^- co-transport (TRIS, which disrupts H^+ extrusion) in *C. serrulata* under all light levels, indicating greater CO_2 dependence in low light. This was confirmed when, after two weeks exposure, DIC enrichment stimulated maximum photosynthetic rates (P_{max}) more in *C. serrulata* grown under lower light levels (36 – 60% increase) than for those in high light (4% increase). However, *C. serrulata* growth increased with both DIC enrichment and light levels. Growth, NPP and photosynthetic responses in *H. uninervis* increased with higher light treatments and were independent of DIC availability. Furthermore, *H. uninervis* was

[2] Chapter 3 has been adapted into and published as Ow Y.X., Uthicke S. and Collier C.J. (2016) Light Levels Affect Carbon Utilisation in Tropical Seagrass under Ocean Acidification. PLoS ONE 11(3): e0150352.

found to be more flexible in HCO_3^- uptake pathways. Here, light availability influenced productivity responses to DIC enrichment, via both carbon fixation and acquisition processes, highlighting the role of water quality in future responses to OA.

3.1 Introduction

Seagrass meadows are highly productive habitats that offer a wide range of ecologically and economically valuable ecosystem services (Costanza et al. 1997). Especially important is their ability to capture and convert light energy into organic matter, which then become available to other trophic levels (Duarte and Chiscano 1999; Hemminga and Duarte 2000). This primary production is strongly determined by the amount of light available in the aquatic environment (Dennison 1987). When coastal water quality declines, eutrophication and high turbidity in the water column reduce the light available to these primary producers (Schaffelke et al. 2005). Chronic light limitation lowers seagrass productivity and contributes to the accelerating areal loss of these habitats (Orth et al. 2006; Ralph et al. 2007; Waycott et al. 2009).

Seagrass productivity may also be limited by the present day seawater composition of dissolved inorganic carbon (DIC) (Beer and Koch 1996; Invers et al. 2001). However, with the rising emissions of carbon dioxide (CO_2) from anthropogenic activities being continually absorbed by the ocean, there is a shift in the relative proportion of each DIC species (HCO_3^-, CO_3^{2-}, CO_2) in seawater (Raven et al. 2005; Gattuso and Hansson 2011) as the pH is reduced (Ocean acidification, or OA). The proportion of CO_2 will have the greatest percent increase (>250%, compared to 24% for HCO_3^-) among the DIC constituents under the projected pH decrease by 2100 (Koch et al. 2013). Studies have indicated that greater availability of DIC under future OA conditions may benefit seagrasses (Short and Neckles 1999; Fabricius et al. 2011; Koch et al. 2013). An increase in photosynthesis and growth following exposure to increased DIC in both tropical and temperate seagrass species was previously demonstrated (Zimmerman et al. 1997; Invers et al. 2002; Palacios and Zimmerman 2007; Jiang et al.

2010; Alexandre et al. 2012; Burnell et al. 2014a; Ow et al. 2015). Moreover, since both tropical and temperate seagrasses displayed a higher photosynthetic affinity for CO_2 than HCO_3^-, an increased availability of CO_2 can further boost productivity (Beer and Waisel 1979; Durako 1993). At natural CO_2 vent sites, growth and areal cover of seagrasses were observed to be much greater than at adjacent non-CO_2 enriched sites (Hall-Spencer et al. 2008; Fabricius et al. 2011). Hence, given sufficient light and nutrients, seagrasses could utilise the extra provisioning of DIC to enhance productivity (Durako 1993; Beer and Koch 1996; Bjork et al. 1997; Burnell et al. 2014a).

Seagrasses can use bicarbonate (HCO_3^-), the dominant DIC species, for photosynthesis (Hellblom et al. 2001). Bicarbonate utilization involves the dehydration and transport of HCO_3^- into the plant cells (Hellblom et al. 2001; Beer et al. 2002). Various HCO_3^- utilization pathways that have been proposed involved the enzyme carbonic anhydrase (CA) and the presence of acidic zones maintained by proton (H^+) gradients (Bjork et al. 1997; Beer et al. 2002; Hellblom and Axelsson 2003). Extracellular CA dehydrates HCO_3^- to CO_2, allowing CO_2 to diffuse into the cell. Another uptake pathway involves H^+ extrusion-driven co-transport of H^+ and HCO_3^- (Beer and Rehnberg 1997). In general, active extrusion of H^+ into localized regions of the leaf boundary layer (acidic zones) for HCO_3^- utilisation is energetically costly compared to passive CO_2 uptake, and thus could be limited by low light levels (Burnell et al. 2014a).

Light availability can influence the ability of seagrasses to exploit enriched DIC conditions for photosynthesis. Fluctuating light availability (Masini and Manning 1997; Enriquez and Pantoja-Reyes 2005; Schubert et al. 2015) could vary the carbon demand for the downstream carbon fixation cycle. Elevated DIC (with CO_2 as substrate) increases photosynthetic efficiency and reduces light requirements of seagrasses (Zimmerman et al. 1997; Burnell et al. 2014a). So far, the interaction of light and DIC availability on seagrass photosynthesis has only been explored in a few studies (Zimmerman et al. 1997; Schwarz et al. 2000; Liu et al. 2005; Palacios and Zimmerman 2007; Burnell et al. 2014b). Productivity responses of seagrasses to DIC enrichment under low light conditions have been variable, ranging from no change in growth rates in *Zostera marina* (Palacios and Zimmerman 2007), to increased growth in *Amphibolis antarctica* (Burnell et al. 2014b) and enhanced photosynthesis in *Halophila ovalis*, *Cymodocea serrulata* (Schwarz et al.

2000) and *Thalassia hemprichii* (Liu et al. 2005). Studies demonstrating positive effects of DIC enrichment under low light (Schwarz et al. 2000; Burnell et al. 2014b) suggested that seagrasses growing under low light may benefit more from DIC enrichment, through a lowered epiphyte load and/or reduced energetic demand from HCO_3^- use with an increase in CO_2 supply. However, some of these studies did not directly measure light levels, but used water depth (Schwarz et al. 2000) and epiphyte cover (Liu et al. 2005) as proxies for light reduction. It is unclear if this DIC limitation stemmed directly from a reduced HCO_3^- utilization due to low light availability, or was compounded with reduced mixing or competitive DIC uptake by epiphytic algae.

This study aimed to examine the effects of light availability on carbon utilisation in two tropical seagrasses, *C. serrulata* and *Halodule uninervis*. Growth, net primary productivity and photosynthetic responses to DIC enrichment under different light treatment levels were characterised in a two-week aquaria experiment. DIC levels were chosen to represent present day and end-of-century (CO_2 ~ 1000ppm) emission scenarios (RCP8.5) (Collins et al. 2013). It was hypothesized that while DIC enrichment (7.5% increase relative to ambient seawater) will increase photosynthesis and growth of seagrasses, the extent of increase will be greater under lower light levels. To further test the influence of light availability on HCO_3^- use in tropical seagrasses, HCO_3^- utilization mechanisms were investigated using CA and H^+ gradient inhibitors (CA-mediated and/or H^+ co-transport mediated) under contrasting light levels (Durako 1993; Uku et al. 2005). Energetic demand from using HCO_3^- may make it unfavourable as a photosynthetic substrate under limiting light levels. Hence the hypothesis was that under limiting light availability, the ability to use of HCO_3^- as a carbon substrate would be reduced (Spalding and Ogren 1982; Kubler and Raven 1995).

3.2 Materials and Methods

3.2.1 Experimental species

Two common tropical seagrass species, *Cymodocea serrulata* and *Halodule uninervis,* were collected from an intertidal meadow at Cockle Bay, Magnetic Island, northern Great Barrier Reef (19°10.88'S, 146°50.63'E). Photosynthetically active radiation (PAR) was measured at Cockle Bay using planar irradiance collectors (Odyssey Photosynthetic Irradiance Recording System, Dataflow Systems Pty Ltd, New Zealand) installed at seagrass canopy height from 2009 to 2014. The mean integrated daily irradiance at Cockle Bay from September 2009 to July 2014 was 14.9 mol m^{-2} d^{-1}. Calculated over the light period, the average PAR at this site was 385 μmol m^{-2} s^{-1}, the average maximum PAR was 961 μmol m^{-2} s^{-1}, while the median PAR was 196 μmol m^{-2} s^{-1} (McKenzie et al. 2015a). Variation in light levels between the wet and dry season was low (Supplementary Fig. 3.1). Average water temperature at this site (2005 – 2012) was around 26ºC (McKenzie et al. 2015a). Cores of *H. uninervis* and sediment were collected as intact plugs and *C. serrulata* as intact horizontal rhizomes (Ow et al. 2015), first in July 2012 for the DIC and light aquaria study, and later in May 2014 for the inhibitors study (detailed below). On both occasions, seagrasses were collected under a limited impact research permit (MTB41), which was assessed and issued by the Department of Employment, Economic Development and Innovation (Fisheries Queensland Code MP05) for the removal of marine plants from the Great Barrier Reef Marine Park. The potted seagrasses (average 40 shoots per pot for *H. uninervis*, 10 shoots per pot for *C. serrulata*) were kept in an outdoor flow-through aquarium (1000 L) with filtered seawater (5 μm) for a three-week acclimation period. Maximum light level in the outdoor aquarium was 350 μmol m^{-2} s^{-1} with seawater temperature of 23 – 25°C and salinity at 35 – 36.

3.2.2 Light and DIC effects on photosynthetic and growth response

3.2.2.1 Experimental set-up

The seagrasses were exposed to three different light levels (35, 100 and 380 μmol m^{-2} s^{-1}) and two seawater DIC concentrations (high vs control; Table 3.1) in an indoor flow-through system over two weeks. Two weeks exposure were shown to be sufficient for inducing photosynthetic changes in the two species in a previous experiment (Ow et al. 2015). The three light levels chosen for the experiment (35, 100 and 380 μmol m^{-2} s^{-1}) provided 1.5, 4.3 and 16.4 mol m^{-2} d^{-1} of light per day over a 12:12 h light:dark cycle. The two lower light levels represented low light conditions that were less common, but still ecologically relevant at the source meadow. The highest light level (380 μmol m^{-2} s^{-1}) provided an integrated daily irradiance which was similar to that most commonly encountered at Cockle Bay (12 – 14 mol m^{-2} d^{-1}). The experiment was conducted at the Australian Institute of Marine Sciences, Townsville. Eighteen glass aquaria (working volume 18 litres) with three replicates for each treatment were supplied with fresh filtered (5 μm) seawater from header tanks. One pot of each seagrass species was placed into each aquarium. Leaves were cleaned of epiphytes every two days by gentle rubbing between fingers. Light and CO_2 levels were randomly assigned to each aquarium. LED lights mounted over the top of each aquarium provided illumination to cover the full sunlight spectrum (Aqua Illumination LED, USA). The LED lights were set to provide 380 μmol m^{-2} s^{-1}. Irradiance was determined using a planar irradiance meter (LICOR, USA). Light reduction was achieved by placing neutral density shade-cloth over individual aquaria. DIC concentrations were manipulated by means of feedback-regulated CO_2 input (AquaMedic, Germany) into the header tanks, as described in Vogel and Uthicke (2012). In all the header and aquarium tanks, diffusers and pumps were installed to ensure thorough mixing of DIC-enriched water. Additional pH and temperature measurements were taken manually (pH probe: Eutech, USA; console: Oakton, USA) and pH levels calibrated to TRIS seawater standards (Batch 10, Supplied by A. Dixon, Scripps Institute of Oceanography). Salinity was measured with a handheld refractometer. Every four days, water samples were taken from each aquarium and analysed for dissolved inorganic carbon (DIC) and total alkalinity (AT) concentrations using a Vindta 3C analyser. Measured values of DIC, AT, temperature and salinity were

used to calculate carbonate system parameters in USGS CO2calc software (Robbins et al. 2010). Water samples for inorganic nutrient (NH_4^+ and NO_3^-) measurements were collected in duplicate, every five days, from each aquarium, filtered (0.45 μm) and analysed (Ryle et al. 1981).

3.2.2.2 Growth measurements

Growth rates of seagrass shoots in the experiment were measured following (Short and Duarte 2001). At the start, all shoots were marked at the top of the bundle sheath with a needle. After two weeks of growth, the shoots were harvested; new tissue growth was excised and dried at 60°C for 48 hours before weighing. Biomass of new tissue growth was normalised to the total above-ground biomass in each pot to calculate relative growth rates (RGR, g g^{-1} DW day^{-1}).

3.2.2.3 Photosynthesis vs irradiance curves

To characterise photosynthetic parameters, dark respiration and photosynthetic rates over a light range were measured to construct photosynthesis vs irradiance (P-E) curves two weeks after the initiation of the experiment. Oxygen consumption and evolution rates of seagrass leaves in seawater from their respective treatment aquaria (DIC enriched: 1077 ± 104 μatm; control: 447 ± 23 μatm) were monitored using optical oxygen sensors (Sensor spots-PSt3, PreSens) and a fibre-optic oxygen meter (PreSens Oxy 4) in 200 mL volume incubation chambers (Ow et al. 2015). The chambers were incubated at 25°C water temperature in a flow-through water bath system (Lauda, Ecoline RE 106). Magnetic stirrers (2 cm × 0.8 cm, 128 revolutions per minute) provided even mixing in each chamber. One mature epiphyte-free (rank 2) leaf was held upright in the chamber to mimic natural orientation. Respiration was measured over a 20-min period in the dark. Photosynthetic rates were measured on the same leaf over a series of light steps (10, 20, 35, 70, 100, 200, 380, 520, 600 μmol m^{-2} s^{-1}), with each light step lasting 25 min. Adjustable LED lights were used to provide the different light intensities (Aqua Illumination LED). Light spectra of LED lights are provided in Supplementary Fig. 3.2.

Incubation media in chambers were replaced with fresh filtered seawater from respective aquaria prior to measurements at these light steps: 35, 100, 380 and 600 μmol m^{-2} s^{-1}. Initial trials showed that low photosynthetic rates at low light levels, and short incubation times between replacements of incubation media at higher light levels reduced the possibility of DIC limitation during incubations (Supplementary Table 3.1). Dark respiration and photosynthetic rates were derived by fitting a linear regression to the logged oxygen concentration data in the each chamber. Only steady-state measurements of respiration and photosynthesis (obtained after ~5 min) were used for regressions. After incubation, leaves were dried at 60°C for 48 h and weighed. Rates were normalised to the dry weight of the leaf. Each optical oxygen sensor was calibrated prior to use according to the method described in Collier et al. (2011).

Net primary productivity (NPP) was taken to be the photosynthetic rate measured at the respective experimental light levels (35, 100 and 380 μmol m^{-2} s^{-1}). To derive photosynthetic parameters, photosynthetic rates were fitted to the adapted hyperbolic tangent model of Jassby and Platt (1976). The model describes the linear increase (photosynthetic efficiency, α, mg O_2 μmol^{-1} photons) in photosynthetic rates with irradiance, up until the saturating irradiance (E_k, μmol m^{-2} s^{-1}) where photosynthesis plateaus at the maximum rate (P_{max}, mg O_2 g^{-1} DW h^{-1}). Compensation irradiance (E_c, μmol m^{-2} s^{-1}) is the light level when photosynthetic rate is equal to respiration rate.

3.2.3 Effect of light levels on HCO_3^- uptake

To assess the effect of light on HCO_3^- utilization, net photosynthesis under specific inhibition of HCO_3^- uptake mechanisms was measured under two contrasting light levels. The light levels represented limiting (40 μmol m^{-2} s^{-1}) and saturating (600 μmol m^{-2} s^{-1}) light as determined from the previous experiment where the derived saturating light (E_k) was 100 and 98 μmol m^{-2} s^{-1} for *C. serrulata* and *H. uninervis* respectively. Therefore, the limiting light level was well below E_k, and the saturating light level was above E_k for both species.

Bicarbonate utilization pathways can be elucidated by the effect of specific inhibitors on photosynthetic rates, as described in Beer et al. (2002). One pathway

involves the membrane-bound extracellular CA dehydrating HCO_3^- into CO_2, which then diffuses into the cell. Addition of acetalzolamide (AZ) inhibits extracellular CA activity (Beer et al. 2002). Another possible pathway for HCO_3^- utilization consists of localised active H^+ extrusion to create H^+ gradients that facilitate the inward co-transport of H^+ and HCO_3^-. Addition of a buffer, e.g. Tris (hydroxymethyl) aminomethane (TRIS), dissipates the H^+ gradient as the buffer reacts with the extruded H^+, thus altering photosynthetic rates (Hellblom et al. 2001). A third HCO_3^- utilization pathway consists of extracellular CA-mediated HCO_3^- conversion to CO_2 in acidic zones created at H^+ extrusion sites. The acidic zones help concentrate CO_2 and facilitate diffusion into the cells. Strong inhibition of photosynthetic rates by the combined addition of buffer and AZ indicates a strong dependence on this pathway (Axelsson et al. 2000; Hellblom and Axelsson 2003). Thus, we used AZ, TRIS and their combination in the experiments described below.

Net photosynthesis was determined by measuring rates of oxygen evolution in 60 mL incubation chambers, similar to the procedure described above. During measurements, incubation media (fresh filtered seawater (pH 8.0) with or without inhibitor) in each chamber was stirred and maintained at 25ºC. Mature and non-epiphytised leaves from non-connected shoots collected fresh from the field, were used for the measurements. Prior to the addition of the inhibitor(s), control rates of dark respiration and photosynthesis for each leaf were measured in fresh filtered seawater (pH 8.0). Dark respiration rates of the leaves were measured over 20 min, before the lights were switched on to provide 40 μmol m^{-2} s^{-1} of light. Net photosynthesis was then measured for 30 min. Subsequently, light levels were increased to 600 μmol m^{-2} s^{-1} and photosynthetic rates of the same leaves were measured. After the control measurements, incubation media for all chambers were replaced with fresh filtered seawater mixed with inhibitor solutions (AZ, TRIS or AZ+TRIS). The second set of incubations used the same leaves and followed the same sequence of measurements (in dark, at limiting light, then at saturating light). Oxygen production and consumption rates were calculated and normalised to leaf dry weight as above. For this experiment, net photosynthetic rates to TRIS and/or AZ were expressed as percentages of the control net photosynthetic rates.

Three inhibitor solutions were used: 1) 0.1 mM AZ, 2) 45 mM TRIS, and 3) combination of 0.1 mM AZ and 45 mM TRIS (Uku et al. 2005). A stock solution of 20

mM AZ was prepared by dissolving the powder in 50 mM sodium hydroxide (NaOH). An aliquot of 350µl AZ stock solution was added to each individual chamber to achieve a final concentration of 0.1 mM. TRIS was prepared as 1 M stock solution and adjusted to pH 8.0. The buffer yielded pH 8.0 when mixed with seawater to a final concentration of 45 mM. The final TRIS concentration used here, although lower than that commonly found in literature (Uku et al. 2005; Burnell et al. 2014a), was sufficient in eliciting a reduction in net photosynthetic rates.

3.2.4 Statistical analyses

To evaluate effects of DIC and light for each species, growth, net primary productivity and photosynthetic parameters were analysed using univariate two-factor ANOVA to test for significance in fixed effects of light (three levels) and DIC (two levels) and their interaction. Data were checked for homogeneity of variance using Levene's test and for normality using Q-Q plots. Where the assumptions of ANOVA were violated, data were square root transformed. However when the assumptions were still not met, as observed in the E_c and α data for *H. uninervis*, the alpha-value was lowered to 0.01 to minimize the risk of a Type I error (Underwood 1997). In case of significant light effects, Tukey's HSD test was carried out to test which of the treatment levels were significantly different from each other. Similarly, Tukey's HSD was used to test for significance between selected groups in case of significant light and DIC interactions.

To determine if the addition of AZ and/or TRIS significantly affects net productivity of each seagrass species in fresh seawater (control), raw net productivity data were compared using paired t-tests. Further, to test if light levels affect HCO_3^- utilization, paired t-tests were used to compare the change in percentage net photosynthesis between the two light levels for each inhibitor type. Arcsine transformation was not performed on the percentage net photosynthesis data, since the data exceeded the range of 0 to 100. Instead, percentage net photosynthesis responses were square root transformed prior to analysis to meet the assumption of normality for the test. All statistical analyses were conducted using R statistical software (R Development Core Team 2014).

3.3 Results

3.3.1 Light and DIC effects on photosynthetic and growth response

3.3.1.1 Experimental parameters

Water temperature and salinity in the experimental aquaria did not vary considerably throughout the experiment (average ± S.D.: 23.9 ± 0.3°C and 35.9 ± 0.5). Manipulation of the CO_2 system resulted in a 140% increase in CO_2 concentration, a 10% increase in HCO_3^- concentration, and a corresponding decrease of ~0.3 units in pH, compared to present day control conditions (Table 3.1). Carbonate system parameters of the DIC treatments remained in the target range. Nutrient concentrations were similar between aquarium tanks; with an average (± S.D.) water column ammonium concentration of 0.12 ± 0.03 μM and nitrate concentration of 1.30 ± 0.11 μM.

3.3.1.2 Productivity responses to light and DIC enrichment

For *C. serrulata*, DIC enrichment and light availability both increased relative growth rates (RGR) significantly (ANOVA: DIC $P = 0.015$; light $P = 0.002$) (Table 3.2; Fig. 3.1a). When light levels were at 100 μmol m^{-2} s^{-1} and 380 μmol m^{-2} s^{-1}, RGRs increased by 20% and 26% respectively, relative to that at 35 μmol m^{-2} s^{-1} (Tukey HSD: 100 μmol m^{-2} s^{-1} $P = 0.015$; 380 μmol m^{-2} s^{-1}: $P = 0.002$). DIC enrichment raised RGR by 13%. There was no significant interaction between light and DIC enrichment on RGR in *C. serrulata*. In *H. uninervis*, effect of DIC enrichment on RGR varied with light levels (ANOVA: $P = 0.009$) (Table 3.2; Fig. 3.1b). DIC enrichment increased RGR only for *H. uninervis* growing at 100 μmol m^{-2} s^{-1} (Tukey HSD: $P = 0.027$), but not at lower (Tukey HSD: $P = 0.955$) or higher (ANOVA: $P = 0.905$) light levels.

Table 3.1: Measured and calculated carbonate system parameters for high DIC and control treatments. Temperature and pH readings in the aquaria tanks were measured with a hand held pH probe calibrated on the NBS scale. Dissolved inorganic carbon (DIC) and total alkalinity (AT) concentrations were measured from water samples were taken every four days from each aquaria tank. Carbonate system parameters were calculated using USGS CO2calc software (Robbins et al. 2010). Average values and S.D. (in brackets) were given.

	Measured parameters					Calculated parameters					Nutrient concentrations		
DIC treatment	DIC (µmol kg^{-1}SW)	pH [NBS]	A_T (µmol kg^{-1} SW)	Temp (^oC)	Salinity (psu)	pH [NBS]	pCO_2 (µatm)	CO_2 (µmol kg^{-1}SW)	HCO_3^- (µmol kg^{-1} SW)	CO_3^{2-} (µmol kg^{-1} SW)	NH_4^+ (µmol/L)	NO_3^- (µmol/L)	NO_2^- (µmol/L)
High	2215 (16.1)	7.85 (0.1)	2327 (8.4)	23.8 (0.3)	36.4 (0.7)	7.82 (0.04)	1077 (104.1)	31.7 (3.1)	2083 (20.6)	101 (8.2)	0.12 (0.01)	1.35 (0.11)	0.07 (0.01)
Control	2063 (9.8)	8.26 (0.1)	2327 (10.0)	23.9 (0.3)	36.4 (0.8)	8.15 (0.02)	447 (23.1)	13.1 (0.7)	1858 (14.1)	192 (7.1)	0.13 (0.01)	1.26 (0.11)	0.07 (0.01)

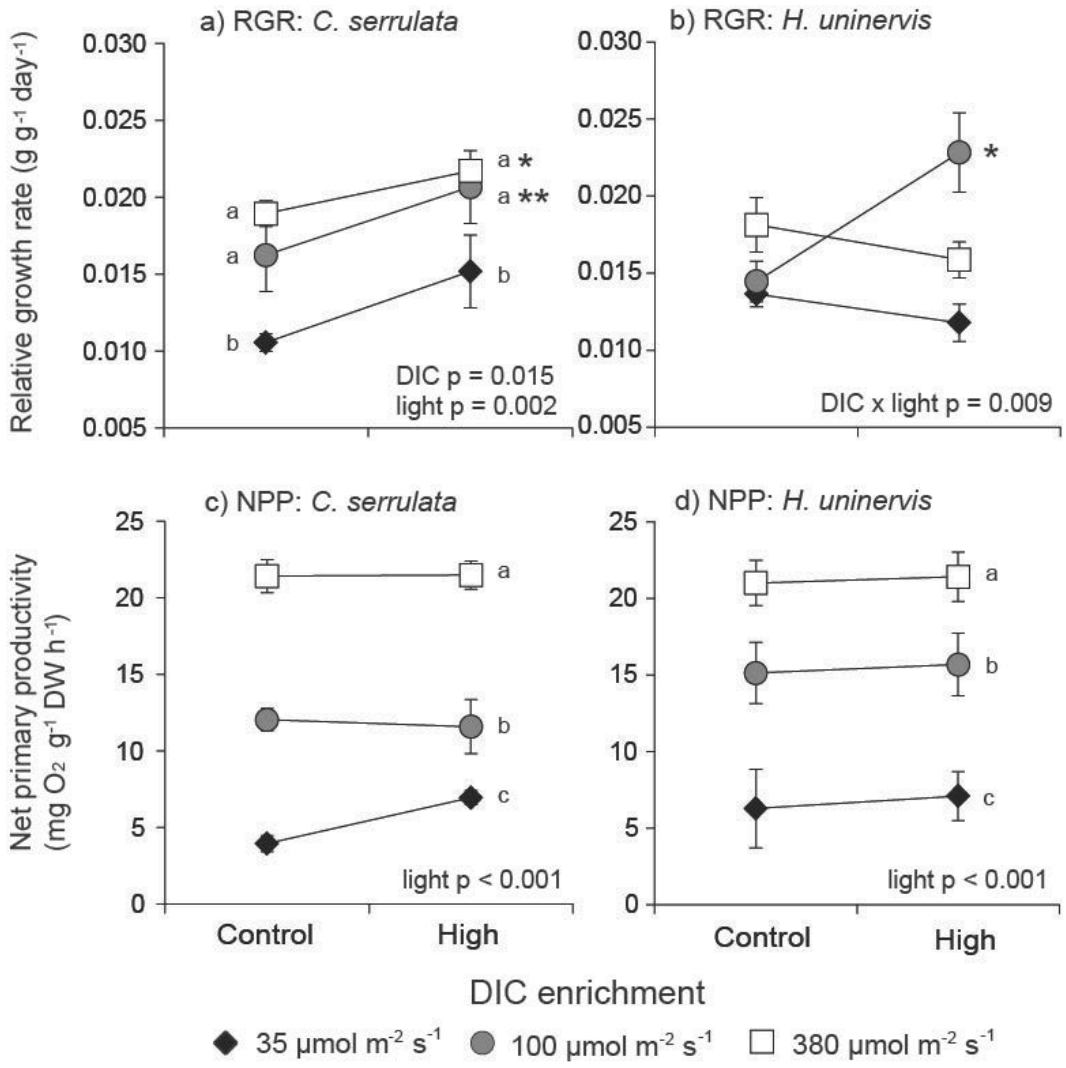

Fig. 3.1: Comparison of relative growth rates and net primary productivity of *C. serrulata* and *H. uninervis* after two weeks exposure to light and DIC. (a-b) RGR- relative growth rate, (c-d) NPP – net primary productivity. Letters indicate significant differences between light treatments based on Tukey's HSD test; asterisks indicate significant differences between DIC treatments based on ANOVA results where there is no interaction between light and DIC, or based on Tukey's HSD test if an interaction was detected (* $P < 0.05$; ** $P < 0.01$). Control DIC = 2063 µM; high DIC = 2215 µM. Means (± S.E.) are given (n = 3).

Table 3.2: Two-way ANOVA results. All parameters were analysed with DIC and light treatments as fixed factors. n = 3. Significant *P*-values are in bold. Prior analysis, square-root transformation had been applied to RGR for *Cymodocea serrulata*, and to E_c, α and respiration for *Halodule uninervis*. RGR- relative growth rate, P_{max}- maximum photosynthetic rate, E_k- saturating irradiance, E_c- compensation irradiance, α- photosynthetic efficiency.

		Cymodocea serrulata				*Halodule uninervis*			
		df	MS	F	*P*	df	MS	F	*P*
RGR	DIC	1	1.07×10^{-3}	8.115	**0.015**	1	8.94×10^{-6}	1.189	0.297
	Light	2	1.43×10^{-3}	10.793	**0.002**	2	5.63×10^{-5}	7.488	**0.008**
	DIC x light	2	3.84×10^{-5}	0.290	0.753	2	5.45×10^{-5}	7.238	**0.009**
NPP	DIC	1	3.400	1.116	0.312	1	1.600	0.142	0.713
	Light	2	389.100	126.762	$\mathbf{8.52 \times 10^{-9}}$	2	320.800	29.000	$\mathbf{2.54 \times 10^{-5}}$
	DIC x light	2	5.200	1.705	0.223	2	0.100	0.006	0.994
Dark respiration	DIC	1	18.924	2.033	0.179	1	0.002	0.003	0.957
	Light	2	23.395	2.513	0.123	2	2.584	4.697	**0.031**
	DIC x light	2	0.163	0.018	0.983	2	0.545	0.991	0.400
P_{max}	DIC	1	60.320	27.800	$\mathbf{1.97 \times 10^{-4}}$	1	4.320	0.514	0.487
	Light	2	190.160	87.630	$\mathbf{6.92 \times 10^{-8}}$	2	126.060	14.992	$\mathbf{5.45 \times 10^{-4}}$
	DIC x light	2	8.500	3.920	**0.049**	2	2.550	0.304	0.744
E_k	DIC	1	8181	39.080	$\mathbf{4.25 \times 10^{-5}}$	1	150	0.293	0.598
	Light	2	3475	16.597	$\mathbf{3.50 \times 10^{-4}}$	2	3668	7.149	**0.009**
	DIC x light	2	1132	5.409	**0.021**	2	2547	4.964	**0.027**
E_c	DIC	1	21.500	0.204	0.660	1	1.315	0.789	0.392
	Light	2	534.500	5.053	**0.026**	2	8.073	4.843	0.029
	DIC x light	2	29.200	0.276	0.763	2	8.977	5.385	0.021
α	DIC	1	1.53×10^{-2}	5.513	**0.037**	1	1.14×10^{-2}	2.413	0.146
	Light	2	2.24×10^{-4}	0.081	0.923	2	2.22×10^{-3}	0.469	0.637
	DIC x light	2	1.91×10^{-3}	0.686	0.522	2	0.024	5.049	0.026

In *C. serrulata*, net primary productivity (NPP) was significantly influenced by light levels (ANOVA: $P < 0.001$) (Table 3.2; Fig. 3.1c). DIC enrichment did not significantly raise NPP (ANOVA: $P = 0.312$) (Table 3.2). NPP in *C. serrulata* increased with light, by 12 % (at 100 μmol m^{-2} s^{-1}) and 29 % (at 380 μmol m^{-2} s^{-1}) (Tukey HSD: 100 μmol m^{-2} s^{-1}: $P < 0.001$; 380 μmol m^{-2} s^{-1}: $P < 0.001$). Similarly for *H. uninervis*, NPP increased significantly with light but not with DIC enrichment (ANOVA: light $P < 0.001$; DIC $P = 0.713$) (Table 3.2; Fig. 3.1d). NPP increased by 13 to 22 % in *H. uninervis* with light (Tukey HSD: 100 μmol m^{-2} s^{-1}: $P = 0.002$; 380 μmol m^{-2} s^{-1}: $P < 0.001$). Dark respiration rates did not vary with light or DIC treatments in *C. serrulata* (Table 3.2). In *H. uninervis*, dark respiration rates responded to light levels only (ANOVA: $P = 0.031$). Dark respiration rates increased by 67 % at 380 μmol m^{-2} s^{-1} relative to at 35 μmol m^{-2} s^{-1} (Tukey HSD: $P = 0.032$).

3.3.1.3 Photosynthetic-irradiance (P-E) curves

The adapted hyperbolic tangent model provided a good fit for all P-E curves ($R^2 > 0.85$; $P < 0.050$). Photosynthetic rates increased linearly (initial slope, α) with irradiance before plateauing off at the maximum photosynthetic rate (P_{max}) above saturating irradiance (E_k).

The increase in maximal photosynthetic rates (P_{max}) in *C. serrulata* with DIC enrichment depended on light levels (ANOVA: $P = 0.049$) (Table 3.2; Fig. 3.2a). The observed increase in P_{max} due to DIC enrichment became smaller with increasing light availability (Fig. 3.2). Post-hoc tests indicated that P_{max} significantly increased with DIC in seagrasses growing at 35 μmol m^{-2} s^{-1} (60% increase) and 100 μmol m^{-2} s^{-1} (36% increase (Tukey HSD: 35 μmol m^{-2} s^{-1}: $P = 0.014$; 100 μmol m^{-2} s^{-1}: $P = 0.011$). There was no significant increase in P_{max} at 380 μmol m^{-2} s^{-1} ($P = 0.969$). For *H. uninervis*, P_{max} increased with light treatments but not with DIC enrichment (ANOVA: light $P < 0.001$; DIC $P = 0.487$) (Table 3.2; Fig. 3.2b). P_{max} was significantly higher at 380 μmol $m^{-2}s^{-1}$ than at 35 (71% increase) and 100 μmol m^{-2} s^{-1} (35% increase) (Tukey HSD: 35 μmol m^{-2} s^{-1}: $P < 0.001$; 100 μmol $m^{-2}s^{-1}$: $P = 0.014$).

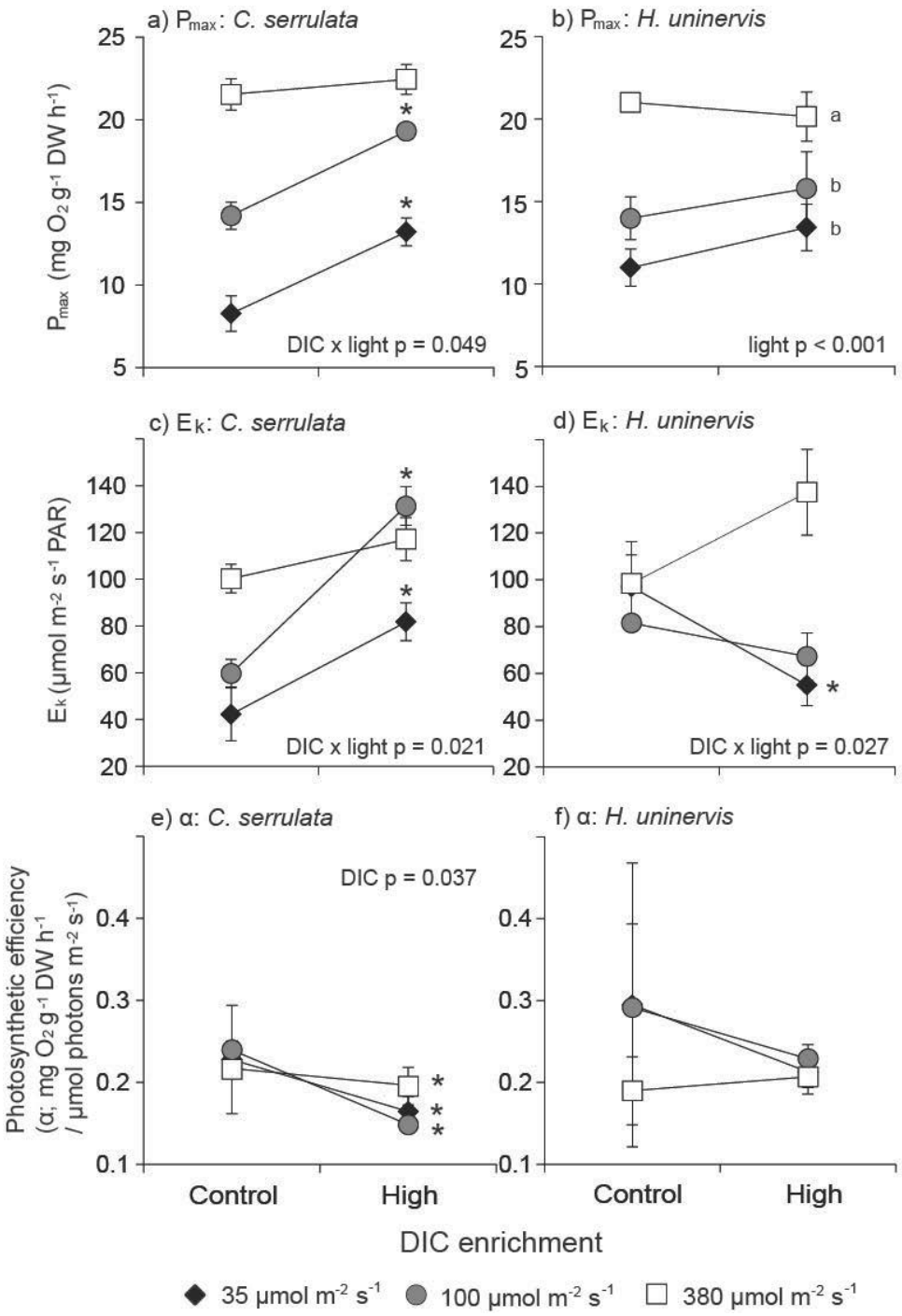

Fig. 3.2: Comparison of photosynthetic parameters between light and DIC for *C. serrulata* and *H. uninervis* after two weeks exposure. (a-b) P_{max} - maximal photosynthetic rate, (c-d) E_k - saturating irradiances, (e-f) α - photosynthetic efficiency. Letters indicate significant differences between light treatments based on Tukey's HSD test; asterisks indicate significant differences between DIC treatments based on ANOVA results where there is no interaction between light and DIC, or based on Tukey's HSD test if an interaction was detected (* $p < 0.05$). Control DIC = 2063 µM; high DIC = 2215 µM. Means (± S.E.) are given (n = 3).

The response in E_k to DIC enrichment was dependent on light levels in *C. serrulata*, as evidenced by a significant light × DIC interaction (ANOVA: $P = 0.021$) (Table 3.2; Fig. 3.2c). DIC enrichment resulted in a greater increase in E_k at lower light levels (Tukey HSD: 35 μmol m^{-2} s^{-1}: $P = 0.051$; 100 μmol m^{-2} s^{-1}: $P < 0.001$) than at high light level (Tukey HSD: $P = 0.713$) (Fig. 3.2). For *H. uninvervis*, there was an interactive effect of light and DIC enrichment on E_k (ANOVA: $P = 0.027$) (Table 3.2; Fig. 3.2d). In low light, DIC enrichment reduced E_k by 43% (Tukey HSD: $P = 0.028$). However, at and above saturating light (100 and 380 μmol m^{-2} s^{-1}) DIC enrichment had no significant effect on E_k.

Compensation irradiance (E_c) in *C. serrulata* varied with light (ANOVA: $P = 0.026$) but not with DIC enrichment (Table 3.2). At 380 μmol m^{-2} s^{-1}, E_c was 102% higher than at 35 μmol m^{-2} s^{-1} (Tukey HSD: $P = 0.020$), but was not significantly different from that at 100 μmol m^{-2} s^{-1} (Tukey HSD: $P = 0.236$). In *H. uninervis*, there was no main or interactive effect of DIC enrichment and light on E_c (ANOVA: $P = 0.021$; alpha lowered to 0.01) (Table 3.2).

Photosynthetic efficiency (α) was significantly reduced by DIC enrichment (ANOVA: $P = 0.037$), but not among light levels (Table 3.2). No interaction of DIC enrichment and light was detected for α in *C. serrulata* (Table 3.2; Fig. 3.2e). Overall, DIC addition reduced α by 34 % for *C. serrulata*. For *H. uninervis*, there were no main or interactive effects of light and DIC enrichment on α (ANOVA: $P = 0.026$; alpha lowered to 0.01) (Table 3.2; Fig. 3.2f).

In summary, both DIC enrichment and light levels influenced the response of photosynthetic parameters in *C. serrulata*. The change in parameters such as P_{max} and E_k depended on either DIC enrichment or light levels, and also the interaction of both factors. Conversely, photosynthesis in *H. uninervis* - P_{max} and E_k - seemed to be strongly influenced by light treatment but was independent of DIC enrichment.

3.3.2 Effects of light levels on HCO_3^- uptake

In *C. serrulata*, addition of AZ significantly reduced net photosynthesis (paired t-test: t = 4.261, df = 11, $P < 0.001$) (Fig. 3.3a), indicating that activity of external CA is an important mechanism aiding in HCO_3^- uptake. The reduction in net photosynthesis due to the inhibition of CA-catalysed conversion of HCO_3^- (AZ treatment) was not significantly different between light levels (paired t-test: t = 1.851, df = 5, $P = 0.123$) (Fig. 3.3a). Addition of TRIS to *C. serrulata* also resulted in a strong reduction in net photosynthesis (paired t-test: t = 3.962, df = 11, $P = 0.001$) (Fig. 3.3a). This suggests that presence of the H^+ gradient is essential for HCO_3^- transport into the cell. Dissipation of H^+ gradient alone (TRIS addition) resulted in a higher reduction in net photosynthesis under saturating light (>90 %), compared to that under limiting light (60%) (paired t-test: t = 10.126, df = 5, $P < 0.001$) (Fig. 3.3a). When both CA and H^+ extrusion were inhibited (addition of both AZ and TRIS), net photosynthesis in *C. serrulata* was reduced significantly (paired t-test: t = 4.095, df = 11, $P < 0.001$) (Fig. 3.3a). Net photosynthesis was reduced to a greater extent under saturating light than under limiting light (paired t-test: t = 7.855, df = 5, $P < 0.001$) (Fig. 3.3a).

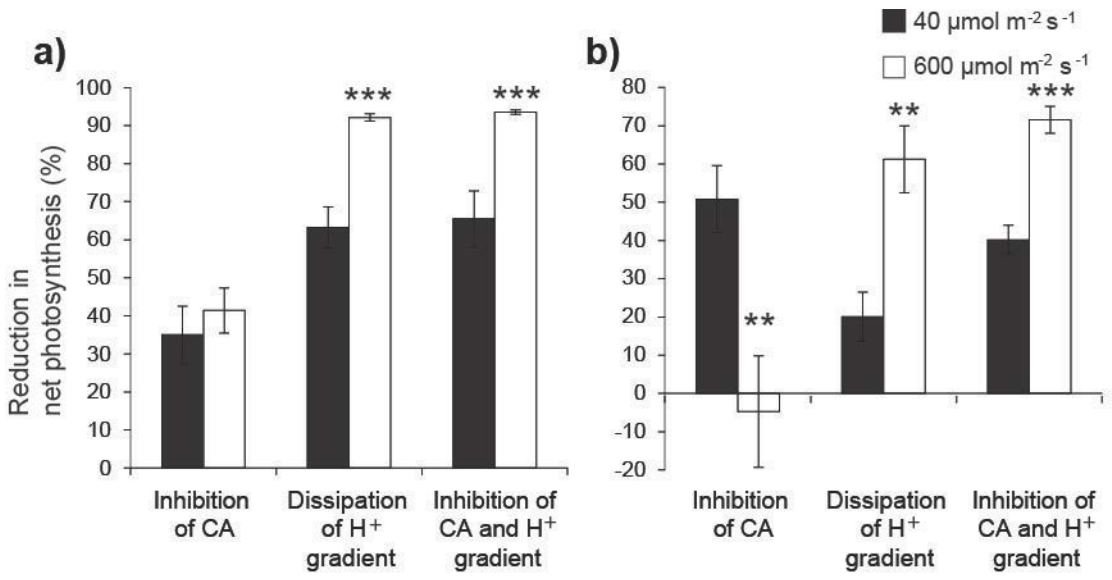

Fig. 3.3: Reduction in net photosynthetic responses of (a) *C. serrulata* and (b) *H. uninervis*, when subjected to HCO_3^- uptake inhibitors. Net photosynthetic responses were expressed relative to control rates in normal seawater. 0.1 mM acetazolamide (inhibition of extracellular CA), 45 mM TRIS buffer at pH 8.0 (dissipation of H^+ gradient), and a combination of TRIS and acetazolamide (inhibition of CA and H^+ gradient). Asterisks indicate significant differences between light treatments for each inhibitor or inhibitor combination (** $P < 0.01$; *** $P < 0.001$). Means (± S.E) are given (n = 6).

In *H. uninervis*, inhibition of extracellular CA (AZ treatment) reduced net photosynthesis by 50% in limiting light (paired t-test: t = -4.188, df = 5, $P = 0.009$) (Fig. 3.3b), but had no effect in saturating light. This indicated that when light is limiting, CA played a more significant role in HCO_3^- uptake. The dissipation of H^+ gradient (TRIS addition) decreased net photosynthesis in *H. uninervis* (paired t-test: t = 2.755, df = 11, $P < 0.001$) (Fig. 3.3b). Net photosynthesis decreased more under saturating light conditions (60%) than under low light (20%) (paired t-test: t = 4.380, df = 5, $P = 0.007$) (Fig. 3.3b). Inhibition of both CA and H^+ extrusion (addition of both AZ and TRIS) lowered net photosynthesis under both light levels (paired t-test: t = 4.079, df = 11, $P <$

0.001) (Fig. 3.3b). The extent of inhibition was greater under saturating light than in limiting light (paired t-test: t = 8.315, df = 5, $P < 0.001$).

3.4 Discussion

Light availability can affect the ability of tropical seagrasses to respond to increased DIC provisioning under OA. The present study examined whether *C. serrulata* and *H. uninervis* were able to adjust growth and photosynthesis responses when exposed to an enriched DIC concentration approximating predicted end-of-century pCO_2 level (1077 μatm) (Collins et al. 2013) over a range of light levels. Growth of *C. serrulata* was stimulated by both DIC enrichment and light availability while growth of *H. uninervis* was strongly influenced by light availability only. Interactive effects of DIC enrichment and light treatment were evident in P-E curve parameters in *C. serrulata*, while photosynthetic potential in *H. uninervis* was affected by the experimental light treatment. The use of CA and H^+ gradient inhibitors highlighted important differences in carbon uptake mechanisms which may explain some of the differences in responses of the species on a physiological level.

3.4.1 Growth and net productivity response

The experimental light treatments represented a range of ecologically relevant light conditions that occur in the natural environment. Both *C. serrulata* and *H. uninervis* were grown in light-saturated conditions under both moderate (100 μmol m^{-2} s^{-1}) and high (380 μmol m^{-2} s^{-1}) light treatments, where light levels were generally at or above E_k. The only exception to this was for *C. serrulata* at 100 μmol m^{-2} s^{-1} under DIC enrichment, in which E_k was 120 μmol m^{-2} s^{-1}. In contrast, the lowest light treatment (35 μmol m^{-2} s^{-1}) was below E_k for both species under all DIC levels, and so photosynthesis was light-limited.

All light treatments were above E_c, and so both seagrass species were in net carbon surplus.

For *C. serrulata*, the effect of light and DIC enrichment on growth appeared to be additive, meaning that increasing both DIC and light increased their growth rates. Thus, the highest growth rate was observed at the high light level under DIC enrichment. Further, short term exposure to DIC enrichment meant that plants growing at 35 and 100 μmol m^{-2} s^{-1} were able to grow as fast as plants without DIC enrichment at 100 and 380 μmol m^{-2} s^{-1} respectively, such that DIC enrichment somewhat compensated for lower light availability (Zimmerman et al. 1997). While a previous *ex-situ* study showed that growth rates of *C. serrulata* did not respond to CO_2 enrichment (Ow et al. 2015), our current study showed an increase in growth rates for this species with DIC enrichment, albeit at a very modest 13%. Higher increases in shoot density (194 – 350%) and above-ground biomass (32 – 987%) of *C. serrulata* were observed in natural CO_2 vent sites with considerably greater DIC enrichment, compared to adjacent non-CO_2 enriched sites (Russell et al. 2013). Net primary production of *C. serrulata* in this study appeared to be strongly limited by light, not DIC concentration. However, previous studies have indicated an increase in net primary productivity with CO_2 enrichment in this species (Russell et al. 2013; Ow et al. 2015). Here, the results suggest that light availability plays an upstream role, relative to DIC, in the hierarchical control of seagrass photosynthesis.

Net production and growth in *H. uninervis* did not appear to be DIC-limited, as it was less sensitive to an increase in DIC than *C. serrulata* under all light treatments. However, previous work has shown that the same population of *H. uninervis* can respond to DIC addition by increasing net productivity and growth under similar treatment conditions (Ow et al. 2015). Seasonal variation in carbon demand for growth and metabolism might have contributed to the observed differences in response to DIC enrichment between studies (Kaldy and Dunton 2000; Lee et al. 2005).

Both DIC enrichment and light availability stimulate productivity and growth in seagrasses (Zimmerman et al. 1997; Jiang et al. 2010; Collier et al. 2012b; Burnell et al. 2014a). However, while *C. serrulata* growth rates increased with DIC enrichment and light availability, *H. uninervis* did not demonstrate a growth response to DIC enrichment. The growth response of *H. uninervis* here was limited by light availability, consistent

with its net productivity response. Growth responses to DIC enrichment can also be influenced by nutrient availability (Stitt and Krapp 1999) and water temperature (Touchette and Burkholder 2000a). Sediment pore water nutrients were not measured in the present study and hence it was not possible to assess if overall nutrient availability was limiting seagrass productivity. Knowledge of the interactive effects of environmental factors (light, temperature, nutrients) with DIC enrichment is needed to predict future seagrass productivity responses in the field.

3.4.2 Photosynthetic potential

Photosynthetic response of *C. serrulata* to DIC enrichment depended on treatment light levels. Photosynthetic capacity (P_{max}) was higher in *C. serrulata* exposed to higher DIC levels for two weeks. *C. serrulata* at similar CO_2 and light conditions to those used here - DIC enrichment and at 400 μmol m^{-2} s^{-1}- did increased P_{max} by ~20% (Ow et al. 2015). Increases in P_{max} in response to CO_2 enrichment have also been observed in *Z. marina* (Zimmerman et al. 1997) and *Z. noltii* (Alexandre et al. 2012). A larger increase in P_{max} was observed in plants from the limiting (60% increase) compared to the saturating light treatments (0% increase). DIC enrichment can enhance maximum photosynthetic capacity by providing more substrate for fixation and simultaneously lowering photorespiration rates (Long et al. 2004). Light increases maximum photosynthetic capacity by boosting the production of reducing intermediates (e.g. NADPH and ATP) for the carbon reduction cycle (Hall and Rao 1994). In theory, since both factors have independent modes of actions, their combined effect should be synergistic (Bliss 1939; van Dam et al. 2012). The combined sub-additive effect on P_{max} observed in results suggested that the extent of DIC limitation was greater under low light than high light.

Saturating irradiance (E_k) in *C. serrulata* increased with DIC enrichment, with a greater rise in E_k observed at lower light levels. Higher saturating light requirements could be driven by the higher photosynthetic capacity due to greater DIC availability, as similarly observed in *Thalassia hemprichii* (Jiang et al. 2010). The lowering of photosynthetic efficiency with DIC enrichment was unexpected, as increased CO_2 availability would mean less resources (i.e. ATP) were needed to procure HCO_3^- for photosynthesis (Kubler and Raven 1995). Under CO_2 enrichment, *Zostera marina* tripled

the rate of light-saturated photosynthesis (i.e. P_{max}) to reduce the daily photoperiod required for a positive carbon balance (Zimmerman et al. 1997). Overall, the studies conducted so far suggest that while DIC enrichment increases the intensity of saturating irradiance required to reach maximum photosynthetic rates, it also reduces the daily period of saturating irradiance required to achieve a net carbon surplus.

Photosynthesis in *H. uninervis*, in contrast was limited by light, and not by DIC availability. Photosynthetic capacity (P_{max}) and E_k increased with increasing light levels. Increased DIC concentration lowered E_k for *H. uninervis* shoots growing under low light (35 and 100 μmol m^{-2} s^{-1}). This implies that DIC enrichment could, to a certain extent, compensate for low light levels by reducing light requirements in this species. However, the increase in productivity with light availability was higher than the increase with DIC enrichment (Zou and Gao 2009), with maximum photosynthetic rates remaining the highest under high light treatment. Similarly in *Z. marina*, CO_2 enrichment only increased shoot production and below-ground biomass under light-replete but not light-deplete treatments (Palacios and Zimmerman 2007).

3.4.3 Effects of light on DIC utilization

Normally the supply of CO_2, the preferred DIC species for seagrasses (Beer and Waisel 1979; Durako 1993), is limited by low free CO_2 concentration and diffusion rates, and slow conversion of HCO_3^- to CO_2 (Stumm and Morgan 2013). While most seagrasses can utilise HCO_3^- as a DIC source (Bjork et al. 1997; Hellblom et al. 2001) and the concentration of total DIC is non-limiting, the high energetic cost of HCO_3^- uptake makes it a less preferred substrate under low light levels (Beer et al. 2002; Hepburn et al. 2011). Light fuels the generation of ATP for both carbon fixation and HCO_3^- uptake (Hall and Rao 1994; Axelsson et al. 2000). This may explain the apparent paradox that *C. serrulata* in our experiment was more DIC-limited at lower light levels than at higher levels. This finding was consistent with the postulation that HCO_3^- utilization is limited at lower light levels (Schwarz et al. 2000). In Schwarz et al. (2000), both deep water (i.e. low light) *Halophila ovalis* and *C. serrulata* showed a greater increase in relative electron transport rates (100 % and 66 % respectively) compared to their intertidal (i.e. high light)

counterparts (30 % and 20 % respectively) when subjected to an 180 % increase in DIC concentration. Low light availability can lower HCO_3^- utilization, and OA conditions could boost DIC supply by providing more dissolved CO_2 (Campbell and Fourqurean 2013b; Burnell et al. 2014a).

Application of CA and H^+ gradient inhibitors showed that, in general, both CA and H^+ gradients are important mechanisms to allow utilisation of HCO_3^- as a carbon source for the two species investigated. This was observed previously in several other seagrass species (Beer et al. 2002; Uku et al. 2005; Burnell et al. 2014a). For *C. serrulata*, the inhibition of carbonic anhydrase (CA) and H^+ extrusion both reduced net photosynthesis. The strong dependence on H^+ extrusion for HCO_3^- utilisation in *C. serrulata* meant that adequate light levels were needed before HCO_3^- can be used efficiently for photosynthesis. Therefore, at limiting light levels this species probably depends more on CO_2 diffusion. CA-mediated HCO_3^- dehydration in acidic zones was thought to be a more efficient means of HCO_3^- utilization than CA conversion alone, as the CO_2 concentration at equilibrium is higher within acidic zones than at normal seawater pH, thus driving the inward diffusion of CO_2 (Beer et al. 2002). Despite this, Bjork et al. (1997) and Uku et al. (2005) demonstrated that extracellular CA-catalysed HCO_3^- conversion to CO_2 (without H^+ extrusion sites) was enough to support photosynthetic demand in *C. serrulata*. Differences between their findings and ours could be due to long term acclimation of conspecifics to different source meadow environments, or that there were genotypic variation among seagrass populations (Reusch et al. 2008; Salo et al. 2015).

Halodule uninervis appeared to vary HCO_3^- uptake mechanisms, enabling it to use HCO_3^- over a wider light range for photosynthesis. Under low light conditions, CA-mediated conversion of HCO_3^- contributed substantially to the carbon supply for photosynthesis (Beer et al. 2002); when light became saturating, HCO_3^- uptake was supplemented by H^+ co-transport with HCO_3^-. Extracellular CA-mediation of HCO_3^- uptake is less likely to depend on light availability, when compared to the H^+ extrusion-driven co-transport of HCO_3^- (Bjork et al. 1997; Hellblom and Axelsson 2003). This flexibility between HCO_3^- utilization pathways suggested *H. uninervis* was able to mediate, to a certain extent, DIC limitation under low light conditions. Similarly, efficient use of HCO_3^- utilization mechanisms was thought to account for the lack of

photosynthetic responses to DIC enrichment in marine macroalgae (Israel and Hophy 2002).

Differential sensitivity to photosynthetic carbon among seagrass species could be due to species variation in DIC utilization mechanisms (Uku et al. 2005; Campbell and Fourqurean 2013b) and extent of carbon-limitation (Beer and Koch 1996; Campbell and Fourqurean 2013b). Species such *C. serrulata* (this study) and *Thalassia testudinum* (Campbell and Fourqurean 2013b) would benefit more from increased CO_2 supply under OA conditions since they are limited in their ability to utilise the dominant HCO_3^- in seawater. Species less limited by DIC, such as *H. uninervis* (this study), *Halodule wrightii* and *Syringodium filiforme* might show a smaller response to DIC enrichment (Campbell and Fourqurean 2013b).

3.4.4 Light and OA as drivers of seagrass productivity

Under future scenarios of OA, marine macrophytes like seagrasses could benefit, from both increased DIC concentration and a proportional rise in CO_2 (Hall-Spencer et al. 2008; Fabricius et al. 2011). While short-term (e.g. this study) and long-term (Takahashi et al. 2015) studies have documented varying degrees of DIC limitation in seagrasses, physiological processes that could regulate responses to increased DIC over intermediate time-scales remain unexplored for seagrasses. Epiphytic algae may also benefit from higher CO_2 (Burnell et al. 2014b), and in turn compete with seagrasses for the same resources. Their effects would vary with their composition, biomass and the turnover rates of seagrass leaves (Campbell and Fourqurean 2014). Furthermore, many seagrass habitats are primarily light-limited (Ralph et al. 2007). The range of light levels used in this experiment, representing the recent light exposure history (~ five years) of the seagrasses at their source meadow, was already much reduced compared to pre-European settlement due to a four-times increase in sediment runoff (Kroon et al. 2012). Reduced light availability, due to increased sedimentation and epiphytic algal growth, can negate positive OA effects on seagrass growth (Burnell et al. 2014b). Our study showed that the rise in light availability elicited a greater increase in seagrass productivity than DIC enrichment. Compared to OA, variation in water clarity occurs over shorter frequencies

and with greater intensities, thus playing a more imminent role in controlling seagrass productivity. Hence, to ensure continued productivity in seagrass meadows in the future, changes in water clarity and OA have to be studied and managed in unison.

CHAPTER 4

NITRATE FERTILISATION DOES NOT ENHANCE CO_2 RESPONSES IN TWO TROPICAL SEAGRASS SPECIES [3]

Abstract

Seagrasses are often considered "winners"' of ocean acidification (OA); however, seagrass productivity responses to OA could be limited by nitrogen availability, since nitrogen-derived metabolites are required for carbon assimilation. We tested nitrogen uptake and assimilation, photosynthesis, growth, and carbon allocation responses of the tropical seagrasses *Halodule uninervis* and *Thalassia hemprichii* to OA scenarios (428, 734 and 1213 µatm pCO_2) under two nutrients levels (0.3 and 1.9 µM NO_3^-). Net primary production (measured as oxygen production) and growth in *H. uninervis* increased with pCO_2 enrichment, but were not affected by nitrate enrichment. However, nitrate enrichment reduced whole plant respiration in *H. uninervis*. Net primary production and growth did not show significant changes with pCO_2 or nitrate by the end of the 24-day experiment in *T. hemprichii*. However, nitrate incorporation in *T. hemprichii* was higher with nitrate enrichment. There was no evidence that nitrogen demand increased with pCO_2 enrichment in either species. Contrary to our initial hypothesis, nutrient increases to levels approximating present day flood plumes only had small effects on metabolism. This study highlights that the paradigm of increased productivity of seagrasses under ocean acidification is not valid for all species under all environmental conditions.

[3] Chapter 4 has been adapted into and published as Ow Y.X., Vogel N., Collier C.J., Holtum J.A.M., Flores F. and Uthicke S. (2016) Nitrate fertilisation does not enhance CO2 responses in two tropical seagrass species. Scientific Reports 6: 23093.

4.1 Introduction

Ocean acidification (OA) increases oceanic carbon dioxide (CO_2) concentration and alters the relative proportion of dissolved inorganic carbon (DIC) species in seawater (Caldeira and Wickett 2003). Seawater concentrations of CO_2 and bicarbonate were projected to rise by 250% and 24%, respectively, up from current levels of 8 and 1650 μmol kg^{-1} seawater by the end of the century (Koch et al. 2013). Seagrass productivity, thought to be limited by current seawater DIC composition, could benefit from the increased availability of carbon (Beer et al. 2002). Photosynthetic rates of most seagrasses are enhanced by elevated partial pressure of CO_2 (pCO_2) (Zimmerman et al. 1997; Ow et al. 2015), which is the preferred DIC substrate (Beer and Koch 1996; Beer et al. 2002). Carbon fixed in the leaves through photosynthesis has a number of sinks, therefore under increased pCO_2, growth, respiration, storage, biomass, and reproductive output may be increased (Zimmerman et al. 1997; Touchette and Burkholder 2000a; Palacios and Zimmerman 2007; Jiang et al. 2010; Ow et al. 2015).

Coastal seagrass systems can be subjected to fluctuations in water column nutrient levels (Schaffelke et al. 2012). While strong and sustained nutrient enrichment can stimulate the growth of competing macroalgae and epiphytes and in turn inhibit seagrass growth (Burkholder et al. 2007), moderate increases in nutrients can promote seagrass growth, which demonstrates nutrient limitation (Udy et al. 1999; Kelaher et al. 2013). The paradigm that OA benefits seagrass meadow productivity assumes that other environmental parameters, such as nutrient levels are not co-limiting productivity (Beer and Koch 1996; Zimmerman et al. 1997). In terrestrial plants, nutrient availability can affect responses to elevated CO_2; they initially respond by increasing productivity and growth but photosynthesis and growth are subsequently downregulated as nitrogen becomes limited (Stitt and Krapp 1999).

Responses to elevated pCO_2 are affected by nutrient availability because carbon and nitrogen metabolism are strongly coupled (Touchette and Burkholder 2007). Nitrate and nitrogen metabolites regulate processes such as photosynthesis (Nielsen et al. 1998), organic acid synthesis and starch accumulation (Scheible et al. 1997). In turn, energy stored in carbon metabolites plays a role in regulating the uptake and incorporation of nitrogen (Touchette and Burkholder 2000b); leading some authors to speculate that moderate increases in dissolved

inorganic nitrogen (DIN) may augment CO_2 responses in tropical seagrasses (Palacios and Zimmerman 2007; Apostolaki et al. 2014).

The demand for nitrogen could be affected by the rate of carbon assimilation, and therefore be affected by CO_2 enrichment (Stitt and Krapp 1999). In marine macroalgae, productivity responses to CO_2 enrichment are enhanced under increased nutrient availability when compared to non-enriched nutrient conditions (Gordillo et al. 2003; Russell et al. 2009; Hofmann et al. 2014). With increased nutrient availability, marine macroalgae in enriched pCO_2 conditions increased photosynthetic efficiency (Gordillo et al. 2003; Russell et al. 2009), growth (Gordillo et al. 2003) and nitrogen uptake and assimilation (Hofmann et al. 2014), observations consistent with an increase in the demand for nitrogen driven by enhanced productivity.

Nitrogen incorporation involves both the uptake and assimilation of nitrogen species (Touchette and Burkholder 2000b). Both uptake and assimilation are inducible processes that may reflect instantaneous nitrogen demand in the plant (Touchette and Burkholder 2000b). For seagrasses, inorganic nitrate and ammonium are considered the most significant sources of nitrogen, supplying over 90% of externally acquired nitrogen (Touchette and Burkholder 2000b; Nayar et al. 2010). Sediment pore-water can potentially supply the majority of nitrogen for seagrass as the sediment contains higher concentrations than does the water column, but seagrasses will rapidly absorb DIN from the water column (Romero et al. 2006). Furthermore, the uptake affinity (K_m) of leaves is greater than that of rhizomes, meaning that a small increase in supply to the water column will trigger rapid uptake (Romero et al. 2006). Nitrogen assimilation involves the enzymatic conversion of nitrate to nitrite by nitrate reductase (NR), and ammonium to glutamine through the glutamine synthetase (GS)/ glutamate synthase pathway (Touchette and Burkholder 2000b). The activities of NR and GS, key in amino acids synthesis (Pregnall et al. 1987), occur primarily in leaves and to a much smaller degree, in the rhizomes and roots (Kraemer et al. 1997). Therefore, increasing external inorganic nitrogen may promote nitrogen uptake and assimilation in seagrasses (Pregnall et al. 1987; Kraemer et al. 1997).

The partitioning of fixed carbon to sinks is affected by nitrogen availability (Palacios and Zimmerman 2007; Apostolaki et al. 2014) and other environmental cues (Zimmerman et al. 1995; Brun et al. 2003). The flux of fixed carbon in each tissue organ is controlled by key

enzymes. For example, sucrose-phosphate synthase (SPS) in mature photosynthetic leaves primes the conversion of carbon into sucrose, which is subsequently transported to sinks (Touchette and Burkholder 2007). The import of sucrose into sinks is controlled by sucrose synthase (SS). Under CO_2 enrichment, reduced nitrogen availability could direct more carbon into below-ground biomass for storage, reducing nutrient imbalances in the leaves (Werf and Nagel 1996).

Across the Great Barrier Reef (GBR), DIN (nitrate, ammonium and nitrite) levels in the water column over seagrass meadows are relatively low, averaging 0.13 µM (Schaffelke et al. 2012). However, terrestrial run-off into coastal areas can deliver DIN loads that are an order of magnitude or more higher (1.54 to 7.02 µM, or 2.20 µM averaged across the GBR) (Devlin et al. 2011). The increase in local availability of nitrogen, similar to CO_2 enrichment, would likely promote seagrass productivity. I hypothesized that 1) pCO_2 and nitrate enrichment can have additive effects on seagrass productivity and biomass and 2) pCO_2 enrichment drives nitrogen demand. To test this, I increased DIN and pCO_2 levels in seawater, to simulate DIN levels in flood plumes and predicted end-of-century levels under RCP 2.6 and RCP 8.5 CO_2 emission scenarios (Collins et al. 2013). To allow wider inference I examined species with different growth and storage strategies, the fast-growing species *Halodule uninervis* and the slow-growing species *Thalassia hemprichii* (Kilminster et al. 2015). Assessment of growth and productivity permitted the testing of the first hypothesis, and measurement of nitrogen incorporation processes (uptake and assimilation) enabled testing of the second hypothesis.

4.2 Methods

4.2.1 Plant collection and experimental setup

The experiment was carried out at Lizard Island, GBR, Australia, in March 2014. *Halodule uninervis* was collected from an intertidal meadow and *Thalassia hemprichii* from the subtidal zone (2 – 3 m depth) of One Tree Coconut beach (14° 41.370'S, 145° 27.392'E) following protocol described in Ow et al. (2015). Seagrasses were potted up within 48 h of collection in the same sediments from their source meadows (*H. uninervis* in 20:80 carbonate sand:site mud

mixture, *T. hemprichii* in carbonate sand). Potted seagrasses were stored in outdoor flow-through aquaria (50 L) for three to six days prior to the initiation of the experiment. Experimental treatments consisted of three pCO_2 levels (ambient ~428 µatm, moderate ~734 µatm and high ~1213 µatm pCO_2) and two nitrate treatments (ambient ~0.3 µM and enriched ~1.9 µM) crossed in a fully factorial design. Each treatment comprised of three replicate 25 L aquaria leading to a total of eighteen aquaria, supplied with seawater at 24 L h^{-1} directly from the adjacent lagoon. Two sub-replicate pots of each species were placed in each aquarium. The aquaria were situated outdoors under a solid translucent roof, which attenuated 50% of down-welling light. 2π light loggers (Odyssey, New Zealand) were randomly allocated to aquaria to record photosynthetically active radiation (PAR) at canopy height. Over the course of the experiment, the net daily PAR in aquaria ranged from 1.2 to 5.2 mol m^{-2} d^{-1}, averaging 3.8 mol m^{-2} d^{-1}. Mid-day maximum PAR averaged 480 µmol m^{-2} s^{-1}. Treatments were randomised among the aquaria to eliminate any potential environmental effects within the set-up area. The experiment ran for 24 days before it had to be terminated due to an approaching cyclone.

pCO_2 concentrations were manipulated by injecting different amounts of CO_2 gas into sump tanks. pH levels in the sump tanks were monitored with six potentiometric sensors (±0.01 pH unit) calibrated on the NIST (National Institute of Standards and Technology) scale as a proxy to control for CO_2 input. The sensors provide feedback to a control system that regulates pH levels via CO_2 gas injection (AquaMedic, Germany) (Ow et al. 2015).

Nitrate enrichment was achieved by dripping sodium nitrate solution (Sigma-Aldrich, Australia) into individual aquaria. Peristaltic pumps (Cole Palmer, USA) delivered 2 mM of $NaNO_3$ solution into the individual aquaria at a rate of 0.5 ml min^{-1}. Small aquaria pumps (Hailea, China) in each aquarium provided mixing.

4.2.2 Seawater chemistry

pH_{total} in treatment tanks was monitored by spectrometric determination of m-cresol absorbance (Dickson et al. 2007), and additionally checked against TRIS seawater standard (A. G. Dickson, Scripps Institute of Oceanography, Batch 106). Weekly water samples were analysed for total alkalinity (A_T) by gran titration with 0.5 M HCl on a Metrohm 855 titrosampler (Metrohm, Switzerland), and for total dissolved inorganic carbon (DIC) by acid

titration on a VINDTA 3C. Carbonate system parameters were calculated using measured values of A_T, DIC, temperature and salinity on CO2calc software (Robbins et al. 2010). Duplicate water samples for dissolved inorganic nutrient analysis were filtered through 0.45 μm cellulose acetate filters and stored at -20°C before determination of seawater ammonium, nitrate, and phosphate concentrations according to standard procedures outlined in Ryle et al. (1981). Temperature in the treatment tanks was logged by HOBO tidbit loggers (Onset, USA) every 5 min. Salinity readings were taken from an IMOS weather buoy (Integrated Marine Observing System; www.aims.gov.au) situated in the lagoon.

4.2.3 Productivity

After 22 days, photosynthetic and respiration rates were measured using the second youngest leaf of a shoot from each sub-replicate pot using optical oxygen sensors ("optodes", PreSens, Germany) and a fiber-optic oxygen meter (PreSens Oxy 4, Germany). Respiration rates of below-ground rhizome with associated roots (~2.5 cm) from each pot were quantified similarly. Measurements were conducted in 70 mL chambers at constant 28°C water temperature following procedures described in Ow et al. (2015). Respiration of the leaves and below-ground rhizome-roots were measured separately over a 20-min period in the dark while photosynthetic rates were measured on the same leaf at 400 μmol m^{-2} s^{-1} PAR over 30 min. Plant material was dried (60ºC for 48 h) and weighed after incubation. Photosynthetic and respiration rates were normalised to the dry weight of the leaf and rhizome. Optodes were calibrated according to the protocol described in Collier et al. (2011).

Growth rates were measured according to the method described in Short and Duarte (2001). On day 0 and day 14 of the experiment, all shoots were marked at the top of the bundle sheath with a needle. Length of new tissue growth was measured with vernier callipers regularly throughout the experiment, totalled and normalised to the number of shoots and days since marking. Growth rates of plants, from three separate plots in each source meadow, were also obtained using the same method from day 13 to day 17.

4.2.4 Nitrogen uptake

Leaf nitrate uptake rates were estimated at the end of the experiment. Seagrass shoots were incubated in seawater enriched with ^{15}N labelled potassium nitrate (atom% = 98; Novachem, Australia), and the final ^{15}N in the leaf tissue was used to calculate the uptake of $^{15}NO_3^-$. Incubations were carried out on individual shoots in their pots, in their respective treatment tanks, using a method similar to that described in Prado et al. (2011). Individual shoots were enclosed within a plastic bag (~250 mL volume) fitted with a filter cassette and a plug that could be sealed. No leakage was detected when tested using a food dye. Potassium nitrate solution was injected into the chambers to achieve around 20% $^{15}NO_3^-$ enrichment of the initial ambient DIN concentration (Apostolaki et al. 2012). The shoots were incubated for one hour at ambient mid-day temperature (28°C) and light (450 μmol m^{-2} s^{-1}). After one hour, the shoots were excised from the rhizomes and rinsed with deionized water to remove excess adherent label. Non-incubated leaf samples were collected from each tank to provide background leaf ^{15}N levels for each species. Leaf material was processed and measured for total nitrogen content and atom% ^{15}N according to method described in Takahashi et al. (2015). Uptake rates (μmol N g^{-1} dry weight h^{-1}) of $^{15}NO_3^-$ were calculated following equations outlined in Nayar et al. (2010). The atom% ^{15}N of ^{15}N enriched seawater was calculated based on the amount of atom% $^{15}NO_3^-$ added and background DIN concentrations (assumed to reflect ^{15}N concentration of atmospheric N ~ 0.37 atom% ^{15}N).

4.2.5 Nitrogen assimilation and carbon translocation

Plant material used for measuring nitrogen assimilation and carbon translocation (i.e. enzyme analyses), except for nitrate reductase (NR), were collected at the end of the experiment and stored in liquid nitrogen until analysis.

NR activity in fresh shoot tissue was determined using the *in vivo* assay described in Roth and Pregnall (1988) for *Zostera marina*. The *in vivo* technique was shown to yield consistently higher activity than the *in vitro* assay, which often gave negligible readings (Touchette and Burkholder 2007). Extraction and assay for glutamine synthetase (GS) activity in new and fully extended leaf tissue was carried out following the method developed for *Z.*

marina (Pregnall et al. 1987), except that the incubation was carried out nearer to the aquaria temperature (30ºC).

To study carbon translocation, sucrose-phosphate synthase (SPS) from young but fully extended shoot tissue and sucrose synthase (SS) from the root–rhizome complex were extracted using a technique described in Brun et al. (2003) and assayed according to the protocol outlined in Zimmerman et al. (1995). The sucrose produced was quantified colourimetrically using anthrone assay (Huber et al. 1991).

4.2.6 Shoot and rhizome-root biochemistry

Shoot tissue nutrients (carbon and nitrogen) of ashed samples were analysed using an elemental analyser (Elementar Vario EL, Germany) interfaced to an isotope-ratio-mass-spectrometer (PDZ Europa 20-20, Sercon Ltd; Cheshire UK), as described in Takahashi et al. (2015). To study carbon storage, ground rhizome-roots samples were analysed for non-structural carbohydrates content according to procedure described in Collier et al. (2012b). The summed amount of soluble carbohydrates and starch gave total non-structural carbohydrates (TNSC) content, expressed as milligrams dry weight^{-1} of tissue.

4.2.7 Statistical analysis

Parameters were analysed using linear mixed effects models with pCO_2 as a continuous predictor, and nitrate (ambient and enriched) as a categorical factor. Individual tanks were included as replicates, with sub-replicate pots nested within tanks. The nested factor was omitted for parameters without sub-replicate measurements (*T. hemprichii*: net primary production, respiration; both species: $^{15}NO_3^-$ uptake). For these parameters, measurements were terminated prematurely due to an unforeseen evacuation of the research station caused by a cyclone, and therefore the second sub-replicate could not be measured. Assumptions of normality and homogeneity of variances were tested with Shapiro-Wilks' and Bartlett's tests, respectively. Percentage data (%C and %N) were arcsine square-root transformed to meet the assumptions (Underwood 1997). All statistical tests were assessed at $\alpha = 0.05$ and analysed using R statistical software (R Development Core Team 2014).

4.3 Results

4.3.1 Experimental parameters

Water temperature (daily range 27.8 – 29.8°C) and salinity (34.6 – 34.9) were similar among experimental tanks and throughout the experiment (Table 4.1). Carbonate system parameters of the enriched pCO_2 treatments remained well within the target range of 428, 734 and 1213 μatm for the three treatments (Table 4.1). Average ammonium (0.59 μM; S.D. = 0.28 μM) and phosphate (0.05 μM; S.D. = 0.02 μM) concentrations were similar among treatments. Nitrate concentration was 0.29 ± 0.18 μM (S.D.) and 1.91 ± 0.33 μM (S.D.) in ambient and nutrient enriched treatments, respectively.

Table 4.1: Experimental parameters. Values are given as mean ± S.D. Carbonate system parameters were calculated using measured values of total alkalinity (A_T), total dissolved inorganic carbon (DIC), temperature and salinity on CO2calc software (Robbins et al. 2010).

		Measured parameters					Calculated parameters				Nutrient levels		
pCO_2 treatment	Nutrient	DIC (μmol kg^{-1} SW)	A_T (μmol kg^{-1} SW)	pH (NIST)	Temperature (°C)	Salinity	pCO_2 (μatm)	HCO_3^- (μmol kg^{-1} SW)	CO_2 (μmol kg^{-1} SW)	CO_3^{2-} (μmol kg^{-1} SW)	NH_4^+ (μM)	PO_4^{3-} (μM)	NO_3^- (μM)
Control	-	1945.82 (9.59)	2234.77 (4.71)	8.01 (0.01)	28.47 (0.55)	34.74 (0.13)	434.56 (12.44)	1721.66 (13.66)	11.27 (0.42)	187.33 (2.45)	0.78 (0.37)	0.05 (0.02)	0.39 (0.25)
Control	+	1937.83 (9.49)	2233.82 (5.96)	8.02 (0.01)	28.53 (0.49)	34.74 (0.13)	421.50 (15.07)	1710.36 (14.13)	10.93 (0.43)	192.32 (2.02)	0.67 (0.32)	0.04 (0.02)	1.98 (0.34)
Intermediate	-	2045.79 (12.22)	2238.79 (5.25)	7.83 (0.04)	28.63 (0.76)	34.74 (0.13)	730.60 (78.43)	1875.54 (21.95)	18.95 (2.09)	129.41 (13.06)	0.55 (0.18)	0.04 (0.02)	0.24 (0.13)
Intermediate	+	2047.41 (12.88)	2238.27 (4.76)	7.82 (0.05)	28.80 (0.72)	34.74 (0.13)	737.95 (88.59)	1877.37 (24.40)	19.10 (2.33)	130.97 (12.94)	0.60 (0.29)	0.04 (0.02)	1.80 (0.23)
High	-	2134.96 (18.95)	2240.40 (4.89)	7.63 (0.04)	28.70 (0.61)	34.74 (0.13)	1235.32 (129.49)	2001.11 (22.17)	32.00 (3.15)	92.85 (5.91)	0.76 (0.55)	0.05 (0.02)	0.29 (0.16)
High	+	2130.62 (14.89)	2239.85 (5.20)	7.64 (0.04)	28.73 (0.58)	34.74 (0.13)	1189.85 (110.60)	1994.66 (18.18)	30.79 (2.67)	87.79 (4.85)	0.54 (0.18)	0.04 (0.02)	1.71 (0.68)

4.3.2 Productivity and growth

In *H. uninervis*, net primary production increased with pCO_2 levels (LME: $P = 0.049$) (Fig. 4.1; Table 4.2). The linear model predicted an increase of 1.071 units in net primary production for every 100 µatm rise in pCO_2. There was no effect of nitrate enrichment on primary production (Table 4.2). Leaf respiration was not affected by pCO_2 levels, but decreased by 34 % with nitrate enrichment (LME: $P = 0.025$) (Fig. 4.1; Table 4.2). Rhizome respiration responses to pCO_2 depended on nitrate enrichment (LME pCO_2 × nitrate interaction: $P = 0.009$) (Fig. 4.1; Table 4.2). Under ambient DIN conditions, rhizome respiration increased with pCO_2; under enriched DIN, rhizome respiration decreased with pCO_2 (Fig. 4.1).

Growth rates of *H. uninervis* shoots increased with pCO_2 enrichment after 10 days (LME: $P = 0.006$) (Fig. 4.2; Table 4.2). At day 10, growth rates increased from 3.3 mm shoot^{-1} day^{-1} in control pCO_2 aquaria (428 µatm) to 4.2 mm shoot^{-1} day^{-1} in high pCO_2 aquaria (1213 µatm). The enhancement of growth rates with pCO_2 was sustained after 24 days (LME: $P = 0.001$) as growth rates in control pCO_2 aquaria were 4.1 mm shoot^{-1} day^{-1}, while those in high pCO_2 aquaria were elevated by 52 % (6.2 mm shoot^{-1} day^{-1}). There was no significant effect of nitrate enrichment on growth (Fig. 4.2; Table 4.2). Shoot growth of *H. uninervis* in the source meadow at day 13 – 17 of the experiment was in a similar range (7.0 mm shoot^{-1} day^{-1}; S.E. = 1.24 mm shoot^{-1} day^{-1}).

Net primary production in *T. hemprichii* did not increase with pCO_2 or nitrate enrichment (Fig. 4.1; Table 4.2). In addition, no significant changes in leaf and rhizome respiration with pCO_2 and nitrate enrichment were detected.

In *T. hemprichii*, at day 10, leaf growth rates responded to pCO_2 enrichment and no effect of nitrate enrichment was detected (LME: pCO_2 $P = 0.024$; nitrate $P = 0.252$) (Fig. 4.2; Table 4.2). Growth rates increased by 28 % with pCO_2 enrichment. By day 24, no change in growth rate to pCO_2 or nitrate was detected (Fig. 4.2; Table 4.2). Overall, growth of *T. hemprichii* in the experimental aquaria (global average = 2.98 mm shoot^{-1} day^{-1}; S.E. = 0.12 mm shoot^{-1} day^{-1}) was lower than that measured in the source meadow (5.95 mm shoot^{-1} day^{-1}; S.E. = 0.57 mm shoot^{-1} day^{-1}).

Table 4.2: Linear mixed effects models for measured productivity response variables. Variables were analysed with pCO_2 as a continuous predictor and nitrate as a categorical factor. Individual aquarium tanks were included as replicates (n = 3), with two sub-replicate pots nested within aquaria. For net primary production, shoot and rhizome-root respiration, linear models were used for analysis, with aquaria as replicates (n = 3) and without nested sub-replicate pots. *P*-values < 0.05 are in bold.

		Halodule uninervis			*Thalassia hemprichii*		
Parameter	Source	df	F	*p*	df	F	*p*
Net primary production	pCO_2	1	4.669	**0.049**	1	0.184	0.675
	Nitrate	1	0.091	0.767	1	2.745	0.120
	pCO_2 × Nitrate	1	0.721	0.410	1	3.648	0.077
Shoot respiration	pCO_2	1	3.785	0.072	1	0.849	0.373
	Nitrate	1	5.199	**0.039**	1	0.226	0.642
	pCO_2 × Nitrate	1	1.861	0.194	1	1.756	0.206
Rhizome-root respiration	pCO_2	1	1.818	0.199	1	3.082	0.101
	Nitrate	1	8.593	**0.011**	1	0.584	0.458
	pCO_2 × Nitrate	1	9.037	**0.009**	1	1.607	0.226
Growth rate (10 days)	pCO_2	1	10.430	**0.006**	1	6.376	**0.024**
	Nitrate	1	3.418	0.086	1	1.427	0.252
	pCO_2 × Nitrate	1	0.003	0.961	1	1.575	0.230
Growth rate (24 days)	pCO_2	1	19.218	**0.001**	1	0.068	0.799
	Nitrate	1	1.014	0.331	1	0.870	0.367
	pCO_2 × Nitrate	1	1.544	0.234	1	0.077	0.786
Sucrose phosphate synthase	pCO_2	1	1.556	0.233	1	0.534	0.477
	Nitrate	1	5.109	**0.040**	1	0.436	0.520
	pCO_2 × Nitrate	1	0.275	0.608	1	0.062	0.806
Sucrose synthase	pCO_2	1	0.002	0.967	1	3.619	0.078
	Nitrate	1	1.677	0.216	1	0.389	0.543
	pCO_2 × Nitrate	1	3.291	0.091	1	0.251	0.624
Total non-structural carbohydrates	pCO_2	1	0.003	0.959	1	0.548	0.471
	Nitrate	1	0.053	0.821	1	0.994	0.336
	pCO_2 × Nitrate	1	0.152	0.702	1	4.66×10^{-4}	1.000

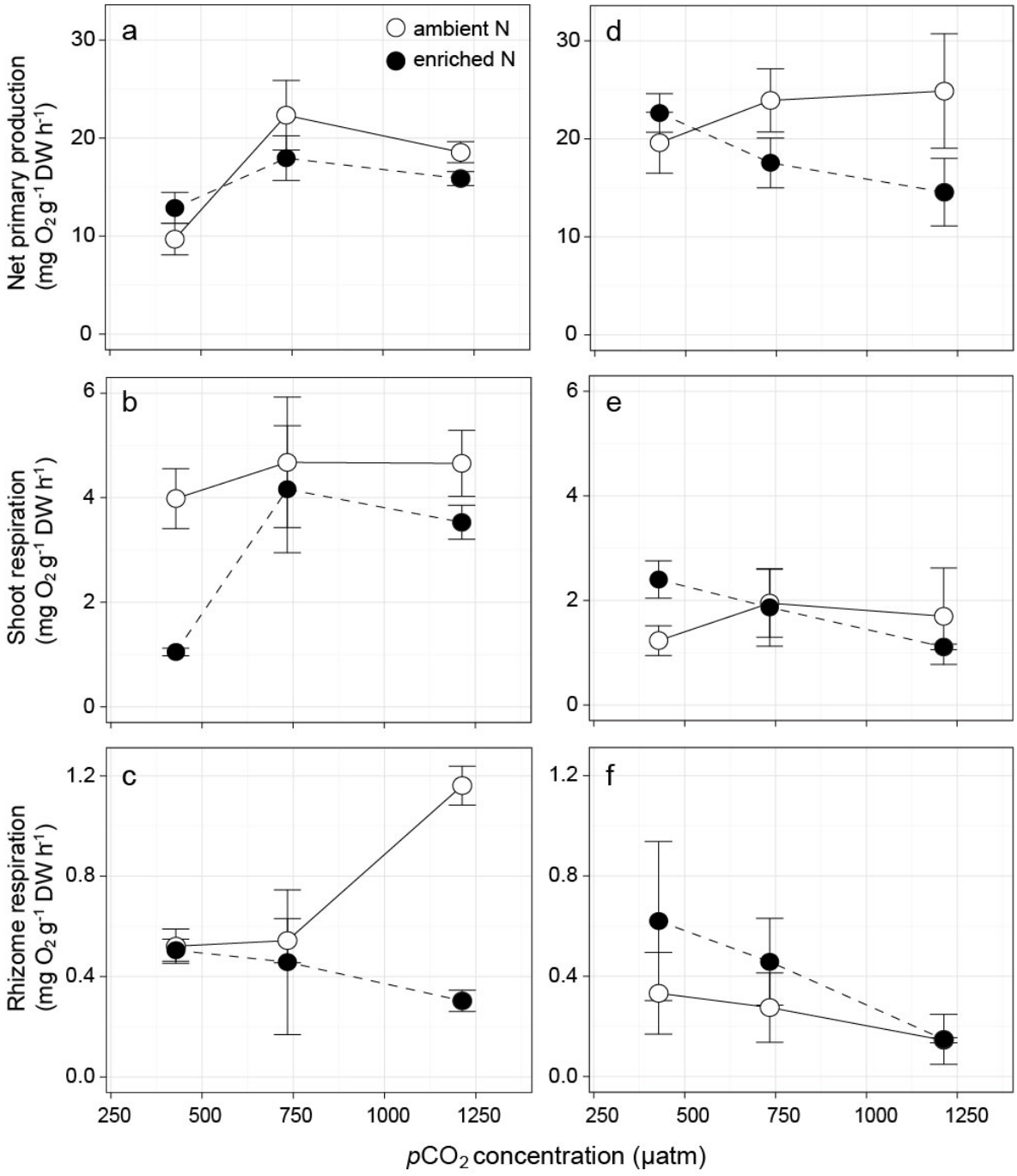

Fig. 4.1: Net primary production and respiratory responses of (a – c) *H. uninervis* and (d – f) *T. hemprichii* measured after 22 days exposure to treatment. Values are average ± S.E. n = 3.

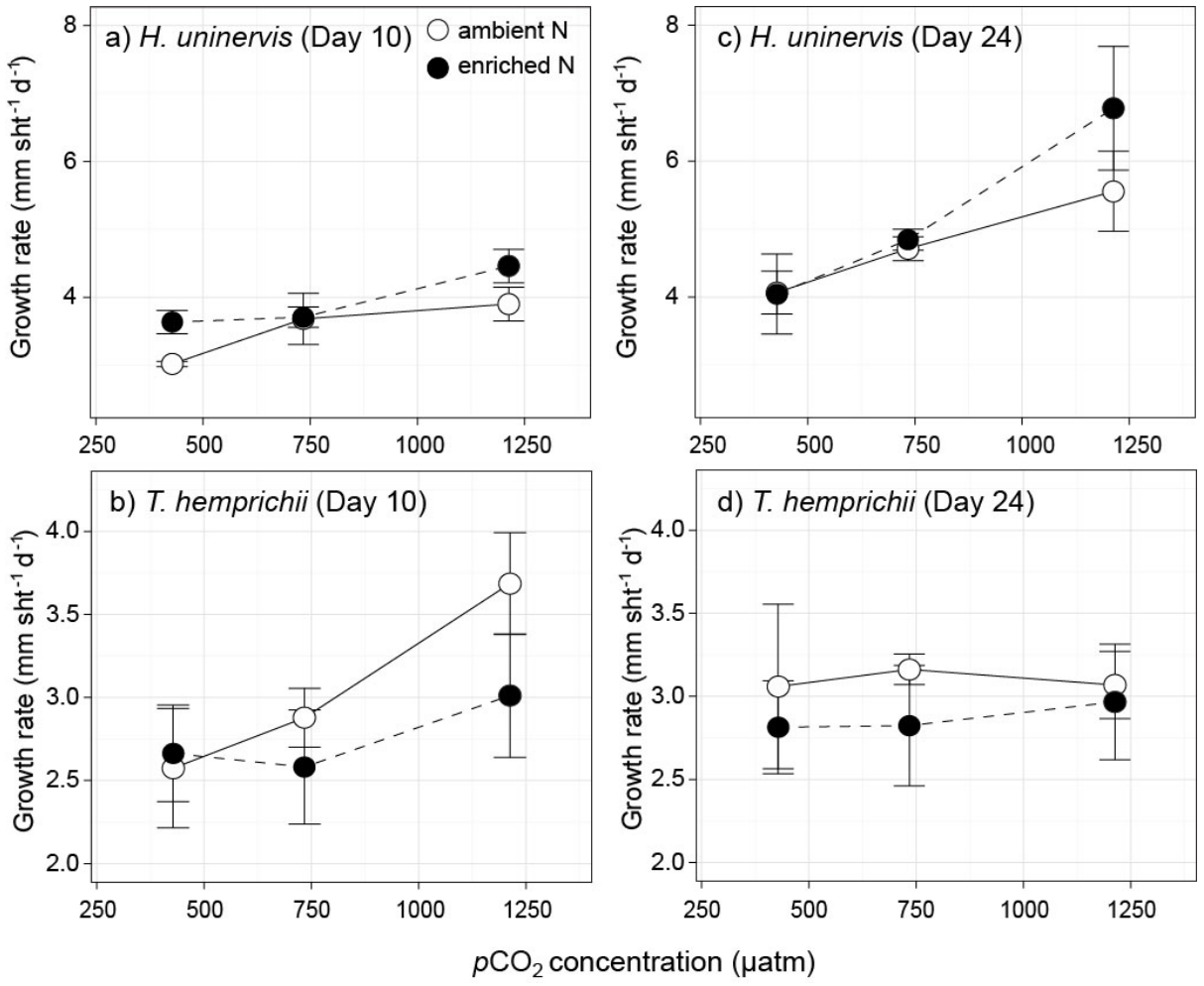

Fig. 4.2: Growth rates of (a, c) *H. uninervis* and (b, d) *T. hemprichii* after 10 and 24 days exposure to treatments. Values are average ± S.E. n = 3.

4.3.3 Carbohydrates translocation and storage

For both *H. uninervis* and *T. hemprichii*, pCO_2 manipulation did not affect sucrose-phosphate synthase (SPS) or sucrose synthase (SS) activity indicative of carbohydrate translocation (Table 4.2). Nutrient enrichment reduced SPS activity in *H. uninervis* leaves (LME: $P = 0.040$) (Table 4.2), but overall the effects were of limited consequence for our hypotheses (Supplementary Fig. 4.1). Non-structural carbohydrates in *H. uninervis* and *T. hemprichii* rhizomes showed no change to CO_2 and nitrate enrichment (Table 4.2).

4.3.4 Nitrogen uptake and assimilation

In *H. uninervis*, leaf uptake of nitrate, determined by ^{15}N incorporation, did not vary with pCO_2 or nitrate enrichment in *H. uninervis* (10.91 μmol N g^{-1} DW h^{-1}; S.E. = 1.35 μmol N g^{-1} DW h^{-1}) (Fig. 4.3; Table 4.3). No significant changes in nitrogen assimilation (enzymatic activity) in *H. uninervis* with pCO_2 and nitrate enrichment were detected (Table 4.3). Furthermore, there were no changes in leaf tissue nutrient content (means ± S.E.: C – 41 ± 0.2 %; N – 2.5 ± 0.03 %; C:N – 16.3 ± 0.2) (Table 4.2).

In *T. hemprichii*, nitrate uptake was increased with pCO_2, but only in the nitrate enriched treatment (LME pCO_2 × nitrate interaction: $P = 0.017$) (Fig. 4.3; Table 4.3). Nitrate uptake rates increased by 117 % at the highest pCO_2 relative to ambient levels. In *T. hemprichii* leaves, NR activity was higher with nitrate enrichment (linear model $P = 0.019$) but was not affected by pCO_2 levels (Table 4.3). NR activity in *T. hemprichii* leaves in ambient seawater (0.61 μmol NO_2 g^{-1} FW h^{-1}; S.E. = 0.14 μmol NO_2 g^{-1} FW h^{-1}) was ~50 % of that in enriched nitrate conditions (1.12 μmol NO_2 g^{-1} FW h^{-1}; S.E. = 0.25 μmol NO_2 g^{-1} FW h^{-1}). GS activity in *T. hemprichii* leaves did not change significantly with pCO_2 or nitrate (69.50 μmol g^{-1} FW h^{-1}; S.E. = 5.06 μmol g^{-1} FW h^{-1}) (Table 4.3). There were no significant changes in leaf carbon content (39 ± 0.3 %), but there were marginal increases in leaf nitrogen (LME: $P = 0.056$) and a significant reduction in C:N ratio (LME: $P = 0.045$) with nitrate enrichment in *T. hemprichii* (Table 3). Under ambient nitrate levels, nitrogen content and C:N were 2.7 ± 0.06 % and 14.7 ± 0.27 respectively; with nitrate enrichment, nitrogen content was 2.8 ± 0.08 % and C:N was 14.0 ± 0.41 (means ± S.E.).

Table 4.3: Linear mixed effect models for all nitrogen uptake and metabolism variables. Variables were analysed with pCO_2 as a continuous predictor and nitrate as a categorical factor. Individual aquaria were included as replicates (n = 3), with two sub-replicate pots nested within aquaria. *P*-values < 0.05 are in bold.

		Halodule uninervis			*Thalassia hemprichii*		
Parameter	Source	df	F	*p*	df	F	*p*
Nitrate uptake	pCO_2	1	0.157	0.698	1	1.014	0.331
	Nitrate	1	0.000	0.984	1	0.156	0.698
	pCO_2 x Nitrate	1	0.380	0.548	1	7.392	**0.017**
Nitrate reductase	pCO_2	1	3.076	0.101	1	1.144	0.303
	Nitrate	1	2.523	0.135	1	8.092	**0.013**
	pCO_2 x Nitrate	1	0.526	0.480	1	4.061	0.064
Glutamine synthetase	pCO_2	1	0.142	0.712	1	0.089	0.769
	Nitrate	1	0.376	0.550	1	0.289	0.600
	pCO_2 x Nitrate	1	3.443	0.085	1	0.466	0.506
Carbon content	pCO_2	1	1.420	0.253	1	0.928	0.352
	Nitrate	1	1.390	0.259	1	0.094	0.764
	pCO_2 x Nitrate	1	0.330	0.576	1	0.094	0.764
Nitrogen content	pCO_2	1	0.310	0.584	1	2.180	0.162
	Nitrate	1	1.350	0.266	1	4.363	0.056
	pCO_2 x Nitrate	1	0.510	0.487	1	3.113	0.099
C:N ratio	pCO_2	1	1.571	0.231	1	1.436	0.251
	Nitrate	1	3.227	0.094	1	4.846	**0.045**
	pCO_2 x Nitrate	1	0.282	0.604	1	3.258	0.093

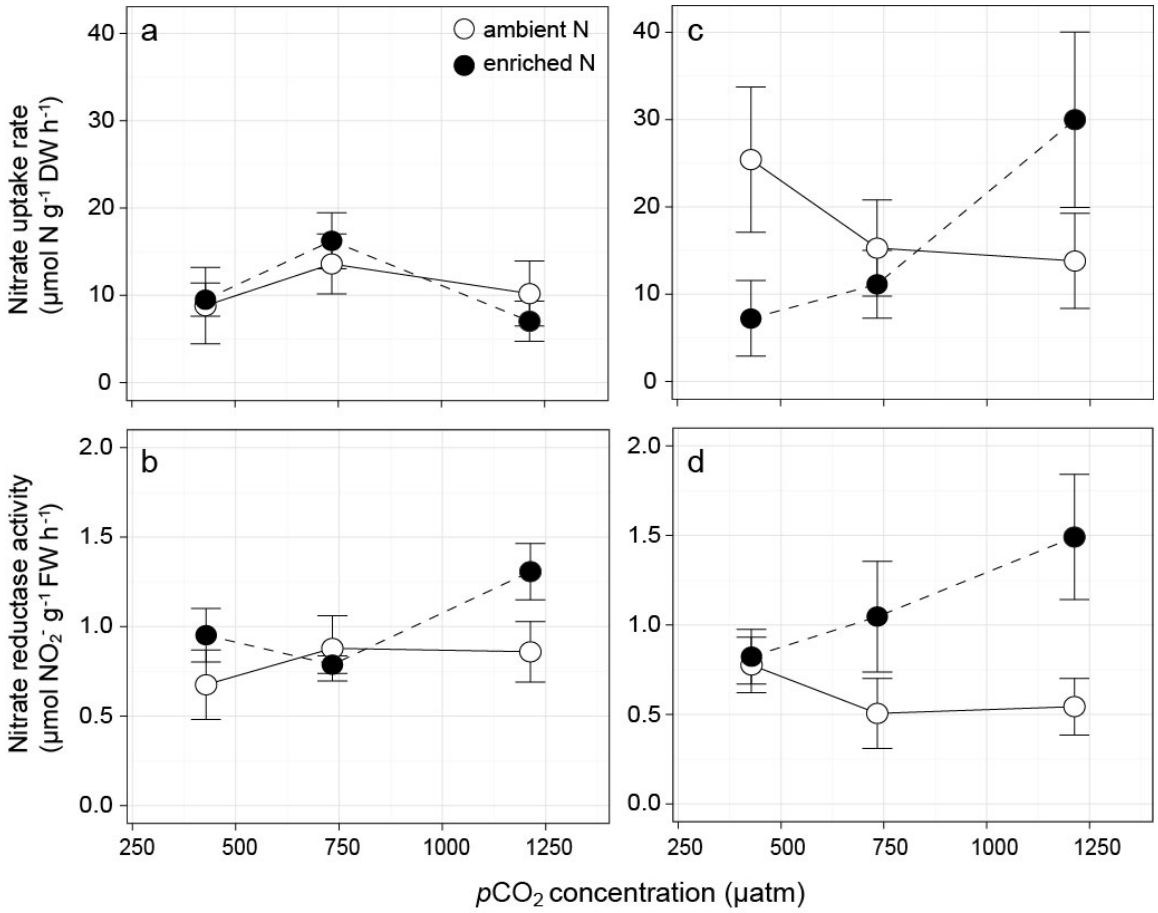

Fig. 4.3: Nitrate incorporation (uptake and assimilation) in leaves of (a – b) *H. uninervis* and (c – d) *T. hemprichii* across a range of pCO_2 concentrations. Values are average ± S.E. n = 3.

4.4 Discussion

This study aimed to test whether seagrass productivity is affected by pCO_2 and nitrate (NO_3^-) enrichment, and whether pCO_2 drives demand for nitrogen in seagrasses. In *H. uninervis*, net primary production (NPP) and growth rates increased with higher pCO_2 but were not affected by nitrate enrichment. However, in *T. hemprichii*, NPP and growth were not affected by either pCO_2 or nitrate enrichment. In *H. uninervis*, pCO_2 enrichment did not increase nitrate uptake or assimilation while nitrate uptake was higher in CO_2-enriched (simulating end of century RCP 8.5 emission scenario) (Collins et al. 2013) *T. hemprichii*. In addition, nitrate enrichment

(1.9 μM compared to 0.3 μM in ambient) raised leaf nitrate reductase (NR) activity in *T. hemprichii*. Therefore, productivity responses to pCO_2 and nitrate enrichment varied between species with different growth strategies.

H. uninervis and *T. hemprichii* differed in productivity responses to pCO_2 enrichment after 24 days exposure. In *H. uninervis*, NPP increased by 1.1 units for every 100 μatm rise in pCO_2, an increase slightly higher than the 0.9 units measured in the same species by Ow et al. (2015). This difference might be due to variation between seagrass populations. Other fast-growing seagrass species that have increased photosynthetic rates with pCO_2 enrichment include *Z. marina* (250 % increase at ~35,800 μatm pH 6.2) (Zimmerman et al. 1997) and *Z. noltii* (34% increase at ~760 μatm pH 7.9) (Alexandre et al. 2012). Leaf growth rates in *H. uninervis* were also enhanced in pCO_2 enriched treatments, with the highest leaf growth rates [6.2 ± 0.40 (S.E.) mm $shoot^{-1}$ day^{-1}] being slightly lower than that measured in the field [7.0 ± 1.24 (S.E.) mm $shoot^{-1}$ day^{-1}]. Aquaria experiments may impose potential artefacts on leaf growth due to transplantation stress, which were minimised by allowing for acclimation prior to experiments. However, as described below, light levels within experimental tanks, which were lower than that of nearby shallow reef systems, most likely explained the lower growth rates in aquaria.

In *T. hemprichii*, CO_2 enrichment had no effect on NPP and growth rates after three weeks, in contrast to previous work on this species (Jiang et al. 2010; Ow et al. 2015). Jiang et al. (2010) studied *T. hemprichii* from a nutrient-enriched meadow (0.8 – 4.6 μM $NO_3^- + NO_2^-$) (Zhang et al. 2014) and exposed to much higher CO_2 concentrations (25 – 1005 μM) compared to the present study (19 – 31 μM). *T. hemprichii* grown under high nitrogen might have utilised its pre-existing nutrients store (Touchette and Burkholder 2000b) to supplement a rapid growth increase during strong CO_2 enrichment (Jiang et al. 2010). In the present study, *T. hemprichii* productivity did not appear to be nitrogen-limited (discussed below), indicating that light levels in experimental tanks, or phosphate availability in carbonate sediments (Erftemeijer and Middelburg 1993) could have limited its growth response. Interestingly, leaf growth of *T. hemprichii* showed a transient rise with pCO_2 at day 10, but subsequently stabilised. This growth response to initial (short-term) pCO_2 exposure has been reported for *T. hemprichii* after 14 days of exposure (Ow et al. 2015). However, NPP measured at the end of the experiment (22 days) suggest a downregulation in response to pCO_2 over time.

Nitrate addition did not increase NPP in *H. uninervis*. This was despite respiration rates of the rhizome-root complex in enriched pCO_2 being lowered with nitrate enrichment. Given the relatively large proportion of below-ground biomass for this species (Collier et al. 2012b), a reduction in rhizome-root respiration could be substantial for improving carbon use (Hemminga 1998). In the present study, the export of fixed carbon from leaves, which was correlated with sucrose phosphate synthase (SPS) activity (Brun et al. 2003; Touchette and Burkholder 2007), was lower with nitrate enrichment, probably due to reduced metabolic demand in the rhizome-root biomass (Touchette and Burkholder 2000a). Further quantification of nitrate uptake rates and of the activities of the key enzymes in the nitrogen assimilation pathway, nitrate reductase and glutamine synthetase (Pregnall et al. 1987; Touchette and Burkholder 2000b), revealed no effect of nitrate enrichment on nitrogen incorporation in *H. uninervis*.

Productivity in *T. hemprichii* did not increase with nitrate enrichment, even though nitrate enrichment increased nitrate uptake and assimilation at high pCO_2 and assimilation in the leaves of *T. hemprichii*. Increased nitrate uptake and assimilation under water-column nitrate enrichment could be advantageous for seagrasses acclimatised to growing in a low-nitrogen environment (Touchette and Burkholder 2007). This allows the plant to sequester and store nitrogen rapidly when it becomes available. Higher nitrogen content and a lowered C:N ratio were observed in nitrate-enriched *T. hemprichii* leaves. In general, nitrate enrichment appeared to have a greater influence on nitrogen incorporation in *T. hemprichii* than *H. uninervis*.

Overall there was no evidence in the present study that nitrate enrichment enhanced productivity responses to pCO_2 for either species. This was surprising as nitrogen had been suggested (Alexandre et al. 2012) and shown to limit the productivity of marine macrophytes to pCO_2 enrichment (Russell et al. 2009) in subtidal rocky habitats. The experiment duration might not have been long enough for pCO_2 enrichment to induce a significant change in nitrogen demand (24 days vs 5 months; Alexandre et al. 2012), which may still be covered by pre-existing nitrogen-resources. Previous work reported increases in leaf tissue carbon-to-nitrogen (C:N) ratios in CO_2 enriched seagrasses (Jiang et al. 2010; Campbell and Fourqurean 2013a), which suggested nitrogen limitation in these plants. However, C:N ratios in both *H. uninervis* and *T. hemprichii* here revealed no evidence that pCO_2 enrichment led to the

seagrasses requiring more nitrogen. In the Great Barrier Reef (GBR) region, seagrass growth was limited by nitrogen at some sites (Udy et al. 1999; Mellors et al. 2005). In the present study, leaf nitrogen content and C:N ratios of *H. uninervis* (N = 2.53 %; C:N = 16.3) and *T. hemprichii* (N = 2.75 %; C:N = 14.4) were similar to previous values measured in GBR seagrasses (McKenzie et al. 2015b). These were well above the values assumed to indicate nitrogen limitation (N = 1.8%; C:N = 20) (Duarte 1990; McKenzie et al. 2015b) and suggest that the two species were not nitrogen limited. DIN levels in sediment pore-water and that adsorbed to sediments were not quantified here, but typical concentrations can be 200 times higher than in the water column (Romero et al. 2006). Thus sediment pore-water may have supplied sufficient DIN to maintain productivity rates measured here. Another possible explanation for apparent nutrient sufficiency (C:N < 20) (Collier et al. 2009) is that light levels during the experiment, averaging 9 mol m^{-2} d^{-1}, were low compared to longer-term monitoring from shallow seagrass meadows in far north Queensland which typically reach 15 – 20 mol m^{-2} d^{-1}. Furthermore light levels dropped in the region of the study site (Cape York) in early 2014 (McKenzie et al. 2015b). Lowered levels of natural light, relative to the typical levels available (McKenzie et al. 2015b), may also explain the limited productivity responses to pCO_2.

Carbon dioxide enrichment did not drive nitrogen demand in *H. uninervis* or *T. hemprichii*. In other marine macrophytes, CO_2 enrichment increases nitrate reductase activity (Zou 2005; Alexandre et al. 2012). Here, increased CO_2 availability did not affect nitrate uptake and assimilation (measured as nitrate reductase and glutamine synthetase activity) in *H. uninervis*, whereas the effect was dependent on nitrate enrichment in *T. hemprichii*. This is interesting as water column DIN concentrations at northern mid-shelf GBR (e.g. Lizard Island) are typically lower than that at inshore reefs (Furnas 2003), where the majority of seagrasses grow (Coles et al. 2015). Perhaps experiments on longer time-scales are needed to evaluate the effects of nitrogen availability on productivity, as seagrasses possess mechanisms to improve nitrogen-use efficiency, likely through recycling or re-allocation of nitrogen within the plant (Romero et al. 2006). At natural CO_2 seeps with elevated pCO_2, no difference in tissue nutrients were found between seagrasses growing around, and away from the CO_2 seeps, suggesting CO_2-induced nitrogen limitation was not present (Takahashi et al. 2015). Coastal habitats are continually subjected to fluxes in nutrients, which may be supplemented by nitrogen fixation in the sediments (Welsh 2000). Low level renewal of nitrogen supplies may enable seagrasses to be more productive without facing nitrogen limitation with future OA.

In conclusion, the tropical seagrasses, *H. uninervis* and *T. hemprichii,* did not appear to be strongly nitrogen limited despite being collected from a mid-shelf reef where ambient water column nitrogen concentrations were low (0.13 μmol DIN). Consequently, nitrate fertilization of the water column did have some effect on nitrate uptake rates, but did not enhance seagrass productivity or leaf growth rates. Furthermore, in contrast to our initial hypothesis, responses to pCO_2 enrichment, simulating future ocean acidification scenarios, were also unaffected by nitrate fertilisation. To better reconcile the effects of nutrient enrichment on seagrass CO_2 responses with previous studies, there is the need to account for differences in background light, nutrient levels and durations among experiments. Ocean acidification can also promote the growth of epiphytic filamentous algae, outweighing the influence of nutrient addition on seagrass epiphytes (Campbell and Fourqurean 2014). Hence, while seagrass meadows may potentially flourish in a future where the oceans are enriched in CO_2, negative ecological effects of ocean acidification and nutrient fertilisation, such as competition from macroalgae and epiphytes, may prevent net gains to seagrass productivity.

CHAPTER 5

DO CO_2 RESPONSES DEPEND ON TEMPERATURE? A STUDY OF CARBON METABOLISM IN TWO TROPICAL SEAGRASS SPECIES [4]

Abstract

Anthropogenic carbon dioxide (CO_2) emissions can increase seagrass productivity by increasing CO_2 availability in seawater. However, the combined effects of temperature and increasing CO_2 availability (simulating OA) on seagrass metabolism are not known. Here, the effects of pCO_2 enrichment and temperature on carbon metabolism in two tropical seagrass species (*Cymodocea serrulata* and *Halodule uninervis*) were investigated. Physiological processes, such as net productivity (NP), biomass allocation and enzyme activity as a proxy for carbon translocation (sucrose-phosphate synthase SPS and sucrose synthase SS), were quantified to determine carbon assimilation and utilization under different pCO_2/temperature conditions. Seagrasses were exposed to three temperatures (20°C, 25°C and 30°C, spanning seasonal variation) and three target pCO_2 levels (present day 353 – 485 μatm; high 915 – 1102 μatm; extreme 1658 – 2297 μatm) for seven weeks. Net productivity, respiration and above-ground biomass increased with temperature for both species. At warmer treatments, *C. serrulata* exported more carbohydrates to its rhizomes, while *H. uninervis* invested in increasing shoot density. Above-to below-ground ratio increased with pCO_2 levels in *C. serrulata*. CO_2 enrichment increased NP in *H. uninervis*, but the effects were greatest at 30ºC. Overall, this study demonstrates that temperature exerts a much stronger control over carbon metabolism than CO_2 enrichment in tropical seagrasses. Species varied in the allocation

[4] Data from Chapter 5 has been written into a manuscript in preparation for submission.

of fixed carbon in response to temperature and CO_2, highlighting the importance in accounting for species differences when predicting meadow-scale productivity responses.

5.1 Introduction

Seagrass meadows are highly productive (Duarte and Chiscano 1999), which forms the basis of their many ecological functions. Carbon fixed during photosynthesis is converted and partitioned to above- (leaves) or below-ground (rhizomes and roots) structures for various processes such as growth, respiration or storage (Touchette and Burkholder 2000a). Distribution of fixed carbon to above-ground biomass affects plant photosynthetic potential (Farrar and Williams 1991), growth and nutritional contents of leaves (Aragones et al. 2006) and the quality of detrital export to adjacent habitats (Duarte et al. 2005). Similarly, the growth of below-ground biomass influences the carbon sequestration potential of seagrass meadows (Fourqurean et al. 2012; Macreadie et al. 2014). Hence, carbon metabolism and partitioning is highly influential in the functional ecology of seagrass meadows.

The ocean is becoming enriched in carbon dioxide (CO_2) due to the continued absorption of atmospheric carbon emissions (ocean acidification, OA). Partial pressure of CO_2 (pCO_2) in seawater is projected to rise by a moderate 500 μatm (-0.14 pH; RCP 2.6) to a high 1000 μatm (-0.4 pH; RCP 8.5) from current levels of ~ 400 μatm (Collins et al. 2013). Seagrass photosynthesis is carbon-limited under current *p*CO2 levels (Beer and Koch 1996) and is shown to increase with CO_2 enrichment (Zimmerman et al. 1997; Ow et al. 2015). Increased productivity from CO_2 enrichment has led to higher tissue carbon content (Campbell and Fourqurean 2013a), greater increase in below-ground biomass (Palacios and Zimmerman 2007; Takahashi et al. 2015) and non-structural carbohydrates content (Jiang et al. 2010) in seagrasses.

Seagrass response to CO_2 enrichment may be modulated by ambient growth temperatures (Koch et al. 2013). In terrestrial C3 plants, CO_2 responses result from

increased photosynthesis from higher CO_2 (Mott 1990; Long et al. 2004). On the other hand, temperature responses manifest from the combination of metabolic (e.g. enzyme) and physical (e.g. diffusion) changes that take place among various processes (Berry and Bjorkman 1980; Farrar and Williams 1991). Since temperature has an over-riding effect on carbon metabolism - from assimilation to utilization (Touchette and Burkholder 2000a) - it is expected that changes in ambient growth temperature would affect the ability to utilize CO_2 and also affect carbon partitioning.

Carbon assimilation, or photosynthesis, is limited by low temperatures and CO_2 availability (Sage and Kubien 2007). At low temperatures, photosynthesis is limited by the regeneration of inorganic phosphate, which is needed for photophosphorylation (Harley and Sharkey 1991). The regeneration of inorganic phosphate comes from the synthesis of starch and sucrose from triose phosphates, and is not affected by CO_2 supply (Harley and Sharkey 1991). Hence, at low temperatures, the plant might not increase photosynthesis with CO_2 availability (Sage and Kubien 2007). As temperatures increase and approach the thermal optima of each individual species, enhanced photosynthetic efficiency and capacity result in higher photosynthetic rates (Taiz and Zeiger 1991; Masini and Manning 1997). However, higher temperatures also raise photorespiration rates (Taiz and Zeiger 1991). Carbon dioxide enrichment could enhance the carboxylase function of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) while simultaneously inhibiting its oxygenase function (Bowes and Ogren 1972; Sage and Kubien 2007). This helps to reduce photo-respiratory loss. Furthermore, the rate of increase in photosynthesis with CO_2 concentration was predicted to be greater than that with temperature in seagrass (Zimmerman et al. 2015). Hence with temperature rise and CO_2 enrichment, the increase in carboxylation could compensate for photo-respiratory loss and result in an overall increase in net productivity (Long 1991; Stitt 1991).

Carbon utilization among various sink processes could also be influenced by temperature and CO_2 availability. Metabolic respiratory demands in both seagrass leaf and rhizome tissues increase with higher temperatures (Zimmerman et al. 1989; Masini et al. 1995). As a result, there should be increased transport of carbon assimilate (i.e. carbohydrates) and increased utilisation in sink tissues (Farrar and Williams 1991). Decreased carbohydrate accumulation could trigger sucrose and starch synthesis, thereby creating a positive feedback for more carbon assimilation (Harley and Sharkey 1991;

Long 1991). Biomass accumulation under warmer environments would tend to favour above-ground biomass (higher shoot-to-root ratio) (Zimmerman et al. 1989; Farrar and Williams 1991) in order to increase photosynthetic potential while reducing respiratory burden (Hemminga 1998). Carbon dioxide enrichment may lead to a different carbon utilization pattern from that during raised temperatures. Under elevated CO_2, increased productivity leads to greater increase in below-ground biomass (lower shoot-to-root ratio) (Palacios and Zimmerman 2007; Takahashi et al. 2015) and an accumulation of non-structural carbohydrates content in the rhizomes (Jiang et al. 2010). Hence, rising temperatures appears to increase the utilization of fixed carbon, while CO_2 enrichment appears to increase storage of fixed carbon.

As a result of the physiological responses to temperature, seagrass response to CO_2 availability may depend on temperature. During cooler (sub-optimal) winter months, productivity from CO_2 enrichment may be limited by lowered sink capacity due to lower respiratory demands, as well as reduced carbohydrate storage and biomass accumulation capacity (Terrados and Ros 1995; Masini and Manning 1997). Over hotter summer months that encompass the optimal growth temperature range, productivity with CO_2 enrichment should increase from enhanced photosynthetic rates and higher carbohydrates demand to offset increased respiratory and growth requirements (Long 1991; Perez and Romero 1992). The interaction between increasing temperature and CO_2 on seagrass carbon utilization needs investigation, as these two variables profoundly influence the biochemistry and physiology of marine plants (Koch et al. 2013).

Species with different growth strategies (e.g. fast- vs slow-growing) might display specific resource allocation patterns (Kilminster et al. 2015). *Cymodocea serrulata* and *Halodule uninervis* are two common tropical seagrass species in the Great Barrier Reef (Waycott et al. 2004) with different shoot turnover rates and responses to disturbances (Kilminster et al. 2015; McKenzie et al. 2015b). Different species may adjust their carbon allocation strategies differently under CO_2 and temperature (Koch et al. 2013). Understanding the allocation and metabolism of fixed carbon provides valuable insights about their adaptive strategies to cope with future climate change.

The present study tested a) temperature responses over a known seasonal range and b) if responses to CO_2 enrichment are temperature dependent. It was hypothesized

that warmer temperatures will increase net productivity, biomass production and carbohydrates storage in CO_2-enriched seagrasses. In addition to ambient (present day) CO_2 concentrations, two CO_2 enriched treatments were used (high 915 – 1102 μatm and extreme 1658 – 2297 μatm). Also, species-specific carbon partitioning responses in response to pCO_2 and temperature were examined for species with contrasting growth strategies, i.e. *C. serrulata* and *H. uninervis* (Kilminster et al. 2015; McKenzie et al. 2015b).

5.2 Methods

5.2.1 Plant collection

Two tropical seagrass species species, *Halodule uninervis* and *Cymodocea serrulata*, were collected from Cockle Bay at Magnetic Island (19°10.88'S, 146°50.63'E) in April 2014. Average water temperature at this site (2005 – 2013) was 26.2ºC, with daily means ranging from 20 – 30ºC across a year (McKenzie et al. 2015b). *H. uninervis* was collected as 10 cm diameter plugs with intact leaves, rhizome/roots and sediments and placed in pots lined with plastic bags that are sealed to prevent moisture loss. *C. serrulata* was collected by digging up intact shoots with connected horizontal rhizomes and placed into seawater-filled containers. The seagrasses were transported to National Sea Simulator (SeaSim) facilities at the Australian Institute of Marine Science, Townsville. Within 3 days, the seagrasses were potted in a mixture of coarse beach sand and mud (~ 80:20) from the collection site (pot size 15 cm length, 15 cm wide and 7 cm height), and allowed to recover from transplantation stress for four weeks prior to the start of the experiment. Holding tanks (1000 L) were housed under a 50 % light-reducing roof on flow-through seawater.

5.2.2 Experimental setup

The experiment was conducted in the SeaSim facility. Water temperature in tanks was controlled through heat-exchanges and mixers to a 0.1°C precision. Temperature changes within each tank were buffered by a controlled temperature water jacket around the tank. After the temperatures were regulated, pCO_2 levels in each tank was adjusted for each temperature treatment. pCO_2 levels were computer-regulated through in-line injection of CO_2 gas using membrane contactors (Liqui-Cel Extra-Flow 2.5 x 8, USA). The fully factorial experiment design consisted of three temperatures (20°C, 25°C and 30°C) and three pCO_2 levels (nominal treatments 400, 1000, and 2000 μatm), with three replicate tanks for each combination, resulting in a total of 27 tanks. Each tank had a working volume of 49 L in a flow-through system, with water replacement once every hour. Two sub-replicate pots of each species were placed in each tank. Treatments were randomly allocated throughout the experimental set-up. Light was delivered through two sets of white-light LED lamps over each tank set to 12 hours of illumination (400 μmol m^{-2} s^{-1}) plus 30 min ramp up and ramp down. The experiment was run for seven weeks (29 May to 24 July 2014).

5.2.3 Water chemistry and temperature

pH and temperature were monitored continuously with ISFET type pH probes (Endress Hauser CPS-471D, Switzerland) installed in one representative tank per treatment combination. The $pH_{[total\ scale]}$ values in all tanks over the course of the experiment were determined using a spectrophotometric method with m-cresol purple (Dickson et al. 2007). Water samples for total alkalinity (A_T) were collected from each tank in parallel and analysed by gran titration on a Metrohm-dosino analyser. Furthermore, samples for dissolved inorganic carbon (DIC) and A_T were collected three times from each tank. Salinity was measured with a refractometer at the point of water sampling. A_T samples were analysed on a Vindta 3C by acid titration (TA) and DIC samples by acidification and coulometric detection (UIC 5105 Coulometer) of the evolved CO_2. Certified reference seawaters (A. G. Dickson, Scripps Institute of Oceanography, Dixon, Batch 106) were used for calibration. Carbon chemistry parameters of the treatment waters were

calculated using DIC, A_T, temperature and salinity values using USGS CO2calc software (Robbins et al. 2010). Water samples for nutrient analysis were collected in duplicate, filtered through 0.45 μm cellulose acetate filters, and stored at -20°C. The concentrations of ammonium, nitrate, and phosphate in the seawater were determined according to standard procedures by Ryle et al. (1981).

5.2.4 Photosynthesis and respiration

Seagrass leaf photosynthesis and respiration were measured in small incubation chambers (70 mL) using optical oxygen sensors ("optodes", PreSens, Sensor spots-Pst3) and two PreSens Oxy 4 four-channel fiber-optic oxygen meters (Ow et al. 2015). Two arrays of four chambers were run at each time. Two blank chambers were included on each measuring run to account for blank respiration or production. The chambers were incubated at treatment water temperature using a flow-through water system connected to a water bath (Lauda, Ecoline RE 106). A magnetic stirrer bar within each chamber ensured even mixing. The leaves, scrapped clean of epiphytes with a razor blade, were held upright in the chamber to mimic natural orientation. Oxygen consumption (dark respiration) was measured over a 20-min period in the dark. Photosynthetic rates were then measured on the same leaf at 400 μmol m^{-2} s^{-1} PAR for 20 – 40 min. Oxygen concentration in the chambers was logged every 15 s, and respective respiration and production rates were derived with a fitted linear regression to the data. After the incubation, leaves were rinsed in freshwater, dried (48 hrs at 60°C) and weighed. The photosynthetic and respiration rates were normalised to the dry weight of the leaf. Initial periods of incubation (~5 min) prior to stabilization of photosynthetic and respiration rates were not included in regressions.

Respiration rate in the rhizome-root complex (below-ground) was measured on one sub-replicate pot from each tank. A single rhizome section was randomly chosen from each pot. This approach (only three replicates) was taken to minimise the damage caused to the plants in harvesting a small piece of rhizome. The rhizome was incubated in 5 mL chambers in the dark for 20 – 30 min, and otherwise following the same procedure as described for leaves.

5.2.5 Biomass measurements

Shoot counts in each pot were conducted prior to the experiment (week 0) and then at the end of 7 weeks. Shoot density change for each pot was calculated as the shoot difference between the start (week 0) and end (week 7) of the experiment, relative to the shoot density at Week 0. At the end of the experiment, all seagrass biomass was harvested from each pot and separated into above- and below-ground components. Biomass collected was dried (60ºC, 48 h) and weighed.

5.2.6 Enzyme assays

Plant material used for enzyme analyses was collected at the end of the experiment, snap frozen in liquid nitrogen and stored at -80ºC until analysis. The action of sucrose-phosphate synthase (SPS) in mature photosynthetic leaves primes the conversion of fixed carbon into sucrose, which is subsequently transported to various sink tissues (Touchette and Burkholder 2007). Sucrose synthase (SS) activity controls the import of sucrose into sink tissues. Sucrose-phosphate synthase from young but fully extended shoot tissue and SS from the rhizome-root complex were extracted using a technique described by Brun et al. (2003) and assayed according to Zimmerman et al. (1995). The sucrose produced was quantified using anthrone assay (Huber et al. 1991).

5.2.7 Tissue nutrients and non-structural carbohydrates (NSC)

At the end of the experiment, seagrass shoots were separated from the rhizomes and roots. Both above- and below-ground biomass were washed, dried at 60°C for 48 h and weighed before being finely ground. Ground leaf samples were analysed for carbon and nitrogen content with a Leco Truspec Micro CHNS analyser (Leco, UK). Ground rhizome-roots samples were analysed for non-soluble carbohydrates and starch content using the procedure outlined in Ow et al. (2015).

5.2.8 Statistical analysis

All parameters were analysed using two-way analysis of variance (ANOVA), with temperature and pCO_2 as fixed factors. Individual tanks were included in the model as replicates, with sub-replicate pots nested within tanks. Shapiro-Wilks' and Levene's tests were used to check for normality and homogeneity of variances respectively. When necessary, values were natural log transformed to meet the assumptions of ANOVA (Underwood 1997). Percentage data (% C and % N) were arcsine square-root transformed. When significant treatment effects were detected, Tukey HSD post-hoc tests were used to compare among treatment groups. Interaction plots were used to examine the effect of temperature at each pCO_2 level when significance of the interaction was detected. All statistical tests were assessed at $\alpha = 0.05$. All analyses were performed in R (R Development Core Team 2014).

5.3 Results

5.3.1 Experimental parameters

Water temperature within the treatment tanks was relatively stable over time and consistent among tanks and pCO_2 treatments. The lowest temperature treatment was consistently above the target temperature, ranging from 20.6°C to 20.7°C (Table 5.1). Other temperature treatments were within target (Table 5.1). CO_2 treatments were successful at creating distinct pCO_2 treatments representing present-day pCO_2 (range of treatment averages: 353 – 485 μatm, for ease of reading referred to as 400 μatm), high pCO_2 (915 – 1102 μatm; 1000 μatm), and extreme pCO_2 (1658 – 2297 μatm, 2000 μatm). The highest pCO_2 treatment showed the greatest variability amongst tanks, but it nonetheless remained well above the moderate treatment. Dissolved inorganic nutrient concentrations were consistently low, with overall averages of 0.57 ± 0.02 μM (S.D.) nitrogen (nitrite, nitrate and ammonium) and 0.07 ± 0.002 μM (S.D.) phosphate.

Table 5.1: Experimental parameters. Carbonate system parameters were calculated from measured total dissolved inorganic carbon (DIC), total alkalinity (A_T), temperature and salinity at 36 using CO2calc software (Robbins et al. 2010). Values are given as means (± SD).

Nominal Treatment		Measured parameters			Calculated parameters				
pCO_2 (µatm)	Temperature (ºC)	DIC (µmol kg^{-1} SW)	A_T (µmol kg^{-1} SW)	pH total	Temperature (ºC)	pCO_2 (µatm)	HCO_3^- (µmol kg^{-1} SW)	CO_2 (µmol kg^{-1} SW)	CO_3^{2-} (µmol kg^{-1} SW)
400	20	2041 (27)	2328 (9)	8.08 (0.04)	20.7 (0.3)	365 (36)	1824 (39)	11.6 (1.1)	205 (14)
400	25	1999 (25)	2328 (8)	8.09 (0.03)	25.1 (0.2)	359 (35)	1755 (37)	10.1 (1.0)	233 (13)
400	30	1991 (37)	2329 (8)	8.03 (0.05)	29.8 (0.2)	419 (56)	1741 (55)	10.6 (1.4)	240 (20)
1000	20	2200 (46)	2329 (8)	7.75 (0.12)	20.6 (0.3)	925 (285)	2061 (61)	29.3 (9.0)	110 (25)
1000	25	2187 (42)	2327 (7)	7.71 (0.09)	25.2 (0.1)	1006 (206)	2042 (58)	28.3 (5.8)	117 (22)
1000	30	2169 (42)	2329 (7)	7.69 (0.08)	30.0 (0.1)	1066 (201)	2013 (59)	26.8 (5.1)	130 (22)
2000	20	2325 (17)	2331 (6)	7.40 (0.05)	20.7 (0.3)	2155 (242)	2206 (15)	68.4 (7.5)	51 (5)
2000	25	2269 (48)	2329 (8)	7.50 (0.13)	25.2 (0.1)	1772 (516)	2143 (56)	50.0 (14.6)	76 (22)
2000	30	2278 (43)	2331 (10)	7.42 (0.11)	29.9 (0.2)	2157 (590)	2150 (44)	54.2 (14.7)	74 (16)

5.3.2 Productivity and biomass responses

In *C. serrulata*, net productivity (NP) significantly increased with temperature (ANOVA: $F = 31.76$, $P < 0.001$). NP at 25ºC and 30ºC were 133 % and 109 % higher, respectively, than that at 20ºC (Tukey HSD: 25ºC $P < 0.001$, 30ºC $P = 0.014$; Fig. 5.1). Leaf respiration increased with temperature (ANOVA: $F = 27.90$, $P < 0.001$). Leaf respiration was 80 – 83 % higher at 25ºC and 30ºC, compared to at 20ºC (Tukey HSD: 25ºC $P < 0.001$, 30ºC $P < 0.001$; Fig. 5.1). Rhizome-root complex respiration did not vary significantly with temperature (Table 5.2). CO_2 enrichment had no significant effect on NP, leaf respiration and rhizome-root complex respiration.

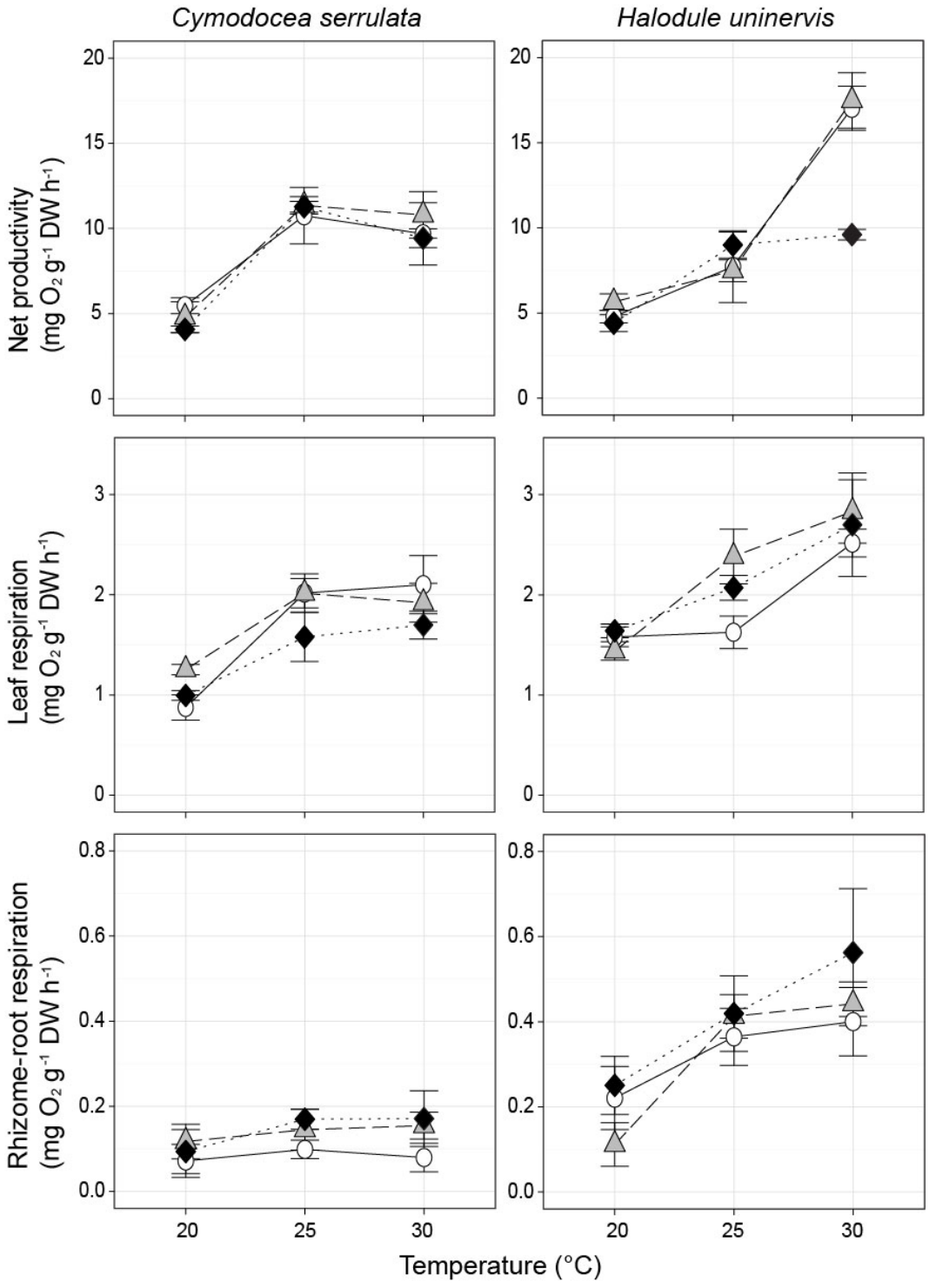

Fig. 5.1: Net productivity and respiratory responses to temperature and CO_2 enrichment treatments after seven weeks. pCO_2 levels are represented by different symbols (400 µatm - white circles; 1000 µatm - grey triangles and 2000 µatm - black diamonds). Means ± S.E. n = 3.

Table 5.2: Two way ANOVA results. All parameters were analysed with temperature and pCO_2 as fixed factors. Individual tanks were included in the model as replicates, with sub-replicate pots nested within tanks. Significant *P*-values ($P < 0.05$) are in bold. n = 3. AB:BG – above- to below-ground biomass ratio; NSC – total non-structural carbohydrates; SPS – sucrose phosphate synthase; SS – sucrose synthase.

		Cymodocea serrulata			*Halodule uninervis*		
Parameter	Source	df	F	*P*	df	F	*P*
Net Productivity	Temperature	2	31.760	**<0.001**	2	42.896	**<0.001**
(mg O_2 g^{-1} DW h^{-1})	pCO_2	2	0.362	0.702	2	1.917	0.176
	pCO_2 × Temp	4	0.335	0.851	4	2.980	**0.047**
Leaf respiration	Temperature	2	27.899	**<0.001**	2	20.915	**<0.001**
(mg O_2 g^{-1} DW h^{-1})	pCO_2	2	2.692	0.095	2	1.336	0.288
	pCO_2 × Temp	4	1.445	0.260	4	1.627	0.211
Rhizome	Temperature	2	1.452	0.260	2	6.567	**0.007**
respiration	pCO_2	2	3.321	0.059	2	1.733	0.205
(mg O_2 g^{-1} DW h^{-1})	pCO_2 × Temp	4	0.442	0.777	4	0.821	0.528
Change in shoot	Temperature	2	2.370	0.122	2	8.123	**0.003**
density (%)	pCO_2	2	0.602	0.559	2	0.983	0.394
	pCO_2 × Temp	4	0.422	0.791	4	0.373	0.825
Above-ground	Temperature	2	5.653	**0.012**	2	6.913	**0.006**
Biomass	pCO_2	2	2.258	0.133	2	2.384	0.121
(g dry weight)	pCO_2 × Temp	4	0.990	0.438	4	0.863	0.505
Below-ground	Temperature	2	2.036	0.160	2	0.427	0.659
biomass	pCO_2	2	2.041	0.159	2	0.777	0.475
(g dry weight)	pCO_2 × Temp	4	1.347	0.291	4	0.896	0.487
AB: BG	Temperature	2	2.515	0.109	2	17.071	**<0.001**
ratio	pCO_2	2	8.049	**0.003**	2	3.004	0.075
	pCO_2 × Temp	4	0.669	0.622	4	2.625	0.069
Soluble	Temperature	2	7.822	**0.004**	2	0.930	0.413
carbohydrates	pCO_2	2	0.465	0.636	2	1.710	0.209
(mg g^{-1} DW)	pCO_2 × Temp	4	0.151	0.960	4	1.480	0.250
Starch	Temperature	2	0.154	0.858	2	1.121	0.348
(mg g^{-1} DW)	pCO_2	2	2.582	0.103	2	0.175	0.841
	pCO_2 × Temp	4	2.023	0.134	4	0.899	0.485
NSC	Temperature	2	7.811	**0.004**	2	1.456	0.260
(mg g^{-1} DW)	pCO_2	2	0.451	0.644	2	0.258	0.776
	pCO_2 × Temp	4	0.152	0.960	4	0.880	0.495
% C	Temperature	2	4.845	**0.021**	2	0.446	0.647
	pCO_2	2	2.718	0.093	2	4.358	**0.029**
	pCO_2 × Temp	4	0.976	0.445	4	0.743	0.575

(Table 5.2 continued)

		Cymodocea serrulata			*Halodule uninervis*		
Parameter	Source	df	F	*P*	df	F	*P*
% N	Temperature	2	3.567	**0.050**	2	3.049	0.072
	pCO_2	2	1.780	0.197	2	0.066	0.936
	pCO_2 × Temp	4	1.785	0.176	4	2.512	0.078
C:N	Temperature	2	1.260	0.308	2	5.746	**0.012**
	pCO_2	2	0.973	0.397	2	5.002	**0.019**
	pCO_2 × Temp	4	3.136	**0.040**	4	4.165	**0.015**
Shoot SPS	Temperature	2	5.256	**0.016**	2	2.129	0.148
(μmol sucrose g^{-1}	pCO_2	2	0.392	0.681	2	1.507	0.248
FW mg^{-1} protein)	pCO_2 × Temp	4	0.382	0.819	4	1.278	0.315
Rhizome SS	Temperature	2	2.333	0.126	2	2.721	0.093
(μmol sucrose g^{-1}	pCO_2	2	3.631	**0.047**	2	0.492	0.620
FW mg^{-1} protein)	pCO_2 × Temp	4	1.100	0.387	4	5.675	**0.004**

Above-ground biomass of *C. serrulata* increased significantly with temperature (ANOVA: F = 5.65, $P = 0.012$) but not with pCO_2. At 30ºC, above-ground biomass was 26 – 35 % higher than at the lower temperatures (Tukey HSD: 20ºC $P = 0.002$, 25ºC $P = 0.029$; Fig. 5.2). The change in shoot density and below-ground biomass were not significantly different among treatments (Table 5.2; Fig, 5.2). The ratio of above- to below-ground (AB:BG) biomass was higher with increased pCO_2 (ANOVA: F = 8.05, $P = 0.003$) but was unaffected by temperature. At 2000 μatm pCO_2, AB:BG was 26 – 35 % greater than at 400 and 1000 μatm (Tukey HSD: 400 μatm $P = 0.003$, 1000 μatm $P = 0.032$; Fig 5.2).

In *H. uninervis*, the change in NP with pCO_2 level was dependent on temperature, as suggested by a significant interaction term between the main effects (ANOVA: F = 2.98, $P = 0.047$) (Table 5.2; Fig. 5.1). At 20ºC and 25ºC, NP was similar among all pCO_2 levels; at 30ºC, NP was 77 – 82 % lower at 1950 μatm pCO_2 than at 400 and 1000 μatm pCO_2 (Tukey HSD: 400 μatm $P = 0.027$; 1000 μatm $P = 0.014$; Fig. 5.1). Leaf respiration increased with temperature in *H. uninervis* (ANOVA: F = 20.92, $P < 0.001$). At 30ºC, leaf respiration was 32 – 73 % higher than at 20ºC and 25ºC (Tukey HSD: 20ºC $P = 0.002$, 25ºC $P = 0.005$; Fig. 5.1). Respiration rates in the rhizome-root complex were at raised

temperatures were significantly higher (ANOVA: F = 6.57, P = 0.007). At higher temperatures, rhizome-root respiration was 105 –141 % higher than at 20ºC (Tukey HSD: 25ºC P = 0.015, 30ºC P < 0.001; Fig. 5.1). There was no effect of CO_2 enrichment on leaf or rhizome-root respiration in *H. uninervis* (Table 5.2).

Above-ground biomass of *H. uninervis* increased with temperature (ANOVA: F = 6.91, P = 0.006) but not with pCO_2 (Table 5.2). Above-ground biomass increased by 42 – 88 % at higher temperatures, compared to at 20ºC (Tukey HSD: 25ºC P = 0.008, 30ºC P < 0.001; Fig. 5.2). Changes in shoot density varied with temperature in *H. uninervis* (ANOVA: F = 8.12, P = 0.003). At 20ºC, a reduction in shoot density over seven weeks (-8%) was observed; however at 25ºC and 30ºC, there was an 8 – 16 % increase in shoot density relative to Week 0 (Fig. 5.2). Below-ground biomass did not vary with temperature or pCO_2 treatments (Table 5.2). Above- to below-ground biomass was significantly higher with temperature rise (ANOVA: F= 17.07, P < 0.001). At 30ºC, AB:BG was 50 – 100 % higher than at 20ºC and 25ºC (Tukey HSD: 20ºC P < 0.001, 25ºC P < 0.001; Fig. 5.2).

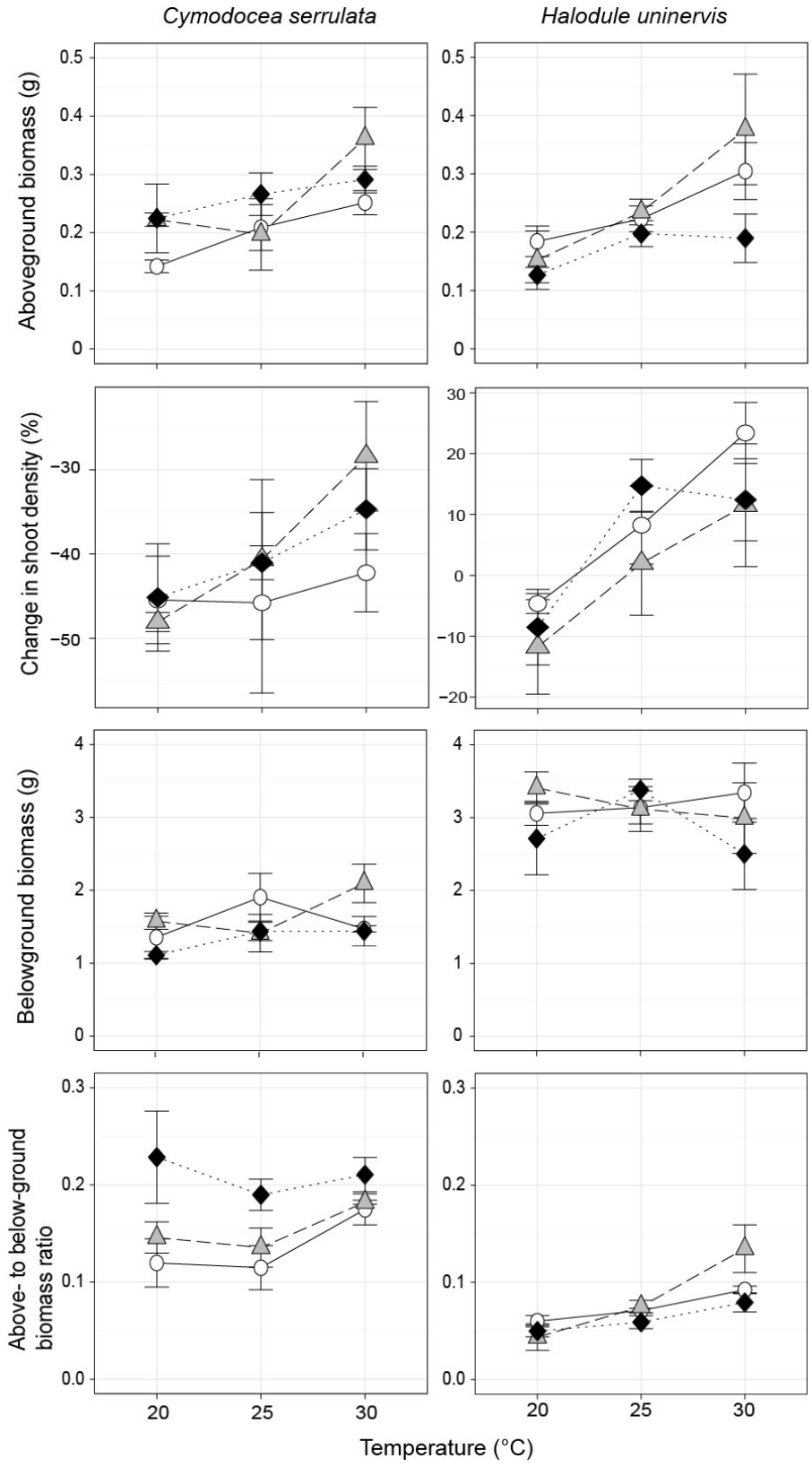
Cymodocea serrulata
Halodule uninervis
Aboveground biomass (g)
Change in shoot density (%)
Belowground biomass (g)
Above- to below-ground biomass ratio
Temperature (°C)

Fig. 5.2: Changes in biomass production and allocation after seven-week exposure to temperature and CO_2 enrichment treatment. pCO_2 levels are represented by different symbols (400 μatm - white circles; 1000 μatm - grey triangles and 2000 μatm - black diamonds). Means ± S.E. n = 3.

5.3.3 Tissue biochemical properties

Leaf tissue carbon content (% C) decreased with higher temperatures in *C. serrulata* (ANOVA: F = 4.85, $P = 0.021$) (Table 5.2; Fig. 5.3). Leaf % C was 7.8 % lower in plants grown at 30ºC, compared to those grown at 25ºC (Tukey HSD: $P = 0.009$). Nitrogen content (% N) in *C. serrulata* leaves decreased with increasing temperature (ANOVA: F = 3.57, $P = 0.050$) (Table 5.2). At 30ºC, % N in leaves were 14% lower than at 25ºC (Tukey HSD: $P = 0.032$) (Supplementary Fig. 5.1). There was an interactive effect of temperature and pCO_2 on carbon-to-nitrogen ratio (C:N) in *C. serrulata* leaves (ANOVA: F = 3.14, $P = 0.040$) (Table 5.2). At 20ºC and 25ºC, leaf C:N did not vary with pCO_2; however at 30ºC, C:N was 32 % higher at 2000 μatm than at 1000 μatm pCO_2 (Tukey HSD: $P = 0.039$) (Supplementary Fig. 5.1). In *C. serrulata* rhizomes, total non-structural carbohydrates (NSC) increased with temperature (ANOVA: F = 7.81, $P = 0.004$). At 25ºC and 30ºC, NSC contents increased by 18 – 20 % relative to that at 20ºC (Tukey HSD: 25ºC $P < 0.001$, 30ºC $P < 0.001$) (Fig. 5.4). Changes in soluble carbohydrates mirrored that of NSC, increasing with temperature rise (ANOVA: F = 7.82, $P = 0.004$) (Table 5.2). At higher temperatures, soluble carbohydrate contents were 18 – 20 % greater than at 20ºC (Tukey HSD: 25ºC $P < 0.001$, 30ºC $P < 0.001$) (Fig. 5.4). Starch content in rhizomes did not change significantly with both pCO_2 and temperature (Table 5.2; Fig. 5.4).

In *H. uninervis*, leaf carbon content varied with pCO_2 (ANOVA: F = 4.36, $P = 0.029$) but not with temperature (Table 5.2). % C was significantly lower at 1000 μatm than at 400 or 2000 μatm (Tukey HSD: 400 μatm $P = 0.043$; 2000 μatm $P = 0.008$) (Supplementary Fig. 5.1). Leaf nitrogen content in *H. uninervis* was not significantly different among treatments (ANOVA: $P > 0.05$) (Table 5.2). Variation in leaf C:N with pCO_2 treatment depended on temperature (ANOVA: F = 4.17, $P = 0.015$) (Table 5.2). At 20ºC and 25ºC, leaf C:N did not vary with pCO_2; at 30ºC, C:N was 18 – 22 % higher in

plants grown in 2000 μatm than those grown in lower pCO_2 levels (Tukey HSD: 400 μatm $P = 0.025$, 1000 μatm $P = 0.005$) (Supplementary Fig. 5.1). Total non-structural carbohydrates, soluble carbohydrates and starch content in *H. uninervis* rhizomes showed no significant change with temperature and pCO_2 (Table 5.2; Fig. 5.4).

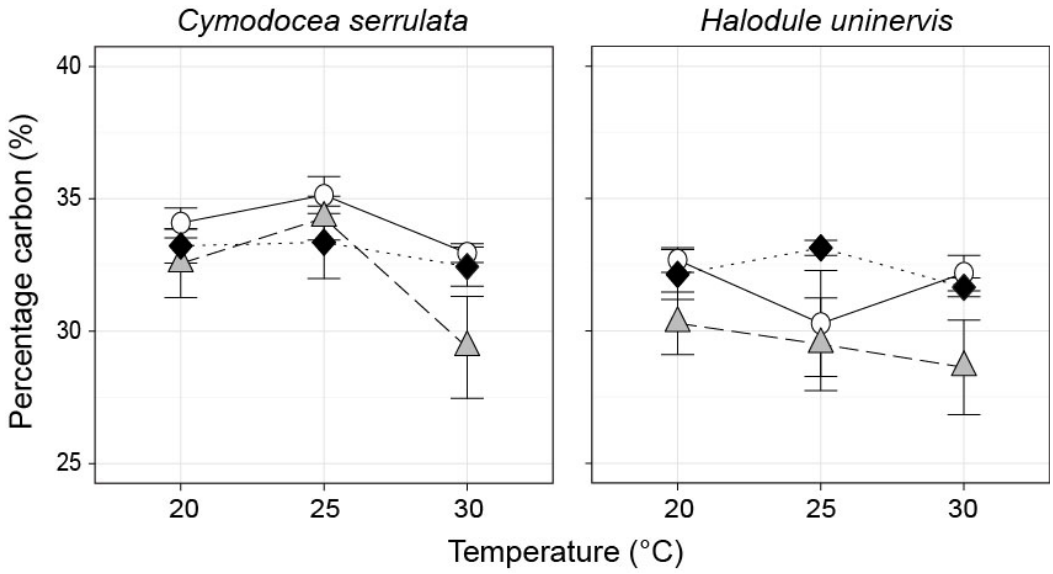

Fig. 5.3: Leaf tissue carbon content (%) after seven-week exposure to temperature and CO_2 enrichment treatments. pCO_2 levels are represented by different symbols (400 μatm - white circles; 1000 μatm - grey triangles and 2000 μatm - black diamonds). Means ± S.E. n = 3.

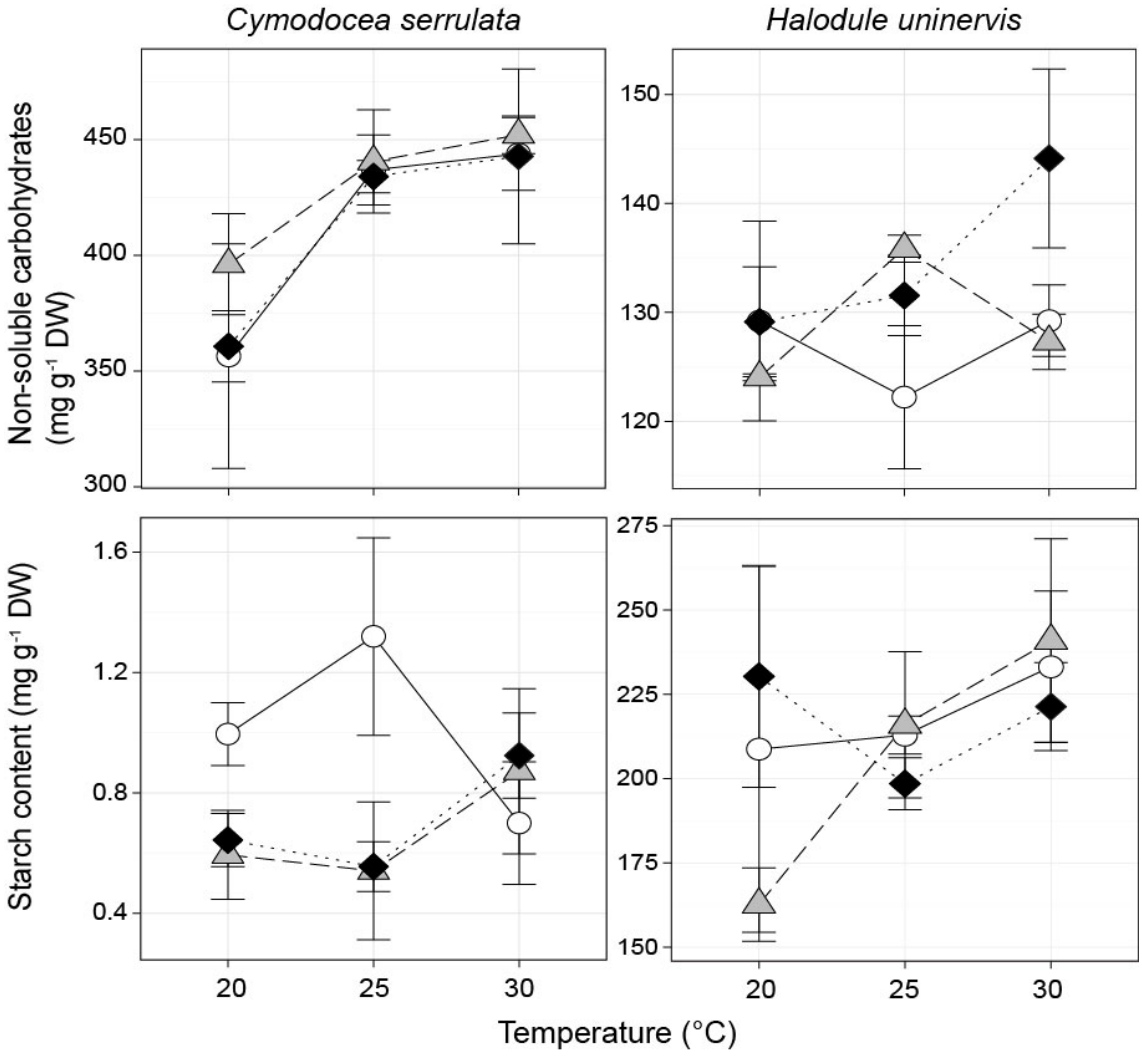

Fig. 5.4: Changes in the concentration of non-structural carbohydrates (non-soluble carbohydrates and starch) in rhizome tissue after seven-week exposure to temperature and CO_2 enrichment treatment. Axes differ between species for non-soluble carbohydrates and starch. pCO_2 levels are represented by different symbols (400 µatm - white circles; 1000 µatm - grey triangles and 2000 µatm - black diamonds). Means ± S.E. n = 3.

Across both species, sucrose phosphate synthase (SPS) and sucrose synthase (SS) activity yielded no significant change with treatments when expressed over tissue fresh weight. However when normalized to tissue protein concentration, significant differences in enzyme activities among treatments emerged (Table 5.2). Hence, results described here related to the enzyme activity normalized to tissue protein concentration.

In *C. serrulata*, SPS activity in the leaves increased with temperature only (ANOVA: F = 5.26, *P* = 0.016). At 30ºC, leaf SPS activity was 100 % higher than at 20ºC (Tukey HSD: *P* = 0.002; Supplementary Fig. 5.2). Rhizome SS activity varied with pCO_2 (ANOVA: F= 3.63, *P* = 0.047) but not with temperature (Table 5.2). *C. serrulata* rhizome SS activity was higher at 1000 μatm than at 2000 μatm (Tukey HSD: *P* = 0.022) (Supplementary Fig. 5.2).

H. uninervis leaves displayed no significant change in SPS activity with both temperature and pCO_2 (Table 5.2; Supplementary Fig. 5.2). Rhizome SS activity responded to pCO_2 enrichment in a temperature-dependent manner (ANOVA: F = 5.68, *P* = 0.004) (Table 5.2). At 20ºC, rhizome SS activity was lowered as pCO_2 increased from 400 to 2000 μatm (Tukey HSD: *P* = 0.001). At 25ºC, rhizome SS activity was much higher at 1000 μatm than at 400 μatm pCO_2 (Tukey HSD: *P* = 0.004). SS activity did not vary with pCO_2 at 30ºC (Supplementary Fig. 5.2).

5.4 Discussion

This study investigated if carbon metabolism in tropical seagrasses was influenced by temperature and CO_2 enrichment. Increasing water temperature, over natural seasonal ranges (20 – 30ºC) in the central Great Barrier Reef, had a much greater and more widespread effect on metabolism of the seagrasses investigated than increasing pCO_2, even though pCO_2 was increased to levels beyond those predicted at end of century (Collins et al. 2013). Compared to previous studies (Jiang et al. 2010; Ow et al. 2015), the expected "positive" effects of CO_2 enrichment were relatively small. The effects of temperature occurred at the whole plant level: increasing net productivity, respiration, storage of carbohydrates and biomass production. However, responses were variable between the two species. Specifically, *C. serrulata*, which can form persistent communities (Kilminster et al. 2015; McKenzie et al. 2015b), exported additional carbon (increased SPS activity) and increased carbohydrate concentrations in rhizomes when net productivity was increased at higher temperatures. In contrast, *H. uninervis*, which is a

colonizing species and rapidly grows and expands when conditions allow, did not increase sucrose export or invest in storage but rather increased above-ground biomass by producing new shoots.

5.4.1 Effects of temperature on carbon metabolism

Temperature increase from 20 to 30ºC enhanced net productivity, leading to a carbon surplus for biomass production and carbohydrate storage in both *C. serrulata* and *H. uninervis*. This agrees with observations of higher productivity and biomass during summer months in field conditions, supplemented by longer photoperiods (Olesen and Sand-Jensen 1993; Hillman et al. 1995; Lee and Dunton 1997). The increase in net productivity was consistent with photosynthesis-temperature curves derived for *C. serrulata* and *H. uninervis* (Adams et al. unpubl. data.). Net photosynthesis of *C. serrulata* and *H. uninervis* rapidly increases up to around 30°C, before the rate of increase starts to plateau. Net photosynthesis peaks at around 35ºC, and declines sharply thereafter (Adams et al. unpubl. data.). Therefore, photosynthetic rates measured here at 30°C approximate the maximum rate. In the present study, photosynthesis in *C. serrulata* and *H. uninervis* increased by 109 % and 197 % over the 10ºC rise (20 – 30ºC), respectively. *Halodule wrightii* growing in sub-tropical regions increased net productivity by a comparable 49 % over a 4ºC rise in temperature (Dunton and Tomasko 1994). Temperate species, such as *Zostera marina* (36 %) (Marsh et al. 1986), *Posidonia australis* (171 %), *Amphibolis antarctica* (67 %), *Amphibolis griffithii* (244 %) and *Posidonia sinuosa* (100 %) (Masini and Manning 1997) showed a wider range of productivity increases over 10ºC rise. Q_{10}, which is the ratio of the enzyme reaction rates at one temperature to that at 10ºC lower, is widely accepted for terrestrial plants at a value of two, i.e. photosynthetic rates would increase by 100% with a 10ºC rise in temperature (Tjoelker et al. 2001). Photosynthetic responses of seagrasses were more variable, probably as a result of species-specific changes in respiratory demands over the range of measurement temperatures (Tjoelker et al. 2001).

Respiratory demand increases with temperature (Perez and Romero 1992), with the change in respiration rates being stronger in *H. uninervis* than in *C. serrulata*. Rhizome-root complex respiration rates in *H. uninervis* increased by 147 % between 20

to 30ºC. This was much higher compared to that in *C. serrulata* (non-significant, this study) and in *Z. marina* (40 % between 10 to 20ºC; Zimmerman et al. 1989). However, the overall increase in net productivity indicates a carbon surplus for biomass accumulation and possible storage, despite higher respiration rates.

Above-ground biomass increased for both species with temperature, likely enabled by higher photosynthetic rates (Bulthuis 1987). Having more photosynthetic tissue, i.e. leaves, is useful in balancing whole plant respiratory demand (Zimmerman et al. 1989). Above-ground biomass was increased by reducing shoot loss and by production of new shoots (*H. uninervis*) with higher temperature. Carbon (% C) and nitrogen (% N) content of *C. serrulata* leaves were reduced at higher temperatures, likely due to carbon assimilate being directed towards storage in below-ground tissue (discussed below). This could have implications for the nutritional quality of leaves which are eaten by green turtles (Aragones et al. 2006).

Higher productivity did not necessarily translate into higher storage reserves in the below-ground biomass. In *C. serrulata*, non-soluble carbohydrates (the dominant storage form) increased with higher temperature, while in *H. uninervis*, starch (dominant storage form in this species) showed no evidence of increase with temperature. An increase in rhizome carbohydrate content in hotter summer months has been documented in species such as *Zostera noltii* (Pirc 1989), *Thalassia testudinum* (Lee and Dunton 1997), *P. sinuosa* (Collier et al. 2008) and *P. oceanica* (Alcoverro et al. 2001). Storage of carbohydrate reserves in below-ground structures safeguards them from herbivory and ensures sufficient reserves are available to sustain the persistent shoots during cooler months (Larkum et al. 2006), or during short-term events that affect their capacity to photosynthesize (Unsworth et al. 2015). For *H. uninervis*, the results suggest that under higher temperatures, growth (shoot production) and meeting respiratory demand are given “priority” over the accumulation of carbohydrate stores. Thus, higher productivity during warmer periods is partitioned according to the growth strategy of individual species.

5.4.2 Limited effects of CO_2 enrichment on carbon metabolism

Carbon dioxide enrichment in the present experiments had limited effects on plant metabolism in both seagrasses, which was unexpected. In previous studies, CO_2 enrichment enhanced photosynthetic rates, growth, biomass production and carbohydrate contents in seagrasses (Zimmerman et al. 1997; Jiang et al. 2010; Alexandre et al. 2012; Ow et al. 2015). In Ow et al. (2015), a three-fold increase in CO_2 concentration resulted in an 43 – 46 % increase in *H. uninervis* and *C. serrulata* productivity. In contrast, a five-fold increase in CO_2 concentration here failed to elicit significant changes in productivity. In the present study, higher CO_2 treatments were associated with higher above- to below-ground biomass ratio and increased import of carbon into the rhizome in *C. serrulata*. These changes were not related to changes in net productivity (since NP was not affected by pCO_2), and may be the result of other physico-chemical changes (e.g. changes to sediment biogeochemistry) (Burdige and Zimmerman 2002). However this cannot be determined from these results and effects of increasing pCO_2 on sediments warrant further investigation.

The response to CO_2 enrichment in seagrasses may be limited by nutrient supply (Alexandre et al. 2012; Hofmann et al. 2014). Over time, photosynthetic and growth responses to CO_2 enrichment would be reduced as nutrients become limiting (Stitt and Krapp 1999). Nutrient limitation has been postulated to account for the lack of growth response (Alexandre et al. 2012), a reduction in leaf nitrogen content and an increase in carbohydrates contents (Jiang et al. 2010; Campbell and Fourqurean 2013a) in seagrasses. Although dissolved inorganic nitrogen values in experimental tanks (0.57 μM) was higher than average inshore water values (0.24 μM) (Schaffelke et al. 2012), leaf tissue nutrient contents suggested both species in the present experiment were nitrogen limited. Leaf tissue nitrogen (% N: 1.3 – 1.6 %) content in both species after seven weeks of treatment were much lower than measured at the source meadow (% N: 2 – 2.1 %) (McKenzie et al. 2015b). Overall leaf C:N ratio (19.4 – 27.2) was also higher than in the source meadow (19.6 – 20.0), which could indicate nitrogen limitation (McKenzie et al. 2015b). In other studies that found a significant effect of CO_2 enrichment, leaf % N content were much higher, ranging from 2.3 – 2.6 % for *C. serrulata*, *H. uninervis* and *Thalassia hemprichii* (Jiang et al. 2010; Table 6.1, Chapter 6). Further, in *Cymodocea rotundata* at CO_2 enriched volcanic seeps (pCO_2 = 548 – 5098 μatm) where leaf biomass

and density were considerably increased compared to nearby ambient CO_2 sites, % N ranged from 1.7 – 2.1 % (Takahashi et al. 2015) and was higher than the % N content in this experiment. Apart from nutrient status, there was no other obvious factors (e.g. light, experimental duration) which could have caused the discrepancy in findings from previous studies (see Table 6.1, Chapter 6). Temperature responses (increased productivity and biomass), however, appear to be unaffected by nitrogen limitation, which is interesting and requires examination in detail.

5.4.3 Effects of temperature and CO_2

The limited extent to which seagrass responses to CO_2 enrichment depended on temperature in this study was surprising. Since CO_2 enrichment promotes carboxylation, it might offset photo-respiratory loss and elevate photosynthetic rates under higher temperatures (Sage and Kubien 2007; Koch et al. 2013). Previously, Connell and Russell (2010) demonstrated that CO_2 enrichment (550 ppm) and temperature increase (+3ºC) synergistically increased biomass and cover in marine turf algae. For seagrasses, in contrast, interactive effects of temperature and CO_2 enrichment were only observed in NP, % C and rhizome sucrose synthesis activity of *H. uninervis*. Overall results suggest temperature had a stronger effect on seagrass physiology than CO_2 enrichment.

Seagrass carbon metabolism is modulated by temperature, from assimilation to utilization in sinks (Farrar and Williams 1991; Touchette and Burkholder 2000a). However, the effects of CO_2 enrichment, according to current literature, occur primarily as a result of increasing photosynthesis (Zimmerman et al. 1997; Jiang et al. 2010; Ow et al. 2015). Furthermore, its effect on seagrass metabolism is dependent on the carbon demand in the plant, which could be limited by availability of light and nutrients (Palacios and Zimmerman 2007; Alexandre et al. 2012; Burnell et al. 2014b). Acclimation of respiration and growth to temperature in seagrasses could also affect carbon demand (Zimmerman et al. 1989; Collier et al. 2011). Hence, even though in *Z. marina*, the Q_{10} for photosynthesis is < 2 under present-day seawater (Zimmerman et al. 1989), and photosynthetic Q_{10} is predicted to increase exponentially with CO_2 concentration (Zimmerman et al. 1997; Zimmerman et al. 2015), our results still suggested that

temperature plays an over-riding role over CO_2 availability in the control of seagrass carbon metabolism. Seagrasses also possess effective carbon concentrating mechanisms (CCMs) to cope with photosynthetic carbon demand (Beer et al. 2002; Uku et al. 2005). The presence of CCMs helps procure bicarbonate (the dominant DIC source in seawater) and reduce the reliance on CO_2 diffusion. Whether temperature can affect CCMs and any subsequent effect on carbon assimilation in marine macrophytes remains to be explored (Raven et al. 2011).

5.4.4 Species-specific responses

Cymodocea serrulata is classified as an opportunistic species on a global assessment of seagrasses (Kilminster et al. 2015), but can form foundational or persistent communities in the Great Barrier Reef (McKenzie et al. 2015b). When productivity increased at higher temperatures, *C. serrulata* exported sucrose (SPS increased), and carbohydrate concentrations in rhizomes were increased. This is consistent with responses in persistent species, such as *Posidonia* spp. and *Thalassia* spp., which increase storage reserves in summer, when photosynthetic rates are faster and photoperiods are longer (Lee and Dunton 1997; Alcoverro et al. 2001; Collier et al. 2008). Accumulation of storage reserves is important as a "resistance" strategy for maintaining resilience particularly in persistent species (Unsworth et al. 2015).

Halodule uninervis is a colonizing species, although it can also be considered opportunistic, as it can rapidly grow and expand under appropriate environmental conditions (Kilminster et al. 2015). It has a high proportion of below-ground biomass (BG:AB *C. serrulata*: 7.2 vs *H. uninervis*: 16.8), and hence, sink strength. *H. uninervis* showed greater increase (197 % over 10ºC) in NP with temperature than *C. serrulata* (109 % over 10ºC), suggesting the ability to upregulate photosynthesis effectively (Zimmerman et al. 1989; Collier et al. 2011). This species also increased above-ground biomass with temperature, and specifically, this was driven by the production of new shoots, a pre-cursor to branching and expansion. Rapid shoot production and expansion is important as a "recovery" strategy for maintaining resilience in colonizing species in particular (Unsworth et al. 2015).

5.5.5 Conclusion

The present study demonstrates that temperature exerts a much stronger control over carbon metabolism than CO_2 enrichment in tropical seagrasses. This implies that future patterns of carbon metabolism (assimilation and partitioning) might closely follow seasonal temperature variations and be less influenced by OA. A caveat to this result was that the nutrient status of the seagrass population in the present study could have limited CO_2 response. Both *C. serrulata* and *H. uninervis* displayed different carbon allocation strategies with respect to temperature variation and CO_2 enrichment. Such differences allude to the variety of carbon allocation strategies present in tropical seagrass meadows. Understanding such differences is important for predicting meadow-scale productivity and the long term carbon sequestration potential of seagrass beds.

Chapter 6

GENERAL DISCUSSION

Ocean acidification (OA) will impact marine and coastal ecosystems. Even with reduced carbon emissions under a stringent emissions scenario (RCP 2.6), marine organisms will experience physiological changes to some extent from elevated CO_2 levels (Gattuso et al. 2015). While research has advanced our knowledge of OA effects on marine calcifiers, such as corals and planktonic coccolithophores, work on seagrasses has been limited. Studies have demonstrated that photosynthetic rates of seagrasses at present day concentrations of dissolved carbon dioxide (CO_2) are under-saturated, and these rates increase with CO_2 enrichment. In turn, this can lead to some other plant-level modifications, such as increasing biomass and growth (Beer and Koch 1996; Zimmerman et al. 1997; Jiang et al. 2010; Alexandre et al. 2012; Campbell and Fourqurean 2013a). Beyond that, attempts to quantify interactive effects of local-scale changes in temperature and water quality (e.g. turbidity, nutrients) on photosynthetic response to CO_2 enrichment, or to compare CO_2 responses among species with varying growth strategies, are lacking. Given that seagrasses meadows are important ecosystems and may also be able to mitigate effects of climate change on neighbouring ecosystems (Unsworth et al. 2012; Macreadie et al. 2014), it is imperative to better understand their responses to CO_2 enrichment, temperature and local water quality, starting from the physiological level.

My research aimed to investigate the responses of tropical seagrass species to OA alone, and in combination with key water quality parameters (i.e. light, nutrients), with a particular focus on processes that affect seagrass productivity. This thesis focussed on tropical seagrass species in the Great Barrier Reef, Australia (GBR). The GBR is situated in the biodiverse Indo-Pacific bioregion, which hosts around 20% of the world's seagrass species (Short et al. 2011). High seagrass diversity in this region facilitates and necessitates the examination of inter-specific seagrass response.

In this final chapter, I present a synthesis of the major findings presented in Chapters 2 to 5 to illustrate the complex interactions between other environmental factors and OA on seagrass physiology. Limitations of the studies are discussed, with the objective to reconcile apparent differences in results among experiments and to identify key parameters that influence seagrass responses to OA. I also discuss the implications of the results for future seagrass productivity and considerations for management of seagrass meadows for a changing climate. Lastly, directions for future work are suggested.

6.1 Physiological controls of seagrass productivity: revisited

Tropical seagrass physiological responses to OA were tested in isolation (increased pCO_2 only; Chapter 2), in a range of water quality scenarios (low light and elevated nutrients, Chapters 3 and 4), and over their seasonal temperature range (Chapter 5). Overall findings indicate that seagrass responses did not always adhere to the paradigm that seagrass productivity increases with OA (Zimmerman et al. 1997; Jiang et al. 2010; Alexandre et al. 2012).

6.1.1 Effects of OA (CO_2 enrichment)

All seagrass species investigated (*Cymodocea serrulata*, *Halodule uninervis* and *Thalassia hemprichii*) were able to (though did not always, as outlined below) upregulate photosynthetic rates with CO_2 enrichment over the short term (Chapters 2, 3 and 4). The findings are generally consistent with earlier experiments (Zimmerman et al. 1997; Jiang et al. 2010; Alexandre et al. 2012) and observations at CO_2 seep sites (Russell et al. 2013; Apostolaki et al. 2014), which demonstrated that seagrasses may benefit from OA. As a result of increased photosynthesis, carbon budgets (P_G:R) also increased under CO_2 enrichment. Having greater carbon surplus could enable downstream effects to plant-scale responses, such as growth and shoot production (Invers et al. 2002; Palacios and

Zimmerman 2007). In Chapters 2, 3 and 4, growth rates increased as a response to enhanced photosynthesis and energetic surplus under CO_2 enrichment.

Photosynthetic parameters (P_{max}, α) responded similarly to CO_2 enrichment among species (Chapter 2). However, not all species increased plant-scale responses in the same way: *H. uninervis* and *T. hemprichii* increased leaf growth with CO_2 enrichment while no such increase occurred in *C. serrulata* (Chapter 2). Leaf growth rate of *Thalassia testudinum* was unaffected by CO_2 enrichment (Campbell and Fourqurean 2013a) but an increased rate occurred in *T. hemprichii* (Jiang et al. 2010). I recognised that responses vary among species with different growth strategies. From the literature I also identified that other sinks for energetic surplus, such as shoot density and below-ground biomass (Fabricius et al. 2011; Apostolaki et al. 2014) or sexual reproduction (Palacios and Zimmerman 2007) exist. Furthermore, light (Chapter 3; Palacios and Zimmerman 2007), nitrogen availability (Chapter 4; Stitt and Krapp 1999) and temperature (Chapter 5; Touchette and Burkholder 2000a) can influence carbon metabolism and hence, growth response (Table 6.1). Therefore, further studies were conducted on the interactive effects of these environmental conditions and OA on seagrass physiology, in particular, on carbon sinks (section 6.1.4).

In this thesis, I also describe differences in carbon utilisation between two seagrass species and the implications of these for responses to CO_2 enrichment (Chapter 3; Mercado et al. 2003; Uku et al. 2005; Campbell and Fourqurean 2013b). *C. serrulata* required high light to utilise bicarbonate (HCO_3^-) while HCO_3^- use in *H. uninervis* was not restricted by light availability (Chapter 3). Seagrass species that utilise HCO_3^- less readily, such as *T. testudinum* (Campbell and Fourqurean 2013b), may benefit more from increased CO_2 supply under OA conditions. Some species of marine algae (Israel and Hophy 2002) and the seagrasses *Halodule wrightii* and *Syringodium filiforme* (Campbell and Fourqurean 2013b), were thought to show reduced photosynthetic responses to CO_2 enrichment due to their efficient use of HCO_3^-. Hence, results from Chapter 3 are consistent with existing literature: *C. serrulata* showed a greater photosynthetic response to CO_2 enrichment, especially at low light levels, while *H. uninervis* displayed limited response to CO_2 enrichment due to efficient carbon use at lower concentrations. Thus, the ability for the efficient use of the dominant HCO_3^- pool for photosynthesis is an important consideration when examining response to CO_2 enrichment among species.

6.1.2 Effects of light

Light availability plays an upstream role, relative to dissolved inorganic carbon (DIC), in the hierarchical control on seagrass photosynthesis (Fig. 1.4, Chapter 1). Light controls photosynthesis by providing reducing intermediates (NADPH and ATP) for the incorporation of CO_2 (Hall and Rao 1994). Photosynthetic rates (Chapter 3) were strongly limited by light and productivity increases (at high pCO_2) did not offset low light effects as productivity remained considerably reduced compared to high light in *C. serrulata* and *H. uninervis*. Similarly, in *Zostera marina*, CO_2 enrichment only increased shoot production and below-ground biomass under light-replete but not light-deplete treatments (Palacios and Zimmerman 2007).

Carbon dioxide enrichment can affect light required to maintain positive whole plant carbon balance (Chapter 2; Zimmerman et al. 1997). Dissolved inorganic carbon (DIC) enrichment stimulated photosynthesis more under lower light levels than under high light in *C. serrulata* (Chapter 3), which was consistent with findings by Schwarz et al. (2000) on deep and shallow seagrass populations. This implies that metabolic balances are met at lower light levels (Schwarz et al. 2000; Long et al. 2004) and CO_2 enrichment potentially increases the tolerance of seagrasses to low light conditions, for example during flood plumes. On the other hand, light required to saturate photosynthesis increased with CO_2 enrichment [*C. serrulata* (Chapter 3), *Z. marina* (Zimmerman et al. 1997), *Z. noltii* (Alexandre et al. 2012) and *T. hemprichii* (Jiang et al. 2010)]. Overall, CO_2 enrichment increases the efficiency of light use (Chapter 2 and 3) but also results in additional light required to saturate photosynthetic capacity.

6.1.3 Effects of nitrogen enrichment

I hypothesised that nitrogen enrichment could potentially augment CO_2 responses in seagrasses, because nitrate and nitrogen metabolites are essential for the assimilation of fixed carbon into amino acids (Fig. 1.4, Chapter 1). Nitrogen limitation limits growth responses in terrestrial plants (Stitt and Krapp 1999; Ainsworth and Long 2005). However, nutrient increases to levels approximating present day flood plumes only had

small effects on seagrass metabolism (Chapter 4). Signs of nitrogen limitation that had been observed in previous CO_2 enrichment studies, such as increased carbohydrates and C:N ratios (Jiang et al. 2010; Campbell and Fourqurean 2013a), were not detected in the study. Assessment of nitrogen demand (nitrogen uptake and enzyme activity) showed that CO_2 enrichment did not drive nitrogen demand in *H. uninervis* and *T. hemprichii*. This contrasts with studies on other marine macrophytes (Zou 2005; Alexandre et al. 2012). Seagrasses are capable of recycling or re-allocation of nitrogen within the plant (Romero et al. 2006), or even "luxury uptake" (Mellors 2003). Coastal habitats are continually subjected to fluxes in nutrients in the water column, which may be supplemented by nitrogen fixation in the sediments (Welsh 2000). Therefore, they might be less prone to nitrogen limitation than terrestrial plants (Stitt and Krapp 1999; Ainsworth and Long 2005).

Comparing results of Chapter 4 with studies that demonstrated possible nitrogen depletion with enhanced CO_2 (Jiang et al. 2010; Campbell and Fourqurean 2013a), it seems likely that CO_2 responses are contingent on local nutrient regimes, and thus, are location-specific. The ability to respond to CO_2 enrichment varied among seagrass populations with different tissue nutrient status (between Chapter 4 and 5, see section 6.2.1), suggesting that CO_2 response might also be dependent on nutrient use efficiency of the population (Romero et al. 2006). Hence, inter-population variation in nutrient use can determine if low-level renewal of nitrogen supplies in GBR meadows would enable seagrasses to be more productive without facing nitrogen limitation with future OA.

6.1.4 Effects of temperature

Increasing temperatures and CO_2 enrichment affect carbon metabolism (assimilation and utilization) in seagrass (Touchette and Burkholder 2000a). Findings in Chapter 5 revealed limited effects of CO_2 enrichment across a range of ambient growth temperatures in tropical seagrasses. This is surprising because 1) previous chapters demonstrated effects of CO_2 enrichment on productivity and growth (under average temperatures also covered in the range investigated in this chapter), and 2) fundamental links have been proposed to exist between elevated CO_2 and temperature on photorespiration, enzyme systems and carbohydrate production in marine macrophytes (Koch et al. 2013). Models predicted

that CO_2 increases projected for the next century should stimulate photosynthesis sufficiently to offset the negative effects of thermal stress (+5ºC above thermal optimum) on *Z. marina* (Zimmerman et al. 2015). Given that the highest temperature treatment in Chapter 5 was within the range of their thermal optima (Chapter 1), it was expected that CO_2 enrichment and temperature rise should stimulate photosynthesis in an additive or synergistic manner. The limited effects of CO_2 enrichment observed in this experiment might be due to other limiting parameters, such as nutrients (see discussion below, Table 6.1). As there is very limited literature on temperature and CO_2 effects on marine macrophytes (Koch et al. 2013), with this study being the first, more studies are needed to draw further conclusions.

6.2 Experimental limitations

This study is one of the first few to systematically examine the interactive effects of CO_2 enrichment and water quality parameters on seagrasses. Some limitations have been identified across the series of experiments and are discussed below for the benefit of future work exploring this subject.

6.2.1 Low replication

In *ex-situ* aquaria settings, space and logistical constraints had limited the number of replicates within experiments. This is especially pertinent for factorial design experiments, where the number of experimental units increases as a factor of the number of treatments. For Chapters 3, 4 and 5, the number of replicates (i.e. independent tanks) was at the minimum (n = 3), but still resulted in large experiments with 18 to 27 tanks. Reduced statistical power could limit the ability to detect interactive effects. Ways around this include 1) using a larger facility with larger numbers of tanks, for example, the AIMS SeaSimulator is now available for use in Townsville, but was not available at the time of experiments for Chapters 2, 3, and 4; 2) repeating the same experiment in order to

compare responses; and 3) using sub-replication. Experiments should also be repeated over a wide spatial scale, i.e. among localities, as environmental histories and genetic diversity may result in different responses among seagrass populations (Reusch et al. 2008; Salo et al. 2015).

6.2.2 Optimisation of experimental conditions

Responses to CO_2 enrichment varied among the four experiments described in this thesis. This was despite the fact that CO_2 treatments were comparable (ranging from 442 to 1235 μatm, and with an extreme also included in chapter 5), the methods used to mimic enhanced pCO_2 levels were the same in principle (via bubbling of CO_2 gas, except Chapter 5 where membrane contactors were used), and in most experiments (except Chapter 4) the same population of seagrass was used. To identify key experimental parameters that could have contributed to variation in responses, I compiled the experiments from this thesis in Table 6.1, together with published studies listing the responses observed, experimental treatment conditions, and the other environmental conditions that also occurred, but were not targeted. Table 6.1 is a subset of the full data compilation, presented in Appendix 6. The information considered most pertinent to this discussion has been condensed below and focusses on the two main species *C. serrulata* and *H. uninervis*.

Table 6.1: Summary of studies on CO_2 enrichment on tropical seagrasses, listing down responses and corresponding experimental treatments. Growth strategies of species are listed as "C" for colonizing, "O" for opportunistic and "P" for persistent (Kilminster et al. 2015). Tissue nutrients (% nitrogen, and carbon-to-nitrogen ratios) on control plants are given. Net productivity (NP) and growth responses are grouped as "+" for significant increase, "=" for no significant change, and "na" for not measured in that particular study.

Species	Growth strategy	Duration (days)	Experiment pCO_2 (µatm)	Experiment light (mol m^{-2} d^{-1})	Experiment temperature (ºC)	Control plant % N	Control plant C/N	NP	Growth	Reference
C. serrulata	O	14	694	17.28	25.0	2.32	18.02	+	=	Chapter 2
C. serrulata	O	14	884	17.28	25.0	2.32	18.02	+	=	Chapter 2
C. serrulata	O	14	1204	17.28	25.0	2.32	18.02	+	=	Chapter 2
C. serrulata	O	14	1077	1.51	24.0	1.93	19.01	+	+	Chapter 3
C. serrulata	O	14	1077	4.32	24.0	2.06	17.58	=	+	Chapter 3
C. serrulata	O	14	1077	16.42	24.0	1.76	20.81	=	+	Chapter 3
C. serrulata	O		1200	25.3	30.0			+	+	Russell et al 2013
C. serrulata	O	49	925	17.28	20.0	1.35	26.89	=	=	Chapter 5
C. serrulata	O	49	2155	17.28	20.0	1.35	26.89	=	=	Chapter 5
C. serrulata	O	49	1006	17.28	25.0	1.34	26.69	=	=	Chapter 5
C. serrulata	O	49	1772	17.28	25.0	1.34	26.69	=	=	Chapter 5
C. serrulata	O	49	1066	17.28	30.0	1.33	25.34	=	=	Chapter 5
C. serrulata	O	49	2157	17.28	30.0	1.33	25.34	=	=	Chapter 5
H. ovalis	C/O		1200	25.3	30.0			+	na	Russell et al 2013

(Table 6.1 continued)

Species	Growth strategy	Duration (days)	Experiment pCO_2 (µatm)	Experiment light (mol m^{-2} d^{-1})	Experiment temperature (ºC)	Control plant % N	Control plant C/N	NP	Growth	Reference
H. uninervis	C/O	14	694	17.28	25.0	2.56	16.15	+	+	Chapter 2
H. uninervis	C/O	14	884	17.28	25.0	2.56	16.15	+	+	Chapter 2
H. uninervis	C/O	14	1204	17.28	25.0	2.56	16.15	+	+	Chapter 2
H. uninervis	C/O	14	1077	1.51	24.0	2.05	19.19	=	=	Chapter 3
H. uninervis	C/O	14	1077	4.32	24.0	2.26	16.13	=	+	Chapter 3
H. uninervis	C/O	14	1077	16.42	24.0	2.09	17.08	=	=	Chapter 3
H. uninervis	C/O	24	731	3.8	28.5	2.47	16.58	+	+	Chapter 4
H. uninervis	C/O	24	738	3.8	28.5	2.57	15.74	+	+	Chapter 4
H. uninervis	C/O	24	1235	3.8	28.5	2.47	16.58	+	+	Chapter 4
H. uninervis	C/O	24	1190	3.8	28.5	2.57	15.74	+	+	Chapter 4
H. uninervis	C/O	49	925	17.28	20.0	1.78	18.56	=	=	Chapter 5
H. uninervis	C/O	49	2155	17.28	20.0	1.78	18.56	=	=	Chapter 5
H. uninervis	C/O	49	1006	17.28	25.0	1.50	20.37	=	=	Chapter 5
H. uninervis	C/O	49	1772	17.28	25.0	1.50	20.37	=	=	Chapter 5
H. uninervis	C/O	49	1066	17.28	30.0	1.65	19.65	=	=	Chapter 5
H. uninervis	C/O	49	2157	17.28	30.0	1.65	19.65	=	=	Chapter 5

(Table 6.1 continued)

Species	Growth strategy	Duration (days)	Experiment pCO_2 (μatm)	Experiment light (mol m^{-2} d^{-1})	Experiment temperature (ºC)	Control plant % N	Control plant C/N	NP	Growth	Reference
T. hemprichii	P	21	903	10.8	25.0	2.43	14.17	+	+	Jiang et al 2010
T. hemprichii	P	21	1688	10.8	25.0	2.43	14.17	+	+	Jiang et al 2010
T. hemprichii	P	21	35350	10.8	25.0	2.43	14.17	+	+	Jiang et al 2010
T. hemprichii	P	14	694	17.28	25.0	2.56	14.82	+	+	Chapter 2
T. hemprichii	P	14	884	17.28	25.0	2.56	14.82	+	+	Chapter 2
T. hemprichii	P	14	1204	17.28	25.0	2.56	14.82	+	+	Chapter 2
T. hemprichii	P	24	731	3.8	28.5	2.60	15.07	=	=	Chapter 4
T. hemprichii	P	24	738	3.8	28.5	2.90	13.60	=	=	Chapter 4
T. hemprichii	P	24	1235	3.8	28.5	2.60	15.07	=	=	Chapter 4
T. hemprichii	P	24	1190	3.8	28.5	2.90	13.60	=	=	Chapter 4
T. testudinum	P	180	1982	38.28	18.1	2.35	18.27	na	=	Campbell & Fourqurean 2013
T. testudinum	P	330	1982	38.28	34.5	2.35	18.27	na	=	Campbell & Fourqurean 2013
T. testudinum	P	57	700	6.3	27.0			na	=	Durako & Sackett 1993
T. testudinum	P	57	1400	6.3	27.0			na	=	Durako & Sackett 1993
T. testudinum	P	57	3500	6.3	27.0			na	=	Durako & Sackett 1993

Table 6.1 suggests that nutrient limitation may modulate seagrass response to CO_2 enrichment. From the analysis of the compiled studies, in *C. serrulata* and *H. uninervis*, average % N in control leaves differed between plants that increased productivity to CO_2, and those that did not (t-test: *C. serrulata* t = -5.47, df = 8.40, $P < 0.001$; *H. uninervis* t = -7.97, df = 8.59, $P < 0.001$; details in Fig. 6.1). As a rough estimate, *C. serrulata* with control % N < 2.0 % and *H. uninervis* with control % N < 2.4 % seemed to correspond with having limited or no response to CO_2 enrichment (Fig. 6.1). The % N value that was indicative of nutrient limitation (1.8) in Duarte (1990)'s assessment was lower than the values found here that limited *C. serrulata* and *H. uninervis* productivity, and this might reflect lower investment in structural material in these species. *H. uninervis* used in Chapter 4 was nutrient-sufficient (% N = 2.47), and hence did not require nitrate enrichment to respond to CO_2. Tissue % N content in *T. hemprichii* was high across experiments (Table 6.1), but its productivity responses to CO_2 enrichment varied. In fact, in the experiment where control % N was the highest (2.6 – 2.9; Chapter 4), *T. hemprichii* showed no response to CO_2 enrichment. In such a case, other factors such as light could have affected the results. Light availability also affects CO_2 responses in seagrass (Chapter 3 and 4), given its role in controlling carbon assimilation (Chapter 3; Mercado et al. 2003) and its influence on tissue nutrients (Collier et al. 2009).

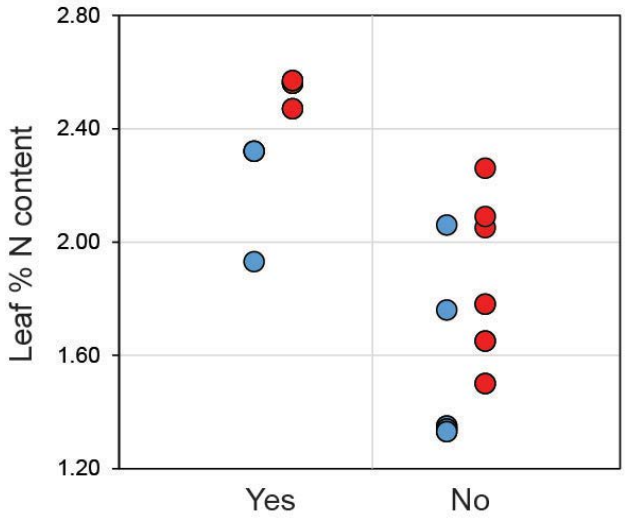

Fig. 6.1: Experiments in this thesis were compiled to compare productivity response to CO2 enrichment with % N content in control leaves. Graph shows results for *C. serrulata* (blue) and *H. uninervis* (red). In order to visualise and statistically test the apparent trend of % N limiting productivity responses, I broadly classified productivity response "Yes" for increased productivity (n = 12) , and "No" for no significant change (n = 17) for each species, and tested whether % N content in control leaves differed between the two groups using Welch's t-test.

Apart from % N, the summary and analysis in Table 6.1 did not reveal any further factors influencing the results (see Appendix 6). The influence of experiment duration on CO_2 responses could not be inferred with confidence due to the limited number of studies conducted so far (Table 6.1; Supplementary Fig. 6.1). Studies on terrestrial plants have shown that the longer the plants were exposed to enriched CO_2, the more likely they are to develop nutrient limitation and eventually minimise or stop responding to enriched CO_2 (Luo et al. 2004). However around CO_2 seep sites, seagrasses are still showing enhanced biomass with CO_2 enrichment (Takahashi et al. 2015). The influence of exposure time on acclimation response would be an interesting premise to explore as more studies emerge.

To effectively elucidate responses to CO_2 enrichment, other experimental parameters (that are not tested) need to be controlled and optimised. References for these optimal, i.e. non-limiting, conditions can be derived from monitoring data, if available,

or by measuring environmental conditions and taking note of nutrient and growth status of plants *in-situ*. One can go a step further by establishing tissue nutrient contents over a range of nutrient availability, with finer resolution in nutrient concentrations. Given that seagrasses can access nutrients from both water column and sediments, nutrient availability can be a complex issue and requires careful consideration when planning future experiments.

6.3 Relevance and implications

Findings in this thesis research indicate that seagrasses are likely to succeed (maintain productivity and growth) in a future with OA, hence they may be considered "winners" (sensu. Fabricius et al. 2011) compared to other tropical marine organisms. However, productivity responses differ among species and across the tropical distribution range. Since their responses to OA are so dependent on other environmental factors, how much seagrass ecological functions (e.g. carbon sequestration, food and habitat for dependant species) can be realised depends on whether other environmental conditions that maintain optimum productivity occur.

6.3.1 Seagrass productivity changes

Productivity increase with CO_2 enrichment differs among seagrass species (Chapters 2-5; Borum et al. 2015). Subsequent differences in carbon surplus could indicate flow-on effects to plant scale responses, such as growth and shoot production (Invers et al. 2002; Palacios and Zimmerman 2007). Plant-scale responses would be subjected to light and nutrient use efficiency in each species (Romero et al. 2006; Silva et al. 2013). Over time, differences could manifest on a community scale, such as response to physical disturbances including grazing by mega-herbivores (Eklöf et al. 2009), abundance and spatial distribution (Dennison et al. 1993; Zimmerman et al. 1997), and even reproductive output (Palacios and Zimmerman 2007). A change in seagrass species composition across

a gradient of pCO_2 levels (475 – 2754 μatm) around CO_2 seep sites has been shown by Takahashi et al. (2015).

Productivity responses to OA might differ across tropical seagrass distribution range. For example in the GBR system, the rate of pCO_2 rise in seawater varied from inshore to the mid-shelf reefs (Uthicke et al. 2014). Hence, expected productivity increase could differ between inshore and mid-shelf seagrasses in the future. Furthermore, productivity responses will be modulated by environmental conditions that vary across the tropical to sub-tropical range. CO_2 enrichment increased photosynthetic capacity for plants growing in low light more than those growing in high light (Chaper 2; Schwarz et al. 2000). Such findings posit that deep-water seagrasses, or those growing in turbid environments, might benefit more under OA. For seagrasses that are growing close to their thermal limit (Collier et al. 2011), CO_2 enrichment might increase their thermal optima (Sage and Kubien 2007) and enhance their tolerance to ocean warming, given other environmental factors are not limiting.

6.3.2 Management of local environmental conditions

Seagrasses can thrive and continue to provide vital ecosystem services in more acidic seas (Chapter 2). However, reduced light availability would still negatively impact tropical seagrass even with OA, although higher CO_2 did result in increases in efficiency and lower compensation irradiances. My findings showed that the rise in seagrass productivity with CO_2 enrichment could not compensate for the reduction in light availability (Chapter 3). Reduced light availability (from epiphytic cover on leaves) can negate positive OA effects on seagrass growth (Burnell et al. 2014b). Compared to OA, variation in water clarity occurs over shorter frequencies and with greater intensities, thus playing a more imminent role in controlling seagrass productivity. In the GBR, light fields have already been reduced from coastal run-off and dredging activities since pre-European settlement (Kroon et al. 2012), and acute turbidity/low light events have been linked to seagrass loss (Collier et al. 2012b; Petus et al. 2014).

While moderate nutrient enrichment boosts seagrass productivity (Udy et al. 1999), seagrass epiphytes and macroalgae also benefit from nutrient enrichment.

Findings demonstrated that nutrient enrichment did not enhance productivity in CO_2-enriched seagrasses (Chapter 4); however, addition of nutrients boosted growth of CO_2-enriched algae (Russell et al. 2009; Hofmann et al. 2014). Furthermore, temperature rise increased the biomass of CO_2-enriched non-calcareous algae (Connell and Russell 2010) but did not enhance responses for CO_2-enriched seagrasses (Chapter 5). Taken together, seagrass epiphytes and macroalgae might benefit more from OA than seagrasses. To prevent algae from out-competing seagrass, it is necessary to study and manage the effects of water quality and OA changes on marine macrophytes and their interactions (Burnell et al. 2014b).

6.4 Future research directions

This study was one of the first to examine the physiological responses of seagrasses to interactive effects of CO_2 enrichment and key water quality parameters. To this end, the scope was restricted to examining short- to mid-term responses in tropical seagrasses of the Great Barrier Reef. To further develop the conceptual understanding of OA responses in tropical seagrasses, several directions for future research have been identified.

Experiments in this thesis were mostly short- to mid-term (two weeks to two months). In a number of terrestrial plants, long term exposure (> 1 year) to enhanced CO_2 led to acclimation responses, due to other resources becoming limiting (Stitt and Krapp 1999; Ainsworth and Long 2005). Seagrasses can acclimate to their environment, such as to changes in light and temperature (Olesen et al. 2002; Peralta et al. 2005). In the few "long-term" experiments, growth responses varied from enhanced shoot production (1 year, Palacios and Zimmerman 2007) to no increase in growth rates (5 months, Alexandre et al. 2012; 1 year, Campbell and Fourqurean 2013a). Hence, more long term experiments are need to help elucidate acclimation responses to ocean acidification and identify possible limiting resources. Also, long-term experiments allow for the examination of how physiological responses scale up into morphological or even community responses. While surveys at underwater CO_2 seeps sites provide insights into long-term responses

in seagrasses (Hall-Spencer et al. 2008; Fabricius et al. 2011; Russell et al. 2013; Apostolaki et al. 2014), results are sometimes contradictory (Apostolaki et al. 2014). This points out that responses to acidification in seagrasses depend on species and geochemical characteristics of the site. Therefore, longer term experiments on interactive effects of CO_2 enrichment and environmental parameters are still necessary to predict long-term CO_2 effects.

The restricted spatial scale over which the studies took place meant that population differences are not accounted for. Genotypic differences among populations (Reusch et al. 2008) could significantly influence experimental outcomes. The experiments could be repeated for different seagrass populations across their distributional range. This is especially pertinent for populations that are already near their thermal or light limits, since they might benefit most from OA. Replicating studies across a wide spatial scale could also help establish if findings in the GBR are applicable to other regions in the Indo-Pacific.

Co-occurrence of epiphytes and macroalgae in seagrass meadows means that they would be subjected to the same environmental changes. These marine macrophytes compete for the same resources as seagrasses, and also benefit from CO_2 enrichment (Russell et al. 2009; Hofmann et al. 2014). Documenting how OA and declining water quality affect the interaction between seagrass and algae is an important step to determining factors that trigger phase-shifts in future seagrass meadows.

Top–down control is important to global change stressors in affecting seagrass ecosystems (Duffy et al. 2015). Seagrass grazers (e.g. dugongs and turtles) and mesograzers help increase the turnover of leaves (Valentine and Duffy 2006; Christianen et al. 2012) and control epiphytic algae proliferation (Duffy 2002). The intensity of grazing might influence OA effects on seagrass productivity. However, the operating processes in top-down control of seagrass populations remain poorly understood. Investigating changes in top-down control, such as changes to mesograzer communities under OA, is essential to develop a holistic understanding of OA effects on seagrass meadows.

6.5 Conclusion

The systematic series of experimentation developed in this thesis was critical in providing a comprehensive overview of the physiological responses of tropical seagrasses to OA and key water quality parameters. In summary, I found that OA can increase productivity of tropical seagrasses in the short term, and this was more beneficial for seagrasses growing under low light, such as deep-water species. Increased seawater pCO_2 reduced the energetic effort of using the alternative substrate bicarbonate (HCO_3^-) by providing more CO_2 substrate to light-limited plants. However, plants in high light without CO_2 enrichment still grew more, when compared to plants growing in low light with enriched CO_2. Ocean acidification did not increase demand for nutrients or induce nutrient limitation, leading to the surprising find that nitrate fertilization did not enhance productivity response for CO_2-enriched seagrasses. This was especially relevant when they are not nutrient-limited. Carbon metabolism is regulated by temperature and, to a much lesser extent, by CO_2 availability. Tropical seagrass responses under OA are expected to vary with seasonal temperature variation and across their distribution range. Collectively, the information and technical know-how generated in this thesis serve as a starting platform for future efforts into the research and conservation of tropical seagrass meadows under a changing climate.

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APPENDIX 1

Supplementary information to Chapter 1

Supplementary Table 1.1: Studies that examined photosynthetic responses to temperature compiled from ISI database prior to 2014.

Region	Species	Location	Type of study	Reference
Temperate	*Amphibolis antarctica*	Spencer Gulf, S Australia	Mesocosm	Seddon & Cheshire 2001
Temperate	*A. antarctica*	Perth, W Australia	Lab incubation	Masini & Manning 1997
Temperate	*A. griffithii*	Perth, W Australia	Lab incubation	Masini & Manning 1997
Temperate	*Cymodocea nodosa*	Mar Menor, Spain	Lab incubation	Terrados & Ros 1995
Temperate	*C. nodosa*	Alfaques Bay, Spain	Lab incubation	Perez & Romero 1992
Temperate	*Halophila ovalis*	Swan Canning Estuary, W Australia	Lab incubation	Hillman et al 1995
Temperate	*H. ovalis*	Taylor's Bay, Australia	Lab incubation	Ralph 1998
Temperate	*Posidonia australis*	Spencer Gulf, S Australia	Mesocosm	Seddon & Cheshire 2001
Temperate	*P. australis*	Perth, W Australia	Lab incubation	Masini & Manning 1997
Temperate	*P. sinuosa*	Perth, W Australia	Lab incubation	Masini & Manning 1997
Temperate	*P. sinuosa*	Princess Royal Harbour, W Australia	Lab incubation	Masini et al 1995
Temperate	*Ruppia maritima*	Chesapeake Bay, USA	Lab incubation	Evans et al 1986
Temperate	*Zostera marina*	Del Monte Beach, California	Mesocosm	Zimmerman et al, 1989
Temperate	*Z. marina*	Aarhus Bight, Denmark	Lab incubation	Olesen & Sand-Jensen 1993
Temperate	*Z. marina*	Woods Hole, USA	Lab incubation	Marsh et al 1986
Temperate	*Z. marina*	Chesapeake Bay, USA	Lab incubation	Evans et al 1986
Temperate	*Z. noltii*	Palmones Estuary, Spain	Lab incubation	Perez-Llorens & Niells 1993

(Supplementary Table 1.1 continued)

Region	Species	Location	Type of study	Reference
Tropical	*Cymodocea rotundata*	Green island, Australia	Lab incubation	Campbell et al 2006
Tropical	*C. serrulata*	Green island, Australia	Lab incubation	Campbell et al 2006
Tropical	*C. serrulata*	Juno Bay, Fantome Island	Lab incubation	Collier & Waycott 2014
Tropical	*Halophila ovalis*	Green island, Australia	Lab incubation	Campbell et al 2006
Tropical	*H. ovalis*	Juno Bay, Fantome Island	Lab incubation	Collier & Waycott 2014
Tropical	*Halodule uninervis*	Green island, Australia	Lab incubation	Campbell et al 2006
Tropical	*H. uninervis*	Juno Bay, Fantome Island	Lab incubation	Collier & Waycott 2014
Tropical	*H. wrightii*	Laguna Madre, Texas USA	Field	Dunton & Tomasko 1994
Tropical	*Syringodium isoetiofolium*	Green island, Australia	Lab incubation	Campbell et al 2006
Tropical	*Thalassia hemprichii*	Green island, Australia	Lab incubation	Campbell et al 2006
Tropical	*T. hemprichii*	Juno Bay, Fantome Island	Lab incubation	Collier & Waycott 2014
Tropical	*Zostera capricorni*	Cairns Harbour, Australia	Lab incubation	Campbell et al 2006

APPENDIX 2

Copyright release for Chapter 2

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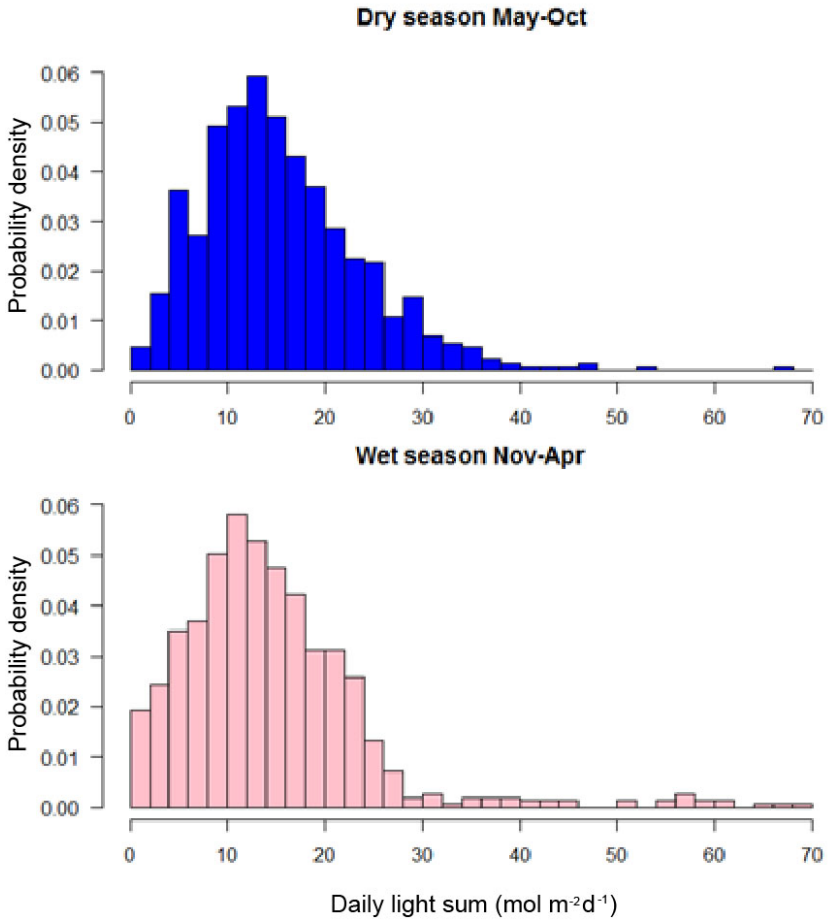
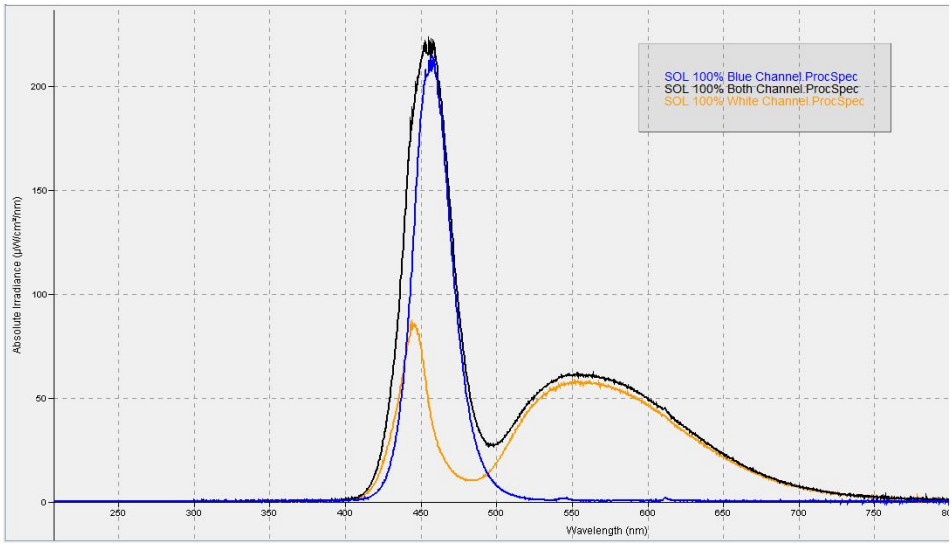
APPENDIX 3

Supplementary information to Chapter 3



Supplementary Fig. 3.1: Distribution of daily light sums (photosynthetically active radiation levels) at Cockle Bay, Magnetic Island, Great Barrier Reef during the dry and wet seasons. Distribution of PAR was measured at seagrass canopy height using planar 2π PAR loggers from September 2009 to July 2014.



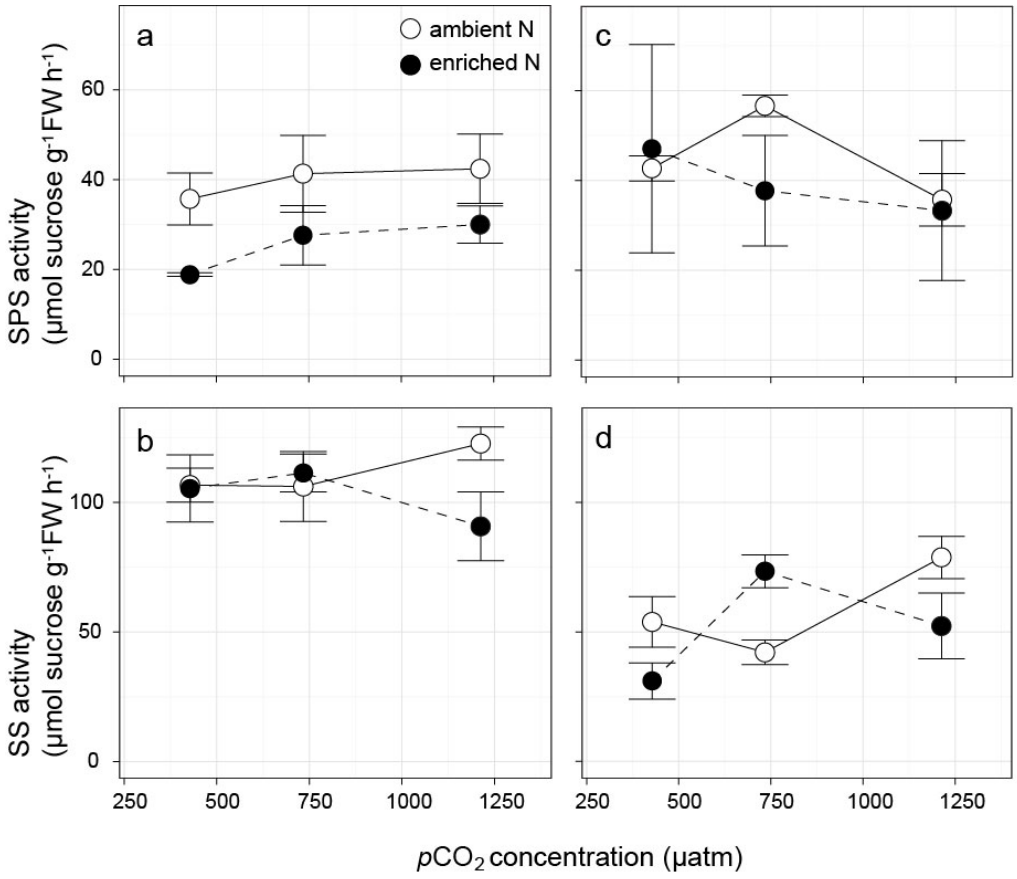
Supplementary Fig. 3.2: Light spectra of Aqua Illumination LED Sol at different settings. At 100% blue channel only (blue), 100% white channel only (orange) and 100% both blue and white channels (black). Light spectra were measured using Jazz spectrometer on SpectraSuite software (OceanOptics, USA).

Supplementary Table 3.1: Average incubation time, and approximate time range for DIC limitation to occur in 200 mL of ambient seawater during trials, for each light step of P-E curve. Leaf material used during trials was 5 – 12 mg in dry weight. Light steps in bold indicate a replacement of fresh filtered seawater prior to incubation during actual measurements.

Light step	Average incubation time (min)	Time for DIC limitation (min)
10	25	Not measured
20	25	400 – 480
35	25	160 – 170
70	25	70 – 80
100	25	50 – 55
200	25	40 – 50
380	25	40 – 50
520	25	35 – 40
600	25	30 – 50

APPENDIX 4

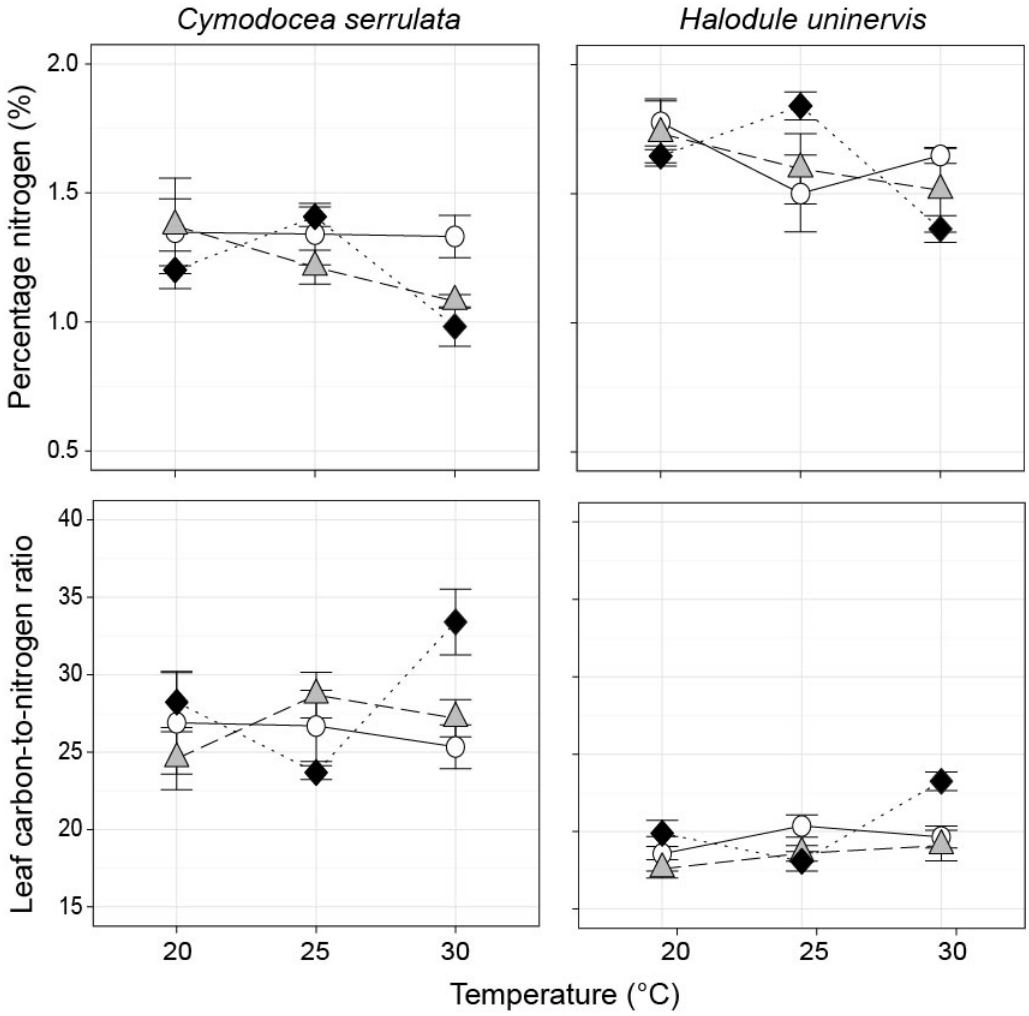
Supplementary information to Chapter 4



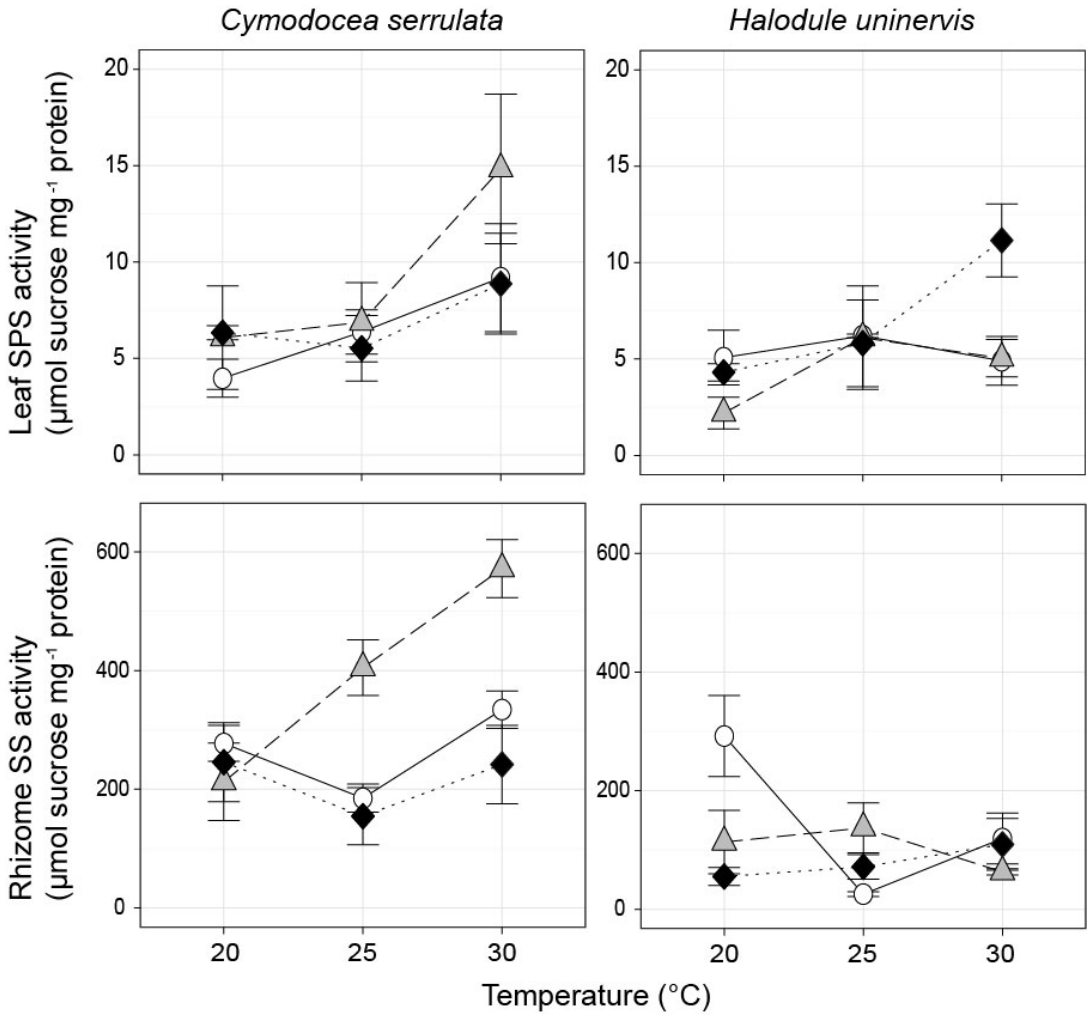
Supplementary Fig. 4.1: Leaf sucrose-phosphate synthase (SPS) and rhizome sucrose synthase (SS) assayed from (a – b) *Halodule uninervis* and (c – d) *Thalassia hemprichii* across a range of pCO_2 concentrations. Values are average ± S.E. n = 3.

APPENDIX 5

Supplementary information to Chapter 5



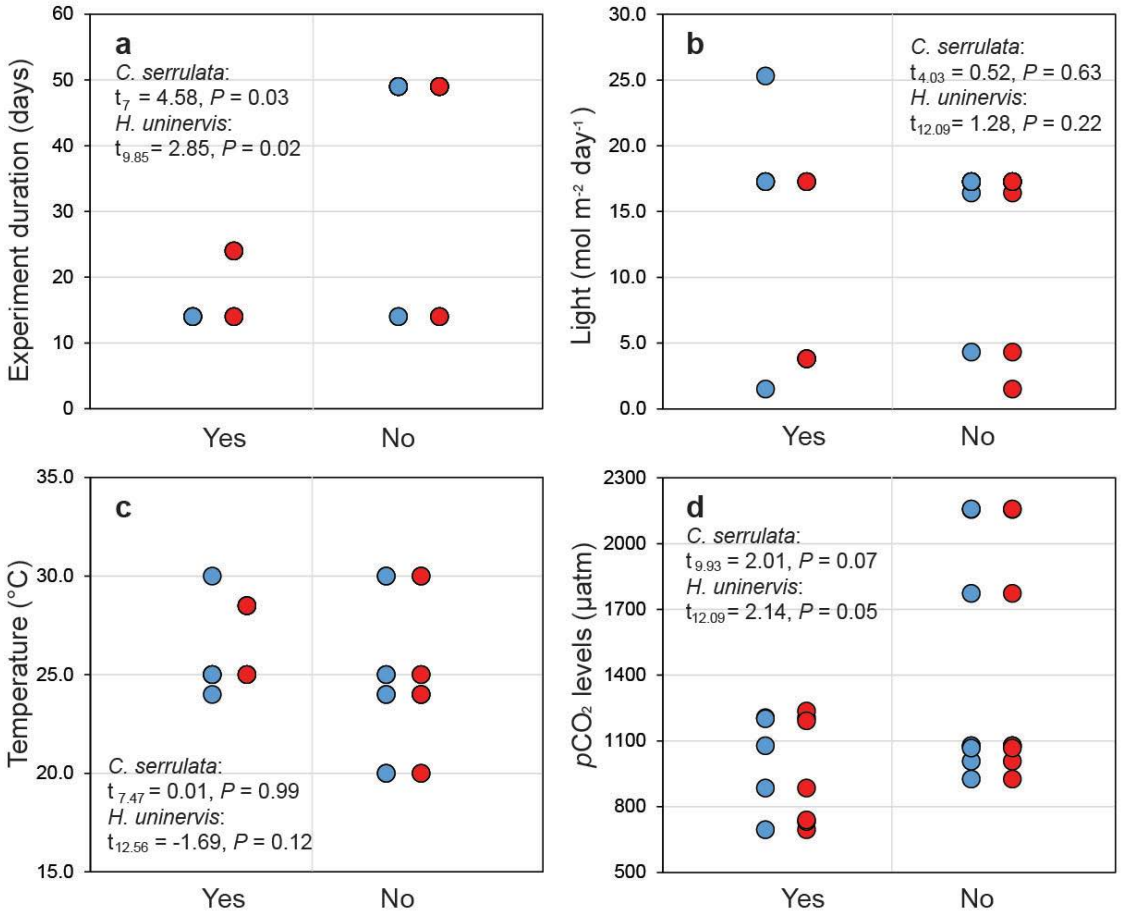
Supplementary Fig. 5.1: Leaf tissue percentage nitrogen content (%N) and carbon-to-nitrogen (C:N) ratio after seven-week exposure to temperature and CO_2 enrichment treatments. pCO_2 levels are represented by different symbols (400 μatm - white circles; 1000 μatm - grey triangles and 2000 μatm - black diamonds). Means ± S.E. n = 3.



Supplementary Fig. 5.2: Activities of carbon translocation enzymes in the leaves (sucrose-phosphate synthase, SPS) and rhizomes (sucrose synthase, SS) after seven-week exposure to temperature and CO_2 enrichment treatments. pCO_2 levels are represented by different symbols (400 µatm - white circles; 1000 µatm - grey triangles and 2000 µatm - black diamonds). Means ± S.E. n = 3.

APPENDIX 6

Supplementary information to Chapter 6



Supplementary Fig. 6.1: Experiments in this thesis were compiled to compare productivity response to CO_2 enrichment with (a) experimental duration, (b) light, (c) temperature and (d) pCO_2 levels. Graphs show results for *C. serrulata* (blue) and *H. uninervis* (red). Productivity response was broadly classified into "Yes" for increased productivity, and "No" for no significant changes and average values for experimental treatments were compared between the two groups using Welch's t-test. α-value for experimental duration (a) has been lowered to 0.01, due to non-normality of data even after transformation.

Supplementary Table 6.1: Summary of studies on CO_2 enrichment on tropical and temperate seagrasses, listing down responses and corresponding experimental treatments. Growth strategies of species are listed as "C" for colonizing, "O" for opportunistic and "P" for persistent (Kilminster et al. 2015). Tissue nutrients (% nitrogen, and carbon-to-nitrogen ratios) on control plants are given. Net productivity (NP) and growth responses are grouped as "+" for significant increase, "=" for no significant change, and "na" for not measured in that particular study.

Distribution range	Species	Growth strategy	Duration (days)	Experiment pCO_2	Experiment light (mol m^{-2} d^{-1})	Experiment temperature (ºC)	Control plant % N	Control plant C/N	NP	Change in NP (%)	Growth	Growth change (%)	Reference
Tropical	*C. serrulata*	O	14	694	17.28	25.0	2.32	18.02	+	24	=	-16	Chapter 2
Tropical	*C. serrulata*	O	14	884	17.28	25.0	2.32	18.02	+	28	=	-16	Chapter 2
Tropical	*C. serrulata*	O	14	1204	17.28	25.0	2.32	18.02	+	50	=	-12	Chapter 2
Tropical	*C. serrulata*	O	14	1077	1.51	24.0	1.93	19.01	+	76	+	41	Chapter 3
Tropical	*C. serrulata*	O	14	1077	4.32	24.0	2.06	17.58	=	-4	+	9	Chapter 3
Tropical	*C. serrulata*	O	14	1077	16.42	24.0	1.76	20.81	=	0	+	62	Chapter 3
Tropical	*C. serrulata*	O		1200	25.3	30.0			+	31	+	1023	Russell et al 2013
Tropical	*C. serrulata*	O	49	925	17.28	20.0	1.35	26.89	=	-12	=	-1	Chapter 5
Tropical	*C. serrulata*	O	49	2155	17.28	20.0	1.35	26.89	=	-25	=	-51	Chapter 5
Tropical	*C. serrulata*	O	49	1006	17.28	25.0	1.34	26.69	=	6	=	-29	Chapter 5
Tropical	*C. serrulata*	O	49	1772	17.28	25.0	1.34	26.69	=	5	=	43	Chapter 5
Tropical	*C. serrulata*	O	49	1066	17.28	30.0	1.33	25.34	=	11	=	-17	Chapter 5
Tropical	*C. serrulata*	O	49	2157	17.28	30.0	1.33	25.34	=	-3	=	7	Chapter 5
Tropical	*H. ovalis*	C/O		1200	25.3	30.0			+	182	na		Russell et al 2013

(Supplementary Table 6.1 continued)

Distribution range	Species	Growth strategy	Duration (days)	Experiment pCO_2	Experiment light (mol m^{-2} d^{-1})	Experiment temperature (ºC)	Control plant % N	Control plant C/N	NP	Change in NP (%)	Growth	Growth change (%)	Reference
Tropical	*H. uninervis*	C/O	14	694	17.28	25.0	2.56	16.15	+	10	+	28	Chapter 2
Tropical	*H. uninervis*	C/O	14	884	17.28	25.0	2.56	16.15	+	18	+	17	Chapter 2
Tropical	*H. uninervis*	C/O	14	1204	17.28	25.0	2.56	16.15	+	46	+	38	Chapter 2
Tropical	*H. uninervis*	C/O	14	1077	1.51	24.0	2.05	19.19	=	13	=	21	Chapter 3
Tropical	*H. uninervis*	C/O	14	1077	4.32	24.0	2.26	16.13	=	4	+	25	Chapter 3
Tropical	*H. uninervis*	C/O	14	1077	16.42	24.0	2.09	17.08	=	2	=	-22	Chapter 3
Tropical	*H. uninervis*	C/O	24	731	3.8	28.5	2.47	16.58	+	130	+	20	Chapter 4
Tropical	*H. uninervis*	C/O	24	738	3.8	28.5	2.57	15.74	+	39	+	8	Chapter 4
Tropical	*H. uninervis*	C/O	24	1235	3.8	28.5	2.47	16.58	+	91	+	3	Chapter 4
Tropical	*H. uninervis*	C/O	24	1190	3.8	28.5	2.57	15.74	+	23	+	1	Chapter 4
Tropical	*H. uninervis*	C/O	49	925	17.28	20.0	1.78	18.56	=	18	=	-14	Chapter 5
Tropical	*H. uninervis*	C/O	49	2155	17.28	20.0	1.78	18.56	=	-8	=	-14	Chapter 5
Tropical	*H. uninervis*	C/O	49	1006	17.28	25.0	1.50	20.37	=	-3	=	-14	Chapter 5
Tropical	*H. uninervis*	C/O	49	1772	17.28	25.0	1.50	20.37	=	16	=	20	Chapter 5
Tropical	*H. uninervis*	C/O	49	1066	17.28	30.0	1.65	19.65	=	3	=	-15	Chapter 5
Tropical	*H. uninervis*	C/O	49	2157	17.28	30.0	1.65	19.65	=	-43	=	-9	Chapter 5
Tropical	*T. hemprichii*	P	21	903	10.8	25.0	2.43	14.17	+	17	+	16	Jiang et al 2010
Tropical	*T. hemprichii*	P	21	1688	10.8	25.0	2.43	14.17	+	29	+	23	Jiang et al 2010
Tropical	*T. hemprichii*	P	21	35350	10.8	25.0	2.43	14.17	+	54	+	40	Jiang et al 2010

(Supplementary Table 6.1 continued)

Distribution range	Species	Growth strategy	Duration (days)	Experiment pCO_2	Experiment light (mol m^{-2} d^{-1})	Experiment temperature (ºC)	Control plant % N	Control plant C/N	NP	Change in NP (%)	Growth	Growth change (%)	Reference
Tropical	*T. hemprichii*	P	14	694	17.28	25.0	2.56	14.82	+	29	+	6	Chapter 2
Tropical	*T. hemprichii*	P	14	884	17.28	25.0	2.56	14.82	+	46	+	24	Chapter 2
Tropical	*T. hemprichii*	P	14	1204	17.28	25.0	2.56	14.82	+	64	+	15	Chapter 2
Tropical	*T. hemprichii*	P	24	731	3.8	28.5	2.60	15.07	=	22	=	-3	Chapter 4
Tropical	*T. hemprichii*	P	24	738	3.8	28.5	2.90	13.60	=	-23	=	32	Chapter 4
Tropical	*T. hemprichii*	P	24	1235	3.8	28.5	2.60	15.07	=	27	=	-3	Chapter 4
Tropical	*T. hemprichii*	P	24	1190	3.8	28.5	2.90	13.60	=	-36	=	30	Chapter 4
Tropical	*T. testudinum*	P	180	1982	38.28	18.1	2.35	18.27	na		=	33	Campbell & Fourqurean 2013
Tropical	*T. testudinum*	P	330	1982	38.28	34.5	2.35	18.27	na		=	87	Campbell & Fourqurean 2013
Tropical	*T. testudinum*	P	57	700	6.3	27.0			na		=	-11	Durako & Sackett 1993
Tropical	*T. testudinum*	P	57	1400	6.3	27.0			na		=	-34	Durako & Sackett 1993
Tropical	*T. testudinum*	P	57	3500	6.3	27.0			na		=	22	Durako & Sackett 1993
Temperate	*A. antarctica*	O/P	84	650	7.21	20.0			=	-24	=	10	Burnell et al 2014
Temperate	*A. antarctica*	O/P	84	900	7.21	20.0			=	9	=	-14	Burnell et al 2014

(Supplementary Table 6.1 continued)

Distribution range	Species	Growth strategy	Duration (days)	Experiment pCO_2	Experiment light (mol m^{-2} d^{-1})	Experiment temperature (ºC)	Control plant % N	Control plant C/N	NP	Change in NP (%)	Growth	Growth change (%)	Reference
Temperate	*A. antarctica*	O/P	84	650	1.84	20.0			+	28	+	5	Burnell et al 2014
Temperate	*A. antarctica*	O/P	84	900	1.84	20.0			+	33	+	14	Burnell et al 2014
Temperate	*P. oceanica*	P	14	1026	2.91	14.5			=	-3	na		Cox et al 2015
Temperate	*P. oceanica*	P	14	2610	2.91	14.5			=	18	na		Cox et al 2015
Temperate	*P. oceanica*	P	28	1026	2.91	14.5			=	19	na		Cox et al 2015
Temperate	*P. oceanica*	P	28	2610	2.91	14.5			+	35	na		Cox et al 2015
Temperate	*Z. marina*	C/O	45	35806	8.64	14.0			+	290	+	900	Zimmerman et al 1997
Temperate	*Z. marina*	C/O	10	475	15.12	14.5			+	14	+	7	Thom 1996
Temperate	*Z. marina*	C/O	10	570	15.12	14.5			+	20	+	13	Thom 1996
Temperate	*Z. marina*	C/O	10	760	15.12	14.5			+	37	+	36	Thom 1996
Temperate	*Z. marina*	C/O	10	950	15.12	14.5			=	53	na		Thom 1996
Temperate	*Z. marina*	C/O	10	1520	15.12	14.5			=	41	na		Thom 1996
Temperate	*Z. marina*	C/O	10	1900	15.12	14.5			=	18	na		Thom 1996
Temperate	*Z. marina*	C/O	365	958	9.90	15.0			na		+	33	Palacios & Zimmerman 2007
Temperate	*Z. marina*	C/O	365	1778	9.90	15.0			na		+	24	Palacios & Zimmerman 2007

(Supplementary Table 6.1 continued)

Distribution range	Species	Growth strategy	Duration (days)	Experiment pCO_2	Experiment light (mol m^{-2} d^{-1})	Experiment temperature (ºC)	Control plant % N	Control plant C/N	NP	Change in NP (%)	Growth	Growth change (%)	Reference
Temperate	*Z. marina*	C/O	365	23621	9.90	15.0			na		+	113	Palacios & Zimmerman 2007
Temperate	*Z. marina*	C/O	365	958	1.50	15.0			na		=	-38	Palacios & Zimmerman 2007
Temperate	*Z. marina*	C/O	365	1778	1.50	15.0			na		=	-47	Palacios & Zimmerman 2007
Temperate	*Z. marina*	C/O	365	23621	1.50	15.0			na		=	25	Palacios & Zimmerman 2007
Temperate	*Z. noltii*	C/O	300	700			1.40		+	33	=	-5	Alexandre et al 2012

APPENDIX 7

Papers published and seminars presented from this thesis

Publications

1. Ow Y.X., Collier C.J. and Uthicke S. (2015) Responses of three tropical seagrass species to CO_2 enrichment. Marine Biology. 162: 1005-1017
2. Ow Y.X., Uthicke S. and Collier C.J. (2016) Light Levels Affect Carbon Utilisation in Tropical Seagrass under Ocean Acidification. PLoS ONE 11(3): e0150352
3. Ow Y.X., Vogel N., Collier C.J., Holtum J.A.M., Flores F. and Uthicke S. (2016) Nitrate fertilisation does not enhance CO2 responses in two tropical seagrass species. Scientific Reports 6: 23093.

Conferences

1. Ow, Y. X., Collier C. and Uthicke S. Effects of light and carbon dioxide on seagrass physiology and growth. *Australian Marine Science Association 2013* Oral presentation.
2. Ow, Y. X., Uthicke S. and Collier C. Productivity Changes of Three Tropical Seagrasses to Carbon Dioxide Enrichment. *Asia-Pacific Coral Reef Symposium 2014* Oral presentation.
3. Ow, Y. X., Vogel N., Collier C. and Uthicke S. Nutrient enrichment does not enhance CO_2 response in tropical seagrasses. *International Seagrass Biology Workshop 2014* Oral presentation.