Assessing the risk of ocean acidification for scleractinian corals on the Great Barrier Reef

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School of the Environment

Certificate of original authorship

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature of Student:

Date:

This thesis is dedicated to my son, Javier Patrick Lloyd. His arrival into this world during my PhD was a timely reminder that we do not inherit this earth from our ancestors; we borrow it from our children. Javier, I wrote this thesis in the hope that it will serve to maintain the beauty of the Great Barrier Reef for your generation and many more to come.

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Chapter 4:

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Abbreviations

α: photosynthetic efficiency at sub-saturating irradiance

ATP: adenosine triphosphate

ADP: adenosine diphosphate

A_T: total alkalinity

CA: carbonic anhydrase

CAP: canonical analysis of principle coordinates

CCA: crustose coralline algae

CCM: carbon concentration mechanism

Chl *a*: Chlorophyll *a*

CPCe: Coral Point Count with Excel extensions

DBL: diffusion boundary layer

DGGE: denaturing gradient gel electrophoresis

DbRDA: distance based redundancy analysis

Dd: diadinoxanthin

DIC: dissolved inorganic carbon

DISTLM: distance based linear model

Dt: diatoxanthin

E_k: minimum saturating irradiance

ECM: extracellular calcifying medium

fCO_2: fugacity CO_2

F_0: minimum fluorescence

F_m: maximum fluorescence

F_m': light-adapted maximum fluorescence

F_t: steady-state fluorescence

ΔF: light-adapted variable fluorescence

F_v: variable fluorescence

F_v/F_m: maximum dark-adapted quantum yield of PSII

$\Delta F/F_m'$: effective dynamic quantum yield of PSII

FP: fluorescent proteins

G3P: glyceraldehyde 3-phosphate

GFP: green fluorescent protein

HIRS: Heron Island Research Station

HPLC: high performance liquid chromatography

iPAM: imaging pulse amplitude modulation fluorometer

IPCC: Intergovernmental Panel on Climate Change

ITS1/2: internal transcribed spacer regions of rDNA

K'_{sp}: solubility constant

LEC: light enhanced calcification

LEDR: light enhanced dark respiration

LHC: light harvesting complex

LIRS: Lizard Island Research Station

LR: Loomis Reef

MC: Mermaid Cove

$NAD(P)^+$: nicotinamide adenine dinucleotide (phosphate)

NADPH: nicotinamide adenine dinucleotide phosphate

NPQ: non-photochemical quenching

OA: ocean acidification

Ω_{calc}: saturation state of calcite

Ω_{arag}: saturation state of aragonite

P_{680}: PSII primary donor

PCR: polymerase chain reaction

PG: phsophoglycolate

PGA: phosphoglycerate

PGPase: phosphoglycolate phosphatase

Phe: phaeophytin

P_{net}: net photosynthesis

$P_n$max: maximum rate of net photosynthesis

pCO_2: partial pressure of carbon dioxide

P:E curve: photosynthesis-irradiance curve

PERMANOVA: permutational analysis of variance

P_g: gross photosynthesis

P_g:R: gross photosynthesis to respiration ratio

$pH = -\log [H^+]$

pH_T: total pH

PQ: plastoquinone

PRIMER: Plymouth routines in multivariate ecological research

PSII: photosystem II

PSU: practical salinity unit

Q_A: quinone acceptor

Q_m: excitation pressure

RC: reaction centre

R_{dark}: dark respiration

ROS: reactive oxygen species

RuBisCO: ribulose bisphosphate carboxylase/oxygenase

RuBP: ribulose-1,5-bisphosphate

SR: Station Reef

SST: sea surface temperatures

Φ_{NO}: yield of fluorescence

Φ_{NPQ}: yield of non-photochemical quenching

Abstract

Ocean acidification will impact the photo-physiology of reef-building corals as it can lead to dysfunction of the symbiosis and loss of productivity. The major objective of this thesis was to provide insight into the mechanism of CO_2-induced bleaching and productivity loss across multiple life-history stages and interpret these findings in an ecological context.

Chapter 1 provides a review of the literature investigating the photo-physiological impact of ocean acidification, emphasizing the experimental conditions in studies that observed *Symbiodinium* dysfunction and productivity loss. Chapter 2 presents a working hypothesis to describe the fundamental physiological aspects of coral bleaching under ocean acidification. This research investigates the response of *Acropora aspera* using pulse amplitude modulation (PAM) fluorometry and oxygen respirometry under increased pCO_2 with concomitant high light conditions. The dinoflagellate density and HPLC pigment analysis are utilised to characterise the CO_2-induced bleaching response. We present a conceptual model linking photorespiration to CO_2-induced bleaching and productivity loss.

The impact of ocean acidification on coral reef ecosystems is likely to deviate from oceanic climate models due to diel modification of carbonate chemistry by community metabolism. Chapter 3 characterises the diurnal variation in carbonate chemistry at sites around Lizard Island and links this to the ocean acidification response of *Acropora millepora* collected from these sites. Furthermore, we utilise permutational multivariate statistical analyses to partition the variation in carbonate chemistry attributable to community composition at these sites. It was hypothesized that greater diurnal variation in carbonate chemistry may improve resilience of scleractinian corals to future ocean acidification conditions. This chapter highlights that site-specific physiological trade-offs may influence the response of reef-building corals to future ocean acidification scenarios.

Chapter 4 reports a visual bleaching response in *A. millepora* juveniles under future ocean acidification conditions. The effect of ocean acidification on coral juveniles is

hypothesised to impact *Symbiodinium* uptake and photochemical efficiency. We utilised the iPAM to align the photochemistry in the juveniles with their visual bleaching response and *Symbiodnium* type, as assessed by denaturing gradient gel electrophoresis (DGGE) of the internal transcribed spacer region 1 (ITS1) of the ribosomal genes. This study links the bleaching response with recruits containing a dominant population of *Symbiodinium* type D1 or D1-4, with potential implications for post-settlement survivorship and population dynamics.

Lastly, in Chapter 5 the key findings of this thesis are discussed in light of the ecological implications for the Great Barrier Reef. The synopsis outlines the effect of ocean acidification on the photo-physiology, productivity, calcification, reproduction and symbiont acquisition of reef-building corals. Future avenues for research are suggested based on new research gaps revealed by this thesis with the aim to continue to provide up-to-date scientific information to policy makers and reef managers.

Chapter 1: General Introduction

Reef-building corals and their threatened ecosystem

The value of coral reefs span biological, social and economic boundaries. They are among the most productive and diverse ecosystems on the planet, providing humanity with services such as coastal protection, building materials and novel biochemical compounds and providing economic returns through the fisheries and tourism industries (Costanza et al. 1997; Moberg and Folke 1999). In addition, coral reefs provide aesthetic and cultural benefits, which remain difficult to quantify. Nevertheless, the economic value of the Great Barrier Reef fisheries and tourism industries were estimated to be AU$ 912 million in 1996 (Driml 1999) and the global economic value of coral reefs has been estimated up to US$ 600,000 per km^2 (UNEP-WCMC 2006). Therefore, it is not surprising that the loss of coral reefs would amount to hundreds of billions of dollars per year worldwide (Bryant et al. 1998; Hoegh-Guldberg 1999). Yet despite their value and importance, coral reefs are declining due a number of direct anthropogenic influences including eutrophication, sedimentation, destructive fishing, overfishing and mining (Bruno and Selig 2007). In addition, anthropogenic CO_2 emissions threaten coral reefs due to i) the radiative scattering effect of CO_2 in the atmosphere, which raises the sea surface temperature (SST) and ii) ocean acidification (OA) as CO_2 dissolves into oceanic waters and reduces the availability of dissolved inorganic carbon (DIC) species to marine organisms (Caldeira and Wickett 2003; Canadell et al. 2007; Hoegh-Guldberg et al. 2007).

Scleractinian corals are largely responsible for the reef framework through deposition of their limestone skeleton, creating a structurally complex habitat for a diverse range of reef organisms (Smith 1983). Branching corals in the family Acroporidae are known to be fast growing, averaging 10 cm^2 growth per year and therefore significantly contributing to reef formation (Connell 2012). The taxonomic classification of the Acroporids investigated in the experimental chapters of this thesis is shown below:

Kingdom: Animalia
Phylum: Cnidaria
Class: Anthozoa
Subclass: Zoantharia
Order: Scleractinia
Family: Acroporidae
Genus: *Acropora*

These reef-building corals are also referred to as the coral holobiont which encompasses all symbionts of the coral animal, including dinoflagellates (genus: *Symbiodinium*), bacteria, fungi, protozoa and endolithic algae (Rohwer et al. 2002; Rosenberg et al. 2007). The taxonomic classification of the symbiotic dinoflagellates is listed below:

Kingdom: Protozoa
Division: Dinoflagellata
Subdivision: Dinokaryota
Subclass: Gymnodiniphycidae
Order: Suessiales
Family: Symbiodiniaceae
Genus: *Symbiodinium*

The exchange of nutrients and energy between the symbiotic dinoflagellate and the host coral is a fundamental factor contributing to the success of this symbiosis, and enables the widespread formation of coral reefs around the world's tropical and sub-tropical seas. The dinoflagellates are protected in the gastrodermal cells of the coral where they receive the CO_2, nitrogen and phosphorus essential for their photosynthesis and growth (Muscatine and D'Elia 1978). In return, the symbionts can translocate up to 95% of their photosynthate, principally as glucose or glycerol, which significantly contributes to the energy budget of the coral host (Muscatine 1967). This mutualistic symbiosis can be disrupted under certain physical and chemical conditions including changes in light, temperature or CO_2. During these suboptimal conditions, the coral may "bleach" due to expulsion of the dinoflagellates or loss of their photosynthetic pigments. This process represents a major threat to coral reef

ecosystems, as coral growth, survivorship and reproduction are diminished during bleaching episodes (Little et al. 2004; Jones and Berkelmans 2011).

Acropora aspera (blue morph) (Dana, 1846)

Acropora aspera is a staghorn coral with digitate, upright branches, which divide infrequently and taper towards the end (Figure 1.1). The axial corallites are dome-shaped while the radial corallites, exhibiting a variety of sizes, are appressed and thick-walled with small openings (Veron 2000). *A. aspera* branch ends may be pale brown or pale blue due to the presence of host pigment, pocilloporin, a type of Green Fluorescent Protein (GFP) (Dove 2004). In general, fast-growing corals such as arborescent acroporids are more susceptible to thermal bleaching than slow-growing corals (Marshall and Baird 2000), while *A. aspera* (blue morph) may be hypersensitive due to the vulnerability of pigment synthesis during heat stress (Dove 2004).

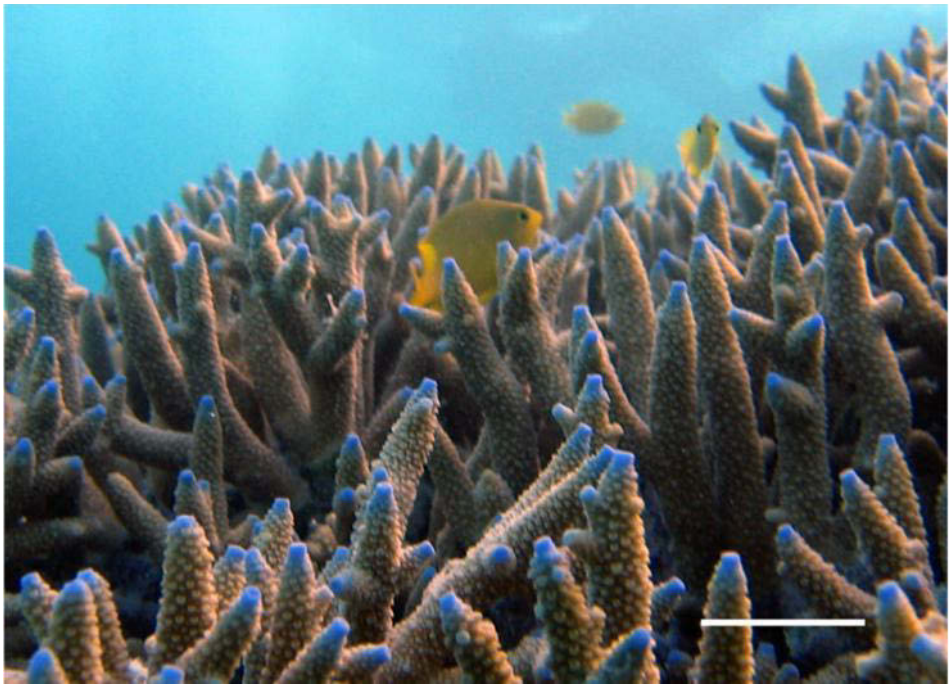

Figure 1.1: Colony of *Acropora aspera* (blue morph) at Heron Island showing varied size of the radial corallites and blue colouration around the axial corallite due to the host pigment, pocilloporin. Scale bar = 5cm. (Photo: R Middlebrook).

Acropora millepora (red morph) (Ehrenberg, 1834)

Acropora millepora is a corymbose coral with short branches of uniform shape (Figure 1.2). The axial corallites are distinctly tubular while the radial corallites are compact and of the same size with a scale-like appearance (Veron 2000). The branches of *A. millepora* may be green, yellow or red due to the presence of host fluorescent proteins (FP) with the red morph containing the least amount of FP and being most susceptible to thermal bleaching (Paley and Bay 2012). Overall, it has been shown that *A. millepora* is susceptible to thermal bleaching, but not to as great an extent as *A. aspera* (Fisher et al. 2012).

Figure 1.2: Colony of *Acropora millepora* (red morph) at Lizard Island showing corymbose colony shape and tubular axial corallites. Scale bar = 5cm.

Ocean Acidification on Coral Reefs

Increased anthropogenic CO_2 emissions, due to fossil fuel burning, cement production and land-use changes, are causing global climate change and ocean acidification (Caldeira and Wickett 2003; Feely et al. 2004; Orr et al. 2005). Atmospheric CO_2 equilibrates with the sea surface through air-sea gas exchange and this leads to an increase in DIC and a decline in the pH of seawater (Sabine et al. 2004; Doney et al. 2009). The seawater carbonate chemistry is governed by the following reversible chemical reactions:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+ \quad (1)$$

The dissolution of CO_2 in seawater forms carbonic acid ($H_2CO_3^-$), a weak acid that rapidly dissociates into hydrogen (H^+), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions. To balance the equilibrium, some of the additional H^+ reacts with CO_3^{2-} forming more HCO_3^-. Overall, the net effect of adding CO_2 to seawater is an increase in HCO_3^- and a decrease in CO_3^{2-}, along with lowered pH (pH = -log [H^+]) (Doney et al. 2009). The Intergovernmental Panel on Climate Change (IPCC) predicts that oceanic pH may drop 0.3 - 0.5 pH units by the end of the century, depending on the CO_2 emission scenario (Zeebe and Wolf-Gladrow 2001; Caldeira and Wickett 2005; IPCC 2007). Prior to the industrial revolution the atmospheric partial pressure of CO_2 (pCO_2) was 280 µatm and Total pH (pH_T) was 8.16; yet today the pCO_2 has risen to 400 µatm, which represents a 43% increase in pCO_2 (Jones 2013). In addition, pH has declined to 8.04 due to the ~30% increase in H^+ (Kleypas et al. 2006). These changes in H^+ and pCO_2 may have important implications for extracellular and intracellular acid-base regulation, ion-transport mechanisms and the occurrence of hypercapnia in marine organisms (Pörtner 2008). As the pH and CO_3^{2-} concentration decline, so too does the saturation state of the seawater with respect to calcite (Ω_{calc}) and aragonite (Ω_{arag}). Calcium carbonate ($CaCO_3$) mineral formation, imperative to the shells and skeletons of many marine organisms, is dependent on Ω, which is defined as:

$$\Omega = [Ca^{2+}] [CO_3^{2-}] / K'_{sp} \quad (2)$$

where K'_{sp} is the apparent solubility constant for calcite or aragonite, which depends on temperature, salinity and pressure. Aragonite is the $CaCO_3$ polymorph found in scleractinian coral skeletons and as K'_{sp} (aragonite) is approximately double K'_{sp} (calcite), aragonite is ~50% more soluble than calcite (Doney et al. 2009). Although dissolution of aragonite is expected to occur in under-saturated waters ($\Omega_{arag} < 1$), calcification of particular species and net accretion of coral communities can cease even in super-saturated water (Langdon and Atkinson 2005; Silverman et al. 2009).

Throughout the oceans, marine life has adapted to the ambient CO_2 conditions, which includes the high concentrations at underwater volcanic vents and the highly variable levels in shallow reef waters (Pörtner 2008); yet the current rate of increase in pCO_2 is

approximately 1000x faster than has occurred in the past 420,000 years (Hoegh-Guldberg et al. 2007). Actually, there are no geological records of a decline in Ω_{arag} comparable to the present rate, although the pCO_2 has previously reached 20x pre-industrial levels during steady state conditions whereby slow geochemical feedbacks (ie. rock-weathering) allowed Total Alkalinity (A_T) to increase (Veron 2008; Pandolfi et al. 2011). A_T represents the ability of the seawater to buffer changes in pH upon addition of acid and is equal to the sum of all proton acceptors less proton donors (Dickson et al. 2007):

$$\begin{aligned} A_T = \; & [HCO_3^-] + 2[CO_3^{2-}] + [B(OH)_4^-] + [OH\text{-}] + [HPO_4^{2-}] \\ & + 2[PO_4^{3-}] + [SiO(OH)^{3-}] + [NH_3] + [HS^-] + \ldots \\ & - [H^+]_F - [HSO_4^-] - [HF] - [H_3PO_4] - \ldots \end{aligned} \quad (3)$$

Despite this potential buffering, coral reefs have ceased to accrete in the past in alignment with geological records of mass extinction during OA and warming events (Veron 2008).

Large diel variability in carbonate chemistry has been observed on coral reefs (Ohde and vanWoesik 1999; Yates and Halley 2006; Shamberger et al. 2011; Shaw et al. 2012) and with increasing anthropogenic pCO_2, the magnitude of this fluctuation is projected to increase due to changes in the ratio of DIC to A_T (Egleston et al. 2010; Shaw et al. 2013). The carbonate chemistry within the benthic boundary layer will be affected by upstream community metabolism and this may have implications for the pH gradient within the diffusive boundary layer (DBL) (Shashar et al. 1996; Hurd et al. 2011; Jokiel 2011). During the day, net photosynthesis on coral reefs removes CO_2 from the water, which leads to an increase in pH (Figure 1.3). In contrast, the net respiration occurring at night releases CO_2 into the water and reduces the pH. Calcification and dissolution affects both the pH and A_T as calcification releases CO_2 and removes CO_3^{2-} from the water. Overnight accumulation of CO_2 in shallow reef waters can drop the pH to levels as low as those predicted by the end of the century by the IPCC (Kline et al. 2012; Shaw et al. 2012).

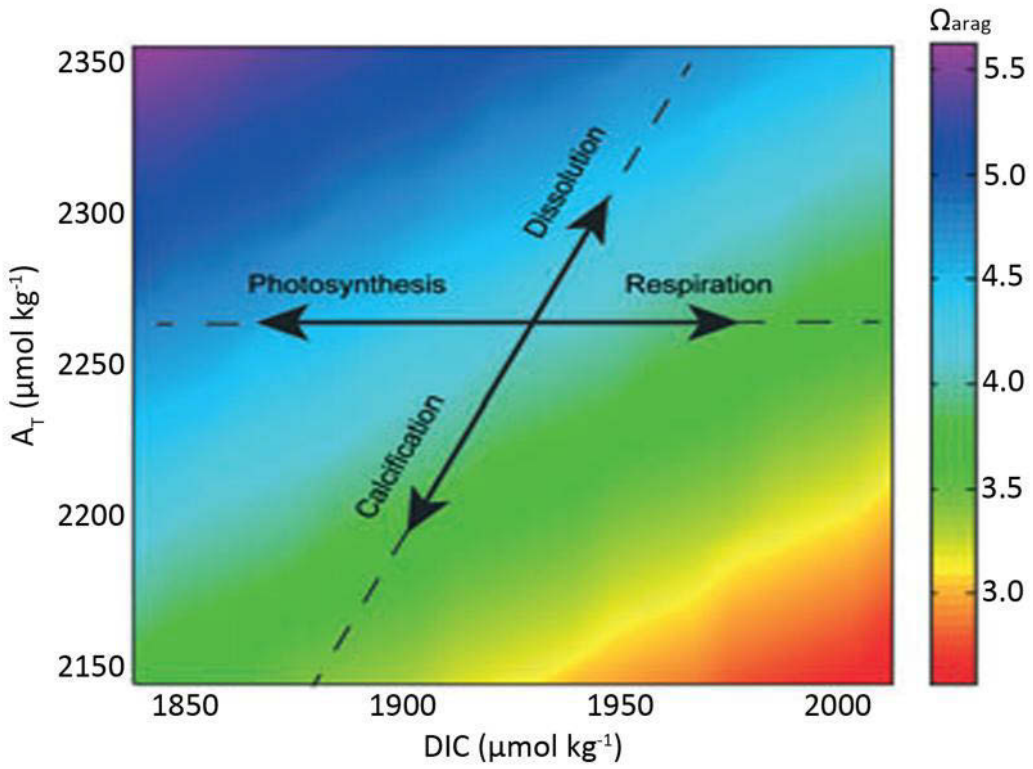

Figure 1.3: Deffeyes diagram showing iso-contours of the saturation state of aragonite (Ω_{arag}) plotted as a function of Dissolved Inorganic Carbon (DIC) and Total Alkalinity (A_T) (Anthony et al. 2011a). Photosynthesis removes CO_2 thereby reducing DIC, whereas calcification removes HCO_3^- and CO_3^{2-} which impacts both DIC and A_T.

The large diel variability of carbonate chemistry on coral reefs is at odds with climate models of OA that are based on constant A_T (Kleypas et al. 1999; Silverman et al. 2009; Shaw et al. 2013), hence the risks associated with OA are difficult to predict. Recent models and experiments have begun to investigate the diel variation, with evidence that day-time decreases in pCO_2 may provide some relief (Dufault et al. 2012) and predictions that the diel variation will increase on shallow reef flats (Shaw et al. 2013). Previous models that did not incorporate these diel changes are likely invalid (Silverman et al. 2009), as are models without projected changes in community composition, as this is now known to have strong implications for the diel variation in carbonate chemistry (Anthony et al. 2011b; Kleypas et al. 2011) and will occur under future OA conditions (Hall-Spencer et al. 2008; Inoue et al. 2013).

Impact of ocean acidification on the coral symbiosis and bleaching

OA has the potential to impact the coral symbiosis directly through increased pCO_2, HCO_3^- and H^+, which are important to physiological processes such as photosynthesis, acid-base regulation and ion-transport mechanisms (Badger et al. 1998; Pörtner 2008). Previous research has predominately focused on the radiative forcing of atmospheric CO_2, which increases the SST and is linked to thermal coral bleaching events (Glynn 1991; Hoegh-Guldberg 1999). Yet recent research has indicated that OA may act synergistically with warming to lower the thermal bleaching threshold of corals (Pecheux 2002; Anthony et al. 2008).

For thermally induced bleaching, the initial sites of dysfunction are proposed to be either the D1 protein within the reaction centre (RC) of Photosystem II (PSII) or impairment of CO_2 fixation by Ribulose bisphosphate carboxylase/oxygenase (RuBisCO), both of which lead to damage to PSII within the symbiotic dinoflagellates (Iglesias-Prieto et al. 1992; Warner et al. 1996; Jones et al. 1998; Hill et al. 2004; Lilley et al. 2010). Under these conceptual models of bleaching, there is reduced translocation of photosynthates to the host and production of reactive oxygen species (ROS) due to overwhelmed photo-protective and anti-oxidant mechanisms (Iglesias-Prieto et al. 1992; Shick et al. 1995). Following this damage, a signal transduction is initiated that ends with host cell death or apoptosis (Dunn et al. 2004; Dunn et al. 2007). In general, however, this signal transduction is preceded by reduction in the flow of electrons and rate of photosynthesis, which would lead to the accumulation of excess irradiance within the dinoflagellates (Brown 1997).

In line with these hypotheses, a CO_2-induced bleaching mechanism has been proposed which implicates a decline in the photoprotective mechanism, photorespiration, as the initial site of dysfunction (Anthony et al. 2008; Crawley et al. 2010). *Symbiodinium* possess a Form II RuBisCO, which lacks discrimination between CO_2 and O_2 fixation, the latter of which leads to the photorespiratory cycle rather than the Calvin-Benson cycle. Under elevated CO_2 conditions, genetic expression of the first enzyme in the photorespiratory cycle is down regulated, suggesting that the potential to use photorespiration for photoprotection is reduced (Crawley et al. 2010).

Impacts on productivity

An increase in CO_2 may have beneficial implications for photosynthetic organisms but the situation is complicated for dinoflagellates residing within the endoderm of corals. Corals have the ability to transport HCO_3^-, a carbonate species that also increases under OA conditions (Goiran et al. 1996). This ability arises from the presence of a carbon concentrating mechanism (CCM) consisting of isoforms of carbonic anhydrase (CA), which catalyze the inter-conversion of HCO_3^- and CO_2 (Bertucci et al. 2013). Furthermore, H^+-ATPase plays a role in CO_2 protonation (Bertucci et al. 2013). The CCM allows CO_2 to readily diffuse into host ectodermal cells, remain within the cytosol as HCO_3^- and be transported across the endoderm and symbiosome as CO_2 to the dinoflagellates for photosynthesis (Al-Moghrabi et al. 1996).

The impact of OA on the productivity of reef-building corals has been shown to be either positive (Langdon and Atkinson 2005; Crawley et al. 2010; Suggett et al. 2012), negative (Langdon and Atkinson 2005; Anthony et al. 2008; Iguchi et al. 2012; Kaniewska et al. 2012; Anthony et al. 2013) or have no effect (Leclercq et al. 2002; Reynaud et al. 2003; Schneider and Erez 2006; Marubini et al. 2008; Rodolfo-Metalpa et al. 2010; Takahashi and Kurihara 2013) (Table 1.1). It is difficult to ascertain how much of this variation in results is due to variable species resilience or different experimental procedures. Experiments that measure productivity on the same coral species are rare, the duration of the experiments range from hours to months and the experimental light fields range from 30 – 3800 μmol quanta m^{-2} s^{-1} (Table 1.1). As a general rule, it is apparent that the decline in productivity usually coincides with high temperature or high light, indicating that increased CO_2 may exacerbate these stressful conditions.

Impacts on calcification

The biomineralisation mechanism is enhanced in hermatypic corals due to the phenomenom of Light-Enhanced Calcification (LEC) (Yonge and Nicholls 1931), yet its link to carbonate chemistry renders the process vulnerable under OA conditions. LEC is proposed to occur due to photosynthetic removal of CO_2 by the symbiotic dinoflagellates in accordance with the following stoichiometric equations:

$$\begin{array}{l} \text{calcification} \rightarrow \qquad \text{photosynthesis} \rightarrow \\ Ca^{2+} + 2\,HCO_3^- \rightleftharpoons CaCO_3 + H_2O + CO_2 \rightleftharpoons CaCO_3^- + CH_2O + O_2 \\ \qquad\qquad \leftarrow \text{dissolution} \qquad\quad \leftarrow \text{oxidation} \end{array} \qquad (4)$$

Although LEC has been observed in many coral species, the mechanism remains elusive and probably includes the contribution of several factors in addition to CO_2 removal, including increasing O_2 (Colombo-Pallotta et al. 2010), buffering H^+ (Bertucci et al. 2013), removing inhibitors (Ferrier-Pagès et al. 2000), providing energy to the host (Colombo-Pallotta et al. 2010) and supplying precursors for organic matrix synthesis (Muscatine and Cernichiari 1969). While there are numerous studies describing the decline of calcification under increased pCO_2 (Kroeker et al. 2010; Erez et al. 2011) the mechanism is more complicated than the direct effect of the seawater at the site of calcification, due to the abovementioned processes in connection to photosynthesis.

Potential for adaptation or acclimatization

In relation to the ability of scleractinian corals to adapt to climate change and OA, the general concensus among scientists is that the rate of environmental change is too rapid for adaptation, due to their slow generational time (Kleypas et al. 1999; Hoegh-Guldberg et al. 2007; Veron 2008). Nevertheless, field acclimatization or phenotypic plasticity is evident in corals as indicated by reciprocal transplant experiments (Coles and Jokiel 1978; Foster 1979) and studying species across environmental gradients (Falkowski and Dubinsky 1981). A further promising aspect of coral evolution, however, is their association with *Symbiodinium*, which have short generation times (Wilkerson et al. 1988) and have shown the ability to adapt to local conditions (Howells et al. 2012). Furthermore, evidence of acclimatization has been shown in adult coral colonies under thermal stress through shuffling the proportion of genetic types of *Symbiodinium* (Jones et al. 2008). In addition, coral juveniles have an opportunity to establish symbiosis with many types of *Symbiodinium* during early ontogeny and this represents an avenue for adaptation to climate change and OA (Abrego et al. 2009; Cumbo et al. 2013).

Table 1.1: Summary of the current literature investigating the impact of OA on the productivity of scleractinian corals. Change in net photosynthesis (P_{net}) and dark respiration (R_{dark}) relative to the control and normalised to branch surface area or equivalent. Control pCO_2 approximately 380 µatm, mid pCO_2 ranges 500 - 800 µatm and high pCO_2 ranges 800 – 2100 µatm.

Reference	Coral	CO_2 manipulation	Duration	µmol quanta $m^{-2}s^{-1}$	P_{net} at mid CO_2	P_{net} at high CO_2	R_{dark} at mid CO_2	R_{dark} at high CO_2	Comments
Leclercq et al., 2002	Community	Bubbling CO_2	4 weeks	220	0%	0%	0%	22%	
Reynaud et al., 2003	*Stylophora pistillata*	Bubbling CO_2	5 weeks	380	0%	-	0%	-	At +3°C Pnet declined 32% at mid CO_2
Langdon & Atkinson, 2005	Community	Acid addition	1.5 hours	1300-1700	34%	-8%	-	-	Based on O_2 method reported
Schneider & Erez, 2006	*Acropora eurystoma*	Acid addition	3 hours	6m depth, Red Sea	-	0%	-	0%	
Marubini et al., 2008	*Stylophora pistillata*	Acid addition	8 days	300	0%	0%	-	-	DIC enrichment increased Pnet by 70%
Anthony et al., 2008	*Acropora intermedia*	Bubbling CO_2	8 weeks	700-1200	*0%	*-88%	*P_{net} here is actually Daily Productivity which incorporates R_{dark}		At +3°C productivity increased 52% at mid CO_2 and declined 85% at high CO_2
Anthony et al., 2008	*Porites lobata*	Bubbling CO_2	8 weeks	700-1200	*-11%	*-42%			At +3°C productivity declined 86% at mid CO_2 and declined 95% at high CO_2
Crawley et al., 2010	*Acropora formosa*	Bubbling CO_2	4 days	110	38%	0%	0%	0%	
Rodolfo Metalpa et al., 2010	*Cladocora caespitosa*	Bubbling CO_2	1 month- 1 year	30-60	0%	-	0%	-	Temperate coral
Iguchi et al., 2012	*Porites australiensis*	Bubbling CO_2	8 weeks	120-140	-	*-12-33%	-	-	*P_{net} here is actually Fv/Fm
Kaniewska et al., 2012	*Acropora millepora*	Bubbling CO_2	4 weeks	860	0%	-60%	-25%	-75%	
Suggett et al., 2012	*Acropora horrida*	Bubbling CO_2	5-6 weeks	100-400	*60%	-	0%	-	*P_{net} here is actually P_{gross} which incorporates R_{dark}
Suggett et al., 2012	*Porities cylindirca*	Bubbling CO_2	5-6 weeks	100-400	*20%	-	-10%	-	
Anthony et al., 2013	*Acropora aspera*	Bubbling CO_2	1 day	1000	-30%	-	50%	-	
Takahashi & Kurihara, 2013	*Acropora digitifera*	Bubbling CO_2	5 weeks	152-3789	0%	0%	0%	0%	Based on DIC method (no O_2 measurement)

Understanding CO_2-induced coral bleaching

Coral bleaching under elevated CO_2 conditions has recently been observed in *A. millepora* (Kaniewska et al. 2012), *Acropora intermedia* and *Porites lobata* (Anthony et al. 2008). Differentiation of the bleaching as either loss of pigments or loss of symbiont cells could not be ascertained in Anthony et al. (2008) due to loss of the biological samples in a fire. For *A. millepora* the bleaching was due to loss of symbiont cells (Kaniewska et al. 2012). These studies have highlighted the impact of increased CO_2 on *Symbiodinium*, as well as increased H^+ on the coral. While it is likely that acidosis plays a role in disruption of host cellular mechanisms, the disruption of the symbiosis and cellular processes within *Symbiodinium* are likely to be linked to inhibition of photosynthesis due to loss of photoprotection, as exemplified by down-regulated expression of the photorespiratory enzyme, phosophoglycolate phosphatase (PGPase) (Takahashi et al. 2007; Crawley et al. 2010). An alternative hypothesis states that OA may cause coral bleaching through dinoflagellate cell division upon release from CO_2-limitation and subsequent changes in carbon translocation leading to disruption of the host CCM (Wooldridge 2013).

Photodamage and photoinhibition

Photodamage occurs in the photosynthetic apparatus when it is exposed to excess light, as the electron transport chain cannot process any further excitons. To prevent photodamage, the first line of defense is dynamic photoinhibition through reversible photoprotective processes such as non-photochemical quenching (NPQ) (Brown et al. 1999). Should excitons overwhelm NPQ, this may lead to the formation of ROS such as singlet oxygen ($^1O_2$), the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH·) (Niyogi 1999). There are several photoprotective mechanisms that involve electron transport such as PSI cyclic electron transport, the water-water cycle and photorespiration; all of these result in less photodamage. Yet, under dynamic light conditions, the inevitable production of ROS necessitates antioxidant and repair systems in order to scavenge ROS and replace damaged proteins (Nishiyama et al. 2006). Environmental stress may tip the balance between photodamage and repair mechanisms, thereby leading to chronic photoinhibition (Takahashi and Murata 2008). Given that previous OA experiments have only shown

bleaching or productivity loss with concomitant high light or temperature (Table 1), it is likely that high pCO_2 affects photoprotection and repair mechanisms, while the photodamage itself may be caused by exposure to excess light.

Photoprotective processes

Photoprotective mechanisms can function to quench light prior to photosynthesis (e.g. xanthophyll cycle), to cycle electrons during photosynthesis (e.g. PSI cyclic electron transport and the water-water cycle), or to increase substrate availability downstream of the photosynthetic light reactions (e.g. photorespiration) (Demmig-Adams and Adams 1996; Niyogi 1999; Endo and Asada 2006). In a previous OA experiment (Crawley et al. 2010), xanthophyll cycling increased despite sub-saturating light conditions, which suggests that the quenching was required due to a reduction in downstream electron transport and the resultant exposure to excess light. Xanthophyll pigment de-epoxidation is triggered by the change in pH (ΔpH) across the thylakoid membrane (Demmig-Adams and Adams 1996). In addition, NPQ can occur in functionally detached LHCs of PSII due to the pH-sensing protein, PsbS (Holzwarth et al. 2009). The water-water cycle and PSI cyclic electron transport are linked here as they can maintain the ΔpH in order to trigger thermal dissipation of excess light (Schreiber and Neubauer 1990). These photoprotective processes may change in order to assist photoacclimation of *Symbiodinium* to OA conditions.

The ability of dinoflagellates to utilize the photorespiratory pathway is largely dependant on the proportion of CO_2 and O_2 surrounding the enzyme RuBisCO, which catalyses both carboxylation and oxygenation reactions (Figure 1.4). As dinoflagellates possess a Form II Rubsico with a low affinity for CO_2, a CCM is required to increase the proportion of CO_2 surrounding this enzyme (Badger et al. 1998; Leggat et al. 1999). Carboxylation is preferred due to the subsequent formation of sugars through the Calvin-Benson Cycle, yet the evolutionary persistence of the Form II RuBisCO suggests a role for oxygenation (Badger et al. 2000). Indeed, the fact that high temperature reduces the affinity of RuBisCO for CO_2 implies that the photoprotective role of photorespiration can be harnessed during thermal stress (Badger and Collatz 1977; Jordan and Ogren 1984). Increasing pCO_2 due to OA is at

odds with this fact, however, as the oxygenase reaction will be suppressed thus reducing the photoprotective role of photorespiration (Crawley et al. 2010).

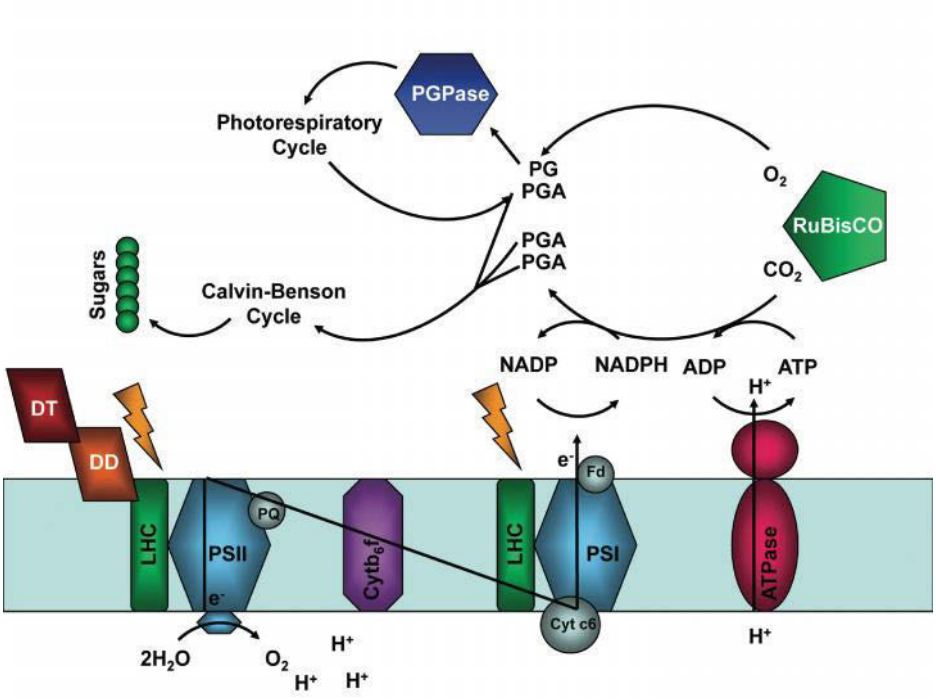

Figure 1.4: Diagram showing the photosynthetic "light reactions" across the thylakoid membrane and the "dark reactions" of carbon fixation by Ribulose bisphosphate carboxylase/oxygenase (RuBisCO). Also depicted are the photoprotective mechanisms photorespiration (beginning with phosphoglycolate phosphatase [PGPase]) and the xanthophyll cycle (de-epoxidation of diadinoxanthin [DD] to diatoxanthin [DT]). Image from Crawley et al. (2010).

Chlorophyll *a* fluorescence

Chlorophyll *a* (chl *a*) fluorescence is a powerful non-invasive tool for investigating photosynthetic performance, which relies on the fact that light energy absorbed by PSII has three possible fates (Bulter 1978). Firstly, photons may drive photochemistry as resonance energy allows electrons to be transferred from the PSII RC chl *a*, known as P_{680}, through the electron carrier, Phaeophytin (*Phe*), to the quinone acceptor (Q_A). Secondly, absorbed light energy may be dissipated as heat through non-photochemical quenching (NPQ) or thirdly, as chl *a* fluorescence (Figure 1.5). Chl *a* fluorescence measurements are commonly used in coral bleaching research as the relative change in chl *a* fluorescence can be used to ascertain the proportion of closed

PSII RCs, which may indicate acute or chronic photoinhibition (Krause 1988; Iglesias-Prieto 1995; Warner et al. 1999; Fitt et al. 2001; Ralph et al. 2001).

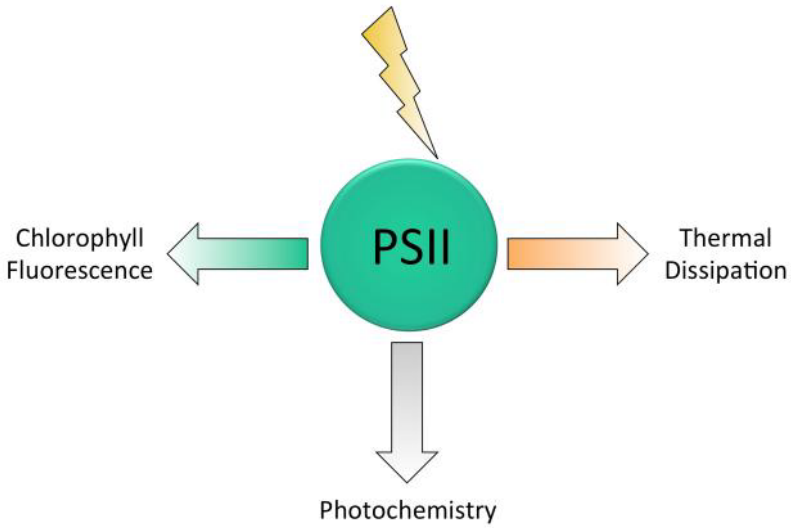

Figure 1.5: The three potential fates of light energy absorbed by Photosystem II (PSII) include: (1) Photochemistry, whereby an electron is transferred from P_{680} to the quinone acceptor (Q_A); (2) thermal dissipation as heat through non-photochemical quenching; or (3) chlorophyll fluorescence.

The imaging-Pulse Amplitude Modulated fluorometer (iPAM) (Walz GmbH, Effeltrich, Germany), utilised throughout this thesis, applies saturating pulses of light and captures the resultant fluorescence image. Initially, samples are dark-adapted for one hour to determine the minimum fluorescence (F_0). Following the saturating pulse, the maximum fluorescence (F_m) is measured and the difference, $F_m - F_0$, is the variable fluorescence (F_v). The ratio F_v/F_m gives an indication of the maximum quantum yield of PSII. In addition, samples can be studied after exposure to the iPAM actinic light, which may close some RCs through NPQ. Here, the steady-state fluorescence (F_t) is higher due to the actinic light. After application of the saturating pulse, the light-adapted maximum fluorescence (F_m') is less than F_m due to NPQ. The light-adapted variable fluorescence (ΔF) is similarly calculated as $F_m' - F_t$ and the ratio, $\Delta F/F_m'$, is the effective quantum yield of PSII. Acute photoinhibition through a decrease in F_m' may indicate the use of photoprotective mechanisms, whereas chronic photoinhibition indicates that photodamage may have occurred and results in persistent loss of quantum yield through time (weeks or months). Excitation pressure (Q_m) is a measure of the relative change in the quantum yield of PSII:

$$Q_m = 1 - [(\Delta F/F_m') \cdot (F_v/F_m)^{-1}] \quad (5)$$

where $\Delta F/F_m'$ is measured at midday and F_v/F_m is measured at dusk. An increase in Q_m suggests an increased need for NPQ to reduce over-excitation of PSII, while acclimation to high Q_m can lead to resistance to photoinhibition (Maxwell et al. 1995).

Understanding CO_2-induced productivity loss

Reef-building corals may acquire carbon via autotrophy, utilising the photosynthate of their mutualistic symbiont or via heterotrophic feeding, although the latter is considered to be a compensatory carbon acquisition strategy (Muscatine and Porter 1977; Muscatine et al. 1981). Research suggests that the ability for scleractinian corals to live autotrophically depends on the light regime and therefore the photosynthetic capacity of the symbiont, with heterotrophy being up regulated during episodes of poor water clarity or coral bleaching (Muscatine et al. 1984; Anthony and Fabricius 2000; Grottoli et al. 2006). Despite this ability to compensate, the autotrophy of the coral symbiosis is highly important to the growth, reproduction and survival the holobiont (Hatcher 1988; Hoegh-Guldberg 1999). Respiration is needed for the growth of new biomass and for the maintenance and repair of existing biomass (Amthor 2000). Up to 95% of the photosynthetically fixed carbon can be translocated to the host (Falkowski et al. 1984; Muscatine et al. 1984) while the contribution of dinoflagellate photosynthates may even exceed the host metabolic requirements (Muscatine et al. 1981; Davies 1984). The energy budget is defined by the level that gross photosynthesis (P_g) exceeds respiration (R), with P_g:R > 1 indicating the potential to operate autotrophically and P_g:R < 1 indicating that heterotrophic feeding is required. The P_g:R ratio for the coral symbiosis operates within the range of 0.5 – 5.0 and on average at 2.4 (Hatcher 1988; Battey 1992).

Intuitively, increased pCO_2 should be an advantage for photosynthetic organisms as this could increase the size of the sink for photon energy thereby allowing production of more glucose through the Calvin-Benson cycle. Yet increased pCO_2, with resultant increased HCO_3^- and H^+, will also affect other cellular mechanisms of the coral holobiont and thus complicates the response of their symbiotic dinoflagellates

(Pörtner 2008; Kaniewska et al. 2012). OA has been shown to reduce the host metabolism and change the expression of genes involved in acid-base regulation, ion/molecular transport, apoptosis, calcium homeostasis, cell signaling and cell structure (Kaniewska et al. 2012; Moya et al. 2012). A model was proposed implicating increased ROS as the starting point for the coral bleaching response and this oxidative stress may originate from the *Symbiodinium* cells or host mitochondria, or both (Weis 2008; Kaniewska et al. 2012). Overall, it appears that the paradox of decreased productivity of photosynthetic dinoflagellates under OA conditions (Langdon and Atkinson 2005; Anthony et al. 2008; Iguchi et al. 2012; Kaniewska et al. 2012; Anthony et al. 2013) may linked to the phenomenon of CO_2-induced coral bleaching.

Coral bleaching impacts on productivity

Reducing the population of symbiotic dinoflagellates within the coral host might ultimately lead to a reduction in the photosynthate transferred to the host with implications for the energy budget of the holobiont (Baird and Marshall 2002). Indeed, previous research has indicated that bleached corals consumed their own biomass in order to survive thermal bleaching episodes (Szmant and Gassman 1990). Prolonged coral bleaching conditions have been shown to inhibit gametogenesis (presumably due to the lack of lipids available for eggs) (Szmant and Gassman 1990; Ward et al. 2000), decrease growth rates (Goreau and Macfarlane 1990) and lead to coral mortality (Baird and Marshall 2002). Coral bleaching may be indicated by a decline in coral productivity, which can be investigated with respirometry chambers that measure the rate of photosynthesis oxygen evolution.

Coral respirometry techniques

Coral productivity is estimated from rates of net photosynthesis (P_{net}), dark respiration (R_{dark}) and light enhanced dark respiration (LEDR) measured by the oxygen evolution of a coral sample within respirometry chambers. These chambers sit within a temperature-controlled water bath, contain filtered seawater and a magnetic stirrer for recirculation and are connected to an optical oxygen electrode (Oxy-4, Presens, Germany). The electrodes measure the oxygen flux, which is regressed against time in order to ascertain the steady-state rates of P_{net}, R_{dark} and LEDR. The coral sample is initially dark-acclimated for one hour prior to the respirometry assay, which

commences in darkness to measure R_{dark}. P_{net} is then measured under various light levels to determine both the sub-saturating rate of photosynthesis, α, and the maximum rate of photosynthesis ($P_n$max). Lastly, the coral samples are returned to darkness to measure LEDR. Data from the respirometry assays can be used to generate Photosynthesis-Irradiance (P:E) curves to estimate productivity or to express the ratio of gross photosynthesis to respiration (Pg:R) (Jassby and Platt 1976; Chalker 1981).

Understanding OA impacts on calcification

Calcification occurs in the extracellular calcifying medium (ECM), which exists in close contact with the calicoblastic cells of the coral (Allemand et al. 2004). In accordance with Reaction 4, Ca^{2+} and HCO_3^- must be transported to the ECM while CO_2 and H^+ must be transported away from the ECM, or converted to HCO_3^-. For calcification to occur, it is imperative that pH remains elevated in the ECM relative to seawater (Al-Horani et al. 2003; Reis 2011; Venn et al. 2011; McCulloch et al. 2012; Venn et al. 2013). In addition, Ca-ATPase actively transports $Ca^{2+}$to the ECM (Al-Horani et al. 2003). As biomineralisation requires both passive and active epithelial transport of those molecules to and from the ECM, any environmental condition that weakens the chemical gradient driving passive transport will lead to an increased requirement for active transport, thereby increasing energy costs of calcification (Allemand et al. 2004). Similarly, any environmental condition that leads to a decrease in productivity will reduce the energy available for active transport and therefore decrease the rate of calcification (Erez et al. 2011). Although the exact mechanism is not known, OA research has indicated that coral calcification rates may decline up to 70% once pCO_2 levels double the pre-industrial levels (Renegar and Riegl 2005; Kroeker et al. 2010; Erez et al. 2011). This is a significant issue for coral reefs, as calcification must exceed the rate of bioerosion in order for net accretion to occur (Hoegh-Guldberg et al. 2007).

Metrics for coral calcification

Coral calcification is commonly assessed by measuring the linear growth rate (Lamberts 1978), using the buoyant weight technique (Jokiel et al. 1978) or the

alkalinity anomaly technique (Smith and Key 1975). The linear growth rate is measured by staining the skeleton with alizarin red and requires a long time (> monthly intervals) between measurement points in order to get accurate readings (Allemand et al. 2011). Accordingly, the buoyant weight technique is generally preferred over the linear growth rate as it can be used for short-term experiments (> weekly intervals) and has been shown to have a direct correlation with the skeletal dry weight, although correction may be required for tissue weight (Jokiel et al. 1978; Davies 1989; Langdon et al. 2010). The alkalinity anomaly technique can be used to measure the calcification rate during short-term incubation (< 1 h) due to the fact that A_T decreases two moles for every one mole of $CaCO_3$ produced, however it is limited to very short-term rates of calcification (Smith and Key 1975; Langdon et al. 2010). In Chapter 3, the skeletal dry weight normalised to the surface area of the branch is used to assess calcification, and this measurement gives a proxy for the skeletal density or branch compactness. This unit of measurement is relevant given that calcification and dissolution processes may change the skeletal density or branch compactness over the long-term and this may not be detectable from short-term measurements of the calcification rate (Kaniewska et al. 2012).

Metrics for community calcification

Community scale measurements of calcification are often employed to project calcification rates under future OA conditions (Silverman et al. 2009; Anthony et al. 2011a; Kleypas et al. 2011; Anthony et al. 2013; Shaw et al. 2013). The change in A_T can be assessed during a slack-water low tide or as the water flows across the reef using Eulerian or Langrangian approaches yet these techniques are restricted to shallow reef flats (Barnes 1983; Langdon et al. 2010). Furthermore, the flow methods require unidirectional flow and do not account for lateral mixing, which limits their application to certain ecosystems, such as reef flats with broad biological zones. In Chapter 3, the community calcification rate could not be assessed as wind direction changed the current seasonally. In addition, lateral mixing would have been significant, as the sites were not characterised by broad biological zones. Accordingly, here the diurnal variation in carbonate chemistry is the best metric to characterise the metabolism of the benthic communities.

Characterising reef acidification

A distinction lies between the process of OA and its effect on coral reef ecosystems as community metabolism in shallow reef waters leads to a diel pattern of reef acidification. Factors that will influence reef acidification include the community assemblage, the water depth, the tidal cycle, the current speed and the current direction (Anthony et al. 2011a; Kleypas et al. 2011; Price et al. 2012; Shaw et al. 2012; Anthony et al. 2013; Shaw et al. 2013).

Carbonate chemistry parameters

All carbonate chemistry parameters can be derived from two of four measurable parameters:

1. Total Alkalinity (A_T);
2. Dissolved Inorganic Carbon (DIC), also called Total CO_2 (TCO_2);
3. pH, and
4. Partial pressure of CO_2 (pCO_2) or fugacity of CO_2 (fCO_2).

In addition, the temperature, pressure and salinity must be entered and dissociation constants specified in order for the calculations to run in the program CO2calc (Robbins et al. 2010). This program draws on the dissociation constants for carbonic acid (K_1 and K_2) (Mehrbach et al. 1973; Dickson and Millero 1987), boric acid (K_B) (Dickson 1990a), water (K_W) (Millero 1995), bisulfate ion (K_{SO4}) (Dickson 1990b), hydrogen fluoride (K_F) (Dickson and Riley 1979), phosphoric acid (K_{P1}, K_{P2} and K_{P3}) and silicia acid (K_{Si}) (Millero 1995) as determined by various researchers. The K'_{sp} for calcite and aragonite are from Mucci (1983) and the solubility of CO_2 in seawater (K_0) is from Weiss (1974) and Murray & Riley (1971). The concentrations of boron (Uppstrom 1974), sulfate (Morris and Riley 1966), fluoride (Riley 1965) and calcium (Riley and Tongudai 1967) are assumed to be proportional to salinity. The activity coefficient of the hydrogen ion (f_H) is necessary for converting between pH scales (Takahashi et al. 1982). Values are also needed for the pressure dependence of the dissociation constants (Millero 1995) and solubility products (Ingle 1975; Millero 1979). The virial coefficients of CO_2 and CO_2-air are needed to correct for the non-ideal nature of the gas (Weiss 1974) and the vapour pressure of water above seawater is from Weiss & Price (1980). There are 4 pH scales (Total, Free, Seawater and NBS)

that have been used in previous research and the NBS scale was used throughout this thesis (Dickson 1984).

Carbonate chemistry techniques

In order to characterise the carbonate chemistry of field sites and experimental treatment aquaria, water samples can be collected and processed for A_T and pH measurements following the standard operating procedures of Dickson et al. (2007). The 500 mL glass sample bottles are rinsed 3 times with sample water before collection and sealed underwater to prevent atmospheric gas exchange. The samples are then fixed with 100 μL of saturated mercuric chloride, which is 0.02% of the total sample volume, for later processing.

A potentiometric titration procedure can be used whereby the 20 g of sample seawater is placed in open-cell and titrated with hydrochloric acid (HCl). Firstly, the pH is reduced to approximately 3.5 and stirred to allow CO_2 to escape (Dickson et al. 2003). For this small volume titration, the pH data collection is optimal at an incremental addition of 0.015 mL of 0.1 M HCl until pH 3.0 is reached. A_T is calculated from the titrant volume and pH measurements using a Gran approach, which derives the equivalence point from a non-linear least-square fit of the data (Gran 1952). Each daily run on the titrator (T50, Mettler Toledo, Langacher, Switzerland) is preceded by pH sensor (DGi101-SC, Mettler Toledo) calibration using NBS scale buffers (Mettler Toledo) and titrator calibration with Certified Reference Materials (AG Dickson, SIO, Oceanic Carbon Dioxide Quality Control).

Coral Reproduction under OA

Sexual reproduction in reef-building corals is inherently important to coral reef ecosystems by providing scope for evolutionary processes and population growth, yet many aspects of this process are threatened by future OA conditions (Albright 2011; Harrison 2011). The processes of gametogenesis, fertilisation, larval dispersal, settlement, metamorphosis, post-settlement growth, calcification, symbiont uptake and post-settlement survival may all experience potential impacts from increasing pCO_2 (Suwa et al. 2010; Albright 2011; Doropoulos et al. 2012b).

Gametogenesis and fertilisation are critical aspects of sexual reproduction, yet there are limited studies on the impact of OA on these processes. Given that gametogenesis is affected by coral bleaching due to depleted energy reserves (Szmant and Gassman 1990) and OA can increase bleaching susceptibility (Anthony et al. 2008), it is likely that gametogenesis will be indirectly affect by OA. While a previous study did not see an effect of OA on gametogenesis, colonies with partial mortality were excluded from the analysis, which may have confounded the results (Jokiel et al. 2008). Coral that survived as decalcified fleshy polyps were still able to undergo gametogenesis (Fine and Tchernov 2007) whereas in another study, female colonies were impacted more than male colonies, suggesting that the energy budget is a key factor determining the impact of OA on gametogenesis (Holcomb et al. 2010). Acid-base regulation is an another important process that may be affected by OA with implications for sperm motility, egg secretion of a motility-suppressing substance to prevent polyspermy and subsequent embryo development (Morita et al. 2010).

The success of the coral planulae larvae in dispersal, settlement and metamorphosis may be impacted by OA directly through suppression of larvae metabolism and indirectly through decreasing settlement cues (Albright and Langdon 2011; Nakamura et al. 2011). While temporary metabolic suppression may be advantageous during acute stress from hypercapnia, OA is a chronic stressor and subsequent decreases in growth and motility may have implications for dispersal and settlement (Pörtner 2008; Albright 2011). Furthermore, OA has been shown to interfere with the settlement cues from crustose coralline algae (CCA) and microbial biofilms. Firstly, CCA substrate coverage declines in OA conditions and secondly, the normally preferred CCA are avoided under increasing pCO_2 (Kuffner et al. 2008; Doropoulos et al. 2012b). These changes in larval physiology and behavior, in combination with ecological community shifts, will have significant implications for successful coral recruitment.

Many studies have investigated the effect of OA on post-settlement growth, calcification and survival as this early life-history stage may be particularly vulnerable to increasing pCO_2 (Cohen et al. 2009; Anlauf et al. 2011; dePutron et al. 2011; Doropoulos et al. 2012a). Initially, a direct impact on calcification may occur due to the change in carbonate chemistry of the surrounding seawater. Thereafter, for corals that acquire their symbiotic dinoflagellates horizontally (environmental

transmission) rather than vertically (maternal transmission), calcification and growth may be further impacted by symbiont fitness. A previous study has shown that *Symbiodinium* uptake was delayed and polyp growth declined under future OA conditions (Suwa et al. 2010). While symbiont fitness has been shown to increase the thermal tolerance of the holobiont, the differential response of the coral holobiont to the effects of OA in relation to the type of *Symbiodinium* they harbour is largely unknown.

Establishment of symbiosis with *Symbiodinium*

The eggs and larvae of *A. millepora* are devoid of *Symbiodinium* but uptake occurs from the environment approximately 5-13 days after settlement (Babcock 1985; Babcock and Heyward 1986). *Symbiodinium* are ingested while the coral host is feeding and are subsequently phagocytosed by the endodermal cells (Schwarz et al. 1999). While vertical transmission ensures symbiont infection, horizontal transmission allows for flexible establishment of symbiosis with different types of *Symbiodinium*, which may have important implications to the adaptation of the coral holobiont to changing environmental conditions (Baird et al. 2007; Abrego et al. 2009; Cumbo et al. 2013). In addition, the short life cycle of dinoflagellate cells (1-3 days) allows *Symbiodinium* to locally adapt to environmental conditions, which may explain instances of phylogenetic similarities across ecological zones (Fitt and Trench 1983; Howells et al. 2012; Noonan et al. 2013).

Techniques for assessing *Symbiodinium* types

There are currently nine broad genetic clades of *Symbiodinium* (A-I) described (Rowan and Powers 1991; Pochon and Gates 2010), which contain genetic and ecologically distinct types, some of which are considered species (LaJeunesse et al. 2004; Sampayo et al. 2009). The clade lineages were originally designated using chloroplast and mitochondrial DNA sequences yet these classifications were not ecologically distinct (Sampayo et al. 2007; Frade et al. 2008). Utilising more rapidly evolving regions of DNA, such as internal transcribed spacer regions of the rDNA (ITS1 and ITS2), has revealed significant phylogenetic *Symbiodinium* types within these clades (Sampayo et al. 2009).

Denaturing gradient gel electrophoresis (DGGE) analysis of the ITS1 rDNA region is a technique found to differentiate high species diversity in comparison to single stranded conformation polymorphism (SSCP) of the ITS1/2 regions or restriction fragment length polymorphism (RFLP) of the large subunit (LSU or 28S) and small subunit (SSU or 18S) of rDNA (Sampayo et al. 2009). Accordingly, DGGE analysis of ITS1 was used in chapter 3 of this thesis. The double stranded DNA is separated by an increasing gradient of denaturing chemicals and migrates across the gel at a different rate with sensitivity for a single-base pair mutation (Muyzer 1999). Bands are subsequently excised from the gels for direct sequencing in order to confirm the *Symbiodinium* type (LaJeunesse et al. 2003). Although quantitative polymerase chain reaction (qPCR) techniques have recently been developed to assess background type populations, the primers developed to date can only differentiate to the clade level (Mieog et al. 2007; Correa et al. 2009; Mieog et al. 2009).

Research Objectives and Thesis Outline

The major objective of this research thesis is to improve our understanding of the physiological response of scleractinian corals to future pCO_2 conditions in an ecological context. In particular, this project investigates the impact of ocean acidification on the productivity and bleaching response of reef-building corals at various life-history stages and across spatial scales. This research will provide insight to policy makers and reef managers in relation to coral reef ecosystem changes that may occur over the coming century.

Chapter 1 provides a review of the literature and techniques relevant to this thesis, including its significance and research objectives.

Chapter 2 presents a working hypothesis towards the mechanism of coral bleaching under ocean acidification. This research investigates the response of *A. aspera* to increasing pCO_2 with concomitant high light conditions and presents a conceptual model linking photorespiration to CO_2-induced bleaching and productivity loss.

Chapter 3 characterises the diurnal variation in carbonate chemistry at sites around Lizard Island and links this to the OA response of *A. millepora* collected from each of these sites. It was hypothesized that greater diurnal variation in carbonate chemistry may improve resilience of scleractinian corals to future OA conditions. This chapter highlights that site-specific physiological trade-offs may influence the response of reef-building corals to future OA scenarios.

Chapter 4 reports a bleaching response in *A. millepora* juveniles under future OA conditions. The effect of OA on coral juveniles is hypothesised to impact *Symbiodinium* uptake and photochemical efficiency. This study links the photo-physiology and bleaching response with recruits containing a dominant population of *Symbiodinium* type D1 or D1-4, with potential implications for post-settlement survivorship and population dynamics.

Chapter 5 recapitulates the key findings of this thesis and discusses the results in the light of ecological implications for the Great Barrier Reef. The synopsis outlines the effect of OA on the photo-physiology, productivity, calcification, reproduction and symbiont acquisition of reef-building corals. Future avenues for research are suggested based on new research gaps identified in this thesis with the aim to continue to provide up-to-date scientific information to policy makers and reef managers.

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Chapter 2: A working hypothesis to describe the mechanism of coral bleaching under ocean acidification

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Ocean acidification, climate change, photorespiration, coral bleaching, productivity, photosynthesis, respiration, photoacclimation, *Symbiodinium*

Abstract

Ocean Acidification has been shown to lower the thermal bleaching threshold and reduce productivity of reef building corals, yet the mechanism of this process remains unclear. To clarify this issue and to better understand if a CO_2 bleaching threshold exists, we investigated the photoacclimatory response of *Acropora aspera* to CO_2 enrichment under high light conditions. Pulse Amplitude Modulation (PAM) fluorometry indicated that there was no treatment effect on the excitation pressure of Photosystem II (PSII), suggesting that PSII is not the initial site of dysfunction. We suggest that a CO_2-induced bleaching mechanism may be initiated by dinoflagellate cell proliferation due to relaxation of carbon limitation, as exhibited by *A. aspera* exposed to the conservative (IPCC A2) CO_2 scenario (pH 7.8 ± 0.1). In contrast, under the business-as-usual (IPCC A1FI) CO_2 scenario (pH 7.6 ± 0.1), dinoflagellate cell density decreased by 72% and the maximum rate of areal net photosynthesis ($P_n$max) declined significantly by 25% in comparison to controls. Overall, the photosynthesis: respiration ratio did not significantly change and remained greater than one for all treatments, suggesting that the host retained autotrophic capacity and that down-regulation of the host carbon concentrating mechanism is not the likely cause of CO_2-induced bleaching. Despite high light conditions, increasing CO_2 did not trigger increased thermal dissipation through the xanthophyll cycle nor increased capacity for thermal dissipation through the xanthophyll pool. The loss of productivity under the A1FI scenario may be partially explained by the significantly increased Chlorophyll *a* (Chl *a*) per cell yet decreased Chl *a* per phaeophytin in the remnant dinoflagellate cells, representing a change in the photosynthetic electron transport pathway. We describe a conceptual model linking the potential decline in photorespiration, a key photoprotective mechanism, with CO_2-induced bleaching. Overall, this study describes fundamental physiological aspects of CO_2-induced bleaching and productivity loss and recommends that management strive to cap anthropogenic CO_2 emissions below IPCC A2 CO_2 levels predicted for the end of the century in order to retain the socio-economic benefits of coral reefs.

Introduction

Atmospheric carbon dioxide (CO_2) levels have recently reached 400 ppm (Jones 2013), compared to 280 ppm before the Industrial Revolution, presenting a significant challenge to Scleractinian corals firstly due to warming associated with radiative forcing in the atmosphere and secondly by changing the carbonate chemistry of the oceans (Caldeira and Wickett 2003; Canadell et al. 2007; Hoegh-Guldberg et al. 2007). The former is implicated in rising sea surface temperatures and the phenomenon of coral bleaching, which is the loss of photosynthetic symbionts (dinoflagellates, genus: *Symbiodinium*) or their pigments from coral tissue (Brown 1997; Hoegh-Guldberg 1999). The second effect leads to the process of ocean acidification (OA), a decrease in pH and carbonate ions (CO_3^-) that is detrimental to formation of the coral calcium carbonate ($CaCO_3$) skeleton (Erez et al. 2011). While little research has looked at the effect of OA on the primary productivity of *Symbiodinium*, some studies have reported a decline (Reynaud et al. 2003; Anthony et al. 2008) while others have reported no change (Leclercq et al. 2002; Schneider and Erez 2006), which is likely a reflection of diversity amongst species and different experimental manipulations. It has recently been suggested that OA can lower the thermal bleaching threshold of *Acropora intermedia* and *Porites lobata*, two ubiquitous reef-building corals on the Great Barrier Reef (Anthony et al. 2008). A further study demonstrated that OA caused down-regulation of the first enzyme involved in photorespiration and increased thermal dissipation, which are photoprotective mechanisms of the symbiont; however, this experiment was performed under low light (Crawley et al. 2010). In the present study, we assess the response of *Acropora aspera* under high light conditions and future IPCC projections of OA in order to determine the photo-physiological pCO_2 threshold. Furthermore, we explore the potential mechanism of CO_2-induced bleaching using a conceptual model.

The mechanism of thermal coral bleaching is well studied, although debate still exists about whether the site of dysfunction lies within Photosystem II (PSII) (Warner et al. 1999) or downstream at the site of carbon fixation by the enzyme RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase) (Jones et al. 1998; Leggat et al. 2004). Light-induced photodamage is also exacerbated by thermal suppression of the protein synthesis involved in PSII repair (Takahashi et al. 2008), which was shown to

occur when Adenosine Triphosphate (ATP) synthesis and/or RuBisCO were chemically inhibited (Takahashi and Murata 2008). Furthermore, RuBisCO has reduced activity under thermal stress (Lilley et al. 2010). Regardless of the starting point, a blockage in the photosynthetic pathway will ultimately lead to similar symptoms, such as an increase in excitation pressure (Q_m) (Iglesias-Prieto et al. 2004). An increase in light beyond the capacity of PSII or RuBisCO, leads to an increase in reactive oxygen species (ROS) and by-products of photosynthesis. There are several photoprotective cycles to prevent build up of these ROS, such as non-photochemical quenching (NPQ), photorespiration, chlororespiration, Photosystem I (PSI) cyclic electron transport and the water-water cycle (Heber et al. 1996; Niyogi 1999; Badger et al. 2000; Nixon 2000). In addition, photoinhibition itself may be an indication of inadequate photoprotection (Matsubara and Chow 2004; Murchie and Niyogi 2011; Hill et al. 2012). Once the capacity of these photoprotective mechanisms along with the antioxidant and scavenging systems are exceeded, ROS damage occurs to the photosynthetic apparatus and surrounding pigments and lipids (Weis 2008). The ROS may also damage the host coral tissue, thereby triggering a cell signalling cascade leading to programmed cell death or necrosis of host cells containing the symbiont (Dunn et al. 2004) or leading to expulsion of the dinoflagellate from the coral host (Weis 2008). While thermal bleaching may occur due to the concomitant high photon flux, which exceeds the rate of photoprotective, scavenging and repair mechanisms, the process of CO_2-induced bleaching has not been elucidated.

Photoprotective mechanisms can function to quench light prior to primary photochemistry (e.g. xanthophyll cycle) (Demmig-Adams and Adams 1996), to cycle electrons during photosynthesis (e.g. PSI cyclic electron transport and the water-water cycle) (Endo and Asada 2006), or to increase substrate availability downstream of the photosynthetic light reactions (e.g. photorespiration) (Niyogi 1999). The xanthophyll cycle contributes to NPQ that thermally dissipates excess light energy within dinoflagellates through the de-epoxidation of diadinoxanthin (Dn) to diatoxanthin (Dt), and therefore diverts energy from downstream assimilatory carbon fixation. Cyclic electron transport is also a non-assimilatory but functions to produce the pH gradient across the thylakoid membrane, which generates ATP and partly triggers the xanthophyll cycle (Miyake et al. 2005). On the other hand, as photorespiration generates 19% more Adenosine diphosphate (ADP) and 50% more Nicotinamide

adenine dinucleotide (phosphate) ($NAD(P)^+$) than carbon fixation, it can relieve blockage downstream from the light reactions of photosynthesis (Badger et al. 2000; Ort and Baker 2002) (Figure 1). This is explained by the ADP and NAD(P)+ outputs of the following equations:

O_2 fixation by RuBisCO: 12 O_2 + 12 RuBP + 57 ATP + 18 $NADH_2$ + 18 NADP = 18 G3P + 57 ADP + 57 P + 36 $NAD(P)^+$ + 6 CO_2

CO_2 fixation by RuBisCO: 12 CO_2 + 12 RuBP + 48 ATP + 24 NADPH = 24 G3P + 48 ADP + 44 P + 24 $NAD(P)^+$

Where RuBP is Ribulose-1,5-bisphosphate, the substrate acted upon by the RuBisCO enzyme and G3P is Glyceraldehyde 3-phosphate, a triose from which all hexose sugars are synthesised, and P is inorganic phosphate. The primary role of photorespiration is to remove the toxic compound glycolate (Figure 1). Additional benefits of photorespiration include removal of O_2, which could otherwise potentially form ROS, and regeneration of CO_2, thereby improving ability for carbon fixation. Photorespiration is dependent on the ratio of CO_2:O_2 at the site of RuBisCO and previous research has shown that high CO_2 conditions in the seawater (1100 ppm CO_2) led to a 50% down-regulation of the first enzyme in the *Symbiodinium* photorespiratory pathway in *Acropora formosa* (Crawley et al. 2010). Here we consider the impact of this significant loss of an alternate electron sink under high light, as opposed to low light conditions.

In addition to their photoprotective mechanisms, dinoflagellates have the ability to photoacclimate in order to reduce photoinhibition and photodamage (Niyogi 1999); through differential survival over generations this could lead to photoadaption (Ralph et al. 2002). Photoacclimation can occur on short time scales (seconds to minutes), for example through a change in enzyme activity (Lilley et al. 2010). Over longer time scales (hours to days), photoacclimation may occur through reduced Chlorophyll *a* (Chl *a*) concentration in the Light Harvesting Complexes (LHCs) to decrease light captured (Ralph et al. 2002) or increased xanthophyll to Chl *a* ratio to increase ability for thermal dissipation (Warner and Berry-Lowe 2006; Kramer et al. 2013) or changes in the photorepair process (Takahashi et al. 2008; Takahashi et al. 2013). In

this experiment, we have captured short-term and long-term photoacclimatory changes and although long-term, trans-generational OA experiments would be required to assess the capacity for photoadaptation, an inability to photoacclimate effectively would not provide a selective advantage.

The energetic costs of photoprotection, photoinhibition, photoacclimation and repair of photodamage represents a significant trade-off to the energy obtained under high light conditions (Hoogenboom et al. 2009). Despite the fact that CO_2 is usually a limiting substrate for photosynthesis, decreased coral productivity has been observed under CO_2 enrichment, a result likely confounded by either increased dinoflagellate cell density leading to increased competition for resources (Reynaud et al. 2003; Wooldridge 2009) or bleaching (Anthony et al. 2008). A decrease in areal productivity under high CO_2 may represent a conundrum for the holobiont as the symbionts incur increased energetic costs of photoacclimation, such as pigment synthesis, and additional energy is required to calcify the coral skeleton under OA conditions (Jokiel 2011).

To form a better understanding of coral acclimation to high CO_2 conditions, we examined branches of the reef flat coral, *Acropora aspera*, during exposure to future predicted levels of pCO_2 enrichment over 10 days. We assessed the photo-physiology using Pulse Amplitude Modulation (PAM) fluorometry throughout the experiment and measured O_2 respirometry upon completion. In order to clarify the role of OA in coral bleaching and productivity loss, we propose a conceptual model linking the potential loss of photorespiration in a high CO_2 world. This model provides mechanistic knowledge to assist resource managers in defining the pCO_2 threshold for future coral survival.

Materials and Methods

Experimental Approach

In October 2008, 36 branches of *Acropora aspera* (blue morph) were collected from Heron Island reef flat (23°26.77' S, 151°54.80' E), which is exposed at low tide (maximum irradiance ranging 1400 – 2300 μmol quanta m^2 s^{-1}). The branches were hung by nylon line in shaded outdoor aquaria (maximum irradiance ranging 720 -

1040 μmol quanta m^2 s^{-1} and average daily light 300 μmol quanta m^2 s^{-1}) to acclimate for 5 days. Following acclimation, the branches were divided among 4 replicate tanks for the control and treatments (n = 3). The seawater carbonate chemistry was maintained via a computer-controlled solenoid valve system (Aquatronica-AEB Technologies, Cavriago, Italy), which bubbled CO_2 to levels representing Intergovernmental Panel on Climate Change (IPCC) CO_2 scenarios for the year 2100 (IPCC 2007). The A1FI treatment was pH 7.6 ± 0.1, the A2 treatment was pH 7.8 ± 0.1 and the control measured pH 8.1 ± 0.2. Variation in the controls was naturally higher (McElhany and Shallin Busch 2013) due to diel fluctuation in carbonate chemistry on Heron Island reef flat (Kline et al. 2012). The pH sensor (InPro4501VP, Mettler Toledo, Langacher, Switzerland), calibrated with NBS scale standard buffers (Mettler Toledo), was used to continuously monitor the pH in the 200 L sumps and routinely verify the pH in treatment aquaria. We measured the Total Alkalinity (A_T) throughout the experiment following the standard operating procedures of Dickson et al (2007) performing the Gran titration (Gran 1952; Dickson et al. 2007) as described in Kline et al (2012) using a titrator (T50, Mettler Toledo, Langacher, Switzerland). In order to characterise the carbonate chemistry of the treatments (Table 1), this data along with the ambient temperature (25°C), pressure (10.16 dbars) and salinity (35 PSU), was input into the CO2calc program (Robbins et al. 2010). For the calculations we specified the total hydrogen ion scale, the carbonic acid dissociation constants of Mehrbach et al (1973) as refit by Dickson & Millero (1987) and the sulfonic acid dissociation constant of Dickson (1990).

Chlorophyll Fluorescence

An imaging-PAM (iPAM, Walz GmbH, Effeltrich, Germany) was used to measure Chl fluorescence. We measured the maximum dark-adapted quantum yield of PSII photochemistry (F_v/F_m) at dusk and the effective quantum yield of PSII photochemistry ($\Delta F/F_m'$) at noon with the actinic light set at 925 μmol quanta m^{-2} s^{-1}. Excitation pressure (Q_m) was calculated as $Q_m = 1 - [(\Delta F/F_m') \cdot (F_v/F_m)^{-1}]$ (Iglesias-Prieto et al. 2004), This was conducted on days 1, 5 and 7 of the experiment with the same branch region selected across all time points (Ralph et al. 2005).

Respirometry

After 10 days, we measured the O_2 evolution and consumption within custom-made acrylic respirometry chambers following the method of Crawley et al (2010). The water bath was set to 25°C, the ambient seawater temperature at Heron Island in October 2008. Corals were dark-acclimated for one hour and the chambers were filled with 0.22 μm filtered treatment water prior to the start of the respirometry. To obtain steady-state dark respiration (R_{dark}), respirometry began with 10 minutes in the dark. Then the actinic light from the iPAM followed 10 minutes steps at 20, 55, 110 μmol quanta m^{-2} s^{-1} to obtain an estimate of alpha (α), the photosynthetic efficiency at sub-saturating irradiance. Next, the maximum rate of net photosynthesis ($P_n$max) was determined as the maximum rate of O_2 evolution following 10 minutes at 925 μmol quanta m^{-2} s^{-1}, and 5 minute steps at 1075 and 1250 μmol quanta m^{-2} s^{-1}. Shorter steps at the higher irradiances were sufficient to obtain steady-state rates of O_2 evolution. Finally, Light-Enhanced Dark Respiration (LEDR) was determined as the rate of O_2 consumption during 10 minutes in the dark, which began after approximately 1 minute of darkness. Respirometry rates were normalized to the respective surface area of the coral branches.

Data from the respirometry assays were used to generate Photosynthesis-Irradiance (P: E) Curves using the hyperbolic tangent function (Jassby and Platt 1976)

$$Pg = (P_n\text{max} + \text{LEDR}) \tanh (E/E_k) \quad (1)$$

Where Pg is Gross Photosynthesis in μmol O_2 cm^{-2} h^{-1}, E is Irradiance in μmol quanta m^{-2} s^{-1} and E_k is the minimum saturating irradiance at which $P_n$max and α intercept (Jassby and Platt 1976; Chalker 1981). We calculated daily Pg by integrating Eq. 1 over the average hourly light curve recorded by the light loggers throughout the experiment.

$$\text{Daily Pg} = \int_{t=0}^{24} (P_n\text{max} + \text{LEDR}) \tanh [E(t)/E_k]\, dt \quad (2)$$

Furthermore, we determined the ratio of daily Pg to daily respiration (daily Pg:R), based on LEDR occurring during the 14 hours of daylight and R_{dark} occurring for the 10 hours at night.

Cell Counts and Pigment Profile

The coral branches were frozen in liquid nitrogen on the last day of the experiment at midday and were later water-piked using a high pressure airgun with injection of 0.22 μm filtered seawater. The samples were centrifuged (4,500 x *g*, 5 min) to separate the dinoflagellate pellet from host tissue. One aliquot was made for dinoflagellate cell counts using a 0.100 mm Tiefe Depth Profondeur hemocytometer (0.0025 mm^2). A second aliquot was made to quantify the photosynthetic pigments, including Chl *a*, Dd, Dt and phaeophytin (*phe*), using High Performance Liquid Chromatography (HPLC) following the methods of Zapata et al (2000) and Dove et al. (2006). Cells and pigments were normalized to the surface area of the branch, which was measured using a double-dipping wax method appropriate for skeletons with deep corallites (Stimson and Kinzie 1991; Veal et al. 2010).

Statistical Analysis

The CO_2 treatment effect was assessed by the PERMANOVA procedure in PRIMER using a mixed model with treatment fixed and tank nested in treatment (Anderson 2001; McArdle and Anderson 2001). The variables were log transformed and Euclidean distance resemblance matrices were created. For the respirometry, pigments and cell count data the Pseudo-F distribution was created by unrestricted permutation of the raw data. For the chlorophyll fluorescence data, we used a repeated measures design with the highest-order interaction term excluded to overcome the issue of temporal replication within cells (Gurevitch and Chester 1986) and the Pseudo-F distribution was created by permutation of residuals under a reduced model. Monte-Carlo P-values were used for all pair-wise comparisons (Anderson and Robinson 2003).

Results

Chlorophyll Fluorescence

Overall Q_m increased with time and there was a significant treatment x time interaction (Figure 2) (See Supplementary Materials S1 for full statistic results). Pair-wise comparisons for the interaction at the level of treatment indicated that on day five the A1FI scenario had reduced Q_m compared to the A2 scenario (Pseudo-$F_{2,9}$ = 3.32, $p_{perm} < 0.02$, pairwise A1FI < A2, $p_{MC} < 0.03$). However, there were no significant differences in Q_m due to treatment after seven days. Pairwise comparisons for the interaction at the level of time revealed that excitation pressure was highest on day seven for all treatments. For the control and A2 scenario, Q_m was higher on day five compared to day one (Pairwise, Control and A2 day 1 < day 5 < day 7, $p_{MC} < 0.02$, A1FI day 5 < day 7, $p_{MC} < 0.03$).

Respirometry

$P_n$max significantly declined by 29% under the A1FI scenario in comparison to the A2 scenario (Pseudo-$F_{2,9}$ = 4.73, $p_{perm} < 0.04$, pairwise A1FI < A2, $p_{MC} < 0.04$) (Figure 3A). Although LEDR declined under the A1FI scenario, high variation precluded statistical significance (Pseudo-$F_{2,9}$ = 2.83, $p_{perm} > 0.07$) (Figure 3B). Furthermore, there was no significant effect of the CO_2 treatment on R_{dark} (Pseudo-$F_{2,9}$ = 0.63, $p_{perm} > 0.5$). E_k averaged 682 ± 138 μmol quanta m^{-2} s^{-1} and was not significantly different across CO_2 treatments (Pseudo-$F_{2,9}$ = 1.02, $p_{perm} > 0.4$). Similarly, CO_2 treatment did not significantly affect α, the efficiency of photosynthesis at sub-saturating light (Pseudo-$F_{2,9}$ = 0.50, $p_{perm} > 0.6$) nor the daily Pg:R ratio, which was 1.2 ± 0.1 (Pseudo-$F_{2,9}$ = 2.99, $p_{perm} > 0.08$) (Table S1).

Cell Counts and Pigment Profile

Areal dinoflagellate cell density significantly decreased by 72% under the A1FI scenario compared to the control (Pseudo-$F_{2,9}$ = 14.60, $p_{perm} < 0.01$, pairwise A1FI < A2 = Control, $p_{MC} < 0.03$). The estimated component of variation analysis attributed 80% of the variation in dinoflagellate cell density to CO_2 treatment (Figure 4). In contrast, Chl *a* per cell significantly increased by 50% in the remnant dinoflagellate cells under the A1FI scenario compared to the control (Pseudo-$F_{2,9}$ = 14.98, $p_{perm} < 0.01$, pairwise A1FI < A2 = Control, $p_{MC} < 0.03$). There were no changes in the

xanthophyll pool, at 0.20 ± 0.01 (Dt + Dd) Chl a^{-1}, nor the xanthophyll cycle, at 0.19 ± 0.01 Dt / (Dt + Dd) (Pseudo-$F_{2,9}$ = 0.60, p_{perm} > 0.55; Pseudo-$F_{2,9}$ = 0.61, p_{perm} > 0.64) (Figure 5). Yet, there was a significant, 23% decline in the xanthophyll pool normalised to *phe* under the A1FI scenario (Pseudo-$F_{2,9}$ = 7.40, p_{perm} < 0.02, pairwise A1FI < A2 = Control, p_{MC} < 0.01). Similarly, Chl *a*: *phe* significantly declined by 22% under the A1FI scenario (Pseudo-$F_{2,9}$ = 7.69, p_{perm} < 0.02, pairwise A1FI < A2 = Control, p_{MC} < 0.01).

Discussion

This study has attempted to elucidate the mechanisms behind coral bleaching and productivity loss due to OA. It appears that CO_2-induced bleaching may initially begin with dinoflagellate proliferation upon relaxation of CO_2-limitation as observed in *A. aspera* branches exposed to IPCC A2 scenario CO_2 conditions (pH 7.8) (Figure 4). Under the high light conditions of this experiment, symbiont density decreased in *A. aspera* branches under the IPCC A1FI CO_2 conditions (pH 7.6) and this was most likely due to photoinhibition, as exemplified by high Q_m values (Iglesias-Prieto at al. 2004). It is plausible that the increased symbiont cell density at pH 7.8 increased the susceptibility of the coral to bleaching, damage or stress, which was observed at pH 7.6. Increased symbiont cell density has similarly been shown as a precursor to thermal bleaching (Cunning and Baker 2013). Verification of this mechanism could be observed by following symbiont density and productivity through time during increased pCO_2 conditions. As described in Crawley et al (2010), the loss of photorespiration, a key photoprotective mechanism, may have led to increased photoinhibition and photodamage, thereby triggering the expulsion of *Symbiodinium* cells. In addition, the photoacclimation process undertaken by the *A. aspera* branches may explain the loss of productivity under the A1FI scenario. A decrease in Chl *a*: *phe*, where *phe* is the first electron acceptor in the reaction centre of PSII, indicates a change in electron transport capacity (Klimov and Krasnovskii 1981; Vredenberg 2011) (Figure 5). Alternatively, as *phe* is a degradation product of Chl *a*, the increased *phe*: Chl *a* may represent loss of functional Chl *a* (Matile et al. 1999). This study confirms the detrimental effect of CO_2 enrichment under high light conditions, which could impact future ecological services provided by structurally complex, fast-growing, branching corals such as *Acropora aspera*.

Under conceptual models of thermal bleaching, ROS production occurs due to damaged PSII or impairment of CO_2 fixation by Rubisco, both of which lead to over-reduction in the photosystems of dinoflagellates (Iglesias-Prieto et al. 1992; Jones et al. 1998; Warner et al. 1999; Hill et al. 2004). In support of this, CO_2-induced bleaching has only been observed with concomitant high irradiance (Anthony et al. 2008; Kaniewska et al. 2012) and not under low light conditions (Crawley et al. 2010; Putnam et al. 2013; Wall et al. 2014). Just as environmental stressors can tip the balance between photodamage and repair mechanisms (Takahashi and Murata 2008), a CO_2-induced decline in photorespiration represents a loss of photoprotection and may also suppress repair mechanisms (Takahashi et al. 2007; Crawley et al. 2010). In recent studies, a decrease in PGPase gene expression was only observed under the business-as-usual (IPCC A1FI) CO_2 scenario (Crawley et al. 2010) rather than conservative CO_2 scenarios (Crawley et al. 2010; Ogawa et al. 2013; Putnam et al. 2013) suggesting a CO_2 threshold at approximately 1000 μatm, but only in the absence of other stressors. For example, when considering that excess dinoflagellate cell density may increase the susceptibility of corals to thermal bleaching (Cunning and Baker 2013), the IPCC A2 CO_2 conditions are also detrimental to coral considering the predicted rise in global SSTs. Therefore; we suggest that the ecological impact of increasing pCO_2 will be intricately dependent on concomitant environmental factors.

An alternative hypothesis for CO_2-induced bleaching implicates the carbon concentrating mechanism (CCM) of the coral host as the initial site of dysfunction (Wooldridge 2013), yet the results of this study do not align with this perspective. A generally stable symbiosis is characterized by the uncoupling of dinoflagellate photosynthesis and growth due to nutrient-limitation, which hinders amino acid synthesis needed for cell division (Dubinsky and Berman-Frank 2001). Despite high variability, dinoflagellate cell density did increase under the A2 scenario (pH 7.8) (Figure 4), which suggests that cell division was promoted due to release from CO_2-limitation in nutrient-replete conditions. The assumption of nutrient-replete conditions at Heron Island is valid considering the high nutrient inputs associated with the seabird population and human effluent treatment on this coral cay (Staunton-Smith and Johnson 1995) in addition to the nutrients provided from fish defecation (Meyer

and Shultz 1985). The host-CCM hypothesis then postulates that this cell division leads to less carbon translocated to the host and therefore less energy to maintain the CCM, paradoxically resulting in CO_2-limitation (Wooldridge 2013). At odds with this hypothesis, carbon translocation per symbiont cell has been shown to increase under increased pCO_2 (pH 7.2) (Tremblay et al. 2013). In the present study, we did not observe a decrease in the daily Pg:R ratio, which remained above one for all treatments suggesting that the coral retained the capacity to operate using autotrophically derived carbon, although the values in this study were slightly lower than the average of 2.4 reported among 70 species of corals (Battey 1992). Nevertheless, a Pg:R ratio greater than one is necessary but not sufficient to conclude autotrophy, as the actual carbon translocated must meet the energy requirements of the host (Muscatine et al. 1981), and a significant amount of carbon may be excreted as mucus and not utilised by host metabolism (Wild et al. 2004). Yet, if carbon translocation to the host declined without heterotrophic supplementary feeding, respiration rates would also decline, and this was not observed in our experiment. In addition, Kaniewska et al. (2012) did not observe a change in expression of the carbonic anhydrase (CA) genes of the host CCM in a similar OA experiment conducted under high irradiance. Ogawa et al. (2013) reported decreased CA expression, yet this occurred subsequent to the decrease in dinoflagellate cell density and was therefore not the trigger of coral bleaching, although CA enzyme activity is needed for verification. Overall, it is highly unlikely that CO_2-limitation due to host CCM dysfunction played a role in CO_2-induced bleaching.

A recent study has implicated the role of nutrient imbalance, specifically phosphate starvation, in coral bleaching due to substitution of phospholipids with presumably less stable sulpholipids (Wiedenmann et al. 2012), yet this is unlikely to have played a role in the CO_2-induced bleaching observed here. These authors suggested that, just as uncoupling of photosynthesis from growth occurs due to departure from the C:N aspect of the "Redfield ratio" (Redfield et al. 1963; Dubinsky and Berman-Frank 2001), departure from the N:P aspect of the "Redfield ratio" may alter the composition of lipid membranes thereby disrupting the functionality of the photosynthetic apparatus (Wiedenmann et al. 2012). A comprehensive seasonal water sampling regime at Heron Island reported benthic N:P ratios ranging from approximately 7:1 in summer to 2:1 in winter, therefore indicating adequate

phosphate levels for balanced growth (Staunton-Smith and Johnson 1995). Accordingly, despite proliferation of the dinoflagellate cells under the IPCC A2 scenario, the nutrient levels were adequate to support the building of stable thylakoid membranes and it is unlikely that this phenomenon influenced in the mechanism of CO_2-induced bleaching.

The increase in Q_m throughout the experiment confirms that the treatments were conducted in high light and indicates that the proportion of closed PSII reaction centres was gradually increasing with time (Maxwell et al. 1995; Iglesias-Prieto et al. 2004) (Figure 2). A previous thermal stress experiment has shown that *Symbiodinium* cell density in *A. aspera* decreased by 50% while Q_m increased by 50% to approximately 0.8 (Fisher et al. 2012), which is in agreement with the Q_m levels in the present study and signifies potential bleaching conditions. After 5 days, the A1FI CO_2 treatment temporarily alleviated Q_m, but this effect was no longer apparent after 7 days suggesting that high CO_2 levels do not permanently increase the proportion of open PSII reaction centres. Exposure to high excitation pressure can improve resistance to photoinhibition provided that there is capacity to reduce the light harvested or increase thermal dissipation (Maxwell et al. 1995). In the A1FI CO_2 treatment, however, the dinoflagellate cells increased Chl *a* per cell, presumably due to the significantly reduced cell density and increased capacity for carbon fixation. Despite high light levels, the xanthophyll cycle may not have been running at full capacity, which has been reported as high as 0.8 Dt / (Dt + Dd) in *A. aspera* under thermal stress (Middlebrook et al. 2008). In the present study, however, increasing CO_2 did not lead to up-regulation in the xanthophyll pool or de-epoxidation, which is in contrast to the increased thermal dissipation observed due to CO_2 enrichment under low light conditions (Crawley et al. 2010). While these studies estimated the daytime xanthophyll cycling conditions, a unique feature of the dinoflagellate Dd-Dt xanthophyll cycle is that it may also function at night. Accordingly, future studies should quantify the nocturnal xanthophyll cycle, which may increase under OA conditions. In the present study, it appears that exposure to high excitation pressure did not allow improved resistance to photoinhibiton.

Photoacclimation can occur via changes in the LHC pigment concentration or alternatively through changes in the number of PSII reaction centres (Falkowski et al.

1981). In the A1FI CO_2 treatment, we observed a decrease in Chl *a: phe*, which implies a decrease in light-harvesting relative to the subsequent transport of excitation energy from P_{680} to Plastoquinone (PQ), assuming the *phe* is located within the PSII reaction centre (Klimov and Krasnovskii 1981; Vredenberg 2011) (Figure 5). In addition, the increased chlorophyll concentration observed in the high CO_2 treatment most likely decreased the efficiency of light capture due to self-shading and increasing the path of excitation energy flow to the PSII reaction centre (Walters 2005) (Figure 4). On the other hand, increased light may have been experienced by the dinoflagellates due to multiple scattering in the coral tissue (Wangpraseurt et al. 2014) and especially by the coral skeleton (Enriquez et al. 2005; Wangpraseurt et al. 2012). Overall, It appears that CO_2 enrichment leads to a cascade of events resulting in suboptimal photoacclimation of the dinoflagellates, which may in turn explain the decline in areal productivity (Figure 3).

This study has described the photo-physiological response of a reef-building coral to enriched CO_2 conditions. In doing so, we have taken significant steps towards unfolding a new mechanism of CO_2-induced bleaching and productivity loss as previously proposed by Anthony et al (2008). It is now clear that increased anthropogenic CO_2 emissions also represent a significant challenge to the photosynthesis of symbiotic dinoflagellates in reef-building corals. While we only observed bleaching in the A1FI CO_2 scenario, even emissions under the A2 CO_2 scenario may increase bleaching susceptibility due to increased dinoflagellate density, as recently shown by Cunning and Baker (2013). The activity and regulation of RuBisCO may also contribute to the response of *Symbiodinium* to CO_2 enrichment and this warrants future investigation. As the present study did not incorporate the thermal effect of increased anthropogenic CO_2 emissions, the results should be interpreted with caution as the additional impact of temperature-induced bleaching may further lower coral resilience. Overall, our findings support the view that CO_2 emissions should be capped to ensure we do not reach the IPCC A2 CO_2 level in order to preserve reef-building corals and their socio-economic benefit for future generations.

Acknowledgements

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Table 1: Carbonate chemistry parameters calculated from the pH, total alkalinity (A_T), temperature (25°C), salinity (35 PSU) and pressure (10.16 dbars) using the program CO2calc (Robbins et al. 2010).

pH	A_T (μmol kg^{-1})	pCO_2 (μatm)	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	CO_2 (μmol kg^{-1})
8.1 ± 0.2	2296 ± 39	372 ± 190	1704 ± 234	241 ± 80	11 ± 5
7.8 ± 0.1	2295 ± 39	789 ± 208	1962 ± 100	136 ± 28	22 ± 6
7.6 ± 0.1	2295 ± 41	1319 ± 335	2073 ± 84	91 ± 19	37 ± 9

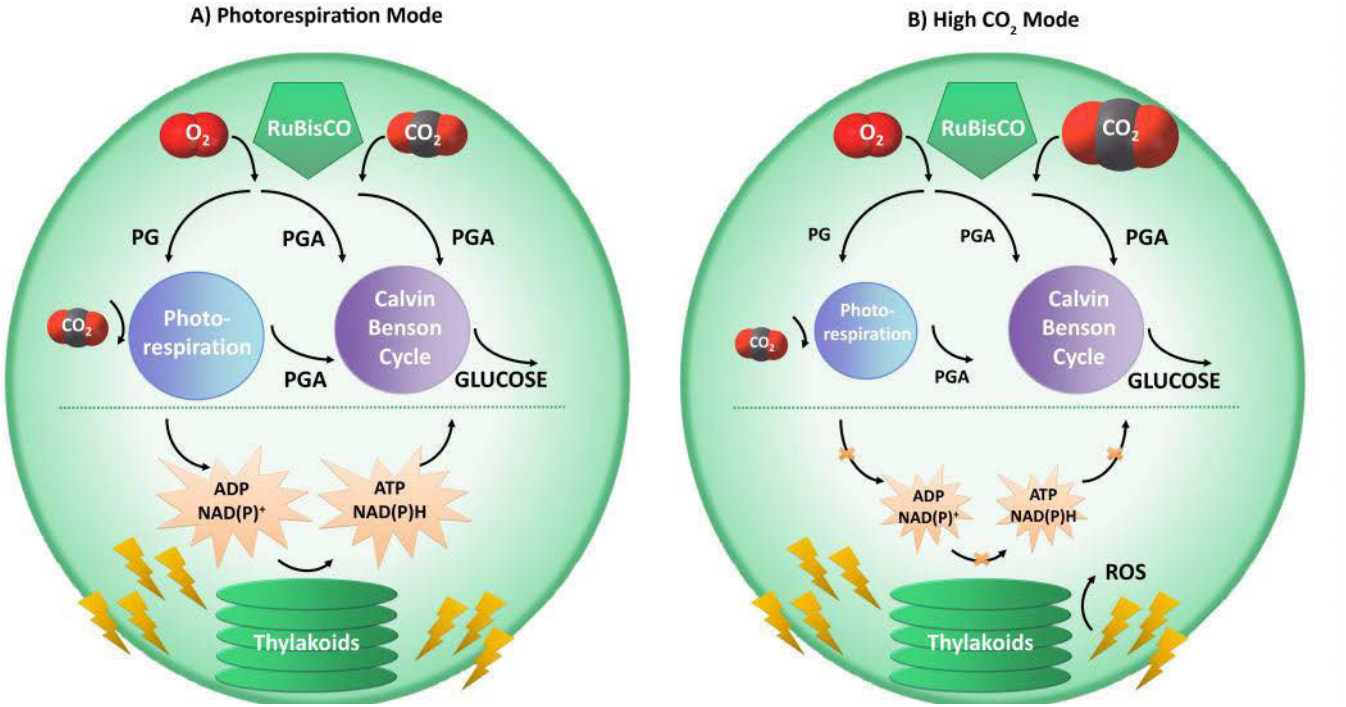

Figure 1: Conceptual model illustrating the photoprotective role of photorespiration. The *Symbiodinium* Form II (Ribulose-1,5-bisphosphate carboxylase oxygenase) does not discriminate between fixation of O_2 or CO_2. O_2 fixation leads to 19% more ADP (Adenosine diphosphate) and 50% more $NAD(P)^+$ (Nicotinamide adenine dinucleotide (phosphate)) than through CO_2 fixation and this can relieve the photosynthetic apparatus from additional photon excitation energy. Under high CO_2 conditions, photorespiration is reduced which leads to less availability of ADP and NAD(P)+ to accept the excitation energy flow from the photosystems in the thylakoid membrane. Therefore, excitation energy reacts with oxygen species leading to formation of Reactive Oxygen Species (ROS), which starts the signalling cascade that may lead to coral bleaching. PGA = Phosphoglycerate; PG = Phosphoglycolate.

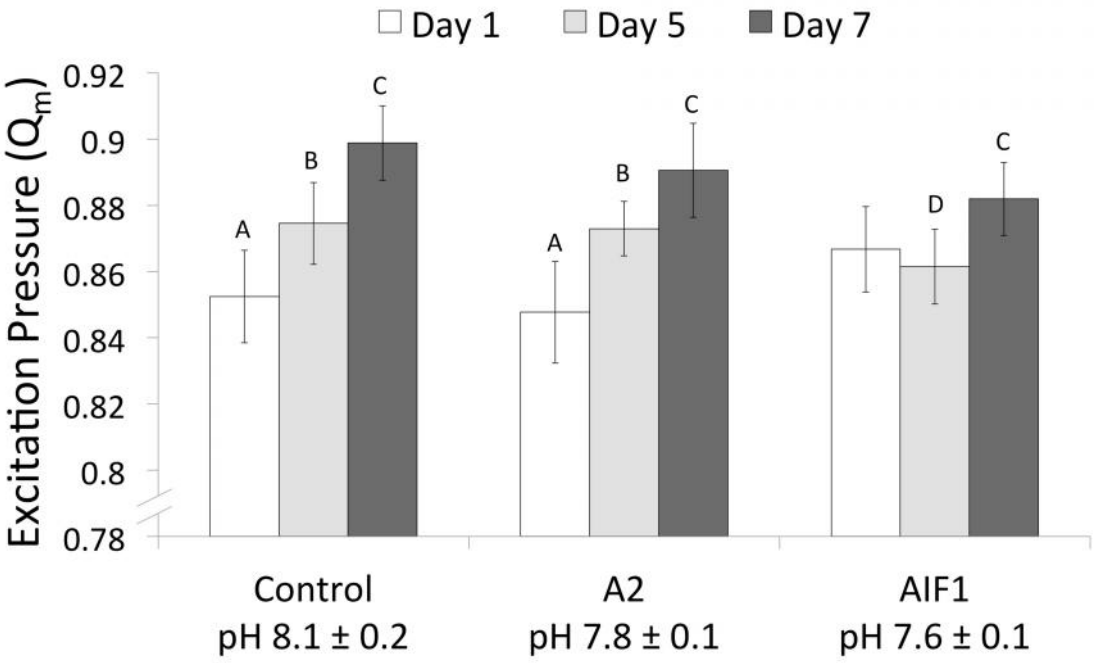

Figure 2: Excitation pressure (Q_m) of photosystem II (PSII) throughout the experiment. On Day 5, A1FI < A2 ($p < 0.03$). For the control and A2 treatment Day 1 < Day 5 < Day 7 ($p < 0.02$). For the A1FI treatment Day 5 < Day 7 ($p < 0.03$). Error bars are standard error (n = 3).

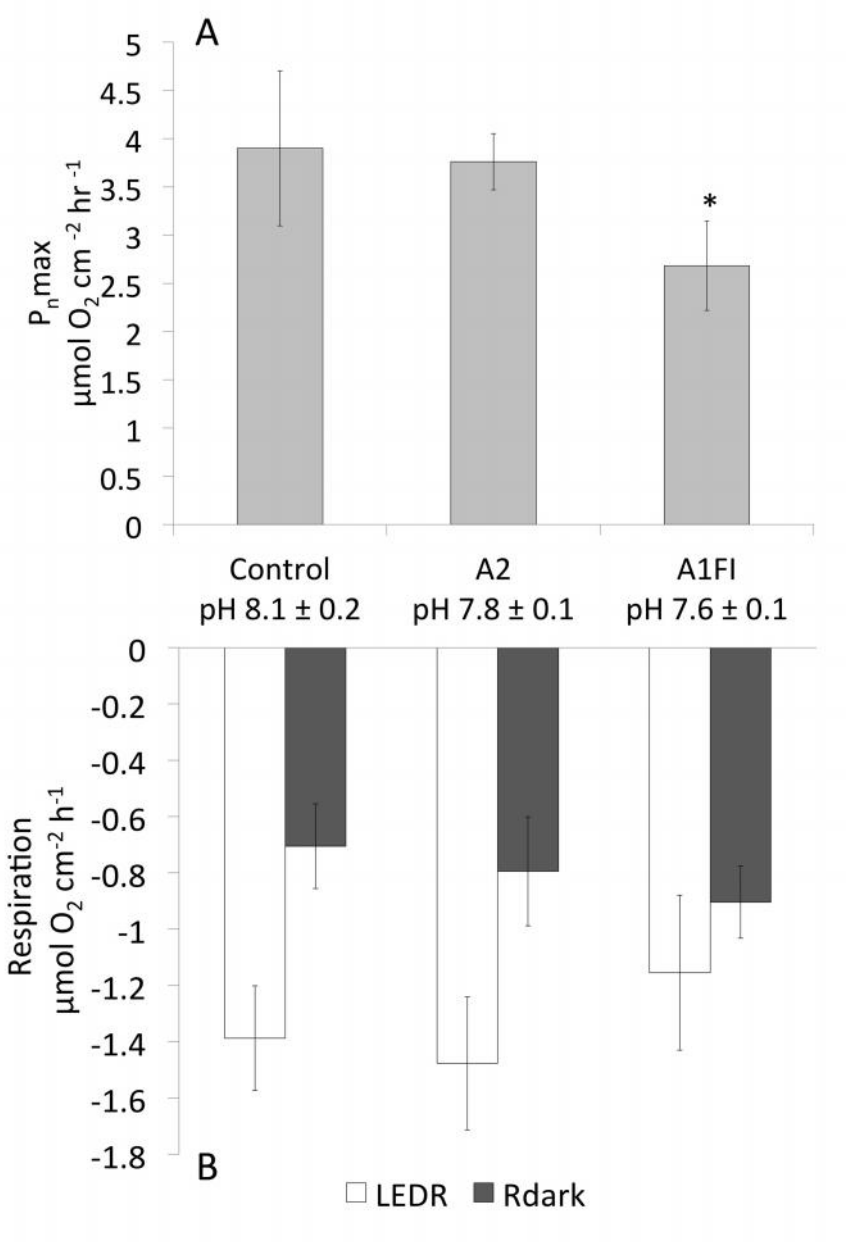

Figure 3: Changes in respirometry under the CO_2 treatments. A) $P_n$max significantly declines under the IPCC A1FI CO_2 scenario ($p < 0.04$). B) There were no significant changes to LEDR or Rdark. Error bars are standard error (n = 3).

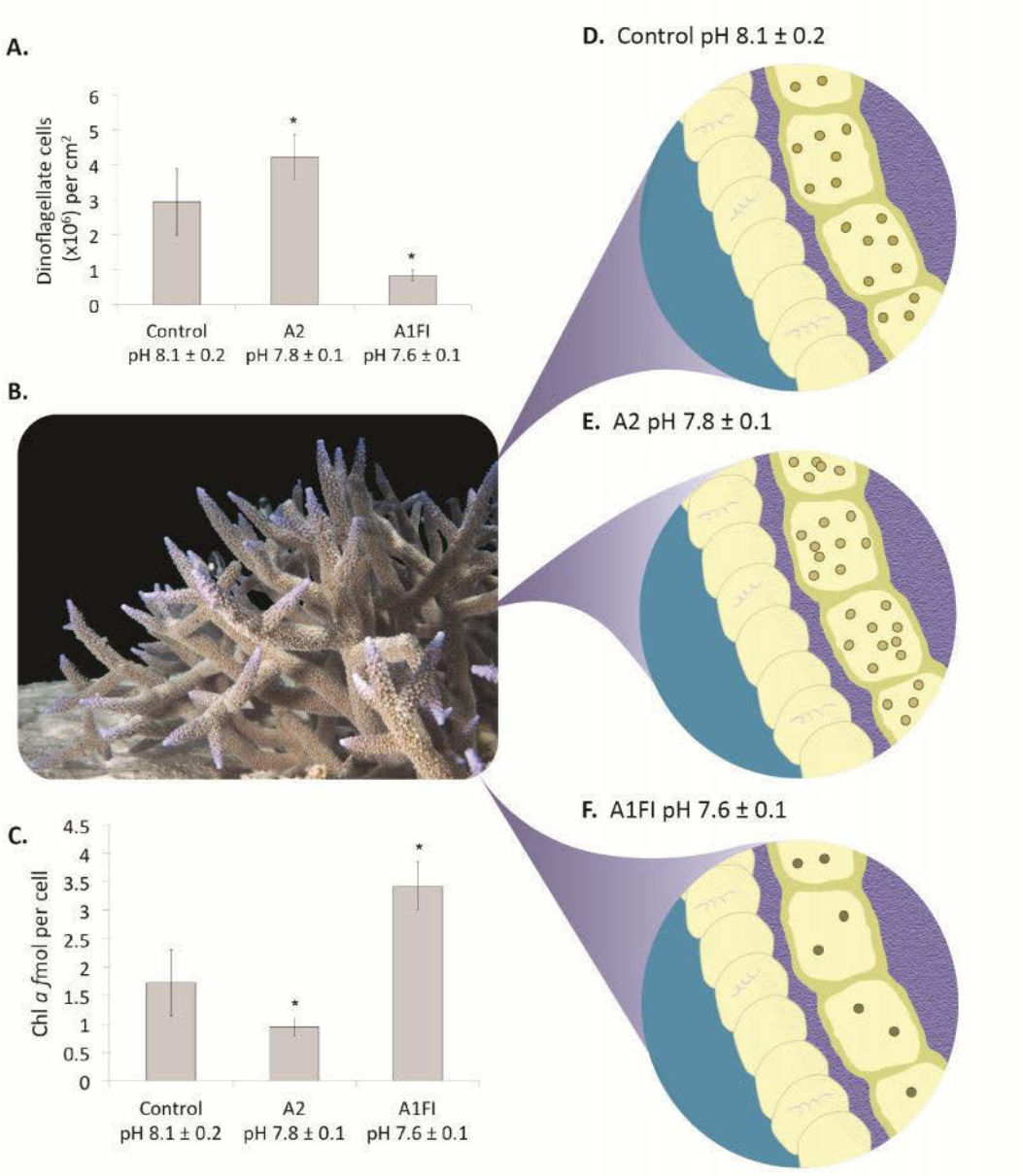

Figure 4: The physiological response of (B) *Acropora aspera* to the CO_2 treatments. (A) Dinoflagellate cell density is significantly decreased under the A1FI IPCC scenario ($p < 0.03$). (C) Chlorophyll *a* (chl *a*) per cell is significantly increased under the A1FI IPCC scenario ($p < 0.03$). Error bars are standard errors ($n = 3$). Insets show conceptual coral tissue cross sections of the ectoderm and mesoglea with dinoflagellate cell density under (D) ambient conditions, (E) with increased dinoflagellate cell density under the A2 scenario and (F) after expulsion of dinoflagellates yet increased chl *a* per cell under the A1FI scenario (Photo: Mark Priest; Drawings not to scale).

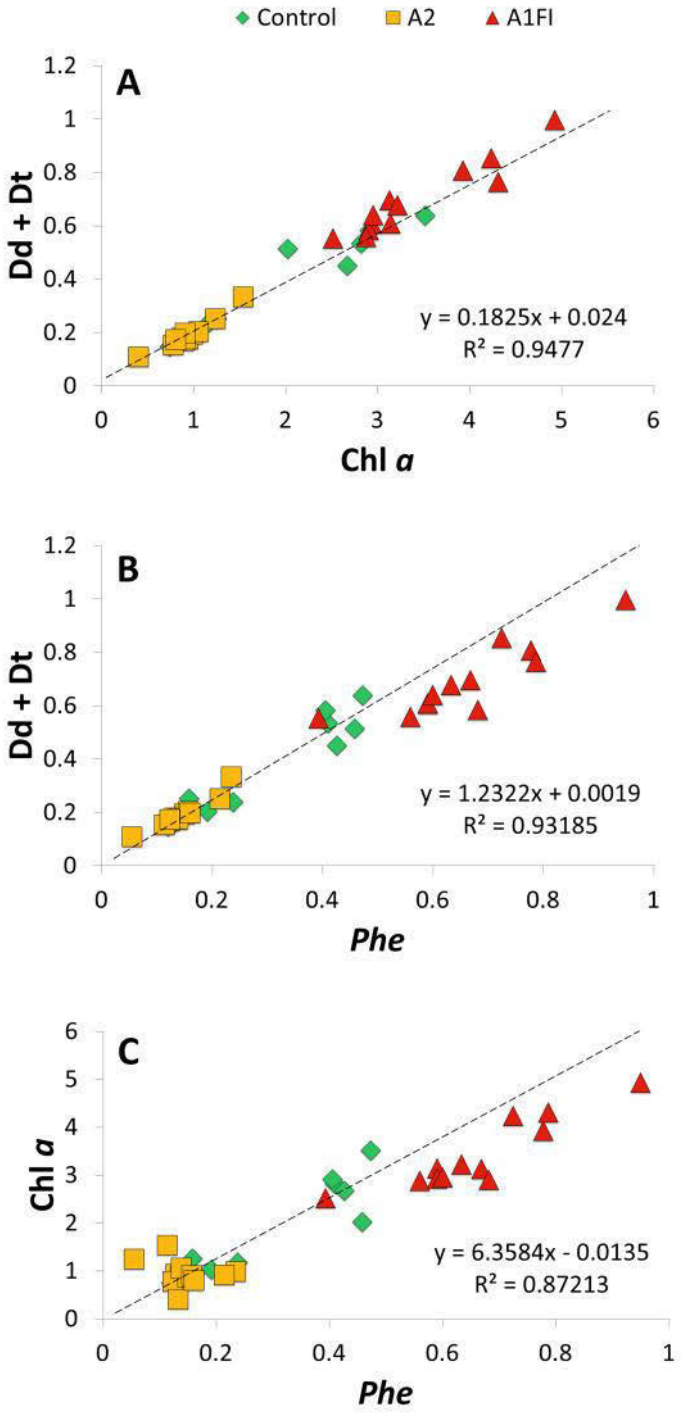

Figure 5: Ratios of photosynthetic pigments in *f*mol per cell. A) The xanthophyll pool, Diadinoxanthin + Diatoxanthin (Dd + Dt), remained in proportion to the quantity of Chlorophyll a (Chl *a*) across all treatments. Yet in the A1FI scenario, both B) the ratio of (Dd +Dt) to pheophytin (*phe*) and C) the ratio of Chl *a* to *phe* significantly declined. Linear regression of control data points overlaid on all graphs.

Table S1: Table of statistical results

Variable	Source of Variation	df	Pseudo F	P(perm)	Pairwise (MC)
R_{dark} (µmol O_2 cm^{-2} h^{-1})	Treatment	2	0.62585	0.5231	
	Tank (Treatment)	**9**	**5.1246**	**0.0011**	
$P_n$max (µmol O_2 cm^{-2} h^{-1})	**Treatment**	**2**	**4.7285**	**0.0352**	A2 > A1FI p=0.0303
	Tank (Treatment)	9	1.987	0.0805	
LEDR (µmol O_2 cm^{-2} h^{-1})	Treatment	2	2.8303	0.0711	
	Tank (Treatment)	9	1.598	0.1669	
Ek (µmol quanta m-2 s-1)	Treatment	2	1.0199	0.4047	
	Tank (Treatment)	**9**	**3.7384**	**0.0048**	
Alpha (µmol O_2 cm^{-2} h^{-1})	Treatment	2	0.50201	0.6672	
	Tank (Treatment)	**9**	**3.7585**	**0.0078**	
Daily Pg:R	Treatment	2	2.9935	0.0895	
(µmol O_2 cm^{-2} day^{-1})	Tank (Treatment)	9	1.0795	0.408	
Cells per cm^2	**Treatment**	**2**	**14.595**	**0.0079**	Control > A1FI p=0.0266
	Tank (Treatment)	**9**	**5.7983**	**0.0007**	A2 > A1FI p=0.0002
Chl *a* *f*mol per cell	**Treatment**	**2**	**14.979**	**0.002**	Control < A1FI p=0.0243
	Tank (Treatment)	**9**	**4.8168**	**0.0013**	A2 < A1FI p=0.0002
Chl *a* per cm^2	Treatment	2	1.7478	0.2249	
	Tank (Treatment)	**9**	**3.5688**	**0.0041**	
Xanthophyll Cycle	Treatment	2	0.60768	0.6451	
Dt (Dd + Dt)$^{-1}$	Tank (Treatment)	9	1.6808	0.0799	
Xanthophyll Pool per Chl *a*	Treatment	2	0.60224	0.5566	
(Dd + Dt) Chl a^{-1}	Tank (Treatment)	9	0.73264	0.6934	
Xanthophyll Pool per Pheophytin	**Treatment**	**2**	**7.3984**	**0.0153**	A2 > A1FI p=0.0022
(Dd + Dt) Pheophytin^{-1}	Tank (Treatment)	9	1.7646	0.1219	
Chl *a* per Pheophytin	**Treatment**	**2**	**7.6892**	**0.0155**	A2 > A1FI p=0.0011
	Tank (Treatment)	9	2.2337	0.0572	Control > A1FI p=0.0501
Fv/Fm	Treatment	2	0.016125	0.9672	Control Time 1 < 5 < 7 p <0.004
	Time	**2**	**25.721**	**0.0001**	A2 Time 1< 5< 7 p<0.03
	Tank (Treatment)	**9**	**5.8978**	**0.0001**	A1FI Time no sig diff
	Treatment x Time	**4**	**4.0468**	**0.0044**	
$Q_m = 1 - [(\Delta F/Fm') \bullet (Fv/Fm)^{-1}]$	Treatment	2	0.052335	0.966	At time 5, A1FI < A2 p=0.0248
	Time	**2**	**30.939**	**0.0001**	Control Time 1 < 5 < 7 p<0.008
	Tank (Treatment)	**9**	**6.3031**	**0.0001**	A2 Time 1 < 5 < 7 p<0.02
	Treatment x Time	**4**	**3.3183**	**0.0133**	A1FI Time 5 < 7 p<0.03

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Chapter 3: Diurnal carbonate chemistry variability impacts the sensitivity of *Acropora millepora* to ocean acidification

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Abstract

The predicted decline in oceanic pH facilitated by increasing anthropogenic CO_2 emissions is expected to challenge the calcification and primary productivity of reef-building corals. Previous research has either focused on the physiological or ecosystem responses with little understanding of the combined impacts. This study aimed to characterise intra-specific variation in the response of *Acropora millepora* to constant ocean acidification (OA) treatments in the context of their exposure to diurnal variation in carbonate chemistry at distinct sites around Lizard Island. Corals collected from the lagoon sites were exposed to more extreme daily changes in carbonate chemistry than corals collected from outside the lagoon. After subsequent exposure to constant CO_2 levels that correspond to IPCC scenario A1FI (pH 7.7 ± 0.05; pCO_2 1020 ± 140 μatm) in an aquaria-based OA experimental system, corals from outside the lagoon increased net photosynthesis per cm^2 (P_{net}), yet there were no significant improvements to the gross photosynthesis to respiration ratio (Pg:R). Furthermore, a site-specific physiological trade-off was exhibited in the form of differential investment in skeletal morphology and tissue thickness with corals from a lagoon-based site having a lower skeletal dry-weight to surface-area ratio, but more protein per cm^2. Overall, these results indicate that corals collected from reefs with varying diurnal fluxes in carbonate chemistry may respond differently to future OA scenarios, suggesting that the biogeochemical cycles of the collection site must be considered in future experimental studies with implications for the predictive power of climate models when applied to coral reefs.

Introduction

The calcification and primary productivity of marine organisms will be affected by changing marine carbonate chemistry, which is occurring due to the increased oceanic absorption of anthropogenic carbon dioxide (CO_2) emissions, leading to the decline in oceanic pH known as ocean acidification (OA) (Caldeira and Wickett 2003; Sabine et al. 2004; Canadell et al. 2007). Since the industrial revolution there has already been a ~ 30% increase in the oceanic concentration of hydrogen, [H^+], with a further 55% rise expected once pCO_2 doubles from 280 μatm to 560 μatm (Jokiel 2011). Under these conditions, research has shown that calcification of scleractinian coral may decline by 10-40%, whereas productivity impacts are variable and the overall response is species-specific, with higher sensitivity to OA occurring during early life stages (Kroeker et al. 2010). The variable response of reef-building coral species to OA has made it difficult to model accurate future projections. This research responds to the critical need to characterise intra-specific variation in the response of the common coral species, *Acropora millepora*, to future OA conditions and investigates key carbonate chemistry variables defining their resilience.

Climate models assume a constant oceanic total alkalinity (A_T), as the predicted rate of pCO_2 increase is too rapid for the slower geochemical feedbacks to achieve carbonate chemistry equilibrium (Raven and Falkowski 1999; Kump et al. 2009; Pandolfi et al. 2011). However, as coral reefs exist in the surface layer of the ocean with community metabolism changing the pCO_2 over diel and seasonal timescales, the impact of increased anthropogenic pCO_2 on the carbonate chemistry of coral reefs is likely to deviate from oceanic models with static A_T (Hofmann et al. 2011; Shaw et al. 2013). The process of calcification reduces the A_T by removing carbonate (CO_3^{2-}) or bicarbonate (HCO^{3-}) ions, but A_T is not affected by the CO_2 byproduct of calcification, which is considered to be a source of CO_2 to the atmosphere around coral reefs (Bates et al. 2001; Fagan and Mackenzie 2007; Kleypas et al. 2011). In addition, this byproduct is also coupled to photosynthesis whereby CO_2 is removed from the water during the day, leading to a rise in the seawater pH, which contributes to the phenomenon known as light enhanced calcification (Yonge and Nicholls 1931). The diel cycle of carbonate chemistry has been well documented on coral reefs and consists of daytime higher pH due to photosynthesis with lowered A_T due to

calcification compared to nighttime lowered pH due to respiration with higher A_T due to calcium carbonate dissolution (Ohde and vanWoesik 1999; Yates and Halley 2006; Silverman et al. 2007; Santos et al. 2011). As this diel oscillation is driven by residence time of surface waters, shallow reef flats exhibit extreme variability in carbonate chemistry, with seasonal tide and metabolic changes also playing a role (Shamberger et al. 2011; Shaw et al. 2012).

Early research utilized the relationship between A_T and O_2 to derive calcification and primary production measurements of individual corals or whole reef communities (Smith and Key 1975; Barnes 1983). Coupled with future predicted pCO_2 levels, models have described an overall decline in coral community calcification (Silverman et al. 2009; Shaw et al. 2013), yet spatial and temporal variability must be considered to accurately determine the vulnerability of each reef community (Hofmann et al. 2011). A recent study has related the temporal variability in the carbonate chemistry to recruitment success and calcification, with high-Mg calcite coralline algae and bryozoans being particularly sensitive (Price et al. 2012). While *a priori* expectations suggest increased resilience of organisms nocturnally exposed to pH levels predicted by 2100, the potential for carbonate chemistry variability to be used as a predictor of the physiological response of reef-building corals to OA remains largely unexplored.

Despite an abundance of published work, the key drivers of coral calcification remain equivocal (Allemand et al. 2011; Jokiel 2011) and few studies have investigated the potential changes in primary productivity under future OA conditions (Schneider and Erez 2006; Anthony et al. 2008; Crawley et al. 2010). Coral skeletal morphological plasticity is evidence of the response of biomineralisation to environmental heterogeneity, traditionally constrained by flow and light (Lesser et al. 1994; Bruno and Edmunds 1997; Hoogenboom et al. 2008), while OA research has focused on the skeletal mineralogy and rate of calcification (Ries 2011). Furthermore, physiological plasticity is evident by the corals ability to photoacclimate through mechanisms such as manipulating the symbiotic dinoflagellate (genus: *Symbiodinium*) population size or distribution in the tissue (Iglesias-Prieto and Trench 1994; Enriquez et al. 2005) or by the symbionts optimising their photosynthetic pigments (Hoogenboom et al. 2009). Investigating photoacclimatory processes are relevant to this study, as increasing the CO_2 substrate for photosynthesis has been shown to drive photo-physiological

changes (Anthony et al. 2008; Crawley et al. 2010). As photosynthesis and calcification are tightly coupled, it is imperative to consider these together when investigating the response reef-building corals to OA.

Studies investigating the effects of increased pCO_2 on coral physiology have been carried out in aquaria (Ohde and Hossain 2004; Jury et al. 2009), in mesocosms (Langdon et al. 2000; Leclercq et al. 2002; Reynaud et al. 2003) and on the reef using *in situ* carbon enrichment facilities (Kline et al. 2012). Alternatively, community metabolism calculations and modeling have been used to address this problem (Silverman et al. 2009; Shaw et al. 2012). Although aquaria-based experiments provide an ability to investigate the detailed mechanism behind changes in calcification and productivity in individual species, there may be difficulties in extrapolating this relationship to the ecosystem level due to inter-species variation and interactions. On the other hand, the use of mesocosms and community metabolism models offer insight into community responses, but are often limited to a particular community assemblage. For example, the prediction that reefs will begin to dissolve at 560 ppm CO_2 (Silverman et al. 2009) assumes that the community composition will remain the same, which is very unlikely due to competition, mortality and successional processes, and therefore compromises the future relevance of these types of predictions. Recent studies near naturally occurring CO_2 vents have confirmed that community shifts will occur with increasing pCO_2 (Kroeker et al. 2012; Inoue et al. 2013). In this study, the response of individual corals to future OA conditions has been placed in the context of ecosystem variability. In order to improve future projections of the spatial variability of the response of reef-building corals to OA, the intra-species variation of *Acropora millepora* was investigated from a range of sites with inherent differences in carbonate chemistry and then a manipulative experiment was performed using samples from these distinct habitats.

In addition to the temporal variation caused by reef metabolism, the carbonate chemistry may exhibit spatial variation due to factors such as the particular community assemblage, water depth, tidal fluctuations and current dynamics (Hofmann et al. 2011; Price et al. 2012). The impact of this temporal and spatial variation in carbonate chemistry on the response of reef-building corals to OA is largely unknown. In this study, the diurnal and seasonal variability in carbonate

chemistry and the community assemblage was assessed at three sites around Lizard Island on the Great Barrier Reef. From these sites, the physiology of *A. millepora* was assessed under future (constant) pCO_2 scenarios in a manipulative, aquaria-based experiment. This study design enabled determination of the coral physiological response to OA in relation to their site of origin, an important consideration to improve the predictive power of climate models as applied to coral reefs.

Materials and Methods

Study Area

The study was conducted at three sites around Lizard Island, Great Barrier Reef, Australia (Figure 1). Station Reef (SR) (14.6798° S, 145.4451° E) is located in front of Lizard Island Research Station (LIRS) in the open lagoon. Loomis Reef (LR) (14.6830° S, 145.4494° E) is also located within the lagoon, while Mermaid Cove (MC) (14.6457° S, 145.4537° E) is located outside the lagoon on the northern side of the Island. While the crest of each reef is exposed at low tide, the reef slopes extend to different depths; approximately 1 m at SR, 3 m at LR and 6 m at MC. The tidal fluctuation of this open lagoon only plays a minor role in the water current dynamics except during spring tides, which occurred during the September 2009 sample time point (see below). The current direction is strongly influenced by the south easterly trade winds which are prevalent from March to November and the intermittent northerly wind from December to February (Crossland and Barnes 1983). Wind speed and direction data was provided via the Australian Institute of Marine Science (AIMS) Integrated Marine Observing System (IMOS).

Carbonate Chemistry

The carbonate chemistry at the sites was determined during three separate trips in September 2009, February 2010 and January 2011. At 3 locations within each site, water samples were collected in 500 mL glass bottles for A_T and pH measurements. The water was collected equidistant from the reef crest at each site to avoid depth bias. Sample frequency was every two hours during the day between the hours of 6:00 to 18:00 except due to logistical constraints. Collection, storage and analysis followed the standard operating procedures as described by Dickson et al. (2007). The bottles were rinsed with sample water three times before collection, sealed underwater to

prevent atmospheric gas exchange and then poisoned with 100 µL of saturated mercuric chloride solution if analysis was to take place more than 12 hours later. A_T was determined by Gran titration (Gran 1952; Dickson et al. 2007) with 0.1M HCl using a titrator (T50, Mettler Toledo, Langacher, Switzerland) modified with a 1 mL burette, pH sensor (DGi101-SC) and small volume stirrer. The pH electrode was calibrated with NBS scale standard buffers (Mettler Toledo) and the titrator was calibrated with Certified Reference Materials (AG Dickson, SIO, Oceanic Carbon Dioxide Quality Control) prior to daily sample analysis. The pH was measured using a pH sensor (DGi101-SC, Mettler Toledo). Measurements were repeated until precision of ± 3 µmol kg^{-1} was achieved for A_T and ± 0.005 for pH. The data was input into the CO2calc program (Robbins et al. 2010) along with ambient temperature, pressure and salinity in order to obtain total dissolved inorganic carbon (DIC), and saturation states for aragonite (Ω_{arag}) and calcite (Ω_{calc}). The calculations were performed using the total hydrogen ion scale, the carbonic acid dissociation constants of Mehrbach et al (1973) as refit by Dickson & Millero (1987) and the sulfonic acid dissociation constant of Dickson (1990).

Community Assemblage

Twenty x 1 m^2 photo quadrats were randomly taken at each water sample location within the sites and analysed using 100-point intercept method with the Coral Point Count with Microsoft Excel extensions (CPCe) software (Kohler and Gill 2006). The coral, algae and substrate coverage was classified in terms of functional groups and also to genus level, or species level where possible, with the assistance of the Kraft (2007) and the Indo Pacific Coral Finder (Kelley 2009).

OA Experiment

At LIRS in November 2009, 36 branches of *A. millepora* collected near the reef crest at each site (1 - 3 m depth) were hung with nylon line in aquaria with ambient seawater to acclimate for five days before beginning CO_2 treatment. The CO_2 dosing was controlled through a computer interface (Aquatronica-AEB Technologies, Cavriago, Italy) operating solenoid valves, which injected CO_2 gas based on a pH threshold in line with IPCC scenarios B2 (pH 7.9 ± 0.05; pCO_2 600 ± 80 µatm) and A1FI (pH 7.7 ± 0.05; pCO_2 1020 ± 140 µatm) (IPCC 2007). The control aquaria had ambient seawater from Lizard Island lagoon (pH 8.1 ± 0.05; pCO_2 350 ± 50 µatm).

The pH was continuously measured using a pH sensor precise to 0.01 pH units (InPro4501VP, Mettler Toledo) in 200 L sumps and pumped into 4 replicate aquaria for each treatment. The aquaria were maintained outdoors with neutral density shade cloth, which resulted in maximum light levels of 400 μmol quanta m^{-2} s^{-1} as measured by light loggers (Odyssey, Dataflow Systems, Christchurch, New Zealand). The experiment ran for 3 weeks with physiological measurements taken upon completion.

Coral Physiology after OA experiment

Respirometry was performed on the coral samples using the procedure of Crawley et al (2010) with the following modifications. The water bath was set to 27°C, the ambient seawater temperature at Lizard Island in November 2009. Corals were dark-acclimated for one hour prior to the start of respirometry analysis. The light program was 10 minutes in darkness to obtain steady-state dark respiration (R_{dark}) followed by 30 minutes at 600 μmol quanta m^{-2} s^{-1} to obtain steady-state net photosynthesis (P_{net}), then 10 minutes in darkness to obtain light-enhanced dark respiration (LEDR), which began approximately 1 minute post illumination. Linear regression of the bulk oxygen (O_2) measurements, obtained with O_2 optodes (Oxy4 v2, PreSens, Regensburg, Germany), provided the rate of O_2 evolution or consumption. The Pg:R ratio was calculated as the ratio of gross photosynthesis ($Pg = P_{net} + LEDR$) to respiration ($R = R_{dark} + LEDR$), under a 12 hour light : 12 hour dark cycle. A Pg:R ratio greater than 1 denotes that the coral has the potential to grow phototrophically, assuming equal translocation of photosynthates from the dinoflagellates to the coral and equal use of the photosynthates for growth (Muscatine et al. 1981; Amthor 2000). Alternatively, a Pg:R ratio < 1 indicates that the coral must resort to heterotrophy in order to grow.

After completion of the respirometry assays, the branches were frozen in liquid nitrogen the following day at midday, at which time light loggers recorded 300 μmol quanta m^{-2} s^{-1}, which was 25% lower than the average maximum daily light level due to intermittent cloud cover. The branches were later water-piked using 0.22 μm filtered seawater and centrifuged (4,500 x *g*, 5 min) to separate the dinoflagellate pellet from host tissue. Two aliquots were made; one for dinoflagellate cell counts using a 0.100 mm Tiefe Depth Profondeur hemocytometer (0.0025 mm^2), and the other for pigment analysis using High Performance Liquid Chromatography (HPLC) following the methods of Zapata et al (2000) and Dove et al. (2006). The supernatant

from centrifuging was diluted with 0.22 μm filtered seawater and analysed with a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, California, United States) to determine host protein using the equations of Whitaker & Granum (1980). In a previous OA experiment, the buoyant weight technique may have been too insensitive to detect a change in the calcification rate of *A. millepora* (Kaniewska et al. 2012). Skeletal dry weight has previously been shown to have perfect correlation with buoyant weight, which has 1-2% error due to the negative buoyancy of the organic matrix and tissue (Jokiel et al. 1978). We therefore measured the skeletal dry-weight to surface-area ratio, which represents a change in the aragonite density and/or branch compactness. The coral surface area was determined using a double-dipping wax method appropriate for skeletons with deep corallites (Stimson and Kinzie 1991; Veal et al. 2010).

Statistical analysis

Site differences in the diurnal carbonate chemistry range were assessed using a one-way PERMANOVA in PRIMER with Site as a fixed factor (Anderson 2001; McArdle and Anderson 2001). We corrected for seasonal baseline differences using the diurnal range in A_T, DIC and pH, which were normalized and projected in Euclidean space for analysis. The diurnal range in Ω_{arag} and Ω_{calc} were similarly analysed. For all PERMANOVA procedures, unrestricted permutation of the raw data created the Pseudo-F distribution for the main test and Monte Carlo P-values were used for pairwise comparisons. We used distance-based linear models (DISTLM) to assess the relationship between the community assemblage and carbonate chemistry predictor variables (Legendre and Anderson 1999; McArdle and Anderson 2001). For this procedure, the community assemblage data was log transformed and a zero-adjusted Bray-Curtis resemblance matrix was generated. Canonical analysis of principle coordinates (CAP) (Anderson and Robinson 2003; Anderson and Willis 2003) was used to determine the best axes through the multivariate data cloud to depict *a priori* sites. The carbonate chemistry predictor variables found to explain a significant proportion of the data through DISTLM marginal and sequential tests were then overlaid on the CAP model as Pearson correlation coefficients with the CAP axes. We assessed the coral physiological parameters with the PERMANOVA procedure using Euclidean resemblance matrices (Anderson 2001; McArdle and

Anderson 2001). Treatment and Site were specified as fixed factors, while Tank was random and nested within Site. The source of variation was pooled when $p > 0.25$.

Results

Carbonate Chemistry

The diurnal range in the carbonate chemistry variables was significantly higher at SR compared to MC (Pseudo-$F_{2,24} = 2.79$; $p_{perm} < 0.03$, pairwise MC < SR, $p_{MC} < 0.03$) (Figure 2). Across all seasons sampled the diurnal A_T range was 46 ± 4 μmol kg^{-1} at SR, 33 ± 4 μmol kg^{-1} at LR and 28 ± 4 μmol kg^{-1} at MC. Similarly, the pH range was higher in the lagoon sites than outside the lagoon with SR and LR ranging 0.11 ± 0.01 pH units and 0.12 ± 0.01 pH units, respectively, while MC only 0.08 ± 0.01 pH units. Overnight accumulation of respiratory CO_2 meant that the pH was usually lowest at the morning sample times (See Supplementary Material Figure S1), corresponding with the lowest Ω_{arag} and Ω_{calc} values (Table 1).

Community Assemblage

At the functional group level, there were clear differences in the community assemblage at each site (Figure 3). SR and LR were differentiated from MC by an abundance of soft corals, sea fans and sponges with approximately 55% coverage at SR, 25% coverage at LR and less that 2% coverage at MC. This group was largely composed of the soft corals *Sarcophyton* sp, *Sinularia* sp and *Lobophyton* sp (data not shown). MC was also distinguished by a high abundance of crustose coralline algae (CCA) with up to 20% coverage, whereas SR and LM had less than 3% coverage. Furthermore, branching corals were at approximately 17% cover at MC compared to only approximately 10% at SR and LR.

The DISTLM analysis showed that the community assemblage was distinct at each site and that the seasonal and diurnal carbonate chemistry variables were able to explain a significant proportion of the variation (Figure 4). In September 2009, the maximum daily pH (pH max) significantly explained 25% of the total variation in the community assemblage (Pseudo-$F = 2.33$, $p_{perm} < 0.02$). In February 2010, the daily minimum A_T (A_T min) and daily maximum A_T (A_T max) significantly explained 26% and 21% of the community assemblage variation, respectively (Pseudo-$F = 2.5$, p_{perm}

< 0.03). In January 2011, marginal tests showed that the daily DIC minimum (DIC min) and pH max significantly explained 26% and 28% of the variation (Pseudo-F = 2.57, p_{perm} < 0.02), although sequential tests indicated overlap in the variation explained as indicated by the plane of the vector on the CAP analysis.

Coral Physiology after OA Experiment

Overall, P_{net} was not significantly affected by the treatment (Pseudo-$F_{2,18}$ = 3.16; p_{perm} = 0.07), but a one-way PERMANOVA on samples from each site showed that P_{net} increased significantly in MC corals from the B2 to the A1FI scenario (Pseudo-$F_{2,9}$ = 5.66; p_{perm} < 0.03, pairwise B2 < A1FI, p_{MC} <0.02) (Figure 5). Under the B2 scenario, LEDR significantly increased in corals from SR and MC, but declined in corals from LR (Pseudo-$F_{2,22}$ = 3.59; p_{perm} < 0.04, pairwise Present ≠ B2, p_{MC} <0.03). R_{dark} was significantly higher in corals from SR compared to MC (Pseudo-$F_{2,22}$ = 3.95; p_{perm} < 0.03, pairwise MC < SR, p_{MC} < 0.02). Overall, the Pg:R ratio remained > 1 and there were no significant differences due to treatment or site factors.

Dinoflagellate cell density was significantly higher in SR corals compared to MC across all treatments (Pseudo-$F_{2,9}$ = 4.94; p_{perm} < 0.05, pairwise SR > MC, p_{MC} < 0.03) although there were also some tank effects that may have been related to the diurnal sun angle for which the variability could not be partitioned (Figure 6A). There were no changes in photosynthetic pigments; however, the photoprotective pigment, Diatoxanthin (DT), was upregulated significantly in corals from LR under the B2 scenario (Pseudo-$F_{4,90}$ = 2.55; p_{perm} < 0.04, pairwise LR: B2 > A1FI, p_{MC} < 0.02) (Figure 6B).

Corals from SR had significantly higher protein per surface area than MC corals, although there were some tank effects (Pseudo-$F_{2,18}$ = 5.80; p_{perm} < 0.03, pairwise MC < SR, p_{MC} < 0.02) (Figure 6C). Irrespective of treatment, the skeletal dry-weight to surface-area ratio was higher in corals from MC compared to SR and LM (Pseudo-$F_{2,18}$ = 10.65; p_{perm} < 0.01, pairwise LR < MC, p_{MC} < 0.01, MC > SR, p_{MC} < 0.01) (Figure 6D).

Discussion

This study has illustrated the link between benthic community composition and diurnal fluctuations in carbonate chemistry, indicating that the latter may be useful as predictor variables for future models of reef susceptibility to OA. At MC, a site outside Lizard Island lagoon, the diurnal range in carbonate chemistry was significantly smaller than SR, a site inside the lagoon, and this factor could be used to partition the variation in site community composition. A further key finding of this study was the site-specific physiological trade-off demonstrated by variable skeletal morphology and tissue thickness in *A. millepora*. The skeletal dry-weight to surface-area ratio, a proxy for aragonite density or branch compactness, was higher in MC coral samples in comparison to SR and LR whereas protein per cm, a proxy for tissue thickness, was higher in SR coral samples compared to MC and LR. In addition, this study has demonstrated that the resilience of *A. millepora* to future OA may be differentially impacted by the life-history strategies employed by the coral. After three weeks under the pCO_2 conditions of the A1FI scenario, the corals from MC increased P_{net}, yet there were no significant improvements to the Pg:R ratio. This may have been a necessary shift in energy harvest for MC corals in order to increase protein production and tissue thickness in an attempt to buffer the skeleton from acidifying seawater; a response which has been shown in long-term experiments (Fine and Tchernov 2007; Krief et al. 2010). Despite these strategies for survival, a future with less calcification on coral reefs, either through individual decalcification or a shift in community assemblage, seems likely in an elevated-pCO_2 world.

Diurnal variation in carbonate chemistry

The diurnal range in carbonate chemistry reported in this study is comparable to other studies on reef metabolism (Frankignoulle et al. 1996; Gattuso et al. 1996; Bates et al. 2001; Fagan and Mackenzie 2007) although not as extreme as some shallow reef flat studies (Ohde and vanWoesik 1999; Shaw et al. 2012). To our knowledge, the most extreme recording of diel oscillations in Ω_{arag} is 1.83 to 6.36 at Lady Elliot Island reef flat (Shaw et al. 2012) and assuming half the diel range occurred during the daytime, the diurnal range reported here at Lizard Island is still considerably less. It is well understood that coral reef communities can alter the carbonate chemistry of their seawater through the processes of photosynthesis, respiration, calcification, and

dissolution (Smith and Key 1975). As this composite signal is amplified in shallow waters, reef flat studies have subsequently dominated this research. However, it is imperative to understand the full range of diurnal variation in carbonate chemistry and the associated implications for coral reef communities. This study has shown that daily maximum and minimum A_T values can explain up to 40% of the variation in the community assemblage. Although direct causality cannot be attributed, it is highly likely that the carbonate chemistry at MC played a role in the enhanced settlement, growth or survivorship of branching scleractinian corals and CCA in comparison to SR and LR leading to the observed higher percent coverage at MC. This result is not surprising considering the work of Chisholm (2000) suggesting that night-time dissolution lowered the actual accretion rates of CCA on the windward reefs of Lizard Island despite high calcification rates. Previous studies have also predicted enhanced sensitivity of branching scleractinian corals and especially CCA to future elevated pCO_2 scenarios (Anthony et al. 2008; Hall-Spencer et al. 2008; Jokiel et al. 2008; Hurd et al. 2011). On the other hand, the carbonate chemistry at SR and LR were likely more amenable to the life-history characteristics of soft corals, which appear to be physiologically unchanged by elevated pCO_2 conditions (Gabay et al. 2013). Future models of coral reef ecology must consider that reef community composition will be strongly influenced by the diurnal range in their carbonate chemistry.

Coral physiological response to carbonate chemistry

In addition to defining the community, the diurnal variation in carbonate chemistry may also play a role in the underlying morphology of ubiquitous species as exemplified by the site differences in skeletal morphology and protein per cm^2 in *A. millepora*. Given that calcification responds to changes in atmospheric CO_2 forcing (Kleypas et al. 2011), it is not surprising that skeletal morphology is also potentially shaped by diurnal fluctuations in carbonate chemistry. In this study, the aragonite density or branch compactness of coral samples from outside Lizard Island lagoon was significantly higher than those samples from inside the lagoon at SR and LR. Calcification is enhanced during the day when CO_2 is removed by the process of photosynthesis and hindered each night by the build up of CO_2 from respiration leading to the process of $CaCO_3$ dissolution. Accordingly, coral skeletal structure will be impacted differentially depending on the extremities of the range in the diel carbonate chemistry and the ability of the coral to maintain optimal conditions in the

extracellular calcifying medium (ECM) (Cohen et al. 2009; Allemand et al. 2011). In order for calcification to occur, the pH must remain elevated in the ECM relative to seawater while Ca^{2+} and HCO_3^- must be transported to the ECM. It is likely that the smaller diurnal range in carbonate chemistry was a contributing factor impacting the skeletal morphological plasticity leading to denser aragonite or more compactness with coral samples from outside the lagoon.

One possible avenue to improve the maintenance of optimal calcifying conditions in the ECM is to increase tissue thickness as a means to increase the potential buffering capacity. Increased tissue thickness may restrict entry of seawater and/or result in greater capacity to actively transport ions to and from the ECM. However, as energy allocation to the growth and maintenance of tissue thickness represents a trade-off to skeletal deposition (Anthony et al. 2002), this strategy therefore comes at an energetic cost. In this study, *A. millepora* at MC had less protein per cm^2, but a different skeletal morphology in comparison to *A. millepora* from SR, which suggests that MC corals were able to build and maintain their skeleton with comparatively less energetic cost. On the other hand, SR corals may have utilized their increased tissue thickness to protect their skeleton from the nocturnal corrosive pH conditions (Chisholm, 2000). While the SR corals aragonite density or branch compactness was still significantly lower than MC, it may have been worse without this additional effort. The higher R_{dark} at SR compared to MC is a testimony to the increased respiratory cost of maintaining thicker tissue (Figure 6C). Given that this trade-off is a life-history strategy that may already be utilized by present day diurnal variation in carbonate chemistry, its future benefit to reef-building corals under OA conditions should be reassessed.

The ability of corals to utilize increased tissue thickness in an attempt to protect their skeleton in the face of future elevated pCO_2 levels also depends on the availability of energy to be allocated to this function. It seemed fortunate for MC corals that P_{net} significantly increased under the A1FI scenario, yet there were no significant differences in the Pg:R ratio, suggesting that the increased energy was being fully utilized. Although we did not detect an increase in protein per cm^2 in our 3 week study, other long-term experiments have shown that tissue thickness increases under future elevated pCO_2, yet the rate of calcification by those scleractinian corals still

declined (Fine and Tchernov 2007; Krief et al. 2010). So, despite potential increases in photosynthetic output and potential improvements to tissue thickness, the future of coral reefs may still be threatened by decreased skeletal calcification.

Implications for future models

Current research has started to investigate the ecological consequences of OA on coral reefs, with some studies modeling the future carbonate chemistry considering different community composition (Anthony et al. 2011; Shaw et al. 2013). While previous studies have focused on a shift to macroalgal dominance, the observed differences in community composition in the present study indicate that soft coral is also a likely candidate for future reef dominance, which has also been shown near volcanically-acidified waters in Japan (Inoue et al. 2013). This will have cascading effects on the ecology of reefs with different invertebrate, fish and algal communities associated with the habitat of soft corals, which provide overall less structural and topographical complexity.

The overlaid vectors on the CAP model depict the quintessential carbonate chemistry variables that distinguish the community composition of the sites, although these were not common between or within seasons. We believe the driver of this difference was the particular alignment of tidal height and photoperiod. As previous discussed, longer seawater residence times lead to a greater signal of community metabolism, therefore the coincidence of low tide around dawn and dusk will give different values to a low tide regime at midnight and midday. During the September 2009 and February 2010 sample regimes, low tide occurred around midday/midnight whereas January 2011 sampling occurred with low tide on dawn/dusk. In order to partition the variation due to alignment of tidal height and photoperiod, future studies should be designed with comprehensive sampling within seasonal and tidal ranges (Shaw et al. 2012).

Additional factors contributing to the site variability and coral physiology could be included in future studies to further partition the variation. These include current speed and direction, tidal fluctuations, water depth, light attenuation, temperature, nutrients, community metabolism and heterotrophic capacity. This study aimed to investigate the relationship between diurnal variation in carbonate chemistry and

community composition and assess the response of a common reef-building coral from these sites under future OA conditions. Nevertheless, we do not discount the role of the abovementioned factors at these sites. For example, increased heterotrophic feeding at SR could also lead to increased protein and thicker tissue. Furthermore, reflected light from the lagoon sandy bottom could play a greater role in the coral photophysiolgy at SR and LR, in comparison to MC. Similarly, temperature oscillations are likely to be greater at the lagoon sites due to shallow pooled water at low tide with decreased proximity to deeper waters upon inundation at high tide. As our study is purely correlative, we do not exclude these factors as potential drivers and encourage future studies to further investigate.

The natural diel variation in carbonate chemistry on coral reefs offers a unique opportunity to investigate mechanisms of survival under future pCO_2 conditions, akin to studying communities near natural CO_2 vents (Fabricius et al. 2011; Rodolfo-Metalpa et al. 2011). The present study is unique as it investigated the intra-species variation in *A. millepora* in the context of sites with significantly different carbonate chemistry and community assemblage. Site-specific factors have previously been shown to influence the life-history of the dinoflagellates (genus: *Symbiodinium*) growing within *A. millepora* from different sites on the Great Barrier Reef and this led to variability in their response to thermal stress conditions (Howells et al. 2012). Here, the site-specific diurnal variation in carbonate chemistry correlated with differential investment in skeletal morphology and tissue thickness, but this trade-off may be of limited future benefit especially considering the suite of additional factors such as temperature, eutrophication, overfishing, that will impact reef resilience.

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Table 1: Diurnal range of the saturation state of aragonite (Ω_{arag}) and calcite (Ω_{calc}) at the sampling sites around Lizard Island as calculated using the CO2calc program (Robbins et al. 2010).

Site	Sample Timepoint	Range of Ω_{arag}	Range of Ω_{calc}
Station Reef	September 2009	3.38 - 4.00	5.12 - 6.05
	February 2010	4.23 - 4.40	6.33 - 6.58
	January 2011	4.04 - 4.40	6.06 - 6.59
Loomis Reef	September 2009	3.94 - 4.00	5.96 - 6.05
	February 2010	3.72 - 3.88	5.56 - 5.81
	January 2011	4.30 - 4.36	6.45 - 6.52
Mermaid Cove	September 2009	3.85 - 4.20	5.82 - 6.35
	February 2010	3.88 - 4.23	5.81 - 6.34
	January 2011	4.41 - 4.50	6.61 - 6.73

Table 2: PERMANOVA results for *Acropora millepora* physiology after 3 weeks in an OA experiment and the diurnal range in carbonate chemistry at their site of collection.

Variables	Source of Variation	P	Pairwise P
R_{dark} (μmol O_2 cm^{-2} hr^{-1})	Treatment	0.25	
	Site	**0.02**	**MC > SR p = 0.0119**
	Pooled	0.66	
P_{net} (μmol O_2 cm^{-2} hr^{-1})	Treatment	0.07	
	Site	0.17	
	Tank (Site)	**0.03**	
	Treatment x Site	0.23	
	Treatment x Tank (Site)	0.73	
P_{net} MC (μmol O_2 cm^{-2} hr^{-1})	**Treatment**	**0.0214**	**A1FI > B2 p = 0.0196**
	Tank (Treatment)	0.3975	
LEDR (μmol O_2 cm^{-2} hr^{-1})	**Treatment**	**0.03**	**Present ≠ B2 p = 0.021**
	Site	0.67	
	Tank (Site)	0.15	
	Pooled	0.17	
Cells per cm^2	Treatment	0.4307	
	Site	**0.0323**	**MC < SR p = 0.0205**
	Tank (Site)	**0.004**	
	Treat x Site	0.1868	
	Treat x Tank (Site)	0.2349	
Dt *p*g per cell	Treatment	0.1234	
	Site	0.1438	
	Tank (Site)	0.3879	
	Treat x Site	**0.039**	**LR: B2 > A1FI p=0.0135**
Protein mg cm^{-2}	Treatment	0.2865	
	Site	**0.028**	**MC < SR p = 0.0147**
	Tank (Site)	**0.0141**	
	Treatment x Site	0.01911	
Skeleton g cm^{-2}	Treatment	0.4598	
	Site	**0.0061**	**LR < MC p=0.0099; SR < LR p=0.0021**
	Tank (Site)	0.6494	
A_T, DIC, pH diurnal range	**Site**	**0.0263**	**SR > MC p = 0.0248**
			LR > MC P = 0.0794
Ω_{arag} Ω_{calc} diurnal range	Site	0.12	

Figure 1: Map of Lizard Island and surrounding reefs showing the location of sampling sites at Mermaid Cove (MC) 14.6457° S, 145.4537° E, Station Reef (SR) 14.6798° S, 145.4451° E and Loomis Reef (LR) 14.6830° S, 145.4494° E. Image Copyright GeoEye 2005.

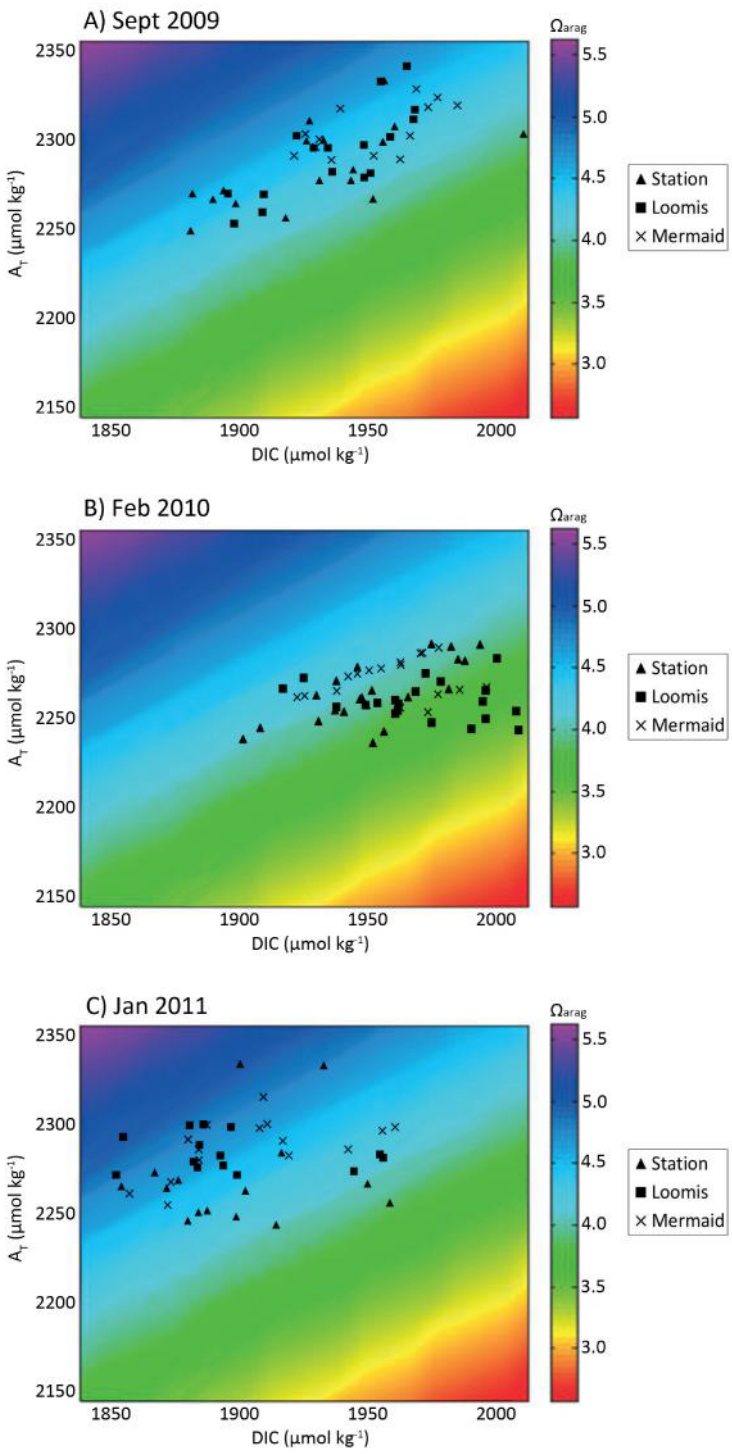

Figure 2: Deffeyes plots depicting Total Alkalinity (A_T) and Dissolved Inorganic Carbon (DIC) at the lagoon sites, Station Reef (SR) and Loomis Reef (LR) and outside the lagoon at Mermaid Cove (MC) during the sampling in A) September 2009, B) February 2010 C) January 2011. Data are overlaid on the contours of aragonite saturation (Ω_{arag}).

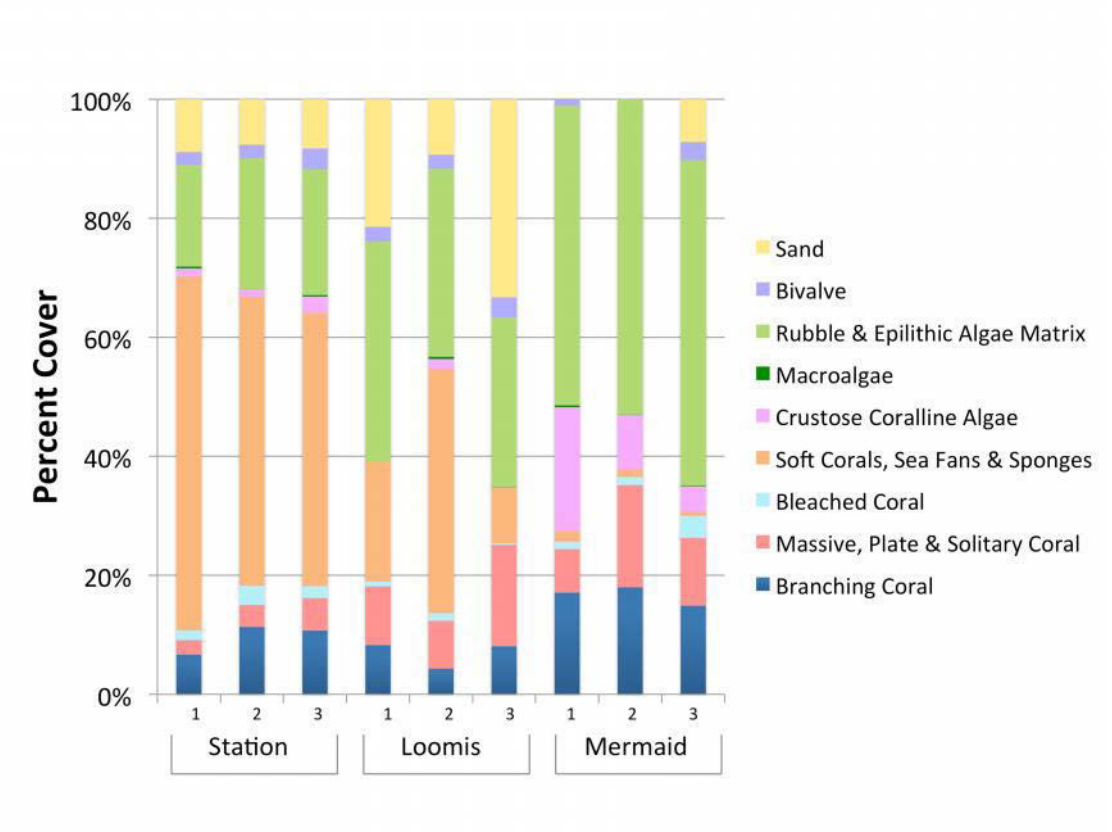

Figure 3: Functional group community assemblage determined from the percent cover at three locations within each site at Lizard Island.

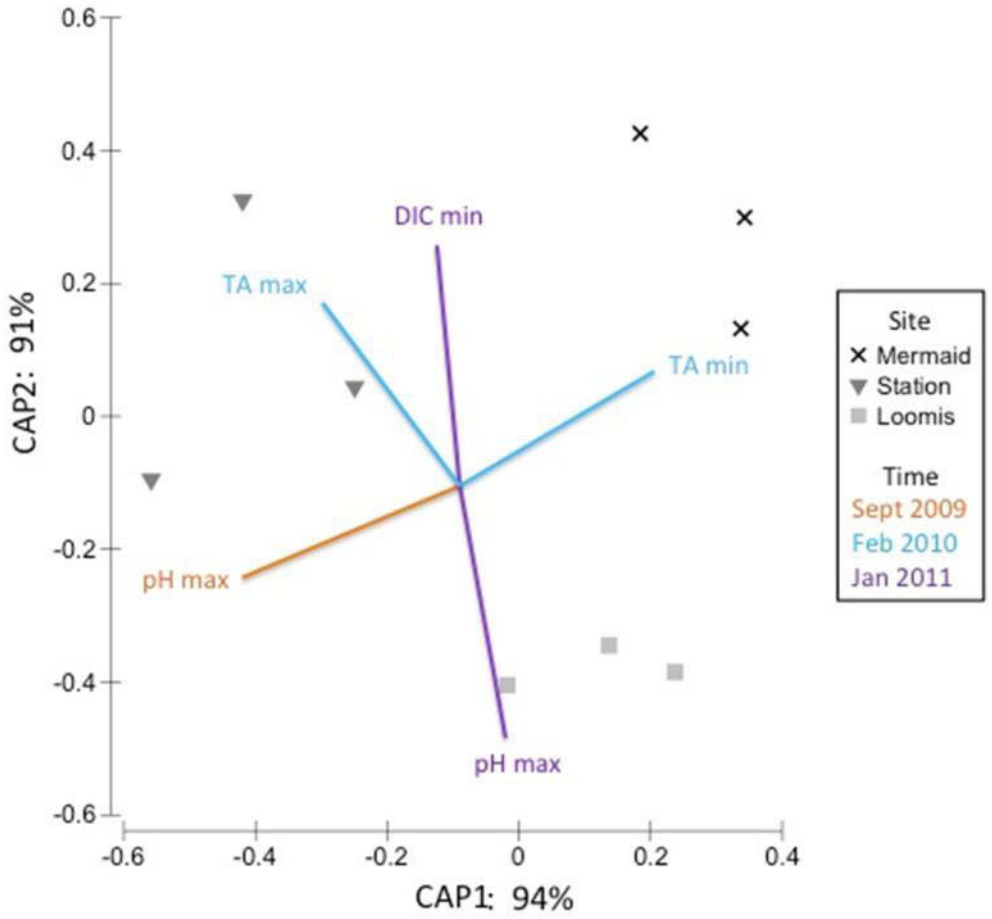

Figure 4: Canonical analysis of principal coordinates (CAP) discriminating the *a priori* sites based on community assemblage. Overlaid vectors are Pearson correlations depicting carbonate chemistry parameters that represent a significant proportion of the variation as partitioned by distance-based linear modelling (DISTLM).

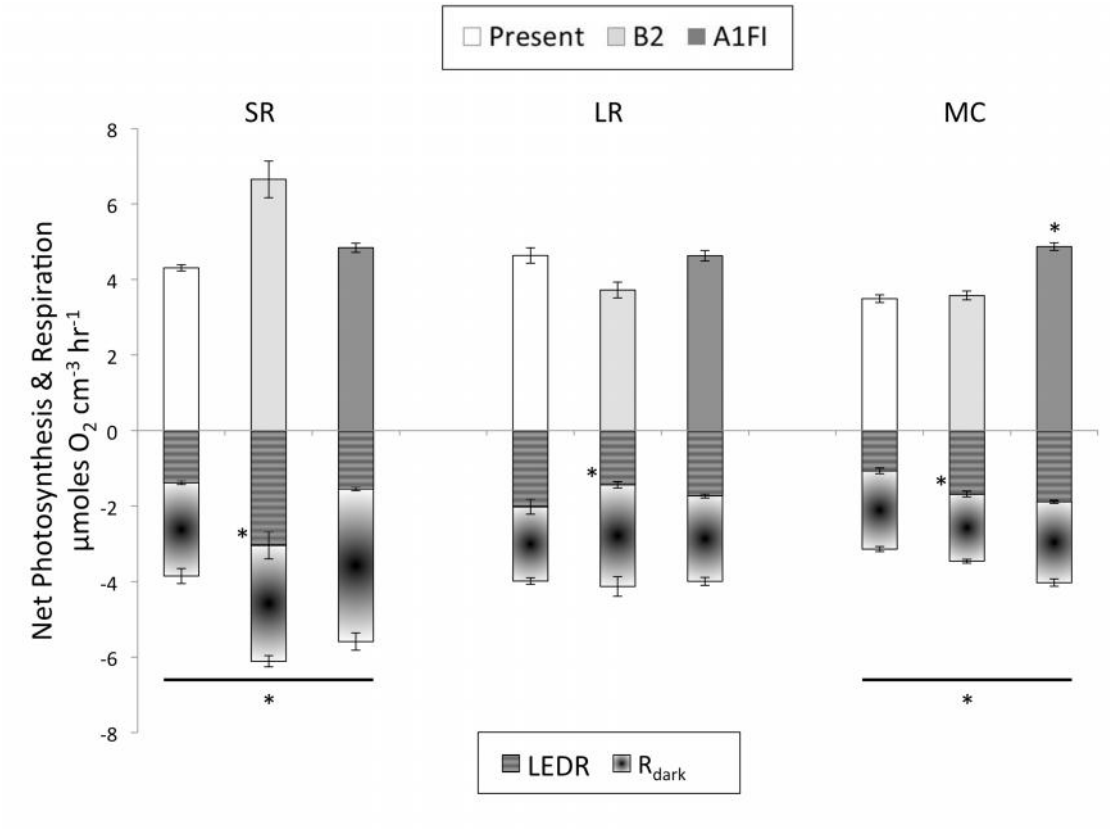

Figure 5: Net Photosynthesis (O_2 production) and Respiration (O_2 consumption) of *Acropora millepora* branches from 3 sites around Lizard Island after 3 weeks in the OA experiment. SR = Station Reef and LR = Loomis Reef are sites inside the lagoon. MC = Mermaid Cove is situated outside the lagoon. LEDR = Light Enhanced Dark Respiration. R_{dark} = Dark Respiration. * = Significant differences ($p_{MC} < 0.05$). Error bars are standard error (n=3).

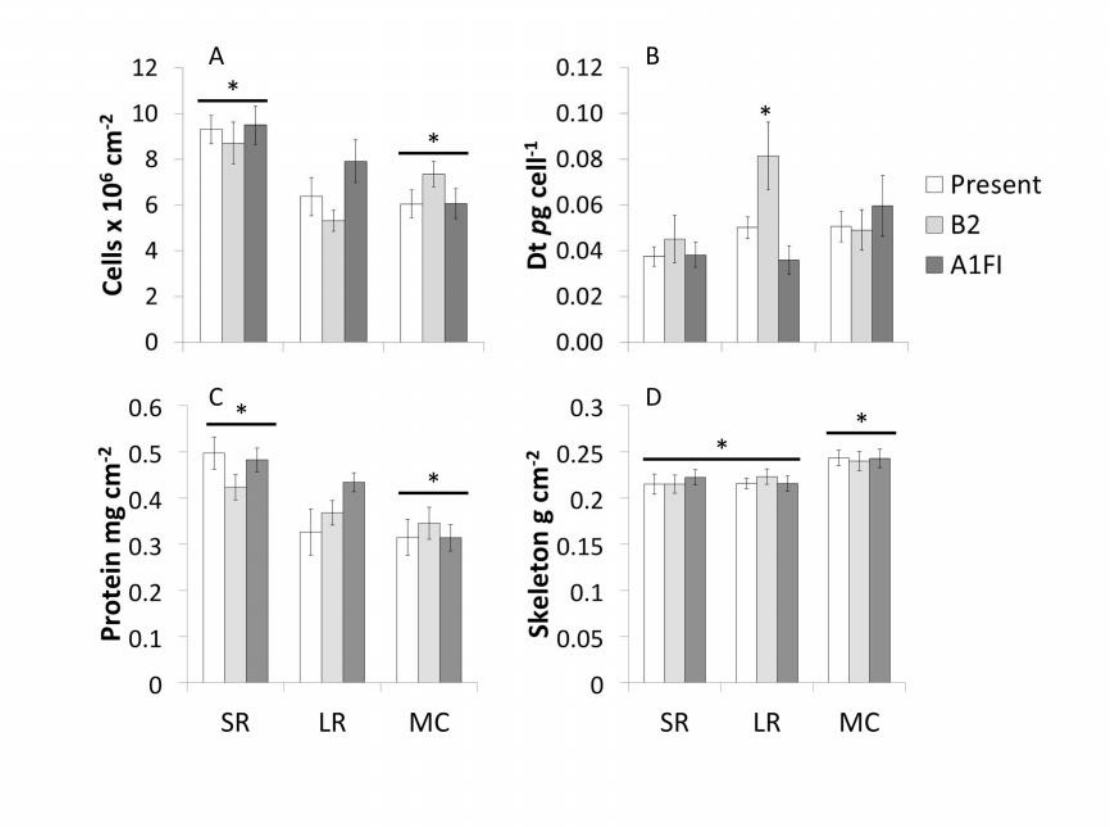

Figure 6: Physiology of *Acropora millepora* collected from 3 sites around Lizard Island after 6 weeks in OA experiment. SR = Station Reef and LR = Loomis Reef are sites inside the lagoon. MC = Mermaid Cove is situated outside the lagoon. Dt = Diatoxanthin * = Significant differences ($p_{MC} < 0.05$). Error bars are standard error (n=3).

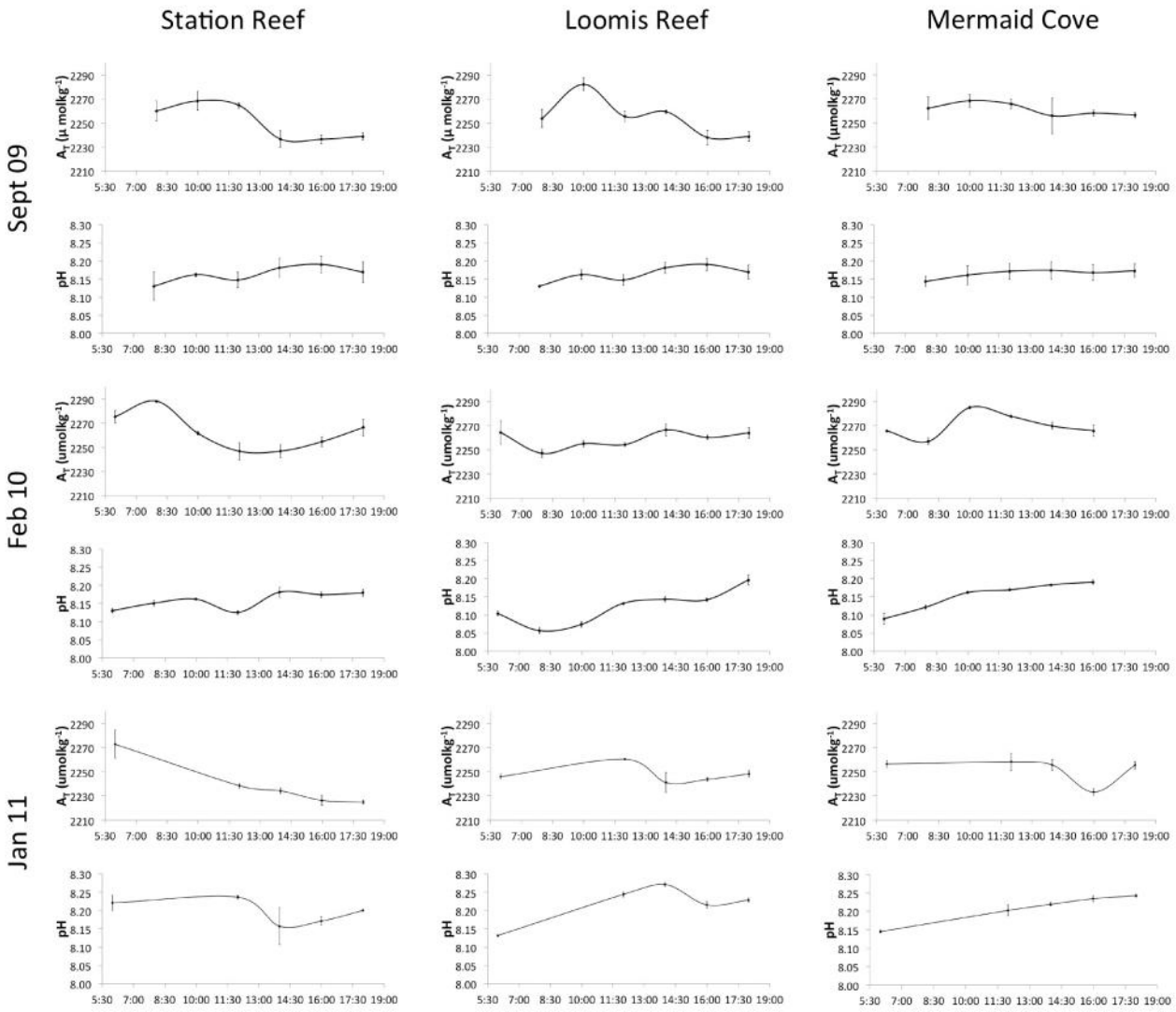

Figure S1: Total Alkalinity (A_T) and pH data from three sites at Lizard Island (Station Reef, Loomis Reef and Mermaid Cove) sampled at three timepoints (September 2009, February 2010 and January 2011). Error bars are standard error (n=3).

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Chapter 4: Coral bleaching in *Acropora millepora* juveniles under ocean acidification

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Abstract

Ocean acidification (OA) presents a challenge to the productivity of reef-building corals, with recent studies indicating that OA can cause coral bleaching (loss of dinoflagellate endosymbionts or photosynthetic pigments). While certain dinoflagellate types (genus *Symbiodinium*) can infer increased thermal tolerance to the holobiont, other types are more sensitive to heat stress. However, the effects of OA on coral bleaching in relation to symbiont type are largely unknown. In this study, we investigated the impact of OA on the photo-physiology of *Symbiodinium* within newly settled *Acropora millepora* recruits during the establishment of endosymbiosis. After two months under increased CO_2 conditions (pH 7.81 ± 0.10 and pH 7.60 ± 0.10), coral recruits were significantly bleached > 2.5 fold relative to controls, using a visual bleaching index at the centre of the polyp. This bleaching response correlated with recruits containing a dominant population of *Symbiodinium* type D1 or D1-4, indicating that OA may exhibit a phenotypic selective pressure on *A. millepora* recruits, with implications for post-settlement survivorship and population dynamics. The benthic functional groups surrounding the recruits significantly explained the coral photo-physiological response with crustose coralline algae corresponding with ambient CO_2 levels, while turf and endolithic algae correlated with high CO_2 levels. Overall, this indicates that the additional impact of CO_2 on benthic functional groups may indirectly affect post-settlement survivorship as the diel metabolic cycles of turf and endolithic algae could potentially exacerbate OA conditions.

Introduction

Over the past century, human activities have become more carbon intensive, leading to an increase in atmospheric carbon dioxide (CO_2) emissions and subsequent reduction in oceanic pH (Sabine et al. 2004; Canadell et al. 2007). This shift in carbonate chemistry, termed ocean acidification (OA), is a result of oceanic uptake of CO_2, having already led to a ~ 30% increase in the concentration of hydrogen ions [H^+] and ~ 15% decline in carbonate ions (CO_3^{2-}) in oceans worldwide (Kleypas et al. 2006). The ocean pH has not dropped this low for the last two million years and while OA events have coincided with coral mass extinctions in the fossil record, the current rate of pH decline is unprecedented (Veron 2008; Kump et al. 2009; Pandolfi et al. 2011). Should the remaining fossil fuel resources be utilised, oceanic pH may drop a further 0.7 units with critical implications for marine calcification and primary productivity (Caldeira and Wickett 2003; Kroeker et al. 2010; Jokiel 2011). Recent studies have indicated that OA will present a challenge for the calcification of reef-building corals (Schneider and Erez 2006; Marubini et al. 2008), especially in their early life history stages (Albright and Langdon 2011; Doropoulos et al. 2012b). Furthermore, some adult corals have exhibited a bleaching response (loss of endosymbionts and/or their pigments) to elevated CO_2 (Anthony et al. 2008) potentially due to changes in photoprotection (Crawley et al. 2010), yet there are very few studies investigating the photo-physiology of juvenile corals under OA conditions. Here we describe the response of *Acropora millepora* recruits after post-settlement exposure to CO_2 levels predicted under Intergovernmental Panel on Climate Change (IPCC) scenarios.

Disruption of the mutualistic symbiosis between corals and their algal symbionts results in coral bleaching, which occurs in response to a variety of stressors including temperature (Jokiel and Coles 1990), light (Falkowski et al. 1984), CO_2 (Anthony et al. 2008), salinity (Hoegh-Guldberg and Smith 1989), nutrients (Wiedenmann et al. 2012) and sedimentation (Anthony et al. 2007). One of the first symptoms of coral bleaching is impairment of the photosynthetic apparatus, which can be measured through changes in the photochemical efficiency of Photosystem II (PSII) (Warner et al. 1999). A decrease in the variable fluorescence yield in relation to the maximum fluorescence yield (F_v/F_m) indicates closure of the PSII reaction centres, which may

reduce the rate of photosynthesis and lead to production of damaging reactive oxygen species (ROS) (Hill and Ralph 2008). Dinoflagellate density and pigments may be adjusted in order to photoacclimate to changing conditions (Falkowski and Dubinsky 1981; Walters 2005). The differential ability to photoacclimate, between (Iglesias-Prieto et al. 2004) and within *Symbiodinium* types (Howells et al. 2012), may have important implications for the future survival of the coral holobiont. In this study, we consider the photochemical efficiency of *Symbiodinium* types at the onset of symbiosis under OA conditions.

In shallow reef flat areas characterised by a smaller volume of water, large diel oscillations in carbonate chemistry are detectable, driven by community metabolism and calcification (Shaw et al. 2012; Anthony et al. 2013). The proportion of photosynthetic and calcifying organisms within the coral reef community can significantly influence the carbonate chemistry (Anthony et al. 2011; Kleypas et al. 2011). Diel variation in carbonate chemistry occurs as photosynthesis and calcification are coupled during the day, with photosynthesis removing CO_2 from the seawater, thereby increasing the pH and providing optimal conditions for the precipitation of calcium carbonate. On the other hand, as night respiration increases CO_2, this creates conditions optimal for dissolution of calcium carbonate. Due to the small size of coral recruits, the impact of OA may vary depending on the surrounding community composition that can alter carbonate chemistry within the diffusive boundary layer (DBL) (Shashar et al. 1996; Hurd et al. 2011; Jokiel 2011). In addition, OA has previously been shown to indirectly affect coral settlement due to changes in the community composition of the substrate (Albright and Langdon 2011; Doropoulos et al. 2012a) and interactions among coral larvae and crustose coralline algae (CCA) (Doropoulos et al. 2012a; Doropoulos and Diaz-Pulido 2013). Accordingly, in this study we characterise the community composition surrounding the coral recruits in order to assess whether this impacts the photo-physiological response of the symbiont to OA conditions.

Small variations in factors that influence recruit success, such as competition or physiological defects, can have profound impacts on coral community structure and their evolutionary development (Gosselin and Qian 1997). Juvenile mortality is naturally high among benthic marine invertebrates, with a less than 20% survival rate

past the first four months, while post-settlement survivorship of corals can be as low as 0.2-6.0% (Gosselin and Qian 1997; Fairfull and Harriott 1999; Wilson and Harrison 2005). Stochastic events and chronic stressors dictate survivorship during these early life history stages (Vermeij and Sandin 2008). The situation is complicated for corals as selective pressures can act phenotypically or genetically on the coral host (Marfenin 1997) or their symbiotic dinoflagellates (genus: *Symbiodinium*) (Howells et al. 2012) and potential combinations of both (Little et al. 2004; LaJeunesse et al. 2010). Nine major genetic clades (A-I) exist within the genus *Symbiodinium* (Rowan and Powers 1991; Pochon and Gates 2010) and these clades contain genetically and ecologically distinct types (LaJeunesse et al. 2004a; LaJeunesse et al. 2004b; Warner et al. 2006; Pochon et al. 2007; Sampayo et al. 2007). Differences in physiological traits among *Symbiodinium* include cell size (LaJeunesse et al. 2005), pigment composition (Frade et al. 2008), photosynthetic performance (Iglesias-Prieto et al. 2004); photosynthate composition (Loram et al. 2007) and tolerance to heat stress (Rowan and Knowlton 1995; Iglesias-Prieto et al. 2004; Berkelmans and van Oppen 2006; Sampayo et al. 2008). In particular, *Symbiodinium* types D1 and D1-4 (D1-4 provisionally named *Symbiodinium trenchi*) have been related to increased thermal tolerance of the coral holobiont, while certain C types are more sensitive to heat stress than other types (Berkelmans and van Oppen 2006; Wham et al. 2011). In contrast, the differential response of the coral holobiont to the effects of OA in relation to the type of *Symbiodinium* they harbour is largely unknown [except see Noonan et al. (2013)]. While symbiont community shifts may occur in some adult reef-building corals in response to stress (Berkelmans and van Oppen 2006; Jones et al. 2008), the population reverts back to the original symbiont community when the stress subsides and is generally stable over time (Thornhill et al. 2006; Finney et al. 2010). There is, however, greater opportunity for selective pressures to act during early ontogeny due to significant flexibility in the establishment of endosymbiosis (Gómez-Cabrera et al. 2008; Abrego et al. 2009; Cumbo et al. 2013). In the present study, we investigate the potential for the chronic stressor, OA, to exhibit selective pressure on *A. millepora* through association with *Symbiodinium*.

Methods

Coral spawning and settlement

Five gravid adult colonies of *Acropora millepora* were collected from Heron Island reef flat (23°26.73' S, 151°54.77' E) and placed in separate aquaria with ambient flow-through seawater. Spawning occurred seven nights after the full moon on 29 November 2010 and egg-sperm bundles were collected from each colony. Gametes were released by gentle agitation of the bundles and cross-fertilised for two hours. Embryos were then placed in 200 L tubs with aeration and for the first day approximately half the ambient seawater was changed every four hours to avoid fouling. Thereafter, water changes occurred as needed every 6 - 12 hours.

Five nights after spawning, terracotta and limestone tiles were added to the tubs for settlement. The tiles measured approximately 5 x 5 x 0.5 cm, were stacked in pairs and separated by a 0.5 cm spacer. In the six months prior to the experiment, those tiles had been pre-conditioned on Heron Island reef flat to develop a community of crustose coralline algae (CCA) and microbial biofilms necessary to cue settlement and metamorphosis of the planulae larvae (Negri et al. 2001; Webster et al. 2004). Fouling organisms had been carefully removed from the tiles using plastic scrapers, toothbrushes and tweezers. Following three days settlement time, we scored the number of recruits on each tile using a dissecting microscope, and the tiles were randomly allocated to CO_2 treatments. Each CO_2 treatment had seven replicate flow-through aquaria randomly placed on an outdoor table.

Experimental Protocol

Seawater carbonate chemistry was maintained via a computer-controlled solenoid valve system (Aquatronica-AEB Technologies, Cavriago, Italy), which bubbled CO_2 to levels representing IPCC (Intergovernmental Panel on Climate Change) CO_2 scenarios for the year 2100. The water temperature remained ambient (mean 27 ± 1°C) throughout the experiment. The A1FI treatment was pH 7.60 ± 0.10, the B2 treatment was pH 7.81 ± 0.10 and the control measured pH 8.04 ± 0.14 (Table 1). The pH sensors (InPro4501VP, Mettler Toledo, Langacher, Switzerland), calibrated with NBS scale standard buffers (Mettler Toledo), were used to continuously monitor the

pH in the 200 L tubs and the seven replicate aquaria. The aquaria were maintained outdoors and covered with neutral density filter (Lee 298 ND 0.15; LEE Filters Limited, Andover, UK) and thin shade cloth which resulted in light averaging 140 μmol quanta m^{-2} s^{-1} during a 12 hour photoperiod. These low light levels were maintained as corals commonly recruit to cryptic, low-light habitats (Babcock and Mundy 1996; Baird and Hughes 2000).

In order to fully characterise the carbonate chemistry, the treatment water was sampled every six hours for 48 hours during a spring and neap tidal regime for Total Alkalinity (A_T) measurement via Gran titration (T50 titrator, Mettler Toledo) (Gran 1952; Dickson et al. 2007). The A_T and pH were entered, along with ambient temperature (27 °C), pressure (10.16 dbars) and salinity (35 PSU), into the CO_2calc program (Robbins et al. 2010) (Table 1). For the calculations, we specified the total hydrogen ion scale, the carbonic acid dissociation constants of Mehrbach (1973) as refit by Dickson and Millero (1987) and the sulfonic acid dissociation constant of Dickson (1990). We tested the statistical significance of the treatments using a one-way PERMANOVA in PRIMER-e (v 6.1.13), which indicated that A_T was not statistically significant between CO_2 treatments (Pseudo $F_{2,47} = 0.0005$, $p = 0.999$) and all other parameters were statistically distinct between CO_2 treatments (Pseudo $F_{2,47} > 43.25$, $p = 0.0001$) (Anderson et al. 2008).

To determine whether CO_2 treatment would change *Symbiodinium* uptake, the recruits were offered a variety of cultured *Symbiodinium* to supplement those that would naturally reside in the environment (Coffroth et al. 2006; Porto et al. 2008) (Table 2). *A. millepora* recruits have been known to establish symbiosis within 5 - 13 days of settlement (Babcock 1985; Babcock and Heyward 1986). Beginning five days after settlement, we isolated the flow-through system for one hour to disperse approximately 1.2 x 10^6 cells of each *Symbiodinium* clade diluted in 5 mL culture media [Guillard's (F/2) Marine Water Enrichment Solution, Sigma-Aldrich, Australia] throughout the 15 L aquaria. This process was repeated for three days and then 23 days after settlement we randomly selected one tile per tank and scored the percentage of recruits with endosymbionts using the dissecting microscope. These tiles were subsequently returned to their respective treatments. For all tile analyses,

we only used the underside of the tile pairs to avoid confounding effects due differential to light levels.

Response Variables

After two months in the OA treatments we randomly selected three tiles per tank and five recruits per tile and to assess photochemical efficiency of Photosystem II (PSII) in *Symbiodinium* with the imaging Pulse Amplitude Modulating Fluorometer (iPAM, Walz, Effeltrich, Germany). Prior to commencement of the induction curves, the tiles were placed in a dark container with treatment water to dark-acclimate for 15 minutes. An initial saturating pulse enabled calculation of the maximum quantum yield of PSII, F_v/F_m. The actinic light was then set for one minute at 110 µmol quanta m^{-2} s^{-1}, which was similar to the average ambient light in the experimental aquaria. Another saturating pulse applied after this illumination enabled partitioning of the absorbed excitation energy between the light used for photochemistry, and excitation energy dissipated either through non-photochemical quenching (Φ_{NPQ}) or fluorescence (Φ_{NO}). Furthermore, we calculated $\Delta F/F_m'$ and used this to derive Excitation Pressure (Q_m = $1 - [(\Delta F/F_m') \cdot (F_v/F_m)^{-1}]$) (Iglesias-Prieto et al. 2004). Thereafter, the recruits recovered for five minutes in the dark and a final saturating pulse was applied. The recruit positions were mapped from high-resolution digital images of the tiles and aligned with the iPAM images in order to define the areas of interest in the iPAM software (ImagingWin, Walz).

Using the same recruits, bleaching was quantified as a reduction in luminescence relative to the maximum, signifying symbiont or chlorophyll density, following the method of Anthony et al. (2008). We used the C1 - C6 scale of luminescence on the CoralWatch Coral Health Chart (CoralWatch 2001), with C1 being 100% bleached and C6 being 0% bleached. The luminescence measurement was performed for areas of interest defined at the centre of the polyp and the whole recruit from the digital images of the tiles in Photoshop (Adobe Systems Software).

In addition, the benthic community at the edge of these recruits was quantified using the point-intercept method by placing an eight-point circular grid around the recruits on the digital images of the tiles in Photoshop (Adobe Systems Software, Ireland). We classified the substrata into the following benthic functional groups: bare tile; live

CCA; dead CCA; encrusting fleshy algae; turf + endolithic algae (where endoliths were within the surrounding substrata as opposed to the coral recruit skeleton); shell (including empty polychaete tubes and gastropod shells) and coral recruit. For images of the recruits surrounded by examples of the benthic functional groups see Figure 1.

Genotyping

Following the iPAM assays, the recruits were scraped from the tiles using a sterile surgical blade and preserved in salt-saturated 20% dimethyl sulfoxide (SS-DMSO) (Seutin et al. 1991). DNA was extracted using the PowerBiofilm DNA isolation kit (Mo Bio Laboratories Inc, California, USA) according to the manufacturers protocol and amplified using *Symbiodinium* specific primers 'ITS1CLAMP' (5' CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGGATCCGTTTC CGTAGGTGAACCTGC 3') and 'ITS1intrev2' (5' TTCACGGAGTTCTGCAAT 3') targeting the internal transcribed spacer region 1 (ITS1) of the ribosomal genes (LaJeunesse et al. 2008). PCR amplification (initialization: 94°C, 3 min; denaturation of 35 cycles: 94°C, 40 sec: annealing: 62°C, 40 sec; elongation: 72°C, 30 sec; final step: 72°C, 10 min) was followed by polymorphism screening using denaturing gradient gel electrophoresis (DGGE) in a vertical system (CBS Scientific, San Diego, USA) (LaJeunesse 2001; LaJeunesse et al. 2003) and run for 14 hours on 8% polyacrylamide gels (37.5:1 acrylamide/bis) with a gradient of 35-60% denaturants (formamide and urea). DGGE was used to give resolution at the level of *Symbiodinium* types rather than at clade level although it is unable to detect background types < 10% of the population (Fabricius et al. 2004; Thornhill et al. 2006). Up to five representative samples of each characteristic ITS1-DGGE fingerprint were used to identify the *Symbiodinium* type by stabbing the dominant DNA bands from the denaturing gel using a 10 µl pipette tip and placing the tip in 30 µl H_2O for two hours. The DNA was then re-amplified using the 'ITS1intfor' primer (without the GC-clamp) and reverse primer 'ITS1intrev' using the same PCR thermal cycle profile as mentioned above. PCR products were cleaned using ExoSap-IT (GE Healthcare Limited, Buckinghamshire, UK) according to the manufacturers' protocol and sequenced at the Macrogen (Seoul, Korea) using an ABI 3730*xl* sequencer. Sequence chromatograms were visually checked using Codoncode Aligner version

3.5.3. (Codoncode Corporation). The resulting sequences were blasted against previously recorded *Symbiodinium* types on GenBank (http://www.ncbi.nih.gov).

Statistical Analysis

To determine the effect of CO_2 treatment, multivariate analyses were run on the groups of response variables (iPAM, bleaching, benthic functional groups, *Symbiodinium* types) in PRIMER-e (v 6.1.13) with PERMANOVA add-on (v 1.03) (Anderson et al. 2008). A mixed effects model was used in which CO_2 treatment was specified as a fixed factor, tank as a random factor nested in treatment and tile as a random factor nested in tank (Anderson 2001). The iPAM and bleaching data were normalised and resemblance matrices were based on Euclidean distance due to the different scales used for response variables. Benthic substrate was square root transformed and Bray Curtis similarity with dummy variable (+1) added to define joint zeros was used for the resemblance matrix. *Symbiodinium* data consisted of type presence/absence data and type dominance data, which were used to create a Jaccard resemblance matrix. The *Symbiodinium* types were designated dominant when the band intensity was > 30% luminescence relative to the maximum as quantified from the gel images in Photoshop (Adobe Systems Software), using the same method described above for the bleaching index (Anthony et al. 2008). The effect of CO_2 treatment on individual variables was also assessed using a univariate PERMANOVA using the same mixed effect model previously described. Type (III) Partial Sums of Squares were specified and the Pseudo-F distribution was created by 9999 permutations of the residuals under a reduced model for the main test, whereas subsequent pairwise tests used Monte Carlo simulations (Anderson and Robinson 2001).

Distance-based linear models (DISTLM) and distance-based redundancy analyses (dbRDA) were performed in PRIMER with PERMANOVA to assess the relationship between groups of predictor variables and groups of response variables (Legendre and Anderson 1999; McArdle and Anderson 2001). In this way, the variation in photo-physiology (iPAM and bleaching response) was partitioned in response to benthic substrate and CO_2 level. In addition, we assessed the significance of *Symbiodinium* types present (detectable at any band intensity), *Symbiodinium* type dominance (detectable at band intensities > 30% luminescence threshold), benthic substrate and

CO_2 level on the recruit bleaching response. Furthermore, we tested the hypothesis that CO_2 level and benthic substrate may influence *Symbiodinium* types present and/or *Symbiodinium* type dominance.

Results

Photo-physiology

The photochemical efficiency of PSII was not significantly affected by the CO_2 treatment when assessed as a multivariate (Pseudo $F_{2,228} = 0.72$; $p = 0.67$) or univariate response (See Supplementary Material Table S1). However, at the centre of the polyp, bleaching significantly increased more than 2.5 fold under the B2 and A1FI IPCC CO_2 scenarios in comparison to controls (Pseudo $F_{2,232} = 5.84$, $p = 0.01$). Similarly, bleaching of the whole recruit significantly increased by 30% under the B2 IPCC CO_2 scenario (Pseudo $F_{2,232} = 4.36$, $p = 0.03$) but there was no significant increase under the A1FI CO_2 scenario, although there was an increasing trend relative to the control treatment (Figure 2).

Symbiodinium

The percentage of recruits that had established endosymbiosis 23 days after settlement was not significantly affected by the CO_2 treatments, although there was a declining trend with increasing CO_2 (Pseudo $F_{2,18} = 1.48$, $p = 0.25$; See Supplementary Material Table S2) (Figure 3). After two months in the CO_2 treatments, *Symbiodinium* from clades A, C and D were detected in the recruits with 64.7% hosting multiple clades. At the clade level, the percentage of *Symbiodinium* present in the coral recruits was Clade A at 92.5%, Clade C at 24.8% and Clade D at 59.4%. The *Symbiodinium* types detected were A3, A5 and two unofficial clade A types that, for the purpose of this study were named A_EF455526 and A_AF333508 according to their Genbank accession numbers (EF455526 and AF333508 respectively), C3, D1 and D1-4 (provisionally named *Symbiodinium trenchi* and also known as D1a) (LaJeunesse et al. 2010a; LaJeunesse et al. 2010b). Due to a high degree of sequence homology in A5 and A_AF333508 these types were pooled and as type D1-4 was only present in two recruits we pooled this data with type D1 for subsequent analyses. The recruits may have taken up the cultured *Symbiodinium* type D1 but did not take up types A2, C_AF360576 (Genbank accession number AF360576) or C1 (within the detection

limits of DGGE). There were no significant differences in the *Symbiodinium* types present in the recruits (Pseudo $F_{2,103} = 0.51$, $p = 0.83$) and there was no change in the dominant *Symbiodinium* type due to CO_2 treatment (Pseudo $F_{2,103} = 1.58$, $p = 0.15$) although there were phenotypic differences of the types within treatment (See Combined Effects).

Benthic Substrate

CO_2 treatment significantly affected the composition of benthic functional groups around the edge of the recruits (Pseudo $F_{2,232} = 3.27$, $p = 0.02$; See Supplementary Material Table S3). Upon further analysis of the individual functional groups, we found that this response was characterised by a 33% decline in CCA at the edge of the recruits in the A1FI CO_2 scenario in comparison to the controls (Pseudo $F_{2,232} = 2.99$, $p = 0.07$) (Figure 4).

Combined Effects

Together, the benthic functional group surrounding the recruits and CO_2 treatments explained 10% of the variation in photo-physiology data (iPAM and bleaching response) (Figure 5). The dbRDA ordination clearly shows separation of the control group from the IPCC B2 and A1FI CO_2 treatments. When assessed separately in the marginal tests of the DISTLM analysis, the benthic functional groups bare tile, CCA, encrusting fleshy algae, and turf + endolithic algae along with the CO_2 treatments significantly explained the photo-physiological response ($p < 0.05$; See Supplementary Material Table S4). The vectors on the dbRDA ordination indicate that a similar photo-physiological response was found among recruits surrounded by CCA and encrusting fleshy algae or maintained under control conditions. In contrast, recruits in the IPCC B2 or A1FI CO_2 treatments displayed a similar photo-physiological response as recruits surrounded by bare tile or turf + endolithic algae, respectively (Figure 5).

Overall, the *Symbiodinium* type ('presence' indicating the type was detectable at any band intensity and 'dominance' indicating detectable at band intensities > 30% luminescence threshold), benthic substrate and CO_2 treatments explained 25% of the variation in the bleaching response (Figure 6). The marginal tests of the DISTLM analysis indicated that statistically significant predictors of the bleaching response

were *Symbiodinium* type D1 + D1-4 dominance along with the A1FI CO_2 scenario and Control conditions ($p < 0.05$; See Supplementary Material Table S5). On average, recruits that were dominant with *Symbiodinium* types D1 + D1-4 were 56% (n = 22) and 47% (n = 18) more bleached at the centre of the polyp in the B2 and A1FI CO_2 treatments, respectively. Similarly, the D1 + D1-4 dominant whole recruits were 28% and 17% more bleached in the B2 and A1FI CO_2 treatments, respectively. *Symbiodinium* type C3 presence also explained a relatively high proportion of the variation in the bleaching response but was not statistically significant (Pseudo F = 2.67, p = 0.10). An additional DISTLM analysis indicated that the substrate and CO_2 levels were not significant predictors of the uptake of *Symbiodinium* types (presence and dominance) (See Supplementary Material Figure S1 and Table S6).

Discussion

In this study, we set out to investigate the potential for OA to exhibit selective pressure on *Acropora millepora* through establishment of endosymbiosis with *Symbiodinium* during early ontogeny. As a result of increasing CO_2, the recruits were bleached at the centre of the polyp > 2.5 fold relative to controls, which may represent changes in light-harvesting pigment density or changes in *Symbiodinium* population density. Similarly, the whole recruit was 30% more bleached under IPCC B2 pCO_2 conditions (pH 7.80 ± 0.10) in comparison to the controls yet not under the IPCC A1FI pCO_2 conditions (pH 7.61 ± 0.10), which may be related to the benthic functional groups surrounding the recruits (see below). Recruits containing a dominant population of *Symbiodinium* type D1 or D1-4 were more bleached than recruits containing *Symbiodinium* type C3 and visual bleaching increased under the CO_2 treatments. Interestingly, these results are the opposite of the effect of increased SST on adult colonies harbouring the more thermo-tolerant D1 versus the bleaching susceptible C3 (Jones et al. 2008). Together, these findings indicate that OA does exhibit a phenotypic selective pressure on *A. millepora* recruits, which may have implications for post-settlement survivorship and population dynamics. In addition, the benthic functional groups surrounding the recruits were significant in explaining the coral photo-physiological response with certain substrata (turf + endolithic algae) correlating with the response of recruits under the A1FI pCO_2 conditions while other substrata (live CCA) correlated with control conditions. Unfortunately, under the

A1FI pCO_2 conditions, the percentage of live CCA surrounding the edge of the recruits significantly declined. These findings highlight the importance of benthic functional groups, which could potentially exacerbate OA conditions in the DBL of the coral recruits.

The phenomenon of coral bleaching, caused by the loss of *Symbiodinium* cells or degradation of their photosynthetic pigments, is generally associated with changes in sea surface temperature (SST) and light intensity (Hoegh-Guldberg 1999). More recently, OA has been shown to contribute to the bleaching response under high irradiance in adult reef-building corals, although the change in luminescence may be due to changes in either pigment or cell density (Anthony et al. 2008). Similarly, the bleaching response observed in the present study may be a result of changes in light-harvesting pigments such as chlorophyll *a* (chl *a*) and chlorophyll *c2*, or changes in pigments associated with thermal dissipation such as the xanthophyll cycle pigments, diadinoxanthin and diatoxanthin (Demmig-Adams and Adams 1996). Yet, in a study conducted at sub-saturating light intensities, the chl *a* and the xanthophyll pool per symbiont cell in *Acropora formosa* increased in response to increasing CO_2 (Crawley et al. 2010). This was presumably due to an increased capacity of the symbiont to process products of the photosynthetic "light-reactions" through the carboxylation of Rubsico and decreased photoprotection through photorespiration (Crawley et al. 2010). Similarly, as the present study was conducted under low irradiance, unlike Anthony et al. (2008), the bleaching response is more likely to be due to decreased symbiont cell density, rather than pigments. A decrease in *Symbiodinium* cells may have significant implications for the growth rate of *A. millepora* as there could be less carbon transferred to the host, although this depends on the initial symbiont density and whether the coral can continue to operate within a range of optimal energy acquisition (Hoogenboom et al. 2010). If the bleaching observed led to a shift in the dominant symbiont type, then this may have additional consequences for the energy budget. Although *Symbiodinium* D1 and D1-4 are generally regarded as more thermally tolerant, there are energetic trade-offs associated to harbouring these types such as reduced growth rates, with downstream effects on coral lipids, reproductive capacity (Little et al. 2004; Jones and Berkelmans 2011) and disease susceptibility (Bruno et al. 2007). In fact, *Symbiodinium* type D1-4 has been described as an opportunistic endosymbiont that outcompetes the optimal symbiont under acute or

chronic stressors (Stat and Gates 2011). These trade-offs could explain that whilst *Symbiodinium* D1 and D1-4 are known to perform better under increased SST conditions, this advantage is not necessarily sustained under other stressful conditions, such as increased pCO_2.

Noteworthy in the context of the juvenile growth rate is the need to exceed a size threshold in order to avoid mortality from disturbances such as fish grazing (Doropoulos et al. 2012b). It has previously been demonstrated that the risk of juvenile mortality is inversely proportional to growth rate and colony size (Babcock 1985; Hughes and Jackson 1985; Gosselin and Qian 1997). The delayed uptake of *Symbiodinium*, while not statistically significant in this study (Figure 3), has previously been demonstrated in *Acropora* spp. (Suwa et al. 2010) and may contribute to reduced growth rates. While the dominance of *Symbiodinium* D1 and D1-4 may allow recruits to survive OA-induced bleaching, reduced growth rates (Cohen et al. 2009; Albright et al. 2010; Doropoulos et al. 2012b) may render those recruits vulnerable to increased mortality due to predation (Doropoulos et al. 2012b) and presumably other acute disturbances such as increased SSTs (Stat and Gates 2011).

Horizontal transmission of *Symbiodinium* creates a window of opportunity for new recruits to harbour diverse symbionts, yet in many cases adult colonies exhibit high specificity with strong influence from the local environment (LaJuenesse et al. 2004a, LaJuenesse et al. 2004b; Abrego et al. 2009; LaJuenesse et al. 2010a). In this study, the recruits did not take up the cultured *Symbiodinium* types A2, C_AF360576 or C1 (above the detection limits of DGGE), as these types were not likely adapted to the local environment due to their distant geographical origin (van Oppen 2004). Adult *A. millepora* colonies predominantly harbour *Symbiodinium* ITS2 types C1, C3, D1 and more rarely types A, C3k and D1-4 across the Great Barrier Reef (Cooper et al. 2011; Tonk et al. 2013) while at Heron Island the dominant type is C3 (Tonk et al. 2013). The *A. millepora* juveniles in this study contained a high diversity of *Symbiodinium* types within Clade A and although this type was not significant in explaining the photo-physiological response, it may have been an opportunistic symbiont. A recent study has shown that *A. millepora* adults hosted types C1 and C3 near a volcanic seep site and at a nearby low pCO_2 site, while other corals hosted type D1 signifying its potential availability in the local environment (Noonan et al. 2013). This aligns with

the results of this study indicating that type D1 may not suitable for *A. millepora* under high pCO_2 conditions and also suggests that local adaptations will play an important role under future environmental perturbations (Howells et al. 2012). Long-term research is required to verify whether environmental factors such as increased pCO_2 can lead to local adaptation or a persistent change in *Symbiodinium* types.

A number of environmental factors have been shown to drive the distribution of *Symbiodinium* in *A. millepora* across the Great Barrier Reef, including SST anomalies, mean summer SST, mud and carbonate content of the sediments and distance from coastline (Cooper et al. 2011; Tonk et al. 2013). Both studies indicate additional environmental variables are at play, accounting for the unexplained variation in the *Symbiodinium* distribution data in their analyses. Interestingly, shallow reef flats that become isolated from oceanic water on low tide exhibit large fluctuations in SST, as well as large diel oscillations in carbonate chemistry (Shaw et al. 2012). In the present study, we have shown that OA alone can exert phenotypic selective pressure on *Symbiodnium* spp. *in hospite.* Conceivably, this factor may already be acting *in situ*, potentially masked by a correlation with SST anomalies. Future studies are needed to fully characterise site carbonate chemistry in order to confirm this hypothesis. Additionally, carbonate chemistry should be included as a potential predictor in multivariate analyses that aim to assess factors driving *Symbiodinium* distribution and the vulnerability of reef-building corals to a *Symbiodinium* D1 or D1-4 community shift.

The impact of community composition on carbonate chemistry presents an additional challenge for growth and survivorship of newly settled recruits under OA conditions. In our model, the photo-physiological response of recruits surrounded by turf + endolithic algae was significantly different from recruits surrounded by bare tile, as exhibited by the polarisation of these vectors in the dbRDA (Figure 5). It is not surprising that this axis only explained a small proportion of the total variation as there were no statistical differences in the fluorescence parameters and stochastic variation in recruit survival may be high during post-settlement phases (Gosselin and Qian 1997; Vermeij and Sandin 2008). Over time, however, the community composition of settlement substrate may have ecologically significant implications on the growth and survival of coral juveniles (Gosselin and Qian 1997). Interestingly,

turf + endolithic algae aligns with the highest pCO_2 treatment, the IPCC A1FI scenario, implying that this substrate produces a similar photo-physiological response. Indeed, the diel photosynthesis and respiration cycles could exacerbate the pCO_2 conditions creating even lower pH conditions at night but increasing the pH during the day (Shaw et al. 2013). A recent short-term study has demonstrated oscillating carbonate chemistry, as opposed to stable conditions, can improve growth and survivorship of recruits of the brooding coral, *Seriatopora caliendrum* presumably due to daytime relief from low pH (Dufault et al. 2012). The incidence of turf + endolithic algae surrounding recruits in the IPCC A1FI pCO_2 scenario may have improved the daytime pCO_2 conditions and may explain the slight decrease in the bleaching response for the whole recruit (Figure 2B). Dufault et al. (2012) hypothesised that nocturnal increases in pCO_2 due to respiration can accumulate and increase the intracellular dissolved inorganic carbon (DIC) pool available for calcification and photosynthesis the following day.

A. millepora recruits surrounded by live CCA demonstrated a similar photo-physiological response to recruits under control conditions, as indicated by the similar direction of the vectors in the dbRDA (Figure 5). CCA provides an important settlement cue for *A. millepora* planulae larvae (Heyward and Negri 1999; Negri et al. 2001), which has previously been shown to perform poorly in high pCO_2 conditions (Albright et al. 2010; Doropoulos et al. 2012a). Our results align with previous studies, as after two months in the IPCC A1FI pCO_2 conditions, the percentage of live CCA surrounding the edge of the recruits significantly declined (Figure 4). The impact of OA on CCA represents an indirect mechanism by which OA may reduce *A. millepora* settlement. Furthermore, as coral recruits are less likely to exhibit a bleaching response when surrounded by CCA, the loss of CCA substrate due to OA may reduce the growth and survivorship of *A. millepora* post-settlement.

This research demonstrates that the chronic stressor, OA, causes bleaching in *A. millepora* recruits and may exhibit selective pressure through *Symbiodnium* association. Reduced symbiont density or association with particular *Symbiodinium* types may diminish growth rates thereby rendering the recruits more susceptible to acute stressors (Stat and Gates 2011; Doropoulos et al. 2012b). This will have implications for subsequent life history stages with less capacity to store lipids and

reduced reproductive output (Little et al. 2004). In addition, this study has also identified critical interactions with the benthic community, which warrant further investigation. In the field, a fine line exists between the benefits obtained from favourable manipulation of carbonate chemistry by algal photosynthesis and the potential disadvantage due to competitive interactions. Overall, the impact of OA represents a substantial threat to *A. millepora* recruitment with significant implications for the resilience of reef-building corals to both acute and chronic stressors.

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Table 1: Carbonate chemistry of the seawater in the CO_2 treatments. Total Alkalinity (A_T) and pH were measured during the experiment and are reported as mean ± standard deviation. These values along with ambient temperature (27°C), salinity (35 PSU) and pressure (10.16 dbars) were input into the CO_2calc program (Robbins et al. 2010) to derive the remaining parameters. We used the total hydrogen ion scale, the carbonic acid dissociation constants of Mehrbach (1973) as refit by Dickson and Millero (1987) and the sulfonic acid dissociation constant of Dickson (1990). A_T was not statistically significant between CO_2 treatments (Pseudo $F_{2,47} = 0.0005$, $p = 0.999$) and all other parameters were statistically distinct between CO_2 treatments (Pseudo $F_{2,47} > 43.25$, $p = 0.0001$).

Treatment	pH	A_T μmol kg^{-1}	pCO_2 μatm	HCO_3^- μmol kg^{-1}	CO_3^{2-} μmol kg^{-1}	Ω arag
Control	8.04 ± 0.14	2291 ± 34	399 ± 169	1743 ± 162	223 ± 51	3.56 ± 0.81
B2	7.81 ± 0.10	2291 ± 33	752 ± 214	1934 ± 98	145 ± 26	2.33 ± 0.42
A1FI	7.60 ± 0.10	2290 ± 33	1297 ± 352	2056 ± 79	95 ± 18	1.53 ± 0.29

Table 2: Details of the *Symbiodinium* cultures offered to the *Acropora millepora* recruits five - eight days after settlement.

Culture ID	Clade	Type / Genbank Accession no.	Isolated from Host	Origin of Culture
A001	D	D1	*Acropora* sp.	Okinawa
A013	D	D1	*Porites annae*	Okinawa
Sin	C	AF360576	*Sinularia* sp.	Guam
A2	A	A2	*Zoanthus sociatus*	Jamaica
C1	C	C1	*Diccospina sanctithomae*	Jamaica

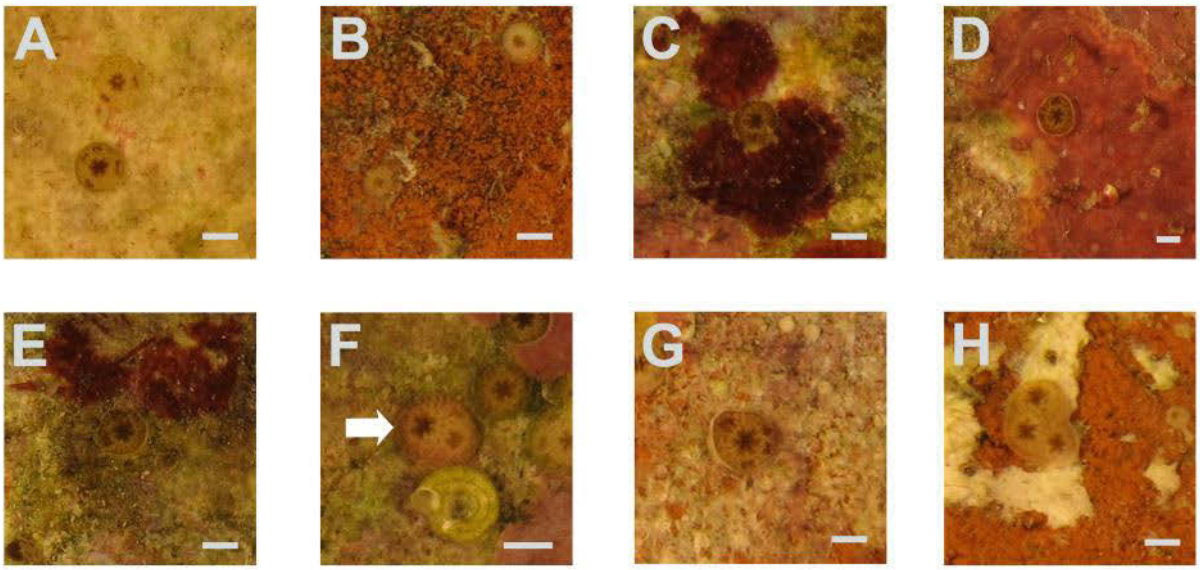

Figure 1: Examples of the benthic functional groups surrounding the *Acropora millepora* recruits (indicated by the arrow where necessary). Scale bar = 1.0 mm. A. turf + endolithic algae; B. bare tile (also some turf + endolithic algae); C. encrusting fleshy algae (also some dead crustose coralline algae [CCA]); D. live CCA; E. turf + endolithic algae (also some encrusting fleshy algae); F. shell and coral (also some turf + endolithic algae); G. dead CCA; H. dead CCA and bare tile.

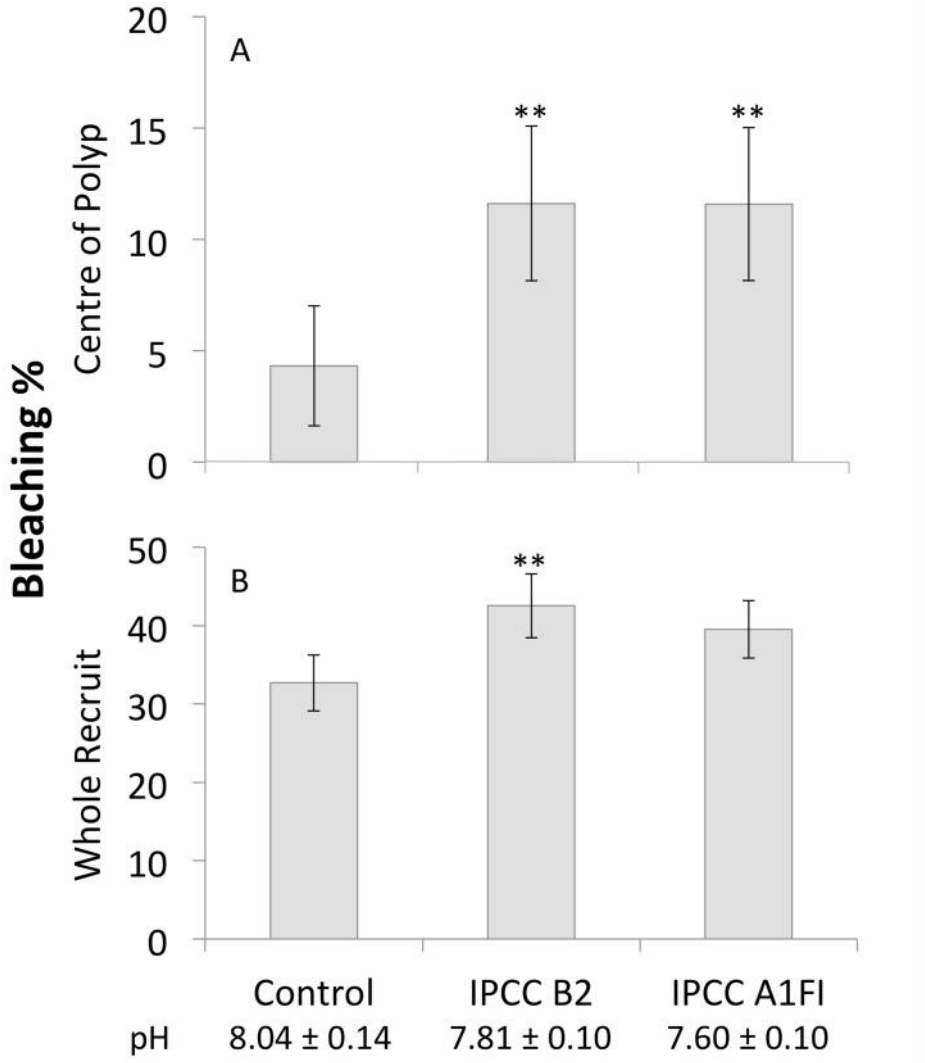

Figure 2: Bleaching % of *Acropora millepora* assessed A. at the centre of the polyp and B. for the whole recruit. Bleaching was quantified as a reduction in luminescence relative to the maximum, signifying symbiont or chlorophyll density, following the method of Anthony et al. (2008). Error bars indicate standard error (n = 15) and ** denotes significantly different to the control ($p < 0.01$).

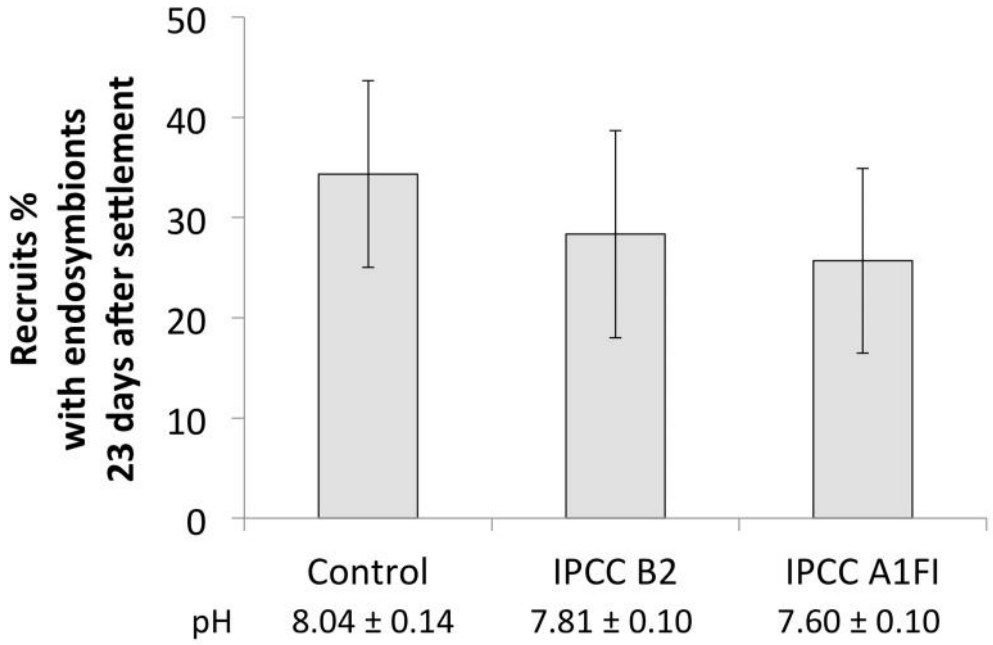

Figure 3: The percentage of *Acropora millepora* recruits that had established endosymbiosis with *Symbiodinium* 23 days after settlement. Error bars indicate standard deviation (n = 7).

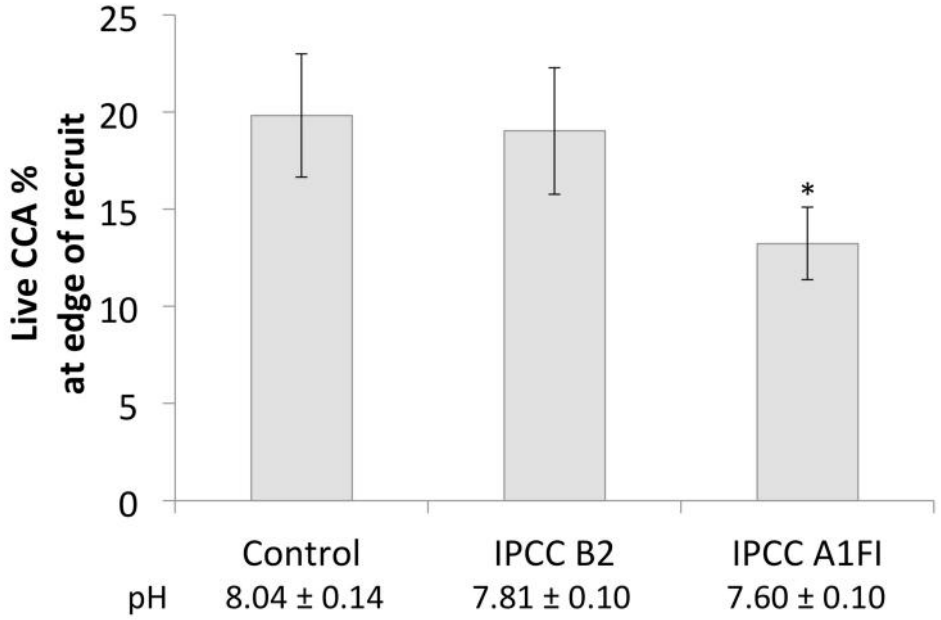

Figure 4: Percentage of live crustose coralline algae (CCA) at the edge of the *Acropora millepora* recruits after 2 months in IPCC CO_2 treatments. Error bars indicate standard error (n = 15) and * denotes significantly different from the control ($p < 0.05$).

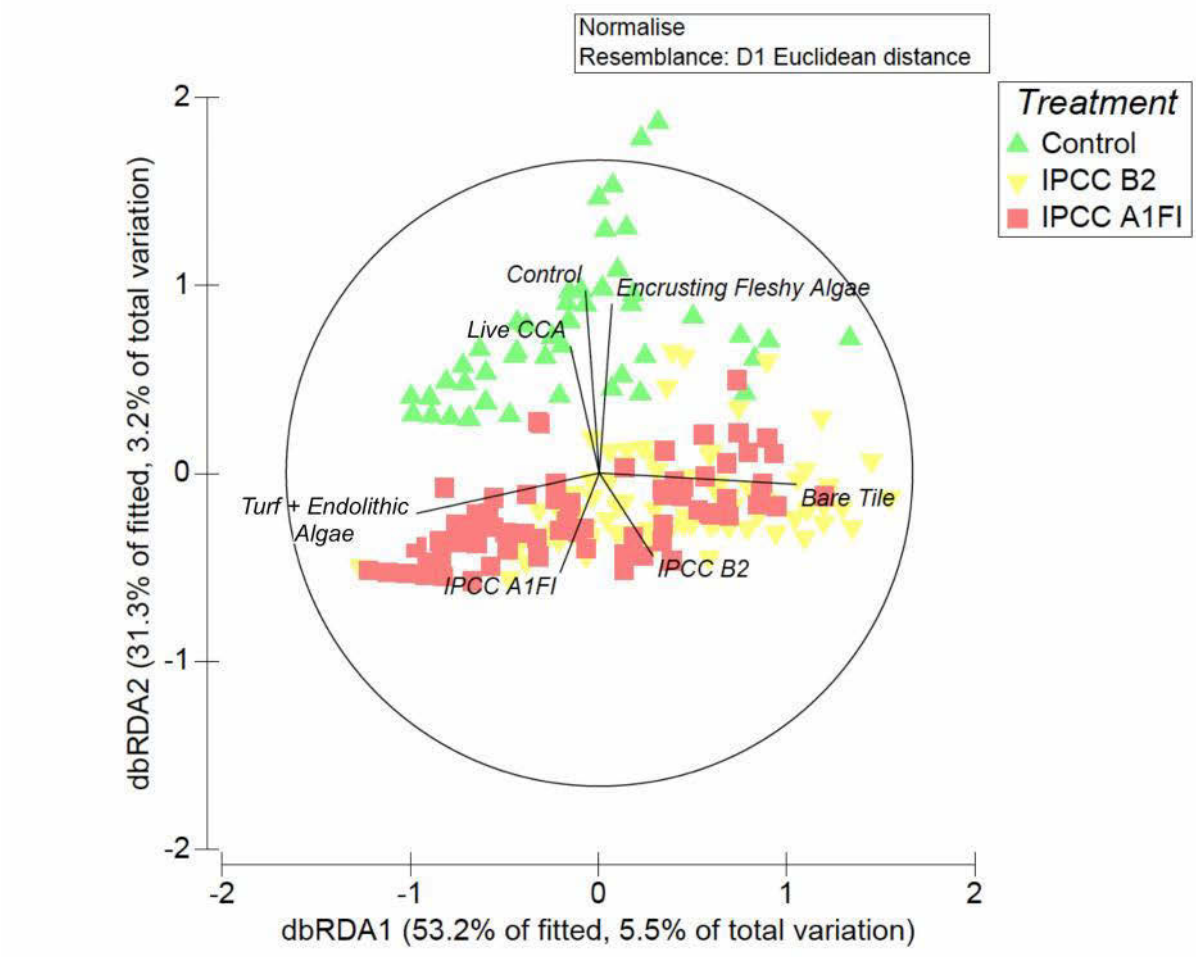

Figure 5: Distance-based redundancy analysis (dbRDA) showing ordination of the fitted values from the model of variation in photo-physiological response variables (dark-adapted *Fv/Fm*, at 110 µmol quanta m^{-2} s^{-1}: Φ_{NPQ}, Φ_{NO}, $\Delta F/F_m'$, Excitation Pressure (Q_m), Bleaching % at Centre of Polyp and Bleaching % for the Whole Recruit) explained by the benthic functional groups and CO_2 treatments. Vectors are predictor variables explaining a statistically significant proportion of the response ($p < 0.05$), as assessed by the marginal tests of distance-based linear models (DISTLM) (See Supplementary Material Table S4 for Full Statistics).

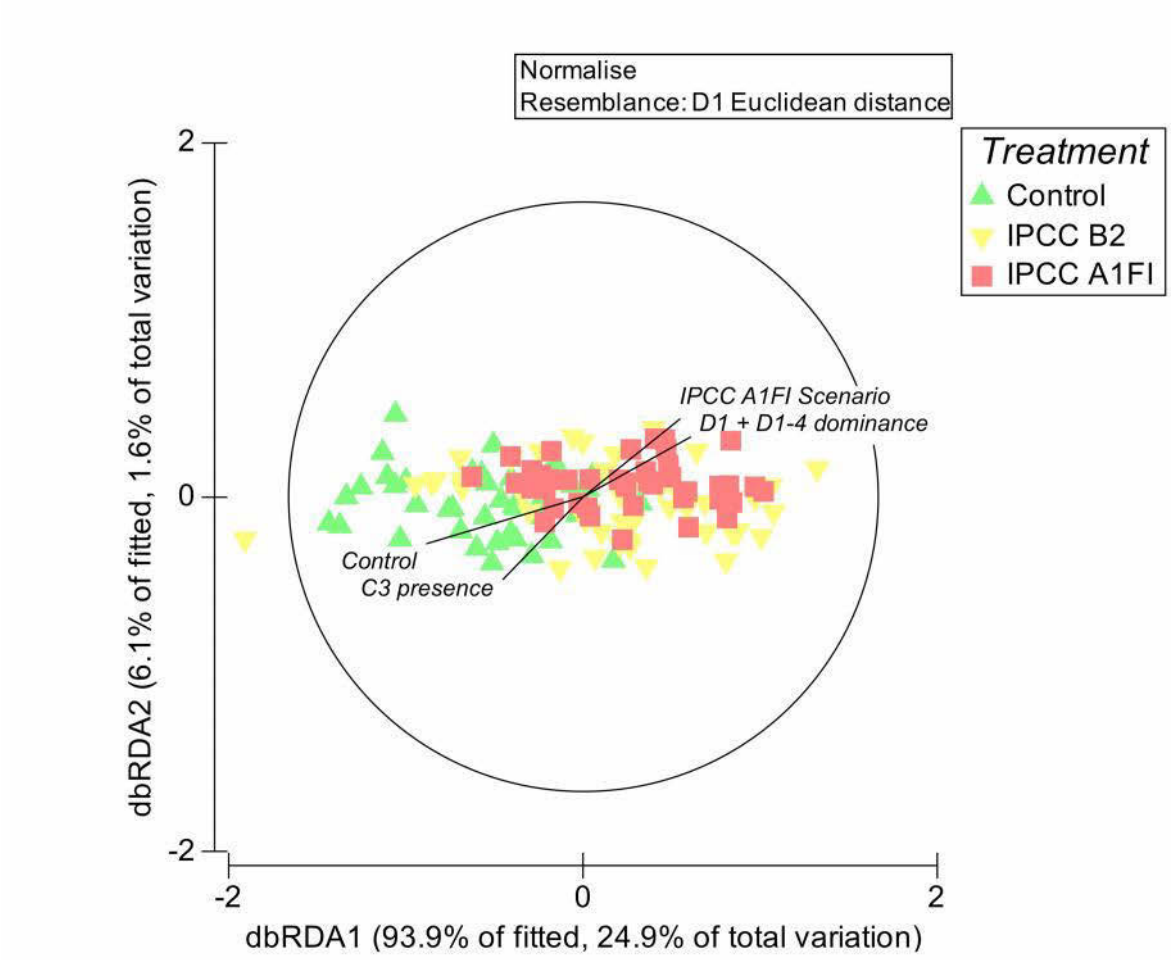

Figure 6: Distance-based redundancy analysis (dbRDA) showing ordination of the fitted values from the model of variation in bleaching response variables (Bleaching % at Centre of Polyp and Bleaching % for the Whole Recruit) explained by the *Symbiodinium* type (presence and dominance), benthic functional groups and CO_2 treatments. Vectors are predictor variables explaining a large proportion of the response ($p < 0.10$), as assessed by the marginal tests of distance-based linear models (DISTLM) (See Supplementary Material Table S5 for Full Statistics).

Table S1: PERMANOVA results of the photochemical efficiency of Photosystem II in *Symbiodinium* within *Acropora millepora* recruits after two months in CO_2 treatments. All models are univariate except the final model, iPAM multivariate, which includes all variables.

Variables	Source of Variation	df	Pseudo F	P (Permutation)
Fv/Fm	Treatment	2	0.87947	0.4379
dark-adapted	Tank (Treatment)	17	2.4578	0.0152
	Tile (Tank(Treatment))	38	21.145	0.0001
$\Delta F/Fm'$	Treatment	2	0.31794	0.7383
110 µmol quanta s^{-1}	Tank (Treatment)	17	1.2415	0.2811
	Tile (Tank(Treatment))	38	7.2193	0.0001
Q_m	Treatment	2	0.20475	0.8134
$1 - (\Delta F/Fm') \cdot (Fv/Fm)^{-1}$	Tank (Treatment)	17	1.0497	0.4253
	Tile (Tank(Treatment))	38	9.8168	0.0001
Fv/Fm	Treatment	2	0.015579	0.986
dark recovery	Tank (Treatment)	17	0.80958	0.6851
	Tile (Tank(Treatment))	38	5.4629	0.0001
Φ_{NPQ}	Treatment	2	0.86704	0.4328
110 µmol quanta s^{-1}	Tank (Treatment)	17	1.0805	0.4009
	Tile (Tank(Treatment))	38	2.5335	0.0001
Φ_{NPQ}	Treatment	2	1.8009	0.1936
dark recovery	Tank (Treatment)	17	0.55684	0.9077
	Tile (Tank(Treatment))	38	3.1206	0.0001
Φ_{NO} minimum	Treatment	2	0.888	0.4376
dark-adapted	Tank (Treatment)	17	2.3921	0.0178
	Tile (Tank(Treatment))	38	21.575	0.0001
Φ_{NO}	Treatment	2	1.2185	0.3196
110 µmol quanta s^{-1}	Tank (Treatment)	17	1.3991	0.187
	Tile (Tank(Treatment))	38	3.9426	0.0001
Φ_{NO}	Treatment	2	0.43264	0.6732
dark recovery	Tank (Treatment)	17	0.73565	0.7689
	Tile (Tank(Treatment))	38	7.3319	0.0001
iPAM multivariate	Treatment	2	0.72297	0.6717
(all of the above)	Tank (Treatment)	17	1.2935	0.0992
	Tile (Tank(Treatment))	38	6.1931	0.0001

Table S2: PERMANOVA results for the percentage of *Symbiodinium* uptake 23 days after settlement and the *Symbiodinium* type (presence and dominance) within *Acropora millepora* recruits after two months in CO_2 treatments.

Variables	Source of Variation	df	Pseudo F	P (Permutation)
Symbiodinium uptake 23 days after settlement	Treatment	2	1.4762	0.2536
		18		
Symbiodinium type presence	Treatment	2	0.51402	0.8308
	Tank (Treatment)	14	0.8878	0.645
	Tile (Tank(Treatment))	13	1.6868	0.0039
Symbiodinium type dominance	Treatment	2	1.5766	0.1466
	Tank (Treatment)	14	1.4751	0.1229
	Tile (Tank(Treatment))	13	1.0946	0.3056
Symbiodinium type presence + dominance	Treatment	2	0.90752	0.5122
	Tank (Treatment)	14	1.1207	0.3465
	Tile (Tank(Treatment))	13	1.4189	0.0256

Table S3: PERMANOVA results for the benthic functional groups surrounding the edge of the *Acropora millepora* recruits after two months in CO_2 treatments.

Variables	Source of Variation	df	Pseudo F	P (Permutation)	Pairwise P (Monte Carlo)
Benthic Substrate	Treatment	2	3.272	0.02	Control ≠ B2; p = 0.0035
	Tank (Treatment)	17	1.4668	0.0705	Control & A1FI; p = 0.0664
	Tile (Tank(Treatment))	38	3.6818	0.0001	B2 & A1FI; p = 0.2145
Live Crustose Coralline Algae	Treatment	2	2.993	0.0703	
	Tank (Treatment)	17	1.1144	0.376	
	Tile (Tank(Treatment))	38	2.7048	0.001	
Bare Tile	Treatment	2	4.4939	0.0196	Control < B2; p = 0.0017
	Tank (Treatment)	17	1.5412	0.1283	Control & A1FI; p = 0.0545
	Tile (Tank(Treatment))	38	5.3557	0.0001	B2 & A1FI; p = 0.426
Turf + Endolithic Algae	Treatment	2	1.5623	0.2296	
	Tank (Treatment)	17	1.9667	0.0267	
	Tile (Tank(Treatment))	38	2.8185	0.0001	
Encrusting Fleshy Algae	Treatment	2	0.27728	0.7962	
	Tank (Treatment)	17	0.77459	0.7266	
	Tile (Tank(Treatment))	38	1.3165	0.0986	
Shell	Treatment	2	2.4835	0.1033	
	Tank (Treatment)	17	0.69438	0.8044	
	Tile (Tank(Treatment))	38	1.9882	0.0012	
Coral	Treatment	2	2.4835	0.1033	
	Tank (Treatment)	17	0.69438	0.8044	
	Tile (Tank(Treatment))	38	1.9882	0.0012	
Dead Crustose Coralline Algae	Treatment	2	0.51146	0.6097	
	Tank (Treatment)	17	1.2942	0.2519	
	Tile (Tank(Treatment))	38	2.0434	0.0005	

Table S4: Distance-based linear model (DISTLM) marginal tests indicating the proportion of photo-physiology variation (dark-adapted *Fv*/*Fm*, at 110 µmol quanta m^{-2} s^{-1}: Φ_{NPQ}, Φ_{NO}, $\Delta F/F_m'$, Excitation Pressure (Q_m), Bleaching % at Centre of Polyp and Bleaching % for the Whole Recruit) explained by the benthic functional groups and CO_2 treatments.

Predictor Variables	Pseudo F	P	Proportion of variation explained
Bare Tile	**11.983**	**0.0001**	**0.0406**
Shell	0.97681	0.4066	0.0034
Coral	0.36075	0.8321	0.0013
Live CCA	**2.7931**	**0.0283**	**0.0098**
Dead CCA	1.5021	0.2014	0.0053
Encrusting Fleshy Algae	**2.4852**	**0.0457**	**0.0087**
Turf + Endolithic Algae	**7.6351**	**0.0001**	**0.0263**
Control	**7.5963**	**0.0001**	**0.0261**
IPCC B2	**4.552**	**0.0017**	**0.0158**
IPCC A1FI	**3.9961**	**0.0048**	**0.0139**

Table S5: Distance-based linear model (DISTLM) marginal tests indicating the proportion of bleaching variation (Bleaching % at Centre of Polyp and Bleaching % for the Whole Recruit) explained by the *Symbiodinium* type (presence and dominance), benthic functional groups and CO_2 treatments.

Predictor Variables	Pseudo F	P	Proportion of variation explained
A3 presence	0.061096	0.8851	0.0005
A5 + A_AF333508 presence	0.55502	0.4750	0.0042
A_EF455526 presence	0.36332	0.5694	0.0028
C3 presence	2.6674	0.0986	0.0200
D1 + D1-4 presence	0.55423	0.4736	0.0042
A3 dominance	2.2815	0.1266	0.0171
A5 + A_AF333508 dominance	0.23219	0.6741	0.0018
A_EF455526 dominance	1.9345	0.1553	0.0146
C3 dominance	1.2674	0.2567	0.0096
D1 + D1-4 dominance	**4.5884**	**0.0313**	**0.0338**
Bare Tile	1.6106	0.2020	0.0121
Shell	0.81592	0.3745	0.0062
Coral	2.488	0.1086	0.0186
Live CCA	0.91213	0.3449	0.0069
Dead CCA	1.47	0.2232	0.0111
Encrusting Fleshy Algae	0.33804	0.5962	0.0026
Turf + Endolithic Algae	2.6126	0.1050	0.0196
Control	**10.042**	**0.0028**	**0.0712**
IPCC B2	1.2166	0.2681	0.0092
IPCC A1FI	**3.7872**	**0.0495**	**0.0281**

Table S6: Distance-based linear model (DISTLM) marginal tests indicating the proportion of *Symbiodinium* type (presence and dominance) variation explained by the benthic functional groups and CO_2 treatments.

Predictor Variables	Pseudo F	P	Proportion of variation explained
Bare Tile	1.4541	0.2065	0.0110
Shell	1.1028	0.3493	0.0083
Coral	**2.8031**	**0.0212**	**0.0210**
Live CCA	0.62605	0.6810	0.0048
Dead CCA	1.5633	0.1715	0.0118
Encrusting Fleshy Algae	0.88448	0.4731	0.0067
Turf + Endolithic Algae	1.4721	0.1961	0.0111
Control	0.97031	0.4256	0.0074
IPCC B2	1.1851	0.3119	0.0090
IPCC A1FI	0.62431	0.6784	0.0047

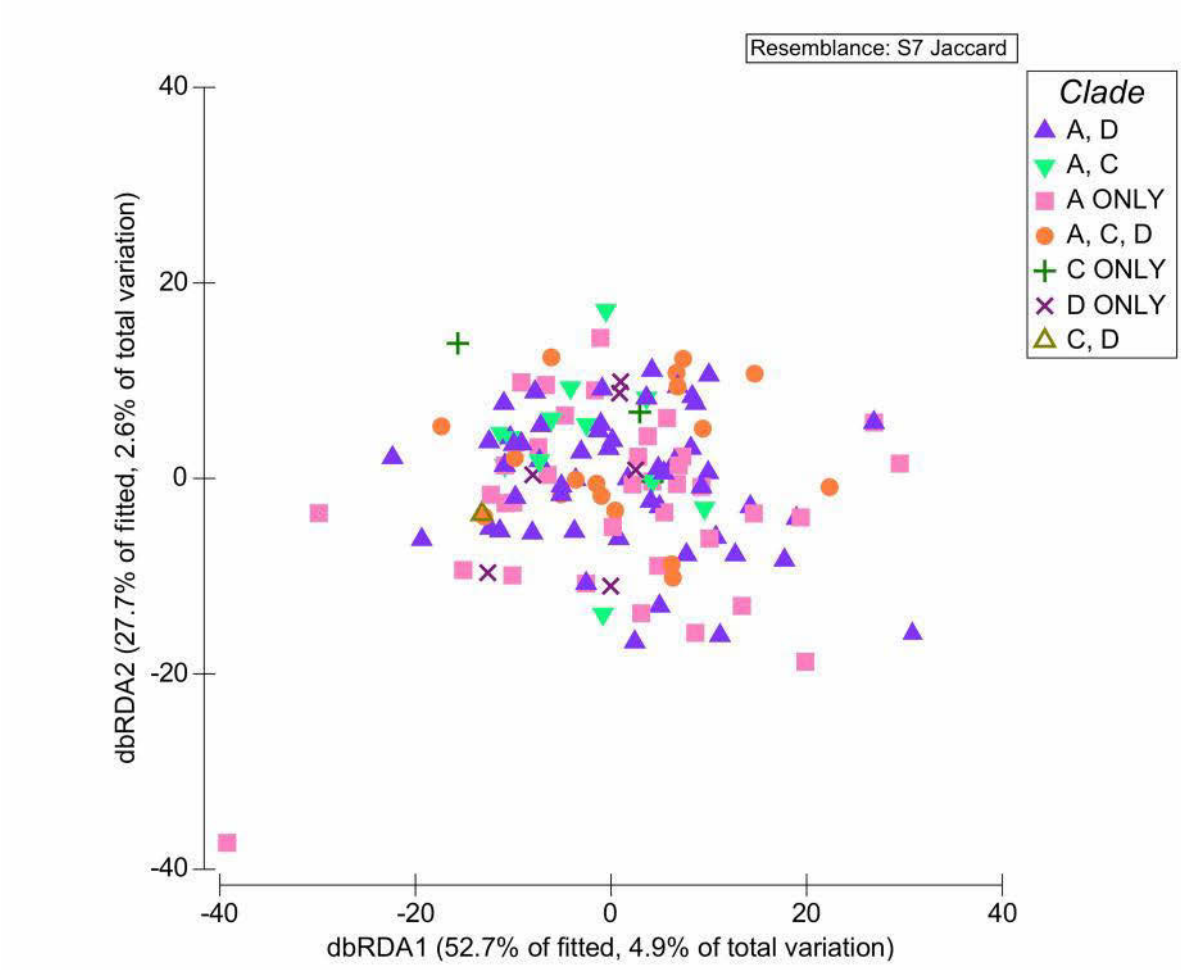

Figure S1: Distance based redundancy analysis (dbRDA) showing ordination of the fitted values from the model of variation in *Symbiodinium* type (presence and dominance) explained by the benthic functional groups and CO_2 treatments.

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Chapter 5: General Discussion

The survival of reef-building corals is threatened by the physiological impacts of ocean acidification (OA) operating across several life-history stages. While most of the current literature has focused on the decline in calcification, characterising the effect of OA on photo-physiological processes is essential to understanding the phenomenon of CO_2-induced bleaching and productivity loss (Anthony et al. 2008). The mechanisms of thermal coral bleaching are well understood, albeit the site of dysfunction is still debated (Jones et al. 1998; Warner et al. 2006), yet little is known about how increased pCO_2 lowers the thermal bleaching threshold. Furthermore, the ecological ramifications of the impact of OA must be considered in order to improve the predictive power of climate models on coral reef ecosystems. The resilience of adult corals to OA is fundamentally dependent on coral reef carbonate chemistry, which is shaped by the community composition and residence time of reef waters (Anthony et al. 2011; Kleypas et al. 2011; Shaw et al. 2013). Overall, coral reef communities are likely to respond to selective pressures, such as OA, with a shift towards more resilient species of the coral host (Inoue et al. 2013) or resilient species of their symbiotic dinoflagellates (genus *Symbiodinium*) (Jones et al. 2008). In relation thereto, coral juvenile uptake of *Symbiodinium* represents a life history stage amenable to adaption of the symbiosis (Baird et al. 2007; Abrego et al. 2009b; Van Oppen et al. 2011). The first objective of this thesis was to investigate the photo-physiological response of reef-building corals to OA during the adult phase and at the onset of symbiosis during the juvenile phase. The second objective was to address these physiological changes in an ecological setting by assessing the coral response to future OA treatments in relation to their surrounding community composition and diurnal fluctuation in carbonate chemistry.

A review of the literature investigating the impact of OA on productivity (**Chapter 1**) examined the fact that increased pCO_2 only leads to a decline in productivity under relatively high light and/or temperature conditions (Reynaud et al. 2003; Langdon and Atkinson 2005; Anthony et al. 2008; Kaniewska et al. 2012; Anthony et al. 2013). This is confirmed by experiments in this thesis whereby CO_2-induced bleaching and productivity loss occurred in *Acropora aspera* under an average maximum daily irradiance of 860 μmol quanta m^2 s^{-1} (**Chapter 2**), but not in *Acropora millepora*

exposed to an average maximum daily irradiance of 400 μmol quanta m^2 s^{-1} (**Chapter 3**). In contradistinction to this analysis is the fact that juvenile *A. millepora* exhibited a bleaching response under very low light conditions (averaging 140 μmol quanta m^2 s^{-1}) (**Chapter 4**); here, the bleaching observed may have been partially due to the delayed uptake of *Symbiodinium* or may have been related to the difference in dominant *Symbiodinium* type. The divergence between adult and recruit responses may be associated with their differential skeletal morphology and light scattering processes. Recruits are small and less complex than adult colonies and this may partially explain their cryptic behaviour and settlement in low light areas. In contrast, adult have the ability to self-shade due to the complexity and orientation of their branches. Furthermore, adult colonies have a greater pool of energetic resources, which may result in increased capacity for photoprotection and photorepair.

Coral bleaching is a loss of symbionts or their pigments but these characteristics can also be features of the mechanism of photoacclimation (Brown 1997; Niyogi 1999). The differentiation lies in whether the bleaching represents a loss of health or fitness (Fitt et al. 2000). In *A. aspera*, the decrease in *Symbiodinium* cells per cm^2 led to a decrease in areal net maximum photosynthesis ($P_n$max), which would have implications for the energy budget of the coral holobiont (**Chapter 2**). In the *A. millepora* juveniles, the photochemical efficiency of photosystem II (PSII) was not significantly affected by OA treatments despite the bleaching response (**Chapter 4**); future studies are needed employing oxygen microsensors to fully characterize the energy budgets of these juveniles. A decline in growth has been observed in *A. millepora* juveniles under OA conditions (Doropoulos et al. 2012), although this might be related to the increased energy cost of maintaining optimal conditions for skeletogenesis rather than decreased energy production, hence warranting the need for assays which would clearly partition photosynthetic and respiration changes in juveniles.

Photoacclimation optimises the light entering the photosystems in order to maximise the rate of photosynthesis and minimise photodamage (Niyogi 1999; Walters 2005). The potential decline of photorespiration as a photoprotective mechanism may contribute to CO_2-induced bleaching (Crawley et al. 2010) (**Chapter 2**). Indeed, this may explain the fact that CO_2-induced bleaching only occurs with concomitant high

light or temperature, as loss of photoprotection at sub-saturating light intensities would not be an issue for photosynthetic electron transport. Further molecular studies are needed to determine the expression and activation of Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) under increased pCO_2 conditions: the prediction from work in Chapter 2 would be that Rubisco levels may decline under increased pCO_2. In addition, the expression of enzymes downstream of phosphoglycolate phosphatase (PGPase) in the photorespiratory pathway, such as glycolate oxidase (GLO), should be assessed to confirm that the glycolate indeed enters that pathway. Furthermore, future experiments should compare the expression of these genes and proteins between *Symbiodinium* types in order to determine whether this led to the increased susceptibility of types D1 and D1-4.

Relaxation from carbon limitation may lead to dinoflagellate cell proliferation, as observed in *A. aspera* under the IPCC A2 CO_2 (pH 7.8 ± 0.1) conditions (**Chapter 2**). This would indicate that the host and symbiont carbon concentrating mechanisms (CCM) remain functional and that delivery of additional CO_2 occurs under OA conditions (Wooldridge 2013). Yet excess algal symbionts can increase the susceptibility of corals to thermal bleaching presumably due to subsequent CO_2 limitation under high light or temperature conditions (Cunning and Baker 2013). In the case of high pCO_2 conditions, the symbiont may photoacclimate to the new pCO_2 levels by reducing the use of the photorespiratory pathway, which would render the holobiont more susceptible to CO_2-induced bleaching with concomitant high light or temperature. This hypothesis requires further testing potentially through molecular analysis of carbonic anhydrase enzymes with an experiment designed to partition the variation caused by light, temperature and pCO_2.

Just as the susceptibility of corals to thermal bleaching depends on the physiological state of the coral prior to the thermal stress (Cunning and Baker 2013), the impact of OA may differ depending on the life history strategies of the coral and this aspect must be considered in future reef models. In **Chapter 3**, the *A. millepora* branches collected outside the lagoon at Lizard Island, characterised by a less dense population of symbionts, were the only coral branches to show a significant increase in net photosynthesis under OA conditions. Furthermore, comparison with coral branches

from a lagoon site showed a physiological trade-off between protein per surface area and skeletal density or branch compactness. Although there was no effect of OA on these characteristics in this short-term aquaria-based experiment, previous work has shown that increased protein and decreased calcification occur under long-term OA conditions (Fine and Tchernov 2007). A key finding in **Chapter 3** is that coral branches may be less susceptible to the detrimental effects of OA in an environment that favours historical maintenance of a relatively smaller symbiont population. Moreover, allocation of resources to protein thickness at the expense of skeletal density or branch compactness may be an early indicator of the impact of OA, given that this life-history strategy is already presently utilized under the diurnal variation in carbonate chemistry. While recent studies have highlighted the need to incorporate diurnal variation in future experiments (Dufault et al. 2012; Kline et al. 2012; Shaw et al. 2013), **Chapter 3** has highlighted the importance of life-long exposure to biogeochemical cycles at the coral collection sites, with implications for both experimental results and modeling. Future mechanistic and ecological models should be developed considering these physiological trade-offs under multiple anthropogenic stressors, such as temperature and pCO_2 in line with ecologically relevant diel oscillations.

In a rapidly changing environment, reproduction represents an important mode for recombination and adaption yet the uptake of *Symbiodinium* types appears to be non-specific during early ontogeny (Abrego et al. 2009a; Cumbo et al. 2013). In addition, differential holobiont mortality can drive community shifts both at the symbiont and the host level (Sampayo et al. 2008). In some species, juveniles may revert to the homologous adult *Symbiodinium* type after > 3 years but other species, such as *A. millepora*, may lack specificity and therefore have greater opportunity for adaptation to new environmental conditions (Abrego et al. 2009b). The fitness and survival of the juvenile holobiont is key to this process. In **Chapter 4**, we observed a correlation between juvenile bleaching and the establishment of symbiosis with *Symbiodinium* types D1 or D1-4. Several questions arise from this finding. Firstly, does this level of bleaching indicate a change in fitness of the juveniles? If so, will this change the ratio of juvenile survival with certain *Symbiodinium* types? And lastly, what is the ecological importance of this mode of adaptation in comparison to differential adult survival, which may lead to community shifts as discussed in **Chapter 3**? Future OA

coral reproduction experiments should be carried out long term in order to determine the persistence of the shift in symbiont population and the implications for holobiont fitness. The hypotheses generated in **Chapters 3 & 4** suggest that OA should have profound effects on symbiont populations of any given coral species. However, recent research has shown that symbiont populations were similar regardless of distance from a volcanic CO_2 seep site (Noonan et al. 2013) and this warrants further investigation particularly in combination with the associated temperature increases predicted under future climate change.

In conclusion, this thesis has corroborated the OA-induced physiological changes of reef-building corals at various life history stages and examined the ecological implications of these findings. The increased susceptibility of corals to the detrimental impacts of increased pCO_2 is likely to coincide with additional environmental stressors such as high light and temperature, potentially due to the impact of OA on the photoprotective mechanism, photorespiration. In relation thereto, qualification of coral bleaching is required in order to differentiate bleaching detrimental to the health and fitness of the holobiont as opposed to photoacclimation. The research in this thesis has captured the link between bleaching and loss of productivity under high pCO_2 and high light conditions, particularly in adult colonies rather than juvenile recruits. The physiological changes induced by OA are likely to lead to processes of natural selection. Noteworthy in this context is the fact that diurnal variation in carbonate chemistry plays a significant role in shaping coral reef community composition. This suggests that anthropogenic OA may similarly place selective pressure on communities and these findings must be incorporated into future reef models. The rapid climate change predicted to occur over the coming century is likely to exhibit selective pressure and potentially cause differential mortality of both juvenile and adult reef-building corals. While this represents an adaptive mechanism, the resultant community shift may hinder the critical functions and services of coral reef ecosystems.

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