CLIMATE CHANGE AND TROPICAL SPONGES: THE EFFECT OF ELEVATED pCO_2 AND TEMPERATURE ON THE SPONGE HOLOBIONT

HOLLY M. BENNETT

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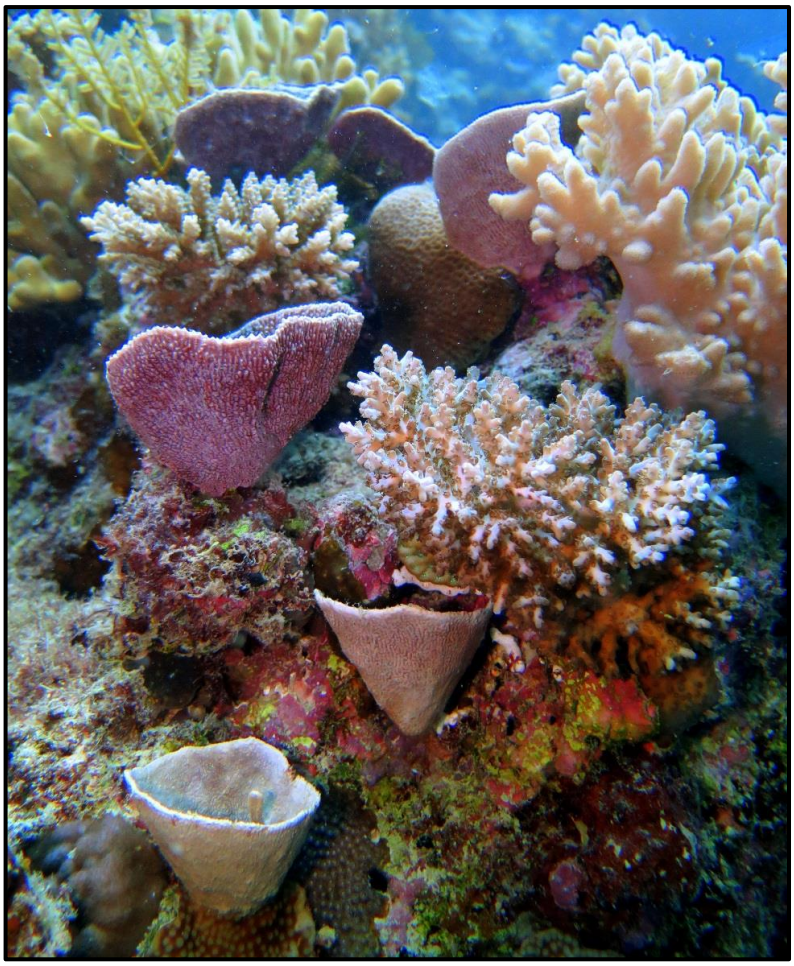

Carteriospongia foliascens on the Great Barrier Reef, Australia.

“I can mention many moments that were unforgettable and revelatory. But the most single revelatory three minutes was the first time I put on scuba gear and dived on a coral reef.”

David Attenborough

This thesis was conducted under the supervision of:

Associate Professor James J. Bell
(*Primary supervisor*)
Victoria University of Wellington
Wellington, New Zealand

+

Doctor Nicole S. Webster
(*Co-Supervisor*)
Australian Institute of Marine Science
Townsville, Australia

+

Professor Simon K. Davy
(*Co-Supervisor*)
Victoria University of Wellington
Wellington, New Zealand

Abstract

As atmospheric CO_2 concentrations rise, associated ocean warming (OW) and ocean acidification (OA) are predicted to cause declines in reef-building corals globally, shifting reefs from coral-dominated systems to those dominated by less sensitive species. Sponges are important structural and functional components of coral reef ecosystems, but despite increasing field-based evidence that sponges may be 'winners' in response to environmental degradation, our understanding of how they respond to the combined effects of OW and OA is limited. This PhD thesis explores the response of four abundant Great Barrier Reef species – the phototrophic *Carteriospongia foliascens* and *Cymbastela coralliophila* and the heterotrophic *Stylissa flabelliformis* and *Rhopaloeides odorabile* to OW and OA levels predicted for 2100, under two CO_2 Representative Concentration Pathways (RCPs). The overall aim of this research is to bridge gaps in our understanding of how these important coral reef organisms will respond to projected climate change, to begin to explore whether a sponge dominated state is a possible future trajectory for coral reefs.

To determine the tolerance of adult sponges to climate change, these four species were exposed to OW and OA in the Australian Institute of Marine Science's (AIMS) National Sea Simulator (SeaSim) in a 3-month experimental study. The first data chapter explores the physiological responses of these sponges to OW and OA to gain a broad understanding of sponge holobiont survival and functioning under these conditions. In this chapter I also address the hypothesis that phototrophic and heterotrophic sponges will exhibit differential responses to climate change. In the second and third data chapters I explore the cellular lipid and fatty acid composition of sponges, and how these biochemical constituents vary with OW and OA. Lipids and fatty acids are not only vital energy stores, they form the major components of cell membranes, and the structure and composition of these biochemical constituents ultimately determines the integrity and physiological competency of a cell. Therefore through these analyses I aimed to determine how OW and OA affects the metabolic balance of sponges, and to understand mechanisms underpinning observed systemic sponge responses. Finally, to provide greater insight into the population level impacts of climate change on tropical sponges, in the last data chapter I explore the response of the phototrophic species *Carteriospongia foliascens* to OW/OA throughout its developmental stages.

I found that while sponges can generally tolerate climate change scenarios predicted under the RCP6.0 conditions for 2100 (30ºC/ pH 7.8), environmental projections for the end of this century under the RCP8.5 (31.5ºC/ pH 7.6) will have significant implications for their survival. Temperature effects were much stronger than OA effects for all species; however, phototrophic and heterotrophic species responded differently to OA. Elevated pCO_2 exacerbated temperature stress in heterotrophic sponges but somewhat ameliorated thermal stress in phototrophic species. Furthermore, sponges with siliceous spiculated skeletons resisted the RCP 8.5 conditions for longer than the aspiculate species. Biochemical analysis revealed that spiculated species also have greater cell membrane support features, which is likely to contribute to the observed stress tolerance. I also found that the additional energy available to phototrophic sponges under OA conditions may be used for investment into cell membrane support, providing protection against thermal stress. Finally, larval survival and settlement success of *C. foliascens* was unaffected by OW and OA treatments, and juvenile sponges exhibited greater tolerance than their adult counterparts, again with evidence that OA reduces OW stress for some of these life stages.

Based on the species studied here, this thesis confirms that sponges are better able to deal with OW and OA levels predicted for 2100 under RCP6.0, compared to many corals for which survival in a high CO_2 world requires OW to remain below 1.5°C. This suggests sponges may be future 'winners' on coral reefs under global climate change. However, if $CO_{2\ atm}$ concentrations reach levels predicted under RCP8.5, the prognosis for sponge survival by the end of this century changes as inter-species sponge tolerances to OW and OA differ. Under this projection it is likely we will also start to see a shift in sponge populations to those dominated by phototrophic sponges with siliceous spiculated skeletons. Overall, this thesis gives a holistic view of OW and OA impacts on tropical sponges and provides the basis from which to explore the potential for a sponge-coral regime shift in a high CO_2 world.

This thesis is dedicated to my grandparents; Hugh, Mary and Ana.

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Contributions and Publications

The data chapters that make up this thesis are based on two manuscripts, chapters two and five are published in *Global Change Biology* and chapters three and four are *in preparation* to be submitted for publication.

Chapter two:

Study design: Holly M. Bennett, A/Prof James J. Bell, Dr Nicole S. Webster and Prof Simon K. Davy.
Data collection: H.M.B with support from Christine Altenrath (AIMS).
Analyses: H.M.B with guidance from Lisa Woods (VUW).
Writing and editing: H.M.B with editing by A/Prof James J. Bell, Prof Simon K. Davy and Dr Nicole S. Webster and proofreading from Lisa Woods and Christine Altenrath.
Publication: Bennett HM, Altenrath C, Woods L, Davy SK, Webster NS, Bell JJ (2016) Interactive effects of temperature and pCO_2 on sponges: from the cradle to the grave. Global Change Biology. DOI: 10.1111/gcb.13474.

Chapter three:

Study design: H.M.B with guidance from A/Prof James J. Bell, Dr Nicole S. Webster, Prof Simon K. Davy and Dr David Francis (Deakin University).
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Laboratory analyses: H.M.B with Dr David Francis.
Data analyses: H.M.B with guidance from Dr David Francis.
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Study design: H.M.B with guidance from A/Prof James J. Bell, Dr Nicole S. Webster and Prof Simon K. Davy.
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Chapter five:

Study design: H.M.B with guidance from A/Prof James J. Bell, Dr Nicole S. Webster and Prof Simon K. Davy.

Data collection: H.M.B with support from Christine Altenrath.

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Chapter one

General introduction: Climate Change and Tropical Sponges

The National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (AIMS) where all experimental work was undertaken.

1.1 Climate change

Present day atmospheric carbon dioxide levels ($CO_{2\ atm}$) now exceed 400 ppm (NOAA, 2016), more than 100 ppm higher than levels prior to the Industrial Revolution (Siegenthaler *et al.*, 2005). Global $CO_{2\ atm}$ concentrations are expected to increase at unprecedented rates (Fabry *et al.*, 2008), exceeding 1000 ppm by 2100 if no additional efforts are made to moderate greenhouse gas emissions (IPCC, 2014). Earth's oceans initially seemed suitable dumping grounds for these increasing emissions (Pörtner, 2008), serving as global sinks for at least half of the anthropogenic $CO_{2\ atm}$ over the past 200 years (Sabine *et al.*, 2004). However, as the partial pressure of CO_2 (pCO_2) in seawater increases with increasing $CO_{2\ atm}$ (Pörtner, 2008), the pH of the upper layers of the world's oceans is predicted to decrease 0.3-0.5 units by 2100 (IPCC, 2014), in a phenomenon referred to as Ocean Acidification (OA). Such a shift in the carbonate chemistry of the ocean has significant implications for marine organisms including reduced calcification rates and disruptions to acid-base physiology with subsequent impacts on organism physiology, survival and ecosystem functioning (Fabry *et al.*, 2008; Pörtner, 2008). Furthermore, rising $CO_{2\ atm}$ has increased global surface temperatures by ~0.2°C *per* decade over the past 30 years, with the years 2011 to 2015 being the warmest five-year period on record globally (Hansen *et al.*, 2006, WMO, 2016). As the World's oceans absorb a significant amount of this excess energy (Hoegh-Guldberg & Bruno, 2010), the Intergovernmental Panel on Climate Change (IPCC) predicts that global warming resulting from this rising $CO_{2\ atm}$ will see mean sea surface temperatures increase a further 1.1-4.0°C by the end of this century (IPCC, 2014). Due to the fundamental effect of temperature on biological processes, organisms live in environments where the average temperature facilitates optimal physiological performance (Hoegh-Guldberg & Bruno, 2010). Outside of these optimum temperatures, biological processes are likely to be compromised, resulting in reduced organism fitness with subsequent implications for population survival and species persistence (Hoegh-Guldberg & Bruno, 2010). As a result, temperature ultimately drives the large scale geographical distributions of marine organisms (Pörtner, 2008) and climate change related OW has already been implicated in shifts in species distributions, mass mortalities, reductions in ocean productivity, disruptions to food web dynamics, declines in important habitat-forming species, as well as increased invasive species and disease incidences (Pörtner & Farrell, 2008; Hoegh-Guldberg & Bruno, 2010; Byrne & Przeslawski, 2013).

1.2 Climate change and coral reefs

Despite OW and OA occurring concurrently, and increasing evidence from meta-analyses that multiple climate stressors have a greater deleterious impact on marine organisms than stressors applied in isolation (Wernberg *et al.*, 2012), few experimental studies have considered their combined effects (Dupont & Pörtner, 2013; Boyd *et al.*, 2015; Lesser *et al.*, 2016). Furthermore, the major focus of most combined OW and OA research in tropical marine ecosystems has been on reef-building corals (Hughes et al., 2003; Hoegh-Guldberg et al., 2007; Erez et al., 2011). The implications of a high CO_2 ocean for corals include increased mass bleaching events (Hughes *et al.*, 2003; Hoegh-Guldberg *et al.*, 2007), reduced coral calcification rates (Erez *et al.*, 2011) and disruption to reproduction and larval development (Heyward & Negri, 2010; Albright & Langdon, 2011). While coral bleaching occurs in response to a number of environmental stressors including reduced salinity, shifts in light and temperature regimes, toxicants, and disease, elevated temperature is the root cause of the most destructive, wide-spread, mass bleaching events to date (Hoegh-Guldberg, 1999). As water temperatures increase above a coral's thermal threshold, symbiotic zooxanthellae or their photosynthetic pigments are lost from the coral tissue, resulting in reductions in coral growth, reproduction and resilience i.e. increased susceptibility to disease, and in many cases, coral mortality (Glynn, 1993; Hoegh-Guldberg, 1999; Hughes *et al.*, 2003, Pandolfi *et al.*, 2003; Hoegh-Guldberg *et al.*, 2007). Temperature related mass bleaching events will increase with OW in a high CO_2 world, whilst OA changes will further stress corals, as alterations to ocean carbonate chemistry reduce coral calcification rates (Erez *et al.*, 2011). The carbonic acid formed as CO_2 dissolves in seawater dissociates rapidly, forming hydrogen ions which react with carbonate ions to form bicarbonate (Fabry *et al.*, 2008). The resultant effect is a decrease in the abundance of carbonate ions available for the formation of calcium carbonate skeletons (Erez *et al.*, 2011).

These implications, combined with further proposed impacts to reproduction and early-life history success for corals under OW and OA conditions (Heyward & Negri, 2010; Albright & Langdon, 2011) suggest a bleak future for a group of organisms that are already experiencing global declines in abundance (Hoegh-Guldberg, 1999; Pandolfi *et al.*, 2003; Hoegh-Guldberg *et al.*, 2007; Pandolfi *et al.*, 2011; De'ath *et al.*, 2012). Competition for space on coral reefs is high (Colvard & Edmunds, 2011) and where coral abundance has declined, ''winners'' and ''losers'' are beginning to emerge. Most regime-shifts away from coral-dominated states

demonstrate changes to macro-algal dominated reefs (Kroeker *et al.*, 2013a; Enochs *et al.*, 2015), especially when the coral decline occurs in combination with over-fishing and increased nutrient input (McClanahan, 2002; McManus & Polsenberg, 2004; Hughes *et al.*, 2010). However, coral reefs can also become dominated by other benthic fauna (Norström *et al.*, 2009), with sponges being identified as potential "winners" in the face of environmental change (Bell *et al.*, 2013). A shift from coral to sponge dominance on coral reefs is no novel phenomenon. The Triassic-Jurassic mass-extinctions saw the disappearance of all reef-building fauna and the ultimate destruction of coral reefs. There is evidence to suggest that this extinction was followed by a radiation of prehistoric siliceous sponges, which led to sponge dominance on reefs for over two million years (Delecat *et al.*, 2011). Interestingly, this regime-shift was the result of changes to ocean chemistry, similar to those we expect to witness in the ocean as a result of predicted $CO_{2\ atm}$ increases (Delecat *et al.*, 2011; Bell *et al.*, 2013).

1.3 Sponges

Sponges, the most ancient multicellular animals on Earth, are the exclusive representatives of the phylum Porifera. Porifera is divided into four extant classes of sponges; Calcarea, Hexactinellida, Homoscleromorpha and Demospongiae, with 83% of all known sponges belonging to the latter class (Van Soest *et al.*, 2012). Between these classes sponge species demonstrate significant differences in skeletal composition, morphology and modes of life including reproductive and nutritional strategies; however several generalisations can be made regarding the basic biological functioning of sponges. The sponge body plan is comprised of three distinct cell layers (Bergquist, 1978): the external epithelium (pinacoderm), a single celled layer, isolates the sponge from its external enviornement; the internal epithelium (choanoderm), a single layer of flagellated cells, facilitates the flow of water through the sponge; the third layer is the mesohyl, located between the pinacoderm and choanoderm, containing mobile cells and skeletal elements (Simpson, 2012). This simple body plan is specialised for the sponge's sedentary, filter-feeding lifestyle and has changed very little since early sponge origins (Bergquist, 1978). Water enters through many small openings (ostia) on the sponge surface and flows through the canal system lined with choanocyte chambers, using flow created by the beating flagella of the choanocyte cells. The collar filters of sponge choanocytes retain suspended particles (e.g. bacteria and picoplankton) as the inhalant water is drawn through the canal system and out of the sponge's exhalent oscules (Riisgård *et al.*, 1993;

Leys *et al.*, 2011). Such a simple, yet effective way of life has undoubtedly facilitated sponge persistence through historical mass extinction events, enabling them to prevail today within benthic ecosystems across all aquatic environments; spanning temperate, tropical and polar habitats (Van Soest *et al.*, 2012).

1.4 Importance of sponges in coral reef ecosystem

Sponges fulfil a number of important functional roles in benthic ecosystems globally (Diaz & Rutzler, 2001; Wulff, 2006a; Bell, 2008), no more so than in coral reef ecosystems in which they are an integral structural and functional component, and contribute significantly to coral survival and reef health (Diaz & Rutzler, 2001; Wulff, 2006a). Their efficient filtration system, combined with high particle retention efficiencies (Reiswig, 1971), make sponges a critical link in the coral reef food chain, facilitating the flow of carbon between trophic levels (Wulff, 2006a; Bell, 2008; de Goeij *et al.*, 2008; McMurray *et al.*, 2016) through feeding on picoplankton and subsequent predation by higher organisms. The recent discovery of the "sponge loop" reinforces this importance, demonstrating how sponge-facilitated carbon flow contributes to the productivity of oligotrophic coral reef waters (de Goeij *et al.*, 2013; Rix *et al.*, 2016). In addition, sponge associated microbial symbionts contribute further to reef primary productivity and nutrient cycling (Cheshire & Wilkinson, 1991; Maldonado *et al.*, 2012). Bioeroding sponges play an important role in reef bioerosion processes, excavating coral skeletons and reworking their solid carbonate skeletons into finer sediments (Glynn & Manzello, 2015). Meanwhile studies have demonstrated that some sponges actually contribute to the consolidation of the reef framework, increasing coral survival by binding live coral to the reef and deterring excavation from other organisms (Wulff, 2001). Finally, various chemical and physical adaptations make sponges one of the top spatial competitors in space competitive reef ecosystems (Hill, 1998; Diaz & Rutzler, 2001; Rützler, 2002), as a result they also form an important microhabitat for coral reef organisms (Bell, 2008).

1.5 Sponge-coral regime shifts

Coral cover is already declining globally (Veron *et al.*, 2009). Extensive time series data collected from Great Barrier Reef surveys (GBR, Australia) have demonstrated a mean decline in coral cover from 28 to 13.8% between 1985 to 2012 (De'ath *et al.*, 2012). A meta-analysis

of coral cover in the Caribbean highlights similar levels of coral decline within this region, with coral cover declining from 50% in the 1970's to 10% in 2003 (Gardner *et al.*, 2003). Globally, there have also been reports of increased sponge abundance on coral reefs as coral cover has declined (Aronson *et al.*, 2002; McMurray *et al.*, 2010; Colvard & Edmunds, 2011; Schils, 2012; Bell *et al.*, 2013; Kelmo *et al.*, 2013; Kelmo *et al.*, 2014; Bell *et al.*, 2015b). For example, in 1998 severe coral bleaching caused a decline in coral cover along the Barrier Reef in Belize. Hard coral cover decreased from 40% to 5% in just a few months, and subsequently sponge cover increased from 25% to 43% from 1998 to 2001 (Aronson *et al.*, 2002). This rise in sponge dominance was mainly attributed to an increase in the cover of the encrusting sponge *Chondrilla* cf. *nucula* as a result of space opened by coral mortality. Similarly, in St John, Virgin Islands, coral cover declined to around 5%, while sponge cover increased 64% from 1997-2002 (Colvard & Edmunds, 2011). In the Wakatobi Marine National Park (SE Sulawesi, Indonesia) higher sponge dominance was recorded in areas where hard coral dominance was low (Bell & Smith, 2004). In the Caribbean the giant barrel sponge *Xestospongia muta* increased by up to 46% on some reefs from 2000-2006; meanwhile, average coral cover halved (McMurray *et al.*, 2010). Bioeroding sponge abundance increased by at least 150% on reefs near Orpheus Island (GBR, Australia) from 1997/8 to 2003/4 in response to a major decline in coral as a result of a mass bleaching event (Schönberg, 2008).

It is estimated that many of the reef building corals we see today will be extinct by the end of this century, as a consequence of environmental degradation (Solomon *et al.*, 2007). Sponges already appear to be moving into areas of coral reef where large coral mortality has occurred and reef conditions no longer favor coral survival. The effects of climate change are likely to weaken these stressed coral reefs further (Hughes *et al.*, 2007; Frieler *et al.*, 2013), creating even more space into which sponges may disperse. However, many of the described increases are associated with environmental degradation, particularly nutrient influx and sedimentation (Bell *et al.*, 2015b), and are associated with single-species proliferations, in response to a release from space competition as coral cover declines (Bell *et al.*, 2015b). However, despite the reports of widespread increases in sponge abundance, declines in sponge abundance have also been reported within reef ecosystems (Cebrian *et al.*, 2011; Di Camillo *et al.*, 2013). Declines in sponge species and biomass were described in a 14-year study on a coral reef in San Blass, Panama (Wulff, 2006a). More than 50% of sponge species and more than 40% of sponge biomass was lost from the study site between 1984 and 1998. A 16-year study (1995-2011) in Bahia, Brazil reported an increase in the abundance of bioeroding *Cliona* and

Siphonodictyon species following the 1997-8 El Niño Southern Oscillation (ENSO) event (Kelmo *et al.*, 2013). Following this same event the abundance of at least 12 other sponge species declined (Kelmo *et al.*, 2014). Whilst some species may benefit from increased substrate availability as a result of coral declines, other sponge species may not fare so well in a changing climate, especially when faced with a multitude of other stressors.

1.6 Sponges and climate change

The effects of climate change, specifically OW and OA, on sponges have been largely overlooked in comparison to reef-building corals. The direct importance of corals to coral reef functioning plays a large part in this imbalance, although the implications of a changing climate are also less obvious for sponges than for hard corals. For example, in an acidifying ocean, any organism with calcium carbonate skeletal elements may be affected. The class Calcarea, consisting solely of sponges with calcium carbonate skeletons, represents just 8% of the total extant sponges (Van Soest *et al.*, 2012). Therefore it is expected that the impact of OA to skeletal systems of sponges overall, compared to hard corals, will be minimal.

Another obvious and widely reported impact to reef building corals as a result of elevated temperatures is coral bleaching. Sponges too harbour microbial communities, which can contribute up to 40% of sponge biomass and are crucial to the health and functioning of the sponge host (Webster & Taylor, 2012). This microbial partnership is collectively referred to as the sponge "holobiont" (Webster & Taylor, 2012). While all sponges contain microbes, the holobiontcan be categorised into two nutritional types based on the sponges association with phototrophic symbionts, i) phototrophic i.e. >50% of energy requirements are acquired from photosynthetically-fixed carbon, and ii) heterotrophic i.e. primarily reliant on suspension feeding for carbon requirements (Cheshire & Wilkinson, 1991). Contrary to reef-building corals, in which all species rely primarily upon photosynthetic dinoflagellates to satisfy nutritional requirements (Muscatine & Porter, 1977), not all sponges are phototrophic (Taylor *et al.*, 2007). In fact, phototrophic sponges comprise only half of the total sponge biomass on outer reefs of the GBR (Taylor *et al.*, 2007), and slightly lower percentages of phototrophic species have been reported on Caribbean reefs (Vicente, 1990). Interestingly, while smaller scale sponge bleaching, in response to elevated temperatures, has been reported on coral reefs globally (Vicente, 1990; Fromont & Garson, 1999; López-Legentil *et al.*, 2008, McMurray *et*

al., 2011; Hill *et al.*, 2016), there have been no widespread bleaching events recorded for sponges to date (Bell *et al.*, 2013) suggesting that the impacts of bleaching are less of a threat to sponges overall than for corals.

Whilst the majority of sponge species skeletal elements are unlikely to be impacted by OA, just one cell layer separates a sponge from the external environment (Bergquist, 1978), so changes to ambient water temperature and pH may strongly influence cellular processes (Pörtner & Farrell, 2008). Further to this, although the effect of bleaching events on sponges has been negligible to date, implications of a warming climate indicate this may become an increased risk. In addition, sponges are not just reliant upon phototrophic symbionts; other sponge microbes play a significant role in host fitness and have been shown to benefit sponges through host defense and metabolic functions (Taylor *et al.*, 2007; Webster & Thomas, 2016). Disruptions to the functioning of these symbioses have implications for the overall survival of the sponge holobiont (Webster & Taylor, 2012). Despite their ecological importance, the increasing evidence that some sponges are able to benefit from environmental change, and their natural predisposition to changing ocean conditions, little is known about how sponges respond to predicted OW and OA scenarios as a result of increasing $CO_{2\ atm}$.

1.7 Response of sponges to ocean warming and ocean acidification

Studies focussing exclusively on the effects of OW have demonstrated that some sponges are sensitive to increasing temperature predicted under future climate change scenarios. For example, the Caribbean sponge *Xestospongia muta* exhibits high heat shock protein expression and mortality when exposed to 30°C (López-Legentil *et al.*, 2008) and the Great Barrier Reef sponge *Rhopaloeides odorabile* exhibits a thermal threshold of 32°C (Webster *et al.*, 2008; Pantile & Webster, 2011; Massaro *et al.*, 2012) with a breakdown in host-symbiont molecular interactions occurring at elevated temperature (Fan *et al.*, 2013). Mass mortalities of the phototrophic *Ircinia fasciculata* in the Mediterranean, as a result of a loss of symbiotic cyanobacteria, were correlated with temperatures 1 to 2 °C above those previously recorded for the region (Cebrian *et al.*, 2011). Studies focussing exclusively on the effects of OA at volcanic CO_2 seep sites have reported contrasting results. While sponge diversity was found to increase at low pH sites (pH 7.8-7.9) in the Mediterranean (Goodwin *et al.*, 2014), sponge diversity decreased at low pH sites (pH 7.73-8.00) in Papua New Guinea (Fabricius *et al.*,

2011), although particular species did become significantly more abundant at sites with active CO_2 bubbling (Morrow *et al.*, 2015).

Although these studies provide some insight into sponge responses to a changing climate, the co-occurrence of OW and OA requires studies on organismal responses to investigate the effects of these parameters in conjunction with one another (Dupont & Pörtner, 2013). Research into the combined effects of elevated temperature and pCO_2 has predominantly focused on the response of bioeroding sponge species i.e. *Cliona celata* (Duckworth & Peterson, 2013), *C. orientalis* (Fang *et al*., 2013, Wisshak *et al*., 2012) and *C. varians* (Stubler *et al.*, 2015), and specifically the effect of OW and OA on sponge bioerosion rates. Bioeroding sponges account for up to 90% of all macro-bioerosion and up to 40% of all sediments produced within coral reef ecosystems (Fang *et al.*, 2013; Stubler *et al.*, 2015). Currently, growth and bioerosion processes are more-or-less balanced in healthy coral reef ecosystems (Glynn & Manzello, 2015). However, as increasing pCO_2 reduces coral calcification rates, and weakens existing calcium carbonate structures (Stubler *et al.*, 2014) this balance is likely to shift in favour of reef erosion (Enochs *et al.*, 2015). As many of the recent reports of increasing sponge abundance on coral reefs have been attributed to bioeroding species (Aronson *et al.*, 2002; Schönberg, 2008; Kelmo *et al.*, 2013), determining how OW and OA will impact their abundance and erosion capabilities is important in assessing the effect of climate change on coral reefs. These studies demonstrated that for all species, except *C. varians*, sponge bioerosion rates increase under future OW and OA scenarios (Wisshak et al., 2012; Duckworth & Peterson, 2013; Fang et al., 2013). Such an increase in erosional processes on coral reefs at a time when accretion processes are declining has serious implications for the persistence of these ecosystems in a high CO_2 world (Enochs *et al.*, 2015).

Combined OW and OA studies looking at the interactive effect on non-bioeroding sponges also suggest a degree of sponge tolerance to the effects of a changing climate (Duckworth *et al.*, 2012; Vicente *et al.*, 2015; Lesser *et al.*, 2016). Research exposing six sponge species to OW and OA suggested no impact on growth, survival and secondary metabolite biosynthesis (Duckworth *et al.*, 2012). Furthermore, despite slight negative effects of elevated pCO_2 on spicule biomineralisation, OW and OA had little effect on overall survival or growth rates in *Mycale grandis* (Vicente *et al*., 2015). Finally, in *Xestospongia muta* phototrophic cyanobacterial symbiont productivity was reported to decline with exposure to elevated temperature and pCO_2, although no evidence of bleaching or associated host stress was

reported despite a reduction in holobiont carbohydrate levels and reduced stability of the sponge microbiome (Lesser *et al.*, 2016). However, these short-term (12 to 26 d) studies provide little insight into the effects of environmental change at the cellular level required for a mechanistic understanding, especially when stressors are impacting in synergy (Dupont & Pörtner, 2013; Enochs *et al.*, 2015).

The survival of early life stages is fundamental for population persistence, yet for non-coral species there is a paucity of climate change research considering multiple life history stages of the same species (Dupont & Pörtner, 2013). Early life-history stages of benthic marine invertebrates are potential bottlenecks for the ecological success of species, due to their proposed sensitivity to environmental stressors (Kurihara, 2008; Gibson *et al.*, 2011). These life stages are therefore of particular concern for species persistence in a changing ocean (Byrne & Przeslawski, 2013). Environmental factors that disrupt larval dispersal, recruitment and early development have wide implications for population dynamics and the ultimate survival of a species (Albright & Langdon, 2011). Whilst previous studies have demonstrated that larvae of the sponge *R. odorabile* have a greater thermal tolerance than the adults (Whalan *et al.*, 2008b; Webster *et al.*, 2011), there are no studies to date which look at the early-life history response of sponges to the combined effect of OW/ OA (Przeslawski *et al.*, 2015).

1.8 Thesis outline

The aim of this thesis is to address these gaps in our understanding of the response of the sponge holobiont (that is, the sponge host and its symbionts together) to the combined effects of OW and OA. In a series of experiments, carried out in the Australian Institute of Marine Science's National Sea Simulator (SeaSim), I exposed a number of sponge species and life-history stages to the OW and OA conditions predicted under the latest IPCC CO_2 representative concentration pathways (RCP, IPCC) for 2100. OW and OA levels were simulated under two RCP CO_2 concentrations under which no additional efforts are made to constrain global greenhouse gas emissions (IPCC, 2014); RCP6.0 and RCP8.5, both of which are likely scenarios based on current CO_2 emissions (Peters *et al.*, 2013).

The first data chapter determines the immediate physiological response of four abundant Great Barrier Reef demosponge species - the phototrophic *Carteriospongia foliascens* and

Cymbastela coralliophila and the heterotrophic *Rhopaloeides odorabile* and *Stylissa flabelliformis* - to a rise in pCO_2 and seawater temperature (Fig. 1.1). I measured the survival, growth, respiration rates and photosynthetic parameters (phototrophic species only) including oxygen production and photosynthetic efficiency of these four important sponge species in response to the OW and OA scenarios predicted under RCP6.0 and RCP8.5.

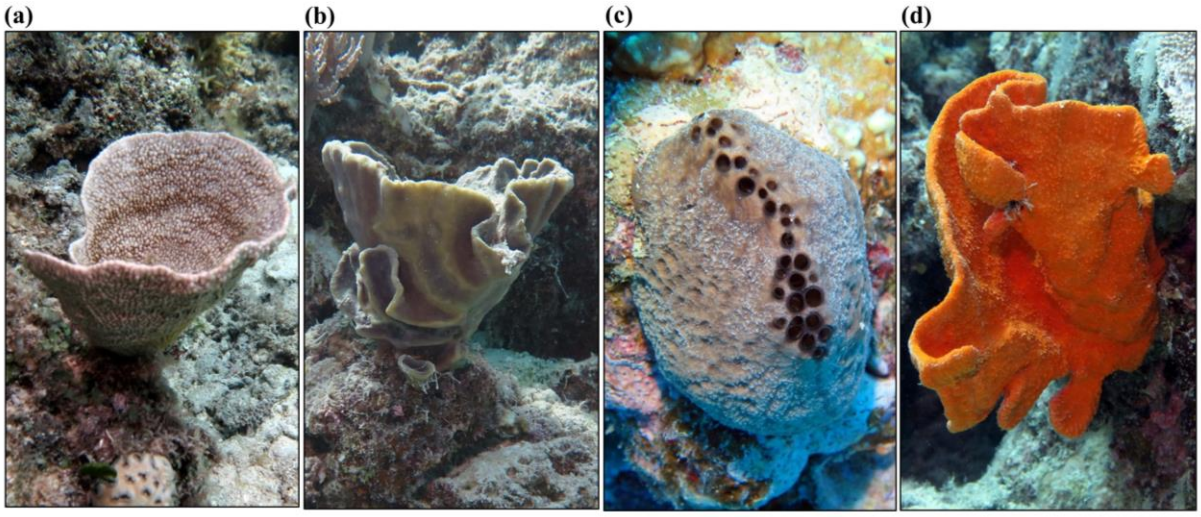


Fig. 1.1 Great Barrier Reef sponge species experimentally exposed to OW and OA scenarios predicted for 2100 - the phototrophic (a) *Carteriospongia foliascens* and (b) *Cymbastela coralliophila* and the heterotrophic (c) *Rhopaloeides odorabile* and (d) *Stylissa flabelliformis*.

In the second data chapter, the lipid and lipid class composition of these same sponges exposed to the OW and OA scenarios above is explored. Lipids are a major source of metabolic energy, they protect and support cell membranes, are important in physiological and reproductive processes of marine organisms, and play a vital role in organism tolerance to physiological stressors (Singh *et al.*, 2002; Bergé & Barnathan, 2005). The total lipid content, and lipid class composition of these sponges under different OW and OA scenarios is analysed to determine whether lipid content, and sponge utilisation of lipids under OW and OA relates to tolerance and to understand how OW and OA affects the metabolic balance of sponges in terms of lipid content.

Fatty acids (FA) are the main constituents of lipids and, in addition to being an important energy reserve, form the hydrophobic barrier of cell membranes; they are particularly important for environmental stress resistance due to the role they play in maintaining the fluidity of this membrane in response to changing conditions (Bergé & Barnathan, 2005; Parrish, 2013). In this chapter I examined the fatty acid (FA) composition of sponges before and after exposure

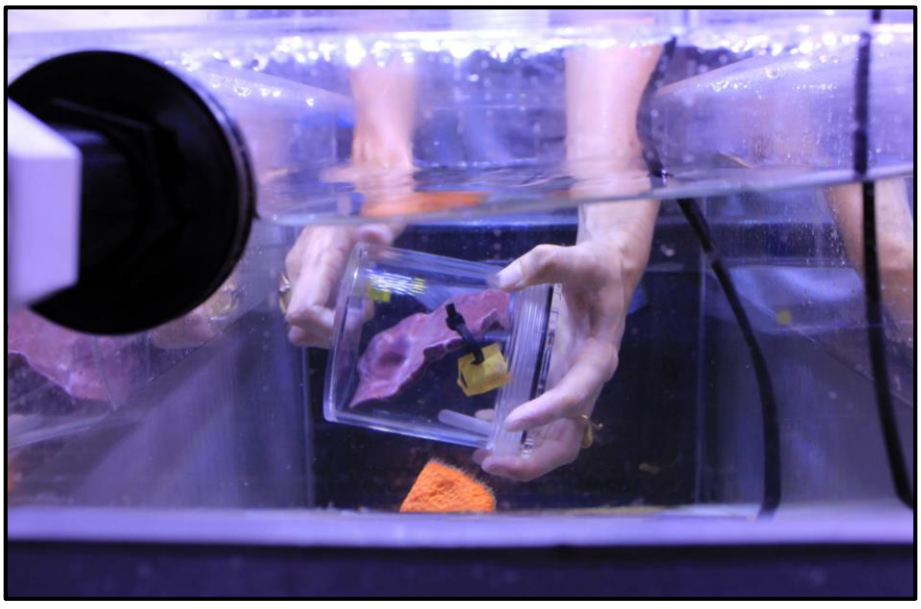
to OW and OA to explore potential tolerance mechanisms and to understand cellular level impacts of OW and OA.

The fourth data chapter explores the response of one of the most widespread Dictyoceratid sponges on the GBR (Wilkinson, 1988), the phototrophic *Carteriospongia foliascens*, to OW and OA throughout its developmental stages. In this chapter I ran a series of experiments exposing *C. foliascens* to OW and OA pre-settlement, one-month; one-year; and two-years post-settlement, to determine the effect of OW and OA on this important species across all life-stages.

In the final chapter I combined the findings of this research to discuss how tropical sponges are likely to respond to OW and OA as a result of increasing $CO_{2\ atm}$ and the mechanisms driving these responses. An overview of measured responses is discussed to determine physiological indicators that determine sponge species sensitivities to OW and OA. Differences in species tolerances are then considered to provide insight into the composition of future sponges communities in a high CO_2 world. I then explored the response of these sponge species to projected OW and OA conditions for 2100 to determine whether a sponge-dominated state is a possible future trajectory for coral reefs. Finally, insight into how this alternate state might function in terms of alterations to coral reef productivity and biodiversity is discussed based on projected changes in sponge dominance.

Chapter two

The physiological response of the sponge holobiont to elevated pCO_2 and temperature: *Nutritional strategy matters*



Cymbastela coralliophila inside an incubation chamber in which metabolic rates were measured.

2.1 Introduction

Through the burning of fossil fuels, atmospheric carbon dioxide levels ($CO_{2\ atm}$) are 100 ppm higher than levels prior to the Industrial Revolution, and are increasing at unprecedented rates (Fabry *et al.*, 2008; NOAA, 2016). This rising $CO_{2\ atm}$ is predicted to cause global mean sea surface temperatures to increase 1.1-4.0°C (ocean warming) and the pH of the world's oceans to decrease 0.3-0.5 units (ocean acidification) by the end of this century (IPCC, 2014). Coral reef ecosystems, the biodiversity hubs of the ocean, already experience a number of natural and anthropogenic stressors, and as a result coral cover is declining globally (Hughes *et al.*, 2003; Pandolfi *et al.*, 2003; De'ath *et al.*, 2012). Ocean warming (OW) and ocean acidification (OA) are expected to cause further reductions in coral abundance, and at the current rate $CO_{2\ atm}$ is rising, it is unlikely these systems will recover (Hoegh-Guldberg *et al.*, 2007; Veron *et al.*, 2009; Pandolfi *et al.*, 2011). Competition for space on coral reefs is high and where coral abundance has declined, ''winners'' and ''losers'' are already beginning to emerge, with sponges being identified as potential winners in the face of environmental change (Bell *et al.*, 2013). Additionally, recently proposed positive feedbacks resulting from dissolved organic carbon cycling between seaweeds and sponges suggest that these alternate states are also likely to persist, as sponge and seaweed growth are enhanced to the detriment of coral recovery (Pawlik *et al.*, 2016). However, despite the importance of sponges to coral reef ecosystem functioning (Wulff, 2006a; Bell, 2008; de Goeij *et al.*, 2013), and the recent trend in increasing sponge abundance on coral reefs globally (Aronson *et al.*, 2002; McMurray *et al.*, 2010; Colvard & Edmunds, 2011; Schils, 2012; Kelmo *et al.*, 2013; Bell *et al.*, 2013; Kelmo *et al.*, 2014; Bell *et al.*, 2015b), there is an obvious lack of understanding as to how these organisms will respond when faced with the imminent threat of ocean warming (OW) and ocean acidification (OA).

Studies show that OW alone may be stressful for sponges; species experiencing thermal stress demonstrate increased heat shock protein expression (Bachinski *et al.*, 1997; Krasko *et al.*, 1997, López-Legentil *et al.*, 2008, Pantile & Webster, 2011), reduced feeding efficiencies (Massaro *et al.*, 2012), bleaching (Vicente, 1990; Fromont & Garson, 1999; López-Legentil *et al.*, 2008; Cebrian *et al.*, 2011; McMurray *et al.*, 2011; Hill *et al.*, 2016) and other disruptions to microbial symbioses (Webster *et al.*, 2008; Fan *et al.*, 2013); and in many cases mortality. Meanwhile, the effects of OA, mainly studied at volcanic CO_2 seep sites, appear less stressful; with sponge diversity increasing at low pH (pH 7.8-7.9) in the Mediterranean (Goodwin *et al.*,

2014), and particular species becoming more abundant at low pH sites (pH 7.73-8.00) in Papua New Guinea (Fabricius *et al.*, 2011; Morrow *et al.*, 2015).

This apparent tolerance of sponges to OA is perhaps due to the majority of species excreting silicate skeletal elements or organic fibres of collagen or spongin (Van Soest *et al.*, 2012; Smith *et al.*, 2013), as such skeletal compositions have resisted previous OA related mass extinction events (Delecat *et al.*, 2011; Vicente *et al.*, 2015). However, lower marine invertebrates such as sponges are thought to be particularly susceptible to hypercapnic effects of elevated pCO_2 under OA, due to their limited capacity for acid-base regulation (Pörtner, 2008). Maintenance of extracellular pH is proposed to be the first line of defence against elevated pCO_2 (hypercapnia) induced disturbances of metabolic and tissue functioning i.e. metabolic depression, reduced growth and calcification. Therefore, despite the majority of sponge species containing OA resistant skeletal components, their simple body plan means they are literally bathed in seawater, and therefore are likely to have very little control over extracellular pH (Pörtner, 2008; Pörtner & Farrell, 2008; Melzner *et al.*, 2009; Goodwin *et al.*, 2014), making them vulnerable to pCO_2 induced cellular disturbances.

Ocean warming and OA will increase concurrently with projected rises in $CO_{2\ atm}$, therefore the effects of these parameters must be studied in conjunction (Dupont & Pörtner, 2013). Where sponges are concerned, bioeroding sponge species have been the focus of this research (Wisshak *et al.*, 2012; Duckworth & Peterson, 2013; Fang *et al.*, 2013; Wisshak *et al.*, 2013; Stubler *et al.*, 2015), with bioerosion generally increasing under future OW and OA scenarios. The mechanisms underpinning increased sponge bioerosion rates in a high CO_2 world are fairly well understood. Sponge bioerosion takes place extracellularly *via* biochemical dissolution and sponges use specialised etching cells to extract calcium carbonate as chips (Rützler, 1975; Wisshak *et al.*, 2012). There is some evidence to suggest that sponges lower the pH at the interface between their tissue and the calcium carbonate substrate to extract these chips (Schönberg, 2008). Therefore lower pH environments under OA conditions are likely to reduce the pH gradient between the seawater and dissolution substrate, reducing the cost associated with bioerosion for the sponge (Wisshak *et al.*, 2012). Elevated pCO_2 also facilitates increased sponge bioerosion rates as abiotic dissolution takes place in the low pH environment, weakening calcium carbonate substrates and making erosional processes easier (Duckworth & Peterson, 2013; Fang *et al.*, 2013). Moreover, as the sponge does not need to expend as much energy into bioerosion under high pCO_2 conditions, they are able to etch more whilst using the

same amount of energy, and therefore have increased scope for growth (Fang *et al.*, 2013). These results all suggest the current increase in bioeroding sponges evident on coral reefs globally may continue in a high CO_2 world, essentially to the detriment of reef-building corals.

For the majority of sponge species, however, the combined effect of OW and OA are largely unknown. The few studies that focus on the response of non-bioeroding sponges to the combined effects of OW and OA allude toward an overall tolerance of sponges to climate change (Duckworth *et al*., 2012; Vicente *et al*., 2015; Lesser *et al*., 2016). However, the short duration of these exposures combined with a lack of information on the basic biological functioning of sponges under these conditions means we still have little overall understanding of sponge responses to OW and OA. For example, although growth, survival and secondary metabolite production of six sponge species exposed to OW and OA for 24 days was unaffected (Duckworth *et al.*, 2012), the experimental duration may have been too short to elicit significant treatment driven changes in the measured parameters (Pörtner, 2008). Similarly, it is possible that sub-lethal metabolic costs were incurred during the exposures that were not observed in the measured responses (Byrne & Przeslawski, 2013). In laboratory experiments, where representative time-frames are not achievable, sensitive physiological performance indicators e.g. metabolic and pumping rates are likely to provide greater insight into the health of sponges under such conditions, rather than e.g. growth rates which have notoriously low and variable response times (Koopmans & Wijffels, 2008). Similarly, after 26 d of exposure to OW and OA there was no effect on survival or growth rates of *Mycale grandis* (Vicente *et al*., 2015). *M. grandis* biomineralisation was also measured in response to the treatments, and although OA was found to have a slight negative effect on this process it was considered insignificant. While skeletal formation and maintenance is crucial to sponge development and ultimate survival (Uriz *et al.*, 2003), measuring physiological performance indicators fundamental to biological functioning would provide immediate feedback on the effect of the treatments, as well as provide greater mechanistic understanding on sponge sensitivities. Finally, the response of *Xestopongia muta* to OW and OA was assessed during a 12 d exposure. Cyanobacterial symbiont productivity and holobiont carbohydrate levels were depleted under OW and OA conditions, as was the stability of the sponge microbiome. The parameters measured meant that although there was no evidence of bleaching or associated visual host stress, the authors were able to conclude that such environmental changes negatively impact *X. muta* (Lesser *et al.*, 2016). Regardless, the short (12 d) duration of this experiment makes it difficult to completely assess the degree to which OW and OA will impact *X. muta* in the longer-term.

Temperature and CO_2 both exert strong control over fundamental biological processes, with slight increases in temperature increasing metabolic rates (Hoegh-Guldberg & Bruno, 2010) and acid-base imbalances under high CO_2 levels depressing metabolic processes (Fabry *et al.*, 2008). Disturbances to oxygen transport pathways indicate important processes such as protein synthesis and other crucial energetic processes, including pumping, skeletal and tissue growth, reproduction, and metabolism as well as behavioural responses required for predator avoidance or competition, are compromised in favour of maintaining basic biological functioning (Fabry *et al.*, 2008; Pörtner & Farrell, 2008; Leys *et al.*, 2011). Measuring such performance parameters gives greater insight into the response of the study organism, regardless of the duration of the exposure and enables more meaningful conclusions into the long-term health, survival and ecological fitness of a species (Pörtner & Farrell, 2008). In this chapter I will therefore focus on the physiological performance of coral reef sponges exposed to OW and OA, in a bid to elucidate mechanisms underpinning observed responses.

In this chapter I will also consider how physiological functioning differs under OW and OA between sponge nutritional strategies. The sponge holobiont is broadly categorised into two nutritional types; phototrophic and heterotrophic sponges (Cheshire & Wilkinson, 1991; Taylor *et al.*, 2007; Webster & Taylor, 2012), with an almost even divide in the occurrence of these nutritional strategies existing between described coral reef sponges (Wilkinson, 1987a; Vicente, 1990). Most phototrophic sponges harbour photosynthetic cyanobacteria, however associations with other photosymbionts including dinoflagellates (Clionid sponges in particular), diatoms and unicellular chlorophytes can also occur (Wilkinson, 1987a; Davy *et al.*, 2002; Erwin & Thacker, 2008; Weisz *et al.*, 2010; Hill *et al.*, 2011). Photosymbionts are likely to benefit the sponge host *via* the translocation of photosynthetically derived carbon, as well as through other nutritional exchanges, metabolite production, competitive interactions and defence mechanisms (Wilkinson, 1979; Wilkinson, 1980; Hill, 1996; Roberts *et al.*, 2006; Taylor *et al.*, 2007; Weisz *et al.*, 2007; Erwin & Thacker, 2008; Freeman & Thacker, 2011; Thacker & Freeman, 2012). However, the nutritional interactions and dependencies between sponge associated photosymbionts and the sponge host remain poorly defined, compared to those of the coral-zooxanthellae symbiosis (Davy *et al.*, 2002; Webster & Blackall, 2009; Weisz *et al.*, 2010). Nevertheless, bleaching, the loss of symbiotic photosymbionts, is likely to have negative implications as photosystem damage and subsequent photoinactivation of the

photosymbionts at high temperatures leads to a loss of translocated photosynthetic products to the host (Hoadley *et al.*, 2015).

Whilst recorded mass bleaching events to date have been restricted to reef-building corals (Bell *et al.*, 2013), smaller scale sponge bleaching, in response to elevated temperatures, has been reported on coral reefs globally (Vicente, 1990; Fromont & Garson, 1999; López-Legentil *et al.*, 2008; McMurray *et al.*, 2011). The percentage of sponges within a population and geographic area reported to have bleached during these episodes is low, and in most of these examples bleaching does not eventuate in sponge mortality. However a recent report of a mass sponge bleaching event involving *Cliona varians* from the Caribbean, during a period in which water temperatures exceeded 31 °C for more 10 days, suggests the extent of these events may increase as temperatures increase under OW (Hill *et al.*, 2016). This is the first report of a mass sponge bleaching and also demonstrates that phototrophic sponges may actually be more susceptible to temperature anomalies than heterotrophic sponges, due to their reliance upon carbon transfer from associated photosynthetic symbionts. Contrasting responses to temperature anomalies have been recorded for phototrophic and heterotrophic sponge species in the Mediterranean, whereby mass mortalities of the phototrophic *Ircinia fasciculata* were correlated with a loss of symbiotic cyanobacteria, whilst the co-occurring heterotrophic *Sarcotragus spinosulum* was unaffected (Cebrian *et al.*, 2011). Whilst no bleaching was reported for *I. fasciculata*, experimentally induced temperatures similar to those experienced during the anomaly, demonstrated a reduction in measured photosynthetic parameters confirming a breakdown in this symbiosis to be the root-cause of mortalities. It is important to note that temperature related mortality of the heterotrophic sponge *Rhopaloeides odorabile* was also correlated with a breakdown in the relationship between the host and its associated symbionts (Webster *et al.*, 2008; Fan *et al.*, 2013). This confirms that carbon transfer from phototrophic symbionts is just one of many important roles of sponge microorganisms in the functioning of the holobiont, and that heterotrophic sponges are also sensitive to disruptions in this symbiosis. The implications of OA also differ for phototrophic sponges in that the productivity of associated photosymbionts may increase with elevated pCO_2, providing additional photosynthates to the sponge host. For example, a positive feedback of elevated temperature and pCO_2 on photosynthetic and growth rates in free-living *Synechococcus* (*Synechococcus* species are common sponge-associated cyanobacteria) suggests that some sponge symbionts may benefit from increased carbon availability (Fu *et al.*, 2007). Moreover, in sponge species that are particularly successful at low pH sites in Papua New Guinea CO_2

vent seep sites, shifts in the associated microbial communities towards photosynthetic symbionts have been reported (Morrow *et al.*, 2015), and it is hypothesised that these symbiotic shifts may provide better scope for sponge growth under OA conditions (Morrow *et al.*, 2015). Additionally, despite no effect of OW and OA on *Cliona varians* bioerosion rates, the photosynthetic yields of the sponge associated zooxanthellae increased under OA conditions (Stubler *et al.*, 2015). Such an increase in symbiont productivity may enable phototrophic sponges to have higher productivity in an environment where more carbon is available for photosynthetic production, potentially ameliorating any negative effects of temperature stress.

In this chapter, the physiological response of four abundant Great Barrier Reef (GBR) demosponge species - the phototrophic *Carteriospongia foliascens* and *Cymbastela coralliophila* and the heterotrophic *Stylissa flabelliformis* and *Rhopaloeides odorabile* - to a rise in pCO_2 and seawater temperature over 3 months was examined. The observed and measured physiological performance of sponges under OW and OA was assessed to gain a better understanding of sponge energetics and the implications for ecological performance, in a rapidly changing climate. I specifically addressed the hypothesis that phototrophic and heterotrophic sponges will exhibit differential responses to OW and OA to determine whether sponge nutritional mode matters in a high CO_2 world. Overall the aim of this research was to provide insight into how tropical sponges are likely to respond to the combined effects of OW and OA and to begin to explore whether a sponge-dominated state is a possible future trajectory for coral reefs.

2.2 Materials and Methods

2.2.1 Experimental treatments

Nine combined temperature and pH treatments were tested based on present day $CO_{2\ atm}$ levels (~400 ppm) and projected $CO_{2\ atm}$ increases for 2100 under the IPCC "baseline" emission scenarios RCP6.0 (~800 ppm) and RCP8.5 (~1200 ppm) (IPCC, 2014). All experiments were performed within the National Sea Simulator at the Australian Institute of Marine Science (AIMS). Target treatments for the experiment were 28.5 °C, 30 °C and 31.5 °C and pH (Total scale) 8.1, 7.8, and 7.6. All factors were fully crossed resulting in nine experimental treatments and three replicates of each treatment. Temperature treatments were created by mixing streams of 22 °C and 36 °C 0.04μm filtered seawater using automatic valves controlled through a Siemens PCS7 SCADA system. Accurate temperature control (±0.14 to 0.23; Table 2.1) within each treatment tank was assisted by use of individual water baths, as described in appendix one. Subsequent to the temperature manipulation, each stream of temperature controlled seawater was dosed with CO_2 to reach the three OA treatment levels (in-tank pH ±0.05 to 0.08; and pCO_2 ± 27 to 93 ppm; Table 2.1); note that pCO_2 manipulated treatments will be described as pH, pCO_2 or OA treatments hereafter. CO_2 dosing was achieved *via* mass flow controllers (Aalborg GFC17), delivering a precise flow of CO_2 to a series of membrane contactors (Liqui-Cell Extra-Flow 2.5 9 8; 3M Industrial Business Group, Charlotte, NC, USA), where the gas was dissolved in a constant flow of temperature-controlled seawater. Treated seawater was delivered to three separate, randomly distributed 50 l flow-through aquaria *per* treatment, at a rate of 0.9 l min^{-1} ensuring 100% water replacement every hour. Water circulation within each aquarium was provided by a submersible aquarium pump. Treatment levels were monitored continuously with in-tank pH and temperature loggers. Light was provided by Aqua Illumination SOL Blue LED (75 W) aquarium lights maintained on a 12-h dark: 12-h light cycle, with one hour ramping either side of the light: dark cycle. Maximum irradiance averaged 80 μmol photons m^{-2} s^{-1} (±20 μmol photons m^{-2} s^{-1}) of photosynthetically active radiation (PAR), which is within the range of naturally occurring PAR levels at 10-15 m depth on inshore and mid-shelf reefs of the Central Great Barrier Reef (Cooper *et al.*, 2007). Temperature, pH, pCO_2, salinity, alkalinity, dissolved inorganic carbon (DIC) and PAR were measured weekly (Table 2.1); see appendix one - *Experimental treatments* - for sampling methods, and Table A1.1 for replicate tank temperature and pH means (SD) derived from weekly in-tank measurements.

Table 2.1 Summary of measured (*) and calculated (**) seawater chemistry parameters represented as the mean (±SD) of measurements taken weekly (n= 22 sampling periods). Reported values of temperature and in-tank total scale pH (pH_T) were measured in all aquaria weekly (see Table A1.1 for replicate tank means). Salinity, pCO_2 (ppm), alkalinity and DIC were measured in one tank *per* treatment weekly. pH_T and pCO_2 (µatm) were calculated by inputting salinity, alkalinity and DIC measurements into CO2calc software (Robbins, 2010).

Treatment	**Temp [°C] ***	**pH_T [in-tank] ***	**pCO_2 [ppm] ***	**Salinity [ppm] ***	**A_T [µmol/kg SW] ***	**DIC [µmol/kg SW] ***	**pH_T ****	**pCO_2 [µatm] ****
28.5°C/ pH 8.1	28.63 (0.16)	8.01 (0.05)	464 (27)	36.7 (0.4)	2325 (57)	2025 (37)	7.98	483
28.5°C/ pH 7.8	28.53 (0.20)	7.83 (0.07)	765 (64)	36.7 (0.4)	2326 (56)	2113 (61)	7.82	743
28.5°C/ pH 7.6	28.49 (0.18)	7.68 (0.07)	1125 (92)	36.8 (0.5)	2327 (57)	2171 (80)	7.70	1031
30°C/ pH 8.1	30.02 (0.14)	8.00 (0.05)	469 (30)	36.6 (0.5)	2327 (58)	2022 (35)	7.96	497
30°C/ pH 7.8	30.08 (0.17)	7.81 (0.07)	797 (56)	36.7 (0.5)	2325 (56)	2103 (54)	7.81	752
30°C/ pH 7.6	30.03 (0.22)	7.67 (0.07)	1115 (90)	36.7 (0.5)	2326 (57)	2168 (55)	7.68	1074
31.5°C/ pH 8.1	31.51 (0.19)	8.02 (0.08)	452 (43)	36.8 (0.5)	2328 (57)	2005 (38)	7.97	487
31.5°C/ pH 7.8	31.52 (0.23)	7.83 (0.07)	803 (79)	36.7 (0.4)	2325 (58)	2104 (30)	7.79	797
31.5°C/ pH 7.6	31.48 (0.19)	7.65 (0.07)	1164 (93)	36.8 (0.5)	2325 (57)	2152 (71)	7.69	1039

2.2.2 Sponges

Sponges were collected from 10 to 15 m depth at Davies Reef on the GBR, Australia (18°82'S, 147°65'E) in 2014 between June and September. For all species, except *C. foliascens*, ~20 larger specimens were cut to form ~60 smaller "clones" to make experimental handling more feasible. For *C. foliascens*, ~30 small individuals and 15 larger sponges (each cut into 2-3 "clones") were collected. At least four weeks of acclimation post-cloning allowed recovery from cloning stress (recovery assessed as pinacoderm growth over the cut surface) prior to taking time zero (hereafter referred to as T0) measurements and starting the experiment. Once healed, each sponge was treated as an individual (termed "sponge") with all sponges being randomly allocated across experimental treatments and tanks. After the acclimation period, sponges were transferred from holding tanks to the experimental tanks, which were maintained at 27 °C and pH 8.1 (hereafter referred to as ambient seawater). Six sponges of each species were distributed across the three replicate tanks *per* treatment (n = 6 sponges *per* species *per* treatment, except *S. flabelliformis* where n = 5). Temperature was gradually ramped at 0.5 °C *per* day and pCO_2 levels increased 1.4 to 3.1 ml/min *per* day, depending on the treatment, resulting in 100 - 200 ppm increments of pCO_2 in the experimental tanks (see Table A1.2 for ramping schedule), so as to reach the desired pH once target temperatures were reached. A 12-

week exposure period post-ramping was planned for all adult sponges, with experiments terminated on a species-by-species basis as lethal effects were observed.

2.2.3 Visual assessment

To provide an overall assessment of sponge responses to the treatment conditions, photographs were taken before (T0) and after (T-end) experimental exposure. ImageJ software (ImageJ, U. S. National Institutes of Health, USA) was used to trace individual sponges, and to determine the percentage of tissue affected by necrosis and bleaching. Buoyant weights were taken at T0 and T-end, and used to calculate change in sponge mass (final mass minus initial mass) as well as to normalise metabolic measurements.

2.2.4 Physiological measurements

2.2.4.1 Oxygen flux

Rates of oxygen consumption (respiration) were measured in each treatment, to assess how OW/ OA impact the host sponge. Oxygen production (net photosynthesis) was measured for the two phototrophic species to determine how OW/OA impact the sponge-associated cyanobacteria and their autotrophic energetic contribution to sponge holobiont functioning.

To determine rates of net photosynthesis (*C. foliascens* and *C. coralliophila* only) and dark respiration (all species), six individuals of each species (except n = 5 for *S. flabelliformis*) in each treatment were incubated in the light (net photosynthesis) and dark (dark respiration) in a controlled environment, using the set-up and a method adapted from Strahl and colleagues (Strahl *et al.*, 2015b). T0 measurements were taken in O_2-saturated ambient seawater. For all other time points (every two weeks), incubations were carried out in the sponges' respective O_2-saturated treatment seawater. For each treatment, a chamber without a sponge was used to control for potential changes in O_2 due to metabolic activity of microorganisms in the seawater. Sponges were attached vertically onto the inverted lids of clear acrylic 0.64 l incubation chambers. To determine treatment effects on photosynthetic potential, light incubations were carried out under 350 μmol photons m^{-2} s^{-1} (provided by SOL Blue LED aquarium lights). This irradiance was chosen as preliminary studies demonstrated that it was sufficient to saturate photosynthesis without inducing photoinhibition. Incubations were then run in the dark to determine the treatment effect on holobiont respiration. After 30 min acclimation at 350 μmol photons m^{-2} s^{-1} for net photosynthesis (or in the dark for respiration), chambers were sealed and placed onto submersible stands in 68 l black flow-through bins at the respective treatment

temperatures. The motorised stands continuously mixed the seawater in each chamber with a magnetic stir bar. After 30 min either at saturating irradiance (see above) or in darkness, the chambers were opened and the dissolved oxygen (DO) concentration of each was analysed with a portable Optical Dissolved Oxygen meter (HQ30d equipped with LDO101 IntelliCAL dissolved oxygen probe, Hach, Loveland, CO, USA). Rates of net photosynthesis and dark respiration were normalised to buoyant mass, then calculated in mg O_2 g^{-1} h^{-1} after subtracting the values measured in the blank chambers for each run.

2.2.4.2 Photosynthetic efficiency

Pulse Amplitude Modulated Fluorometry (PAM) was used as an additional assessment of the effect of OW and OA on the photosynthetic efficiency of sponge-associated cyanobacteria. Chlorophyll fluorescence yield measurements were taken weekly using a red LED diving PAM (Walz, Germany) with a 5.5 mm (active diameter) fibre optic cable with spacer (maintaining 1 cm between optic probe and sponge). The saturation pulse method was applied to determine changes in the quantum yield of photosystem II (PSII) in which a weak pulse-modulated red measuring light (0.15 μmol photons m^{-2} s^{-1}) determined the initial fluorescence (F_t in illuminated samples, F_0 in dark-adapted samples), and a 0.8 s saturating pulse of white light (> 10, 000 μmol photons m^{-2} s^{-1}) then determined the maximum fluorescence ($F_{m'}$, in illuminated samples, F_m in dark-adapted samples) (Ralph *et al.*, 2005; Cosgrove & Borowitzka, 2010). The effective quantum yield (ΔF/Fm'), measured under experimental light levels (between 1100 – 1300 h), was determined as ($F_{m'}$-F_t)/$F_{m'}$, where $F_{m'}$ is the light-adapted maximum fluorescence and F_t is the initial fluorescence before the saturating pulse is applied, and provides an estimate of the efficiency of photochemical energy conversion under a given light intensity (Ralph *et al.*, 2005; Cosgrove & Borowitzka, 2010).

Maximum fluorescence yields were determined by applying the saturation pulse method described above to samples following 30 min dark adaptation. However, cyanobacterial photosystems undergo a transition to "state II" in the dark, reflected by lower F_v/F_m and high nonphotochemical quenching (Campbell *et al.*, 1989). Therefore, to obtain true maximal fluorescence yields, measurements must be taken in the presence of far-red light (Schreiber *et al.*, 2011) or in conjunction with the use of an inhibitor such as DCMU (El Bissati *et al.*, 2000), to prevent the state II transition. Neither option was practical here, however dark-adapted yields were still measured weekly as above and determined as (F_0 - F_m)/ F_m, where F_m is the

"maximum" fluorescence yield and F_0 is the initial fluorescence in a dark-adapted sample (Cosgrove & Borowitzka, 2010). These values are not representative of true maximum fluorescence and are thus provided in appendix two (Fig. A2.1).

2.2.5 Data analysis

Data analyses were performed by SPSS (IBM Corp., SPSS Statistics for Windows, V22) and PRIMER-E (PRIMER version 6.0, PERMANOVA+, Plymouth Marine Laboratory, Plymouth, UK). All graphs were generated using GraphPad Prism (GraphPad Software, version 6.00 for Windows, La Jolla California USA). To account for repeated measures over time, generalised linear mixed models (GLMM), with an identity link and normally distributed errors were used to analyse respiration, net photosynthesis and ΔF/Fm' for each species. A diagonal covariance structure was fitted for all models, selected based on the Akaike information criterion (AICc) values. Fixed effects were temperature, pH and time, and random effects were tank and sponge individual (i.e. post "cloning" all sponges were treated as individual sponges). Where significant treatment effects were revealed, the model's *post hoc* pairwise comparisons test (with the sequential Sidak correction applied) was used to determine which treatments differed significantly. The results of the *post hoc* test for each time point are reported in appendix two (Table A2.4a-h), however, only significant differences at T-end will be discussed here. Equal variance and normal distribution assumptions were evaluated *via* analysis of the residuals. Continuous data were log-transformed as necessary to meet these assumptions and ΔF/Fm' data were arcsine-square root transformed prior to analysis as these data are ratios and therefore assumed to not be normally distributed. For the remaining response variables (sponge growth, tissue necrosis and bleaching) a two-way PERMANOVA was employed to test the effects of temperature and pH. For all response variables, Euclidean distances were used to generate a resemblance matrix. Permutational *post hoc* comparisons were used to determine which treatments differed significantly. A 5% significance level was used for all tests.

2.3 Results

2.3.1 Survival

C. foliascens and *R. odorabile* were the two most sensitive species to OW and OA, with experiments terminated after two weeks following high mortalities at 31.5°C. 89% of the total *C. foliascens* mortality occurred at 31.5°C, with the highest levels at pH 8.1 (Fig. 2.1a). Temperature significantly affected tissue necrosis (Pseudo-$F_{(2, 48)}$ = 16.415, P = 0.001; Table A2.1a-b) with higher levels of necrosis at 31.5 °C compared to 28.5°C (P = 0.001) and 30°C (P = 0.001). A significant interaction between temperature and pH affected *C. foliascens* bleaching (Pseudo-$F_{(4, 48)}$ = 1.9002, P = 0.011; Table A2.1a), with the percentage of bleached tissue greatest in sponges at 31.5°C/ pH 8.1, compared to 28.5°C (P = 0.001) and 30°C (P = 0.003), and no significant temperature effect at pH 7.6 (Fig. 2.1a; Table A2.1b). 100% of *R. odorabile* mortality occurred in the 31.5°C/ pH 7.6 treatment, with mortalities occurring after 12 days. A significant interaction between temperature and pH was observed for *R. odorabile* tissue necrosis (Pseudo-$F_{(4, 54)}$ = 5.1629, P = 0.003). Levels of tissue necrosis were significantly higher at 31.5° than at 28.5°C and 30°C, at pH 7.8 (28.5°C, P = 0.010; 30°C, P = 0.006) and pH 7.6 (28.5°C, P = 0.004; 30°C, P = 0.038), with no visible temperature effect at pH 8.1 (Fig. 2.1b; Table A2.1a, c). No mortality was observed for *S. flabelliformis* or *C. coralliophila*. However, there was a significant effect of temperature (Pseudo-$F_{(2, 36)}$ = 11.794, P = 0.001; Fig. 2.1c; Table A2.1, d), which resulted in significantly higher levels of tissue necrosis in *S. flabelliformis* at 31.5°C such that the experiment was terminated after eight weeks. Despite high levels of bleaching, *C. coralliophila* remained in the experimental system for 12 weeks, with low levels of tissue necrosis in sponges at 31.5°C/ pH 8.1 and 7.8 following bleaching (Pseudo-$F_{(4, 45)}$ = 2.6507, P = 0.031; Fig. 2.1d; Table A2.1a, e). Despite significant tissue loss across all species in the 31.5°C treatments (Fig. 2.1a-d), PERMANOVA revealed no significant difference in the change in sponge mass between treatments (Table A2.1a).

2.3.2 GLMM

Models were fitted with tank as a random effect, however AICc values indicated that the variation between tanks was small enough to be excluded from the model. Sponge individual was a significant random effect for all response variables (again determined by AICc values), and this remained in all models to account for variation between sponges (see Table A2.2 for report of random effects for sponge individual). All GLMM coefficients are reported in the

appendix, (Table A2.3a-h), and significant differences between treatments based on the estimated means are discussed below (*post hoc* results reported in Table A2.4a-h).

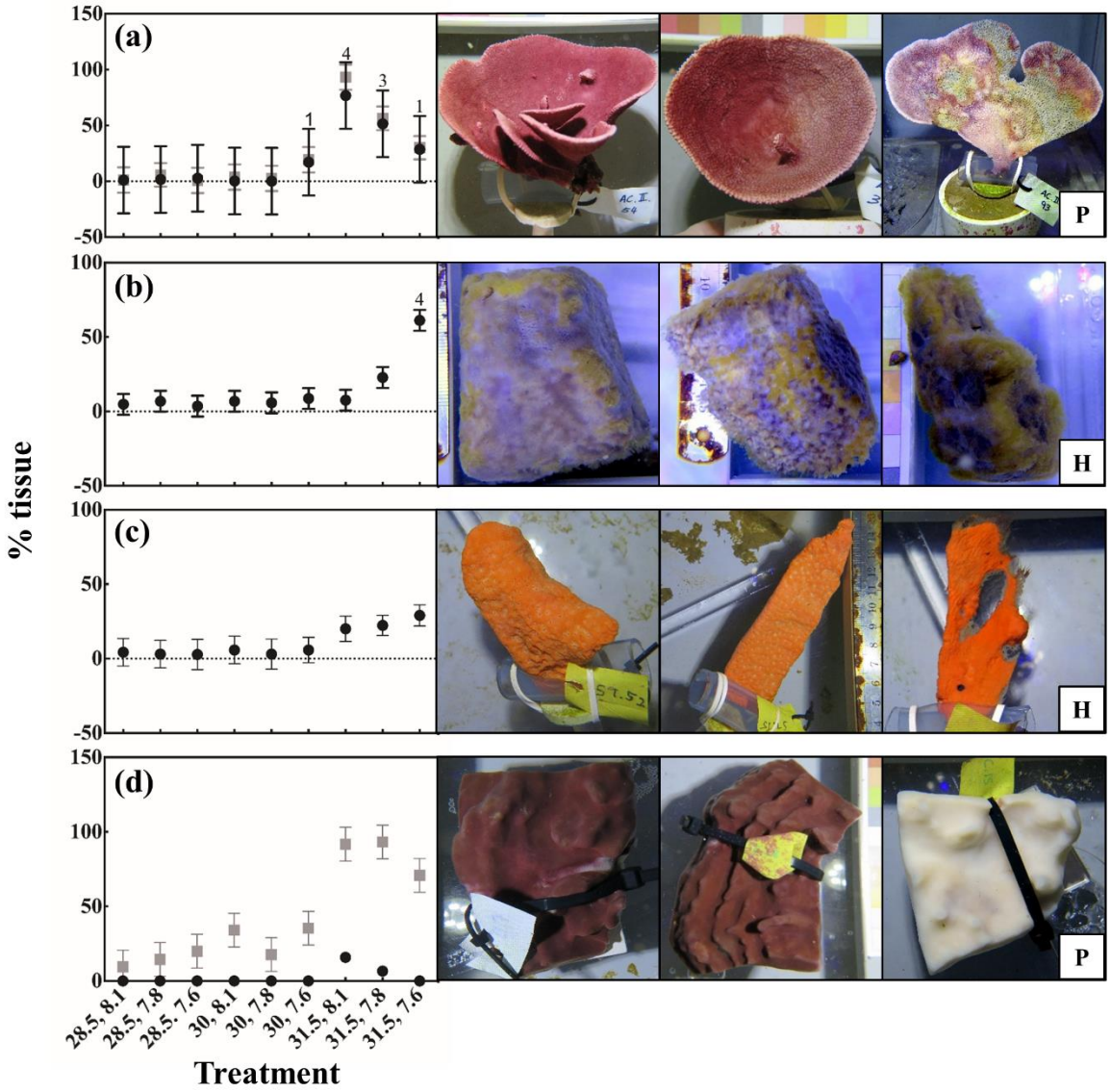


Fig. 2.1 Health state of sponges following exposure to treatments. (a) *Carteriospongia foliascens* (b) *Rhopaloeides odorabile* (c) *Stylissa flabelliformis* (d) *Cymbastela coralliophila* (note: P = phototrophic species and H = heterotrophic species).The first plot in each panel is % tissue necrosis ● and % tissue bleached (phototrophic species only) ■ for each species in each treatment, calculated using ImageJ (y-axis = % tissue and x-axis = treatment). Values are mean % tissue *per* sponge *per* treatment ± SE (n = 6 *C. foliascens*, *R. odorabile* and *C. coralliophila*, and n = 5 *S. flabelliformis per* treatment). Numbers above treatments for *C. foliascens* and *R. odorabile* represent the total mortalities in the corresponding treatment. Following each plot is a representative sponge from each temperature treatment at pH 8.1, left to right: 28.5°C, 30°C and 31.5°C. Treatment exposure times vary between species where T=2 weeks for *C. foliascens* and *R. odorabile*, T=8 weeks for *S. flabelliformis* and T=12 weeks for *C. coralliophila*.

2.3.3 Respiration

Respiration rates in *C. foliascens* were significantly influenced by temperature over time ($F_{(2, 69)}$ = 15.011, $P < 0.001$; Fig. 2.2a), increasing with temperature from T0. *Post hoc* analysis revealed that the average respiration rate of *C. foliascens* was significantly higher at 31.5°C than 30°C ($P = 0.024$) and 28.5°C ($P < 0.001$), and significantly higher at 30°C than 28.5°C ($P = 0.024$) at T-end (Fig. 2.2a; Table A2.4a). A significant pH effect on *C. foliascens* respiration rates over time was also detected ($F_{(2, 69)}$ = 3.226, $P = 0.046$; Fig. 2.2a), although *post hoc* analysis did not elucidate this effect (Table A2.4a). Respiration rates at 31.5°C/ pH 8.1 were elevated compared to other treatments, which potentially drives this pH effect. However, this trend was driven by a visibly stressed sponge, and due to high *C. foliascens* mortalities in this treatment only two sponges remained at 31.5°C/ pH 8.1 at T-end. *R. odorabile* respiration rates also increased with temperature over time ($F_{(2, 89)}$ = 5.025, $P = 0.009$; Fig. 2.2b). *Post hoc* analysis revealed that the average respiration rate of *R. odorabile* was significantly higher for sponges at 31.5°C than at 28.5°C ($P = 0.007$) and 30°C ($P = 0.007$) at T-end (Fig. 2.2b; Table A2.4b). A significant interaction between temperature, pH and time was observed on respiration rate for *S. flabelliformis* ($F_{(16, 177)}$ = 3.676, $P < 0.001$; Fig. 2.2c). *Post hoc* analysis revealed that at T-end the average respiration rate of *S. flabelliformis* at pH 7.6 was significantly higher in sponges at 31.5°C than at 30°C ($P = 0.023$) and 28.5°C ($P = 0.050$; Fig. 2.2c; Table A2.4c). Respiration rates in *S. flabelliformis* generally decreased between T0 to T2, and although highly variable throughout the experiment, were elevated at 31.5°C and lower at pH 7.6 (Fig. 2.2c). The average respiration rate declined with pH at all temperatures except at 31.5°C, in which respiration rates were elevated regardless of pH. A significant interaction between temperature, pH and time was also observed on respiration rates for *C. coralliophila* ($F_{(20, 256)}$ = 2.098, $P = 0.005$; Fig. 2.2d). Respiration rates of *C. coralliophila* at 31.5°C/ pH 8.1 increased from T2 (Fig. 2.2d). At T-end, *post hoc* analysis revealed that, at pH 8.1, the average *C. coralliophila* respiration rate was significantly higher at 31.5°C than 28.5°C ($P = 0.021$) and 30°C ($P < 0.001$), while at lower pH, temperature had no statistically significant effect on *C. coralliophila* respiration rate (Fig. 2.2d; Table A2.4d).

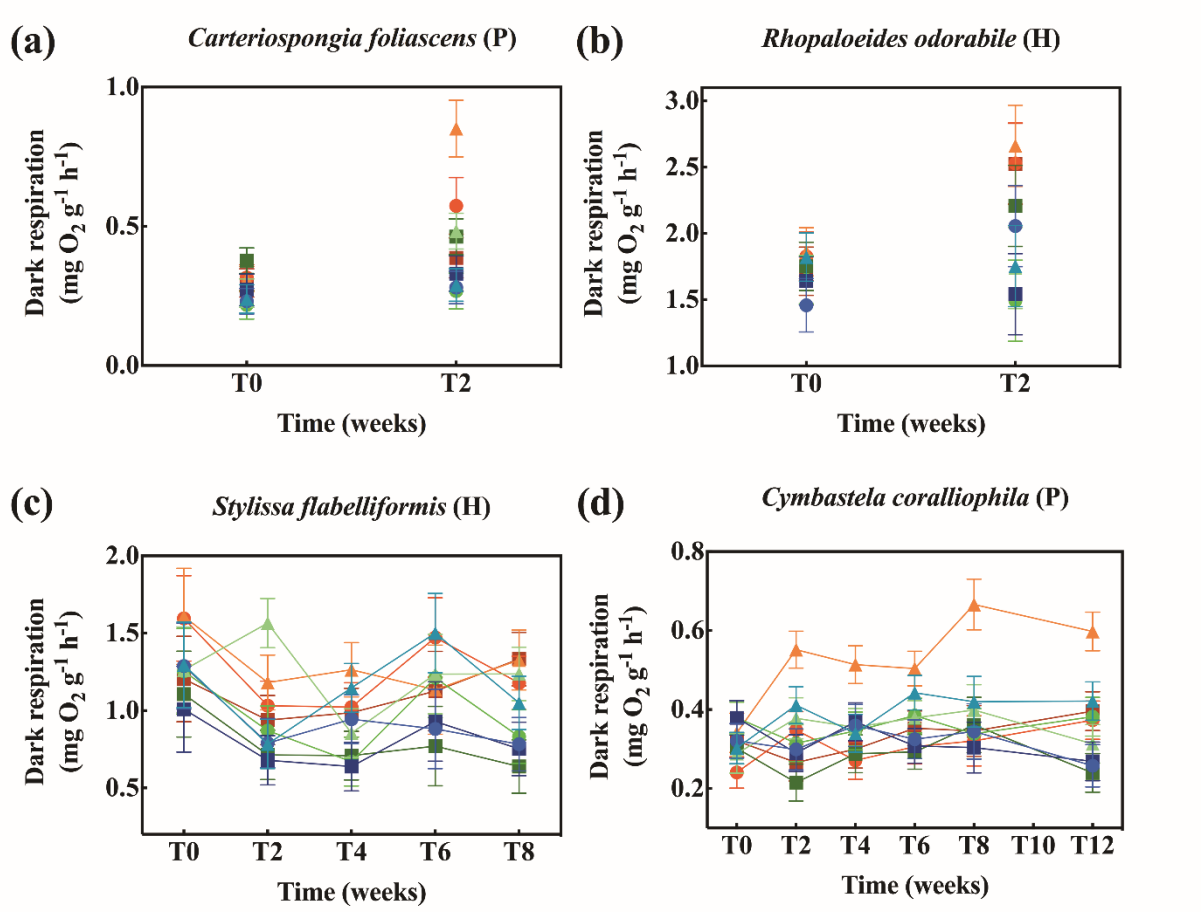


Fig. 2.2 Respiration rate of (a) *Carteriospongia foliascens* (b) *Rhopaloeides odorabile* (c) *Stylissa flabelliformis* (d) *Cymbastela coralliophila*, in each treatment (note: P = phototrophic and H = heterotrophic species). ▲ 28.5°C/pH 8.1, ● 28.5°C/pH 7.8, ■ 28.5°C/pH 7.6, ▲ 30°C/pH 8.1, ● 30°C/pH 7.8, ■ 30°C/pH 7.6, ▲ 31.5°C/pH 8.1, ● 31.5°C/pH 7.8, ■ 31.5°C/pH 7.6. Values are mean respiration rate *per* treatment ± SE *per* sampling time point (n = 6 C. foliascens, R. odorabile and C. coralliophila and n = 5 S. flabelliformis *per* treatment, *per* time point - except where mortalities occurred). Note: x and y-axis scales differ between species.

2.3.4 Net photosynthesis

A significant interaction between temperature and pH was observed for rates of net photosynthesis over time in *C. foliascens* ($F_{(4, 70)}$ = 3.014, P = 0.024; Fig. 2.3a). Average net photosynthetic rates of sponges exposed to 31.5°C decreased from T0, with the greatest decline in net photosynthesis occurring in sponges exposed to 31.5°C/ pH 8.1 (Fig. 2.3a). *Post hoc* analysis revealed that at T-end net photosynthetic rates at 31.5°C were significantly lower than net photosynthetic rates at 28.5°C (pH 8.1, P < 0.001; pH 7.6, P = 0.011) and 30°C (pH 8.1, P = 0.001; pH 7.6, P < 0.001). The only exception in which temperature had no significant effect (Fig. 2.3a; Table A2.4e) was at pH 7.8, however due to high mortalities in this treatment only

two sponges remained at T-end. Net photosynthetic rates in *C. coralliophila* were significantly influenced by temperature over time ($F_{(10, 259)}$ = 9.943, P < 0.001; Fig. 2.3b), with declines in net photosynthesis at 31.5°C after two weeks (Fig. 2.3b; Table A2.4f). *Post hoc* analysis revealed that, at T-end, the average *C. coralliophila* net photosynthetic rate was significantly lower at 31.5°C than in sponges exposed to 28.5°C (P < 0.001) and 30°C (P < 0.001) (Fig. 2.3b; Table A2.4f). The model also revealed a significant pH and time interaction ($F_{(10, 259)}$ = 1.984, P = 0.035; Fig. 2.3b) for *C. coralliophila*, with the average net photosynthetic rate of *C. coralliophila* at pH 8.1 being nearly half that of *C. coralliophila* in the lower pH treatments at T-end (Fig. 2.3b). Regardless, *post hoc* analysis detected no significant pH effect (Table A2.4f).

2.3.5 Effective quantum yield (ΔF/Fm')

A significant interaction between temperature and time affected ΔF/Fm' in *C. foliascens* ($F_{(4, 117)}$ = 18.574, P < 0.001; Fig. 2.3c). ΔF/Fm' declined at 31.5°C and *post hoc* analysis revealed that the average *C. foliascens* ΔF/Fm' was significantly lower at 31.5°C than at 28.5°C (P < 0.001) and 30°C (P < 0.001; Table A2.4g). A significant interaction between temperature and pH affected ΔF/Fm' over time in *C. coralliophila* ($F_{(24, 310)}$ = 1.906, P = 0.007; Fig. 2.3d), with ΔF/Fm' declining at 31.5°C from T0. *Post hoc* analysis revealed that, at T-end, ΔF/Fm' was significantly lower in sponges at 31.5°C compared to 28.5°C and 30°C at all pH levels tested, except for pH 8.1, in which ΔF/Fm' also declined at 30°C. ΔF/Fm' did not decline in the low pH treatments at 30°C (Table A2.4h), suggesting an ameliorative effect of pH on photosynthetic processes at this temperature. Whilst true F_v/F_m could not be obtained, it is noteworthy that both light (Fig. 2.3c-d) and dark (Fig. A2.1a-b) quantum yield measurements responded similarly to the treatments over time, increasing our confidence that temperature was responsible for photoinhibition rather than variable fluorescence and/or state transitions.

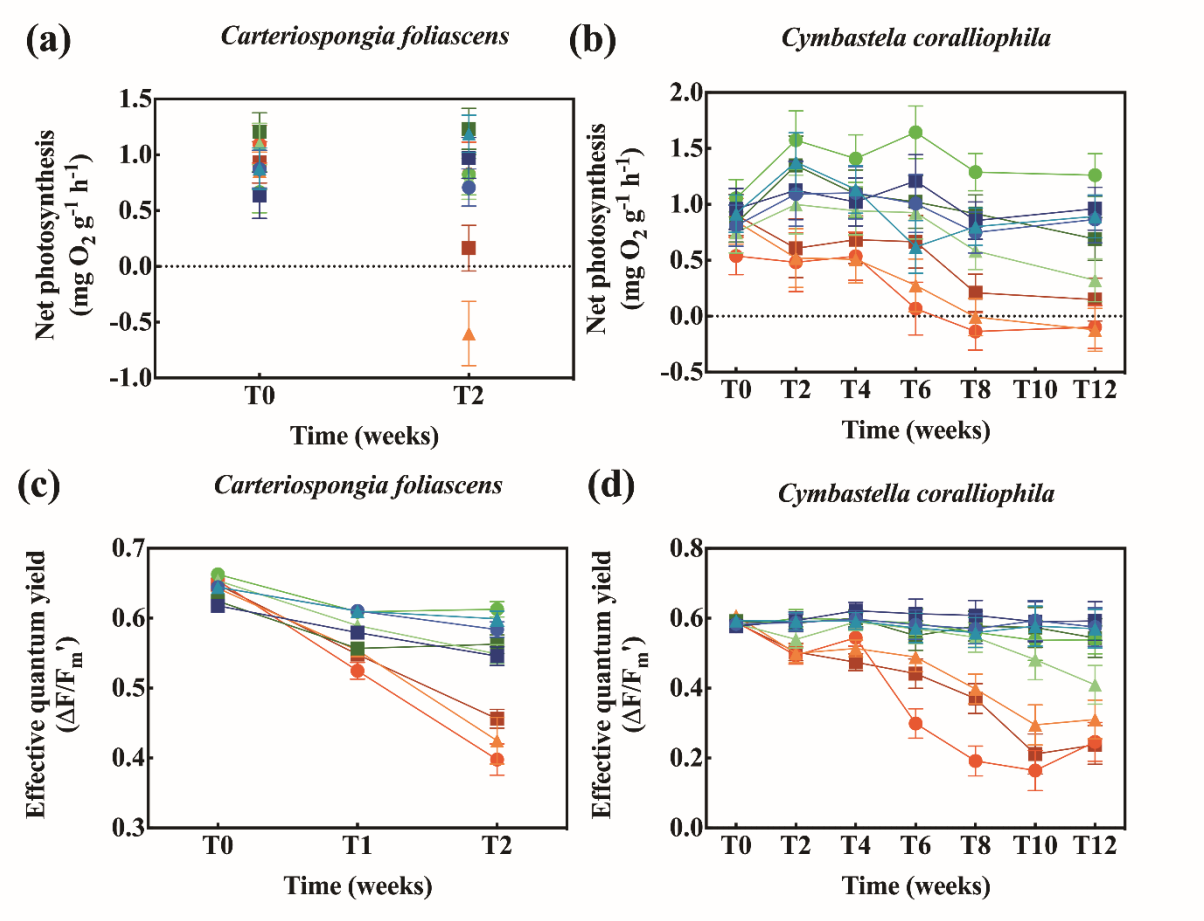


Fig. 2.3 Rates of net photosynthesis in (a) *Carteriospongia foliascens* and (b) *Cymbastela coralliophila*, and effective quantum yield in (c) *C. foliascens* and (d) *C. coralliophila* in each treatment. ▲ 28.5°C/pH 8.1, ● 28.5°C/pH 7.8, ■ 28.5°C/pH 7.6, ▲ 30°C/pH 8.1, ● 30°C/pH 7.8, ■ 30°C/pH 7.6, ▲ 31.5°C/pH 8.1, ● 31.5°C/pH 7.8, ■ 31.5°C/pH 7.6. Values are mean net photosynthetic rate and effective quantum yield *per* treatment ± SE *per* sampling time point (n = 6 *per* treatment *per* time point - except where mortalities occurred). Note: x and y-axis scales differ between species and response variables.

2.4 Discussion

This chapter provides evidence from a 3-month experimental study that, whilst tropical sponges are unaffected by climate change scenarios predicted under the RCP6.0 conditions for 2100, environmental projections for the end of this century under the RCP8.5 have significant implications for their survival. Elevated temperature under RCP8.5 was found to negatively affect the health and survival of all four GBR sponge species, with mortality (*C. foliascens* and *R. odorabile*), high levels of tissue necrosis (all species), elevated respiration (all species) and bleaching (phototrophic species) evident at 31.5°C. Elevated pCO_2 exacerbated the symptoms of thermal stress in the heterotrophic species, yet overall, it reduced the impact of elevated temperature on the phototrophic species.

2.4.1 The response of heterotrophic sponges to OW and OA

Differing degrees of sensitivity to elevated temperature and lowered pH were apparent between the two heterotrophic species. High mortality of *R. odorabile* was evident after two weeks of exposure to the highest temperature and lowest pH treatment. *R. odorabile* has previously been shown to have a strict thermal limit of 32°C (Webster *et al.*, 2008; Pantile & Webster, 2011; Massaro *et al.*, 2012), after which point disruption to nutritional interdependence and molecular interactions between the host and its microbial symbionts destabilises the holobiont (Fan *et al.*, 2013), resulting in rapid mortality. Importantly, however, this thermal threshold, which is already close to seawater temperatures currently experienced on the northern GBR, is further lowered by the combined effect of OA. Meanwhile, *S. flabelliformis*, although visibly stressed (necrosis) and having elevated (albeit highly variable) respiration rates at 31.5°C, experienced no mortality even after eight-weeks of exposure. Tissue necrosis was highest at 31.5°C/ pH 7.6, suggesting that low pH exaggerates thermal stress in *S. flabelliformis*. *S. flabelliformis* respiration rates were also depressed at low pH, irrespective of temperature.

Metabolic depression has been observed in a wide range of marine invertebrates following exposure to elevated pCO_2 (Pörtner *et al.*, 1998; Michaelidis *et al.*, 2005; Pörtner, 2008; Albright & Langdon, 2011; Dupont & Pörtner, 2013; Putnam *et al.*, 2013). This response is thought to be a short term adaptive strategy to protect against acidosis and abnormally high levels of carbonic acid in bodily fluids (Fabry *et al.*, 2008; Przeslawski *et al.*, 2008). However, the physiological or molecular mechanisms causing metabolic depression in sponges are still uncertain, as sponges do not possess bodily fluids (Duckworth & Peterson, 2013) and like

many lower marine invertebrates, have limited capacity for acid-base regulation. Whatever the mechanism, a depressed metabolic state is likely to be detrimental to the long term survival of a species as processes such as growth and reproduction become compromised as energy gets diverted into sustaining basic biological processes (Pörtner *et al.*, 2004). In the heterotrophic species examined here, it is apparent that mechanisms for coping with thermal stress become disrupted under elevated pCO_2, to the detriment of the health of sponges at 31.5°C. Similarly, higher mortality in the bioeroding sponge *C. celata* has been reported after exposure to pH 7.8 than to pH 8.1 (Duckworth & Peterson, 2013), providing further support that some sponge species are negatively affected by OA.

2.4.2 The response of phototrophic sponges to OW and OA

Respiration rates of the phototrophic sponge species increased following exposure to 31.5°C, however the increases were only observed under present day pH conditions. This is in contrast to the heterotrophic species, and indicates a buffering effect of increased pCO_2. While net photosynthetic rate and effective quantum yield in the two phototrophic species declined significantly at 31.5°C, the rate of decline in net photosynthesis over time was less at low pH, demonstrating an ameliorative effect of pCO_2 on photosynthetic processes. This "positive feedback" of increased pCO_2 in thermally-stressed phototrophic sponges was also observed in the final visible health state of these species, with mortality and bleaching of *C. foliascens* at the highest temperature decreasing with increasing pCO_2 and no evidence of tissue necrosis in *C. coralliophila* exposed to 31.5°C/ pH 7.6.

The role of sponge photosymbionts in ameliorating the impact of climate change warrants further investigation. For many marine phytoplankton species, photosynthetic rates remain unaffected by experimental increases in pCO_2 suggesting they are not carbon limited, most likely due to their ability to accumulate inorganic carbon intracellularly (Doney *et al.*, 2009). However, previous studies suggest that some sponge associated phototrophic symbionts may benefit from increased carbon availability (Fu *et al.*, 2007; Stubler *et al.*, 2015). The transfer of this increased symbiont productivity under elevated pCO_2 conditions has been observed in organisms residing at CO_2 vent seeps. Photosynthesis, respiration and subsequent growth of the sea anemone *Anemonia viridis* increased along a natural CO_2 gradient in Italy (Suggett *et al.*, 2012). Similarly, for three coral species at a CO_2 vent seep in Papua New Guinea, net photosynthetic rates increased compared to the same species at control sites (Strahl *et al.*,

2015b). This additional DIC appeared to compensate for any negative effects of elevated pCO_2 on net coral calcification, which remained unaffected in two out of the three species. At the same CO_2 seep site in Papua New Guinea, the sponges *Coelocarteria singaporensis* and *Cinachyra* sp. are 40-fold more abundant and host a higher relative abundance of symbiotic *Synechococcus* than the same species at nearby control sites (Morrow *et al.*, 2015). While photosynthetic rates were not measured, these authors postulated that the increased abundance of photosynthetic microbes in sponges at the seep provides these sponges with a nutritional benefit under future climate scenarios (Morrow *et al.*, 2015). Furthermore, photosynthetic yields of symbiotic dinoflagellates associated with the bioeroding sponge *Cliona varians* increased under OA conditions (Stubler *et al.*, 2015). Such an increase in symbiont productivity may enable phototrophic sponges to have higher productivity in an environment where more inorganic carbon is available for photosynthesis. For both species in our experiment, elevated inorganic carbon availability apparently facilitated the sponge symbionts' 'resistance' to bleaching for longer, and in turn lessened the degree of stress on the sponge host, perhaps through the continued provision of translocated products of photosynthesis. A similar ameliorative effect of elevated pCO_2 has been demonstrated for the bioeroding sponge *Cliona orientalis* during temperature stress (Wisshak *et al.*, 2013), although when exposed to temperatures predicted under RCP8.5, the additional carbon was not sufficient to offset increased metabolic demand (Fang *et al.*, 2014).

Sensitivity to the various OW and OA treatments also varied between the two phototrophic species. Bleaching of *C. foliascens* commenced within one week of exposure to 31.5°C with subsequent rapid mortality of this species. In contrast, despite near complete bleaching of the majority of *C. coralliophila* individuals at 31.5°C after six-weeks, no mortality occurred even after 12-weeks of exposure, indicating that *C. coralliophila* is either, i) less reliant upon photosynthetically-acquired carbon, or ii) has the potential to switch to acquiring carbon heterotrophically when bleached. Commensal relationships have been suggested between other sponges containing phototrophic symbionts. For example, despite declines in the photosynthetic cyanobacterium *Synechococcus spongiarum* during shading experiments on *Xestospongia exigua*, there was no decline in host mass (Thacker, 2005). Similarly, it is suggested that cyanobacteria associated with the *Xestospongia muta* holobiont provide little benefit to the host sponge (McMurray *et al.*, 2011). Alternatively, a switch to heterotrophically acquiring carbon during bleaching is also possible, and would be highly beneficial as the thermal anomalies that drive bleaching events today become increasingly frequent. The ability

to switch feeding strategy following the loss of zooxanthellae has been demonstrated in the thermally tolerant coral *M. capitata*. Total lipid levels of this species *in situ* following a natural bleaching event did not differ, suggesting nutritional demands were met elsewhere (Grottoli *et al.*, 2006). Experimentally induced bleaching confirmed that the ability of this species to acquire carbon heterotrophically during bleaching enables recovery from this stressor (Grottoli *et al*., 2006). Future work examining lipid levels and bacterial consumption rates under OW and OA conditions will help to elucidate the mechanisms underlying *C. coralliophila* tolerance to bleaching.

2.4.3 Skeletal composition matters

While it is widely accepted that skeletal mineralogy will play an important role in the sensitivity of marine organisms to OW and OA (Vicente *et al.*, 2015), most of the research to date focuses on the sensitivity of calcifying organisms to OA (Fabry *et al.*, 2008; Doney *et al.*, 2009). However, this study reveals differences in thermal tolerance between sponges with different skeletal compositions. The two species most tolerant of the RCP8.5 OW levels were *C. coralliophila*, and *S. flabelliformis*; both of which are spiculated demosponges. In conrast, the two most sensitive sponge species were *C. foliascens* and *R. odorabile*; aspiculate demosponges with fibrous skeletons of collagen or spongin. This suggests that similar to calcium carbonate skeletal elements and ocean acidification, skeletal mineralogy may play a role in the thermal tolerance of sponges. Recent research exploring the response of sponge skeletal mineralogy under climate change confirmed that the spicule biomineralization of *Mycale grandis* is tolerant to OW and OA (Vicente *et al.*, 2015), although it remains unclear what the advantage of being spiculated is under these conditions. Regardless, sponges with siliceous spicules have resisted previous mass extinction events as a result of changes in ocean chemistry similar to those we expect to witness in the ocean under predicted $CO_{2\ atm}$ increases (Maldonado *et al.*, 1999; Delecat *et al.*, 2011), and once again it appears these robust skeletal forms will facilitate the adaptation of spiculated sponges to a rapidly changing climate.

2.4.4 Summary

This chapter demonstrates that four abundant GBR sponge species are unaffected by climate change scenarios predicted under the RCP6.0 model, but that they are likely to be adversely affected by environmental conditions represented under the RCP8.5 model. Importantly, elevated pCO_2 differentially affects sponges with different nutritional modes, exacerbating the impact of elevated temperature in heterotrophic species but ameliorating the effect of elevated

temperature in phototrophic species. Space is a limiting resource on coral reefs (Jackson, 1977), and if coral cover continues to decline globally, some sponge species have the potential to increase their cover on coral reefs as space is made available. Beyond 2100, the OW and OA conditions predicted under RCP8.5 may result in a shift in abundance towards a dominance of phototrophic sponge species. In addition, this chapter provides evidence to support previous suggestions that sponges with siliceous skeletons are more resistant to environmental stressors than keratose species. This demonstrates that skeletal form is not just important with respect to carbonate accretion processes in a high CO_2 world, the combined effect of OW and OA create an environment that can be stressful for other less resilient skeleton forms. Therefore, not only might future OW and OA see a shift in abundance of sponge holobiont type towards phototrophic species with greater scope for growth, but also a reduction in sponges with less resilient skeletal elements. In addition to providing insight into the response of an essential reef organism to climate change, this study highlights the need to measure the effect of applied stressors using relevant physiological performance indicators. In particular, the selection of physiological processes fundamental to organism function and survival provide significant insight to organismal responses, especially in *ex situ* experiments where representative time-frames are unachievable. Finally, this study also emphasises the importance of running multi-species, multi-factorial, long-term exposure experiments when determining an organism's response to environmental change *ex situ.*

Chapter three

The biochemical response of sponges to elevated pCO_2 and temperature (Part 1): *Lipid class composition reveals potential tolerance mechanisms in sponges*

Lipid extracts from four Great Barrier Reef sponge species.

3.1 Introduction

Under climate change, the prognosis for many marine organisms is negative (Kroeker *et al.*, 2013b). Coral reef ecosystems are particularly vulnerable to the effects of ocean warming (OW) and ocean acidification (OA), primarily due to the sensitivity of coral to the effects of these stressors (Hoegh-Guldberg *et al.*, 2007; Doney *et al.*, 2012). Coral cover is already declining globally due to a number of natural and anthropogenic stressors (De'ath *et al.*, 2012), with space being made available for other more tolerant organisms (Norström *et al.*, 2009; Bell *et al.*, 2013, Kroeker *et al.*, 2013a). The previous chapter demonstrated that some coral reef sponge species are able to tolerate high oceanic pCO_2, suggesting certain species have the capacity to increase on coral reefs as space is made available through coral decline. However, while physiological responses provide an understanding of the systemic tolerance of these sponges to OW and OA, these stressors are also known to influence other cellular and molecular processes that may not be reflected by host physiology (Pörtner, 2008). In particular, temperature and CO_2 exert strong control over fundamental metabolic processes, influencing cellular metabolic pathways with implications for energy storage (Pörtner, 2008; Pörtner & Farrell, 2008; Fabry *et al.*, 2008; Hoegh-Guldberg & Bruno, 2010). Stress exposure can have significant impacts on an organism's energy reserves, as energy intake is reduced whilst stores are depleted to support defence and repair processes (Maltby, 1999). An organism's performance is ultimately determined by its energy status, due to the importance of this resource to all physiological and biological processes (Brown *et al.*, 2004). Therefore a decrease in energy reserves not only compromises important processes, such as growth and reproduction, but is likely to increase an organism's susceptibility to stressors (Brown *et al.*, 2004; Bachok *et al.*, 2006; Anthony *et al.*, 2007). Furthermore, chapter two found that elevated pCO_2 can provide phototrophic sponges with protection from thermal stress; however the pathways underlying this ameliorative effect remain unclear. Additionally, the tolerance of sponges to OW and OA is species-specific, yet the mechanisms underpinning such differences remain unresolved. Determining the energy status of sponges under OW and OA conditions will not only increase our understanding of the implications of climate change for sponges, it will also help elucidate factors contributing to the tolerance of these ancient organisms.

Energy can be stored as lipids, proteins and carbohydrates (Lesser, 2013). Lipids are regarded as being amongst the most important nutritional factors affecting the fitness of marine organisms (Kainz & Fisk, 2009). They are often considered the most effective energy store,

and as a major source of metabolic energy lipids are essential for somatic growth and reproduction (Parrish, 2013). Lipids also play a crucial role in cell membrane structure, protection and function (Hofmann & Todgham, 2010), and are involved in intracellular signalling pathways, including signal transduction and molecular recognition processes (Van Meer *et al.*, 2008). Lipids are therefore very important to an organism's physiological and biological processes (Bergé & Barnathan, 2005), and often play a role in determining its environmental tolerance (Anthony *et al.*, 2009). Physically, lipids are esters of fatty acids and alcohols that comprise a large group of structurally distinct, organic compounds (Singh *et al.*, 2002). Depending on their function, lipids can be grouped into broad categories, those which are primarily utilised for storage, and those with structural functions (Rod'kina, 2005). Storage lipids predominantly serve as an energy reserve as they are rich in fatty acids that are oxidised to produce energy (Van Meer *et al.*, 2008). The major structural lipids in eukaryotes are the phospholipids (Rod'kina, 2005; Van Meer *et al.*, 2008), and together with sterols these serve as the building blocks for the lipid bilayer that forms cell membranes (Bergé & Barnathan, 2005), with essential roles in regulating biophysical properties, protein sorting and cell signalling pathways (Genin *et al.*, 2008), as well as controlling cell membrane fluidity and permeability (Simons & Vaz, 2004; Parrish, 2013).

The biomolecular lipid layer of cell membranes is sensitive to environmental stressors, and in particular temperature change (Vígh *et al.*, 2007). At an organism's adapted temperature, lipids in this bilayer are mobile, and facilitate vital cellular functions such as nutrient transport and enzyme activity (Neidleman, 1987). The fluidity of this lipid bilayer is dependent on an organism's "body" temperature, and changes in temperature have serious implications for cellular functioning. As a result, the physical properties of membrane lipids ultimately define an organism's thermal limits (Hazel, 1995). Therefore, the ability of an organism to regulate and adapt cell lipid bilayers in response to environmental stressors, such as temperature change, plays a significant role in stress tolerance (Geider, 1987; Hazel, 1995). Organisms have developed different strategies to maintain appropriate membrane fluidity in response to thermal stress, through the compositional adaptation of membrane lipids (homeoviscous adaptation) that specifically involve changes to the saturation state of these lipids (Vígh *et al.*, 2007). This can be achieved through alterations in fatty acid composition (Neidleman, 1987), shifts in the relative proportion of sterols, glycolipids and phospholipids in the lipid bilayer (Weirich & Reigh, 2001; Horváth *et al.*, 2012; Parrish, 2013), and increased biosynthesis of wax esters to compensate for temperature changes (Neidleman and Geigert, 1984). Alteration in membrane

lipid content is of major importance in the response to environmental stressors (Singh *et al.*, 2002). For example, membrane fluidity and lipid composition are fundamental to the survival of bacteria at extreme temperatures and under other adverse conditions including salinity changes and desiccation (Ritter & Yopp, 1993), while sensitivity of the yeast *Saccharomyces cerevisiae* to both heat and oxidative stress is dependent on membrane lipid composition (Steels *et al.*, 1994). Lipids also play a role in the resistance of plant cells to environmental stressors (Yordanov *et al.*, 2000), including maintenance of membrane stability *via* alterations to membrane lipid composition in response to thermal stress (Wahid *et al.*, 2007). In plants exposed to elevated temperature, the xanthophylls interact with thylakoid membrane lipids to reduce membrane fluidity, ultimately lowering susceptibility to lipid peroxidation under high temperatures (Havaux, 1998).

Other adaptive responses to environmental stressors through alteration of lipid content and composition have also been identified. For example, total lipids are known to accumulate under stressful conditions for a number of algal species (Gordillo *et al.*, 1998). This phenomenon is most widely reported in response to decreased nutrient availability, in particular nitrogen. Under such conditions, cell division slows down whilst photosynthetic carbon dioxide fixation continues. Due to the low lipid demand with suppressed growth, lipids accumulate and can be used to support rapid growth following release from the stressor (Solovchenko, 2012). This response has also been demonstrated in some algal species as a result of temperature change, with a number of species having higher lipid content when grown under elevated temperatures (Zhu *et al.*, 1997; Renaud *et al.*, 2002). Coral lipids have been used to study the effect of environmental stressors (Yamashiro *et al.*, 2005; Imbs & Yakovleva, 2012; Strahl *et al.*, 2015a) and are useful indicators of coral mortality risk (Anthony *et al.*, 2007). For example, under thermal stress, coral colonies with high levels of energy storage show greater rates of survival and recovery from bleaching than starved corals (Rodrigues & Grottoli, 2007; Anthony *et al.*, 2009; Strahl *et al.*, 2015a). Additionally, species-specific differences in the metabolism of lipid reserves have contributed to the differential resistance of coral species under acute heat exposures (Yamashiro *et al.*, 2005). For instance, species with a preferential consumption of structural lipids in the earlier stages of bleaching have a lower capacity to cope with environmental stress than those that are able to conserve structural lipids (Imbs & Yakovleva, 2012).

As with most organisms, the abundant storage lipids found in sponges are triacylglycerides (Bergé & Barnathan, 2005), along with sterol esters and free fatty acids (Marsden, 1975). Storage lipids are generally accumulated in an organism's fat tissue as an energy reserve (Bergé & Barnathan, 2005), however, as sponges have no real tissues or organs (Rod'kina, 2005), storage lipids comprise just a small proportion of total sponge lipid content (Marsden, 1975). Conversely, structural lipids, including phospholipids, sterols (e.g. cholesterol), and acetone mobile polar lipids (e.g. pigments, glycolipids and monoacylglycerols; Parrish, 2013) are the most abundant sponge lipids (Marsden, 1975). Sponge lipid composition is distinctive among marine organisms (Lawson *et al.*, 1988), with specific features particularly in respect to their cell membranes, which have an abundance of novel phospholipids, and sterols (Santalova *et al.*, 2004; Bergé & Barnathan, 2005). Due to the role of sterols and phospholipids in maintaining correct membrane fluidity, these features are thought to play at least some part in the persistence of sponges throughout time, and their survival in what are often unpredictable and variable environments (Lawson *et al.*, 1988; Djerassi & Lam, 1991; Santalova *et al.*, 2004; Genin *et al.*, 2008). Furthermore, there is evidence to suggest that sponges are able to control the fluidity of their cell membranes under changing temperatures, for example by increasing lipids that have a higher melting point in summer, as a form of homeoviscous adaptation (Lawson 1984). While sponge lipids have been shown to vary seasonally with important processes such as reproduction (Elvin, 1979; Barthel, 1986), there is a lack of studies considering the effects of environmental change on sponge lipid content (Arillo *et al.*, 1993).

As with reef building corals, many reef sponges form symbiotic relationships with photosynthetic microorganisms that are important to the health of the sponge (Taylor *et al.*, 2007). However, the nutritional interactions between sponge associated photosymbionts and the sponge host remain poorly defined (Davy *et al.*, 2002; Webster & Blackall, 2009). As a result, inferences surrounding the exchange of nutrients, specifically photosynthetically translocated products of carbon fixation to the sponge, are drawn from studies of coral-algal symbiosis. While there is some uncertainty about the forms in which photosynthetic carbon is translocated to the coral host, common compounds known to be transferred include glycerol (a major component of lipids) and glucose as well as amino acids and lipids (Muscatine, 1967; Yellowlees *et al.*, 2008; Davy *et al.*, 2012). Coral bleaching, the loss of symbiotic algae, induces changes in lipid and fatty acid composition (Yamashiro *et al.*, 2005), with lipid levels declining in thermally sensitive species (Bachok *et al.*, 2006) as a result of a loss of translocated photosynthetic products from the symbiotic algae caused by photosystem damage and

subsequent photoinactivation of the algae at high temperatures (Hoadley *et al.*, 2015). To account for this loss of translocated carbon and to support elevated metabolic rates under temperature stress, the host must utilise its catabolic pathways, resulting in a decline in energy reserves (Hoadley *et al.*, 2015; Hillyer *et al.*, 2016).

It is well established that nutritional exchange occurs between sponges and their associated photosynthetic symbionts, including algae and cyanobacteria (Wilkinson, 1979; Wilkinson, 1980; Roberts *et al.*, 2006; Taylor *et al.*, 2007; Weisz *et al.*, 2007; Erwin & Thacker, 2008; Freeman & Thacker, 2011; Freeman *et al.*, 2013). However, only indirect evidence exists to support the incorporation of translocated carbon into sponge lipid, with reduced accumulation of lipid and other metabolites in the host tissues when the phototrophic sponge *Chondrilla nucula* was shaded (Arillo *et al.*, 1993). Interestingly, while metabolic collapse occurs in light deprived *C. nucula*, lipid content of the sponge *Petriosia ficiformis* was not correlated to light, suggesting that this species can activate heterotrophic metabolism when required (Arillo *et al.*, 1993). Such metabolic flexibility may be used by phototrophic sponges to survive bleaching. Using evidence from coral symbiosis in which lipid content is directly related to phototrophy, I hypothesise that changes in phototrophy due to OW and OA will be reflected in the lipid content of the sponge holobiont. Furthermore, while the link between energy stores and carbon acquisition is even less clear for sponges containing heterotrophic bacteria, it is likely that significant changes to sponge nutrition as a result of the OW and OA treatments will be reflected in the lipid content.

This chapter explored the primary differences in lipid content between sponge species to determine whether particular compositions reflect sponge tolerance to OW and OA. I hypothesised that lipid composition would differ between different nutritional modes, and also proposed that lipid composition, particularly membrane lipids, would differ with species sensitivities, due to the important role lipids play in organism stress tolerance. Lipid content and composition following exposure to OW and OA was then explored between the four species. An understanding of how the sponge holobionts alter and consume lipid reserves under such conditions would not only provide insight into the impact of climate change on sponges, but would also help to elucidate mechanisms underlying different species tolerances. Due to the plausible link between cyanobacteria photosynthetic rates and holobiont lipid responses, I hoped to further understand the ways in which pCO_2 provides protection from thermal stress in phototrophic species. Finally, I used differential responses in lipid class composition

between the two phototrophic sponges to OW and OA and explored the possibility of heterotrophic metabolic activation during sponge bleaching.

3.2 Materials and Methods

The following analyses were carried out on four Great Barrier Reef (GBR) sponge species - the phototrophic *Carteriospongia foliascens* and *Cymbastela coralliophila*, and the heterotrophic *Stylissa flabelliformis* and *Rhopaloeides odorabile*, experimentally exposed to nine combined temperature (OW; 28.5, 30 and 31.5ºC) and pH (OA; pH 8.1, 7.8, 7.6) treatments as described in chapter two.

3.2.1 P:R (phototrophic species only)

Respiration and gross photosynthetic rates measured for the two phototrophic species in chapter two were used here to determine gross photosynthesis/respiration (P:R ratios). P:R is a measure of whether symbiont productivity can compensate for holobiont respiration and is used to assess the potential energetic contribution of photosymbionts to the sponge host (Freeman *et al.*, 2013, Wilkinson, 1987a). P:R therefore enables changes in sponge lipid composition to be attributed to changes in phototrophy.

3.2.2 Tissue sampling

Six sponges of each phototrophic species and nine sponges of each heterotrophic species were sacrificed at T0 for the initial analysis of tissue samples. Tissue was then taken from experimental sponges at the final sampling point (n = 4 to 6 individuals *per* species *per* treatment, except where mortalities occurred for: *C. foliascens* where n = 2 at 31.5ºC/pH 8.1 and 31.5ºC /pH 7.8; and *R. odorabile* where n = 2 at 31.5ºC /pH 7.6). The final sampling point varied for each species, due to differing sensitivities to treatment conditions (T = 2 weeks for *C. foliascens* and *R. odorabile*; T = 8 weeks for *S. flabelliformis*; T = 12 weeks for *C. coralliophila*). Sponge tissue was cryopreserved in liquid nitrogen, in 1.5 ml vials for subsequent pigment, total lipid and lipid class analysis.

3.2.3 Chlorophyll a *determination (phototrophic species only)*

Chlorophyll *a* (Chl *a*) concentrations were determined for the two phototrophic species as a proxy for phototrophic symbiont presence (Wilkinson, 1983a), following the methods of (Pineda *et al.*, 2016). Vials (1.5 ml) of cryopreserved sponge tissue were thawed, cut into ~1 mm pieces and then weighed (~50 mg samples). Three stainless steel beads and 1 ml 95% ethanol were added to each vial and shaken in a Bead Beater (Bio Spec Products Inc., Bartlesville, USA) for 3 min. Vials were centrifuged at 10,000 x g for 5 min, and 700 μl of the

pigment extract was recovered from each tube. Another 1 ml 95% ethanol was added and the process was repeated, with a further 700 μl of pigment extract recovered following the second round. Triplicate 300 μl extracts of each sample, and a 95% ethanol blank, were then pipetted into a 96-well microplate. Absorbance at 470, 632, 649, 665, 696 and 750 nm (i.e. turbidity) was read on a Power Wave Microplate Scanning Spectrophotometer (BIO-TEKw Instruments Inc., Vermont, USA). Using the blank corrected absorbance readings minus the absorbance at wavelength 750 nm, Chl *a* concentration was calculated using the calculation below from Pineda *et al*. (2016), which uses a path length correction factor of 0.794. Pineda *et al*. (2016) determined this as the ratio of the absorbance of the microplate measurement divided by the absorbance of a 1 cm cuvette for a given wavelength, using 95% ethanol and 300 μl of sample.

$$\text{Chl } a \ (\mu g/ml^{-1}) = [(-0.9394 \times E_{632}) + (-4.2774 \times E_{649}) + (13.3914 \times E_{665})]/0.794$$

Chl *a* concentrations were then normalised to sponge wet weight as: Chl *a* (mg ml^{-1}) × extraction volume (ml)/ wet weight (g).

3.2.4 Total lipid

Lipids were extracted according to the method described by Folch *et al*. (1957), following modifications by Conlan *et al.* (2014). Cryopreserved sponge tissue was dried for 48 hr in a freeze dryer to remove all moisture, crushed using a mortar and pestle, and weighed (20 to 600 mg *per* sample). Samples were sonicated (Vibracell, Sonics and Materials, Newtown, USA) in 3 ml of dichloromethane: methanol (DCM:MeOH) for 5 min. The liquid portion of the sample was filtered through solvent-extracted sterile cotton, and the remaining solid sample was resuspended in 3 ml DCM:MeOH, sonicated for another 5 min, and re-filtered. This process was repeated three times, resulting in ~ 9 ml of filtrate, to which 4.5 ml of a potassium chloride sample wash [KCl (0.44%) in H_2O (3)/ CH3OH (1)] was added. The mixture was shaken and left overnight for lipids to settle. The following morning, the bottom layer containing the extracted lipid was recovered and the solvent was evaporated under nitrogen. Total lipid content was quantified on a five decimal balance and standardised to dry weight and expressed as a percentage of freeze dried sponge dry weight. Once lipid content was determined, the lipid fraction was resuspended in 1 ml DCM:MeOH for subsequent lipid class analysis (Conlan *et al.*, 2014).

3.2.5 Lipid class analysis

Lipid class analysis followed the method described by Nichols *et al*. (2001), with modifications by Conlan *et al.*, (2014). A 100 μl aliquot of the total lipid fraction was taken and analysed for lipid class composition using thin layer chromatography and flame ionisation detection (Iatroscan MK 6s, Mitsubishi Chemical Medience, Tokyo Japan). Samples were spotted in duplicate onto silica gel S4-chromarods (5 μm particle size) and developed in a glass tank containing pre-extracted filter paper. Lipid separation followed a two-step elution sequence: 1) the elution of the phospholipids (PL), phosphatidylcholine (PC), phosphatidylserine (PSE), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) in a dichloromethane/methanol/water (50:20:2, by volume) solvent system run to half height for 15 min; and 2) after air drying, the elution of the acetone mobile polar lipids (AMPL), sterol (ST), sterol ester (SE), triacylglycerol (TG), free fatty acid (FFA), and 1,3-diacylglycerol (DG) in a hexane/diethyl ether/formic acid (60:15:1.5, by volume) solvent system run to full height for 30 min. Rods were then placed in an oven at 100°C for 10 min prior to analysis. The Iatroscan MK 6s was calibrated using known compound classes in the range of 0.1–10 μg (Sigma-Aldrich, Inc., St. Louis, MO, USA and from Nu-Chek Prep Inc., Elysian, MN, USA) and peaks were quantified using PowerChrom version 2.6.15 (eDAQ Pty Ltd.). The contribution of each lipid class was standardized to mg lipid and expressed as a proportion of total lipid. Lipid classes were grouped as 'structural' and 'storage', depending on their primary functional roles. PL (PC, PSE, PI, PE), AMPL and ST were combined as the structural lipid component, and SE, TG, FFA and DG were combined as the storage lipids. A ratio of structural to storage lipid (structural: storage) was then calculated to determine how OW and OA affect sponge lipid content. The effects of OW and OA on individual lipid classes were also explored.

3.2.6 Data analysis

Principal components analysis (PCA) and cluster analysis was performed by PRIMER-E (PRIMER version 6.0, Plymouth Marine Laboratory, Plymouth, UK) using standardised data to summarise lipid composition across the four study species. To test the effect of OW and OA on pigment content, P:R, total lipid content, lipid ratio and lipid class composition for each species, a two-way analysis of variance (2-way ANOVA) was employed, with temperature and pH as fixed factors. Where significant treatment effects were revealed, *post hoc* pairwise comparisons (with the holm correction applied) were used to determine which treatments differed significantly. Equal variance and normal distribution assumptions were evaluated *via* analysis of the residuals. Data were log-transformed (log+1) as necessary to meet these

assumptions. A 5% significance level was used for all tests. ANOVA were performed by R (R: A Language and Environment for Statistical Computing, R Core Team, R Foundation for Statistical Computing, Vienna, Austria) and all graphs were generated using GraphPad Prism (GraphPad Software, version 6.00 for Windows, La Jolla California USA).

3.3 Results

3.3.1 Chl a and P:R - phototrophic energy contribution

Chl *a* concentrations in the two phototrophic sponges declined with increasing temperature (Fig 3.1 a-b; Table A3.1). *C. foliascens* Chl *a* concentrations were significantly lower at 31.5°C compared to 28.5°C (P = 0.011) and *C. coralliophila* Chl *a* concentrations declined significantly at both 30°C (P = 0.004) and 31.5°C (P < 0.001). *C. foliascens* P:R declined significantly as temperature increased, with the greatest decline at 31.5°C / pH 8.1 (Fig 3.1 c; Table A3.1). The average P:R in this treatment declined to 0.5, indicating that host energy demands were no longer supported by photosynthetically-acquired carbon. *Post hoc* analysis revealed that, at T-end, P:R at 31.5°C was significantly lower than P:R at 28.5°C (pH 8.1, P < 0.001; pH 7.6, P = 0.004) and 30°C (pH 8.1, P < 0.001; pH 7.6, P = 0.004). The only exception in which temperature had no significant effect was at pH 7.8, where P:R remained above 1.5. *C. coralliophila* P:R declined below 1.5 in all treatments at 31.5°C, with the greatest declines at 31.5ºC/ pH 8.1 and pH 7.8 (Fig 3.1d; Table A3.1). *Post hoc* analysis revealed that, at T-end, the average *C. coralliophila* P:R ratio was significantly lower at 31.5°C than at 28.5°C (P < 0.001) and 30°C (P < 0.001). There was also a significant effect of pH on *C. coralliophila* P:R (Fig 3.1d; Table A3.1), with P:R elevated in the low pH treatments (Fig. 3.1d), however *post hoc* analysis detected no significant differences between treatments.

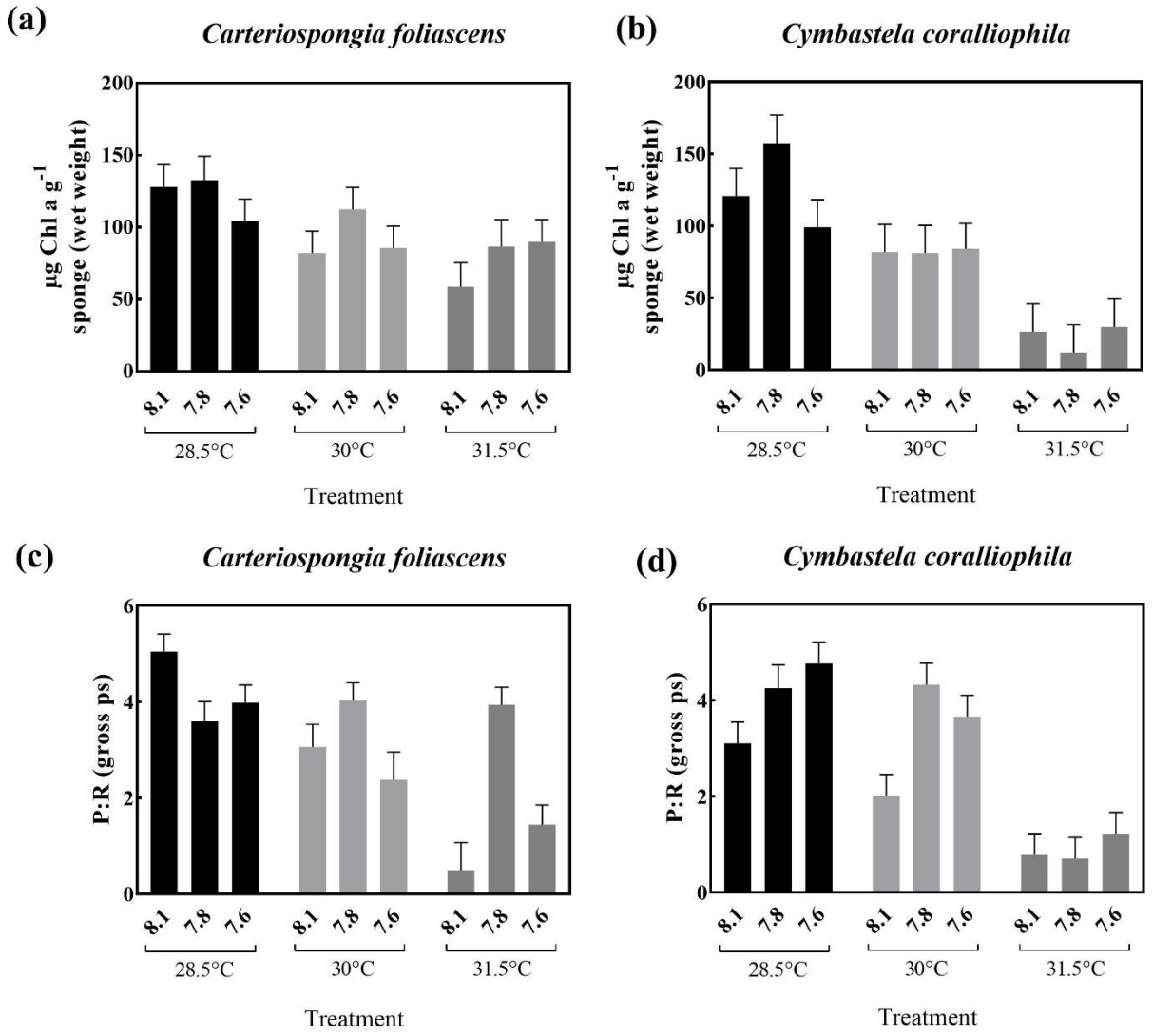


Fig. 3.1 Chlorophyll *a* concentration for (a) *Carteriospongia foliascens* and (b) *Cymbastela coralliophila*, and P:R ratios for (c) *C. foliascens* and (d) *C. coralliophila* in each treatment. Values are mean µg chlorophyll a *per* gram of sponge wet weight (n = 4 to 6 for *C. foliascens*, and n = 6 for *C. coralliophila*) and mean P:R ratio (n = 6 *per* treatment – except where mortalities occurred) ± SE for each treatment.

3.3.2 Species lipid composition

Total lipids accounted for approximately 5% of dried *C. foliascens* and 5.5% of dried *C. coralliophila* weight at T0 (Fig. 3.2). The contribution of total lipid was at least double this for the heterotrophic species, with lipid accounting for 13.8% of dried *R. odorabile* and 10.2% of dried *S. flabelliformis* at T0 (Fig. 3.2). Structural lipid accounted for 82-93% of the total lipid (Fig. 3.2); the main structural lipid grouping across all four species was AMPL, followed by sterol and PI and PC. Storage lipid accounted for only 7-18% of the total lipid content, with SE as the main storage lipid (Fig. 3.2).

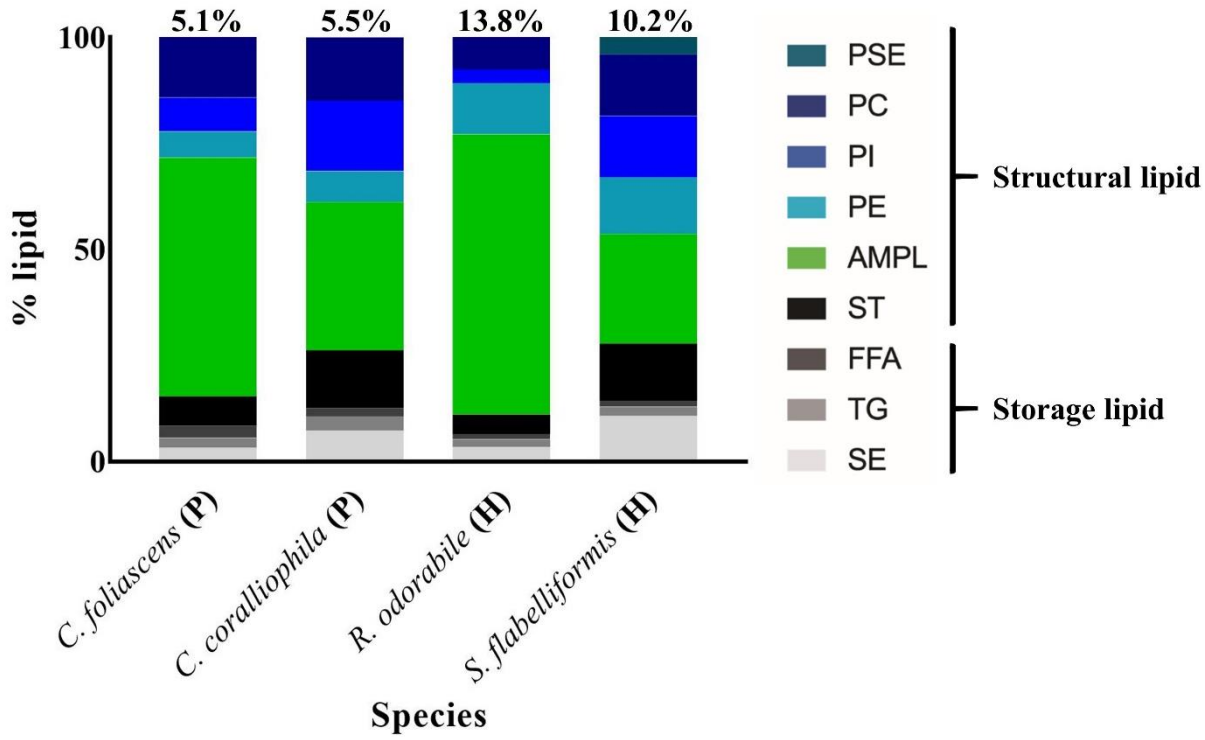


Fig. 3.2 Time zero (T0) lipid class composition presented as the % contribution of each class to the total lipid content for each species (y-axis = % lipid and x-axis = species). Above each bar is the % contribution of lipid to dried sponge for each species (note: P = phototrophic species and H = heterotrophic species). Lipid classes are coded as follows: **i)** structural lipid: PSE = Phosphatidylserine, PC = Phosphatidylcholine, PI = Phosphatidylinositol, PE = Phosphatidylethanolamine, AMPL = Acetone Mobile Polar Lipid, ST = Sterol; and **ii)** storage lipid: FFA = Free Fatty Acids, TG = Triacylglycerol, SE = Sterol Ester (note 1,3-diacylglycerol (DG) was only detectable in trace amounts in *C. coralliophila* at T0). n = 5 for *C. foliascens*, n= 6 for *C. coralliophila*, and n = 9 for *R. odorabile* and *S. flabelliformis*.

Principal components analysis (PCA) revealed a high degree of difference in lipid composition across the four species (PC1, 78.4%; Fig. 3.3). *C. coralliophila* and *S. flabelliformis* lipid composition is distinguished by a high proportion of sterol (ST) and phospholipid (PL), as well as a higher overall storage lipid contribution, primarily in the form of sterol ester (SE), compared to *C. foliascens* and *R. odorabile*. These more thermally tolerant species (chapter two) also have low average ratios of structural to storage lipid, with ratios being 7 and 5.2 for *C. coralliophila* and *S. flabelliformis*, respectively. The lipid composition of the two more sensitive species, *C. foliascens* and *R. odorabile*, is primarily characterised by a high proportion of Acetone Mobile Polar Lipid (AMPL) and high ratios of structural to storage lipid (12.3 and 21 for *C. foliascens* and *R. odorabile*, respectively).

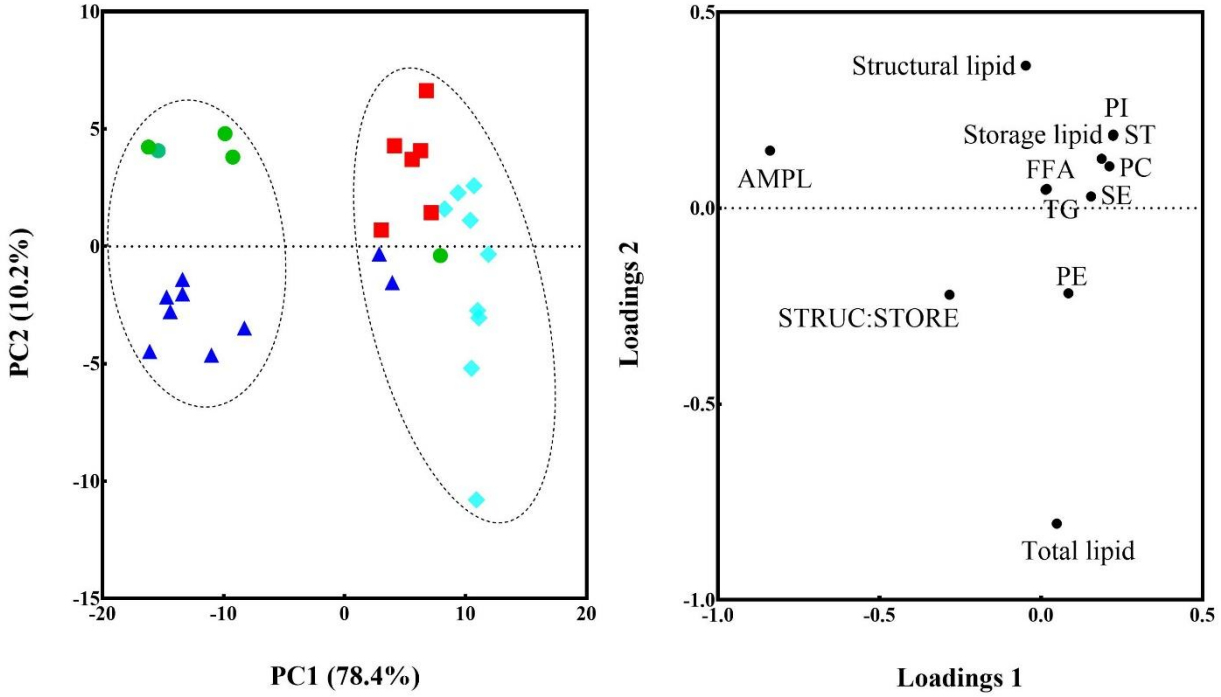


Fig. 3.3 Principal component analysis (PCA) with clusteres based on similarity scores of 20 (left) and loadings plot (right), showing the lipid composition of four GBR sponge species prior to exposure to experimental treatments. Each point on the PCA represents an individual sponge where ● = *Carteriospongia foliascens* (P); ■ = *Cymbastela coralliophila* (P); ▲ = *Rhopaloeides odorabile* (H); ♦ = *Stylissa flabelliformis* (H).

3.3.3 Treatment effects

3.3.3.1 Lipid content

A significant interaction was found between temperature and pH on total percent lipid content of *C. foliascens* (Fig. 3.4a; Table A3.2). Total lipid was higher at 28.5°C/pH 7.6 than at 28.5°C/pH 8.1 ($P = 0.02$; Fig. 3.4a). The percentage lipid content of *C. foliascens* also increased

with temperature at ambient pH. *C. foliascens* at 28.5°C/pH 8.1 had a significantly lower average lipid content than at 30°C/ pH 8.1 (P = 0.0084; Fig. 3.4a) and 31.5°C/pH 8.1 (P = 0.0039; Fig. 3.4a). There was no effect of temperature or pH on the total percent lipid content of *C. coralliophila*, *R. odorabile*, or *S. flabelliformis* (Fig. 3.4b-d; Table A3.2).

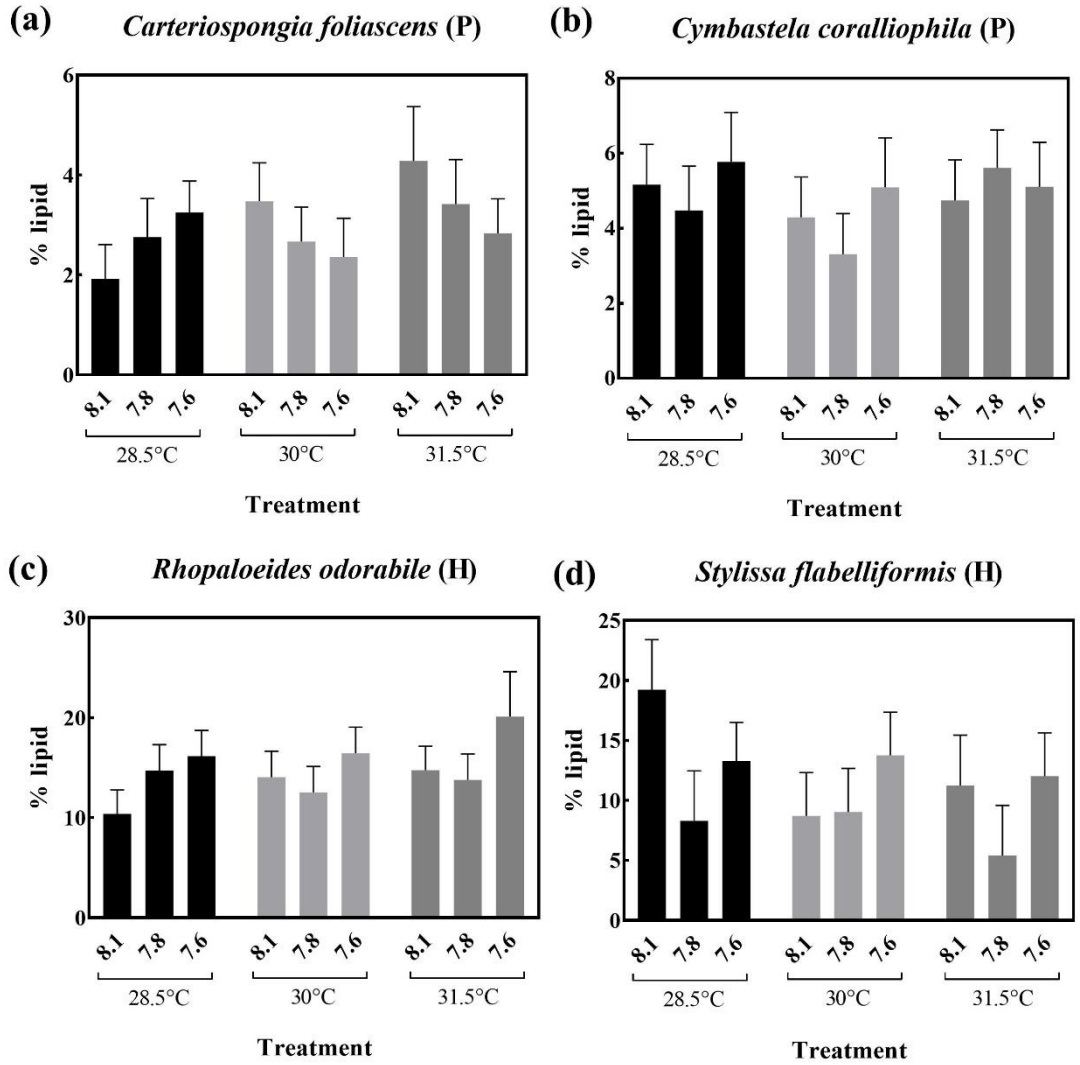


Fig. 3.4 Total % contribution of lipid to dried sponge for (a) *Carteriospongia foliascens* (b) *Cymbastela coralliophila* (c) *Rhopaloeides odorabile* (d) *Stylissa flabelliformis* in each treatment (note: P = phototrophic species and H = heterotrophic species). Values are mean % lipid in dry sponge *per* species for each treatment (y-axis = % lipid and x-axis = treatment) ± SE (n = 4 to 6 individuals *per* species *per* treatment, except where mortalities occurred for: *C. foliascens* where n = 2 at 31.5ºC/pH 8.1 and 31.5 ºC /pH 7.8; and *R. odorabile* where n = 2 at 31.5/pH 7.6). Note: y-xis scales differ between species.

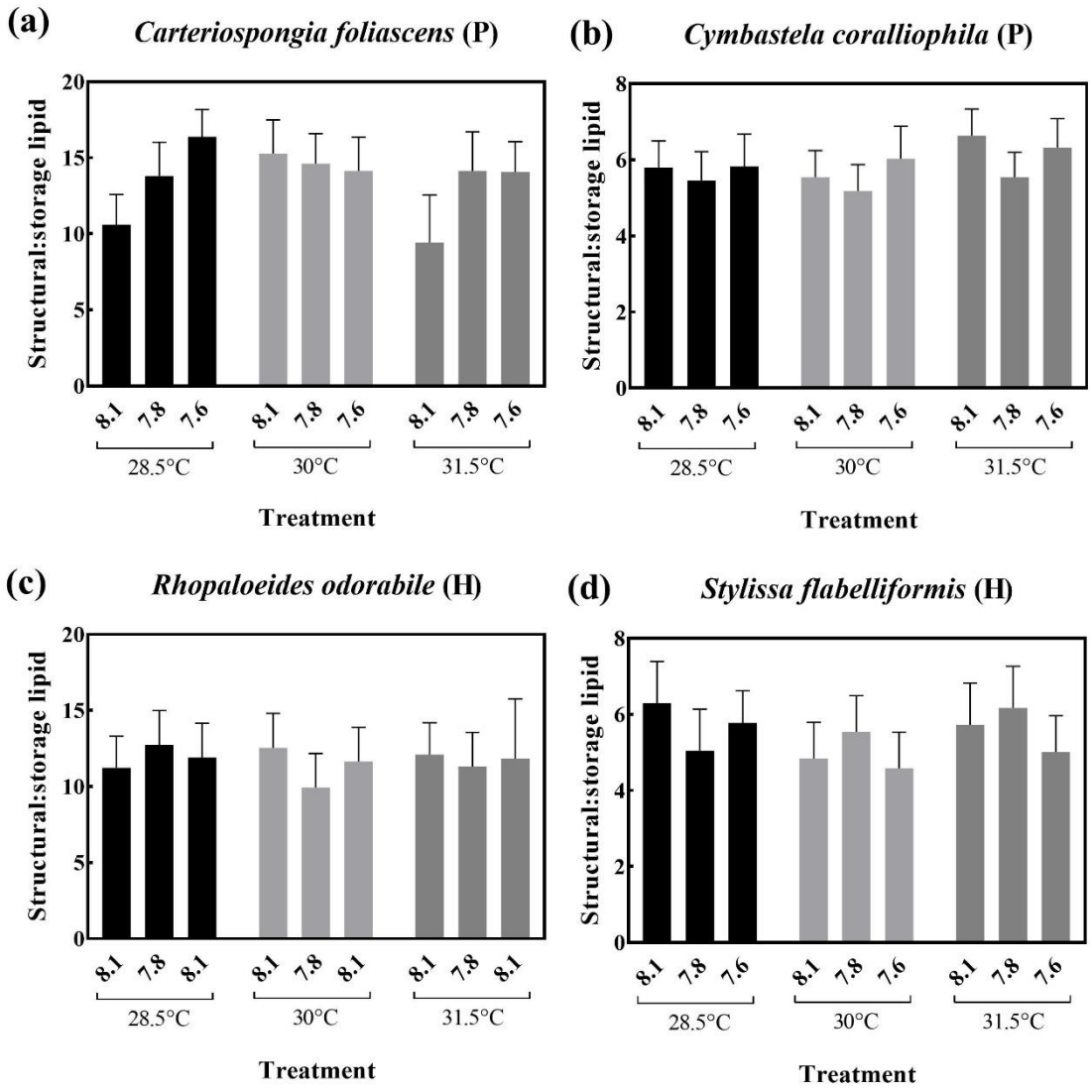


Fig. 3.5 Ratio of structural lipid to storage lipid for (a) *Carteriospongia foliascens* (b) *Cymbastela coralliophila* (c) *Rhopaloeides odorabile* (d) *Stylissa flabelliformis* in each treatment (note: P = phototrophic species and H = heterotrophic species). Values are mean ratio of structural lipid to storage lipid sponge *per* species for each treatment (y-axis = % lipid and x-axis = treatment) ± SE (n = 4 to 6 individuals *per* species *per* treatment, except where mortalities occurred for: *C. foliascens* where n = 2 at 31.5ºC/pH 8.1 and 31.5 ºC /pH 7.8; and *R. odorabile* where n = 2 at 31.5/pH 7.6). Note: y-xis scales differ between species.

3.3.3.2 Lipid class composition

The overall contribution of storage and structural lipids to total lipid composition did not change significantly as a result of any of the treatments in any of the four species (Table 3.2; Fig 3.5a-d), however significant shifts in the specific lipid class composition were observed (Fig. 3.6a-d; Table A3.3). There was a significant interactive effect of temperature x pH on the

contribution of the structural lipid sterol (ST) to the total lipid content of *C. foliascens* (Fig. 3.6a; Table A3.3). The contribution of ST increased with declining pH in the high temperature treatments. At 30°C, the proportion of ST was higher at pH 7.6 (3.6%) than at pH 8.1 (2.3%, $P = 0.045$), and at 31.5°C, the proportion of ST was higher at pH 7.6 (4.4%) than at pH 7.8 (3.5%; $P = 0.014$) and pH 8.1 (3.5%; $P = 0.034$). At pH 7.6, the proportion of ST at 31.5°C was also significantly higher than 28.5°C (3%; $P = 0.0041$). There was a significant temperature effect on the contribution of the storage lipid sterol ester (SE) to the total lipid content of *C. coralliophila* (Fig. 3.6b; Table A3.3). The proportion of SE was significantly higher at 31.5°C (9.7%) than at 28.5°C (8.5%; $P = 0.0043$). A significant effect of temperature was found for the contribution of the phospholipid phosphatidylcholine (PC) to the total percent lipid content of *Stylissa flabelliformis* (Fig. 3.6d; Table A3.3). The proportion of PC to the total lipid content of *S. flabelliformis* decreased with increasing temperature, with significantly higher levels in sponges exposed to 28.5°C (14.3%) than 30°C (11.8%, $P = 0.015$) and 31.5°C (12.7%, $P = 0.0268$).

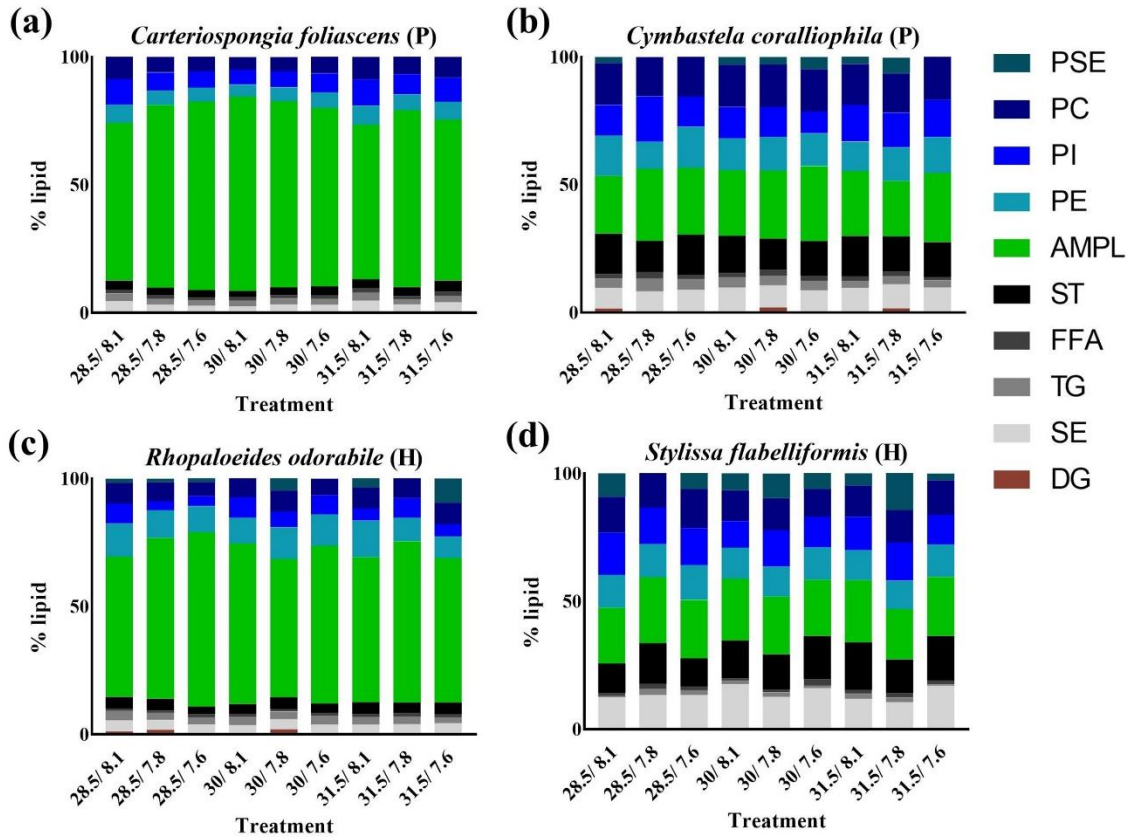


Fig. 3.6 Lipid class composition for: (a) *Carteriospongia foliascens* (b) *Cymbastela coralliophila* (c) *Rhopaloeides odorabile* (d) *Stylissa flabelliformis* in each treatment (note: P = phototrophic species and H = heterotrophic species). Values are mean % contribution of each lipid class to the total lipid content of each species in each treatment (y-axis = % lipid and x-axis = treatment) ± SE (n = 4 to 6 individuals *per* species *per* treatment, except where mortalities occurred for*: C. foliascens* where n = 2 at 31.5ºC/pH 8.1 and 31.5 ºC /pH 7.8; and *R. odorabile* where n = 2 at 31.5/pH 7.6). PSE = Phosphatidylserine, PC = Phosphatidylcholine, PI = Phosphatidylinositol, PE = Phosphatidylethanolamine, AMPL = Acetone Mobile Polar Lipid, ST = Sterol, FFA = Free Fatty Acids, TG = Triacylglycerol, SE = Sterol Ester, DG = 1,3-diacylglycerol (detectable in trace amounts in a few *C. coralliophila* and *R. odorabile* samples). n = 5 for *C. foliascens*, n= 6 *C. coralliophila*, and n = 9 for *R. odorabile* and *S. flabelliformis*.

3.4 Discussion

The lipid composition of four abundant Great Barrier Reef (GBR) sponge species exposed to ocean warming (OW) and ocean acidification (OA) levels predicted for 2100 under two IPCC RCP $CO_{2\ atm}$ emission scenarios was determined. This study found that, while total lipid content is not related to sponge tolerance to OW and OA, sponges with a greater contribution of storage lipids, and a higher proportion of phospholipids and sterols, exhibit greater resistance to these stressors. Such intrinsic cellular protection is especially important to sessile organisms such as sponges, for which potential behavioural responses to stressors are limited. Exposure to OW and OA did not reduce sponge lipid reserves, suggesting that for these species other energy reserves i.e. protein and carbohydrate may be drawn upon under stress. However, shifts in specific lipid classes under OW and OA occurred for all species, except *R. odorabile* for which mortalities may have occurred too rapidly for changes in lipid composition. As classes of lipid have known functions in cells (Lawson *et al.*, 1988), different compositions and changes in composition in response to stressors provide insight to a sponge's physiological state. For example, a reduction in phospholipid content reflects an overall stressed state for *S. flabelliformis* under OW. Moreover, additional energy available under OA due to the stimulatory effect of CO_2 may facilitate the resistance of the phototrophic *C. foliascens* to OW through increased sterol biosynthesis. Finally, despite bleaching for up to three months, there was no decline in *C. coralliophila* lipid levels. The increased sterol ester content may indicate an ability of *C. coralliophila* to receive energy from sources other than phototrophically-acquired carbon. Overall, certain characteristics of lipid class composition can be used as criteria to determine sponge sensitivities to climate change. Furthermore, although total lipid is conserved under OW and OA across all four species, specific shifts in lipid classes reveal essential information on sponge responses, including tolerance and adaptive mechanisms, providing a greater understanding of how OW and OA affect sponges.

3.4.1 Lipid as an indicator of tolerance

Lipids play a vital role in stress tolerance for a number of organisms (Singh *et al.*, 2002); for example, coral species with a high proportion of total lipid are likely to tolerate stressful conditions better than their low lipid counterparts (Rodrigues & Grottoli, 2007; Anthony *et al.*, 2009; Strahl *et al.*, 2015a). However, for the sponge species in this study, total lipid content was unrelated to species-specific tolerance to OW and OA. Of note is that total lipid content differed significantly between the two nutritional sponge types irrespective of treatment, with

the percentage contribution of total lipid to dry sponge weight being much lower in the two phototrophic species than for the heterotrophic sponges. It is possible that these phototrophic species have a reduced need for lipid storage given that they have a constant supply of organic carbon *via* photosynthesis. However, it is also possible that phototrophic sponges simply have a higher metabolic demand, and therefore higher lipid consumption than the heterotrophic sponges due to the increased availability of metabolic substrate from photosynthesis (Cheshire *et al.*, 1997). Metabolic rates of the phototrophic sponge *Phyllospongia lamellosa* over 24 hr were significantly higher than those measured for the heterotrophic *Ianthella basta* (Cheshire *et al.*, 1997), and while dark respiration rates measured in chapter two were significantly lower for the two phototrophic species, compared with the heterotrophic sponges, respiration rates are likely to be much higher in the light (Kühl *et al.*, 1995). Unfortunately, the accurate quantification of light respiration is difficult without the use of microsensors, due to the intimate association between autotrophic and heterotrophic processes (Kühl *et al.*, 1995).

In contrast to total lipids, specific lipid class compositions differed significantly between sponge species with different tolerances to OW and OA. The more tolerant spiculated species - *S. flabelliformis* and *C. coralliophila* - both had lower ratios of structural to storage lipids. The higher storage lipid content likely provides energy during periods of adversity and may explain the ability of these species to survive under OW and OA for longer (Anthony *et al.*, 2009). Furthermore, the high proportion of storage lipid in both species was due to a greater contribution of the storage lipid, sterol ester (SE). SE is not only important with respect to long-term energy reserves (Kattner & Hagen, 2009), but it forms components of cell membranes (Nevenzel, 1970; Parrish, 1988) and may serve as a fatty acid carrier in the biosynthesis of structural lipids (Marsden, 1975), and hence is likely to facilitate resistance to environmental stress. While the less OW-tolerant species had a greater proportion of structural lipid, this was predominantly in the form of acetone mobile polar lipids (AMPL). This group of lipids contains pigments, glycolipids and monoacylglycerols (Siegenthaler & Murata, 2006; Parrish, 2013), which are found in abundance in bacterial lipids (Shaw, 1974), and likely reflects the higher microbial content of these sponge species (Webster & Hill, 2001; Luter *et al.*, 2015). In contrast, the structural lipid composition of the two more tolerant species had a much higher proportion of phospholipids and sterols, the main components of the cell membrane lipid bilayers. These lipids are fundamental for cell support and protection, and to maintain appropriate membrane fluidity under stressful conditions (Siegenthaler & Murata, 2006; Paulucci *et al.*, 2011). The predominance of phospholipids (PL) in bacteria, for example, may

facilitate their adaptation to the surrounding environment, including resistance to antibiotics (Lawson *et al.*, 1988). Therefore, this high proportion of membrane lipid provides a key indicator of environmental stress tolerance in sponges (Tchernov *et al.*, 2004).

3.4.2 Sponge lipids under OW and OA

The total lipid content in *C. foliascens* increased with declining pH at 28.5°C. An increase in total lipids under elevated CO_2 as a result of increased photosynthesis has been observed for a number of microalgal species (Chrismadha & Borowitzka, 1994; Carvalho & Malcata, 2005; Leu *et al.*, 2013), with such an increase also explaining an increase in lipid concentration for a number of coral species under OA as excess carbon is stored as lipid (Schoepf *et al.*, 2013; Hoadley *et al.*, 2015). *C. foliascens* obtains up to 80% of its daily energy requirements from carbon fixed by associated photosynthetic cyanobacteria (Wilkinson, 1987b), and so an increase in total lipid may be the result of increased productivity of sponge-associated cyanobacteria under elevated pCO_2 conditions in the low pH treatments, providing additional photosynthate to the sponge host. While Chl *a* concentrations did not differ between pH treatments at 28.5°C, there was a non-significant increase in the average contribution of AMPL with declining pH at 28.5°C (increased from 62% to 74% of total lipid). As cyanobacteria contain a high proportion of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and chlorophyll (Sato & Murata, 1988; Wada & Murata, 1998), which make up the AMPL (Gordillo *et al.*, 2001), it is likely that this increase in total lipid reflects increased translocation of photosynthetically-fixed carbon to the sponge host, or an increase in lipid content of host associated-photosynthetic cyanobacteria. Moreover, it appears that *C. foliascens* utilises the additional energy available under OA to increase sterol biosynthesis (or to spare it from catabolism) with elevated temperature. Sterols are essential nutrients in marine bivalves and crustaceans (Martin-Creuzburg *et al.*, 2005; Martin-Creuzburg & Elert, 2009; Parrish, 2013) and as with sponges, bivalves contain a number of different sterols (Copeman & Parrish, 2004). It has been proposed that bivalve species living in variable environments, such as surface waters, utilise their wide array of sterols to alter the sterol content of their cell membranes in response to seasonally varying temperatures (Parrish, 2013). Alterations to the sterol content of phospholipid bilayers represent a major method by which eukaryotes control membrane fluidity and permeability (Martin-Creuzburg & Elert, 2009), and may represent a form of homeoviscous adaptation i.e. changes in the composition of membrane lipids to correct membrane fluidity, in thermally-stressed *C. foliascens* (Nes, 1974; Volkman, 2003; Martin-Creuzburg & Elert, 2009).

Surprisingly, total lipids in *C. foliascens* increased with temperature at ambient pH. *C. foliascens* exposed to 31.5°C, especially in the ambient pH treatment, was stressed as evidenced by high levels of necrosis and bleaching, elevated rates of respiration, photosynthesis and mortality (chapter two) and lower Chl *a* concentration and P:R than individuals in other treatments. While increased symbiont productivity under elevated pCO_2 explains the increase in total lipid levels at 28.5°C, an increase in total lipid in sponges exposed to what is obviously a stressful environment is perplexing. At 28.5ºC, the increasing lipid content appears to be in the form of AMPL, suggesting that photosynthetic processes are the site of impact. However, as lipid content increases with increasing temperature, the contribution of AMPL reduces, suggesting that the mechanism underpinning this increasing lipid content is different. Moreover, the only treatment in which the ratio of structural to storage lipid declined was at 31.5ºC/ pH 8.1. While this was not statistically significant, changes in this ratio are a key indicator of stress in a number of organisms (Guckert *et al.*, 1992; Imbs & Yakovleva, 2012), suggesting that a change in total lipid with elevated temperature reflects a stressful state, rather than one in which lipid production is optimal. For example, declines in the ratio of structural to storage lipids, mainly due a reduction in phospholipids and a subsequent increase in triglycerides, were observed in the Redbreast sunfish *Lepomis auritus* in response to contaminant stress (Adams *et al.*, 1990).

While the exact mechanism behind this increased lipid content in apparently stressed *C. foliascens* remains unclear, a few possible explanations exist. Lipid production rates for some cyanobacteria, including *Synechococcus* which is the dominant cyanobacterium associated with *C. foliascens* (Luter *et al*., 2015), are optimal between 30°C to 33°C (Sheng *et al.*, 2011). However as lipid biosynthesis and photosynthesis are likely to be intricately linked (Arillo *et al.*, 1993), and as photosynthetic rates and P:R declined with temperature, it is possible that suppressed growth processes under stress allowed for the intracellular accumulation of lipid stores, as demonstrated for a number of microalgal species (Zhu *et al.*, 1997; Gordillo *et al.*, 1998; Renaud *et al.*, 2002; Solovchenko, 2012). An alternative explanation is that the sponge host is able to increase its lipid content to compensate for the reduction in translocated carbon from symbionts at these stressful temperatures, potentially *via* heterotrophic feeding, as demonstrated for some corals in response to bleaching (Grottoli *et al.*, 2006; Hoadley *et al.*, 2015). However, *C. foliascens* mortalities occurred very rapidly (days) following bleaching at 31.5°C, demonstrating that for this species, survival without its phototrophic symbionts at such temperatures is not possible, even if heterotrophic carbon acquisition is elevated.

In contrast to *C. foliascens*, no change in sponge biomass, tissue necrosis or survival (chapter two) was evident in *C. coralliophila*, despite up to three months of near-complete bleaching at 31.5°C, as evidenced by reduced photosynthesis (chapter two); Chl *a* concentrations; and P:R ratios. This tolerance is further supported here as total lipid content did not change in bleached sponges, while P:R reduced below 1, confirming that the host is no longer receiving sufficient carbon transfer from photosynthesis to meet cellular respiration demands. Therefore, *C. coralliophila* likely has the potential to obtain carbon heterotrophically from the water column, to compensate for reduced photosynthate translocation (Grottoli *et al.*, 2004; Grottoli *et al.*, 2006; Hoadley *et al.*, 2015). Increased oxygen consumption rates at 31.5°C may reflect enhanced pumping and filtration activity, as heterotrophic feeding mechanisms are activated. An increase in the proportion of sterol ester at 31.5°C provides further support for this hypothesis, as it demonstrates that bleached individuals of *C. coralliophila* are able to meet their energy demands from a source other than translocated photosynthates (Rodrigues *et al.*, 2008). Heterotrophic metabolic activation in the absence of cyanobacteria has been confirmed in the usually phototrophic sponge *Petrosia ficiformis* during shading experiments (Arillo *et al.*, 1993). However, it is important to note that, while lipid reserves represent an important source of storage energy (Singh *et al.*, 2002; Rodrigues *et al.*, 2008), it is possible that during this bleaching period the host *C. coralliophila* instead draws upon other energy reserves i.e. protein and carbohydrate stores (Harriott, 1993; Lesser, 2013).

No effect of temperature or pH on total percent lipid content was evident for either heterotrophic species: *R. odorabile* and *S. flabelliformis*. Furthermore, despite high mortality at 31.5ºC/pH 7.6, and increased respiration and tissue necrosis at 31.5 ºC (chapter two), *R. odorabile* maintained a stable lipid class composition regardless of treatment. It is possible that *R. odorabile* mortality occurred so rapidly that changes in lipid composition were not evident, and therefore lipid is not a determining factor in the survival of this species under OW and OA. It is also possible that other energy reserves are utilised under stress to support physiological defence processes in this species, again reinforcing the importance of including both protein and carbohydrate in future biochemical analyses to gain a more complete understanding of the impact of environmental stressors on sponge energetics. For *S. flabelliformis*, however, despite no reduction in lipid content there was a decrease in the important phospholipid (PL), phosphatidylcholine (PC), with increasing temperature. A reduction in PC is observed in Rainbow Trout (*Salmo gairdneri*) when acclimating to increased water temperatures, and is suggested to be a form of homeoviscous adaptation in response to thermal stress (Hazel &

Carpenter, 1985). In corals, however, the preferential utilisation of structural lipid in the early stages of stress is suggestive of a lower capacity to cope with environmental pressure, rather than an adaptive mechanism (Imbs & Yakovleva, 2012). While a reduction in PC may be a mechanism for thermal acclimation in *S. flabelliformis*, minor changes in PL structure can lead to changes in membranes that have major consequences for cell biology (Genin *et al.*, 2008), and high levels of tissue necrosis in this sponge at elevated temperatures suggest the response is likely to reflect a stressed state for this species.

3.4.3 Summary

Specific sponge lipid compositions may facilitate resistance to environmental stress. In particular, sponges with a greater contribution of storage lipid, and a higher proportion of phospholipids and sterols, the main constituents of cell membrane lipid bilayers, demonstrate greater resistance to OW and OA. Such attributes are likely to contribute to the acclimatisation and adaptive potential of these species under climate change. Furthermore, shifts in specific lipid classes under OW and OA provide a greater mechanistic understanding of the effects of these stressors on sponges, and provide support for increased heterotrophy in response to photosynthetic dysfunction. While ipid analysis is a useful tool for assessing the impact of environmental stress on sponges, responses are species-specific and no universal shifts are evident from which common conclusions can be drawn. As a source of energy, lipid content in these species is low and variable (5 to 14% of dry weight). Lesser *et al*., (2013) highlighted in a review of coral energetics that, during stress, organisms are likely to draw on all biochemical pools for energy, therefore future work should also consider how sponge lipid, protein and carbohydrate stores are utilised under OW/ OA scenarios.

Chapter four

The biochemical response of sponges to elevated pCO_2 and temperature (Part 2): *A mechanistic understanding of sponge tolerance to OW and OA.*

Fatty acid extracts in gas chromatography vials, from four Great Barrier Reef sponge species.

4.1 Introduction

As the climate changes, ocean warming (OW) and ocean acidification (OA) pose a number of threats to marine organisms (Harley *et al.*, 2006; Hoegh-Guldberg & Bruno, 2010; Kroeker *et al.*, 2013b). Benthic marine invertebrates, such as sponges, are limited in their ability to directly escape unfavourable conditions (Przeslawski *et al.*, 2008) and must therefore survive and adapt *via* alternative mechanisms. The majority of climate change research on marine sponges has, to date, focused on the response of these sessile organisms to predicted changes, whereas the mechanisms underlying responses remain unclear. While the whole animal response reflects the ability of an organism to adapt to environmental change, parallel adjustments at the molecular or membrane level ultimately define tolerance limits (Pörtner, 2002). The lipid bilayer of cell membranes is sensitive to environmental stressors (Vígh *et al.*, 2007). Changes in environmental conditions can alter the fluidity and permeability of these membranes (Hazel, 1995), which are vital to maintain cell homeostasis, including the regulation of transmembrane activities such as nutrient transport and the maintenance of solute gradients for energetic processes (Hazel, 1995; Guillot *et al.*, 2000). Therefore, the ability of an organism to maintain appropriate membrane function in the face of environmental change plays a significant role in stress tolerance (Geider, 1987; Hazel, 1995; Singh *et al.*, 2002; Arts & Kohler, 2009), and may provide the potential for acclimatisation required for species survival in a high CO_2 world.

Ocean warming affects membrane permeability and fluidity (Guerzoni *et al.*, 2001). As temperature increases, membranes become hyperfluid, changing their physical properties and functionality, with subsequent negative implications for cellular functioning and organism survival (Hazel, 1995). In the previous chapter, it was reported that sponges with a higher storage lipid content, and a greater proportion of phospholipids and sterols, the main constituents of cell membrane lipid bilayers, have greater resistance to ocean warming (OW) and ocean acidification (OA). Fatty acids (FA) are the "building blocks" of lipids and play a number of fundamental biological and biochemical roles in cells (Iverson, 2009; Řezanka & Sigler, 2009). FA are not only vital for energy storage and metabolism, they are particularly important for environmental stress resistance due to the role that they play in maintaining the fluidity of cell membranes and controlling cellular responses to external stimuli, including changing temperatures (Bergé & Barnathan, 2005; Arts & Kohler, 2009; Parrish, 2013). Moreover, FA have specific biological functions and changes in the FA composition of an organism are likely to signal physiological changes at a level that might otherwise not be

observed with more coarse measurements of e.g. growth and mortality (Muradyan *et al.*, 2004). FA have also been used extensively as trophic biomarkers and changes in FA "signatures" may be used to explore variations in energy acquisition under different conditions. This is particularly valuable for exploring nutritional switching under conditions of OW and OA scenarios.

Most organisms are capable of biosynthesising the saturated fatty acids (SFA) 16:0 (palmitic acid), 14:0, 18:0 and 20:0 *de novo*, *via* the Type I FA synthetase pathway, from carbohydrates and proteins (Mueller-Navarra, 1995; Dalsgaard *et al.*, 2003; Bergé & Barnathan, 2005). SFAs are commonly used as sources of metabolic energy (Mueller-Navarra, 1995) and are precursors for the biosynthesis of longer chain and unsaturated FA (Bergé & Barnathan, 2005). Monounsaturated FA (MUFA) are then formed *via* aerobic desaturation, catalysed by the Δ9 desaturase enzyme, resulting in the introduction of a double bond in the carbon chain to form 16:1n-7 (palmitoleic acid), 18:1 n-9 (oleic acid) and 20:1 n-11. Polyunsaturated FA (PUFA) on the other hand are involved in a number of physiological processes and are mainly synthesised by microalgae as well as heterotrophic protists and bacteria; however higher trophic levels, including sponges, also have metabolic pathways that can produce PUFA (Dalsgaard *et al.*, 2003; Müller-Navarra, 2008; Desvilettes & Bec, 2009; Iverson, 2009; Monroig *et al.*, 2013; Koopmans *et al.*, 2015). The n-3 and n-6 PUFAs are biosynthesised from 18:1n-9 (oleic acid) by photosynthetic microalgae and bacteria, which are typically the only organisms with the enzymes Δ12 and Δ15 desaturase that are required to insert double bonds between existing double bonds (Wada & Murata, 1998; Dalsgaard *et al.*, 2003). The product of this biosynthesis are the PUFA 18:2n-6 (alpha linoleic acid) and 18:3n-3 (linoleic acid), referred to as essential fatty acids (EFA) due to both their necessity and the likely inability of most heterotrophic organisms, including sponges, to synthesise them *de novo* (Rod'kina, 2005; Parrish, 2009; Monroig *et al.*, 2013). These EFA are the precursors for the essential long chain n-3 and n-6 PUFA 20:4n-6 (Arachidonic; ARA); 20:5n-3 (Eicosapentaenoic; EPA) and 22:6n-3 (Docosahexaenoic; DHA) (Bergé & Barnathan, 2005), which are fundamental for membrane structure and function (Parrish, 2009).

The n-3 and n-6 PUFA play a central role in cell membrane structure and function (Dalsgaard *et al.*, 2003; Müller-Navarra, 2008; Arts & Kohler, 2009; Koopmans *et al.*, 2015), and are required for a number of fundamental biological processes including growth and reproduction, with their presence ultimately determining the survival of many organisms (Kattner *et al.*,

2007; Parrish, 2013). EFA are required for important processes including the production and regulation of eicosanoids (tissue hormones), stress resistance and immunity, and are involved in the regulation of behaviours including buoyancy control in many marine invertebrates, as well as schooling behaviours in fish (Müller-Navarra, 2008; Parrish, 2013). Depletion of these important FA therefore has serious implications for the health and survival of an organism (Koven *et al.*, 2001; Bachok *et al.*, 2006).

As with most heterotrophic organisms, the biosynthesis of n-3 and n-6 EFA in sponges relies upon exogenous precursors that are primarily obtained from food and phototrophic symbionts (Djerassi & Lam, 1991). Sponges are distinguished from other marine organisms by their high diversity of FA, and in particular an abundance of long chain C_{22}-C_{30} fatty acids with branched, odd-chain, or hydroxyl groups present (Koopmans *et al.*, 2015). These FA are referred to as the "demospongic acids", despite more recent evidence confirming that their biosynthesis is not specific to sponges (Djerassi & Lam, 1991; Bergé & Barnathan, 2005; Rod'kina, 2005; Kornprobst & Barnathan, 2010; Koopmans *et al.*, 2015). Nevertheless, the relative proportion of these "demospongic acids" in sponges has been shown to vary with season, and has been suggested to play a role in the maintenance of sponge membrane fluidity with changes in temperature (Hahn *et al.*, 1988).

The lipid bilayer of cell membranes, which forms a permeability barrier for cells and subcellular organelles, is particularly sensitive to the effects of thermal stress on membrane fluidity and cytotoxicology (Parrish, 2013). Ectothermic organisms can counteract the effects of increased temperature through the compositional adaptation of membrane lipids in a process called homeoviscous adaptation, which involves changes in mechanical and chemical properties of the lipid bilayer (Šajbidor, 1997; Weirich & Reigh, 2001; Vígh *et al.*, 2007; Horváth *et al.*, 2012; Parrish, 2013). A number of organism-specific responses are observed in response to thermal stress in an attempt to prevent membrane structure destabilisation and to maintain an ideal functional state of the cell membrane (Guerzoni *et al.*, 2001), enabling organisms to retain membrane function in the face of environmental change. Such adaptive mechanisms may involve: (1) changes to the degree of FA saturation; (2) alterations in the proportion of saturated and polyunsaturated FA; and (3) changes in FA chain length (Hochachka & Somero, 1984; Renaud *et al.*, 2002).

PUFA are the primary target of lipid peroxidation during thermal stress (Renaud *et al.*, 2002; Lesser, 2006) while SFA are more resistant and can increase the rigidity of biological membranes (Wada *et al.*, 1994). It has been suggested that an increased proportion of SFA to PUFA with increasing temperature will increase membrane integrity (Sinensky, 1974; Imbs & Yakovleva, 2012), and reduce the susceptibility of the membrane lipid bilayer to oxygen toxicity (Tchernov *et al.*, 2004). For example, an increase in the level of SFA in membrane lipids stabilised photosynthetic processes in the plant *Nerium oleander* when grown at elevated temperature (Raison *et al.*, 1982). Similarly, SFA content increases with growth temperature in a number of marine microalgae, whilst the proportion of n-3 and n-6 PUFA decreases (Renaud *et al.*, 2002). Furthermore, the soft coral *Sinularia capitalis* is more thermally tolerant than the hard coral *Montipora digitata* due to an observed increase in SFA and a lower proportion of PUFA following thermal exposure (Imbs & Yakovleva, 2012).

An increase in the activity of systems associated with the oxidation of FA was observed in the tropical sea anemone *Aiptasia* sp. and its associated dinoflagellate symbiont following exposure to thermal stress (32°C) (Hillyer *et al.*, 2016). Metabolite profiles revealed that while SFA increased in the anemone host tissue in response to increased temperature, PUFA increased in the symbiont (Hillyer *et al.*, 2016). Such changes in FA levels would likely result in an overall decrease in the degree of FA unsaturation (DoU) in the anemone holobiont, yet an increase in symbiont FA DoU as double bonds are added (Guerzoni *et al.*, 2001). Increased DoU is recognised as an important response of microorganisms to thermal stress, with reports of species adding double bonds to compensate for the changes in membrane fluidity with increasing temperature (Wada *et al.*, 1994). In the cyanobacterium *Synechocystis* PCC6803, an increase in the unsaturation of lipids stabilised photosynthesis with increased temperature (Gombos *et al.*, 1994). Furthermore, thermotolerance in *Saccharomyces cerevisiae* is associated with a response mechanism involving an increased degree of FA unsaturation in cells exposed to elevated temperatures (Guerzoni *et al.*, 1997). A similar response was observed in the thermotolerant bacterial species *Lactobacillus helveticus* and *Lactococcus lactis* in which the unsaturation level of FA increases with elevated temperature (Guillot *et al.*, 2000; Guerzoni *et al.*, 2001).

Sponges harbour microbial communities, which can contribute up to 40% of the sponge biomass and are crucial to the health and functioning of the sponge host (Webster & Taylor, 2012). This microbial partnership is collectively referred to as the sponge "holobiont" (Webster

& Taylor, 2012) and can be categorised into two nutritional types: i) phototrophic and ii) heterotrophic i.e. primarily reliant on suspension feeding for carbon requirements (Cheshire & Wilkinson, 1991). FA consumed with 14 carbons or more are generally deposited into an organism's tissue unmodified (Iverson, 2009). Due to the conservative incorporation of these FA into higher trophic level organisms, FA content is also directly related to diet (Rod'kina, 2003). FA analysis has therefore been applied extensively to determine the diet of different organisms (Dalsgaard *et al.*, 2003; Kattner *et al.*, 2007; Thurber, 2007; Koopmans *et al.*, 2015). As the sponge host is heterotrophic and therefore likely unable to synthesise all required FA, such as the n-3 and n-6 precursors (18:2n-6 and18:3 n-3) (Rod'kina, 2005), the FA composition of sponges is determined to some extent by the composition of its food FA. FA analysis is likely to provide useful insights into sponge diet and elucidate the contribution of phototrophic and heterotrophic sources of nutrition to these species (Koopmans *et al.*, 2015).

Although it is well established that direct nutritional exchange occurs between phototrophic sponges and their photosynthetic symbionts, including algae and cyanobacteria (Wilkinson, 1979; Wilkinson, 1980; Roberts *et al.*, 2006; Taylor *et al.*, 2007; Weisz *et al.*, 2007; Erwin & Thacker, 2008; Freeman & Thacker, 2011), the specific nutritional interactions between sponge associated photosymbionts and the sponge host remain poorly defined (Davy *et al.*, 2002; Webster & Blackall, 2009). Moreover, examining the FA profile of these species under different OW and OA scenarios, and particularly the FA profile of bleached *versus* non-bleached sponges will further our understanding of the relative importance of photosynthetically derived carbon to sponge nutrition (Imbs & Yakovleva, 2012). In the previous chapters I demonstrated that the phototrophic sponge *C. coralliophila* is able to survive bleaching for up to three months. Therefore, here I explored the potential for this phototrophic sponge species to activate heterotrophic carbon metabolism to supplement reduced photosynthate translocation during bleaching (Grottoli *et al.*, 2004; Grottoli *et al.*, 2006; Rodrigues *et al.*, 2008; Hoadley *et al.*, 2015). Primary producers synthesise specific FA and therefore FA trophic "biomarkers" can be used to identify the contribution of specific taxa to an organism's diet (Thurber, 2007). The abundance of known biomarkers was explored to determine the possibility of a phototrophic species supplementing their diet with heterotrophy during bleaching.

The FA profile of phototrophic and heterotrophic sponge species was characterised to understand the contribution of phototrophic *versus* heterotrophic sources of nutrition to these

different holobionts. I hypothesised that the FA profile of sponges that harbour phototrophic symbionts would be markedly different to that of the heterotrophic sponge holobionts. The FA composition of each species was also determined to assess whether particular compositions reflect host tolerance to OW and OA. I proposed that the more tolerant species would contain higher concentrations of structural FA such as PUFA, and long-chain PUFA (of the n-3 and n-6 series). Quantitative and qualitative changes in FA composition of the four species following exposure to OW and OA were then measured. I proposed that the FA composition and abundance would change as phototrophic sponge species bleach, however, due to observed differences in tolerance between these species I hypothesised that species specific differences in the metabolism and biosynthesis of FA would contribute to differential species resistance to these stressors. The role of FA in sponge thermal stress tolerance was then determined further by measuring changes in: i) the ratio of SFA and PUFA; ii) the degree of FA saturation; and iii) FA chain length, with the aim to understand whether more tolerant species are able to alter membranes to adapt to OW through changes in FA composition. Finally, FA trophic markers were employed to assess whether heterotrophic metabolism is activated during sponge bleaching.

4.2 Materials and Methods

The following analyses were carried out on four Great Barrier Reef (GBR) sponge species - the phototrophic species *Carteriospongia foliascens* and *Cymbastela coralliophila*, and the heterotrophic species *Stylissa flabelliformis* and *Rhopaloeides odorabile*, experimentally exposed to nine combined temperature (OW; 28.5, 30 and 31.5ºC) and pH (OA; pH 8.1, 7.8, 7.6) treatments as described in Chapter Two.

4.2.1 Tissue sampling

Six *C. coralliophila* and *R. odorabile* and seven *C. foliascens* and *S. flabelliformis* individuals were sacrificed at time zero (T0) for the initial analysis of tissue samples. Tissue was then taken from experimental sponges at the final sampling point (n = 3 to 6 individuals *per* species *per* treatment, except where mortalities occurred for *R. odorabile* where n = 2 at 31.5°C/ pH 7.6). The final sampling point varied for each species, due to differing sensitivities to treatment conditions (T = 2 weeks for *C. foliascens* and *R. odorabile*; T = 8 weeks for *S. flabelliformis*; T = 12 weeks for *C. coralliophila*). Sponge tissue was cryopreserved in liquid nitrogen, in 1.5 ml vials for subsequent FA analysis.

4.2.2 Fatty acid analysis

FAs were esterified using the acid-catalysed methylation method (Christie *et al*., 2001) with modifications described by Conlan *et al.*, (2014), following the extraction of total lipid (described in chapter three). 100 μl of the internal standard (C23:0; Sigma-Aldrich, Inc., St. Louis, MO, USA) were added to a 100 μl aliquot of the total lipid fraction with 2.0 ml of the methylation catalyst, acetyl chloride: Methanol (MeOH). Samples were then vortexed and placed in an oven at 100ºC for 1 h. Once cool, 2.0 ml potassium carbonate (1M) and 1.7 ml hexane were added. Samples were then centrifuged at 180 x g for 3 min. The hexane supernatant, containing the FA extraction, was recovered into a gas chromatography (GC) vial for GC injection. Fatty acid methyl esters were isolated and identified using an Agilent Technologies 7890B GC System (Agilent Technologies, USA) equipped with a BPX70 capillary column (120 m × 0.25 mm internal diameter, 0.25 mm film thickness, SGE Analytical Science, Australia), a flame ionization detector (FID), an Agilent Technologies 7693 auto sampler, and a splitless injection system. The injection volume, injector and detector temperatures, and temperature programmes followed Conlan *et al*., (2014); the carrier gas was helium at 1.5 ml min^{-1} at a constant flow. Individual fatty acids were then identified using

known external standards (a series of mixed and individual standards from Sigma-Aldrich, Inc., St Louis, USA and Nu-Chek Prep Inc., USA), using the software GC ChemStation (Rev B.04.03, Agilent Technologies). The resulting peaks were corrected by theoretical relative FID response factors (Ackman, 2002) and quantified relative to the internal standard C23:0 (Conlan *et al.*, 2014).

FA content was standardised to weight of total lipid content for each sample and expressed as mg FA g^{-1} lipid. Total FA and sums of major FA classes (SFA; MUFA; PUFA; TRANS-FA; n-6 PUFA; n-3 PUFA; n-6 LC PUFA and n-3 LC PUFA) were calculated to explore how sponge FA content varied between species and how the FA profiles of sponges was affected by OW and OA. The ratio of SFA to PUFA was calculated as a thermal stress indicator. The unsaturation degree (DoU) and mean chain length (MCL) were also calculated as stress indicators following (Guerzoni *et al.*, 2001):

DoU = [Σ monoenes + 2*(Σ dienes) + 3*(Σ trienes) + 4*(Σ tetraene) + 5*(Σ pentaene) + 6*(Σ hexaene)]/ total FA

MCL = Σ (mg FA g^{-1} lipid x C)/ total mg FA g^{-1} lipid

4.2.3 Data analysis

Data analyses were performed with PRIMER-E (PRIMER version 6.0, PERMANOVA+, Plymouth Marine Laboratory, Plymouth, UK). All graphs were generated using GraphPad Prism (GraphPad Software, version 6.00 for Windows, La Jolla California USA). To determine whether FA profiles were significantly different between species at time zero a one-way Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted on a matrix of all individual FAs, total FA and FA classes with species as a fixed factor. Permutational *post hoc* comparisons were used to determine which species differed significantly and results are reported in Table A4.1. To then test the effect of temperature and pH on sponge FA profiles a two-way PERMANOVA was conducted on a matrix of all individual FAs for each species following exposure to the treatments, with temperature and pH as fixed factors. Euclidean distances were used to generate resemblance matrices. Permutational *post hoc* comparisons were used to determine which treatments differed significantly and results are reported in Table A4.2a-c. Canonical analysis of principal coordinates (CAP) was used to display significant differences in FA profiles among the different species and between the treatments for each

species, based on PERMANOVA results. Individual FAs with a correlation greater than 0.45 were plotted separately to visualise FAs contributing to the variation between groups. Where PERMANOVA did not detect a significant treatment effect, the FA profile was instead visualised using an unconstrained PCA plot.

Finally, to test the effect of temperature and pH on total FA content, the major FA classes, stress indicators, and individual FAs a two-way PERMANOVA was employed, with temperature and pH as fixed factors. For each response variable, Euclidean distances were used to generate a resemblance matrix. Permutational *post hoc* comparisons were used to determine which treatments differed significantly. A 5% significance level was used for all tests. All results are reported in Tables A4.3-5, and only the significant/ relevant results are discussed below.

4.3 Results

4.3.1 Species FA composition

At T0 the two phototrophic sponge species *C. foliascens* and *C. coralliophila* had almost twice the total FA content *per* gram of lipid than the heterotrophic species, *R. odorabile* and *S. flabelliformis* (Fig. 4.1; Table 4.1). SFA accounted for approximately one third of the total FA content of the two phototrophic species (Fig. 4.1; Table 4.1). *C. foliascens* PUFA content was 2-fold that of *C. coralliophila*, which had a higher MUFA content. The two heterotrophic species had similar levels of PUFA, however, *R. odorabile* MUFA content was 5-fold that of *S. flabelliformis*, which had 2-fold more SFA. Although *C. foliascens* had a high concentration of PUFA compared to the other species (Fig. 4.1b), the important n-3 and n-6 PUFAs contributed less than a third of this PUFA content. Similarly, the n-3 and n-6 PUFA contribution to the total PUFA content was low in *R. odorabile* (Fig. 4.2). Meanwhile *C. coralliophila* and *S. flabelliformis* had a much greater proportion of the n-3 and n-6 PUFAs (Fig. 4.2). In *S. flabelliformis* almost all PUFA was of the n-3 and n-6 series. It is important to note that an unknown compound was identified in very high levels in *S. flabelliformis*, and while this is likely to be a biologically significant PUFA, unidentified FA have not been included in the subsequent analyses.

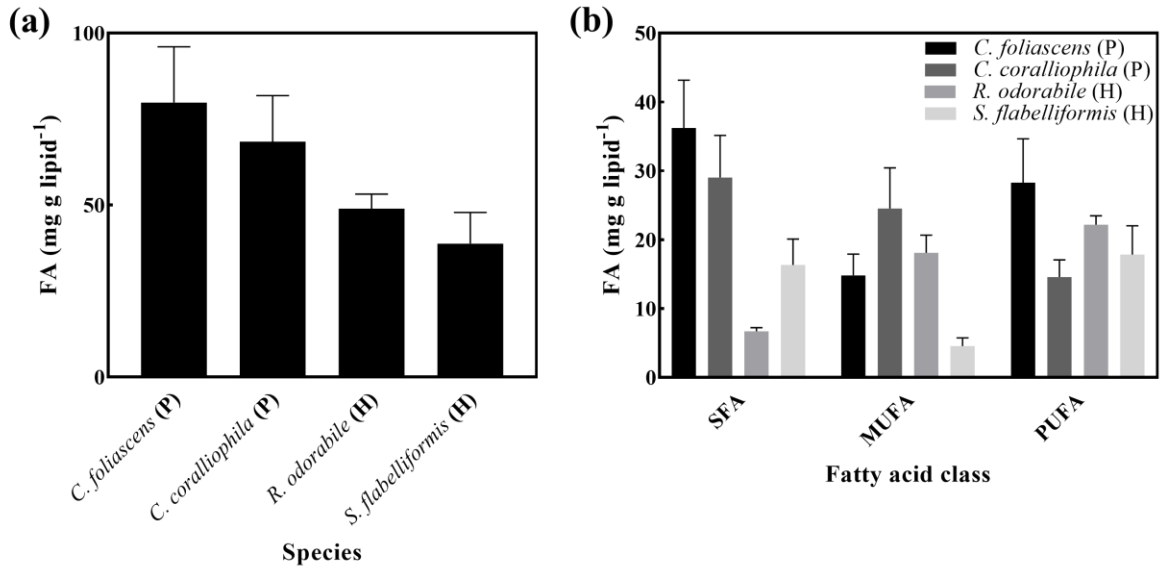


Fig 4.1 Time zero (T0) fatty acid composition for each species presented as (a) Total FA and (b) Major FA classes. SFA = saturated fatty acid; MUFA = monounsaturated fatty acid and PUFA = polyunsaturated fatty acid). Values are mean mg FA g^{-1} lipid (+SE) *per* species (n = 6 *Cymbastela coralliophila* and *Rhopaloeides odorabile*; n = 7 *Carteriospongia foliascens* and *Stylissa flabelliformis*). Note: P = phototrophic species and H = heterotrophic species.

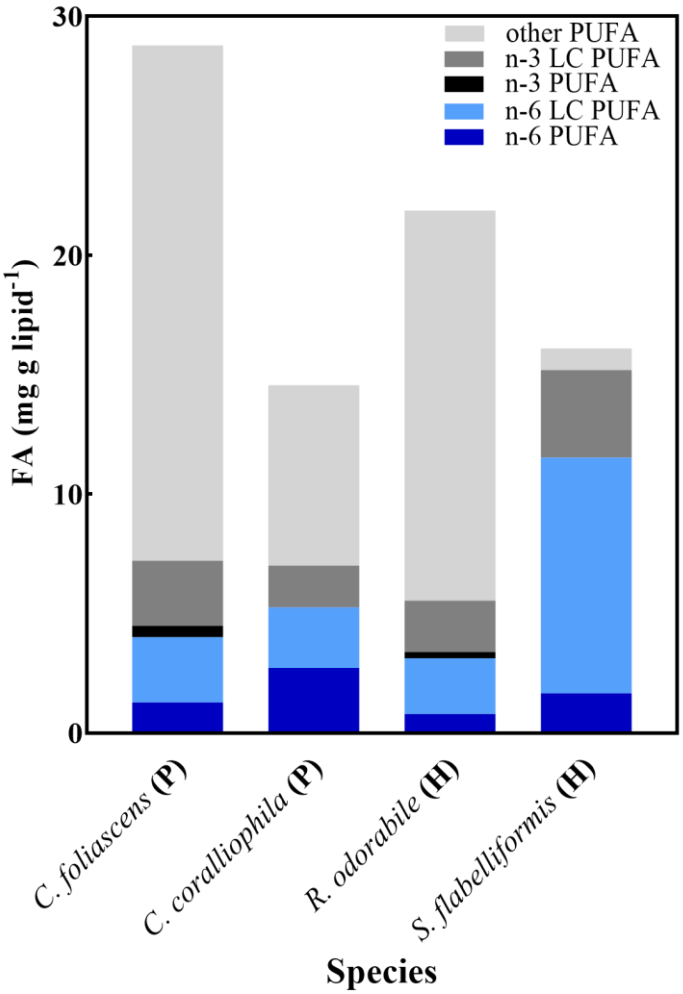


Fig 4.2 Time zero (T0) polyunsaturated fatty acid (PUFA) composition demonstrating the contribution of n-6 and n-3 essential FA (EFA), including n-6 and n-3 long chain (LC) PUFAs, to total PUFA content for each species. Values are mean FA mg g lipid^{-1} per species (n = 6 *Cymbastela coralliophila* and *Rhopaloeides odorabile*; n = 7 *Carteriospongia foliascens* and *Stylissa flabelliformis*). Note: P = phototrophic species and H = heterotrophic species.

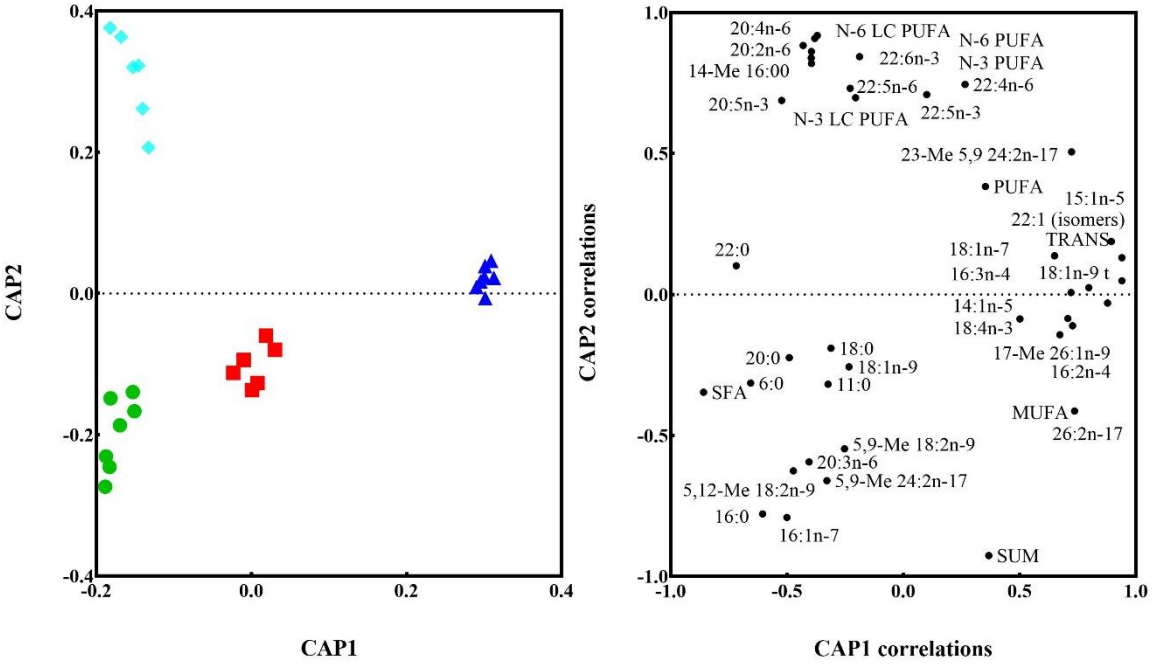


Fig. 4.3 Canonical analysis of principal coordinates (CAP) ordination and correlations plot (right), showing the FA composition of four GBR sponge species prior to exposure to experimental treatments. One-way PERMANOVA and post hoc pairwise comparisons identified significant differences in FA composition between the four species. Each point on the CAP represents an individual sponge where ● = *Carteriospongia foliascens*; ■ = *Cymbastela coralliophila*; ▲ = *Rhopaloeides odorabile*; ♦ = *Stylissa flabelliformis*. The correlations plot shows the FA correlated with the differences between groups.

PERMANOVA and CAP ordination revealed strong differentiation in FA profiles between the four sponge species (Pseudo-$F_{(3, 22)}$ = 41.9, *P* = 0.001; Fig. 4.3; Table A4.1). *C. foliascens* was largely composed of the SFA 16:0 (palmitic acid) as well as other SFA including 6:0, 18:0, 20:0, and 22:0 (Fig. 4.3; Table 4.1). *C. foliascens'* high PUFA content was due to the contribution of *5, 12-Me* 18:2n-9 and *5, 9-Me* 24:2n-17, while the dominant MUFA were 16:1n-7 (palmitoleic) and 18:1n-9 (oleic) acids (Fig. 4.3; Table 4.1). As with *C. foliascens, C. coralliophila* had an abundance of SFA, predominantly 16:0 and 14:0 (myristic acid). The dominant MUFA were again 16:1n-7 and 18:1 n-9 (Fig. 4.3; Table 4.1). *C. coralliophila* had a much higher MUFA content than *C. foliascens*, predominately due to the branching long chain demospongic acid *17-Me* 26:1n-9, which was found only in trace amounts in *C. foliascens* (Fig. 4.3; Table 4.1).

The FA profile of *R. odorabile* was characterised by a high abundance of MUFA (*17-Me* 26:1n-9; 22:1 isomers; and 14:1n-5) and PUFA (the demospongic acids 26:2n-17 and *23-Me 5, 9*

24:2n-17), as well as a low abundance of SFA (Fig. 4.3; Table 4.1). *S. flabelliformis* on the other hand had a high abundance of n-6 PUFA, especially long chain n-6 PUFA including arachidonic acid (ARA; 20:4n-6) and decosapentaenoic acid (DPA; 22:5 n-6), and also a demospongic acid *23-Me 5, 9* 24:2n-17 (Fig. 4.3; Table 4.1). *S. flabelliformis* also had a high concentration of *14-Me* 16:0, but a very low MUFA content when compared to the other species, and no 26:2n-17, a "demospongic acid" present in all other species (Fig. 4.3; Table 4.1). PERMANOVA and CAP also separated sponges by nutritional mode. The FA profile of the two phototrophic species was characterised by high total FA content, with a predominance of 16:0, 16:1n-7 and 18:1n-9. The heterotrophic species were similar in that they both had an abundance of the demospongic acid *23-Me 5, 9* 24:2n-17. However, the FA profile of the heterotrophic species remained distinct, due to the high MUFA content of *R. odorabile* and the high n-6 PUFA content of *S. flabelliformis*.

Table 4.1 Fatty acid composition of each species at T0 (mean mg FA g^{-1} lipid ± SE).

Fatty acid	*C. foliascens* (P)	*C. coralliophila* (P)	*R. odorabile* (H)	*S. flabelliformis* (H)
6:0	4.18 ± 0.8	-	-	0.85 ± 0.2
8:0	0.23 ± 0.1	0.28 ± 0.2	-	-
10:0	-	0.34 ± 0.2	-	-
11:0	0.11 ± 0.0	-	-	-
12:0	0.08 ± 0.0	0.22 ± 0.1	0.02 ± 0.0	-
13:0	0.29 ± 0.1	0.47 ± 0.1	-	-
14:0 [P; D]	2.22 ± 0.8	7.12 ± 1.4	0.39 ± 0.1	0.22 ± 0.1
14:1n-5	0.20 ± 0.1	1.49 ± 0.8	3.08 ± 0.3	0.14 ± 0.1
15:0 [B]	0.36 ± 0.1	0.63 ± 0.3	0.39 ± 0.1	0.33 ± 0.1
15:1n-5	0.09 ± 0.0	0.24 ± 0.1	0.74 ± 0.1	0.19 ± 0.1
16:0 [p] (Palmitic)	18.56 ± 4.0	9.38 ± 2.0	2.31 ± 0.2	2.59 ± 0.5
16:1n-7 [p] (Palmitoleic)	10.78 ± 2.9	4.54 ± 1.2	0.36 ± 0.0	0.29 ± 0.1
14-Me 16:0 [B]	1.37 ± 0.4	0.26 ± 0.1	0.77 ± 0.1	6.79 ± 1.7
3,7,11,15-tetra-Me 16:0 [B]	0.47 ± 0.1	2.71 ± 1.7	0.04 ± 0.0	-
17:0 [B]	0.90 ± 0.2	0.71 ± 0.2	0.44 ± 0.1	0.28 ± 0.1
16:2n-4	0.08 ± 0.1	-	0.17 ± 0.0	-
17:1n-7	0.04 ± 0.0	0.22 ± 0.1	0.16 ± 0.0	0.16 ± 0.1
16:3n-4	0.12 ± 0.1	-	0.63 ± 0.2	-
18:0 [p]	3.46 ± 0.6	3.95 ± 1.3	1.52 ± 0.1	1.65 ± 0.3
18:1n-9 t	0.24 ± 0.1	0.02 ± 0.0	1.47 ± 0.3	0.05 ± 0.0
18:1n-7 t	0.03 ± 0.0	-	0.06 ± 0.0	-
18:1n-9 [p] (Oleic)	2.16 ± 0.5	4.52 ± 1.7	0.49 ± 0.1	1.00 ± 0.2
5,12-Me 18:2n-9 [B]	4.61 ± 1.2	0.48 ± 0.4	-	-
18:1n-7	-	1.57 ± 0.5	1.05 ± 0.1	0.53 ± 0.3
18:2n-6 t	0.05 ± 0.0	3.35 ± 1.4	0.16 ± 0.1	0.27 ± 0.1
5,9-Me 18:2n-9 [B]	1.88 ± 0.6	0.85 ± 0.5	0.14 ± 0.0	-
18:2n-6 [C] (Linoleic; LA)	0.24 ± 0.1	0.39 ± 0.1	0.15 ± 0.0	0.32 ± 0.1
18:3n-6 [C] (γ-linolenic; GLA)	0.83 ± 0.4	0.45 ± 0.1	0.44 ± 0.1	0.28 ± 0.1
18:3n-4 [C]	0.88 ± 0.7	0.32 ± 0.1	0.16 ± 0.0	0.35 ± 0.1
18:3n-3 [C] (α-linolenic; ALA)	0.30 ± 0.2	0.01 ± 0.0	0.01 ± 0.0	-
20:0 [p]	1.36 ± 0.2	1.22 ± 0.3	0.28 ± 0.0	0.47 ± 0.1
18:4n-3 [C; D]	0.67 ± 0.1	-	0.24 ± 0.0	-
20:1 (isomers)	0.33 ± 0.1	3.85 ± 1.5	0.32 ± 0.0	0.53 ± 0.2
20:2n-7	0.26 ± 0.1	1.68 ± 0.5	0.23 ± 0.0	-
21:0	0.48 ± 0.1	0.74 ± 0.2	0.53 ± 0.0	0.30 ± 0.1
20:2n-6	0.19 ± 0.0	0.17 ± 0.1	0.15 ± 0.0	0.82 ± 0.1
20:3n-6	0.64 ± 0.2	0.39 ± 0.1	0.08 ± 0.0	0.10 ± 0.1
20:4n-6 (ARA) [ME]	0.76 ± 0.3	0.69 ± 0.2	0.42 ± 0.1	6.50 ± 1.9
20:3n-3	0.02 ± 0.2	0.13 ± 0.1	0.03 ± 0.0	-
22:0	2.64 ± 0.4	0.72 ± 0.2	0.46 ± 0.1	1.71 ± 0.7
22:1 (isomers)	0.30 ± 0.1	1.31 ± 0.5	5.11 ± 0.2	0.70 ± 0.2
20:4n-3	0.12 ± 0.1	0.16 ± 0.1	-	-
20:5n-3 (EPA) [D; ↓C]	0.45 ± 0.1	0.29 ± 0.1	0.15 ± 0.0	0.75 ± 0.3

22:2n-6	0.02 ± 0.0	1.71 ± 1.0	0.06 ± 0.0	0.25 ± 0.1
24:0	-	0.45 ± 0.2	-	1.15 ± 0.2
22:3n-3	0.26 ± 0.3	-	-	-
22:4n-6	0.86 ± 0.3	1.30 ± 0.3	1.54 ± 0.1	1.51 ± 0.3
22:5n-6	0.49 ± 0.1	0.16 ± 0.1	0.30 ± 0.0	1.74 ± 0.3
24:1n-9	0.54 ± 0.2	1.02 ± 0.4	0.31 ± 0.0	1.02 ± 0.3
5,9-Me 24:2n-17	13.09 ± 3.1	0.19 ± 0.1	1.79 ± 0.2	-
22:5n-3 (DPA)	1.16 ± 0.5	0.83 ± 0.2	1.37 ± 0.1	1.66 ± 0.4
22:6n-3 (DHA) $^{\downarrow C;\ DF;\ \downarrow D}$	0.30 ± 0.1	0.21 ± 0.1	0.47 ± 0.0	1.22 ± 0.3
23-Me 5,9 24:2n-17 ^{h}	0.33 ± 0.1	0.04 ± 0.0	3.82 ± 0.2	2.03 ± 0.5
17-Me 26:1n-9	0.10 ± 0.0	5.73 ± 2.0	6.48 ± 2.0	-
26:2n-17	0.26 ± 0.1	0.79 ± 0.3	9.52 ± 0.5	-
24:5n-3	0.41 ± 0.1	0.06 ± 0.1	0.13 ± 0.0	-
24:6n-3	0.01 ± 0.0	0.05 ± 0.0	0.01 ± 0.0	0.04 ± 0.0

Names of common/ abundant/ essential FA in brackets. Letters in superscript indicate FA that are distinctive to p = phototrophic sponges and h = heterotrophic sponges as determined in this study. Furthermore, FA that are markers for particular taxa as reported in the literature are also indicated in superscript where D = diatom; C = cyanobacteria; ME = microeukaryote; DF = dinoflagellate; B = bacteria; S = sponge. For all assume that an increase reflects the presence of this taxon unless ↓ is used, in which case an absence of that particular FA is indicative of a particular taxon. N = 6 *C. coralliophila* and *R. odorabile*; n = 7 *C. foliascens* and *S. flabelliformis*.

4.3.2 Total FA content of sponges exposed to OW and OA

There was a significant temperature effect on total FA concentration for *C. coralliophila* (Fig. 4.4b; Table A4.2a). Total FA abundance was significantly lower in sponges at 31.5ºC, compared to the other temperature treatments (Table A4.2b). These sponges were bleached as determined in Chapters Two and Three. The decline in total FA content was primarily due to a significant decline in SFA and MUFA (Fig. 4.4b; Table A4.2a). There was no significant effect of temperature or pH on the total FA content or major FA classes of *C. foliascens*, *R. odorabile*, or *S. flabelliformis* (Fig. 4.4; Table A4.2a).

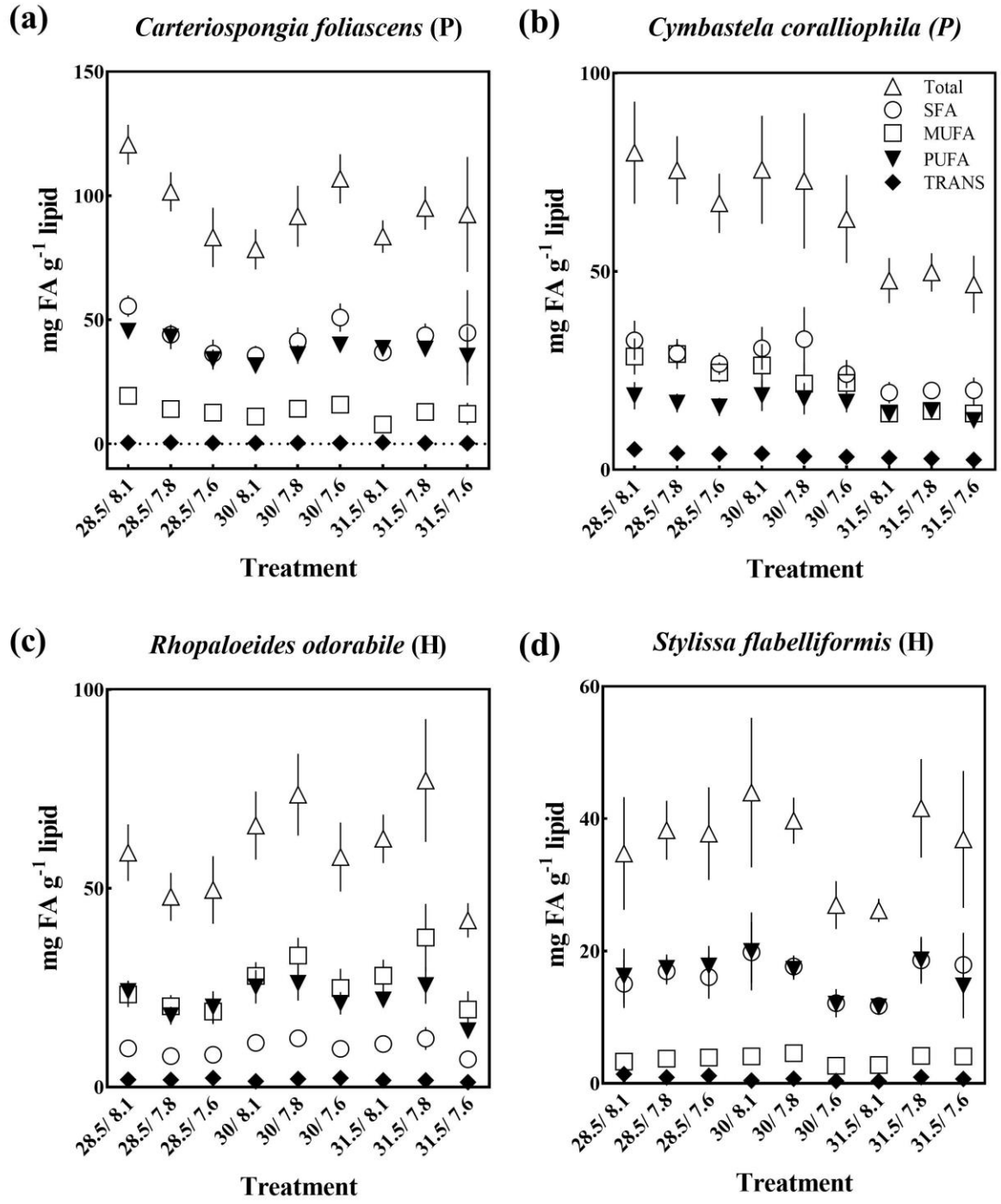


Fig 4.4 Total fatty acid and major fatty acid classes for (a) *Carteriospongia foliascens* (b) *Cymbastela coralliophila* (c) *Rhopaloeides odorabile* (d) *Stylissa flabelliformis* in each treatment. Total = total FA; SFA = saturated FA; MUFA = Monounsaturated FA and PUFA = Polyunsaturated FA (note: P = phototrophic species and H = heterotrophic species). Values are mean mg FA g^{-1} lipid of each species in each treatment ± SE (n = 3 to 6 individuals *per* species *per* treatment, except where mortalities occurred for *R. odorabile* where n = 2 at 31.5°C/pH 7.6). Note: y-xis scales differ between species.

4.3.3 FA profile of sponges exposed to OW and OA

While total FA and the major classes of FA remained similar for all species exposed to the different treatments with the exception of *C. coralliophila,* significant changes in the abundance of specific FAs were found in response to both OW and OA. PERMANOVA revealed significant treatment effects on the FA profile for all species, except *S. flabelliformis*. To explore treatment effects on the overall FA composition, CAP ordination was employed.

There was a significant interaction between temperature and pH on the FA profile of *C. foliascens* (Pseudo-$F_{(4, 28)}$ = 2.2, *P* = 0.043; Fig. 4.5a-b). FA profiles differed significantly between sponges at 28.5ºC and the elevated temperature treatments, at pH 8.1 (Table A4.3a); with *C. foliascens* being significantly bleached at 31.5ºC/ pH 8.1 in particular (Chapters Two and Three). *C. foliascens* at 28.5ºC remained similar to sponges at T0, with high concentrations of SFA (16:0; *3, 7, 11, 15-tetra-Me* 16:0; *14-Me* 16:0; and 13:0); the MUFA 16:1 n-7 (Palmitoleic acid); the PUFAs 20:2 n-6, 20:2 n-7 including EFA 20:5n-3 (EPA); and demospongic PUFA *5,9-Me* 18:2n-9; *5,12-Me* 18:2n-9; and *5,9-Me* 24:2n-17; all of which declined at 31.5ºC/ pH 8.1 (Fig. 4.5a-b). Further to declines in the aforementioned FA, sponges exposed to 31.5ºC/ pH 8.1 were characterised by higher concentrations of n-6 PUFA (18:2 n-6; 18:3 n-6; 20:4 n-6 and 22:4 n-6) and n-3 PUFA (18:3 n-3; 24:5 n-3) including 22:5n-3 (DPA) and 22:6n-3 (DHA), as well as SFAs (15:0 and 21:0), MUFAs (14:1 n-5 and 17:1 n-7) and demospongic FA (*23-Me 5,9* 24:2n-17 and 26:2 n-17) (Fig. 4.5a-b).

There was a significant effect of temperature on the FA profile of *C. coralliophila* (Pseudo-$F_{(2, 40)}$ = 3.9, *P* = 0.003; Fig. 4.5c-d). The FA profile of *C. coralliophila* exposed to 31.5ºC differed significantly from that at both 28.5ºC and 30ºC (Table A4.3b). The FA profile of sponges at 28.5ºC and 30ºC was characterised by high concentrations of the SFA 16:0 (Palmitic) and 10:0, as well as the; MUFA 16:1n-7 (Palmitoleic); 18:1n-9 (Oleic) and 24:1 n-9, all of which declined in bleached sponges at 31.5ºC. *C. coralliophila* exposed to 30ºC also differed from when it was exposed to 28.5ºC with an increased concentration of *5, 9-Me* 18:2n-9 (Fig. 4.5c-d).

There was also a significant effect of temperature on the FA profile of *R. odorabile* (Pseudo-$F_{(2, 43)}$ = 3.0, *P* = 0.042; Fig. 4.5e-f). The FA profile of *R. odorabile* exposed to 28.5ºC differed significantly from that at 30ºC and 31.5ºC (Table A4.3c). Sponges exposed to higher

temperatures had an increased MUFA content due to an increase in the concentration of the demospongic MUFAs *17-Me* 26:1n-9 and 24:1 n-9, as well as an increased content of the SFAs 21:0 and 22:0 (Fig. 4.5e-f). The n-6 PUFA 20:4n-6 (ARA) declined at 30°C, while 22:5n-6 increased at this temperature but declined at 31.5 ºC. There was no significant treatment effect on the FA profile of *S. flabelliformis*, so CAP could not be performed and a PCA was instead used to visualise the data (Fig. 4.5g-h). Two-way PERMANOVA revealed some trends in specific FAs where 14:1 n-5 increased at 31.5 ºC /pH7.6; 16:3 n-4 increased at 31.5ºC/pH 8.1; 24:1n-9 increased then decreased with increased temperature at pH 8.1 (Fig. 4.5g-h; Table A4.5**).**

While PERMANOVA did not detect any significant pH effects on the overall FA composition for any species, significant main effects of pH on individual FA were identified whereby the SFA 8:0, the n-3 PUFA 18:4 n-3 as well as 20:4 n-6 (ARA) increased significantly at low pH for *C. foliascens*. Similarly, the SFA 10:0, MUFA 18:1n-7, and PUFA 20:3 n-6 (DGLA) increased significantly when *C. coralliophila* was exposed to low pH (Table A4.5)

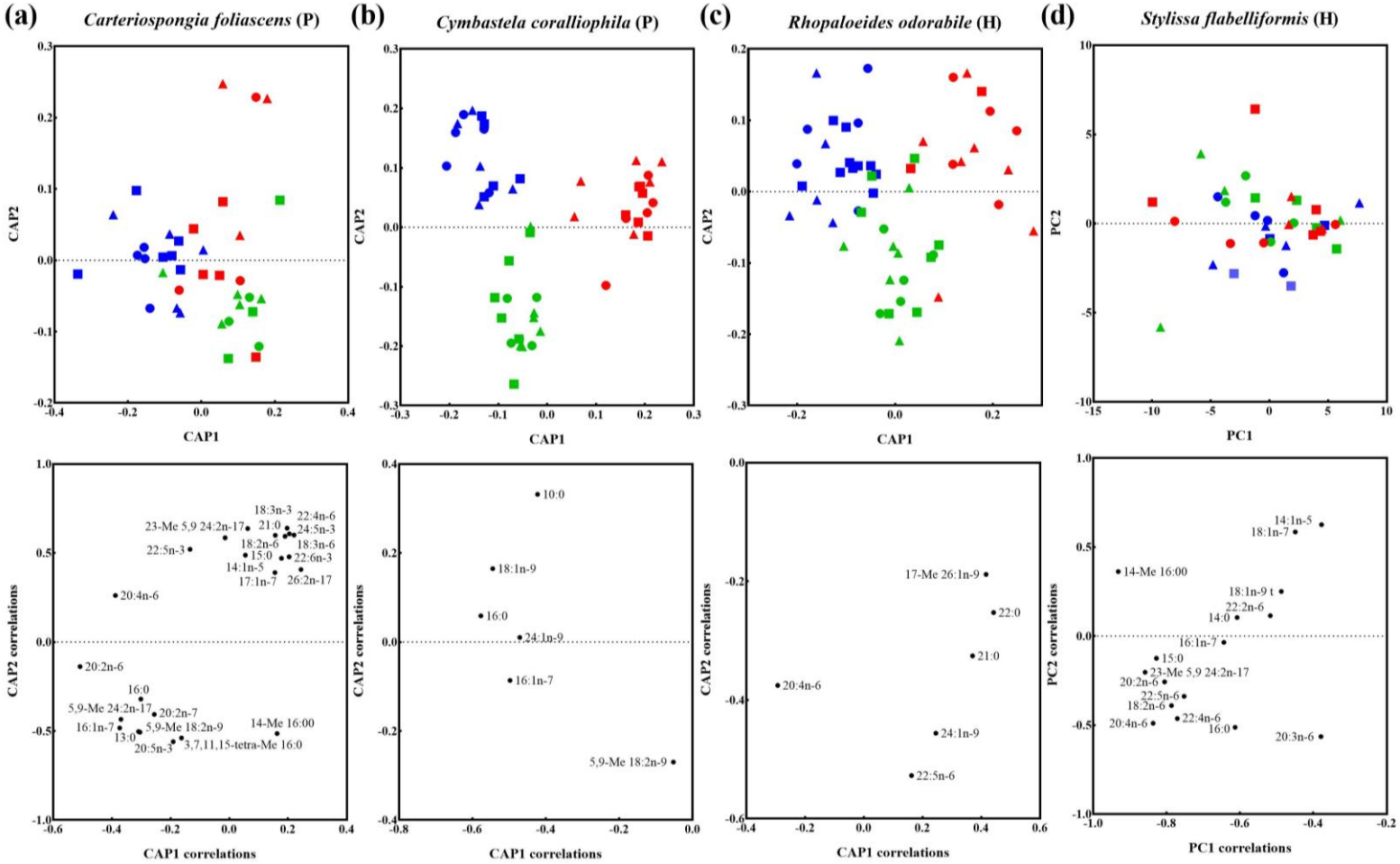


Fig. 4.5 Ordination analysis of sponge FA profiles following exposure to OW and OA. Canonical analysis of principal coordinates (CAP, left) for (a) *Carteriospongia foliascens* (b) *Cymbastela coralliophila* and (c) *Rhopaloeides odorabile* with correlations plot showing FA correlations greater than 0.45 (right). Principal components analysis (PCA, left) for (d) *Stylissa flabelliformis* with correlations plot showing FA correlations greater than 0.45 (right). Each point on the CAP/ PCA represents an individual sponge exposed to a different OW/ OA treatment where ▲ = 28.5°C/pH 8.1, ● = 28.5°C/pH 7.8, ■ = 28.5°C/pH 7.6, ▲ = 30°C/pH 8.1, ● = 30°C/pH 7.8, ■ = 30°C/pH 7.6, ▲ = 31.5°C/pH 8.1, ● = 31.5°C/pH 7.8, ■ = 31.5°C/pH 8.6 (n = 3 to 6 individuals per species per treatment, except where mortalities occurred for *R. odorabile* where n = 2 at 31.5°C/ pH 7.6). Note: P = phototrophic species and H = heterotrophic species; y-xis and x-axis scales differ between species.

4.3.4 Stress response indicators

The degree of FA unsaturation (DoU) increased significantly with temperature for *C. foliascens*, with the greatest increase at 31.5ºC/ pH 8.1 (Fig. 4.6a; Table A4.4a-b). The ratio of SFA to PUFA decreased at 31.5ºC/ pH 8.1 (Fig. 4.6b; Table A4.4a, c). Fatty acid DoU increased significantly with temperature for *C. coralliophila* (Fig. 4.6a; Table A4.4a, d), while the ratio of SFA to PUFA declined with increasing temperature (Fig. 4.6b; Table A4.4a, e). The response for the two heterotrophic sponges was the opposite: DoU declined significantly with increasing temperature for *R. odorabile* (Fig. 4.6a; Table A4.4a, f) and, while SFA:PUFA increased slightly with increasing temperature, this trend was not significant (Fig. 4.6b; Table A4.4a). *S. flabelliformis* FA DoU appeared to decline with temperature at pH 7.6 (Fig. 4.6a), with subsequent declines in the ratio of SFA to PUFA (Fig. 4.6b), however, neither trend was statistically significant (Table A4.4a). The average FA chain length increased significantly with temperature for *R. odorabile* (Fig. 4.6c; Table A4.4a, g). There was no significant treatment effect on MCL for the other species (Fig. 4.6c; Table A4.4a).

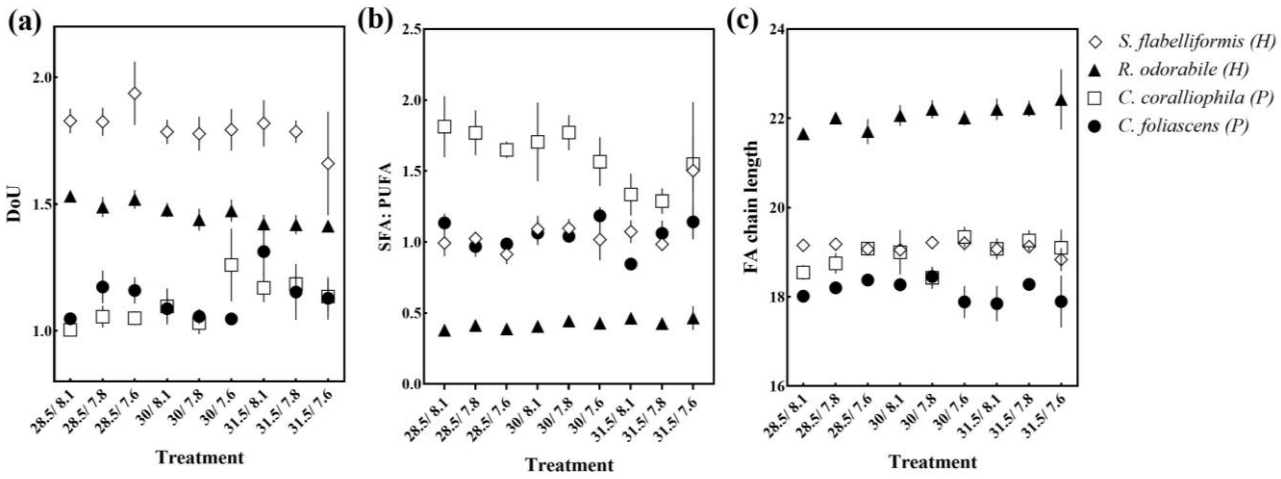


Fig. 4.6 Effects of the combination of OW and OA on (a) the degree of FA unsaturation (DoU) (b) the ratio of SFAs to PUFAs and (c) the average FA carbon chain length for each species exposed to OW and OA (n = 3 to 6 individuals *per* species *per* treatment, except where mortalities occurred for *Rhopaloeides odorabile* where n = 2 at 31.5°C/pH 7.6). Note: P = phototrophic species and H = heterotrophic species.

4.4 Discussion

The FA composition of four abundant Great Barrier Reef (GBR) sponge species exposed to ocean warming (OW) and ocean acidification (OA) levels predicted for 2100 under two IPCC RCP $CO_{2\ atm}$ emission scenarios was explored. This study first compared the FA profiles of the four sponge holobionts prior to experimental exposure. It was determined that while the quantitative and qualitative FA composition of sponges is species-specific, the FA profile of phototrophic sponges varies considerably from the heterotrophic sponge holobionts. Moreover, sponges identified as more resistant to OW and OA stress in the previous chapters contained a higher proportion of n-3 and n-6 PUFA, and long-chain essential FAs of the n-3 and n-6 series in particular. Changes in the FA profile of the four sponge holobionts were then examined following exposure to OW and OA. Potential links between phototrophic symbionts and sponge host nutrition were made as particular FAs declined in bleached phototrophic sponge species. Furthermore, increased pCO_2 resulted in a significant increase in a number of individual FAs. While OA effects were less prominent than the influence of OW on sponge FA profiles, ameliorative effects of OA on thermal stress in phototrophic species were reflected in FA profiles, as well as increases in particular FAs with elevated pCO_2. The use of FAs to explore compositional adaptation mechanisms of cell membranes to OW and OA revealed that phototrophic and heterotrophic holobionts respond differently to thermal stress: phototrophic sponges increase the unsaturation of their FA, while heterotrophic sponges decrease FA unsaturation. Further work should consider the sponge host and associated symbiont responses in isolation, to understand the mechanisms underlying these responses. Finally, although there was no evidence for the activation of heterotrophic metabolism in the thermally resistant bleached specimens of *C. coralliophila*, FA trophic markers detected possible shifts in diet (or microbial community composition) in *C. foliascens* following bleaching at 31.5°C. For this species, the observed shifts reflect a deterioration in health in response to OW, even if heterotrophic carbon acquisition is activated.

4.4.1 Nutritional differences in the FA profile of the sponge holobiont

Total FA content differed significantly between the four species, and most notably between the two nutritional modes with phototrophic sponges having almost double the total FA content than heterotrophic sponges, primarily due to higher levels of SFAs and MUFAs. Both phototrophic species had high levels of palmitic acid (16:0), as well as 14:0, 18:0 and 20:0. These SFAs are likely to reflect contributions from both the photosynthetic cyanobacterial

symbionts and the sponge host to the holobiont FA profile, as both sponges and cyanobacteria are able to synthesise these FAs *de novo* (Mueller-Navarra, 1995; Wada & Murata, 1998; Dalsgaard *et al.*, 2003; Bergé & Barnathan, 2005). The FA profiles of phototrophic sponge holobionts were further characterised by high concentrations of palmotoleic (16:1 n-7) and oleic (18:1 n-9) acids, both of which are prevalent MUFAs in many sponge species, including heterotrophic sponges (Rod'kina, 2005). The abundance of these FAs is very likely to be related to the presence of phototrophic symbionts in these sponge holobionts, however future work exploring the FA profiles of the sponge host and associated symbionts in isolation will confirm this. Oleic acid is particularly important, as this is the FA from which associated photosynthetic cyanobacteria biosynthesise n-3 and n-6 PUFA precursors (Wada & Murata, 1998; Bergé & Barnathan, 2005).

The FA profile of the phototrophic sponge holobionts were not only compositionally distinct from those of heterotrophic holobionts, but they differed from one another due to an abundance of different specific demospongic acids. *C. foliascens* had very high concentrations of *5, 9-Me* 24:2n-17, as well as an abundance of *5, 12-Me* 18:2n-9. Meanwhile, *C. coralliophila* contained *17-Me* 26:1n-9, which was found only in trace amounts in *C. foliascens*. Interestingly, this long chain PUFA was also present in *R. odorabile* along with 26:2n-17 and *23-Me 5, 9* 24:2n-17, which was the only FA present in relatively high amounts in both heterotrophic holobiont FA profiles. *S. flabelliformis* also had an abundance of *14-Me* 16:0; this is likely synthesised by bacteria associated with *S. flabelliformis* and used as a precursor for the biosynthesis of longer chain demospongic acids (Gillan *et al.*, 1988). These FAs are major components of sponge phospholipids, and are important in sponge cell membrane functioning (Gillan *et al.*, 1988; Hahn *et al.*, 1988; Djerassi & Lam, 1991). Sponges adjust the relative abundance of their dominant demospongic acids seasonally to maintain constant membrane fluidity with changing temperature (Hahn *et al.*, 1988), and such unique features of sponge membranes may have enabled sponges to survive and adapt to environmental change throughout time (Djerassi & Lam, 1991).

4.4.2 Essential FAs - an indicator of tolerance

While the most obvious difference between the four species' FA profiles was the distinction between the phototrophic and heterotrophic methods of carbon acquisition, additional differences were observed primarily related to EFA concentrations. Although *C. foliascens* had a particularly high concentration of PUFAs compared to the other species, the important long-

chain n-3 and n-6 PUFAs contributed to less than a third of this PUFA content. Similarly, the n-3 and n-6 PUFA content was low in *R. odorabile*. Meanwhile, the more thermally tolerant *C. coralliophila* and *S. flabelliformis* had a much greater proportion of n-3 and n-6 PUFA. Sponges, like most heterotrophic organisms, are thought to be unable to biosynthesise these FAs *de novo* (Rod'kina, 2005), or in sufficient quantities to meet nutritional requirements (Kainz & Fisk, 2009). They therefore represent an essential resource that must be obtained or supplemented from the sponge's diet, or from associated phototrophic symbionts (Mueller-Navarra, 1995). This not only reflects differences in sponge nutrition, but likely reflects physiological differences that may facilitate the resistance of these species to environmental stress, due to the role that long chain PUFA play as the primary constituents of phospholipids in sponge cellular membranes (Lawson *et al.*, 1988; Mueller-Navarra, 1995; Tocher, 2003).

S. flabelliformis, in particular, had a high concentration of the EFAs: ARA, DPA and DHA. While the presence of these FA likely reflects a substantial contribution of microalgae, bacteria or heterotrophic protists as a food source for this sponge (See Table 4.1 for specific biomarkers), (Monroig *et al.*, 2013; Koopmans *et al.*, 2015), it may also provide some insight into the tolerance of this species to thermal stress. These FAs are biologically valuable and their presence is consistently associated with increased stress resistance across a range of taxa, with deficiencies correlated with reduced growth, as well as increased mortality and susceptibility to stressors and disease (Müller-Navarra *et al.*, 2000; Koven *et al.*, 2001; Parrish, 2013). For example, growth and egg production of the herbivorous zooplankter *Daphnia magna* were reduced as the EPA content of its diet declined (Müller-Navarra *et al.*, 2000). Likewise, *post*-larval stages of the prawn *Penaeus indicus* that were fed on a diet enriched with EFAs, displayed higher survival rate, specific growth rate and stress resistance (Immanuel *et al.*, 2001). Similarly, survival of gilthead seabream (*Sparus aurata*) larvae increased when fed a diet enriched in ARA prior to handling stress (Koven *et al.*, 2001). Interestingly, *S. flabelliformis* had a particularly high content of ARA. This EFA is involved in eicosanoid synthesis; these are metabolites involved in cellular regulation processes such as the fluid and electrolyte fluxes that are likely to be important in the regulation of membranes during a thermal stress response (Koven *et al.*, 2001).

4.4.3 The FA composition of sponges exposed to OW and OA

The only species for which a significant decline in total FA was observed in response to OW was *C. coralliophila*. This is an interesting finding as all previous response variables measured

for this species have indicated that it is the most tolerant species to high OW/OA. This reduction in FA concentration was primarily due to a decline in SFAs (16:0 and 10:0) and MUFAs (16:1 n-7; 18:1n-9; and 24:1 n-9) in bleached sponges. This reduction suggests that these FAs may have been translocated directly from the symbionts to the sponge host's tissues or synthesised by the sponge from other photosynthetic products (Imbs & Yakovleva, 2012). However, it's more likely these FAs were catabolised by the sponge for energy production to maintain functioning in the absence of symbionts, this may reflect sub-lethal stress and longer-term experiments are required to confirm if FA decline precedes mortality.

However, the ability of *C. coralliophila* to survive this bleached state with a reduction in FA content for an extended period of time still suggests this species is less reliant upon the translocation of photosynthate than is the phototrophic *C. foliascens*. While it is possible that this sponge obtains carbon heterotrophically from the water column to compensate for reduced photosynthate translocation during bleaching (Grottoli *et al.*, 2004; Grottoli *et al.*, 2006; Hoadley *et al.*, 2015), there were no significant increases in specific FAs or groups of FAs to support this (See Table 1 for FA trophic markers). Other mechanisms such as physical properties of the cell membrane are likely to be responsible for the observed differences in tolerance of the phototrophic sponges to bleaching. *C. foliascens* has a very high PUFA content, while *C. coralliophila* has a much higher MUFA content. While a high content of essential PUFA is likely to provide stress resistance, as in *S. flabelliformis*, PUFA of thylakoid membranes in photosynthetic organisms are particularly sensitive to temperature and oxidative stress (Wada *et al.*, 1994; Wada & Murata, 1998). This is due to the excessive production of radical oxygen species as a result of the inactivation of the oxygen-evolving capability of PSII at stressful/photoinhibiting temperatures (Wada & Murata, 1998). Therefore a high PUFA content (of the non-essential nature) may, at least partially, explain why *C. foliascens* is more sensitive to OW than *C. coralliophila*.

4.4.4 Stress indicators

An increase in the ratio of SFA to PUFA in response to thermal stress has been reported for a number of organisms (Sheng *et al.*, 2011). In the thermally sensitive *C. foliascens*, the ratio of SFA to PUFA declined at 31.5ºC/pH 8.1. It appears that increased CO_2 availability in the low pH treatments either enabled the retention or facilitated the replenishment of SFAs, providing protection from elevated temperature in this species. However, a number of FA that are characteristic of the healthy *C. foliascens* holobiont declined with temperature, while odd chain

FAs characteristic of bacteria (15:0 and 21:0) (Bergé & Barnathan, 2005) increased, alongside the EFAs ARA, DPA, and DHA and the precursors for these (18:2n-6 and 18:3n-3). These EFAs and their precursors are unlikely to be synthesised by the sponge and may therefore reflect a shift in diet or microbial community composition for this species with elevated temperature (Rod'kina, 2003; Rod'kina, 2005). Nevertheless, *C. foliascens* mortalities occurred very rapidly (days) following bleaching at 31.5°C, demonstrating that this shift in FAs reflects a deterioration of health for this species in response to OW, even if heterotrophic carbon acquisition is activated. Similarly *C. coralliophila* SFA to PUFA declined as temperature increased. However, the only significant shifts in the FA profile of *C. coralliophila* were the substantial declines in SFA due to a loss of photosynthetic symbionts, and the catabolism of this energy source with no subsequent replacement. There were no significant changes in the ratio of SFA to PUFA for either heterotrophic sponge species.

The degree of FA unsaturation, on the other hand, increased significantly with elevated temperature for both phototrophic species, while DoU declined with increasing temperature for *R. odorabile* and for *S. flabelliformis* (however this trend was not significant). The increase in the proportion of unsaturated FAs has been observed for a number of microorganisms (Wada *et al.*, 1994; Guerzoni *et al.*, 1997; Guillot *et al.*, 2000; Guerzoni *et al.*, 2001) and likely reflects a desaturase activation either in the host or symbiont as a means to increase membrane rigidity and/ or protect cells from toxic oxygen species at high temperatures (Guerzoni *et al.*, 1997; Guerzoni *et al.*, 2001). The activation of this desaturase system is especially important for phototrophic organisms due to the excessive production of radical oxygen species as a result of the inactivation of the oxygen-evolving capability of PSII at stressful/photoinhibiting temperatures (Wada & Murata 1998). A decline in DoU, on the other hand, may reflect a response by the heterotrophic sponge host to increase membrane rigidity, mitigate peroxidation, or more likely is a result of host metabolism (Hillyer *et al.*, 2016). Future work exploring the responses of the host and associated symbionts is required to elucidate these responses. In addition, previous reports suggest that the average chain length of FA can alter the fluidity of cell membranes (Hochachka & Somero, 1984). Here it was found that the average FA chain length increased significantly with temperature for *R. odorabile*. However, no significant effect of OW/ OA was observed on the chain length for the other three species; this is similar to studies exploring the response to microalgae grown at different temperatures, for which no significant change in the MCL was observed (Renaud *et al.*, 2002).

4.4.5 OA effects

Finally, whilst OW had a greater overall effect on the FA composition of all four species than OA, some significant positive OA effects were observed in the phototrophic species. Specifically, short-chain SFAs 8:0 and 10:0 increased with increasing pCO_2. These short-chain SFAs were only present in the phototrophic sponge FA profiles, and at low concentrations (0.23 to 0.34 mg FA g^{-1} lipid). Therefore, while such SFAs are not major constituents of cyanobacterial FA pools (Wada & Murata, 1998), it appears that they are either produced directly *via* photosynthesis, or that they are synthesised using photosynthetically-fixed carbon. Furthermore, the MUFA 18:1n-7 and PUFAs 18:4 n-3 and 22:3 n-3, including EFAs 20:3 n-6 (DGLA), ARA and DHA also increased significantly with increasing pCO_2. Positive correlations with a number of PUFAs were observed in a large mesocosm experiment that exposed natural plankton communities to OA, again suggesting that the response observed here for the phototrophic sponges is directly related to photosynthetic organisms, as a result of increased productivity under elevated pCO_2 (Leu *et al.*, 2013). An increase in these important long chain PUFAs is likely to increase stress resistance (Müller-Navarra *et al.*, 2000; Koven *et al.*, 2001; Parrish, 2013) and begins to explain the mechanisms by which OA may ameliorate some of the negative effects of OW for phototrophic sponges (Bennett *et al.*, 2016).

4.4.6 Summary

It is well established that the ability of an organism to maintain appropriate membrane function in the face of environmental change is intimately linked to tolerance. Here I discovered that long-chain FAs of the n-3 and n-6 series (the omegas) are important components of the sponge stress response and facilitate sponge resistance to environmental change. The high n-3 and n-6 PUFA content of *C. coralliophila* and *S. flabelliformis* likely contributes to their tolerance to OW. These species also retained their essential fatty acid content in response to OW and OA. Meanwhile, significant shifts in important FAs in *C. foliascens* following bleaching at 31.5°C, from FAs associated with a healthy holobiont to those associated with bacteria and larger microorganisms, likely reflects changes in the microbial community composition, potentially due to decay and/ or disease. Finally, phototrophic and heterotrophic sponge holobionts respond differently to thermal stress, with phototrophic sponges increasing the unsaturation of their FAs and heterotrophic sponges decreasing FA unsaturation in response to thermal stress. This likely reflects different compositional adaptation methods between associated photosymbionts and sponge hosts, and highlights the need for future work to consider the effect of OW and OA on the different partners of the sponge holobiont in isolation.

Chapter Five

The response of early-life stages of sponges to elevated pCO_2 and temperature: *Tolerant sponge larvae and juveniles provide a means for adaptation and species persistence in a high CO_2 world.*

Aragonite plugs on top of glass vials containing sponge larvae. During settlement larvae swim toward the plug, then settle and metamorphose into juveniles.

5.1 Introduction

For many marine species, juvenile and adult life stages have different tolerances to environmental stressors (Wernberg *et al.*, 2012; Przeslawski *et al.*, 2015). Early-life stages of marine invertebrates are generally considered to be more sensitive to environmental change than adult stages (Thorson, 1950; Kurihara, 2008; Albright, 2011), and as a result are potential bottlenecks for species persistence and ecological success in a changing ocean (Gibson *et al.*, 2011). Environmental factors that disrupt larval dispersal, recruitment and early development have wide implications for population dynamics and the ultimate survival of a species (Albright & Langdon, 2011; Gibson *et al.*, 2011; Byrne & Przeslawski, 2013). Compromised development in these early-life stages has knock on effects and may result in localised species extinctions (Gibson *et al.*, 2011). Exploring the response of an organism to stressors across different life stages is therefore essential if we are to fully understand ecological consequences of climate change on a species (Webster *et al.*, 2013).

Ocean warming (OW) and ocean acidification (OA) both exert strong control over the development, survival and success of early-life history stages of a number of marine invertebrates (Przeslawski *et al.*, 2015). Temperature in particular plays a key role in organism development through its direct influence on molecular and physiological processes, and indirectly as a stimulus in the timing of important reproductive processes, such as the onset of gametogenesis and larval release (Hoegh-Guldberg & Pearse, 1995; Gibson *et al.*, 2011; Byrne, 2012; Byrne & Przeslawski, 2013). Due to the influence of temperature on these fundamental processes, OW is predicted to have a profound effect on marine organism reproduction and development (Byrne & Przeslawski, 2013). Co-occurring OA will result in reduced ocean pH and hypercapnia as pCO_2 increases with rising $CO_{2\ atm}$, with negative implications for early-life stages of marine organisms (Byrne, 2011). The effects of OA have mainly been studied for organisms with calcifying life-stages, and is observed to reduce calcification rates with subsequent implications for growth and survival (Kroeker *et al.*, 2010). However, direct pH effects including disruptions to acid-base physiology and the repercussions of this on metabolism, growth and development confirm it is not only organisms with calcifying stages that are vulnerable (Albright & Langdon, 2011; Byrne, 2012). Less is known, however, in regards to the interactive effects of OW and OA on early life history stages of marine invertebrates, especially when considering multiple life history stages of the same species (Byrne, 2012; Dupont & Pörtner, 2013; Przeslawski *et al.*, 2015).

To date, sponge climate change research has focused almost exclusively on the adult life-history stage, with little known about the responses of larvae and recently-metamorphosed juveniles. The sensitivity of early-life stages is of particular concern for benthic marine invertebrates, such as sponges, for which successful recruitment and population persistence rely upon both pelagic and benthic developmental stages being completed successfully (Marshall & Morgan, 2011). It is essential that all stages of development are tolerant as failure, regardless of stage, has negative flow on effects in terms of recruitment, population growth and ecosystem maintenance (Byrne, 2012).

Sponges reproduce *via* both sexual and asexual processes, however the vast majority of sponges undergo indirect development sexually, through a larval stage (Maldonado, 2006; Maldonado & Riesgo, 2008). Temperature plays a key role in the timing of important sponge reproductive processes such as the onset of gametogenesis (Fromont, 1994; Ettinger-Epstein *et al.*, 2007; Whalan *et al.*, 2007; Riesgo & Maldonado, 2008), with spawning (Fromont & Bergquist, 1994; Mariani *et al.*, 2001) and peak larval release occurring in warmer months for many sponge species (Ereskovsky, 2000; Bautista-Guerrero *et al.*, 2010; Wahab *et al.*, 2014a). There is a lack of studies which explicitly test the effect of OW on sponge reproduction, however strong correlations between sponge reproductive processes and seasonal variation in seawater temperature (Fromont & Bergquist, 1994; Ettinger-Epstein *et al.*, 2007; Whalan *et al.*, 2007; Abdo *et al.*, 2008; Ereskovsky *et al.*, 2013; Zarrouk *et al.*, 2013) suggest changing ocean temperatures will have an influence on sponge reproduction. Furthermore, elevated temperatures during a strong La Niña event were linked to a reduction in sexual productivity of the abundant GBR sponge, *Carteriospongia foliascens* (Wahab *et al.*, 2014a), highlighting the likely implications of a changing climate for sponge reproduction.

Sponges have a bi-phasic lifecycle and may be gonochoristic or hermaphroditic, with embryo development occurring either oviparously or viviparously, to produce a free swimming larval stage (Maldonado, 2006). Once larvae are "competent" i.e. physiologically and morphologically ready for settlement, the dispersive larval stage settles and metamorphoses into a sessile juvenile sponge (Maldonado, 2006). Whilst eight distinct poriferan larval forms are recognised, a few commonalities exist whereby the majority of sponge larvae can be summarised as lecithotrophic, ciliated forms with relatively short planktonic durations (Mariani *et al.*, 2006). The planktonic larval duration (PLD) is important for supply side ecology and genetic connectivity between populations of benthic marine invertebrates, such as

sponges for which this larval stage is the only means of migration in an otherwise sessile lifestyle (Gibson *et al.*, 2011). This sessile lifestyle makes benthic marine organisms especially vulnerable to environmental perturbations, including local disturbances as well as climate change, and as a result these dispersive larval stages are crucial for population persistence and recovery (Wahab *et al.*, 2014a). Larval development is a temperature dependent process, and elevated temperatures are demonstrated to increase larval development, therefore reducing the PLD of some marine invertebrates (O'Connor *et al.*, 2007). While a shortened PLD may increase the retention of larvae within a region, and increase survival initially as the vulnerable dispersive phase is reduced, it has negative implications for genetic diversity and population dynamics, as well as population persistence in a changing ocean (Gibson *et al.*, 2011).

A reduction in PLD was demonstrated in pre-settlement stages of the Great Barrier Reef sponge *Rhopaloeides odorabile* exposed to temperatures of 32-38°C (Whalan *et al.*, 2008b). Metamorphosis of larvae exposed to such temperatures was completed much faster than for larvae at temperatures of 22–26°C (Whalan *et al.*, 2008b). In sponges, the average PLD is already very short, ranging from just minutes to days (Wahab *et al.*, 2014b; Whalan *et al.*, 2008a); this, combined with a sponge larva's poor swimming ability (Whalan *et al.*, 2008a), results in restricted dispersal potential for most species (Wahab *et al.*, 2014b; Whalan *et al.*, 2008a). Changes in the PLD of sponges have implications for population connectivity and maintenance, gene flow and community structure, as well as regional-to-global scale patterns of sponge biodiversity (Maldonado & Young, 1996; O'Connor *et al.*, 2007; Whalan *et al.*, 2008b).

Despite an observed reduction in *R. odorabile* PLD, the overall pre-settlement survival, metamorphosis and settlement success were unaffected by OW (Whalan *et al.*, 2008b). Futhermmore, larval microbial communities remained stable when exposed to temperatures up to 34°C, and significant shifts did not occur until 36°C (Webster *et al.*, 2011). This is in stark contrast to adult stages of *R. odorabile*, which exhibit a thermal threshold of 32°C (Webster *et al.*, 2008; Pantile & Webster, 2011; Massaro *et al.*, 2012; Fan *et al.*, 2013) that is further reduced under combined OA conditions (Bennett *et al.*, 2016). While the PLD of *R. odorabile* may be shortened under OW, tolerant larval stages may still enable the persistence of this species *via* current-driven dispersal into more favourable environments (Webster *et al.*, 2011). However, it is important to note that subsequent consequences of a reduced PLD have resulted in the development of smaller or abnormal juveniles of some marine organisms (Byrne &

Przeslawski, 2013), in what is a direct result of developmental rates under elevated temperatures exceeding growth rates and/or diverting energy away from growth and into other biological processes such as reproduction (Sheridan & Bickford, 2011). For example, while the development of the Elkhorn Coral *Acropora palmata* accelerated with elevated temperature, the number of abnormalities increased and larval survivorship and settlement decreased significantly (Randall & Szmant, 2009).

As well as driving recruitment dynamics, temperature affects the survival of juvenile marine invertebrates (Gibson *et al.*, 2011; Talmage & Gobler, 2011; Byrne, 2012). While development processes such as growth are likely to be facilitated under moderate temperature increases, temperatures beyond an organism's thermal threshold have negative implications for survival (Byrne, 2011). In the one study to have considered the response of juvenile sponges to increased temperature, growth rates of the marine sponge *Hymeniacidon perlevis,* increased when exposed to higher temperatures (23°C) compared to juveniles kept at a constant temperature (18°C) (Xue & Zhang, 2009). This is in contrast to primary polyps of the juvenile coral *Porites panamensis*, for which zooxanthellae densities and coral biomass were reduced with a temperature increase of just 1°C (Anlauf *et al.*, 2011). Furthermore, juvenile bivalves are also likely to be negatively affected by temperature, as demonstrated by reductions in survival, development and growth of larva and juvenile *Mercenaria mercenaria* and *Argopecten irradians* exposed to 24 and 28°C, although *Crassostrea virginica* was not affected (Talmage & Gobler, 2011). These results for bivalves demonstrate that tolerances are very likely to be species specific and further work exploring the response of sponge juveniles to OW is required.

Furthermore, there are no studies to date which look at OA effects on early-life stages of sponges. However, as with adult sponges the implications for OA on skeletal formation is minor compared to organisms with calcifying life stages i.e. corals and crustaceans (Byrne & Przeslawski, 2013; Kroeker *et al.*, 2013b; Talmage & Gobler, 2011). While it is suggested that the development of marine species without calcifying stages in their development may be more robust to near-future OA (Byrne & Przeslawski, 2013), OA can also affect physiological and biological processes independent of calcification, with negative repercussions for a number of non-calcifying organisms across multiple life-history stages (Albright, 2011; Przeslawski *et al.*, 2015). Among other processes, sperm motility is reduced under OA in a number of organisms including the sea urchin *Heliocidaris erythrogramma* (Havenhand *et al.*, 2008), the

coral *Acropora digitifera* and the sea cucumber *Holothuria* spp. (Morita *et al.*, 2010). OA also decreases fertilisation success in non-calcifying stages of the sea urchin *Echinometra mathaei*, with increasing pCO_2 reducing fertilisation and cleavage rate, developmental speed, and larval size (Kurihara & Shirayama, 2004). Elevated pCO_2 also reduces metabolic rates of non-calcifying pre-settlement stages of the coral *Porites astreoides*, subsequent to that OA decreases settlement success and impedes post-settlement growth (Albright & Langdon, 2011).

Nevertheless, OW and OA will occur in combination with one another and for many marine invertebrates the combined effect of OW and OA has greater deleterious impacts on the success of early life stages, than these stressors applied in isolation (Kurihara, 2008; Gibson *et al.*, 2011; Byrne & Przeslawski, 2013; Harvey *et al.*, 2013). It is therefore crucial to study the effects of OW and OA in combination to gain a more ecologically realistic understanding of the effects of these stressors on early-life history stages (Przeslawski *et al.*, 2015). While the survival of early life stages is fundamental for population persistence, no studies have explicitly tested the early life-history response of sponges to the combined effects of OW/OA (Przeslawski *et al*., 2015). Therefore, to provide greater insight into population level impacts of climate change this chapter will explore the response of the phototrophic *Carteriospongia foliascens* to OW and OA throughout its developmental stages. *C. foliascens* is one of the most widespread Dictyoceratid sponges on the GBR (Wilkinson, 1988), is prevalent across a range of habitats including mesophotic reefs (Wilkinson, 1988; Bridge *et al.*, 2011; Wahab *et al.*, 2014a), and contributes significantly to reef primary productivity (Wilkinson, 1983b). The previous chapters demonstrate *C. foliascens* is sensitive to OW, however elevated pCO_2 available under combined OW and OA conditions actually mitigates thermal stress to an extent for this important GBR species. This suggests that, perhaps, *C. foliascens* may persist into a high CO_2 world, with increased productivity of associated symbionts providing the energy required to buffer the stress associated with rising temperatures.

Successful recruitment and persistence of populations require that all ontogenetic stages be completed successfully (Byrne, 2011), and we have no understanding of how early-life stages of *C. foliascens* are likely to be impacted by OW and OA. *C. foliascens* is an internal brooder, the female sponge releases fully developed parenchemella larvae year round, with peak larval release during summer months (Wahab *et al.*, 2014a). This larval stage is central to population maintenance, connectivity and genetic diversity and is likely to serve as as a vector for moving

populations away from unfavourable environments and into more tolerable conditions, making it central to the potential adaptive capacity of this species in a changing climate. *C. foliascens* larvae have a relatively limited dispersal potential (Wahab *et al.*, 2014a), therefore implications of OW for PLD are likely to have negative consequences for *C. foliascens* supply side ecology. However, should *C. foliascens* larvae and early life stages exhibit tolerance to such conditions, there is potential for phenotypic and genotypic adaptation, providing a basis for species persistence in a changing climate. Determining the response of these life stages is important in identifying potential 'winners and losers' as we enter a high CO_2 world (Gibson *et al.*, 2011).

In this chapter I ran a series of experiments in which *C. foliascens* was exposed to OW and OA pre-settlement, 1-month; 1-year; and 2-years post-settlement, to determine the effect of OW and OA on this important species at the population level. I proposed that early-life history stages of sponges will be more tolerant to OW than adult life stages, based on previous research demonstrating remarkable tolerance to OW in these life-stages. *C. foliascens* larvae are released with phototrophic cyanobacteria provisioned, therefore I also proposed that, as with adult life-stages of *C. foliascens*, additional pCO_2 would provide more energy to sponges under OA, potentially facilitating their resistance to OW.

5.2 Materials and Methods

5.2.1 Experimental treatments

The following experiments were carried out using the same experimental set-up of nine combined temperature (OW; 28.5. 30 and 31.5ºC) and pH (OA; pH 8.1, 7.8, 7.6) treatments as described in chapter two. All modifications are discussed below.

5.2.2 Pre-settlement

To determine the effect of OW and OA on *C. foliascens* pre-settlement survival and settlement success, 10 reproductive individuals of *C. foliascens* were collected from Little Pioneer Bay, Orpheus Island Research Station (OIRS) on the GBR, Australia (18°38'S, 146°29'E) on 29th November 2014 and transported back to AIMS where they were kept in ambient seawater. At 0900 h on 20 January 2015, adult sponges were transferred into bins without flow- through, as the peak larval release window for this sponge is 0900 - 1200 h (Wahab *et al.*, 2014b). After three hours, all released larvae were collected from the bin and the adult sponges were returned to flow-through aquaria.

Specially designed floating six-well plates with a mesh base were used to assess survival, swimming duration and settlement in *C. foliascens* larval stages in the different temperature and pH treatments. Due to the quantity of sponge larvae released, the number of treatments was reduced to four for this life-history stage (28.5°C and 31.5°C/ pH 8.1 and 7.6). All larvae released were pooled to form a "larval stock". One six-well plate was floated in each tank, with three replicate tanks *per* treatment. Ten larvae from the stock were pipetted into each well (n = 180 larvae *per* treatment). Larval motility, survival and settlement were then scored at T6, T12 and T24 h (Fig. 5.1). The percentages of larvae that were still motile, settled and dead were calculated for each well at each sampling time point as a proportion of the total initial larvae in each well.

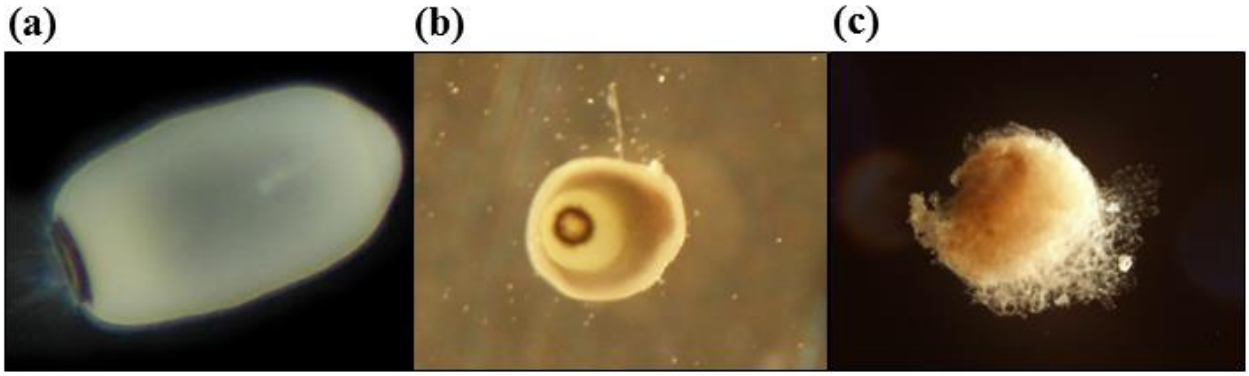


Fig. 5.1 Survival, swimming duration and settlement was assessed in *Carteriospongia foliascens* larval stages exposed to four temperature and pH treatments 6, 12 and 24 hours after larval release (n = 180 larvae *per* treatment). *C. foliascens* larvae were scored as (a) motile, (b) settled, and (c) dead at each time point.

5.2.3 Post-settlement

To determine the post-settlement response of *C. foliascens* to the treatments, on the 9th December 2014 larvae were collected from the same adults as described above. Larvae were settled onto aragonite plugs with a two-week microbial biofilm to stimulate settlement (Whalan & Webster, 2014). To reduce the effects of naturally high post-settlement mortality, settled *C. foliascens* were left for four weeks in ambient seawater prior to treatment exposure. On 15th January, a census of "one-month-old recruits" (hereafter referred to as 1-month recruits) on each plug was carried out (Fig. 5.6b – *photograph of one-month recruit*), and the plugs were photographed and randomly allocated to each of four treatments: 28.5°C and 31.5°C/ pH 8.1 and 7.6, (n = 14 plugs *per* treatment, except 28.5°C/pH 7.6 where n = 15, with a total of 39 to 59 recruits *per* treatment). One-month recruits were exposed to treatment conditions for four weeks, after which a final census was conducted and an end-point photograph of each plug was taken. Photographs were used to measure growth and bleaching. Recruit size was measured using ImageJ and growth was determined as change in area (mm^2) between T0 and T-end. Colour score (used as an indicator of bleaching) was measured using ImageJ grayscale. The area of each recruit was selected and the mean grayscale was calculated (0 = pure black and 255 = pure white). Difference in photo lighting was corrected for by using a white reference grid in each photo. The use of colour change as an index for photophysiological changes was corroborated with a blue LED Maxi Imaging-PAM (I-PAM) fluorometer (Walz Germany) using the saturation-pulse method described above. However, studies on cyanobacteria PSII

generally use red excitation light, as blue light preferentially activates PSI in cyanobacteria (El Bissati *et al*., 2000). Therefore, these data are reported in appendix five (see *post-settlement bleaching*; Fig. A5.1), and as they provide only an approximation of treatment effects on quantum yield (and therefore photophysiology), absolute values should be interpreted with caution.

5.2.4 Juvenile sponges

To determine if sponge sensitivity to OW and OA differs at varying developmental stages, *C. foliascens* was exposed to the treatments one and two years post-settlement (hereafter referred to as 1-year recruits and 2-year recruits). During these life stages, morphology changes significantly (Fig. 5.6c-d). *C. foliascens* recruits reared at OIRS in ambient seawater were transported to AIMS in December 2013. Sponges were acclimatised in ambient seawater for two months and then transferred into experimental tanks (using the same 3 x 3 experimental design and ramping schedule as for the adult sponge experiment), with n = 6 1-year recruits *per* treatment and n = 11-15 2-year recruits *per* treatment. Recruits were exposed to treatment conditions for six weeks and photographs were taken at T0 and T-end. ΔF/Fm' was measured following the method described for adult sponges in chapter two, however a 2.0 mm active diameter plastic fibre replaced the standard 5.5 mm fibre to measure ΔF/Fm' in the 1-year recruits due to their size. As with the adult sponges, F_v/ F_m was also measured and are reported in appendix five (Fig. A5.2a-b). ImageJ software was used to determine the percentage of necrotic or bleached tissue in each sponge, following the same methods described for the adult sponges.

5.2.5 Data analysis

Data analyses were performed by PRIMER-E (PRIMER version 6.0, PERMANOVA+, Plymouth Marine Laboratory, Plymouth, UK). All graphs were generated using GraphPad Prism (GraphPad Software, version 6.00 for Windows, La Jolla California USA). To test the effect of temperature and pH on 1-year recruit and 2-year recruit ΔF/Fm' over time, a permutational three-way analysis of variance (PERMANOVA) was employed with tank and sponge individual as random effects. Where significant treatment effects were revealed, permutational *post hoc* pairwise comparison tests were used to determine which treatments differed significantly. The results of the *post hoc* test for each time point are reported in Appendix Table A5.3a-b, however only significant differences at T-end will be discussed here. For the remaining response variables (pre-settlement survival, motility and settlement success;

post-settlement growth and colour score/bleaching; 1-year and 2-year recruit tissue necrosis and bleaching) a two-way PERMANOVA was employed to test the effects of temperature and pH. For all response variables, Euclidean distances were used to generate a resemblance matrix. Permutational *post hoc* comparisons were used to determine which treatments differed significantly. A 5% significance level was used for all tests.

5.3 Results

5.3.1 Pre-settlement effects

An interactive effect of temperature and pH on larval mortality was observed after six hours of exposure to treatment conditions (Pseudo-$F_{(1, 68)}$ = 6.0863, *P* = 0.008; Fig. 5.2c, Table A5.1), with the average percentage of dead larvae being highest at 31.5°C/pH 8.1 (*P* = 0.005), compared to the other temperature and pH treatments. An interactive effect of temperature and pH was also detected on larval motility after six hours of exposure (Pseudo-$F_{(1, 68)}$ = 5.0732, *P* = 0.029; Fig. 5.2a), with more motile larvae at 28.5°C/pH 8.1 (*P* = 0.026). Throughout the experiment, more larvae remained motile at 28.5°C than 31.5°C, as evidenced by a higher average percentage of motile larvae at 28.5°C after both 12 (Pseudo-$F_{(1, 68)}$ = 20.76, *P* = 0.001; Fig. 5.2a) and 24 hours of exposure (Pseudo-$F_{(1, 68)}$ = 4.7232, *P* = 0.032; Fig. 5.2a). However, after 24 hours, no effect of temperature or pH on overall larval survival and settlement success was detected (Fig. 5.2; Table A5.1).

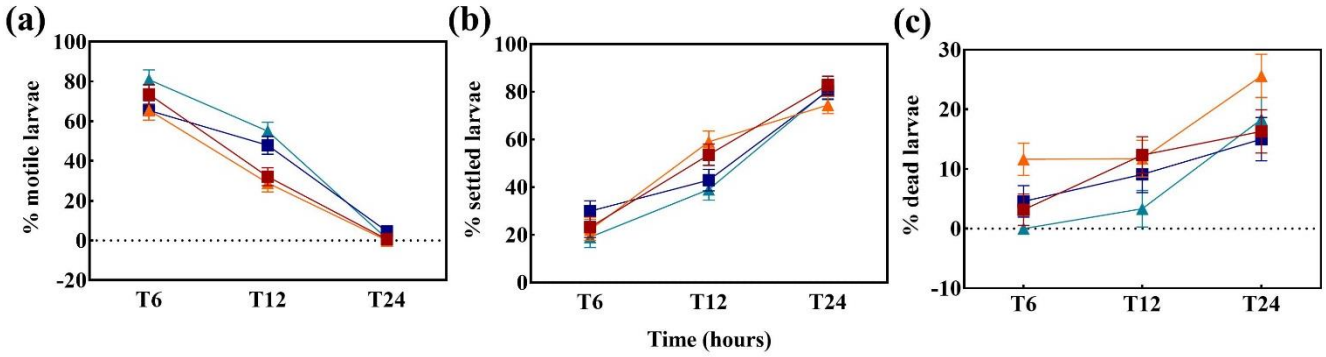


Fig. 5.2 Percentage of larvae (a) motile (b) settled and (c) dead at 6, 12 and 24 hours post-exposure to experimental treatments. ▲ 28.5°C/pH 8.1, ■ 28.5°C/pH 7.6, ▲ 31.5°C/pH 8.1, ■ 31.5°C/pH 7.6. Values are % larvae motile, settled or dead in each well at each sampling time point (calculated as a proportion of the total initial larvae in each well), averaged across n = 18 wells *per* treatment. Note: y-axis scales differ between response variables.

5.3.2 Post-settlement mortality and bleaching

Eighty percent of post-settlement mortality occurred at 31.5°C (Total mortalitiy *per* treatment: 28.5ºC/ pH 8.1 = 2; 28.5 ºC/ pH 7.6 = 0; 31.5 ºC/ pH 8.1 = 5; 31.5 ºC/ pH 7.6 = 3). A significant effect of temperature was observed on recruit growth (Pseudo-$F_{(1, 184)}$ = 20.092, *P* = 0.001; Fig. 5.3a), with average growth in recruits exposed to 31.5°C being significantly less than in recruits at 28.5°C. There was also a significant effect of pH on recruit growth (Pseudo-$F_{(1, 184)}$ = 3.924, *P* = 0.043; Fig. 5.3a), with growth significantly higher at pH 7.6 than at pH 8.1. A significant

interaction between temperature and pH affected recruit bleaching ($F_{(1, 185)} = 54.643$, $P = 0.001$; Fig. 5.3b). Colour score increased significantly at 31.5°C/pH 8.1 ($P = 0.001$), compared to the other temperature and pH treatments. On a grayscale, where 0 = black and 255 = white, recruits at 31.5°C/pH 8.1 had a significantly higher average colour score than recruits in the other treatments (Fig. 5.3b), i.e. they had colours more towards the white end of the scale, indicative of bleaching. These results are supported by ΔF/Fm' measures (see appendix five – *Post-settlement bleaching*).

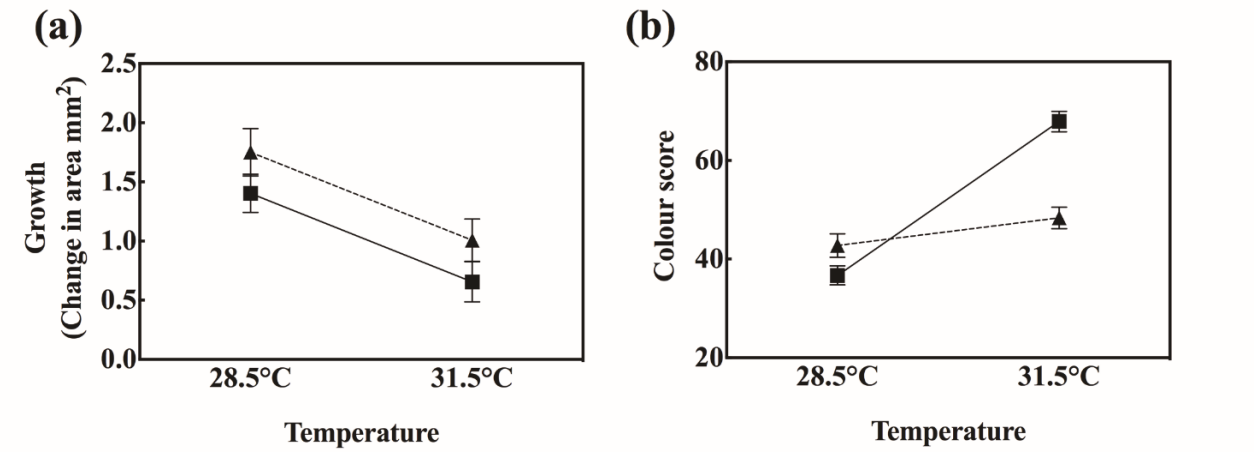


Fig. 5.3 *Carteriospongia foliascens* (a) growth (b) colour score one-month post-settlement at 28.5°C and 31.5°C where -■- = pH 8.1 and --▲-- = pH 7.6. Values are mean growth and colour score (measured using ImageJ). Growth was measured as change in surface area of each recruit (mm^2) between T0 and T-end. Colour score (used as an indicator of bleaching) was measured using ImageJ grayscale. The area of each recruit was selected and the mean grayscale was calculated (0 = pure black and 255 = pure white). n = 14 recruits *per* treatment, except 28.5°C/pH 7.6 where n = 15 and except where mortalities occurred. Note: y-axis scales differ between response variables.

5.3.3 Survival of juvenile sponges

While 80% of the mortality in 1-year recruits occurred at 31.5°C, no mortality occurred at 31.5°C/pH 7.6 (Fig. 5.4a). A significant effect of temperature on tissue necrosis (Pseudo-$F_{(2, 45)} = 20.049$, $P = 0.001$) and bleaching (Pseudo-$F_{(2, 45)} = 159.14$, $P = 0.001$) was detected in 1-year recruits. The percentage of tissue necrosis and bleaching was significantly higher at 31.5°C than 30°C ($P = 0.001$) and 28.5°C ($P = 0.001$; Fig. 5.4a; Table A5.2a-b). Ninety one percent of 2-year recruit mortality occurred at 31.5°C, with 50% of those mortalities occurring at pH 8.1 (Fig. 5.4b). A significant effect of temperature (Pseudo-$F_{(2, 107)} = 19.979$, $P = 0.001$) and pH (Pseudo-$F_{(2, 107)} = 3.7441$, $P = 0.022$) on tissue necrosis was also observed in 2-year recruits. The percentage of tissue necrosis was significantly higher at 31.5°C than 30°C ($P =$

0.001) and 28.5°C (P = 0.001; Fig. 5.4b; Table A5.2a, c), and overall tissue necrosis levels were lower at pH 7.8 than pH 8.1 (P = 0.020). A significant interaction between temperature and pH affected 2-year recruit bleaching (Pseudo-$F_{(4, 107)}$ = 2.4388, P = 0.049; Fig. 5.4b; Table A5.2a, c), with the percentage of tissue bleached being higher in recruits at 31.5°C/pH 8.1 than 31.5°C/pH 7.8.

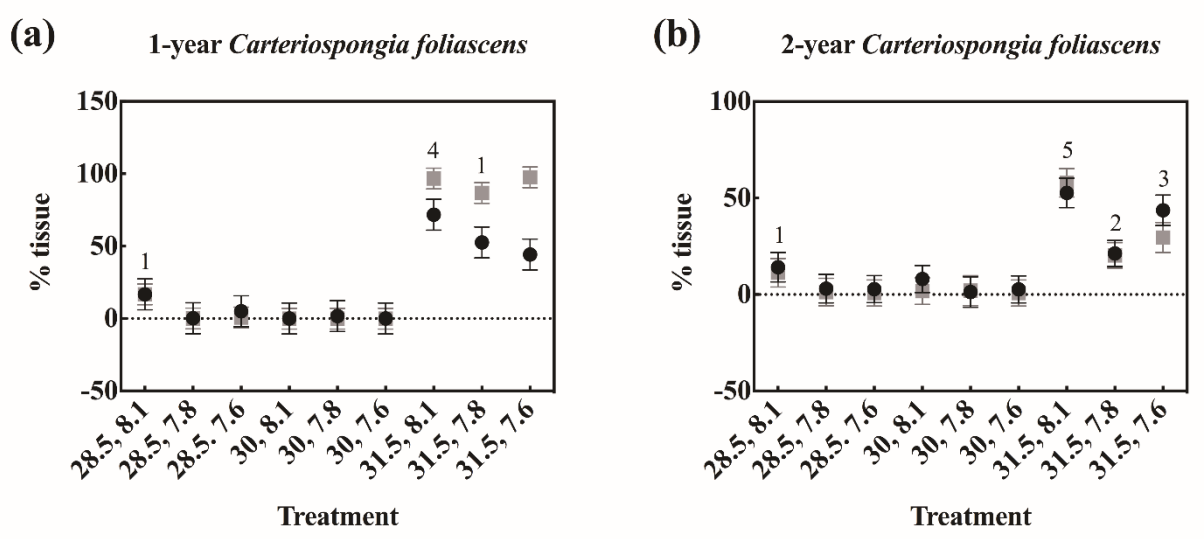


Fig. 5.4 Health state of juvenile sponges following exposure to treatments: (a) 1-year *Carteriospongia foliascens* recruits; (b) 2-year *Carteriospongia foliascens* recruits. Percent tissue necrosis ● and % tissue bleached ■ in each treatment, calculated using ImageJ (y-axis = % tissue, x-axis = treatment). Values are means ± SE (n = 6 for 1-year *C. foliascens* recruits and n = 11-15 for 2-year *C. foliascens* recruits). Numbers above treatments represent the total mortalities in the corresponding treatment.

5.3.4 Juvenile sponge effective quantum yield

ΔF/Fm' declined significantly at 31.5°C for both 1-year and 2-year recruits. For the 1-year recruits, a significant interaction was detected between temperature and time (Pseudo-$F_{(10, 164)}$ = 19.841, P = 0.001; Fig. 5.5a), with declines in the 31.5°C treatment occurring after one week of exposure. *Post hoc* analysis revealed that, at T-end, ΔF/Fm' was lower (zero) in all sponges exposed to 31.5°C, compared to the average ΔF/Fm' of sponges at 28.5°C (P = 0.001) and 30°C (P = 0.001; Table A5.3a). A significant interaction between temperature and pH affected ΔF/Fm' over time for the 2-year recruits (Pseudo-$F_{(24, 657)}$ = 1.9845, P = 0.005; Fig. 5.5b). ΔF/Fm' declined significantly at 31.5°C, with the greatest decline at pH 8.1. *Post hoc* analysis revealed that, at T-end, average ΔF/Fm' at 31.5°C was lower at pH 8.1 than at pH 7.8 (P = 0.002) and pH 7.6 (P = 0.049; Table A5.3b). A similar interactive effect occurred at 30°C, with

average ΔF/Fm' being significantly lower in the sponges exposed to pH 8.1 than those at pH 7.8 (P = 0.017; Table A5.3b). Interestingly, while ΔF/Fm' did not decline at 28.5°C, average ΔF/Fm' was significantly higher in the sponges exposed to pH 7.6 than those at pH 7.8 (P = 0.008) and pH 8.1 (P = 0.001), and at pH 7.8 compared to pH 8.1 (P = 0.018; Table A5.3b). Whilst true F_v/F_m could not be obtained, it is noteworthy that both light (Fig. 5.3c-d) and dark (Fig. A5.2a-b) quantum yield measurements responded similarly to the treatments over time, increasing our confidence that temperature was responsible for photoinhibition rather than variable fluorescence and/or state transitions.

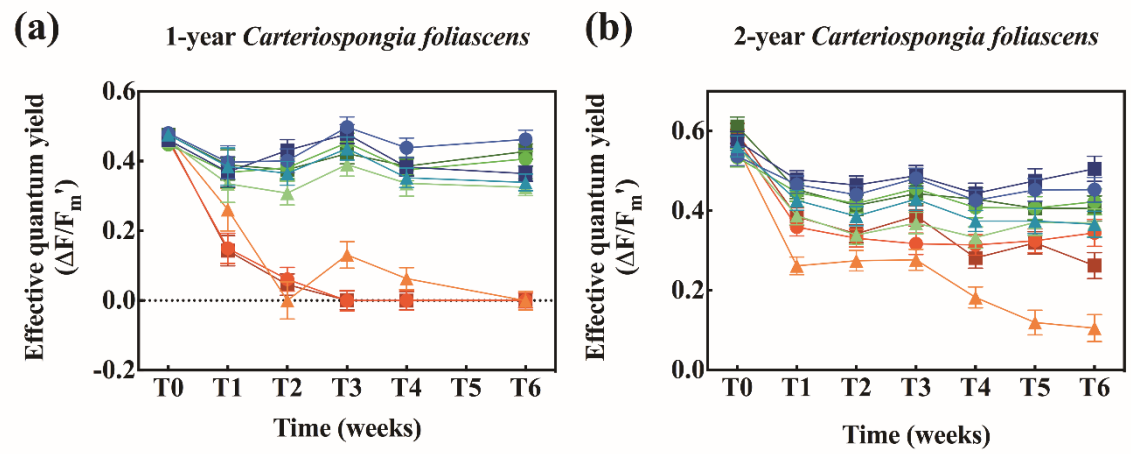


Fig. 5.5 Effective quantum yield for (a) 1-year *Carteriospongia foliascens* recruits and (b) 2-year *Carteriospongia foliascens* recruits in each treatment. ▲ 28.5°C/pH 8.1, ● 28.5°C/pH 7.8, ■ 28.5°C/pH 7.6, ▲ 30°C/pH 8.1, ● 30°C/pH 7.8, ■ 30°C/pH 7.6, ▲ 31.5°C/pH 8.1, ● 31.5°C/pH 7.8, ■ 31.5°C/ pH 7.6. Values are mean effective quantum yield *per* treatment ± SE *per* sampling time point (n = 6 for 1-year *C. foliascens* recruits and n = 14-15 for 2-year *C. foliascens* recruits *per* treatment per time point, except where mortalities occurred). Note: y-axis scales differ between species.

5.4 Discussion

Despite the survival of early life history stages being fundamental for the persistence of an organism, there is a paucity of climate change research considering the interactive effects of OW and OA on multiple life history stages of the same species (Byrne, 2012; Dupont & Pörtner, 2013). In the first study to test the combined effects of Ocean Warming (OW) and Ocean Acidification (OA) on early-life history stages of sponges, I examined the response of larval and juvenile stages of the abundant Indo-Pacific phototrophic sponge *Carteriospongia foliascens* to OW and OA levels predicted under climate change scenarios projected for 2100. Pre-settlement larvae experienced mortalities sooner when exposed to elevated temperatures, and more larvae remained motile under present day temperatures for longer, suggesting reduced larval planktonic durations (PLD) for *C. foliascens* under future OW. However, OW and OA had no effect on the overall pre-settlement survival and settlement success of *C. foliascens* larvae. This is in contrast to responses reported for early life stages of many other marine invertebrates, and may facilitate the persistence of sponges in a changing climate *via* dispersal; or as a source for genetic adaptation as the climate changes. *C. foliascens* sensitivity to OW increased with age, with bleaching, reduced growth and mortality at 31.5°C. However, juvenile sponges still exhibited much greater tolerance to OW than their adult counterparts (chapter two), again with evidence that OA reduces OW stress for some of these life stages. Tolerant early-life history stages, combined with elevated pCO_2 providing *C. foliascens* with protection from elevated temperature across these different life stages, suggests this species has the capacity to survive in a high CO_2 world.

5.4.1 Pre-settlement

A remarkable tolerance of the GBR sponge *Rhopaloeides odorabile* larvae to environmental stress has previously been demonstrated for both elevated temperature (Whalan *et al.*, 2008b; Webster *et al.*, 2011) and anthropogenic contamination (Negri *et al.*, 2016). However, to date no study has focused on the response of larval stages of sponges to the combined effect of OW and OA. Here I found that although *C. foliascens* PLD were shorter, and mortalities occurred earlier for sponges exposed to high temperatures, overall the pre-settlement survival and settlement success of *C. foliascens* larvae were unaffected by temperature and pH. While a reduction in PLD and increased mortality risk have implications for dispersal capability of this species *in situ* (Whalan *et al.*, 2008b), the tolerance of this life stage may provide a means for acclimation/adaptation, enabling species persistence in a changing climate. These findings are

in stark contrast to the OW and OA sensitivity reported for many marine invertebrate larval stages (Przeslawski *et al.*, 2015), which potentially represent a bottleneck for these populations under future OW and OA conditions. In corals, elevated temperature negatively affects larval duration, survivorship and productivity (Edmunds *et al.*, 2001; Heyward & Negri, 2010). Additoinally, OA conditions create unfavourable settlement substrates, reducing settlement cues and larval settlement, and also depress coral larval metabolic rates (Albright & Langdon, 2011; Doropoulos *et al.*, 2012; Webster *et al.*, 2012). Larval stages of other marine invertebrates such as the oysters *Crassostrea gigas* and *Saccostrea glomerata* are also sensitive to OW and OA. While larval development is accelerated for both species under OW, OA lowers development success and a combination of these stressors results in an increase in the development of morphologically abnormal larvae (Parker *et al.*, 2010). Similar sensitivities were observed for the abalone *Haliotis coccoradiata*, in which exposure to OW and OA caused the development of unshelled larvae, and abnormal juveniles (Byrne *et al.*, 2010).

Post release larval behaviours are suggested to explain the tolerance of *R odorabile* larval stages to OW. These larvae exhibit positive phototaxis in which upon release they migrate to the upper water column, where temperatures tend to be warmer and more highly variable than near the benthos (Whalan *et al.*, 2008a; Whalan *et al.*, 2008b). As a result, planktonic life stages are posited to have physiological traits that provide them with greater thermal tolerance than adult stages, likely *via* maternal provisioning (Hamdoun & Epel, 2007). While *C. foliascens* larvae display negative phototaxis *post* release, a high percentage are demonstrated to vertically migrate at nightfall, if settlement is yet to occur (Wahab *et al.*, 2014b). As with *R. odorabile*, this exposes these larval stages to the variable and potentially extreme temperatures of the surface waters, for at least some of their PLD. Similarly, *C. foliascens* releases larvae year round, consequently these life-stages are subjected to, and must be adapted to tolerate a range of hydrodynamic processes and currents (Wahab *et al.*, 2014b), which may explain in part the tolerance observed for these life-stages to OW and OA.

The tolerance of planktonic larval stages may facilitate the migration of sessile sponges into cooler environments as the ocean warms (Walther *et al.*, 2002; Byrne, 2011). However, due to short competency periods and poor swimming abilities of sponge larvae (Whalan *et al.*, 2007; Wahab *et al.*, 2014b), it is uncertain whether sponge dispersal capabilities and subsequent range expansion will keep up in a warming world. These tolerant larval stages may also act as a source for gradual acclimatisation and genetic adaptation as the climate changes, and are likely

to prove critical in the survival and adaptive capacity of sponges in a high CO_2 world. As the environmental selection of resistant populations is influenced by generational turnover time, genetic adaption capacity is likely to be species dependent (Harley *et al.*, 2006; Byrne, 2011). For example, short-lived sponge species with fast generation times such as the opportunistic *Mycale fistulifera* (Meroz & Ilan, 1995), are likely to have a greater capacity for evolutionary adaption compared to longer lived sponges such as the slow growing giant barrel sponge *Xestospongia muta* (McMurray *et al.*, 2008).

5.4.2 Post-settlement

As sponges age, their sensitivity to OW and OA apparently increases, as evidenced by highly tolerant *C. foliascens* larval stages that become increasingly vulnerable between one month, one year, two years, and finally after they have developed into adult sponges (Fig. 5.6). Whilst post-settlement survival, growth and productivity of 1-month, 1-year and 2-year old *C. foliascens* were adversely impacted by elevated temperature, the response of these life-history stages was still far less severe than what was observed in the adult sponges (chapter two). While differences in tolerance between developmental stages of the same species have been demonstrated for a number of marine invertebrates (Byrne, 2011), early developmental stages tend to be more vulnerable to warming than later stages (Randall & Szmant, 2009; Przeslawski *et al.*, 2015), for *C. foliascens* the opposite is true.

Furthermore, while 1-month recruits at 31.5°C bleached and experienced lower growth rates than those in the 'present day' temperature treatment, recruits at 31.5°C/pH 7.6 did not bleach and exhibited less change in growth rates compared to the controls, highlighting an ameliorative effect of elevated pCO_2 at high temperatures. While additional pCO_2 under OA buffers the negative effects of OW for sponge recruit bleaching, this same positive feedback is not observed for post-settlement success of other marine invertebrates, particularly those with calcifying stages as a consequence of the direct effect of OA on calcification processes, which is not a factor in these demosponges. For example, while OW alone did not impact post-larval stages of the barnacle *Semibalanus balanoides*, OA negatively impacted growth (Findlay *et al.*, 2010b). Moreover, the combined effect of OW and OA resulted in reductions in shell calcium carbonate content as well as overall survival of *S. balanoides* juveniles (Findlay *et al.*, 2010a). In corals, elevated pCO_2 reduces post-settlement growth of recruits, likely due to decreased calcification (Albright & Langdon, 2011; Anlauf *et al.*, 2011). Furthermore, while primary polyp growth of the coral *Porites panamensis* was reduced under OA, the combination of OW

and OA reduced polyp growth further as associated zooxanthellae were lost with elevated temperatures (Anlauf *et al.*, 2011).

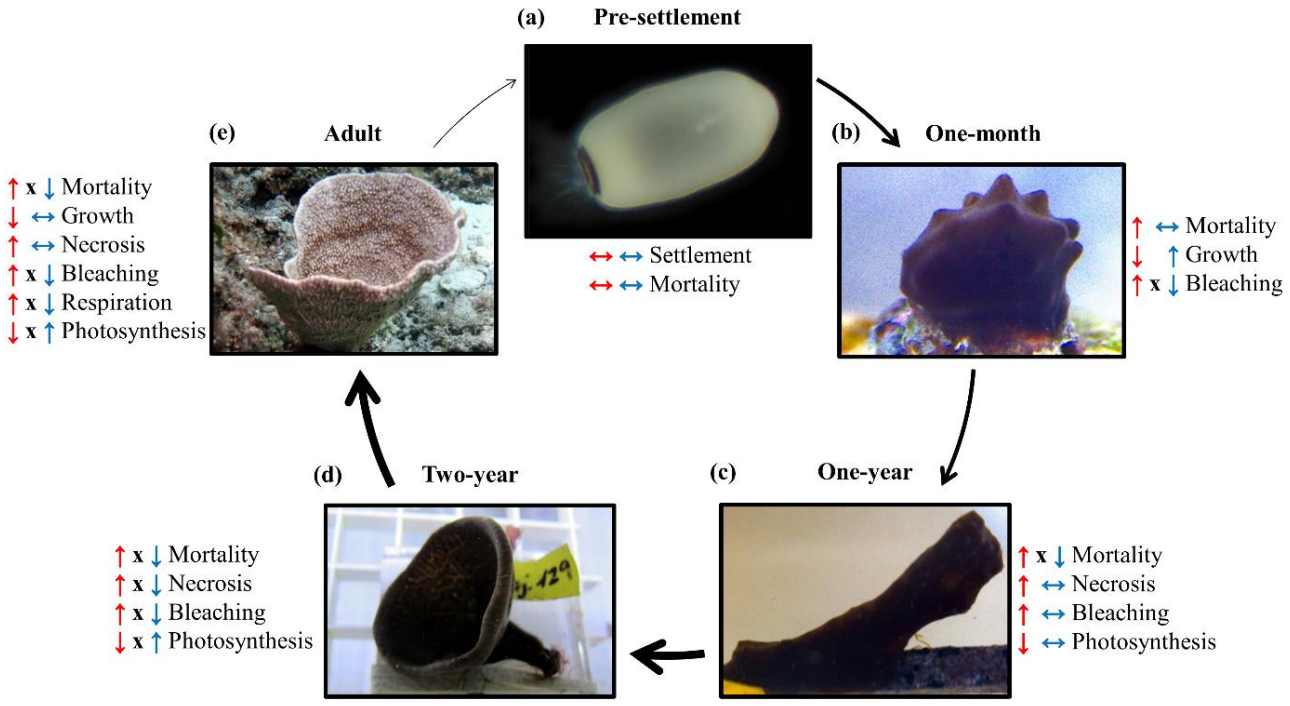


Fig. 5.6 Summary of response of *Carteriospongia foliascens* across different life-history stages to the OW and OA conditions predicted for 2100 under RCP8.5 (31.5°C/pH 7.6): (a) pre-settlement; (b) 1-month recruit; (c) 1-year recruit; (d) 2-year recruit; (e) adult (chapter two). Arrows indicate the effect that temperature (red) and pH (blue) have on the measured sponge response, where ↑ = increased, ↓ = decreased, ↔ no effect and **x** = an interaction between temperature and pH. Thickness of arrow between life-history stages indicates a change in sensitivity to OW, whereby thicker arrows = increased sensitivity.

Increased sensitivity with sponge age was further evidenced by comparing the responses of 1-year and 2-year recruits with those of adult sponges. Whilst all of the 1-year-old *C. foliascens* recruits bleached within a week of exposure to 31.5°C, many sponges survived for the full 6-week experiment. Sensitivity further increased in the 2-year recruits, with mortalities occurring soon after bleaching at 31.5°C, although a positive effect of elevated pCO_2 on 2-year recruit survival, tissue necrosis and bleaching and effective quantum yield at 31.5°C was observed. This ameliorative effect of OA is likely due to an increase in symbiont productivity enabling phototrophic sponges to have higher productivity in an environment where more inorganic carbon is available for photosynthesis as demonstrated for sponge associated cyanobacteria (Fu

et al., 2007; Morrow *et al.*, 2015) and symbiotic dinoflagellates (Fang *et al.*, 2014, Stubler *et al.*, 2015). Such an increase in productivity with elevated pCO_2 has been observed in other holobionts including in corals, sea anemones and sponges residing at CO_2 vent seeps (Suggett *et al.*, 2012; Morrow *et al.*, 2015; Strahl *et al.*, 2015b), however this is the first time this has been observed in early-life stages of sponges.

In a review of OW and OA impacts on marine invertebrate life-histories, it was concluded that whilst antagonistic effects of OW and OA are common, in most elevated temperatures modulate the negative effects of OA (Byrne & Przeslawski, 2013). For example, larval development of the tropical sea urchin *Tripneustes gratilla* was accelerated under OW, and reduced with OA. When these stressors were applied in combination, OW reduced the negative effect of OA on growth processes (Brennand *et al.*, 2010). Similarly, for the sea urchin *Heliocidaris erythrogramma* OA resulted in abnormal juvenile development, again however OW reduced this negative effect (Byrne *et al.*, 2010). Here I demonstrated an antagonistic interaction between temperature and pH for *C. foliascens*, with elevated pCO_2 reducing the negative effect of temperature stress on all life stages, except the 1-year morphologies for which bleaching occurred so rapidly that pCO_2 effects were not observed (Fig. 5.6).

5.4.3 Summary

Environmental factors that disrupt larval dispersal, recruitment and early development have wide implications for population dynamics and the ultimate survival of a species (Albright & Langdon, 2011). Here I found that while PLD of *C. foliascens* may be reduced under future OW and OA, survival and settlement success is unaffected. The tolerance of this planktonic stage is likely to prove critical in the survival and adaptive capacity of *C. foliascens* in a high CO_2 world, not only as a vector into more suitable environments, but as a source for genetic adaptation as temperatures rise. While we see an increase in sensitivity of *C. foliascens* with age, the tolerance of one-month and 1-year juvenile benthic stages further increases *C. foliascens* adaptation potential. However, further investigations involving transgenerational studies are required to understand the acclimation potential of these species *via* such tolerant early-life stages. Finally, while *C. foliascens* 2-year (and adult, chapter two) life stages are more thermally sensitive, a mitigative effect of OA is observed, thus increasing acclimation potential further. Overall these results demonstrate the importance of whole development studies to fully understand the response of an organism to environmental change (Byrne, 2012). Additionally, while previous research supports the finding that early-life stages of sponges are

generally tolerant to environmental stress (Whalan *et al.*, 2008b; Webster *et al.*, 2011; Negri *et al.*, 2016), tolerance thresholds can differ between even closely related species (Byrne, 2011). Therefore future work should consider the response of early-life stages of other sponge species to the combined effect of OW and OA, to get a more comprehensive understanding of how these important organisms will fare in a high CO_2 world.

Chapter six

General discussion

Sponges in the National Sea Simulator at the Australian Institute of Marine Science.

This PhD thesis explores the response of tropical sponges to Ocean Warming (OW) and Ocean Acidification (OA), caused by increasing atmospheric carbon dioxide ($CO_{2\ atm}$). The aim of this thesis was to bridge gaps in our understanding of how these important coral reef organisms will respond to projected climate change. In a series of experiments, carried out in the Australian Institute of Marine Science's (AIMS) National Sea Simulator (SeaSim), a number of sponge species and life-history stages were exposed to the OW and OA conditions predicted under the latest Intergovernmental Panel on Climate Change (IPCC) CO_2 representative concentration pathways (RCP, IPCC) for 2100. OW and OA levels were simulated under two RCP CO_2 concentrations (RCP6.0 and RCP8.5), whereby mean sea surface temperatures are predicted to increase 1.5-3.0°C alongside declines in global average ocean pH of 0.3-0.5 units by 2100 (IPCC, 2014). The response of the sponge holobiont to these conditions was examined over multiple scales, from survival to sponge function, in which physiological processes including growth and metabolism were measured. Biochemical responses were subsequently assessed to determine the effect of these stressors on sponge energy stores and membrane functioning, with the aim to understand mechanisms that underpin sponge tolerance to OW and OA. To provide greater insight into the population level impacts of climate change on tropical sponges I also explored the response of the phototrophic species *Carteriospongia foliascens* to OW/OA throughout its developmental stages. Here I will revisit the major findings of my research and apply this enhanced mechanistic understanding of sponge responses to OW and OA to address the prognosis for sponge survival in a high CO_2 world. Differences in species tolerances will then be considered to provide insight into the composition of future sponge assemblages. Finally, an assessment of the potential for sponge-coral regime shifts in a changing climate will be made with an appraisal of how this alternate state might function in terms of alterations to ecosystem productivity and biodiversity.

6.1 The Response of Tropical Sponges to Ocean Warming and Ocean Acidification

My research demonstrates that whilst tropical sponges are mostly unaffected by climate change scenarios predicted under the RCP6.0 conditions for 2100, environmental projections for the end of this century under the RCP8.5 have significant implications for their survival. Elevated temperature under RCP8.5 negatively affected the health and survival of all four GBR sponge species, with mortality, high levels of tissue necrosis, elevated respiration and bleaching evident at 31.5°C. Importantly, elevated pCO_2 differentially affects sponges with different

nutritional modes, exacerbating the impact of elevated temperature in heterotrophic species but ameliorating the effect of elevated temperature in phototrophic species (Fig. 6.1). Mechanisms underpinning these different responses were then explored. Lipid class and fatty acid (FA) analysis revealed that additional energy available under OA, due to the stimulatory effect of CO_2, may facilitate the resistance of phototrophic sponges to OW through increased membrane lipid and essential FA biosynthesis (Fig. 6.1). Additional tolerance mechanisms unrelated to nutritional mode but instead pertaining to cell homeostasis were also revealed (Fig. 6.1). Finally, my research found that early life-history stages of sponges are more tolerant to OW and OA than adult sponges.

6.2 Acclimation and Adaptation Strategies for Sponge Survival in a High CO_2 Ocean

Benthic marine invertebrates, including sponges, are limited in their ability to directly escape unfavourable conditions (Przeslawski *et al.*, 2008) and in order to survive adversity they must either acclimate or adapt (Hoegh-Guldberg, 1999). Acclimation involves a direct response by the organism in which various physiological processes are modified to perform better under the new environmental conditions. Adaptation subsequently occurs *via* the selection of individuals within populations that are better able to cope with the new environment, be it through acclimatory responses or as a result of inherent tolerance (Hoegh-Guldberg, 1999).

My thesis reveals a number of indicators of innate sponge tolerance to OW and OA that provide insight into the types of sponges that can survive and therefore adapt in a high CO_2 ocean (Fig. 6.1). In Chapter Two it was demonstrated that sponges with spiculated skeletons are more resistant to environmental stressors than aspiculate species with keratose skeletons (Fig. 6.1). This confirms the importance of skeletal mineralogy in the sensitivity of sponges to OW and OA (Vicente *et al.*, 2016), and demonstrates that skeletal forms other than calcium carbonate mineralogies are less resilient to climate change. While it is still unclear what the advantage of being spiculated is under these conditions (Vicente *et al.*, 2016), a robust skeleton is likely to facilitate the adaptation of such sponges to a rapidly changing climate. Another key indicator of sponge tolerance of OW and OA identified in this thesis is that sponges with a greater contribution of storage lipids, as well as a higher proportion of phospholipids and sterols, and higher concentrations of n-3 and n-6 essential PUFAs exhibit greater resistance to OW and OA (Fig. 6.1). These lipids are the main constituents of the lipid bilayer of cell membranes and will likely contribute to the ability of sponges to maintain appropriate membrane function and subsequent cell homeostasis in the face of environmental change (Geider, 1987; Hazel, 1995;

Guillot *et al.*, 2000; Singh *et al.*, 2002). Sponges can also persist in a changing climate *via* dispersal of tolerant planktonic larval stages. These early life stages may migrate into more suitable environments, or act as a source for genetic adaptation as the climate changes, and will prove critical in the survival and adaptive capacity of sponges in a high CO_2 world (Albright & Langdon, 2011; Przeslawski *et al.*, 2015).

A number of acclimatory responses were observed in sponges exposed to OW and OA, which likely contribute to the capacity of these organisms to survive environmental adversity. In particular, elevated pCO_2 provides phototrophic sponges with protection from thermal stress. Lipid analysis revealed a positive feedback and possible acclimation pathway underlying this ameliorative effect for the phototrophic *Carteriospongia foliascens.* Additional energy available to sponges under OA, due to the stimulatory effect of CO_2, may facilitate the tolerance of *C. foliascens* to OW through increased sterol biosynthesis (Fig. 6.1). As sterols are important components of the lipid bilayer that forms cell membranes, alterations to the sterol content of this bilayer can correct membrane fluidity in a form of homeoviscous adaptation (Nes, 1974; Volkman, 2003; Bergé & Barnathan, 2005; Martin-Creuzburg & Elert, 2009). The ability of phototrophic sponges to utilise additional energy available under OA to maintain appropriate membrane function under thermal stress reveals an acclimation pathway for the survival of these sponges in a changing climate (Geider, 1987; Hazel, 1995; Singh *et al.*, 2002).

Another acclimatory response was observed for the heterotrophic sponge *Stylissa flabelliformis* in which respiration rates were depressed at low pH. Metabolic depression is suggested to be a short term strategy to protect against acidosis and elevated carbonic acid in interstitial fluids (Fabry *et al.*, 2008; Przeslawski *et al.*, 2008). While the active suppression of metabolism in response to reduced pH may reduce the negative effect of OA, at least in the short term, respiration rates were elevated under RCP 8.5 conditions, indicating this strategy is ineffective when reduced pH is combined with thermal stress. In addition, although metabolic depression can provide some respite from the potentially toxic effects of CO_2, this state is likely to be detrimental to long term survival as important processes such as growth and reproduction are compromised to support basic biological functioning (Pörtner *et al.*, 2004). Interestingly, previous research suggests that increased productivity of phototrophic symbionts under OA can offset the cellular acidosis that leads to metabolic depression (Gibbin & Davy, 2014), possibly explaining why metabolic depression was not observed in the phototrophic sponge species.

It has been suggested that phototrophic species may tolerate OW and OA better than heterotrophic species if they can switch to acquiring carbon heterotrophically to account for lost photosynthetically translocated carbon which occurs during bleaching (Bell *et al.*, 2013). Lipid and FA profiles were used to determine how energy stores varied under OW and OA to see if energy demands in bleached phototrophic sponges were sustained and FA trophic markers were considered to look for specific changes in sponge nutrition. While there was some evidence to support this mechanism in both phototrophic species, this hypothesis requires further exploration as well as a much greater understanding of the nutritional interactions between sponges and their associated phototrophic microorganisms.

Finally, fatty acid analysis revealed two important responses to OW and OA that have enhanced our understanding of tolerance mechanisms and acclimation potential of different sponge species to global climate change. Firstly, phototrophic sponges increase the degree of lipid unsaturation (DoU) in response to thermal stress, while lipid unsaturation decreases in thermally stressed heterotrophic sponges (Fig. 6.1). An increase in DoU reflects a desaturase activation either in the host or symbiont as a means to increase membrane rigidity and/ or protect cells from toxic oxygen species at high temperatures (Guerzoni *et al.*, 1997; Guerzoni *et al.*, 2001). A decline in DoU, on the other hand, indicates there is some stress associated with lipid peroxidation in the cells as the unsaturated lipid content (the primary target for oxidative stress) is reduced. However, further work exploring the sponge host and associated symbiont responses in isolation is required before the mechanisms underlying these responses can be fully elucidated. Secondly, more thermally resistant sponges not only have a higher long chain PUFA content as mentioned previously, but are capable of retaining these essential FAs under OW, compared to the more sensitive sponges for which PUFAs declined with thermal stress (Fig. 6.1). PUFAs are the primary constituents of phospholipids in sponge cellular membranes, and this capability likely provides thermal resilience and identifies yet another mechanism of environmental acclimation (Lawson *et al.*, 1988; Mueller-Navarra, 1995; Tocher, 2003).

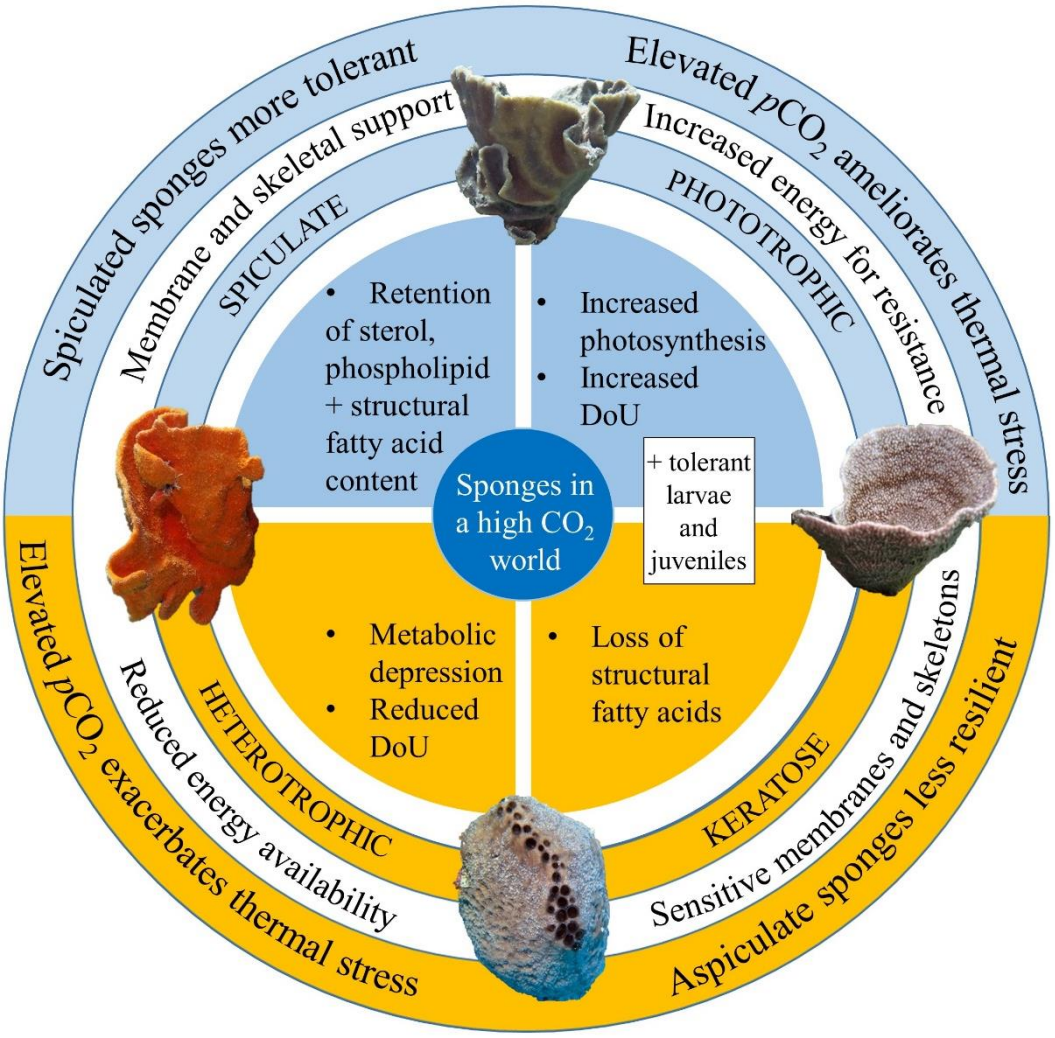


Fig. 6.1 Sponges in a high CO_2 world. Significant differences in tolerance were observed between four main "types" of sponges; heterotrophic and phototrophic sponges, as well as spiculated and aspiculated sponges. For each sponge "type" observed responses that likely contribute towards OW and OA tolerance are listed in the centre. Mechanisms for tolerance and implications for sponge survival are presented as outer rings for each sponge type where orange denotes sensitivity and blue designates tolerance. These results suggest that future sponge assemblages will be populated by phototrophic sponges and sponge species with a skeleton formed of siliceous spicules. Two other important types of sponges that were not considered in this study, for which sensitivity levels have been identified in the literature, are bioeroding species and sponges with calcareous skeletal elements. Bioeroding sponges are likely to thrive under future OW and OA as productivity and bioerosion rates increase (Wisshak *et al*., 2012; Duckworth & Peterson, 2013; Wisshak *et al*., 2013; Fang *et al*., 2013; Stubler *et al*., 2015). Meanwhile calcareous sponges are predicted to be sensitive to climate change as lowered pH under OA impacts skeletal formation (Bell *et al*., 2013).

6.3 Potential for future coral-sponge regime shifts

It is expected that many of the reef building corals we see today will be extinct by the end of this century, as a consequence of environmental degradation (Frieler *et al.*, 2013). Sponges already appear to be moving into areas of coral reef where large coral mortality has occurred or reef conditions no longer favour coral survival (Bell *et al.*, 2013; Bell *et al.*, 2015b), and the effects of climate change are likely to weaken these stressed coral reefs further (Hughes *et al*. 2007). Reef-building corals already live close to their thermal limits, with bleaching occurring when temperatures exceed the summer maxima by just 1 to 2°C (Hoegh-Guldberg, 1999; Hoegh-Guldberg *et al.*, 2007). While interspecific variation between tolerance and the adaptive capacities of corals exists (Frieler *et al.*, 2013), even the most thermally tolerant species are unlikely to endure temperature increases exceeding 2° to 3°C above their adapted maxima. In fact, to preserve greater than 10% of the world's coral reefs it has been suggested that OW must be limited to less than 1.5°C (Frieler *et al.*, 2013), suggesting the likely RCP6.0 (+1.5°C) and RCP8.5 (+3°C) scenarios tested in this thesis are beyond the survival capacity of most coral species (Veron *et al.*, 2009). At the same time, corals are faced with OA, with experiments showing coral calcification decreases as pH is reduced (Hoegh-Guldberg *et al.*, 2007), likely increasing the sensitivity of corals to thermal stress (Frieler *et al.*, 2013). Furthermore, increases of just 2°C negatively affect coral larval duration, development, survival and productivity (Edmunds *et al.*, 2001; Randall & Szmant, 2009; Heyward & Negri, 2010). *In situ* studies confirm that OA conditions have negative implications for early-life stages of corals too, leading to unfavourable settlement substrates, reductions in settlement cues and larval settlement, as well as decreasing post-settlement growth of coral recruits (Albright & Langdon, 2011; Anlauf *et al.*, 2011; Doropoulos *et al.*, 2012; Webster *et al.*, 2012).

At least for the phototrophic and heterotrophic species studied here, sponges are able to cope with OW and OA levels predicted for 2100 under RCP6.0, a CO_2 concentration pathway that is predicted to surpass the survival capacity of most coral species. Furthermore, tolerant early-life history stages of sponges are likely to increase sponge survival potential, providing means for migration and adaptation. These results suggests that sponges may be future 'winners' on coral reefs under global climate change. While competition between sponges, corals and other organisms such as algae, was not assessed in this thesis, various chemical and physical adaptations make sponges one of the top spatial competitors in these incredibly space competitive ecosystems (Diaz & Rutzler, 2001; Rützler, 2002). Release from space

competition as coral declines may therefore result in a proliferation of sponges on coral reefs. As $CO_{2\ atm}$ concentrations continue to rise and reach levels predicted under the RCP8.5 scenario, inter-species differences in sponge tolerance will likely alter sponge assemblage composition. With additional CO_2 providing energy to buffer the negative effects of temperature it is likely we will start to see a shift in sponge populations to those dominated by phototrophic species. Additionally, it is also likely that sponge species with siliceous skeletal elements will become more abundant than those with less resistant skeletal mineralogy i.e. those with calcium carbonate skeletal elements or aspiculate sponges with spongin or collagenous fibres.

By determining whether sponges are able to withstand predicted OW and OA, and comparing this with reported coral sensitivities, my research has provided a basis for understanding the likelihood of coral-sponge regime shifts on coral reefs in the future (Bell *et al.*, 2013). This is not only relevant in light of the reported increases in sponge abundance on coral reefs globally (Aronson *et al.*, 2002; McMurray *et al.*, 2010; Colvard & Edmunds, 2011; Schils, 2012; Kelmo *et al.*, 2013; Kelmo *et al.*, 2014; Bell *et al.*, 2013; Bell *et al.*, 2015b), but also due to the major role sponges play on coral reefs (Wulff, 2006a; Bell, 2008; de Goeij *et al.*, 2013). This information can now be used for building ecosystem models to understand how sponge reefs might function, as it provides the basis for the "types" of sponges these future ecosystems may be dominated by. Importantly, however, elevated temperatures are reportedly responsible for a number of sponge declines globally (Cebrian *et al.*, 2011; Di Camillo *et al.*, 2013; Kelmo *et al.*, 2014) and sponge declines have also been reported in response to local stressors including sediment, disease, cyanobacterial blooms and algal outbreaks (Gaino *et al.*, 1992; Butler *et al.*, 1995; Davis *et al.*, 1997; Wulff, 2006b; Bell *et al.*, 2015a). Hence, not all sponges are likely to persist in a high CO_2 world, especially when faced with a multitude of other stressors.

6.4 Implications for sponge dominance on coral reefs

Shifts in sponge-coral dominance on coral reefs in present times have been primarily attributed to declines in coral cover as a result of degraded environmental conditions, to which sponges are tolerant (Aronson *et al.*, 2002; McMurray *et al.*, 2010; Colvard & Edmunds, 2011, Schils, 2012; Kelmo *et al.*, 2013; Bell *et al.*, 2015b). Here I show that some sponges can also tolerate the climate change scenarios predicted for 2100, suggesting that the trend of increasing sponge dominance may continue throughout the century as oceans warm and acidify. Changes to the abundance of sponges on coral reefs have implications for coral reef functioning. High

pumping and particle retention rates mean that sponges impact the water column by depleting nutrients, such as oxygen, nitrogen and silica, as well as microorganisms (Reiswig, 1971; Diaz & Rutzler, 2001). Increased sponge abundance is therefore likely to modify water column characteristics and have a large influence on reef ecosystem functioning. Sponges are shown to consume plankton in the 0.1 to 70 μm size range (Reiswig, 1971; Thomassen & Riisgård, 1995; Ribes *et al.*, 1999), preferably utilisating particles < 2 μm (Reiswig, 1975; Pile *et al.*, 1996; Turon *et al.*, 1997). Therefore, alterations to reef plankton communities are likely to occur as more of the nano- and picoplankton are consumed with increased sponge biomass. Sponges are also a critical link between dissolved organic carbon (DOC) in the surrounding seawater and the benthos. The recent discovery of the "sponge loop" reinforced this importance, demonstrating how sponge-facilitated carbon flow contributes to the productivity of oligotrophic coral reef waters (de Goeij *et al.*, 2013). Sponge associated microbial symbionts also contribute to reef primary productivity and other nutrient fluxes including nitrogen, phosphorous and silicon cycling (Reiswig, 1971; Cheshire & Wilkinson, 1991; Taylor *et al.*, 2007; Southwell *et al.*, 2008; Koopmans *et al.*, 2010, Maldonado *et al.*, 2012). Therefore, while increased water filtration may deplete nutrients and microorganisms from the water column, an increase in sponge biomass on coral reefs may concomitantly increase the carbon and sponge derived nutrients available for higher trophic levels, particularly in the form of recycled nutrients, waste products including sponge detritus and *via* direct predation on sponges themselves. Sponge derived nutrients facilitate the health and biodiversity of coral reefs (Slattery, 2013), suggesting that increased sponge abundance on coral reefs will continue to support high levels of species diversity. However, not all organisms are likely to benefit from increased sponge activity on reefs; recently proposed positive feedbacks resulting from increased DOC cycling between seaweeds and sponges on reefs where coral has declined are suggested to actually impede coral recovery. It has been suggested that alterations to nutrient dynamics through increased DOC cycling change the microbial activity of the water column, to the detriment of the coral microbiome which subsequently prevents coral recovery (Pawlik *et al.*, 2016).

With elevated pCO_2 providing phototrophic sponges with some protection from elevated temperature, climate change may ultimately drive a shift in the composition of sponge assemblages towards a dominance of phototrophic species. Tropical ecosystems dominated by sponges with phototrophic symbionts are likely to support an array of life as nutrients and photosynthetic energy produced by these symbionts is transferred through the food web,

supporting life in the nutrient-poor waters of the tropics (Wulff, 2006a; Bell, 2008; Bell *et al.*, 2013). However, while sponges can provide sufficient energy and nutrients to support highly diverse ecosystems, and although sponges provide habitat for coral reef organisms (Bell 2008), a reef dominated by sponges will certainly be less structurally complex than habitat provided by reef-building corals (Bell *et al.*, 2013). This has implications for the overall biodiversity of sponge dominated ecosystems as niche diversification potential is reduced (Bell *et al.*, 2013). Sponges play a key role in bioerosion and substrate consolidation on coral reefs. Consolidation of the reef framework following storm damage, which is predicted to become more frequent under RCP 8.5, will have positive implications for increased survivability of corals. However, research into the effects of elevated temperature and pCO_2 on bioeroding sponges demonstrates the bioerosion capacity of these sponges will increase under future OW and OA scenarios (Duckworth & Peterson, 2013; Fang *et al.*, 2013, Stubler *et al.*, 2014, Wisshak *et al.*, 2013). There are also a number of reports of bioeroding sponges already increasing in abundance on reefs where coral cover has declined (Bell *et al.*, 2015b). Therefore while many sponge species may serve as reef binders (Wulff and Buss 1979), an increase in excavating sponges and their bioerosion rates is likely to prompt an imbalance in erosional processes on coral reefs (Glynn & Manzello, 2015). Ultimately, as increasing pCO_2 reduces coral calcification rates, weakens existing calcium carbonate structures, and increases the capacity for bioerosion, the current balance between reef growth, consolidation and erosion is likely to shift in favour of reef erosion (Enochs *et al.*, 2015).

6.5 Future directions

While significant headway into defining symbiont derived nutrient transfer to the sponge host has been made (Freeman *et al.*, 2013), our current understanding of nutritional interactions between sponge associated photosymbionts and the sponge host remains limited (Davy *et al.*, 2002; Webster & Blackall, 2009). Many reef sponges form symbiotic relationships with photosynthetic microorganisms (Taylor *et al.*, 2007) and research focusing on defining nutrient exchange between the sponge and its phototrophic microorganisms under different environmental conditions will increase our understanding of the implications of OW and OA on holobiont functionality. Additionally, further work should consider the sponge host and associated symbiont responses in isolation to better understand the mechanisms underlying observed responses. This is also critically important for identifying mechanisms for survival during bleaching as the current research was unable to determine whether the tolerant phototrophic sponge species was simply less reliant on phototrophic carbon translocation, or

whether it was able to switch to heterotrophic nutrition during bleaching. Moreover, diverse communities of non-photosynthetic microbes play a significant role in sponge host fitness and have been shown to benefit sponges through host defense and other metabolic processes such as nitrogen cycling (Taylor *et al.*, 2007; Thomas *et al.*, 2016; Webster & Thomas, 2016). Disruptions to the functioning of this symbiosis has implications for the overall survival of both phototrophic and heterotrophic sponge holobiont models (Webster & Taylor, 2012; Fan *et al.*, 2013). Therefore to complete our understanding of the sponge holobiont response to OW and OA, future research should focus on the response of sponge associated microbial communities to climate change. Furthermore, the survival of a species in a changing climate requires that all stages of development are completed successfully, future work considering other important reproductive processes such as gametogenesis, spawning, fertilization, larval development and post-settlement success need to be undertaken. Additionally, while previous research supports my finding that early-life stages of sponges are generally tolerant to environmental stress (Whalan *et al.*, 2008b; Webster *et al.*, 2011; Negri *et al.*, 2016), my research also confirms that responses to environmental stressors can be highly species specific. Future work comparing the response of early-life stages of the different sponge "types" identified in this thesis is required. Finally, although my research makes a significant contribution to the global understanding of the effects of OW and OA on sponges, there are more than 8, 500 described sponge species (Van Soest et al., 2012), for most of which there is no information regarding survival in a high CO_2 world. Future work considering other important sponge species is required to increase our understanding of the implications of climate change on sponges as a phyla. In particular, although sponges of the class Calcarea comprise just a small proportion of the extant sponges species (Van Soest *et al.*, 2012), future work should consider the effect of OW and OA on these species as it is expected that sponges with calcium carbonate skeletal elements will be more susceptible to OA than sponges with siliceous skeletons.

6.6 Conclusion

As the climate changes, the prognosis for many marine species is negative. However, this thesis provides evidence that for sponges, the most ancient multicellular animals on Earth, some species may be able to survive under conditions of OW and OA. In particular, increased CO_2 may enable the survival of phototrophic sponge species in a high CO_2 world. Furthermore, thermal tolerance varied across sponge species with different skeletal compositions whereby

sponges with spiculated skeletons were more robust to OW and OA. Sponges with siliceous skeletal elements have survived pre-historical mass extinction events caused by OA and recent studies have also shown that they are more tolerant to thermal stress than keratose species (Webster *et al.*, 2008; Cebrian *et al.*, 2011; Duckworth *et al.*, 2012; Vicente *et al.*, 2016), supporting the importance of skeletal minerology in times of climate change. Finally, in the first study to examine the combined effect of OW and OA on early-life history stages of sponges, this research found that these stages are more tolerant to such stressors than adult life-stages. In addition to providing information on the response of an essential reef organism to climate change, this research highlights the importance of exploring organism responses to stressors across a number of levels of organisational complexity. I show how organisation and functional specificity at the cellular level translates into significant systemic effects at the macroscopic level. Sponge lipid composition and organisation is revealed to be fundamental to the health and function of sponges, due to the influence the composition of these groups has on the integrity and physiological competency of cells (Arts *et al.*, 2009). Finally, it emphasises the need to run multi-species, multi-factorial, long-term exposure experiments, across different life-history stages when determining an organism's response to environmental change. Overall, this thesis gives a holistic view of OW and OA impacts on tropical sponges and provides a basis from which to explore the potential for a sponge-coral regime shift in a high CO_2 world. Importantly however, OW and OA are just two aspects of environmental change currently facing marine ecosystems. The World's oceans will warm and acidify in conjunction with a plethora of direct anthropogenic threats including overfishing, pollution, eutrophication and sedimentation as well as increased storm events and UVB radiation, sea-level rise and subsequent changes in salinity. To fully understand the fate of sponges in future oceans, we first need to assess how they will respond to these cumulative environmental pressures.

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Appendices

Appendix one

Experimental treatments

Precise in-tank temperature control (±0.14 to 0.23; Table 1; Table S1) was facilitated by the addition of water baths surrounding each tank and monitored with in-tank temperature loggers. Temperatures in the water baths were maintained by circulation of the water through Alfa Laval Heat exchangers (controlled by a Siemens PCS7 SCADA systems), with the ability to maintain temperatures within ±0.1°C. The SCADA systems allowed for the parallel control of both the experimentally manipulated seawater and the water bath temperatures, delivering highly accurate experimental temperatures. Temperature loggers were checked and calibrated regularly against a calibrated thermometer (NIST Traceable Non Mercury Calibrated Thermometer). In-tank pCO_2 levels were maintained to within ± 27 to 93 ppm (Table 1), accuracy was ensured using a digital non-glass pH sensor (Tophit CPS471D), calibrated regularly against a Licor LI-840A near infra-red gas analyser (calibrated with certificated CO_2 gas standards). Water samples from these same tanks were taken weekly to measure DIC and alkalinity levels. Water samples were fixed with $HgCl_2$ and measured using a Versatile INstrument for the Determination of Total inorganic carbon and titration Alkalinity (VINDTA 3C from MARIANDA, calibrated against CO_2 in seawater reference material purchased from Professor Andrew Dickson, *Marine Physical Laboratory, University of California, San Diego*). Dissolved oxygen (DO), measured with a portable Optical Dissolved Oxygen meter (HQ30d equipped with LDO101 IntelliCAL dissolved oxygen probe, Hach, USA) remained saturated (~100%) in all tanks. In-tank PAR levels were checked weekly using a Licor LI-250A Light Meter with LI-193 Spherical Quantum Sensor and adjusted as required.

Table A1.1 Summary of replicate tank temperature and total scale pH (pH_T) means (SD) derived from weekly in-tank measurements (n= 22 sampling periods).

Treatment	Tank replicate	Temperature	pH
28.5°C/ pH 8.1	**1**	28.67 (0.10)	8.01 (0.05)
28.5°C/ pH 8.1	**2**	28.60 (0.20)	8.00 (0.05)
28.5°C/ pH 8.1	**3**	28.61 (0.16)	8.01 (0.05)
28.5°C/ pH 7.8	**1**	28.42 (0.18)	7.83 (0.07)
28.5°C/ pH 7.8	**2**	28.58 (0.18)	7.82 (0.07)
28.5°C/ pH 7.8	**3**	28.60 (0.20)	7.83 (0.07)
28.5°C/ pH 7.6	**1**	28.49 (0.13)	7.66 (0.05)
28.5°C/ pH 7.6	**2**	28.48 (0.18)	7.69 (0.08)
28.5°C/ pH 7.6	**3**	28.50 (0.22)	7.69 (0.08)
30°C/ pH 8.1	**1**	29.97 (0.12)	8.00 (0.06)
30°C/ pH 8.1	**2**	30.04 (0.18)	8.00 (0.06)
30°C/ pH 8.1	**3**	30.06 (0.10)	8.00 (0.05)
30°C/ pH 7.8	**1**	30.00 (0.13)	7.81 (0.07)
30°C/ pH 7.8	**2**	30.03 (0.16)	7.81 (0.07)
30°C/ pH 7.8	**3**	30.20 (0.14)	7.81 (0.07)
30°C/ pH 7.6	**1**	29.89 (0.25)	7.67 (0.06)
30°C/ pH 7.6	**2**	30.08 (0.18)	7.68 (0.08)
30°C/ pH 7.6	**3**	30.11 (0.14)	7.67 (0.07)
31.5°C/ pH 8.1	**1**	31.45 (0.18)	8.01 (0.11)
31.5°C/ pH 8.1	**2**	31.64 (0.17)	8.03 (0.05)
31.5°C/ pH 8.1	**3**	31.45 (0.15)	8.02 (0.06)
31.5°C/ pH 7.8	**1**	31.30 (0.18)	7.82 (0.06)
31.5°C/ pH 7.8	**2**	31.64 (0.16)	7.83 (0.08)
31.5°C/ pH 7.8	**3**	31.62 (0.17)	7.83 (0.06)
31.5°C/ pH 7.6	**1**	31.41 (0.19)	7.66 (0.05)
31.5°C/ pH 7.6	**2**	31.58 (0.15)	7.66 (0.08)
31.5°C/ pH 7.6	**3**	31.45 (0.20)	7.64 (0.07)

Table A1.2 Rate of temperature and pCO_2 increase (per day) during experimental ramping. Values are °C for temperature and ml/ min of CO_2 for each pH treatment.

Day	Time	Temperature			28.5°C			30°C			31.5°C		
		28.5°C	30°C	31.5°C	pH 8.1	pH 7.8	pH 7.6	pH 8.1	pH 7.8	pH 7.6	pH 8.1	pH 7.8	pH 7.6
	17:00	27.3°C	27.3°C	27.3°C									
1	20:00	27.6°C	27.6°C	27.6°C	---	2.8	3	---	2	3	---	1.4	2.8
	23:00	27.9°C	29.9°C	29.9°C									
	8:00	28.1°C	28.1°C	28.1°C									
	11:00	28.3°C	28.3°C	28.3°C									
	14:00	28.5°C	28.5°C	28.5°C									
2	17:00		28.8°C	28.8°C	---	5.5	6.1	---	4	5.9	---	2.8	5.6
	20:00		29.1°C	29.1°C									
	23:00		29.3°C	29.3°C									
	8:00		29.6°C	29.6°C									
	11:00		29.9°C	29.9°C	---	---	9.2	---		8.9	---		8.2
	14:00		30°C	30.2°C									
3	17:00			30.5°C									
	20:00			30.8°C									
	23:00			31.1°C									
	8:00			31.3°C									
4	11:00			31.5°C									

Appendix two

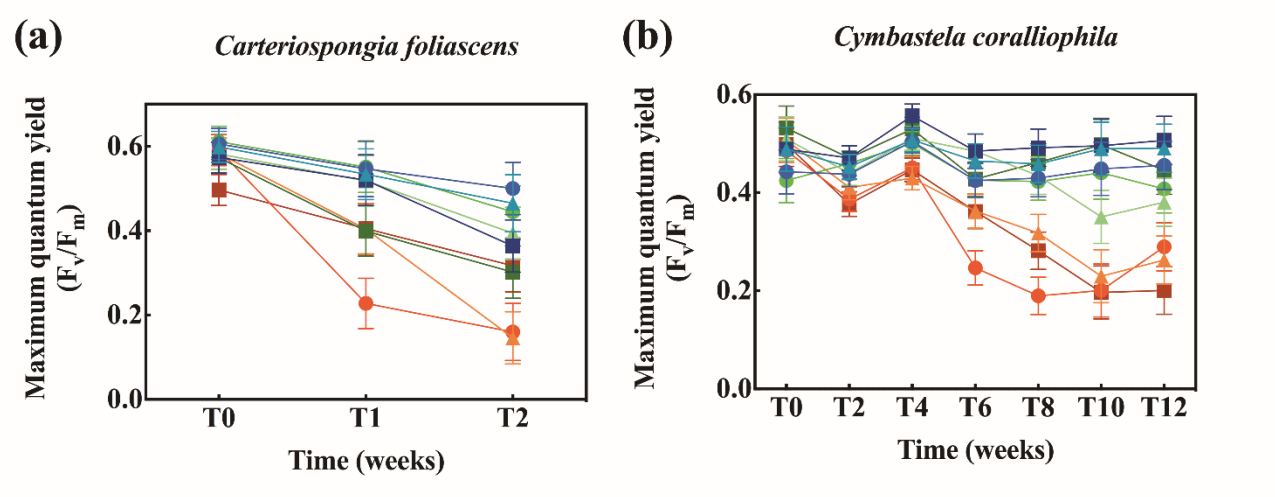

Fig. A2.1 Dark adapted quantum yield for **(a)** *C. foliascens* **(b)** *C. coralliophila* in each treatment. ▲ 28.5°C/pH 8.1, ● 28.5°C/pH 7.8, ■ 28.5°C/pH 7.6, ▲ 30°C/pH 8.1, ● 30°C/pH 7.8, ■ 30°C/pH 7.6, ▲31.5°C/pH 8.1, ● 31.5°C/pH 7.8, ■ 31.5°C/pH 7.6. Values are mean quantum yield determined after 30 min dark adaptation, per treatment ± SE per sampling time point (n = 6 except where mortalities occurred).

Table A2.1a Results of PERMANOVA analysis; the interactive and main effects of temperature and pH are evaluated for % sponge tissue with necrosis and bleaching (phototrophic species), and change in sponge mass (growth) throughout the duration of the experiment. F = Psuedo-F and *P* = p-value derived from permutational comparison. Significant effects are in bold.

a.	***C. foliascens***			***R. odorabile***			***S. flabelliformis***			***C. coralliophila***		
	df	**F**	***P***	***df***	**F**	***P***	***df***	**F**	***P***	***df***	**F**	***P***
Necrosis												
Temp	2, 48	16.415	**0.001**	2, 54	12.142	**0.001**	2, 36	11.794	**0.001**	2, 45	8.9257	**0.002**
pH	2, 48	0.5447	0.574	2, 54	5.2160	**0.010**	2, 36	0.2042	0.838	2, 45	2.6507	0.080
temp*pH	4, 48	1.9002	0.106	4, 54	5.1629	**0.003**	4, 36	0.2472	0.925	4, 45	2.6507	**0.031**
Bleaching												
Temp	2, 48	24.517	**0.001**	---	---	---	---	---	---	2, 45	33.118	**0.001**
pH	2, 48	1.6307	0.183	---	---	---	---	---	---	2, 45	0.0972	0.913
temp*pH	4, 48	3.8384	**0.011**	---	---	---	---	---	---	4, 45	1.1769	0.294
Growth												
Temp	2, 43	0.0474	0.940	2, 43	1.4792	0.231	2, 36	0.4240	0.640	2, 42	0.1553	0.850
pH	2, 43	0.0220	0.976	2, 43	0.7023	0.512	2, 36	1.4174	0.260	2, 42	0.4351	0.640
temp*pH	4, 43	1.4119	0.241	4, 43	0.5308	0.706	4, 36	0.5419	0.690	4, 42	0.5392	0.720

Table A2.1b-e Results of permutational *post hoc* tests where PERMANOVA analysis (above) detected significant interactive and main effects of temperature and pH. Significant effects are in bold.

b. *C. foliascens*

Necrosis	**temp**		**t**	***P***
	28.5°C	30°C	0.7011	0.701
	28.5°C	31.5°C	4.7370	**0.001**
	30°C	31.5°C	3.8410	**0.001**
Bleaching	**temp**		**t**	***P***
	28.5°C	30°C	0.8213	0.725
8.1	28.5°C	31.5°C	24.2080	**0.001**
	30°C	31.5°C	21.3950	**0.003**
	28.5°C	30°C	0.9338	0.501
7.8	28.5°C	31.5°C	2.4573	0.070
	30°C	31.5°C	2.6135	**0.027**
	28.5°C	30°C	1.0698	0.448
7.6	28.5°C	31.5°C	1.5021	0.308
	30°C	31.5°C	0.4304	0.858

c. *R. odorabile*

Necrosis	**temp**		**t**	***P***
	31.5°C	30°C	0.3631	0.903
8.1	31.5°C	28.5°C	1.8088	0.160
	30°C	28.5°C	1.0888	0.387
	31.5°C	30°C	1.7539	**0.006**
7.8	31.5°C	28.5°C	1.6503	**0.010**
	30°C	28.5°C	0.6244	0.688
	31.5°C	30°C	2.8289	**0.038**
7.6	31.5°C	28.5°C	3.1334	**0.004**
	30°C	28.5°C	2.0093	0.086

d. *S. flabelliformis*

Necrosis	**temp**		**t**	***P***
	28.5°C	30°C	0.2229	0.823
	28.5°C	31.5°C	3.4877	**0.001**
	30°C	31.5°C	3.4461	**0.001**

e. *C. coralliophila*

Necrosis	**temp**		**t**	***P***
8.1	30°C	28.5°C	Denominator is 0	
	30°C	31.5°C	Denominator is 0	
	28.5°C	31.5°C	Denominator is 0	
7.8	30°C	28.5°C	Denominator is 0	
	30°C	31.5°C	Denominator is 0	
	28.5°C	31.5°C	Denominator is 0	
7.6	30°C	28.5°C	Denominator is 0	
	30°C	31.5°C	2.3346	0.058
	28.5°C	31.5°C	2.3346	**0.050**
Bleaching	**temp**		**t**	***P***
	30°C	28.5°C	1.7074	0.093
	30°C	31.5°C	5.1990	**0.001**
	28.5°C	31.5°C	9.7132	**0.001**

Table A2.2 Generalised linear mixed model results for random effect "sponge individual". The AICc, estimate of the effect and P-value for each species and response variable; dark respiration, net photosynthesis and effective quantum yield is reported.

Species	**Dark respiration**			**Net photosynthesis**			**Effective quantum yield**		
	AICc	**Estimate**	***P***	**AICc**	**Estimate**	***P***	**AICc**	**Estimate**	***P***
C. foliascens	72	0.113	<0.001	94	0.127	0.001	-402	0.001	<0.001
R. odorabile	185	0.046	0.006		---	---		---	---
S. flabelliformis	148	0.114	<0.001		---	---		---	---
C. coralliophila	-447	0.009	<0.001	250	0.175	0.001	-461	0.002	<0.001

Table A2.3a-h Generalised linear mixed model fixed coefficient estimates. The fixed model term, coefficient estimate, standard error, t-value, P-value and 95% confidence interval for each species and response variable; dark respiration, net photosynthesis and effective quantum yield is reported. Note: redundant parameters (set to zero) are excluded.

Table A2.3a Generalised linear mixed model fixed coefficient estimates for *C. foliascens* dark respiration. Note: redundant parameters (set to zero) are excluded.

Model Term	Coefficient	Std. Error	t	*P*	CI (lower)	CI (Upper)
Intercept	-.835	.1631	-5.120	<0.001	-1.160	-.510
temp=28.5	-.344	.2302	-1.496	.139	-.804	.115
temp=30	.059	.2243	.265	.792	-.388	.507
pH=8.1	.519	.2566	2.022	.047	.007	1.031
pH=7.8	.252	.2566	.983	.329	-.260	.764
Time=T0	-.492	.1387	-3.551	.001	-.769	-.216
[temp=28.5]*[pH=8.1]	-.640	.3380	-1.893	.063	-1.314	.034
[temp=28.5]*[pH=7.8]	-.400	.3380	-1.182	.241	-1.074	.275
[temp=30]*[pH=8.1]	-.477	.3406	-1.402	.165	-1.157	.202
[temp=30]*[pH=7.8]	-.797	.3406	-2.339	.022	-1.476	-.117
[temp=28.5]*[Time=T0]	.404	.1880	2.151	.035	.029	.780
[temp=30]*[Time=T0]	.273	.1783	1.533	.130	-.082	.629
[pH=8.1]*[Time=T0]	-.401	.2175	-1.841	.070	-.835	.033
[pH=7.8]*[Time=T0]	-.226	.2175	-1.040	.302	-.660	.208
[temp=28.5]*[pH=8.1]*[Time=T0]	.421	.2764	1.524	.132	-.130	.973
[temp=28.5]*[pH=7.8]*[Time=T0]	.222	.2764	.803	.425	-.329	.773
[temp=30]*[pH=8.1]*[Time=T0]	-.022	.2700	-.081	.935	-.561	.517
[temp=30]*[pH=7.8]*[Time=T0]	.185	.2700	.684	.496	-.354	.723

Table A2.3b Generalised linear mixed model fixed coefficient estimates for *R. odorabile* dark respiration. Note: redundant parameters (set to zero) are excluded.

Model Term	Coefficient	Std. Error	t	*P*	CI (lower)	CI (Upper)
Intercept	2.526	.3068	8.234	<0.001	1.917	3.136
temp=28.5	-.986	.4339	-2.271	.026	-1.848	-.123
temp=30	-.318	.4339	-.733	.465	-1.180	.544
pH=8.1	.135	.4339	.312	.756	-.727	.997
pH=7.8	.002	.4339	.004	.997	-.860	.864
Time=T0	-.812	.2800	-2.900	.005	-1.368	-.256
[temp=28.5]*[pH=8.1]	.078	.6136	.127	.899	-1.141	1.297
[temp=28.5]*[pH=7.8]	.513	.6136	.836	.405	-.706	1.732
[temp=30]*[pH=8.1]	-.604	.6136	-.985	.327	-1.824	.615
[temp=30]*[pH=7.8]	-.718	.6136	-1.170	.245	-1.937	.501
[temp=28.5]*[Time=T0]	.912	.3959	2.305	.024	.126	1.699
[temp=30]*[Time=T0]	.356	.3959	.899	.371	-.431	1.142
[pH=8.1]*[Time=T0]	.012	.3959	.031	.975	-.774	.799
[pH=7.8]*[Time=T0]	.111	.3959	.279	.781	-.676	.897

[temp=28.5]*[pH=8.1]*[Time=T0]	-.046	.5599	-.082	.935	-1.158	1.067
[temp=28.5]*[pH=7.8]*[Time=T0]	-.851	.5647	-1.507	.135	-1.973	.271
[temp=30]*[pH=8.1]*[Time=T0]	.358	.5599	.639	.525	-.755	1.470
[temp=30]*[pH=7.8]*[Time=T0]	.503	.5599	.899	.371	-.609	1.616

Table A2.3c Generalised linear mixed model fixed coefficient estimates for *S. flabelliformis* dark respiration. Note: redundant parameters (set to zero) are excluded.

Model Term	Coefficient	Std. Error	t	*P*	CI (lower)	CI (Upper)
Intercept	1.333	.1819	7.331	<0.001	.974	1.692
temp=28.5	-.580	.2572	-2.256	.025	-1.088	-.073
temp=30	-.694	.2572	-2.697	.008	-1.201	-.186
pH=8.1	.042	.2630	.159	.874	-.477	.561
pH=7.8	-.155	.2572	-.602	.548	-.662	.353
Time=T0	-.129	.1966	-.659	.511	-.517	.258
Time=T2	-.395	.1233	-3.206	.002	-.639	-.152
Time=T4	-.347	.1199	-2.893	.004	-.583	-.110
Time=T6	-.207	.1551	-1.336	.183	-.513	.099
[temp=28.5]*[pH=8.1]	.255	.3679	.693	.489	-.471	.981
[temp=28.5]*[pH=7.8]	.185	.3637	.509	.611	-.533	.903
[temp=30]*[pH=8.1]	.556	.3679	1.511	.132	-.170	1.282
[temp=30]*[pH=7.8]	.347	.3637	.954	.341	-.371	1.065
[temp=28.5]*[Time=T0]	.385	.2780	1.385	.168	-.164	.934
[temp=28.5]*[Time=T2]	.322	.1744	1.846	.067	-.022	.666
[temp=28.5]*[Time=T4]	.232	.1695	1.369	.173	-.102	.567
[temp=28.5]*[Time=T6]	.384	.2193	1.749	.082	-.049	.817
[temp=30]*[Time=T0]	.597	.2780	2.148	.033	.049	1.146
[temp=30]*[Time=T2]	.470	.1744	2.695	.008	.126	.814
[temp=30]*[Time=T4]	.416	.1695	2.456	.015	.082	.751
[temp=30]*[Time=T6]	.338	.2193	1.543	.125	-.094	.771
[pH=8.1]*[Time=T0]	.335	.2834	1.182	.239	-.224	.894
[pH=8.1]*[Time=T2]	.278	.1829	1.521	.130	-.083	.639
[pH=8.1]*[Time=T4]	.280	.1782	1.569	.118	-.072	.631
[pH=8.1]*[Time=T6]	.015	.2326	.065	.948	-.444	.474
[pH=7.8]*[Time=T0]	.547	.2780	1.968	.051	-.001	1.096
[pH=7.8]*[Time=T2]	.248	.1744	1.424	.156	-.096	.592
[pH=7.8]*[Time=T4]	.190	.1695	1.121	.264	-.144	.525
[pH=7.8]*[Time=T6]	.502	.2193	2.287	.023	.069	.934
[temp=28.5]*[pH=8.1]*[Time=T0]	-.345	.3970	-.870	.385	-1.129	.438
[temp=28.5]*[pH=8.1]*[Time=T2]	-.470	.2527	-1.862	.064	-.969	.028
[temp=28.5]*[pH=8.1]*[Time=T4]	-.067	.2460	-.273	.785	-.553	.418
[temp=28.5]*[pH=8.1]*[Time=T6]	.260	.3197	.814	.417	-.371	.891
[temp=28.5]*[pH=7.8]*[Time=T0]	-.298	.3931	-.758	.449	-1.074	.478
[temp=28.5]*[pH=7.8]*[Time=T2]	-.168	.2466	-.681	.497	-.655	.319
[temp=28.5]*[pH=7.8]*[Time=T4]	.086	.2397	.358	.721	-.387	.559

[temp=28.5]*[pH=7.8]*[Time=T6]	-.580	.3102	-1.868	.063	-1.192	.033
[temp=30]*[pH=8.1]*[Time=T0]	-.777	.3970	-1.957	.052	-1.560	.006
[temp=30]*[pH=8.1]*[Time=T2]	-.025	.2527	-.098	.922	-.523	.474
[temp=30]*[pH=8.1]*[Time=T4]	-.732	.2460	-2.975	.003	-1.217	-.246
[temp=30]*[pH=8.1]*[Time=T6]	-.149	.3197	-.466	.642	-.780	.482
[temp=30]*[pH=7.8]*[Time=T0]	-.593	.3931	-1.508	.133	-1.369	.183
[temp=30]*[pH=7.8]*[Time=T2]	-.282	.2466	-1.145	.254	-.769	.204
[temp=30]*[pH=7.8]*[Time=T4]	-.422	.2397	-1.759	.080	-.895	.051
[temp=30]*[pH=7.8]*[Time=T6]	-.665	.3164	-2.102	.037	-1.289	-.041

Table A2.3d Generalised linear mixed model fixed coefficient estimates for *C. coralliophila* dark respiration. Note: redundant parameters (set to zero) are excluded.

Model Term	Coefficient	Std. Error	t	*P*	CI (lower)	CI (upper)
Intercept	.346	.0578	5.981	<0.001	.232	.460
temp=28.5	-.042	.0818	-.515	.607	-.203	.119
temp=30	-.016	.0839	-.185	.854	-.181	.150
pH=8.1	.320	.0818	3.919	<0.001	.159	.481
pH=7.8	-.025	.0818	-.309	.758	-.186	.136
Time=T0	-.022	.0506	-.439	.661	-.122	.077
Time=T12	.050	.0486	1.025	.306	-.046	.146
Time=T2	-.079	.0495	-1.602	.110	-.177	.018
Time=T4	-.046	.0450	-1.018	.309	-.135	.043
Time=T6	.007	.0463	.161	.872	-.084	.099
[temp=28.5]*[pH=8.1]	-.204	.1156	-1.762	.079	-.431	.024
[temp=28.5]*[pH=7.8]	.067	.1185	.562	.574	-.167	.300
[temp=30]*[pH=8.1]	-.252	.1172	-2.153	.032	-.483	-.022
[temp=30]*[pH=7.8]	.034	.1172	.292	.771	-.197	.265
[temp=28.5]*[Time=T0]	.085	.0716	1.193	.234	-.056	.226
[temp=28.5]*[Time=T12]	-.084	.0687	-1.225	.222	-.219	.051
[temp=28.5]*[Time=T2]	.065	.0700	.932	.352	-.073	.203
[temp=28.5]*[Time=T4]	.111	.0637	1.739	.083	-.015	.236
[temp=28.5]*[Time=T6]	-.004	.0654	-.055	.956	-.132	.125
[temp=30]*[Time=T0]	-.006	.0729	-.080	.936	-.149	.138
[temp=30]*[Time=T12]	-.141	.0712	-1.974	.049	-.281	.000
[temp=30]*[Time=T2]	-.036	.0725	-.492	.623	-.179	.107
[temp=30]*[Time=T4]	.004	.0664	.053	.958	-.127	.134
[temp=30]*[Time=T6]	-.044	.0681	-.652	.515	-.178	.090
[pH=8.1]*[Time=T0]	-.306	.0733	-4.170	<0.001	-.450	-.161
[pH=8.1]*[Time=T12]	-.118	.0687	-1.712	.088	-.253	.018
[pH=8.1]*[Time=T2]	-.035	.0700	-.505	.614	-.173	.103
[pH=8.1]*[Time=T4]	-.106	.0637	-1.666	.097	-.232	.019
[pH=8.1]*[Time=T6]	-.170	.0654	-2.592	.010	-.298	-.041
[pH=7.8]*[Time=T0]	-.058	.0704	-.821	.412	-.197	.081
[pH=7.8]*[Time=T12]	.003	.0687	.040	.968	-.133	.138

[pH=7.8]*[Time=T2]	.107	.0700	1.522	.129	-.031	.244
[pH=7.8]*[Time=T4]	-.004	.0637	-.057	.955	-.129	.122
[pH=7.8]*[Time=T6]	-.022	.0654	-.329	.742	-.150	.107
[temp=28.5]*[pH=8.1]*[Time=T0]	.126	.1017	1.236	.218	-.075	.326
[temp=28.5]*[pH=8.1]*[Time=T12]	.154	.0972	1.582	.115	-.038	.345
[temp=28.5]*[pH=8.1]*[Time=T2]	.040	.0990	.407	.684	-.155	.235
[temp=28.5]*[pH=8.1]*[Time=T4]	-.039	.0901	-.437	.662	-.217	.138
[temp=28.5]*[pH=8.1]*[Time=T6]	.189	.0925	2.040	.042	.007	.371
[temp=28.5]*[pH=7.8]*[Time=T0]	-.029	.1020	-.285	.776	-.230	.172
[temp=28.5]*[pH=7.8]*[Time=T12]	-.055	.0996	-.555	.579	-.251	.141
[temp=28.5]*[pH=7.8]*[Time=T2]	-.139	.1015	-1.370	.172	-.339	.061
[temp=28.5]*[pH=7.8]*[Time=T4]	-.047	.0923	-.505	.614	-.228	.135
[temp=28.5]*[pH=7.8]*[Time=T6]	-.002	.0948	-.020	.984	-.189	.185
[temp=30]*[pH=8.1]*[Time=T0]	.247	.1046	2.357	.019	.041	.453
[temp=30]*[pH=8.1]*[Time=T12]	.123	.0990	1.241	.216	-.072	.318
[temp=30]*[pH=8.1]*[Time=T2]	.120	.1017	1.183	.238	-.080	.321
[temp=30]*[pH=8.1]*[Time=T4]	.105	.0920	1.144	.254	-.076	.287
[temp=30]*[pH=8.1]*[Time=T6]	.187	.0944	1.983	.048	.001	.373
[temp=30]*[pH=7.8]*[Time=T0]	.126	.1005	1.254	.211	-.072	.324
[temp=30]*[pH=7.8]*[Time=T12]	.131	.0990	1.322	.187	-.064	.326
[temp=30]*[pH=7.8]*[Time=T2]	-.016	.1008	-.159	.874	-.215	.183
[temp=30]*[pH=7.8]*[Time=T4]	.052	.0920	.570	.569	-.129	.234
[temp=30]*[pH=7.8]*[Time=T6]	.104	.0944	1.099	.273	-.082	.290

Table A2.3e Generalised linear mixed model fixed coefficient estimates for *C. foliascens* net photosynthesis. Note: redundant parameters (set to zero) are excluded.

Model Term	Coefficient	Std. Error	t	*P*	CI (lower)	CI (Upper)
Intercept	.187	.1966	.952	.344	-.205	.579
temp=28.5	.784	.2743	2.860	.006	.237	1.331
temp=30	1.160	.2688	4.316	<0.001	.624	1.696
pH=7.6	-.590	.3167	-1.862	.067	-1.221	.042
pH=7.8	.340	.3167	1.074	.287	-.292	.972
time=T0	.757	.1785	4.242	<0.001	.401	1.113
[temp=28.5]*[pH=7.6]	.807	.4091	1.973	.052	-.009	1.623
[temp=28.5]*[pH=7.8]	-.605	.4091	-1.478	.144	-1.420	.211
[temp=30]*[pH=7.6]	.027	.4129	.065	.949	-.797	.850
[temp=30]*[pH=7.8]	-.867	.4129	-2.100	.039	-1.690	-.043
[temp=28.5]*[time=T0]	-.982	.2414	-4.068	<0.001	-1.463	-.501
[temp=30]*[time=T0]	-.897	.2306	-3.891	<0.001	-1.357	-.437
[pH=7.6]*[time=T0]	.501	.2849	1.759	.083	-.067	1.069
[pH=7.8]*[time=T0]	-.190	.2849	-.666	.507	-.758	.378
[temp=28.5]*[pH=7.6]*[time=T0]	-.475	.3589	-1.323	.190	-1.191	.241
[temp=28.5]*[pH=7.8]*[time=T0]	.580	.3546	1.636	.106	-.127	1.287
[temp=30]*[pH=7.6]*[time=T0]	-.048	.3525	-.137	.892	-.751	.655
[temp=30]*[pH=7.8]*[time=T0]	.174	.3525	.494	.623	-.529	.877

Table A2.3f Generalised linear mixed model fixed coefficient estimates for *C. coralliophila* net photosynthesis. Note: redundant parameters (set to zero) are excluded.

Model Term	Coefficient	Std. Error	t	*P*	CI (lower)	CI (upper)
Intercept	.209	.1917	1.092	.276	-.168	.587
temp=28.5	.645	.2711	2.380	.018	.111	1.179
temp=30	.709	.2711	2.613	.009	.175	1.242
pH=8.1	-.216	.2711	-.797	.426	-.750	.318
pH=7.8	-.346	.2711	-1.277	.203	-.880	.188
Time=T0	.618	.1251	4.945	<0.001	.372	.865
Time=T12	-.060	.1206	-.501	.617	-.298	.177
Time=T2	.398	.1650	2.409	.017	.073	.722
Time=T4	.476	.1317	3.611	<0.001	.216	.735
Time=T6	.457	.1546	2.954	.003	.152	.761
[temp=28.5]*[pH=8.1]	.162	.3834	.423	.673	-.593	.917
[temp=28.5]*[pH=7.8]	.241	.3929	.613	.540	-.533	1.015
[temp=30]*[pH=8.1]	-.116	.3834	-.303	.762	-.871	.639
[temp=30]*[pH=7.8]	.718	.3834	1.872	.062	-.037	1.473
[temp=28.5]*[Time=T0]	-.567	.1769	-3.206	.002	-.915	-.219
[temp=28.5]*[Time=T12]	.167	.1706	.977	.329	-.169	.503
[temp=28.5]*[Time=T2]	-.127	.2333	-.543	.587	-.586	.333
[temp=28.5]*[Time=T4]	-.308	.1863	-1.656	.099	-.675	.058
[temp=28.5]*[Time=T6]	-.102	.2186	-.465	.642	-.532	.329
[temp=30]*[Time=T0]	-.706	.1721	-4.100	<0.001	-1.045	-.367
[temp=30]*[Time=T12]	-.167	.1706	-.980	.328	-.503	.169
[temp=30]*[Time=T2]	.033	.2333	.142	.888	-.426	.492
[temp=30]*[Time=T4]	-.301	.1863	-1.614	.108	-.667	.066
[temp=30]*[Time=T6]	-.352	.2186	-1.612	.108	-.783	.078
[pH=8.1]*[Time=T0]	.203	.1838	1.102	.271	-.159	.564
[pH=8.1]*[Time=T12]	-.054	.1706	-.317	.751	-.390	.282
[pH=8.1]*[Time=T2]	.129	.2333	.553	.581	-.330	.589
[pH=8.1]*[Time=T4]	.041	.1863	.221	.825	-.326	.408
[pH=8.1]*[Time=T6]	-.174	.2186	-.794	.428	-.604	.257
[pH=7.8]*[Time=T0]	.056	.1721	.325	.745	-.283	.395
[pH=7.8]*[Time=T12]	.101	.1706	.591	.555	-.235	.437
[pH=7.8]*[Time=T2]	.222	.2333	.950	.343	-.238	.681
[pH=7.8]*[Time=T4]	.197	.1863	1.055	.292	-.170	.563
[pH=7.8]*[Time=T6]	-.252	.2186	-1.152	.250	-.682	.179
[temp=28.5]*[pH=8.1]*[Time=T0]	-.137	.2518	-.543	.587	-.633	.359
[temp=28.5]*[pH=8.1]*[Time=T12]	.039	.2412	.163	.870	-.436	.514
[temp=28.5]*[pH=8.1]*[Time=T2]	.178	.3300	.541	.589	-.471	.828
[temp=28.5]*[pH=8.1]*[Time=T4]	.126	.2635	.477	.634	-.393	.645
[temp=28.5]*[pH=8.1]*[Time=T6]	-.362	.3092	-1.169	.243	-.970	.247
[temp=28.5]*[pH=7.8]*[Time=T0]	-.048	.2491	-.192	.848	-.538	.443

[temp=28.5]*[pH=7.8]*[Time=T12]	-.090	.2472	-.364	.716	-.577	.397
[temp=28.5]*[pH=7.8]*[Time=T2]	-.148	.3381	-.439	.661	-.814	.517
[temp=28.5]*[pH=7.8]*[Time=T4]	-.011	.2700	-.039	.969	-.542	.521
[temp=28.5]*[pH=7.8]*[Time=T6]	.154	.3168	.487	.627	-.470	.778
[temp=30]*[pH=8.1]*[Time=T0]	.097	.2518	.386	.700	-.399	.593
[temp=30]*[pH=8.1]*[Time=T12]	.019	.2412	.077	.939	-.456	.494
[temp=30]*[pH=8.1]*[Time=T2]	-.147	.3300	-.445	.657	-.797	.503
[temp=30]*[pH=8.1]*[Time=T4]	.141	.2635	.537	.592	-.377	.660
[temp=30]*[pH=8.1]*[Time=T6]	.409	.3092	1.323	.187	-.200	1.018
[temp=30]*[pH=7.8]*[Time=T0]	-.204	.2400	-.848	.397	-.676	.269
[temp=30]*[pH=7.8]*[Time=T12]	.099	.2412	.410	.682	-.376	.574
[temp=30]*[pH=7.8]*[Time=T2]	-.368	.3300	-1.116	.266	-1.018	.282
[temp=30]*[pH=7.8]*[Time=T4]	-.251	.2635	-.954	.341	-.770	.268
[temp=30]*[pH=7.8]*[Time=T6]	.502	.3092	1.625	.105	-.106	1.111

Table A2.3g Generalised linear mixed model fixed coefficient estimates for *C. foliascens* effective quantum yield. Note: redundant parameters (set to zero) are excluded.

Model Term	Coefficient	Std. Error	t	*P*	CI (lower)	CI (upper)
Intercept	.745	.0215	34.633	<0.001	.702	.787
temp=28.5	.086	.0304	2.838	.005	.026	.146
temp=30	.109	.0304	3.587	<0.001	.049	.169
pH=8.1	-.032	.0385	-.835	.405	-.109	.044
pH=7.8	.036	.0386	.934	.352	-.040	.113
time=T0	.192	.0200	9.590	<0.001	.152	.231
time=T1	.078	.0225	3.471	.001	.034	.123
[temp=28.5]*[pH=8.1]	.087	.0485	1.791	.076	-.009	.183
[temp=28.5]*[pH=7.8]	.003	.0485	.061	.952	-.093	.099
[temp=30]*[pH=8.1]	.014	.0491	.283	.777	-.083	.111
[temp=30]*[pH=7.8]	.010	.0485	.201	.841	-.086	.106
[temp=28.5]*[time=T0]	-.118	.0283	-4.170	<0.001	-.174	-.062
[temp=28.5]*[time=T1]	-.044	.0313	-1.391	.167	-.105	.018
[temp=30]*[time=T0]	-.134	.0283	-4.732	<0.001	-.190	-.078
[temp=30]*[time=T1]	-.084	.0316	-2.667	.009	-.147	-.022
[pH=8.1]*[time=T0]	.027	.0369	.729	.467	-.046	.100
[pH=8.1]*[time=T1]	.047	.0395	1.184	.239	-.031	.125
[pH=7.8]*[time=T0]	-.030	.0370	-.824	.411	-.104	.043
[pH=7.8]*[time=T1]	-.042	.0405	-1.037	.302	-.122	.038
[temp=28.5]*[pH=8.1]*[time=T0]	-.054	.0458	-1.170	.244	-.144	.037
[temp=28.5]*[pH=8.1]*[time=T1]	-.070	.0494	-1.416	.160	-.168	.028
[temp=28.5]*[pH=7.8]*[time=T0]	.019	.0459	.418	.677	-.072	.110
[temp=28.5]*[pH=7.8]*[time=T1]	.034	.0502	.686	.494	-.065	.134
[temp=30]*[pH=8.1]*[time=T0]	.022	.0465	.474	.636	-.070	.114
[temp=30]*[pH=8.1]*[time=T1]	-.001	.0502	-.013	.990	-.100	.099
[temp=30]*[pH=7.8]*[time=T0]	.025	.0459	.539	.591	-.066	.116

[temp=30]*[pH=7.8]*[time=T1]	.044	.0504	.880	.381	-.055	.144

Table A2.3h Generalised linear mixed model fixed coefficient estimates for *C. coralliophila* effective quantum yield. Note: redundant parameters (set to zero) are excluded.

Model Term	Coefficient	Std. Error	t	*P*	CI (lower)	CI (upper)
Intercept	.647	.0468	13.835	<0.001	.555	.739
temp=28.5	.248	.0661	3.749	<0.001	.118	.378
temp=30	.217	.0661	3.278	.001	.087	.347
pH=8.1	.029	.0661	.441	.660	-.101	.159
pH=7.8	-.215	.0661	-3.250	.001	-.345	-.085
time=T0	.227	.0509	4.466	<0.001	.127	.327
time=T10	-.310	.0826	-3.747	<0.001	-.472	-.147
time=T12	-.215	.0803	-2.679	.008	-.373	-.057
time=T2	.139	.0458	3.042	.003	.049	.230
time=T4	.108	.0450	2.406	.017	.020	.197
time=T6	.076	.0616	1.235	.218	-.045	.197
[temp=28.5]*[pH=8.1]	-.080	.0935	-.851	.395	-.264	.104
[temp=28.5]*[pH=7.8]	.176	.0935	1.881	.061	-.008	.360
[temp=30]*[pH=8.1]	-.062	.0935	-.658	.511	-.246	.122
[temp=30]*[pH=7.8]	.197	.0935	2.102	.036	.013	.381
[temp=28.5]*[time=T0]	-.259	.0710	-3.654	<0.001	-.399	-.120
[temp=28.5]*[time=T10]	.291	.1169	2.489	.013	.061	.521
[temp=28.5]*[time=T12]	.199	.1135	1.752	.081	-.025	.422
[temp=28.5]*[time=T2]	-.154	.0648	-2.381	.018	-.282	-.027
[temp=28.5]*[time=T4]	-.095	.0637	-1.485	.139	-.220	.031
[temp=28.5]*[time=T6]	-.071	.0871	-.817	.415	-.243	.100
[temp=30]*[time=T0]	-.213	.0710	-2.999	.003	-.353	-.073
[temp=30]*[time=T10]	.306	.1169	2.618	.009	.076	.536
[temp=30]*[time=T12]	.181	.1135	1.595	.112	-.042	.405
[temp=30]*[time=T2]	-.131	.0648	-2.021	.044	-.259	-.003
[temp=30]*[time=T4]	-.086	.0637	-1.345	.180	-.211	.040
[temp=30]*[time=T6]	-.103	.0871	-1.186	.237	-.275	.068
[pH=8.1]*[time=T0]	-.010	.0710	-.138	.890	-.150	.130
[pH=8.1]*[time=T10]	.195	.1169	1.672	.096	-.035	.425
[pH=8.1]*[time=T12]	.122	.1135	1.077	.282	-.101	.346
[pH=8.1]*[time=T2]	-.030	.0654	-.455	.649	-.158	.099
[pH=8.1]*[time=T4]	.014	.0637	.226	.821	-.111	.140
[pH=8.1]*[time=T6]	.022	.0871	.251	.802	-.150	.193
[pH=7.8]*[time=T0]	.216	.0710	3.046	.003	.077	.356
[pH=7.8]*[time=T10]	.261	.1169	2.231	.026	.031	.491
[pH=7.8]*[time=T12]	.250	.1135	2.201	.028	.026	.473
[pH=7.8]*[time=T2]	.207	.0648	3.200	.002	.080	.335
[pH=7.8]*[time=T4]	.289	.0637	4.542	<0.001	.164	.415
[pH=7.8]*[time=T6]	.032	.0871	.364	.716	-.140	.203

[temp=28.5]*[pH=8.1]*[time=T0]	.077	.0997	.775	.439	-.119	.274
[temp=28.5]*[pH=8.1]*[time=T10]	-.157	.1653	-.951	.342	-.482	.168
[temp=28.5]*[pH=8.1]*[time=T12]	-.095	.1606	-.589	.556	-.411	.221
[temp=28.5]*[pH=8.1]*[time=T2]	.078	.0921	.848	.397	-.103	.259
[temp=28.5]*[pH=8.1]*[time=T4]	.006	.0901	.067	.947	-.171	.183
[temp=28.5]*[pH=8.1]*[time=T6]	-.013	.1232	-.104	.917	-.255	.230
[temp=28.5]*[pH=7.8]*[time=T0]	-.170	.0997	-1.706	.089	-.366	.026
[temp=28.5]*[pH=7.8]*[time=T10]	-.218	.1653	-1.318	.189	-.543	.107
[temp=28.5]*[pH=7.8]*[time=T12]	-.229	.1606	-1.423	.156	-.544	.087
[temp=28.5]*[pH=7.8]*[time=T2]	-.175	.0917	-1.907	.057	-.355	.006
[temp=28.5]*[pH=7.8]*[time=T4]	-.275	.0901	-3.055	.002	-.452	-.098
[temp=28.5]*[pH=7.8]*[time=T6]	-.025	.1232	-.203	.839	-.268	.217
[temp=30]*[pH=8.1]*[time=T0]	.039	.1004	.388	.699	-.159	.237
[temp=30]*[pH=8.1]*[time=T10]	-.260	.1683	-1.546	.123	-.591	.071
[temp=30]*[pH=8.1]*[time=T12]	-.238	.1606	-1.485	.139	-.554	.078
[temp=30]*[pH=8.1]*[time=T2]	.015	.0921	.166	.868	-.166	.196
[temp=30]*[pH=8.1]*[time=T4]	.007	.0901	.082	.935	-.170	.185
[temp=30]*[pH=8.1]*[time=T6]	.030	.1232	.240	.811	-.213	.272
[temp=30]*[pH=7.8]*[time=T0]	-.217	.1004	-2.163	.031	-.415	-.020
[temp=30]*[pH=7.8]*[time=T10]	-.280	.1653	-1.693	.091	-.605	.045
[temp=30]*[pH=7.8]*[time=T12]	-.237	.1606	-1.478	.140	-.553	.079
[temp=30]*[pH=7.8]*[time=T2]	-.175	.0917	-1.906	.058	-.355	.006
[temp=30]*[pH=7.8]*[time=T4]	-.275	.0901	-3.057	.002	-.453	-.098
[temp=30]*[pH=7.8]*[time=T6]	.022	.1232	.178	.858	-.220	.264

Table A2.4a-h Results of *post hoc* pairwise comparisons test (with the sequential Sidak correction applied) based on significant treatment effects revealed by Generalised linear mixed models. The model estimated mean is reported in brackets and italics beneath each treatment for which significant differences exist.

Table A2.4a Results of temperature * time, and pH * time *post hoc* pairwise comparisons test (with the sequential Sidak correction applied) for *C. foliascens* dark respiration.

Time	Temp		Contrast Estimate	Std. Error	T	df	*P*	CI (lower)	CI (upper)
T0	***ns***								
T2	**30°C** (*-0.943*)	**28.5°C** (*-1.269*)	0.326	0.128	2.548	69	**0.024**	-0.690	-0.041
	31.5°C (*-0.578*)	**28.5°C** (*-1.269*)	0.691	0.140	4.947	69	**<0.001**	0.349	1.033
	31.5°C (*-0.578*)	**30°C** (*-0.943*)	0.365	0.142	2.572	69	**0.024**	0.041	0.690

Time	pH	Contrast Estimate	Std. Error	T	df	*P*	CI (lower)	CI (upper)
T0	***ns***							
T2	***ns***							

Table A2.4b Results of temperature * time *post hoc* pairwise comparisons test (with the sequential Sidak correction applied) for *R. odorabile* dark respiration.

Time	Temp		Contrast Estimate	Std. Error	t	df	*P*	CI (lower)	CI (upper)
T0	***ns***								
T2	**31.5°C** (2.572)	**28.5°C** (1.784)	0.788	0.251	3.148	89	**0.007**	0.179	1.398
	31.5°C (2.572)	**30°C** (1.813)	0.759	0.251	3.029	89	**0.007**	0.184	1.333

Table A2.4c Results of temperature * pH * time *post hoc* pairwise comparisons test (with the sequential Sidak correction applied) for *S. flabelliformis* dark respiration.

Time	pH	Temp		Contrast Estimate	Std. Error	t	df	*P*	CI (lower)	CI (upper)
T0	**8.1**	***ns***								
T2		**30°C** (*1.565*)	**28.5°C** (*0.784*)	0.781	0.236	3.307	177	**0.003**	0.212	1.351
T4		***ns***								
T6		***ns***								
T8		***ns***								
T0	**7.8**	***ns***								
T2		***ns***								
T4		***ns***								
T6		**31.5°C** (*1.473*)	**30°C** (*0.799*)	0.673	0.278	2.420	177	**0.049**	0.003	1.344
T8		***ns***								
T0	**7.6**	***ns***								
T2		***ns***								
T4		***ns***								
T6		***ns***								
T8		**31.5°C** (*1.333*)	**28.5°C** (*0.753*)	0.580	0.257	2.256	177	**0.050**	0.000	1.160
		31.5°C (*1.333*)	**30°C** (*0.639*)	0.694	0.257	2.697	177	**0.023**	0.074	1.314

Table A2.4d Results of temperature * pH * time *post hoc* pairwise comparisons test (with the sequential Sidak correction applied) b for *C. coralliophila* dark respiration.

Time	pH	Temp		Contrast Estimate	Std. Error	t	df	*P*	CI (lower)	CI (upper)
T0		***ns***								
T2		**31.5°C** (*0.552*)	**28.5°C** (*0.411*)	0.176	0.069	2.569	256	**0.021**	-0.017	0.298
		31.5°C (*0.552*)	**30°C** (*0.368*)	0.286	0.069	4.159	256	**<0.001**	0.012	0.354
T4		**31.5°C** (0.514)	**28.5°C** (*0.340*)	0.174	0.064	2.741	256	**0.020**	0.021	0.327
		31.5°C (0.514)	**30°C** (*0.355*)	0.159	0.064	2.498	256	**0.026**	0.016	0.302
T6	**8.1**	***ns***								
T8		**31.5°C** (*0.666*)	**28.5°C** (*0.420*)	0.246	0.082	3.007	256	**0.006**	0.062	0.430
		31.5°C (*0.666*)	**30°C** (*0.399*)	0.268	0.082	3.275	256	**0.004**	0.071	0.464
T12		**31.5°C** (*0.598*)	**28.5°C** (*0.422*)	0.176	0.069	2.569	256	**0.021**	0.022	0.331
		31.5°C (*0.598*)	**30°C** (*0.313*)	0.286	0.069	4.159	256	**<0.001**	0.121	0.451
T0		***ns***								
T2		***ns***								
T4	**7.8**	***ns***								
T6		***ns***								
T8		***ns***								
T12		***ns***								
T0		***ns***								
T2		***ns***								
T4	**7.6**	***ns***								
T6		***ns***								
T8		***ns***								
T12		***ns***								

Table A2.4e Results of temperature * pH * time *post hoc* pairwise comparisons test (with the sequential Sidak correction applied) for *C. foliascens* net photosynthesis.

Time	pH	Temp		Contrast Estimate	Std. Error	t	df	*P*	CI (lower)	CI (upper)
T0	**8.1**	***ns***								
T2		**31.5°C** (*-0.403*)	**28.5°C** (*1.189*)	-1.592	0.303	-5.245	70	**<0.001**	-0.709	0.440
		31.5°C (*-0.403*)	**30°C** (*0.784*)	-1.187	0.313	-3.788	70	**0.001**	-0.870	0.387
T0	**7.8**	***ns***								
T2		***ns***								
T0	**7.6**	***ns***								
T2		**31.5°C** (*0.187*)	**28.5°C** (*0.972*)	-0.784	0.274	-2.860	70	**0.011**	-1.411	-0.157
		31.5°C (*0.187*)	**30°C** (*1.347*)	-1.160	0.269	-4.316	70	**<0.001**	-1.818	-0.503

Table A2.4f Results of temperature * time, and pH * time *post hoc* pairwise comparisons test (with the sequential Sidak correction applied) for *C. coralliophila* net photosynthesis.

Time	Temp		Contrast Estimate	Std. Error	t	df	*P*	CI (lower)	CI (upper)
T0	***ns***								
T2	**31.5°C** (*0.536*)	**28.5°C** (*1.199*)	-0.663	0.183	-3.617	259	**0.001**	-1.075	-0.251
	31.5°C (*0.536*)	**30°C** (*1.307*)	-0.770	0.180	-4.273	259	**<0.001**	-1.204	-0.337
T4	**31.5°C** (*0.577*)	**28.5°C** (*1.087*)	-0.510	0.164	-3.113	259	**0.004**	-0.878	-0.141
	31.5°C (*0.577*)	**30°C** (*1.149*)	-0.572	0.161	-3.550	259	**0.001**	-0.959	-0.185
T6	**31.5°C** (*0.337*)	**28.5°C** (*0.946*)	-0.609	0.177	-3.441	259	**0.001**	-1.007	-0.211
	31.5°C (*0.337*)	**30°C** (*1.197*)	-0.860	0.174	-4.944	259	**<0.001**	-1.279	-0.442
T8	**31.5°C** (*0.022*)	**28.5°C** (*0.801*)	-0.780	0.159	-4.899	259	**<0.001**	-1.138	-0.422
	31.5°C (*0.022*)	**30°C** (*0.931*)	-0.909	0.157	-5.807	259	**<0.001**	-1.285	-0.533
T12	**31.5°C** (*-0.022*)	**28.5°C** (*0.906*)	-0.929	0.158	-5.895	259	**<0.001**	-1.308	-0.550
	31.5°C (*-0.022*)	**30°C** (*0.758*)	-0.781	0.155	-5.034	259	**<0.001**	-1.130	-0.432

Table A2.4g Results of temperature * time *post hoc* pairwise comparisons test (with the sequential Sidak correction applied) for *C. foliascens* effective quantum yield.

Time	Temp		Contrast Estimate	Std. Error	t	df	*P*	CI (lower)	CI (upper)
T0	***ns***								
T1	**31.5°C** (*0.826*)	**28.5°C** (*0.886*)	-0.061	0.014	-4.426	117	**<0.001**	-0.094	-0.028
	31.5°C (*0.826*)	**30°C** (*0.873*)	-0.047	0.014	-3.410	117	**0.002**	-0.079	-0.016
T2	**31.5°C** (*0.746*)	**28.5°C** (*0.862*)	-0.116	0.020	-5.670	117	**<0.001**	-0.166	-0.067
	31.5°C (*0.746*)	**30°C** (0.863	-0.117	0.021	-5.664	117	**<0.001**	-0.167	-0.067

Table A2.4h Results of temperature * pH * time *post hoc* pairwise comparisons test (with the sequential Sidak correction applied) for *C. coralliophila* effective quantum yield.

Time	pH	Temp		Contrast Estimate	Std. Error	t	df	*P*	CI (lower)	CI (upper)
T0		***ns***								
T2		**31.5°C** (*0.786*)	**28.5°C** (*0.878*)	-0.092	0.036	-2.568	310	**0.032**	-0.178	-0.006
T4		**31.5°C** (0.799)	**28.5°C** (*0.879*)	-0.080	0.033	-2.439	310	**0.045**	-0.158	-0.001
		31.5°C (0.799)	**30°C** (*0.876*)	-0.077	0.033	-2.353	310	**0.045**	-0.153	-0.001
T6	**8.1**	***ns***								
T8		**31.5°C** (*0.676*)	**28.5°C** (*0.844*)	-0.168	0.066	-2.546	310	**0.034**	-0.327	-0.010
		31.5°C (*0.676*)	**30°C** (*0.831*)	-0.155	0.066	-2.347	310	**0.039**	-0.304	-0.007
T10		**31.5°C** (*0.562*)	**28.5°C** (*0.864*)	-0.302	0.103	-2.924	310	**0.011**	-0.550	-0.054
T12		**31.5°C** (*0.583*)	**28.5°C** (*0.856*)	-0.273	0.100	-2.740	310	**0.019**	-0.512	-0.034
T0		***ns***								
T2		**31.5°C** (*0.779*)	**28.5°C** (*0.874*)	-0.095	0.035	-2.715	310	**0.014**	-0.173	-0.016
		31.5°C (*0.779*)	**30°C** (*0.886*)	-0.107	0.035	-3.082	310	**0.007**	-0.191	-0.024
T4		***ns***								
T6	**7.8**	**31.5°C** (*0.540*)	**28.5°C** (0.867)	-0.328	0.068	-4.827	310	**<0.001**	-0.483	-0.172
		31.5°C (*0.540*)	**30°C** (*0.872*)	-0.332	0.068	-4.891	310	**<0.001**	-0.495	-0.169
T8		**31.5°C** (*0.432*)	**28.5°C** (*0.856*)	-0.424	0.066	-6.410	310	**<0.001**	-0.583	-0.265
		31.5°C (*0.432*)	**30°C** (*0.845*)	-0.413	0.066	-6.250	310	**<0.001**	-0.562	-0.265
T10		**31.5°C** (*0.383*)	**28.5°C** (*0.880*)	-0.497	0.103	-4.810	310	**<0.001**	-0.745	-0.249

		31.5°C (*0.383*)	**30°C** (*0.822*)	-0.439	0.103	-4.253	310	**<0.001**	-0.672	-0.207
T12		**31.5°C** (*0.467*)	**28.5°C** (*0.861*)	-0.394	0.100	-3.961	310	**<0.001**	-0.633	-0.155
		31.5°C (*0.467*)	**30°C** (*0.824*)	-0.357	0.100	-3.587	310	**0.001**	-0.581	-0.133
T0		***ns***								
T2		**31.5°C** (*0.786*)	**28.5°C** (*0.880*)	-0.094	0.035	-2.684	310	**0.023**	-0.177	-0.010
		31.5°C (*0.786*)	**30°C** (*0.872*)	-0.086	0.035	-2.458	310	**0.029**	-0.164	-0.007
T4		**31.5°C** (*0.755*)	**28.5°C** (*0.909*)	-0.153	0.033	-4.688	310	**<0.001**	-0.232	-0.075
		31.5°C (*0.755*)	**30°C** (*0.886*)	-0.131	0.033	-4.008	310	**<0.001**	-0.205	-0.058
T6		**31.5°C** (*0.723*)	**28.5°C** (*0.900*)	-0.177	0.068	-2.604	310	**0.029**	-0.340	-0.014
T8	**7.6**	**31.5°C** (*0.647*)	**28.5°C** (*0.895*)	-0.248	0.066	-3.749	310	**0.001**	-0.407	-0.089
		31.5°C (*0.647*)	**30°C** (*0.864*)	-0.217	0.066	-3.278	310	**0.002**	-0.365	-0.068
T10		**31.5°C** (*0.337)*	**28.5°C** (*0.876*)	-0.539	0.103	-5.216	310	**<0.001**	-0.787	-0.291
		31.5°C (*0.337)*	**30°C** (*0.860*)	-0.523	0.103	-5.060	310	**<0.001**	-0.755	-0.291
T12		**31.5°C** (*0.432*)	**28.5°C** (*0.879*)	-0.394	0.100	-3.961	310	**<0.001**	-0.633	-0.155
		31.5°C (*0.432*)	**30°C** (*0.830*)	-0.357	0.100	-3.587	310	**0.001**	-0.581	-0.133

Appendix three

Table A3.1 Results of the two-way ANOVAs testing the effects of temperature and pH on phototrophic sponge P:R and chlorophyll *a* concentration at T-end.

	C. foliascens			*C. coralliophila*		
	d.f.	***F***	***p***	***d.f.***	***F***	***p***
P:R						
Temp	2	28.922	< **0.001**	2	40.496	< **0.001**
pH	2	1.190	0.318	2	7.203	**0.002**
Temp*pH	4	6.872	< **0.001**	4	2.022	0.108
Residuals	31			44		
Chl *a*						
Temp	2	5.026	**0.011**	2	24.496	< **0.001**
pH	2	1.267	0.292	2	0.378	0.687
Temp*pH	4	0.805	0.529	4	1.305	0.282
Residuals	41			46		

Table A3.2 Results of the two-way ANOVAs testing the effects of temperature and pH on sponge total lipid content, total storage and total structural lipid content, and the ratio of structural to storage lipid.

	C. foliascens			*C. coralliophila*			*R. odorabile*			*S. flabelliformis*		
	d.f.	***F***	***p***	***d.f.***	***F***	***p***	***d.f.***	***F***	***p***	***d.f.***	***F***	***p***
Total lipid												
Temp	2	3.912	**0.031**	2	0.573	0.569	2	0.423	0.658	2	1.337	0.277
pH	2	0.887	0.423	2	0.926	0.405	2	1.625	0.209	2	1.592	0.219
Temp*pH	4	4.804	**0.004**	4	0.882	0.484	4	0.441	0.778	4	0.574	0.683
Residuals	29			36			43			33		
Storage lipid												
Temp	2	1.525	0.235	2	0.146	0.865	2	0.567	0.767	2	1.055	0.360
pH	2	1.896	0.168	2	1.206	0.311	2	0.308	0.737	2	0.682	0.513
Temp*pH	4	1.643	0.190	4	0.233	0.918	4	0.291	0.882	4	0.663	0.622
Residuals	29			36			43			33		
Structural lipid												
Temp	2	1.525	0.235	2	0.146	0.865	2	0.267	0.767	2	1.055	0.360
pH	2	1.896	0.168	2	1.206	0.311	2	0.308	0.737	2	0.682	0.513
Temp*pH	4	1.643	0.190	4	0.233	0.918	4	0.291	0.882	4	0.663	0.622
Residuals	29			36			43			33		
Structural:Storage												
Temp	2	0.599	0.556	2	0.182	0.835	2	0.111	0.896	2	1.145	0.331
pH	2	1.433	0.225	2	1.043	0.363	2	0.136	0.873	2	0.174	0.841
Temp*pH	4	0.815	0.526	4	0.247	0.910	4	0.462	0.763	4	0.503	0.734
Residuals	29			36			43			33		

Table. A3.3 Results of the two-way ANOVAs testing the effects of temperature and pH on sponge lipid class composition for each class.

	C. foliascens			*C. coralliophila*			*R. odorabile*			*S. flabelliformis*		
	d.f.	***F***	***p***	***d.f.***	***F***	***p***	***d.f.***	***F***	***p***	***d.f.***	***F***	***p***
Sterol ester												
Temp	2	2.291	0.119	2	5.344	**0.009**	2	0.138	0.872	2	0.801	0.457
pH	2	1.147	0.332	2	0.814	0.451	2	0.046	0.955	2	0.637	0.535
Temp*pH	4	1.767	0.163	4	1.074	0.384	4	0.658	0.625	4	0.612	0.657
Residuals	29			36			43			33		
Triacylglycerol												
Temp	2	0.411	0.667	2	2.436	0.102	2	0.561	0.575	2	0.712	0.498
pH	2	2.259	0.127	2	0.347	0.709	2	0.414	0.664	2	0.760	0.476
Temp*pH	4	1.318	0.287	4	0.663	0.622	4	0.734	0.574	4	0.021	0.999
Residuals	29			36			43			33		
Free fatty acid												
Temp	2	0.324	0.726	2	0.908	0.412	2	1.061	0.355	2	0.209	0.812
pH	2	0.810	0.455	2	0.835	0.442	2	0.562	0.574	2	1.318	0.282
Temp*pH	4	0.274	0.893	4	2.354	0.072	4	0.701	0.595	4	0.705	0.594
Residuals	29			36			43			33		
Sterol												
Temp	2	3.804	0.034	2	1.064	0.356	2	0.367	0.695	2	2.485	0.099
pH	2	2.458	0.103	2	3.169	0.054	2	0.871	0.426	2	0.384	0.685
Temp*pH	4	2.710	**0.049**	4	0.336	0.852	4	0.865	0.493	4	0.979	0.432
Residuals	29			36			43			33		
AMPL												
Temp	2	2.967	0.067	2	0.823	0.447	2	0.330	0.721	2	0.760	0.476
pH	2	1.015	0.375	2	1.090	0.347	2	0.390	0.679	2	0.515	0.602
Temp*pH	4	1.740	0.168	4	0.814	0.525	4	1.272	0.296	4	2.445	0.066
Residuals	29			36			43			33		
Phosphatidylethanolamine												
Temp	2	2.686	0.085	2	1.650	0.206	2	0.100	0.905	2	2.744	0.079
pH	2	0.523	0.598	2	0.404	0.670	2	0.681	0.512	2	0.841	0.440
Temp*pH	4	1.385	0.264	4	0.534	0.712	4	1.008	0.414	4	0.303	0.874
Residuals	29			36			43			33		
Phosphatidylinositol												
Temp	2	3.201	0.055	2	0.614	0.590	2	1.181	0.317	2	0.396	0.676
pH	2	1.142	0.332	2	0.319	0.639	2	0.571	0.569	2	0.627	0.541
Temp*pH	4	1.475	0.236	4	0.294	0.890	4	1.203	0.323	4	0.085	0.987
Residuals	29			36			43			33		
Phosphatidylcholine												
Temp	2	2.850	0.074	2	0.536	0.590	2	0.386	0.682	2	7.855	**0.002**
pH	2	0.998	0.381	2	0.454	0.639	2	0.480	0.622	2	0.367	0.696
Temp*pH	4	1.490	0.231	4	0.278	0.890	4	0.465	0.761	4	1.302	0.290
Residuals	29			36			43			33		
Phosphatidylserine												
Temp				2	5.754	0.022	2	3.033	0.059	2	0.308	0.737

pH	2	0.791	0.461	2	1.175	0.319	2	0.400	0.674
Temp*pH	4	0.240	0.788	4	5.042	**0.002**	4	0.874	0.487
Residuals	36	0.999	0.421	43			33		

Appendix four

Table A4.1 Results of permutational *post hoc* tests where PERMANOVA analysis (reported in text) detected a significant main effect of species on sponge fatty acid profiles. Significant effects are in bold.

Species		t	*P*
C. foliascens	*C. coralliophila*	3.93	0.001
C. foliascens	*R. odorabile*	8.38	0.001
C. foliascens	*S. flabelliformis*	7.51	0.001
C. coralliophila	*R. odorabile*	5.46	0.001
C. coralliophila	*S. flabelliformis*	5.86	0.001
R. odorabile	*S. flabelliformis*	8.85	0.002

Table A4.2a Results of PERMANOVA analysis; the interactive and main effects of temperature and pH are evaluated for total FA (TFA) and the major FA classes for each species where SFA = saturated FA; MUFA = monounsaturated FA; PUFA = polyunsaturated FA). F = Psuedo-F and *P* = p-value derived from permutational comparison. Significant effects are in bold.

	C. foliascens			*C. coralliophila*			*R. odorabile*			*S. flabelliformis*		
	df	**F**	***P***	***df***	**F**	***P***	***df***	**F**	***P***	***df***	**F**	***P***
Total FA												
Temp	2	0.74	0.49	2	5.17	**0.01**	2	1.92	0.15	2	0.16	0.86
pH	2	0.02	0.97	2	0.52	0.61	2	2.01	0.14	2	0.89	0.40
temp*pH	4	2.21	0.10	4	0.10	0.99	4	0.97	0.46	4	1.08	0.39
SFA												
Temp	2	0.26	0.79	2	4.53	**0.01**	2	1.82	0.20	2	0.03	0.98
pH	2	0.04	0.97	2	0.70	0.49	2	1.53	0.23	2	0.83	0.48
temp*pH	4	2.54	0.07	4	0.36	0.85	4	0.65	0.62	4	1.24	0.32
MUFA												
Temp	2	2.21	0.13	2	8.07	**0.00**	2	3.03	0.06	2	0.02	0.97
pH	2	0.12	0.89	2	0.35	0.70	2	2.49	0.08	2	1.26	0.30
temp*pH	4	2.32	0.08	4	0.22	0.91	4	0.84	0.47	4	0.96	0.43
PUFA												
Temp	2	1.11	0.32	2	1.79	0.20	2	1.01	0.39	2	0.42	0.64
pH	2	0.28	0.75	2	0.38	0.69	2	1.56	0.20	2	0.78	0.47
temp*pH	4	1.52	0.23	4	0.07	0.99	4	0.92	0.43	4	0.96	0.46
TRANS												
Temp	2	2.25	0.10	2	2.36	0.13	2	0.66	0.51	2	2.27	0.11
pH	2	2.73	0.07	2	0.63	0.53	2	0.22	0.79	2	0.01	0.99
temp*pH	4	1.00	0.44	4	0.05	1.00	4	0.45	0.77	4	2.21	0.08
N6 PUFA												
Temp	2	2.72	0.08	2	2.75	0.07	2	1.24	0.32	2	0.23	0.83
pH	2	0.74	0.49	2	0.81	0.44	2	0.78	0.46	2	0.60	0.57

temp*pH	4	1.90	0.13	4	0.57	0.67	4	0.48	0.76	4	1.17	0.36
N6 LC PUFA												
Temp	2	1.61	0.22	2	0.83	0.42	2	1.75	0.16	2	0.26	0.79
pH	2	0.16	0.84	2	0.34	0.70	2	0.60	0.56	2	0.47	0.63
temp*pH	4	1.88	0.15	4	1.31	0.26	4	0.34	0.85	4	1.20	0.35
N3 PUFA												
Temp	2	2.17	0.13	2	0.86	0.43	2	1.53	0.23	2	0.57	0.57
pH	2	0.04	0.96	2	1.59	0.22	2	1.66	0.21	2	1.39	0.25
temp*pH	4	0.55	0.71	4	0.93	0.47	4	0.64	0.63	4	0.33	0.87
N3 LC PUFA												
Temp	2	1.49	0.24	2	0.77	0.49	2	1.33	0.28	2	0.56	0.59
pH	2	0.69	0.51	2	1.63	0.23	2	1.19	0.31	2	1.31	0.28
temp*pH	4	0.14	0.98	4	0.88	0.47	4	0.51	0.74	4	0.30	0.89

Table A4.2b Results of permutational *post hoc* tests where PERMANOVA analysis (above) detected significant interactive and main effects of temperature and pH. Significant effects are in bold.

C. coralliophila

	temp		**t**	***P***
TFA	28.5°C	30°C	0.35	0.73
	28.5°C	31.5°C	4.04	**0.00**
	30°C	31.5°C	2.47	**0.01**
SFA	28.5°C	30°C	0.08	0.94
	28.5°C	31.5°C	3.86	**0.00**
	30°C	31.5°C	2.38	**0.03**
MUFA	28.5°C	30°C	1.05	0.29
	28.5°C	31.5°C	5.32	**0.00**
	30°C	31.5°C	2.63	**0.01**

Table A4.3a-c Results of permutational *post hoc* tests where PERMANOVA analysis (reported in text) detected significant interactive and main effects of temperature and pH on sponge fatty acid profiles. Significant effects are in bold.

a. *C. foliascens*

pH	**temp**		**t**	***P***
8.1	28.5°C	30°C	3.07	**0.02**
	28.5°C	31.5°C	3.75	**0.02**
	30°C	31.5°C	1.81	0.06
7.8	28.5°C	30°C	0.68	0.60
	28.5°C	31.5°C	0.76	0.67
	30°C	31.5°C	0.51	0.74
7.6	28.5°C	30°C	1.10	0.28
	28.5°C	31.5°C	0.66	0.75
	30°C	31.5°C	0.78	0.61

b. *C. coralliophila*

	temp		t	*P*
	28.5°C	30°C	1.02	0.34
	28.5°C	31.5°C	3.55	**0.00**
	30°C	31.5°C	1.74	**0.04**

c. *R. odorabile*

	temp		t	*P*
	28.5°C	30°C	2.163	0.026
	28.5°C	31.5°C	2.1367	0.027
	30°C	31.5°C	0.53727	0.743

Table A4.4a Results of PERMANOVA analysis; the interactive and main effects of temperature and pH are evaluated for FA stress indicators for each species where DoU = degree of unsaturation; SFA:PUFA = saturated FA/ polyunsaturated FA; MCL = mean chain length). F = Psuedo-F and P = p-value derived from permutational comparison. Significant effects are in bold.

	C. foliascens			*C. coralliophila*			*R. odorabile*			*S. flabelliformis*		
	df	**F**	***P***	***df***	**F**	***P***	***df***	**F**	***P***	***df***	**F**	***P***
DoU												
Temp	2	3.71	**0.04**	2	3.23	**0.04**	2	4.00	**0.02**	2	0.86	0.45
pH	2	0.33	0.72	2	0.75	0.47	2	0.45	0.63	2	0.02	0.98
temp*pH	4	2.11	0.08	4	1.42	0.24	4	0.10	0.98	4	0.52	0.71
SFA:PUFA												
Temp	2	1.11	0.38	2	3.53	**0.03**	2	2.21	0.12	2	0.94	0.46
pH	2	1.47	0.24	2	0.02	0.99	2	0.11	0.91	2	0.28	0.83
temp*pH	4	3.05	**0.03**	4	0.59	0.67	4	0.49	0.76	4	1.16	0.35
MCL												
Temp	2	0.58	0.61	2	1.08	0.38	2	3.61	**0.04**	2	1.20	0.31
pH	2	1.21	0.32	2	1.22	0.29	2	0.52	0.60	2	1.00	0.40
temp*pH	4	1.09	0.40	4	1.09	0.35	4	0.33	0.85	4	0.61	0.65

Table 4.4b-g Results of permutational *post hoc* tests where PERMANOVA analysis (above) detected significant interactive and main effects of temperature and pH. Significant effects are in bold.

b. C. foliascens

DoU	temp		t	*P*
	28.5°C	30°C	1.7498	0.086
	28.5°C	31.5°C	1.2888	0.214
	30°C	31.5°C	2.6241	**0.015**

c. C. foliascens

SFA:PUFA	temp		t	*P*
8.1	28.5°C	30°C	0.66	0.53
	28.5°C	31.5°C	3.12	**0.04**
	30°C	31.5°C	1.86	0.14
7.8	28.5°C	30°C	0.85	0.38
	28.5°C	31.5°C	0.81	0.43
	30°C	31.5°C	0.23	0.90
7.6	28.5°C	30°C	2.69	**0.03**
	28.5°C	31.5°C	1.59	0.15
	30°C	31.5°C	0.36	0.73

d. C. coralliophila

DoU	temp		t	*P*
	28.5°C	30°C	1.56	0.14
	28.5°C	31.5°C	3.58	**0.00**
	30°C	31.5°C	0.74	0.48

e. C. coralliophila

SFA:PUFA	temp		t	*P*
	28.5°C	30°C	1.56	0.14
	28.5°C	31.5°C	3.58	**0.00**
	30°C	31.5°C	0.74	0.48

f. R. odorabile

DoU	temp		t	*P*
	28.5°C	30°C	1.67	0.10
	28.5°C	31.5°C	2.85	**0.01**
	30°C	31.5°C	1.26	0.23

g. R. odorabile

MCL	temp		t	*P*
	28.5°C	30°C	1.97	0.07
	28.5°C	31.5°C	2.53	**0.02**

	30°C	31.5°C	0.89	0.37

Table A4.5 Results of PERMANOVA analysis; the interactive and main effects of temperature and pH are evaluated for individual FA concentration for each species. F = Psuedo-F and P = p-value derived from permutational comparison. Significant effects are in bold.

Fatty acid	*C. foliascens*			*C. coralliophila*			*R. odorabile*			*S. flabelliformis*		
	df	**F**	***P***	***df***	**F**	***P***	***df***	**F**	***P***	***df***	**F**	***P***
6:0												
Temp	2	1.85	0.17	2	0.03	0.98				2	0.32	0.75
pH	2	1.59	0.20	2	1.25	0.33		*na*		2	0.00	1.00
temp*pH	4	1.12	0.37	4	3.31	**0.02**				4	1.08	0.38
8:0												
Temp	2	1.02	0.40	2	1.18	0.35						
pH	2	3.13	**0.05**	2	0.77	0.60		*na*			*na*	
temp*pH	4	1.45	0.22	4	1.04	0.43						
10:0												
Temp	2	1.66	0.22	2	9.28	0.00						
pH	2	3.30	0.05	2	2.42	0.13		*na*			*na*	
temp*pH	4	3.76	**0.01**	4	2.94	**0.03**						
11:0												
Temp	2	1.70	0.21	2	0.64	0.57						
pH	2	0.42	0.68	2	0.40	0.71		*na*			*na*	
temp*pH	4	0.23	0.92	4	2.69	**0.05**						
12:0												
Temp	2	0.53	0.60	2	0.23	0.79						
pH	2	0.12	0.88	2	0.32	0.72		*na*			*na*	
temp*pH	4	0.53	0.72	4	0.27	0.89						
13:0												
Temp	2	2.66	0.09	2	1.18	0.33						
pH	2	0.35	0.72	2	2.51	0.07		*na*			*na*	
temp*pH	4	1.76	0.18	4	0.79	0.53						
14:0												
Temp	2	0.38	0.68	2	1.36	0.31	2	0.57	0.57	2	0.70	0.50
pH	2	0.19	0.83	2	0.83	0.46	2	0.34	0.73	2	0.86	0.44
temp*pH	4	1.66	0.17	4	0.40	0.82	4	0.93	0.47	4	1.27	0.29
14:1n-5												
Temp	2	2.12	0.13	2	1.12	0.36	2	0.42	0.66	2	3.41	**0.04**
pH	2	1.13	0.36	2	0.89	0.50	2	0.54	0.58	2	0.37	0.73
temp*pH	4	1.36	0.30	4	0.38	0.88	4	1.03	0.40	4	2.14	0.08
15:0												
Temp	2	1.48	0.26	2	2.19	0.12	2	0.66	0.49	2	0.13	0.89
pH	2	0.71	0.50	2	0.76	0.52	2	1.12	0.32	2	0.51	0.59

temp*pH	4	1.75	0.15	4	0.30	0.91	4	0.79	0.54	4	1.01	0.43
15:1n-5												
Temp	2	0.39	0.68	2	0.51	0.61	2	0.70	0.50	2	0.09	0.93
pH	2	6.35	0.01	2	0.22	0.79	2	0.79	0.46	2	0.73	0.47
temp*pH	4	3.71	**0.02**	4	2.36	0.07	4	1.00	0.43	4	0.78	0.55
16:0												
Temp	2	1.99	0.16	2	8.93	**0.00**	2	1.85	0.19	2	0.71	0.51
pH	2	0.36	0.71	2	0.01	1.00	2	1.63	0.20	2	0.44	0.63
temp*pH	4	2.49	0.07	4	0.13	0.97	4	0.45	0.79	4	0.62	0.66
16:1n-7												
Temp	2	3.58	**0.04**	2	6.39	**0.01**	2	4.14	**0.03**	2	1.10	0.36
pH	2	0.46	0.64	2	0.97	0.41	2	0.40	0.66	2	0.61	0.56
temp*pH	4	1.99	0.12	4	0.38	0.82	4	0.19	0.94	4	0.84	0.52
14-Me 16:00												
Temp	2	2.01	0.16	2	0.55	0.59	2	0.80	0.44	2	0.38	0.69
pH	2	0.97	0.37	2	1.35	0.26	2	1.01	0.37	2	0.61	0.56
temp*pH	4	2.56	0.06	4	1.03	0.37	4	0.92	0.47	4	1.26	0.31
3,7,11,15-tetra-Me 16:0												
Temp	2	3.31	0.06	2	1.86	0.15	2	1.27	0.29			
pH	2	3.96	0.03	2	0.84	0.50	2	0.46	0.64		*na*	
temp*pH	4	2.80	**0.05**	4	0.28	0.93	4	0.52	0.72			
17:0												
Temp	2	0.83	0.45	2	0.80	0.42	2	0.52	0.61	2	0.63	0.51
pH	2	0.11	0.89	2	0.72	0.50	2	2.10	0.13	2	1.10	0.35
temp*pH	4	2.78	**0.04**	4	0.23	0.93	4	1.04	0.38	4	0.27	0.90
16:2n-4												
Temp	2	0.77	0.53				2	0.48	0.64			
pH	2	0.93	0.43		*na*		2	1.15	0.35		*na*	
temp*pH	4	0.62	0.69				4	1.42	0.26			
17:1n-7												
Temp	2	2.99	0.07	2	0.12	0.90	2	0.26	0.79	2	0.27	0.78
pH	2	4.57	0.02	2	1.82	0.17	2	1.21	0.29	2	1.80	0.18
temp*pH	4	2.97	**0.04**	4	0.24	0.92	4	1.05	0.39	4	1.98	0.12
16:3n-4												
Temp	2	0.32	0.73				2	0.26	0.79	2	0.08	0.93
pH	2	0.48	0.65		*na*		2	2.15	0.13	2	2.13	0.15
temp*pH	4	0.94	0.48				4	2.51	**0.05**	4	3.92	**0.02**
18:0												
Temp	2	0.53	0.59	2	2.25	0.12	2	0.85	0.43	2	0.62	0.58
pH	2	0.09	0.92	2	0.70	0.53	2	1.41	0.27	2	0.57	0.58
temp*pH	4	2.24	0.10	4	1.06	0.42	4	0.90	0.47	4	1.04	0.42
18:1n-9 t												
Temp	2	5.19	0.02	2	1.01	0.41	2	0.58	0.56	2	0.88	0.45
pH	2	3.75	0.04	2	0.90	0.50	2	0.21	0.80	2	0.95	0.40
temp*pH	4	3.29	**0.02**	4	0.19	0.96	4	0.43	0.78	4	1.22	0.34

18:1n-7 t												
Temp	2	0.80	0.50				2	0.96	0.38			
pH	2	3.79	**0.02**		*na*		2	0.44	0.66		*na*	
temp*pH	4	0.71	0.58				4	0.74	0.60			
18:1n-9												
Temp	2	1.26	0.33	2	8.54	**0.00**	2	2.54	0.09	2	0.63	0.54
pH	2	0.65	0.53	2	0.17	0.83	2	1.54	0.20	2	2.06	0.15
temp*pH	4	1.24	0.31	4	0.23	0.92	4	0.48	0.77	4	0.66	0.61
5,12-Me 18:2n-9												
Temp	2	2.93	0.07	2	0.05	0.96						
pH	2	0.54	0.58	2	3.43	**0.05**		*na*			*na*	
temp*pH	4	1.40	0.26	4	0.45	0.77						
18:1n-7												
Temp				2	2.59	0.08	2	0.93	0.39	2	0.08	0.93
pH		*na*		2	5.42	**0.01**	2	0.92	0.45	2	0.38	0.65
temp*pH				4	1.28	0.28	4	0.67	0.60	4	1.21	0.31
18:2n-6 t												
Temp	2	0.21	0.80	2	2.04	0.14	2	2.33	0.10	2	2.82	0.08
pH	2	1.35	0.28	2	0.71	0.50	2	0.47	0.64	2	0.08	0.93
temp*pH	4	0.99	0.41	4	0.06	1.00	4	0.24	0.92	4	2.26	0.08
5,9-Me 18:2n-9												
Temp	2	2.68	0.09	2	1.28	0.33	2	0.14	0.85			
pH	2	0.48	0.62	2	1.82	0.19	2	1.05	0.37		*na*	
temp*pH	4	1.10	0.37	4	0.41	0.82	4	2.36	0.06			
18:2n-6												
Temp	2	3.16	**0.05**	2	2.63	0.08	2	0.87	0.42	2	1.10	0.32
pH	2	0.34	0.72	2	0.41	0.68	2	1.21	0.31	2	0.95	0.41
temp*pH	4	0.85	0.53	4	0.61	0.67	4	0.60	0.68	4	1.71	0.17
18:3n-6												
Temp	2	2.67	0.08	2	0.04	0.97	2	2.27	0.13	2	1.98	0.17
pH	2	1.43	0.28	2	0.68	0.52	2	1.34	0.27	2	2.58	0.09
temp*pH	4	2.48	0.07	4	0.35	0.86	4	0.57	0.67	4	0.92	0.46
18:3n-4												
Temp	2	0.83	0.45	2	1.03	0.37	2	2.33	0.12	2	0.41	0.68
pH	2	0.46	0.62	2	0.29	0.77	2	1.26	0.30	2	0.00	1.00
temp*pH	4	1.20	0.32	4	0.10	0.98	4	0.75	0.56	4	1.81	0.13
18:3n-3												
Temp	2	3.55	0.05				2	0.80	0.48			
pH	2	2.37	0.11		*na*		2	1.67	0.20		*na*	
temp*pH	4	4.04	**0.01**				4	0.44	0.78			
20:0												
Temp	2	0.48	0.64	2	3.89	**0.04**	2	4.75	**0.02**	2	2.24	0.14
pH	2	0.57	0.57	2	0.46	0.63	2	2.24	0.12	2	2.29	0.14
temp*pH	4	1.84	0.15	4	0.91	0.52	4	0.29	0.89	4	0.80	0.57
18:4n-3												

Temp	2	1.98	0.14				2	2.34	0.12			
pH	2	5.67	0.01		*na*		2	1.76	0.18		*na*	
temp*pH	4	3.77	**0.03**				4	0.95	0.47			
20:1 (isomers)												
Temp	2	1.72	0.19	2	4.10	**0.02**	2	0.88	0.40	2	0.57	0.57
pH	2	0.29	0.73	2	0.24	0.80	2	1.61	0.22	2	1.21	0.31
temp*pH	4	2.29	0.08	4	0.13	0.98	4	1.22	0.31	4	1.84	0.14
20:2n-7												
Temp	2	2.60	0.11	2	3.54	**0.04**	2	4.03	**0.02**			
pH	2	0.30	0.74	2	0.27	0.80	2	2.42	0.12		*na*	
temp*pH	4	1.65	0.21	4	0.24	0.92	4	0.41	0.81			
21:0												
Temp	2	4.24	**0.03**	2	3.52	**0.02**	2	3.84	**0.03**	2	0.01	1.00
pH	2	1.31	0.28	2	0.47	0.70	2	1.09	0.34	2	1.45	0.25
temp*pH	4	0.95	0.45	4	0.70	0.64	4	0.25	0.90	4	1.79	0.16
20:2n-6												
Temp	2	6.94	0.01	2	1.04	0.40	2	0.09	0.91	2	0.04	0.97
pH	2	1.81	0.20	2	0.82	0.48	2	1.34	0.27	2	0.30	0.76
temp*pH	4	2.72	**0.04**	4	0.53	0.75	4	4.22	**0.01**	4	0.54	0.71
20:3n-6												
Temp	2	8.77	**0.00**	2	1.12	0.37	2	0.13	0.90	2	0.35	0.73
pH	2	1.15	0.35	2	3.16	**0.04**	2	0.47	0.64	2	0.30	0.80
temp*pH	4	2.43	0.07	4	0.63	0.69	4	1.02	0.40	4	2.47	0.07
20:4n-6												
Temp	2	4.43	0.02	2	1.41	0.24	2	2.44	0.12	2	0.17	0.84
pH	2	2.50	0.09	2	0.13	0.86	2	0.08	0.92	2	0.43	0.66
temp*pH	4	4.09	**0.01**	4	0.55	0.67	4	0.50	0.74	4	1.26	0.31
20:3n-3												
Temp	2	0.35	0.80	2	1.74	0.12						
pH	2	0.40	0.73	2	1.11	0.39		*na*			*na*	
temp*pH	4	1.09	0.40	4	1.36	0.20						
22:0												
Temp	2	0.30	0.78	2	0.88	0.41	2	4.15	**0.02**	2	0.90	0.42
pH	2	1.72	0.19	2	0.25	0.78	2	1.66	0.21	2	0.62	0.53
temp*pH	4	2.26	0.10	4	0.03	1.00	4	0.71	0.59	4	1.46	0.23
22:1 (isomers)												
Temp	2	1.23	0.29	2	0.55	0.58	2	1.26	0.30	2	0.83	0.48
pH	2	1.43	0.26	2	0.42	0.68	2	1.29	0.27	2	0.05	0.96
temp*pH	4	0.82	0.53	4	1.05	0.39	4	1.46	0.24	4	0.70	0.60
20:4n-3												
Temp	2	0.06	0.93	2	2.06	0.12						
pH	2	1.07	0.38	2	2.00	0.14		*na*			*na*	
temp*pH	4	1.36	0.25	4	0.81	0.54						
20:5n-3												
Temp	2	2.14	0.13	2	1.24	0.29	2	1.86	0.18	2	0.13	0.87

pH	2	1.20	0.32	2	0.95	0.42	2	0.15	0.86	2	0.61	0.55
temp*pH	4	1.57	0.19	4	1.48	0.21	4	1.20	0.33	4	0.76	0.59
22:2n-6												
Temp	2	0.85	0.44	2	3.72	**0.03**	2	1.23	0.29	2	0.96	0.40
pH	2	0.89	0.44	2	1.06	0.36	2	1.56	0.23	2	2.15	0.15
temp*pH	4	0.58	0.66	4	1.00	0.43	4	0.58	0.68	4	0.89	0.50
24:0												
Temp				2	0.64	0.53				2	0.20	0.82
pH		*na*		2	1.14	0.34		*na*		2	1.19	0.31
temp*pH				4	0.06	0.99				4	1.10	0.39
22:3n-3												
Temp	2	0.93	0.40	2	0.07	0.93						
pH	2	0.48	0.59	2	2.05	0.13		*na*			*na*	
temp*pH	4	2.26	0.10	4	1.57	0.18						
22:4n-6												
Temp	2	2.42	0.10	2	0.05	0.95	2	1.34	0.28	2	0.32	0.75
pH	2	0.12	0.89	2	0.02	0.99	2	0.98	0.37	2	0.51	0.60
temp*pH	4	1.64	0.20	4	1.64	0.19	4	0.59	0.70	4	1.26	0.30
22:5n-6												
Temp	2	0.55	0.59	2	1.67	0.15	2	3.48	**0.05**	2	0.47	0.65
pH	2	1.12	0.32	2	1.11	0.37	2	0.63	0.54	2	0.54	0.61
temp*pH	4	0.24	0.92	4	0.77	0.69	4	0.19	0.94	4	0.76	0.58
24:1n-9												
Temp	2	0.21	0.81	2	5.30	**0.01**	2	4.17	**0.02**	2	1.73	0.20
pH	2	0.62	0.57	2	0.03	0.97	2	1.43	0.25	2	1.22	0.30
temp*pH	4	2.09	0.11	4	0.13	0.96	4	0.68	0.60	4	3.01	**0.04**
5,9-Me 24:2n-17												
Temp	2	3.77	0.04	2	5.30	**0.01**	2	1.18	0.33			
pH	2	0.95	0.41	2	0.22	0.80	2	1.48	0.22		*na*	
temp*pH	4	3.45	**0.02**	4	0.17	0.95	4	0.55	0.71			
22:5n-3												
Temp	2	1.81	0.19	2	2.14	0.14	2	0.85	0.43	2	0.31	0.73
pH	2	1.08	0.38	2	0.74	0.49	2	1.51	0.24	2	1.42	0.24
temp*pH	4	0.49	0.76	4	0.13	0.97	4	0.69	0.62	4	0.73	0.57
22:6n-3												
Temp	2	1.18	0.31	2	0.76	0.48	2	1.26	0.30	2	1.17	0.32
pH	2	1.52	0.23	2	0.27	0.74	2	0.27	0.76	2	0.81	0.46
temp*pH	4	0.43	0.80	4	0.78	0.56	4	0.31	0.86	4	0.84	0.53
23-Me 5,9 24:2n-17												
Temp	2	3.12	**0.05**	2	1.81	0.17	2	0.84	0.44	2	0.75	0.51
pH	2	0.41	0.69	2	0.64	0.55	2	1.24	0.30	2	1.58	0.23
temp*pH	4	0.73	0.60	4	0.68	0.67	4	0.69	0.61	4	0.40	0.81
17-Me 26:1n-9												
Temp	2	1.39	0.26	2	0.82	0.45	2	3.69	**0.03**		*na*	
pH	2	1.49	0.23	2	0.46	0.66	2	2.37	0.11			

temp*pH	4	2.76	**0.05**	4	0.23	0.93	4	0.33	0.83	
26:2n-17										
Temp	2	1.67	0.19	2	1.84	0.18	2	0.99	0.37	
pH	2	2.09	0.14	2	0.36	0.71	2	1.29	0.27	*na*
temp*pH	4	1.96	0.10	4	0.46	0.76	4	0.79	0.54	
24:5n-3										
Temp	2	2.72	0.07				2	0.88	0.43	
pH	2	2.29	0.12		*na*		2	0.94	0.40	*na*
temp*pH	4	1.20	0.34				4	1.93	0.14	
24:6n-3										
Temp	2	2.19	0.13	2	0.08	0.91				
pH	2	0.61	0.59	2	1.61	0.22		*na*		*na*
temp*pH	4	1.63	0.20	4	0.89	0.49				

Appendix five

Post-settlement bleaching

ΔF/Fm' values were measured for each recruit at T-end with a blue LED Maxi Imaging-PAM (I-PAM) fluorometer (Walz Germany) using the saturation pulse method described above. Studies on cyanobacteria PSII generally use red excitation light, as blue light preferentially activates PSI in cyanobacteria (El Bissati *et al.*, 2000) due to the phycobiliprotein complexes that function as light-harvesting complexes for cyanobacterial PSII, not absorbing blue light (Solhaug *et al.*, 2014). The result is that yields tend to be lower in blue light due to the transfer of excitation energy to PSI at the expense of PSII (Campbell *et al.*, 1998) as PSI has significantly more chlorophyll than PSII, therefore the blue light is mainly absorbed by PSI (Solhaug *et al.*, 2014). While ΔF/Fm' measured here were not lower than values determined using red light PAM, absolute values should be interpreted with caution. These values are provided only as an approximation of treatment effects on quantum yield to confirm pigment change is due to photosphysiologcal changes (i.e. Lesser *et al.*, 2016). As with pigment change, a significant interaction between temperature and pH affected recruit ΔF/Fm' ($F_{(1, 185)} = 22.368$, $P = 0.001$; Fig. A5.1c). ΔF/Fm' at 31.5°C/ pH 8.1 was significantly lower than for recruits in the other treatments ($P = 0.001$; Fig. A5.1c). While these ΔF/Fm' values suggest that the loss of colour in these recruits is due to photophysiological changes, further work should be carried out using red light iPAM to confirm these changes are due to treatment effects.

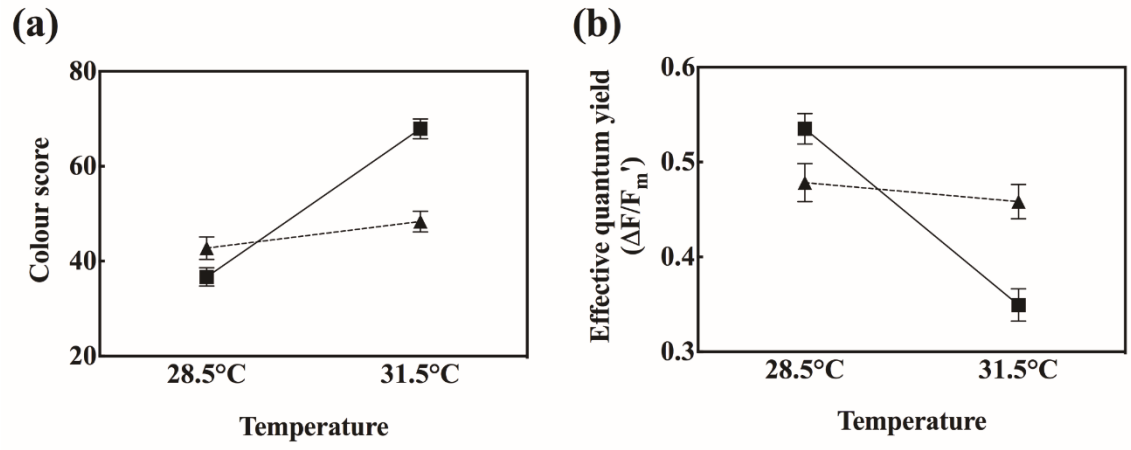

Fig. A5.1/ S4 *C. foliascens* **(a)** colour score **(b)** ΔF/Fm' one-month post-settlement at 28.5°C and 31.5°C where -■- = pH 8.1 and --▲-- = pH 7.6. Values are mean colour score (measured using ImageJ) and ΔF/Fm' (±SE) after one-month exposure to the treatments. Colour score (used as an indicator of bleaching) was measured using ImageJ grayscale. The area of each recruit was selected and the mean grayscale was calculated (0 = pure black and 255 = pure white). ΔF/Fm' values were measured with a blue LED Maxi Imaging-PAM (I-PAM) fluorometer (described above). Colour score and ΔF/Fm' demonstrate a similar response to

temperature and pH and provide evidence to conclude than the visual loss of recruit colour is due to photophysiological changes. n = 14 recruits *per* treatment, except 28.5°C/ pH 7.6 where n = 15 and except where mortalities occurred. Note: y-axis scales differ between response variables.

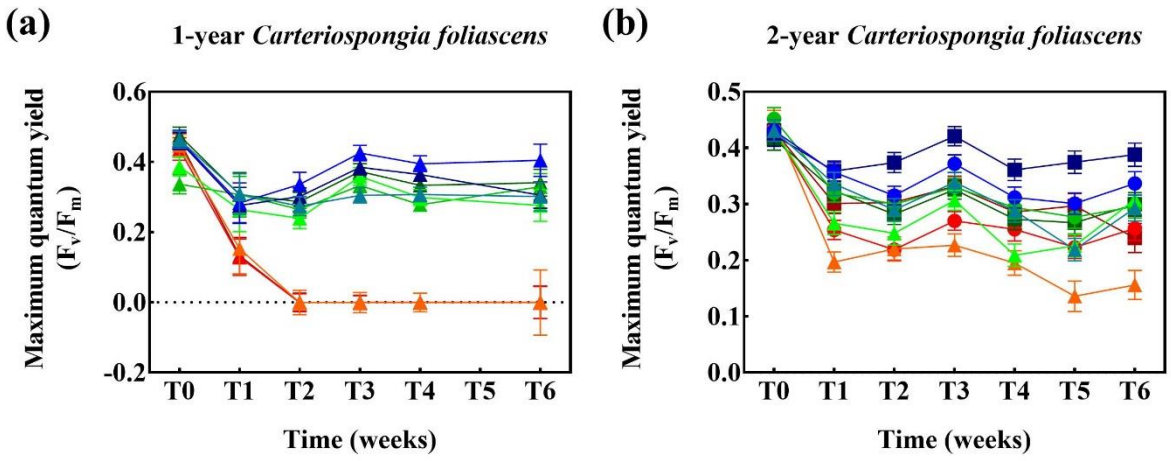

Fig. A5.2 Dark adapted quantum yield for **(a)** 1-year *C. foliascens* recruits and **(b)** 2-year *C. foliascens* recruits in each treatment. ▲ 28.5°C/pH 8.1, ● 28.5°C/pH 7.8, ■ 28.5°C/pH 7.6, ▲ 30°C/pH 8.1, ● 30°C/pH 7.8, ■ 30°C/pH 7.6, ▲31.5°C/pH 8.1, ● 31.5°C/pH 7.8, ■ 31.5°C/pH 7.6. Values are mean quantum yield determined after 30 min dark adaptation, per treatment ± SE per sampling time point (n = 6 1-year *C. foliascens* recruits and n = 14-15 for 2-year *C. foliascens* recruits per treatment per time point, except where mortalities occurred).

Table A5.1 Results of PERMANOVA analysis; the interactive and main effects of temperature and pH are evaluated for pre-settlement larval survival, motility and settlement at 6, 12 and 24 hours post-exposure to experimental treatments. F = Psuedo-F and *P* = p-value derived from permutational comparison. Significant effects are in bold.

Time (hours)	Response	Temp			pH			Temp*pH		
		df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
6	Motile	1, 68	0.3715	0.571	1, 68	0.7737	0.389	1, 68	5.0732	**0.029**
6	Settled	1, 68	0.2399	0.630	1, 68	2.1304	0.153	1, 68	1.1572	0.291
6	Dead	1, 68	3.7713	**0.043**	1, 68	0.5371	0.498	1, 68	6.0863	**0.008**
12	Motile	1, 68	20.7600	**0.001**	1, 68	0.1509	0.718	1, 68	1.9362	0.161
12	Settled	1, 68	0.2486	0.638	1, 68	1.2821	0.253	1, 68	1.4950	0.241
12	Dead	1, 68	3.8076	0.056	1, 68	1.1933	0.289	1, 68	0.5920	0.421
24	Motile	1, 68	4.7232	**0.032**	1, 68	3.6927	0.051	1, 68	1.7315	0.200
24	Settled	1, 68	0.2486	0.632	1, 68	1.2821	0.252	1, 68	1.4950	0.242
24	Dead	1, 68	1.3815	0.242	1, 68	2.9768	0.085	1, 68	0.6415	0.444

Table A5.2a Results of PERMANOVA analysis; the interactive and main effects of temperature and pH are evaluated for change in % of 1-year *C. foliascens* recruits and 2-year *C. foliascens* recruit tissue with necrosis and bleaching. F = Psuedo-F and P = p-value derived from permutational comparison. Significant effects are in bold.

a.	***1 year***			***2 year***		
	df	**F**	***P***	**df**	**F**	***P***
Necrosis						
Temp	2, 45	20.049	**0.001**	2, 107	19.979	**0.001**
pH	2, 45	1.4047	0.255	2, 107	3.7441	**0.022**
temp*pH	4, 45	0.6353	0.636	4, 107	1.2390	0.292
Bleaching						
Temp	2, 45	159.14	**0.001**	2, 107	20.683	**0.001**
pH	2, 45	1.1532	0.406	2, 107	4.4899	**0.012**
temp*pH	4, 45	0.6634	0.756	4, 107	2.4388	**0.049**

Table A5.2b-c Results of permutational *post hoc* tests where PERMANOVA analysis (above) detected significant interactive and main effects of temperature and pH. Significant effects are in bold.

b. 1-year C. foliascens

Necrosis	**temp**		**t**	***P***
	30°C	28.5°C	1.0507	0.375
	30°C	31.5°C	5.3799	**0.001**
	28.5°C	31.5°C	4.2033	**0.001**
Bleaching	**temp**			
	30°C	28.5°C	1.0000	0.434
	30°C	31.5°C	20.3660	**0.001**
	28.5°C	31.5°C	12.2120	**0.001**

c. 2-year C. foliascens

Necrosis	**temp**		**t**	***P***
	30°C	28.5°C	0.9414	0.386
	30°C	31.5°C	4.3493	**0.001**
	28.5°C	31.5°C	5.0324	**0.001**
Necrosis	**pH**		**t**	***P***
	7.6	8.1	1.2173	0.252
	7.6	7.8	1.6425	0.116
	8.1	7.8	2.6330	**0.020**
Bleaching	**temp**		**t**	***P***
8.1	28.5°C	30°C	1.1680	0.177
	28.5°C	31.5°C	2.9591	**0.010**
	30°C	31.5°C	4.4020	**0.001**
7.8	28.5°C	30°C	0.3633	0.786
	28.5°C	31.5°C	1.7128	0.058
	30°C	31.5°C	1.5393	0.148
7.6	28.5°C	30°C	0.0000	1.000
	28.5°C	31.5°C	2.5745	**0.002**
	30°C	31.5°C	2.5782	**0.001**

Table A5.3a-b Results of permutational *post hoc* pairwise comparisons test for a) 1-year *C. foliascens* recruits and b) 2-year *C. foliascens* recruit effective quantum yield (ΔF/Fm') based on significant treatment effects revealed by permutational three-way analysis of variance.

a.

Time	Temp		t	*P*
T0	31.5°C	30°C	0.450	0.657
	31.5°C	28.5°C	1.127	0.252
	30°C	28.5°C	1.569	0.136
T1	31.5°C	30°C	3.814	**0.001**
	31.5°C	28.5°C	4.010	**0.001**
	30°C	28.5°C	1.186	0.257
T2	31.5°C	30°C	10.880	**0.001**
	31.5°C	28.5°C	11.159	**0.001**
	30°C	28.5°C	2.226	**0.027**
T3	31.5°C	30°C	29.658	**0.001**
	31.5°C	28.5°C	43.747	**0.001**
	30°C	28.5°C	2.835	**0.009**
T4	31.5°C	30°C	15.939	**0.001**
	31.5°C	28.5°C	36.750	**0.001**
	30°C	28.5°C	1.071	0.306
T6	31.5°C	30°C	28.508	**0.001**
	31.5°C	28.5°C	18.441	**0.001**
	30°C	28.5°C	0.041	0.964

b.

Time	Temp	pH		t	*P*
T0	28.5°C	ns			
T1		7.6	8.1	2.870	**0.014**
		7.6	7.8	0.783	0.451
		8.1	7.8	2.317	**0.027**
T2		7.6	8.1	4.191	**0.002**
		7.6	7.8	1.984	0.061
		8.1	7.8	3.462	**0.003**
T3		7.6	8.1	2.877	**0.014**
		7.6	7.8	0.471	0.648
		8.1	7.8	2.719	**0.016**
T4		7.6	8.1	3.124	**0.004**
		7.6	7.8	0.864	0.390
		8.1	7.8	2.734	**0.011**
T5		7.6	8.1	4.550	**0.001**
		7.6	7.8	1.371	0.172
		8.1	7.8	3.476	**0.001**
T6		7.6	8.1	4.808	**0.001**
		7.6	7.8	3.150	**0.008**

		8.1	7.8	2.505	**0.018**
T0				ns	
T1		7.6	8.1	4.617	**0.001**
		7.6	7.8	0.563	0.588
		8.1	7.8	4.153	**0.001**
T2		7.6	8.1	3.338	**0.003**
		7.6	7.8	0.244	0.804
		8.1	7.8	3.628	**0.003**
T3		7.6	8.1	3.003	**0.013**
		7.6	7.8	0.649	0.543
	30°C	8.1	7.8	4.276	**0.001**
T4		7.6	8.1	4.905	**0.001**
		7.6	7.8	1.381	0.177
		8.1	7.8	4.765	**0.001**
T5		7.6	8.1	1.387	0.188
		7.6	7.8	0.022	0.989
		8.1	7.8	1.471	0.169
T6		7.6	8.1	1.476	0.161
		7.6	7.8	0.840	0.423
		8.1	7.8	2.563	**0.017**
T0				ns	
T1		7.6	8.1	2.377	**0.029**
		7.6	7.8	0.594	0.516
		8.1	7.8	1.835	0.089
T2		7.6	8.1	1.216	0.241
		7.6	7.8	0.223	0.843
		8.1	7.8	1.203	0.226
T3		7.6	8.1	1.892	0.079
		7.6	7.8	1.139	0.273
	31.5°C	8.1	7.8	0.734	0.465
T4		7.6	8.1	1.637	0.120
		7.6	7.8	0.580	0.557
		8.1	7.8	2.339	**0.021**
T5		7.6	8.1	2.939	**0.004**
		7.6	7.8	0.077	0.944
		8.1	7.8	3.489	**0.004**
T6		7.6	8.1	2.099	**0.049**
		7.6	7.8	1.036	0.329
		8.1	7.8	3.817	**0.002**