The effects of a changing marine environment on the bioeroding sponge *Cliona orientalis*

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Abstract

Bioeroding sponges are a unique group of coral reef sponges. They transform dissolved nutrients into particulate nutrients via active filter feeding whilst also eroding the coral reef framework that they inhabit. Despite their ecological importance, we know little about their distribution or abundance, especially along the inshore Great Barrier Reef (GBR). In addition, bioeroding sponges are often considered to be thermally tolerant, even though their thermal thresholds are unknown. Bioeroding sponges also occur in high abundance in polluted or eutrophic habitats, but it is unclear whether these conditions directly benefit sponges through accelerated growth or improved condition or benefit bioeroding sponges indirectly via negative effects on corals. To address these knowledge gaps, this thesis investigated whether bioeroding sponges and their photosynthetic symbionts can tolerate changing environmental conditions on coral reefs. Research focused on *Cliona orientalis* as it is a conspicuous bioeroding sponge on the GBR. Field surveys were used to measure the abundance of *C. orientalis* on the inshore GBR and laboratory experiments were performed to investigate the response of *C. orientalis* to ocean warming and nutrient enrichment.

Decreasing coral cover on the GBR may provide opportunities for rapid growth and expansion of other taxa. The bioeroding sponges *Cliona* spp. may increase in abundance after coral bleaching, damage, and mortality as they withstand elevated temperatures without bleaching. In Chapter 2, I analysed benthic surveys of the inshore GBR (2005–2014) which revealed that the percent cover of *C. orientalis* has not increased in the past decade, as would be expected if the sponge benefited from coral bleaching or mortality. I found that the proportion of

fine particles in benthic sediments was negatively associated with the presence-absence and the percent cover of this sponge, indicating that *C. orientalis* requires wave-exposed habitats where fine sediments are absent. The fastest increases in *C. orientalis* cover coincided with the lowest macroalgal cover and chlorophyll *a* concentration, highlighting the importance of macroalgal competition and local environmental conditions for this sponge. Given the observed distribution and habitat preferences of *C. orientalis*, bioeroding sponges likely represent site-specific rather than regional threats to corals and reef accretion.

Coral reefs face many stressors associated with global climate change, including increasing sea surface temperature and ocean acidification. In Chapters 3 and 4, I exposed *C. orientalis* to temperature increments increasing from 23 to 32 °C to define the thermal tolerance threshold of the sponge and its associated microbiome. At 32 °C, or 3 °C above the maximum monthly mean (MMM) temperature, sponges bleached and the photosynthetic capacity of *Symbiodinium* was compromised, consistent with sympatric corals. *Cliona orientalis* demonstrated little capacity to recover from thermal stress, remaining bleached with reduced *Symbiodinium* density and energy reserves after one month at reduced temperature. While *C. orientalis* can withstand current temperature extremes (<3 °C above MMM) under laboratory and natural conditions, this species would not survive ocean temperatures projected for 2100 without acclimatisation or adaptation (≥3 °C above MMM). In Chapter 4, I demonstrated that bleaching of *C. orientalis* is preceded by a change in its microbial community, which is not restored after the thermal stress is removed. In Chapter 5, I investigated the effects of dissolved inorganic nutrients and light intensity on the growth and condition of five common Great Barrier Reef sponges, including *C. orientalis*, to test whether *C. orientalis* responds differently than other sponge species. Dissolved nutrients up to 7

μM total DIN did not significantly affect the growth, condition, or chlorophyll content of any sponge species after 10 weeks of exposure. Light (80 vs 160 μmol quanta m^{-2} s^{-1}) did not affect four of the five sponge species, but higher irradiance resulted in higher organic content and chlorophyll levels in *C. orientalis*.

Hence, as ocean temperatures increase above local thermal thresholds, *C. orientalis* will have a negligible impact on reef erosion, and nutrient enrichment is unlikely to alter these effects.

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Chapter 1. Introduction

Coral reefs are in decline across the world. On the Great Barrier Reef (GBR), coral cover decreased by 50% just from 1985 to 2012 (De'ath et al. 2012), and decreased further following widespread coral bleaching in 2016-2017 (Hughes, Kerry, et al. 2017). In the Caribbean, coral cover is currently 20% of what it was in the 1970s (Gardner et al. 2003). By itself, the loss of coral fails to capture the extent of reef ecosystem degradation, as carnivores, herbivores, filter feeders, and seagrasses are all declining (Pandolfi et al. 2003) coincident with coral loss. As corals decline, understanding how other taxa respond to changing environmental conditions will provide a glimpse of what reef communities may look like in the future (Graham et al. 2014, Hughes, Barnes, et al. 2017).

Coral reefs are threatened by multiple anthropogenic stressors (Hughes, Barnes, et al. 2017), although much of the recent decline has been attributed to ocean warming (Hoegh-Guldberg 1999, Hughes, Kerry, et al. 2017). Warming is caused by carbon dioxide emissions produced from burning fossil fuels (IPCC 2014). For corals, ocean warming disrupts the mutualism with their endosymbiotic dinoflagellate *Symbiodinium*, termed coral bleaching (Baird et al. 2009). Severe coral bleaching events occurred in 2016 and 2017, with bleaching particularly intense in the northern and central GBR which experienced up to 83% coral mortality (Hughes et al. 2016). Ocean warming also has the potential to affect non-coral invertebrates that rely on *Symbiodinium* for their energy requirements, including anemones, clams, foraminifera, octocorals, and sponges (McClanahan et al. 2008, Hill et al. 2016), although less is known about bleaching in these taxa.

While the outlook for corals is bleak, sponges potentially tolerate changing environmental

conditions, including ocean warming, ocean acidification, and nutrient enrichment, better than reef-building corals (Bell et al. 2013). Sponges are multicellular animals found in all aquatic habitats where they filter dissolved and particulate material out of the water and play an integral role in nutrient cycling (Bell 2008, de Goeij et al. 2013, Rix et al. 2018). Sponges may be resistant to ocean warming since few species associate with *Symbiodinium* (Hill et al. 2011) and those with *Symbiodinium* appear to tolerate exposure to elevated temperature (Vicente 1990, Schönberg & Ortiz 2008). In addition to warming, oceans are becoming more acidic as the excess carbon dioxide reacts with seawater, releasing protons through a series of equilibrium reactions (Guinotte & Fabry 2008). However, sponge growth (Bell et al. 2017), respiration, and survival are unlikely to be affected by ocean acidification(Bennett et al. 2016). Sponges may also benefit from nutrient enrichment of coastal reefs, which lead to increased concentrations of food as organic matter available for nutrition (Dinsdale & Rohwer 2010, Pawlik et al. 2016).

While all sponges may tolerate ocean warming and nutrient enrichment, bioeroding sponges in particular may benefit from changing environmental conditions (Schönberg, Fang, & Carballo 2017), and this could tip the balance from accretion to erosion on future reefs (Kennedy et al. 2013, Perry et al. 2014). This thesis investigates whether bioeroding sponges benefit from warming and nutrient-enriched oceans. The primary objectives are to determine whether the abundance of bioeroding sponges is increasing on the GBR, to assess whether bioeroding sponges have high thermal tolerance, and to evaluate whether bioeroding sponges benefit from elevated dissolved nutrients more than other sponge species.

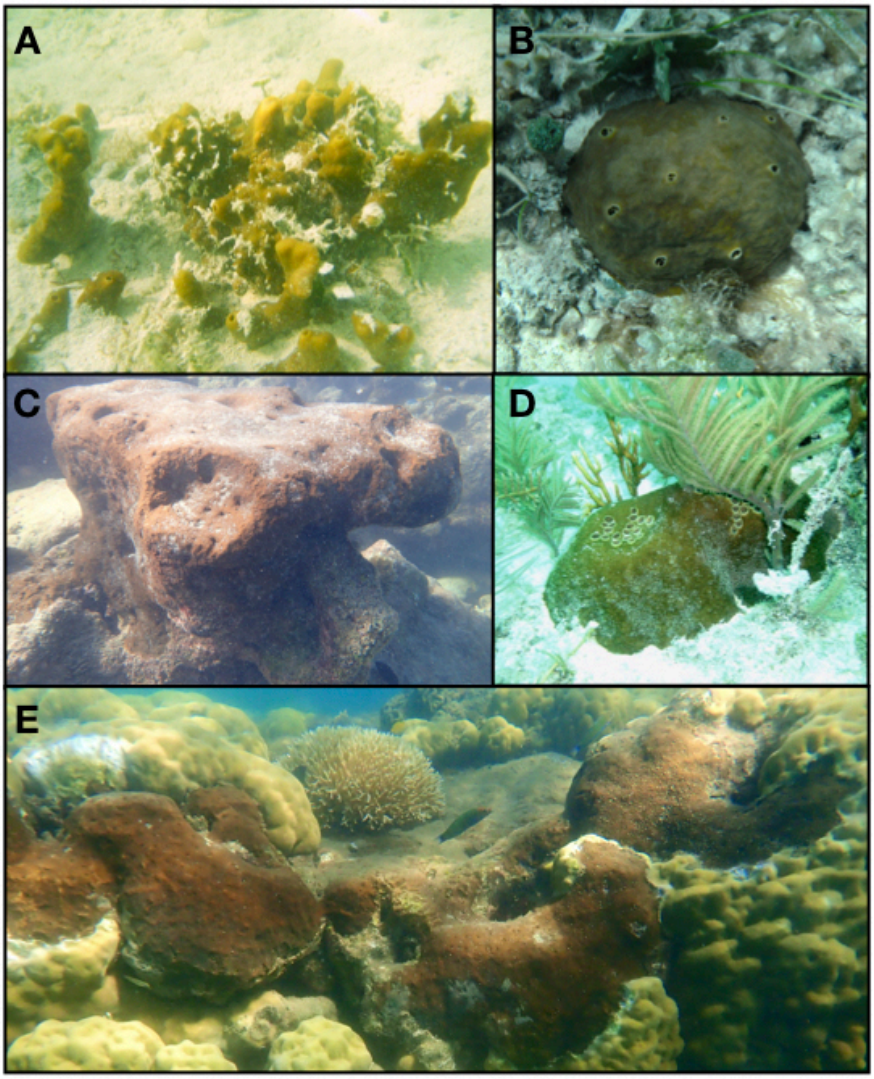

Figure 1-1. Three bioeroding sponge species with *Symbiodinium*. A) *Cliona varians* forma *varians* (Florida, USA). B) *Cliona varians* forma *incrustans* (Quintana Roo, Mexico). C) *Cliona orientalis* (Queensland, Australia). D) *Cliona tumula* (Florida, USA). E) *Cliona orientalis* (Queensland, Australia).

1.1. Bioeroding sponges

Bioerosion is the erosion of substrata by biological agents and is performed by many species of invertebrates and vertebrates in reef ecosystems (Perry & Harborne 2016). Bioeroding sponges erode coral skeleton through physical processes that break apart calcium carbonate and chemical processes that dissolve it (Fang et al. 2013, de Bakker et al. 2018). Specialized etching cells facilitate sponge erosion by extending pseudopodia into the substratum and secreting

enzymes (Pomponi 1979, Glynn 1997), thereby reducing the pH and saturation state of calcium carbonate and weakening the substrate beneath the sponge (Schönberg 2002). The molecular agent of chemical erosion has yet to be determined (Schönberg 2008).

Only a few sponge species erode the reef substratum, most of which belong to the families Clionaidae and Spirastrellidae and the genera *Cervicornia, Cliona, Pione*, *Diplastrella*, and *Spirastrella* (Schönberg, Fang, & Carballo 2017). In particular, *Cliona* species are conspicuous bioeroders in many ecosystems, including tropical coral reefs (Holmes et al. 2000, Rützler 2002, Nava et al. 2014), temperate oyster beds (Duckworth & Peterson 2013), and coastal caves (Mariani et al. 2000). *Cliona* spp. are of specific interest on the GBR, as they tolerate elevated temperatures and have increased in cover at one location (Schönberg & Ortiz 2008, Schönberg et al. 2008). In addition to eroding calcium carbonate, bioeroding sponges are the only group of sponges that associate with the dinoflagellate *Symbiodinium* (Hill 1996, Weisz et al. 2010, Achlatis et al. 2018). Carbon translocation from *Symbiodinium* to *Cliona varians* provides autotrophic energy for the sponge (Weisz et al. 2010, Achlatis et al. 2018), in the same way that *Symbiodinium* fuels coral growth (Muscatine et al. 1984). Thus, *Symbiodinium* photosynthates likely represent a major component of the energy budget of bioeroding sponges (Fang et al. 2014). The *Symbiodinium* in *Cliona* are most closely related to *Symbiodinium* found in Foraminifera (clade G), rather than the *Symbiodinium* of Cnidarians ((clades A-D); Schönberg & Loh 2005, Hill et al. 2011). *Cliona* species associate with distinct *Symbiodinium* species, as *Cliona orientalis* harbors *Symbiodinium endoclionum*, while *Cliona varians* associates with *Symbiodinium spongiolum* (Appendix B: Ramsby, Hill, et al. 2017).

This thesis will focus primarily on *C. orientalis* which has been a focal point of sponge bioerosion research (Schönberg 2002, 2006, Holmes et al. 2009) and a model for how sponge

erosion will be affected by ocean warming and acidification (Schönberg et al. 2008, Wisshak et al. 2013, Fang et al. 2013, 2014, Achlatis et al. 2017).

1.2. Monitoring bioeroding sponges

As space is limiting on coral reefs, declining coral cover could allow for increases in the abundance of other taxa, such as algae and sponges (Ward-Paige et al. 2005, Hughes et al. 2010, Bell et al. 2013). While trends in coral and algal cover have been extensively documented (Bruno et al. 2009), very few reef surveys monitor sponges, likely because sponge species can be difficult to identify and are often cryptic (Berman et al. 2013, Schönberg 2015, Bell et al. 2017).

Encrusting sponge populations can proliferate following disturbance, highlighting their importance for monitoring efforts. In an extreme example, following massive coral bleaching, the abundance of the encrusting sponge *Chondrilla caribensis* (not bioeroding) increased until it comprised virtually all of the living cover on the reef (Aronson et al. 2012). Multiple reports also describe increases in encrusting bioeroding sponges following coral mortality (Cortés et al. 1984, Rützler 2002, Lopez-Victoria & Zea 2005, Carballo et al. 2013). These observations raise two important points: i) bioeroding sponges appear to respond quickly to changes in the benthic community and ii) bioeroding sponges appear to be relatively resilient to stress compared to other reef taxa. Given the decline in coral cover on the GBR and changing community composition (De'ath et al. 2012, Hughes et al. 2018), further understanding and monitoring of bioeroding sponges is clearly warranted.

Abundance data for bioeroding sponges on the GBR is sparse. For bioeroding sponges that occupy the surface of the reef substratum, like *Cliona orientalis*, their abundance is accurately quantified via percent cover. Surveys at Orpheus Island indicate that *Cliona* spp. cover increased

from 10 to 15% between 1997 and 2004, which encompasses two coral bleaching events (Schönberg & Ortiz 2008). Unfortunately, little is known about *C. orientalis* cover elsewhere on the GBR. Increased cover of *Cliona* spp. has also been reported on the Mexican Pacific Coast (Nava et al. 2014) and in Belize (Rützler 2002). In other areas however, little change has occurred in *Cliona* spp. (Ruzicka et al. 2010, Bautista Guerrero et al. 2013). Articles entitled "…*Cliona vermifera*: a threat to Pacific coral reefs?" and "Boring sponges, an increasing threat for coral reefs affected by bleaching events" emphasize that dramatic changes are occurring in the abundance of bioeroding sponges (Carballo et al. 2013, Bautista Guerrero et al. 2013), but the extent of the threat within the GBR and elsewhere is still largely unknown.

Sponge distributions can also be influenced by depth, sedimentation, and light (Wulff 2012). Total sponge abundance typically increases with depth (Wilkinson & Evans 1989, Wilkinson & Cheshire 1989), but this is generally driven by increases in upright or massive sponge species rather than encrusting morphologies, like *Cliona* spp. (Roberts & Davis 1996). In contrast, the abundance of encrusting sponges has been shown to decrease with depth (Zea 1993, Roberts & Davis 1996). However, it is challenging to untangle the effects of environmental variables that co-vary with depth, including distance from shore, light, water movement, and organic matter (Wilkinson & Cheshire 1989). In particular, light can limit the maximum depth of sponges with photosynthetic symbionts (Cheshire & Wilkinson 1991, Cheshire et al. 1997). Since *C. orientalis* depends upon *Symbiodinium* photosynthesis, light likely contributes to the shallow distribution of *C. orientalis* (Schönberg 2001). Moreover, bioeroding sponges require suitable calcium carbonate substratum, which may also be correlated with biotic or abiotic gradients (Schönberg 2015).

Biotic factors can also influence the distribution of sponges, including competition and predation (Wulff 2012). Sponges can be strong competitors, but macroalgae can outcompete sponges for space on coral reef (Cebrian & Uriz 2006, Wulff 2006). In addition, predation can also exclude sponges from reef or seagrass habitats (Hill 1998, Ruzicka & Gleason 2009). Thus, the abundance and distribution of sponges are influenced by a combination of biotic and abiotic factors.

1.3. Thermal tolerance of bioeroding sponges

Oceans have warmed by 0.6 °C in the last 100 years (IPCC 2014), with significant adverse consequences for coral reefs (Pandolfi et al. 2011). Most evidence suggests that corals cannot increase thermal tolerance by acclimation to warmer conditions (Middlebrook et al. 2012, Howells et al. 2013, Rodolfo-Metalpa et al. 2014, Silverstein et al. 2015), leading to bleak projections regarding the future of coral-dominated reefs under current climate scenarios (Pandolfi et al. 2011). In contrast, bioeroding sponges may benefit from climate change by growing faster (Fang et al. 2013), eroding faster (Stubler et al. 2014), or becoming stronger competitors.

Laboratory studies have investigated the effects of elevated temperature on bioeroding sponges (Schönberg et al. 2008, Duckworth & Peterson 2013, Fang et al. 2013, Achlatis et al. 2017), with the available evidence suggesting that *Cliona* spp. can tolerate ocean warming. In one study, four months of exposure to elevated temperature (+5 °C) did not induce mortality or affect the growth of the temperate sponge *Cliona celata* (Duckworth & Peterson 2013). In addition, the rates of bioerosion by *C. celata* and *C. orientalis* were not affected by exposure to elevated temperature (Duckworth & Peterson 2013, Wisshak et al. 2013). Field observations also suggest

bioeroding sponges may be tolerant of elevated temperature, as *Cliona aprica* and *C. varians* were unaffected by a thermal stress event in 1990 that induced coral bleaching (Vicente 1990). Furthermore, *Cliona* spp. can increase in abundance following coral mortality post bleaching (Rützler 2002, Lopez-Victoria & Zea 2005, Carballo et al. 2013). If bioeroding sponges are more tolerant of ocean warming than scleractinian corals, sponge bioerosion should increase in the future (Perry et al. 2014). Importantly however, the thermal limits and bleaching thresholds of bioeroding sponges are unknown, in part due to the use of factorial experimental designs to study interactive effects (Fang et al. 2013, 2014, Achlatis et al. 2017) rather than functional responses to identify threshold temperatures. As these sponges contribute to reef erosion, understanding the functional response of bioeroding sponges to temperature will enhance our understanding of reef communities and reef growth in the future.

Elevated temperature can impair *Symbiodinium* photosynthesis, which can precede coral bleaching (Warner et al. 1999). It has been suggested that the *Symbiodinium* of *C. orientalis*, *S. endoclionum* (Ramsby, Hill, et al. 2017), may be resistant to thermal stress as it maintains higher yields during heat and light stress than *Symbiodinium* from *Acropora palifera* (Schönberg et al. 2008). In another study, net photosynthetic production of oxygen by *Symbiodinium* in *C. orientalis* increased under moderate warming and acidification, suggesting that it is thermally-tolerant, but photosynthesis decreased under more extreme conditions (Fang et al. 2014). However, *C. orientalis* can bleach like corals, which may limit its ability to tolerate future climate conditions (Fang et al. 2014, Achlatis et al. 2017). Thus, the response of *C. orientalis* to ocean warming will also depend on the response of its *Symbiodinium*, yet the amount of thermal stress required to impair either partner is largely unknown.

In addition to *Symbiodinium*, *C. orientalis* hosts a diverse bacterial community that may be affected by elevated sea temperatures (Pineda et al. 2016). Sponges associate with a diverse group of microorganisms that can perform an array of metabolic functions and are linked to sponge health(Webster & Thomas 2016). Many sponge species experience a rapid shift in the composition and function of their microbial communities during thermal stress (Simister, Taylor, Tsai, Fan, et al. 2012, Fan et al. 2013), which can lead to disease, necrosis, and mortality (Webster & Taylor 2012, Fan et al. 2013). Hence, understanding the effects of warming on the microbiome of *C. orientalis* is requisite for a complete understanding the response of this sponge to climate change.

1.4. Nutrient enrichment and bioeroding sponges

Bioeroding sponges can be abundant on degraded or polluted reefs (Rose & Risk 1985, Holmes et al. 2000, Nava et al. 2014), which is a concern for much of the inshore GBR as pollution pressure increases (Brodie et al. 2012). Runoff from agricultural lands carries inorganic fertilizer and organic material to the inshore GBR (Fabricius 2005). Flood plumes reach inshore reefs during the wet season, reducing salinities and carrying inorganic nutrients and organic matter which reduce water quality and light (Brodie et al. 2011). Currently, the waters of the inshore GBR contain over 5 times more nitrogen and phosphorus than before Western settlement (Kroon et al. 2012), which can produce algal blooms and lead to coral disease (Fabricius 2005). However, while these conditions are unfavourable for corals, they may benefit sponges and other filter-feeding organisms (Smith et al. 1981, Fabricius 2005, Uthicke et al. 2014). For example, nutrient enrichment leads to increased food availability for sponges, which can metabolize dissolved nutrients via microbial symbioses or directly consume microalgae and plankton (Fiore, Baker, & Lesser 2013a, Mueller et al. 2014).

In other regions, bioeroding sponges occurred in higher abundance in areas with high nutrient levels (Rose & Risk 1985, Holmes et al. 2000, Nava et al. 2014). For example, *Cliona* spp. colonies were larger and more abundant at sites with high nutrient levels in the Florida Keys, USA (Ward-Paige et al. 2005). In addition to abundance, elevated nutrient levels may affect the physiology of bioeroding sponges, as *C. orientalis* eroded calcium carbonate faster in nutrient-rich reefs, although the study did not manipulate nutrient levels directly (Holmes et al. 2009). Despite many claims that nutrient-rich water benefit bioeroding sponges (Rose & Risk 1985, Holmes et al. 2000, Nava et al. 2014), direct experimental evidence that bioeroding sponges benefit from these conditions is lacking.

1.5. Thesis overview

The aim of this thesis is to determine the impact of ocean warming and nutrient enrichment on bioeroding sponges, with a focus on the bioeroding sponge *Cliona orientalis*. Chapter 2 describes the abundance of *C. orientalis* across the inshore GBR and identifies the environmental factors that limit its distribution. In addition, locations with increasing *C. orientalis* abundance are identified and discussed in the context of nutrient enrichment and competition with macroalgae. Chapter 3 defines the functional response to temperature for *C. orientalis*, including the threshold and physiological effects of sponge bleaching. The bleaching threshold is discussed in the context of the thermal sensitivity of sympatric corals with implications for future reef erosion. Chapter 4 expands upon Chapter 3 by investigating how warming and sponge bleaching affect the microbiome of *C. orientalis*. Destabilisation of the microbiome may contribute to bleaching and the breakdown of the symbiosis between *C. orientalis* and *Symbiodinium*. In addition, abundant microbial taxa are identified that may play important roles within the *C. orientalis* holobiont. Chapter 5 takes a broader approach and

investigates whether the growth or condition of GBR sponges, including *C. orientalis*, are affected by nutrient enrichment or changes in irradiance. Investigating how *C. orientalis* responds to ocean warming and nutrient enrichment furthers our understanding of how bioeroding sponges will affect erosion on future reefs.

Chapter 2. A decadal analysis of bioeroding sponge cover on the inshore Great Barrier Reef

2.1. Abstract

Decreasing coral cover on the Great Barrier Reef (GBR) may provide opportunities for rapid growth and expansion of other taxa. The bioeroding sponges *Cliona* spp. are strong competitors for space and may take advantage of coral bleaching, damage, and mortality. Benthic surveys of the inshore GBR (2005–2014) revealed that the percent cover of the most abundant bioeroding sponge species, *Cliona orientalis*, has not increased. However, considerable variation in *C. orientalis* cover, and change in cover over time, was evident between survey locations. We assessed whether biotic or environmental characteristics were associated with variation in *C. orientalis* distribution and abundance. The proportion of fine particles in the sediments was negatively associated with the presence-absence and the percent cover of *C. orientalis*, indicating that the sponge requires exposed habitat. The cover of corals and other sponges explained little variation in *C. orientalis* cover or distribution. The fastest increases in *C. orientalis* cover coincided with the lowest macroalgal cover and chlorophyll *a* concentration, highlighting the importance of macroalgal competition and local environmental conditions for this bioeroding sponge. Given the observed distribution and habitat preferences of *C. orientalis*, bioeroding sponges likely represent site-specific – rather than regional – threats to corals and reef accretion.

2.2. Introduction

Loss of coral cover has led to dire predictions for the future of coral reef ecosystems (Pandolfi et al. 2011, Kennedy et al. 2013, Spillman 2014), including the Great Barrier Reef (GBR) (De'ath et al. 2012). A number of processes compromise coral health and the broader health of

coral reefs, including increased sea surface temperatures, ocean acidification, pollution, cyclones, and crown of thorns starfish outbreaks (Dubinsky & Stambler 2010, De'ath et al. 2012). All of these stressors are predicted to intensify over coming decades, potentially shifting the coral reef benthic community from coral-dominated systems to those dominated by less-sensitive species (Hughes et al. 2010, Bell et al. 2013). Some community changes have already been documented on coral reefs, including changes along acidification gradients at CO_2 seeps (Fabricius et al. 2011) and the octocoral and sponge dominance of shallow habitat of the Florida Keys, USA (Ruzicka et al. 2013, Loh et al. 2015).

Changes to reef communities may reduce reef accretion, the balance of calcification and consolidation with erosional processes (Glynn 1997). Increased abundance of eroding organisms (bioeroders) or decreased abundance of calcifying organisms already suggest that some reefs are eroding rather than growing (Perry et al. 2013, 2014). Bioeroding sponges break down coral skeleton and other calcium carbonate structures, oyster shells, and cave walls. The sponges grow several mm to several cm into the coral skeleton and some species can quickly overgrow adjacent live coral tissue (Schönberg 2003). While sponges erode calcium carbonate at fast rates (Neumann 1966, Rützler 1975, Acker & Risk 1985), bioeroding sponges are patchily-distributed, which currently limits their impact on regional carbonate budgets (Perry et al. 2014, Enochs et al. 2015).

In some locations, bioeroding sponges (mostly *Cliona* spp.) have recently increased in abundance (Rützler 2002, Ward-Paige et al. 2005, Schönberg & Ortiz 2008, Kelmo et al. 2013). While these reports are largely restricted to single reefs, the rates of increase are notable: *Cliona caribbaea* cover doubled between 1979 and 1998 at one location in Belize (Rützler 2002) and *Cliona* spp. abundance doubled between 1996 and 2001 in the Florida Keys, USA (Ward-Paige et

al. 2005). In addition, the abundance of *Cliona orientalis* more than doubled between 1998 and 2004 at one location in Queensland, Australia (Schönberg & Ortiz 2008). These changes gave rise to the hypothesis that the abundance of bioeroding sponges has increased over time, but the geographic extent and rate of these increases are largely unknown.

Several physiological and ecological hypotheses have been proposed to explain the observed increases in abundance of bioeroding sponges. *Cliona* is thought to be a robust sponge taxon that is tolerant of disturbances and changing environmental conditions (Vicente 1990, Schönberg et al. 2008, Fang et al. 2013, Stubler et al. 2014) as well as benefitting from the poor water quality that can adversely affect corals (Holmes 2000, Ward-Paige et al. 2005). Based on the success of *Cliona* spp. in similar habitats, the inshore GBR was expected to be optimal habitat for bioeroding sponges where site-specific increases in cover may represent regional trends (Schönberg & Ortiz 2008). However, poor water quality is also associated with low light conditions that may negatively impact growth of photo-symbiotic bioeroding sponges such as *C. orientalis* and *C. varians* (Hill 1996, Schönberg 2006).

Increases in the abundance of bioeroding sponges will have implications for coral reefs in addition to the erosion of substratum (Rützler 2002). Bioeroding sponges weaken reef substrata, produce carbonate sediments (Hutchings 1986, Glynn 1997, Carballo et al. 2017), and are strong competitors against live corals (Schönberg & Wilkinson 2001, Rützler 2002, Lopez-Victoria & Zea 2004, Márquez & Zea 2006, Chaves-Fonnegra & Zea 2007, González-Rivero et al. 2012, Halperin et al. 2015), particularly following coral bleaching events (Carballo et al. 2013). However, the growth of *Cliona* spp. can be limited by macroalgae (Cebrian 2010, González-Rivero et al. 2012), suggesting that the composition of the reef community may influence the success of *Cliona*.

Given that sponge erosion is expected to accelerate as oceans become more acidic (Fang et al. 2013, Wisshak et al. 2014, Enochs et al. 2015, Stubler et al. 2015), there is a clear need to monitor bioeroding sponge populations (Schönberg 2015, Murphy et al. 2016). The most conspicuous bioeroding sponge on the GBR is *Cliona orientalis* but percent cover has only been reported for a single GBR site (Schönberg 2001, Schönberg & Ortiz 2008, Schönberg 2015). Here, we quantified the abundance and trajectory of *C. orientalis* cover on the inshore GBR over a 10-year period (2005-2014) to resolve whether environmental conditions are drivers of change in sponge abundance. Our sampling covers a wide geographic area to assess whether previous reports of increasing *Cliona* abundance represent a GBR-wide trend or site-specific responses (Schönberg & Ortiz 2008).

2.3. Methods

2.3.1. Benthic surveys

Benthic cover was surveyed at 35 locations on the inshore GBR between 2005 and 2014 as part of the Inshore Water Quality and Coral Reef Monitoring program at the Australian Institute of Marine Science (Thompson et al. 2016). Briefly, at each location, two sites and two depths (2 and 5 m) were surveyed using five, fixed, 20 m transects. Every 0.5 m along each transect, photographs were taken of the benthos, which were used to determine presence-absence, percent cover, and change in percent cover. Survey data were pooled across sites and depths to relate to environmental variables measured at each location.

Percent cover was measured from digital photographs of the benthos. Five markers were overlaid onto each photograph and percent cover was calculated as the proportion of points occupied by each taxon. Percent cover of *C. orientalis* (encrusting ß form), other sponges,

scleractinian corals, octocorals, and macroalgae was calculated for each of the four within-location survey sites. The influence of other benthic taxa on *C. orientalis* cover was explored using biplots of cover at each within-location site.

Trends in cover were analysed for each within-location site where *C. orientalis* was detected in at least three survey years. Trends were estimated for each location separately as the locations were surveyed at different frequencies over the course of the study. Change in percent cover was estimated for each within-location site using linear regression. Thus, change in percent cover represents the average of the within-location sites (1-4) where *C. orientalis* was detected. Analysis of presence-absence of *C. orientalis* at each location followed the same criterion as change in percent cover, whereby *C. orientalis* was considered present if it occurred in at least three survey years at any of the sites.

2.3.2. Environmental variables

Survey data were related to environmental variables collected at the location scale (not sites or transects). Water quality was assessed using satellite-derived data from the eReefs Marine Water Quality Dashboard (http://ereefs.org.au/ereefs), including chlorophyll *a* concentration, coloured dissolved organic matter, and non-algal particulates (1 km resolution). The data nearest each survey location were analysed for each survey year. Sediment was collected from the 5 m survey sites and the proportion of fine particles, carbon content, and nitrogen content in the sediment were measured as described in (Thompson et al. 2016), with average values compared to *C. orientalis* cover. Fine particles in the sediments were defined as all particles smaller than 63 μm and expressed as a proportion of the total sediment (Cooper et al. 2007).

2.3.3. Data analysis

Exploratory plots were prepared to identify correlations amongst the environmental predictors and to compare the effects of different benthic taxa on *C. orientalis* cover. Note that only fine sediment, chlorophyll *a*, and total C in sediment were included in the model, as other environmental variables were strongly correlated with either the proportion of fine sediments or chlorophyll *a* (all r>0.7). Uncorrelated environmental variables were used to predict the presence-absence of *C. orientalis* (generalized linear model (GLM) with binomial errors and logit link), the percent cover of *C. orientalis* (GLM with negative binomial errors and log link), and changes in *C. orientalis* cover per year (linear model). Latitude was used to account for the spatial relationships among locations. Model fit was evaluated by plotting residual and fitted values. For generalized linear models, model fit was also evaluated using the chi-square probability of the residual deviance and residual degrees of freedom and by comparing observed and simulated residuals from each model.

Three models were used to assess whether other taxa, the environment, or geography explained patterns in *C. orientalis* distribution. Models were compared using AIC and R^2 values. For the GLM, R^2 was calculated as the deviance ratio of models with and without predictors. The most parsimonious model was identified as the model that maximized explanatory power with the fewest predictors.

Analyses were conducted in R statistical software (R core team). The map in Figure 6 was produced using R statistical software and the packages, "ggplot2" (Wickham), "mapdata" (Becker et al.), and "oz" (Venables & Hornik) packages.

2.4. Results and Discussion

C. orientalis was present in at least three survey years at 16 of the 35 inshore GBR locations. Where present, *C. orientalis* occupied as much surface substratum (0.73% ± 0.97 SD) as all other sponges combined (0.56% ± 1.11 SD). Havannah Island had the highest average cover at 3.6% (Figure 2-1A), although *C. orientalis* cover reached as high as 5% at Fitzroy Island and High Island (Figure 2-2). *C. orientalis* percent cover was lower than previously reported from Orpheus Island (>6%)(Schönberg & Ortiz 2008), possibly due to a greater area surveyed or the untargeted design in the current study. When absences are included (i.e., zero cover), the average percent cover of *C. orientalis* on the inshore GBR was 0.14% (±0.51 SD), which is comparable to the average cover of *C. delitrix* in the Florida Keys, USA (~0.1%) (Ruzicka et al. 2010) and southeast Florida (~0.08%)(Gilliam 2010), but lower than *C. delitrix* cover in Colombia (~2%)[47]. Additional studies have assessed the abundance of bioeroding sponges (Holmes 2000, Carballo et al. 2013, Bautista Guerrero et al. 2013, Nava et al. 2014), although it is challenging to reliably compare these measures of abundance with percent cover.

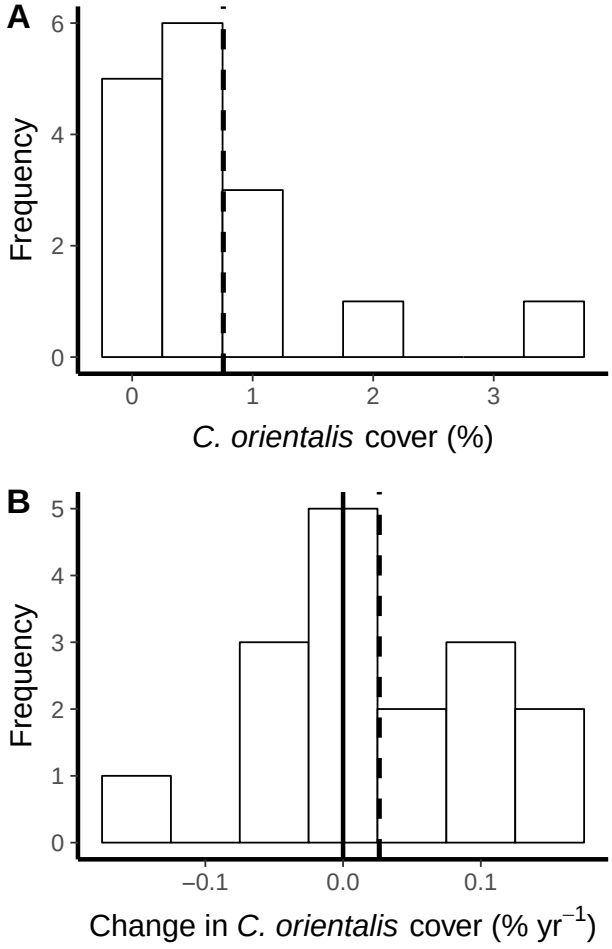

Figure 2-1. (A) Average percent cover of *Cliona orientalis* at the 16 sites used to measure changes over time. **(B)** Changes in *C. orientalis* cover per year. Changes in percent cover were estimated using linear regression and represent the average of 1–4 trends at each location. Dashed vertical lines indicate means and the solid vertical line indicates a value of zero.

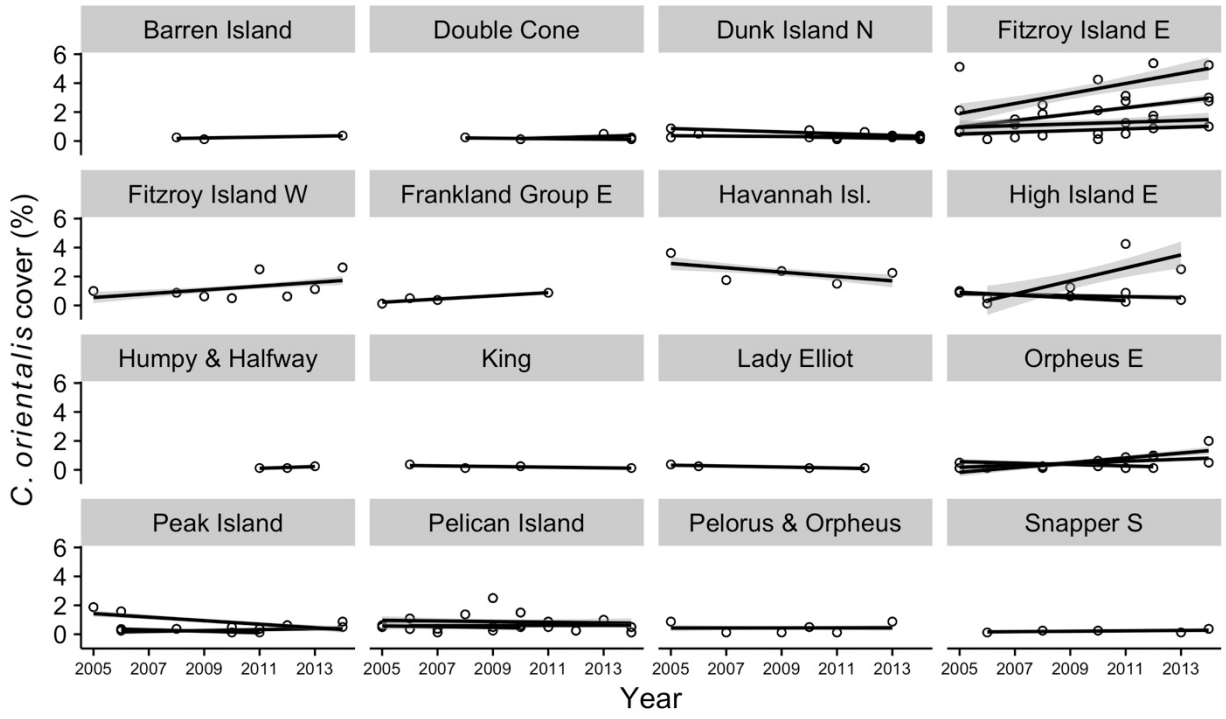

Figure 2-2. Trends in *Cliona orientalis* cover from 16 locations between 2005 and 2014. *C. orientalis* was found at 1–4 of the sites at each location. Linear regressions were fit to each site. Lines indicate the linear fits for each site and grey shading represents the standard error of the fit.

C. orientalis occurred less frequently at locations with high accumulation of fine sediments. The model predicted a 50% probability of *C. orientalis* occurrence at 17% fine sediments, suggesting that even moderate accumulation of silt and clay sized particles prevents the establishment of *C. orientalis* (Figure 2-3A); further evidenced by the low percent cover of *C. orientalis* at sites with large accumulations of fine sediments (Figure 2-3B). The amount of fine sediments distinguishes exposed and sheltered locations, as waves and currents resuspend fine particles and prevent accumulation (Wolanski et al. 2005). Both suspended and deposited sediments can influence the composition of sponge communities (Knapp et al. 2013). Suspended and deposited sediments have negative effects on sponges (Bell et al. 2015), including reduced reproductive output (Whalan et al. 2007) and increased respiration (Bannister et al. 2012). The deposition of fine sediment may hinder filter-feeding or reduce the light available for

photosynthesis (Fabricius 2005). The negative correlations observed between fine sediments and the distribution and abundance of *C. orientalis* suggest that sediments have negative physiological effects on *C. orientalis*, although these effects have not been demonstrated experimentally.

As coral cover declines on the GBR (De'ath et al. 2012), changes in the cover of bioeroding taxa may dictate future reef growth (Kennedy et al. 2013, Perry et al. 2014, Enochs et al. 2015). In

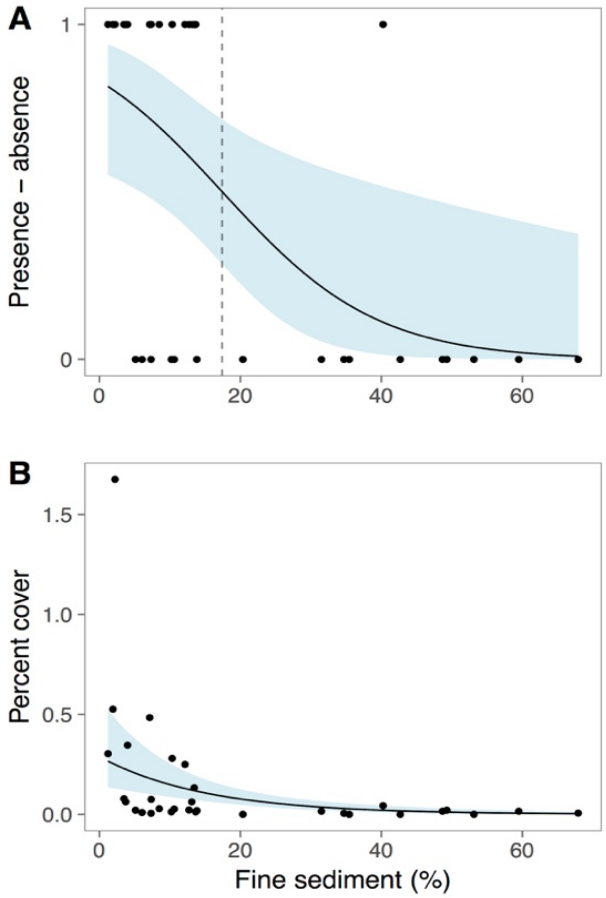

Figure 2-3. (A) *Cliona orientalis* was more likely to occur at sites with the lowest proportion of fine sediments (z = −2.5, P = 0.01; Wald test). The line represents the probability of occurrence using a binomial generalized linear model and shading represents the 95% confidence interval. The dashed line indicates the proportion of ne sediments (17%) with a 50% predicted probability of *C. orientalis* occurrence. Points represent presence- absence and the average fine sediment proportion for each location. (**B**) *C. orientalis* cover significantly decreased as a function of the proportion of fine particles in the sediment (z = −4.9, p < 0.01; Wald test). The line represents the predicted cover from a negative binomial generalized linear model and shading represents the 95% confidence interval. Points represent average cover and fine sediment proportion for each location.

this study, the average change in *C. orientalis* percent cover was 0.03% yr^{-1} (± 0.08 SD). Cover

increased at 10 out of 16 locations (Figure 2-1B), although only one trend was statistically significant (0.2% yr^{-1} at Fitzroy Island (East); t=2.8, $p<0.05$). *C. orientalis* cover exhibited non-linear patterns at some sites, possibly due to disturbances such as cyclones or outbreaks of crown of thorns starfish (Thompson et al. 2016), which altered community composition and potentially increased the detectability of *C. orientalis*. The rate of change in *C. orientalis* cover was similar to the rate of change in sponge cover at the same locations (0.03% yr^{-1} ±0.10 SD), but slower than the changes in other benthic groups (Figure 2-4). These time series indicate that cover of *C. orientalis* and other sponges has remained largely stable over the past decade on the inshore GBR despite changes to the reef community, such as a decline in octocoral cover (Figure 2-4).

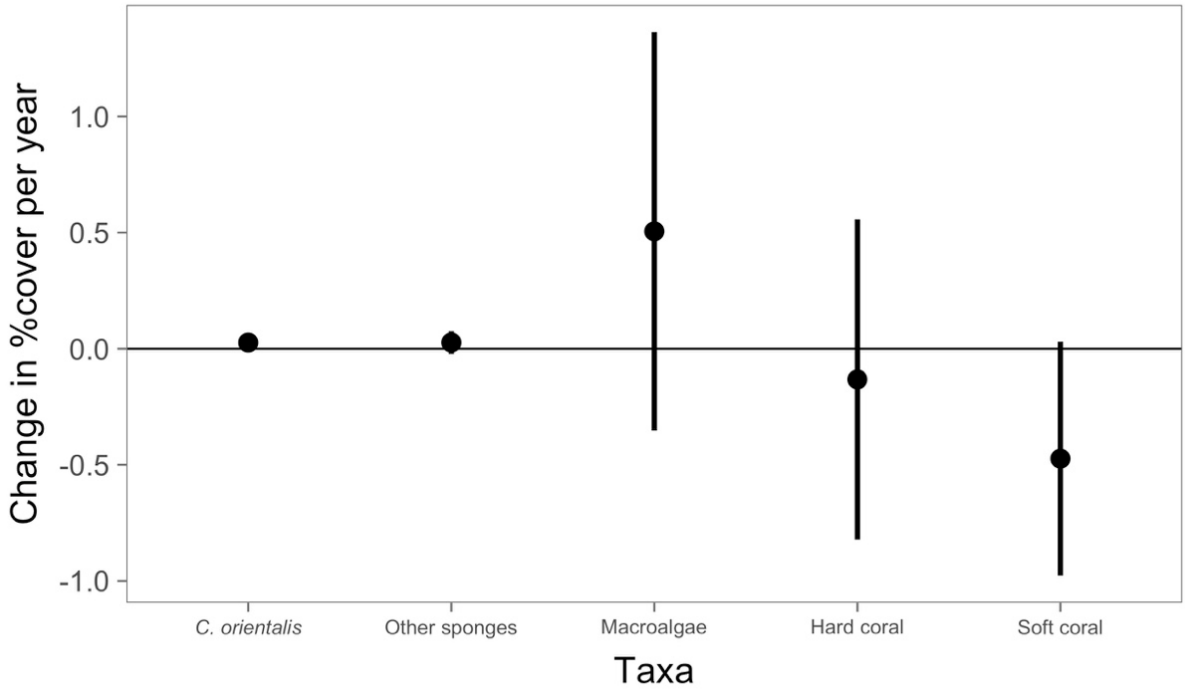

Figure 2-4. Changes in *Cliona orientalis* and sponge cover were near-zero, despite changes in other benthic groups. Points represent the average change in cover for the 16 locations where *C. orientalis* was present and error bars represent the 95% confidence interval.

Few studies have reported the rate of change in percent cover of bioeroding sponges. Therefore, we estimated rates of change in cover of other *Cliona* spp. to provide context for the rates of change in *C. orientalis* cover measured in this study. The fastest estimated rate of change was for *C. orientalis* cover from 1998 to 2004 at Orpheus Island on the GBR (~0.9% yr^{-1})(Schönberg & Ortiz 2008). Slower rates of increase were reported from the Caribbean, where *C. caribbaea* cover increased ~0.14% yr^{-1} from 1979 to 1998 in Belize (Rützler 2002), bioeroding sponge cover increased ~0.05 % yr^{-1} from 2005 to 2009 in southwest Florida (Makowski & Keyes 2011) and *C. delitrix* cover changed <0.01% yr^{-1} from 2003 to 2009 in southeast Florida (Gilliam 2010). In contrast, *C. delitrix* cover decreased (-0.03% yr^{-1}) in the Florida Keys (Ruzicka et al. 2010). The rate of change reported here (0.03% yr^{-1} for *C. orientalis*) is relatively low in the context of these estimates, but also encompassed a comparatively large number of survey locations. It is worth noting that many of the observations of increased cover of bioeroding

sponges were initiated prior to 2001(Rützler 2002, Ward-Paige et al. 2005, Schönberg & Ortiz 2008) and that subsequent studies have not observed increased cover (Gilliam 2010, Ruzicka et al. 2010, Makowski & Keyes 2011, Carballo et al. 2013).

Changes in *C. orientalis* cover are best explained by the abundance of macroalgae (Figure 2-5, Table 2-1). Increases in *C. orientalis* cover occurred at locations with low macroalgal cover (t=-3.0, P=0.01). However, these locations also had low average chlorophyll *a* concentration in the water (Figure 2-5), which also significantly affected the change in *C. orientalis* cover (t=-2.4, P=0.03). Therefore, the fastest increases in *C. orientalis* cover occurred at locations with a combination of low macroalgal cover and low chlorophyll concentrations, which were clustered near Cairns (Figure 2-6). When analysed together, neither macroalgal cover nor chlorophyll concentration was significantly associated with change in *C. orientalis* cover (P>0.05), likely due to the positive correlation between macroalgal cover and chlorophyll concentration (r=0.56). While macroalgal cover explained 39% of the variation in change in *C. orientalis* cover (Table 2-1), macroalgal cover (or chlorophyll *a*) did not predict the distribution or abundance of *C. orientalis* (Table 2-1, Figure 2-7).

These results suggest that macroalgae outcompete bioeroding sponges for space: all but one of the locations with increased *C. orientalis* cover had less than 10% macroalgal cover and all had less than 0.45 µg/L chlorophyll *a* (Figure 2-5), a water quality threshold that separates reefs with low and high macroalgal abundance (De'ath & Fabricius 2010). Previous work observed that macroalgal cover was negatively correlated with *C. orientalis* cover (Cebrian 2010) and macroalgae have also been reported to outcompete *C. tenuis* for substratum in the Caribbean (González-Rivero et al. 2012). In addition, several studies have observed that large colonies of bioeroding sponges occur where macroalgal cover is low (Lopez-Victoria & Zea 2005, González-

Rivero et al. 2012). By extension, controls on macroalgal growth, such as fish and urchin herbivory (Cebrian 2010) as well as dissolved nutrient levels (De'ath & Fabricius 2010), may indirectly affect the growth of bioeroding sponges.

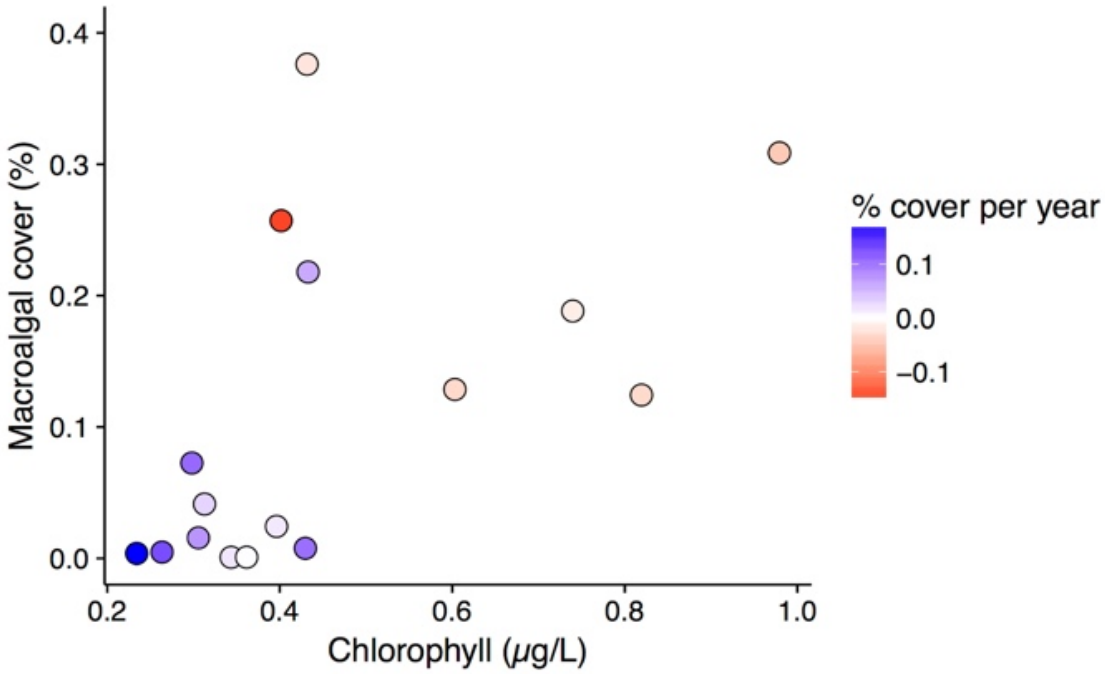

Figure 2-5. Change in *Cliona orientalis* cover was highest at locations with low chlorophyll concentration and low macroalgal cover. Points represent average macroalgal cover and chlorophyll *a* concentrations for each location and the colour indicates the direction of change in *C. orientalis* cover.

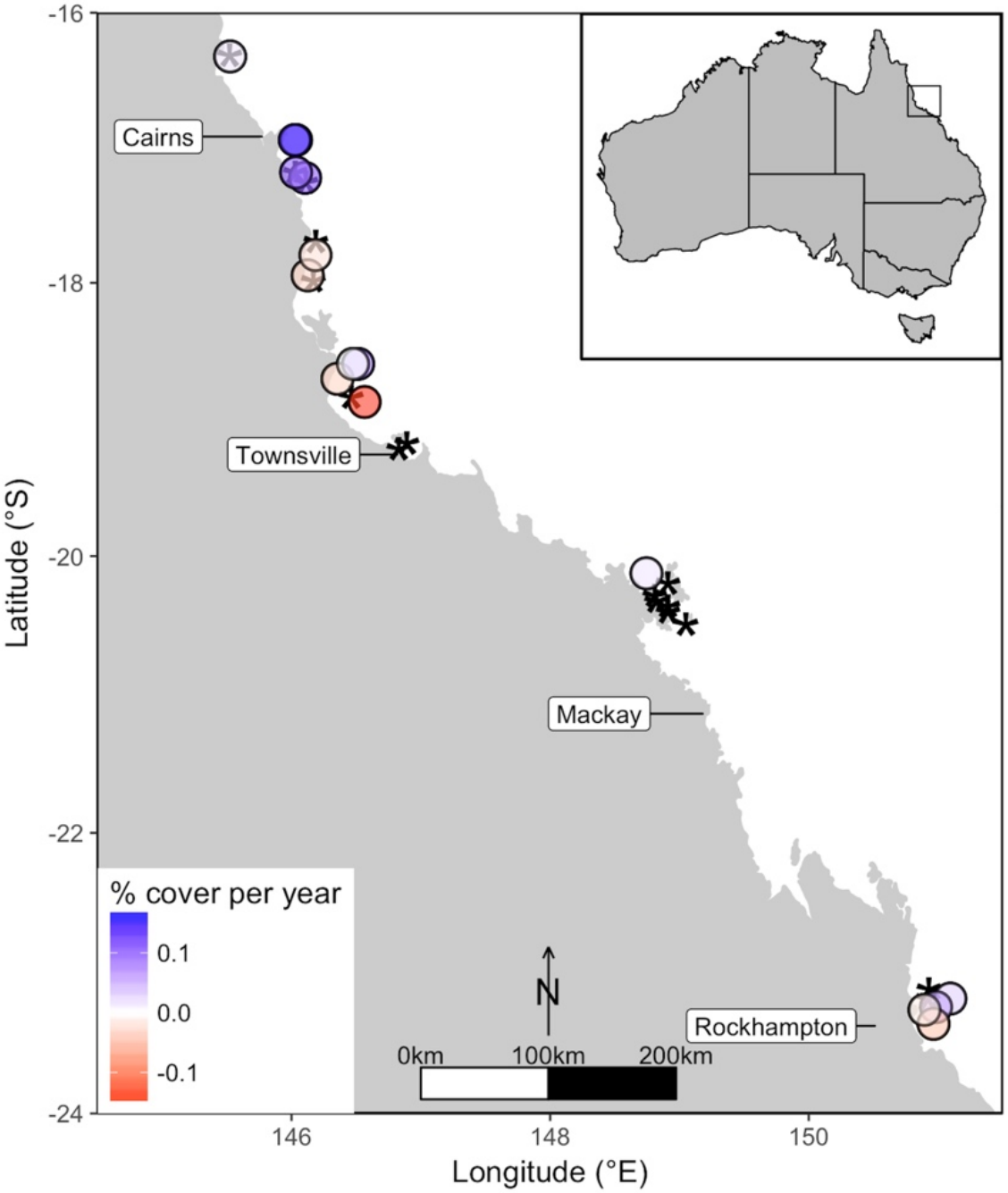

Figure 2-6. The spatial distribution of changes in *Cliona orientalis* cover on the inshore GBR. Circles represent locations where change over time was measured and an * represent locations where *C. orientalis* was absent (not detected in at least 3 survey years). Blue circles indicate increases in cover; white circles indicate zero change in cover; and red circles indicate decreases in cover.

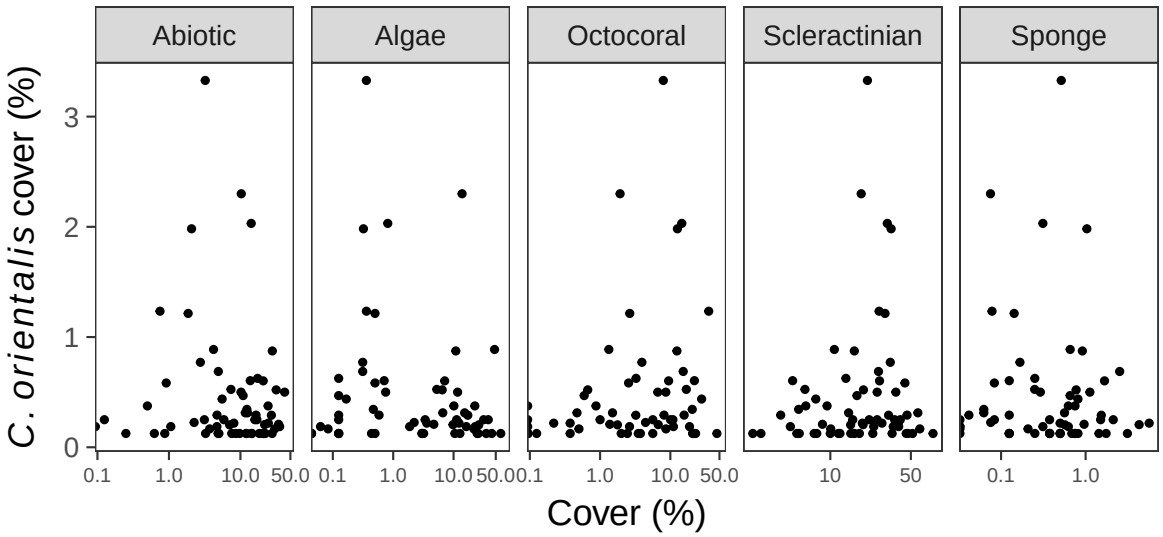

Figure 2-7. The relationship between *C. orientalis* cover and the cover of other taxa is similar for algae, scleractinian corals, soft corals, and other sponges. Points represent the average cover for all survey sites (within each location) where *C. orientalis* is present.

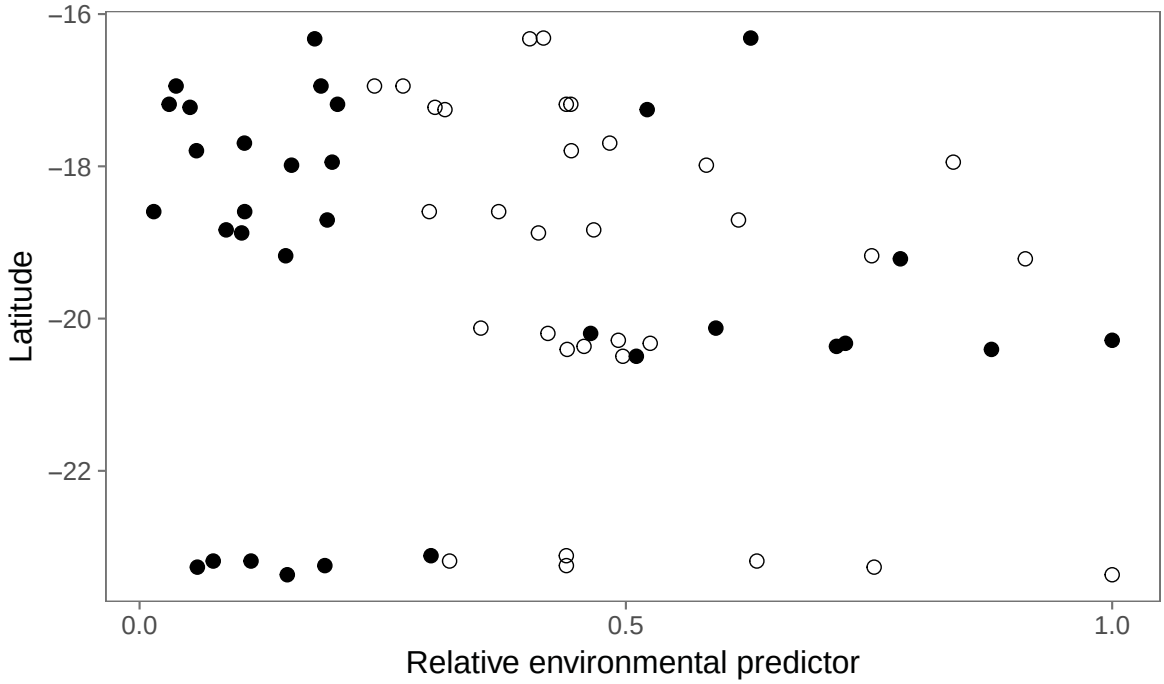

Figure 2-8. Relative chlorophyll (open circles) and fine sediment (closed circles) as a function of latitude. Chlorophyll was similar across latitudes, but sediment values were highest at intermediate latitudes.

The gradual increases in *C. orientalis* cover observed at multiple locations suggest that broader ecological changes may be responsible for increases in *C. orientalis* cover. Water quality is declining across the inshore GBR, driven by inputs of terrestrial nutrients that are delivered during seasonal flood events (Brodie et al. 2011, Schaffelke et al. 2012). Dissolved nutrient levels increase during floods (Brodie et al. 2011), which can lead to phytoplankton blooms and higher concentrations of organic material in the water (Furnas et al. 2005), which is a primary food for some *Cliona* species (Mueller et al. 2014). Nutrient levels likely increased over the survey period, as river flows were high, particularly during the middle of the study (Thompson et al. 2016, Fabricius et al. 2016). At locations with high nutrient levels, additional nutrients would have benefited the already high macroalgal cover (De'ath & Fabricius 2010). However, at locations with low nutrient levels and little cover of macroalgae, additional nutrients may have contributed to increases in *C. orientalis* cover (Figure 2-5). Thus, increases in *C. orientalis* cover may reflect additional nutrient loads entering the GBR lagoon, but are restricted to locations where nutrient concentrations are insufficient to support high macroalgal cover.

Table 2-1. Environmental variables are stronger predictors of *Cliona orientalis* distribution and abundance than biotic variables. The table contains a comparison of models with two categories of predictors, representing the hypotheses that the C. orientalis response was influenced by the percent cover of other taxa or environmental conditions. The table includes the C. orientalis response variable; the category, number and description of predictors; the proportion of deviance explained by the predictors (R^2); and the Aikaike Information criterion score (AIC). An * indicates predictors which were statistically significant and statistics are reported in figure legends. The most parsimonious models, in terms of R^2 and AIC, are highlighted bold.

Response	Predictors Category	n	Description	R^2	AIC	ΔAIC
Presence-absence	Environmental	3	Chlorophyll *a*, fine sediment*, total carbon in sediment	0.29	39.5	3.4
		1	Chlorophyll *a*	0.01	47.8	11.7
		1	Total carbon in sediment	0.12	43.1	10.0
		1	**Fine sediment***	**0.28**	**36.1**	**0**
	Biotic	4	Coral, macroalgae, sponge, and abiotic percent cover	0.11	44.2	7.9
	Geography	1	Latitude	0	52.1	16.0
Percent cover	Environmental	3	Chlorophyll *a*, fine sediment*, total carbon in sediment	0.40	260.0	36.0
		1	**Fine sediment***	**0.38**	**256.8**	**32.8**
		1	Total carbon in sediment*	0.19	266.6	42.6
		1	Chlorophyll *a*	0	275.7	51.7
	Biotic	4	Coral, macroalgae, sponge, and abiotic percent cover	0.10	224.0	0
	Geography	1	Latitude	0.05	272.9	48.9
Change in percent cover	Environmental	3	Chlorophyll *a*, fine sediment, total carbon in sediment	0.33	-32.6	5.4
		1	**Chlorophyll *a****	**0.30**	**-35.9**	**2.1**
		1	Total carbon in sediment	0.12	-32.3	5.7
		1	Fine sediment	0.03	-30.7	7.3
	Biotic	4	Coral, macroalgae*, sponge, and abiotic percent cover	0.42	-33.0	5.0
		1	Coral	0.01	-30.4	7.6
		1	**Macroalgae***	**0.39**	**-38.0**	**0**
		1	Sponge	0.05	-31.0	7.0
		1	Abiotic	0.11	-32.1	5.9
	Geography	1	Latitude	0.10	-31.8	6.2

While the response of *C. orientalis* to high nutrient levels has not been investigated experimentally, several other *Cliona* species exhibit positive associations with elevated nutrients, including *C. delitrix* and *C. vastifica* (Rose & Risk 1985, Holmes 2000, Ward-Paige et al. 2005). However, not all *Cliona* species respond the same way, as several exhibited either positive or negative responses to a chlorophyll *a* gradient in Mexico (Nava et al. 2014). On the GBR, observation of higher abundance of bioeroding sponges on inshore versus offshore reefs suggests that bioeroding sponges benefit from high nutrient conditions (Sammarco & Risk 1990). The correlations reported here suggest that *C. orientalis* is affected by local environmental conditions, specifically fine sediments, dissolved nutrients (chlorophyll *a*), and macroalgal cover, but experimental evidence of how these conditions affect *Cliona* species is lacking.

Factors other than fine sediments, macroalgal cover, and chlorophyll *a* explained little variation in the cover or distribution of *C. orientalis*. The cover by other taxa (corals, sponges, macroalgae) did not influence the distribution or abundance of *C. orientalis* (Table 2-1; Figure 2-7), suggesting that competition with these groups does not exclude *C. orientalis* from its habitat (Preciado & Maldonado 2005). Total carbon in the sediment explained some variation in *C. orientalis* abundance, but the effect was not significant in a model that included both total carbon and fine sediments (Table 2-1). Latitude explained little variation in *C. orientalis* abundance or distribution (Table 2-1) or in the environmental predictors (Figure 2-8). Importantly however, processes affecting *C. orientalis* at small spatial scales were not accounted for. For example, whilst the presence-absence of *C. orientalis* varied between nearby locations (i.e., kilometres; Figure 2-6), presence-absence also varied within locations (i.e., 250 m). Much of the unexplained variation in the distribution and cover of *C. orientalis* may be due to small-scale factors, such as the availability of hard substratum (Schönberg 2001).

Here, we present a large-scale monitoring effort to assess temporal changes in the abundance of the bioeroding sponge *Cliona orientalis* on the inshore GBR. Whilst *Cliona* abundance increased at 11 of 16 locations, increases in macroalgal cover and decreases in scleractinian and octocoral cover all outpaced changes in *Cliona* abundance. Low deposition of fine sediments was strongly associated with both the presence and abundance of *C. orientalis*, suggesting that the sponge requires exposed habitat. Increased cover of *C. orientalis* was only observed where mean chlorophyll *a* concentration was less than 0.45 μg/L and macroalgal cover was low, suggesting that *C. orientalis* can only increase in habitats where macroalgae are nutrient-limited. Experimental work that identifies the limiting environmental conditions (light, suspended sediment, nutrients) for *C. orientalis* is clearly warranted. Given the clumped distribution and strong association with local environmental conditions (e.g., sediment, macroalgae), bioeroding sponges such as *C. orientalis* likely represent site-specific – rather than regional – threats to coral health and reef accretion on the GBR.

Chapter 3. Defining the thermal tolerance of the bioeroding sponge *Cliona orientalis*

3.1. Abstract

Coral reefs face many stressors associated with global climate change, including increasing sea surface temperature and ocean acidification. Excavating sponges, such as *Cliona* spp., are expected to break down reef substrata more quickly as seawater becomes more acidic. However, increased bioerosion requires that *Cliona* spp. maintains physiological performance and health under continuing ocean warming. In this study, we exposed *C. orientalis* to temperature increments increasing from 23 to 32 °C. At 32 °C, or 3 °C above the maximum monthly mean (MMM) temperature, sponges bleached and the photosynthetic capacity of *Symbiodinium* was compromised, consistent with sympatric corals. *Cliona orientalis* demonstrated little capacity to recover from thermal stress, remaining bleached with reduced *Symbiodinium* density and energy reserves after one month at reduced temperature. In comparison, *C. orientalis* was not observed to bleach during the 2017 coral bleaching event on the Great Barrier Reef, when temperatures did not reach 32 °C threshold. While *C. orientalis* can withstand current temperature extremes (<3 °C above MMM) under laboratory and natural conditions, this species would not survive ocean temperatures projected for 2100 without acclimatisation or adaptation (≥3 °C above MMM). Hence, as ocean temperatures increase above local thermal thresholds, *C. orientalis* will have a negligible impact on reef erosion.

3.2. Introduction

Increasing global temperatures are requiring organisms to acclimate to greater thermal extremes, migrate, or suffer reduced fitness and, potentially, local extirpation. The earth's climate

is already estimated to be 0.85°C warmer than it was in 1880, which is affecting both terrestrial and marine ecosystems (IPCC 2014). Much of the thermal energy (~60%) associated with warming has been absorbed by the oceans, resulting in melting sea ice, rising sea levels (IPCC 2014), and record temperatures in tropical waters (Heron et al. 2016, Hughes, Kerry, et al. 2017). Ocean warming has already resulted in extensive coral mortality (Doney et al. 2012, Hughes, Kerry, et al. 2017), as evidenced in 2015/2016, when extreme temperatures led to consecutive mass coral bleaching events around the world (Heron et al. 2016, Hughes, Kerry, et al. 2017).

Corals contain photosynthetic dinoflagellates (genus *Symbiodinium*) that provides them with organic carbon (Yellowlees et al. 2008). However, the symbiosis is thermally sensitive and exposure to elevated temperature disrupts *Symbiodinium* photosynthesis (Warner et al. 1999) and causes coral bleaching (Baird & Marshall 2002, McClanahan et al. 2008). Some coral species and *Symbiodinium* 'types' are more thermally tolerant than others (Abrego et al. 2008, Grottoli et al. 2014, Díaz-Almeyda et al. 2017), but even tolerant genotypes can be overwhelmed by severe temperature stress (Hughes, Kerry, et al. 2017). Nonetheless, in some cases, previous exposure to high temperature or association with tolerant *Symbiodinium* can lead to greater thermal tolerance of the coral symbiosis (Berkelmans & van Oppen 2006, Abrego et al. 2008, Palumbi et al. 2014, Grottoli et al. 2014), or accelerate recovery following bleaching (Grottoli et al. 2014, Silverstein et al. 2015, Bay et al. 2016).

In comparison to reef-building corals, sponges are thought to be relatively tolerant of increasing sea surface temperatures (Przeslawski et al. 2008, Bell et al. 2013). In particular, some bioeroding sponges can tolerate temperatures that induce bleaching in sympatric corals (Cortés et al. 1984, Vicente 1990, Rützler 2002, Schönberg & Ortiz 2008, Carballo et al. 2013). Bioeroding

sponges, principally the genus *Cliona*, are important members of coral reef communities as they erode the limestone substratum by reducing the pH at the sponge:substratum interface (Hatch 1980), dissolving the substratum, and extracting microscopic 'chips' of calcium carbonate (Rützler & Rieger 1973, Zundelevich et al. 2007). Like corals, many bioeroding sponge species form symbioses with photosynthetic *Symbiodinium* and photosynthesis enhances their growth and bioerosion (Hill 1996, Schönberg 2006, Weisz et al. 2010). However, while dependence on *Symbiodinium* may increase the thermal sensitivity of *Cliona*, little is known about how these sponges will tolerate predicted incremental temperature increases or whether they can recover from extreme thermal stress (Fang et al. 2014, Achlatis et al. 2017).

Experimental research combining elevated temperature and reduced pH has shown that sponge bioerosion rates will likely increase under conditions of ocean acidification (Wisshak et al. 2012, Duckworth & Peterson 2013, Wisshak et al. 2013, Fang et al. 2013, Enochs et al. 2015). However, warming can have negative effects on bioeroding sponges, including bleaching or necrosis; and it is likely that these negative effects will override all other environmental factors (Wisshak et al. 2013, Fang et al. 2014, Achlatis et al. 2017). For instance, under temperature and pH conditions predicted for 2100, the bioeroding sponge *Cliona orientalis* bleaches, and the associated reduction in photosynthetic productivity results in a negative energy budget for the sponge despite accelerated rates of erosion (Fang et al. 2013, 2014). Warming was subsequently identified as the primary stressor inducing bleaching in a bioeroding sponge (Achlatis et al. 2017). However, temperature tolerance appears to vary among bioeroding sponge species as bleaching or mortality was not observed in all studies (Duckworth & Peterson 2013, Stubler et al. 2015). Therefore, the net effect of climate change on bioeroding sponges, and on their erosion rates, appears to be species-specific.

Identifying thermal thresholds under near-future warming requires measurement of performance across a broad range of incremental temperature changes. This incremental approach enables a holistic understanding of temperature effects by allowing quantification of the optimal temperature for peak physiological performance, along with derivation of sub-lethal and lethal temperature thresholds (Pörtner 2001, Angilletta 2009). A similar approach has been applied to corals to quantify adaptation to local thermal regimes (Rodolfo-Metalpa et al. 2014), and to determine how coral respiration and photosynthesis varies with temperature (Coles & Jokiel 1977). Here, we experimentally assessed the ability of *C. orientalis* to tolerate incrementally increasing sea surface temperatures between 23-32°C, which represents the annual temperature range for the studied sponges (22-30°C), and warmer temperatures predicted for 2100 (31, 32°C)(IPCC 2014). In addition, we monitored recovery from temperature stress to evaluate the impact of thermal exposure on sponge survival. Photosynthetic performance of *Symbiodinium* and energy reserves of the sponge were quantified to identify temperature optima and define thermal thresholds. To contextualize the laboratory experiment, we assessed bleaching severity for *C. orientalis* during the 2017 mass coral bleaching event.

3.3. Methods

3.3.1. Laboratory experiment

Thirteen *Cliona orientalis* (Thiele, 1900) sponges were collected at 2 - 4 m depth from Little Pioneer Bay on Orpheus Island, Queensland, Australia (18°37'40" S, 146°29'36" E) in June 2015 (Marine Parks Permit G12/35236.1). Sponges were transported by road in 60 L plastic aquaria to the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science in Townsville, Queensland, where they were maintained in outdoor flow-through aquaria at ambient temperature (23.0°C ± 0.1 SD). After seven days in aquaria, 3.5 cm-diameter cores

(n=151) were drilled from the 13 sponges. Each sponge produced between 4 and 24 cores, depending on the size of the sponge (median=9). Each core was labelled with the identity of the original sponge to control for genotype differences. Sixteen days after drilling, the cores from each sponge were haphazardly divided amongst nine indoor aquaria (50 L), resulting in 15-18 cores per aquarium. Ten sponges were represented in every aquarium, but three sponges were not, as they were represented by fewer than nine cores (Table 3-1).

Each aquarium was continuously supplied with 0.04 µm filtered seawater at 0.8 L/min. Water temperature was regulated by a SeaSim computer-controlled system to reach target temperatures; tanks were additionally buffered against temperature fluctuations using water jackets. LED lighting illuminated aquaria with 300 µmol quanta m^{-2} s^{-1} light for 11 h with an additional 1 h ramping period after dawn and before dusk. The irradiance level was less than the saturation irradiance (400 µmol quanta m^{-2} s^{-1}) that was determined via rapid light curves. The irradiance level (300 µmol quanta m^{-2} s^{-1}) is comparable to a cloudy day on the reef (302 µmol quanta m^{-2} s^{-1}), but less than the average irradiance on a clear day (653 µmol quanta m^{-2} s^{-1}) (calculated from Hoogenboom et al. 2011). In terms of the total irradiance, irradiance in the aquaria was 12 mol d^{-1} compared to 15 or 31 mol d-1 on a cloudy or sunny day on the reef, respectively (calculated from Hoogenboom et al. 2011). Cores were maintained at 23.2 ± 0.3°C (±SD) for 17 days, after which sponge photosynthesis and respiration were measured and tissue samples were taken (detailed below). The sponge cores were not fed and therefore their response may have differed from sponges on the reef.

Table 3-1. The number of cores and original sponges sampled for oxygen flux and tissue contents at each timepoint.

Time point	Target temperature	Heated	Control

		Sponges	Cores	Sponges	Cores
17	23.0	9	9	6	6
43	25.0	10	12	6	6
56	27.0	9	12	6	6
71	29.0	9	12	6	6
84	30.0	8	12	6	6
96	31.0	7	12	5	6
108	32.0	8	16	4	5
144	*30.0	8	16	4	6

To measure changes in *C. orientalis* condition and performance as a function of temperature, two temperature treatments were established: three aquaria were maintained under their initial conditions at 23°C for the duration of the experiment (control) and six aquaria had the temperature increased every two weeks, first by 2°C increments (23 to 29°C) then by 1°C increments (30 to 32°C). Thus, the temperature targets were 23, 25, 27, 29, 30, 31, and 32°C. These span the average range of temperatures at the collection site: 22.4°C in July to 29.1°C in February, with a maximum monthly mean of 30.5°C between 2002 and 2010 (Source: Australian Institute of Marine Science; http://weather.aims.gov.au; 1.9 m depth). Each temperature increment was achieved via ramping by 0.5°C per day up to the target temperature followed by 10 days of exposure to that temperature. The rate of temperature increase is similar to daily changes in mean temperature at the collection site (calculated from Hoogenboom et al. 2011). The 25°C temperature increment was extended from 14 to 24 days due to a logistical issue with the heating and cooling system. With this exception, temperatures were finely controlled throughout the experiment, typically within 0.1°C of the target temperature and with ~0.1°C s.d. among aquaria (Table 3-2). Exposure of sponges to these temperature increments occurred from July to November and coincided with the natural winter to summer temperature increase during the austral summer.

Table 3-2. Target and actual temperatures during the laboratory experiment. For each target temperature, tank temperatures were recorded every 5 minutes over 8 days (mean ± SD°C). # indicates where the SD was less than 0.05°C. °C above the maximum monthly mean (MMM) at Orpheus island (29.1+1.0°C) indicates the thermal anomaly above long-term summer temperatures which was used to calculate the accumulated degree heating weeks (DHW) as the product of the thermal exposure and the thermal duration (°C-weeks).

Time point	Target temperature	Heated (n=6)	Control (n=3)	°C above MMM+1	Accumulated DHW
17	23.0	23.5 ± 0.1	23.4 ± 0.2	0	0
43	25.0	25.2 ± 0.1	23.1 ± 0.1#	0	0
56	27.0	27.0 ± 0.1	23.3 ± 0.1	0	0
71	29.0	29.0 ± 0.2	23.0 ± 0.2	0	0
84	30.0	30.0 ± 0.1	23.1 ± 0.1	0	0
96	31.0	30.9 ± 0.1#	23.2 ± 0.1	0.8	1.3
108	32.0	32.0 ± 0.1	23.1 ± 0.2	1.9	4.4
144	*30.0	30.1 ± 0.1	23.1 ± 0.3	0	4.4

To evaluate the potential for *C. orientalis* to recover from bleaching, cores exposed to 32°C were returned to 30°C (by 0.25°C per day) and monitored for a further 28 days. Hereafter, '*30°C' is used to distinguish the recovery period at 30°C from the incremental increase to 30°C. To compare the thermal exposure in the laboratory to exposure under natural conditions, degree heating weeks (DHW) of thermal exposure were calculated for each temperature target above the 29.1+1.0°C bleaching threshold for Orpheus Island. DHW was calculated as the product of the °C above 30.1°C and the duration of temperature ramping (2-4 days) and exposure to the target temperature (10 days). 30°C was chosen as the recovery temperature as it is below the bleaching threshold and represents a typical summer temperature at the collection site.

For each temperature increment, photosynthetic measurements were taken on two days near the completion of each temperature exposure. Photochemical efficiency of all cores was measured at least twice during exposure to each increment, after one and eight days of exposure,

but photochemical efficiency was measured more frequently for the 23°C, 25°C, 31°C, and 32°C temperature increments. For other photosynthetic parameters and tissue contents, 2-3 cores were selected from each tank at each temperature increment, resulting in ~6 and ~12 samples for the control and heated treatments, respectively. Oxygen flux and sponge surface area were measured after nine days at each temperature increment (due to the time required to measure oxygen flux) except for *30°C, where measurements were taken after 28 days. After 10 days acclimation to each temperature increment (28 days at *30°C), sponges were frozen in liquid nitrogen for DNA extraction and measurement of tissue contents.

3.3.1.1. Photosynthesis and respiration

Photochemical efficiency is an indicator of electron transport during photosynthesis (Baker 2008). For *Symbiodinium*, decreases in photochemical efficiency can precede bleaching and coincide with damage to photosystems (Warner et al. 2010). The photochemical efficiency of photosystem II was measured using a mini-PAM fluorometer (Walz, Effeltrich, Germany) using standard settings (MI=8; SI=6; SW=0.6; G=2; D=2). Clear tubing was used to maintain a constant distance between the PAM fibre optic cable and the sponge. Photochemical efficiency was measured at two timepoints: before the lights turned on (Fv/Fm; maximum efficiency) and after two hours of constant light exposure (ΔF/Fm'; effective efficiency). Changes in these variables over the course of each temperature increment reflect the severity of the stress on the *Symbiodinium*.

Oxygen flux is an integrative measure of respiration by the sponge (and symbionts) and photosynthesis by the *Symbiodinium* (Osinga et al. 2012). For sponges, stress can manifest in altered respiration, decreased photosynthesis, or a reduced ratio of photosynthesis to respiration

(Fang et al. 2014, Bennett et al. 2016, Achlatis et al. 2017). Oxygen flux was measured using an optical dissolved oxygen meter (Hach HQ30d; Hach, Colorado, USA). Sponge cores were sealed in a darkened chamber (500 mL) for one hour. Water within each chamber was mixed with a magnetic stir bar and the target temperature was maintained using an external water jacket. Respiration rates were calculated by the difference in oxygen concentration between the beginning and end of the incubations. After measurement of respiration, sponges were returned to the aquaria for one hour at 300 μmol quanta m^{-2} s^{-1} then sealed in a chamber at 400 μmol quanta m^{-2} s^{-1} to measure oxygen production. The photosynthetic rate was calculated using the change in oxygen concentration in the chamber over 40 minutes. Both respiration and photosynthetic rates were adjusted by the amount of oxygen flux in a chamber without a sponge core to account for oxygen flux by microorganisms in the water. The surface area of the selected cores was measured using the aluminium foil method (Marsh 1970), sponge tissue was removed (top 1 cm), the cores were frozen in liquid nitrogen and stored at -80°C.

3.3.1.2. Sponge condition (*Symbiodinium*, chlorophyll, protein, organic content)

Invertebrates that harbour *Symbiodinium* can bleach by losing symbionts or their photosynthetic pigments (Fitt et al. 2001). *Symbiodinium* cells were extracted by incubating sponge tissue in 1M NaOH at 37°C for 1 h (Zamoum & Furla 2012). Cells were counted using 4 replicate counts of 0.45 cm^2 on a hemocytometer and standardised to the wet weight of sponge tissue. To quantify the photosynthetic pigments within *C. orientalis* tissues, chlorophylls were extracted in two consecutive extractions of 1 mL of ethanol (95%) to ensure complete extraction of pigments. During each extraction, the tissue was homogenized for 3 min in a bead beater, centrifuged for 5 min (10,000 g), after which the two extracts were pooled. Absorbance of the extract was measured at 630, 647, 664, and 750 nm using a Power Wave Microplate Scanning

Spectrophotometer (BIO-TEK Instruments Inc., Vermont, USA). The concentrations of chlorophylls *a*, *b*, and *c* were estimated using the equations of Ritchie (2008) and standardised to sponge wet weight.

Protein content and tissue organic matter were measured as proxies for sponge condition as these parameters have been shown to decline in bleached corals (Fitt et al. 1993). Frozen sponges were weighed (wet weight), lyophilized, and weighed again (dry weight). Proteins were extracted from homogenized dried sponge in 2 mL of 0.125 M NaOH over 24 h at room temperature. Protein concentration was estimated using the Red660 Protein Assay with five concentrations of BSA protein standard and then standardised to the dry weight of the sponge tissue. Organic matter was measured using lyopholized sponge tissue (see Protein content). Dried sponge was combusted at 450°C for 16 h. Organic matter was estimated as the difference between the dry weight and the ash weight and standardised to the dry weight of the sponge.

3.3.1.3. *Symbiodinium* identity

To determine whether the sponges switched *Symbiodinium* types during temperature stress, we identified the *Symbiodinium* associated with cores from 9 of the original sponge fragments as temperatures increased. In total, 35 cores were analysed including cores from the same genotype in each temperature increment. DNA was extracted from frozen sponge tissue (~0.2 g) using the Powerplant Pro DNA Isolation Kit (Mo Bio), including the beadbeating, RNAse, and proteinase K procedures as per the manufacturer's instructions. DNA extracts were sent to the Australian Centre for Ecogenomics at the University of Queensland, Australia for sequencing. The ITS2 region of ribosomal rDNA was amplified using *Symbiodinium*-specific ITS2 primers (Pochon et al. 2001) and sequenced using Illumina MiSeq 250 bp chemistry.

Sequences were analysed in Mothur v.1.38.0 (Schloss et al. 2009). Paired reads were combined and screened for quality and chimeric sequences were identified using Uchime in Mothur. The remaining sequences were clustered into 97% similar operational taxonomic units (OTU). The dataset was reduced to 2000 sequences per sample and the relative abundance and prevalence were calculated for each OTU. Representative sequences were defined using the sequence with the smallest distance to all other sequences within the OTU. Sequences were compared against a curated database of ITS2 sequences including all clades of *Symbiodinium* using the BLAST algorithm (Arif et al. 2014). Blast results with bit scores less than 100 were discarded.

3.3.2. Bleaching surveys

Field surveys were conducted to assess the thermal tolerance of *C. orientalis* during a natural thermal bleaching event, and to contrast bleaching responses between *C. orientalis* and corals. In March 2017, video transects were filmed at six sites within the Palm Islands Group, three of which were at Orpheus Island where the experimental samples were collected. Survey sites were chosen as replicate exposed and protected locations, as well as to span the depth gradient over which *Acropora* cover is relatively high at Orpheus Island. At each site, two transects (50 m long and parallel to shore) were filmed at 0-4 m below the lowest astronomical tide. *Cliona orientalis* sponges and scleractinian coral colonies were manually counted along each video transect. Coral colonies were categorized as either branching or massive and we used a simple 'bleached' or 'unbleached' categorisation due to the absence of a reliable colour reference in the videos. White individuals, as well as individuals with fluorescent discolouration (e.g., blue or pink *Acropora* spp.) were considered bleached.

To compare the thermal exposure during the natural bleaching event to the laboratory experiment, daily mean and maximum temperatures at Orpheus Island (5.8 m depth) were downloaded from the Australian Institute of Marine Science (http://weather.aims.gov.au).

3.3.3. Statistical analysis

Photosynthesis and sponge condition data were analysed using linear mixed models with treatment, time point, and treatment * time point interaction as well as a random intercept to account for between-sponge differences using the R packages lme4 (Bates et al. 2015), lmerTest (Kuznetsova et al. 2016), and multcomp (Hothorn et al. 2008). Only the final photochemical efficiency was analysed statistically. *Symbiodinium* density was analysed with an additional random intercept to account for correlations between samples from the same aquarium. Boxplots and residual plots were used to assess whether the data met the assumptions of linear models. Samples with large deviations from fitted values (>1.5*interquartile range) were removed from the analysis. Some response variables were transformed using log (respiration, chl *a*:*c*, protein, organic matter) or odds ratios (Fv/Fm, ΔF/Fm') to meet these assumptions. Planned contrasts were used to test whether heated sponges differed from control sponges at each time point, as well as whether changes occurred after the reduction in temperature following exposure to 32°C. Results from the planned contrasts are reported with z statistics and *P* values. *P* values were corrected using a single-step correction for multiple comparisons.

For each benthic category in the bleaching surveys, individuals were pooled between replicate transects at each site. The proportion of bleached individuals was calculated out of the total number of individuals encountered at each site. For each category of taxa, the proportion of bleached individuals was calculated using proportion of bleached individuals relative to the total

number of colonies and then weighted according to the number of individuals at each site.

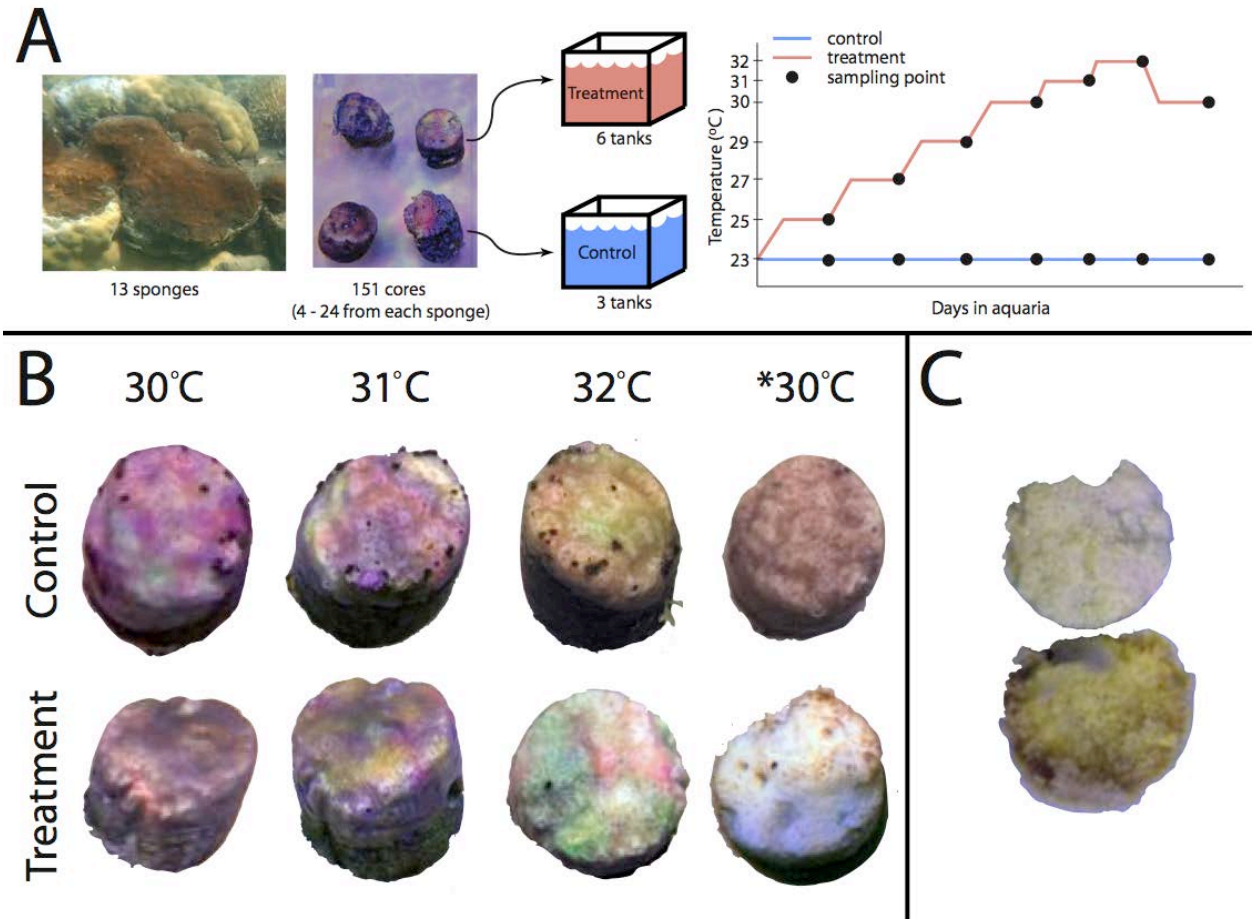

Figure 3-1. (A) Sponge sampling and temperature treatments. Healthy and bleached *Cliona orientalis* cores. **(B)** A time-series of two cores from the control and heated treatments, respectively. Cores in the heated treatment visibly bleached at 32°C and had not recovered four weeks later at *30°C. **(C)** The top surface and pinacoderm of a bleached core at the end of the experiment. The pinacoderm of bleached cores (interior) remained healthy despite the absence of *Symbiodinium* for four weeks.

3.4. Results

3.4.1. Response to laboratory thermal exposure

3.4.1.1. Bleaching and *Symbiodinium* identity

Cliona orientalis survived temperatures up to 31°C with no visual signs of bleaching and little evidence of compromised health, such as discolouration or tissue regression (Figure 3-1B).

However, at 32°C, more than 50% of the cores became visibly bleached within three to five days and this increased to 70% bleached after 8 days (Figure 3-1B). The condition of some cores also visibly deteriorated in the four weeks following bleaching, including the presence of algae at the margin of the cores (Figure 3-1C). The narrow bleaching threshold of 32°C identified for *C. orientalis* is 2.9°C above the maximum monthly mean at Orpheus Island. A low level of mortality occurred in both temperature treatments but was constrained to cores from a few specific sponge genotypes.

Only a single *Symbiodinium* ITS2 type was associated with *C. orientalis*, regardless of temperature treatment or bleaching state. ITS2 sequences clustered into 61 OTUs at 97% sequence similarity, but only 21 of these matched the *Symbiodinium* database, and only 9 were confidently matched with bitscores greater than 100. Of the nine *Symbiodinium* OTUs, one OTU comprised 96% of the *Symbiodinium* sequences and was the most abundant OTU in every sponge core. The ITS2 sequence of the dominant OTU was identical to *Symbiodinium* clade G previously sequenced from *C. orientalis* (Genbank accession JQ247051) and which was recently described as *Symbiodinium endoclionum* (Ramsby, Hill, et al. 2017). One other OTU occurred in 97% of the cores and was also most similar to *Symbiodinium* clade G from *C. orientalis*, differing from the dominant ITS2 sequence by one insertion of eight nucleotide substitutions. The remaining *Symbiodinium* OTUs were most similar to *Symbiodinium* clades A, B, or C, however these OTUs comprised <1% of total sequences and occurred in <20% of the samples.

3.4.1.2. Photosynthesis and respiration

Temperature effects were interpreted as significant temperature*treatment interactions which indicate the temperatures at which heated sponge cores responded differently than control

cores (Table 3-3). Photosynthesis was largely unaffected by temperatures up to and including 31°C, but became inhibited at 32°C. Sponges at 27 and 29°C had higher effective (ΔF/Fm'), but not maximum (Fv/Fm), photochemical efficiency than sponges maintained at 23°C (Figure 3-2; eff. 27°C: z=-4.5, $p<0.01$; eff. 29°C: z=-3.2, $p=0.01$). At 29 - 31°C, sponges had significantly lower maximum photochemical efficiencies than control sponges ($p<0.01$), but the differences were small, and *C. orientalis* maintained 93% of maximum photochemical efficiency of control sponges at 31°C. However, at 32°C, maximum and effective photochemical efficiencies were 52% and 50% of control sponges, respectively (Table 3-4). As with photochemical efficiency, the photosynthetic rate (gross oxygen production) did not differ from controls at temperatures up to and including 31°C (Figure 3-3A; $p>0.86$) but was 43% lower than controls in sponges exposed to 32°C (Table 3-4).

In contrast to photosynthesis, the effect of thermal exposure on sponge respiration was greatest at 29°C, where heated sponges had 47% higher respiration rates than control sponges (Figure 3-3B; z=-3.4, $p<0.01$). At temperatures close to 29°C (27, 30, and 31°C), respiration was 28-35% higher than controls, but these differences were not significant ($0.09<p<0.29$). For sponges at 32°C, respiration rates were similar to control sponges (Table 3-4). The ratio of gross photosynthesis to respiration (P/R) was affected at a similar temperature as the respiration rates (Figure 3-3C). Sponges at 29, 30, and 31°C had 79% of the sponge P/R of control sponges, coinciding with faster respiration rates (Figure 3-3B), but the difference was only statistically significant at 30°C (z=3.1, $p=0.01$). Sponges at 32°C had 37% of the P/R of control sponges (Table 3-4), indicating a loss of productivity of the symbiosis.

Table 3-3. Statistical results of the laboratory experiment. Parameters tested include maximum photochemical efficiency (Fv/Fm), and effective photochemical efficiency (ΔF/Fm'), photosynthetic rate (P), respiration rate (R), the ratio P:R, *Symbiodinium* density (*Symb.*), chlorophyll *a* and *c* density (chl), protein content, and ash-free dry weight (AFDW). Units for each measure are provided in Figs 3-4. Data were analysed using a linear mixed model with four components: temperature treatment, temperature, treatment:temperature interaction, and a random intercept for the *C. orientalis* sponge. *Symbiodinium* density was analysed with an additional random intercept for each aquarium. Parameters that were significantly affected by the temperature exposure have a significant treatment:temperature interaction term. The table includes the transformation used (Trans.), whether any outlying observations were removed, and statistical estimates: sums of squares (SS), mean square (MS), denominator degrees of freedom (D. df), F ratio (F), and P value (*p*).

			Treatment (num. df=1)					**Temperature (num. df=7)**					**Treatment : Temperature (num. df=7)**				
	Trans.	**Out. rem.**	**SS**	**MS**	**D.df**	**F**	***p***	**SS**	**MS**	**D. df**	**F**	***p***	**SS**	**MS**	**D. df**	**F**	***p***
Photosynthesis																	
Fv/Fm	p/(1-p)	N	18.9	18.9	686.1	676.4	<0.01	35.5	5.1	679.0	181.9	<0.01	25.1	3.6	677.9	128.5	<0.01
ΔF/Fm'	p/(1-p)	N	0.7	0.7	9.8	106.2	<0.01	23.1	3.3	674.0	106.2	<0.01	11.3	1.6	672.5	52.0	<0.01
Gross P	-	N	<0.1	<0.1	121.4	4.0	<0.01	0.3	<0.1	24.1	128.3	<0.01	<0.1	<0.1	7.6	124.7	<0.01
R	-	Y	<0.1	<0.1	121.0	18.9	<0.01	<0.1	<0.1	128.8	14.9	<0.01	<0.1	<0.1	125.0	3.0	0.01
Gross P:R	-	Y	14.3	14.3	119.9	41.3	<0.01	25.3	3.6	10.5	126.2	<0.01	12.9	1.8	5.3	122.8	<0.01
Sponge condition																	
Symb.	-	N	18944	18944	125.2	8.5	<0.01	173824	24823	129.7	11.1	<0.01	89443	12778	127.4	5.7	<0.01
Chl *a*	-	N	25624	25624	123.2	19.6	<0.01	121098	17300	13.2	128.4	<0.01	94888	13556	10.4	13556	<0.01
Chl *c*	-	N	990	990	123.1	20.3	<0.01	7072	1010	20.8	128.1	<0.01	4372	624.6	12.8	125.2	<0.01
Chl *a*:*c*	Log	Y	<0.1	<0.1	123.3	0.4	0.52	0.8	0.1	5.3	128.6	<0.01	0.1	<0.1	0.8	126.1	0.59
Protein	Log	N	0.8	0.8	120.5	10.4	<0.01	4.0	0.6	7.9	130.2	<0.01	2.3	0.3	4.6	124.8	<0.01
AFDW	Log	Y	0.3	0.3	122.1	14.3	<0.01	0.7	0.1	5.8	129.2	<0.01	0.7	0.1	5.5	125.0	<0.01

Table 3-4. Results of post-hoc comparisons. To compare the sensitivity of different parameters, the table includes the lowest temperature increment with a significant difference between heated and control sponges. While post-hoc tests were used to test for differences after each temperature increase, detailed results are presented from 3 post-hoc tests that indicate 1) whether cores heated to 32°C differed from control cores, 2) whether cores heated to 32°C differed from cores returned to *30°C (i.e., recovery), and 3) whether cores reduced to *30°C differed from controls (i.e., recovery). For each test, the table indicates the direction of the difference between groups, the z value, and *p* value. *p* values were corrected for multiple comparisons using a single-step correction. - indicates where there was no difference between treatments. Parameter abbreviations are listed with Table 3.

	Exposure				**Recovery**					
		32°C: Heated vs Ctrl.			**Heated: *30 vs 32°C**			***30°C: Heated vs Ctrl.**		
	Min. sig. temp		**z**	***p***		**z**	***p***		**z**	***p***
Photosynthesis										
Fv/Fm	29	C>32	27.8	<0.01	*30>32	11.3	<0.01	C>H	14.2	<0.01
ΔF/Fm'	32	C>32	15.8	<0.01	*30>32	12.2	<0.01	C>H	5.4	<0.01
Gross P	32	C>32	7.8	<0.01	*30>32	-5.6	<0.01	C>H	3.7	<0.01
R	-	-	-0.8	0.99	*30>32	-4.8	<0.01	-	1.2	0.89
Gross P:R	30	C>32	6.1	<0.01	*30>32	5.8	<0.01	C>H	3.2	<0.01
Sponge condition										
Symb.	32	C>32	4.3	<0.01	-	1.9	0.42	C>H	5.3	<0.01
Chl *a*	32	C>32	3.7	<0.01	32>*30	12.8	0.33	C>H	8.6	<0.01
Chl *c*	32	C>32	3.9	<0.01	32>*30	3.33	<0.01	C>H	9.5	0.01
Chl *a*:*c*	-	-	-	-	-	-	-	-	-	-
Protein	32	C>32	3.0	0.03	32>*30	3.2	0.01	C>H	4.8	<0.01
AFDW	32	C>32	2.8	0.05	32>*30	4.3	<0.01	C>H	6.6	<0.01

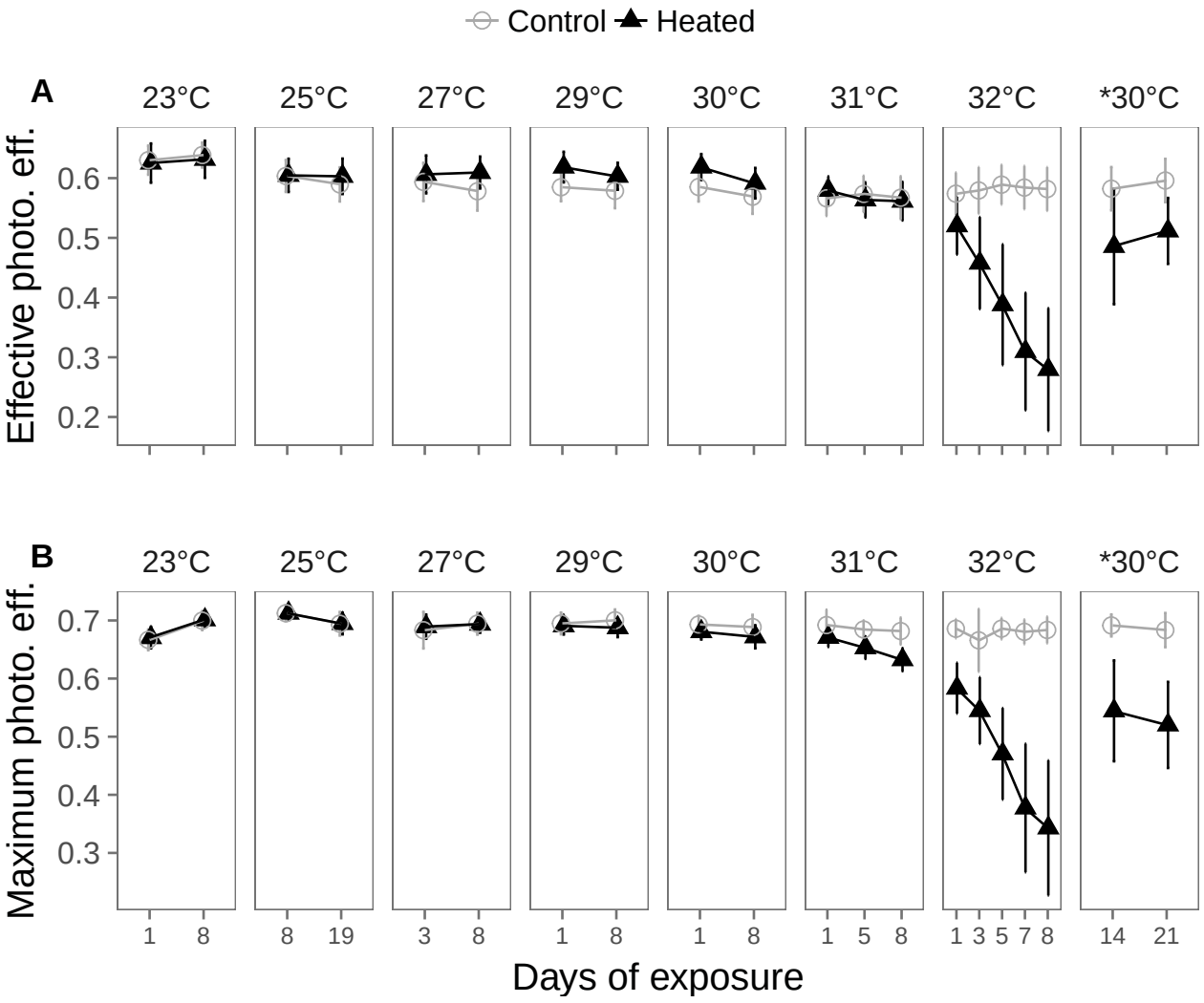

Figure 3-2. Photochemical efficiency of *Symbiodinium* within *C. orientalis*. Effective **(A)** and maximum **(B)** photochemical efficiencies are shown for control cores measured at 23°C (grey open circles and lines) and heated cores at elevated temperature (black triangles and lines). Panels separate the temperature of the heated treatment. Points represent means and error bars indicate one s.d..

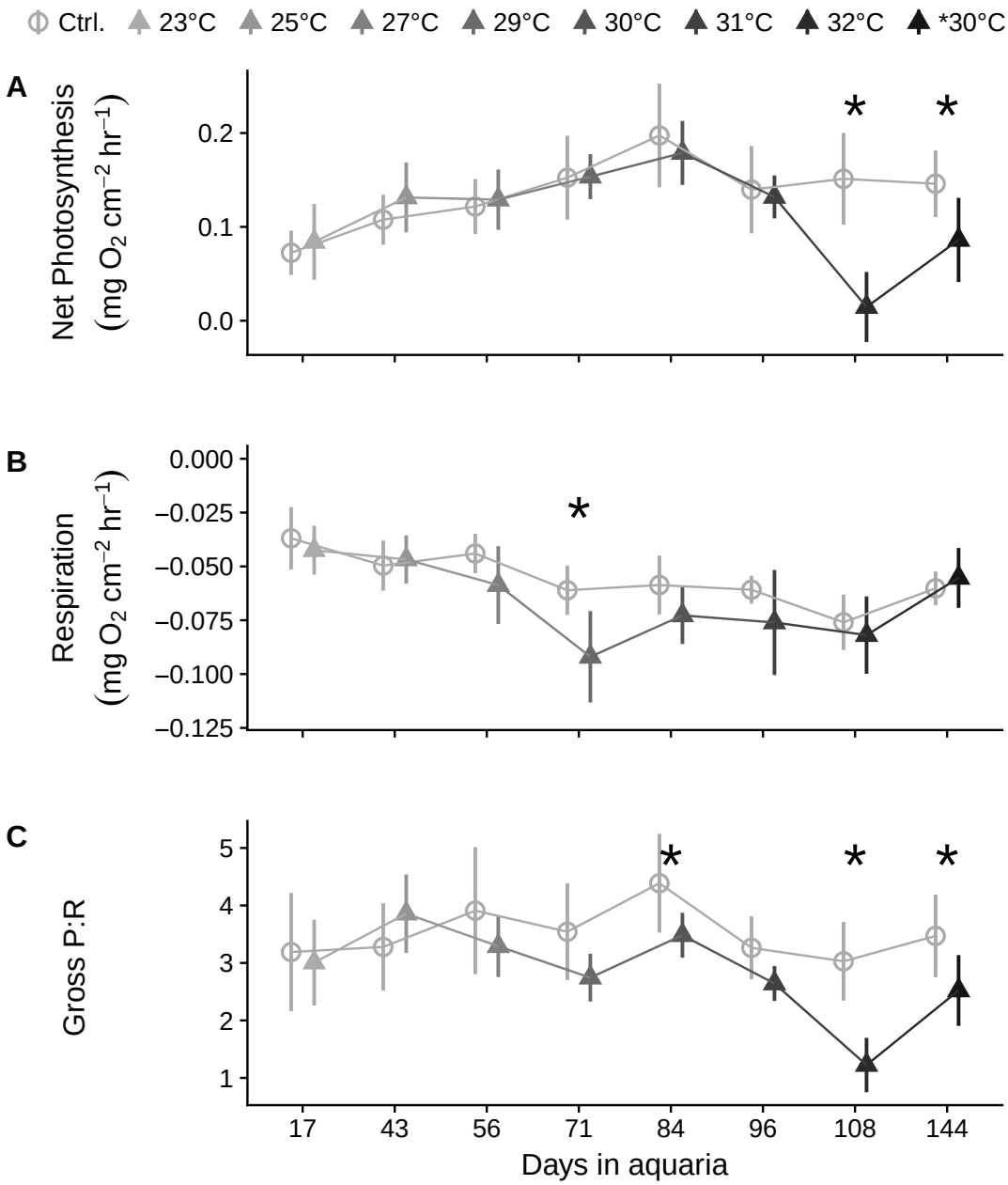

Figure 3-3. Oxygen flux rates for net photosynthesis **(A)**, respiration **(B)**, and the ratio of gross photosynthesis to respiration **(C)** for *C. orientalis* between 23 and 32°C. Control cores (grey open circles and lines) were all measured at 23°C, while heated cores (triangles and lines) were sampled at the temperature indicated in the legend. The shading of the heated treatment intensifies as the temperature increases. *30 indicates samples that were exposed to 32°C and then returned to 30°C for four weeks following bleaching. Points represent means and error bars indicate one standard error. Asterisks indicate temperature increments where sponges in control and heated treatments had significantly different responses.

3.4.1.3. Sponge condition

Temperatures less than 32°C did not significantly affect the condition of *C. orientalis* and the density of *Symbiodinium,* chlorophylls *a* and c_2, protein, and organic matter were similar to control sponges (Figure 3-4; $p>0.20$). At 32°C, the bleached sponges contained 25% of the *Symbiodinium*, 35% of chlorophyll *a*, and 42% of chlorophyll c_2 of the control sponges (Table 3-4). Moreover, sponges at 32°C contained 83% of the organic matter and 66% of the protein found in control sponges (Table 3-4), signifying reduced condition of the sponge. Notably, control sponges did not exhibit reductions in *Symbiodinium*, chlorophylls, protein, or organic matter over the course of the experiment, suggesting that the sponges had adequate nutrition even though they were not fed (Figure 3-4).

While the a:c_2 ratio decreased over the course of the experiment (Table 3-4, Temperature), it was not strongly affected by temperature, as heated sponges were 91-107% that of control sponges throughout the experiment and differences between treatments were not significant (Table 3-4, Treatment:Temperature).

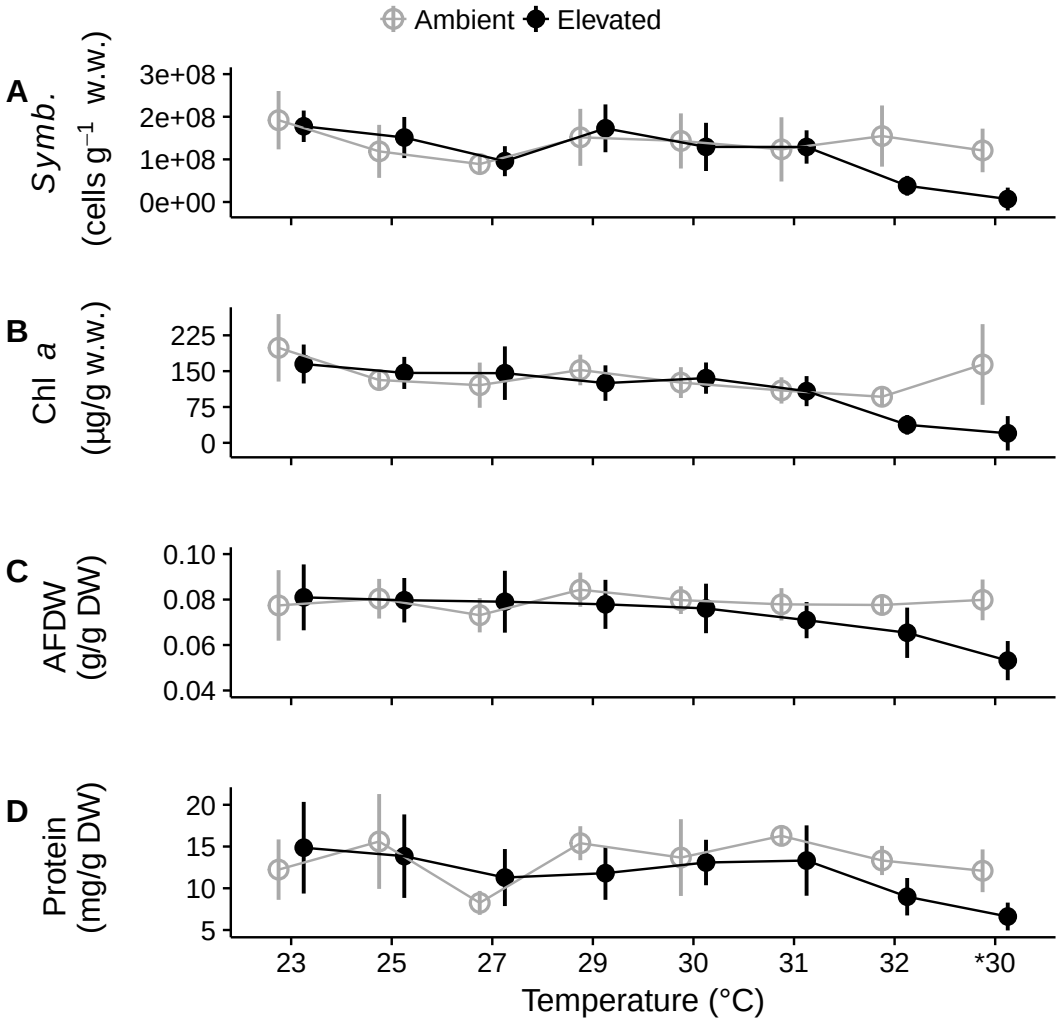

Figure 3-4. Tissue contents of *Symbiodinium* **(A)**, chlorophyll *a* **(B)**, ash-free dry weight **(C**; AFDW), and protein content **(D)** for *C. orientalis* between 23 and 32°C. Controls (grey open circles and lines) were all sampled at 23°C while heated cores (triangles) were sampled at the temperature indicated in the legend. *30 indicates samples that were exposed to 32°C, bleached, and were returned to 30°C for four weeks. Points represent means and error bars indicate standard error. Asterisks indicate temperature increments where cores in control and heated treatments had significantly different responses.

3.4.2. Recovery from laboratory thermal exposure

3.4.2.1. Bleaching and *Symbiodinium* identity

The *Symbiodinium* associated with *C. orientalis* did not change following bleaching, with all cores dominated by *Symbiodinium endoclionum*. Four weeks following bleaching, *C. orientalis* cores appeared white, and both the *Symbiodinium* density and chlorophyll content revealed that

the sponges had not recovered from their bleached state, indicating prolonged holobiont disruption (Figure 3-4).

3.4.2.2. Photosynthesis and respiration

C. orientalis recovered some photosynthetic capacity when returned to *30°C, with photochemical efficiencies, photosynthetic rates, and P/R being higher than when sponges were at 32°C (Figure 3-3, Table 3-4). However, this response was likely due to other photosynthetic colonisers as *Symbiodinium* densities remained low in sponges at *30°C (Figure 3-3). Regardless, recovery of photosynthesis was incomplete, as photochemical efficiencies, photosynthetic rates, and P/R remained lower than control sponges (Figure 3-3, Table 3-4). The respiration rates of sponges returned to *30°C were higher than the sponges at 32°C, but not significantly different from controls (Figure 3-3, Table 3-4).

3.4.2.3. Sponge condition

Tissue contents indicated that the condition of the 32°C sponges continued to deteriorate after they were returned to *30°C (Figure 3-4, Table 3-4). Chlorophyll content, organic matter, and protein content were lower in sponges at *30°C than at 32°C, but *Symbiodinium* density was not significantly different (Table 3-4). In addition, all measured tissue contents remained lower in sponges returned to *30°C than control sponges (Figure 3-4, Table 3-4).

3.4.3. Field bleaching surveys

A total of 133 *C. orientalis* sponges, 1891 branching corals, and 1068 massive corals were counted among the six survey sites. No bleached *C. orientalis* sponges were observed in any of the video transects. In contrast, 83% (±6.0 SD) of branched coral colonies and 51% (±3.2 SD) of massive coral colonies were bleached (Figure 3-5B).

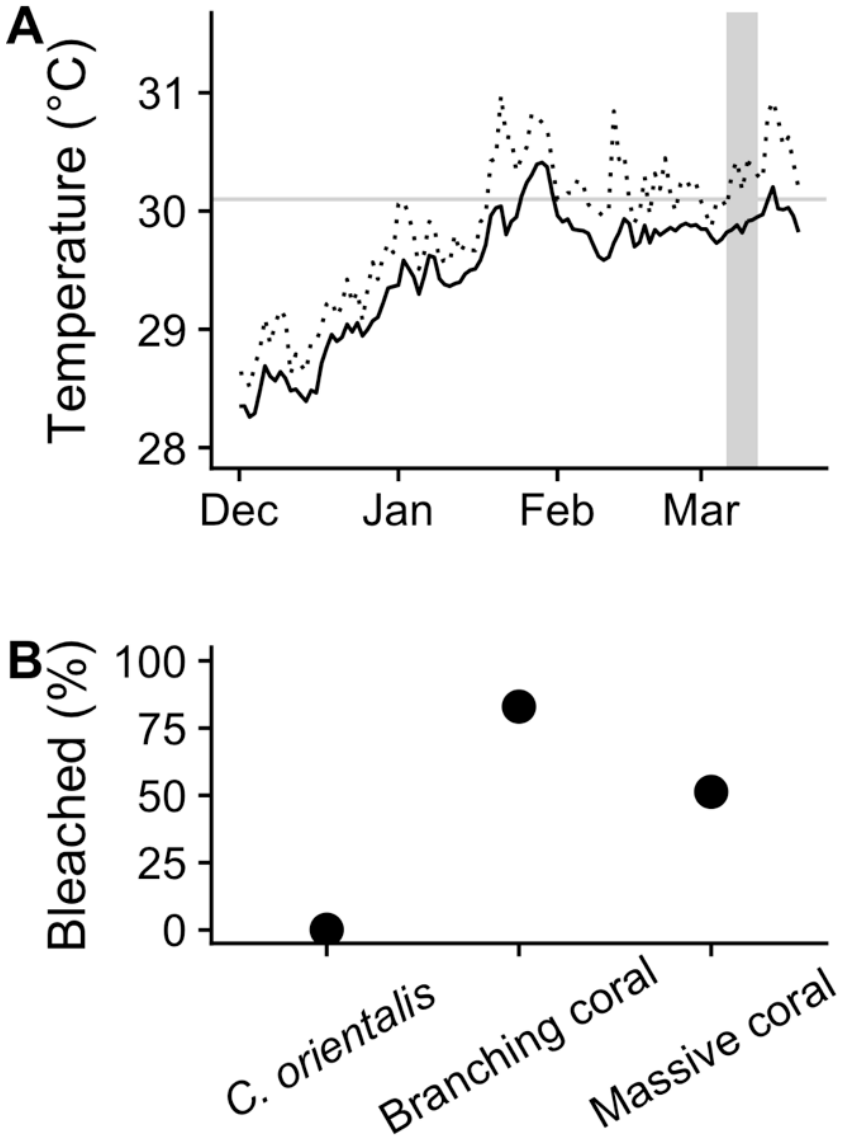

Figure 3-5. The temperature **(A)** and bleaching severity **(B)** during a natural bleaching event in the Palm Islands, Great Barrier Reef, Australia in February-March 2017. The daily mean (solid black line) and maximum (dotted line) temperature for the twelve weeks preceding the surveys are shown. The horizontal grey line indicated the local coral bleaching threshold (30.1°C) and the vertical grey bar denotes the survey period.

Temperatures did not reach 32°C during the 2017 bleaching event at Orpheus Island (Figure 3-5A). During the two weeks preceding the surveys, daily mean temperatures averaged 29.8°C at 5.8 m depth (Source: Australian Institute of Marine Science; http://data.aims.gov.au) and, during the 12 weeks preceding the surveys, the cumulative thermal exposure summed to 1 degree heating week (weeks above 30.1°C). In comparison, the laboratory experiment indicated that *C. orientalis* bleached at 32°C after accumulating 2.5 degree heating weeks (3 days at 32°C; Table 3-1).

3.5. Discussion

Bioeroding sponges are generally thought to be tolerant of environmental stressors, including elevated temperature, ocean acidification, and eutrophication, raising concerns about increased reef erosion under future projected climate scenarios (Wulff 2016, Schönberg, Fang, Carreiro-Silva, et al. 2017). Here, we show that incremental increases in ocean temperature up to 30°C have negligible effects on *C. orientalis*, but *C. orientalis* bleaches when exposed to 32°C, and exhibits little potential for recovery. At the collection site, 32°C represents an increase of 3°C above the maximum monthly mean temperature and corresponds to the increase expected under very high greenhouse gas emissions by 2100, but could represent the mean temperature as soon as 2078 (RCP 8.5) (IPCC 2014), suggesting that *C. orientalis* could bleach regularly by the end of this century. The results of this study do not support the hypothesis that bioeroding sponges (particularly those species with photosynthetic symbionts) will play a larger role in structuring future reefs.

Temperature exposure in the laboratory revealed a narrow thermal threshold for *C. orientalis*, with sponges appearing visibly healthy after 10 days at 31°C, but bleaching after only 3

days at 32°C. This narrow threshold is similar to several sympatric coral species that bleached following 1°C temperature increases between 31 and 33°C (Berkelmans & Willis 1999). In thermally sensitive corals, bleaching coincides with reduced condition and growth (McClanahan et al. 2008) and *C. orientalis* exhibited similar negative responses, including a 75% reduction in *Symbiodinium* density, 17% reduction in organic matter and 44% reduction in protein content of *C. orientalis*. Few bleached cores exhibited necrosis which had been previously reported in *C. orientalis* from Orpheus Island after exposure to only 2°C above MMM. The discrepancy between studies likely results from the faster temperature increases or acute exposures (3-72 h) used in previous research (Schönberg et al. 2008, Wisshak et al. 2013). The thermal threshold identified here is consistent with findings for *C. orientalis* in the southern GBR, which tolerates exposure to +2.0°C above MMM (27.3 °C)(MMM = 27.3 °C; Fang et al. 2013), but bleaches at +2.7°C (Achlatis et al. 2017), and dies at +3.5°C (Fang et al. 2013). Taken together, these experimental and field results suggest that *C. orientalis* can tolerate current ocean temperatures, but will have little capacity to cope with the warmer oceans projected for 2100.

The primary cause of coral bleaching is exposure to extreme ocean temperature, although longer exposure to moderate increases in temperature can also induce bleaching (Berkelmans 2002, Baker et al. 2008). Cumulative thermal exposure is a product of the amount and the duration of stress, which is incorporated into the degree heating weeks (DHW) index, which can be used to accurately predict bleaching (Hughes, Kerry, et al. 2017). In the laboratory, *C. orientalis* bleached in the laboratory after 2.5 degree heating weeks (DHW), similar to corals that bleach after 2 DHW under natural conditions (Hughes, Kerry, et al. 2017). Consistent with our field observations at Orpheus Island, there are few reports of *C. orientalis* bleaching under natural conditions. In most cases, other *Cliona* species (*C. aprica*, *C. caribbaea, C. varians*, and *C.*

vermifera) have tolerated periods of elevated temperature better than neighbouring corals (Cortés et al. 1984, Vicente 1990, Carballo et al. 2013), including exposures exceeding 31°C (Carballo et al. 2013) and even 33°C (Vicente 1990). In our surveys, *C. orientalis* did not bleach although temperatures did not exceed 31°C, which is below the 32°C thermal threshold identified during our experiment. A 32°C threshold is consistent with other *Cliona-Symbiodinium* symbioses, as *C. varians* was recently reported to bleach when mean temperatures exceeded 31°C for 10 days (Hill et al. 2016). The combination of a 32°C laboratory bleaching threshold with the lack of bleaching during the 2017 coral bleaching event suggests that current summer temperatures could lead to faster local erosion rates in the near future.

Coral bleaching is often preceded by disruption of *Symbiodinium* photosynthesis (Warner et al. 1999, Smith et al. 2005) which leads to the production of toxic oxygen radicals, which must be neutralized to prevent damage to lipids, proteins and DNA (Baird et al. 2009). The mechanisms of bleaching in sponges may be similar, however, if damage to the photosystems was responsible for triggering bleaching in *C. orientalis*, the response must have been very rapid: when *C. orientalis* bleached, *Symbiodinium* still retained ~66% of Fv/Fm which had only declined for 3 days. After eight days of exposure to 32°C, the photosynthetic capacity of the symbiosis was diminished, coinciding with a loss of *Symbiodinium* and chlorophyll. Similar effects have previously been observed in bleached *C. orientalis* (but see Fang et al. 2013, Achlatis et al. 2017) and scleractinian corals, where a loss of *Symbiodinium* coincides with a loss of lipids, proteins, and organic matter (Fitt et al. 1993, Rodrigues & Grottoli 2007). In addition, bleaching can disrupt the bacterial symbioses in *C. orientalis* (B. Ramsby et al. *Mol Ecol in press*) and scleractinian corals (Bourne et al. 2016).

Prior to *C. orientalis* bleaching, there was some evidence that respiration rates increased (29-31°C) and that energy reserves were reduced (31°C), suggesting that the sponges expend resources to maintain their symbiosis at sub-bleaching temperatures. Respiration in *C. orientalis* was fastest at intermediate temperatures, likely contributing to the significant decline in the productivity of the symbiosis. Nevertheless, bleached *C. orientalis* had similar respiration rates to control sponges despite their reduced condition (Achlatis et al. 2017). The absence of an effect of bleaching (i.e., absence of *Symbiodinium*) on respiration rates highlights the need to separate measurement host and *Symbiodinium* respiration (Hawkins et al. 2016). Based on their low biomass relative to the biomass of the sponge tissue, it is likely that *Symbiodinium* makes a minor contribution to overall respiration, and other factors such as pumping or feeding rates may dictate energetic demand and respiration in thermally stressed sponges (Riisgård et al. 1993).

The ability to persist in warming oceans will depend upon recovery of symbionts and energy reserves, before exposure to any subsequent bleaching-inducing temperatures (Rodrigues & Grottoli 2007). After *C. orientalis* bleached at 32°C, the sponges did not recover during four weeks at *30°C, with no recovery of the symbiosis or sponge condition. The only parameter that changed during recovery was photosynthesis, where the rates of oxygen production and photochemical efficiency were higher in sponges returned to *30°C than in sponges at 32°C. However, based on visual observations and the lack of recovery of *Symbiodinium*, relatively high photochemical efficiency was likely due to fouling by photosynthetic epibionts rather than a re-establishment of the *Symbiodinium* population. In corals, recovery can take between 1.5 and 10 months and some species do not recover within 12 months (Szmant & Gassman 1990, Fitt et al. 1993, Grottoli et al. 2006, Rodrigues & Grottoli 2007, Grottoli et al. 2014). Our experiment indicated that *C. orientalis* did not recover *Symbiodinium* under aquarium conditions, but the

availability of *Symbiodinium* may have limited recovery. In other laboratory studies, *C. orientalis* have recovered *Symbiodinium* following irradiance-induced bleaching (Riesgo et al. 2014, Pineda et al. 2016), but further study is necessary to determine whether *C. orientalis* can regain symbionts following thermal bleaching under natural conditions. Observations in the Florida Keys, USA indicate that *C. varians* can recover from thermal bleaching (M. Hill pers. comm.), although some *Symbiodinium* likely remained within the sponge (Hill et al. 2016).

Association with tolerant *Symbiodinium*, especially multiple types of *Symbiodinium*, can aid recovery from coral bleaching (Bay et al. 2016). Here, all *C. orientalis* cores harboured the same symbiont, *S. endoclionum* (Ramsby, Hill, et al. 2017), and exhibited little flexibility in their symbiotic association before or after bleaching. This may make *C. orientalis* more vulnerable to warming than reef taxa that can associate with multiple *Symbiodinium* clades (Berkelmans & van Oppen 2006, Abrego et al. 2008), as *C. orientalis* harbours *S. endoclionum* over a large geographic range (Ramsby, Hill, et al. 2017). Little is known about the genetic diversity or physiology of clade G *Symbiodinium*, which have only have been found in bioeroding sponges (Schönberg & Loh 2005, Granados et al. 2008, Hill et al. 2011), foraminifera (Pochon et al. 2006), and one octocoral species (Bo et al. 2011). A physiological comparison of Clade G to other *Symbiodinium* has suggested that the Clade G from *C. orientalis* are more thermally tolerant than the clade C or D inhabiting scleractinian corals (Schönberg et al. 2008). However, here we have refined the thermal threshold, showing that while the clade G symbiont in *C. orientalis* can tolerate current summer temperatures (<32°C), photosynthesis is impaired at predicted future temperatures (≥32°C).

Recent mass coral bleaching events are a clear indication that ocean warming is a primary threat to reef corals (Hughes, Kerry, et al. 2017) and accelerated bioerosion by *Clionaid* sponges

under ocean acidification would further compound the adverse outcomes of climate change. However, here we show that while the symbiosis between *C. orientalis* and its associated *Symbiodinium* tolerates current maximum sea surface temperatures, the partnership breaks down as sea surface temperatures reach 32°C. A relatively high tolerance of present day temperature extremes may benefit *C. orientalis* via coral mortality and increased substratum availability in the short term (Schönberg & Ortiz 2008, Chaves-Fonnegra et al. 2018), however bioeroding sponges with *Symbiodinium* will be severely affected by ocean temperatures expected by 2100.

Chapter 4. Elevated seawater temperature disrupts the microbiome of *Cliona orientalis*

4.1. Abstract

Bioeroding sponges break down calcium carbonate substratum, including coral skeleton, and their capacity for reef erosion is expected to increase in warmer and more acidic oceans. However, elevated temperature can disrupt the functionally important microbial symbionts of some sponge species, often with adverse consequences for host health. Here, we provide the first detailed description of the microbial community of the bioeroding sponge *Cliona orientalis* and assess how the community responds to seawater temperatures incrementally increasing from 23°C to 32°C. The microbiome, identified using 16S rRNA gene sequencing, was dominated by *Alphaproteobacteria*, including a single operational taxonomic unit (OTU; *Rhodothalassium* sp.) that represented 21% of all sequences. The "core" microbial community (taxa present in >80% of samples) included putative nitrogen fixers and ammonia oxidizers, suggesting that symbiotic nitrogen metabolism may be a key function of the *C. orientalis* holobiont. The *C. orientalis* microbiome was generally stable at temperatures up to 27°C; however, a community shift occurred at 29°C, including changes in the relative abundance and turnover of microbial OTUs. Notably, this microbial shift occurred at a lower temperature than the 32°C threshold that induced sponge bleaching, indicating that changes in the microbiome may play a role in the destabilization of the *C. orientalis* holobiont. *C. orientalis* failed to regain *Symbiodinium* or restore its baseline microbial community following bleaching, suggesting that the sponge has limited ability to recover from extreme thermal exposure, at least under aquarium conditions.

4.2. Introduction

Corals and sponges harbour abundant and diverse communities of microbial symbionts (Bourne & Webster 2013), often hosting thousands of distinct operational taxonomic units (OTUs) that span a broad range of bacterial and archaeal phyla (Blackall et al. 2015, Thomas et al. 2016, Bourne et al. 2016). These microorganisms perform a range of functions that benefit their hosts, including photosynthesis (Venn et al. 2008) and nutrient cycling (carbon, nitrogen, sulphur and phosphate) (Webster & Thomas 2016, reviewed in Bourne et al. 2016). Many corals and some sponge species also form obligate symbiotic partnerships with *Symbiodinium*, a dinoflagellate which provides the host with essential photosynthates (Yellowlees et al. 2008). *Symbiodinium* also produces dimethylsulfoniopropionate, which can provide energy for other coral-associated microbes (Bourne et al. 2016). The loss of *Symbiodinium* during stress-induced bleaching reduces coral fitness (Szmant & Gassman 1990, Baird & Marshall 2002), and in the same way, shifts in prokaryotic symbionts are often associated with compromised coral or sponge health (Webster et al. 2008, Fan et al. 2013, Bay et al. 2016, Bourne et al. 2016).

The bacterial communities of corals and sponges tend to be dominated by *Proteobacteria*, typically the classes *Gammaproteobacteria* and/or *Alphaproteobacteria*, although *Actinobacteria* and *Cyanobacteria* are also commonly reported symbionts (Blackall et al. 2015, Thomas et al. 2016). While corals can harbour abundant *Bacteroidetes* and *Deltaproteobacteria* (Blackall et al. 2015), sponge microbiomes often contain larger proportions of *Acidobacteria*, *Chloroflexi*, and *Poribacteria* than corals (Webster et al. 2012, Schmitt et al. 2012, Thomas et al. 2016). Some of this disparity in microbiome composition has been explained by the presence of *Symbiodinium*, whereby reef invertebrates hosting *Symbiodinium* tend to be dominated by *Gammaproteobacteria* and invertebrates without *Symbiodinium* have more abundant *Alphaproteobacteria* (Bourne et al. 2013).

While *Symbiodinium* is the primary photosymbiont of many reef-building corals, most phototrophic sponge species form symbiotic partnerships with *Cyanobacteria* (Simister, Deines, et al. 2012). The bioeroding sponge family *Clionaidae* is a notable exception, with *Symbiodinium* reported to associate with at least 13 different Clionaid species (Rosell 1993, Zundelevich et al. 2007, Granados et al. 2008, Hill et al. 2011, Friday et al. 2013), where they enhance the growth and erosion capacity of the sponge host (Hill 1996, Schönberg 2006). Clionaid sponges are important members of reef communities, as they break down calcium carbonate substratum, including coral skeleton (Rützler 2002). Bioeroding sponges associate almost exclusively with Clade G *Symbiodinium* (Schönberg & Loh 2005, but see Granados et al. 2008, Hill et al. 2011), which is only common within the *Porifera* and *Foraminifera* (Pochon et al. 2006), and is understudied relative to clades commonly found in corals (A-D) (LaJeunesse et al. 2003). Two species have been described within Clade G *Symbiodinium*, *S. endoclionum* from *Cliona orientalis* on the Great Barrier Reef (GBR) and *S. spongiolum* from *Cliona varians* in the Caribbean (Ramsby, Hill, et al. 2017). The Clade G *Symbiodinium* from sponges is thought to be more thermally tolerant than the *Symbiodinium* symbionts of corals (Schönberg et al. 2008), which might explain why bioeroding sponges are generally less sensitive to elevated seawater temperatures than corals (Vicente 1990, Schönberg & Ortiz 2008, Carballo et al. 2013, Wulff 2016), although *Cliona varians* has been observed to bleach during thermal stress (Hill et al. 2016).

For both corals and sponges, exposure to elevated temperature can cause the loss of specific bacterial and archaeal taxa and an increase in opportunistic microorganisms (Fan et al. 2013, Sweet & Bulling 2017). Interestingly, in corals, the microbiome can shift prior to bleaching (Bourne et al. 2008, Lee et al. 2015), suggesting that a stable and/or specific bacterial community may be important for thermal tolerance. In addition, the response of the coral bacterial

community can differ depending on the identity of the associated *Symbiodinium* (Littman et al. 2010). The sponge microbiome can also shift upon exposure to elevated temperature (Lemoine et al. 2007, López-Legentil et al. 2008, Webster et al. 2008). However, some sponge species are able to maintain stable communities regardless of temperature (Webster, Botté, et al. 2011, Strand et al. 2017), while others retain their symbionts until the very late stages of heat stress when the sponge itself exhibits necrosis (Webster et al. 2008, Luter et al. 2012, Simister, Taylor, Tsai, Fan, et al. 2012). Little is known about the microbial communities of bioeroding sponges and, in particular, the sensitivity of the microbiome to elevated sea surface temperatures. Previous investigations have provided a phylum-level overview of the microbial communities within *Cliona* species, including *C. celata*, *C. delitrix*, *C. orientalis*, and *C. viridis* (Blanquer et al. 2013, Rodrigues Soares 2015, Jeong et al. 2015, Pineda et al. 2016, Thomas et al. 2016), but have not presented detailed accounts of species-level community dynamics. Here, we first defined the 'common' and 'core' microbial associates of *C. orientalis* before assessing how the microbiome responded to increasing temperatures between 23-32°C in order to ascertain how the *C. orientalis* holobiont may be impacted by ocean warming.

4.3. Methods

4.3.1. Experimental design

Cliona orientalis sponges were collected from Little Pioneer Bay on Orpheus Island, Queensland, Australia (18°37'40" S, 146°29'36" E) in June 2015. Sponges (n=13) were transported to the National Sea Simulator at the Australian Institute of Marine Science in Townsville, Queensland, where they were maintained at 23 °C. The microbiome of *C. orientalis* has been shown to be stable following transfer to this aquarium system (Pineda et al. 2016). After seven days, 151 cores were extracted from the 13 sponges using a hole saw (3.5 cm diameter) and these

were allowed to heal for 16 days at which point the margin of exposed choanosome was no longer visible. Cores were labelled with the identity of the original donor sponge to track cores from the same individual.

Cores from each sponge were distributed haphazardly amongst nine indoor aquaria (50 L), resulting in 15-18 cores per aquarium. Each aquarium was continuously provided with filtered seawater (0.04 μm) at 0.8 L/min which was mixed within aquaria using a small pump. Light was provided using Aqua Illumination Sol LEDs (Iowa, USA; maximum: 300 μmol quanta m^{-2} s^{-1}; 11 h photoperiod). Water temperature was controlled using a programmable logic controller (Siemens, Munich, Germany). The sponge cores were not fed and therefore their response may have differed from sponges on the reef. Cores were maintained at 23.2 ± 0.3 °C (SD) for 17 days, consistent with the ambient temperature at the time of collection and the minimum annual temperature at Orpheus Island. Then, one core from each sponge was sacrificed (i.e., 1-2 cores per tank): sponge tissue was removed with a sterile knife, frozen in liquid nitrogen, and stored at -80 °C.

After the acclimation period, two temperature treatments were established: a control treatment (3 aquaria) was maintained at 23 °C for the experimental duration and a heated treatment (6 aquaria), which increased by 2 °C increments (from 23 to 29 °C) and subsequently by 1 °C increments (from 30 to 32 °C) every 14 days. The rate of each temperature increase was 0.5 °C per day, resulting in a 2-4 day period of warming followed by 10 days of acclimation at each temperature. However, after the first temperature increment (25 °C), conditions were maintained for an additional 10 days due to logistical issues with the heating system. Following exposure to 32 °C, seawater temperature was reduced by 0.25 °C per day to 30 °C at which point

the temperature was maintained for four weeks and cores were sampled to assess recovery from elevated temperature.

In total, the temperature treatment lasted four months (July-November) and the temperature range approximated the winter to summer temperature change at the collection site: from a minimum of 22.4 °C in July to a maximum of 29.1 °C in February (source: http://weather.aims.gov.au). Thus, the 30, 31, and 32 °C temperature increments represented increases of 1, 2, and 3 °C above mean summer values, consistent with mean temperatures projected for 2100 (RCP 8.5; IPCC, 2014).

After the first temperature increment, cores were sampled from each of the nine sponges regardless of temperature treatment, leading to different sample sizes between treatments (Table 4-1). After subsequent temperature increments, 1-2 cores per tank were sampled and, where possible, cores from the same sponge were samples across both treatments. Cores from only 9 of the 13 sponges were used for DNA sequencing in order to maximize the number of cores per sponge and to minimize the effects of sampling different sponges. The final sample sizes (after DNA quality screening) are listed in the supplementary information (Table 4-1).

Table 4-1. Sample sizes for each temperature treatment and time point after DNA sequence analysis.

Time point	Heated temp. (°C)	Control (n)	Heated (n)
17	23	1	8
43	25	3	6
56	27	5	5
71	29	5	7
84	30	4	7
96	31	5	6
108	32	4	7
144	30 (recovery)	4	8

4.3.2. DNA sequencing

DNA was extracted from frozen sponge tissue (~0.2 g) using the Powerplant Pro DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's protocols, with additional bead-beating, RNAse, and proteinase K procedures. DNA samples were sent to the Australian Centre for Ecogenomics at the University of Queensland, Australia for sequencing. The V4 region of the 16S rRNA gene was amplified with primers 515f and 806r primers (Caporaso et al. 2012) using Illumina MiSeq 250 bp chemistry. Twenty-five PCR cycles were used during library preparation.

4.3.3. Sequence analysis

Sequence data was processed in Mothur 1.36 following the MiSeq standard operating procedure (Schloss et al. 2009). Briefly, demultiplexed paired-end reads were quality screened (max. ambiguous bases=0, max. homopolymers=8, 100<length<350), assembled, and sequences appearing only once within a sample were removed. The dataset was reduced to 8297 sequences per sample to account for differences in sampling depth. Identical sequences were combined into unique sequences, PCR chimeras were identified using Uchime (Edgar et al. 2011), and sequences

were classified taxonomically to the SILVA taxonomic reference file v.123 (Quast et al. 2013) using the needleman algorithm in Mothur. Sequences that matched chloroplast, mitochondrial, or eukaryote sequences, or that failed to match sequences in the SILVA database, were discarded. The remaining sequences were clustered into OTUs with 97% sequence similarity using the furthest neighbour algorithm and the Mothur function cluster.split. The sequence with the smallest maximum pairwise distance within each OTU was used for taxonomic assignment. Representative sequences for OTUs of interest (*Rhodothalassium* sp., *Nitrosopumilus* sp.) were blasted against the 16S sequences from 268 sponge species in the EMP-Porifera in order to determine their occurrence in other sponges and environmental samples (Moitinho-Silva *et al.* 2017b).

The bacterial community was analysed as two subsets according to the prevalence of OTUs amongst samples (Ainsworth et al. 2015). OTUs present in more than 10% of the samples in either temperature treatment (control: >3 samples; heated: >5 samples) were considered the 'common' microbial community. OTUs that were present in ≥ 80% of the samples in either treatment (control: >24 samples; heated: >43 samples) were considered the 'core' component of the 'common' community. To minimize the effect of the temperature treatment on community membership, each community was defined within each treatment and then pooled to form the 'common' and 'core' communities. Singleton OTU were excluded during this process. OTU subsetting was performed using the R package phyloseq (McMurdie & Holmes 2013).

To investigate changes at a broad taxonomic level, the relative abundances of bacterial phyla and classes were compared by pooling samples within each treatment and calculating the relative number of DNA sequences belonging to each taxon. To investigate species-level changes,

community similarity of OTUs was investigated using nonmetric multidimensional scaling (NMDS) with Bray-Curtis and Jaccard distance metrics using the R package vegan (Oksanen et al. 2017). Significant differences in the community composition between temperature treatments, time points, and treatment*time point interactions were tested using PERMANOVA on square root transformed distances using the adonis function in the R package vegan (Anderson 2001, Oksanen et al. 2017). Marginal sums of squares were used in all tests. Significant treatment*time point interactions were interpreted as an effect of warming on the microbial community. Permutations were constrained to account for correlations between cores from the same sponge and there was no significant difference in multivariate dispersion amongst cores from different sponges (pseudo-$F_{7,77}$=2.1, P=0.09). Tank was not included as an independent variable as there were no significant differences in microbial communities among tanks (Table 4-2). Following significant treatment*time interactions, 14 PERMANOVA post-hoc comparisons were used to determine the temperature increment that induced microbial changes among samples in the heated treatment (e.g., 23 vs. 25 °C, 25 vs. 27 °C, etc.) for the 'common' and 'core' communities (Table 4-3). Post-hoc P-values were corrected using Benjamini Hochberg adjustment.

To facilitate interpretation of the NMDS ordination, OTU network diagrams were constructed using Cytoscape 3.4.0 (Shannon et al. 2003). Three groups of OTUs were depicted: 1) OTUs from control samples at 23 °C, 2) OTUs from the heated treatment at 29-32 °C (pre-bleaching), and 3) OTUs from bleached sponges exposed to 32 °C and then 30 °C. OTU abundance was averaged within each group and only abundances >0.1% were depicted.

Microbial community characteristics, including richness, turnover, and mean rank shift, were compared between treatments and temperatures using the R package codyn (Hallett et al.

2015). Richness was calculated as the number of OTUs per sample. Turnover was calculated as the proportion of OTUs gained or lost between sampling points (i.e., total turnover). Mean rank shift also reflects stability of the microbial community and was calculated as the average change in rank abundance of OTUs. Significant differences in richness were tested using a linear mixed model for treatment* time point with a random intercept that accounted for correlations between cores from the same sponge using the R package lme4 (Bates et al. 2015).

To define the phylogenetic position of the primary bacterial symbiont, sequences (800-1400 bp) of the genus *Rhodothalassium* were downloaded from SILVA (https://www.arb-silva.de) and NCBI databases (https://blast.ncbi.nlm.nih.gov). Reference sequences were aligned with the representative sequence (252 bp) of the dominant microbial OTU derived from *C. orientalis* using the SILVA Incremental Aligner v1.2.11 (Pruesse et al. 2012). The alignment was used to build a neighbour-joining phylogenetic tree (Jukes-Cantor) in Geneious 9.1.8 (Kearse et al. 2012). Support for phylogenetic nodes was calculated using 500 bootstrap replicates.

Table 4-2. Test for similarities among *Cliona orientalis* cores from the same tank or from the same sponge. PERMANOVA results for differences in the 'common' microbial community between sponge cores from different tanks and between cores from different sponge genotypes (i.e., individuals). No significant differences were found between cores from different tanks, but there were significant differences between cores originating from different sponge genotypes. As a result, 'sponge' individual was included in the statistical analysis of microbial communities and richness. The table includes the degrees of freedom (Df), sums of squares (SS), mean squares (MS), pseudo-F ratio, proportion of variance explained (R2), and P-value (P) from PERMANOVA with 999 permutations.

	Df	SS	MS	Pseudo-F	R^2	P
Tank	8	2.1	0.3	1.0	0.09	0.59
Sponge	9	4.6	0.5	2.2	0.20	≤0.01
Residuals	84	22.7				

Table 4-3. Post-hoc PERMANOVA comparisons to determine the effect of each temperature increment on the heated samples. Differences between communities were summarized using square-root transformed Bray-Curtis distances. Due to the few samples present within each comparison, the random permutations were not restricted to cores from the same sponge. The

table includes the two elevated temperature increments for each comparison, the degrees of freedom (Df), sums of squares (SS), mean squares (MS), pseudo-F ratio (F), proportion of variance explained (R^2), and Benjamini Hochberg adjusted *P*-values for 14 comparisons (*P*). *P*-values less than 0.05 are indicated in bold.

		Common						Core					
Comparison		**Df**	**SS**	**MS**	**F**	**R^2**	**P**	**SS**	**MS**	**F**	**R^2**	**P**	
23°C vs 25°C	Temp.	1	0.2	0.2	0.8	0.06	0.70	0.1	0.1	1.1	0.08		0.44
	Resid.	12	2.9	0.2				0.8	0.1				
25°C vs 27°C	Temp.	1	0.5	0.5	1.8	0.17	0.12	0.6	0.6	5.8	0.39		**0.01**
	Resid.	9	2.5	0.3				0.9	0.1				
27°C vs 29°C	Temp.	1	0.6	0.6	2.3	0.18	**0.01**	0.5	0.5	4.6	0.31		**0.01**
	Resid.	10	2.7	0.3				1.1	0.1				
29°C vs 30°C	Temp.	1	0.4	0.4	1.6	0.12	0.13	0.1	0.1	1.2	0.09		0.44
	Resid.	12	2.8	0.2				1.1	0.1				
30°C vs 31°C	Temp.	1	0.3	0.3	1.3	0.10	0.33	0.1	0.1	0.7	0.06		0.63
	Resid.	11	2.6	0.2				1.1	0.1				
31°C vs 32°C	Temp.	1	0.2	0.2	1.0	0.08	0.44	0.1	0.1	1.1	0.09		0.44
	Resid.	11	2.4	0.2				0.9	0.1				
32°C vs 30°C	Temp.	1	0.6	0.6	2.6	0.17	**0.02**	0.4	0.4	2.7	0.17		0.11
	Resid.	13	3.2	0.2				1.8	0.1				

4.4. Results

The sponge *C. orientalis* was largely unaffected by increasing temperature up to 32 °C, at which point the symbiosis with *Symbiodinium* became disrupted and the sponge tissue appeared white (Fang et al. 2013). Bleached sponges were allowed to recover at 30 °C for four weeks but did not regain their brown pigmentation. Notably, control sponges did not exhibit reductions in *Symbiodinium*, chlorophylls, protein, or organic matter over the course of the experiment, suggesting that the sponges had adequate nutrition even though they were not fed (Figure 3-4).

4.4.1. Sequence processing

A total of 1.9 million sequences passed quality filtering, with an average of 22,184 reads per sample (±12,632 SD). The single most abundant OTU accounting for 21% of the total

sequences (Figure 4-1). The 'common' microbial community consisted of 3198 OTUs that represented 89% of the total sequences. The 'core' community consisted of only 45 OTUs that

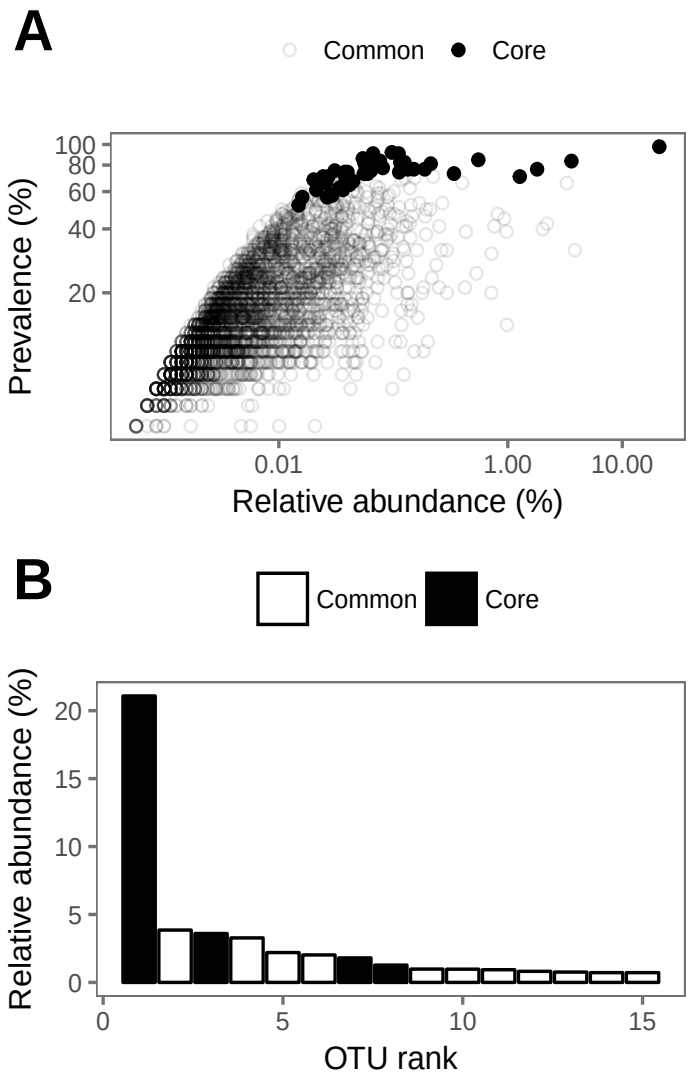

Figure 4-1. (A) OTUs are plotted according to their relative abundance amongst all sequences (x-axis) and their occurrence amongst all samples (y-axis). The 'core' subset of the 'common' community is indicated with filled circles. **(B)** Rank abundance of the 15 most abundant OTUs (1. *Rhodothalassium* sp., *Alphaproteobacteria*; 2. Unclassified *Deltaproteobacteria*; 3. Unclassified *Bacteria*; 4. Unclassified *Alphaproteobacteria*; 5. Unclassified *Alphaproteobacteria*; 6. *Rivularia* sp., *Cyanobacteria*; 7. Unclassified *Deltaproteobacteria*; 8. Unclassified *Gammaproteobacteria*; 9. Unclassified *Deltaproteobacteria*; 10. Unclassified *Alphaproteobacteria*; 11. Unclassified *Chloroflexi*; 12. Unclassified *Bacteria*; 13. *Pir4 lineage*, *Planctomycetes*; 14. Unclassified *Cyanobacteria*; 15. Unclassified *Alphaproteobacteria*). White bars are 'common' OTUs and dark bars represent 'core' OTUs. Abundance and ranks were calculated using all sequences.

represented 31% of the total sequences. OTUs that occurred in less than 10% of samples generally

represented less than 0.1% of total sequences and were excluded from further analyses.

4.4.2. Composition of the microbial community

The 'common' *C. orientalis* microbiome was dominated by *Proteobacteria*, specifically the classes *Alphaproteobacteria* (42%), *Deltaproteobacteria* (8%) and *Gammaproteobacteria* (5%) (Figure 4-2A). Other abundant phyla included *Cyanobacteria* (7%) and *Bacteroidetes* (2%) (**Figure 4-2**Figure 4-2A). *Alphaproteobacteria* and *Planctomycetes* were the most OTU-rich taxa, together comprising nearly 50% of all OTUs (**Figure 4-2**Figure 4-2B).

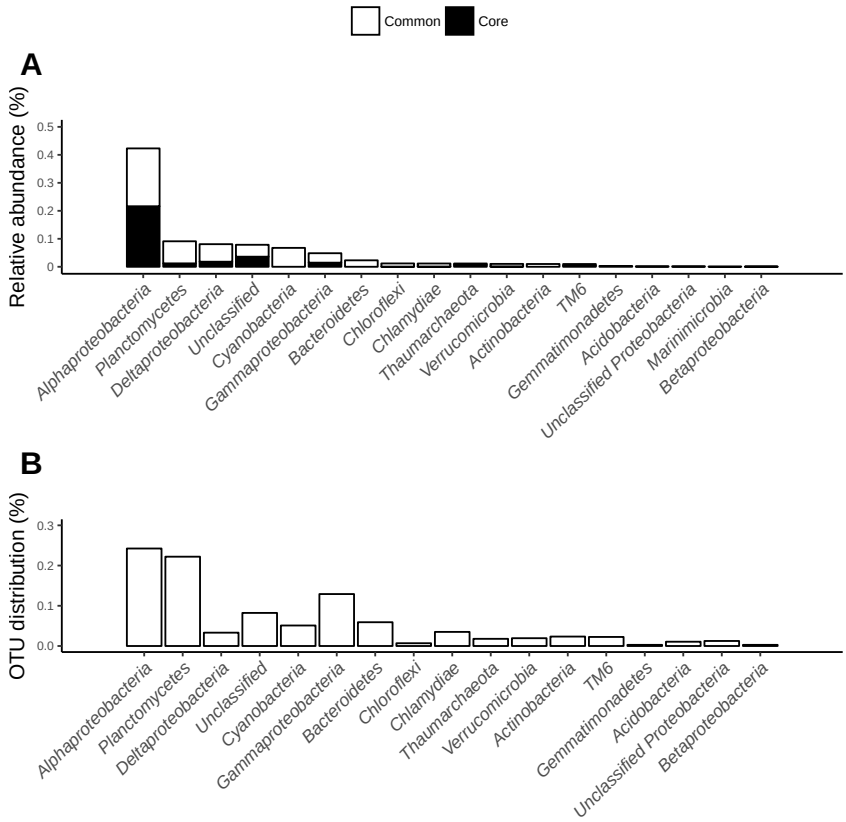

Figure 4-2. (A) Relative abundance of microbial phyla and *Proteobacteria* classes. Bar height indicates the relative abundance of each taxa and the black portion indicates the relative abundance of the 'core' subset within that phylum. **(B)** Proportion of all OTUs belonging to each phylum or *Proteobacteria* class.

'Core' OTUs included *Bacteria* and *Archaea* spanning 13 different phyla or candidate phyla (Figure 4-2**Figure 4-2**A). Consistent with the 'common' community, the 'core' was

dominated by OTUs within the *Alphaproteobacteria* and *Planctomycetes* (Figure 4-2A, Table 4-4). However, the 'core' microbiome contained lower abundance of *Deltaproteobacteria* and lacked the *Actinobacteria*, *Bacteroidetes*, and *Cyanobacteria* present in the 'common' community (Figure 4-2A). Several rare phyla/classes, including the *Betaproteobacteria*, *TM6*, and the *Thaumarchaeota*, had low abundances in both the 'common' and 'core' communities (Figure 4-2A). Three 'core' OTUs belonged to the *Thaumarchaeota* and all were most similar to the candidate genus *Nitrosopumilus*. The most abundant 'core' *Nitrosopumilus* OTU was similar to four OTUs in the EMP-Porifera dataset, which occurred in 108 sponge species (mean rel. abundance 2.5%), seawater (0.9%), and sediment (0.4%) from the EMP-Porifera dataset.

Table 4-4. The taxonomy of the 24 'Core' OTU that were identified to genus level. + symbols indicate genera that met the 'core' definition (occur in ≥80% of samples) in both temperature treatments. n indicates the number of OTUs identified for each genus.

Domain	Phylum	Class	Order	Family	Genus		n
Archaea	Thaumarchaeota	Marine Group I	Unknown	Unknown	Nitrosopumilus	+	3
Bacteria	Chlamydiae	Chlamydiae	Chlamydiales	Simkaniaceae	Simkania		1
	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Blastopirellula	+	4
					Bythopirellula	+	2
					Pir4 lineage	+	4
					Planctomyces		3
					Rhodopirellula	+	1
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Filomicrobium		1
			Rhodobacterales	Rhodobacteraceae	Labrenzia	+	1
					Rhodothalassium	+	1
			Rhodobacterales	Rhodospirillaceae	Magnetospira		1
		Betaproteobacteria	Methylophilales	Methylophilaceae	OM43 clade		1
		Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Pseudospirillum		1

While *C. orientalis* hosted a wide diversity of microorganisms, a single OTU dominated the microbial community, comprising 21% of the total sequences and occurring in 97% of the sponges (Figure 4-1B). This dominant OTU was most closely related to the genus

Rhodothalassium, in particular *Rhodothalassium* sequences retrieved from corals and sponges, with highest sequence similarity to a *Rhodothalassium* from the scleractinian coral *Orbicella faveolata* (Figure 4-3). This *Rhodothalassium* OTU was an identical match to an OTU within the EMP-Porifera database which was primarily derived from *C. orientalis* (<0.1% rel. abundance across the 267 other sponge species) and was not observed in biofilm, sediment, or seawater samples. The *C. orientalis* samples in the EMP-Porifera database were collected 10 km from the site of collection for sponges in this study.

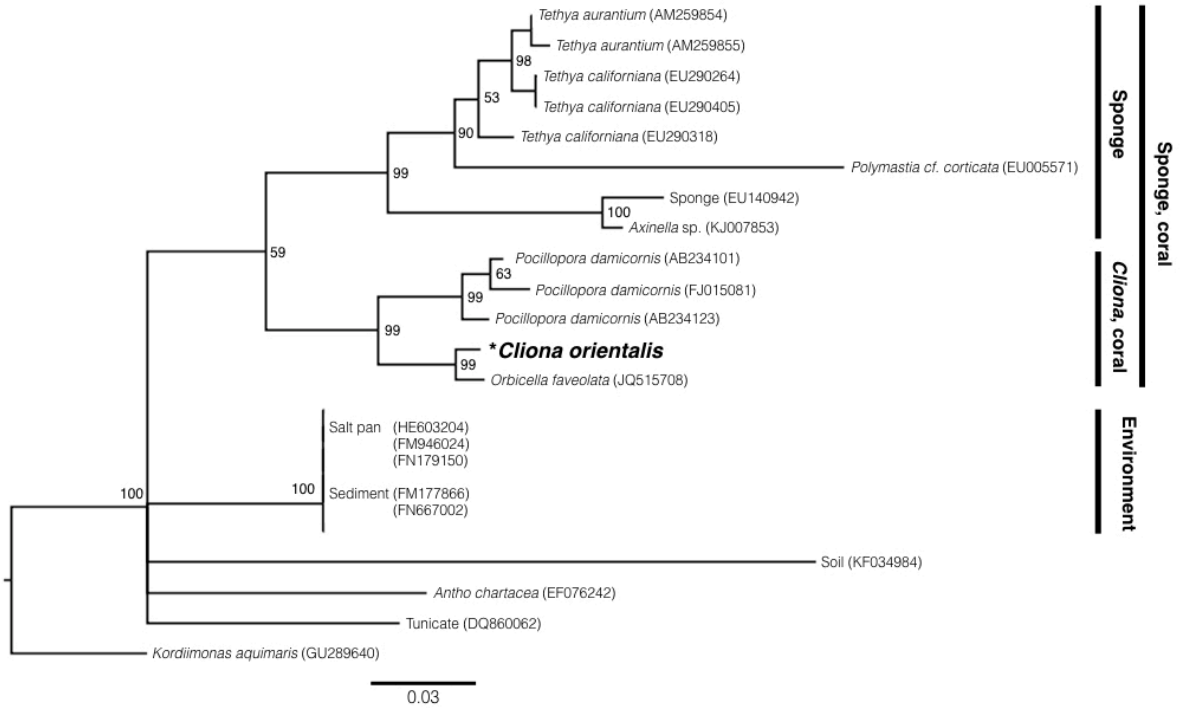

Figure 4-3. Neighbour-joining phylogenetic tree from analysis of 252 bp of the 16s rRNA gene from the dominant *Cliona orientalis* OTU, *Rhodothalassium* sp. Branch tips are labelled with the *Rhodothalassium* source and NCBI accession number (except for the outgroup *K. aquimaris*). *Rhodothalassium* from *C. orientalis* is in bold and indicated with an asterisk. The numbers at nodes are percentages of bootstrap support using 500 resampled datasets and the scale bar represents 0.03 substitutions per nucleotide position. The *C. orientalis* OTU is positioned within a clade of *Rhodothalassium* from corals that is distinct from the *Rhodothalassium* isolated from other sponges and environmental samples.

4.4.3. Response to elevated temperature

Having established the reference microbiome of *C. orientalis*, we assessed how it was impacted by elevated temperature. At the phylum level, both the 'common' and 'core' components of the community showed little variability with increasing temperature until the samples failed to recover from bleaching at 32 °C and a marked increase in *Cyanobacteria* was observed in the 'common' community (Figure 4-4A and Figure 4-4B). A gradual decrease in *Alphaproteobacteria* also occurred in both temperature treatments over the course of the experiment (Figure 4-4A). In contrast, at the OTU-level, the 'common' community underwent significant changes in response to the temperature treatment, both in terms of relative abundance

(Figure 4-4A; PERMANOVA; Table 4-5: treatment*time) and presence-absence (PERMANOVA; Table 4-5: treatment*time). In particular, the communities of samples exposed to 29 °C and above were different to the communities in control samples (Figure 4-4C and Figure 4-4D). However, there was no significant difference in the relative abundance of OTUs between treatments at any time point (Table 4-7), perhaps due to differences in sample size between treatments. Comparisons within the heated treatment indicated that two significant microbial shifts occurred: i) when the seawater temperature increased from 27 to 29 °C and ii) when samples that had bleached at 32 °C were maintained at 30 °C (Table 4-3). These shifts distinguished *C. orientalis* samples that associated with significantly different 'common' OTU communities (Table 4-6). In part, these differences were driven by a lower abundance of *Alphaproteobacteria* (unclass.) and *Bacteroidetes* (unclass.) OTUs in 29-32 °C samples and novel *Cyanobacteria* (*Rivularia* sp., unclass.) and *Verrucomicrobia* (*Roseibacillus* sp.) OTUs that occurred in samples post-bleaching (Figure 4-4C). Within the 'core' community, 29-32 °C samples were associated with a higher relative abundance of an *Thaumarchaeota* (*Nitrosopumilus* sp.) OTU and bleached samples at 30 °C were associated with a higher abundance of OTUs affiliated to *Planctomycetes* (*Planctomyces* sp.) and *Alphaproteobacteria* (*Labrenzia* sp.; Figure 4-4D).

Consistent with the 'common' microbial OTUs, 'core' OTUs were also impacted by exposure to elevated temperature. Changes in the relative abundance of OTUs led to significantly different 'core' communities in heated compared with control sponges (Figure 4-4D; PERMANOVA; Table 4-5: treatment*time), although differences were less clear than for the larger community (Figure 4-4C). Presence-absence in the 'core' OTU community was not affected by exposure to elevated temperature, unlike the 'common' community (PERMANOVA; Table 4-5: treatment*time). However, changes in the relative abundance of 'core' OTUs was

detected after temperature increases to 27°C and 29°C, but not at higher temperatures (Table 4-3). Heated samples at 23 and 25°C had significantly different 'core' OTU composition than heated samples at 27 °C, but the 27°C samples were similar to those at higher temperatures (Table 4-6). However, at any time point, there was no significant difference in the relative abundance of 'core' OTUs between treatments (Table 4-7).

Table 4-5. PERMANOVA results for main effects of heating treatment, time point, and the treatment * time point interaction. Two PERMANOVA were conducted on the 'Common' and 'Core' communities according to the relative abundance (Bray-Curtis distance) or presence-absence of OTUs (Jaccard distance). The table includes the degrees of freedom (Df), marginal sums of squares (SS), mean squares (MS), pseudo-F ratio (F), proportion of variance explained (R^2), and *P*-values (*P*). *P*-values less than 0.05 are indicated in bold.

	Common						**Core**				
Factor	**Df**	**SS**	**MS**	**F**	**R^2**	**P**	**SS**	**MS**	**F**	**R^2**	**P**
Rel. abundance											
Treatment	1	0.5	0.5	2.4	0.02	**<0.01**	0.3	0.3	2.4	0.02	0.06
Time point	7	3.1	0.4	2.1	0.14	**<0.01**	1.5	0.2	2.1	0.14	**<0.01**
Individual	8	3.6	0.5	2.1	0.16	0.20	1.1	0.1	1.3	0.10	0.62
Treat.*Time	7	2.4	0.3	1.6	0.10	**<0.01**	1.7	0.2	2.4	0.16	**<0.01**
Residual	61	13.1	0.2		0.58		6.4	0.1		0.58	
Pres.-absence											
Treatment	1	0.9	0.9	2.9	0.03	**<0.01**	0.7	0.7	8.5	0.08	**<0.01**
Time point	7	4.0	0.6	1.8	0.13	**<0.01**	1.7	0.2	2.9	0.19	**<0.01**
Individual	8	3.5	0.4	1.4	0.12	0.15	0.8	0.1	1.2	0.09	0.645
Treat.*Time	7	2.6	0.4	1.2	0.09	**<0.01**	0.5	0.1	0.9	0.06	0.572
Residual	61	18.9	0.31		0.63		4.9	0.1		0.58	

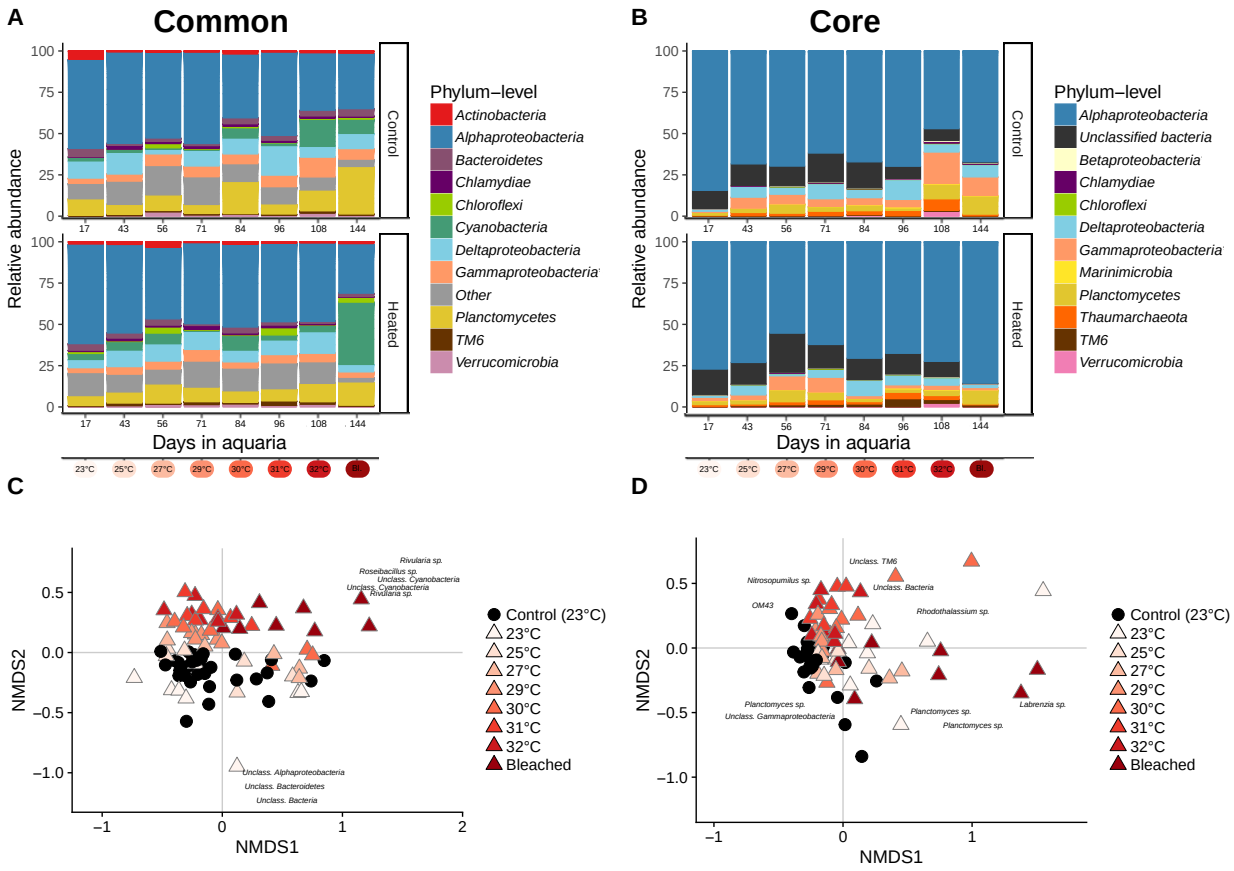

Figure 4-4. The proportion of microbial phyla or *Proteobacteria* classes represented by (A) 'common' and (B) 'core' OTUs under different temperature treatments. Proportions were calculated by pooling samples within each time point and treatment combination. Time as well as the temperature of the heated treatment are indicated on the x-axis. Non-metric multidimensional scaling of samples in each time point and treatment combination based on relative abundance within the (C) 'common' (stress=0.17) and (D) 'core' (stress=0.18) communities. Black circles indicate samples from the control treatment and red triangles indicate samples from the heated treatment. The colour of the triangle represents the temperature of the heated treatment. For all panels, the control treatment was sampled at 23 °C.

Table 4-6. Post-hoc PERMANOVA comparisons to compare samples across the thermal thresholds identified in Table 4-3. Differences between communities were summarized using square-root transformed Bray-Curtis distances. Due to the few samples present within each comparison, the random permutations were not restricted to cores from the same sponge. The table includes the temperature for each comparison, the degrees of freedom (Df), sums of squares (SS), mean squares (MS), pseudo-F ratio (F), proportion of variance explained (R^2), and Benjamini Hochberg adjusted *P*-values for 14 comparisons (*P*). Corrected *P*-values less than 0.05 are indicated in bold.

Comparison				**Df**	**SS**	**MS**	**F**	**R^2**	**P**
Common community									
23-27°C	vs.	29-32°C		1	0.5	0.5	2.1	0.04	**0.05**
			Resid.	53	12.8	0.2		0.96	
29-32°C	vs.	30°C Rec.		1	1.0	1.0	4.0	0.07	**0.03**
			Resid.	51	13.0	0.3		0.93	
30°C Rec.	vs.	23-27°C		1	1.0	1.0	3.8	0.10	**0.03**
			Resid.	34	8.8	0.3		0.90	
Core community									
23-25°C	vs.	27°C		1	0.7	0.7	8.7	0.29	**0.03**
			Resid.	21	1.7	0.1		0.71	
27°C	vs.	29-32, 30°C Rec.		1	0.3	0.3	1.9	0.04	0.07
			Resid.	43	5.9	0.1		0.96	
29-32, 30°C Rec.	vs.	23-25°C		1	0.1	0.1	1.4	0.03	0.08
			Resid.	51	5.1	0.1		0.97	

Despite several 'core' OTUs being maintained in both heated and bleached sponges, the heating treatment affected 'core' OTU prevalence (Figure 4-5). Control samples and samples heated to 29-32 °C shared more OTUs than either treatment did with the bleached samples at the end of the experiment (Figure 4-5). Bleached samples had a larger diversity of *Cyanobacteria* OTUs (unclass. family I and II, *Rivularia* sp., *Phormidium* sp.) and higher abundance of one *Cyanobacteria* OTU (family I unclass.) than control samples. In addition, bleached samples had a larger diversity of *Planctomycetes* OTUs (family *Planctomycetaceae*) and fewer

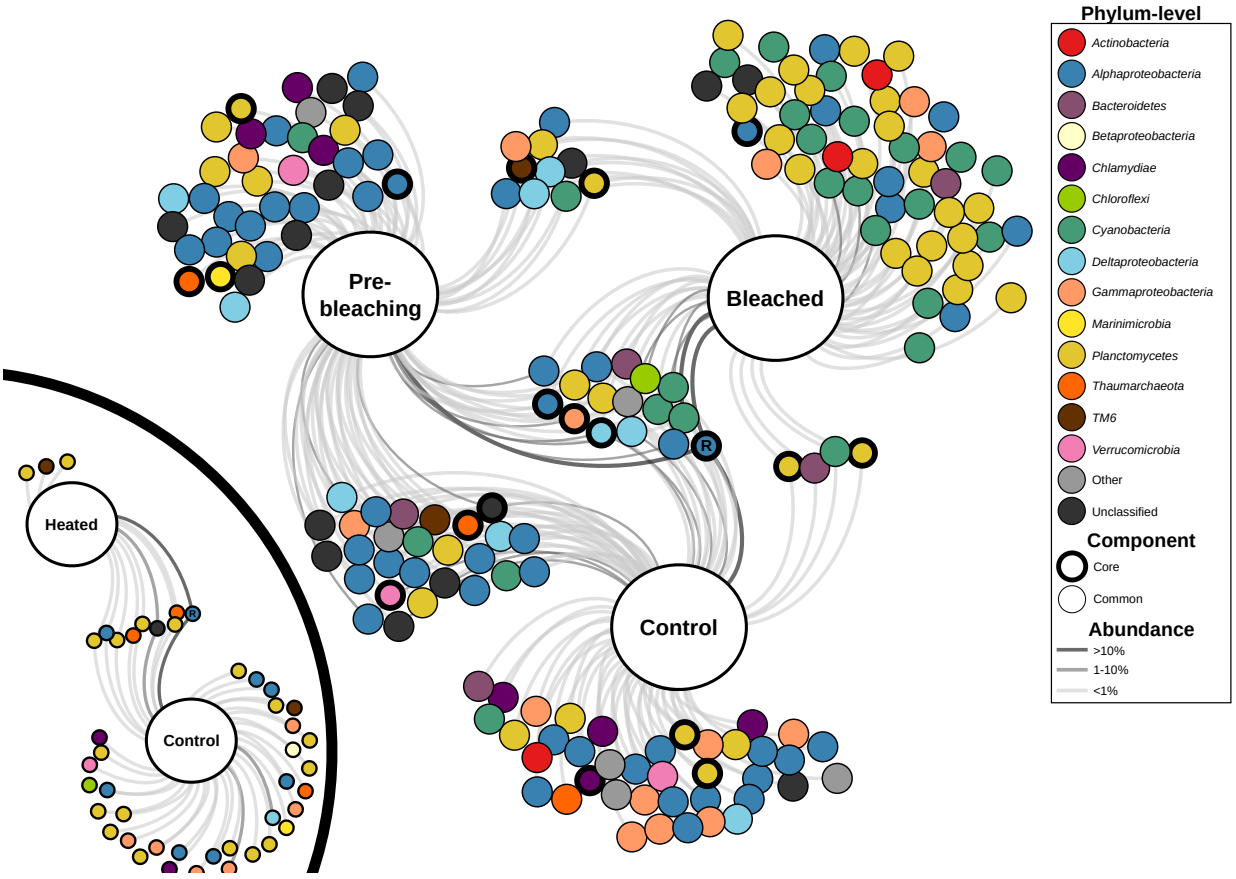

Figure 4-5. Cytoscape network of the 196 OTUs with highest relative abundance in the 'common' community (present in > 10% of samples in either treatment and > 0.1% abundance) from the controls, 29-32 °C (pre-bleaching) or 32 °C (bleaching) treatments. Some OTUs are restricted to specific treatments whereas others are shared between treatment groups. 'Core' OTUs (present in > 80% of samples in either treatment) are indicated using bold circle margins. Node colours represent the OTU phylum or *Proteobacteria* class and the edge intensity indicates OTU abundance. **INSET**: Network of the 45 'core' OTUs and their prevalence in sponges from either control or heated temperature treatments. Taxonomic information for 'core' OTUs can be found in Table 4-4. The most abundant OTU, *Rhodothalassium* sp., is indicated with an R.

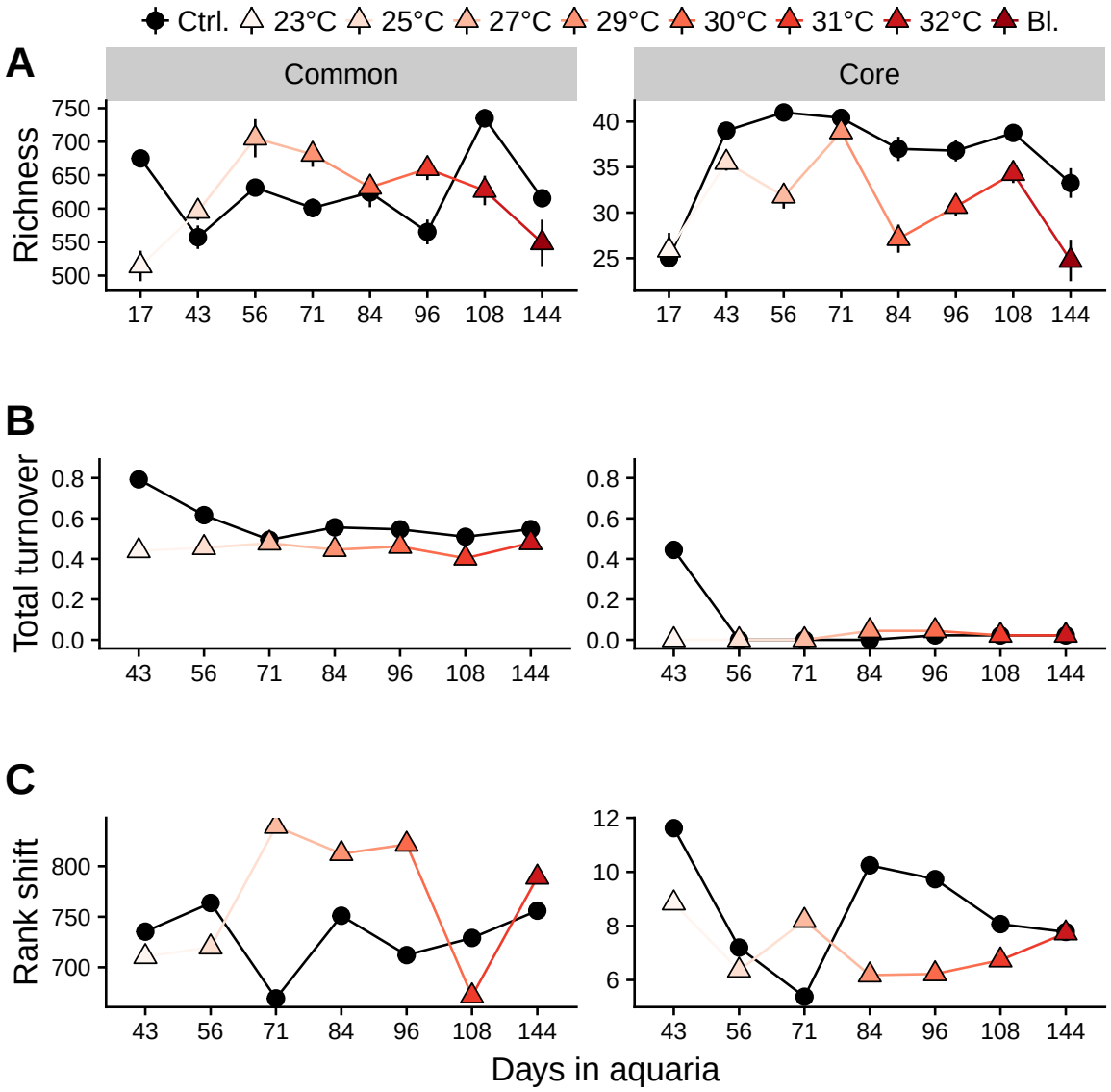

Figure 4-6. Dynamics within the 'common' and 'core' components of the microbial community. **(A)** Richness (number of OTU per sample), **(B)** OTU turnover (proportion of OTU gained or lost) and **(C)** mean change in OTU rank abundance (rank shift) are shown for each community. Black lines and circles indicate the control treatment (all 23 °C). Red lines and triangles indicate the heated treatment. The colour indicates the temperature of the heated treatment. Error bars in **(A)** represent standard error.

Alphaproteobacteria OTUs than control or 29-32 °C samples (Figure 4-5). Only 16 OTUs were present in sponges from all three groups, including the dominant OTU *Rhodothalassium* (Figure 4-5). Two 'core' *Thaumarchaeota* OTUs, closely related to the candidate genus *Nitrosopumilus*, were present (>0.1% abundance) in either the control or 29-32 °C heated samples, but were absent (<0.1% abundance) from the bleached samples at the end of the experiment (Figure 4-5).

Within the 'core' community, the majority of OTUs only met the 'core' definition within control samples (Figure 4-5 inset), likely contributing to the significant differences in the 'core' community observed in heated sponges. Nine 'core' OTUs met the 'core' definition in both temperature treatments, including the dominant OTU *Rhodothalassium* and the *Thaumarchaeota* candidate genus *Nitrosopumilus* (Figure 4-5 inset; Table 1). Only three OTUs, including two *Plancytomycetes* (*Pir4 lineage*, one unclass.) and one TM6 OTU (unclass.), met the 'core' definition within sponges at elevated temperature but not in control samples (Figure 4-5 inset).

Diversity metrics were used to summarize the effect of elevated temperature on the *C. orientalis* microbiome (Cleland et al. 2013, Hallett et al. 2016). Diversity metrics varied greatly between the 'common' and 'core' components of the community (Figure 4-6). In the 'common' community, increasing seawater temperature did not significantly affect the number of OTUs per sponge (ANOVA treatment*time: $F_{7, 64.0}$=1.4, P=0.22), despite consistently greater OTU richness in sponges exposed to sub-bleaching temperatures (27-31 °C) and lower richness in bleached samples (Figure 4-6A). Analysis of OTU turnover in the 'common' community indicated that sponges sampled at different time points shared ~50% of 'common' OTUs regardless of temperature treatment (Figure 4-6B). While richness and turnover stabilized at intermediate temperatures, the 'common' community was not static, and changes to OTU rank abundance (rank shift) were highest in sponges exposed to intermediate temperatures (Figure 4-6). Rank shift decreased in sponges exposed to 31 °C and 32 °C, indicating fewer changes in the structure of the 'common' component of the community. However, rank shift increased in bleached sponges at the end of the experiment, revealing a second restructuring of the microbial community as sponges failed to recover from bleaching (Figure 4-6C). The 'core' component of the microbiome exhibited different patterns in diversity metrics than the larger 'common'

community. In general, 'core' OTU richness was significantly lower in the heated treatment (ANOVA treatment: $F_{7, 63.0}$=12.3, P<0.01), although this difference did not vary as heated samples were exposed to increasingly higher temperature (Figure 4-6A; ANOVA treatment*time: $F_{7, 62.2}$=0.7, *P*=0.63). In addition, exposure to elevated temperature did not trigger large changes in the turnover or rank abundance of 'core' OTUs (Figure 4-6B and Figure 4-6C).

Table 4-7. Post-hoc PERMANOVA comparisons to determine the effect of the heating treatment at each time point (i.e., heated temperature). Differences between communities were summarized using square-root transformed Bray-Curtis distances. Due to the few samples present within each comparison, the random permutations were not restricted to cores from the same sponge. The table includes the temperature for each comparison, the degrees of freedom (Df), sums of squares (SS), mean squares (MS), pseudo-F ratio (F), proportion of variance explained (R^2), and Benjamini Hochberg adjusted *P*-values for 14 comparisons (*P*).

		Common						Core				
Temp.		**Df**	**SS**	**MS**	**F**	**R^2**	**P**	**SS**	**MS**	**F**	**R^2**	**P**
25°C	Treat.	1	0.2	0.2	0.8	0.10	0.78	<0.1	<0.1	0.4	0.05	0.81
	Resid.	7	1.5	0.2		0.90		0.29	<0.1		0.95	
27°C	Treat.	1	0.7	0.7	2.9	0.26	0.10	0.7	0.7	6.0	0.43	0.10
	Resid.	8	1.9	0.2		0.74		1	0.1		0.57	
29°C	Treat.	1	0.3	0.3	1.4	0.12	0.36	0.3	0.3	1.4	0.12	0.52
	Resid.	10	2.2	0.2		0.88		2.2	0.2		0.88	
30°C	Treat.	1	0.3	0.3	1.1	0.11	0.44	0.3	0.3	1.1	0.11	0.54
	Resid.	9	2.5	0.3		0.89		2.5	0.3		0.89	
31°C	Treat.	1	0.2	0.2	1.2	0.12	0.34	0.2	0.2	1.2	0.12	0.36
	Resid.	9	1.8	0.2		0.88		1.8	0.2		0.88	
32°C	Treat.	1	0.5	0.5	2.1	0.19	0.09	0.5	0.5	2.1	0.19	0.08
	Resid.	9	2.2	0.2		0.81		2.2	0.2		0.81	
30°C	Treat.	1	0.4	0.4	1.4	0.12	0.35	0.4	0.4	1.4	0.12	0.55
	Resid.	10	2.9	0.3		0.88		2.9	0.3		0.88	

4.5. Discussion

4.5.1. Composition of the microbial community

The 'common' and 'core' components of the *C. orientalis* microbial community were dominated by *Alphaproteobacteria,* but also included abundant *Planctomycetes*,

Deltaproteobacteria, and *Gammaproteobacteria*, consistent with previous findings for this species (Pineda et al. 2016). Unlike *C. orientalis*, many sponge species associate with *Cyanobacteria* rather than *Symbiodinium* and these species tend to be dominated by groups other than *Alphaproteobacteria* (Luter et al. 2014, Burgsdorf et al. 2015, Thomas et al. 2016), such as *Gammaproteobacteria*, *Chloroflexi*, *Acidobacteria*, and *Actinobacteria* (Thomas et al. 2016). Within the sponge family Clionaidae, the *C. orientalis* microbiome is most similar to that of *Cliona viridis*, which also associates with *Symbiodinium*, and is dominated by *Alphaproteobacteria* (Blanquer et al. 2013, Thomas et al. 2016). Several other *Cliona* species associate with *Symbiodinium*, but the composition of their microbiomes has not yet been described. The *Cliona* species that lack *Symbiodinium* do not contain high proportions of *Alphaproteobacteria* and are instead dominated by *Firmicutes* (Rodrigues Soares 2015), *Betaproteobacteria* (Jeong et al. 2015), *Gammaproteobacteria* (Thomas et al. 2016), or unclassified bacteria (Thomas et al. 2016).

Nearly one-quarter of all 16S rRNA genes from *C. orientalis* belonged to a single OTU, with highest similarity to *Rhodothalassium* sp. (*Alphaproteobacteria*). The taxonomic assignment of *Rhodothalassium* is uncertain, but the genus may represent a novel order of *Alphaproteobacteria* (Venkata Ramana et al. 2013). *Rhodothalassium* found in soils perform a range of important metabolic functions, including photosynthesis (Drews 1981), sulphur oxidation (Xia et al. 2015), and nitrogen fixation (Madigan et al. 1984). Interestingly, *Rhodothalassium salexigans* only fixes nitrogen (*sensu* nitrogenase production) when glutamate, a molecule involved in nitrogen recycling within *Symbiodinium*-coral associations (Yellowlees et al. 2008), is available (Madigan et al. 1984). Analysis of the global EMP-Porifera dataset showed that while this OTU is prevalent in *C. orientalis* (from the central Great Barrier Reef), it is extremely

rare among other sponge species and is not found in the surrounding environment. The abundance and stability of the *Rhodothalassium* OTU seen here therefore suggests an intimate partnership with *C. orientalis*, although further genomic and transcriptomic work is required to confirm the functional basis of any symbiotic interaction.

Using prevalence rather than abundance to examine the *C. orientalis* microbiome facilitated identification of additional taxa likely to perform important symbiotic functions (Ainsworth et al. 2015, Thomas et al. 2016, Hernandez-Agreda et al. 2016, Astudillo-García et al. 2017). Multiple members of the 'core' community matched microbial taxa involved in nitrogen metabolism and may play a role in nitrogen cycling within the *C. orientalis* holobiont. In addition to the dominant OTU being highly similar to nitrogen fixing *Rhodothalassium*, the 'core' community contained three OTUs affiliated with the candidate genus *Nitrosopumilus* (Archaea), which is known to oxidize ammonia (Gaidos et al. 2010) and is involved in nitrogen cycling within the sponge *Cymbastela concentrica* (Moitinho-Silva et al. 2017) and possibly *Xestospongia muta* (Morrow et al. 2016). Moreover, the 'core' community contained 17 *Planctomycetes* OTUs (38% of 'core' OTU), a group that contributes to nitrogen cycling in other sponges (Mohamed et al. 2010). The presence of these taxa in the 'core' *C. orientalis* microbiome suggests that nitrogen metabolism is a key function of the *C. orientalis* microbiome, although detailed genomic and experimental isotope work would be required to validate and quantify the role of symbionts in holobiont nitrogen cycling. In addition, broader geographic sampling would confirm the ubiquity of these 'core' OTUs.

4.5.2. Response to elevated temperature

In general, both the 'common' and 'core' components of the *C. orientalis* microbiome

were stable upon exposure to temperatures up to 27 °C, however changes to the relative and rank abundance of microbial OTUs occurred at temperatures of 29 °C and above. Overall, a large number of 'core' OTUs were less prevalent in sponges at elevated temperatures, although the functional implications of this community shift remain to be determined. While the most abundant *Rhodothalassium* OTU and one 'core' *Nitrosopumilus* OTU were apparently unaffected by elevated temperatures, a second 'core' *Nitrosopumilus* OTU occurred in higher abundance in sponges at 29-32 °C than in control sponges, perhaps due to a greater availability of ammonia in the stressed holobionts. The maintenance of these 'core' members of the microbiome suggests that the different components of nitrogen metabolism (nitrogen fixation, ammonia oxidation) may continue after bleaching, as has been shown in the giant barrel sponge *Xestospongia muta* (López-Legentil et al. 2010).

Notably, the significant shift in the microbial community occurred at a lower temperature than the 32 °C threshold that induced sponge bleaching. Hence, it is possible that changes in the microbiome contribute to the destabilisation of the *C. orientalis* holobiont, ultimately resulting in the loss of *Symbiodinium* and sponge bleaching. The microbial community of corals has also previously been shown to shift at sub-lethal temperatures prior to visual signs of bleaching (Lee et al. 2015, reviewed in Bourne et al. 2016), but in some cases, the coral microbiome can be restored following the thermal event (Bourne et al. 2008). In contrast, *C. orientalis* failed to regain *Symbiodinium* or restore its baseline microbial community following bleaching, suggesting that it has limited ability to recover from exposure to temperatures above its thermal threshold However, the availability of *Symbiodinium* or microbial taxa might have been limited within the aquarium system, and future analyses should assess the potential for recovery under field conditions.

In many sponge species, microbial shifts at high temperatures coincide with declining host health (Lemoine et al. 2007, Webster et al. 2008, López-Legentil et al. 2010, Luter et al. 2012, Simister, Taylor, Tsai, Fan, et al. 2012, Fan et al. 2013), whereas other sponges are able to maintain stable microbial communities irrespective of seawater temperature (Webster, Botté, et al. 2011, Pita et al. 2013, Lesser et al. 2016, Strand et al. 2017). Microbial shifts can lead to dysbiosis, including reduced expression of genes related to nutrient transport, substrate utilisation, sugar metabolism and cellular integrity in the symbionts and increased expression of stress response genes in the sponge (Fan et al. 2013). Disruption to nutritional interdependence and molecular interactions between the host and symbionts was proposed to further destabilize the holobiont ultimately leading to the loss of the archetypal sponge symbionts and the necrotic sponge phenotype that becomes apparent in the latter stages of heat stress (Fan et al. 2013).

Exposure of *C. orientalis* to 32 °C led to sponge bleaching and the subsequent establishment of a community dominated by *Cyanobacteria*. *Cyanobacteria* were present within the 'common' community throughout the experiment and are frequent symbionts of sponges (Thacker & Freeman 2012). However, the increase in *Cyanobacteria* did not correspond to known sponge symbionts, such as *Oscillatoria spongeliae* or *Synechococcus spongiarum* (Erwin & Thacker 2007, Lemloh et al. 2009), but were instead driven largely by a tenfold increase in filamentous, heterocystous *Cyanobacteria* similar to the genus *Rivularia*. In another sponge species, *Rivularia* is associated with unhealthy tissues (Gao et al. 2014). Thus, the increase in filamentous *Cyanobacteria* is likely opportunistic, occurring in response to greater nutrient availability in bleached sponges or changes in the host surface chemistry, which may have facilitated cyanobacterial colonisation, as has been seen in other reef organisms (Webster, Soo, et al. 2011).

Examining changes to the microbial community of *C. orientalis* across a range of temperatures enables prediction of how the bioeroding sponge might fare in future, warmer, oceans. At Orpheus Island, *C. orientalis* regularly experience temperatures up to 29-30 °C and hence the microbial changes observed at these temperatures therefore likely represent seasonal variation. Other sponge species exhibit minimal seasonal variability in their microbial associates (Erwin et al. 2015), although seasonal summer warming can increase the variability between individuals (Erwin et al. 2012). However, when exposed to temperatures above current summer averages, the microbial community of *C. orientalis* is disrupted, filamentous *Cyanobacteria* become dominant and the metabolic exchange between host and symbionts is likely to be disrupted (Fan et al. 2013). The thermal sensitivity of the microbiome suggests that ocean temperatures predicted to occur by 2100 will negatively impact the *C. orientalis* holobiont.

The *C. orientalis* microbiome is dominated by a single *Alphaproteobacteria* OTU with high sequence similarity to *Rhodothalassium* sp. retrieved from other reef invertebrates. In addition, the 'core' microbiome comprises ammonia oxidising *Thaumarchaeota* (*Nitrosopumilus* sp.) and numerous *Planctomycetes* OTUs (family Planctomycetaceae), both of which are common features of sponge microbiomes. The taxonomic affiliation of these 'core' members of the microbiome indicate a potential role in nitrogen metabolism within the sponge, although further functional analysis is required to validate these pathways. For *C. orientalis*, the thermal threshold occurred at 32 °C, when the sponges irreversibly bleached. Importantly however, the microbiome showed evidence of destabilisation at sub-lethal temperatures (29 °C), suggesting that microbial shifts may play a role in the holobiont stress response. Given the importance of clionaid sponges to reef bio-erosion, understanding the role microbial symbionts play in holobiont resilience during ocean warming is imperative. This work has provided a valuable platform for future

research to explore the functional basis of microbial symbiosis in *C. orientalis* and assess how microbial shifts contribute to bleaching sensitivity.

Chapter 5. Dissolved inorganic nutrient enrichment does not affect sponge growth or condition

5.1. Abstract

Changing land use and an increasing human population have led to increased terrestrial runoff, delivering nutrients, pesticides, and heavy metals into aquatic ecosystems. Elevated nutrient levels can exacerbate coral disease and coral bleaching as well as stimulate algal growth, but the effects on other reef taxa are poorly understood. Here, we investigated the effects of dissolved inorganic nutrients and light intensity on the growth and condition of five common Great Barrier Reef sponges, including one heterotrophic species and four species with photosynthetic symbionts. Dissolved nutrients up to 7 μM total DIN did not significantly affect the growth, condition, or chlorophyll content of any sponge species after 10 weeks of exposure. Light (80 vs 160 μmol quanta m^{-2} s^{-1}) did not affect four of the five sponge species, but higher irradiance resulted in higher organic content and chlorophyll levels in the bioeroding sponge *Cliona orientalis*, the only species that associates with the photosynthetic dinoflagellate *Symbiodinium*. Our findings indicate that sponges tolerate moderate increases in dissolved inorganic nutrients and that nutrient enrichment does not accelerate sponge growth or improve sponge condition. Only *C. orientalis* responded to higher irradiance, suggesting that sponge-*Symbiodinium* associations may be more sensitive to environmental conditions than sponge-*Cyanobacteria* associations. While elevated nutrient levels are exacerbating the decline of reef-building corals, they appear to have negligible effects on reef sponges, providing further support for the environmental tolerance of this ecologically important phylum.

5.2. Introduction

Intensified agricultural land use and modification of coastal landscapes has increased terrestrial runoff, carrying nutrients, sediments, and pollutants into the marine environment (Brodie et al. 2011, 2012, Waterhouse et al. 2012). For example, rivers now carry over five times more nutrients into the Great Barrier Reef (GBR) lagoon than before European settlement (Kroon et al. 2012). Seasonal floods cause acute runoff-related stresses (Schaffelke et al. 2012, Fabricius et al. 2016), but chronic nutrient enrichment (dissolved inorganic N and P) can also occur in nearshore locations with limited exposure to oligotrophic water (Smith et al. 1981, Goreau 1992). While rivers and streams represent point sources of nutrient pollution, plumes of sediments and nutrients can cover hundreds of km^2 and increase turbidity for prolonged periods (Bainbridge et al. 2012, Fabricius et al. 2016). Increased levels of nutrients and sediments are not just problematic for GBR reefs, but for coral reefs around the world (Haas et al. 2016, Pawlik et al. 2016).

Terrestrial runoff contains dissolved and particulate nutrients that may be inorganic or organic. Each nutrient type has different lifetimes and impacts on coral reef ecosystems (Fabricius 2005). In particular, dissolved inorganic nitrogen (DIN; ammonia, nitrite, and nitrate) forms a large component of the nitrogen pollution in the GBR lagoon (Brodie et al. 2012). However, DIN is rapidly taken up by phytoplankton, leading to phytoplankton blooms (Furnas et al. 2005, Bainbridge et al. 2012) which potentially contribute to outbreaks of crown of thorns starfish (Brodie et al. 2017, Pratchett et al. 2017). While coral reefs do occur in nutrient-rich habitats, the benthic community differs from that found in oligotrophic locations, including higher macroalgal cover and richness of heterotrophic taxa (De'ath & Fabricius 2010). Overall, DIN enrichment contributes to poor coral health (Wiedenmann et al. 2013), however, the effects of DIN on other reef taxa are less well known.

Sponges are highly efficient filter feeders and may benefit from DIN enrichment via increased dissolved or particulate carbon food sources (Bainbridge et al. 2012), or from enhanced nitrification activity by sponge-associated microbes (Southwell et al. 2008, Fiore et al. 2010, Fiore, Baker, & Lesser 2013b). Increased food availability is thought to be a primary driver of the high abundance of heterotrophic sponges on coastal reefs (Wilkinson & Cheshire 1989) and the greater abundance of heterotrophic sponges in the Caribbean relative to the Pacific (Pawlik et al. 2016). The abundance of bioeroding sponges in particular, is strongly related to nutrient gradients (Rose & Risk 1985, Holmes 2000, Ward-Paige et al. 2005, Chaves-Fonnegra & Zea 2007, Nava et al. 2014). However, while enhanced heterotrophy or microbial metabolism could lead to accelerated growth or greater energy reserves if the products of microbial metabolism are translocated to the sponge host, studies to date suggest that nutrient enrichment does not directly benefit sponge growth (Roberts et al. 2006, Gochfeld et al. 2012, Easson et al. 2014), protein content (Gochfeld et al. 2012, but see Easson et al. 2014), or alter the microbial community composition (Simister, Taylor, Tsai, & Webster 2012, Luter et al. 2014).

In resource exchange mutualisms, nutrient enrichment can remove nutrient limitation (e.g., nitrogen or phosphorus) of the phototroph to the detriment of the heterotroph (Kiers et al. 2010, Shantz & Burkepile 2014, Shantz et al. 2016). DIN enrichment may have adverse consequences for phototrophic sponges if the symbiosis is destabilised by increasing symbiont density. For instance, one study suggested that DIN enrichment upset the symbiosis between *Cyanobacteria* and the sponge *Aplysina cauliformis*, as chlorophyll levels were found to increase while sponge protein levels decreased (Easson et al. 2014). However, other studies have suggested that chlorophyll levels in sponges hosting *Cyanobacteria* are unaffected by DIN enrichment (Roberts et al. 2006, Gochfeld et al. 2012). Thus, whether DIN enrichment destabilises the

symbiosis between sponges and *Cyanobacteria* is unclear and the effects of elevated DIN on sponges hosting *Symbiodinium* have not been investigated.

Terrestrial runoff also contains sediments and particulate matter (Fabricius et al. 2016). At river mouths, large particles remain suspended in the water column thereby limiting the light reaching the benthos (Bainbridge et al. 2012). However, large particles settle out near the river mouth while flocs of small particles and nutrients travel further, triggering phytoplankton blooms, and limit irradiance on reefs farther from shore (Bainbridge et al. 2012). Reductions in irradiance can slow the growth of sponges with photosynthetic symbionts (Thacker 2005, Roberts et al. 2006, Erwin & Thacker 2008, Freeman & Thacker 2011) and can also reduce chlorophyll levels in some species (Pineda et al. 2016). The growth of bioeroding sponges, in particular, appears to be negatively affected by decreased irradiance (Hill 1996, Cebrian & Uriz 2006, Schönberg 2006, Pineda et al. 2016), likely due to their association with *Symbiodinium* (Weisz et al. 2010, Hill et al. 2011). Since reduced irradiance can cooccur with nutrient enrichment, it is difficult to discern independent effects of irradiance and nutrients during flood events. Moreover, the effects of light and nutrients may vary between sponge species due to their relative dependence on autotrophic production and heterotrophic feeding or their ability to switch between nutritional modes (Anthony & Fabricius 2000, Grottoli et al. 2006, Freeman et al. 2015).

DIN enrichment is thought to benefit sponges indirectly, as the associated increase in dissolved or particulate C which results from N addition, increases the food available to sponges. In contrast, our understanding of how DIN directly affects sponges is limited and may vary between sponges with and without photosynthetic symbionts. To address this, we exposed heterotrophic and phototrophic sponge species to concentrations of dissolved nutrients

simulating flood plume conditions under two light levels and measured effects on sponge growth and energy reserves.

5.3. Methods

5.3.1. Sponge collection and acclimation

In April 2017, whole sponges (*Carteriospongia foliascens*, *Cliona orientalis*, *Cymbastella coralliophila*, *Ircinia ramosa*, and *Stylissa flabelliformis*) were collected using SCUBA between 1 and 9 m (Table 5-1). All species are known to associate with diverse populations of microbial symbionts with *C. foliascens, C. coralliophila*, and *I. ramosa* hosting *Cyanobacteria*; *C. orientalis* hosting *Symbiodinium*; and S. *flabelliformis* lacking photosymbionts (Wilkinson 1982, Pineda et al. 2016). All sponges were transported to the Australian Institute of Marine Science (Townsville, Queensland) and acclimated in outdoor aquaria at 27 °C. After 10 days, each sponge was cut into smaller explants (n = 4-11 dependent on the size of the donor sponge; Table 5-1) to provide sufficient experimental replication. The bioeroding *C. orientalis* and its underlying calcium carbonate substratum was cut into rectangular explants in order to measure growth: the sponge occupied one side of the explant and the top surface was clean substratum.

All explants were labelled according to the donor sponge, allowed to heal for six weeks, and then allocated into 36 experimental aquaria (50 L). Explants from the same sponge were allocated into different treatments; most sponges were represented by one explant per treatment, but some sponges were represented by multiple explants per treatment, and a few sponges did not have enough explants for all treatments. As a result, each aquarium contained explants from 1-3 sponges of each species and 8-11 total explants (Table 5-2). Tank temperature was maintained at 26.9±0.1 (SD) °C using a computer-controlled SCADA system. Sponges were fed daily with a 1.5

x10^6 cells L^{-1} final concentration of cultured microalgae (*Isochyrisis galbana*, *Nanochloropsis oceanica*, *Pavlova lutheri, Dunaliella sp.*) and a diatom (*Chaetocerous muelleri*) ranging from 3 to 10 μm in diameter.

Table 5-1. Sampling location and counts of five sponge species at Orpheus Island (18° 35' 27" S, 146° 28' 47" E) and Rib Reef (18° 28' 54" S, 146° 52' 15" E) on the Great Barrier Reef. Each sponge was cut into explants for use in the experiment.

Species	Photosymbiont	Location	Depth (m)	Sponges (n)	Explants (n)
Carteriospongia foliascens	*Cyanobacteria*	Orpheus Island	1	12	68
Cliona orientalis	*Symbiodinium*	Orpheus Island	1	12	81
Cymbastella coralliophila	*Cyanobacteria*	Orpheus Island	9	12	72
Ircinia ramosa	*Cyanobacteria*	Rib Reef	9	12	70
Stylissa flabelliformis	NA	Orpheus Island	9	10	61

Table 5-2. Sponges per tank and treatment for *Carteriospongia foliascens* (*C. fol.*), *Cliona orientalis* (*C. ori.*), *Cymbastella coralliophila* (*C. cor.*), *Ircinia ramosa* (*I. ram.*), *Stylissa flabelliformis* (*S. fla.*). Total indicates the sum of sponges per species for each treatment.

Nutrients	Light	Tank	*C. fol.*	*C. ori.*	*C. cor.*	*I. ram.*	*S. fla.*
Control	80	Total	12	16	13	12	10
		5	2	3	3	2	1
		6	2	3	2	2	1
		17	2	4	2	2	2
		23	2	2	2	2	2
		27	2	2	2	2	2
		36	2	2	2	2	2
	160	Total	12	14	12	12	10
		2	3	2	2	2	2
		8	1	3	2	2	2
		14	2	2	2	2	2
		25	2	2	2	2	1
		30	2	2	2	2	2
		32	2	3	2	2	1
Medium	80	Total	12	14	12	12	10
		4	2	3	2	2	2
		9	2	3	2	2	1
		11	2	2	2	2	2
		28	2	2	2	2	2
		29	2	2	2	2	1
		31	2	2	2	2	2
	160	Total	12	12	11	12	9
		10	2	2	1	2	1
		18	2	2	2	2	1
		19	2	2	2	2	2
		21	2	2	2	2	2
		33	2	2	2	2	2
		34	2	2	2	2	1
High	80	Total	12	13	13	12	9
		3	2	2	3	2	1
		7	2	2	2	2	2
		13	2	2	2	2	2
		15	2	3	2	2	1
		16	2	2	2	2	1
		20	2	2	2	2	2
	160	Total	12	14	11	11	10
		1	2	2	1	2	2
		12	2	2	2	2	2
		22	2	4	2	2	1
		24	2	2	2	1	1
		26	2	2	2	2	2
		35	2	2	2	2	2

5.3.2. Nutrient and light treatments

Nutrient treatments were designed to enrich seawater DIN to concentrations experienced

on inshore reefs during flood events, from 1 to 10 μM (Devlin et al. 2011). Two dissolved nutrient treatments were established, representing medium and high levels of nutrient enrichment, while a third treatment contained no nutrient amendment (control). Additions of 14.9 or 29.8 g of soluble fertilizer (Yates Thrive; New South Wales, AUS) were added to 60 L reservoirs of filtered seawater (0.04 μM) to achieve the medium and high treatment levels, respectively. Doses were pumped from the reservoirs at 0.01 L/min into filtered seawater entering the experimental aquaria at 0.8 L/min. Nutrient reservoirs were depleted every 3-4 days so were replaced twice weekly.

To distinguish effects of nutrient enrichment from effects of irradiance reduction, the three nutrient treatments were fully-crossed with two light conditions (80 and 160 μmol quanta m^{-2} s^{-1}), resulting in six treatments that were each replicated in six aquaria. Light was provided by Aquaillumination Sol LED lamps (C2 Development; Iowa, USA). Sponges were exposed to treatment conditions for 10 weeks.

5.3.3. Nutrient sampling

Dissolved and particulate nutrients were sampled weekly in both the dosing reservoirs and in the aquaria. Due to pump failure on several occasions, dosing reservoirs were empty on the day of sampling and water samples from these time points have thus been omitted from the analysis, leaving 9 measurements for the control and medium treatments and 5 measurements for the high treatment. Samples for particulate organic C (POC) and particulate N (PN) were filtered onto pre-combusted Whatman glass fibre filters (250 mL), acidified using hydrochloric acid, and analysed on a Shimadzu TOC-V analyser with a Total Nitrogen unit. Particulate values were compared to marine sediment standards. For dissolved nutrients, seawater samples (10 mL) were

taken from each dosing reservoir and aquarium and filtered using 0.45 µm Sartorius Minisart Cellulose Acetate filters (Göttingen, Germany). Samples for dissolved organic C (DOC) and dissolved N (DN) were acidified with hydrochloric acid and measured on a Shimadzu TOC-L analyser. Duplicate samples for dissolved inorganic nitrogen (NH_3, NO_{2+3}) and phosphate (PO_4) were measured on a Seal AA3 segmented flow analyser and referenced against OSIL standards and in-house reference samples. Samples for POC, PN, DIN, and PO_4 were kept frozen at -20°C until measurement while samples for DOC and DN were kept at 4°C.

5.3.4. Sponge growth

To determine the effect of nutrients on growth, sponge volume (to the nearest ±0.5 mL) and surface area (to the nearest ±0.1 mm^2) were measured at the beginning and end of the experiment (10 week interval). Sponge volume was assessed using a standard water displacement technique (Wilkinson & Vacelet 1979) for all species except the bioeroding *C. orientalis*. Sponge growth was estimated as the difference in sponge volume over the course of the experiment as a percentage of the initial volume. Sponges were briefly exposed to air during volume measurement. Due to its encrusting morphology, growth of *C. orientalis* was measured using change in surface area over the course of the experiment. Area was calculated from photographs of the top surface in ImageJ software (Abramoff et al. 2014). *C. orientalis* growth was calculated as the change in surface area relative to the initial area. For all species, sample sizes were ≥9 in each treatment (Table 5-3).

Table 5-3. Sample sizes for all sponge species and treatments. Sponges indicates the number of individual sponges used to create the explants. The counts for each treatment indicate the number of sponge explants.

		80 µmol quanta m^{-2} s^{-1}			160 µmol quanta m^{-2} s^{-1}		
	Sponges (n)	**Control**	**Medium**	**High**	**Control**	**Medium**	**High**
Growth rate							
C. coralliophilia	12	11	11	13	12	10	10
C. foliascens	12	11	11	12	10	11	11
C. orientalis	12	15	13	13	12	9	12
I. ramosa	12	11	12	12	12	12	11
S. flabelliformis	10	10	10	9	10	9	10
Organic matter							
C. coralliophilia	12	12	13	12	12	11	12
C. foliascens	12	11	12	11	10	11	10
C. orientalis	12	14	14	13	13	13	13
I. ramosa	12	12	11	12	12	12	11
S. flabelliformis	10	10	10	9	10	9	10
Chlorophyll *a*							
C. coralliophilia	12	12	13	12	12	12	12
C. foliascens	12	10	12	11	11	11	12
C. orientalis	12	13	14	13	15	12	13
I. ramosa	12	12	11	12	12	12	11

5.3.5. Sponge organic matter and chlorophyll

Sponge organic matter was used as a proxy for sponge condition. Organic matter was measured at the end of the experiment by freeze-drying the sponge tissue, weighing the dried tissue, burning it at 450°C for 3 h, and weighing the remaining ash. Organic matter was calculated as the difference between the dry weight and ash weight as a proportion of the dry weight. For all species, sample sizes were ≥9 in each treatment (Table 5-3).

Chlorophyll was used as a proxy for determining whether treatment affected photosymbiosis. At the end of the experiment, chlorophyll was extracted from frozen sponge tissue following homogenization in a bead beater with 1 mL of 95% ethanol. Pigments were

extracted twice from each sample, both extracts were pooled, and absorbance was recorded at 630, 645, 660, and 750 nm using a Powerwave microplate reader (BIO-TEK Instruments Inc., Vermont USA). For all species, sample sizes were ≥10 in each treatment (Table 5-3).

5.3.6. Statistical analyses

Nutrient levels within dosing reservoirs were analysed using a linear mixed model with nutrient dose and time as predictors. Nutrient levels within sponge aquaria were analysed using linear mixed models with nutrient dose, light treatment, time, as well as nutrient:time and nutrient:light interactions as predictors and aquarium as a random effect to account for autocorrelations between measurements taken on the same tank. Where significant differences were detected among nutrient treatments or nutrient:light combinations, all pairs of treatments were compared using linear contrasts and P-values were corrected using a single step correction.

Sponge responses were analysed using linear mixed models with nutrient treatment, light treatment, and a nutrient:light interaction as predictors. Tank and sponge were included as random effects to account for correlations within aquaria and among measurements on explants from the same donor genotype. Where significant differences were detected among nutrient treatments or nutrient:light combinations, all pairs of treatment levels were compared using linear contrasts and P-values were corrected using single-step correction. All models were verified to meet the assumptions of normality of residuals and heteroscedascity using histograms of the residuals and plots of fitted versus residual values.

5.4. Results

5.4.1. Nutrient and light conditions

The medium and high dosing reservoirs contained higher dissolved organic carbon

(DOC), dissolved nitrogen (DN), and inorganic nutrients (total DIN, NH_4, NO_{2+3}, PO_4) compared to the control reservoir (Table 5-4, Table 5-5, Table 5-8). However, only the medium nutrient reservoir had an altered ratio of DIN:PO_4, with reduced DIN:PO_4 compared to the control reservoir (Table 5-4, Table 5-5, Table 5-8). Concentrations of particulate carbon and nitrogen were consistent across all dosing reservoirs (Table 5-4, Table 5-5, Table 5-8).

Table 5-4. Particulate and dissolved nutrient levels within the dosing reservoirs. The table lists mean and standard deviation for each reservoir.

		Control		Medium		High	
	n	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Particulate (µg/L)							
Organic C	5	258.6	126.6	589.8	796.8	379.9	236.4
N	5	61.9	22.0	173.2	160.0	140.6	64.4
Dissolved (mg/L)							
Organic C	3	1.1	0.1	30.4	1.3	58.1	2.7
N	3	0.1	<0.1	65.8	2.7	128.1	4.4
Dissolved inorg. (µmol)							
Total N	5	1.1	1.0	905.6	276.2	2098.4	95.1
NH_4	5	0.4	0.2	388.8	113.4	950.0	48.5
NO_x	5	0.7	0.8	516.8	166.1	1148.4	57.1
PO_4	5	0.1	0.1	317.7	91.7	681.6	41.4
Total N : PO_4	5	16.0	8.9	2.8	0.8	3.1	0.1

Table 5-5. Results of linear models analysing nutrient levels within dosing reservoirs. The model included nutrient treatments and time. Degrees of freedom (num., denom.), F statistic, and P-values are reported for each measured nutrient. P-values less than 0.05 are indicated in bold.

	Linear model						**Post-hoc tests**		
	Nutrients			**Time**			**C vs M**	**M vs H**	**C vs H**
	df	**F**	**P**	**df**	**F**	**P**	**P**	**P**	**P**
Particulate									
Organic C	2,6	1.3	0.33	4, 6	9.5	**0.01**	-	-	-
N	2,8	1.6	0.24	4, 8	1.4	0.31	-	-	-
Dissolved									
Organic C	2,4	1464.5	**<0.01**	2, 4	3.4	0.14	**<0.01**	**<0.01**	**<0.01**
N	2,4	2455.0	**<0.01**	2, 4	3.2	0.14	**<0.01**	**<0.01**	**<0.01**
Dissolved inorg.									
Total DIN	2,14	464.7	**<0.01**	8, 14	1.4	0.26	**<0.01**	**<0.01**	**<0.01**
NH_4	2,14	335.0	**<0.01**	8, 14	1.4	0.29	**<0.01**	**<0.01**	**<0.01**
NO_x	2,14	432.7	**<0.01**	8, 14	1.6	0.21	**<0.01**	**<0.01**	**<0.01**
PO_4	2,14	118.9	**<0.01**	8, 14	1.0	0.50	**<0.01**	**<0.01**	**<0.01**
Total DIN : PO_4	2,14	432.7	**<0.01**	8, 14	1.6	0.21	**0.02**	0.91	0.08

In the treatment aquaria, DN, DIN, and PO_4 increased (but not DOC) relative to control

aquaria (Figure 5-1, Table 5-6, Table 5-7). Mean total DIN in the sponge aquaria was 1.45, 3.3, and 5.9 μM for the control, medium, and high treatments, respectively. Aquaria in the medium treatment contained significantly higher concentrations of all DIN compounds and PO_4 compared to control aquaria, but aquaria in the high treatment were not significantly different from controls in NO_{2+3} (Figure 5-1, Table 5-7). The ratio of DIN:PO_4 decreased in treatment aquaria compared to control aquaria (Figure 5-1D). Importantly, the concentration of all nutrients varied over time and differences between treatments varied over the course of the experiment, particularly for DIN compounds (Figure 5-1, Table 5-7).

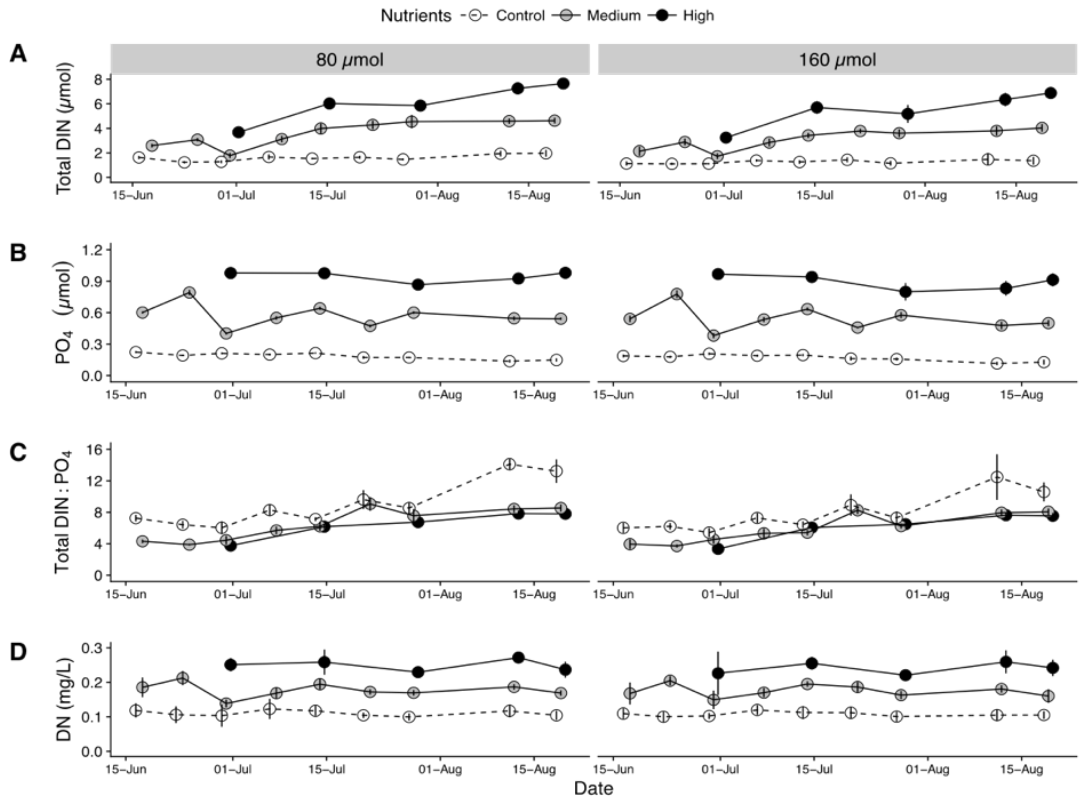

Figure 5-1. Nutrient levels for **(A)** total dissolved inorganic nitrogen (DIN; NH_4+NO_3+NO_2), **(B)** phosphate (PO_4), **(C)** ratio of DIN:PO4, and **(D)** total dissolved nitrogen (DN). Circles represent control aquaria (white), medium dose aquaria (grey), and high dose aquaria (black). Left and right panels separate the 80 and 160 μmol quanta m^{-2} s^{-1}, respectively. Error bars represent ±SD.

Irradiance levels in the low and high light treatments were 79.5 (±5.4 SD) and 157.8

(±13.2 SD) µmol quanta m^{-2} s^{-1}, respectively. The irradiance treatment had small, but significant, effects on nutrient levels within the sponge aquaria (Table 5-6, Table 5-7). The concentration of DN was slightly higher in the higher irradiance treatment, whereas most DIN compounds were slightly lower (Figure 5-1, Table 5-7). The concentration of NH_4 depended on both the irradiance and nutrient treatments: in aquaria from the control and medium treatments, NH_4 was similar between irradiance treatments (Table 5-7; control: z=-0.8, P=0.97; medium: z=-0.6, P=0.99) whereas in aquaria from the high treatment, NH_4 was 33% higher at 80 versus 160 µmol quanta m^{-2} s^{-1} (z=-6.0, P≤0.01).

Table 5-6. Summary of particulate and dissolved nutrients within the experimental aquaria. Table includes weekly means and SD.

		Nutrient treatment						
		Control			Medium		High	
	Irradiance	n	Mean	SD	Mean	SD	Mean	SD
Particulate (µg/L)								
Organic C	80	3	30.4	8.2	39.0	3.0	31.3	2.5
	160	3	27.5	7.0	36.6	4.5	29.0	3.2
N	80	3	9.3	2.0	10.1	0.8	8.2	1.2
	160	3	8.1	2.1	9.6	1.9	6.6	0.4
Dissolved (mg/L)								
Organic C	80	6	1.1	0.1	1.1	0.1	1.3	0.1
	160	6	1.1	0.1	1.1	0.1	1.2	0.1
N	80	6	0.1	<0.1	0.2	<0.1	0.3	<0.1
	160	6	0.1	<0.1	0.2	<0.1	0.2	<0.1
Dissolved inorg. (µmol)								
Total N	80	6	1.6	0.1	3.6	0.2	6.1	0.2
	160	6	1.3	0.1	3.1	0.1	5.6	0.6
NH_4	80	6	0.3	<0.1	0.6	0.1	1.2	0.1
	160	6	0.2	<0.1	0.6	0.1	0.9	0.1
NO_{2+3}	80	6	1.3	0.1	3.0	0.2	4.9	0.2
	160	6	1.0	0.1	2.6	0.1	4.6	0.3
PO_4	80	6	0.2	<0.1	0.6	<0.1	1.0	<0.1
	160	6	0.2	<0.1	0.5	<0.1	0.9	0.1
Total N : PO_4	80	6	9.0	0.2	6.5	0.4	6.5	0.2
	160	6	7.8	0.6	5.9	0.2	6.3	0.5

Table 5-7. Results of linear mixed models analysing nutrient levels within the experimental aquaria. The model included nutrient treatments, light treatments, time, a nutrient*light interaction term, nutrient*time interaction term, and a random intercept for each tank. Degrees of freedom (num., denom.), F statistic, and P-values are reported for each measured nutrient. P-values ≤0.05 are indicated in bold.

	Nutrients			**Light**			**Time**			**Nut. * Light**			**Nut. * Time**		
	df	**F**	**P**	**df**	**F**	**P**	**df**	**F**	**P**	**df**	**F**	**P**	**df**	**F**	**P**
Particulate															
Organic C	2,12	1.0	0.39	1,12	<0.1	0.84	3,45	2.6	0.06	2,12	0.2	0.81	6,45	0.3	0.94
N	2,12	2.1	0.17	1,12	1.7	0.21	2,30	1.8	0.19	2,12	0.1	0.88	4,30	0.3	0.86
Dissolved															
Organic C	2,30	0.5	0.64	1,30	0.4	0.53	8,259	66.3	**<0.01**	2,30	0.3	0.77	16,259	0.6	0.91
N	2,30	77.2	**<0.01**	1,30	0.5	0.48	1,233	0.2	0.62	2,30	0.5	0.60	2,233	5.2	**0.01**
Dissolved inorg.															
Total DIN	2,30	18.8	**<0.01**	1,30	10.5	**<0.01**	1,235	7.7	**0.01**	2,30	1.5	0.24	2,235	122.1	**<0.01**
NH_4	2,30	71.8	**<0.01**	1,30	1.3	0.27	1,235	6.5	**0.01**	2,30	17.2	**<0.01**	2,235	2.0	0.14
NO_{2+3}	2,30	5.3	**0.01**	1,30	10.6	**<0.01**	1,237	16.0	**<0.01**	2,30	1.1	0.35	2,237	146.1	**<0.01**
PO_4	2,30	297.8	**<0.01**	1,30	1.4	0.24	1,237	12.0	**<0.01**	2,30	1.3	0.28	2,237	0.2	0.79
Total DIN : PO_4	2,30	14.3	**<0.01**	1,30	22.0	**<0.01**	1,235	288.5	**<0.01**	2,30	2.9	0.07	2,235	3.2	**0.04**

Table 5-8. Post-hoc results from linear contrasts of nutrient doses. P-values were corrected using single-step correction. P-values less than 0.05 are indicated in bold.

	Control vs. Med.		Med. vs. High		Control vs. High	
	Z	**P**	**Z**	**P**	**Z**	**P**
Particulate						
Organic C	-	-	-	-	-	-
N	-	-	-	-	-	-
Dissolved						
Organic C	-	-	-	-	-	-
N	6.9	**<0.01**	-6.3	**<0.01**	12.3	**<0.01**
Dissolved inorg.						
Total N	5.2	**<0.01**	1.3	0.37	4.9	**<0.01**
NH_4	-	-	-	-	-	-
NO_{2+3}	3.2	**<0.01**	-1.1	0.48	1.1	0.53
PO_4	16.7	**<0.01**	11.1	**<0.01**	22.4	**<0.01**

5.4.2. Sponge growth

None of the sponge species exhibited significantly different growth rates in response to the nutrient treatments, light treatments, or any combination of nutrients and light (Figure 2-2A, Table 5-9). Over the course of the 10 week experiment, *C. coralliophila* increased in volume (11.5%±6.1 SD), while *I. ramosa* (-6.4±7.2%), *C. foliascens* (-25.9±10.9%), and *S. flabelliformis* (-45.0±10.7%) decreased in volume. For *C. coralliophila*, this corresponded to a growth rate of 4.9% volume mo^{-1}. Surface area growth of the encrusting sponge *C. orientalis* was similar amongst all treatments (Table 5-9), with an average increase of 11.1±6.7%, or 4.7% area mo^{-1}.

5.4.3. Sponge organic matter and chlorophyll

No sponge species exhibited significantly different organic content (i.e. condition) in response to the nutrient treatments or combinations of nutrients and light (Figure 5-2B, Table 5-9). However, organic content in *C. orientalis* was significantly affected by irradiance, with 7% more organic content at 160 versus 80 μmol m^{-2} s^{-1} (Table 5-9). *I. ramosa* had the highest organic content (73.8±7.8% SD), followed by *S. flabelliformis* (56.5±5.5%), *C. coralliophila* (41.2±7.9%), *C.*

foliascens (39.4±4.9%), and *C. orientalis* (5.1±0.1%; Figure 5-2B).

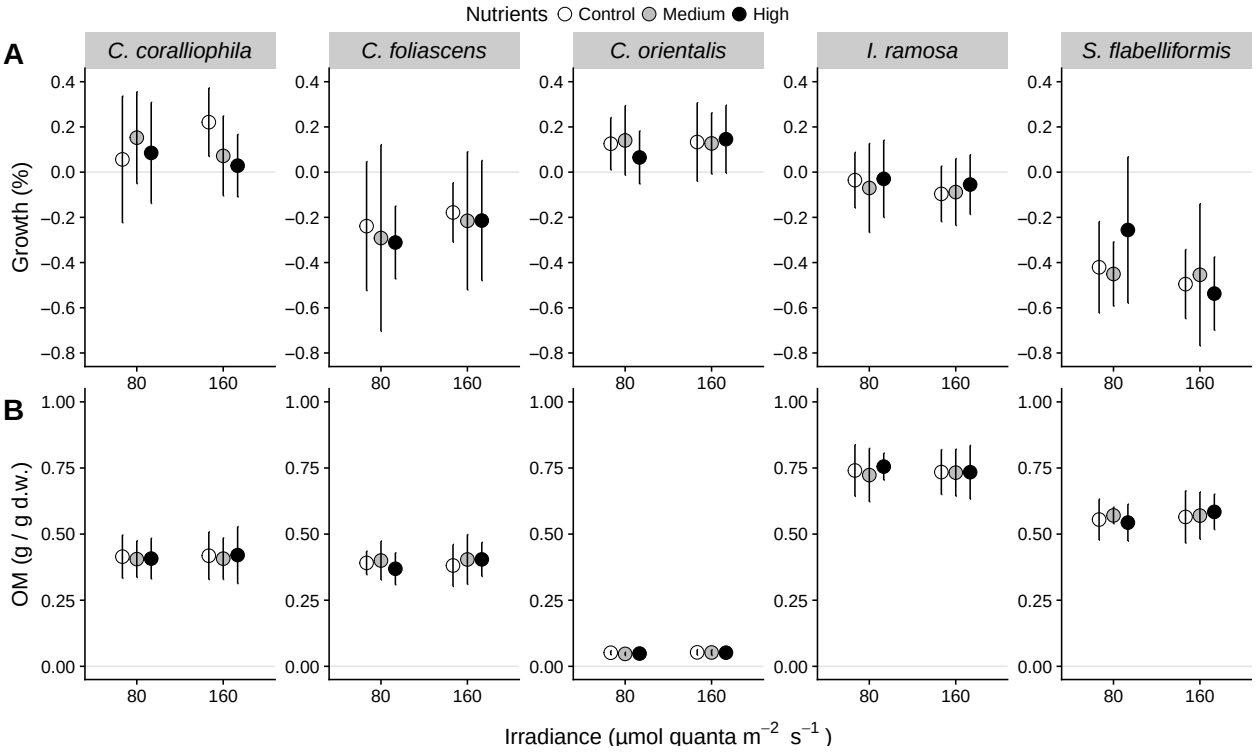

Figure 5-2. **(A)** Sponge growth (% increase) and **(B)** organic matter (OM; % dry weight) under nutrient and light treatments. Circles represent sponges in control treatments (white), medium treatments (grey), and high treatments (black). Light treatments are indicated on the x-axis. Error bars indicate ±SD. Growth of *C. orientalis* is calculated via change in surface area while the growth of all other species is calculated via change in volume.

Sponge chlorophyll content did not differ between nutrient treatments or combinations of nutrient and light for any sponge species (Figure 3, Table 5-9). However, chlorophyll levels in *C. orientalis* were significantly different between light treatments, with 35% more chlorophyll at 160 versus 80 µmol quanta m^{-2} s^{-1} (Figure 3). *I. ramosa* had the highest chlorophyll µg g^{-1} wet weight (99.0±34.3 SD), followed by *C. coralliophila* (82.1±23.2), *C. foliascens* (68.8±20.7), and *C. orientalis* (57.2±10.7; Figure 3).

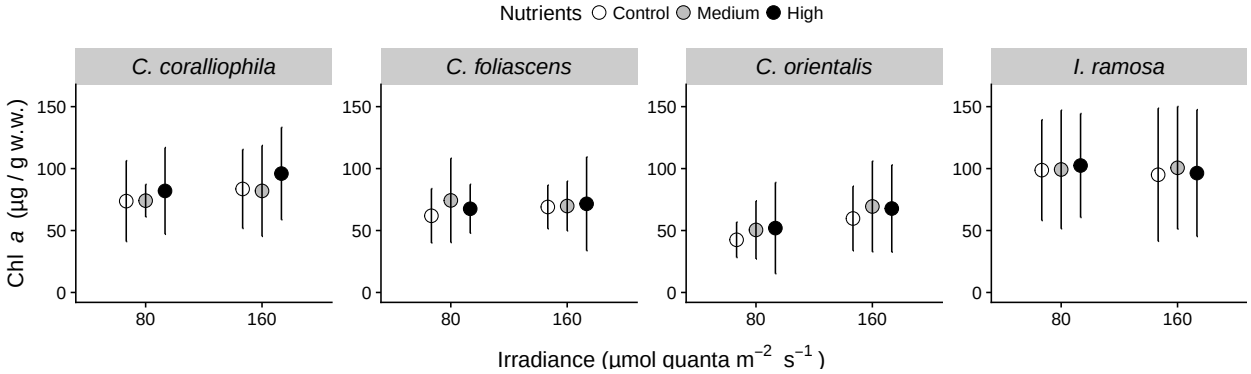

Figure 5-3. Sponge chlorophyll *a* content. Circles represent sponges from control treatments (white), medium treatments (grey), and high treatments (black). Light treatments are denoted on the x-axis. Error bars indicate ±SD.

Table 5-9. Results of linear mixed models for sponge growth, organic matter, and chlorophyll *a* content for each sponge species. The model included nutrient treatments, light treatments, time, a nutrient*light interaction term, and a random intercept for each sponge. Degrees of freedom (num., denom.), F statistic, and P-values are reported for each measured nutrient. P-values less than 0.05 are indicated in bold.

	Nutrients			**Light**			**Nutrients*Light**		
	df	**F**	**P**	**df**	**F**	**P**	**df**	**F**	**P**
Growth rate									
C. coralliophila	2, 61.0	2.3	0.11	1, 61.0	<0.1	0.77	2, 61.0	1.6	0.21
C. foliascens	2, 30.3	0.8	0.47	1, 30.3	3.2	0.08	2, 30.3	0.4	0.70
C. orientalis	2, 59.9	0.3	0.73	1, 61.0	<0.1	0.92	2, 59.4	1.0	0.37
I. ramosa	2, 28.0	0.3	0.74	1, 28.0	0.8	0.37	2, 28.0	0.1	0.89
S. flabelliformis	2, 28.0	0.3	0.74	1, 28.0	0.8	0.37	2, 28.0	0.1	0.89
Organic matter									
C. coralliophila	2, 23.8	0.4	0.66	1, 26.8	1.9	0.18	2, 26.3	0.3	0.73
C. foliascens	2, 21.7	0.6	0.56	1, 21.7	0.7	0.40	2, 22.0	0.7	0.48
C. orientalis	2, 63.1	2.0	0.14	1, 63.1	5.0	**0.03**	2, 63.1	0.3	0.76
I. ramosa	2, 22.4	0.8	0.48	2, 22.5	0.3	0.59	2, 22.4	0.4	0.68
S. flabelliformis	2, 9.8	0.2	0.81	1, 9.9	0.9	0.36	2, 9.9	0.6	0.55
Chlorophyll *a*									
C. coralliophila	2, 25.9	1.2	0.31	1, 27.2	2.8	0.10	2, 28.9	0.1	0.91
C. foliascens	2, 24.6	0.6	0.57	1, 24.4	0.1	0.75	2, 24.5	0.5	0.62
C. orientalis	2, 28.2	0.4	0.65	1, 28.2	4.8	**0.04**	2, 28.2	<0.1	0.99
I. ramosa	2, 53.3	0.1	0.94	1, 53.3	0.1	0.73	2, 53.2	<0.1	0.95

5.5. Discussion

Nutrient enrichment is generally thought to benefit filter feeders and bioeroders (Birkeland 1988, Glynn 1997), with positive correlations between nutrient levels and abundance and/or bioerosion reported for some species (Rose & Risk 1985, Sammarco & Risk 1990, Risk et al. 1995, Ward-Paige et al. 2005, Fabricius & De'ath 2008). Importantly, direct experimental evidence for the advantages provided by nutrient enrichment is lacking. Dissolved inorganic nitrogen (DIN) is an important component of runoff from agricultural or urban areas (Brodie et al. 2012) and previous research has shown that sponges exhibit either no response (Roberts et al. 2006, Gochfeld et al. 2012) or decreased growth / condition (Koopmans & Wijffels 2008, Easson et al. 2014) following DIN enrichment. In this study, addition of DIN at levels experienced on the inshore GBR during flood plume events (Devlin & Schaffelke 2009, Devlin et al. 2011), caused no adverse effects on the health of 5 sponge species but neither did it accelerate growth or improve sponge condition. These findings were consistent across phototrophic and heterotrophic species.

Sponges are fundamental to nutrient cycling on coral reefs due to their efficient filtration of seawater, including the consumption of dissolved and particulate organic matter (DOM and POM) and potentially the production of particulate organic matter (POM) (Richter et al. 2001, de Goeij et al. 2013, McMurray et al. 2018, Rix et al. 2018). Additional inorganic nutrients can increase the dissolved or particulate organic carbon (DOC or POC) pool in seawater, thereby increasing the potential food available for sponges (Maldonado et al. 2012). Numerous studies have demonstrated that sponges consume DOC and/or POC (reviewed in Maldonado et al. 2012, de Goeij et al. 2017) and POC levels have been shown to positively correlate with sponge growth in both the laboratory and in the field (Duckworth & Pomponi 2005, Koopmans & Wijffels 2008). However, in this study nutrient amendment did not result in increased sponge growth or

improved condition, and neither did DOC addition, although DOC was only significantly enriched in the nutrient reservoirs and not in the experimental aquaria. This distinction suggests that DOC was rapidly consumed by the sponges or plankton, although either scenario could have theoretically benefited the sponges by providing increased scope for growth. The scope for sponge growth in this study was limited by duration of the experiment and a longer experiment may have led to larger effects of nutrient addition.

In resource exchange mutualisms, nutrient amendment can upset the nutritional exchange between heterotrophs and autotrophs (Shantz & Burkepile 2014, Shantz et al. 2016). More specifically, nutrient enrichment can remove the resource limitation of the phototrophic partner, who no longer requires the heterotroph to supply nutrients (Shantz et al. 2016). In this way, DIN enrichment leads to increased *Symbiodinium* density in corals which then increases the thermal instability of the symbiotic partnership (Wooldridge 2014). In this study, inorganic nutrient enrichment did not affect the density of chlorophyll *a*, a proxy for photosymbiont density, which is consistent with two previous reports (Roberts et al. 2006, Gochfeld et al. 2012), although nutrient enrichment has been reported to increase chlorophyll and decrease protein in *A. cauliformis* (Easson et al. 2014). Notably, where *Cyanobacteria* density has been measured directly, no effect of DIN enrichment was observed (Gochfeld et al. 2012, Easson et al. 2014), which supports that sponge symbioses are stable under nutrient enrichment.

As nitrogen enrichment can increase microbial growth, nutrient balance can be as important to symbiosis as total nutrient load (Wiedenmann et al. 2013). In corals, high DIN:P ratios can have detrimental effects, with phosphorous limitation increasing the thermal sensitivity of the symbiotic partnership (Ezzat et al. 2016). In the current study, DIN:P was reduced,

potentially mitigating any negative effects on the sponge symbioses. However, it is notable that no change in symbiont density (*sensu* chlorophyll *a*) occurred at elevated DIN, suggesting that the sponge photosymbiont populations are unaffected by nutrient amendment.

During a flood event, DIN enrichment may coincide with reduced irradiance due to particulate material, suspended fine particles, or nutrient-induced phytoplankton blooms reducing the incident light reaching the benthos (Bainbridge et al. 2012, Schaffelke et al. 2012). For all five sponge species, irradiance did not significantly affect sponge growth, although higher irradiance increased organic content and chlorophyll levels in *C. orientalis*, suggesting that the condition of this species is tightly coupled to the performance of its photosynthetic symbiont, *Symbiodinium* (Hill 1996, Schönberg 2006, Fang et al. 2014, Achlatis et al. 2017). This finding supports field experiments where bioeroding sponges grew and eroded faster under higher irradiance (Hill 1996, Schönberg 2006), fuelled by increased carbon translocation from the *Symbiodinium* (Weisz et al. 2010). Irradiance appears to play a role in the success of *C. orientalis* on the inshore GBR, as *C. orientalis* cover has increased at locations with relatively low turbidity (low chlorophyll *a*) and intermediate DIN levels (Ramsby, Hoogenboom, et al. 2017). Whilst most studies indicate that increased irradiance accelerates the growth of phototrophic sponges (Hill 1996, Thacker 2005, Roberts et al. 2006, Schönberg 2006, Freeman & Thacker 2011), some species are not affected (this study; Erwin & Thacker 2008), suggesting that sponge photosymbioses have species-specific responses to irradiance.

Despite the potential for inorganic and organic nutrients to increase the scope for sponge growth, nutrient (DIN+P) and organic (DOC) enrichment had no effect on GBR sponges and their photosymbionts. The DIN exposure in this study was a similar magnitude as previous

studies (Simister, Taylor, Tsai, & Webster 2012, Gochfeld et al. 2012), but the sponges were exposed for more than twice as long in this study. Thus, if sponges are to benefit from coastal eutrophication, it is likely to be via particulate material rather than inorganic nutrient enrichment.

Chapter 6. Discussion

6.1. Summary of findings

Sponges are important members of marine communities, encompassing large amounts of biomass, filtering large volumes of water, and processing and recycling nutrients (Bell 2008). In particular, bioeroding sponges break down calcium carbonate substrata, thereby contributing to reef erosion and reducing reef accretion (Schönberg, Fang, & Carballo 2017). The performance of bioeroding sponges under future ocean conditions will directly affect scleractinian corals through competition (Stubler et al. 2014, Enochs et al. 2015, Chaves-Fonnegra et al. 2018), reef accretion (Murphy et al. 2016), and reef nutrient cycling (Mueller et al. 2014). This thesis includes experiments designed to test whether bioeroding sponges will benefit from changing ocean conditions, including ocean warming and coastal nutrient enrichment, thereby reducing reef accretion (Enochs et al. 2015). In this discussion, I integrate my main findings (Table 6-1), emphasize how they expand and strengthen our understanding of the ecology of bioeroding sponges as potential threats to future reefs, and conclude by highlighting directions for future research.

Chapter 2 described the distribution of *Cliona orientalis* across the inshore GBR and identified sediment deposition and water clarity as primary drivers of its distribution and abundance, respectively. Chapter 3 defined a 32 °C thermal bleaching threshold for *C. orientalis* and measured the effect of bleaching on sponge energy reserves. Chapter 4 identified core microbial taxa, including an abundant *Rhodothalassium* sp., and demonstrated that the *C. orientalis* microbiome shifts prior to bleaching. Chapter 5 determined that dissolved inorganic nutrients do not affect the growth or health of *C. orientalis* or four other common reef sponge

species. Light treatments only affected *C. orientalis*, increasing energy reserves and chlorophyll content. Together, this thesis expands our understanding of how sponges will respond to changing environmental conditions and highlights that the bioeroding *C. orientalis* will not directly benefit from ocean warming or nutrient enrichment.

Table 6-1. Summary of the effects of studied environmental cues on *C. orientalis*.

Environmental cue		Effect on *C. orientalis*	Chapter
Deposition of fine sediment	Negative	Restricts distribution	2
		Limits percent cover	
Chlorophyll concentration	Negative	Associated with decreases in cover	2
Macroalgal abundance	Negative	Associated with decreases in cover	2
Temperature	Negative	Triggers bleaching	3, 4
		Disrupts photosynthesis and microbial community	
		Consumption of energy reserves	
Dissolved inorganic nutrients	NA	No effect on growth, chlorophyll, or energy reserves	5
Irradiance	Positive	Increases chlorophyll content	2, 5
		Increases energy reserves	

6.2. Effects of environmental change on bioeroding sponges

Bioeroding sponges may be stress-tolerant 'winners' on changing reefs (Schönberg, Fang, & Carballo 2017), as their abundance has increased in some regions following coral bleaching events (Schönberg 2001, Schönberg & Ortiz 2008, Chaves-Fonnegra et al. 2018). However, Chapter 2 shows that any increases following bleaching have not translated to increased cover on the inshore GBR, similar to what has been reported from the Caribbean over a similar period (Gilliam 2010, Ruzicka et al. 2010, Marulanda-Gómez et al. 2017). Many of the reefs studied in Chapter 2 bleached prior to the survey period in 1998 and/or 2002 (Berkelmans et al. 2004), suggesting that *C. orientalis* did not proliferate following bleaching on the GBR and that reported increases in cover from individual locations (Ward-Paige et al. 2005, Schönberg & Ortiz 2008)

may be local and episodic rather than a wider long-term trend. Even though bioeroding sponge cover has not increased over the past decade on the GBR, *C. orientalis* may still be tolerant of warming that triggers coral bleaching (Schönberg et al. 2008). In several instances, bioeroding sponges did not bleach during coral bleaching events (Cortés et al. 1984, Vicente 1990), leading some researchers to define them as thermally tolerant. However, laboratory studies indicate that bioeroding sponges can bleach at similar temperatures to corals (Fang et al. 2013, Achlatis et al. 2017). Chapter 3 defined the bleaching threshold for *C. orientalis* as +3 °C above the maximum monthly mean, which also corresponded with a significant shift in the associated microbial community (Chapter 4). These findings reveal that *C. orientalis* cannot tolerate substantially more stress than sympatric corals (Berkelmans & Willis 1999). That said, 1-2°C of warming may benefit *C. orientalis* in the short-term, as *C. orientalis* did not bleach during the 2017 bleaching event on the GBR when temperatures did not reach its bleaching threshold of 32 °C. Few studies have investigated the recovery capacity of bioeroding sponges, but Chapter 3 indicates that their ability to recover from bleaching is poor, at least under aquarium conditions.

Therefore, *C. orientalis* is not a likely 'winner' under changing environmental conditions that threaten coral reefs. While the rate of sponge-mediated erosion will increase as oceans become more acidic (reviewed in Schönberg, Fang, & Carballo 2017), the severe physiological costs of ocean warming and bleaching on these sponges indicate that accelerated erosion is unlikely to be realised (Achlatis et al. 2017). If warming induces a decline in other bioeroders as well, reef growth may continue for a longer period than if bioerosion rates were maintained. Perhaps a decline in bioeroders would buy time to reduce greenhouse gas emissions, slow ocean warming, and prevent the collapse of coral reefs. However, as Caribbean reefs demonstrate, once coral cover declines to less than 10%, reef erosion will likely exceed reef accretion even if

bioeroders decline (Perry et al. 2013, 2014). Other species of bioeroding sponges, such as those without photosynthetic symbionts or those that reside entirely within the reef structure, may respond differently than *C. orientalis*, but little is known about these species. Understanding the response of other bioeroding taxa, in addition to sponges, to changing environmental conditions is critical to predicting future reef erosion.

6.3. Future directions

A number of research questions emerged from the work conducted in fulfilment of this PhD. While the temperature threshold for *C. orientalis* bleaching was clearly defined, it is important to test how other stressors, including ocean acidification, nutrient enrichment and light, interact with warming to alter the bleaching threshold. For example, organic enrichment can mitigate some effects of thermal stress on *C. orientalis* (Achlatis et al. 2017) and ocean acidification can dampen the effects of warming on other phototrophic sponges (Bennett et al. 2016). Whether nutrient enrichment affects the bleaching tolerance of sponges is largely unknown (Achlatis et al. 2017), but nutrient enrichment can make corals more susceptible to bleaching (Wooldridge 2009). While several studies have investigated the combined effects of warming and acidification on bioeroding sponges (Wisshak et al. 2013, Fang et al. 2014, Stubler et al. 2015, Achlatis et al. 2017), it remains unclear whether ocean acidification alters the bleaching threshold.

Despite their recognised critical importance to host health, the functional role of microbes within bioeroding sponges has not yet been investigated. Recent research suggested that the microbial community of *C. orientalis* does not participate in the assimilation of inorganic nutrients, with the Authors positing that the microbial community plays a minor role in the *C.*

orientalis holobiont (Achlatis et al. 2018). However, sponge-associated microorganisms are known to undertake a diverse array of functional roles in addition to carbon and nitrogen cycling, including essential vitamin synthesis, antimicrobial production, elimination of toxic wastes and sulfur metabolism (Webster & Thomas 2016, Hill & Sacristán-Soriano 2017). The microbial community of *C. orientalis* is overwhelmingly dominated by one symbiont, *Rhodothalassium* sp., yet its role in the sponge holobiont remains unknown. Whether the *C. orientalis* microbial community contributes to the erosion capacity of the sponge has also not been considered. Defining the functional interactions between the different components of the *C. orientalis* holobiont will improve our understanding about the ecology and environmental tolerance of this species.

More generally, future research should address whether organic enrichment (DOC or POC) benefits sponges. It's hypothesized that DIN enrichment leads to DOC enrichment which facilitates sponge dominance on reefs (de Goeij et al. 2013, Pawlik et al. 2016). While numerous studies measure DOC and POC fluxes in sponges (reviewed in de Goeij et al. 2017), direct experimental evidence that additional DOC benefits sponges is lacking. In Chapter 5, DIN enrichment had no effect on the growth or condition of *C. orientalis* or other GBR sponges, and DOC also had no apparent effect, although additional experiments are needed to validate the ubiquity of this finding.

6.4. Conclusions

This thesis addressed several common hypotheses bioeroding sponges, including that bioeroding sponges are increasing in cover, have a high thermal tolerance, and benefit from nutrient pollution. These knowledge gaps led to supposition that bioeroding sponges are a threat

to coral reefs. However, this thesis demonstrates that the thermal sensitivity of bioeroding sponges will limit their contribution to future reef erosion and suggests that *C. orientalis* is unlikely to exploit reef decline or anthropogenic influence. Furthermore, the use of a multi-level ocean warming experiment provided key insights, such as the bleaching threshold and multiple microbial shifts, that would not have been detected using a factorial design. The results of this thesis can be incorporated into GBR carbonate budgets by predicting where sponge erosion occurs and where sponge erosion is likely to increase until future ocean warming compromises bioeroding sponges.

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Appendix A: Related publications

1. **Ramsby, B. D.**, Hill, M. S., Thornhill, D. J., Steenhuizen, S. F., Achlatis, M., Lewis, A. M., & LaJeunesse, T. C. (2017). Sibling species of mutualistic *Symbiodinium* clade G from bioeroding sponges in the western Pacific and western Atlantic oceans. *Journal of Phycology*, *11*, 36–10. http://doi.org/10.1111/jpy.12576

   (included as Appendix B)

2. Goulet, T. L., Shirur, K. P., **Ramsby, B. D.**, & Iglesias-Prieto, R. (2017). The effects of elevated seawater temperatures on Caribbean gorgonian corals and their algal symbionts, Symbiodinium spp. *PLoS ONE*, *12*(2), e0171032. http://doi.org/10.1371/journal.pone.0171032

3. Hoogenboom M. O., Frank G.E., Chase T.J., Jurriaans S., Álvarez-Noriega M., Peterson K., Critchell K., Berry K.L. E., Nicolet K.J., **Ramsby B.**, Paley A.S. (2017). Environmental drivers of variation in bleaching severity of *Acropora* species during an extreme thermal anomaly. *Frontiers in Marine Science*, *4*, 376. http://doi.org/10.3389/fmars.2017.00376

Appendix B. Associated publication: genetic description of *Symbiodinium*

Title: Sibling species of mutualistic *Symbiodinium* Clade G from bioeroding sponges in the western Pacific and western Atlantic Oceans[1]

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B.1. Abstract

Dinoflagellates in the genus *Symbiodinium* associate with a broad array of metazoan and protistan hosts. *Symbiodinium*-based symbioses involving bioeroding sponge hosts have received less attention than those involving scleractinian hosts. Certain species of common *Cliona* harbor high densities of an ecologically restricted group of *Symbiodinium*, referred to as Clade G. The relationships of these unusual Clade G *Symbiodinium* with Foraminifera, sponges, and black coral (Antipatharia) are rarely studied. Nonetheless, analyses of genetic evidence indicate that Clade G likely comprises several distinct species. Here we use genetic data in combination with ecological and geographic evidence to formally describe *Symbiodinium endoclionum* sp. nov. obtained from the Pacific boring sponge *Cliona orientalis* and *S. spongiolum* sp. nov. from the congeneric western Atlantic sponge *C. varians*. These species appear to be part of an adaptive radiation of Clade G lineages specialized to the metazoan phyla Porifera and Cnidaria that began prior to the separation of the Pacific and Atlantic Oceans.

Key words: Atlantic Ocean; *Cliona*; Pacific Ocean; Porifera, *Symbiodinium*, systematics.

B.2. Introduction

Dinoflagellates in the genus *Symbiodinium* are often symbiotic with cnidarians including hard corals, soft corals, and sea anemones (Baker, 2003). Less commonly recognized, they also form ecologically important symbioses in other hosts, including sponges, and giant clams, as well as single-celled foraminifera (Pochon *et al.*, 2001, Schönberg & Loh, 2005, Granados *et al.*, 2008). As mutualistic symbionts, *Symbiodinium* occur at high densities within these various hosts and provide them with metabolic energy via photosynthesis (Hill, 1996, Weisz *et al.*, 2010), thus

fuelling important ecological processes such as coral calcification and sponge bioerosion. Despite the importance of these abundant micro-algae to coral reef ecosystems, most *Symbiodinium* lack formal taxonomic designations, which has both constrained and complicated our understanding of their physiology, ecology, and evolution (LaJeunesse *et al.*, 2012).

While it is problematic to differentiate *Symbiodinium* using morphological traits, the analyses of molecular genetics clearly identify a breadth of distantly and closely-related lineages. The genus is divided hierarchically into many subgroupings, or *clades*, that are separated by large nucleotide sequence differences among conserved genes (e.g. small and large subunit rDNA) (Rowan & Powers, 1992, Stern *et al.*, 2010). *Clades* of *Symbiodinium* differ in their geographic distribution, ecological and regional abundance, and range of host associations (Pochon *et al.*, 2006). Moreover, each *clade* contains different numbers of biologically distinctive entities, or species, possessing different ecological niches (e.g., LaJeunesse et al., 2012, Parkinson *et al.*, 2015). Presently, species of *Symbiodinium* are formally described based primarily on genetic evidence (phylogenetic and population genetic) supported by ecological, biogeographic, physiological, and morphological data (Sampayo *et al.*, 2009, LaJeunesse *et al.*, 2014, e.g., Lee *et al.*, 2015, Wham *et al.*, 2017). While ecologically common *clades* (e.g. Clades A, B, C, and D) contain described species (LaJeunesse, 2017), other *clades* have distinct entities that are candidates for species classification.

Symbiodinium Clade G is an evolutionarily divergent and enigmatic group (Pochon *et al.*, 2001). In contrast to those *Symbiodinium* that are common to symbiotic cnidarians, Clade G appears restricted mostly to protistan and poriferan hosts, notably found in certain miliolid foraminifera, primarily in the genus *Marginopora* (Pochon *et al.*, 2001), and in bioeroding

sponges of the family Clionaidae (Schönberg & Loh, 2005, Granados et al., 2008, Hill *et al.*, 2011). Gene sequences diagnostic of Clade G have also been recovered from the water column and benthic substrates, including coral rubble and turtle grass blades (Granados-Cifuentes *et al.*, 2015, Takabayashi *et al.*, 2011, Yamashita *et al.*, 2014). Rarely is this *clade* detected in Cnidaria, but one particular member appears to form stable symbioses with a species of black coral from the Indo-West Pacific (Bo *et al.*, 2011). Clade G has occasionally been observed with other cnidarians as co-dominant symbionts in some intertidal coral colonies (LaJeunesse *et al.*, 2010), or at very low background densities (Van Oppen *et al.*, 2005, Thomas *et al.*, 2014, Ziegler *et al.*, 2017).

Certain sponge species in the family Clionaidae, comprising bioeroding sponges, are known to harbor high densities of *Symbiodinium*. As with symbiotic corals, photosynthesis by these resident *Symbiodinium* appear to enhance growth and bioerosion rates in these sponges through translocation of fixed carbon (Hill, 1996, Schönberg, 2006, Weisz et al., 2010). *Cliona orientalis* from the Indo-Pacific is ecologically abundant and rapidly bioerodes carbonate material including the skeletons of living corals. In contrast to many scleractinian corals, bioeroding sponges may benefit from anthropogenically driven global climate change. *Cliona orientalis* has been observed to increase in abundance after episodes of mass coral mortality from thermal stress (Schönberg & Oritz, 2009). This loss of live coral cover exposes new settlement areas where sponge larvae colonize and grow without competition. Moreover, increasing regional eutrophication and the rise of ocean pCO_2 may accelerate sponge-mediated bioerosion (Holmes *et al.*, 2000, Wisshak *et al.*, 2012). Such observations indicate that the Clionaidae, especially those with Clade G *Symbiodinium*, may increase in abundance in response to anthropogenically driven global change, which may further facilitate coral reef erosion (Bennett *et al.*, 2017).

Given the likelihood of the increasing abundance and ecological significance of bioeroding sponges and their negative effect on reef accretion, their symbioses with dinoflagellates should attract more research interests. Establishing a formal taxonomy for these sponge symbionts will help standardized the reporting of findings from future research projects. Therefore, we seek to formally classify and name species of Clade G *Symbiodinium* commonly associated with sponges within the genus *Cliona*. Recent genetic analysis of the Clade G symbionts obtained from sponges found that they are diverged from those harboured by Foraminifera (Schönberg & Loh, 2005, Hill *et al.*, 2011). Moreover, preliminary genetic analyses have shown that Clade G in clionaids from the West Atlantic are distinguished genetically from closely-related Clade G counterparts in the Pacific Ocean (Hill *et al.*, 2011); and are likely different species. Thus, as with most other recently described *Symbiodinium* spp. (e.g. LaJeunesse et al., 2012), we rely here on the concordance of several independent phylogenetic markers and known ecological and biogeographic attributes to classify two new species of *Symbiodinium*, the first species representing Clade G.

B.3. Materials and Methods

B3.1. Specimen collection

Symbiotic species of clionaid bioeroding sponges were collected from the tropical Atlantic and the Western Pacific. Samples were collected so as not to include any live coral tissue. *Cliona varians* was collected from the Florida Keys and Belize (depths of 1-2 m). *Cliona tumula* was obtained only from the Florida Keys. In the Pacific, *C. orientalis* was collected from Little Pioneer Bay, Orpheus Island, Eastern Australia (< 3m depth) and Okinawa (7-15 m), Japan, in the northwest tropical Pacific Ocean. Tissues from the top 1 cm of the sponge surface were removed

and preserved either by freezing in liquid N_2 and stored at -80˚C, preserved in 70% ethanol, or in high-salt, 20% DMSO buffer (Seutin *et al.*, 1991) and stored at -20°C.

B.3.2. Cell size measurements

Preserved cells from host tissue homogenates were photographed under bright-field illumination at a magnification of 400× using an Olympus BX51 compound microscope (Olympus Corp., Tokyo, Japan) with a Jenoptik ProgRes CF Scan digital camera (Jenoptik, Jena, Germany). The lengths and widths for at least 50 cells per sample were calculated with the program ImageJ (Abramoff *et al.*, 2004).

B3.3. DNA extraction, PCR amplification, sequencing, and phylogenetic analysis

Whole tissue DNA extractions were performed as described by LaJeunesse *et al.* (2003), consisting of a 2 min bead-beating step (0.4-0.6 mm glass beads) and a modified and abreviated DNA wizard extraction protocol (Promega). Nuclear large-subunit ribosomal DNA (LSU), *chloroplast large-subunit* (*cp23S*), mitochondrial cytochrome *b* (*cob*) gene, and the partial coding and entire non-coding region of the *psbA* (*psbA^{ncr}*) were amplified and sequenced to delimit species (LaJeunesse *et al.*, 2012). Conditions for amplifying the LSU are provided in (Zardoya *et al.*, 1995); and conditions for amplifying cp23S and *cob* are provided by Zhang *et al.* (2000) and Zhang *et al.* (2005), respectively; primers and conditions for *psbA^{ncr}* are specified in LaJeunesse and Thornhill (2011).

To amplify DNA, reactions were performed in 25 µL volumes containing 2.5 µL of 2.5 mM dNTPs, 2.5 µL of 25 mM $MgCl_2$, 2.5 µL standard *Taq* Buffer (New England Biolabs, Ipswich, MA, USA), 0.13 µL of 5 U • μL^{-1} *Taq* DNA Polymerase (New England Biolabs), 1 µL of each forward and reverse primer at 10 µM, and 1 µL of 5–100 ng DNA template. Products were

cleaned with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and directly sequenced on an Applied Biosciences sequencer (Applied Biosciences, Foster City, CA, USA) at the Pennsylvania State University Genomics Core Facility. Sequence electropherograms were each examined manually and nucleotide sequences aligned by eye. Raw sequences and alignments for each gene can be found in dryad.

Phylogenetic analyses were performed on aligned data sets in PAUP* v.4.0d151 (Swofford, 2014) under maximum parsimony with indels in rDNA included as a 5th character state. Bootstrap support was calculated based upon 1000 replicates. Bayesian posterior probabilities were calculated with the software MrBayes v.3.2.3 (Ronquist *et al.*, 2012), using the optimal nucleotide substitution model for each gene based on corrected Akaike Information Criterion as calculated with the software ModelTest v.3.7 (Posada & Crandall, 1998).

B.4. Results

B.4.1. Phylogenetic delineation of recently divergent lineages

Distinct lineages of Clade G *Symbiodinium* were identified from Pacific and Atlantic clionaid sponges, respectively. Sequence data from chloroplast *psbA*^{ncr}, *cp23S*, mitochondrial *cob* and nuclear LSU rDNA indicate that there are several closely-related species lineages within Clade G. Genealogies produced from genetic markers, including nuclear (*LSU*), mitochondrial (*cob*), and chloroplast genomes (*cp23S*), differentiated a Pacific and Atlantic lineage of *Symbiodinium* Clade G by few, albeit fixed, sequence differences (Figures 1 and 2). Large sequence differences separating these lineages were found in the plastid *psbA* (Figure B-3). Moreover, there was no indication of recombination among sequence variants (alleles) in samples analysed from each ocean basin. Collectively, these genetic data and patterns are used here to

unambiguously resolve two new *Symbiodinium* species.

B.4.2. Morphology

Micrographs taken using light microscopy show no differences in symbiont cell morphology between samples obtained from Pacific and Atlantic clionaid sponges (Figs 4A and 4B). Furthermore, measurements of cell sizes did not differentiate these lineages (Figures B-4C).

B.4.3. Taxonomic descriptions

B.4.3.1 *Symbiodinium endoclionum*, sp. nov. Ramsby & LaJeunesse

Diagnosis: Coccoid cells ranged in mean size from 8.0 to 9.2 μm at maximum diameter (Figures B-4c). The combined nucleotide sequences of *cp23S* (GenBank Accessions: MF322792, MF322793), nuclear ribosomal and LSU (MF322789, MF322790), mitochondrial *cob* (MF322795), and *psbA–psbA*^{ncr} are diagnostic of this species.

Holotype designation: The type specimen was obtained in July 2015 from Orpheus Island, Australia (18˚35'41.01" S; 146˚29'10.83" E) and deposited in the US Algal Collection of the National Herbarium, Smithsonian Institution, Washington, DC, United States of America; and assigned the catalog number: US Alg. Coll. #223179.

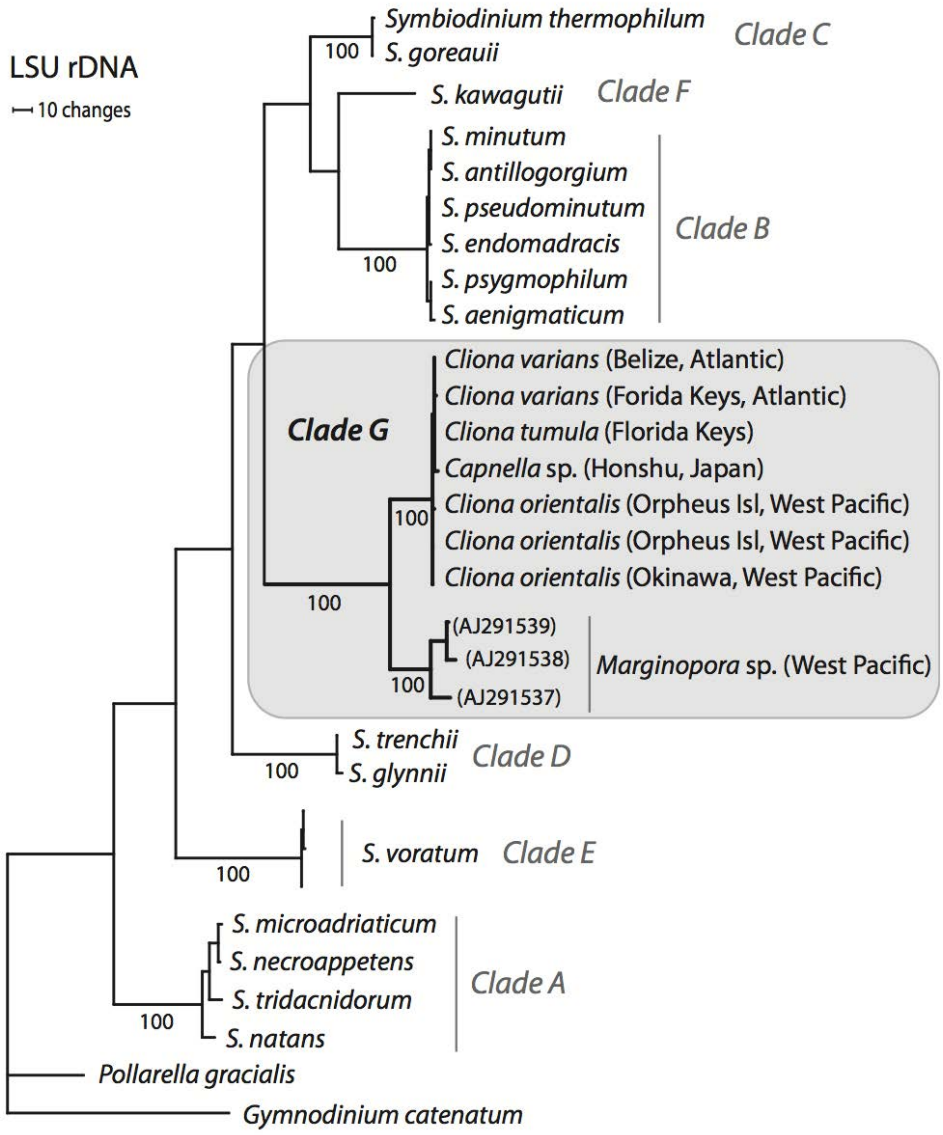

Figure B-1. Phylogeny based on nuclear LSU rDNA comparisons showing the diversity and evolutionary relationships between Clade G *Symbiodinium* relative to other clades containing formally described species. Clade G bifurcates into distinct lineages that exhibit specificity to either metazoan or foraminiferan hosts. Small sequence differences in LSU separate several ecologically and geographically distinct entities associated with particular metazoan hosts, including bioeroding sponges and some cnidarians. Bootstrap values based on 1000 replicates support Clade G as monophyletic as well as each of its subclades.

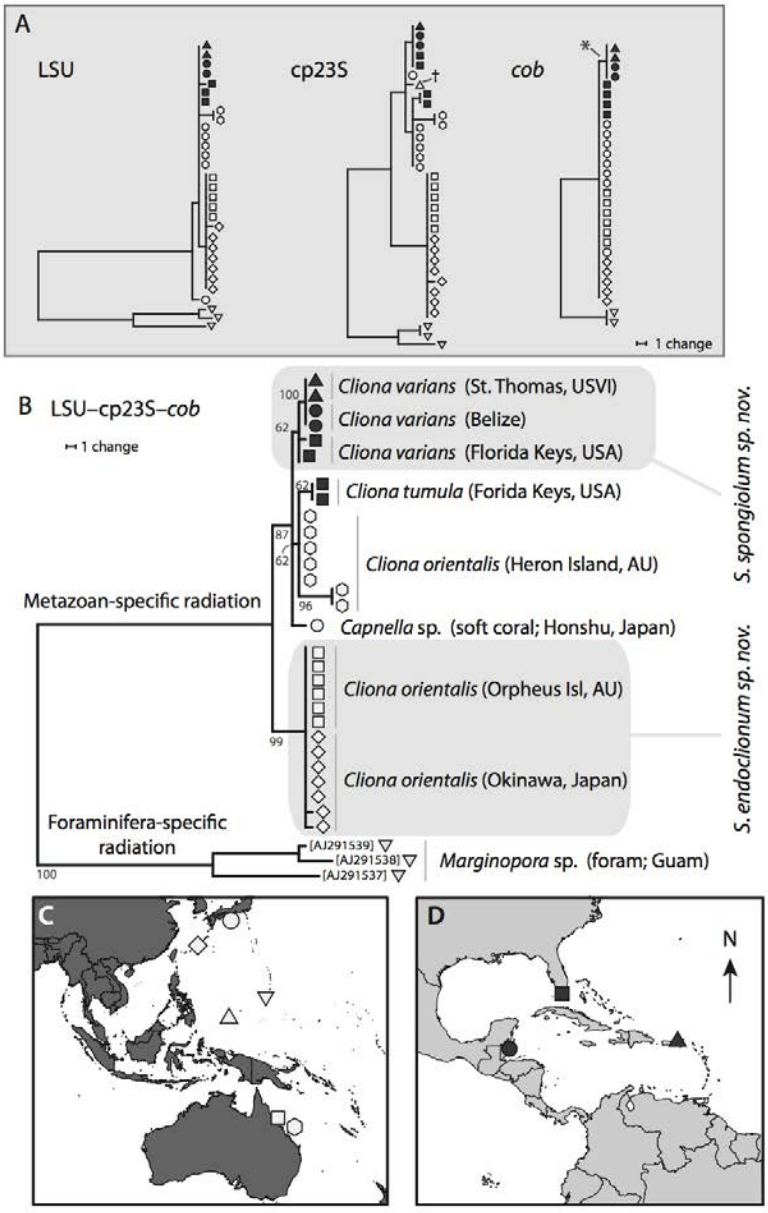

Figure B-2. Genetic partitioning of Clade G lineages from specimens collected in the Pacific and Atlantic Oceans **(A)**. Gene phylogenies of Clade G *Symbiodinium* based on partial sequences of LSU rDNA (~ 610 b.p.), chloroplast cp23S (~ 600 b.p.), and mitochondrial *cob* (~ 900 b.p.). **(B)** Small, albeit fixed, differences based on a concatenated DNA phylogeny identifies host-specific and, in some cases, geographically widespread lineages from the Pacific and Atlantic Oceans. Two of these are described here as *Symbiodinium enodclionum* sp. nov. from the Pacific Ocean and *S. spongiolum* sp. nov. from the Atlantic Ocean. Bootstrap values are based on 1000 iterations. Light-coloured symbols of different shapes identify collection locations from the Pacific **(C)** and dark symbols identify locations from the Greater Caribbean/Atlantic **(D)**. The cross symbol (†) identifies the cp23S sequence from the *Symbodinium* in *Madracis asanoi* obtained from a depth of 75 meters in Palau. The asterisk indicates the non-synonymous mutation found in genotypes of *S. spongiolum* from the tropical Atlantic.

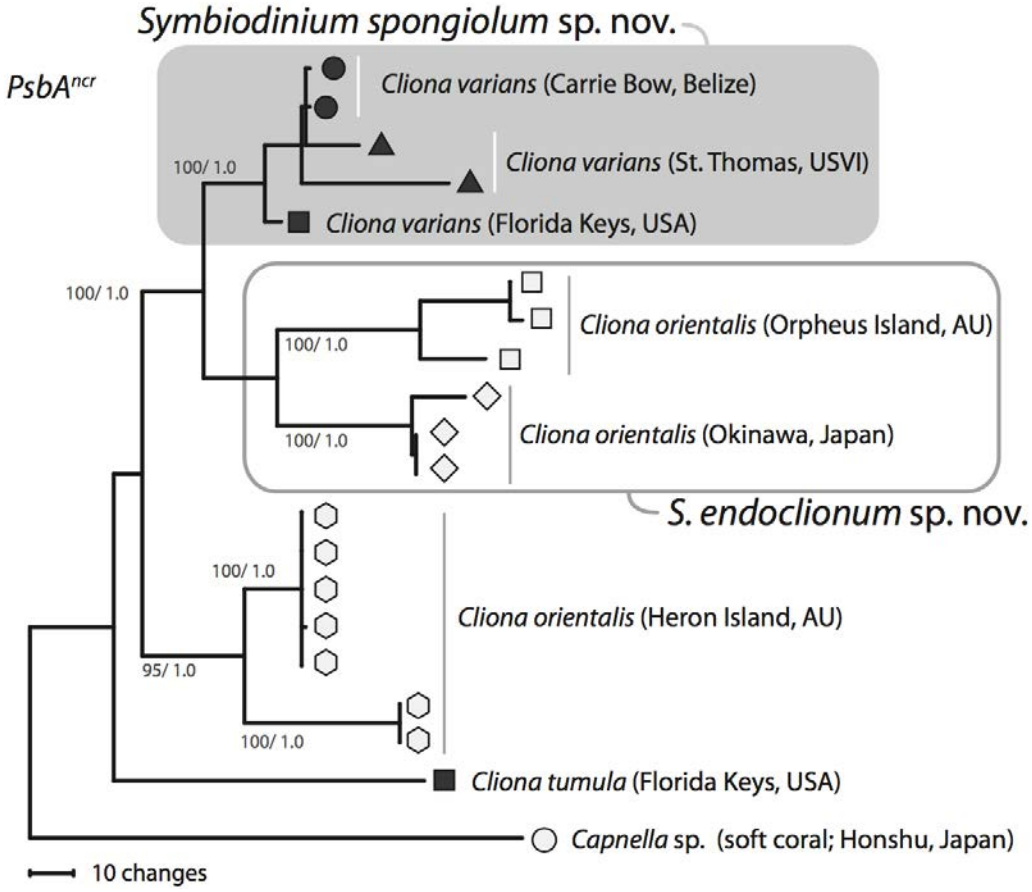

Figure B-3. Phylogeny of Clade G *Symbiodinium* associated with *Cliona* spp. using high-resolution *psbA*^{ncr} sequences (~1000 bases). The sequence haplotypes corresponding to the new species *Symbiodinium endoclionum* and *S. spongiolum* are specified. Symbols placed at end of each terminal branch correspond to locations indicated on the maps shown in Figures 2C and D. Bootstrap values based on 1000 iterations and Bayesian posterior probabilities are indicated for each internal branch.

Type locality: Orpheus Island, Australia collected at a depth of 1-3 meters from the host sponge *Cliona orientalis* (Demospongiae: Porifera).

Etymology: The Latin *endo* (inside) and *cliona* (bioeroding sponge) refers to the ecological specialization of *S. spongiolum*, which occurs in this host species of carbonate-excavating sponge.

Other notes: Populations in Japan appear to be genetically distinct from populations in Australia based on rapidly evolving *psbA*^{ncr} (Figures B-3). However, both populations share the same sequences for conserved chloroplast, mitochondrial and nuclear rDNA genes.

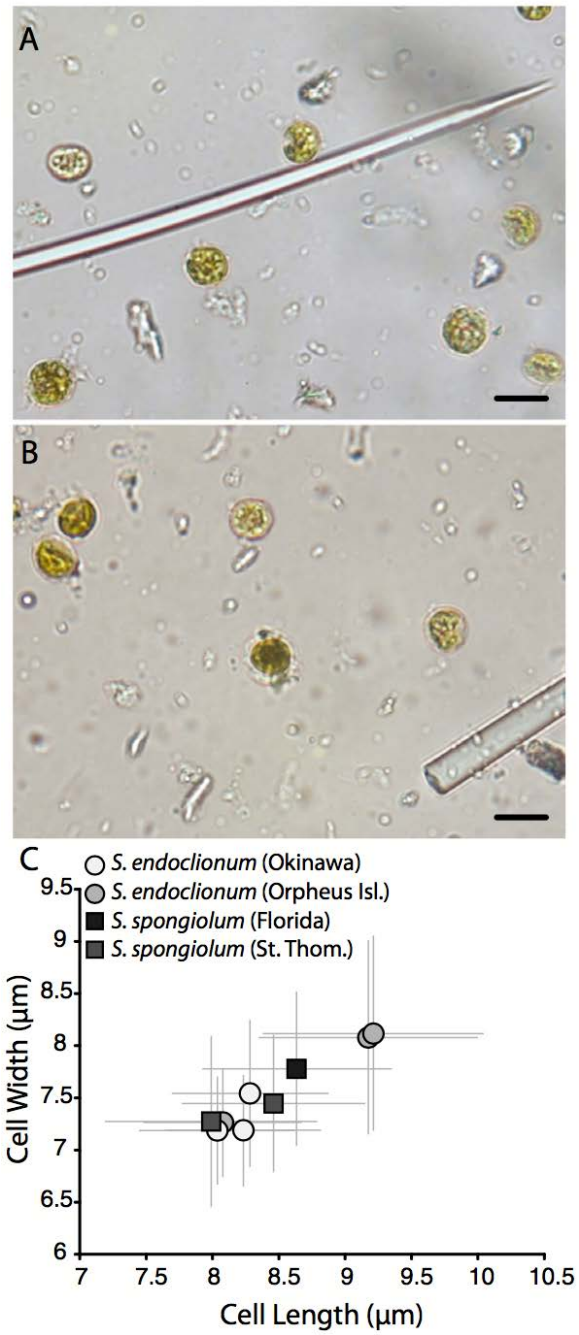

Figure B-4. Light micrographs of *Symbiodinium endoclionum* **(A)** and *S. spongiolum* **(B)** taken at 1000× (scale bar = 10 µm). **(C)** A comparison of mean cell dimensions (length and width) of coccoid cells obtained from independent samples of *Symbiodinium endoclionum* (light and grey colored circles) and *S. spongiolum* (dark colored squares). Error bars represent standard deviations calculated from measurements on ≥ 50 cells.

B.4.3.1. *Symbiodinium spongiolum*, sp. nov. Hill & LaJeunesse

Diagnosis: Coccoid cells have a mean size of 8.6 μm at maximum diameter (Figures B-4C). The combined nucleotide sequences of *cp23S* (GenBank Accession: MF322791), nuclear ribosomal LSU (MF322787, MF322788), mitochondrial *cob* (MF322794), and *psbA–psbA*^{ncr} are diagnostic of this species.

Holotype designation: The type specimen was obtained in July 2015 from the Florida Keys, USA (24˚39'35.44" N; 81˚27'07.41" W) and deposited in the US Algal Collection of the National Herbarium, Smithsonian Institution, Washington, DC, United States of America; and assigned the catalogue number: US Alg. Coll. # 223180.

Type locality: Key Largo, Florida, United State of America in the Greater Caribbean. The host specimen of *Cliona varians* was collected at a depth of 1 meter.

Etymology: From the Latin *spongia* (sponge) and suffix *–ulum* (small one) for the host this species exhibits ecological (i.e. host) specialization.

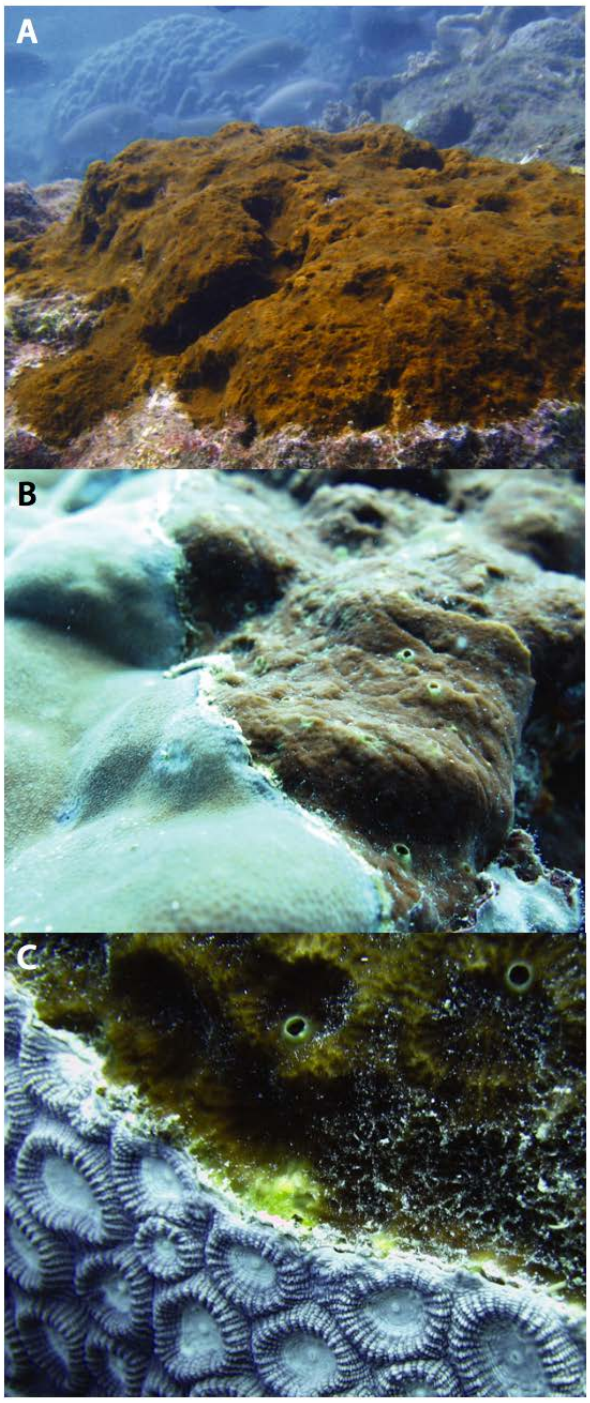

Figure B-5. The zooxanthellate Pacific sponge *Cliona orientalis* harbor *Symbiodinium endoclionum* sp. nov. This photosymbiotic bioeroding sponge bores into calcium carbonate substrate and overgrows live coral colonies by eroding their skeletons. Examples include, **(A)** reef rock, **(B)** *Porites* spp. and **(C)** *Phymastrea* (=*Montastraea*) *magnistellata*. Photos courtesy of the AIMS Long Term Monitoring Program (panels **A** and **C**) and Michelle Jonker (Australian Institute for Marine Science; panel **B**).

B.5. Discussion

B.5.1. Clade G species diversity

The genus *Symbiodinium* as a whole is ecologically widespread. However, the prevalence and abundance of each designated *clade* of *Symbiodinium* differs widely from the overwhelmingly dominant Clade C to the extremely rare Clade I, which is known from only one study (Pochon & Gates, 2010). Clade G is perceived as ecologically uncommon and little is known about its species composition and their distributions. This is mainly because Clade G occurs in understudied host taxa (i.e. sponges, black corals, and foraminifera). Phylogenetically, Clade G is bifurcated into divergent lineages that exhibit disparate host affinities (Schönberg & Loh, 2005, Hill et al., 2011), which are clearly distinguished using LSU and *cp23S* sequences (Figures B1 and 2). One lineage associates primarily with large soritid foraminifera, especially in the genus *Marginopora* (Garcia-Cuetos *et al.*, 2005, Pochon *et al.*, 2007), while the second group associates with the metazoan phyla, Porifera and Cnidaria (Figures B-2B), and thus each evolutionarily divergent lineage possess distinct ecological habits.

Only certain species of clionaid sponge appear to require *Symbiodinium* for their survival and growth (Figure B-5; Hill, 1996). The *Symbiodinium* species described here appear to be ecologically specialized for living in symbiosis with specific species of clionaid sponge. Indeed, *S. endoclionum* has yet to be identified in hosts other than *C. orientalis* (Western Pacific) and *S. spongiolum* may only occur in *C. varians* (Caribbean). The Atlantic *Cliona tumula* also associates with Clade G, but that *Symbiodinium* sp. appears to be a different species from *S. spongiolum* (Figures B-3). Partner specificity from the point of view of the host vs. the symbiont can differ. For example, the Caribbean sponge, *Cliona caribbaea* can associate with either a Clade G or a

Clade A *Symbiodinium* (Granados et al., 2008). Thus, there is some flexibility with the kind of *Symbiodinium* a particular host sponge may associate. More exhaustive sampling is required to determine the full ecological breadth of *S. endoclionum* and *S. spongiolum*, but for now they appear to be highly host-specialized (Thornhill *et al.*, 2014).

Clade G probably contains additional sponge-associated/specialized species, which are closely related to *S. endoclionum* and *S. spongiolum*, that require future classification and naming (Figs 2 and 3). For example, the *Symbiodinium* within *C. orientalis* from Heron Island, Australia, and *C. tumula* from the Florida Keys, USA, had divergent *psbA*^{ncr} sequences diagnostic of them being separate species (Figures B-3). This is further supported by small, but fixed, sequences differences in, for example, *cp23S* rDNA between *S. spongiolum* and the symbiont from *C. tumula* (Figures B-2B) (Hill et al. 2011). Furthermore, additional *cp23S* and ITS2 rDNA sequence data indicate that several other Clade G species appear ecologically specialized for cnidarians, including black corals (Antipatharia)(Bo et al., 2011) and deep-dwelling stony corals (Pacific *Madracis asanoi*; Figures B-2A). Finally, our understanding of the diversity in Clade G associated with Pacific Foraminifera is restricted to a small number of field surveys from essentially one geographic location (Guam)(Pochon *et al.*, 2007). Thus the actual species diversity and prevalence of Glade G will remain under appreciated until further studies and characterizations are conducted.

B.5.2. A fixed genetic difference probably corresponds to a physiological adaptation in populations of *Symbiodinium spongiolum*.

Amino acid (aa) variability among cytochrome *b* sequences is highly conserved among *Symbiodinium* spp., especially in functional regions of this proton-motive transmembrane enzyme. For all known *Symbiodinium* analyzed to date, the amino acid at position 165 (colored

white in Figure B-6) encodes as a polar Cysteine, which possesses a non-charged residue. However, *S. spongiolum* from tropical regions in the Caribbean possess a Tyrosine, which possesses an aromatic residue. This change in amino acid would effect the protein's 3-dimensional conformational structure and influence the redox catalysis of the Qo reaction center (Howell, 1989). While this cytochrome *b* variant is not a fixed trait among members of *S. spongiolum*, the physiological implications of this mutation requires future comparative study.

B.5.3. Stability of sponge-*Symbiodinium* associations

Symbiodinium populations found in clionaids are perceived to resist thermal stressors that otherwise induce bleaching in corals. For example, *Cliona varians* forma *varians* is common in shallow tropical habitats with fluctuating temperatures and turbidity, which may explain the apparent tolerance of their symbiotic partnerships to thermal stress. Symbiotic sponges are sometimes found devoid of algae, but usually only when portions have been buried by sediment (Vicente, 1990). However, recent observations of mass bleaching involving clionaid sponges (Hill *et al.*, 2016) indicate that our understanding of the stability of these associations is incomplete. Future investigations into the functional nature of sponge-dinoflagellate mutualism are warranted. To what extent do resident populations of *S. endoclionum* and *S. spongiolum* contribute to the metabolic demand of the host? Do these *Symbiodinium* have adaptations that make them tolerant of thermal stress, or does resistance to "bleaching" depend more on the sponge's physiology?

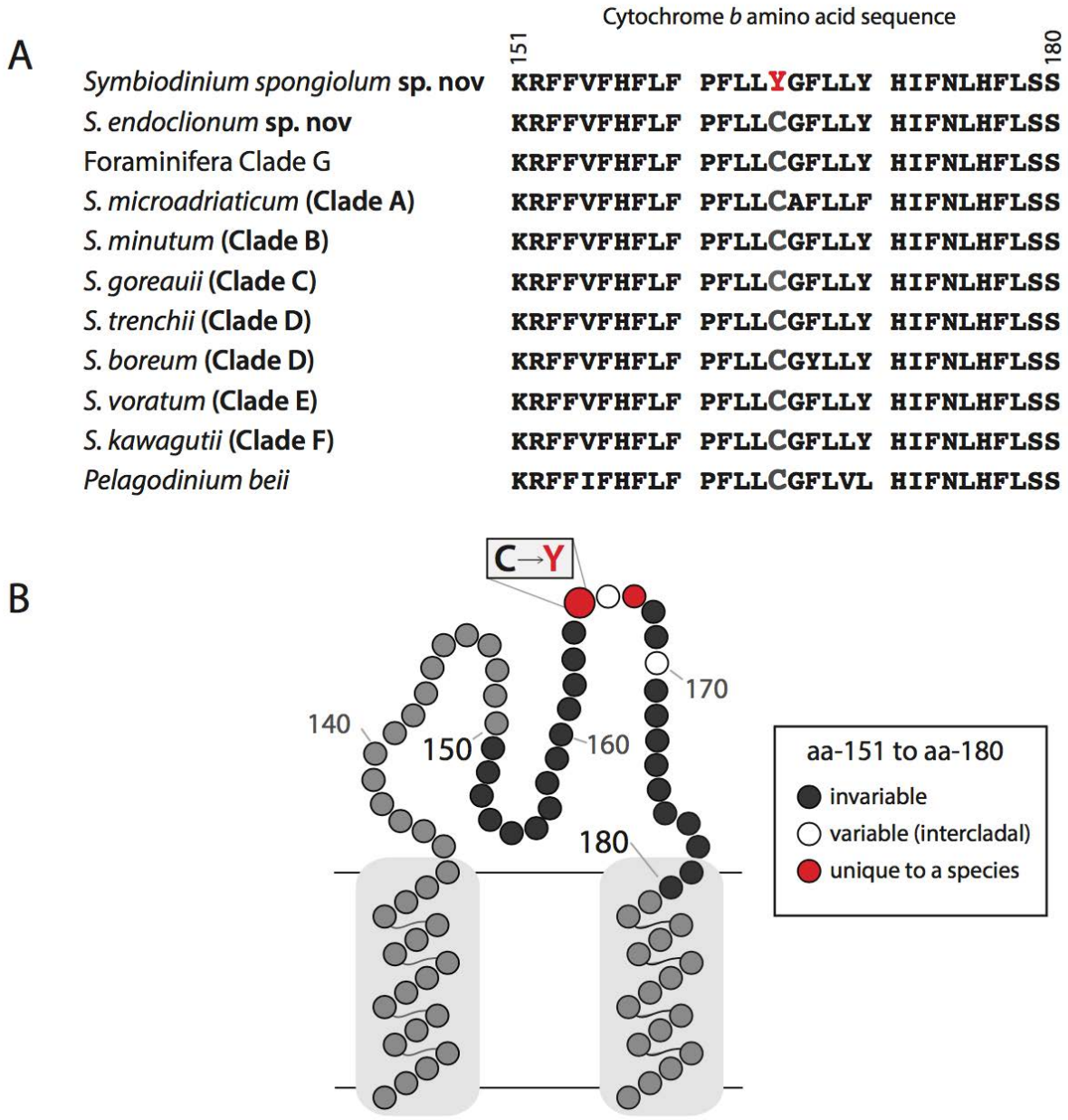

Figure B-6. Nonsynonymous base substitution in the mitochondrial *cob* gene characterizes tropical populations of *Symbiodinium spongiolum* in the tropical western Atlantic Ocean. **(A)** Amino acid sequences (aa 151–180) in the functional region of the Qo reaction centre are highly conserved among divergent clades of *Symbiodinium* and related dinoflagellates (e.g., *Pelagodinium beii*). **(B)** Structural model of the Qo reaction centre of transmembrane cytochrome *b* showing the location of a nonsynonymous mutation (converting a polar Cysteine to an aromatic Tyrosine) unique to some populations of *S. spongiolum* (adapted from Howell 1989).

B.5.4. Persistence of specific host-symbiont combinations over millions of years

The divergence between Clade G and other *clades* is estimated to have occurred tens of

millions of years ago (Pochon *et al.*, 2006). The genetic differences measured between Clade G and its next closest related lineages, Clades D or B, is similar to what is quantified between genera or families of dinoflagellates (Rowan & Powers, 1992). Because *Symbiodinium endoclionum* and *S. spongiolum* both associate with symbiotic clionaids from the Atlantic and Pacific Oceans, it is inferred that their common ancestor was also symbiotic with excavating sponges before the oceanographic and geological separation of these ocean basins. Niche conservation among most extant host-specialized *Symbiodinium* appears to last for many millions of years (Thornhill *et al.*, 2014) even though host-*Symbiodinium* partnerships change dramatically over very long spans of time (Rowan & Powers, 1991), probably in response to extreme changes in regional or global climates (LaJeunesse, 2005).

Symbiodinium endoclionum and *S. spongiolum* both appear to be part of a recent adaptive radiation that produced multiple independent Clade G lineages specialized to Porifera and Cnidaria (Figures B-2). The phylogenetic and ecological concordance indicates that these lineages began diverging (*i.e.* speciating) before separation of the Atlantic and Pacific Oceans (Figures B-3), probably during the Messinian of the Late Miocene Epoch (7.2–5.3 Ma) when global temperatures turned decisively colder (Zachos *et al.*, 2001), and were accompanied by shifts in diversity and community assemblages in marine and terrestrial biota, observed in the fossil record (Cerling *et al.*, 1993, Budd, 2000). The occasional finding of Clade G during broad surveys of host diversity and environmental samples suggest that many more, and possibly some free-living species, from this group exist. Clearly, there are other sponge associated Clade G *Symbiodinium* (Figures B-3)(Hill *et al.*, 2011) and several cnidarian-specific lineages that await formal classification and further ecological study (Bo *et al.*, 2011).

There is mounting awareness that LSU (and ITS) rDNA may not always provide adequate phylogenetic resolution among closely related species of dinoflagellates (Siano *et al.*, 2009, Wham *et al.*, 2017). Little, or no sequence divergence in the LSU rDNA is common among many lineages of *Symbiodinium*, even those separated by the closure of the Central American Seaway (Figures B-2A). For example, *S. tridacnidorum* (ITS2 type *A3*) in the Pacific has an identical LSU to *S. "fitti"* (ITS2 type *A3*) in the Atlantic (Lee *et al.*, 2015). Similarly, a single nucleotide substitution in LSU rDNA differentiates *Symbiodinium endoclionum* from *S. spongiolum* (Figures B-1). Thus, LSU is a marker that often requires millions of years of genetic isolation to evolve diagnostic sequence differences. While LSU rDNA evolves considerably faster than small sub-unit (SSU) rDNA, even faster-evolving markers have been recommended to better assess the phylogenetic relationships between dinoflagellate genera and species (Siano et al., 2009). By comparison, the large number of nucleotide differences between the chloroplast *psbA*^{ncr} from *S. endoclionum* from *S. spongiolum* better chronicles the millions of years that separate these lineages (Figures B-3).

B.6. Acknowledgements

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