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Biotic and Abiotic Influences on Algal Symbiont (Symbiodinium spp.) Community Dynamics in Reef Corals Recovering from Disturbance

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BIOTIC AND ABIOTIC INFLUENCES ON ALGAL SYMBIONT (*SYMBIODINIUM* SPP.) COMMUNITY DYNAMICS IN REEF CORALS RECOVERING FROM DISTURBANCE

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Paul R. Jones

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Reef corals are mutualisms between invertebrate animals and diverse dinoflagellate algae (*Symbiodinium* spp.). Originally thought to be specific relationships between particular corals and *Symbiodinium* types, it is now accepted that many coral species can host different types of symbionts, often simultaneously. This adds complexity to the study of coral-algal symbiosis, because subtle changes in the symbiont community (not just a shift in the dominant symbiont type) may result in a coral host with increased thermal tolerance or resilience. To determine how *Symbiodinium* community dynamics are affected by environmental stressors (abiotic factors) and exogenous sources of algal-symbionts (biotic factors), this project utilized a highly sensitive real-time PCR assay to quantify different *Symbiodinium* in reef corals in response to a number of experimental manipulations. This assay used symbiont to host-cell ratios to standardize algal abundance and generated quantitative data on *Symbiodinium* community dynamics in response to biotic and abiotic factors. They showed that (1) elevated temperatures (and to a lesser extent, elevated pCO_2) drive changes in the *Symbiodinium* communities of the Caribbean coral *Orbicella faveolata* from clade B dominated to clade D dominated, and that this switch corresponds to a decreased growth rate and reduced photosynthetic efficiency; (2) symbiont communities of healthy *Montastraea cavernosa* can be manipulated using transplant plugs (12mm diameter coral cores) hosting alternative

Symbiodinium types; and (3) exogenous sources of symbionts drive the initial recovery from bleaching in *M. cavernosa*. This is contrary to the common assumption that remnant symbiont populations are solely responsible for bleaching recovery and suggests that corals may have some capacity to acquire thermotolerance from neighboring colonies. These findings have significant implications for the study of coral reefs in an era of climate change.

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Chapter 1

Introduction

Coral reefs occupy less than 0.2% of the area of the world's oceans (Smith 1978; Spalding et al. 2001), yet their biological diversity and productivity is unrivalled by any other oceanic system (Veron 1995). They provide crucial ecosystem services beneficial to human societies which provide a net economic benefit of approximately US$375 billion per year (Cesar et al. 2003). While the structures they form are geologically persistent, the building blocks of the world's coral reefs are biologically fragile. The foundation of this diverse and productive ecosystem relies on a mutualistic relationship between coral polyps and endosymbiotic single-celled algae (*Symbiodinium* spp.). This relationship is susceptible to numerous environmental perturbations and stressors such as disease, overfishing and pollution. In Caribbean waters, an 80% reduction (from 50% to 10%) in coral cover has been documented over the last thirty years (Gardner et al. 2003) and world-wide one-fifth of the world's coral reefs have effectively been destroyed and show no immediate signs of recovery (Wilkinson 2004). Further, the threats of climate change are predicted to cause considerable damage to coral reef ecosystems (Hughes et al. 2003; Aronson & Precht 2006).The combined effects of elevated sea surface temperatures and increased atmospheric CO_2 are forecast to increase the incidence and severity of bleaching (reviewed in Hoegh-Guldberg et al. 2007; Baker et al. 2008) and decrease the ability of the corals to calcify (Kleypas et al. 1999, 2005; Langdon & Atkinson 2005). Although these environmental changes are occurring at an alarming rate, corals have exhibited an ability to survive in extreme temperatures (e.g., Oliver & Palumbi 2011;

Coles & Riegl 2013) and thus the future scenarios for coral reefs may include the potential for coral reefs to persist in a globally warmer climate. However, in order to accurately predict the potential for corals to withstand climate change, we must first examine the adaptability of the coral-algal symbiosis.

Coral-algal symbiosis

The exceptional productivity and diversity found on coral reefs would not be possible without the ancient mutualistic symbiosis between heterotrophic cnidarians and phototrophic algae. It has been suggested that hermatypic scleractinian corals have hosted algal symbionts since the mid-Triassic(Stanley & Swart 1995; Trench 1997). The evolution of efficient recycling between the heterotrophic host and the phototrophic symbiont (Muscatine & Porter 1977) permits tropical scleractinian corals to construct massive limestone skeletal structures in nutrient-depleted waters. The photosynthetic endosymbionts are found in host-derived vacuoles (symbiosomes) in the gastrodermal cells of their scleractinian coral host (Wakefield & Kempf 2001). *Symbiodinium* may translocate as much as 95% of their photosynthetically fixed inorganic carbon across the symbiosome to their coral host (Muscatine & Porter 1977; Muscatine 1990). This extra energy source is essential for reef building corals to maintain the calcification rates required for growth and the physical accretion of the reef (Pearse & Muscatine 1971; Gattuso et al. 1999; Allemand et al. 2004; Colombo-Pallotta et al. 2010).

Originally thought to be a single pandemic species, *Symbiodinium microadriaticum* (Freudenthal 1962), due to their similar gross morphology, the genus *Symbiodinium* is in fact very diverse. The genus is currently comprised of nine major

clades (categorized as A-I, Pochon & Gates 2010), with each clade containing multiple subclades, strains or types (reviewed in Coffroth & Santos 2005). The clades (and subclades) are predominantly differentiated based on their ribosomal gene sequences (Rowan & Powers 1991; LaJeunesse 2001, 2002; Baker 2003) and, more recently, with microsatellite and other high resolution analyses (e.g., Thornhill et al. 2009, 2013; Pettay et al. 2011). The high genetic diversity is also reflected in substantial levels of physiological diversity within and between clades. For instance, symbiont types differ in their response to light and temperature (Iglesias-Prieto & Trench 1994, 1997; Warner et al. 1999; Goulet et al. 2005; Loram et al. 2007a) suggesting that they may be suited to contrasting environmental conditions. These physiological differences can equate to a predictable distribution along gradients of light and temperature across reefs (Fabricius et al. 2004; Iglesias-Prieto et al. 2004) and even within single coral colonies (Rowan et al. 1997; Kemp et al. 2008). Certain symbiont types are also regarded as thermally tolerant, particularly members of clade D (Glynn et al. 2001; Rowan 2004; Tchernov et al. 2004; Berkelmans & van Oppen 2006), conferring their hosts with increased resistance to temperature related bleaching. Thermal tolerance is not the only aspect of coral fitness which may be influenced by the *Symbiodinium* type hosted; others include nutritional status and growth rate (Little et al. 2004; Cantin et al. 2009; Jones & Berkelmans 2010). The complex influence of the symbiont community on the coral holobiont (host, endosymbionts and associated microbes) is becoming increasingly clear as newer, more sensitive techniques reveal that many scleractinian corals can associate with multiple *Symbiodinium* taxa simultaneously (Mieog et al. 2007; Baker & Romanski 2007; Loram et al. 2007b; Correa et al. 2009; Silverstein et al. 2012). Furthermore, research indicates

that the whole community (dominant and background symbionts) may impact holobiont fitness, as corals hosting mixed assemblages of *Symbiodinium* spp. may have greater capacity to rapidly acclimatize or adapt to changing environmental conditions (Mieog et al. 2009; Silverstein 2012; Cunning 2013). Despite the historical longevity of this symbiotic partnership, it may still break down, but it is the ability to recover and reform this partnership that determines a corals capacity to survive.

Coral bleaching

The breakdown of the coral-algal symbiosis can be triggered by a range of environmental stressors such as changes in temperature, light intensity, salinity, or pollutants and sedimentation (Brown 1997). The loss of symbionts causes a visible paling of the host due to the reduction of algal and coral pigments. If conditions do not improve sufficiently for the reestablishment of the symbiotic relationship, the coral will die (Glynn 1996). Corals vary in their susceptibility to bleaching by species (Guest et al. 2012) and by symbiotic partnerships. For instance, corals hosting more thermally tolerant symbionts are more resistant to bleaching (Rowan 2004; Berkelmans & van Oppen 2006; Stat & Gates 2011). Consequently bleaching is often patchy across a reef, differentially affecting adjacent colonies (Rowan 1998; Hughes et al 2003).

Bleaching stressors invariably disrupt some portion of the symbionts' photosynthetic machinery, which can uncouple the photosystems (Iglesias-Prieto et al. 1992), causing a buildup of potentially damaging electrons (Jones et al. 1998; Warner et al. 1999) and reactive oxygen species (ROS). These ROS can then trigger a chain of events which ends with the loss of the symbiont (Lesser 1997; Weis 2008), potentially

leading to host mortality. Thermally tolerant *Symbiodinium* may moderate the effect of heat stress by reducing their light harvesting capacity (Jones & Berkelmans 2012) and by altering the lipid composition of their thylakoid membranes to decrease thermal sensitivity (Tchernov et al. 2004). These photochemical modifications may come at the cost of decreased photosynthetic capacity and thus less photosynthate for the host (Jones & Berkelmans 2010; Stat & Gates 2011), but under mildly elevated temperatures these tradeoffs may balance out (Cunning 2013). Therefore, acclimatizing to higher temperatures by shifting symbiont communities to host a more thermally tolerant symbiont may not be as detrimental as previously thought.

Free-living Symbiodinium

The majority of coral species reproduce by broadcast spawning (Baird et al. 2009) and thus begin their lives as aposymbiotic larvae. Each generation must therefore acquire *Symbiodinium* anew from the environment. Such horizontal acquisition of symbionts requires the existence of a diverse community of free-living *Symbiodinium* spp. The free-living *Symbiodinium* can be 15 times more abundant in the sediment than the water column (Littman et al. 2008) however many of the DNA sequences derived from water column samples represent novel *Symbiodinium* not previously found associated with corals (Hirose et al. 2008; Pochon et al. 2010; Zhou et al. 2012; Huang et al. 2013). Corals have been shown to regulate abundance by discharging healthy algal cells on a seasonal (Brown et al. 1999; Fitt et al. 2000; Ulstrup et al. 2008), daily (Jones & Yellowlees 1997; Fitt 2000), and density-dependent (Baghdasarian & Muscatine 2000) basis. The feces of predators feeding on corals have also been shown to contain live

Symbiodinium (Muller-Parker 1984; Castro-Sanguino & Sánchez 2012). These coral-associated *Symbiodinium* have been found in and around reef sediments (Manning & Gates 2008; Yamashita & Koike 2013), have been shown to be sources of symbionts for coral larvae (Adams et al. 2009; Abrego et al. 2009), and could also provide new symbionts for bleached corals (Lewis & Coffroth 2004; Coffroth et al. 2006; Coffroth et al. 2010; see Chapter 5).

Summary

This dissertation examines how environmental disturbances influence the *Symbiodinium* community structure in Caribbean reef corals. The first study examined how elevated temperatures affect the loss of specific symbiont types during bleaching, and how pCO_2 alters the recovery trajectories. Coupled to this is how these symbiont community dynamics alter the photophysiology of the *Symbiodinium* and ultimately, the growth rates of coral. A second study was designed to assess whether residual symbionts remaining in coral tissue after bleaching dictate reassembly of symbiont communities during recovery, or whether exogenous sources of *Symbiodinium* can also influence these recovery trajectories. Additionally, it also examined whether recovery trends are affected by temperature. Finally, after determining whether external sources of symbionts can influence the recovering symbiont community of bleached corals, this study tested whether the symbiont community in healthy adult corals can be altered using tissue transplant plugs from the same coral colony that contain different *Symbiodinium* types.

Collectively, these studies shed light on whether a single symbiont type, or a mixed assemblage of symbionts, improves coral resilience, and what the tradeoffs of hosting

thermally tolerant symbionts are. They also test potential methods for inoculating corals with different *Symbiodinium* that may be better suited to changing environments. In turn, the results of these investigations may assist conservation planners, coral reef resilience modelers, and coral nursery managers to better understand the potential response of coral reefs to climate change, and identify potential intervention actions that may enhance survival.

Chapter 2

Identity and abundance of residual *Symbiodinium* drive recovery trajectories of algal symbiont communities in bleached corals under different pCO_2 conditions

SUMMARY

Patterns of coral recovery from bleaching are often enigmatic, with some colonies, or parts of colonies, recovering from bleaching more quickly than others. To investigate whether these differences can be due to variation in the residual symbiont communities, and how recovery patterns might be affected by increased pCO_2, the recovery dynamics of algal symbiont communities (*Symbiodinium* spp.) were studied in bleached colonies of the Caribbean coral *Orbicella faveolata.* Twelve replicate cores from each of five colonies containing a mixture of *Symbiodinium* clades B+D (n=60 total) were exposed to a two-phase bleaching and recovery experiment in a controlled mesocosm facility. During the exposure phase, all corals were exposed to high light stress (peak irradiance ~850µmol/m^2/sec) as well as different combinations of temperature (27°C or 31°C) and CO_2 (380ppm or 800ppm) to produce bleached corals that contained the same number of residual symbionts (relative to the number of host cells), but different relative proportions of clade B and D *Symbiodinium*. Twelve replicate cores from each of five colonies hosting only *Symbiodinium* clade D were also exposed to the same treatments. After 49 days, light stress was removed and bleached corals were allowed to recover at control temperatures (27°C) under different pCO_2 conditions.

Using an actin-based symbiont:host (S:H) cell ratio as a proxy measure of symbiont density it was found that corals that initially contained only clade D bleached less (i.e., showed lower decreases in S:H ratio during irradiance stress) than corals that

were mixtures of B+D. In addition, temperature shifted symbiont community structure, with higher temperatures favoring retention of clade D over B, and control temperatures favoring clade B. During recovery, clade D symbionts proliferated faster in recovering coral tissue compared to clade B symbionts. However, the rate of recovery was fastest in corals that had originally contained mixtures of B+D, rather than those which were D-only, potentially because D-only corals had not bleached as severely. In the D-only cores, elevated pCO_2 promoted faster recovery rates of the symbiont population, perhaps due to increased availability of CO_2. However, in D-dominated B+D cores, recovery was faster in low pCO_2 than at high pCO_2, potentially reflecting convergence to lower equilibrium density at high pCO_2. Together, these results indicate that biotic factors, namely the identity and abundance of residual *Symbiodinium*, drive the initial recovery trajectories of symbiont communities in bleached corals, while abiotic factors, such as pCO_2, may define the optimal recovery density and influence rate of recovery.

BACKGROUND

Coral reef ecosystems are founded on the symbiotic relationship between scleractinian corals and their endosymbiotic single-celled algae (*Symbiodinium* spp.). This relationship is susceptible to numerous environmental perturbations and stressors including disease and pollution, as well as the threat of climate change, which is predicted to cause considerable damage to coral reef ecosystems (Hughes et al. 2003; Aronson & Precht 2006). The combined effects of elevated sea surface temperatures and increased atmospheric CO_2 are projected to increase the incidence and severity of coral bleaching (reviewed in Hoegh-Guldberg et al. 2007) and decrease the ability of the corals

to calcify (Kleypas et al. 1999, 2005; Reynaud et al. 2003; Langdon and Atkinson 2005). If marine conservation efforts to mitigate these effects are to be successful, it is imperative to gain a stronger understanding of the many processes involved in coral bleaching and, more importantly, recovery, in order to better predict where management and mitigation efforts should be concentrated.

Bleaching susceptibility varies greatly depending on environmental conditions (e.g., temperature and light intensity), coral species (as well as subspecies, morphotype, or genet), and *Symbiodinium* identity. *Symbiodinium* is a genetically diverse genus which is currently divided into 9 distinct clades (categorized as A-I, Pochon & Gates 2010) with each clade containing multiple subcladal types (Stat et al. 2006). The high genetic diversity is complemented by substantial levels of physiological diversity within and between clades. Certain symbiont types may confer greater stress tolerance (Rowan 2004; Berkelmans & van Oppen 2006; Jones et al. 2008; Fitt et al. 2009) while other types may provide more benefits to their host under non-stressful conditions (Little et al. 2004; Loram et al. 2007a). Corals may host mixed assemblages of *Symbiodinium* types, in varying proportions (Rowan et al. 1997; Silverstein et al. 2012), possibly as a way of offsetting the costs and benefits of different symbionts. Consequently there can be widespread variation in the extent of bleaching, both within and between coral colonies on a reef. Patterns of patchiness can also be observed in corals recovering from bleaching; however, the processes which determine why some corals recover faster than others, and why some do not recover at all, are not well characterized (see Baker et al. 2008 for review).

Traditional PCR-based techniques (such as DGGE) are not capable of accurately characterizing members of the *Symbiodinium* community that occur at very low densities and have consequently failed to detect low abundance symbionts in mixed symbiont communities (LaJeunesse et al. 2009). With the advent of more sensitive quantitative-PCR (qPCR) assays (Mieog et al. 2009; Cunning & Baker 2013), it is now possible to identify and quantify remnant symbionts remaining in bleached coral tissues and to examine how they influence recovery. This technique has been successfully used to investigate the effects of temperature on the recovery of the Caribbean coral *Orbicella faveolata* from thermal bleaching, and has shown that higher temperatures favor faster rates of recovery, and a higher relative abundance of thermally tolerant *Symbiodinium* in clade D (Cunning 2013). However, the effects of increased pCO_2 (and the resultant decrease in pH) on coral bleaching and recovery have yet to be investigated using this technique.

Under future climate change scenarios, warming sea surface temperatures are predicted to increase the frequency of bleaching events (Hoegh-Guldberg et al. 2007) and the combined stressor of ocean acidification (OA) is expected to exacerbate this problem (Reynaud et al. 2003; Anthony et al. 2008). Paradoxically, increased pCO_2 has also been predicted to improve the photosynthetic capacity of *Symbiodinium*, by increasing the amount of CO_2 available for their form II Rubisco (ribulose-1,5 bisphosphate carboxy-lase/oxygenase), which has a lower affinity for CO_2 (Rowan et al. 1996; Tortell 2000). Current evidence shows that, as with thermal stress, the effects of elevated CO_2 on cultured *Symbiodinium* are dependent on symbiont type (Brading et al. 2011). Therefore, in order to accurately predict how the different effects of elevated CO_2 may impact a

coral recovering from bleaching, we need to determine how CO_2 can affect different symbiont types *in hospite*.

Future bleaching stressors, such as high temperature and/or high light, will most likely be transient ("pulse" disturbances), but elevated pCO_2 levels are expected to remain elevated, even after the stressor subsides (a "press" disturbance). Therefore, it is important to determine how elevated pCO_2 influences symbiont community reassembly after bleaching, and also to ascertain whether differences in the identity and abundance of remnant *Symbiodinium* modify this influence. Here I undertook the first experiment to use sensitive qPCR techniques to study the effects of elevated pCO_2 on symbiont recovery dynamics in bleached coral colonies. For this experiment, I used *Orbicella faveolata,* an important reef-building scleractinian coral species in the tropical western Atlantic (Caribbean). The goal of this experiment is to improve our ability to predict how bleached corals may respond to elevated pCO_2, and how ocean acidification may influence bleaching recovery. A better understanding of how corals may adapt or acclimatize to forecast climate change (IPCC 2013) is vital for successful conservation efforts.

MATERIALS AND METHODS

Experimental set-up

All experiments were carried out in the Climate Change Laboratory at the University of Miami's Experimental Hatchery (UMEH) at the Rosenstiel School of Marine and Atmospheric Science (RSMAS) located on Virginia Key, Florida. The laboratory consisted of 12 experimental mesocosms shaded by neutral density

shadecloth/plastic-film roof which reduced natural sunlight intensity by ~40% (henceforth referred to as "high-light"). Each mesocosm comprised a 60 L exposure/treatment tank and a 500 L sump tank used as an equilibration chamber. Sump tanks were black to reduce internal algal growth. Mesocosms were independently controlled to maintain specific temperatures and partial pressures of carbon dioxide (pCO_2). Temperature was moderated by a 1500W titanium submersible heater and a chilled water heat exchanger actuated by a solenoid valve within each equilibration tank. Activation of the heating and chilling mechanisms was electronically controlled by temperature sensors in the exposure tanks. Tanks were checked daily for temperature and salinity with a YSI SCT meter and sensors were recalibrated as necessary. The desired partial pressures of carbon dioxide were generated in the equilibration chambers by the direct bubbling of air and CO_2 gas. Different pCO_2 levels were achieved using pairs of mass flow controllers (Sierra Instruments) which regulated the air/CO_2 mix sent to the equilibration chamber to generate the required concentrations. The quantity of dissolved gas was monitored continuously by equilibrator-LiCor infrared gas analyzer systems which fed data directly to the mass flow controllers for dynamic maintenance of the appropriate conditions. The seawater pumped from the equilibration chamber was pumped into the base of the tank and returned via a standpipe. Delivering a high flow of equilibrated water to the exposure tank without further aeration maintained the exposure tanks at the desired experimental conditions by considerably reducing the potential for gas exchange between the exposure tank and the environment. Seawater was dripped directly into the equilibration chambers fitted with an overflow to counteract evaporation

and other potential effects of a recirculating system. Seawater for all tanks was drawn from Bear Cut, Virginia Key, Florida, via a 250,000 L settling tank and a high flow sand filter.

Coral samples

Ten colonies (each >100cm^2) of *Orbicella faveolata* were collected from Key Largo (Ocean Reef Club, July 2008) and Emerald reef (east of Key Biscayne, May 2010) and maintained in shaded (~20% natural sunlight), outdoor, flow-through tanks at the UMEH-RSMAS. In June 2010, these colonies were cored using a 25 mm diameter diamond core drill bit (Stanley, CT, USA), mounted on a drill press equipped with a seawater cooling feed line, to generate 12 experimental coral cores per parent colony. The resultant cores were trimmed with a tile saw to a thickness of 5-10mm and then glued to 8cm^2 neutrally buoyant plastic tags and placed in a shaded flow-through tank at 27^{o}C for four weeks to recover from drilling.

Experimental conditions

The 12 mesocosms were used in a cross-factorial experiment with control (27^{o}C) and high (31^{o}C) temperatures, and control (380ppm) and high (800ppm) pCO_2 levels. Each treatment combination (CTCC - Control Temp, Control CO_2; CTHC - Control Temp, High CO_2; HTCC - High Temp, Control CO_2; HTCC - High Temp, High CO_2) was replicated three times. One randomly-selected core from each parent was assigned to each of the 12 tanks, such that the 10 parent colonies provided 10 cores per tank with a total of 30 cores per treatment. The corals then spent two weeks acclimating to their

respective experimental treatment tanks, which were shaded and set at control conditions of 27°C and ambient pCO_2 (380ppm). Following acclimation, the shading was removed and ramping of the experimental treatments began. Where required, the temperature and CO_2 partial pressure was incrementally raised by 0.5°C and 50ppm, respectively, over 8 days until the experimental treatment levels were reached. Corals were exposed to the full experimental conditions for 41 days, after which the temperatures in the tanks were returned to 27°C at a rate of 0.5°C per day, and the recovery of the corals monitored. The pCO_2 remained at experimental levels during this recovery period. Exposure tank conditions are summarized in Figure 2.1.

Light levels for the acclimation phase were approximately 30% of high-light, with daily maxima not exceeding 300 μmol/m^2/sec^{-1}. Once the ramping period began and the shading was removed, the cores experienced ambient high-light levels, reaching peak irradiance levels of ~850 μmol/m^2/sec^{-1}. The high-light exposure lasted for 7 weeks, after which the tanks were again shaded to 30% of high-light for the recovery period (Figure 2.1).

Once ramping began, water samples were taken every 2-3 days at the same time of day and analyzed for total alkalinity (TA) and pH during the course of the experiment to ensure pCO_2 remained at the desired levels. TA was determined using an automated open-cell Gran titration (Dickson et al. 2007, SOP3b), and compared to certified seawater reference material (A. Dickson, Scripps Institute of Oceanography) to verify accuracy. Seawater pH was determined using an Orion Ross combination pH electrode. Concentrations of CO_3^{2-}, Ca^{2+}, and Ω_a were computed from TA, pH, temperature, and salinity using the program CO2SYS (E. Lewis, Brookhaven National Laboratory).

The mounted coral cores and the tanks were cleaned weekly or as needed to reduce algal growth and limit their potential effect on water chemistry within experimental chambers. Corals were fed (Reef Chili®) every five days. Feeding took place in small plastic open containers which were floated in the tank to maintain the corals at experimental temperature. Feeding was carried out in the evening and lasted for approximately one hour.

Coral symbiont identification

All cores were biopsied at four time points during the experimental period: (1) at the start of the experiment (0 days of exposure); (2) six days after the ramping completion (2 weeks of exposure); (3) at the end of the exposure period (7 weeks of exposure); and (4) at the end of the experiment (5 weeks of shaded recovery). The DNA from each of the samples was extracted using a modified organic extraction protocol (Baker *et al*. 1997) which involved: incubating in 1% SDS at 65^{o}C, digestion with Proteinase K at 55^{o}C, incubation with 1% CTAB at 65^{o}C, mixing with equal volume of chloroform at room temperature, 100% ethanol precipitation at -20^{o}C, re-suspension of dried pellet in 0.3M sodium acetate and subsequent ethanol precipitation at -20^{o}C, washing pellet in 70% ethanol, re-suspension of dried down DNA pellet in Tris-EDTA buffer, and storage at -20^{o}C.

DNA from the *Symbiodinium* internal transcribed spacer-2 (ITS2) region of ribosomal DNA (rDNA) was PCR-amplified using primers ITSintfor2 and ITS2clamp (LaJeunesse and Trench 2000) targeting the conserved flanking regions in the 5.8S subunit and 28S-like large subunit. The reverse primer incorporated a 40 base pair GC-

clamp. 25 μL PCR reactions contained 1 μL template DNA, 10 mM forward and reverse primer, 1 X Promega Go-Taq buffer, 2mM $MgCl_2$ and 0.8mM dNTPs (Fermentas, USA). A touch-down amplification protocol was used with an initial annealing temperature 10°C above the final annealing temperature of 52°C. The annealing temperature was decreased by 1°C every two cycles until 52°C was reached, where it remained for a further 15 cycles. PCR was performed using a DYAD thermocycler (MJ Research). Denaturing gradient gel electrophoresis (DGGE) of the ITS2 amplicons was used to identify the dominant *Symbiodinium* for each coral microcolony during the experiment. PCR products were run on 35-75% denaturing gradient polyacrylamide gels (100% denaturant consisted of 40% deionized formamide and 7M urea) using a CBS Scientific System (Del Mar, CA, USA) for approximately 16 hours at 90 volts. Bands of interest were excised and left in 100 μL of molecular grade H_2O (Rockland, PA, USA) overnight at 4°C. One μL of the resulting DNA solution was then used as the DNA template in a PCR amplification using the same primers (with GC-clamp removed from reverse primer) and reaction mix as above. The thermocycling profile consisted of 3 minutes at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, and a final extension of 5 minutes at 72°C. Amplifications were checked on a 1.5% agarose gel to ensure they produced bright, single bands. Once checked, PCR products were treated with a mixture of Exonuclease I and Shrimp Alkaline Phosphatase to deactivate the polymerase and digest the primers (37°C for 30 minutes and 80°C for 15 minutes). Forward and reverse strands were then amplified using BigDye (Applied Biosystems, Foster City, CA) terminator chemistry. The 10 μL reactions were amplified using 35 cycles of 96°C for 15s, 50°C for 15s and 60°C for 4 minutes. Big Dye amplified sample

sequences were cleaned using Sephadex G-50 (GE Life Sciences), dried down and reconstituted in 10 μL HiDi (Applied Biosystems). Products were sequenced on an ABI 3730. Sequence chromatograms were manually inspected and assembled using Geneious (Biomatters Ltd.). Each sequence type was compared with named sequences in the Genbank database using the BLAST search tool.

Symbiont community characterization

An actin-based quantitative-PCR (qPCR) assay was used to monitor change in symbiont community structure (identity and abundance) in *O. faveolata* during the course of the experiment. Samples were assayed in duplicate using TaqMan-MGB (Life Technolgies) qPCR assays targeting specific actin loci in *O. faveolata* and *Symbiodinium* in clades B, C, and D (Cunning & Baker 2013; Cunning 2013). The *O. faveolata* assay included 150 nM OfavActF (5'- CGCTGACAGAATGCAGAAAGAA-3'), 100 nM OfavActR (5'- CACATCTGTTGGAAGGTGGACA-3'), and 250 nM OfavActProbe (5'-NED-TGAAGATCAAGATCATTGC-MGB-3'). The clade B assay included 200 nM BActF (5'- CGATGGCTTTGCAGCTCAA-3'), 300 nM BActR (5'-TGTCCCGGAATTTGTGCAA-3'), and 100 nM BActProbe (5'-FAM-CTGTAAACCCTGTCATTC-MGB-3'). The clade C and D assays were multiplexed, using the same primers and reaction conditions as described in Cunning & Baker (2013). All reactions were carried out in 10 μL volumes (with 5 μL Taqman Genotyping MasterMix and 1 μL DNA template) on StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Thermal cycling conditions consisted of an initial incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s

and 60°C for 1 min. Cycle threshold (C_T) values were calculated by the StepOnePlus software package with a set fluorescence threshold of ΔR_n=0.01.

Target DNA was considered present when amplification occurred in both technical replicates with no target detected in negative control reactions. C_T values for *O. faveolata* and *Symbiodinium* clade B were reduced by 5.01 and 4.03 cycles, respectively, to normalize for differences in fluorescent signal intensity among TaqMan-MGB fluorophores. Cycle reductions were calculated based on the results of standard curves generated following the methods of Cunning and Baker (2013). Adjusted C_T values were then used to calculate symbiont to host cell ratios using the formula $2^{C_T(\text{host})-C_T(\text{symbiont})}$. Clade C symbionts were not detected in these samples and thus were not involved in subsequent analyses.

Statistical analyses

The software package JMP (SAS Institute Inc.) was used to generate multiple regression models for the analysis of symbiont community dynamics. The models used total symbiont to host cell ratios (clade D S:H + clade B S:H) or percent of clade D (clade D S:H / total S:H) as the response variable, parent as a random variable, and previous total S:H (or %D) as a fixed factor. There was no tank effect (JMP Least squares fit, p-values range from 0.31 to 0.97) so treatment replicates were pooled. Adjusted mean values were calculated that controlled for parent effect and were tested for significance ($p< 0.05$) using *post hoc* Tukey's honestly significant difference (HSD) tests. To

normalize the data prior to statistical analyses, symbiont to host cell ratios were log-transformed and proportional data (*e.g.*, percent D and percent bleached) were arcsine square root transformed.

RESULTS

Initial symbiont communities

DGGE analysis showed that, prior to the start of the experiment, cores identified as D-only hosted *Symbiodinium* ITS2-type D1a, while the mixed assemblage B+D cores hosted types D1a and B1. Both these symbiont types have previously been found in *O. faveolata* (LaJeunesse 2002). QPCR analysis of the initial cores showed that five of the 10 colonies contained 100% clade D (i.e., no other symbionts were detected), and five colonies hosted mixtures of clade B and clade D symbionts ranging from ~20% D to ~90% D (Figure 2.2). No other symbiont types were detected, so the subsequent quantitative PCR (qPCR) analysis used primer and probe sets for clades B and D only. During the course of the experiment, cores from one D-only colony (P10) experienced high mortality across all treatments, and consequently data from P10 cores were excluded from subsequent analyses.

Parental effect on symbiont community composition

Mean symbiont:host-cell (S:H) ratio of D-only cores was ~32% higher than B+D cores at the start of the experiment (0.0745 ± 0.0069 compared to 0.0508 ± 0.0056, p=0.006) (Figure 2.3b). Multiple regression analysis revealed that 65% of the variation in the initial S:H ratio of B+D cores was explained by parent colony, compared to just 18%

in D-cores (Figure 2.3a). Multiple regression models to test for treatment effects controlled for this parental variation, and the following results denote the adjusted means outputs from these models.

Bleaching due to high irradiance

During the first two weeks of the exposure period, high-light stress resulted in visible bleaching in all colonies, across all treatments. Mean S:H ratios in D-only cores and B+D cores declined by 73% and 84%, respectively (Figure 2.4a,b). Bleaching continued during the remaining five weeks of the exposure period, although the rate of symbiont loss decreased. After seven weeks of high-light stress, the mean S:H ratio for D-only cores had declined to 87% of the initial S:H ratio, and by 94% in B+D cores. Mean S:H ratios remained significantly higher ($p<0.0001$) in D-only cores compared to B+D cores throughout the exposure period (Figure 2.3b). However, there was no effect of temperature or CO_2 on either the rate or severity of bleaching (Figure 2.4a,b).

D-only cores remained 100% clade D throughout the course of the experiment, but there were temperature-dependent shifts in the symbiont community structure of B+D cores. The mean initial proportion of clade D in the B+D cores was 53% (± 16%), but after seven weeks exposure to high-light the mean percent D in cores at 31^{o}C had increased to 75% (± 5.9%) (matched pairs test, $p=0.001$). Conversely, at control temperatures, the symbiont community of B+D cores had decreased to 39% (± 5.9%) clade D (Figure 2.5).

To ascertain whether B+D cores lost *Symbiodinium* from clade B and D evenly, the proportion of clade B and clade D lost from each core was calculated using the

following equation: log(S:H bleached / S:H initials) (Figure 2.6). Matched pairs tests (performed in JMP) indicated no significant difference in the decrease of B or D in the control temperature treatments; whereas, at 31^{o}C significantly more clade B symbionts were lost compared to clade D ($p=0.0006$). Matched pairs tests also implied that at higher temperatures, elevated pCO_2 enhanced the disproportionate loss of clade B over D ($p=0.049$).

Symbiont community reassembly

At the start of the 5-week recovery period, temperature and light were returned to acclimation levels (ramped to 27^{o}C at 0.5^{o}C per day, and 30% high-light). During this recovery period, mean S:H ratios increased across all treatments (Figure 2.4a,b; $p<0.01$ all comparisons). For the D-only cores, the temperature experienced during bleaching had no effect on the rate of symbiont community reassembly after bleaching, however elevated pCO_2 did increase the rate of symbiont recovery (as measured by S:H ratio, $p=0.02$, Figure 2.4a). The mean S:H ratios of B+D corals that had been exposed to 31^{o}C were significantly higher than those at 27^{o}C ($p<0.0001$, Figure 2.4b). In addition, among those corals that had been exposed to 31^{o}C, increases in S:H ratios were significantly higher in control $pCO2$ compared to elevated ($p=0.02$, Figure 2.4b). To determine the degree of recovery, S:H ratios were standardized to their initial S:H ratio using the following equation: log(total S:H after recovery / total initial S:H). The B+D cores that experienced high temperatures were the only corals whose mean S:H ratio exceeded their initial value (Figure 2.6).

In the B+D corals under control conditions (CTCC) clade B symbionts reassembled faster after bleaching than clade D (matched pairs test, $p=0.005$). However, the cores in the CTHC treatment had significantly more clade D ($p=0.03$) by the end of the recovery period (Figure 2.5). Corals that had been exposed to 31^{o}C also finished the experiment with significantly more clade D than B ($p<0.0001$), although there was no effect of CO_2 (Figure 2.5).

At the end of the bleaching period there was no difference between the mean total S:H ratios in the B+D cores (Figure 2.4b), but there were significant differences in symbiont community structure (Figure 2.5). To determine how the residual symbiont community structure in bleached cores influenced recovery, the increase in each cores total S:H ratio from "bleached" to "recovering" was plotted against the percent D they hosted at the end of the exposure period (Figure 2.7). Linear regression analysis of the B+D cores showed a positive correlation between the percentage of D hosted when bleached and the extent of the symbiont community reassembly by the end of the recovery period ($p<0.0001$).

DISCUSSION

This experiment used high irradiance stress, combined with additional temperature and/or CO_2 treatments, to create bleached cores of *Orbicella faveolata* that contained similar densities of symbionts (as measured by S:H cell ratio), but which differed in their symbiont community composition. These experimental cores were then allowed to recover under different conditions of temperature and CO_2 to study how these climate change stressors influenced symbiont community recovery.

Response of different symbionts to high irradiance bleaching

The *Orbicella faveolata* hosting D-only had the highest initial S:H ratios, and bleached less than the B+D cores, as measured by reductions in S:H ratio (Figure 2.3b). In the B+D corals, the cores with a higher initial abundance of clade B had greater symbiont loss ($p=0.04$), and the percentage of D in the symbiont community was positively correlated with increased resistance to bleaching ($p=0.0003$). These results are consistent with the characterization of symbionts within clade D being thermally tolerant as they are often present in greater abundance following a bleaching event (Baker et al. 2004; Berkelmans and van Oppen 2006; LaJeunesse et al.2009). A likely explanation is that the ITS2-type D1a *Symbiodinium* are more resistant to photoinhibition than the more thermally sensitive B1 (McGinty et al. 2012), produce less reactive oxygen species (ROS), and elicit a weaker coral bleaching response (Downs et al. 2002; Lesser 2006; Weis 2008).

The fastest rates of bleaching occurred during the first 14 days of high-light stress (Figure 2.4a, b). In the B+D cores this initial bleaching appeared to be indiscriminate, with no apparent selectivity regarding which symbiont type was lost (Figure 2.5, days 0-14). As the rate of bleaching decreased throughout the remaining exposure period, symbiont loss became discriminatory and temperature dependent (Figure 2.5, days 15 to 49). Multiple regression analysis of the bleached cores indicated that symbiont densities in the B+D cores (as measured by S:H ratio) were similar across all treatments (Figure 3b)($p>0.05$), but the proportional losses of clade B and D differed between treatments (Figure 2.6). In the high temperature treatments clade B symbionts were lost more rapidly than clade D (matched pairs test, $p=0.0006$), and elevated pCO_2 caused additional loss,

with even fewer clade B symbionts retained in the HTHC treatment compared to HTCC ($p=0.01$). Proportional changes in the symbiont community occurred during bleaching, which implies that clade B symbionts were preferentially lost in favor of clade D at 31°C. The instability of the association between *O. faveolata* and *Symbiodinium* B1 at 31°C is likely due to the thermal sensitivity of these symbionts at this elevated temperature (Robison & Warner 2006) and the resultant increase in ROS production (McGinty et al. 2012).

Selective preservation and/or release of specific symbiont types has been documented in unstressed corals (Yamashita et al. 2011), but this is the first time that differential rates of loss for distinct symbiont types have been calculated in actively bleaching corals. Further quantification of these changes may improve our understanding of how different symbiont types vary in their stress tolerance.

Effect of residual symbionts on recovery trajectories

In corals that contained mixed communities of symbionts, temperature had a considerable influence on symbiont community structure, with symbiont proportions differing significantly between temperature treatments at the end of the exposure period (Figure 2.5). However, all treatments lost similar proportions of their symbiont populations, with mean S:H ratios ~93% lower than their initial values. Although they bleached less severely than the B+D cores and remained 100% D, the D-only cores also had similar treatment means at the end of the exposure period. Therefore, the influences of symbiont community structure and pCO_2 on recovery from bleaching were examined, having controlled for the effect of differences in remnant symbiont abundance.

B+D corals from the CTCC treatment were the only cores to significantly increase their proportion of clade B during the exposure phase (34% D ± 8%), and the dominance of B increased further during recovery (19% D ± 11%). This increase in clade B suggests that, without added temperature or pCO_2 stress, high light intensity was less detrimental to *Symbiodinium* B1 than to D1a. However, despite a similar abundance of remnant *Symbiodinium* at the beginning of the recovery period, the re-proliferation of symbionts in clade B-dominated corals was slower than D-dominated cores (Figure 2.4b, Figure 2.7). In their natural habitat, this slow recovery could have adverse effects on *O. faveolata* survival after bleaching; and if the corals do fully recover, having a higher density of clade B symbionts may also lower their resistance to future bleaching events, especially at elevated temperatures.

Symbiont communities in *O. faveolata* B+D cores that had been exposed to 31^{o}C recovered faster ($p<0.0001$) than their counterparts at control temperature (Figure 2.4b). Corals at 31^{o}C lost the most clade B during bleaching and also showed no increase in clade B during recovery (Figure 2.6). They also recovered with the same as (or more) symbionts than they had prior to bleaching, because their symbiont communities were now dominated by clade D, which typically has higher equilibrial densities than clade B (Figure 2.4a,b – HTCC and HTHC). Thus, change in symbiont communities may be another reason why corals at higher temperatures appear to recover more quickly than those at lower temperatures. Not only do higher temperatures promote more rapid symbiont proliferation, but they also promote clade D which, due to their relatively smaller sizes (Cunning & Baker 2013) typically occur at higher standing stocks than other symbionts.

Effect of CO2 on recovery

Although elevated pCO_2 did not influence bleaching, it did accelerate symbiont community reassembly in the bleached D-only corals (Figure 2.4a). Symbiont proliferation in D-only cores was significantly faster in the LTHC and HTHC treatments than in the CTCC control. Higher CO_2 may improve photosynthetic efficiency of *Symbiodinium* (Crawley et al. 2010; Brading et al. 2011), particularly since they have an unusual form II Rubisco (Rowan et al. 1996) which has a lower specificity for CO_2. Thus, higher CO_2 may facilitate more rapid recolonization of host coral tissue. However, as noted by Brading et al. (2011), elevated pCO_2 did not increase the recovery of *Symbiodinium* B1, in fact it appeared to hinder it. The mean percent D of B+D cores in the CTHC treatment was significantly higher than in CTCC, but the S:H ratios were similar (Figure 2.4b and 2.5), indicating that D1a was proliferating faster than B1.

In the B+D cores from the high temperature treatments the population of clade B symbionts was significantly reduced, yet these D-dominated corals did not recover in the same manner as the D-only corals. The D-only cores exhibited a positive response to elevated pCO_2, whereas the mean S:H ratio for B+D cores in HTHC was actually lower than the HTCC treatment (Figure 2.4b). At the end of the recovery period the mean S:H ratio for B+D cores in HTCC was the highest of all treatments (including D-only cores in elevated pCO_2). This S:H ratio exceeded the mean initial symbiont density of B+D cores, and it was similar to the mean initial S:H ratio for D-only cores. This implies that these cores had recovered to a new optimal density which was driven by their new clade D dominated symbiont community. The mean S:H ratio of B+D cores in HTHC was also higher than their initial value, yet still lower than HTCC. I hypothesize that the symbiont

communities in these cores had also recovered, and thus the lower S:H ratio at 800ppm reflected convergence to a lower equilibrium density (Muehllehner 2013) due to greater symbiont efficiency under elevated pCO_2.

Conclusions. Symbiont community composition was found to be critical to bleaching recovery following exposure to light, temperature and CO_2 stressors. Corals hosting clades B+ D bleached more severely than the D-only cores, but they also exhibited a temperature-dependent selective retention of clade D symbionts. Once light stress was removed, clade D symbionts proliferated faster in recovering coral tissue compared to clade B. B+D cores exposed to 31^{o}C recovered fastest from high irradiance bleaching, possibly because the D-only cores had not bleached as severely. In the D-only corals, elevated pCO_2 had a positive impact on the rate of recovery, but in the 31^{o}C B+D cores recovery was lower at high pCO_2, perhaps reflecting convergence to lower equilibrium density. This study shows that biotic factors, namely the identity and abundance of residual *Symbiodinium*, drives the recovery trajectories of symbiont communities in bleached corals, while abiotic factors, such as pCO_2, drive recovery at different rates and may define an optimal recovery density.

Figures:

Figure 2.1 Experimental conditions experienced by coral cores within the 4 treatments for the duration of the experiment: CTCC (Control Temp, Control CO_2); CTHC (Control Temp, High CO_2); HTCC (High Temp, Control CO_2); HTCC (High Temp, High CO_2). * denote tissue sampling time points.

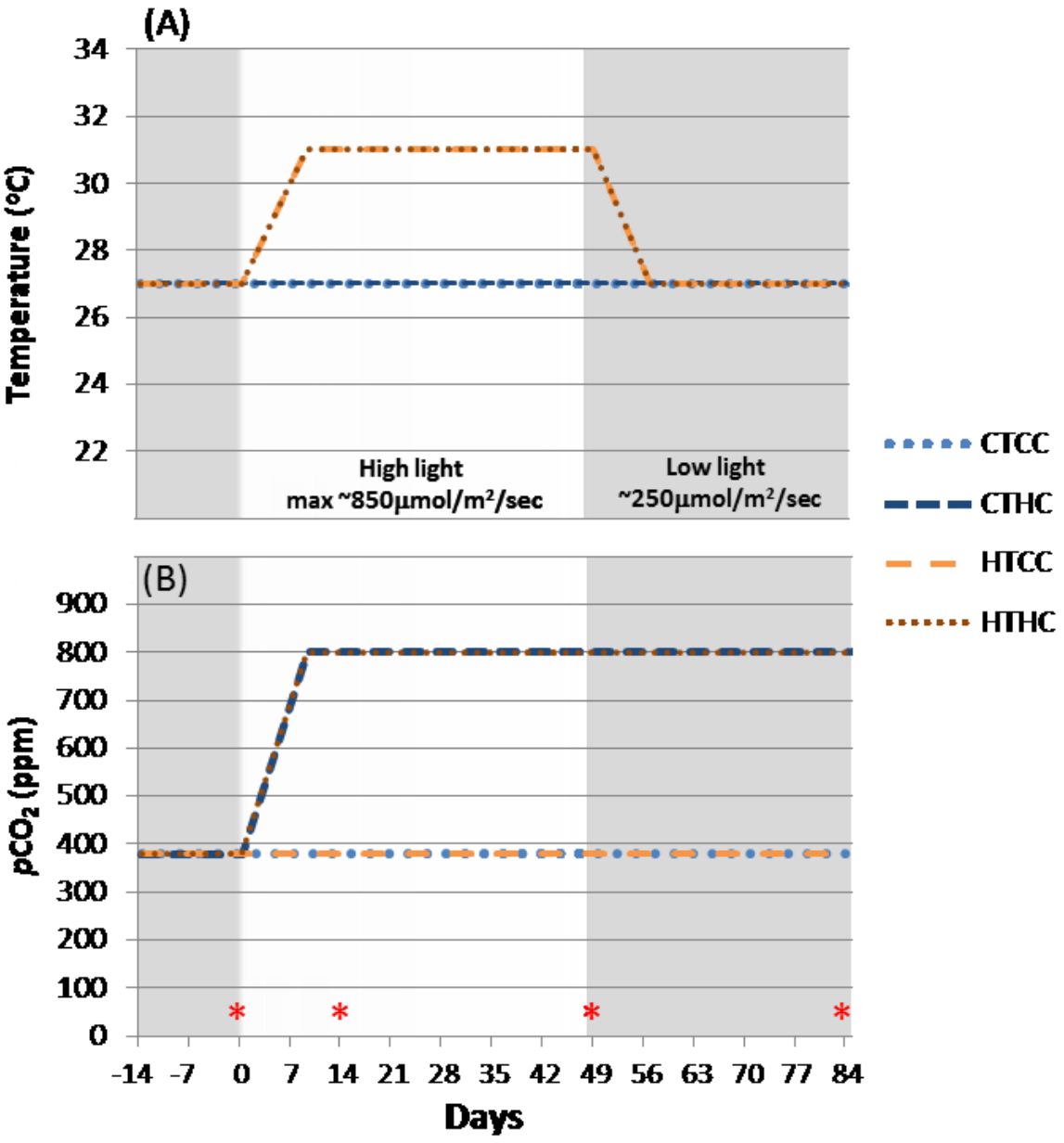


Figure 2.2 Mean symbiont:host (S:H) cell ratios for clade B (blue) and clade D (red) *Symbiodinium* within each of 9 parent colonies (P1-P9) prior to the start of the exposure period. Means are calculated from 12 replicate cores of each parent colony. Error bars display standard errors. Colonies P1-P5 were pooled as mixed "B+D" colonies, while P6-P9 were pooled as "D-only" colonies.

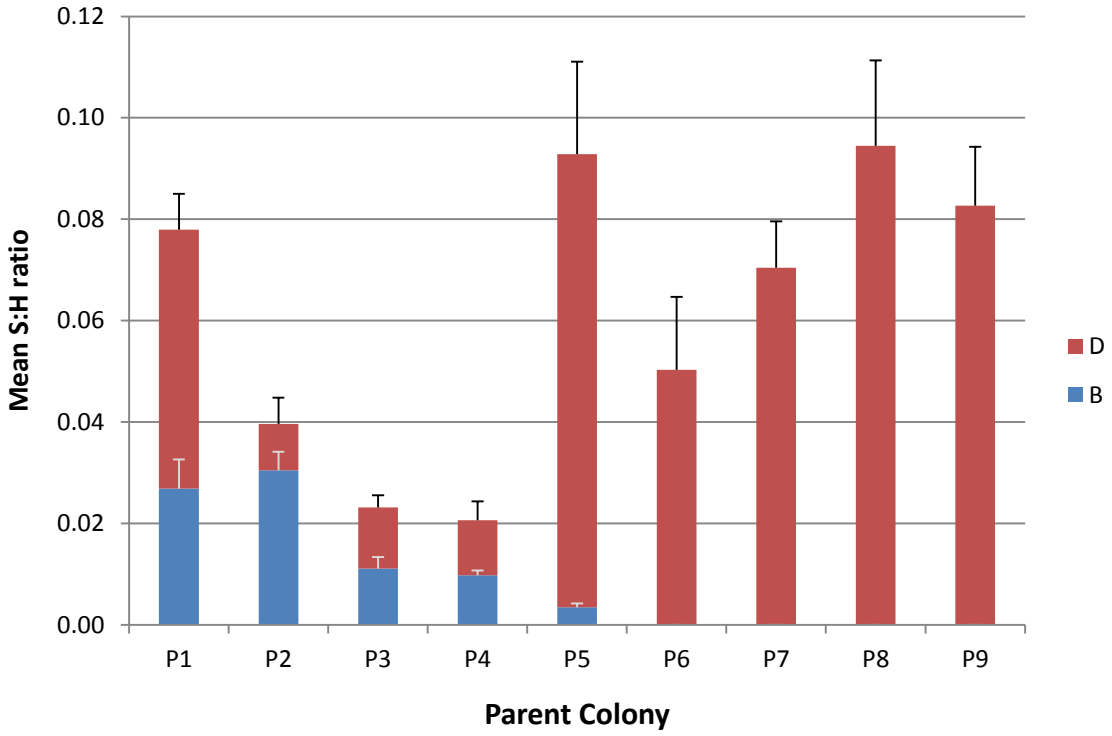


Figure 2.3 Effect of parent colony, as well as symbiont community, on changes in total S:H ratio (sum of clade B and clade D) during exposure (white background) and recovery (grey background) periods. Mean S:H ratios are shown for cores from (A) each parent colony and (B) for corals pooled by D-only (red) and B+D (blue) mixed symbiont communities. Error bars display standard error, B+D n = 60, D-only n=48.

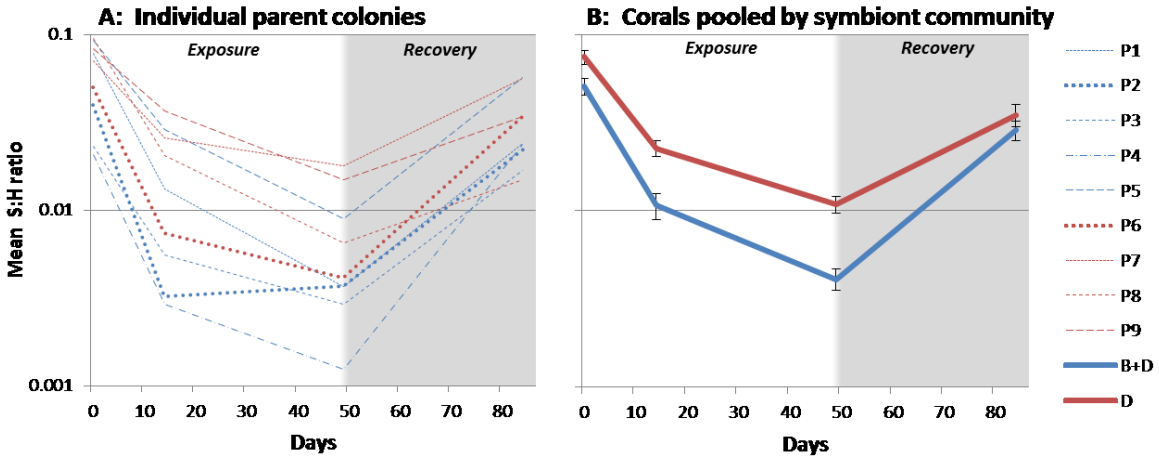


Figure 2.4 Impacts of high-light exposure (white background) on the S:H ratios of *O. faveolata* cores under different temperature and pCO_2 treatments, and influences of elevated pCO_2 on subsequent shaded recovery (grey background). Plots show S:H ratio adjusted means (after controlling for parent effects) for (A) D-only cores and (B) B+D cores in each of the 4 treatments: CTCC (Control Temp, Control CO_2); CTHC (Control Temp, High CO_2); HTCC (High Temp, Control CO_2); HTCC (High Temp, High CO_2). Elevated pCO_2 influences recovery in D-only cores, whereas prior temperature exposure drives community reassembly in B+D cores. Error bars display standard error, D-only n = 12, B+D n = 15

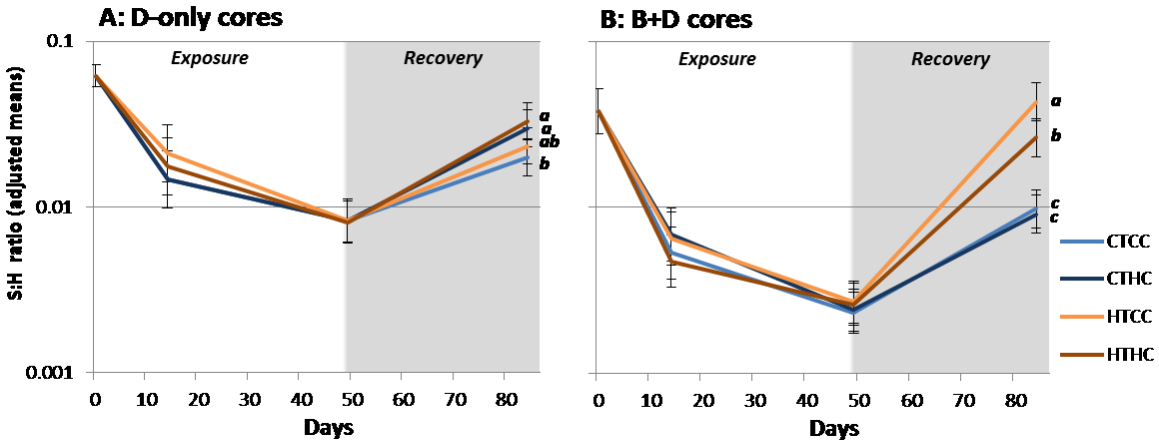


Figure 2.5 Change in proportions of clade D in bleached and recovering *Symbiodinium* communities in response to temperature and pCO_2. Mean of B+D core adjusted means from multiple regression models, pooled by treatments: CTCC (Control Temp, Control CO_2); CTHC (Control Temp, High CO_2); HTCC (High Temp, Control CO_2); HTCC (High Temp, High CO_2). High temperature shifts symbiont community in favor of clade D, whereas control conditions promote dominance of clade B. Error bars display standard error, n = 15.

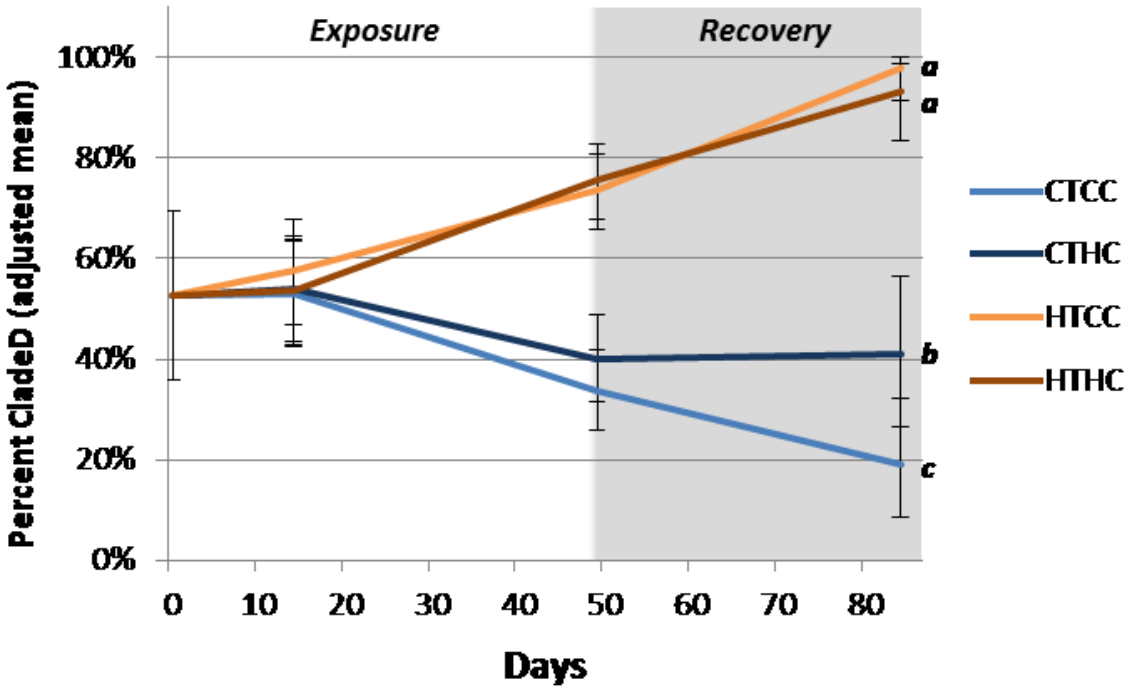


Figure 2.6 Effects of treatment on bleaching and recovery of clade B and D *Symbiodinium* in B+D cores. Graphs show the mean relative proportions of (A) clade B and (B) clade D in bleached (open circles) and recovering (filled circles) cores, standardized to their initial S:H ratio. Proportions were calculated for each core as log(S:H bleached / S:H initial) and log(S:H recovering / S:H initial) and were then pooled by treatment. Positive values signify S:H ratios which were higher than the pre-bleaching values. In elevated temperature treatments clade D recovered with a higher S:H ratio than it had initially, whereas clade B did not increase from its bleached abundance. In control temperature treatments clade B recovered more than D, but neither reached their initial S:H ratios.
CTCC (Control Temp, Control CO_2); CTHC (Control Temp, High CO_2); HTCC (High Temp, Control CO_2); HTCC (High Temp, High CO_2). Error bars display standard error, n=15.

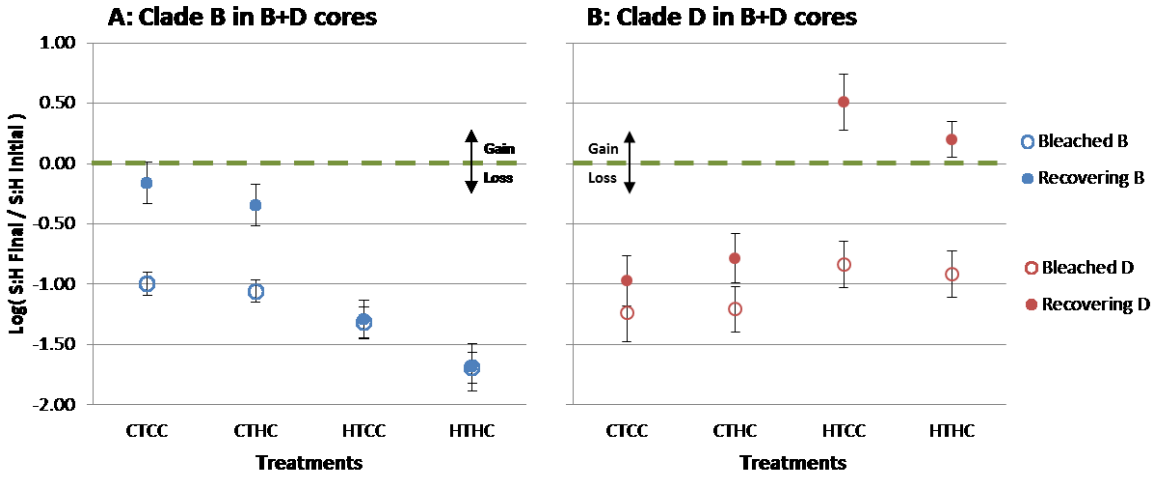
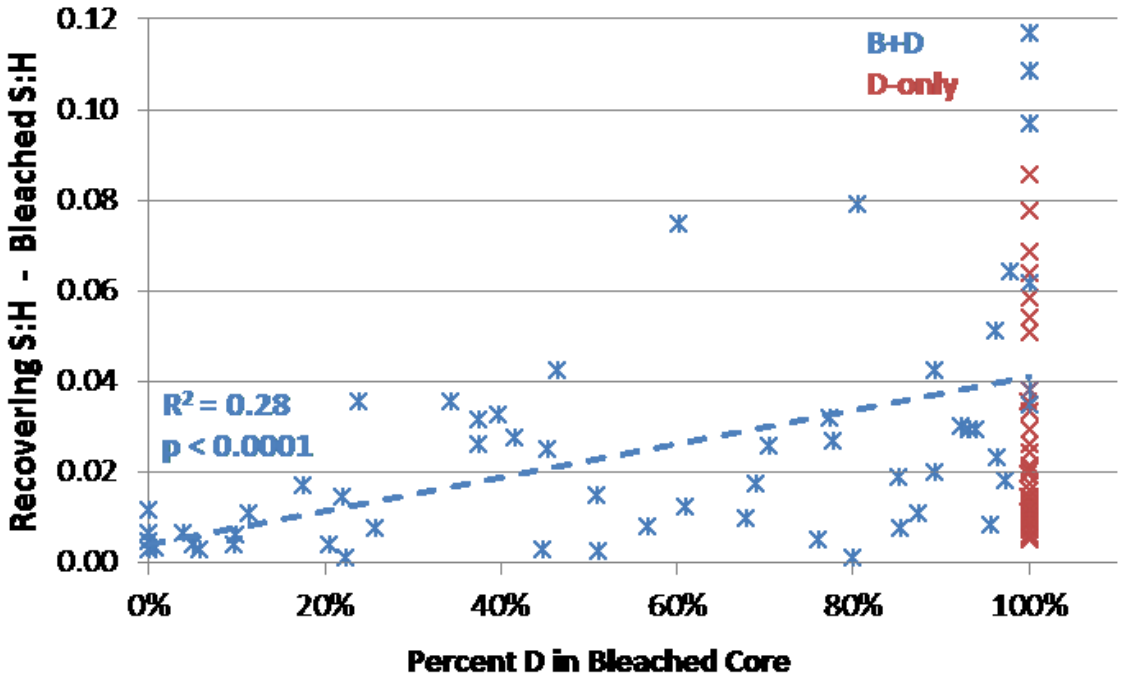


Figure 2.7 Effect of residual symbiont community structure on recovery of bleached cores. Chart shows the increase of each core's total S:H ratio from "bleached" to "recovering", plotted against the percentage of D they hosted at the end of the exposure period. Linear regression analysis (blue dashed line) indicated a positive correlation between the percent D in bleached B+D (blue stars) cores and the extent of their symbiont community reassembly. D-only (red cross) cores hosted 100% D when bleached and recovering, hence no trendline.



Chapter 3

Physiological responses of *Orbicella faveolata* and their symbiont communities to combined effects of light, temperature and pCO_2

SUMMARY

To assess the effect of elevated pCO_2 on bleaching resistance and resilience, 12 replicate cores from each of 10 colonies of *Orbicella faveolata* (hosting either clade D symbionts only or mixtures of clades B and D) were bleached by exposure to high light stress for 7 weeks, while also experiencing different temperatures (27°C and 31°C) and pCO_2 levels (380ppm and 800ppm). Experimental light and temperature stresses were then removed to allow corals to recover under different pCO_2 levels. Experimental cores were monitored for changes in calcification (measured by buoyant weight), symbiont community structure (as measured by symbiont:host cell ratio), and photosystem II efficiency (F_v/F_m).

During the first 2 weeks of exposure to high light stress, symbiont:host (S:H) cell ratios and photochemical efficiency declined dramatically, independent of temperature and CO_2 treatment. D-only cores showed smaller declines in S:H ratio than B+D cores, but a greater decline of F_v/F_m. Coral cores also exhibited skeletal dissolution, this was greatest in D-only cores, and lowest in B+D cores in 27°C/380ppm. After two weeks of exposure, D-only cores at both temperatures, and the B+D cores at 27°C, showed signs of photoacclimation. B+D cores at 31°C shifted their symbiont communities in favor of clade D, but did not photoacclimate during the exposure period. Despite photoacclimating, D-only cores exhibited net skeletal dissolution during the 7 week high-light exposure, independent of temperature, indicating that light-stress was the primary

cause. The B+D cores at 31°C also finished the exposure period with a net weight loss, while those at 27°C were the only cores that increased their weight. Changes in photochemical efficiency matched changes in growth rate, but temperature and symbiont abundance also influenced coral growth. Corals that had been previously exposed to 31°C grew faster during the recovery phase than those that had been maintained at 27°C. This faster growth rate was reduced by elevated pCO_2 in the D-only cores from 31°C. During recovery, the B+D cores hosting >95% D after bleaching exhibited the fastest calcification rates and also had the highest S:H ratios. The B-dominated cores had five-fold lower S:H ratios, but only two-fold lower calcification rates, suggesting that their symbionts translocated more of their photosynthate to the host, rather than utilizing it for proliferation. The results of this study show that elevated pCO_2 increases symbiont proliferation after bleaching in D-only cores, but reduces their photochemical efficiency and calcification rates (relative to other D-only cores at 31°C). In corals hosting clade B and D, higher pCO_2 appeared to reduce calcification in cores from the temperature treatments, and although not significant, this effect was more pronounced in B dominated cores.

Clade D symbionts were shown to be more beneficial than clade B for corals recovering from thermal bleaching stress, but that clade B offer their host more energy per symbiont under non-stressful conditions. Therefore, maintaining diverse symbiont communities allows corals to attain maximal growth rates at normal temperatures, and yet still recover quickly from bleaching. Such symbiont communities may be key components of coral resilience in the face of continued climate change.

BACKGROUND

The symbiotic relationship between reef corals and single-celled dinoflagellates (*Symbiodinium* spp.) forms the foundation of coral reef ecosystems. This essential relationship is susceptible to numerous environmental perturbations and stressors such as disease, overfishing, and pollution, as well as the threats of climate change, which are predicted to cause considerable damage to coral reef ecosystems (Hughes et al. 2003; Aronson & Precht 2006). The combined effects of elevated sea surface temperatures and increased atmospheric CO_2 are projected to increase the incidence and severity of coral bleaching (reviewed in Hoegh-Guldberg et al. 2007; Baker et al. 2008) and decrease the ability of the corals to calcify (Kleypas et al. 1999, 2005; Reynaud et al. 2003; Langdon and Atkinson 2005). It is therefore of paramount importance to gain a stronger understanding of the many processes involved in coral bleaching and, more importantly, recovery, in order to better predict how corals will reacted to climate change conditions, to assist in determining where efforts marine conservation efforts should be concentrated.

Coral bleaching is the visible outcome of the breakdown in symbiosis between *Symbiodinium* and their coral host. Susceptibility to bleaching varies greatly depending on the environmental conditions (e.g. temperature and light intensity), the coral species (as well as subspecies, morphotype, or genet), and the *Symbiodinium* they host. *Symbiodinium* is a genetically diverse genus which is currently divided into 9 distinct clades (categorized as A-I, Pochon & Gates 2010) with each clade containing multiple subclades, strains or types (Stat et al. 2006). The high genetic diversity is complemented by substantial levels of physiological diversity within and between clades. Certain symbiont types may confer greater stress tolerance (Rowan 2004; Berkelmans & van

Oppen 2006; Jones et al. 2008; Fitt et al. 2009) while other types may provide more benefits to their host under non-stressful conditions (Little et al. 2004; Loram et al. 2007a). Therefore, symbiont community structure strongly influences a corals' ability to resist bleaching stressors, as well as its resilience to recover from them. While there may be tradeoffs associated with these benefits, these tradeoffs may be different depending on the prevailing environmental conditions. For example, corals hosting only the thermally tolerant *Symbiodinium* ITS2-type D1a have been shown to grow more slowly under non-stressful conditions (Little et al. 2004; Cantin et al. 2009; Jones and Berkelmans 2010), but even slight elevations in temperature can reduce this disadvantage (Cunning 2013).

Symbiont communities are not static entities (Brown et al. 1999; Ulstrup et al. 2008) and corals can host mixed assemblages of *Symbiodinium* types, in varying proportions (Rowan et al. 1997; Silverstein et al. 2012). A dynamic symbiont community would have a greater capacity to rapidly acclimate to new environmental conditions, and therefore natural environmental variability or stressors may cause shifts in the abundance and/or identity of particular *Symbiodinium*. As a result of the diverse benefits and concessions awarded from hosting particular symbionts, monitoring these community shifts may provide vital information regarding how corals respond to bleaching stressors.

Thermally induced coral bleaching has been observed throughout the range of coral reefs (reviewed in Baker et al. 2008). Elevated pCO_2 alone has also been shown to illicit a bleaching response (Anthony et al. 2008), and therefore the combined stressors of elevated pCO2 and ocean warming is expected to exacerbate the problem (Reynaud et al. 2003). Paradoxically, increased pCO_2 has also been predicted to improve the photosynthetic capacity of *Symbiodinium*, by increasing the amount of CO_2 available for

their form II Rubisco (ribulose-1,5 bisphosphate carboxy- lase/oxygenase), which has a lower affinity for CO_2 (Rowan et al. 1996; Tortell 2000). A recent study showed that changes in gene expression due ocean acidification were consistent with metabolic suppression, an increase in oxidative stress, apoptosis and symbiont loss (Kaniewska et al. 2012). But this study did not account for the effect of symbiont identity. Also, work with cultured *Symbiodinium* indicates that, as with thermal stress, the effects of elevated CO_2 are dependent on symbiont type (Brading et al. 2011). However, this study also notes that *Symbiodinium* in symbioses may behave differently than those that are free-living. Therefore, in order to accurately predict how the paradoxical effects of elevated CO_2 may impact a coral experiencing and recovering from a bleaching episode, we need to determine how these conditions influence the dynamics of the symbiont community.

This study was designed to investigate the factors underlying bleaching resistance and resilience in the Caribbean reef coral, *Orbicella faveolata*. The colonies we studied hosted symbionts from either the thermally tolerant clade D (Rowan 2004) or a mixture of clade B and clade D. The aim was to compare how key physiological characteristics of an actively bleaching coral responded to differences in pCO_2 and/or temperature, and how this response varied depending on symbiont type. A further objective was to ascertain these metrics were affected by elevated pCO_2 as the coral recovered from bleaching. Coral growth was used as a proxy for calcification rates, and chlorophyll fluorescence was measured to assess *Symbiodinium* photochemical efficiency under different conditions. Quantitative-PCR was also used to determine the relative abundance of the symbionts present. Measurements were taken before, during, and after a high-light induced bleaching episode to establish (1) how increased temperature and *p*CO2, alone

and in combination, affects *O. faveolata* physiology during bleaching caused by irradiance stress, and (2) how pCO_2 influences recovery from bleaching. The crossed factorial nature of the experimental design permitted comparisons of the effects of elevated temperature and pCO_2, both alone and in concert. This study improves our understanding of how the response of the symbiont community to different environmental stressors influences a corals ability to recover from bleaching. This better understanding of how corals may adapt or acclimatize to the forecast climate changes (IPCC 2013) is vital for successful conservation efforts.

MATERIALS AND METHODS

Experimental set-up

All experiments were carried out in the Climate Change Laboratory at the University of Miami's Experimental Hatchery (UMEH) at the Rosenstiel School of Marine and Atmospheric Science (RSMAS) located on Virginia Key, Florida. The laboratory consisted of 12 experimental mesocosms shaded by neutral density shadecloth/plastic-film roof which reduced natural sunlight intensity by ~40% (henceforth referred to as "high-light"). Each mesocosm comprised a 60 L exposure/treatment tank and a 500 L sump tank used as an equilibration chamber. Sump tanks were black to reduce internal algal growth. Mesocosms were independently controlled to maintain specific temperatures and partial pressures of carbon dioxide (pCO_2). Temperature was moderated by a 1500W titanium submersible heater and a chilled water heat exchanger actuated by a solenoid valve within each equilibration tank. Activation of the heating and chilling mechanisms was electronically controlled by

temperature sensors in the exposure tanks. Tanks were checked daily for temperature and salinity with a YSI SCT meter and sensors were recalibrated as necessary. The desired partial pressures of carbon dioxide were generated in the equilibration chambers by the direct bubbling of air and CO_2 gas. Different pCO_2 levels were achieved using pairs of mass flow controllers (Sierra Instruments) which regulated the air/CO_2 mix sent to the equilibration chamber to generate the required concentrations. The quantity of dissolved gas was monitored continuously by equilibrator-LiCor infrared gas analyzer systems which fed data directly to the mass flow controllers for dynamic maintenance of the appropriate conditions. The seawater pumped from the equilibration chamber was pumped into the base of the tank and returned via a standpipe. Delivering a high flow of equilibrated water to the exposure tank without further aeration maintained the exposure tanks at the desired experimental conditions by considerably reducing the potential for gas exchange between the exposure tank and the environment. Seawater was dripped directly into the equilibration chambers fitted with an overflow to counteract evaporation and other potential effects of a recirculating system. Seawater for all tanks was drawn from Bear Cut, Virginia Key, Florida, via a 250,000 L settling tank and a high flow sand filter.

Coral samples

Ten colonies (each >100cm^2) of *Orbicella faveolata* were collected from Key Largo (Ocean Reef Club, July 2008) and Emerald reef (east of Key Biscayne, May 2010) and maintained in shaded (~20% natural sunlight), outdoor, flow-through tanks at the UMEH-RSMAS. In June 2010, these colonies were cored using a 25 mm diameter

diamond core drill bit (Stanley, CT, USA) mounted on a drill press equipped with a seawater cooling feed line to generate 12 experimental coral cores per parent colony. The resultant cores were trimmed with a tile saw to a thickness of 5-10mm and then glued to 8cm^2 neutrally buoyant plastic tags and placed in a shaded flow-through tank at 27^{o}C for four weeks to recover from drilling.

Experimental conditions

The 12 mesocosms were used in a cross-factorial experiment with control (27^{o}C) and high (31^{o}C) temperatures, and control (380ppm) and high (800ppm) pCO_2 levels. Each treatment combination (CTCC - Control Temp, Control CO_2; CTHC - Control Temp, High CO_2; HTCC - High Temp, Control CO_2; HTCC - High Temp, High CO_2) was replicated three times. One randomly-selected core from each parent was assigned to each of the 12 tanks, such that 10 parent colonies provided 10 cores per tank with a total of 30 cores per treatment. The corals then spent two weeks acclimating in their respective experimental treatment tanks shaded and set at control conditions of 27^{o}C and ambient pCO_2 (380ppm). Following acclimation, the shading was removed and ramping of the experimental treatments began. Where required, the temperature and CO_2 partial pressure was incrementally raised by 0.5^{o}C and 50ppm, respectively, over 8 days until the experimental treatment levels were reached. Corals were exposed to the full experimental conditions for 41 days, after which the temperatures in the tanks were returned to 27^{o}C at a rate of 0.5^{o}C per day, and the recovery of the corals monitored. The pCO_2 remained at experimental levels during this recovery period. Exposure tank conditions are summarized in Figure 3.1.

Light levels for the acclimation phase were approximately 30% of high-light, with daily maxima not exceeding 300 μmol/m^2/sec^{-1}. Once the ramping period began and the shading was removed, the cores experienced ambient high-light levels, reaching peak irradiance levels of ~850 μmol/m^2/sec^{-1}. The high-light exposure lasted for 7 weeks, after which the tanks were again shaded to 30% of high-light for the recovery period (Figure 3.1).

Once ramping began, water samples were taken every 2-3 days at the same time of day and analyzed for total alkalinity (TA) and pH during the course of the experiment to ensure pCO_2 remained at the desired levels. TA was determined using an automated open-cell Gran titration (Dickson et al. 2007, SOP3b), and compared to certified seawater reference material (A. Dickson, Scripps Institute of Oceanography) to verify accuracy. Seawater pH was determined using an Orion Ross combination pH electrode. Concentrations of CO_3^{2-}, Ca^{2+}, and Ω_a were computed from TA, pH, temperature, and salinity using the program CO2SYS (E. Lewis, Brookhaven National Laboratory).
The mounted coral cores and the tanks were cleaned weekly or as needed to reduce algal growth and limit their potential effect on water chemistry within experimental chambers. Corals were fed (Reef Chili®) every five days. Feeding took place in small plastic open containers which were floated in the tank to maintain the corals at experimental temperature. Feeding was carried out in the evenings and lasted for approximately one hour.

Coral symbiont identification

All cores were biopsied at four time points during the experimental period: (1) at the start of the experiment (0 days of exposure); (2) six days after the ramping completion (2 weeks of exposure); (3) at the end of the exposure period (7 weeks of exposure); and (4) at the end of the experiment (5 weeks of shaded recovery). The DNA from each of the samples was extracted using a modified organic extraction protocol (Baker *et al*. 1997) which involved: incubating in 1% SDS at 65^{o}C, digestion with Proteinase K at 55^{o}C, incubation with 1% CTAB at 65^{o}C, mixing with equal volume of chloroform at room temperature, 100% ethanol precipitation at -20^{o}C, re-suspension of dried pellet in 0.3M sodium acetate and subsequent ethanol precipitation at -20^{o}C, washing pellet in 70% ethanol, re-suspension of dried down DNA pellet in Tris-EDTA buffer, and storage at -20^{o}C.

DNA from the *Symbiodinium* internal transcribed spacer-2 (ITS2) region of ribosomal DNA (rDNA) was PCR-amplified using primers ITSintfor2 and ITS2clamp (LaJeunesse and Trench 2000) targeting the conserved flanking regions in the 5.8S subunit and 28S-like large subunit. The reverse primer incorporated a 40 base pair GC-clamp. 25 μL PCR reactions contained 1 μL template DNA, 10 mM forward and reverse primer, 1 X Promega Go-Taq buffer, 2mM $MgCl_2$ and 0.8mM dNTPs (Fermentas, USA). A touch-down amplification protocol was used with an initial annealing temperature 10^{o}C above the final annealing temperature of 52^{o}C. The annealing temperature was decreased by 1^{o}C every two cycles until 52^{o}C was reached, where it remained for a further 15 cycles. PCR was performed using a DYAD thermocycler (MJ Research).

Denaturing gradient gel electrophoresis (DGGE) of the ITS2 amplicons was used to identify the dominant *Symbiodinium* for each coral microcolony during the experiment. PCR products were run on 35-75% denaturing gradient polyacrylamide gels (100% denaturant consisted of 40% deionized formamide and 7M urea) using a CBS Scientific System (Del Mar, CA, USA) for approximately 16 hours at 90 volts. Bands of interest were excised and left in 100 μL of molecular grade H_2O (Rockland, PA, USA) overnight at 4^{o}C. One μL of the resulting DNA solution was then used as the DNA template in a PCR amplification using the same primers (with GC-clamp removed from reverse primer) and reaction mix as above. The thermocycling profile consisted of 3 minutes at 94^{o}C followed by 35 cycles of 1 min at 94^{o}C, 1 min at 60^{o}C and 1 min at 72^{o}C, and a final extension of 5 minutes at 72^{o}C. Amplifications were checked on a 1.5% agarose gel to ensure they produced bright, single bands. Once checked, PCR products were treated with a mixture of Exonuclease I and Shrimp Alkaline Phosphatase to deactivate the polymerase and digest the primers (37^{o}C for 30 minutes and 80^{o}C for 15 minutes). Forward and reverse strands were then amplified using BigDye (Applied Biosystems, Foster City, CA) terminator chemistry. The 10 μL reactions were amplified using 35 cycles of 96^{o}C for 15s, 50^{o}C for 15s and 60^{o}C for 4 minutes. Big Dye amplified sample sequences were cleaned using Sephadex G-50 (GE Life Sciences), dried down and reconstituted in 10 μL HiDi (Applied Biosystems). Products were sequenced on an ABI 3730. Sequence chromatograms were manually inspected and assembled using Geneious (Biomatters Ltd.). Each sequence type was compared with named sequences in the Genbank database using the BLAST search tool.

Symbiont community characterization

Samples were assayed in duplicate using TaqMan-MGB (Life Technolgies) qPCR assays targeting specific actin loci in *O. faveolata* and *Symbiodinium* in clades B, C, and D (Cunning and Baker 2013; Cunning *et al.* in prep). The *O. faveolata* assay included 150 nM OfavActF (5'- CGCTGACAGAATGCAGAAAGAA-3'), 100 nM OfavActR (5'- CACATCTGTTGGAAGGTGGACA-3'), and 250 nM OfavActProbe (5'-NED-TGAAGATCAAGATCATTGC-MGB-3'). The clade B assay included 200 nM BActF (5'- CGATGGCTTTGCAGCTCAA-3'), 300 nM BActR (5'-TGTCCCGGAATTTGTGCAA-3'), and 100 nM BActProbe (5'-FAM-CTGTAAACCCTGTCATTC-MGB-3'). The clade C and D assays were multiplexed, using the same primers and reaction conditions as described in Cunning & Baker (2013). All reactions were carried out in 10 µL volumes (with 5 µL Taqman Genotyping MasterMix and 1 µL DNA template) on StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Thermal cycling conditions consisted of an initial incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Cycle threshold (C_T) values were calculated by the StepOnePlus software package with a set fluorescence threshold of ΔR_n=0.01.

Target DNA was considered present when amplification occurred in both technical replicates with no target detected in negative control reactions. C_T values for *O. faveolata* and *Symbiodinium* clade B were reduced by 5.01 and 4.03 cycles, respectively, to normalize for differences in fluorescent signal intensity among TaqMan-MGB fluorophores. Cycle reductions were calculated based on the results of standard curves generated following the methods of Cunning and Baker (2013). Adjusted C_T values were

then used to calculate symbiont to host cell ratios using the formula $2^{C_T(\text{host})-C_T(\text{symbiont})}$. Clade C symbionts were not detected in these samples and thus were not involved in subsequent analyses.

Coral physiology

Coral cores were monitored for skeletal growth and *Symbiodinium* photosynthetic efficiency at four time points during the experimental period: (1) at the start of the experiment (0 days of exposure); (2) six days after the ramping completion (2 weeks of exposure); (3) at the end of the exposure period (7 weeks of exposure); and (4) at the end of the experiment (5 weeks of shaded recovery). Growth data were collected using the buoyant weight method. Corals were weighed while fully submerged and hanging on a hook suspended beneath a Mettler Toledo (0.001g accuracy) balance. Water temperature and salinity were constantly monitored (using a YSI 30 SCT meter) to maintain consistent conditions similar to those in the experimental exposure tanks. To account for the tissue removed for DNA extractions, corals were weighed before and after sampling. Buoyant weight equates to skeletal weight (Davies 1989), therefore the rate of calcification for each microcolony was determined by weight differences between weigh-ins, calculated as (Δ wt in g x 10^6)/core area in mm^2, and expressed in $\mu g\ mm^{-2}\ day^{-1}$.

Chlorophyll fluorescence was measured with an Imaging Pulse Amplitude Modulation (I-PAM) (Walz, Germany) fluorometer and was used to calculate photochemical efficiency. Analysis was performed after sunset in order to dark acclimate all samples, thus reducing the time that corals were out of the experimental tanks. The initial fluorescence (F_o) and maximum fluorescence (F_m) of dark adapted algal symbionts

was measured and used to calculate F_v/F_m ($F_v = F_m - F_o$), the maximum quantum yield of photosystem II (PSII) (Genty et al 1989). I-PAM data was collected 1 day prior to growth measurements and tissue sampling, to prevent any erroneous data due to sampling and handling stress.

Statistical analyses

The software package JMP (SAS Institute Inc.) was used to generate multiple regression models for the analysis of treatment effects on symbiont community and coral physiology. The community analysis models used total symbiont to host cell ratios (clade D S:H + clade B S:H) or percentage of clade D (clade D S:H / total S:H) as the response variable, previous total S:H (or percent D) as a fixed factor, and parent as a random variable. There was no tank effect so treatment replicates were pooled. Growth and F_v/F_m analyses used treatments as fixed variables and parent as random. Adjusted mean values were calculated that controlled for parent effect and were tested for significance ($p < 0.05$) using *post hoc* Tukey's honestly significant difference (HSD) tests. To normalize the data prior to statistical analyses, symbiont to host cell ratios were log transformed and proportion data (*e.g.,* percent D and percent bleached) were arcsine square root transformed for use in all statistical models. Growth and F_v/F_m data were normally distributed and thus were untransformed.

During the course of the experiment, cores from one D-only colony (P10) experienced high mortality across all treatments and data associated all P10 cores were therefore removed from subsequent analyses.

RESULTS

Symbiont community structure – (Chapter 2 summary)

Sequencing of ITS2 rDNA fragments revealed four parent colonies hosted D1a (clade D) and five colonies simultaneously hosted types D1a and B1 clade (B+D). Quantitative PCR (qPCR) analysis of the 48 clade D coral cores indicated 100% dominance by D. Symbiont communities in the 60 B+D cores showed higher variation, ranging from 20% D to 90% D (Figure 2.2). Mean S:H ratio remained higher in D-only cores than B+D cores throughout the exposure phase of the experiment ($p<0.0001$), and percent loss of total symbionts resulting from the high-light exposure was significantly less in the D-only cores (82% ± 1.7%) than from the B+D cores (94% ± 1.5%) ($p<0.0001$). There was no significant effect of temperature or CO_2 on either the rate or the severity of bleaching (Figure 2.4).

During the recovery period, S:H ratios increased significantly across all treatments ($p<0.0001$). In the clade D cores, there was no effect of temperature or CO_2 on the extent of symbiont recovery as measured by S:H ratios; however, the B+D corals that had been exposed to 31^{o}C had higher S:H ratios than those at 27^{o}C ($p<0.0001$). In addition, among those corals that had been exposed to 31^{o}C, increases in S:H ratios were significantly higher in control *p*CO2 compared to elevated ($p=0.02$)

D-only cores remained 100% D throughout the course of the experiment, compared to B+D cores, which showed changes in symbiont community structure. In B+D cores, the relative abundance of clade D (%D) changed in response to temperature even though there were no difference in the total S:H ratio ($p<0.0001$). After 7 weeks, the proportion of clade D in corals at 31^{o}C had risen from ~50% to almost 70%, and reached

~90% by the end of the experiment, regardless of pCO_2 (Figure 2.5). The reverse was true for the control temperature treatments, where clade D comprised only 36% of the symbiont community at the end of the recovery period. Matched pairs tests indicated both clades decreased equally in the control temperature treatments; however, at 31°C significantly more clade B symbionts were lost as compared to clade D.

After recovery, the relative abundance of D (%D) in cores in the CTHC treatment was significantly greater than in controls ($p=0.03$). Unlike the S:H ratio, there was no effect of CO_2 on the community composition in the 31°C treatments. Matched pair tests on the recovery data revealed that clade D recovered significantly faster in the cores which had experienced the higher temperature ($p<0.0001$). Data from the CTCC treatment indicate that clade B recovered faster than clade D only when light-stress alone was experienced ($p=0.005$).

Coral calcification

During the first 2 weeks of high light exposure (Figure 3.1 0-14 days), coral cores in all treatments showed negative growth rates, indicating skeletal dissolution (Figure 3.2a-d). The B+D cores implied an influence of pCO_2 at control temperatures as the CTCC treatment showed less dissolution than those at 31°C ($p=0.02$) whereas the CTHC cores did not. There were no differences between treatments during these 2 weeks for the D-only cores, but these cores experienced significantly more decalcification than the B+D corals ($p<0.0001$). Over the next 5 weeks of high light exposure (Figure 3.1 days 15-49) the mean calcification rate of the B+D cores was almost 2 $\mu g\ mm^{-2}\ day^{-1}$, which was significantly higher than the D-only cores ($p=0.001$), and was now independent of

both temperature and pCO_2. However, the D-only cores were now exhibiting treatment effects, as growth rates in the HTHC treatment were significantly greater than HTCC ($p=0.001$), with adjusted means of 1.35 μg mm^{-2} day^{-1} (± 1.09) and -1.89 μg mm^{-2} day^{-1} (± 1.12), respectively. Growth of the D-only cores at 27°C was negligible, although data suggest that cores in elevated CO_2 calcified slightly (0.11 μg mm^{-2} day^{-1} ± 1.1), while those at ambient CO_2 experienced mild dissolution (-0.14 μg mm^{-2} day^{-1} ± 1.1).

All corals grew faster during the 5 week recovery period than during the 7 week exposure period ($p<0.001$). For the B+D corals, there was an effect of both temperature and CO_2 on calcification rates. Cores at 31°C grew 32% faster than those at 27°C ($p=0.006$), and those at 27°C grew 35% more slowly at 800ppm CO_2 compared to 380ppm ($p=0.03$). These B+D cores also exhibited an interaction effect between temperature and CO_2, as the slowest growth rate during recovery was at 27°C and 800ppm (3.7 ± 1.8μg mm^{-2} day^{-1}), and the highest at 31°C and 380ppm (7.3 ± 1.9 μg mm^{-2} day; $p=0.0006$). D-only cores that had been exposed to 31°C showed growth rates that were 36% faster than those which had been exposed to 27°C ($p=0.004$).

Despite bleaching more severely, the mean growth rate of the B+D cores over the complete 12 week experiment was significantly higher than the D-only cores; 2.6 μg mm^{-2} day^{-1} ± 0.4 and 0.7 μg mm^{-2} day^{-1} ± 0.4 ($p=0.003$, data not shown), respectively. The only significant treatment effect was a temperature and CO_2 interaction effect which occurred in B+D cores from the CTHC and HTCC tanks, with the latter growing an averaging of 45% more ($p=0.02$).

Algal photosynthesis

Two weeks after the removal of the acclimation shading and the subsequent increase in light intensity, the maximum quantum yield of photosystem II (F_v/F_m) had dropped significantly in all cores across all treatments ($p<0.0001$) (Figure 3.3a-b). The two weeks of light stress affected the D-only cores more than the B+D, causing decreases in mean F_v/F_m of 34% and 24 %, respectively. At this time point, the mean F_v/F_m of the D-only cores in CTHC (0.348 ± 0.009) was significantly lower than all other cores and treatments. The B+D cores at 27^{o}C also registered their lowest F_v/F_m (0.431 ± 0.022) at 14 days, after which their F_v/F_m began to recover (Figure 3.3b). In contrast, the B+D cores at 31^{o}C continued to decline until the end of the exposure period, where they reached an average low of 0.322 (± 0.019). These temperature-stressed B+D cores were therefore the only corals that required shading in order for their symbionts to recover their photochemical efficiency, rather than photoacclimating.

Once the shading was reapplied, cores in all treatments displayed a significant increase in their F_v/F_m ($p<0.001$). The B+D cores at 27^{o}C were the only corals to attain an F_v/F_m comparable to their initial values, whereas all other treatments finished significantly lower than they started the experiment ($p< 0.01$). However, the B+D cores from 31^{o}C, which did not photoacclimate, displayed the most dramatic increase in F_v/F_m, improving from 0.322 to 0.510 (± 0.012) during the five week recovery period.

DISCUSSION

Influence of symbiont type on bleaching response

Increased light intensity resulted in a significant reduction in the *Symbiodinium* abundance (as measured using the S:H cell ratio metric) and photochemical efficiency (F_v/F_m), and also caused weight loss, which was presumed to be due to skeletal dissolution. These declines during the first 2 weeks of high light exposure occurred in all cores across all treatments, but to differing extents depending on treatment conditions and symbiont community structure.

Cores that hosted only *Symbiodinium* D1a lost fewer symbionts than the B+D cores during the first 2 weeks exposure to high light. However, this appeared to have no benefit to coral growth, because the skeletal dissolution of the D-only cores was actually 85% greater than the B+D corals (Figure 3.2a-b, 14 days). Photochemical efficiency of the D-only cores also dropped more rapidly than B+D cores during this period (Figure 3.3a-b). It is therefore likely that the simultaneous reduction of symbiont abundance and photochemical efficiency, during bleaching, decreased the energy available to the D-only cores and reduced their ability to calcify. Conversely, the B+D cores in the CTCC treatment lost more symbionts than the D-only cores, and yet they showed the lowest rate of skeletal dissolution (Figure 3.2b) and the lowest decline of photochemical efficiency (Figure 3.3b). This emphasizes the potential hazards of drawing conclusions about coral health from singular metrics. In this particular example, symbiont density data (as determined by S:H cell ratio) would suggest the D-only cores were less stressed by the increased light intensity than the B+D cores, whereas the growth and photochemical data imply the opposite.

As the exposure period continued, photochemical efficiency of the D-only cores improved significantly in all but the HTHC treatment (Figure 3.3a). However, this was the only treatment in which *O. faveolata* hosting only *Symbiodinium* D1a exhibited positive growth (Figure 3.2a). Although it was lower the other treatments, the mean F_v/F_m for these cores was similar to its mean F_v/F_m from 5 weeks previously. This suggests that the symbionts were not photoinhibited, and that possibly the lower photochemical efficiency was offset by the greater availability of CO_2 in this treatment. This also indicates that, although the clade D symbionts were capable of at least partially acclimating to high light levels, even at 31^{o}C, photochemical efficiency was not a key indicator of calcification in the bleaching D-only cores. It is probable therefore that heterotrophy was a primary source of energy for growth in these corals (Schoepf et al. 2013, Muellehner 2013). It is also important to note although the growth rates of the D-only cores improved between days 14 and 49, they still exhibited a net loss of skeleton during the exposure period of the experiment. Although it would require a longer exposure period to fully determine whether or not *O. faveolata* hosting D-only are capable of net skeletal growth while exposed to high light intensity, this data hints that it should occur after ~2 months.

Although the B+D cores bleached more severely than the D-only cores, their initial physiological responses to the exposure stress were less extreme. During the first 2 weeks of exposure, the B+D cores exhibited significantly less skeletal dissolution than the D-only cores, and their photochemical efficiency also remained higher. As noted previously, these data would suggest that the B+D corals were experiencing less stress than the D-only cores, which is paradoxical given that their S:H ratio was three times

lower than the D-only corals. Reducing the abundance of photo-damaged symbionts would be beneficial to the coral as it reduces its exposure to damaging reactive oxygen species (Downs et al. 2002; Lesser 2006; Weis 2008). However, the F_v/F_m data does not imply that the B+D symbionts were more damaged than the D-only. It would appear therefore that the S:H ratio may be an important factor in the initial response to bleaching stressors, with lower S:H ratios driving higher F_v/F_m values for symbionts capable of photoacclimating, perhaps as a result of less self-shading.

After 14 days of high light exposure there were no discernible changes in symbiont community structure in the B+D cores, but continued exposure to 31^{o}C resulted in D1a being preferentially retained, while exposure to 27^{o}C retained more B1. By the end of the exposure period the corals with the highest photochemical efficiency were the B+D cores that had remained at 27^{o}C (Figure 3.3b) and were now B1-dominated. These symbionts were the fastest to photoacclimate to the higher light intensity, followed by the D1a in the D-only cores. However, unlike the D-only corals, the D-dominated B+D cores continued to show signs of photoinhibition until the light stress was removed. This response suggests that the presence of thermally stressed B1 *Symbiodinium* may have hindered the photoacclimation of the D1a symbionts, and may also explain the preferential retention of D1a that occurred during the latter portion of the exposure period.

Symbiont community reassembly following bleaching

Once the light stress was removed, the symbiont communities began to recover. Elevated temperatures and/or pCO_2 did not affect the rate of symbiont loss, but they did

influence how the cores recovered. In the D-only corals, cores in the 800ppm CO_2 treatments underwent faster repopulation of symbionts, but this did not translate to higher photochemical efficiency or faster growth rates. The fastest growth rates of D-only cores were found in corals that had experienced 31^{o}C, but, despite their higher S:H ratio, the cores in 800ppm pCO_2 actually grew more slowly than the 380ppm cores (Figure 3.2a). Whether this difference in growth indicates there is an increased energy demand tied to faster symbiont recolonization, or simply emphasizes the difficulties associated with calcifying at higher pCO_2 levels, cannot be distinguished with these data.

While elevated pCO_2 increased the rate of symbiont repopulation in the D-only cores, it was the effects of temperature on community composition during bleaching that dictated recovery trajectories in the B+D corals. Under high light, B+D cores at 31^{o}C experienced increases in *Symbiodinium* D1a, while those at 27^{o}C increased their B1. Without the added stress of elevated temperature the -now predominantly clade B- cores from the 27^{o}C treatments maintained the highest photochemical efficiency throughout the experiment, and were the only cores that reached their initial F_v/F_m values by the end of the experiment (Figure 3.3b). The recovery of the S:H ratio in these B-dominated cores was the slowest observed (Figure 2.4b), yet these B+D corals still grew faster than the D-only cores from the same CTCC treatment, who had a two-fold higher mean S:H ratio. The B+D CTCC cores also grew at a similar rate to the D-dominated B+D cores in the high temperature treatments (Figure 3.3b), which had an almost four-fold higher S:H ratio. Therefore, these *Symbiodinium* B1 dominated cores maintained a comparable growth rate to clade D-dominated (and clade D-only) cores, despite hosting significantly fewer symbionts. However, the B+D cores from the CTHC treatment grew more slowly

than the D-dominated cores, even though they exhibited the same high F_v/F_m and slow symbiont recovery as the cores from CTCC. These results show that corals hosting depleted densities of photosynthetically productive symbionts are still capable of achieving net growth while recovering from bleaching, but that exposure to elevated pCO_2 reduces the rate of calcification.

The fastest growth rates during the recovery phase were found in B+D cores hosting >95% D (Figure 3.4a). These corals grew more than twice as quickly as B+D cores hosting < 70% D, contrary to studies of *Symbiodinium* D1a as a miserly symbiont (Little et al. 2004; Cantin et al. 2009; Jones and Berkelmans 2010). They also grew at about twice the rate of the D-only cores, even though their photochemical efficiency was very similar (Figure 3.4b). The probable explanation for this difference is that the mean S:H ratio of the >95% D cores was almost 50% higher than the D-only corals (Figure 2.7). Thus, it is unlikely that the higher calcification is due to the small number of clade B symbionts present.

Conclusions

Comparing the growth rate responses of the D-only and B+D corals suggests that as long as there is sufficient food available, a greater loss of symbionts during bleaching may actually benefit coral calcification rates. This advantage may be achieved by reducing their vulnerability to phototoxic molecules, such as reactive oxygen species (Downs et al. 2002; Lesser 2006).

The clade D-only cores did not show full recovery of calcification rates before the end of the seven week exposure period. Consequently, hosting just bleaching-resistant

symbionts may only be detrimental to corals if bleaching conditions are experienced for extended periods. Furthermore, symbiont loss in the D-only cores was independent of treatment, and thus it was high-light and not elevated temperature that caused bleaching in these corals. Therefore, if the corals were exposed to more turbid conditions, or if the corals were at a greater depth they might not have bleached.

Having *Symbiodinium* D1a as the dominant component of the symbiont community was not detrimental to the growth of *O.faveolata* recovering from bleaching, and, in fact, these corals grew at twice the rate of cores dominated by *Symbiodinium* B1. The clade B symbionts were the quickest to photoacclimate, but the slowest to repopulate. Despite their lower abundance, their host cores still managed to calcify faster than the D-only corals in the same treatment (CTCC), it is therefore reasonable to assume that the *Symbiodinium* B1 released much of their photosynthate to their host, rather than focusing that energy on their own proliferation, whereas the *Symbiodinium* D1a dedicated more of theirs to repopulating the coral.

B+D cores recovering with a higher percentage of clade D symbionts were the most resilient, showing the highest calcification rates and the most rapid symbiont community reassembly. Nevertheless, a five-fold higher S:H ratio only equated to a two-fold higher calcification rate; consequently a greater abundance of D1a than B1 would probably be required to achieve a similar growth rate in the coral host. Hosting a diverse symbiont community is therefore the best option for the continued survival of *Orbicella faveolata*: productive symbionts for maximal growth under normal temperatures, and thermally tolerant symbionts for rapid recolonization after bleaching.

Figures:

Figure 3.1 Experimental conditions experienced by coral cores within the 4 treatments for the duration of the experiment: CTCC (Control Temp, Control CO_2); CTHC (Control Temp, High CO_2); HTCC (High Temp, Control CO_2); HTCC (High Temp, High CO_2). * denotes weighing and tissue sampling time points, PAM data collected one day prior.

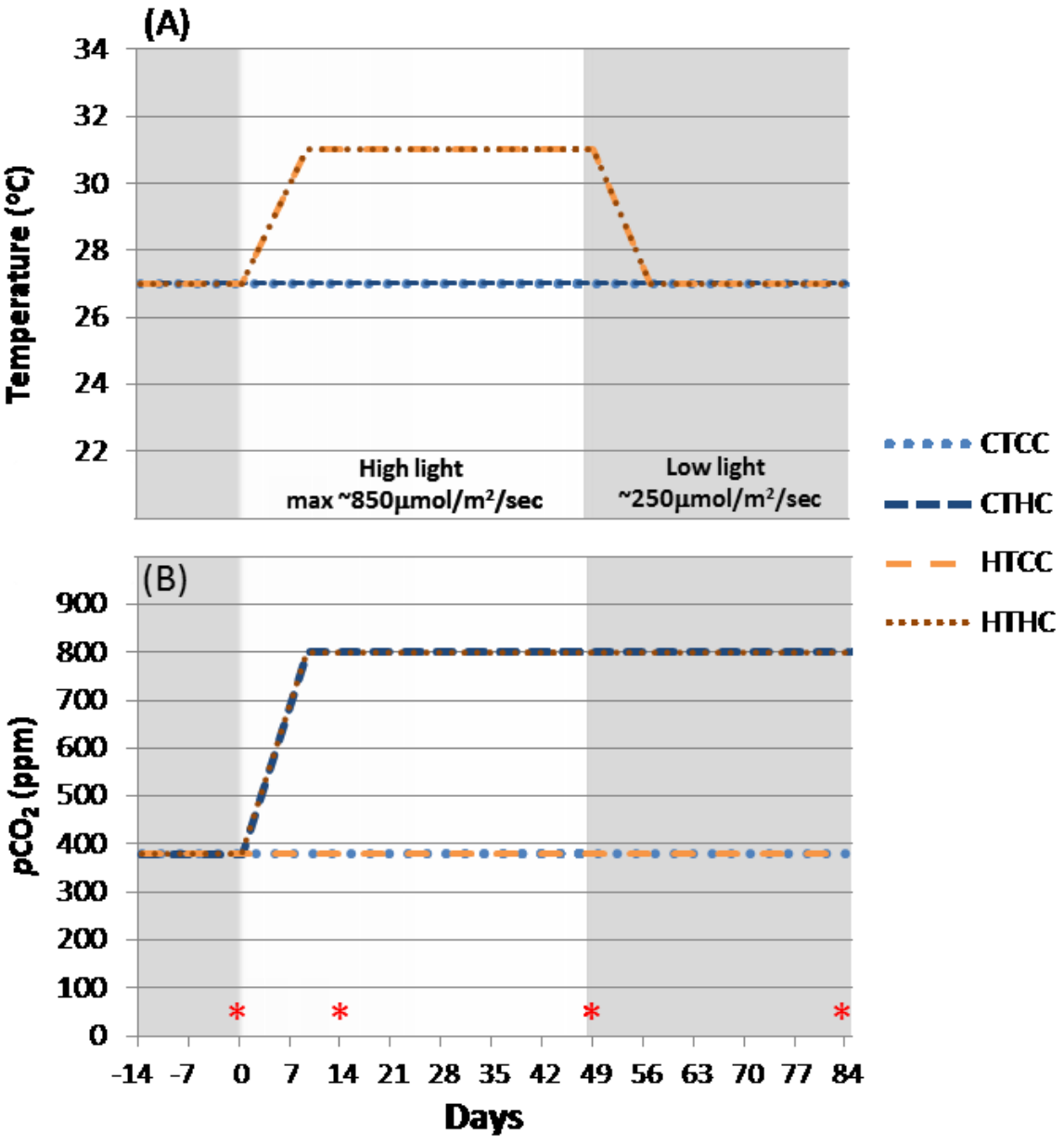


Figure 3.2 Effects of light, temperature, and pCO_2 on the growth of *Orbicella faveolata* hosting (A) clade D-only *Symbiodinium*, and (B) a mixture of clade B and D *Symbiodinium*. Adjusted means of the growth rates (in μg mm^{-2} day^{-1}) are pooled by treatments: CTCC (Control Temp, Control CO_2); CTHC (Control Temp, High CO_2); HTCC (High Temp, Control CO_2); HTCC (High Temp, High CO_2). Initial exposure to high light (white background) caused weight loss in all cores, and growth during recovery (grey background) was influenced by pCO_2 and prior temperature exposure. Letters indicate results of Tukey's HSD test on adjusted means, bars for each time point not sharing a letter are significantly different, $p < 0.05$.
Error bars = StdErr, (A) n = 12, (B) n = 15.

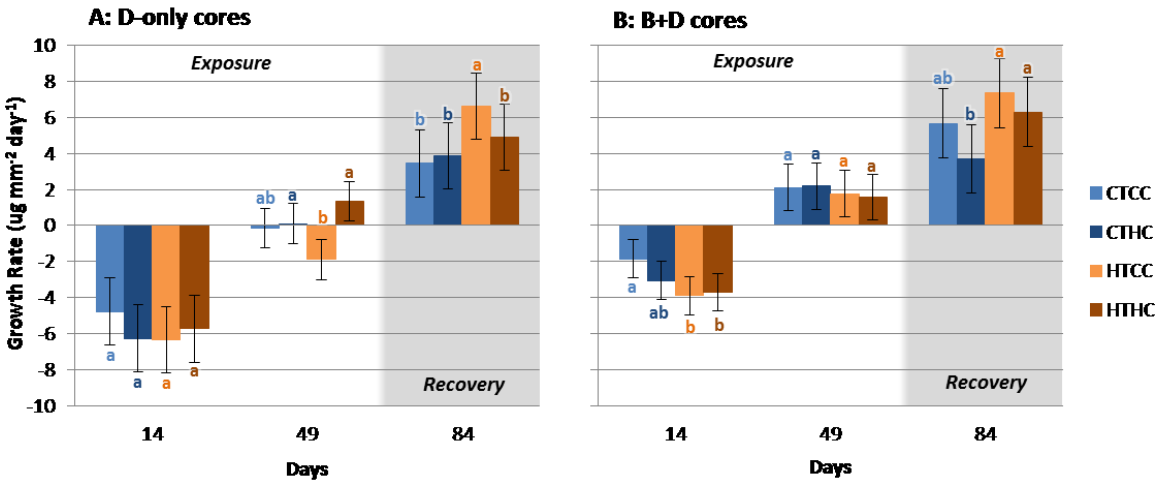


Figure 3.3 Effects of light, temperature, and pCO_2 on the maximum quantum efficiency of PSII (F_v/F_m) of symbionts in (A) D-only cores, and (B) B+D cores. Graphs show adjusted means pooled by treatments: CTCC (Control Temp, Control CO_2); CTHC (Control Temp, High CO_2); HTCC (High Temp, Control CO_2); HTCC (High Temp, High CO_2). Letters indicate results of Tukey's HSD test on adjusted means, lines at each time point not sharing a letter are significantly different, $p < 0.05$. Error bars = StdErr, (A) n = 12, (B) n = 15

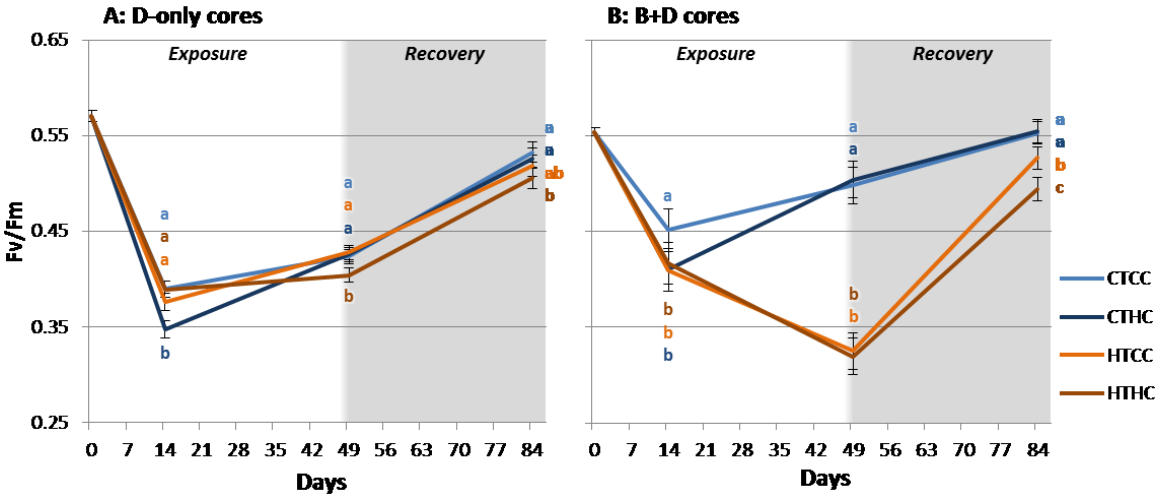
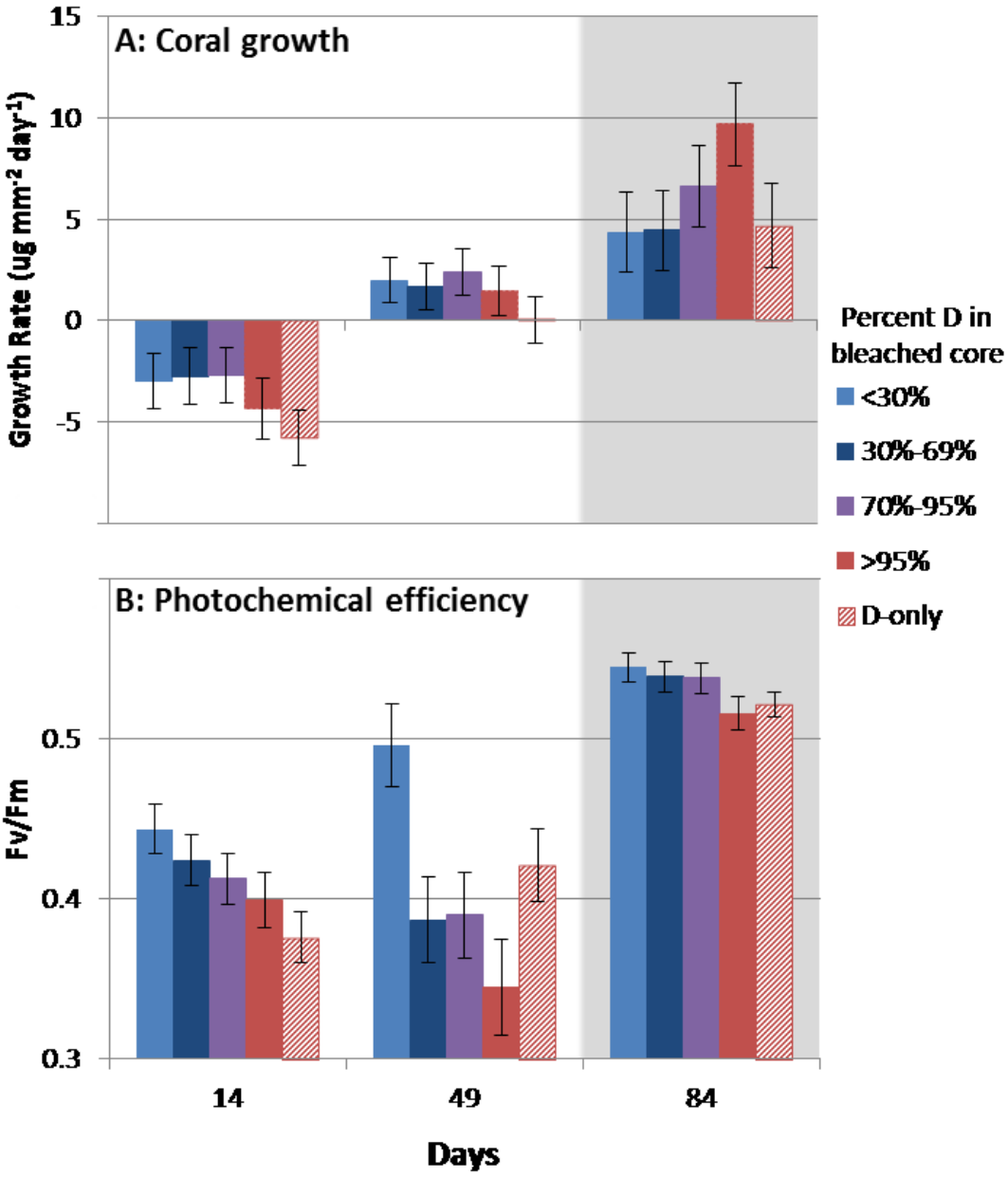


Figure 3.4 Pooling cores based on the percent clade D *Symbiodinium* in bleached symbiont community indicates how remnant symbionts can influence growth and photochemical efficiency. Graph shows the adjusted mean (A) growth rates and (B) F_v/F_m of cores after 14 and 49 days of high light, and at the end of the experiment (84 days). Data are pooled by the % D present in the B+D core at the end of exposure period, i.e. when maximally bleached. Growth and F_v/F_m data for D-only cores (red stripe) are included for reference. Error bars = Standard error, <30% n=18, 30%-69% n=16, 70%-95% n=15, >95% n=11, D-only n=48.



Chapter 4

Biotic vs. abiotic environmental influences on *Symbiodinium* community reassembly in corals recovering from bleaching

SUMMARY

Coral reef response to climate change stressors may be influenced by the community dynamics of algal symbionts (genus *Symbiodinium*) in corals following bleaching events. Different *Symbiodinium* vary in their physiological optima, and certain types can increase a corals resistance to bleaching stressors (such as elevated temperature) or enhance their rate of recovery. Post-bleaching recovery of the symbiont communities occurs through the proliferation of existing remnant symbionts and/or the acquisition of exogenous symbionts. To explore whether the symbiont community composition in recovering corals is influenced by the symbiont communities in nearby colonies, this study monitored replicate cores from three mother colonies of *Montastraea cavernosa*. Experimental cores were bleached through exposure to heat stress (32°C) and then allowed to recover in 1μm filtered seawater at one of two temperatures (22°C or 29°C). During this recovery phase, bleached corals were exposed to (1) healthy, unbleached replicate fragments hosting clade C symbionts; (2) previously bleached replicate fragments that had been allowed to recover at elevated temperatures and had recovered with thermally tolerant symbionts in *Symbiodinium* clade D; (3) both cultured and freshly isolated *Symbiodinium* in clades A, B, C, and D; or (4) no exogenous sources of symbionts (exposure control). Highly sensitive qPCR assays were used to test the hypotheses that exposure to nearby corals with thermally tolerant symbionts promotes

recovery of bleached corals with these symbionts, and that this process is accelerated at higher temperatures.

This study found that bleached corals do not always recover from the residual symbionts remaining in host tissue and that exogenous sources of symbionts may alter the initial recovery trajectory of bleached corals. However, the influence of residual symbionts was dependent on both symbiont type and coral host species, and was further modulated by the abiotic environment, especially later in the recovery process. For example, *Orbicella faveolata* hosting *Symbiodinium* D1a prior to bleaching recovered with D1a at 22^{o}C and 27^{o}C, whereas *Montastraea cavernosa* hosting only *Symbiodinium* C3 recovered as either clade D dominated (at 29^{o}C) or with a mixture of clades A and D (at 22^{o}C).

If exogenous sources of *Symbiodinium* better suited to the current environment can influence initial recovery from bleaching, then founder effects, symbiont availability, and proximity to healthy corals may be more important in determining the early recovery of bleached corals than have previously been assumed. Although abiotic factors, such as temperature, appear to be more influential in determining the eventual symbiont community, the availability of an exogenous source of thermally tolerant symbionts (through inoculations or close proximity of healthy corals) may improve the likelihood of a bleached coral surviving a subsequent thermal anomaly.

BACKGROUND

Due to their similar morphologies, the endosymbiotic unicellular algae hosted by scleractinian corals (and other marine invertebrate species) were originally thought to be

a single pandemic species, *Symbiodinium microadriaticum* (Freudenthal 1962). Subsequent analysis of ribosomal RNA sequences revealed that the genus *Symbiodinium* is in fact highly diverse, currently comprising 9 major clades (A-I) (Rowan & Powers 1992; Santos et al. 2004; Pochon et al. 2006; Stat et al. 2006; Pochon & Gates 2010). The different types of *Symbiodinium* within the 4 clades most commonly associated with scleractinian corals (A-D; LaJeunesse 2002; Baker 2003) have distinct physiological traits (Iglesias-Prieto & Trench 1994; Warner et al. 1999; Goulet et al. 2005; Loram et al. 2007a) and thus they may be suited to different environmental conditions. These differences can equate to a predictable distribution along gradients of light and temperature across reefs (Iglesias-Prieto et al. 2004; Fabricius et al. 2004) and even within single coral colonies (Rowan et al. 1997; Kemp et al. 2008). Certain types of *Symbiodinium* have been associated with corals displaying a higher thermal tolerance (e.g. Clade D, (Rowan 2004; Berkelmans & van Oppen 2006). While these symbionts are geographically widely distributed, they are invariably rare in any given reef system (LaJeunesse et al. 2005). However, the abundance of thermally tolerant symbionts on reefs which have previously experienced a mass bleaching event is often considerably higher (Baker et al. 2004; Jones et al. 2008; LaJeunesse et al. 2009). This community dominance by thermally tolerant *Symbiodinium* may be transient (Thornhill et al. 2006), but maintaining background levels may still improve resistance to bleaching (Silverstein 2013), as these symbionts have been shown to increase their relative abundance within corals as temperatures rise towards bleaching thresholds (LaJeunesse et al. 2009; Cunning & Baker 2013). Therefore, if corals are capable of acquiring thermally tolerant

symbionts from the environment, this could reduce the predicted impacts of climate change on scleractinian corals bleaching (reviewed in Hoegh-Guldberg et al. 2007).

Numerous studies have successfully looked at the uptake of *Symbiodinium* by larvae and newly settled (single polyp recruits) corals (e.g., Schwarz et al. 1999; Weis et al. 2001; Coffroth et al. 2001; Gómez-Cabrera et al. 2007). These studies have indicated some degree of recognition on the part of the host as to which clades/types of *Symbiodinium* successfully form symbioses. Such discrimination by the host at the onset of symbiosis would limit the potential variety of future symbiont communities, and thus may invoke a functional constraint which could hinder the corals ability to respond to climate change. Analysis using denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP) and quantitative polymerase chain reaction (qPCR) indicate that many scleractinian coral colonies have a diverse assemblage of symbionts (Rowan & Knowlton 1995; Rowan et al. 1997; Kemp et al. 2008; Silverstein et al. 2012). Therefore, unless the juvenile host is actually less discerning than some studies suggest, coral colonies may be able to acquire new symbionts from the surrounding environment (Coffroth et al. 2006; Stat & Gates 2007), and thus may not be fully reliant upon this larval seed stock for all their possible responses to environmental perturbations and stressors.

Corals hosting an actively proliferating symbiont community discharge photosynthetically active *Symbiodinium* into the surrounding water column (Hoegh-Guldberg et al. 1987; Jones & Yellowlees 1997; Baghdasarian and Muscatine 2000) and these algae, along with symbionts that survived digestion by corallivores (Muller-Parker 1984; Castro-Sanguino & Sánchez 2012) could act as a seed stock of potential symbionts

for neighboring corals. *Symbiodinium* which occupy the water column, reef sediments and macroalgal beds (Littman et al. 2008; Porto et al. 2008; Pochon et al. 2010) have been shown to be sources of symbionts for aposymbiotic coral larvae (Adams et al. 2009; Abrego et al. 2009), and could also be a source of symbionts for bleached corals (Lewis and Coffroth 2004; Coffroth et al. 2006; Coffroth et al. 2010).

The mechanisms of coral bleaching have been the subject of much research (reviewed in Lesser 2011), although less is known about the dynamics of symbiont community reassembly following a bleaching disturbance (Toller et al. 2001). A recent study (Coffroth et al. 2010) confirmed that bleached adult corals can take up and maintain *Symbiodinium* from the surrounding water column. However, these corals were not left to recover at a different temperature to that experienced prior to bleaching, and quickly reverted back to their original symbionts. This study therefore aims to address the following questions: 1) Do bleached corals recover from remnant symbionts in bleached tissue? 2) Can exogenous sources of *Symbiodinium* influence the rate of recovery and/or the trajectories of symbiont community reassembly? 3) Do different recovery temperatures affect the composition of recovered symbiont communities?

This study investigated these questions by bleaching two different species of Caribbean reef coral (*Montastraea cavernosa* and *Orbicella faveolata*) and allowing them to recover in *Symbiodinium*-free filtered seawater (FSW), or in FSW supplemented with exogenous symbionts by either: a) direct inoculation with freshly isolated and cultured *Symbiodinium*, or b) close proximity (< 1cm) to coral fragments hosting known symbiont types. This will help us understand how thermally tolerant *Symbiodinium* can become

dominant after a bleaching event, and will help test whether it may be possible to artificially increase the abundance of these symbionts before a bleaching event occurs, thereby reducing the severity of the stress response.

MATERIALS AND METHODS

Experimental setup

Experimental manipulations were conducted in three independent, 275 L, indoor, semi-recirculating, temperature controlled, fiberglass tanks supplied with sand-filtered (Hayward S200, Clemmons, NC, USA) seawater from Bear Cut, Biscayne Bay, FL. Temperatures in each of the three tanks were maintained within ±0.5°C of the target temperature using SeaChill TR-20 heater/chillers (TECO US). Artificial light (190-280 μmol quanta m^{-2} s^{-1}) was provided by two 400-W metal halide lamps (IceCap Inc., Hamilton, NJ, USA). Recovery exposures (except for seawater control) were conducted in 20 L glass aquaria, housed within the fiberglass tanks, fed by 1μm filter seawater (FSW) (see Figure 4.1). FSW was produced by filtering the incoming sand-filtered seawater from Biscayne Bay sequentially through 50μm, 5μm, and 1μm cellulose fiber cartridge filters. Water in each aquarium was mixed using a small fountain pump (Aquatic Eco-systems, Apopka, FL, USA). FSW was analyzed for the presence of *Symbiodinium* by filtering 15 liters through a 0.22μm polycarbonate filter (EMD Millepore Corp., Billerica, MA, USA), extracting DNA directly from the filter, and analyzing it using DGGE (see below for methods).

Preparation of coral cores and proximity fragments

Three *Montastraea cavernosa* and two *Orbicella faveolata* colonies were collected from a depth of ~5 m at Emerald Reef (25°40.45' N, 80°5.92'W), near Key Biscayne, Florida, USA. Replicate cores were generated from the parent colonies using a 2.5cm diameter diamond-tipped core drill (Starlite Industries, Rosemont, PA) and core bases were trimmed using a tile saw to produce level cores ~ 1cm high. Cores were glued to plaster reef plugs (Boston Aqua farms, Boston, MA) and left to recover from coring and acclimate to experimental conditions for 6 months in a 275 L seawater tank maintained at 26^{o}C, under artificial light on a 12hr:12hr light:dark cycle.

Randomly selected fragments of *M. cavernosa* parent colonies that remained after coring were experimentally bleached by exposing them to 32^{o}C for 10 days. They were then left to recover at 29^{o}C for 6 months, to promote a shift in symbiont community from the *Symbiodinium* ITS2-type C3 that was dominant at the time of collection, to ITS2-type D1a (see Chapter 5). Control *M. cavernosa* parent colony fragments remained at 26^{o}C.

Randomly selected fragments of *O. faveolata* parent colonies that remained after coring were experimentally bleached by exposing them to 600µg/L DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea, Sigma-Aldrich Co. LLC.) for 7 days under artificial light (~250 µmol quanta m^{-2} s^{-1}). After bleaching, *O. faveolata* fragments were left to recover at 22^{o}C for 6 months.

After the 6-month recovery period, tissue samples were taken from all cores and all parent fragments (controls, as well as bleached & recovered) using a clean razor blade, and samples were heated for 1.5 hours at 65^{o}C in 300µL of 1% SDS in DNA buffer (Rowen and Powers, 1991). DNA was extracted from each sample using a modified

organic extraction protocol (Baker, *et al.* 1997). The same sampling protocol was followed for the remainder of the experiment. ITS2 types were determined through denaturing gradient gel electrophoresis (DGGE) and subsequent sequencing of excised bands (see Chapter 2).

Preparation of Symbiodinium inoculate

Approximately 4cm^2 of *Porites astreoides* coral tissue was removed using an airbrush and artificial seawater, the resultant slurry was used to prepare freshly isolated *Symbiodinium*, following the protocol of Schwarz et al. (1999). Cultured *Symbiodinium* from clade A (BURR cultures Mf10.1 & FLAp1 10AB), clade B (BURR cultures Mf10.14b.01 & Ap4), and clade D (BURR cultures Mf10.8a & A013) were also added to the freshly isolated symbionts. This inoculate was mixed with live *Artemia* nauplii and target fed to the cores in the inoculation treatments (see below for timing of feeding).

Bleaching and recovery of Montastraea cavernosa cores

The three *M.cavernosa* parent colonies generated a total of 100 cores, which were bleached by exposure to 32^{o}C for 12 days under artificial light on a 12hr:12hr light:dark cycle. After 12 days at 32^{o}C, the coral tissue was sampled (using a clean razor blade) and cores were target fed (using a 2mL Pasteur pipette) with *Artemia* nauplii. Tank temperature was decreased to 29^{o}C, at a rate of 0.5^{o}C every ~8 hours. Once at 29^{o}C, corals were cleaned and moved to their randomly assigned recovery treatments (Figure 4.1), which were also set at 29^{o}C. The 5 different recovery exposures were as follows: 1) Exposure control – 11 cores in 1μm FSW; 2) Inoculation – 11 cores in FSW, inoculated

with a mix of freshly isolated and cultured *Symbiodinium* (see section on inoculate preparation); 3) D proximity – 11 cores in FSW, in close proximity (<1cm) to bleached and recovered parent *M. cavernosa* fragments dominated by clade D; 4) C proximity – 11 cores in FSW, in close proximity (<1cm) to unbleached parent fragments dominated by clade C; 5) Seawater control – 6 cores in fiberglass tank fed by sand-filtered Biscayne Bay seawater. Exposure conditions 1 through 5 were duplicated across 2 separate fiberglass tanks. Fifty cores were distributed in recovery exposures 1 through 5 and remained at 29^{o}C, and 50 cores were placed in the same exposure conditions initially set to 29^{o}C, and were ramped to 22^{o}C by 0.5^{o}C every 12 hours.

Three days after being moved to their recovery treatments all cores were fed *Artemia* nauplii, including cores in the 22^{o}C treatments, which had not yet reached their targeted treatment temperature. *Artemia* nauplii target fed to the inoculation treatment corals were supplemented with the mix of freshly isolated and cultured *Symbiodinium*. To maximize the efficiency of the target feeding, lights, incoming water, and pumps were turned off for ~ 2 hours whilst feeding was in progress. This feeding and inoculation process was repeated 4 days later, once all tanks and aquaria were at their designated temperatures. Corals were fed weekly for the remainder of the experimental period. Coral cores were randomly repositioned within their aquaria weekly, and aquaria were repositioned within the fiberglass tanks every 10-14 days, while cores and aquaria were cleaned.

Ten days after the end of the bleaching treatment (i.e., after 8 days in exposure treatments) tissue samples were taken from all cores (described previously). Subsequent tissue samplings were carried out 25 days, 2, 3, and 5 months after the end of bleaching.

Bleaching and recovery of Orbicella faveolata cores

The two *O. faveolata* parent colonies generated a total of 80 cores, which were bleached in 32°C seawater for 8 weeks under artificial light on a 12hr:12hr light:dark cycle. After 8 weeks at 32°C, the coral tissue was sampled (using a clean razor blade) and cores were target fed (using a 2mL Pasteur pipette) with *Artemia* nauplii. Tank temperature was decreased to 27°C, at a rate of 0.5°C every ~12 hours. Once at 27°C, corals were cleaned and moved to their randomly assigned recovery treatments which were also set at 27°C. The 4 different recovery exposures were as follows: 1) Exposure control – 20 cores in 1μm FSW; 2) Inoculation – 20 cores in FSW, inoculated with a mix of freshly isolated and cultured *Symbiodinium* (see section on inoculate preparation); 3) D proximity – 20 cores in FSW, in close proximity (<1cm) to bleached and recovered parent *O. faveolata* fragments dominated by clade D; 4) Seawater control – 20 cores in fiberglass tank fed by sand-filtered Biscayne Bay seawater.

Three days after being moved to their recovery treatments, all cores were fed with freshly hatched *Artemia* nauplii. Inoculation treatment corals were fed *Artemia* supplemented with the mix of freshly isolated and cultured *Symbiodinium*. To maximize the efficiency of the targeted feeding, lights, incoming water, and pumps were turned off for ~ 2 hours while feeding was in progress. This feeding and inoculation process was repeated 4 days later. Corals were fed weekly for the remainder of the experimental period. Coral cores were randomly repositioned within their aquaria weekly, and aquaria were repositioned within the fiberglass tanks every 10-14 days, as cores and aquaria were cleaned.

Two weeks after the end of the bleaching treatment (i.e., after 11 days in exposure treatments) tissue samples were taken from all cores (as described above). Subsequent tissue samplings were carried out 5 weeks, and 3.5 months after the end of the bleaching treatment.

Symbiont community characterization

An actin-based quantitative-PCR (qPCR) assay was used to monitor changes in symbiont community structure in *M. cavernosa* and *O. faveolata* cores during the course of the experiment. Samples were assayed in duplicate using primers and TaqMan-MGB (Life Technologies) probes targeting specific actin loci in *M. cavernosa* and *O. faveolata*, and *Symbiodinium* in clades A, B, C and D. The *O. faveolata* assays followed Cunning (2013), and the clade C and D assays were multiplexed, using the same primers and reaction conditions as described in Cunning & Baker (2013). *M. cavernosa* assays followed Silverstein (2013). All reactions were carried out in 10 µL volumes (with 5 µL Taqman Genotyping MasterMix and 1 µL DNA template) on StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Thermal cycling conditions consisted of an initial incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Cycle threshold (C_T) values were calculated by the StepOnePlus software package with a set fluorescence threshold of ΔR_n=0.01. Target DNA was considered present when amplification occurred in both technical replicates with no target detected in negative control reactions. C_T values for *M. cavernosa* and *O. faveolata* were reduced by 7.03 and 4.97 cycles, respectively, and *Symbiodinium* clades A, B and C by 3.46, 3.98 and 4.52, respectively, to normalize for

differences in fluorescent signal intensity among TaqMan-MGB fluorophores. Cycle reductions were calculated based on the results of standard curves generated following the methods of Cunning and Baker (2013). Adjusted C_T values were then used to calculate symbiont to host cell (S:H) ratios using the formula $2^{C_T(\text{host})-C_T(\text{symbiont})}$. These ratios were then further corrected to account for differences in actin gene copy number between clade A, B, C and D symbionts, 1, 1, 50 and 3 copies, respectively (Silverstein 2012; Cunning 2013).

Statistical analyses

The software package JMP (SAS Institute Inc.) was used to generate multiple regression models for the analysis of treatment effects on symbiont community dynamics. The models used total symbiont to host cell ratios (clade A S:H + clade B S:H + clade C S:H + clade D S:H) or percent of clade (e.g. clade D S:H / total S:H) as the response variable and parent as a random variable. Adjusted mean values were calculated that controlled for parent effect. To normalize the data prior to statistical analyses, symbiont to host cell ratios were log transformed and proportional data (i.e., percent D) were arcsine square root transformed. *Post hoc* Tukey's honestly significant difference (HSD) tests were performed to test for significance ($p < 0.05$).

RESULTS

Initial symbiont communities

DGGE analysis of tissue samples taken prior to bleaching showed that, at the detection resolution of this method, the *Montastraea cavernosa* corals hosted only

Symbiodinium ITS2 type C3, and the *Orbicella faveolata* hosted only type D1a. These symbionts have previously been found in *M. cavernosa* and *O. faveolata* in the Caribbean (LaJeunesse 2002; LaJeunesse et al. 2009). Quantitative PCR (qPCR) analysis of these same tissue samples, taken prior to bleaching, confirmed that *M. cavernosa* hosted only clade C symbionts, with a mean symbiont to host-cell (S:H) ratio of 0.07 (± 0.008) (Figure 4.2). The *O. faveolata* cores were confirmed as hosting only clade D, with mean S:H ratio 0.78 (± 0.16) (Figure 4.2). The symbiont community of the bleached and recovered *M. cavernosa* parent fragments switched to hosting clade D symbionts (99.8% D ± 0.1), while the unbleached control fragments remained 100% clade C. Despite the DCMU bleaching treatment and subsequent recovery temperature of 22^{o}C, the recovered *O. faveolata* parent fragments remained 100% clade D.

Bleaching recovery dynamics in Montastraea cavernosa

After bleaching, the *M. cavernosa* cores exhibited a mean decrease of 97.9% in their S:H ratio (Figure 4.2). The S:H ratios for all cores continued to decrease during the first 10 days of the recovery exposures, after which time most cores (90 %) no longer contained detectable levels of *Symbiodinium* (Figure 4.3a,b). However, by day 25 cores across all treatments had begun to recover their symbiont populations and, although there was no difference in the rate of recovery between treatments (as determined by total S:H ratio), there were differences in community composition (Figure 4.3c,d). At 29^{o}C, parental fragments strongly influenced initial recovery of the bleached cores, with bleached cores close to D-dominated fragments recovering with clade D, and bleached cores close to C-dominated colonies recovering with clade C. However, this effect was

not detected at 22°C, where recovering cores hosted mixtures of clades C and D, regardless of the symbiont type that was dominant in the parental fragment. Initial recovery of cores in the FSW-only treatment at 22°C was with clades A and D, whereas cores in the same treatment at 29°C showed no detectable symbionts, even after 25 days.

After 2 months, the effect of temperature on both the rate of recovery and the symbiont composition was significant (Figure 4.4). Cores in the 22°C treatments hosted predominantly clade A, which had the highest proportions (64-85%) across all treatments, except for the FSW-only treatment which was 73% D. The mean S:H ratio of cores at 29°C after 2 months was 12-fold higher than the cores at 22°C, and they were dominated (82% to 97%) by clade D symbionts. After 3 months, cores in the inoculation, C-proximity, and the seawater control treatments at 29°C appeared to have recovered completely (i.e., had mean S:H ratios which were similar to their initial values, Figure 4.5b), whereas the S:H ratios of the cores at 22°C were ~10-fold lower than initials (Figure 4.5a). Cores in the 22°C treatments did not fully recover (to mean S:H ratios comparable to their initial values) until 5 months after the bleaching treatment, at which point their communities were even mixtures of clades A and D (48.6% A vs. 50.1% D, matched pairs not significantly different, Figure 4.5c). Unfortunately due to equipment failure around day 130, which caused a transient spike in salinity in the 29°C tanks, there is no 5 month data for the corals in the 29°C treatments.

Bleaching recovery dynamics in Orbicella faveolata

After 8 weeks of bleaching, *O. faveolata* cores exhibited a mean decrease in their S:H ratio of 99.8%. Symbiont loss appeared to stop as soon as the high temperature stress

was removed (evidenced by a visible increase in pigmentation). After 2 weeks of recovery cores exhibited more than a 30-fold increase in mean S:H ratios (Figure 4.6b). During this early period of symbiont community reassembly, clade C symbionts constituted ~1% of the *Symbiodinium* in seawater control cores, but after 5 weeks all cores were 100% D1a (Figure 4.6c). Complete dominance by D still remained 15 weeks later, and thus there was no effect of treatment on the rate of recovery, or subsequent community structure.

DISCUSSION

Early biotic influences on M. cavernosa during recovery from bleaching

The continued decrease in S:H ratios during the first 10 days of recovery suggest the experimental cores were still bleaching after removal of heat stress. However, recent research has shown that bleached *Aiptasia pallida* enhance gastrodermal and endodermal cell proliferation during early recovery (Fransolet et al. 2013). This process provides more host cells to accommodate recovering *Symbiodinium*, but it would also decrease the S:H ratio. Therefore it is possible that the continued decreases in S:H ratio observed in *M. cavernosa* is a result of continued symbiont loss and/or post-bleaching proliferation of host-cells.

During the first 10 days of continued symbiont loss (and/or host cell proliferation) there was no change in the types of symbionts within the experimental cores. However, after 10 days, symbionts began to repopulate the coral tissue, and treatment-specific differences were detected (Figure 4.3c,d). After 25 days at 22^{o}C, exposure to healthy corals or unfiltered seawater resulted in the proliferation of clades C and D, commonly

found in symbiosis with *M. cavernosa*, and there was no difference between these treatments in these symbiont communities. However, cores recovering at 29°C showed a clear effect of exposure treatment on their symbiont community. At 29°C, only clade D symbionts were detectable in bleached cores close to healthy D-dominated fragments, and similarly, only clade C was detected in cores from the C-proximity treatment (Figure 4.3d). Cores in the inoculation and seawater control treatments hosted symbionts from clades A & D, and C & D, respectively, but there were no *Symbiodinium* detected in the FSW-only treatment. This suggests that, at 29°C, recovery is influenced by exogenous sources of *Symbiodinium*, whereas at 22°C the residual symbionts are more influential. This may be because remnant symbionts at 29°C are still compromised from the bleaching treatment (or absent), and therefore the exogenous sources of unstressed symbionts have a competitive advantage. Conversely, cooler temperatures and slower recovery rates may initially benefit the remnant symbiont populations, and decrease the ability of exogenous *Symbiodinium* to form new symbioses. Therefore, corals which experienced the same bleaching treatment exhibited different symbiont community founder dynamics depending on the abiotic and biotic conditions they were exposed to.

However, it is important to note that after 25 days, many cores (71%) still had no detectable symbionts. This may be a result of the non-destructive sampling method, which only samples tissue from the top of a coral polyp, and does not survey deeper tissue, which may host remnant *Symbiodinium*.

Abiotic factors influences on final symbiont community reassembly in M. cavernosa

After 2 months of recovery, mean S:H ratios were 12-fold higher in cores at 29°C compared to those at 22°C, indicating that temperature was the principal factor influencing the rate of symbiont community reassembly in the experimental corals. As well as the possible influence of a higher metabolism at the higher temperature, the cores at 29°C were dominated by clade D (Figure 4.4b), which can rapidly proliferate in bleached tissue, especially when in mixed assemblage communities (see Chapter 2). However, rapid proliferation would appear to be temperature dependent, as clade D was also dominant in the FSW-only treatment at 22°C, yet the cores in this treatment had the lowest S:H ratios (Figure 4.4a). The remaining exposure treatments at 22°C were dominated by clade A symbionts, which suggests that exogenous sources of symbionts may reduce the ability of clade D symbionts to dominate recovering tissue at cooler temperatures.

Cores that reached a S:H ratio similar to their initial value were assumed to have recovered. Recovery took almost 2 months longer for cores at 22°C compared to 29°C. However, all corals recovered with symbionts that were different from the ones they had prior to bleaching; all cores were initially dominated by clade C symbionts, whereas they were only a minor component of the community at the end of the recovery period. There was also a strong effect of temperature on recovered symbiont community structure: at 22°C, cores were co-dominated by clades A and D, which is unexpected given the previously reported dominance of clade C over D at this temperature (see Chapter 4). It is possible that clade A symbionts have a competitive advantage over clade C, but not over clade D, leading to mixed assemblages in these experimental cores.

Therefore, although this study did not conclusively show that exogenous sources of symbionts can increase the rate of recovery from bleaching, it did show that bleached *M. cavernosa* are capable of recovering with completely different symbiont communities (contrary to the conclusions of LaJeunesse et al. 2008; Coffroth et al. 2010). It also showed that corals may be capable of acquiring thermotolerance by recovering with symbiont communities dominated by *Symbiodinium* which have been shown to confer greater resistance to elevated temperatures (Rowan 2004; Silverstein 2012).

Bleaching resistance in Orbicella faveolata

Fragments of *O. faveolata* parent colonies which were bleached and recovered for the proximity treatment were created by exposing them to DCMU and high light. Although these fragments appeared to be dead at the end of this treatment, they recovered with clade D after 6 months in a flow-through tank at 22^{o}C. The *O. faveolata* cores also required 8 weeks of exposure to 32^{o}C to fully bleach, and they began to recover after <2 weeks in the recovery treatments. After 5 weeks of recovery these recovered cores were once again dominated by clade D symbionts, regardless of the exposure treatment. The high bleaching resistance (and resilience) of this coral-endosymbiont partnership is remarkable.

Conclusions

This study shows that bleached corals do not always recover from the residual symbionts remaining in host tissue. Exogenous sources of symbionts may alter the initial recovery trajectory of bleached corals, and may produce a coral-symbiont partnership

which is better suited to the prevailing environmental conditions. However, the influence of residual symbionts is dependent on both symbiont type and coral host species, and is further modulated by the abiotic environment, especially later in the recovery process. For example, *Orbicella faveolata* hosting *Symbiodinium* D1a prior to bleaching recovered with D1a at 22^{o}C and 27^{o}C, whereas *Montastraea cavernosa* hosting only *Symbiodinium* C3 recovered as either clade D dominated (at 29^{o}C) or with a mixture of clades A and D (at 22^{o}C). Further analysis of the health of these cores (e.g., calcification and photophysiology) would determine whether these symbiont community shifts had improved the corals fitness for the current environmental conditions, and increased their resistance/resilience to future stressors.

If exogenous sources of *Symbiodinium* better suited to the current environment can influence initial recovery from bleaching, then founder effects, symbiont availability, and proximity to healthy corals may be more important in determining the early recovery of bleached corals than have previously been assumed. Although abiotic factors, such as temperature, appear to be more influential in determining the eventual symbiont community, the availability of an exogenous source of thermally tolerant symbionts (through inoculations or close proximity of healthy corals) may improve the likelihood of a bleached coral surviving a subsequent thermal anomaly.

Figures:

Figure 4.1 Schematic illustrating experimental recovery treatments for bleached 2.5cm diameter cores of *Montastraea cavernosa*. Post-bleaching cores recovered in; 1µm filtered seawater (FSW) (exposure control); FSW + freshly isolated/cultured *Symbiodinium* (inoculation), FSW + parent colony fragments dominated by clade D (D proximity) or C (C proximity); and unfiltered seawater (seawater control), at two different temperatures, 22 and 29°C. *Orbicella faveolata* cores recovered in 4 treatments (exposure control, inoculation, D proximity and seawater control) at 27°C

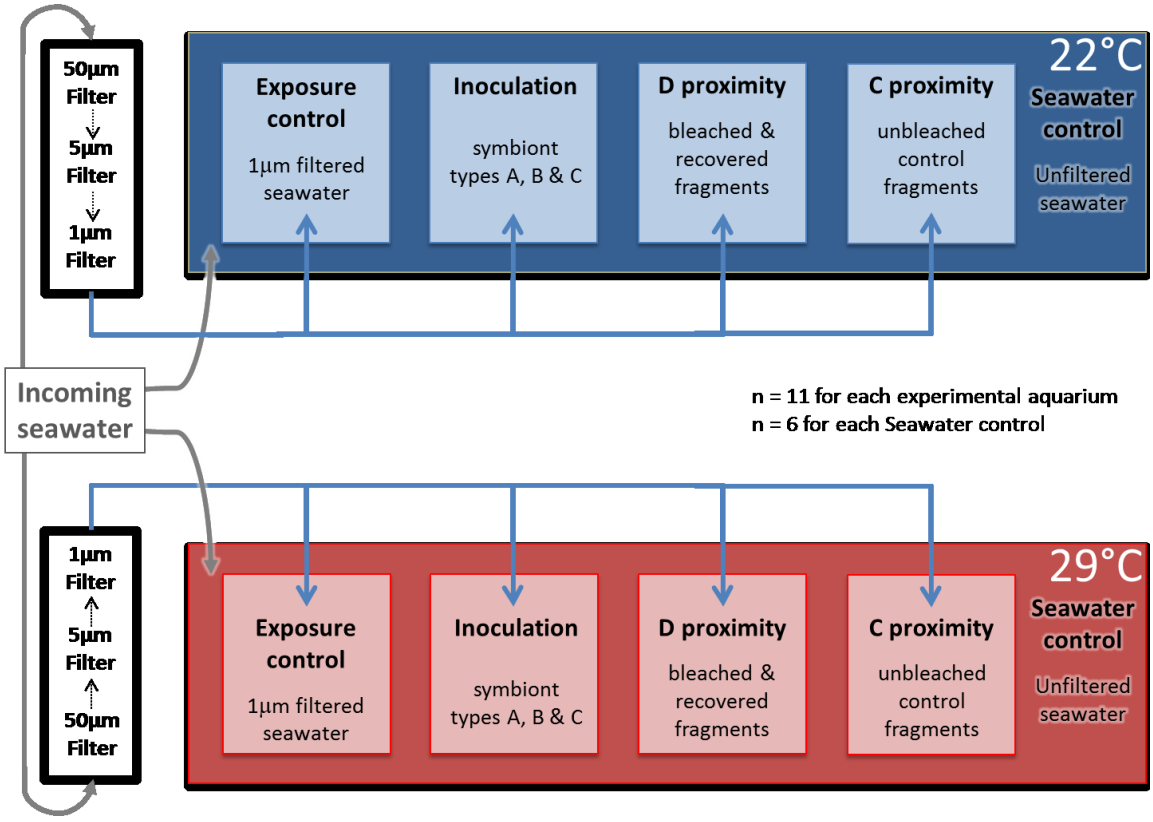


Figure 4.2 Effect of 10-day and 8-week bleaching treatments on total symbiont to host-cell (S:H) ratio in *Montastraea cavernosa* (light grey, dominated by C3*Symbiodinium*) and *Orbicella faveolata* (dark grey, dominated by D1a *Symbiodinium*), respectively. Bars represent the mean S:H ratios of cores prior to- (initial) and immediately post-bleaching (bleached). Error bars display standard error, n = 100 per sampling interval.

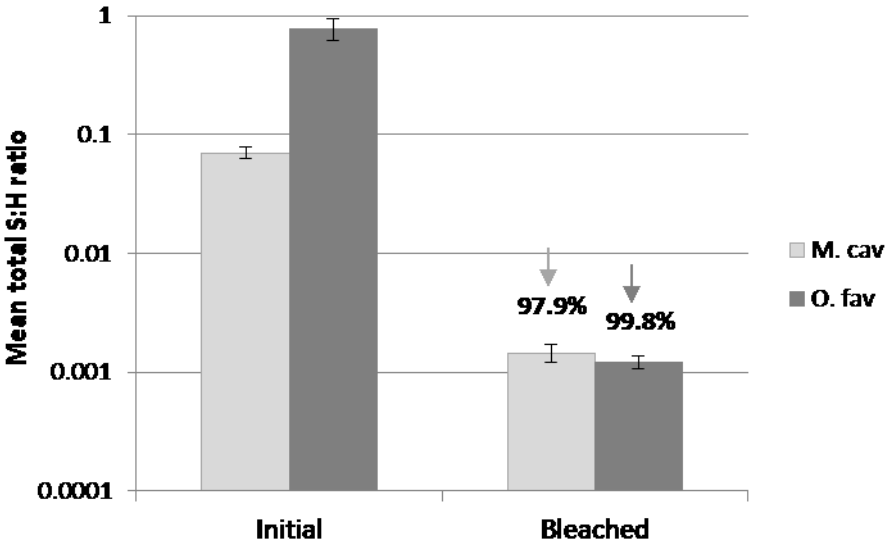


Figure 4.3 Shifts in symbiont community structure in *M. cavernosa* cores after 10 days (panels A & B) and 25 days (panels C & D) of recovery at 22°C (panels A & C) and 29°C (panels B & D). Bars represent the mean S:H ratio of clades D (red), C (blue), and A (green) in each treatment. Error bars display standard error, n = 11 per FSW treatment, n = 6 for seawater control.

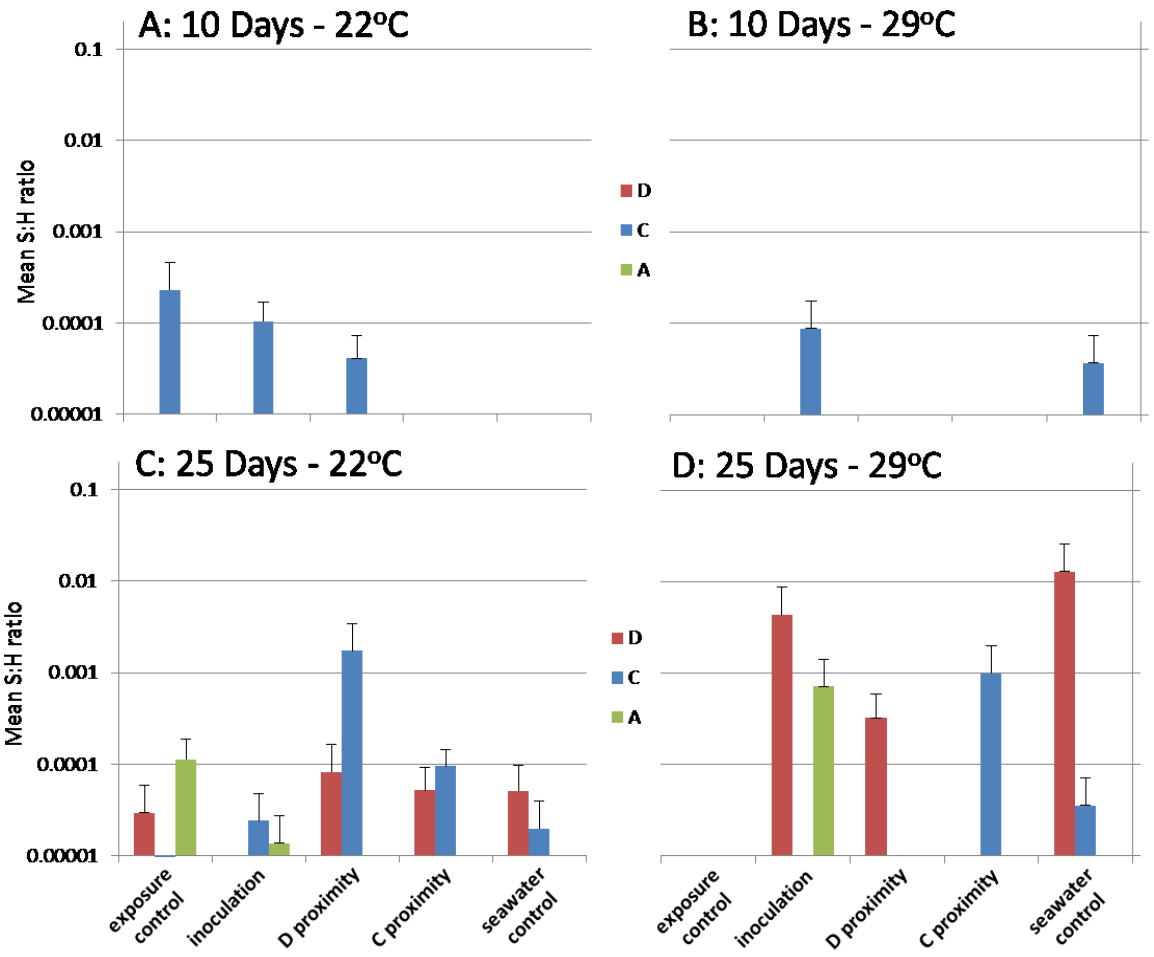


Figure 4.4 Impacts of recovery exposure and temperature on symbiont community structure in *M. cavernosa* cores after 2 months of recovery from bleaching. Stacked bars represent the relative proportions of clades D (red), C (blue), and A (green) to the mean total S:H ratio in each treatment (x-axis) at either 22°C (panel A) or 29°C (panel B). Error bars display standard error, n = 11 for FSW treatments, n = 6 for seawater control.

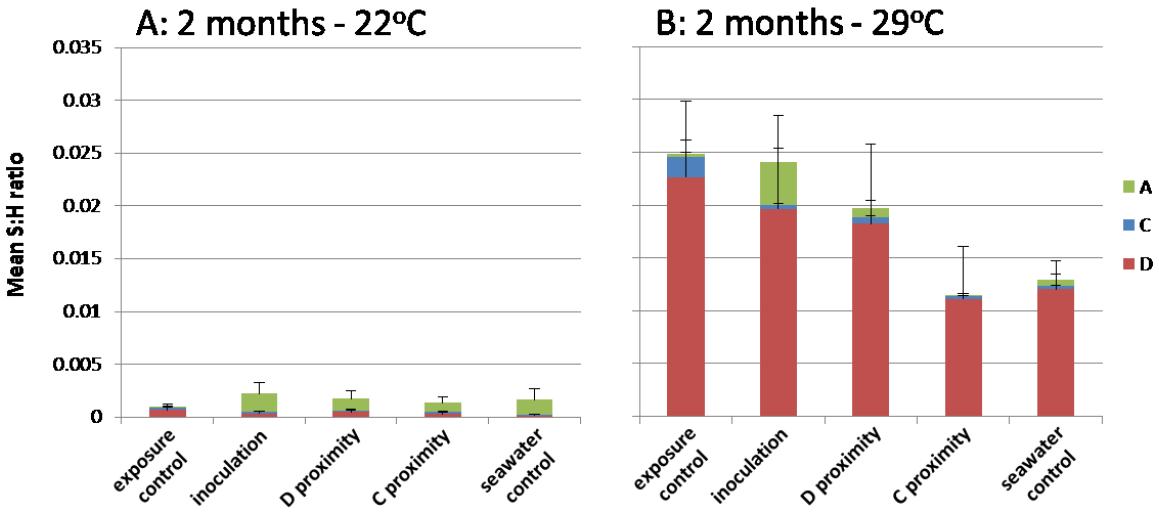


Figure 4.5 Impacts of recovery exposure and temperature on symbiont community structure in *M. cavernosa* cores at 3 months (panel A and B) and 5 months (panel C) post-bleaching. Stacked bars represent the relative proportions of clades D (red), C (blue), and A (green) to the mean total S:H ratio in each treatment (x-axis) at either 22°C (panel A and C) or 29°C (panel B). Error bars display standard error, n = 11 for FSW treatments, n = 6 for seawater control.

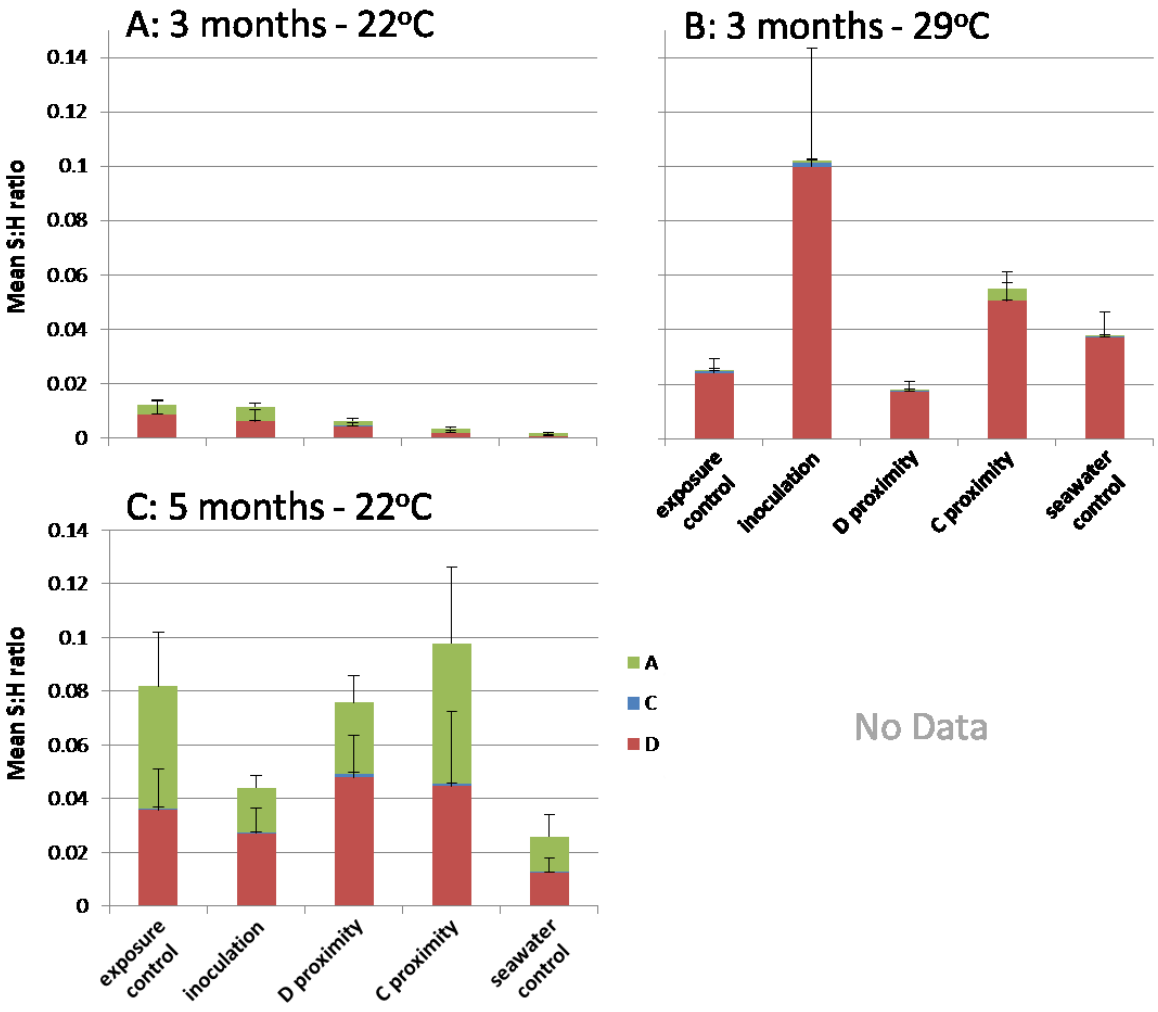
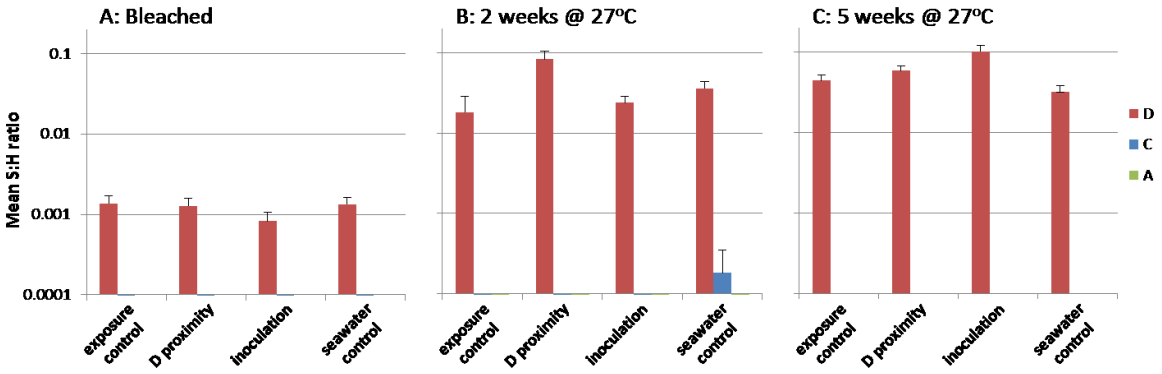


Figure 4.6 Extent of bleaching (panel A) and recovery in *Orbicella faveolata* cores after 8-week bleaching treatment. Bars represent the mean S:H ratios of clades A (green), C (blue), and D (red) in each treatment (x-axis) after 2 weeks recovery (panel B) and 5 weeks recovery (panel C). Error bars display standard error, n = 20 per treatment.



Chapter 5

Vectored inoculation of reef corals with different algal symbionts (*Symbiodinium* spp.) using transplant plugs

SUMMARY

Reef corals can recover from bleaching (the stress-induced expulsion of symbiotic dinoflagellates in the genus *Symbiodinium*) with different symbionts, especially when the recovery environment is different from the original environment. However, the process of symbiont community change has seldom been investigated in healthy corals, and has never been tracked in a quantitative way across colony surfaces. This experiment investigated this process by bleaching replicate 2.5cm cores from 5 colonies of *Montastraea cavernosa* and allowing them to recover at different temperatures to produce experimental microcolonies of the same coral genotype that were dominated (>99%) by either *Symbiodinium* ITS2-types C3 or D1a. A smaller core (1.25cm diameter) was then removed from each experimental microcolony, and these tissue plugs were reciprocally transplanted between microcolonies containing different symbionts. By using tissue plugs of the same genotype as vectors to introduce different symbionts we investigated the invasibility of different *Symbiodinium* into neighboring tissue. Paired cores were randomly assigned to 22^{o}C or 29^{o}C treatments, and tissue biopsies (~1.5mm diameter) were taken along a mini-transect across each core, at a distance of ~2mm and ~10mm from either side of the transplant-core boundary. Samples were taken 1, 2, 3, and 8 months after transplanting, and DNA was analyzed using an actin based qPCR assay. Transplanted corals in the 29^{o}C treatment exhibited displacement of *Symbiodinium* C3 by D1a, but at 22^{o}C *Symbiodinium* D1a was replaced by C3. This change was also more

rapid and more complete than the displacement of C3 by D1a at 29°C, as the mean proportion of clade D in the cores at 22°C was <1%, compared to ~25% clade C remaining at 29°C. The rate of displacement at both temperatures was influenced by core size, with less competitive symbionts being replaced in the smaller transplant cores more rapidly than in the larger host cores, suggesting the greater pool size of symbionts close to the transplant-core boundary accelerated proliferation and spread. At 22°C there was also an effect of distance from the source of C3, with D1a close to the transplant-plug/host-core border being displaced faster than D1a further away. These results indicate that the invasibility of different *Symbiodinium* into neighboring coral tissue is dependent on temperature and symbiont abundance. They also suggest the modes of invasion may differ between symbiont type, with *Symbiodinium* C3 progressing stepwise through coral tissue, while D1a is released to the environment and re-acquired by neighboring polyps, spreading evenly throughout nearby tissue. These findings offer the first insight into the proliferation and spread of different *Symbiodinium* within coral tissue, help understand the response of individual corals to environmental change, and have implications for the resistance and resilience of reef corals to projected climate change.

BACKGROUND

The endosymbiotic unicellular algae hosted by scleractinian corals (and numerous other marine invertebrates) were originally thought to be a single species, *Symbiodinium microadriaticum* (Freudenthal 1962), with a cosmopolitan distribution. Subsequent ribosomal RNA sequence analysis revealed that *Symbiodinium* is highly diverse (Rowan & Powers 1992), consisting of at least 9 different clades (A-I), some of which are more

genetically distinct than some orders of non-symbiotic dinoflagellate (Santos et al. 2004; Coffroth and Santos 2005; Pochon et al. 2006; Pochon and Gates 2010). The relationship between algal symbionts and their coral hosts was also believed to be highly specific, with certain coral species only associating with a particular symbiont type, and vice versa (e.g. LaJeunesse et al. 2004; 2010). However, evidence suggests that, while specificity is still prevalent, many coral species can associate with more than one symbiont type (Rowan and Knowlton 1995; Rowan et al. 1997; Baker 2003; Baker and Romanski 2007), often simultaneously (Mieog et al. 2007; Silverstein et al. 2012) Traditional PCR-based techniques (such as DGGE) are generally only capable of revealing dramatic shifts in symbiont dominance, and often fail to detect unusual symbionts at low abundance in mixed symbiont communities (Thornhill et al. 2006). With the advent of more sensitive quantitative-PCR (qPCR) assays (Mieog et al. 2009; Cunning and Baker 2013), it is now possible to identify and quantify the dynamic changes occurring within symbiont communities in response to variations in environmental conditions.

Characterizing symbiont community structure in corals is important because the high genetic diversity of *Symbiodinium* is complemented by substantial levels of physiological diversity within and between clades. Certain symbiont types may confer greater stress tolerance (Rowan 2004; Berkelmans and van Oppen 2006; Jones et al. 2008; Fitt et al. 2009) while other types may provide more benefits to their host under non-stressful conditions (Little et al. 2004; Loram et al. 2007). Consequently, corals hosting mixed assemblages of *Symbiodinium* types, in varying proportions (Rowan et al. 1997; Silverstein et al. 2012), may have greater capacity to rapidly acclimatize to

changing environmental conditions, by shifting the relative abundance of those symbiont types most favorable to the new environmental conditions (Cunning 2013; see also Chapter 2).

It has been known for some time that symbiont communities are not static. Seasonal (Brown et al. 1999; Fitt et al. 2000; Ulstrup et al. 2008), and even daily (Jones & Yellowlees 1997; Fitt 2000), regulation of symbiont abundance can result in adult corals releasing *Symbiodinium* from their tissue. Discharged algal cells from unstressed corals, as well as symbionts that survived digestion by corallivores (Muller-Parker 1984; Castro-Sanguino & Sánchez 2012), enter the consortium of free-living *Symbiodinium* which occupy the water column, reef sediments and macroalgal beds (Littman et al. 2008; Porto et al. 2008; Pochon et al. 2010). These free-living communities have been shown to be sources of symbionts for aposymbiotic coral larvae (Adams et al. 2009; Abrego et al. 2009), and could also be a source of symbionts for bleached corals (Lewis and Coffroth 2004; Coffroth et al. 2006; Coffroth et al. 2010; see Chapter 5). However, one question which has not yet been addressed is whether healthy (non-bleached) adult corals are capable of obtaining symbionts from their surrounding environment.

Corals hosting an actively proliferating symbiont community discharge photosynthetically active *Symbiodinium* into the surrounding water column (Hoegh-Guldberg et al. 1987; Jones & Yellowlees 1997; Baghdasarian and Muscatine 2000) which could act as a seed stock of potential symbionts for neighboring corals hosting less favorable algae. The acquisition and spread of different *Symbiodinium* could generate a fitter coral that is better suited to the prevailing environmental conditions, without the (high and sometimes fatal) costs associated with bleaching.

This study aimed to investigate symbiont proliferation and spread in healthy adult corals by analyzing symbiont migration between replicate cores taken from individual colonies of the Caribbean coral *Montastraea cavernosa* which had been manipulated to be dominated (>99%) by symbionts from either clade C or from clade D. Transplant plugs (single-polyp sized mini-cores) taken from experimental cores dominated by clade C, were reciprocally swapped with transplant plugs taken from clade D dominated cores. These corals were then exposed to different temperatures and changes in their symbiont community structure were monitored over time at different points across the core surface. This study offers the first insight into the proliferation and spread of different *Symbiodinium* within healthy coral tissue. Determining the potential of symbiont communities in individual corals to respond to environmental change has implications for resolving the potential resistance and resilience of reef corals to projected climate change.

MATERIALS AND METHODS

Preparation of coral cores

In October 2010, five *Montastraea cavernosa* colonies were collected from Breakers Reef, West Palm Beach, Florida USA, from a depth of ~12 m. Replicate cores were generated from the parent colonies using a 2.5cm diamond tipped core drill (Starlite Industries, Rosemont, PA) and core bases were trimmed using a tile saw to produce level cores with a consistent height (approximately 1cm). Cores were glued to plaster reef plugs (Boston Aqua farms, Boston, MA) and left for 2 months to recover in a 75 gallon flow-through seawater tank maintained at 26^{o}C, under artificial light (400W metal halide, IceCap Inc., Hamilton, NJ) on a 12 hour light, dark cycle. Previous research revealed that

recovering bleached *M. cavernosa* corals at 29°C promoted dominance of *Symbiodinium* ITS2-type D1a (Silverstein 2012). Therefore, after the 2-month recovery period, randomly selected cores were experimentally bleached by exposing them to 32°C for 10 days. They were then left to recover at 29°C for 6 months, while the remaining (control) cores remained at 26°C. Tissue samples were taken from each core using a clean razor blade, and samples were heated for 1.5 hours at 65°C in 300µL of 1% SDS in DNA buffer (Rowen and Powers, 1991). DNA was extracted from each sample using a modified organic extraction protocol (Baker, *et al.* 1997). ITS2 types were determined through denaturing gradient gel electrophoresis (DGGE) and subsequent sequencing of excised bands (see Chapter 2). Following the 6 months recovery at 29°C, cores were returned to 26°C at 0.5°C per day, and held for a further 6 months at this temperature. After which, all cores were sampled again and reanalyzed for ITS2 type using DGGE. Control cores were found to be dominated by clade C symbionts, while bleached cores were dominated by clade D symbionts.

Reciprocal transplants

Transplant plugs were generated by coring the 2.5cm diameter *M.cavernosa* cores with a 1.25cm diamond tipped core drill (Starlite Industries, Rosemont, PA), producing mini-cores slightly larger than a single polyp (Figure 5.1a, b). Transplant plugs were randomly paired with larger cores from the same parent colony, but hosting different symbiont type, specifically, a C3-dominated transplant plug paired with D1a-dominated 2.5cm core, and vice versa (N = 20 pairs). Transplants were held in place by gluing the base of the plug to the base of the 2.5cm core, this provided direct contact between the

coral tissue along the core edge. Transplant plugs were also randomly paired with 2.5cm cores from the same parent colony hosting similar symbiont communities (i.e., transplant controls). Initial tissue samples were taken before each core was drilled and DNA was extracted as previously described.

Paired coral cores were randomly assigned to either 22°C or 29°C treatments, which were initially set at 26°C. Once the cores had been assigned to treatments, target temperatures were reached by ramping temperatures at 0.5°C per day, until the required temperature was achieved. After a period of 3 weeks at target temperature, coral cores were sampled in a ~2.0 cm transect across their surface (Figure 5.1c). Due to the need to disturb symbiont communities as little as possible during their recovery, a novel (adapted from Kemp et al. 2008) and minimally-invasive sampling technique was required. A transect of four micro-biopsies was taken across each core using a 2mL syringe fitted with a 200µL pipette tip. This removed a tissue biopsy approximately 1.5mm in diameter, in 1.5mL of seawater. Tissue scars left by this sampling methodology were no longer visible after ~one week. The transect across the transplant pair consisted of micro-samples taken at a distance of ~2mm and ~10mm from either side of the transplant-core boundary (Figure 5.1c). Transects were sampled across each core 1 month (3 weeks after target temperatures were reached), 2 months, 3 months, and 8 months after the reciprocal transplanting. Tissue samples were centrifuged at 10,000g for 10 min, the supernatant was removed and discarded, and 200µL 1% SDS (in DNA buffer, Rowen and Powers, 1991) was added to the sample pellet, which was then heated at 65°C for 1.5 hours. DNA was extracted from each sample as described previously (Baker, *et al.* 1997) and finally eluted in 50µL of 1X Tris-EDTA buffer.

Symbiont community characterization

An actin-based quantitative-PCR (qPCR) assay was used to monitor changes in symbiont community structure in *M. cavernosa* cores during the course of the experiment. Samples were assayed in duplicate using primers and TaqMan-MGB (Life Technolgies) probes targeting specific actin loci in *M. cavernosa*, and *Symbiodinium* in clades C and D. The clade C and D assays were multiplexed, using the same primers and reaction conditions as described in Cunning & Baker (2013), *M. cavernosa* assays followed Silverstein (2012). All reactions were carried out in 10 µL volumes (with 5 µL Taqman Genotyping MasterMix and 1 µL DNA template) on StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Thermal cycling conditions consisted of an initial incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Cycle threshold (C_T) values were calculated by the StepOnePlus software package with a set fluorescence threshold of ΔR_n=0.01. Target DNA was considered present when amplification occurred in both technical replicates with no target detected in negative control reactions. C_T values for *M. cavernosa* and *Symbiodinium* clade C were reduced by 7.03 and 4.41 cycles, respectively, to normalize for differences in fluorescent signal intensity among TaqMan-MGB fluorophores. Cycle reductions were calculated based on the results of standard curves generated following the methods of Cunning and Baker (2013). Adjusted C_T values were then used to calculate symbiont to host cell ratios using the formula $2^{C_T(\text{host})-C_T(\text{symbiont})}$. These ratios were then further corrected to account for differences in actin gene copy number between clade C and D symbionts, 50 and 3 copies, respectively (see Chapter 2).

Analysis of 20 randomly chosen DNA samples, taken prior to transplanting, using qPCR primers and probes for *Symbiodinium* clades A, B, C, and D, determined that A and B were absent (or below the qPCR detection limit) and only C and D symbionts were present these corals. Subsequent qPCR analyses therefore only used primers/probes sets specific for *M. cavernosa*, clade C and clade D

Statistical analyses

The software package JMP (SAS Institute Inc.) was used to generate multiple regression models for the analysis of treatment effects on symbiont community dynamics. Analysis models used percentage of clade D (clade D S:H / (clade D S:H + clade C S:H)) as the response variable, with core type (host or transplant) and sampling location (core or border, Figure 5.1c) as model effects. To normalize the data prior to statistical analyses, percent D data were arcsine square root-transformed for use in all statistical models. *Post hoc* Tukey's honestly significant difference (HSD) tests were performed to test for significance ($p < 0.05$).

RESULTS

Initial symbiont communities

DGGE analysis showed that, at the detection resolution of this method, the unbleached, control, *Montastraea cavernosa* cores hosted only *Symbiodinium* ITS2 type C3, whereas the bleached and recovered cores hosted only type D1a. Both these symbionts have previously been found in *M. cavernosa* in the Caribbean (LaJeunesse et al. 2009). This bleaching and recovery treatment therefore yielded genetically identical

Montastraea cavernosa cores (i.e., from same parent colony) which were dominated by different types of *Symbiodinium*. Quantitative PCR (qPCR) analysis of these same tissue samples taken prior to the generation of transplant plugs revealed that the mean proportion of the dominant symbiont type in both the clade C and clade D corals was 99.9% (± 0.1%, Figure 5.2a-d). Clade C and D corals also hosted similar mean abundance of *Symbiodinium*, with mean symbiont to host cell (S:H) ratios of 0.027 (± 0.005) and 0.029 (± 0.006), respectively. The transplant plugs therefore had a similar symbiont abundance (as measured by S:H ratios) to their adjacent host core, but they contained very different symbiont communities.

Effect of temperature on symbiont proliferation and spread

One month after the reciprocal transplant of plugs into cores, symbiont community structure in the majority of host corals and transplant plugs remained similar to initials (Figure 5.2a-d). However, after 3 months at 22^{o}C, the clade D symbionts in the initially D-dominated corals were being displaced by clade C symbionts. This decline in the proportion of clade D was significantly greater in the smaller D-transplant plugs than in the larger D-host cores (Figure 5.3a). In addition, the mean percent D in tissue close to the border between transplant and host cores was significantly lower than the mean percent D in the distal portions, in both the transplant plug and the host core (Figure 5.3a): D-host core, 82.8% (± 4.0%); D-host border, 63.2% (± 4.9%), D-transplant plug, 19.9% (± 3.5%); D-transplant border, 7.4% (± 2.2%). Throughout this entire 3 month period, corals at 22^{o}C which had started the experiment dominated by clade C exhibit no change in the proportion of C or D hosted by. After 8 months, the initially C-dominated

cores remained C-dominated whereas the mean percent D for the initially D-dominated cores was 0.3% (± 0.2%). There was no significant difference between any of the cores or sampling points after 8 months (Figure 5.2a, c).

In contrast to the corals at 22^{o}C, there were shifts in the symbiont communities of both the clade D-dominated and C-dominated corals at 29^{o}C. Early results suggested that corals were converging toward an equally mixed assemblage of clade C and clade D symbionts; however, this pattern changed in favor of clade D symbionts after 2 to 3 months of exposure to 29^{o}C (Figure 5.2 b, d). Corals originally dominated by clade C exhibited a core size effect, with clade C symbionts in the smaller transplant plug being displaced more rapidly than those in the host core, although there was no difference between the border and core communities (Figure 5.3b). After 8 months at 29^{o}C, the mean percent D within clade C transplant plugs was not significantly different from the clade D corals, 76.4% (± 11.5%), whereas the initially C-dominated host cores contained significantly less D, 29.2% (± 12.9%). After 8 months the initially D-dominated cores appeared to be equilibrated, hosting a symbiont community with a mean percent D of 91.1% D (± 9.6%)

DISCUSSION

Novel experimental approach using manipulated coral symbioses

This study utilized coral cores taken from the same parent colonies and manipulated them to host different symbionts, with a similar symbiont abundance (as measured by S:H ratio). It is the first study of its kind to investigate the invasibility of different symbionts into neighboring tissue by using transplant plugs as vectors. It

investigated proliferation and spread of different *Symbiodinium* across coral surfaces while controlling for the effects of coral genotype, and avoiding potential inter-genotype aggression. The innovative mini-transect syringe-based sampling method developed for this study (modified from Kemp et al. 2008) worked successfully and permitted the characterization of small-scale changes in symbiont community structure across the 2.5cm diameter coral cores. Also, despite the potentially damaging effects of producing single-polyp sized cores (1.25cm) from 2.5cm diameter cores, there was no mortality in any of the reciprocal transplant corals. By the end of the experiment, the borders between the host-cores and the transplant-cores were almost indistinguishable (see Figure 5.4).

Cool temperatures promote the spread of clade C into D-dominated tissue

Symbiodinium ITS2-type C3 appears to be competitively superior to D1a in *Montastraea cavernosa* at 22^{o}C. Corals which started the experiment hosting > 99% clade C showed no change in their symbiont communities, despite being in close proximity to corals containing clade D *Symbiodinium*. In contrast, clade D symbionts were almost completely displaced from tissue that was originally >99% clade D, comprising <1% of the symbiont community in these tissues after 8 months. This displacement of clade D by clade C symbionts is surprising given previous reports of clade D's opportunistic and highly invasive nature (Abrego et al. 2009; LaJeunesse et al. 2009).

It appears that the resistance of *Symbiodinium* D1a to elevated temperatures does not confer an equal resistance to low temperatures (e.g. Tchernov et al. 2004; Ladner et al. 2012). Corals dominated by members of clade D have been found to bleach in

response to low temperatures (Howells et al. 2013), but the 22°C treatment used in this study did not induce bleaching in any of the experimental cores. Furthermore, after 8 months at 22°C all of the control cores (D:D core swaps) remained dominated by clade D (mean 93.4% D), indicating that it was invasion and displacement from adjacent clade C-dominated tissue that drove the observed shifts in community structure.

The rate of displacement of *Symbiodinium* D1a by C3 was influenced by both core size (i.e., host core vs. transplant plug) and the distance from the source of C3 (mini-transect biopsy location). Symbiont communities closer to the border between the transplant plug and host cores showed more rapid displacement of D by C, regardless of core size (Figure 5.5a). Additionally, the overall displacement of D by C occurred more rapidly in the transplant plugs compared to the host cores (Figure 5.3a), indicating that the larger border area, relative to the transplant plug, and/or the greater pool size of host core symbionts accelerated the displacement of clade D.

Warmer conditions favor mixed assemblages

During the first 2-3 months at 29°C, while the corals continued to repair the border between host core and transplant plug, the proportion of clade D in the initially D-dominated corals decreased. This decrease was significantly greater in the border region, compared to the distal portion of the D cores (Figure 5.5b). However, after 8 months, the mean proportion of D in the border tissue had returned to the same level as the distal portion of the core. This suggests that the repair zone may have initially fostered the displacement of clade D symbionts by clade C, but that this effect disappeared once the core tissues were fully merged (Figure 5.4). The results also imply that at 29°C the

optimal symbiont community is not 100% D, because the proportion of clade C in the initially D-dominated cores remained at ~10% for the final 5 months of the experiment.

Symbiodinium C3 was more competitive at 22°C than D1a was at 29°C. In the initially clade C-dominated cores at 29°C, the displacement of clade C symbionts by D was slower and less complete than the displacement of D by C at 22°C. The percent D in the C-transplant plugs was similar to the D-border after 8 months at 29°C (Figure 5.2b), whereas the mean percent D in the larger C host cores was only ~30% (Figure 5.2d). However, the range in the proportion of D within each of these host cores was large (8 – 91%), suggesting that although it takes time for D1a to initiate the displacement of C3 at 29°C, once the community begins to change, it is driven towards clade D dominance. The slower displacement of *Symbiodinium* C3 by D1a at 29°C (compared to displacement of D1a by C3 at 22°C, Figure 5.3a,b) could be due to a reduced competitive advantage i.e., C3 at 29°C is less competitively compromised than D1a at 22°C. Furthermore, the mode of initial uptake for D1a may also be different from that of C3, as suggested by the absence of a diffusive invasion pattern (i.e., no difference between distal vs. border).

Mechanisms of proliferation within coral tissue

Two potential hypotheses for the observed diffusive displacement pattern of *Symbiodinium* D1a by C3 at 22°C are: 1) clade C symbionts invade directly through the coral tissues, i.e., non-oral uptake; 2) symbionts released into the water column from the tissue cores were acquired orally, phagocytized (Schwarz et al. 1999) and, due to their greater fitness (compared to the resident D1a symbionts), were preferentially translocated to areas of maximum growth and calcification, such as the border between the host and

transplant plugs where active repair was occurring (Figure 5.4). These hypotheses need not be mutually exclusive, but which one applies most accurately may depend on symbiont type.

Symbiodinium type C3 is the most common symbiont of *M. cavernosa* in the Caribbean (LaJeunesse 2002; LaJeunesse et al. 2009). As such, the pathways which exist for symbiont translocation throughout the coral tissue (Gladfelter 1983; Gateno et al. 1998; Santos et al. 2009) may be directly accessible, thus bypassing the requirement for initial phagocytic uptake within the gastrovascular cavity of a coral polyp. This method of proliferation and spread might explain why the invasive C3 symbionts were present at higher abundance closer to their source. The diffusive displacement of clade D by clade C symbionts in the border tissues occurred at 29^{o}C during first 2-3 months, after which it ceased (Figure 5.2a,b). Therefore, the direct invasion of C3 (bypassing oral uptake) may only be possible through coral tissue undergoing growth and/or repair, and thus, symbiont acquisition reverted back to gastrovascular uptake once the repair was complete.

In contrast, the mean proportion of clade D proliferating into tissue that was originally C-dominated was similar regardless of where the tissue was sampled (i.e., near border vs. the more distal end of the core), therefore, the change from C3 to D1a at 29^{o}C did not show any effect of distance from source. This even appearance of clade D throughout these tissues suggests that the mode of displacement may differ for D1a compared to C3, with D1a being taken up from the water column after being released from adjacent tissue, i.e., not entering directly through the tissue. *Symbiodinium* D1a has been described as both opportunistic and highly invasive (Abrego et al. 2009; LaJeunesse et al. 2009; Stat and Gates 2011), and has been found in symbioses with numerous

different coral hosts (Silverstein et al. 2012). *Symbiodinium* D1a may therefore have a greater rate of uptake via gastrodermal phagocytosis in a variety of coral species, with the host rapidly distributing it throughout each coral polyp, where they may then proliferate faster than the current resident symbionts (especially in bleached corals, see Chapter 2).

Conclusions

The outcomes of vectored inoculation of *M. cavernosa* with different *Symbiodinium* using transplant plugs tissue was dependent on temperature and symbiont abundance. Transplanted corals in the 29^{o}C treatment exhibited displacement of *Symbiodinium* C3 by D1a. The rate of this displacement was faster in the smaller transplant plug, but there was no effect of proximity to source, indicating that it was the larger localized pool of host core symbionts within the water column which accelerated the displacement of clade C symbionts. At 22^{o}C, *Symbiodinium* ITS2-type C3 rapidly (relative to D1a displacing C3) and thoroughly displaced D1a from the adjacent tissues. Displacement exhibited an effect of core size, but was also influenced by distance from the source of C3. This suggests the modes of invasion may differ between symbiont type, with *Symbiodinium* C3 possibly entering directly through the coral tissue, while D1a is taken up orally and evenly distributed throughout the polyp. These findings offer the first insight into the proliferation and spread of different *Symbiodinium* within coral tissue, and help to understand how environmental changes and exogenous sources of *Symbiodinium* may influence symbiont community dynamics within individual corals. This provides valuable information for researchers and conservationists interested in maximizing the resistance and resilience of reef corals to projected climate change.

Figures:

Figure 5.1 Schematic illustrating reciprocal transplant experimental design: A) 2.5cm diameter cores of *Montastraea cavernosa* originating from the same parent colony and dominated (>99%) by either *Symbiodinium* ITS2 type C3 (blue) or D1a (red); B) Schematic representation of reciprocal transplantation, showing a 1.25cm diameter transplant plug dominated by *Symbiodinium* type C3 transplanted into a 2.5cm core dominated by type D1a, and vice versa; C) Schematic of mini-transect showing approximate location of the 4 micro-biopsies taken from each host core/transplant plug pairing. Tissue samples were taken approximately 2mm and 10mm from the core boundary, in both the transplant plug and the host core.

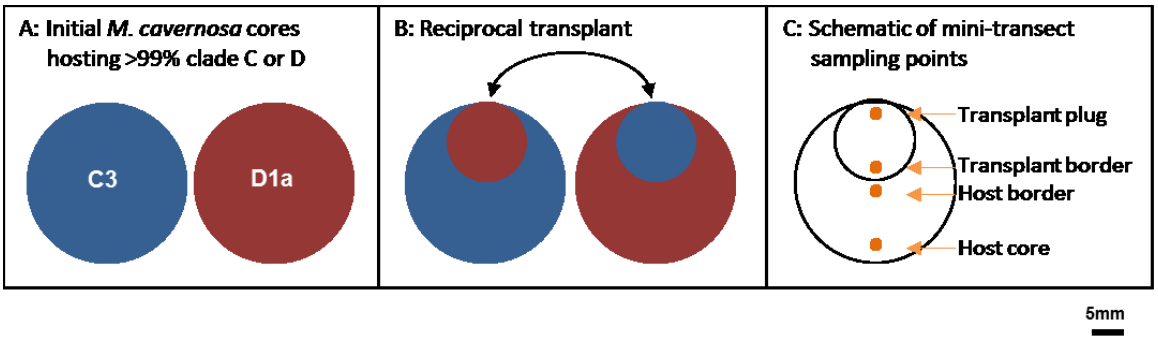


Figure 5.2 Effect of temperature, core size, and transect position on symbiont community change in clade D-dominated (red) and clade C-dominated (blue) tissue in reciprocal transplants of *Montastraea cavernosa*. Data points represent pooled means for each mini-transect sampling location (host-core, host-border, transplant-border, transplant-plug, see Figure 5.1), in D-host/C-transplant (DH/CT) at 22°C (panel A) and 29°C (panel B), and in D-transplant/C-host (DT/CH) at 22°C (panel C) and 29°C (panel D). Black and white symbols represent distal and proximal transect points, respectively, dominated by clade D (squares) or clade C (triangles). Error bars display standard error, n = 5 per sampling interval.

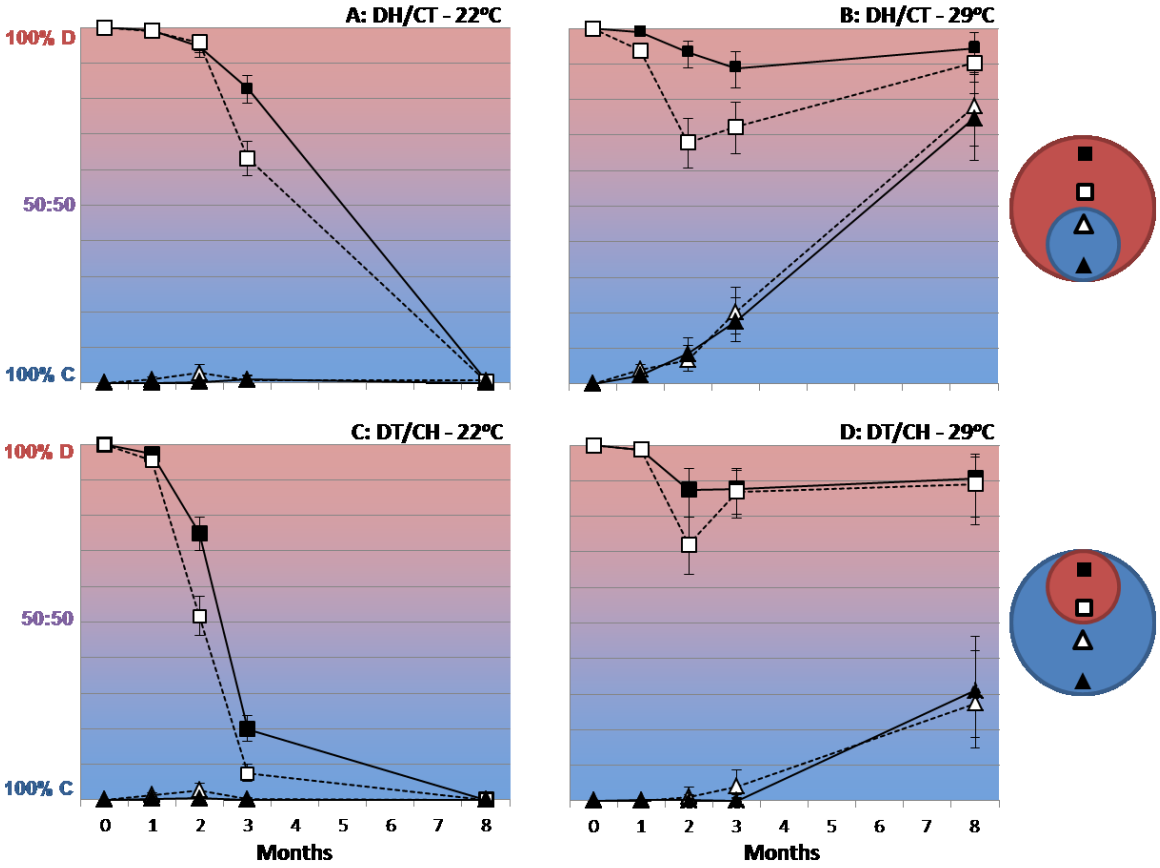


Figure 5.3 Effects of temperature, core size, and transect position on displacement of clade D symbionts by clade C at 22°C (panel A), and of clade C symbionts by clade D at 29°C (panel B). Data points represent pooled means for each mini-transect sampling location (host-core, host-border, transplant-border, transplant-plug), in the initially D-dominated cores at 22°C (panel A) and the initially C-dominated cores at 29°C (panel B). Black and white symbols represent distal and proximal transect points, respectively. Error bars display standard error, n = 5 per sampling interval.

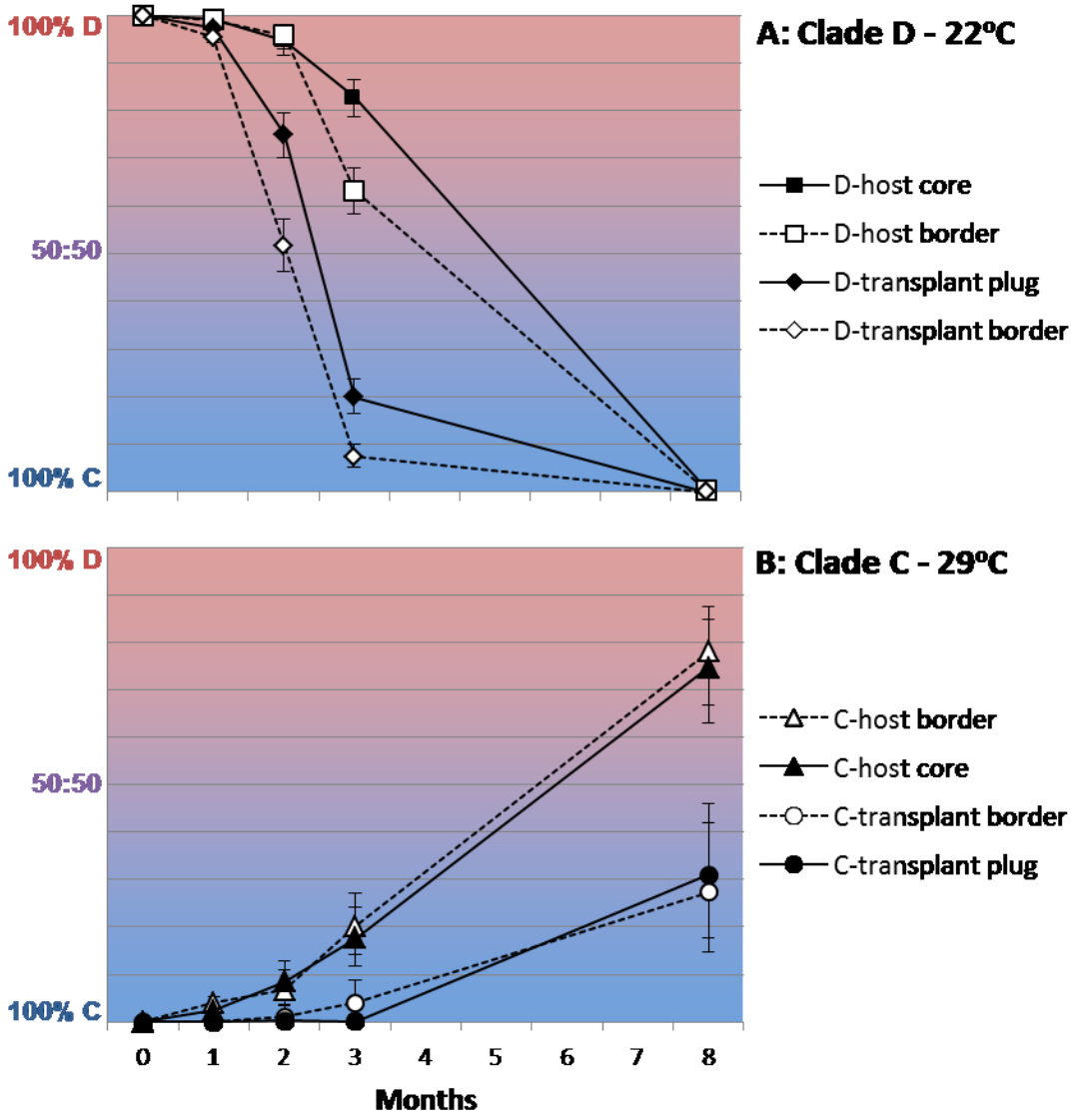


Figure 5.4 Representative images of C3- and D1a-dominated cores prior to transplant coring (initial), immediately after transplanting (transplant), and 8 after months.

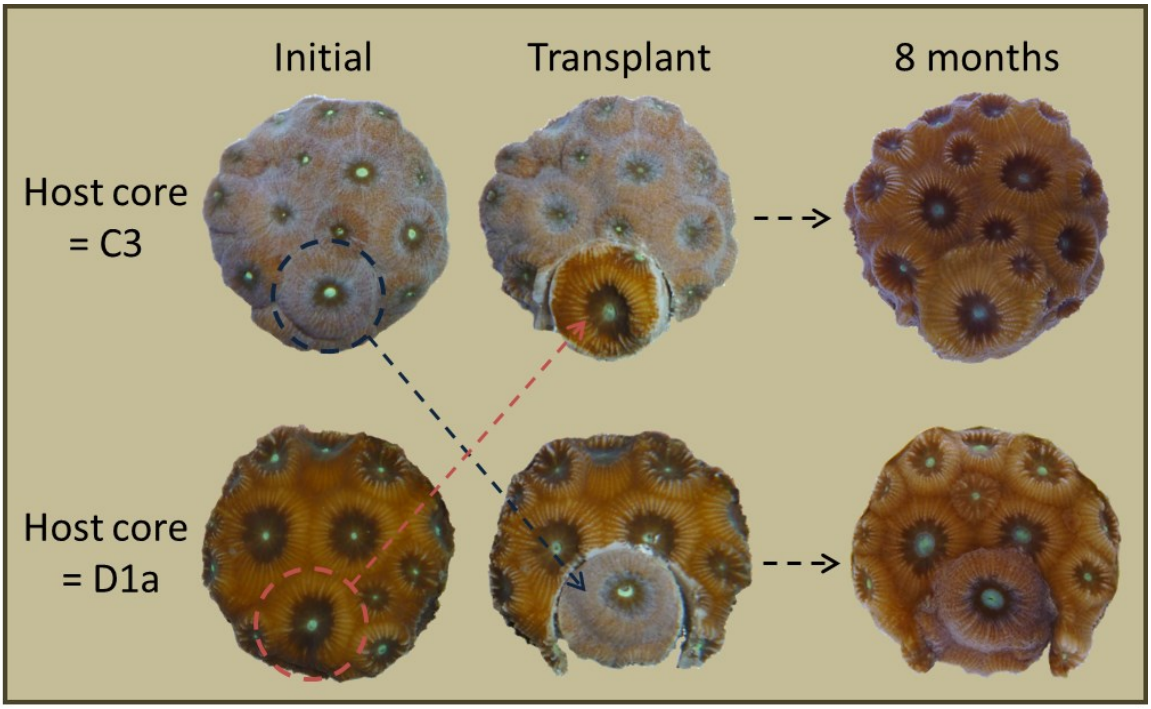
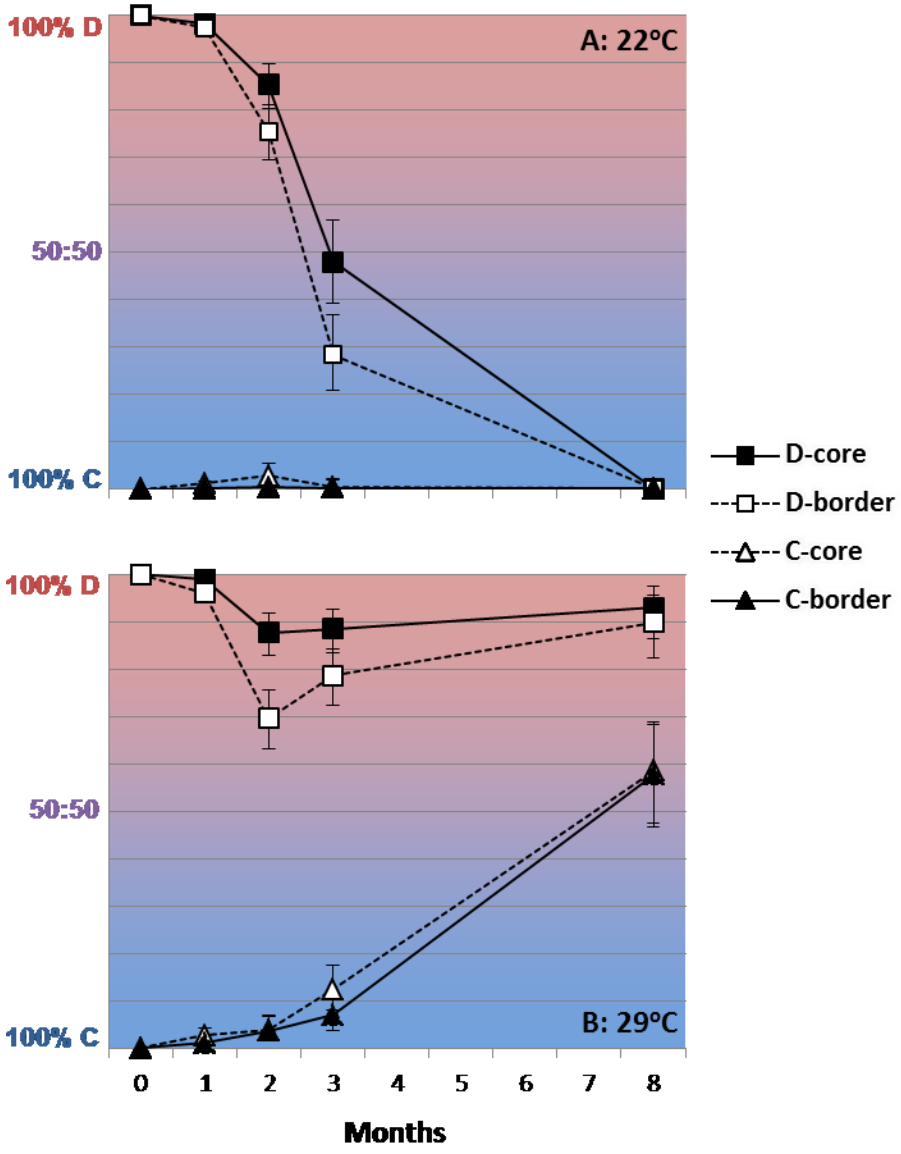


Figure 5.5 Effect of temperature and transect position on symbiont community change in clade D-dominated (square) and clade C-dominated (triangle) tissue in reciprocal transplants of *Montastraea cavernosa*. Data points represent pooled means for the two sampling points within each core type, i.e., core (black symbol) and border (white symbol) of D-dominated cores (squares) and C-dominated cores (triangle) at 22°C (panel A) and 29°C (panel B). Error bars display standard error, n = 10 per sampling interval.



Chapter 6

Synopsis

This dissertation utilized highly sensitive quantitative PCR assays (Cunning & Baker 2013; Cunning 2013; Silverstein 2012) to answer a number of important questions regarding the impact of disturbances on *Symbiodinium* community dynamics in bleached and healthy coral tissue. The studies focused primarily on two important Caribbean reef corals, *Orbicella faveolata* and *Montastraea cavernosa*, and how their symbiont communities respond to different temperatures and pCO_2 for particular studies. In Chapters 2 and 3 it was shown that *O. faveolata* corals hosting only *Symbiodinium* D1a (D-only) bleached less severely in response to elevated light intensities than those hosting a mix of B1 and D1a (B+D); and, in B+D cores at elevated temperatures (31^{o}C), clade B symbionts were lost more rapidly than clade D. During the initial bleaching period all cores exhibited a significant drop in photochemical efficiency, but most cores (with the exception of the B+D cores at 31^{o}C) showed signs of photoacclimation during the exposure period. Similarly, all cores exhibited a rapid decrease in skeletal mass during the initial exposure, but reduced this loss (or regained positive growth) during the subsequent weeks of exposure. B+D cores in control conditions (27^{o}C, 380ppm) had the highest mean growth rates across the full 7 week high-light exposure period (0.56 μg mm^{-2} day^{-1}) and these B1 dominated cores were also the first to photoacclimate. Conversely, the clade D-only cores, which bleached less than the B+D cores and also photoacclimated, showed net skeletal dissolution, with mean growth rates over the same 7 week period between -1.41 μg mm^{-2} day^{-1} (31^{o}C, 800ppm) and -3.24 μg mm^{-2} day^{-1}

(31^{o}C, 380ppm). The combination of high temperature and high pCO_2 therefore produced the best (or least inhibited) growth rate for the high light exposed D-only cores. Consequently, despite experiencing a greater loss of symbionts, corals hosting a mixed assemblage of *Symbiodinium* B1 and D1a grew more than their D-only counterparts, across all treatments.

Once the high-light and high temperature stressors were removed, symbiont communities started to recover, photochemical efficiency improved, and growth rates increased. In the D-only cores, elevated pCO_2 produced faster *Symbiodinium* proliferation compared to the control. However, it was previous exposure to elevated temperature that was the strongest influence on increased growth rates, with those cores in elevated pCO_2 exhibiting lower growth rates. The B+D cores finished the exposure period with similar abundances of symbionts (as measured by S:H ratio), but cores from the high temperature treatments shifted to D1a dominance, while the control temperature cores hosted proportionally more B1. These community shifts had profound effects on the rates of *Symbiodinium* repopulation, which were fastest in the D1a dominated cores in the control pCO_2 treatment. Comparatively, elevated pCO_2 reduced the recovery of photochemical efficiency, the rate of D1a proliferation, and coral growth rate. The B1 dominated cores in the control treatment displayed the slowest symbiont recovery rate, yet they were the only cores to reach the same photochemical efficiency they showed prior to bleaching, and they had a faster growth rate than the D-only cores within the same treatment, which had a 2-fold higher S:H ratio.

These experiments concurred with the hypothesis that corals may shift their symbionts in response to environmental perturbations (e.g., Buddemeier & Fautin 1993;

Baker et al. 2004; Jones et al. 2008) to generate a symbiotic holobiont which is better suited to the prevailing conditions (Jones et al. 2008). They showed that corals hosting a mixed assemblage of symbionts are better able to grow and survive during a bleaching event than corals hosting only bleaching resistant symbionts, despite suffering a greater loss of symbionts. In fact, this greater severity of bleaching appears to promote an increased rate of recovery, at least for *Symbiodinium* D1a.

Having determined that symbiont communities can change during and after bleaching episodes, and that increased severity of bleaching promotes faster recovery, this dissertation project aimed to determine whether this recovery is due to remnant symbionts or exogenous sources of *Symbiodinium* spp. finding greater open niche space within tissue vacated by other symbionts. This study attempted to answer this question by generating experimentally bleached cores of *O. faveolata* and *M. cavernosa* and recovering them in filtered seawater (FSW) alone and FSW containing various sources of exogenous symbionts, at 22^{o}C and 29^{o}C. The investigation revealed that (at 29^{o}C) initial recovery of bleached corals can be influenced by external symbionts. The hypothesis being that symbionts within the coral are physiologically compromised (e.g., producing reactive oxygen species, Weis 2008) and therefore exogenous *Symbiodinium* spp. represent an alternative source of potentially undamaged symbionts to get the coral over the bleaching hump. However, once the symbiont community becomes more established and the coral regains a steady source of photosynthetically derived carbon, winnowing of the *Symbiodinium* may occur (Dunn & Weis 2009; Davy et al. 2012) to optimize the symbiont community structure for the present conditions. Evidence for this hypothesis exists from the differences between the two temperature treatments. Despite being

subjected to the same bleaching conditions and exposed to identical sources of exogenous symbionts, the recovered cores at 22°C displayed a co-dominance of clade A and clade D symbionts, while those at 29°C were dominated (>90%) by clade D. Not only were these communities different from each other, but they were overwhelmingly different from the clade C dominated communities which the corals hosted prior to bleaching.

After verifying that exogenous sources of Symbiodinium can influence the community structure of bleached corals, a final goal of this project was to ascertain whether the symbiont communities of healthy adult corals can also be shifted by exogenous sources of symbionts. The rationale of which was to determine the potential for seeding coral nurseries, and possibly small reefs, with corals hosting symbionts better suited to the prevailing conditions. This particular study utilized cores of *M. cavernosa* from the same parent colony, some of which had been experimentally bleached to host a different symbiont community. By performing reciprocal transplants of tissue plugs from different cores, placing them at two different temperatures (22°C and 29°C) and analyzing the changes in symbiont community across these hybrid cores, this study confirmed that adult corals can also be influenced by exogenous sources of *Symbiodinium*. At 22°C, symbionts from clade C dominated cores (host and transplant) displaced neighboring clade D symbionts from their hosts and eventually dominated (>99%) the previously D dominated coral tissue. At 29°C the shift was not quite so absolute, but clade C symbionts dominating the coral tissue were displaced by neighboring clade D symbionts, which eventually comprised ~70% of the symbiont population. This finding adds validity to the hypothesis that, when given a suitable source of alternative *Symbiodinium* which are better suited to the prevailing conditions, the

symbiont community may shift in favor of the better adapted symbiont. Such an ability could have major implications and benefits for the capability for coral reefs to adapt to global climate change.

These studies suggest that (for corals capable of hosting more than one symbiont type) hosting a mixed assemblage community consisting of the commonly occurring symbiotic partner and a thermally tolerant *Symbiodinium* type, improves resilience and increases the rate of recovery following disturbances. The results also suggest that a healthy and diverse pool of exogenous symbionts may improve the fitness of a coral by providing a source of *Symbiodinium* with which to (potentially) acclimate to changing environmental conditions. By verifying that direct inoculation and close proximity are both viable methods for influencing symbiont community shifts, this research provides valuable data for coral nursery managers interested in improving the resilience of their out-planted corals. Similarly, showing that exogenous symbionts can produce symbiont community shifts without prior bleaching adds another level of complexity to modeling coral reef resilience, but also indicates that corals may have a greater capacity for acclimation than previously assumed.

Anthropogenic climate change is happening and coral reefs will be one of the most heavily impacted ecosystems. But, by reducing the stressors we have some control over (e.g., pollution, overfishing, sedimentation) we can reduce the pressure on corals and increase their chances acclimating and adapting to the extending range of extreme environments that corals have adapted to in the past.

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